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ABNORMAL DEPOSITION OF COLLAGEN-ELASTIC VASCULAR FIBRES AND PROGNOSTIC SIGNIFICANCE IN IDIOPATHIC INTERSTITIAL PNEUMONIAS

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Short Running Title: Collagen/elastic system vessels in IIPs

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SUMMARY

Background: Recently, vascular remodelling has shown to be promising as pathogenetic indicator in Idiopathic Interstitial Pneumonias (IIPs). In this study, we sought to validate the importance of the collagen/elastic system in the vascular remodelling and to study the relationships between collagen/elastic system, survival and the major histological pattern of IIPs. ***Material and Methods:*** We examined collagen/elastic system fibres in 25 acute interstitial pneumonia/diffuse alveolar damage, 22 non-specific interstitial pneumonia/non-specific interstitial pneumonia and 55 idiopathic pulmonary fibrosis/usual interstitial pneumonia cases. We used the Picrosirius-polarization method and Weigert's resorcin-fuchsin histochemistry and morphometric analysis to evaluate the amount of vascular collagen/elastic system fibres, and their association with IIPs histological pattern. In usual interstitial pneumonia (UIP) we also considered the association between vascular remodelling and parenchymal fibrosis degree. ***Results:*** The vascular measurement of collagen/elastic fibres content was significantly higher in UIP than lungs of control, diffuse alveolar damage and non-specific interstitial pneumonia. In addition, the increment of collagen/elastic fibres in UIP varied according to the parenchymal fibrosis degree and activity. The most important predictors of survival in UIP were vascular remodelling classification (p=0.0004) and vascular collagen deposition (p=0.005). ***Conclusion:*** We concluded that a progressive vascular fibroelastosis occurs in IIP histological patterns,

probably indicating evolutionary adapted responses to parenchymal injury. The vascular remodeling classification and the increased of the vascular collagen were related with survival in the idiopathic interstitial pneumonia and possibly play a role in its pathogenesis. Further studies are needed to determine whether this relationship is causal or consequential.
Keywords: Idiopathic interstitial pneumonias, collagen/elastic system fibres, vascular remodelling and survival.

INTRODUCTION

The vascular extracellular matrix (VECM) consists of collagens, elastin, fibrillins, proteoglycans, and others [1]. Collagen fibres are the most abundant component of the vascular pulmonary wall and are found in all three tunicae and especially around smooth muscle cells of the tunicae media, where they provide the necessary mechanical strength and contractility. Collagen fibres are also found in the outer layer (adventitia) where they form large bundles of fibrils, which increase progressively in size from its innermost component, closest to the media, to its outermost aspect [2-3]. The elastic system, a major component of the pulmonary arteries, plays an important role in wall elasticity, facilitated by concentric fenestrated lamellae of elastic fibres in the tunica media layer [1]. The accumulation of extracellular matrix is an important process of pulmonary vascular structural remodelling [4-5]. Elastosis has been well studied in animal models of pulmonary fibrosis and has shown to be transcriptionally regulated by the augment of lytic enzymes [6]. Studies previously conducted by our group showed that lung collagen and elastic fibre content are increased in both acute and chronic interstitial lung diseases, suggesting that significant remodelling of alveolar tissue occurs in both situations [7-11]. We too found evidence of vascular remodeling in lung biopsy specimens from patients with usual interstitial pneumonia. More specifically, a direct relationship between vascular regression— characterized by a progressive reduction in the internal area and internal perimeter, as well as increase in wall thickness of medium or large lung vessels—and parenchymal remodeling [12]. This finding is in agreement with previous reports of vessel ablation in areas of honeycomb lung, regardless of the cause of the pulmonary fibrosis [13-16]**.** Experimental studies have shown that collagen and elastic fibre deposition might be an important event for the understanding of all the alterations in vascular remodelling [17,18], but there has been uncertainty about the changes of collagen/elastic system in lung vessels of patients with IIPs.

We hypothesise that collagen/elastic system in vascular remodelling differ according to evolutionary adapted responses to injury that occur in interstitial pneumonia/diffuse alveolar damage (AIP/DAD), non-specific interstitial pneumonia/non-specific interstitial pneumonia (NSIP/NSIP) and idiopathic pulmonary fibrosis/usual interstitial pneumonia (IPF/UIP).

Therefore, the present study was designed to quantitation of vascular collagen/elastic system in different evolutionary adapted responses to injury that occur in AIP/DAD, NSIP/NSIP and IPF/UIP , as well as their association with survival.

METHODS

Patient selection

Pulmonary specimens were obtained from 109 patients, 25 with AIP/DAD, 22 with NSIP/NSIP and 55 with IPF/UIP, according to the criteria outlined in the *American Thoracic Society/European Respiratory Society* (ATS/ERS) *International Multidisciplinary*

Consensus Classification of the Idiopathic Interstitial Pneumonias [19], by surgical lung biopsy. Only specimens from cases that fulfilled these consensus criteria were included.

We excluded specimens of any other possible etiology (e.g., pneumoconiosis) and/or with histological features suggestive of an alternative diagnosis (e.g., eosinophilic pneumonia). We also excluded biopsy specimens obtained from patients with a concomitant systemic disease (e.g., collagen vascular disease), extensive honeycomb changes (end-stage lung disease), a dual histological pattern (2 different patterns at 2 different biopsy sites), and/or morphologic features not consistent with a specific histological pattern (e.g., bronchocentric distribution in an otherwise classical case of UIP). After excluding specimens with histological and clinical evidence of desquamative interstitial pneumonia, lymphoid interstitial pneumonia or respiratory bronchiolitis, all patients included had exhibited clinical, radiological, and physiological alterations consistent with AIP, NSIP and IPF and had been given the definitive pathological diagnosis of DAD, NSIP and UIP. Two or three biopsies per patient were sampled and the tissue specimens collected according to the High Resolution Computerized Tomography (HRCT) pattern which usually includes normal areas, intermediate and more affected areas in different parts of the lung.

In another words, the apparently clear-cut diagnosis of our patients with IIPs was obtained by clinical, radiological and histological consensus criteria.

Baseline characterization

Median age of patients with AIP (13 males and 12 females) was 51.72 years, ranging from 35 to 74; 50.81 years, ranging from 39 to 76, for NSIP (10 males and 12 females), and 65.35 years, ranging from 50 to 84 yrs for IPF (35 males and 20 females).

A baseline assessment of severity of dyspnoea was made using the level of dyspnoea (LOD) scale [20] (Table 1).

Physiological testing

The pulmonary function tests included FEV_1, FVC, FEV_1/FVC ratio × 100, total lung capacity (TLC), residual volume (RV) and carbon monoxide transfer factor (*D*LCO). TLC, RV and RV/TLC percentages were measured by the helium dilution method with a Master Screen apparatus (Erich Jaeger GmbH, Wuerzburg, Germany), *D*LCO and *D*LCO/VA by the single breath-holding helium dilution method [21]. Lung function measurements (Table 1) were expressed as percentages of predicted values. In all patients, the arterial PaO_2 and $PaCO_2$ were also measured at rest. The cardiac parameters of these patients were normals.

High-resolution computerized tomography

HRCT examinations were performed using 1.0 or 1.5 mm-thick sections taken at 1 cm intervals throughout the entire lungs during inspirations in the supine position and through the caudal 10 cm of the lungs at 2-3 cm increments in the prone position. Two thoracic clinical radiologists prospectively and independently scored all lobes or HRCT for ground-glass opacity (CT alveolar) and interstitial opacity (CT interstitial) on a scale of 0-5; the mean score for each lobe and for the entire lug was calculated [22] (Table 1).

Clinical scoring

Overall clinical severity was assessed using a previously developed clinical and HRCT examinations composite score [20]. The total clinical and HRCT score ranges 0-100 points (100 being the most severe disease) based on variables including the LOD scale, HRCT, and pulmonary function test results (Table 1).

Pathological review of the specimens

Pulmonary specimens were reviewed by three pathologists blinded to their clinical features. Each specimen was assigned a histological diagnosis according to the criteria outlined in the ATS/ERS multidisciplinary consensus classification of the idiopathic interstitial pneumonias [19]. The patients with DAD were defined by involvement and a uniform temporal appearance caused by alveolar collapse, obliterative fibrosis, neosepta formation, and moderately organizing fibrosis [19]. NSIP was characterized by the temporally homogenous septal inflammatory thickening and minimal organizing fibrosis [19] and UIP was defined by alternating areas of normal parenchyma, alveolar collapse, honeycombing, and severe mural organizing fibrosis, defined as sites of active remodeling overlying fibrous airspace walls, and thus showing temporal heterogeneity, or overlying normal rigid pulmonary structures (eg, interlobular septa) in a form of fibroblast foci and granulation tissue [19]. Further demographic data and radiological characteristics are shown in Table 1.

As control, normal lung tissues (NL) from non-pneumonia and non-emphysematous areas were obtained from five individuals (mean age 60 ± 3.6 years), who had died from violent causes. None of these control subjects met any of the histological criteria for IIP.

Morphological Study

Parenchymal remodelling definition. Parenchymal remodelling in UIP was evaluated by semi-quantitative analysis for alternating areas of: 1) minimal fibrosis (figure 1) – when alveolar collapse with relatively unaffected lung tissue or with mild interstitial thickening by fibrosis was present; 2) moderate fibrosis (Figure 1) defined by intermediate mural-organizing fibrosis with fibroblast foci; and 3) severe fibrosis (figure 1) when a severe mural-organizing fibrosis with honeycombing and foci of actively proliferating fibroblasts and myofibroblasts was present [12].

Vascular remodelling definition. To support the possibility of an important vascular contribution to the fibrosis, we make a distinction between vessels within the different areas of fibrosis and compared vessels from severely fibrotic parts to vessels in the far less fibrotic areas. In particular the finding of vascular changes in relatively spared areas of cases with severe fibrosis were compared to similar areas in cases with in general less severe fibrosis. To make a distinction between adventitia and surrounding parenchyma in case of increasing fibrosis.

Then, vascular remodeling in UIP was initially evaluated by semi-quantitative analysis for different levels of vascular obstruction in vessels within the different areas of fibrosis using a grading system as follow: grade I - isolated hypertrophy of the arterial media; grade II - proliferative intimal lesions; grade III - total occlusion of arterial lumen by fibrous tissue and grade IV - plexiform lesions.

After that, collagen and elastic fibre density were evaluated per biopsy specimen in 4 to 8 vessels with diameter ranging from 109,35 to 185,12 µm within the different areas of fibrosis. For the study of collagen, 3 µm paraffin-embedded sections were stained in a 0.2 % solution of Sirius red (Direct Red 80, C. I. 35780, Aldrich, Milwaukee, WI 53233, USA) dissolved in aqueous saturated picric acid [23,24]. Elastic staining was performed by Weigert's Resorcin-Fuchsin method, after previous oxidation [25]. The quantification of collagen/elastic fibres in artery walls was performed by an image analysis system; the system used consists of a CCD Sony DXC-101 camera, coupled to a Zeiss Axioplan microscope, from which the images are sent to a monitor (Trinitron Sony). By means of a digitising system (Oculus TCX, Coreco inc; St Laurent, Quebec, Canada) inserted in a

computer (Pentium 133Mhz), the images are processed by software (Bioscan-Optimas 5.1; Bioscan, Inc; Edmonds, Wash). The enhancement of collagen birefringence promoted by the Picrosirius-Polarization method is specific for collagenous structures composed of aggregates of oriented molecules. Elastic staining was performed by Weigert's resorcin-fuchsin method, following prior oxidation. This method allows the selective identification of the three types of elastic system fibres (oxytalan, elaunin and fully developed elastic fibres). The thresholds for fibres of the collagenous and elastic systems were established for each slide, after enhancing the contrast up to a point at with the fibres were easily identified as black (elastic) or birefringent (collagen) bands. The area occuped by the fibres was determined by digital densitometric reconiting, by adjusting the threshold level of measurement up to the grey density of the fibres of the collagenous and elastic systems. The collagen of the medial layer and elastic fibre content were measured in each vascular wall and were expressed as a relation between the quantity of collagen and elastic fibres divided by the total vascular area studied. Vascular area of each artery analysed was carefully measured in the image analysis system using a cursor that allows the free determination of the area from the internal elastic membrane until external elastic membrame. The results express the amount of fibres of the collagenous and elastic systems (in area) per total area of vascular wall expressed in fraction.

Statistical Analysis

The One-Way ANOVA procedure was used for the analysis of variance of collagen and elastic fibre means and their distribution in the IIPs histological pattern (DAD, NSIP, and UIP). Differences among the means were compared a priori by Levene's test for homogeneity of variance and then by post hoc tests using Bonferroni multiple comparisons for homogenous distribution and Dunnet T3 for non-homogeneous distribution. Survival curves comparing the qualitative changes of the collagen and elastic fibres in the vascular wall and morphological parameters in NSIP, AIP and IPF were initially tested in a univariate model. The significant variables selected on the basis of a univariate model were considered in the multivariate analysis of the Cox regression test, using different model specification.The level of significance was established at 0.05. The data were analysed using the SPSS for Windows program, release 10.0 [26].

RESULTS

Vascular remodelling evaluated by the different levels of vascular obstruction grade was significantly related to UIP parenchymal changes when compared to DAD and NSIP histological patterns. In other words, the alternating areas of minimal, moderate, and severe fibrosis observed in UIP (Figure 1) were significantly related with the vascular occlusion degree (Figure 3). In this regard, high degrees of vascular occlusion (e.g., grade III and IV) coincided with a heavily compromised pulmonary parenchyma by fibrosis. In all UIP surgical lung biopsies, alternating areas of moderate fibrosis were related with intermediate degree of vascular remodelling or obstruction (grade II or III). In the remaining UIP lungs, categorized as minimal fibrosis alternating areas, a significant relationship was found with a minimal degree of vascular remodelling. That is, vascular remodelling was represented by discrete intimal proliferation (grade I) with maintenance of the vascular architecture.

Figures 2 and 3 show the collagen/elastic fibre system in controls and IIPs lungs stained with Picrosirius polarized and Weigert's resorcin-fuchsin. Control lung shows the weak red-orange birefringence in the adventitial tunica of vascular wall in tissue sections

and the maintenance of the vascular wall architecture (Figure 2). In contrast, UIP lung shows distortion of vascular wall architecture and increase of birefringence in vascular medial layer in all four different degrees of vascular obstruction (Figure 3). This is correlated to the increase of parenchymal remodelling activity, septal thickening and increase of vascular collagen fibres (Figure 1). DAD and NSIP show a moderate red-orange birefringence in medial layers (Figure 2). This increase of birefringence is major in NSIP lungs and coincident with moderate parenchymal remodelling and moderate alveolar septal thickening (Figure 1). Equally important alterations of the elastic system are present. Figure 2 shows control groups in which the vascular pattern of the elastic component is preserved in the internal elastic and external elastic lamina. Figure 3 shows major proliferation of elastic fibres in the internal and external elastic lamina of vascular wall in UIP lungs, related to different degrees of vascular remodelling and different stages of parenchymal remodelling activity (Figure 1). A moderate degree of elastic system proliferation is present in vascular wall in the NSIP histological pattern while a minimal degree was observed in DAD pattern (Figure 2).

The qualitative changes of collagen and elastic fibres in the vascular wall are coincident to differences in their quantification in the four groups of patients (Table 2). The density of the collagen and elastic fibres show significantly higher values in artery walls of UIP lungs when compared to controls, NSIP and DAD lungs (p=0.001). In decreasing order, UIP vascular walls were those with the greatest amounts of collagen, followed by NSIP, DAD, and controls (Figure 4A). A statistically significant difference in the amount of collagen was observed among all groups (p=0.001) (Figure 4A). In Figure 4B is shown that elastic fibres showed a similar pattern statistically significant for control vs DAD (p=0.0001); and NSIP vs UIP (p=0.0001).

Parenchymal remodelling in UIP ranged from minimal to moderate and severe fibrosis. In this group, the greater the increase of collagen and elastic fibres, the greater the parenchymal remodelling (Figure 4C and 4D). Major-degree of fibre increase was observed in parenchymal remodelling (severe fibrosis), while intermediate fibre increase was present in minimal and moderate fibrosis activity (p=0.01) (Table 3) related with different degrees of vascular occlusion.

During the follow up of the 102 patients, 52 died and the clinical diagnoses were NSIP (5 patients), AIP (12 patients) and IPF (35 patients). All of patients studied presented a restrictive lung function pattern characterized by a decrease in TLC [mean values were NSIP (63%), AIP (57%), and IPF (72%) of predicted values] and an increased in FEV_1/FVC ratio ×100 [mean values were NSIP (104%), AIP (97%), and IPF (83%) of predicted values]. The mean predicted values of *D*LCO were decreased in NSIP (43%), AIP (46%), and IPF (49%) patients (Table 1). The pulmonary function in the different degrees of fibrosis in IPF weren't significant different in this groups (Table 4). Pulmonary function tests weren't significantly related to vascular oclusion or collagen and elastic vascular density.

In the first statistical test, the individual effect of patient characteristics (age, sex, baseline data, physiological test), parenchymal remodelling (minimal fibrosis, moderate fibrosis and severe fibrosis) and vascular remodelling (grade I, grade II, grade III, grade IV, collagen and elastic fibre density) were examined to estimate curve survival (Figures 5A and 5B). The results of this analysis showed that the prognosis of patients with IPF/UIP was dependent on vascular remodelling classification (log rank 18.24; p=0.0004; Figure 5A) and the collagen vascular density (log rank 10.57; p=0.005; Figure 5B). Multivariate

analysis of overall survival time based on significant factors at univariate analysis was examined by the Cox Regression Model. Initially, the model was constructed with vascular remodelling classification and collagen vascular density. In this situation, only vascular remodeling classification was maintained as independent prognostic factor; collagen vascular density lost significance probably due to joint effects of both vascular variables (Table 5). Thus, total occlusion of arterial lumen by fibrous tissue (grade III) and plexiform lesions (grade IV) increase 4 to 11 times the risk of death for patients with UIP/IPF.

DISCUSSION

Clearly, the likely reason for patients with IIPs to exhibit different outcomes is due to uncontrolled fibrosis progression. The question of interest is whether further information gathered from either the lung parenchyma or interstitial or VECM active remodelling can help us understand the heterogeneous evolution of IIPs. The process of active remodelling undoubtedly comprises a series of complex, sequential steps, but among these, the ECM remodelling is thought to be important because this process facilitates scar formation, which occurs by neovascularization, myofibroblast migration and collagen and elastic fibre deposition. In IIPs, this process takes place in an uncontrolled way, causing excessive fibrosis formations and progressive histo-architectural and vascular alterations [27-33]. The VECM remodelling process is a dynamic process that involves changes in collagen and elastic fibres system resulting in different degree of vascular oclusion. Collagen fibres are distributed diffusely throughout the media and adventitia of vessels, being synthesized by fibroblasts, myofibroblasts, and smooth-muscle cells and providing tensile strength to the vessel wall [34]. Elastic fibres are composed of an amorphous component, called elastin, and a highly structured microfibrillar non-extensible component [17,18]. In normal elastic arteries, the internal and external elastic laminas are composed mainly of fully mature elastic fibres that confer a great elasticity to the vascular tissue under normal circumstances [17,18]. Due to their mechanical properties, elastic fibres provide the elasticity needed for a good vascular function [17,18]. Increased elastin destruction takes place under certain pathological conditions, due to the release of powerful elastolytic proteases by inflammatory cells [35]. Reactivation of elastin synthesis is observed in response to the increased destruction [36], but in a highly disordered manner with deleterious consequences to vascular mechanical properties [37].

Thus, for all these reasons, we should not be surprised to learn that vascular changes provides important information about the active remodelling in IIPs, and our results now confirm the importance of vascular remodelling in DAD, NSIP and UIP, the most important types of IIPs. Whereas only two prior studies were able to show a significant relationship between pathological and structural vascular changes in pulmonary models [37-40], our results suggest that increase of collagen and elastic fibres in vessels probably contributes to vascular changes in fibrotic lung disease. These fibrotic changes cannot be attributed to consequences of any form of pulmonary hypertension because there isn’t real support or evidence in our study. More or less in contrast, when looking at the vessels changes in fibrotic lung disease, these seem mostly secondary to the inflammatory changes and seem therefore not to be the result of increased vascular flow but rather be the result of the effects of many cytokines and other mediators directed to myofybroblasts and smooth muscle cells with possibly also endothelial damage and thrombosis. The examples given in the figures also will support that these are not consistent with changes generally seen in

different stages of plexiform arteriopathy, as observed in pulmonary hypertension. Therefore, the use of a grading system as described in our study was able to discern different levels of severity of obstruction.

UIP was shown to be the prototype of apposition of elastic and collagen fibres in the vascular wall. We demonstrated that there is a correlation between the vascular active remodelling measured by vascular classification, vascular grades of collagen deposition and survival in IPF/UIP. Although we were unable to determine whether vascular remodelling is a primary event or if it is secondary to interstitial fibrosis, collagen/elastic system fibre increase offers us the potential to guide fibrosis progression in patients with UIP. Finally, this conclusion will require further study in randomised and prospective trials, and we also believe it is important to validate our quantitative assessment of collagen/elastic system fibres as well as to extend it to other diffuse parenchymal lung diseases by studying ECM active remodelling in additional patients.

We have also found that collagen/elastic system fibres amount were related to different forms of remodelling in NSIP and DAD. For instance, we found that collagen/elastic system fibre density in DAD and NSIP was significantly related to the histological pattern, and collagen/elastic system fibre quantification provides more information about remodelling state. Collagen/elastic fibre quantitation was also significantly related to evolutionary adapted responses to VECM remodelling, which depend largely, if not solely, on the extent of the destruction of the lung parenchyma as currently seen in DAD and NSIP. Most interesting was the strong, progressive, quantitative increase we established between collagen/elastic system and DAD, NSIP and UIP. Collagen/elastic fibres increased progressively from DAD and NSIP to UIP, emphasizing a temporal increase in IIPs, where fibre deposition and parenchymal activity were considered intermediary between UIP and DAD. In the collagen and elastic vascular area, the adventitial area was not included and the exact delimitation of this area was carefully done to avoid that with increasing interstitial fibrosis in the lung, the distinction between the fibrotic part of the adventitia and the surrounding parenchyma was difficult.

A variety of associations between vascular remodelling and IIPs have been described recently [41]. Not only have collagen and elastic fibre increase in arteries been thought to determine contracture and reduced distensibility of the vessels in IIPs and thus allow chronic hypoxia, but they have also been thought to increase according to parenchymal injury extension. More specifically, in UIP we found a direct relationship between vascular and parenchymal fibrosis degree: minimal fibrosis, moderate fibrosis and severe fibrosis. Peao et al [13] also showed that vascular remodelling regulates lung fibrosis induced by bleomycin in rats. In 1963, Turner-Warwick [16] demonstrated precapillary systemic-pulmonary anastomoses as part of the vascular remodelling in lungs. In addition, these findings are also in agreement with previous reports of vessel ablation in areas of honeycomb lung, regardless of the pulmonary fibrosis cause. Now, we have demonstrated that there is a strong correlation between vascular remodelling and levels of collagen/elastic system fibre deposition in UIP, and to the best of our knowledge, this is the first report of this association in IIPs. The strong association between vascular collagen/elastic system fibre remodelling and IIPs suggests that IIPs secreting higher levels of collagen/elastic fibres facilitate parenchyma destruction and ECM fibroelastosis. Thus, increased collagen/elastic fibre levels may be more of a primary event, and increased vascular remodelling may be more of a secondary event. Regardless of the mechanism, the collagen/elastic system provides important information in IIPs.

During the follow up of the 102 patients, 52 died and the clinical diagnoses were NSIP (5 patients), AIP (12 patients) and IPF (35 patients). The survival analysis showed that the prognosis of patients with IPF/UIP was dependent on vascular remodelling classification and the collagen vascular density. However, when multivariate analysis of overall survival time was examined by the Cox Regression Model, only vascular remodeling classification was maintained as independent prognostic factor; collagen vascular density lost significance probably due to joint effects of both vascular variables. Thus, total occlusion of arterial lumen by fibrous tissue (grade III) and plexiform lesions (grade IV) increase 4 to 11 times the risk of death for patients with UIP/IPF.

We conclude that vascular remodelling present in IIPs differs in terms of collagen and elastic fibre content and characterise a vascular fibroelastosis, which in turns correlates to fibrosis degree, suggesting that vascular remodelling in DAD, NSIP and UIP runs in parallel with the evolutionary adapted responses after injury in these entities, which depend, at least in part, on the extent of collagen and elastic fibre deposition. In UIP lungs, the vascular occlusion is related with survival disease and possibly plays a role in its pathogenesis.

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Table 1. Baseline characteristic in the major histopathological categories

Variables	**Values**		
	NSIP	**AIP**	**IPF**
Age at biopsy (yr)	50.8±16.6	51.7±14.9	65.3±7.4
Sex (F/M)	12/10	13/12	20/35
Dyspnoea			
LOD	5±4	14±5	10±4
Spirometry			
FEV_1 (%pr)	58±6	60±3	81±5
FVC (%pr)	54±5	51±3	71±4
FEV_1/FVC	104±7	97±81	80±6
TLC (%pr)	63±5	57±8	79±4
RV (%pr)	94±17	64±15	93±11
RV/TLC (%pr)	155±16	93±23	40±3
*D*LCO (%pr)	43±14	46±9	60±7
*D*LCO/VA(%pr)	97±2	83±5	63±7
PaO_2 (mmHg)	64±6	78±18	59±2
$PaCO_2$ (mmHg)	35±1	49±5	33±08
HRCT Score			
Alveolar	1.60±1.10	1.60±0.90	1.87±0.90
Interstitial	0.87±0.61	0.73±0.52	1.90±0.56
Total	2.47±0.85	2.33±0.71	3.77±0.73
Clinical Score (CS)			
Total CS	33±20	42±23	53±16

Data are represented as means±SEM; F=Female; M=Male; pr: predicted; LOD=level of dyspnoea; CS=Clinical Score; NSIP=Non-specific Interstitial Pneumonia; AIP=Acute Interstitial Pneumonia; IPF=Idiopathic Interstitial Pneumonia.

Table 2: Descriptive and formal analysis of collagen and elastic vascular fibres stratified by IIP histological patterns in cases of open lung biopsies of patients with AIP/DAD, NSIP/NSIP, IPF/UIP and control lung using the One-Way ANOVA procedure and Bonferroni multiple comparisons post hoc tests for analysis of variance vascular collagen/elastic system fibres and their distribution in IIP histological patterns and control lung with level of significance of 0.05.

Vascular collagen/elastic system		Control Lung and Histological Patterns of IIP				
		Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
*Collagen fibres	Control	0.01	0.005	0.002	0.007	0.02
	DAD	0.13	0.05	0.01	0.11	0.15
	NSIP	0.24	0.03	0.008	0.22	0.26
	UIP	0.29	0.06	0.008	0.27	0.30
**Elastic fibres	Control	0.05	0.01	0.0.7	0.03	0.07
	DAD	0.21	0.02	0.005	0.20	0.22
	NSIP	0.25	0.03	0.006	0.24	0.26
	UIP	0.32	0.07	0.009	0.30	0.33

*(Collagen fibers) control X DAD p=0.0001; DAD X NSIP p=0.0001, NSIP X UIP p=0.009.
**(Elastic fibers) control X DAD p=0.0001; DAD X NSIP p=0.08; NSIP X UIP p=0.0001.

Table 3: Descriptive and formal analysis of collagen and elastic vascular fibres stratified by UIP histological patterns in cases of open lung biopsies of patients with IPF/UIP and control lung using the One-Way ANOVA procedure and Dunnett T3 multiple comparisons post hoc tests for analysis of variance vascular collagen/elastic system fibres and their distribution in UIP histological patterns and control lung with level of significance of 0.05.

Vascular collagen/elastic system		Control Lung and Histological Patterns of UIP				
		Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
*Collagen fibres	Control	0.01	0.05	0.002	0.07	0.02
	Minimal fibrosis	0.22	0.03	0.008	0.20	0.24
	Moderate fibrosis	0.27	0.04	0.01	0.25	0.29
	Severe fibrosis	0.35	0.03	0.007	0.33	0.36
**Elastic fibres	Control	0.05	0.01	0.007	0.03	0.07
	Minimal fibrosis	0.25	0.05	0.01	0.22	0.28
	Moderate fibrosis	0.30	0.05	0.01	0.27	0.33
	Severe fibrosis	0.37	0.04	0.008	0.35	0.39

*(Collagen fibres) control X UIP minimal fibrosis p=0.001; UIP minimal fibrosis X UIP moderate fibrosis p=0.02, UIP moderate fibrosis X severe fibrosis p=0.0001.
**(Elastic fibres) control X UIP minimal fibrosis p=0.001; UIP minimal fibrosis X UIP moderate fibrosis p=0.037, UIP moderate fibrosis X severe fibrosis p=0,01.

Table 4. Baseline characteristic in the IPF histological patterns

Variables	Values of IPF histological patterns		
	Minimal fibrosis	**Moderate fibrosis**	**Several fibrosis**
Age at biopsy (yr)	62.2±7.4	63.5±8.6	67.5±7.8
Sex (F/M)	5/10	7/11	8/14
Dyspnoea			
LOD	8±4	10±2	12±2
Spirometry			
FEV_1 (%pr)	74±18	66±20	90±27
FVC (%pr)	66±18	59±17	76±23
FEV_1/FVC	87±8	72±34	90±10
TLC (%pr)	67±14	70±84	76±21
RV (%pr)	74±15	78±46	90±46
RV/TLC (%pr)	43±9	46±17	44±14
*D*LCO (%pr)	44±17	54±28	48±22
*D*LCO/VA(%pr)	45±17	52±12	60±20
PaO_2 (mmHg)	67±13	63±11	66±14
$PaCO_2$ (mmHg)	36±5	36±3	38±4
HRCT Score			
Alveolar	1.70±0.22	1.75±0.40	1.80±0.50
Interstitial	1.85±0.61	1.87±0.42	1.89±0.56
Total	3.55±0.83	3.52±0.82	3.69±1.06
Clinical Score (CS)			
Total CS	50±20	52±26	53±12

Data are represented as means±SEM; F=Female; M=Male; pr: predicted; LOD=level of dyspnoea; CS=Clinical Score; IPF=Idiopathic Pulmonary Fibrosis

Table 5. Coefficients attributed by Cox Multivariate analysis relating the quantitation of collagen fibres, elastic fibres and vascular occlusion classification to overall survival in idiopathic interstitial pneumonia / usual interstitial pneumonia (IPF/UIP)

Vascular variables	B	SE	Wald	Sig.	Exp(B)	95% CI for Exp(B) Lower	Upper
Collagen fibres quantitation							
</=0.25			0.638	0.727			
0.27 to 0.32	-0.093	0.770	0.015	0.904	0.911	0.201	4.120
>/=0.34	0.314	0.843	0.139	0.710	1.369	0.262	7.145
Elastic fibres quantitation							
</=0.27			0.407	0.816			
0.29 to 0.35	0.391	0.618	0.401	0.527	1.479	0.440	4.969
>/=0.36	0.282	0.695	0.165	0.685	1.326	0.340	5.176
Vascular occlusion							
Grade I			4.815	0.186			
Grade II	0.590	0.584	1.021	0.312	1.804	0.574	5.664
Grade III	1.397	0.714	3.826	0.050	4.045	0.997	16.406
Grade IV	2.408	1.320	3.329	0.068	11.113	0.836	147.667

LEGENDS FOR FIGURES

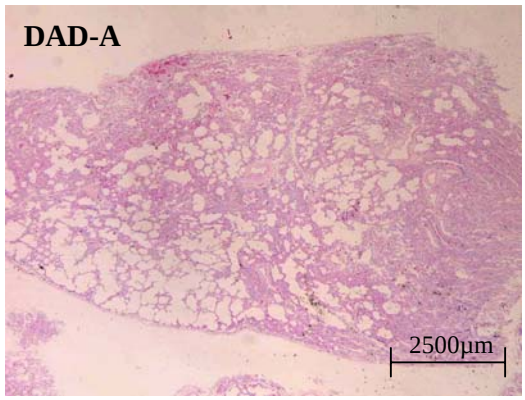
FIGURE 1. Idiopathic Interstitial Pneumonia (Histological Patterns)
DAD-A. Panoramic view showing diffuse involvement and a uniform temporal appearance (Hematoxylin-Eosin X15); **DAD-B.** In a high magnification areas of alveolar collapse, obliterative fibrosis and neoseptal formation (Hematoxylin-Eosin X400).
.**NSIP-A.** Characterized by the temporally homogenous septal inflammatory thickening (Hematoxylin-Eosin X10). **NSIP-B.** In a high magnification septal inflammatory thickening and moderate fibrosis (Hematoxylin-Eosin X400).
UIP - minimal fibrosis. Characterized by alveolar collapse with relatively unaffected lung tissue or with mild interstitial thickening by fibrosis (Hematoxylin-Eosin X10); **UIP - moderate fibrosis.** Pulmonary parenchyma showed high degree of inflammatory activity and moderate mural organizing fibrosis with fibroblast foci (Hematoxylin-Eosin X10); **UIP - severe fibrosis.** Severe mural organizing fibrosis with honeycombing and *foci* of actively proliferating fibroblasts and myofibroblasts (Hematoxylin-Eosin X10)

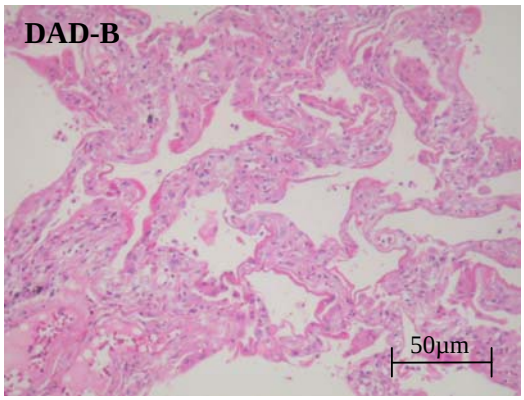
FIGURE 2.
Control lung vessels Collagen/elastic fibres system showed in the adventitial tunica of vascular wall in tissue sections and the maintenance of the vascular wall architecture (Picrosirius-polarization and Weigert's Resorcin-Fuchsin X200).
DAD lung vessels Showed a minimal red-orange birefringence of collagen fibres in medial and adventitial layers and minimal elastic fibres proliferation in vascular wall (Picrosirius-polarization and Weigert's Resorcin-Fuchsin X200).
NSIP lung vessels Showed a moderate red-orange birefringence of collagen fibres and moderate elastic fibres proliferation in the vascular wall (Picrosirius-polarization and Weigert's Resorcin-Fuchsin X200).

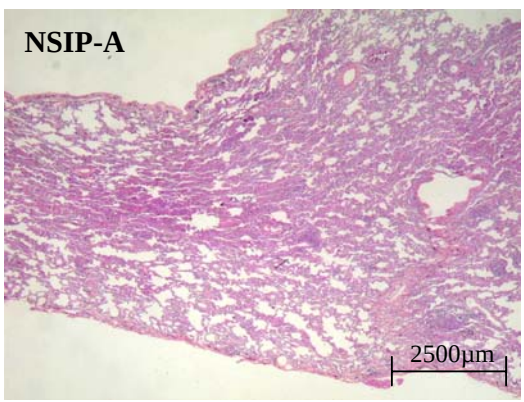
FIGURE 3.
UIP lung vessels. This groups shows distortion of vascular wall architecture and increase of red-orange birefringence of collagen fibres in vascular medial and adventitial layer in all four different degrees of vascular alteration (grade I - isolated hypertrophy of the arterial media, grade II – proliferative intimal, grade III – total occlusion of arterial lumen by fibrosis tissue and grade IV - plexiform lesion) and major proliferation of elastic fibres in the internal and external elastic lamina of vascular wall (Hematoxylin-Eosin, Picrosirius-Polarization and Weigert's Resorcin-Fuchsin X200).

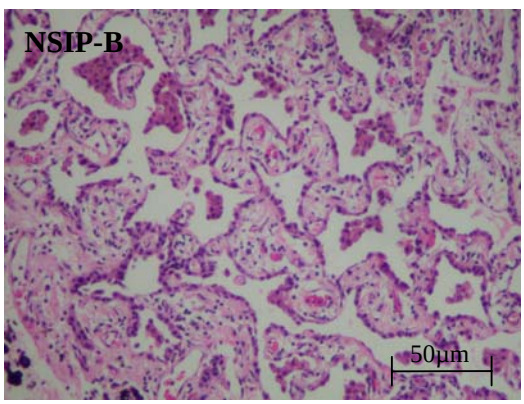
Figure 1

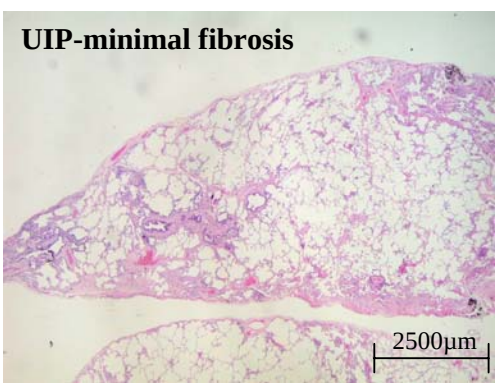
Idiopathic Interstitial Pneumonias (Histologic Patterns)

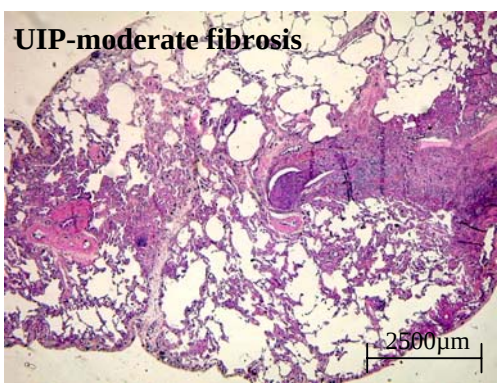












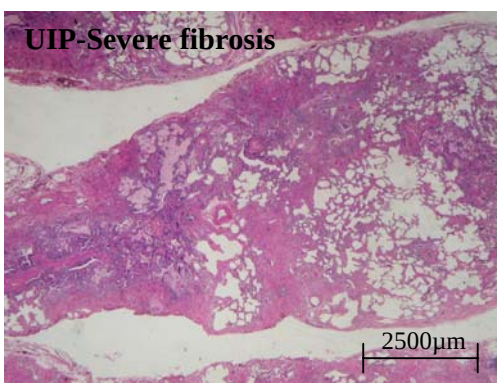
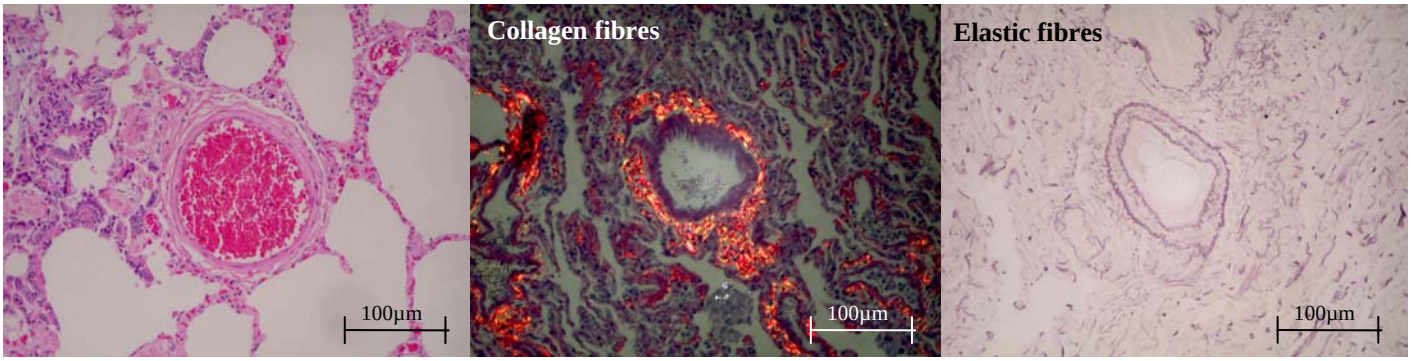
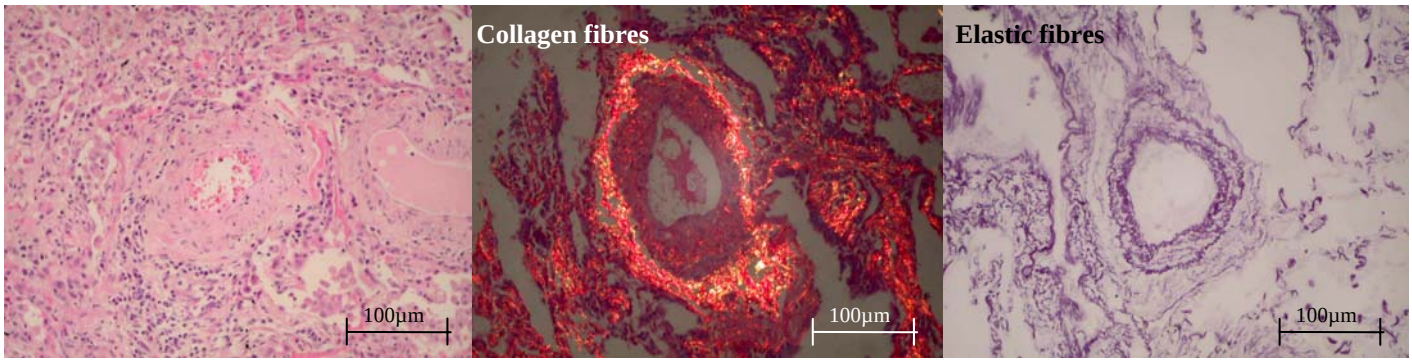


Figure 2

Control Lung - Vessels



DAD Lung - Vessels



NSIP Lung - Vessels

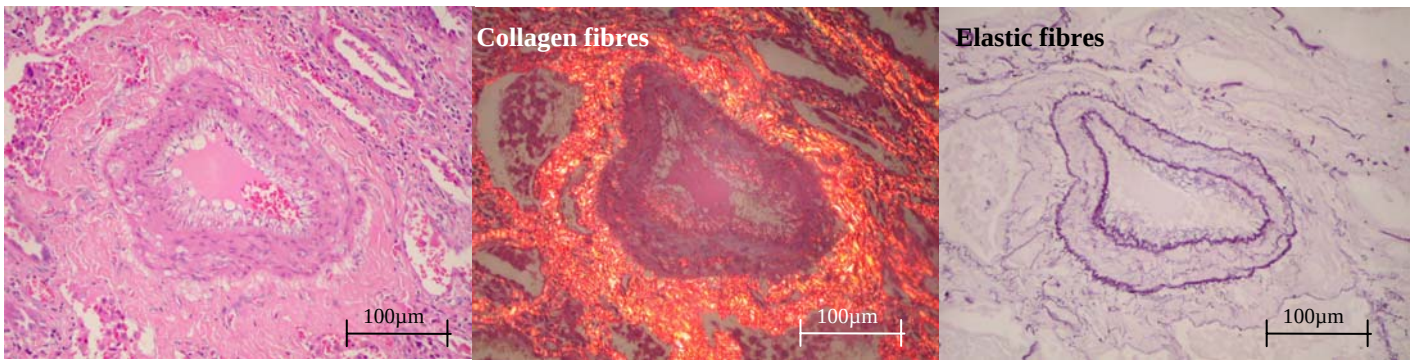


Figure 3

UIP Lung -Vessels

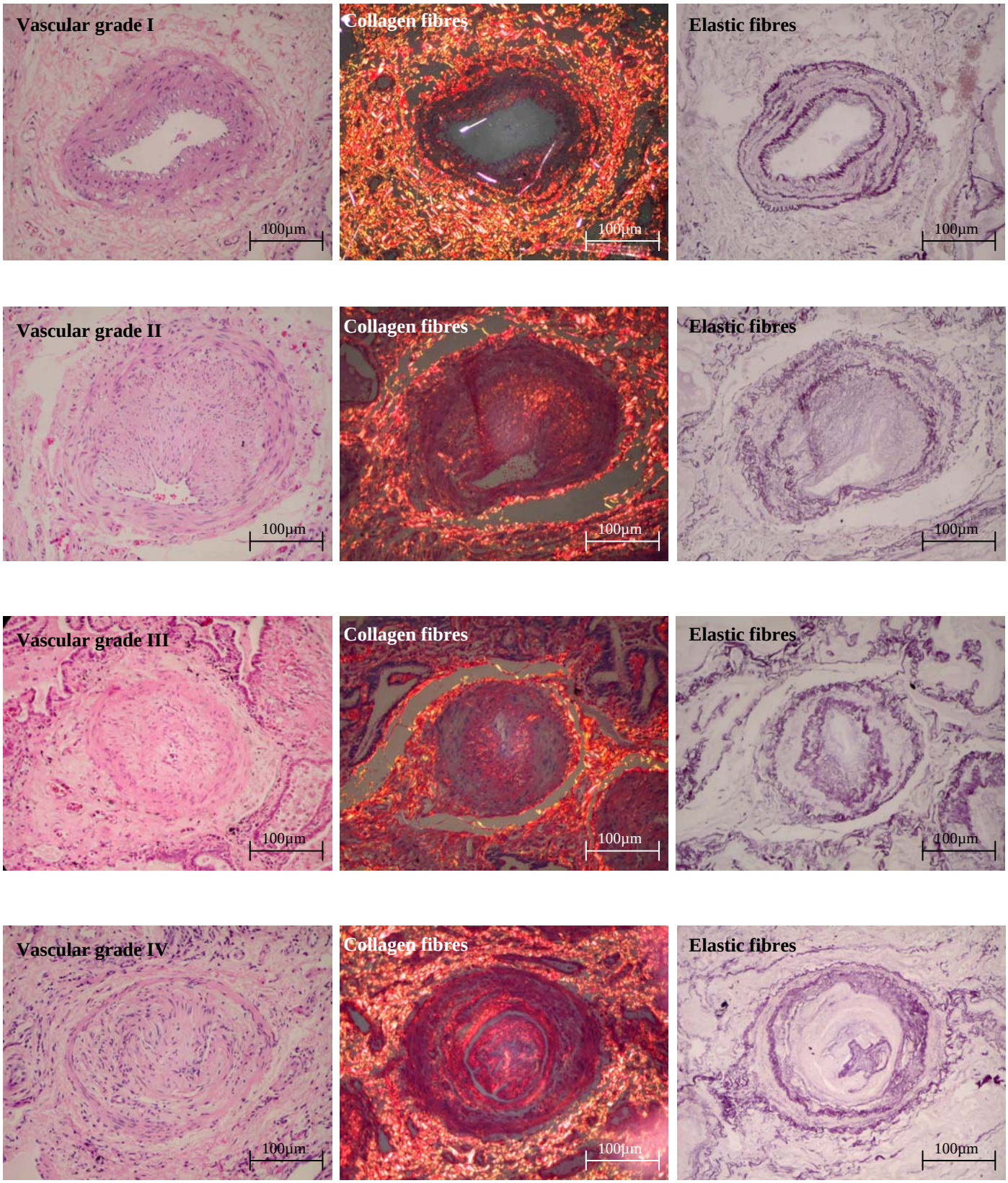
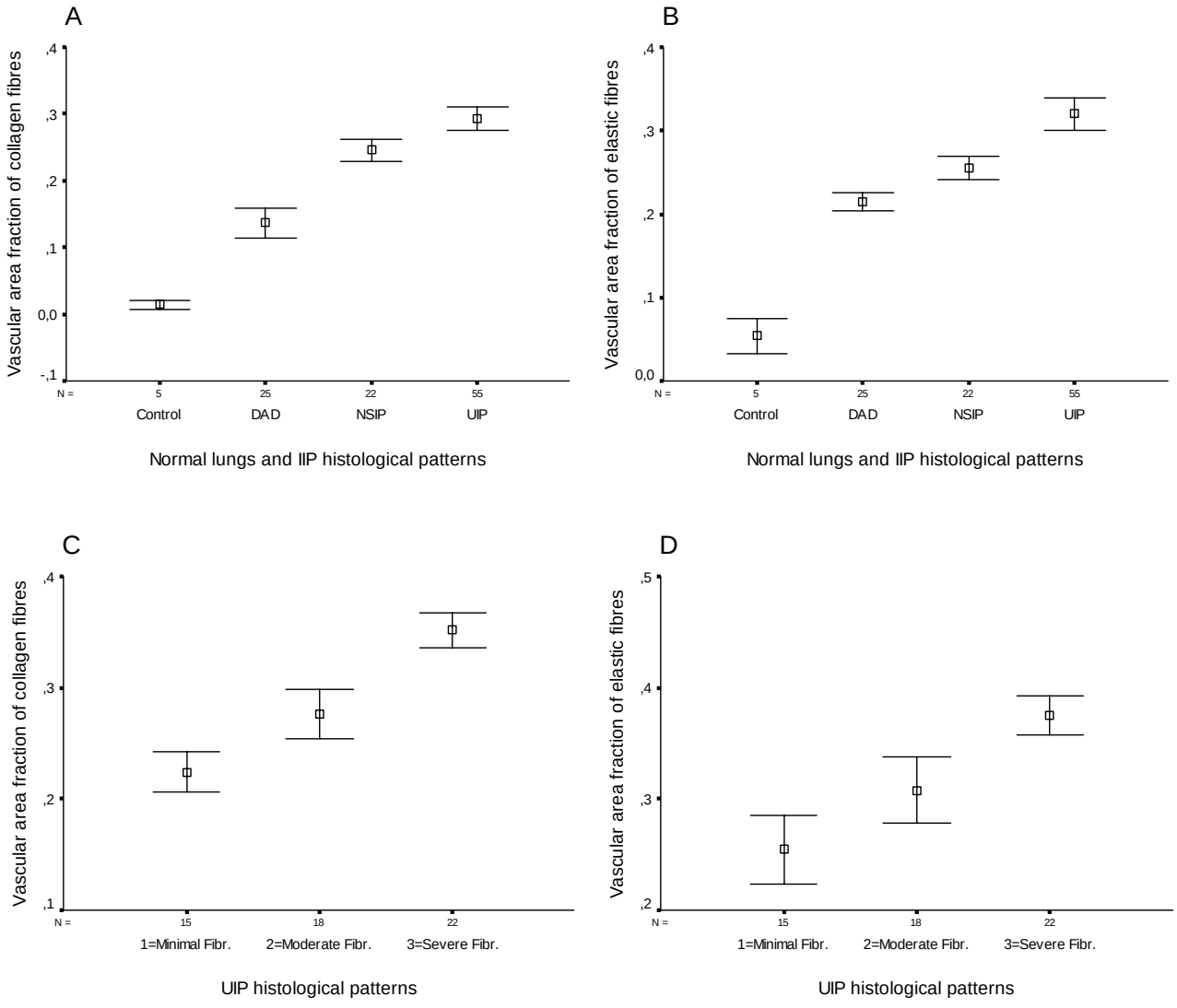


Figure 4. Graphic representation of the confidence interval for means are shown for each group, including vascular area fraction of collagen (A) and elastic fibres (B) as a function of the control lungs *versus* DAD, NSIP, UIP histological patterns and vascular area fraction of collagen (C) and elastic fibres (D) as a function of the UIP histological patterns.



UIP histological patterns: The three grades of fibrosis observed in the usual interstitial pneumonia: 1 = minimal fibrosis, 2 = moderate fibrosis and 3 = severe fibrosis.

Figure 5. Kaplan-Meier survival curve for patients with idiopathic pulmonary fibrosis/usual interstitial pneumonia (IPF/UIP) grouped by A) vascular classification system ($p<0.01$) and B) vascular collagen fibers quantitation grouped by minimal, moderate and severe deposition of vascular collagen $p<0.01$.