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A haplotype of cyclooxygenase-2 gene is associated with idiopathic pulmonary fibrosis

A. Xaubet[1, 2, 3], W.J. Fu[4], M. Li[4], A. Serrano-Mollar[2, 3, 5], J. Ancochea[6], M. Molina-Molina[1, 2, 3], E. Rodriguez-Becerra[7], F. Morell[3, 8], J.M. Rodríguez-Arias[9], J. Pereda[2, 3], A. Casanova[6], L. Molins[10], C. Picado[1, 2, 3]

[1] Servicio de Neumología, Hospital Clínic, Universitat de Barcelona, Spain; [2] IDIBAPS; [3] Centro Investigaciones Biomédicas en Red (CIBER) de Enfermedades Respiratorias; [4] The Computational Genomics Lab, Department of Epidemiology, Michigan State University, East Lansing, Michigan, USA; [5] Departmento de Patología Experimental, IIBB, CSIC, Barcelona. Spain; [6] Servicio de Neumología. Hospital de La Princesa, Madrid, Spain; [7] Servicio de Neumologia. Hospital Virgen de Rocío, Sevilla, Spain; [8] Servicio de Neumología, Hospital Vall d´Hebron, Barcelona, Spain; [9] Departamento de Neumologia. Hospital de Sant Pau, Barcelona, Spain; [10] Servicio de Cirugía Torácica, Hospital Clinic. Barcelona, Spain

Abstract. *Background:* Cyclooxygenase-2, a key regulatory enzyme in the synthesis of the antifibrotic agent prostaglandin E_2, is downregulated in lung tissue from patients with idiopathic pulmonary fibrosis. *Objective:* To investigate the association between COX2.3050 (G → C), COX2.8473 (C → T) and COX2.926 (G → C) single nucleotide polymorphisms (SNP) and the susceptibility to idiopathic pulmonary fibrosis and the progression of the disease. *Design:* Genetic polymorphisms were analyzed in 121 out of 225 available control subjects and in all of 174 patients with idiopathic pulmonary fibrosis by real time polymerase chain reaction. Logistic regression, analysis of covariance and chi-squares test were used for statistical analysis. *Results:* While analysis of disease development did not find any significant association with single SNP genotype, a haplotype analysis revealed a strong association between the disease development and one haplotype [GC] at loci COX2.3050 and COX2.8473, and suggested a recessive genetic effect of this haplotype. Further analysis concluded that subjects having two copies of [GC] haplotype, or equivalently (GG/CC) genotype at the two SNPs, had an increased risk after adjusting for age and sex. Due to the interaction, this elevated risk increased slowly with age, and the estimated odds ratio (OR) decreased with age from OR = 1.4 at age 30 to OR = 1 at age 74 and OR = 0.96 at age 80. The OR was significantly greater than 1 up to age 66, and not significant for age older than 66. Therefore, the recessive effect of [GC] haplotype increased the risk of IPF of subjects younger than 66 years, but its effect diminished for seniors older than 66. One hundred and forty-nine patients with idiopathic pulmonary fibrosis were followed up for 33.7 ± 2.1 months. Further analysis of disease progressions, defined by the changes in pulmonary function tests, did not reveal any association with either SNP genotypes or haplotypes. *Conclusions:* The carriage of double homozygote (GG/CC) at the SNP loci of COX2.3050 and COX2.8473 polymorphisms may increase the susceptibility to idiopathic pulmonary fibrosis, by approximately 1.4 folds at age 30 and by a smaller fold greater than 1 up to age 66 years, but not the progression of the disease. These findings may help to improve our understanding of idiopathic pulmonary fibrosis pathogenesis and may lead to the development of new therapeutic strategies. *(Sarcoidosis Vasc Diffuse Lung Dis 2010; 27: 121-130)*

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Correspondence: Antoni Xaubet, MD, PhD
Servei de Pneumologia. Hospital Clínic
Villarroel 170, Barcelona 08036, Spain
Fax + 34932275455
E-mail: axaubet@clinic.ub.es

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Introduction

Idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic interstitial lung disease, associated with the histological appearance of usual interstitial pneumonia (1). Although the etiology of IPF is largely unknown, it probably involves an interaction between environmental and genetic factors. The genetic predisposition to develop pulmonary fibrosis is suggested by the existence of familial forms of IPF. Surfactant protein C and telomerase gene mutations are involved in the pathogenesis of familial cases of IPF (2, 3). However, the development of sporadic IPF is likely to be determined by multiple genetic factors (4). *In vitro* and experimental studies in animal models have shown that the pathological process of IPF is related to the interactions of several cytokines, chemical mediators and growth factors (5). Therefore, candidate genes may be those that regulate the processes of inflammation, wound healing and repair.

Cyclooxygenase (COX) is the key enzyme that mediates the synthesis of prostaglandins from arachidonic acid. Two distinct isoenzymes of COX have been identified. COX-1 is constitutively expressed in many tissues and it is responsible for the production of prostaglandins in physiological functions. COX-2 may be induced by the effect of a variety of stimuli such as cytokines and growth factors, and it is responsible for the production of prostaglandins under inflammatory conditions (6).

The relevance of COX-2 in the pathogenesis of IPF has been widely demonstrated (7-9). The downregulation of COX-2 expression in IPF has been associated with a failure to synthesize prostaglandin E_2 (PGE_2), a potent inhibitor of fibroblast proliferation which could lead to an altered tissue repair (9-11).

We hypothesized that dysregulation of COX-2 expression in IPF may be due to genetic polymorphisms. The human gene encoding COX-2 is located in chromosome 1q25.2-q25.3 (12). Several SNPs in COX-2 gene have been identified, although only some of them are functionally relevant (13).

While there has been no report on association of haplotypes of COX-2 genes with IPF, it has been reported that certain haplotype of COX-2 genes is strongly associated with diseases in which the altered tissue repair is a main physiopathological process, like colorectal cancer and inflammatory bowel disease (14-15). Since alveolar epithelial wound healing has been demonstrated to trigger lung fibrosis, we have investigated the association of polymorphisms and haplotypes of COX-2 gene with the development and progression of IPF. We have selected gene polymorphisms on the basis of reported functional or biological relevance and/or their appreciable prevalence in Caucasian population: COX2.3050 (G → C) (rs5277) located in the coding region of exon 3, COX2.8473 (C → T) (rs5275), located in the exon 10, 3´-UTR region and COX2.926 (G → C) (rs20417) located in the promoter region. Previous studies have shown that the selected polymorphisms are the more prevalent and have biological relevance in colorectal cancer, non small cell lung carcinoma and the variability of inflammatory responses in patients subjected to coronary artery bypass graft surgery (14, 16, 17).

Methods and materials

Subjects

White subjects from different regions of Spain were recruited. The IPF group had 174 unrelated patients (108 men and 66 women of mean age 67.8 years ± 0.78, range 37-86 years). The control group had 225 unrelated healthy subjects (72 men and 153 women of mean age 36.7 years ± 0.90, range 22-78 years), The control subjects were selected among medical students, medical staffs of our medical centers and unrelated cadaveric renal allograft donors, and were free of medical diseases and diseases related to potential tissue fibrosis. All control subjects were included for examining the Hardy-Weinberg equilibrium (HWE) at the SNPs. Given that IPF rarely develops before the age 30 and that there were no cases younger than 30 years in this study, 104 controls younger than 30 were excluded from the analysis of genetic association. The remaining controls had 121 subjects (51 men and 70 women with mean age 47.4 years ± 1.01, range 30-78). The representative nature of the control group for the Spanish population has been previously demonstrated in HLA genotyping studies (18). The diagnosis of IPF was established according to the American Thoracic Society/European Respiratory Society consensus statement (1). Pulmonary function tests were performed as previously described. Reference values

Table 1. Pulmonary function test findings at diagnosis in patients with idiopathic pulmonary fibrosis.

	Absolute value	Percent predicted
FVC, L	2.36 ± 0.05	71.55 ± 1.31
FEV_1, L	1.94 ± 0.05	78,90 ± 1.42
FEV_1/ FVC, % pred.	83.22 ± 0.006	
TLC, L	3.93 ± 0.09	71.89 ± 1.26
DL_{CO}, mmol/min/kPa	4.44 ± 0.12	58.23 ± 1.54
K_{co}, mmol/min/kPa	1.25 ± 0.03	80.25 ± 2.08
PaO_2, kPa	9.98 ± 0.14	
$P(A\text{-}a)O_2$, kPa	3.68 ± 1.15	

Definition of abbreviations: FVC: forced vital capacity. FEV_1: forced expiratory volume in one second. TLC: total lung capacity. DL_{co}: diffusion capacity of the lung for carbon monoxide; K_{co}: ratio between DL_{co} and alveolar volume. PaO_2: partial pressure of oxygen in arterial blood. $P(A\text{-}a)O_2$: alveolar-arterial oxygen pressure difference.

from our own laboratory were used (19, 20). The findings of pulmonary function tests are represented in Table 1. The study was approved by the ethics committees of the participating hospitals.

Identification of polymorphisms in the COX-2 gene

Genomic DNA was purified from peripheral leukocytes by salting out. Polymorphisms COX2.3050 (G → C) (rs5277), COX2.926 (G → C) (rs20417) and COX2.8473 (C→ T) (rs5275) of the COX-2 gene were assayed by means of TaqMan SNP genotyping assays ("Assay-by-Demand") from Applied Biosystems (Foster City, USA). Real-time PCR was performed in a final reaction of 25 μl, which contained 12.5 μl of 2 Taqman Universal Master Mix, 0.5 μl of Taqman SNP genotyping assay mix (Applied Biosystems), and 15 ng of genomic DNA in 12 μl of distilled water. PCR amplification and detection of amplified fragment were performed on the Real-Time iCycler-IQ Bio-rad equipment (Bio-Rad Laboratories, CA, USA). The amplification conditions were 10 min at 95 °C for Amplitaq Gold activation, followed by 40 cycles of 15 sec at 92°C for denaturation and 1 min at 60°C for annealing and extension. The samples were run together with the nontemplate control in a 96-well plate. Allelic discrimination was performed on the post-PCR product. The fluorescence data of the post-PCR products were analyzed directly using allelic discrimination software of the Bio-rad iCycler-IQ (Bio-Rad Laboratories, CA, USA) and presented in xy scatter dot plot format. Each sample was verified by examining the PCR curve generated to eliminate false-positive results due to aberrant light emission.

Statistical analysis

Single SNP genotype analysis

Using all 225 normal subjects in the control group, the Hardy-Weinberg equilibrium (HWE) was examined by the Chi-square test with 1 degree of freedom (21), or by an exact test, if at least one genotype group was found to have an expected value less than 5. The analysis was conducted with the statistics software R using the package 'genetics' (22). The single SNP genotypes and allele frequencies between groups were analyzed with logistic regression adjusted by patient age and sex. Quantitative variables were tested with the Student´s *t* test or the Wilcoxon rank sum test on the mean. The dependence of the progression of IPF (changes in pulmonary function tests) on the genotype was tested through the analysis of covariance. The model goodness-of-fit was examined to ensure the validity of the model assumption. Data were analyzed with statistical software R version 2.3.1 (The R Project for Statistical Computing), SAS Version 9.1 (SAS Institute Inc, Cary, NC, US) and SPSS 12.0 statistical software (SPSS Inc, Chicago, IL, US). Continuous variables are expressed as means ± SEM. $p < 0.05$ was considered statistically significant.

Haplotype Analysis

Linkage disequilibrium (LD) was examined between SNPs through Chi-squares test: $\chi^2 = 2nD^2/(p_A p_B p_a p_b) \sim \chi^2(1)$ with 1 degree of freedom and the coefficient of LD $D=p_{AB}p_{ab}-p_{aB}p_{Ab}$ for two SNPs denoted as (A/a) and (B/b) SNP. Because of the phase ambiguity of the double heterozygote, the frequencies of the haplotypes were estimated through the E-M algorithm (23). Haplotype analysis was further conducted to examine significant linkage between the haplotype and the disease. In general two-SNP haplotype analysis, 9 possible genotypes (AA/BB), ..., (aa/bb) may be formed (Table 2), and the unknown phase of the double heterozygous genotype (Aa/Bb) may correspond to two different diplotypes [AB][ab] and [bb][aB], leading to 10 possible diplotypes in general. To identify the risk haplotype *H*, e.g. [AB], a powerful binary trait nucleotide (BTN)

Table 2. Mapping genotype into composite diplotype

Genotype	Diplotype			Composite Diplotype
	Haplotype Configuration	Frequency	Relative Freq	
AA/BB	[AB][AB]	p_{11}^2	1	HH
AA/Bb	[AB][Ab]	$2p_{11}p_{12}$	1	$H\bar{H}$
AA/bb	[Ab][Ab]	p_{12}^2	1	$\bar{H}\bar{H}$
Aa/BB	[AB][aB]	$2p_{11}p_{21}$	1	$H\bar{H}$
Aa/Bb	[AB][ab]	$2p_{11}p_{22}$	Φ	$H\bar{H}$
	[Ab][aB]	$2p_{12}p_{21}$	1-Φ	$\bar{H}\bar{H}$
Aa/bb	[Ab][ab]	$2p_{12}p_{22}$	1	$\bar{H}\bar{H}$
aa/BB	[aB][aB]	p_{21}^2	1	$\bar{H}\bar{H}$
aa/Bb	[aB][ab]	$2p_{21}p_{22}$	1	$\bar{H}\bar{H}$
aa/bb	[ab][ab]	p_{22}^2	1	$\bar{H}\bar{H}$

Where $\Phi = \frac{p_{11}p_{22}}{p_{11}p_{22} + p_{12}p_{21}}$

mapping approach (24) was taken while labeling the remaining haplotypes as non-risk haplotype. A logistic regression was employed to model the additive and dominant effects of risk haplotype $\bar{H}$, while controlling the confounding variables that may potentially affect the susceptibility of IPF, such as patient's age and sex (22, 23). (See Appendix 1). The significance of effect estimate was further examined by the bootstrap method with 500 bootstrap samples (25).

Results

Genotype and allele frequencies of COX-2 gene polymorphisms in control subjects and patients with IPF

In controls, the observed genotypes did not deviate from the expected distribution under the Hardy-Weinberg equilibrium in COX2. 3050 (G → C) and COX2.8473 (C → T) polymorphism. However, in COX2.926 (G → C) polymorphism the observed genotype in the control group deviated from that expected under the Hardy-Weinberg equilibrium due to the presence of a high number of CC homozygotes. Thus, this polymorphism was excluded from further analysis. Association analysis of disease phenotype revealed significant associations between disease and age and between disease and sex. However, no significant association between disease phenotype and genotype of individual SNP COX2.8473 or COX2.3050 was revealed through logistic regression with adjustment by age and sex. See Table 3 for the genotype and allele frequencies as well as the p-value for the association test. Although smoking is

Table 3. Genotype and allelic frequencies of cyclooxygenase-2 gene polymorphisms

	IPF (n = 174)	Controls (n = 121)	P *
COX2. 8473			
Genotype			0.16
CC	50.6% (88/174)	39.7% (48/121)	
TT	8.1% (14/174)	14.9% (18/121)	
CT	41.4% (72/174)	45.5% (55/121)	
Allele			
C	71.3% (248/348)	62.4% (151/242)	
T	28.7% (100/348)	37.6% (91/242)	
COX2.3050			
Genotype			0.39
GG	54.6% (95/174)	63.6% (77/121)	
CC	4.6% (8/174)	2.5% (3/121)	
GC	40.8% (71/174)	33.9% (41/121)	
Allele			
G	75.0% (261/348)	80.6% (195/242)	
C	25.0% (87/348)	19.4% (47/242)	

Diplotype	IPF (n=174)	Control (n = 121)	Excluded (n= 104)
(GG/CC)	25.3% (44/174)	16.5% (20/121)	18.3% (19/104)
Non (GG/CC)	74.7% (130/174)	83.5% (101/121)	81.7% (85/104)

* p value for overall difference among three SNP genotypes by logistic regression adjusting for sex and age

well known to be associated with IPF, the heavy missing of smoking status in our data set led to the exclusion of smoking status from the logistic regression model for association analysis.

Association of haplotype with development of IPF

Similar to the above single SNP association, only 174 cases and 121 controls were included in the haplotype analysis. Among the 9 possible genotypes (Table 2), only 8 genotypes were observed, no genotype (CC/TT) was observed in either cases or controls. Out of the 8 observed genotypes, only four haplotypes were possible. Three haplotypes [GC], [GT] and [CC] had a large proportion of subjects, while the fourth haplotype [CT] had a relatively small proportion. Therefore, the haplotype analysis mainly focused on three haplotypes [GC], [GT] and [CC]. The BTN mapping method identified [GC] as a 'risk' haplotype while controlling for sex and age (Table 4). Further, the effect estimates revealed that haplotype [GC] had a significant additive effect (p=0.0021) and a marginally significant dominant effect (p=0.048). Further analysis revealed that the diplotype (GG/CC), or the equivalent 2 copies of the haplotype [GC], remained significant in association with IPF after the Bonferroni's correction for multiple comparison among the genotypes of 2 copies, 1 copy and no copy of haplotype [GC] (p=0.0039), while the 1 copy of [GC] was not significant. The haplotypes [GT] and [CC] were not significantly associated with the development of IPF (p>0.05) by the same test of BTN mapping. Haplotype [CT] was not tested because of its low frequency in the population.

This result suggested a recessive effect of haplotype [GC], or the diplotype (GG/CC) that has an observable double homozygotes. We further conducted a logistic regression to estimate the effect of diplotype (GG/CC) while adjusting for sex, age and the interaction between the diplotype and age or sex, and concluded that the (GG/CC) diplotype had a significant effect (p=0.0065) as well as an interaction effect with age (p=0.024) (Table 5). The results indicated that 1) subjects having (GG/CC) diplotype had an elevated risk for IPF up to age 66 years (Figure 1); 2) males had a higher risk than females (Figure 1); 3) the elevated risk by the (GG/CC) diplotype increased slowly with age (Figure 1), and the OR decreased with age (Figure 2). This implies that the carriers of the diplotype (GG/CC) had an elevated risk than the non-carriers in the younger group up to 66 years, and the risk elevation diminished among older subjects over 66 years due to the dominating effect of age in the development of IPF.

Relationship between genotype and allele frequencies of COX-2 gene polymorphisms and disease progression in patients with IPF

One hundred and forty-nine patients with IPF were monitored for 33.7 ± 2.1 months (range 2 - 114 months), and the contact of the remaining 26 patients was lost. Overall, patients in the follow-up

Table 4. Analysis of development of idiopathic pulmonary fibrosis with [GC] haplotype by logistic regression

Risk haplotype	Parameter Estimate		p-value (LR test)
[GC]	Testing null hypothesis H_0: $\beta_1 = \beta_2 = 0$		0.049
	β_1 additive	0.557 (0.181)	0.0021[a]
	β_2 dominant	0.482 (0.244)	0.048[b]
	β_3 sex	1.022 (0.219)	<0.001[a]
	β_4 age	0.153 (0.0096)	<0.001[a]
[GT]	Testing on genetic components null hypothesis H_0: $\beta_1 = \beta_2 = 0$		0.235
[CC]	Testing on genetic components null hypothesis H_0: $\beta_1 = \beta_2 = 0$		0.75

[a]. Remained significant with Bonferroni's correction for multiple comparison on 4 haplotypes.
[b]. Became not significant after Bonferroni's adjustment for multiple comparison on 4 haplotypes

Table 5. Association test on the double homozygotes (GG/CC), age and sex with the IPF.

Parameter	Estimate (Stderr)	OR (95% CI)	P-value
(GG/CC)	0.57 (0.21)	1.76 (1.17, 2.63)	0.0065
Sex	0.11 (0.04)	1.11 (1.02, 1.21)	0.0081
Age	0.0245 (0.002)	1.02 (1.02, 1.03)	< 0.001
Age * (GG/CC)	-0.0076 (0.003)	0.992 (0.986, 0.999)	0.0237

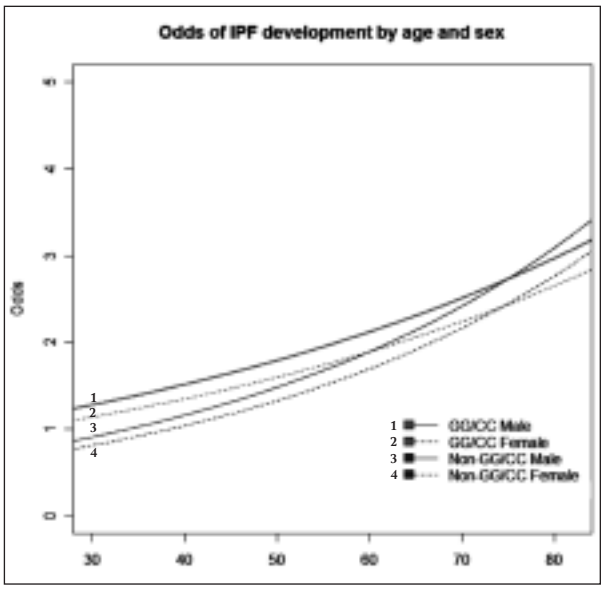

Fig. 1. Odds of idiopathic pulmonary fibrosis development by age and sex.

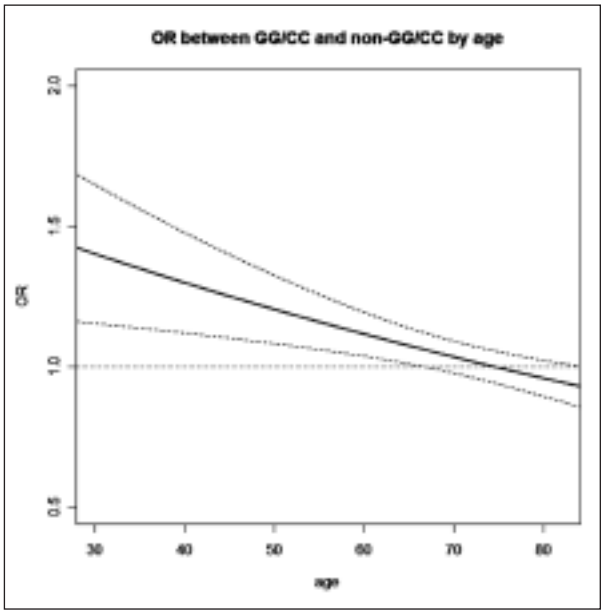

Fig. 2. Plot of OR and 95% confidence interval by age for idiopathic pulmonary fibrosis development by genotype (GG/CC) versus non-(GG/CC) (solid: OR; dash: 95% CI).

showed deterioration in pulmonary functional parameters. To fully determine the changes in pulmonary function tests during the follow-up, we have expressed the changes over time with slope – change per month of the pulmonary function tests (Table 6). There was no significant association between the progression of IPF and the frequencies of genotypes and alleles ($p>0.05$).

Haplotype analysis of the IPF progression

Further analysis revealed no significant association between haplotypes [GC], [GT], [CC] or the diplotype (GG/CC) and the progression of IPF in pulmonary function tests based on each function's per month change ($p>0.05$) (Table 7) adjusting for the effect of sex and age. The haplotype [CT] was not tested in this progression analysis because of the small number of subjects having a potential [CT] haplotype.

Discussion

Table 6. Changes in pulmonary function tests in the follow-up study

Variable	Value
Patients, n	149
Follow-up, months	33.7 ± 2.1
FVC, (%/month)*	- 0.8 ± 0.09
DL_{co}, (%/month)*	- 0.88 ± 0.19
$P(A\text{-}a)O_2$, (kPa/month)†	0.03 ± 0.01

* Percentage change per month in FVC and DL_{co} were calculated by dividing the change in FVC and DL_{co} by the follow-up period in months for each patient

† Values are expressed as the difference in pressure (mm Hg) at the initial assessment and the end of follow-up period divided by the follow-up in months for each patient

Table 7. Testing on association of haplotypes with idiopathic pulmonary fibrosis progression

Progression index	Haplotype	p-value of genetic effects
FVC	[GC]	0.451
	[GT]	0.894
	[CC]	0.245
DL_{co}	[GC]	0.139
	[GT]	0.409
	[CC]	0.340
P (A-a) O_2	[GC]	0.451
	[GT]	0.561
	[CC]	0.986

The association of haplotype [CT] was not studied due to small number of subjects in the IPF progression analysis

The main findings of our study are as follows: a) no specific genotype of either COX2.3050 or COX2.8473 gene polymorphisms was associated with the development of IPF; b) the diplotype (GG/CC) of COX2.3050 and COX2.8473 polymorphisms posted significantly higher risk of development of IPF among subjects of age 66 years or younger. However, no genotype or haplotype was found to be significantly associated with IPF progression. The exclusion of the 104 control subjects younger than 30 years from the association tests served the purpose of reducing the age confounding effect in the analysis, and the frequency of the risk genotype (GG/CC) among these 104 young controls was found not to differ significantly from that of the controls older than 30 years remained in the association test.

The identification of genetic factors that predispose to IPF will improve our understanding of the disease and may lead to the development of new therapeutic strategies. Several mediators and growth factors are critical in the pathogenesis of IPF (5). Therefore, variants in genes encoding these mediators may be involved in the predisposition and evolution of IPF. Interleukin (IL)-1 receptor antagonist, tumor necrosis factor-α (TNF-α), the carriage of IL-6 and TNF receptor II and surfactant A and B protein gene polymorphisms have been associated with an increased risk of IPF. In addition, the presence of transforming growth factor β-1 (TGF β-1) and IL-6 polymorphisms have been linked with the decline of lung function over time (4, 26).

The relevance of COX-2 in the pathogenesis of pulmonary fibrosis has been widely demonstrated. Cultured fibroblasts from patients with IPF may have a markedly reduced capacity to synthesize PGE_2 in both basal and stimulated conditions because of a down-regulation of COX-2 (9, 11, 27). COX-2 is down-regulated in lung tissue from patients with IPF (11). COX-2 deficient mice suffer from greater lung injury in response to fibrogenic agents (7,9). Hodges et al (8) highlighted the importance of up-regulating COX-2 and PGE_2 in the protection against fibrosis in bleomycin induced lung fibrosis in mice. These observations suggest that the downregulation of Cox-2 expression could play an important role in the fibrosing process in IPF. Moreover, decreased COX-2 expression in lung parenchyma has also been reported in sarcoidosis, suggesting that it is not only characteristic of IPF but a phenomenon implicated in other fibrotic lung diseases (28). In a recent study, it has been reported that COX-2 is up-regulated into regenerating metaplastic epithelium in IPF and other interstitial lung diseases, such as asbestosis and cryptogenic organizing pneumonia suggesting that COX-2 may also participate in the re-epithelization of the injured alveolar walls (29).

We hypothesized that the dysregulation of COX-2 expression in IPF may be due to the influence of genetic polymorphisms. Several SNPs in COX-2 gene have been identified, although only a few of them have been studied in association with diseases (13). In our study we have selected polymorphisms on the basis of their reported functional or biological relevance and/or their appreciable prevalence in Caucasian population.

The COX2.843 polymorphism is located in the 3´-UTR region. Although it has been associated with the risk for non-small cell lung cancer (16) and sarcoidosis susceptibility (30), there are no data on the possible effect of this SNP. It has been suggested that the binding of proteins to the 3´-UTR region could regulate mRNA stability and degradation and subsequent protein expression (16, 31].

The COX2.3050 polymorphism is one of the most prevalent in the Caucasian population. It has been analyzed in several pathologic disorders, and associations with disease have been found (16, 32-34). Recently, López-Campos et al have reported that the carriage of the C allele is associated with systemic sarcoidosis (35). The functional impact of this polymorphism on COX-2 activity still remains unknown. We hypothesized that this polymorphism itself or with another linked marker may have biological effects on COX-2 activity.

We have found in this study that the carriage of the double homozygote (GG/CC) of the COX2.3050 and COX2.8473 gene polymorphisms led to significantly increased susceptibility to IPF. This finding is novel since there has been no report on the association between haplotypes of COX2 genes with the development of IPF, although associations of haplotypes of COX2 genes with colorectal cancer and inflammatory bowel disease have been reported (14,15). In colorectal cancer, differently than lung fibrosis, is the presence of PGE2 directly

involved in the pathogenesis of the disease and not its lack as it has been described in IPF. More similar to lung fibrosis is the role played by PGE-2 in inflammatory bowel diseases where its reduction seems to be involved in the pathogenic events leading to the disease. In fact, prostaglandin E-2 exerts its action via four different E prostanoid (EP) receptors. EP 1-4. The EP receptor subtypes exhibit differences in regulation of PGE-2 release. (36).

The next step would be to determine how COX2.8473 and COX2.3050 alleles may influence the expression of COX-2 or its protein function in relevant cells in the pathogenesis of IPF, such as alveolar epithelial cells and fibroblasts.

We also analyzed the COX2.926 polymorphism and did not find any differences in the distribution of genotypes between controls and IPF patients. These findings are of no value because the genotype distribution in the control group did deviate from that expected under Hardy-Weinberg equilibrium, due to the presence of a high number of CC homozygotes (data not shown). Hill et al have reported that the C allele of COX2.926 polymorphism is associated with poor outcome and susceptibility and evolution to fibrosis in patients with sarcoidosis (37). The COX2.926 polymorphism is located in the promoter region within a binding site for stimulatory protein-1 (Sp1), an activator of Cox- 2 gene transcription. The presence of the polymorphism reduces the activity of the promoter of the COX-2 gene (38). It has been shown that the homozygous CC genotype of the COX2.8473 polymorphism is associated with sarcoidosis susceptibility (30). These findings, together with our results support the role of COX-2 genetic variants in the pathogenesis of various fibrotic interstitial diseases.

This study shows that genetic variations in COX-2 are significantly associated with the predisposition to develop IPF. These findings may help to improve our understanding of IPF pathogenesis and may lead to the development of new therapeutic strategies.

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Appendix 1:

A logistic regression was employed to model the additive and dominant effects of risk haplotype *H*, patient sex and age.

$$\log\left(\frac{P(Y=1|X)}{P(Y=0|X)}\right)=\beta_0+\sum_{i=1}^{4}x_i\beta_i$$

The haplotype and environmental variables age and sex are coded as follows.

$$x_1=\begin{cases}1 & \textit{for composite dyplotype } HH\\ 0 & \textit{for composite dyplotype } H\bar{H}\\ -1 & \textit{for composite dyplotype } \bar{H}\bar{H}\end{cases}$$

$$x_2=\begin{cases}1 & \textit{for composite dyplotype } H\bar{H}\\ 0 & \textit{otherwise}\end{cases}$$

$$x_3=\begin{cases}1 & \textit{Male}\\ 0 & \textit{Female}\end{cases}$$

x_4: age as continuous variable,

where β_0 is the model intercept representing the overall log-odds. β_1 is the additive effect of the haplotype *H*, and β_2 the dominant effect. β_3 is the effect of sex, β_4 the age effect. The statistical expectation-maximization algorithm was used to estimate the probability of each unknown phase by maximizing the likelihood function. The standard errors of the parameters for significant haplotypes were computed through statistical bootstrap method with 500 bootstrap samples retaining the same numbers of cases and controls.
The disease progression indices were analyzed through a linear regression model.

$$Y=\beta_0+\sum_{i=1}^{6}x_i\beta_i+\varepsilon$$

where *Y* is the continuous response variable of disease progression index. $x_1 \cdots x_4$ are defined the same as before and

$$x_5=\begin{cases}1 & \textit{Treatment } A\\ 0 & \textit{Treatment } B\end{cases}$$

and x_6 = duration of subject in the study.

Similarly, the haplotype analysis was conducted by maximizing the likelihood function through an EM algorithm for the probability of the unknown phase. The likelihood ratio test was conducted on the model parameters