

# Fibrogenic Polymorphisms (*TGF-β*, *PAI-1*, *AT*) in Mexican Patients With Established Liver Fibrosis. Potential Correlation With Pirfenidone Treatment

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## ABSTRACT

**Background/Aim:** The aim of this work was to establish a potential correlation between specific polymorphisms and presence of hepatic fibrosis in Mexican patients with established liver fibrosis (ELF). Second, necro-inflammatory index improvement was correlated with Pirfenidone (PFD) treatment response and the same polymorphisms.

**Methods:** We analyzed *TGF-β* polymorphisms in codon 25, a single basepair guanine insertion-deletion polymorphism (4G/5G) for *PAI-1* and angiotensin *AT-6* single nucleotide polymorphism located in -6 promoter region. Twenty patients infected with either hepatitis C virus (HCV) (n = 13) or affected by alcohol consumption (n = 7) were included. Thirty subjects with no hepatic damage were included in control group. Blood samples for genomic DNA were obtained and plasminogen activator inhibitor-1 polymorphisms were done by polymerase chain reaction-artificial introduction of a restriction

site, *TGF-β* by polymerase chain reaction-amplification refractory mutation system and *AT* by polymerase chain reaction-restriction fragment length polymorphisms. Liver biopsies were obtained at baseline and after 12 months of PFD treatment.

**Results:** Established liver fibrosis patients had the homozygote G/G *TGF-β* genotype, which has been associated with increased development of fibrosis. None of our patients had the G/C genotype. All pure HCV and pure alcohol abuse subjects carried G/G *TGF-β* genotype (100% vs 37% control) ( $P = 0.0006$ ). The odds of having β G/G genotype was 19.5 for HCV patients and 10.83 for alcohol consumption patients as compared with healthy subjects ( $P < 0.001$ ). Established liver fibrosis patients had an improvement in necroinflammatory index after PFD treatment when correlated with plasminogen activator inhibitor-1 and angiotensinogen-6 genotypes.

**Conclusion:** Our data suggested that a combination of inherited polymorphisms increased the risk of advanced fibrosis in ELF patients. Pure HCV and pure alcohol consumption patients which were homozygous G/G carriers had 19.5- and 10.8-fold higher risk to develop advanced fibrosis respectively.

**Key Words:** Polymorphisms, *TGF-β*, *PAI-1*, *AT*, Pirfenidone, cirrhosis

**Abbreviations:** *TGF-β*, transforming growth factor β; RAS, renin angiotensin system

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## INTRODUCTION

The development of fibrosis in hepatic cirrhosis is a feature of almost all chronic liver diseases, as a consequence of different injuries (alcohol consumption, viral hepatitis, autoimmune processes, genetic diseases, etc.). Recent epidemiologic studies in humans have identified possible polymorphisms in a number of candidate genes that influence the progression of liver fibrosis.<sup>1</sup> The possibility of developing fibrosis varies substantially among

subjects and is influenced by a number of factors including gender, age at infection, alcohol consumption, and hepatic steatosis. These demographic and environmental factors account for only a small proportion of the variability in the rate of disease progression.<sup>2</sup>

Important evidence indicates that genetic factors determine the rate of progression of liver fibrosis.<sup>1</sup> Specifically, transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) has shown to be the most potent master cytokine that promotes hepatic fibrosis through stimulating synthesis and inhibition of degradation of a broad spectrum of extracellular matrix (ECM) proteins.<sup>3</sup>

A coding single nucleotide polymorphism (SNP) in codon 25 of the TGF- $\beta$ 1 gene resulting in an amino acid substitution (Arg/Pro) has been shown to be associated with advanced stages of fibrosis in patients with chronic hepatitis C.<sup>4</sup> These patients were from caucasian origin. The data shown here were obtained from Hispanic people.

Hepatic stellate cells are the major source of ECM production mostly through TGF $\beta$  signaling. Thus, expression of TGF- $\beta$  is useful to evaluate fibrotic disease progression. In this context, hepatic damage markers such as TGF $\beta$  polymorphisms will be of great advantage to evaluate liver disease progression and clinical response to new antifibrogenic agents.

Similar results have been found by analyzing SNP located in the promoter region of the angiotensinogen gene (*AT*, -6 G/A) which results in increased transcription of the gene.<sup>5</sup> Angiotensinogen (*AT*) is the precursor peptide for angiotensin I. Evidence has been published describing an A for G nucleotide substitution of the *AT* gene 6 nucleotides upstream from the start site of transcription appears as a functional polymorphism.<sup>6</sup> An adenine, instead of a guanine, 6 residues upstream from the initiation site for transcription (CCCGGCCAGGG-GAAGAAG), and is found in 97% to 99% of the angiotensin alleles. Plasminogen activator inhibitor-1 (*PAI-1*) is the major inhibitor of tissue type plasminogen activator and urokinase-type plasminogen activator. Recent studies suggest that plasmin cascade plays a significant role related to plasminogen activators.<sup>7</sup> In addition to their well-known fibrinolytic activity, the plasminogen activator/plasmin enzymes also have direct and indirect ECM-degrading actions.<sup>8</sup> A variety of factors have been shown to affect *PAI-1* synthesis and secretion in vitro.<sup>9,10</sup> In vivo, plasma *PAI-1* levels have been related to a common single basepair guanine insertion/deletion polymorphism (4G/5G) within the promoter region of the *PAI-1* gene,<sup>11</sup> with homozygotes for the deleted allele (4G/4G) carrying the highest plasma levels of this inhibitor.<sup>12,13</sup> Both *PAI-1* and *AT* polymorphisms described previously have been associated with the development of fibrosis.

The study of polymorphic genes implicated in ECM production or collagen metabolism is of great importance because this genomic variability could be used to tailor a given antifibrotic therapy according to polymorphisms screening.

Polymorphisms of TGF- $\beta$ 1, *PAI-1*, and *AT* genes have been shown to affect progression to cirrhosis or other chronic liver diseases, in humans from caucasian origin.<sup>4</sup> Thus, one of the aims of this work was to establish a correlation between the mentioned polymorphisms and presence of liver fibrosis in Mexican patients with established liver fibrosis (ELF). The actual Ishak's staging scores showed that patients displayed different scores of hepatic damage. Therefore, we then attempted to correlate the score of modified histological activity index of Knodell (necroinflammatory index) with the gene polymorphisms already discussed.

## ■ MATERIALS AND METHODS

### Study Design and Patients

Patients with ELF were recruited at Institute of Molecular Biology and Medicine. Patients with ELF because of either pure hepatitis C virus (HCV) ( $n = 13$ ) or only alcohol abuse ( $n = 7$ ) were included.<sup>14,15</sup> A control group of 30 healthy volunteers (female  $n = 18$ ; male  $n = 22$ ) was included to compare polymorphisms data (average of age, 56.6 years; range, 30–69 years old).

Because our main issue was to look for a potential correlation between TGF- $\beta$ 1, *PAI-1*, and *AT* gene polymorphisms with presence of liver fibrosis, we selected our patients from a cohort of subjects that had undergone a liver biopsy 1 year before. Liver biopsies analysis showed patients displaying different degrees of fibrosis and necroinflammatory index, according to observations made by 2 different pathologists blinded to the study. Inclusion criteria included age 18 years or older, elevated serum alanine aminotransferase or aspartate aminotransferase activities (alanine aminotransferase  $> 41$  IU/L or aspartate aminotransferase  $> 43$  IU/L), evidence of liver damage by ultrasonography, willingness to use contraceptive measures, prothrombin activity safe enough to perform liver biopsy, absence of alcohol ingestion, and absence of psychotropic medication during the previous 6 months.

Exclusion criteria included therapy with modifiers of collagen metabolism or antiviral drugs (corticoids, silymarin, colchicine, alpha interferon, ribavirin, ursodeoxycholic acid, etc.) within 6 months of enrollment, patients with hemoglobin less than 10 g/ml, platelets count less than 70,000/mL, patients with severe encephalopathy (stages 3–4), concomitant presence of cardiac disease, diabetes mellitus, cancer, any other connective tissue disease or pregnancy. In addition, patients with insufficient blood sample to perform polymorphisms assays were excluded.

Because we wanted to also compare the presence of the mentioned polymorphisms, with liver histology improvement, the 20 patients with hepatic damage were subjected to a second liver biopsy 12 months after treatment with Pirfenidone (PFD) which was administered orally with a dose of 1200 mg/d ( $3 \times 400$  mg) during 12 months. Patients were instructed to use Pirfenidone for three times a day, 30 minutes after meals.

This study was conducted in accordance with the principles of the Declaration of Helsinki, and good clinical practice regulations issued by the US Food and Drug Administration. The institutional Review Boards and the Drug Evaluation Direction from Mexican Ministry of Health approved the protocol. Patients agreed to undergo liver biopsy after written informed consent.

**Biopsy.** Color Doppler echocardiography ultrasound-guided liver biopsies were obtained using an automatic 15-gauge Microvasive core biopsy system (Boston Scientific Corporation, Watertown, MA). A liver specimen of 15 mm was considered adequate for evaluation. Biopsies were performed at baseline and after 12 months of treatment and were processed using standard techniques and evaluated for stage of fibrosis and grade of activity according to modified histological activity index of Knodell and Ishak fibrosis stage.<sup>16-18</sup>

**Screening of TGF $\beta$ , AT and PAI-1 polymorphisms.** Genomic DNA was extracted from 3 mL of EDTA-peripheral blood according to Miller method.<sup>19</sup>

**TGF- $\beta$ :** amplification of a 197 bp polymerase chain reaction (PCR) product of TGF $\beta$  gene corresponding to codon 25, (Arg<sup>25</sup>/Pro<sup>25</sup>) was done by PCR-allele specific (polymerase chain reaction- amplification refractory mutation system) by amplification refractory mutation system in a Thermal Cycler (Cycler BIO-RAD Life Science Research Products) using the following primers: 5'ACTGGTGCTGACGCCTGGCCG3' (Forward) and 5'TGCTGTTGTACAGGGCGAGCA3' (Reverse) where the homozygous can be distinguished from heterozygous based on the presence or absence of a given allele discerned by inspection, based on the principle that primers are complementary to target DNA sequence except for a mismatched 3'. Two complementary reactions were performed for each allele, consisting of allele-specific primer and common primer. Polymerase chain reaction was performed by initial denaturation at 94°C during 3 minutes, 35 cycles of amplification at 94°C for 1 minute, correspond to denaturation step, following at 64°C during 1 minutes for annealing and 72°C during 2 minutes for extension. Finally, incubation at 72°C during 1 minute was used for ending extension (Fig. 1A, B).

**PAI-1:** amplification of -675 PAI-1 promoter region was done by polymerase chain reaction-artificial intro-

duction of a restriction site) in a Thermal Cycler (Cycler BIO-RAD Life Science Research Products) using the following oligonucleotides 5'CACAGAGAGAGTCTGGCCACGTGTT3' (Forward) and 5'CCAACAGAGGACTCTTGGTCT3' (Reverse).

Polymerase chain reaction was performed by initial denaturation at 94°C during 3 minutes, 30 cycles of at 94°C, 30 seconds for denaturation, following at 60°C during 30 seconds for annealing and 72°C during 30 seconds for extension step. Finally, incubation at 72 °C during 1 minute was used for ending extension, resulting in 99 bp for the 5G and 98 bp for the 4G amplified fragments. The initial PCR fragment was analyzed on a 2% agarose gel (Invitrogen Life Technologies, Carlsbad, CA) stained with ethidium bromide. Amplified fragment of PAI-1 promoter polymorphism were digested for 1 hour at 55°C with 3 units of BsiI (New England Biolabs, Boston MA) restriction enzyme. Afterwards, restriction fragments were analyzed by electrophoresis on a 4% agarose gel (Invitrogen Life Technologies) stained with ethidium bromide (Fig. 1C).

**AT:** amplification of -6 AT promoter region was done by polymerase chain reaction-restriction fragment length polymorphisms (restriction fragment length polymorphism) in a Thermal Cycler (Cycler BIO-RAD Life Science Research Products) using the following oligonucleotides: 5'GTGTCGCTTCTGGCATCTGTCCTTCTGG3' (Forward) and 5'CTCAGTTCAATCCTGAGAGAGACAAGACC3' (Reverse).

Polymerase chain reaction was performed by initial denaturation at 94°C during 3 minutes, 35 cycles of at 94°C, 1 minute for denaturation, following at 55°C, during 30 seconds for annealing and 72°C during 2 minutes for extension step. Finally, an incubation at 72°C during 1 minute was used for ending extension, resulting in a 240-bp product which was analyzed on a 2% agarose gel (Invitrogen Life Technologies) stained with ethidium bromide. The amplified fragments of the AT promoter polymorphism were digested for 1 hour at 37°C, with 3 units of BsiI (New England Biolabs) restriction enzyme. Afterwards, restriction fragments were analyzed by electrophoresis on a 4% agarose gel (Invitrogen Life Technologies) stained with ethidium bromide (Fig. 1D).

**Statistical Analysis.** Conventional  $\chi^2$  test and Fisher exact test were used to analyze qualitative variables. The differences between quantitative data were evaluated with the Mann-Whitney *U* test.

Odds ratios (OR) (with 95% confidence intervals) were calculated to estimate the presence of liver fibrosis risk associated with each polymorphism.

Hardy-Weinberg equilibrium is based on asymptotic theory, and its validity is reduced in small samples. In this case, we used the Fisher exact test and 2

approximations (Yates' correction and conditional expectations).<sup>20</sup> Because the  $\chi^2$  test with Yates' correction overestimates the significance level and conditional expectation test underestimate it, we combined these 2 tests to obtain an adequate approximation to the exact significance level. Probability values of less than 0.05 were considered significant. Statistical analysis was performed with SPSS for Windows Version 12.0 (SPSS Inc., Chicago, IL) statistical software.

## RESULTS

### TGF- $\beta$ Polymorphism

Patients chronically infected with hepatitis C (n = 13), and patients by alcohol consumption (n = 7), displayed several degrees of fibrosis as it is showed in Table 1

and Table 2, though all of them had the homozygous G/G TGF- $\beta$  genotype (codon 25; genotype Arg<sup>25</sup>/Arg<sup>25</sup>) with a frequency in each group of 100%. This evidence is relevant to Mexican population because G/G genotype for TGF- $\beta$  has been associated with severe fibrosis<sup>21,22</sup> (Tables 1 and 2). In this regard, recent data from Brazilian population showed that the frequency of allele G at TGF- $\beta$ 1 codon 25 was significantly higher in HCV patients than in healthy controls.<sup>23</sup> Importantly, healthy control subjects (n = 30) displayed predominantly a heterozygous G/C genotype Arg<sup>25</sup>/Pro<sup>25</sup> (frequency 43%). Nonetheless, 37% of them had a homozygous G/G genotype and only 20% of control individuals had a C/C TGF- $\beta$  genotype (P = 0.0006). The allelic distribution of the G allele demonstrated its predominance in cirrhotic patients independently of their etiology (in

**TABLE 1.** Genotype Frequencies and Allelic Distribution of TGF- $\beta$ , AT, and PAI-1 Polymorphism in HCV Patients

<i>TGF-<math>\beta</math> (Arg<sup>25</sup>/Pro<sup>25</sup>) n = 13</i>				
<i>Genotype</i>	<i>HCV-induced cirrhosis</i>	<i>Healthy subjects (n = 30)*</i>	<i>P</i>	<i>OR</i>
G/G	100% (13)	37% (11)	$\chi^2(2) = 14.75$ <i>P</i> = 0.0006 <i>P</i> < 0.001	
G/C	0	43% (13)		
C/C	0	20% (6)		
G	100% (26)	58% (35)	$\chi^2(1) = 15.27$ <i>P</i> = 0.00009 <i>P</i> < 0.001	G→C 19.5 2.53 < 409.7 <i>P</i> = 0.00023 <i>P</i> < 0.001
C	0% (0)	42% (25)		
<i>ANGIOTENSINOGEN (AT-6) (n = 12)</i>				
A/A	17% (2)	47% (14)	$\chi^2(2) = 5.20$ <i>P</i> = 0.0742 <i>P</i> = NS	
A/G	75% (9)	53% (16)		
G/G	8% (1)	0% (0)		
A	54% (13)	73% (44)	$\chi^2(1) = 2.89$ <i>P</i> = 0.089 <i>P</i> = NS	G→A 2.33 0.78–7.00 <i>P</i> = NS
G	46% (11)	27% (16)		
<i>PAI-1 (4G/5G) (n = 13)</i>				
4G/4G	15.4% (2)	10% (3)	$\chi^2(2) = 0.259$ <i>P</i> = 0.878 <i>P</i> = NS	
4G/5G	38.5% (5)	40% (12)		
5G/5G	46.1% (6)	50% (15)		
4G	35% (9)	30% (18)	$\chi^2(1) = 0.18$ <i>P</i> = 0.6719 <i>P</i> = NS	4G→5G 1.24 0.41–3.65 <i>P</i> = NS
5G	65% (17)	70% (42)		

Genotype distribution in healthy subjects (n = 30) was evaluated for Hardy-Weinberg equilibrium.

\*The minimal "n" to calculate the Hardy-Weinberg equilibrium ( $p^2 + 2pq + q^2$ ) was 30. This satisfies the random segregation in all population studied. Yates' correction and conditional expectation test were used to obtain an adequate approximation to Fisher exact test to evaluate Hardy-Weinberg equilibrium with small samples (n = 13).<sup>20</sup>

**TABLE 2.** Genotype Frequencies and Allelic Distribution of TGF- $\beta$ , Angiotensinogen and PAI-1 Polymorphism in Alcohol Abuse Patients

<i>TGF-<math>\beta</math> (Arg<sup>25</sup>/Pro<sup>25</sup>) (n = 7)</i>				
<i>Genotype</i>	<i>Alcohol abuse</i>	<i>Healthy Subjects (n = 30)*</i>	<i>P</i>	<i>OR</i>
G/G	100% (7)	37% (11)	$\chi^2(2) = 9.11$ $P = 0.0105$ $P < 0.05$	
G/C	0%	43% (13)		
C/C	0%	20% (6)		
G	100% (14)	58% (35)	$\chi^2(1) = 8.81$ $P = 0.003$ $P < 0.01$	G→C 10.83 1.34 <233.52 $P = 0.00075$ $P < 0.001$
C	0% (0)	42% (25)		
<i>ANGIOTENSINOGEN (AT-6) (n = 5)</i>				
A/A	60% (3)	47% (14)	$\chi^2(2) = 7.14$ $P = 0.028$ $P < 0.05$	G→A 1.88 0.21–6.04 $P = 0.826$ $P = NS$
A/G	20% (1)	53% (16)		
G/G	20% (1)	0% (0)		
A	70% (7)	73% (44)	$\chi^2(1) = 0.05$ $P = 0.826$ $P = NS$	
G	30% (3)	27% (16)		
<i>PAI-1 (4G/5G) (n = 7)</i>				
4G/4G	14.3% (1)	10% (3)	$\chi^2(2) = 0.17$ $P = 0.920$ $P = NS$	
4G/5G	42.85% (3)	40% (12)		
5G/5G	42.85% (3)	50% (15)		
4G	35.7% (5)	30% (18)	$\chi^2(1) = 0.17$ $P = 0.677$ $P = NS$	4G→5G 1.30 0.32–5.09 $P = NS$
5G	64.3% (9)	70% (42)		

\*Genotype distribution in healthy subjects (n = 30) was evaluated for Hardy-Weinberg equilibrium.

The minimal “n” to calculate the Hardy-Weinberg equilibrium ( $p^2 + 2pq + q^2$ ) was 30. This satisfies the random segregation in all population studied. Yates’ correction and Conditional expectation test were used to obtain an adequate approximation to Fisher exact test to evaluate Hardy-Weinberg equilibrium with small samples (n = 13).<sup>20</sup>

HCV patients, 100% vs 58%;  $P = 0.0009$ ) (and alcohol consumption; 100% vs 58%;  $P = 0.003$ ), but not in the healthy control subjects. The OR value for HCV patients was 19.5 (C to G) ( $P < 0.001$ ) (Table 1) and alcohol consumption patients of 10.83 ( $P < 0.001$ ) (Table 2) meaning that G/G homozygous carriers have 19.5- and 10.8-fold higher risk to develop cirrhosis respectively.

### AT-6 Polymorphism

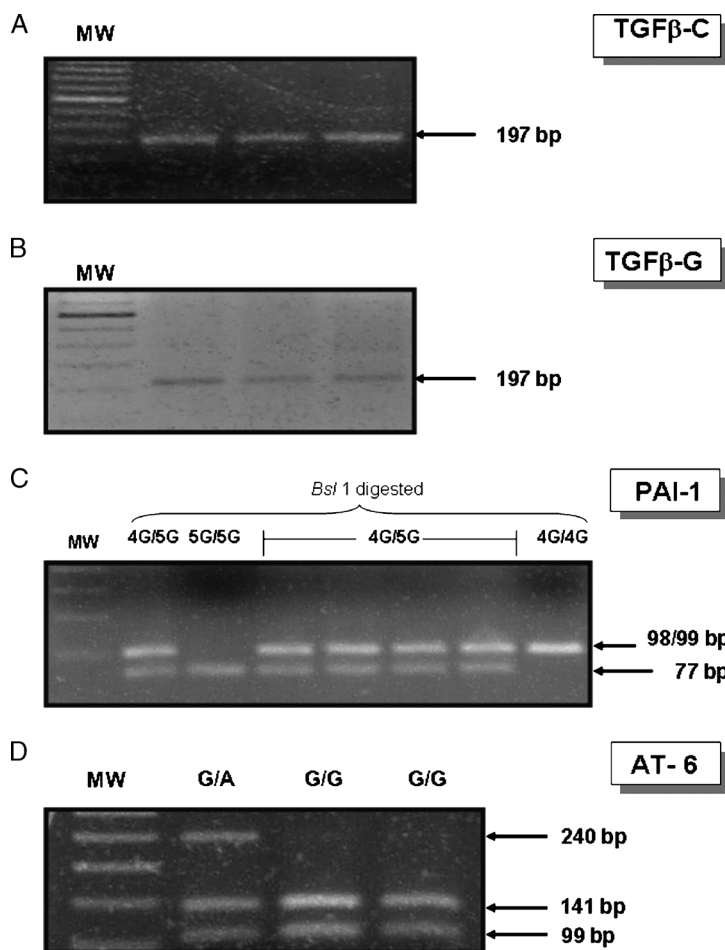
Hepatitis C virus patients displayed predominantly a heterozygous A/G for AT-6 genotype (75%) versus 53% of healthy subjects (Table 1). In contrast, cirrhotic patients by alcohol abuse had predominantly homozygote A/A for AT-6 genotype (60% vs 47% of healthy subjects). Eight percent of HCV patients presented homozygous G/G, 20% of alcohol consumption (Table 2) and healthy

subject did not show G/G genotype (0%). Conversely, 17% of HCV patients displayed a homozygous A/A versus 47% in healthy subjects. However, differences between groups by genotype and alleles were not statistically significant (NS) (Table 1). In ELF patients AT-6 genotypes due to alcohol consumption, a statistical significant difference was found with respect healthy subjects; however, with a small sample (n = 5 vs n = 30), it is not possible to establish a valid conclusion (Table 2).

AT-6 gene polymorphism correlated also with improved fibrosis index after PFD treatment in both alleles (A and G).

### PAI-1 Polymorphism

Patients chronically infected with hepatitis C displayed predominantly a homozygous 5G/5G PAI-1 (46.1%),



**FIGURE 1.** *TGF-β*, *PAI-1*, *AT-6* polymorphisms. In A, *TGFβ-C*, polymorphism is shown, where a 197-pb band is observed in allelic specific hybridization. In B, *TGFβ-G* polymorphism is observed, which a 197-pb band is characteristic. In all gels, the molecular weight marker used was a ladder of 50 bp. (C) Show a picture of representative *PAI-1* polymorphism, (4G/4G, 4G/5G, 5G/5G). Characteristic bands of enzymatic cut can be visualized in 98-99 and 77 bp. (D) Represents *AT-6* polymorphism (G/G, G/A, A/A). Characteristic bands of enzymatic cut can be visualized in 240, 141 and 99 bp.

alcohol intake (42.85%) versus 50% of healthy subjects ( $P = NS$ ).

Comparison between homozygous 4G/4G to *PAI-1* genotype was of 15.4% to HCV, alcohol consumption 14.3% versus 10% of healthy subjects.

In contrast, 38.5% of HCV patients presented heterozygous 4G/5G, 42.85% with alcohol consumption versus 40% that was found in healthy control. However, differences between groups by genotype and alleles were NS (Tables 1 and 2).

Fibrosis index decreased significantly ( $P < 0.05$ ) after PFD treatment in both alleles (4G-5G) of *PAI-1* gene. A tendency to associate 4G/5G and 5G/5G *PAI-1* genotype to improve necroinflammatory index was found at the end of treatment.

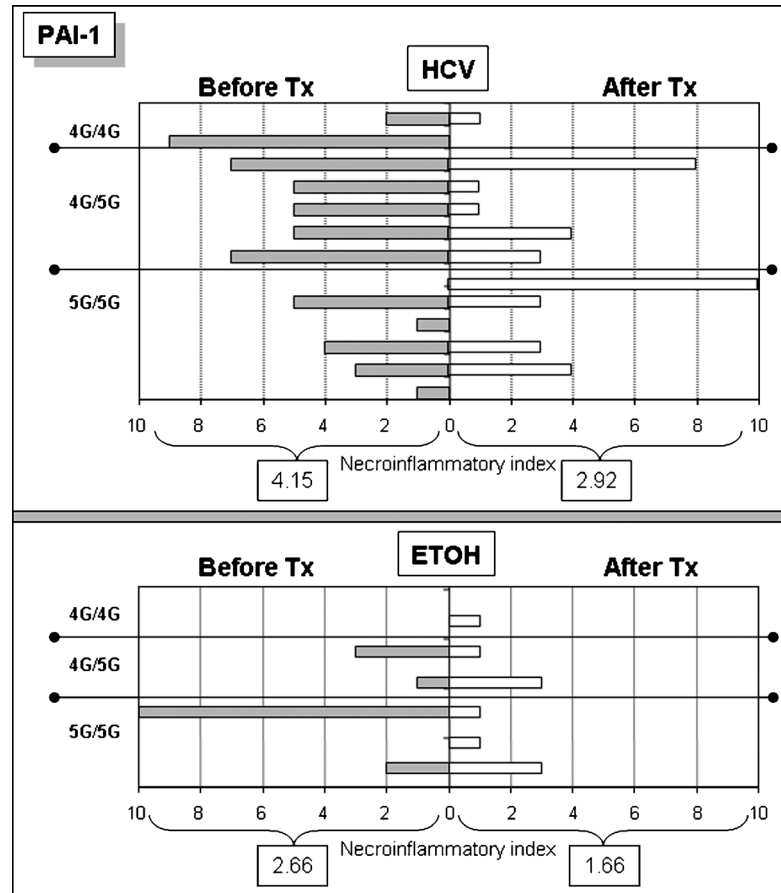
These results suggest that independently of patient genotypes and evolution of time of disease, patients

improved after PFD treatment although *TGF-β1* G/G genotype was present in 100% of patients and this polymorphism has been claimed to be related to fibrosis development (Tables 1 and 2).

Then, we looked for an association between necroinflammatory index, PFD treatment and both polymorphisms: *PAI-1* (Fig. 2) and *AT-6* polymorphisms (Fig. 3).

In general, ELF patients had a good response in necroinflammatory index (HCV: 4.15 → 2.92, and alcohol consumption 2.66 → 1.66) after PFD treatment when correlated with 3 *PAI-1* genotypes (Fig. 2). In a similar way, ELF patients had a good response in necroinflammatory index (HCV: 4.16 → 2.92, and alcohol consumption 3.25 → 2.00) after PFD treatment when correlated with 3 *AT-6* genotypes (Fig. 3).

In this study, we only found 3 patients with highest risk genotype to *PAI-1* (4G/4G) distributed in HCV



**FIGURE 2.** Necroinflammatory index and PAI-1 genotype after Pirfenidone (PFD) treatment for both groups. In this figure, we showed the improvement in necroinflammatory index after PFD treatment. The mean value of necroinflammatory index for 3 genotypes (4G/4G, 4G/5G 5G/5G) is shown. HCV indicates hepatitis C virus; Tx, treatment.

(n = 2) and alcohol consumption (n = 1) patients. Five to AT-6 (A/A) in HCV (n = 2) and alcohol consumption (n = 3) and neither with both polymorphism (Fig. 4).

**DISCUSSION**

Fibrogenesis is a complex process, driven by a number of concurrent pathways involving oxidative stress, inflammation, and steatosis. Genetic variation may occur at various sites within these pathways to influence the development of cirrhosis.

In previous studies, we have demonstrated a relationship between pro-fibrogenic molecules and development of cirrhosis. TGF-β1 has a central role in hepatic fibrosis.<sup>24,25</sup>

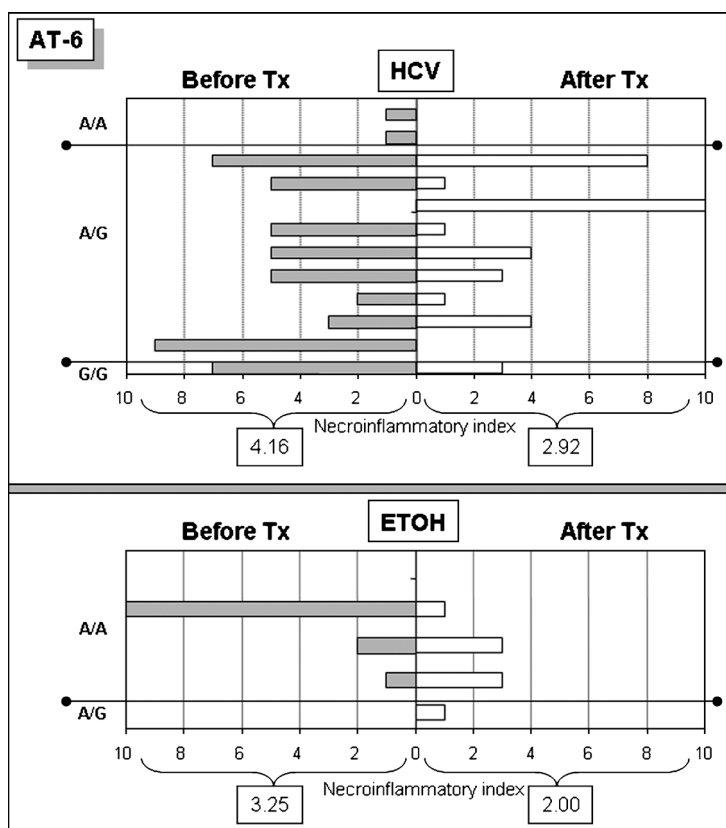
The aim of this work was to establish a potential correlation between specific polymorphisms and presence of hepatic fibrosis in Mexican patients with ELF. We investigated polymorphism of *TGF-β1* gene (codon 25 Arg<sup>25</sup>/Arg<sup>25</sup>), which may modulate progression to fibro-

sis/cirrhosis in homozygous carriers of the G/G genotype of the *TGF-β* gene and a single basepair guanine insertion-deletion polymorphism (4G/5G) for PAI-1 and angiotensin AT-6 SNP located in -6 promoter region.

Regardless of sex, age, and serum biochemistry, established advanced liver fibrosis was common in G/G homozygotes subjects (frequency: 100%), reinforcing the concept that *TGF-β1* codon 25 polymorphism influences fibrogenesis, according with previous observations in patients from non-Hispanic origin.<sup>4,5</sup>

Concerning *TGF-β1* polymorphism, the allelic distribution of the G allele demonstrated its predominance in the cirrhotic, but not in the healthy subjects. Odds ratio value was 19.5 (C to G) meaning that G/G genotype present in HCV carriers promotes a higher risk of 19.5-fold to develop cirrhosis and 10.83-fold in alcoholic consumption patients.

All HCV and alcohol abuse patients presented G/G *TGF-β* genotype (n = 20). Transforming growth factor β polymorphism (G/G) was the only genotype with an odd score greater than 1. This value implicates an association



**FIGURE 3.** Necroinflammatory index and AT-6 genotype after Pirfenidone (PFD) treatment for both groups. In this figure, we showed the improvement in necroinflammatory index after PFD treatment. The mean value of necroinflammatory index for 3 genotypes (A/A, A/G G/G) is shown. HCV indicates hepatitis C virus; Tx, treatment.

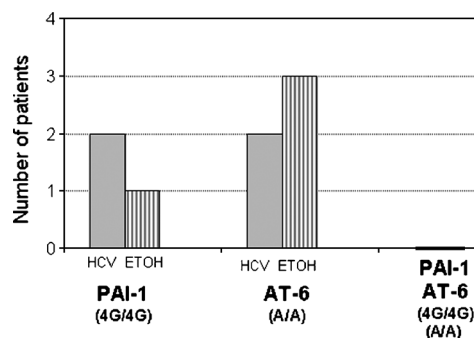
with genotype polymorphism and the development of fibrosis.

Although other investigators have observed association between G/C carriers for the TGF- $\beta$ 1 codon 25 polymorphism that is associated with faster fibrosis progression, we did not find any patient with this heterozygosity.<sup>26</sup> Otherwise, Baogui et al.<sup>27</sup> showed that higher percentage of hypertensives (92%) compared with normotensives (86%) are homozygous for the arginine allele at codon 25. These data suggest that TGF- $\beta$ 1 may play an important role in blood pressure regulation. TGF- $\beta$ 1 hyper-expression resulting in progressive glomerular sclerosis could potentially exacerbate hypertension.<sup>27</sup>

Along with these lines, in a recent study of Albuquerque Pereira et al.,<sup>23</sup> with 128 Brazilian patients, they found statistically significant differences when HCV-infected patients were compared with healthy control group ( $P = 0.0005$ ; OR 2.9). Their findings suggest that the presence of the TGF- $\beta$ 1 G/G genotype could be a host genetic factor associated with susceptibility to HCV infection.<sup>23</sup> The expression of TGF- $\beta$  mRNA and the production of TGF- $\beta$  are also increased in patients with HCV infection, alcohol-induced and autoimmune

hepatic fibrosis, and bleomycin-induced or idiopathic pulmonary fibrosis.<sup>28</sup>

The AT-6 polymorphism of the AT gene in the promoter region has been associated with increased angiotensin plasma levels and mRNA expression in several tissues.<sup>6</sup> Angiotensin II (A-II) is the effector



**FIGURE 4.** Number of patients with established liver fibrosis by pure hepatitis C virus (HCV) and pure ethanol consumption and their PAI-1 and AT-6 genotype. In this figure, we showed the patients who carried the highest risk genotype to PAI-1 (4G/4G) ( $n = 3$ ), AT-6 (A/A) ( $n = 8$ ) and both (neither).



peptide of the renin-angiotensin system (RAS), which is a major regulator of arterial pressure homeostasis in humans. Key components of this system are locally expressed in chronically injured livers, and activated hepatic stellate cells de novo generate A-II.<sup>29</sup>

The *AT-6* polymorphism occurs in the core promoter of the *AT* gene. Mutations in such a gene region could affect DNA interactions with factors involved in transcription initiation and could thus affect gene expression rate.<sup>27,30</sup> Substitution of Adenine alters the binding of a nuclear protein resulting in increased gene transcription compatible with increased AT levels. Although has been reported that *AT-6* homozygotes have major risk to develop heart and renal fibrosis, in this work, only 2 HCV (2/12) and 3 alcoholic (3/5) patients presented A/A homozygous.

*PAI-1* (-675) polymorphisms showed a genotype distribution according to Hardy-Weinberg distribution, without a statistically significant value of OR score that imply risk in the development of cirrhosis. The 4G/5G polymorphism has been related to differential binding of nuclear proteins that affect transcription rate of this fibrinolytic inhibitor.<sup>28,31</sup> Other studies reported that *PAI-1* 4G/4G genotype carriers had the highest plasma values of *PAI-1* antigen ( $14.09 \pm 2.01$  ng/mL) than in 4G/5G and 5G/5G individuals ( $12.56 \pm 1.90$  ng/mL and  $11.78 \pm 1.85$  ng/mL, respectively).<sup>29,32</sup> In the present work, only 2 (2/13) patients with HCV and 1 (1/7) ELF subject by alcohol abuse carried the genotype 4G/4G.

In a previous work, *AT-6* polymorphism was found to be associated with increased hepatic fibrosis<sup>31,33</sup> and was related to RAS. A modest increase in AT basal expression may lead to chronic elevation in baseline AT production, either by the circulating RAS or by tissue RAS. Recent data indicate that A-II may augment the accumulation of ECM.<sup>31,33</sup>

Further exploration of the relationship between the RAS and hepatic fibrosis is urgently required as it may be of immediate practical importance as ACE inhibitors and A-II type I receptor blockers are presently available. In our recent data published,<sup>14,15</sup> we found an important response to PFD treatment that seems to be a TGB- $\beta$ 1 blocker (or antagonist).

Our results are suggestive that G/G *TGF $\beta$*  genotype may be related with a high susceptibility of Mexican population to develop liver fibrosis.

Although the risk attributed to an individual polymorphism is very small, the additive effect of several genetic variants from different loci may account for a greater proportion of the disease risk. Progress in identifying genetic determinants of fibrosis could further refine patient selection for clinical trials and shorten their duration, as well as unearthing new directions of scientific inquiry.<sup>32,34</sup>

Because we do not have, yet, a clear-cut explanation of how polymorphism interactions contribute to hepatic fibrogenesis, further studies will determine at which level they are related. In addition, the response to PFD treatment might be in association with TGF- $\beta$ , AT (-6) A/A and *PAI-1* 4G/4G genotypes. Whereas the proposed mechanism of PFD treatment is repressing TGF- $\beta$  expression,<sup>23,24</sup> our present work is suggestive that the improved reduction points in necroinflammatory index were due to the effect of PFD against *TGF- $\beta$ 1*.

At the end of the treatment, patients that presented a greater necroinflammatory index improvement were HCV patients. The group of patients that showed a smaller improvement average was the one including patients with alcohol abuse. Results shown in this article represent an initial step in our effort to understand susceptibility of ELF patients to a novel treatment of liver disease and will be of importance correlating several types of polymorphisms and development of better therapies.

In conclusion, these data are suggestive that a combination of inherited polymorphisms (*TGF- $\beta$ 1*, *AT* (-6) and *PAI-1*) increase the risk of advanced fibrosis/cirrhosis in patients with HCV and alcohol consumption and may play a role in the impairment of hepatic fibrosis.

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