Clinical Epigenetics

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HCV patients with residual fibrosis after DAA treatment re-establish their epigenetic signature after prolonged-release pirfenidone: MINERVA study

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Abstract

Background & Aims Patients with residual liver fibrosis after hepatitis C virus infection clearance represent an important challenge. The primary objective of this study was to evaluate epigenetic marks in DAA-responders HCV, Hispanic patients with remaining fibrosis who were treated with prolonged-release pirfenidone (PR-PFD).

Methods Forty-four DAA-responders HCV patients presenting remaining fibrosis received PR-PFD (1200 mg/day) for 12 months. Liver biopsies and serum samples were analyzed. Patients were classified as regressive fibrotic profile (RFP), stable fibrosis profile (SFP), or progressive fibrotic profile (PFP) based on liver stiffness (Fibroscan) (± 30% variation). A control cohort of 20 DAA-responders HCV patients received only standard of care treatment. Additionally, six non-fibrotic controls were included to compare epigenetic marks.

Results Thirty-eight patients completed the 12-month treatment; 28.94% showed a reduction in at least one fibrosis stage based on liver biopsies. Fibroscan revealed that 44.73% of patients in the PR-PFD group exhibited RFP. Bilirubin, alkaline phosphatase, AST, INR and APRI values significantly decreased in this group. Noteworthy, 85% of 20 control patients had SFP. Profibrogenic miRNAs displayed a significant increase in expression in advanced fibrosis versus controls without fibrosis. PR-PFD treatment restored the expression of miR-34a, miR-16, miR-192, miR-200a, and miR-122. *PDGFA* CpGs hypermethylation in both cell-free DNA and liver biopsies has been found in advanced fibrosis. Interestingly, four CpGs in *PPARD* were hypomethylated compared to controls. PR-PFD treatment resulted in hypermethylation of three *TGFB1*-CpGs.

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Conclusion These findings indicate for the first time that PR-PFD might exert therapeutic effects in Hispanic patients with residual fibrosis by modulating the expression of miRNAs and methylation of specific CpG sites.

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Keywords Pirfenidone, HCV-liver fibrosis, Epigenetics, miRNAs

Introduction

Hepatitis C virus (HCV) infection has been one of the major causes of cirrhosis worldwide for several decades. However, highly effective oral direct-acting antiviral (DAA) drugs have changed the scenery by curing HCV and other clinical extrahepatic Manifestations in 97–99% of patients, including those with established advanced liver fibrosis, cirrhosis, comorbidities, and even complications of cirrhosis. A patient is considered to have a sustained virologic response (SVR) when the serum levels of HCV RNA are ≤ 15 IU/ml by a sensitive assay determined at 12 weeks post-treatment [1]. SVR is considered equivalent to a cure for HCV infection and is achieved in 92–99% of treated patients. After HCV eradication, patients still need to be monitored for liver disease progression, since fibrosis regression is not warranted. Thus, data indicate that 33–66% of patients who achieved SVR exhibit some degree of fibrosis regression, although severe residual fibrosis will revert in a lesser stage than mild liver fibrosis, and risk evolution after SVR, such as alcoholism, type 2 diabetes mellitus (T2DM), and metabolic comorbidities, could also explain the excess in liver-related morbidity in SVR patients [2]. Available data suggest that in patients with residual advanced fibrosis, as determined by METAVIR scoring system (F3-F4) or compensated cirrhosis (F4), SVR reduces, but does not eliminate, the risk of hepatocellular carcinoma (HCC) development [2]. In this context, METAVIR scoring system was used to assess histological liver biopsies according to necroinflammatory activity and fibrosis stage and was particularly developed for patients with chronic hepatitis C [3]. Thus, sensitive and significant epigenetic markers have become essential for the study of such patients, such as methylation within gene regulatory regions, which is anticipated to modulate gene expression. Reduced methylation (hypomethylation) is generally associated with enhanced transcriptional activity, whereas increased methylation (hypermethylation) tends to suppress transcription. Pioneering work by Zeybel et al. demonstrated that specific *PPARG* CpG islet methylation correlates quite well with fibrosis stage [4]. The same research group showed that methylation densities in hepatic DNA and plasma circulating cell-free DNA (cf-DNA, which represent a liquid biopsy) could be used as potential biomarkers for stratification of liver fibrosis in patients with metabolic dysfunction-associated steatotic liver disease (MASLD) or fibrosis arising from alcohol-related liver disease (ALD) and HBV chronic infection [5, 6]. Dynamic changes in DNA methylation in *PPARG*, *PPARD*, *TGFB1*, and *PDGFA* sequences have been shown to reflect the severity of fibrosis [7] and are involved in key mechanisms of fibrogenesis, such as the transdifferentiation of hepatic stellate cells (HSC) [8]. The augmented *PDGFA* methylation degree could be linked to an evolving fibrotic condition; specifically, CpG3 islet is hypomethylated in severe MASLD samples, but not in liver tissues from ALD patients. The remaining CpGs remained unaltered in the Zeybel study [7]. Interestingly, the level of DNA methylation, in particular *PPARG* CpG sites in liver biopsies and cf-DNA, showed the potential to stratify fibrosis severity in the UK population. Moreover, samples from Turkish patients demonstrated that density methylation was different from that in UK patients. In addition, epigenetic regulation through microRNAs (miRNAs) has been implicated in liver fibrosis; particular attention has been paid to miR-122, the predominant miRNA in the liver, constituting approximately 70% of the total hepatic microRNA pool, and miR-192 is also expressed in hepatic tissue [9, 10]. Multiple studies have found elevated circulating miRNA levels in patients with fibrosis [11], while miR-200a/b, miR-34a, miR-16, and miR-99b have been implicated in fibrosis evolution [12, 13]. In cirrhosis, miR-34a, miR-21, miR-31, and miR-181b have also been found upregulated [14]. On the other hand, pirfenidone is an anti-fibrotic drug approved and licensed for marketing in Europe, Japan, the USA, Canada, and Mexico for the treatment of idiopathic pulmonary fibrosis. Likewise, prolonged-release pirfenidone (PR-PFD) has been approved by COFEPRIS in Mexico since 2013 as a treatment for advanced liver fibrosis (ALF). Strengthening this premise, Poo et al. showed that ALF-patients treated with 1200 mg of PR-PFD plus standard of care for one-year had reduced fibrosis in 35% of the patients as compared with patients managed only with standard of care [15]. Recently, a controlled double-blind multicenter clinical study (ODISEA study), carried on patients with compensated liver cirrhosis, showed that the same PR-PFD formulation used here at a dose of 1200 mg/day for 24 months significantly decreased FibroTest and transient elastography values and induced improvement in serum ALT and AST, MELD score, and quality of life, compared to placebo

plus standardized care [16]. The primary endpoint of the present study was to analyze the epigenetic status regarding DNA CpG methylation and miRNA expression levels in DAA-treated HCV patients with SVR, treated for 12 months with PR-PFD, aiming to diminish residual liver fibrosis.

Methods

Study design and participants

This is a clinical controlled, open-label, proof-of-concept study carried out in a cohort of Hispanic patients infected with HCV who received treatment with direct-acting antiviral (DAAs) therapy regimens (Supplemental Information) according to standard clinical practice. Candidates were recruited at the Hepatitis Clinic of the Medical Specialties Unit at Central Military Hospital in Mexico City. From January 2019 to December 2022, a total of 130 patients treated with DDAs who achieved SVR were scrutinized for residual fibrosis. After initial screening, 62 patients were invited to participate. Eligible candidates for PFD treatment were: (a) ambulatory patients over 18 years old, indistinct sex, and regardless of the presence or absence of cirrhosis; (b) DAA-treated; (c) patients who achieved sustained viral response (SVR) at 12 weeks and continued with SVR for 12 more months; and (d) presence of persistent liver fibrosis ($\geq$ F2) according to liver stiffness to Fibroscan™. Once they understood the objectives and aims of the study, as well as the diagnostic studies and the treatment to be performed during the entire protocol, participants signed an informed consent form. Approval was obtained from the Ethics and Research Committees (ID: 013/2019), and registration was carried out on ClinicalTrial.gov (NCT05542615).

Medication

Treatment consisted of 600 mg twice a day of PR-PFD for 12 months, in addition to standard of care management following national and/or international clinical guidelines. Patients were advised to take pirfenidone 20 min after meals to mitigate gastrointestinal discomfort throughout the 12-month period. Adherence to the prescribed drug regimen was evaluated by using tailored drug registration forms. Additionally, all participants received standard care that included nutritional support and medication adjustment. Hepatic transient elastography was performed semiannually, and endoscopic evaluation was performed annually. Adverse events were recorded and treated until clinical resolution.

Controls

To compare the progression of fibrosis, a control cohort of 20 patients who met the previously described criteria and received only standard of care treatment with no PR-PFD was monitored for one year (Supplemental information). Liver stiffness was evaluated using Fibroscan™ at baseline and one year later.

In addition, six subjects were included as non-HCV, non-fibrotic controls to compare the levels of epigenetic marks with the severity of residual fibrosis against our target population. These individuals (one man and five females) without HCV were scheduled to undergo surgical intervention for reasons unrelated to liver disease and signed to participate in this study as non-fibrotic controls. Previous studies have shown that when significant differences are found (as in our study), the statistical power is satisfied.

Hematological parameters and hepatic function assessments

Blood counts and liver function tests, including bilirubin, albumin, prothrombin time expressed as International Normalized Ratio (INR), serum transaminases, glucose, and creatinine, were determined at baseline and 12 months. All participants had a negative serum HCV RNA evaluation.

Histological assessment and transient elastography

After 12 months of treatment, an end-of-study liver biopsy was performed. Percutaneous liver biopsies were performed using a CareFusion Achieve programmable Soft Tissue automatic Biopsy needle 18G × 20 cm (Becton Dickinson, Becton Drive Franklin Lakes, NJ, USA). Liver biopsies were all > 15 mm in length, covering 10 + portal tracts, and were interpreted by two certified pathologists who were blinded to the clinical status. The stage of fibrosis was evaluated using the METAVIR [3] scoring system (F0-F4). For this study purposes, patients were grouped as mild fibrosis when they showed an F2 METAVIR score and as severe fibrosis when they had an F3 or F4 METAVIR score. Additionally, liver stiffness measure (LSM) was evaluated by using vibration-controlled transient elastography (TE), Fibroscan Expert 630 (Echosens, Paris, France). Fibrosis stage was determined as follows: F0 (0–5 kPa), F1 (> 5–7.1 kPa), F2 (> 7.1–9.5 kPa), F3 (> 9.5–12.5 kPa), and F4 (> 12.5 kPa). A cutoff point of 1 grade in METAVIR score change or ± 30% kPa variation was used to classify patients as regressive fibrotic profile (RFP), stable fibrosis profile (SFP), or progressive fibrotic profile (PFP) [17].

DNA methylation analysis

DNA was extracted from 200 µL of plasma using the QIAamp ccfDNA/RNA Kit and bisulfite treated with the EpiTect Bisulfite Kit (Qiagen, Maryland, USA) according to the manufacturer's protocol. The bisulfite-treated DNA was eluted in 10 µL of elution buffer. Similarly,

DNA was extracted from liver biopsies using the QIAamp DNA micro kit according to the manufacturer's protocol, and bisulfite was treated as above. Methylation of specific cytosines within CpG dinucleotides was quantified by pyrosequencing using a PyroMark Q48 Instrument (Qiagen, Maryland, USA). Predesigned PyroMark CpG assays and a custom-designed assay for *PPARG* based on Zeybel et al. [5], containing PCR and sequencing primers, were obtained from Qiagen (Table S1). Ten microliters of biotin-labeled PCR product was added to each well and combined with streptavidin-coated sepharose beads. Sequencing primers were annealed to the DNA product at 80 °C. Samples were run in duplicate. Assay efficiency was validated using unmethylated and methylated DNA (Qiagen, EpiTect PCR Control DNA Set, 59,695). CpG methylation data were analyzed using PyroMark Q48 Autoprep Software 4.3.3.

miRNAs extraction and expression determination.

Statistical analysis

To assess the normality of variables, we employed the Kolmogorov–Smirnov test. Continuous normally distributed variables are represented as mean ± SD or mean ± SEM. To compare the means of the baseline and 12 months values, we utilized the paired Student's t test for variables demonstrating a normal distribution, while the Wilcoxon matched-pair test was applied for variables that did not conform to a normal distribution.

To determine the differences between more than two groups, parametric one-way analysis of variance (ANOVA) was conducted for variables with a normal distribution, followed by Tukey's post hoc test. Conversely, for variables not adhering to a normal distribution, significance was determined using the nonparametric Kruskal–Wallis test. All statistical analyses were performed using SPSS software V.21.0 (SPSS, Chicago, USA).

Results

Characteristics of patients

Of the initial 62 patients enrolled, 18 patients were excluded from the protocol due to non-compliance with the inclusion criteria (a fibrosis stage lower than F1, 14 subjects). Forty-four patients started PR-PFD treatment, and 38/44 patients completed the one-year treatment period. Reasons for non-compliance of treatment were: 3 deaths (death not attributable to pirfenidone secondary effects) due to advanced liver disease complications (Acute-on-Chronic Liver Failure and HCC) and a traffic accident (Table S3). The flowchart in Fig. 1 shows the total study population. The mean age of the patients was 58.95 ± 9.51 years, and 28 patients were female (73.68%) and 10 were men. All participants

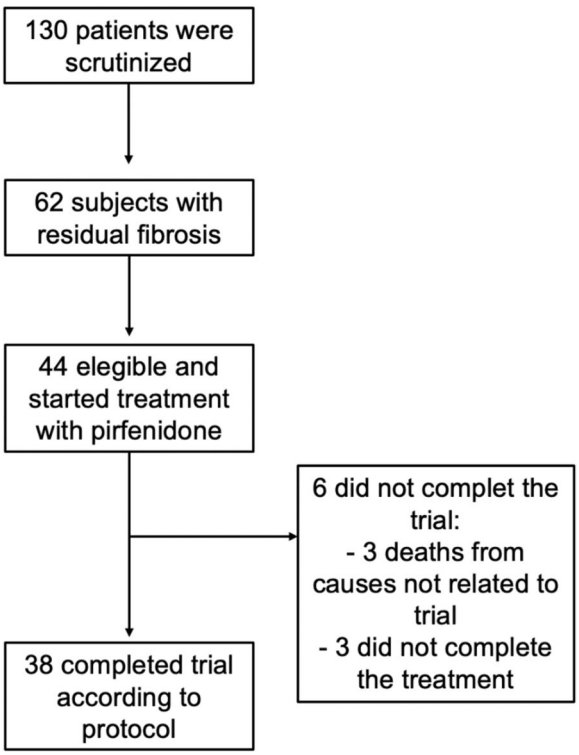

Fig. 1 Clinical study profile

remained enrolled in the study without discontinuation owing to significant adverse reactions to PR-PFD (Table S4). Additionally, a tendency toward a decrease in the presence and size of esophageal varices was observed (Table S5).

Biomarkers modification

Serum markers, including alkaline phosphatase (AP) and AST to Platelet Ratio Index (APRI), were significantly reduced after treatment. AP reduction was achieved in 55.5% of participants with values of 139.3 ± 69.01 IU/L baseline and 117.6 ± 50.95 IU/L after 12 months of treatment ($p < 0.05$). APRI values decreased from 0.95 ± 0.60 to 0.77 ± 0.51 ($p < 0.01$) in 66.5% of the patients, and in 50% of them achieved the cutoff < 0.5 value. In addition, platelets increased in 55.2% of patients from 139.9 ± 79.13 to 150.0 ± 88.09 platelets/mm3, and a significant reduction in bilirubin and INR levels after pirfenidone intervention was noticed ($p < 0.01$). A significant decrease in AST levels (reduction in 45.9% of patients) after PR-PFD treatment was evident. Creatinine, leukocyte, ALT, and glucose values were not significantly modified at the end of the protocol. Hemoglobin levels experienced a negligible change clinically non-relevant (Table 1).

Table 1 Biochemical measurements of patients before and after pirfenidone

	Baseline	12 months	*p* value
Weight	68.32 ± 15.13	68.30 ± 13.46	0.978
BMI	27.87 ± 4.58	27.94 ± 4.46	0.825
Hemoglobin	14.32 ± 1.739	13.87 ± 2.203	**0.028**
Leukocytes	4.981 ± 1.894	5.058 ± 1.943	0.765
Platelets	139.9 ± 79.13	150.0 ± 88.09	0.205
Glucose	115.7 ± 84.10	106.7 ± 47.90	0.551
Creatinine	0.962 ± 1.344	0.954 ± 1.226	0.716
Bilirubin	1.18 ± 0.98	0.81 ± 0.59	**0.034**
Albumin	3.853 ± 0.5469	3.908 ± 0.5633	0.557
ALT	29.57 ± 15.67	29.81 ± 16.04	0.983
AST	38.44 ± 12.42	34.94 ± 13.01	**0.025**
INR	1.16 ± 0.16	1.083 ± 0.15	**0.003**
ALP	139.3 ± 69.01	117.6 ± 50.95	**0.034**
APRI	0.95 ± 0.60	0.77 ± 0.51	**0.004**

Significant values of *p* are written in bold

Table 2 Evolution of liver fibrosis based on biopsy staging and transient elastography after PR-PFD

	Control cohort		PR-PFD	
Biopsy				
SFP		–	22	57.89%
PFP		–	5	13.16%
RFP		–	11	28.94%
Elastography				
	Baseline	12 months	Baseline	12 months
Mean Liver stiffness (kPa)	13.85 ± 8.41	14.3 ± 8.09	12.78 ± 6.04 ^{ns}	8.64 ± 3.99**
SFP	17	85%	17	44.73%
PFP	0	0%	4	10.52%
RFP	3	15%	17	44.73%

Values are presented as the mean ± SD

ns: no significant differences between the control cohort and PR-PFD at baseline, T-student

** : $p < 0.01$ at 12 months in control cohort compared to PR-PFD, T-student

SFP, Stable Fibrosis Profile; PFP, Progressive Fibrosis Profile; RFP, Regressive Fibrosis Profile

Residual fibrosis reduction

The fibrosis stage after 12 months of treatment with PR-PFD showed that 28.94% of patients reduced at least one fibrosis grade compared to their initial scores, 57.89% showed no change, and 13.16% worsened fibrosis by one grade (Table 2). In the context of transient elastography, no significant differences were observed in baseline liver stiffness between the control cohort and the PR-PFD group ($p = 0.56$). Remarkably, after 12 months of follow-up, a significant reduction in liver stiffness was noted in the PR-PFD group compared to that in the control cohort ($p < 0.01$). In addition, patients were classified as having RFP, SFP, or PFP based on liver stiffness (± 30% variation). In the PR-PFD group, 17 patients (44.73%) had RFP, 17 patients had SFP (44.73%), and 4 patients progressed to PFP (10.52%). Notably, in the control cohort, 85% of the patients exhibited SFP (Table 2).

Also, a persistent average kPa baseline of 13.38 ± 6.20 compared to 9.76 ± 4.17 kPa was noted after PR-PFD treatment (Fig. 2A; $p < 0.001$). This reduction was also found for the leftward shift of the distribution frequency curve, indicating a reduction in fibrosis (Fig. 2B). The patients did not present with any hepatic decompensation during follow-up.

Representative photographs of a patient's liver biopsy before and after treatment are shown in Fig. 2C. In the baseline biopsy, thick fibroconnective tissue septa with abundant chronic inflammatory infiltrate and prominent ductular reaction were observed. Important macro- and micro-steatosis was detected in the mid-lobular zone (zone 2) and pericentral area (zone 3). Following treatment, there was a reduction in the thickness of fibrotic septa, as well as in the inflammatory infiltrate and ductular reaction. Patients with reduced fibrosis showed mild fibrosis, as observed in Masson´s Trichrome staining, displaying a noticeable diminution in fibrotic septa and reduction in extracellular matrix thickness. Hematoxylin and Eosin (H&E) staining indicated a reduction in inflammatory cell infiltration and steatosis, along with an improvement in tissue morphology.

PR-PFD modifies miRNA expression while improving liver fibrosis

Several miRNAs involved in liver fibrosis and damage have been explored in liver tissue. miR-34-5p regulates HSC activation. Here, we observed statistically significant differences in miR-34-5p expression between F3 and F4 compared to that in the control group, as well as between mild and severe fibrotic livers (Fig. 3A). miR-21a-5p levels were significantly higher only in severe fibrosis (Fig. 3B). However, miR-192-5p did not show changes among the different fibrosis stages (Fig. 3C). Profibrogenic miR-181b-5p is implicated in HSC proliferation and is highly expressed in the liver of patients with fibrosis [18]. Figure 3D shows increased expression according to fibrosis staging. Additionally, profibrotic miR-16-5p was significantly augmented between mild and severe fibrotic livers. As depicted in Fig. 3F, miR-122-5p was downregulated in hepatic tissue, correlating with fibrosis stage. Lastly, miR-200a-5p and miR-200b-5p overexpression was detected in F4 stage (Fig. 3G) and advanced fibrosis, including F3 and F4 (Fig. 3H). Remarkably, five of these dysregulated miRNAs in fibrosis were significantly restored by pirfenidone

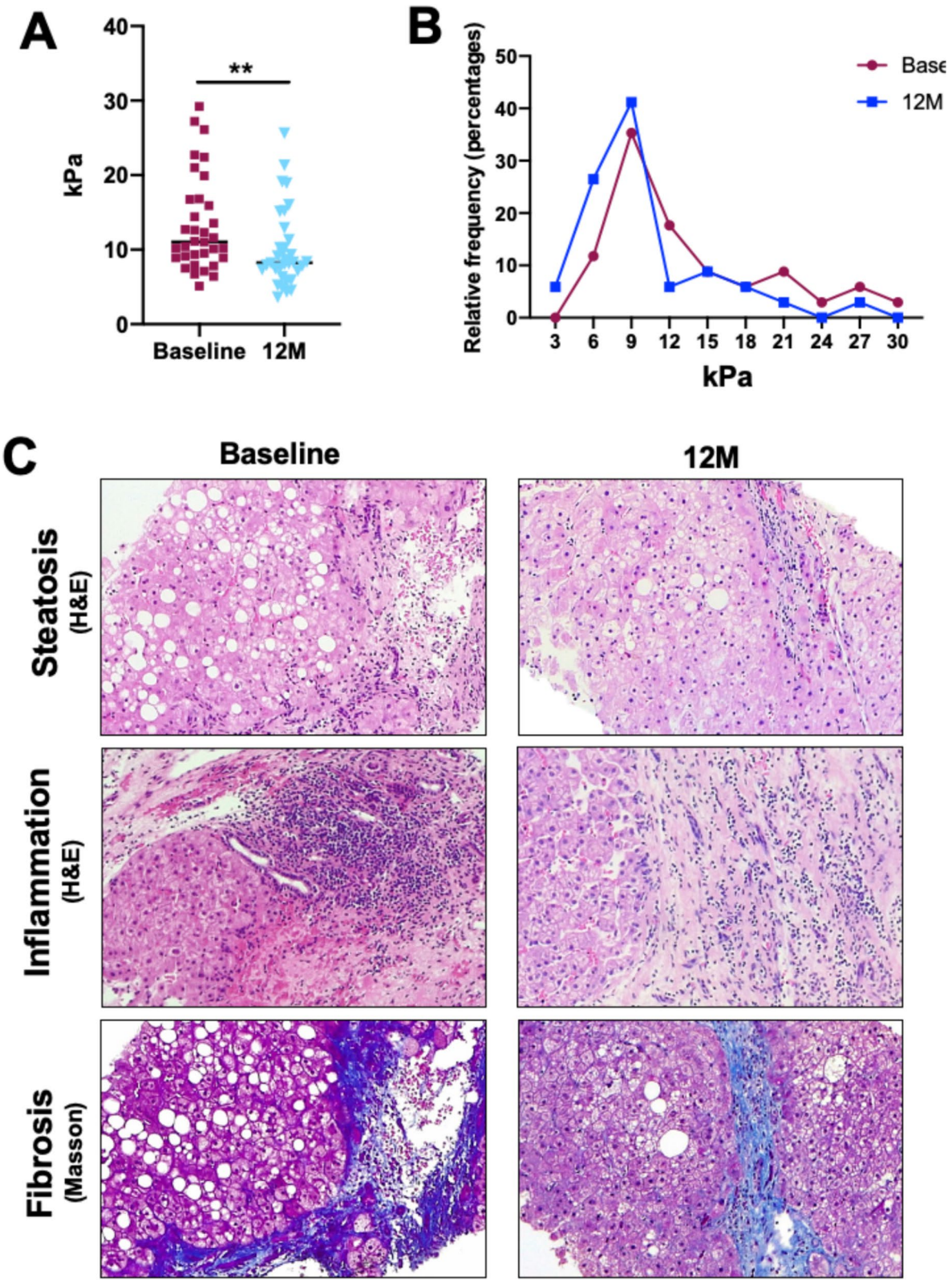

Fig. 2 **A** Liver stiffness in kPa before and after pirfenidone intervention. Data represent mean ± SD (**$p < 0.01$). A paired Student's t test was used. **B** Distribution frequency curve. **C** Representative photomicrographs of liver biopsy that underwent H&E staining to evaluate steatosis and inflammation and trichrome Masson staining to evaluate fibrosis pre- and post-treatment. Photos at × 20 magnification

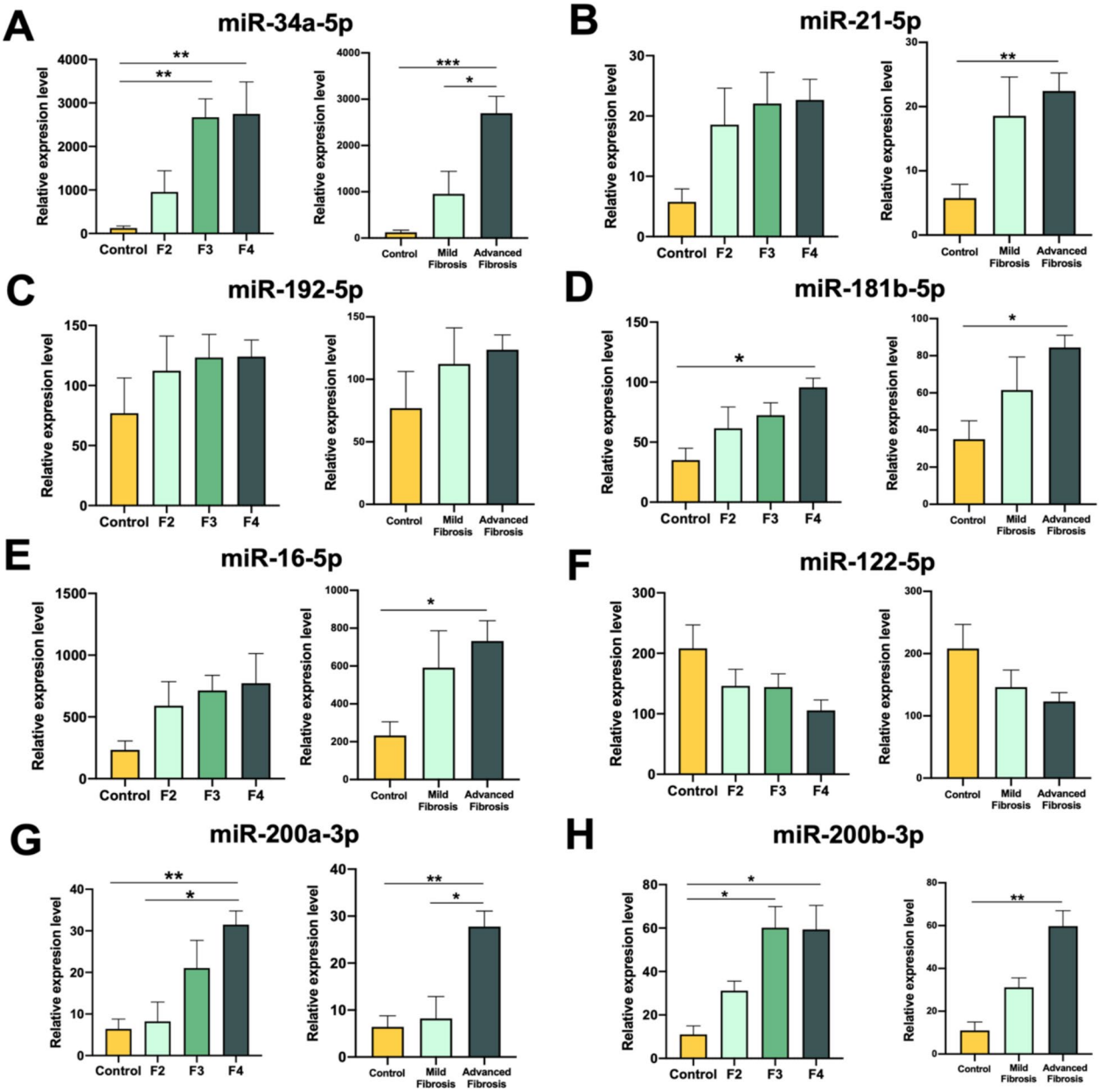

Fig. 3 miRNA expression patterns associated with fibrosis stage and severity in the liver. **A** miR-34a-5p, **B** miR-21-5p, **c** miR-192-5p, **D** miR-181b-5p, **E** miR-16-5p, **F** miR-122-5p, **G** miR-200a-3p and **H** miR-200b-3p. Data represent mean ± SEM (*$p < 0.05$; **$p < 0.01$)

intervention during the 12-month period (Fig. 4). miR-34a, miR-16, miR-192, and miR-200a were downregulated by pirfenidone (Fig. 4A–D), whereas miR-122 was upregulated (Fig. 4E). No significant changes in miR-200b, miR-21, and miR-181b were observed after pirfenidone intervention.

DNA methylation in the liver of patients with residual fibrosis

In Fig. 5A and b, CpG islets of *PDGFA* were analyzed. Liquid biopsy showed an increase in the percentage of methylation in PDGFA cf-DNA in the mild fibrosis cohort of patients in CpG3 and CpG4 (**$p < 0.01$) compared to non-fibrotic controls and the advanced fibrosis cohort (**$p < 0.01$, *** $p < 0.001$). CpG4 also showed an increase in advanced fibrosis, although with no statistical significance. In hepatic tissue, *PDGFA* showed a lower percentage of methylation in all CpGs analyzed in controls versus fibrotic patients classified according to fibrosis severity in mild (F2) and advanced (F3-F4) fibrosis (Fig. 5B, *$p < 0.05$, ***$p < 0.001$). This hypermethylation

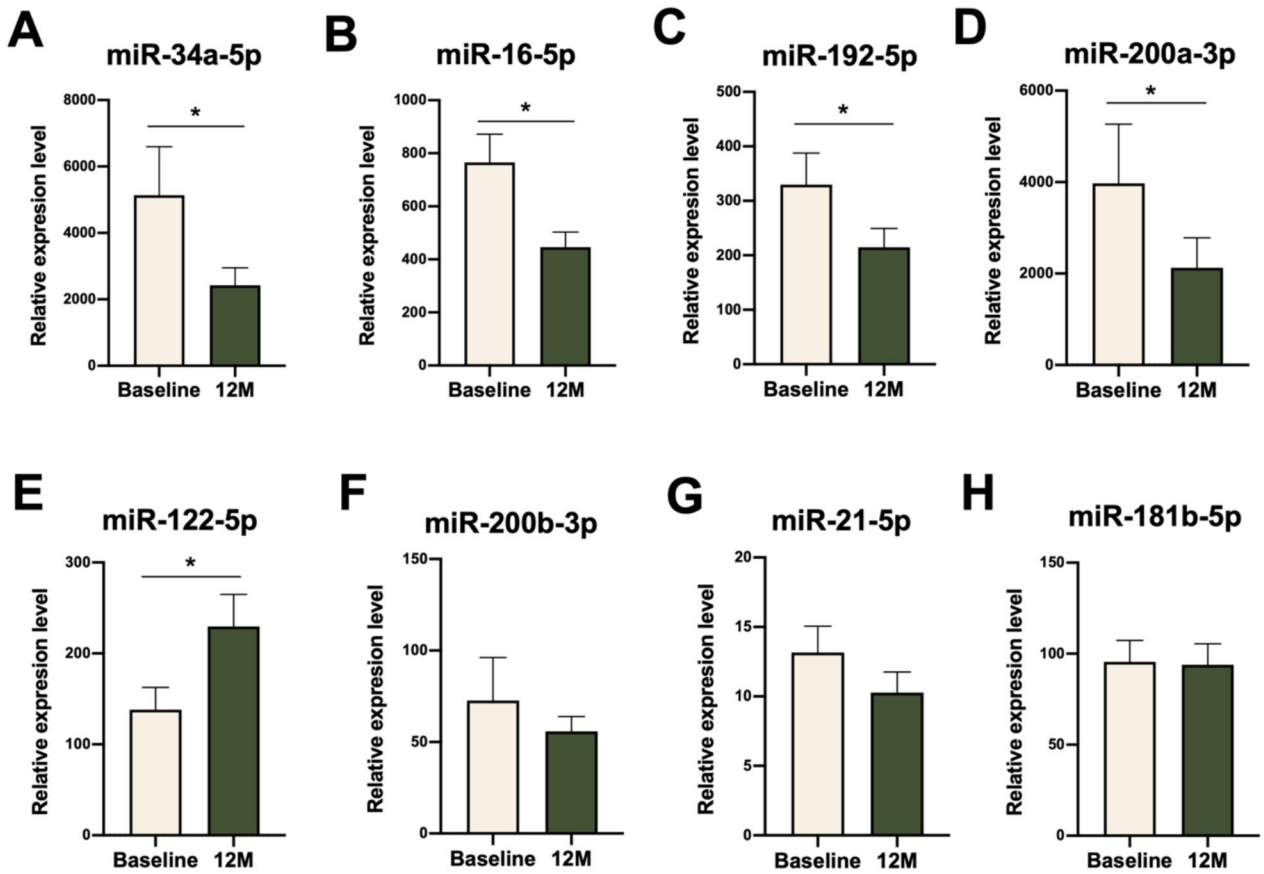

Fig. 4 miRNA expression before and after pirfenidone intervention. **A** miR-34a-5p, **B** miR-16-5p, **C** miR-192-5p, **D** miR-200a-3p, **e** miR-122-5p, **F** miR-200b-3p, **G** miR-21-5p, and **H** miR-181b-5p. Data represent mean ± SEM (*$p < 0.05$). All patients (n = 38) were screened at baseline and 12 months after PR-PFD treatment

observed in both cf-DNA and hepatic samples has not been previously reported. As shown in Fig. 5C, *PPARG* CpGs in cell-free circulating DNA showed a statistical diminution in methylation in CpG1 and an increased methylation in CpG3 in the mild fibrosis cohort of patients (*$p < 0.05$, ***$p < 0.001$). Regarding *PPARD* CpGs in cf-DNA, control non-cirrhotic patients showed a hypermethylation status compared to mild and advanced fibrosis cohorts (Fig. 5D, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Baseline versus 12 months with PR-PFD treatment shows significant differences across several CpGs in fibrosis-related genes in liver tissue

The percentage of methylation in CpGs of genes linked to fibrosis was measured, as far as we know, for the first time after pirfenidone treatment. Figure 5E shows that *PPARG* methylation tended to decrease in CpG2 and CpG3 after PR-PFD treatment. This hypomethylation favors gene expression. Figure 5F shows that *TGFB1*, the *bona fide* profibrogenic cytokine, increased methylation in the three CpGs analyzed after PR-PFD treatment (*$p < 0.05$, **$p < 0.01$). This is expected to influence a decrease in *TGFB1* transcription.

Discussion

A strength of this study is the use of liver biopsy along with transient elastography. Previous studies have shown that pirfenidone has anti-fibrotic properties and has been effective in reducing hepatic fibrosis caused by different etiologies [15, 16, 19]. In this study, we aimed to evaluate the efficacy of prolonged-release pirfenidone in treating HCV-SVR patients with residual advanced fibrosis. After a 12-month treatment with PR-PFD, the results indicated that 28.94% of patients exhibited a reduction in fibrosis stage based on liver biopsy findings. Similarly, 44.73% of patients showed improvement according to transient elastography. In contrast, no improvement was observed in patients receiving only standard of care treatment, where 85% remained fibrotic (SFP). These findings highlight the efficacy of PR-PFD in inducing RFP compared to standard-care treatment only.

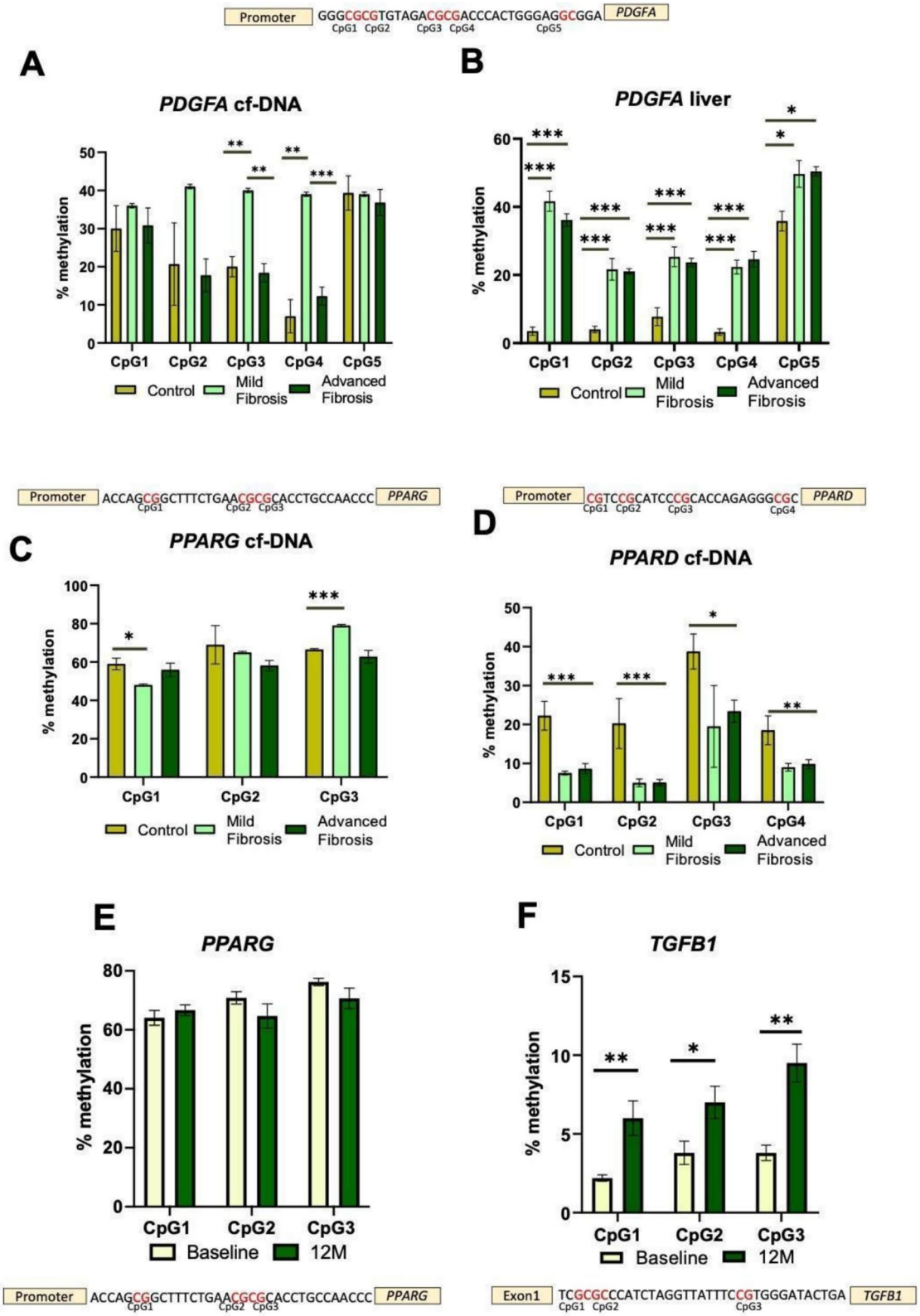

Fig. 5 Methylation in liquid biopsy and liver samples of patients grouped according to baseline fibrosis stage. **A** *PDGFA* cfDNA, **B** *PDGFA* in the liver, **C** *PPARG* cfDNA, and **D** *PPARD* cfDNA. Differential methylation at baseline and after 12 months of PR-PFD. **E** *PPARG* and **F** *TGFB1*. Data represent mean ± SEM (*$p < 0.05$)

The improvement in fibrosis stage was associated with a decrease in serum levels of liver enzymes and bilirubin, indicating improved liver function due to PR-PFD treatment. Differences in percentage of patients with reduced fibrosis observed by the two methods could be explained since elastography comprises a larger and probably more representative area of the liver analyzed, while liver biopsy only evaluates a small area of the liver tissue -50,000th of the entire organ, resulting in a significant variability of up to 40% for fibrosis staging [20]. Nonetheless, liver biopsy remains the gold standard for follow-up of patients included in clinical protocols. A strength of our study is the additional use of transient elastography. Recently, a double-blind randomized study has been reported (ODISEA Protocol) that confirmed previous findings [15] in a clinical scenario that a dose of 1200 mg/day of PR-PFD is effective in regressing advanced liver fibrosis compared with patients treated with placebo [16].

To the best of our knowledge, this is the first time that the percentage of methylation in specific CpGs of key genes implicated in fibrosis development has been evaluated in non-European patients. In addition, this is the first time that these epigenetic marks have been evaluated after treatment with an antifibrogenic medication, such as PR-PFD. Gene expression is expected to be affected by methylation in gene regulatory regions; hypomethylation augments transcription, while hypermethylation decreases transcription. Specifically, in an advanced fibrosis stage, association of hypomethylation of profibrogenic genes like *TGFB1* and *PDGFA*; and on the other hand, hypermethylation of antifibrogenic transcripts like *PPARA* and *PPARG* have been described in liver biopsy samples of MASLD and ALD cohorts in European patients [4–7]. In this study, liver samples from Hispanic patients showed a clear increase in the percentage of methylation in the three CpGs analyzed in *TGFB1* exon 1 after PR-PFD treatment, implying a reduction in *TGFB1* transcription. Previous data from the PROMETEO study and Flores-Contreras et al. showed that PFD treatment significantly decreased serum levels of this profibrogenic cytokine [15, 19]. The epigenetic change observed in our study after PDF treatment could explain the diminution reported by these studies. Our findings suggest that pirfenidone may act as an epigenetic modulator. In *PPARG* CpGs, pirfenidone treatment decreased the percentage of methylation of CpG2 and CpG3. Previous work by Hardy et al. and Zeybel et al. demonstrated that *PPARG* CpGs are hypermethylated in proportion to fibrosis stage [6, 7]. This slight reduction observed in our data correlates with the improvement in fibrosis grading observed in our patients.

In previous animal models, we demonstrated the capacity of pirfenidone to induce epigenetic changes in the liver, particularly affecting miRNA expression [21]. Here, for the first time in human patients, we showed that pirfenidone restores the expression of miR-34a, miR-16, miR-192, miR-200a, and miR-122 in the liver. Previously, serum levels of miRNA-16 were found to be significantly upregulated in early and late stages of liver fibrosis in HCV patients [22]. Additionally, Zhou et al. found elevated levels of miR-16 in individuals with HCV infection, showing a correlation with the expression levels of *HGF* and *SMAD7*. Interestingly, they demonstrated that IFN-α could reverse miR-16 expression in liver cells [23]. However, our findings indicate that its expression continues to remain higher than that in controls without fibrosis after the elimination of the HCV virus. Nevertheless, pirfenidone treatment was able to reduce miR-16 expression.

A previous study revealed that HCV infection induced miR-192 expression and that miR-192 positively regulated the expression of TGF-β1 in hepatocytes [24]. Interestingly, after pirfenidone treatment, there was a significant reduction in miR-192 levels, indicating that pirfenidone could modulate its expression and restore it to normal levels.

miR-200a and miR-200b levels were found to be significantly increased in advanced fibrosis. Earlier studies have shown a strong positive correlation between the levels of 200a and 200b expression and the advancement of liver fibrosis in samples obtained from 105 patients with chronic hepatitis C, as well as in a mouse model of CCl4-induced liver fibrosis [25]. Alternatively, our study revealed that pirfenidone treatment restored miR-200a and miR-200b expression levels.

In contrast, miR-122 expression tended to decrease in advanced fibrosis. Similar to our findings, Halász et al. observed a reduction in miR-122 expression in advanced fibrosis [26]. Importantly, the administration of PR-PFD led to a significant increase in miR-122 levels.

In advanced fibrosis, miR-21 was significantly increased and showed a strong tendency to decrease with pirfenidone treatment. Several studies have reported that miR-21 is positively regulated in patients and mouse models with hepatic fibrosis [27], and miR-21 serum levels were significantly elevated in patients with HCV-induced liver cirrhosis and HCV-related hepatocellular carcinoma [28]. In liver tissues, miR-21 expression was associated with viral load and the level of fibrosis in liver biopsies of patients with HCV infection [29]. These findings suggest that pirfenidone may exert therapeutic effects in patients with residual fibrosis by modulating the expression of these specific miRNAs.

It has been suggested that DNA methylation status at specific CpGs may be useful for patient stratification of liver fibrosis. With this in context, at baseline, patients

were grouped in mild (F2) or advanced (F3-F4) fibrosis, and methylation of specific CpGs was analyzed in liver biopsy and serum cf-DNA as representation of a liquid biopsy. In addition, a control group of patients without fibrosis was analyzed. In cf-DNA samples of the mild fibrosis cohort, CpG3 and CpG4 methylation in *PDGFA* promoter was augmented compared to control and advanced fibrosis cohorts, suggesting that hypermethylation of these precise CpGs could be associated with lower fibrosis development. In liver tissue, *PDGFA* CpG1, CpG2, CpG3, CpG4, and CpG5 are hypermethylated in mild and advanced fibrosis cohorts when compared to non-fibrotic controls. As PDGF plays an important role in fibrogenesis, the observed augmented methylation degree could be linked to an evolving fibrotic condition. Data from Zeybel et al. reported that CpG3 islet is hypomethylated in severe MASLD samples but not in liver tissues from ALD patients. The rest of CpGs maintained unaltered in Zeybel study [7].

Also, in the mild fibrosis cohort, methylation in the cf-DNA *PPARG* promoter at CpG1 significantly decreased, while that in CpG3 significantly increased, compared to the control group. Methylation stages in our patients are not in accordance with those reported by Yigit et al. who found that *PPARG* CpG1, CpG2, and CpG3 methylation degree increased in cf-DNAs and hepatic tissue samples from HBV, HCC, and MASLD patients [6]. In liquid biopsy, hypermethylation correlates proportionally to fibrosis stage in CpG1 and CpG2 of *PPARG* gene promoter in a previous report by Zeybel et al. [7]. Then, this potential marker for liver fibrosis was not corroborated in our samples.

Finally, we observed higher DNA methylation for all four CpGs at the *PPARD* promoter in control tissue, indicating that in our population, any grade of fibrosis is associated with a diminution in these CpGs methylation. *PPARD* was previously analyzed by Zeybel et al. in liver samples, and CpG2 showed an increase in the percentage of methylation in advanced fibrosis and CpG3 in ALD samples [7]. Interestingly, in the control subjects of our study methylation values were around 20–40%: while they were > 5–10% in mild and severe fibrosis patients, values that are similar to those previously reported in MASLD and ALD Caucasian patients [7].

The 4 patients who progressed had comorbidities such as T2DM and systemic arterial hypertension, which are factors of metabolic syndrome, and some studies [30] observed a 43.1% prevalence of MASLD in patients with chronic hepatitis C infection. The composite etiology has a greater risk of developing advanced fibrosis and HCC even after HCV clearance, implying that managing MASLD is as important as HCV clearance to prevent the progression of hepatic disease and death from HCC or cardiovascular disease (31).

We are aware of our study limitations. We acknowledge that life-style changes in between groups were not measured, and its potential implications on the evolution of the disease were overlooked.

An additional limitation resides in the fact that in our clinical settings, women are more willing than men to participate in this kind of study, since they show more willingness to comply with the morning schedule for the clinical appointments. The hormonal issue results in negligible since most patients were already menopausal women.

In the present study, we demonstrated a broader spread of DNA methylation values at specific CpG dinucleotides within the regulatory regions of key genes implicated in fibrosis development. We speculate that this methylation density might reflect the genetic variance that our Hispanic population presents compared to other populations from different genetic backgrounds.

Abbreviations

ALD	Alcohol-related liver disease
ALF	Advanced liver fibrosis
ALT	Alanine transaminase
ALP	Alkaline phosphatase
APRI	AST to Platelet Ratio Index
AST	Aspartate transaminase.
DAA	Direct acting antiviral
EBR	Elbasvir
GLE	Glecaprevir
GZR	Grazoprevir
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HSC	Hepatic stellate cells
INR	International Normalized Ratio
LDV	Ledipasvir
MASLD	Metabolic dysfunction-associated steatotic liver disease
OBV	Ombitasvir
PTV	Paritaprevir
PIB	Pibrentasvir
PFP	Progressive fibrotic profile
PR-PFD	Prolonged-release pirfenidone
RFP	Regressive fibrotic profile
RTV	Ritonavir
SFP	Stable fibrosis profile
SOF	Sofosbuvir
SVR	Sustained virologic response
VEL	Velpatasvir
SD	Standard deviation
SEM	Standard Error of the mean

Supplementary Information

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Supplementary Material 1.

Author contributions

ECR, JRA, JLP and JAB conceived and designed the work. ECR, AT, SCH, AGA, SAP, GGS, and JRA performed the clinical trial. RRB, RRC, REG, and ASR conducted miRNA expression experiments. SAP, GAGS and JBA performed

the transient elastography. RRB, ASR, AVE and JGC carried out DNA methylation analysis. ECR, RRB, ASR, RRC, AM and JAB analyzed and interpreted the data and drafted the original manuscript. JAB wrote, reviewed, and edited the manuscript.

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Data availability
No datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate
This study was approved by the Central Military Hospital Ethics and Research Committee (ID: 013/2019).

Competing interests
The authors declare no competing interests.

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