Liver Angiopoietin-2 Is a Key Predictor of *De Novo* or Recurrent Hepatocellular Cancer After Hepatitis C Virus Direct-Acting Antivirals

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Recent reports suggested that direct acting antivirals (DAAs) might favor hepatocellular carcinoma (HCC). In study 1, we studied the proangiogenic liver microenvironment in 242 DAA-treated chronic hepatitis C patients with advanced fibrosis. Angiopoietin-2 (ANGPT2) expression was studied in tissue (cirrhotic and/or neoplastic) from recurrent, *de novo*, nonrecurrent HCC, or patients never developing HCC. Circulating ANGPT2,vascular endothelial growth factor (VEGF), and C-reactive protein (CRP) were also measured. In study 2, we searched for factors associated with *de novo* HCC in 257 patients with cirrhosis of different etiologies enrolled in a dedicated prospective study. Thorough biochemical, clinical, hemodynamic, endoscopic, elastographic, and echo-Doppler work-up was performed in both studies. In study 1, no patients without cirrhosis developed HCC. Of 183 patients with cirrhosis, 14 of 28 (50.0%) with previous HCC recurred whereas 21 of 155 (13.5%) developed *de novo* HCC. Patients with recurrent and *de novo* HCCs had significantly higher liver fibrosis (LF) scores, portal pressure, and systemic inflammation than nonrecurrent HCC or patients never developing HCC. In recurrent/*de novo* HCC patients, tumor and nontumor ANGPT2 showed an inverse relationship with portal vein velocity (PVv; r = −0.412, *P* = 0.037 and r = −0.409, *P* = 0.047 respectively) and a positive relationship with liver stiffness (r = 0.526, *P* = 0.007; r = 0.525, *P* = 0.003 respectively). Baseline circulating VEGF and cirrhotic liver ANGPT2 were significantly related (r = 0.414, *P* = 0.044). VEGF increased during DAAs, remaining stably elevated at 3-month follow-up, when it significantly related with serum ANGPT2 (r = 0.531, *P* = 0.005). ANGPT2 expression in the primary tumor or in cirrhotic tissue before DAAs was independently related with risk of HCC recurrence (odds ratio [OR], 1.137; 95% confidence interval [CI], 1.044-1.137; *P* = 0.003) or occurrence (OR, 1.604; 95% CI, 1.080-2.382; *P* = 0.019). In study 2, DAA treatment (OR, 4.770; 95% CI, 1.395-16.316; *P* = 0.013) and large varices (OR, 3.857; 95% CI, 1.127-13.203; *P* = 0.032) were independent predictors of *de novo* HCC. *Conclusion:* Our study indicates that DAA-mediated increase of VEGF favors HCC recurrence/occurrence in susceptible patients, that is, those with more severe fibrosis and splanchnic collateralization, who already have abnormal activation in liver tissues of neo-angiogenetic pathways, as shown by increased ANGPT2. (Hepatology 2018; 68:1010-1024).

Direct-acting antivirals (DAAs) have been considered to represent a long-awaited solution in the treatment of hepatitis C. Thus, recent reports of the increased incidence of recurrent and *de novo* hepatocellular carcinoma (HCC) after DAA use[1,2] have raised great concern. Following the publication of these reports, several other studies have confirmed the increased occurrence of HCC after DAA

Abbreviations: AFP, alpha-fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANGPT2, Angiopoietin-2; APRI, AST to Platelet Ratio Index; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; CRP, C-reactive protein; DAAs, direct-acting antivirals; EV, esophageal varices; FIB-4, Fibrosis score 4; GGT, gamma-glutamyl transpeptidase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVPG, hepatic venous pressure gradient; IFN, interferon; IHC, immunohistochemistry; INR, international normalized ratio; kPa, kilopascals; LC, liver cirrhosis; LF, liver fibrosis; MELD, Model for End-Stage Liver Disease; NS, not significant; OR, odds ratio; PD1, programmed death 1; PDL1, programmed death ligand 1; PVv, portal vein velocity; SVR, sustained virological response; US, ultrasound; VEGF, vascular endothelial growth factor.

use,[3,4] whereas others have refuted these findings.[5-7] In a recent meta-analysis of 41 DAA- and interferon (IFN)-based studies (26 of HCC occurrence and 17 of HCC recurrence) including a total of 13,875 patients (90% with liver cirrhosis [LC]), DAA therapy was not associated with increased HCC occurrence or recurrence.[8] In general, these studies show that the rate of recurrence is more variable than that of *de novo* HCC. They also suggest that DAA treatment favored tumor growth in individual patients, although this remains unproven.[9] Most researchers have pointed toward reduced immunosurveillance of neoplastic clones as an explanation for the occurrence of HCC events.[2-4] A rapid change in local immune control might be pivotal for the emergence of tumor clones. Serti et al.[10] showed that the rapid declines in hepatitis C virus (HCV) viremia and inflammatory cytokine levels induced by DAAs were associated with restoration of a normal natural killer cell phenotype, with potential loss of intrahepatic immune activation by IFN-α. In this line, Meissner et al.[11] showed that on-treatment viral clearance by IFN-free regimens was accompanied by rapid down-regulation of IFN-stimulated genes in the liver and blood, regardless of treatment outcome.

Severity of fibrosis/cirrhosis[12-14] and parameters indicative of portal hypertension[15-18] are recognized prognostic factors for HCC, but their roles in tumor onset post-DAA have not been explored thoroughly, although a few findings indicate their potential involvement. Several researchers have suggested that a more advanced stage of liver disease is a key factor for HCC development.[9,19,20] Moreover, Conti et al.[2] reported that patients with HCC after DAA therapy had significantly higher liver stiffness levels than did those without HCC.

We hypothesized that the angiogenic mechanisms associated with structural changes in the liver during fibrosis progression and splanchnic vascular bed hyperplasia/hypertrophy (vasculogenesis) during development and worsening of clinically significant portal

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hypertension were critical in predisposing the hepatic microenvironment to HCC development after DAAs. We thus evaluated key expression markers of local immune control and angiogenesis in hepatic biopsy samples from patients undergoing DAA treatment, and related them to clinical features of fibrosis and portal hypertension.

Patients and Methods

PATIENTS

Study 1

All treatment-naïve and -experienced patients with advanced liver fibrosis (LF) undergoing DAA with or without ribavirin for chronic HCV infection at the Gastroenterology, University Hospital (Modena, Italy) between January 2015 and May 2017 were enrolled in this study (Supporting Table S1; Supporting Fig. S1). Eligibility criteria for treatment with DAAs (according to the priority criteria of the Italian Medicines Agency) were: patients had Child-Pugh class A or B LC; no history of previous HCC or histories of complete response for at least 3 months after surgical resection or locoregional ablation of previous HCC, and/or an F3 METAVIR fibrosis score, determined by liver histology or transient elastography result between 10 and 12.5 kilopascals (kPa).[21] Virological response to therapy was assessed by quantitative HCV-RNA detection, using real-time polymerase chain reaction (RT-PCR) with a detection limit of 15 IU/mL. Before starting antiviral therapy and on a 6-month basis, all patients underwent hepatic ultrasound (US) examination. In cases of suspected liver lesions, computed tomography or magnetic resonance imaging was performed. All patients with HCC underwent US-guided liver biopsy of the tumor and surrounding nontumoral liver tissue at the time of diagnosis. For patients with previously treated HCC, samples from US-guided liver biopsy of the primary HCC and surrounding cirrhotic tissue were available, because they had been collected in a prospective study of HCC aggressiveness (Clinicaltrials.gov: NCT01657695). In case of recurrence, patients underwent repeat US-guided liver biopsy.

Study 2

A prospective cohort of patients with cirrhosis was analyzed to evaluate risk factors (including DAAs treatment) for HCC development and as a source of pre-DAA liver tissue for those HCV patients who developed *de novo* HCC during follow-up (see study 1). It includes 257 consecutive patients (94 [36.6%] HCV positive [63.2% treated with DAAs], 30 [11.7%] hepatitis B virus (HBV) positive, 133 (51.8%) dysmetabolic/alcoholic/nonalcoholic steatohepatitis [NASH] patients [Clinicaltrials.gov: NCT03083 002]). For all patients enrolled in this study, including the 94 HCV subjects, cirrhotic liver tissue, collected 15.9 ± 4.3 months before HCC development and 7.8 ± 3.0 months before DAA treatment was available (Supporting Fig. S2). Twenty-seven patients belonged to both cohorts; 3 patients with *de novo* HCC of study 2 were among the 21 *de novo* HCC of study 1 (Table 2).

Both studies fulfill the guidelines of the Declaration of Helsinki and Good Clinical Practice in clinical trials. All patients provided written informed consent. All authors had access to the data and reviewed and approved the final manuscript.

CARDIOPULMONARY AND HEPATIC HEMODYNAMIC EVALUATION

Hemodynamic examination was performed after an overnight fast under mild sedation with intravenous midazolam (0.02 mg/kg; B Braun, Milan, Italy). Weight and height were recorded before the procedure to calculate body surface area (BSA). After local anesthesia with subcutaneous lidocaine injections (Fisiopharma, Palomonte, Italy), a venous introducer was placed in the right internal jugular vein following the Seldinger technique. During the entire procedure, the patient's heart rate and mean arterial pressure were measured every 5 minutes with an automatic sphygmomanometer (Marquette Electronics, Milwaukee, WI). Cardiopulmonary pressures were measured as described.[22] Cardiac output (CO) was determined by online thermodilution (Marquette Electronics). Cardiac index was calculated as CO/BSA. After the measurement of cardiopulmonary parameters, a balloon catheter (Edwards Lifesciences, Irvine, CA) was introduced into the main right or middle hepatic vein by the inferior vena cava (IVC) for measurement of the wedged hepatic venous pressure (WHVP) and free hepatic venous pressure (FHVP).[22] Permanent tracings were recorded electronically (PowerLab; ADI Instruments, Milford, MA) and analyzed using dedicated software (LabChart 7; ADI Instruments). Hepatic venous pressure gradient (HVPG) was obtained by subtracting the FHVP at the junction with

the IVC from the WHVP. All hemodynamic assessments were performed in triplicate.

LIVER US AND DOPPLER US STUDY OF THE PORTAL VEIN

The liver was examined in standard grayscale using 2- to 5-MHz curvilinear or vector transducers with Esaote myLab 70 XVG US machines. Portal enlargement was defined as the measured portal caliber ≥13 mm. Splenomegaly was defined as length greater than or equal to 12 cm, measured using a left lateral intercostal approach in supine position obtaining the main longitudinal axis and area passing through the splenic hilus. Doppler US examinations were obtained using a right lateral intercostal approach or a subcostal oblique scansion. Peak and mean velocity of portal vein (PVv) were measured in cm/sec at the porta hepatis using a Doppler angle ≤60 degrees for angle correction.

LIVER STIFFNESS MEASUREMENT

Liver stiffness measurement with FibroScan was performed before beginning DAAs using the standard M probe after overnight fasting. Ten valid measurements were recorded, and the result was expressed as the median of the valid measurements.

ANGPT2 RNA LEVELS AND NEO-ANGIOGENIC TRANSCRIPTOMIC SIGNATURE

ANGPT2 mRNA levels and neo-angiogenic transcriptomic signatures were tested by quantitative real-time PCR, as described.[23] ANGPT2 RNA levels were evaluated in terms of fold change in comparison with surrounding nontumoral tissue.

IMMUNOHISTOCHEMISTRY

Tumoral and nontumoral liver tissue samples from all patients with HCC were available for programmed death 1 (PD1), programmed death ligand 1 (PDL1), and ANGPT2 immunohistochemical (IHC) analysis, which was performed as described.[24] For recurrent patients, nontumoral and tumoral tissue from the primary and from the recurrent tumor were examined. ANGPT2 expression was also evaluated in the endothelia of tumoral and nontumoral vessels. Details are reported in Supporting Methods.

DETERMINATION OF CIRCULATING ANGPT2, VASCULAR ENDOTHELIAL GROWTH FACTOR, AND C-REACTIVE PROTEIN

Vascular endothelial growth factor (VEGF)-1 and ANGPT2 levels were determined with a quantikine/high-sensitivity enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Serum level of C-reactive protein (CRP), the most common marker of systemic inflammation, was assessed using a commercially available test (CRPL3-Cobas; Roche Diagnostics, Monza, Italy) with a lower sensitivity limit of 0.49 mg/dL. Levels ≥0.5 mg/dL were considered to be abnormal.

STATISTICAL ANALYSIS

Dichotomous and continuous variables were analyzed using Fisher's exact test, chi-squared test, bivariate (Pearson) correlation analysis, paired and unpaired *t* test, and nonparametric tests for independent samples (Mann-Whitney U or Kruskal-Wallis test), as appropriate. Logistic regression analysis was performed to identify variables associated independently with recurrence or occurrence of HCC after DAAs use (first model), and with *de novo* HCC in the prospective cohort of patients with cirrhosis (second model).

In the first model, the dependent variable (recurrent or *de novo* HCC) was coded as present (1) or absent (0). Candidate risk factors were sex, age, HCV genotype, sustained virological response (SVR), ANGPT2, HVPG value liver stiffness level, splenic diameter, F2-F3 varices, cardiac index, ascites, body mass index (BMI), diabetes, platelet count, and levels of albumin, bilirubin, international normalized ratio (INR), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), and CRP. In the model for recurrence, the semiquantitative ANGPT2 expression level in the primary tumor was used; in the model for *de novo* HCC, ANGPT2 expression in the cirrhotic tissue at the time of enrollment in the prospective study of HCC development (Clinicaltrials.gov: NCT03083002) was used. To avoid effects of collinearity, the single components of the scores evaluated (Child-Pugh and Model for End-Stage Liver Disease [MELD]) were included in the multivariate models individually.

In the second model, the dependent variable (*de novo* HCC) was coded as present (1) or absent (0). Candidate risk factors were DAAs, sex, age, viral etiology, DAA treatment, nucleos(t)ide analogue (NUC) treatment, HVPG value, liver stiffness level, splenic diameter, F2-F3 varices, decompensated cirrhosis, BMI, diabetes, platelet count, INR, ascites, encephalopathy, and levels of albumin, bilirubin, INR, creatinine, AST, ALT, GGT, ALP, and CRP. Variables with *P* values <0.10 in univariate analyses were included in the final multivariate models. To test for relevance of viral etiology, a model excluding alcohol- or NASH-related chronic liver disease was used.

The PASW Statistics 24 program (IBM Corp., Armonk, NY) was used for statistical analyses.

Results

CLINICAL CHARACTERISTICS OF THE PATIENTS

Supporting Table S1 shows the demographic and baseline clinical characteristics of the patients included in study 1. The cohort was composed of 242 patients (153 [63.2%] males); 126 (52.0%) patients had never been treated before, and 116 (48.0%) patients were nonresponders to previous antiviral treatment, which was IFN-based in all but 3 cases. Patients with F3 fibrosis underwent treatment for a mean period of 14 ± 4 weeks (median, 12; range, 5-25) whereas patients with F4 fibrosis/cirrhosis underwent treatment for a mean period of 19 ± 6 weeks (median, 23; range, 1-26). Ribavirin was significantly more used in patients with F3 than F4/cirrhosis stage (F3: 41 of 59, 69.5% vs. F4: 96 of 183, 52.5%; *P* = 0.022). One patient with F3 fibrosis (1 of 59; 1.5%) and 7 of 183 (3.8%) among patients with F4/cirrhosis discontinued treatment (*P* = 0.472). Therapeutic regimens are detailed in Supporting Table S3.

No patient without cirrhosis had or developed HCC. One hundred eighty-three (75.6%) patients had LC (defined by histology, liver stiffness, and/or imaging findings). Analysis for risk factors for HCC recurrence or occurrence has been performed in patients with LC. No significant difference between abstinent and alcohol abusers was found between patients developing or recurring HCC and those who did not (3 of 35, 8.5% vs. 18 of 148, 12.1% respectively; *P* = 0.582). Table 1 shows the demographic and clinical characteristics of patients with cirrhosis, stratified according to HCC events. Fourteen (7.65%) patients had recurrent HCC, 21 (11.5%) *de novo* HCC, 14 (7.65%) nonrecurrent HCC, and 134 (73.2%) did not have history of HCC and did not develop HCC after DAAs. One hundred sixty-six (90.7%) patients had Child-Pugh A disease, 16 (8.8%) had Child-Pugh B, and 1 (0.5%) had Child-Pugh C disease. Esophageal varices (EV) were present in 72 of the 183 (39.3%) patients. Thirty-eight (20.8%) patients had F1, 31 (16.9%) had F2, and 3 (1.6%) had F3 varices.

Twenty-eight of the 183 (14.7%) patients with cirrhosis had histories of HCC when they started antiviral treatment. Twenty had received curative treatment (6 by liver resection [LR], 13 by radiofrequency ablation [RFA], and 1 by liver transplantation), and 8 had undergone transarterial chemoembolization (TACE). Mean interval from HCC curative treatment and DAA initiation was 16.0 ± 11.5 months (median, 11; range, 3-33). The interval between HCC therapy and DAA treatment was not significantly different between recurrent and nonrecurrent cases (recurrent vs. nonrecurrent, 17 ± 12 vs. 28 ± 33 months; *P* = 0.867, Mann-Whitney U test). After DAA use, HCC recurred in 14 of the 28 (50%) patients (6 previously treated with RFA, 4 with LR [with clean surgical margins and no microvascular invasion], and 4 with TACE). Mean recurrence time was 3.9 ± 3.8 months after stopping DAAs. Twenty-one of 155 (13.5%) patients without previous history of HCC developed *de novo* HCC after DAA therapy (Supporting Fig. S1) after a mean period of 3.3 ± 5.3 months after stopping DAAs.

RELATIONSHIPS BETWEEN CLINICAL CHARACTERISTICS OF PATIENTS WITH CIRRHOSIS AND TYPE OF HCC EVENT

Patients with recurrent or *de novo* HCC had significantly higher liver stiffness levels than did the other two subgroups (recurrent HCC, 27.0 ± 13.4 kPa; *de novo* HCC, 24.9 ± 8.5 kPa; no recurrence, 12.3 ± 5.9 kPa; no history of HCC, 15.6 ± 5.5 kPa; *P* = 0.001, Kruskal-Wallis test; Table 1). HVPG distribution also differed (recurrent HCC, 17.4 ± 8.3 mm Hg; *de novo* HCC, 22.8 ± 4.0 mm Hg; no recurrence, 9.5 ± 2.1 mm Hg; no history of HCC, 15.0 ± 5.1 mm Hg; *P* = 0.002, Kruskal-Wallis test; Table 1).

LF (as indicated by AST to Platelet Ratio Index [APRI] and Fibrosis-4 [FIB-4] scores) was correlated significantly with type of HCC event; scores were significantly higher in recurrent and *de novo* cases than

TABLE 1. Demographic and Clinical Characteristics at Baseline of the 183 Patients With LC Treated With DAAs

Variable	Recurrent HCC (n = 14)	*De Novo* HCC (n = 21)	Nonrecurrent HCC (n = 14)	No HCC (n = 134)	*P* Value
Male sex	14 (100)	16 (76.1)	8 (57.1)	88 (65.6)	0.019
Age (years)	62.5 ± 9.3	58.8 ± 9.0	66.2 ± 8.7	62.7 ± 10.1	0.145
HCV genotype					
1a	2 (14.3)	2 (9.5)	0	20 (14.9)	
1b	8 (57.1)	12 (57.1)	9 (64.3)	64 (47.8)	
2	2 (14.3)	1 (4.8)	3 (21.4)	16 (11.9)	0.867
3	2 (14.3)	5 (23.8)	2 (14.3)	23 (17.2)	
4	0	1 (4.8)	0	11 (8.2)	
Previous IFN-based treatment	7 (50.0)	9 (42.8)	6 (42.8)	67 (50.0)	0.964
SVR (n, %)	14 (100)	17 (81.0)	13 (92.9)	126 (94.0)	0.140
BMI (kg/m^2)	25.7 ± 4.5	25.6 ± 4.0	25.9 ± 4.1	26.8 ± 3.7	0.441
Diabetes (n, %)	2 (14.2)	3 (11.8)	5 (35.7)	25 (18.6)	0.457
Ascites (n, %)	0	3 (14.2)	1 (7.1)	19 (14.2)	0.209
Child-Pugh class (n, %)					
Child A	13 (92.9)	16 (76.2)	12 (85.7)	125 (93.3)	
Child B	1 (7.1)	4 (19.0)	2 (14.3)	9 (6.7)	0.172
Child C	0	1 (4.8)	0	0	
MELD score (M ± SD)	9.3 ± 3.8	10.9 ± 4.5	7.1 ± 1.0	8.6 ± 2.8	0.054
EV (n, %)					
F0-F1	7 (50.0)	12 (57.1)	14 (100.0)	115 (85.8)	0.000
F2-F3	7 (50.0)	9 (42.8)	0	19 (14.2)	
HVPG (mm Hg)	17.4 ± 8.3	22.8 ± 4.0	9.5 ± 2.1	15.0 ± 5.1	0.002
Liver stiffness (kPa)	27.0 ± 13.4	24.9 ± 8.5	12.3 ± 3.9	15.6 ± 5.5	0.001
Cardiac index (L/min/m^2)	3.3 ± 0.4	4.0 ± 1.0	2.7 ± 0.5	3.4 ± 0.6	0.040
Peak PVv (cm/sec)	29.6 ± 5.5	27.3 ± 5.9	27.1 ± 5.8	31.4 ± 6.5	0.190
Mean PVv (cm/sec)	19.0 ± 6.4	21.4 ± 4.5	23.2 ± 5.7	23.7 ± 5.5	0.392
Spleen diameter (cm)	14.2 ± 1.6	16.2 ± 3.1	12.9 ± 2.1	13.8 ± 2.8	0.005
Edmondson-Steiner grade (1/2/3) (n)	4/6/4	1/12/8	3/8/3	—	0.347
Microvascular/perineural invasion (n, %)	2 (12.2)	1 (4.7)	0	—	0.504
Serum VEGF (pg/mL)	188 ± 89	156 ± 125	191 ± 49	166 ± 54	0.299
Serum ANGPT2 (pg/mL)	3.029 ± 1,750	11.470 ± 9,436	2.767 ± 1,332	2.940 ± 2,114	0.021
Bilirubin (mg%)	0.9 ± 0.4	1.7 ± 1.9	0.8 ± 0.5	1.0 ± 0.6	0.088
Albumin (g/dL)	3.7 ± 0.4	3.5 ± 0.4	3.8 ± 0.3	3.8 ± 0.3	0.175
AST (IU/mL)	81.2 ± 43.5	59.1 ± 28.9	49.0 ± 43.1	73.0 ± 60.3	0.075
ALT (IU/mL)	101.6 ± 68.3	46.2 ± 22.0	70.1 ± 102.8	80.4 ± 76.0	0.228
GGT (IU/mL)	118.2 ± 93.9	98.6 ± 81.8	110.7 ± 193.9	83.4 ± 67.7	0.260
ALP (IU/mL)	96.2 ± 33.4	139.4 ± 71.8	115.75 ± 59.7	99.0 ± 41.9	0.068
INR	1.3 ± .05	1.2 ± 0.2	1.0 ± 0.1	1.1 ± 0.2	0.054
Creatinine (mg/dL)	0.8 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	0.8 ± 0.2	0.381
Platelets (×10^3/mm^3)	101 ± 45	122 ± 94	140 ± 67	122 ± 58	0.158
CRP (mg/dL)	0.75 ± 0.34	0.80 ± 0.54	0.64 ± 0.50	0.22 ± 0.26	<0.0001
AFP (ng/mL)	37.0 ± 47.4	7.65 ± 6.0	4.77 ± 2.5	11.3 ± 11.2	0.168
APRI	2.7 ± 2.1	2.2 ± 1.1	1.1 ± 0.8	1.6 ± 1.5	0.008
FIB-4	6.7 ± 4.9	7.3 ± 2.9	3.9 ± 2.1	5.3 ± 3.9	0.007
ALBI	0.7 ± 0.1	0.8 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.072

Data are reported as n (%) or mean ± SD.
Abbreviation: ALBI, Albumin-Bilirubin Score.

in patients with no recurrence and those who never developed HCC (APRI, $P = 0.008$; FIB-4, $P = 0.007$; Table 1). Severity of portal hypertension (evaluated by variceal grade) was also significantly related to recurrence or occurrence of HCC. F2-F3 EV was significantly more common among patients with recurrent or *de novo* HCC than among patients with no HCC recurrence and those who did not develop HCC (Table 1; $P < 0.0001$). No significant difference among groups was found in severity of liver disease, evaluated as Child-Pugh class ($P = 0.172$) or MELD score ($P = 0.054$).

CRP level was related significantly to type of HCC event. Baseline CRP level was significantly higher in patients with recurrent and *de novo* HCC than in those with no HCC recurrence and those who never developed HCC at baseline (recurrent HCC, 0.75 ± 0.34 mg/L; *de novo* HCC, 0.80 ± 0.54 mg/L; no recurrence, 0.64 ± 0.50 mg/L; no HCC, 0.22 ± 0.26 mg/L; $P < 0.0001$, Kruskal-Wallis test; Table 1). Similar modification was observed at follow-up after DAA treatment (recurrent HCC, 1.0 ± 1.3 mg/L; *de novo* HCC, 0.88 ± 0.60 mg/L; no recurrence, 0.68 ± 0.50 mg/L; no HCC, 0.23 ± 0.10 mg/L; $P < 0.0001$, Kruskal-Wallis test). CRP levels were also related positively to cardiac index at baseline (r = 0.259; $P = 0.035$, Pearson correlation).

EXPRESSION STUDIES

Immunohistochemical PD1 and PDL1 Expression

PD1 and PDL1 molecules were examined in tumor and nontumoral tissue from recurrent, *de novo*, and nonrecurrent HCCs, in order to evaluate the immune cells infiltrating the tissue microenvironment and immune tolerance, as described.[24]

They were found to be expressed at very low levels in infiltrating lymphocytes and hepatocytes in tumor and nontumoral tissue. In tumor tissue, PD1 was expressed more in *de novo* HCC (optical density, 4.0 ± 3.3) than in recurrent (optical density, 0.4 ± 0.4; $P = 0.031$) and nonrecurrent (optical density, 0.99 ± 0.98; $P = 0.020$) HCC. No significant difference in PD1 expression was observed in nontumoral tissue. PDL1 expression (evaluated in tumor and nontumoral tissue) did not differ significantly among *de novo*, recurrent, and nonrecurrent HCCs (tumor tissue, $P = 0.284$; nontumoral tissue, $P = 0.425$; Kruskal-Wallis test).

Neo-Angiogenic Transcriptomic Signature

In 36 of 49 patients with HCC, frozen tissue (collected during initial liver biopsy in patients with past HCC and during biopsy of newly diagnosed lesions in patients with *de novo* HCC) was available for testing the presence of the neo-angiogenic transcriptomic signature.[23] This signature was observed among patients with recurrent and *de novo* HCC, but not in patients with no HCC recurrence ($P = 0.0002$; Supporting Table S2).

ANGPT2 Expression

In RNA extracted from tumoral hepatic tissue, ANGPT2 levels were significantly higher in patients with recurrent HCC (median fold change, 2.19) and *de novo* HCC (median fold change, 3.91) than in those with nonrecurrent HCC (median fold change, 1.00; $P = 0.005$, Kruskal-Wallis test; Fig. 1).

IHC results for ANGPT2 differed significantly in tumor tissue among patients with recurrent, *de novo*, and nonrecurrent HCC ($P < 0.0001$, Kruskal-Wallis test). The highest expression levels were found in tumoral tissue of patients with recurrent and *de novo* HCCs; significantly lower levels were found in nonrecurrent HCC (Table 2, study 1; Fig. 2). Lower levels were expressed in nontumoral tissue of recurrent and nonrecurrent HCCs in comparison with those found in nontumoral tissue surrounding *de novo* HCCs (Table 2, study 1). In patients with recurrent HCC, paired mean ANGPT2 expression levels did not differ significantly between primary and recurrent HCC tissue (mean optical density, 14.0 ± 4.0 vs. 13.8 ± 4.1 respectively; $P = 0.356$) or primary and recurrent nontumoral tissue surrounding HCC (mean optical density, 12.2 ± 1.9 vs. 12.3 ± 5.2 respectively; $P = 0.955$). All values were in the high range of expression.

In vascular endothelia from tumoral and surrounding nontumoral tissue, ANGPT2 expression was

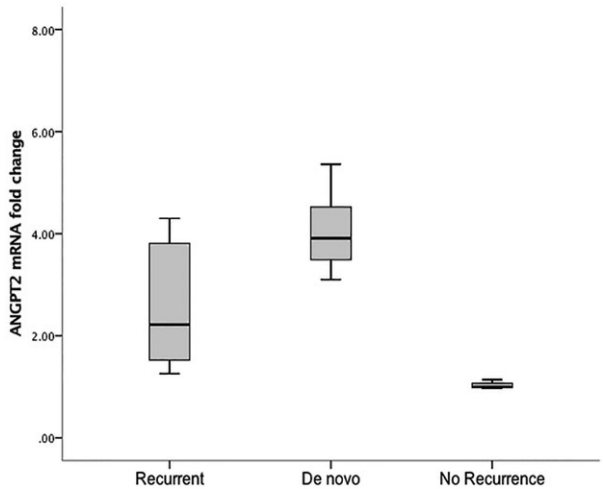

FIG. 1. ANGPT2 mRNA in tumor tissues from patients with recurrent, *de novo*, nonrecurrent HCC. In patients with recurrent and nonrecurrent HCC, primary tumor tissue was tested. In patients with *de novo* HCC, new tumor tissue was examined. Horizontal dark bars represent median values.

TABLE 2. Study 1: Semiquantification of ANGPT2 Expression (Analyzed by IHC) in Hepatocytes and in Vascular Endothelia

Study 1

	Hepatic Tissue Optical Density (int/sq mm)		Vascular Endothelia Optical Density (int/sq mm)	
(n)	NT	T	NT	T
Recurrent HCC (14)	12.2 ± 1.9[a,b]	14.0 ± 4.0[d,e]	8.3 ± 2.3[g,h]	14.1 ± 4.4[l,m]
Nonrecurrent HCC (14)	11.5 ± 3.1[a,c]	10.5 ± 1.9[d,f]	7.7 ± 1.3[g,i]	9.1 ± 3.5[l,n]
De novo HCC (18)[1]	17.7 ± 6.2[b,c]	18.4 ± 4.6[e,f]	9.9 ± 6.3[h,i]	15.3 ± 7.4[m,n]
	[a] P = NS	[d] P = 0.031	[g] P = NS	[l] P = 0.0027
	[b] P = 0.046	[e] P = 0.028	[h] P = NS	[m] P = NS
	[c] P = 0.020	[f] P < 0.0001	[i] P = NS	[n] P = 0.0043

Study 2

	Hepatic Tissue Optical Density (int/sq mm)			Vascular Endothelia Optical Density (int/sq mm)		
(n)	Cirrhotic Tissue	NT	T	Cirrhotic Tissue	NT	T
No HCC (24)	4.8 ± 0.6[a]	—	—	5.1 ± 0.5[b]	—	—
De novo HCC (12)	7.9 ± 1.0[a]	15.9 ± 6.2	17.9 ± 4.3	7.0 ± 0.6[b]	8.0 ± 2.3	15.9 ± 7.4
	[a] P < 0.0001			[b] P < 0.0001		

Study 1: Evaluation of ANGPT2 was made between tumor tissue (primary tumor) and surrounding nontumor tissue of recurrent, nonrecurrent HCC, and *de novo* HCC cases after DAAs. Study 2: Semiquantification of ANGPT2 expression was performed in cirrhotic tissue obtained by transjugular liver biopsy during the prospective study of HCC development, 16.0 ± 11.5 months before DAA treatment. Data regarding patients who either developed or did not develop *de novo* HCC after DAA treatment are reported on. Data were analyzed by unpaired *t* test. Superscript letters indicate the subgroups compared.
**De novo* HCC cases of study 1 are 21, but, because 3 cases belong also to study 2, their IHC values are reported among cases of study 2, in order to display data on cirrhotic tissue before DAA treatment.
Abbreviations: NT, nontumor; T, tumor.

significantly higher in *de novo* and recurrent HCCs than in nonrecurrent HCC (P = 0.046 and P = 0.002 respectively, Kruskal-Wallis test; Table 2, study 1). The highest levels of expression were found in *de novo* and recurrent HCC, with no significant difference between them (P = not significant [NS]).

In prospective study 2, evaluation of the cirrhotic tissue collected at baseline 16.0 ± 11.5 months before DAA treatment showed significantly higher ANGPT2 levels in patients who later develop HCC after DAAs versus those who did not (Table 2, study 2; Fig. 2A,G). Comparable ANGPT2 expression levels were found in nontumor and tumor tissue of *de novo* HCC in both studies (tumor tissue, study 1: 18.4 ± 4.6 vs. study 2: 17.9 ± 4.3; Table 2).

CIRCULATING VEGF AND ANGPT2 LEVELS

Circulating VEGF levels increased from baseline to the end of DAA treatment (144 ± 95 pg/mL vs. 204 ± 116 pg/mL; P = 0.040, paired *t* test) and maintained higher levels at 3-month follow-up (baseline vs. end of follow-up, 199 ± 102 pg/mL; paired *t* test, P = 0.024). Circulating ANGPT2 levels were stable throughout the observation period (baseline, 6.047 ± 4.986 pg/mL; 3-month follow-up, 5.802 ± 4.367 pg/mL; P = 0.958, paired *t* test). However, ANGPT2 levels differed significantly among HCC groups both at baseline (reported in Table 1) and at 3-month follow-up (recurrent, 5.807 ± 5.115 pg/mL; *de novo*, 6.941 ± 2.995 pg/mL; no recurrence, 2.373 ± 1.264 pg/mL; no history of HCC, 3.273 ± 1.426 pg/mL; P= 0.020, Kruskal-Wallis test). Moreover, circulating ANGPT2 levels at 3-month follow-up were positively related to ANGPT2 expression in tumor tissue (0.534; P = 0.022, Pearson correlation). A significant relationship was found between baseline VEGF and ANGPT expression in the non-neoplastic cirrhotic tissue (r = 0.414; P = 0.044, Pearson correlation) and between 3-month follow-up serum VEGF and ANGPT2 levels (r = 0.531; P = 0.005, Pearson correlation).

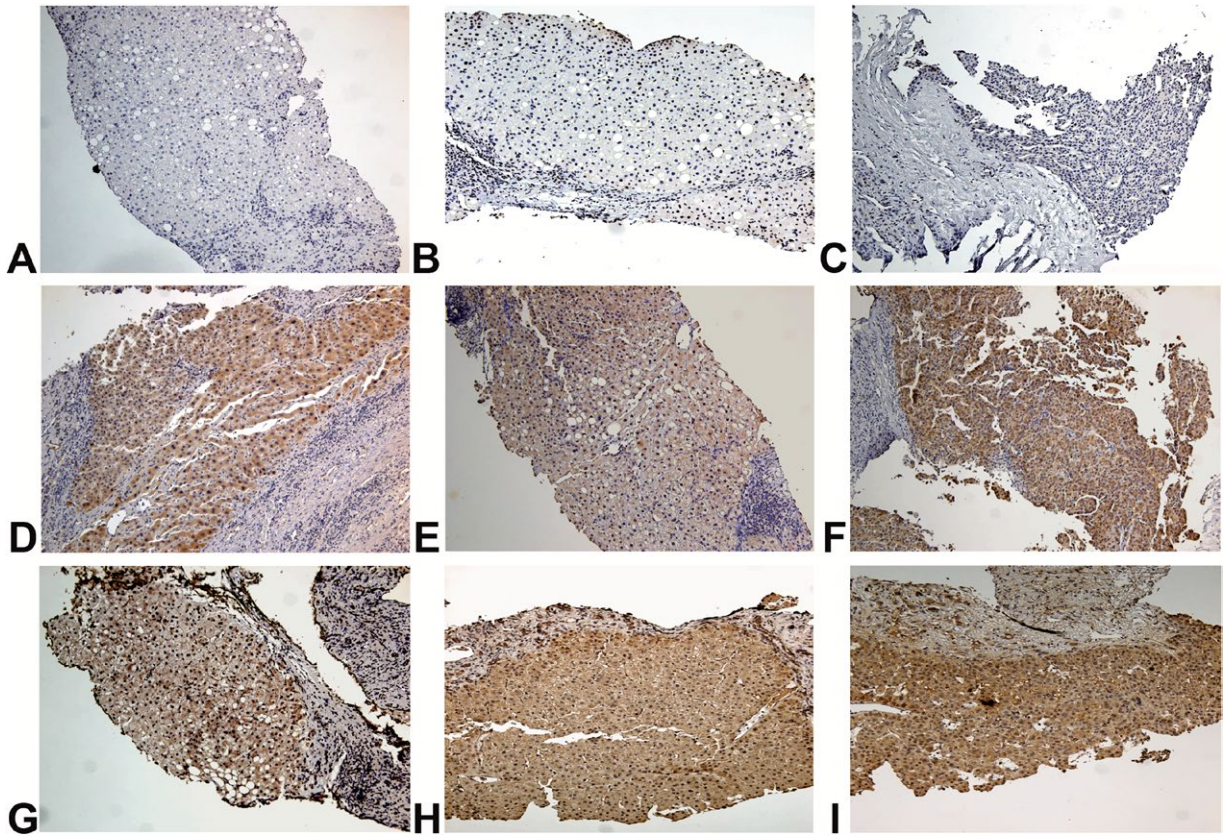

FIG. 2. Representative IHC images of ANGPT2 in tumor tissue and surrounding cirrhotic tissue of recurrent, *de novo*, and nonrecurrent HCC and in cirrhotic tissue of patients who did not develop HCC after DAA treatment. (A) Cirrhotic tissue from a patient enrolled in the prospective study of HCC development, who did not develop HCC after DAA treatment. (B,C) Nontumoral and tumoral tissue, respectively, from a patient in whom HCC did not recur after DAA treatment. (D-F) Tissue from the primary tumor (D), nontumor tissue at the time of primary tumor (E), and recurrent tumor tissue (F) from a patient in whom HCC recurred after DAA treatment. (G-I) Cirrhotic tissue obtained at enrollment in the prospective cohort of HCC development, 2 years before DAA treatment (G), and nontumor (H) and tumor tissue (I) obtained after HCC development after DAA treatment.

RELATIONSHIPS BETWEEN CLINICAL AND MOLECULAR DATA

Hepatic stiffness was related significantly to the expression level of ANGPT2 in tumoral (r = 0.526; *P*= 0.007, Pearson correlation) and nontumoral (r = 0.525; *P* = 0.003, Pearson correlation) tissues (Fig. 3). Histochemical expression level of ANGPT2 in tumor tissue was related to the HVPG, but not significantly (r = 0.545; *P* = 0.067, Pearson correlation). Significant inverse relationships were observed between ANGPT2 expression in tumor tissue and peak PVv (r= –0.412; *P* = 0.037, Pearson correlation) or mean PVv velocity (r = –0.409; *P* = 0.047, Pearson correlation). ANGPT2 expression in cirrhotic nontumoral tissue was inversely correlated with PVv (r = –0.391; *P*= 0.043, Pearson correlation). A significant positive relationship was found between the median ANGPT2 level in tumor tissue and presence of F2-F3 EV (*P* = 0.015, chi-squared test). No significant relationship was found between histochemical expression level of ANGPT2 and biochemical variables (platelet and white blood cell counts; levels of bilirubin, albumin, creatinine, hemoglobin, ALT, AST, Na, and CRP).

RISK FACTORS FOR HCC RECURRENCE AND *DE NOVO* DEVELOPMENT

Study 1: DAA-Treated Cohort

In univariate analyses, HCC recurrence was associated with ANGPT2 expression in primary tumor

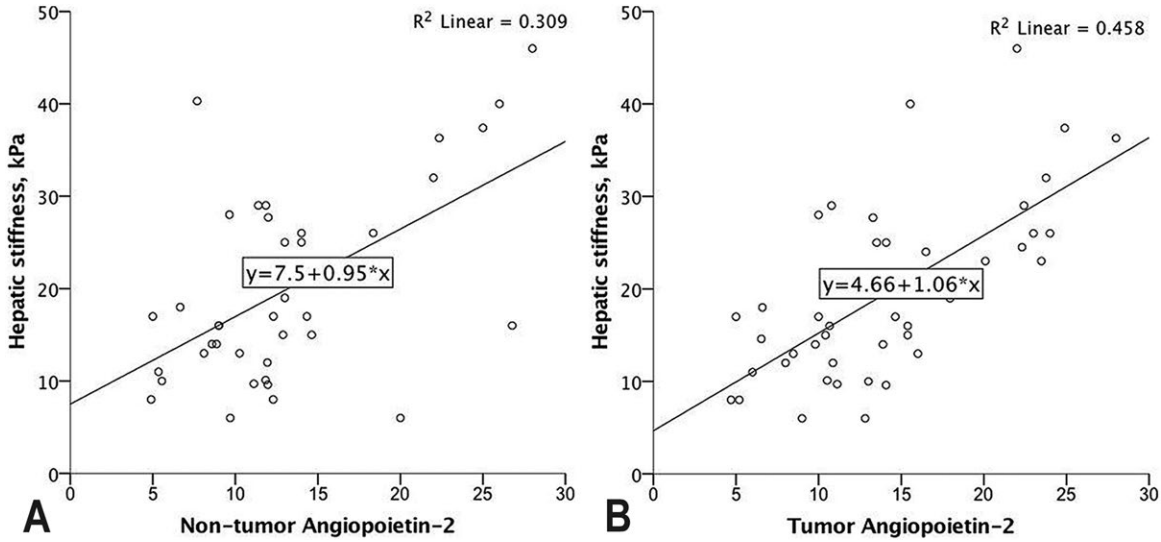

FIG. 3. Relationship between liver stiffness and ANGPT2, tested in cirrhotic nontumoral tissue (A) and tumor tissue (B). A significant relationship was present between expression level of ANGPT2 in tumoral (P = 0.002) and nontumoral (P = 0.026) liver tissues. Lines represent the best-fit regression line for each subgroup. Data were analyzed using the Pearson correlation test.

tissue (odds ratio [OR], 1.103; 95% confidence interval [CI], 1.035-1.176; P = 0.002), F2-F3 EV (OR, 2.514; 95% CI, 1.115-5.666; P = 0.026), and CRP level (OR, 9.534; 95% CI, 2.652-34.279; P = 0.001). In multivariate analysis, only ANGPT2 expression in the primary tumor was associated with the risk of recurrence (OR, 1.120; 95% CI, 1.040-1.207; P = 0.003; Table 3).

In patients with *de novo* HCC, tumor occurrence was associated with ANGPT2 expression in cirrhotic tissue obtained before the start of DAA treatment (OR, 1.496; 95% CI, 1.137-1.969; P = 0.004), CRP level (OR, 7.101; 95% CI, 1.940-25.997; P = 0.003), hepatic stiffness level (OR, 1.094; 95% CI, 1.042-1.269; P < 0.0001), splenic size (OR, 1.394; 95% CI, 1.181-1.646; P < 0.0001), presence of F2-F3 EV (OR, 5.236; 95% CI, 2.002-13.694; P = 0.001), albumin level (OR, 0.186; 95% CI, 0.040-0.863; P = 0.032), bilirubin level (OR, 2.077; 95% CI, 1.178-3.664; P = 0.012), and long-term SVR (OR, 0.189; 95% CI, 0.053-0.669; P = 0.010; Table 3). Given that the following four factors (hepatic stiffness, splenic size, variceal size, and HVPG) were highly correlated among them, only variceal size was tested in multivariate analyses as the most easily available on the routine clinical practice. In these analyses, only ANGPT2 expression in cirrhotic tissue was associated with risk of HCC occurrence (OR, 1.604; 95% CI, 1.080-2.382; P = 0.019; Table 3).

Study 2: Patients With Cirrhosis Enrolled in the Prospective Study of HCC Development

De novo occurrence of HCC was associated in univariate analyses with DAA treatment (OR, 5.406; 95% CI, 1.620-18.032; P = 0.006), viral etiology (OR, 5.938; 95% CI, 1.264-27.892; P = 0.024), AFP (OR, 1.059; 95% CI, 1.004-1.117; P = 0.036), and presence of F2-F3 varices (OR, 4.367; 95% CI, 1.316-14.485; P = 0.016). Because viral etiology and AFP levels were highly correlated with DAA use, only DAA use was tested in the multivariate model. In multivariate analysis, DAA treatment (OR, 4.770; 95% CI, 1.395-16.316; P = 0.013) and large varices (OR, 3.857; 95% CI, 1.127-13.203; P = 0.032) were related independently to HCC occurrence (Table 4). When including in the model HCV or HBV etiologies only, neither virus was significantly associated with *de novo* occurrence of HCC while large varices and DAA treatment remained independently related (data not shown).

Discussion

Recent reports regarding the putative association between DAA use and onset or recurrence of HCC have

TABLE 3. Associations of Study Variables With Recurrent and *De Novo* HCC After DAA Treatment (Study 1)

	Recurrent HCC				*De Novo* HCC			
Variable	Univariate Analysis OR (95% CI)	*P* Value	Multivariate Analysis OR (95% CI)	*P* Value	Univariate Analysis OR (95% CI)	*P* Value	Multivariate Analysis OR (95% CI)	*P* Value
Sex*	0.588 (0.224-1.547)	0.282			0.548 (0.192-1.562)	0.260		
Age (years)	1.023 (0.981-1.066)	0.293			0.973 (0.928-1.020)	0.253		
Genotype	1.001 (0.912-1.099)	0.983			1.031 (0.915-1.163)	0.613		
SVR	1.673 (0.210-13.321)	0.627			**0.189 (0.053-0.669)**	**0.010**		
ANGPT2	**1.103 (1.035-1.176)**	**0.002**	**1.137 (1.044-1.137)**	**0.003**	**1.496 (1.137-1.969)**	**0.004**	**1.604 (1.080-2.382)**	**0.019**
HVPG (mm Hg)	0.868 (0.738-1.021)	0.088			**1.361 (1.074-1.725)**	**0.011**		
Liver stiffness (kPa)	1.013 0.964-1.064)	0.616			**1.094 (1.042-1.269)**	**<0.0001**		
Spleen diameter (cm)	1.040 (0.910-1.188)	0.563			**1.394 (1.181-1.646)**	**<0.0001**		
F2-F3 EV	**2.514 (1.115-5.666)**	**0.026**	6.330 (0.481-8.351)	0.161	**5.236 (2.002-13.694)**	**0.001**	0.068 (0.007-2.057)	0.122
Cardiac index (L/min/m^2)	0.260 (0.073-0.925)	0.037			2.010 (0.835-4.839)	0.119		
Ascites	0.271 (0.036-2.033)	0.204			1.962 (0.645-5.964)	0.235		
BMI (kg/m^2)	0.962 (0.860-1.077)	0.505			0.916 (0.787-1.066)	0.258		
Diabetes	1.969 (0.770-5.036)	0.158			0.667 (0.146-3.039)	0.600		
Platelet count (×10^3/mm^3)	1.000 (1.000-1.000)	0.350			1.000 (1.000-1.000)	0.631		
Albumin (g/dL)	0.639 (0.204-2.005)	0.443			**0.186 (0.040-0.863)**	**0.032**		
Bilirubin (mg%)	0.583 (0.249-1.366)	0.214			**2.077 (1.178-3.664)**	**0.012**		
INR	1.727 (0.504-5.917)	0.384			3.512 (0.889-13.871)	0.073		
Creatinine (mg/dL)	1.310 (0.217-7.894)	0.768			1.383 (0.084-22.729)	0.820		
AST (IU/mL)	0.999 (0.991-1.007)	0.804			0.997 (0.983-1.012)	0.701		
ALT (IU/mL)	1.001 (0.996-1.007)	0.591			0.987 (0.968-1.006)	0.190		
GGT (IU/mL)	1.003 (1.000-1.007)	0.084			1.002 (0.996-1.008)	0.535		
ALP (IU/mL)	1.005 (0.997-1.013)	0.232			**1.013 (1.004-1.023**	**0.006**		
CRP (mg/dL)	**9.534 (2.652-34.279)**	**0.001**	0.397 (0.031-5.076)	0.477	**7.101 (1.940-25.997)**	**0.003**	2.543 (0.210-15.882)	0.463

*Male sex used as reference. Bold characters indicate significant values.

prompted many researchers to try to identify patients undergoing DAA treatment who are at greater HCC risk. In the present study, we mainly focused on neo-angiogenesis, evaluated by testing for the neo-angiogenic transcriptomic signature[24] and hepatic expression of ANGPT2, as potentially favoring HCC onset and recurrence in these patients. In recurring HCC and *de novo* HCC after DAA treatment, we found striking activation of neo-angiogenesis, as shown by the significantly greater frequency of the neo-angiogenic transcriptomic signature (with consistent expression of ANGPT2 at the highest level) and the significantly

TABLE 4. Associations of Study Variables With HCC Occurrence in Patients Enrolled in a Prospective Study of HCC Development in LC (Study 2)

Variable	Univariate Analysis OR (95% CI)	*P* Value	Multivariate Analysis OR (95% CI)	*P* Value
Sex*	0.448 (0.095-2.115)	0.311		
Age (years)	1.016 (0.961-1.074)	0.582		
Viral etiology	**5.938 (1.264-27.892)**	**0.024**		
DAAs	**5.406 (1.620-18.032)**	**0.006**	**4.770 (1.395-16.316)**	**0.013**
NUCs	1.283 (0.393-4.191)	0.680		
HVPG (mm Hg)	1.050 (0.963-1.146)	0.267		
Liver stiffness (kPa)	1.008 (0.965-1.052)	0.724		
Splenic diameter (cm)				
F2-F3 EV	**4.367 (1.316-14.485)**	**0.016**	**3.857 (1.127-13.203)**	**0.032**
Decompensated cirrhosis	0.800 (0.168-3.817)	0.780		
BMI (kg/m^2)	0.888 (0.718-1.098)	0.272		
Diabetes	0.985 (0.829-1.170)	0.859		
Platelets ($\times 10^3/mm^3$)	1.000 (0.990-1.010)	0.965		
Albumin (g/dL)	0.510 (0.197-1.321)	0.165		
Bilirubin (mg/dL)	0.904 (0.599-1.365)	0.632		
INR	3.133 (0.365-26.937)	0.298		
Creatinine (mg%)	0.742 (0.173-3.188)	0.688		
AST (IU/mL)	1.007 (0.994-1.020)	0.301		
ALT (IU/mL)	0.998 (0.989-1.007)	0.686		
GGT (IU/mL)	1.003 (0.990-1.016)	0.626		
ALP (IU/mL)	1.007 (0.995-1.019)	0.233		
AFP (ng/mL)	**1.059 (1.004-1.117)**	**0.036**		
CRP (mg/dL)	0.549 (0.203-1.483)	0.237		

*Male sex used as reference. Bold characters indicate significant values.

greater expression of ANGPT2 in hepatocytes and vessel endothelia in tumoral and surrounding cirrhotic tissue. In patients with recurrence, this pattern was observed in the primary and recurrent tumors whereas in patients with no recurrence, no or slight activation of neo-angiogenesis in tumor tissue and no activation in the surrounding tissue were observed. Also, in patients with *de novo* HCC, ANGPT2 expression was elevated in tumor and nontumoral tissues, as well as in the cirrhotic parenchyma, well before DAA treatment and HCC onset. Cirrhotic tissue from patients with cirrhosis who underwent DAA treatment but did not develop HCC showed little or no expression of ANGPT2.

The extremely high expression of ANGPT2 in recurrent and *de novo* HCCs had its counterpart in the increased levels of circulating VEGF during DAA therapy, which persisted after treatment was stopped. VEGF is known to be a critical factor for induction of ANGPT2 expression, specifically under conditions of slow flow, as in advanced cirrhosis.[25] This property seems to have been confirmed in our study by the observation of an inverse relationship between ANGPT2 expression and portal vein flow. Indeed, we have found the highest levels of ANGPT2 expression in patients with the lowest flow velocity, as measured by Doppler ultrasonography. In addition, these patients had increased VEGF levels. DAAs are capable of inducing an increase in VEGF,[26] which, in turn, is critical for induction of ANGPT2 expression. Baseline ANGPT2 expression, however, is not similar in all patients. Only patients with very advanced fibrotic conditions, as indicated by the strict linear relationship between ANGPT2 hepatic expression and hepatic stiffness, are predisposed. In these patients, the intrahepatic microcirculation is greatly altered, the blood flow is slowed, and shear stress is substantially enhanced. ANGPT2 is a critically shear-stress–regulated gene[25,27]; under experimental conditions that mimic the slow blood flow of portal hypertension arising through progression of chronic liver damage, ANGPT2 mRNA, protein expression, and release are up-regulated after 24 hours of application of shear

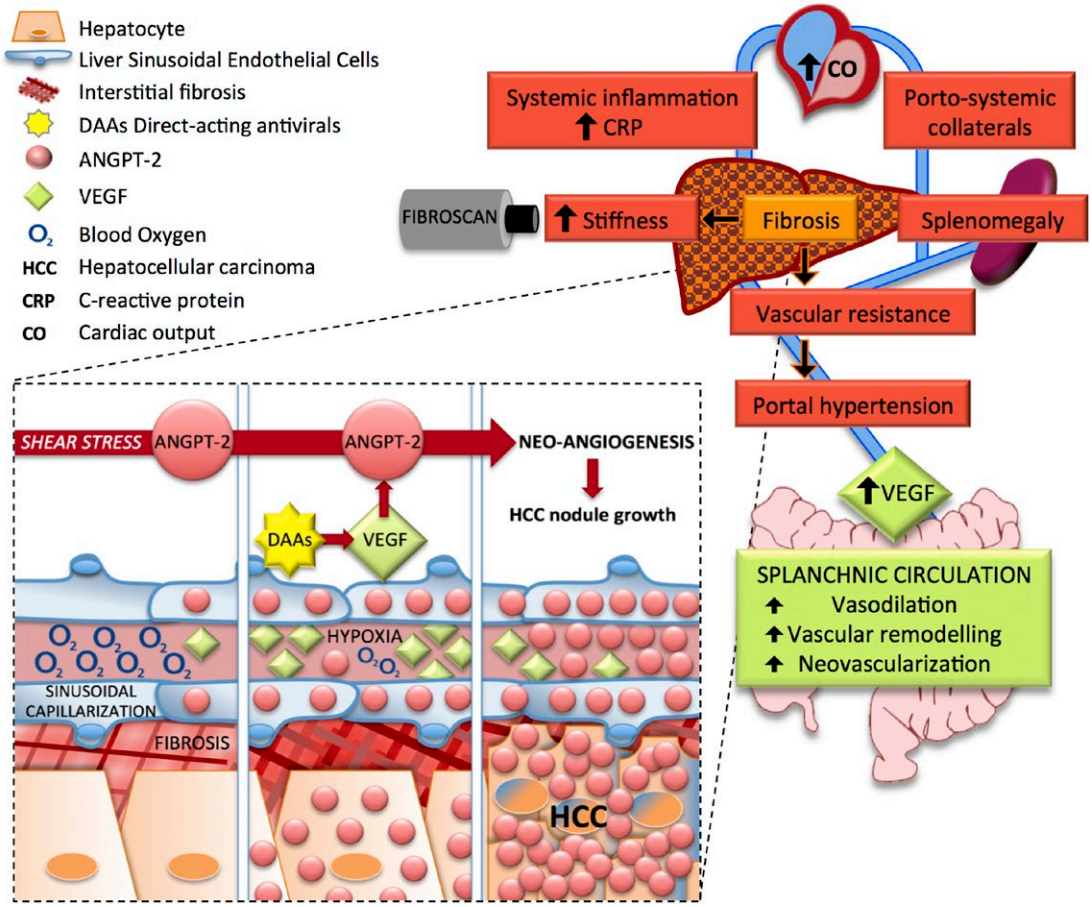

FIG. 4. Hypothesis of sequential events favoring HCC development after DAAs. In patients with cirrhosis with splanchnic collateralization attributed to portal hypertension, DAAs induce an increase of already elevated circulating VEGF, which further activates ANGPT2 expression in livers of predisposed patients, that is, those with severe fibrosis and increased microvascular shear stress, triggering carcinogenesis.

stress.[25] Under these conditions, VEGF elevation plays a substantial role in the induction of ANGPT2 expression.[25] The interaction between local overexpression of ANGPT2 and circulating VEGF has been found to lead to marked increases in vascularization and perfusion of experimental liver tumors.[28]

From this perspective, DAAs might play a minor role through their demonstrated capacity to increase VEGF levels during treatment.[26] The data from patients with cirrhosis enrolled in the prospective study of HCC development indicate that DAAs are capable of significantly favoring HCC onset, given that DAA use was independently related to HCC occurrence in regression analysis. However, the identification of large varices as a factor independently related to HCC onset indicates that DAAs act through the main predisposing condition, that is, severe portal hypertension and the linked modification of hepatic and splanchnic microcirculation, which, in turn, give rise to ANGPT2 activation (Fig. 4). DAAs thus appear to critically activate ANGPT2-mediated angiogenesis only in predisposed patients, that is, those with more fibrosis, extensive splanchnic collateralization, and more altered intrahepatic and splanchnic blood flows. This suggestion is consistent with the hypothesis put forward by Fernandez et al.[29,30] of VEGF-driven splanchnic angiogenesis, based on findings in animals with portal hypertension and patients with cirrhosis. VEGF is known to be overexpressed in splanchnic organs from portal hypertensive animals,[31,32] and several factors present in patients with cirrhosis, that is, tissue hypoxia, cytokine imbalance, and shear stress, are known to promote VEGF expression.[33,34] Not surprisingly, HCC did not develop or recur in any patient

with small varices or a low (<16 kPa) hepatic stiffness value during DAA treatment. Along the same line are the strong relationships found in regression analyses between HCC occurrence or recurrence and factors related to advanced fibrotic disease and severe portal hypertension, such as splenic size and low PVv. This is consistent with the suggestion put forward by Kozbial et al. that the risk of developing HCC is linked with advanced stage of cirrhosis of patients treated with DAAs.[35] Much less evident was the relationship with liver function, given that albumin level was related to *de novo* HCC occurrence only in univariate analysis.

The role of local immunity, evaluated by PD1 and PDL1 expression, was less relevant in this study than in previous ones. No significant difference was found in the expression of these two markers among subgroups, except for a difference in PD1 expression between *de novo* HCC and other tissues. The higher PD1 expression in *de novo* HCC suggests a more relevant role of lower local immune control in this subgroup of cases. This is indeed consistent with the higher presence of the transcriptomic signature (as a marker of high growth speed and biological aggressiveness) and of the clinical aggressive behavior of these HCCs. PDL1 instead does not seem to play a major role in this scenario.

The small number of patients with recurrent and *de novo* HCC in study 1 may represent a limitation of this study. However, the remarkably clear-cut difference in the predictive biomarkers of tumor development and clinical features between HCC patients and the large group of subjects not developing HCC give strength to our data, which derived from the analysis of well-characterized prospective cohorts.

In conclusion, our study findings suggest that DAAs are not *per se* able to determine the occurrence or recurrence of HCC, but that the DAA-mediated increase in VEGF acts as a trigger in predisposed patients, that is, those with severe fibrosis and splanchnic collateralization, who already show high activation of neo-angiogenetic pathways in cirrhotic tissue (Fig. 4). These patients would have had a high probability of developing HCC during the natural history of their liver disease, and, given the intrinsic characteristics of the liver microenvironment,[23,24] their tumors would have had distinctive clinical and biological aggressiveness. The combination of the clinical and biological risk factors that we have identified gives the unique possibility of selecting the patients at real risk of developing HCC after DAAs, without the need of holding back from treatment patients with cirrhosis at low risk of HCC.[36]

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