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High serum angiopoietin-2 level predicts non-regression of liver stiffness measurement-based liver fibrosis stage after direct-acting antiviral therapy for hepatitis C

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Short title: Ang2 predicts changes in liver fibrosis

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Abstract

***Background*:** Factors associated with improvement of liver fibrosis after successful hepatitis C virus (HCV) eradication by interferon (IFN)-free direct-acting antiviral agents (DAAs) have been not clarified well. Angiopoietin-2 (Ang2) is reported to be associated with vascular leak and inflammation observed in patients with advanced liver fibrosis.

Methods: In this retrospective study, patients treated with IFN-free DAAs who underwent transient elastography before and at 24-weeks post-treatment and achieved sustained viral response were enrolled. Baseline serum Ang2 was measured, and its relationship with other clinical factors was analyzed. Liver fibrosis stage was defined based on liver stiffness according to a previous report. Predictive factors for regression of liver fibrosis stage after DAA therapy were evaluated.

Results: Overall, 116 patients were analyzed. Baseline serum Ang2 levels were significantly associated with liver stiffness, spleen index, and liver stiffness-based liver fibrosis stage. Moreover, 75% of patients experienced regression of liver fibrosis stage after DAA therapy. Multivariate analysis revealed that advanced liver fibrosis stage and Ang2 levels were significantly associated with regression of liver fibrosis stage after DAA therapy. In patients with advanced liver fibrosis (F3/4), baseline Ang2 level alone could predict regression of liver fibrosis stage. A baseline Ang2 cutoff value (354 pg/ML) could predict regression of liver fibrosis stage after DAA therapy with high accuracy (sensitivity 0.882, specificity 0.733).

Conclusions: Evaluation of serum Ang2 levels before DAA therapy is important. Our results provide a novel mechanistic insight into non-regression of liver stiffness after DAA therapy. Long-term and larger studies are required.

Keywords: HCV, DAAs, angiopoietin-2, liver stiffness measurement

Introduction

Hepatitis C virus (HCV) infection is the major cause of liver cirrhosis and hepatocellular carcinoma (HCC); thus, effective and safe treatment is crucial. Recently developed direct-acting antiviral agents (DAAs) revolutionized the treatment of HCV-infected patients; thus, even HCV-infected patients with complications such as advanced liver fibrosis, renal dysfunction, and co-infection of human immunodeficiency virus could achieve sustained viral response (SVR) at a high rate [1-4].

Several studies on interferon (IFN)-based therapy for patients with HCV infection revealed that a subset of patients who achieved successful HCV eradication experienced improvement of liver fibrosis [5, 6]. Tachi et al. revealed that 45% of patients who experienced HCV eradication by IFN experienced regression of liver fibrosis stage; however, in 48% and 6% of the patients, the liver fibrosis stage did not change or worsened, respectively [5]. Moreover, a study revealed that progressive liver fibrosis after successful HCV eradication is an independent risk factor of HCC occurrence [5].

Similar to IFN-based therapy, Mauro et al. reported that after SVR by DAA therapy in post-liver transplant recurrent hepatitis C, 43% of patients with liver cirrhosis experienced liver fibrosis regression, that is, more than half of the liver-transplanted patients with liver fibrosis did not improve or progressed even after successful HCV eradication [7]. Additionally, when sofosbuvir (SOF) and velpatasvir were used for HCV-infected patients with decompensated liver cirrhosis, after successful HCV eradication, 42% and 11% of patients experienced no change or had a worse Child-Pugh score [8]. Moreover, Takehara et al. [1] reported that 21% of HCV-infected Japanese patients with decompensated liver cirrhosis treated with SOF and velpatasvir experienced no change of the Child-Pugh score.

Based on these previous reports, even after HCV eradication, some subsets of patients with successful HCV eradication experience liver fibrosis progression, which could be a risk factor for HCC after HCV eradication. However, risk factors of liver fibrosis progression after HCV eradication have not been clarified. Recently, Seko et al. reported that the presence of varices was an independent predictor of liver function after successful HCV eradication by DAA therapy [9]. Thus, we hypothesized that portal hypertension-induced hypoxia might be involved in non-regression of liver fibrosis stage after HCV eradication.

Angiopoietin-2 (Ang2) is a context-dependent antagonist of the Tie2-mediated signaling which is associated with vessel stabilization [10, 11]; thus, increased Ang2 level causes vascular leak and inflammation. Moreover, elevated serum Ang2 level was observed in patients with advanced liver fibrosis and HCC [12]. Importantly, portal hypertension-induced slow blood flow could increase Ang2 expression [13, 14]. Recently, several studies revealed that high Ang2 expression could predict de novo or recurrent HCC after DAA therapy, mortality, and worse kidney outcomes in decompensated cirrhosis and nonalcoholic steatohepatitis (NASH) [12, 14, 15].

The gold standard for liver fibrosis diagnosis is liver biopsy. However, liver biopsy occasionally causes severe complications and is prone to sampling error. Recently, several non-invasive methods for the evaluation of liver fibrosis have been developed. FibroScan (Echosens, Paris, France) can perform liver stiffness measurement (LSM) for liver fibrosis assessment with great accuracy [16]. Thus, in this study, we hypothesized that serum Ang2 might predict non-regression of liver fibrosis stage after successful HCV eradication by DAAs and, by evaluating liver fibrosis using LSM, we aimed to analyze this hypothesis.

Methods

Patients and study design

In this retrospective study at Hokkaido University Hospital conducted between October 2014 and July 2016, a total of 206 patients with HCV infection who received IFN-free DAA therapy were screened. Patients were included if they had complete clinical information, preserved serum samples, and paired FibroScan LSM for liver fibrosis assessment at baseline and had achieved SVR at week 24 (SVR24). Patients were excluded if they did not achieve SVR, had a history of liver transplantation, had missing clinical information, were co-infected with human immunodeficiency virus or hepatitis B virus, had another liver disease, had severe renal dysfunction with hemodialysis, did not undergo paired FibroScan examination at baseline and SVR24, and had a history of HCC.

Patients were assessed via physical examinations and blood tests at baseline and every 2 weeks during the treatment, and every 3 months after treatment termination. In this study, data were collected at baseline, SVR24, and end of treatment (EOT).

Serum Ang2 levels were measured by a commercial enzyme-linked immunosorbent assay according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

The study was approved by the ethics committee of Hokkaido University Hospital. The protocol of this study conformed to the ethical guidelines of the Declaration of Helsinki and was registered at the UMIN Clinical Trials Registry as UMIN000031091.

LSM and controlled attenuation parameter

FibroScan 502 (Echosens, Paris, France) was utilized for LSM and controlled attenuation parameter (CAP) evaluation with the M-probe and XL-probe. As described previously, each patient was placed in the supine position with the right hand at the most

abducted position during the procedure [17]. At least 10 valid measurements were obtained, and effective measurements were defined as those >60% with an interquartile range of <30%.

Definition of liver fibrosis stage according to transient elastography data

In this study, fibrosis stage was defined using transient elastography data (Fibroscan; Echosens, Paris, France); cut-off values were 7.1 kPa for F ≥ 2, 9.5 kPa for F ≥ 3, and 12.5 kPa for F4, according to a previous report [18].

Regression of liver fibrosis stage

In this study, we defined regression of liver fibrosis stage as follows: in patients with liver fibrosis F2 to F4 stage based on FibroScan data, after successful HCV eradication, liver fibrosis stage decreased more than 1 stage, and in patients with liver fibrosis F0/1, liver fibrosis stage did not worsen.

Spleen index

Spleen size was evaluated by ultrasonography according to the spleen index: transverse diameter × vertical diameter × 0.9 [19].

Statistical analyses

Continuous variables were analyzed with the paired Mann-Whitney *U*-test, Wilcoxon test, or one-way analysis of variance, as appropriate. Categorical data were analyzed by the chi-squared test. Cutoff point was based on the receiver operating characteristic (ROC) curve by maximizing the Youden index. The relationship between two variables was analyzed by Spearman's rank correlation. A multivariate logistic regression analysis with stepwise forward selection was performed with variables regarded significant at $P<0.001$ in the

univariate analyses. All *P*-values were two-tailed, and the level of significance was set at $P < 0.05$. Statistical analyses were performed using SPSS version 24.0 (IBM Japan, Tokyo, Japan) and Prism 7.03 (GraphPad Software, Inc., La Jolla, CA).

Results

Patients

A total of 206 HCV-infected patients who received IFN-free DAA therapy between October 2014 and January 2016 were screened. Of these 206 patients, 116 who had paired FibroScan examination data at baseline and SVR24, complete clinical information, and preserved serum were included in this study (**Figure S1**). The baseline characteristics of the enrolled patients are shown in **Table 1**. Mean age was 66 (range, 22–87) years, and 43 (37.1%) were male. Overall, 20, 49, 42, and 5 patients were treated with daclatasvir plus asunaprevir (ASV), SOF plus ledipasvir, SOF plus ribavirin, and ombitasvir plus paritaprevir boosted with ritonavir, respectively. In LSM-based liver fibrosis evaluation, 64, 20, 9, and 23 patients were classified as having F0-1, F2, F3, and F4, respectively. The baseline median HCV-RNA titer was 6.3 Log IU/mL (3.6–7.2), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were 39 IU/L (range, 14–180) and 38 IU/L (range, 6–273), respectively, and the spleen index was 25.9 (11.1–59.1).

Correlation between baseline Ang2, LSM, and liver fibrosis stage based on FibroScan data or spleen index

Because previous studies showed that portal hypertension-induced slow blood flow could cause increased Ang2 expression [13, 14], we evaluated the relationship between baseline serum Ang2 level and factors related to portal hypertension, namely, LSM, spleen index, and LSM-based liver fibrosis stage. As shown in Figure 1A, baseline LSM and serum Ang2 levels were significantly correlated (r =0.35, P=0.01). Additionally, as shown in Figure 1B, this correlation became stronger in patients with advanced liver fibrosis (r =0.56, P<0.001). Similar to LSM, baseline spleen index and serum Ang2 level were significantly correlated (r =0.22, P=0.02; Figure 1C), and this correlation was stronger in patients with advanced liver fibrosis (r =0.41, P =0.03; Figure 1D).

Moreover, baseline Ang2 levels were significantly different among LSM-based fibrosis stages and were significantly higher in patients with F3/4 than in those with F0 to F2 (Figure 1E and 1F). Additionally, we analyzed the relationship between the existence of esophageal varices and baseline serum Ang2 level in patients who underwent endoscopy before DAA therapy (n=10). As shown in Figure S2, Ang2 level had a tendency to be higher in patients with esophageal varices than in those without esophageal varices. These results indicated that serum Ang2 level is associated with portal hypertension-induced clinical features.

On the contrary, as shown in Supplementary Figure 3, Ang2 level did not show significant correlation with liver steatosis (CAP value).

Changes in liver fibrosis stage based on FibroScan data after successful HCV eradication by DAA therapy

As shown in Table 2, 87 (75%) patients experienced regression of LSM-based liver fibrosis stage, and 29 (25%) patients experienced non-regression of liver fibrosis stage.

Factors associated with non-regression of LSM-based liver fibrosis stage after successful HCV eradication

Subsequently, we analyzed the factor associated with regression of LSM-based liver fibrosis stage after successful HCV eradication by DAAs. Univariate analysis revealed that baseline fibrosis stage (P<0.001), baseline platelet count (P<0.001), AST (P<0.001), Ang2 level (P<0.001), FIB-4 index (P<0.001), albumin level (p=0.013), and spleen index (P=0.017) were significantly associated with progression of liver fibrosis stage after successful HCV eradication by DAAs.

Then, we carried out a multivariate logistic regression analysis using the factors with p value <0.001 in the univariate analysis (fibrosis stage (P<0.001), baseline platelet count (P<0.001), AST (P<0.001), FIB-4 index (P<0.001), and Ang2 level (P<0.001)). Multivariate analysis revealed that baseline fibrosis stage (odds ratio 4.474; 95% confidence interval, 1.651–12.125; P=0.003) and Ang2 level (odds ratio 1.004; 95% confidence interval, 1.001–1.007; P=0.006) were significantly associated with regression of liver fibrosis stage based on FibroScan data after DAA therapy (Table 3). Because baseline fibrosis stage affects regression of liver fibrosis stage, we conducted stratified analysis according to baseline fibrosis stage (F0 to F2 and F3/4). As shown in Table S1, in patients with baseline fibrosis stage of F0 to F2, fibrosis stage, white blood cell count, FIB-4 index, and platelet count are significantly associated with regression of liver fibrosis stage after DAA therapy.

Importantly, in patients with baseline fibrosis stage of F3/4, as shown in Table 4, baseline Ang2 alone is significantly associated with regression of liver fibrosis stage after DAA therapy.

Additionally, because the baseline LSM-based liver fibrosis stage might be affected by inflammation due to HCV infection and the transient elastography data were affected by HCV eradication, we conducted additional analysis to minimize the effect of decreased

inflammation due to HCV eradication. We compared the regression of liver fibrosis stage at EOT, SVR24, and SVR96. In this study, a total of 24 patients were evaluated by transient elastography at baseline, EOT, and SVR24. Of those, 22 patients were evaluated by transit elastography data at baseline, EOT, SVR24, and SVR96. Then, we evaluated the regression of LSM-based liver fibrosis stage in those patients at EOT, SVR24, and SVR96. As shown in Supplementary Table 2, regression of LSM-based liver fibrosis stage between EOT and SVR24 was significantly associated with AST level, FIB4 index, and Ang2 level at EOT. Importantly, as shown in Supplementary Table 3, regression of LSM-based liver fibrosis stage between EOT and SVR96 was significantly associated with only the Ang2 level and liver fibrosis stage 3/4 at EOT. Although the number of patients was limited, after minimization of inflammation due to HCV infection, Ang2 level was still significantly associated with LSM-based liver fibrosis stage regression after DAA therapy.

Ang2 level between patients with or without regression of liver fibrosis stage and changes in Ang2-level between baseline and EOT

Subsequently, we compared the baseline Ang2 level stratified according to liver fibrosis stage with or without regression of liver fibrosis stage after DAA therapy. As shown in Figure 2A and 2B, in patients with fibrosis stages F3, F4, and F3/4, the mean Ang2 levels were significantly higher in patients with liver fibrosis non-regression than in patients with liver fibrosis regression. Then, we analyzed changes in Ang2 level before and after DAA treatment. As shown in Figure S4A, overall, the mean Ang2 levels significantly decreased after DAA therapy; however, in patients with baseline advanced liver fibrosis (F3/4), the mean Ang2 levels were similar between before and after DAA treatment (Figure S4B). In the subgroup analysis stratified according to fibrosis stage (F0–F2 and F3/4) with or without regression of liver fibrosis stage after DAA therapy, patients with non-regression of liver

fibrosis stage after DAA therapy and baseline fibrosis stage F3/4 had a tendency to show an increased Ang2 level in contrast to those with regression of liver fibrosis stage and baseline fibrosis stage F3/4 (Figure S4C).

Baseline Ang2 levels as predictive factors of progression of liver fibrosis stage after successful HCV eradication by DAA therapy

Subsequently, we conducted ROC analysis to determine the cutoff baseline Ang2 level associated with non-regression of liver fibrosis stage after successful HCV eradication by DAA therapy. As shown in **Figure 3A**, the cutoff value was set at 354 pg/mL (sensitivity, 0.882; specificity, 0.733; ROC-AUC, 0.855). As shown in **Figure 3B and 3C**, in patients with baseline advanced liver fibrosis (F3/4), 59.4% (19/32) had baseline Ang2 level more than 354 pg/mL. Of those, 79% (15/19) experienced non-regression of liver fibrosis stage (**Figure 3B**). A total of 40.6% (13/32) had baseline Ang2 level <354 pg/mL, of which 85% (11/13) experienced regression of liver fibrosis stage **(Figure 3C).**

Discussion

Recent development in DAAs has dramatically change anti-HCV therapy with high SVR rate and safety, even in patients with other complications and advanced liver fibrosis, including decompensated liver cirrhosis [1, 4, 20-26]. After successful HCV eradication, the risks of HCC occurrence and liver fibrosis progression have generally decreased; however, a definite number of patients experienced liver fibrosis progression even after successful HCV eradication by DAAs [5, 6]. Liver fibrosis progression after successful HCV eradication is a clinically important issue because it predicts HCC occurrence [5], and in patients with decompensated liver cirrhosis, it could cause fatal hepatic failure. Therefore, predicting HCC

and liver fibrosis progression is clinically crucial; however, the prediction methods have not been clarified.

In the present study, 75% (87/116) of the patients experienced improvement of LSM-based liver fibrosis stage (Table 2). The multivariate analysis revealed that baseline liver fibrosis stage based on LSM and baseline serum Ang2 levels were significantly associated with non-regression of liver fibrosis stage after successful HCV eradication by DAA therapy (**Table 3).** The association between baseline advanced fibrosis stage and liver fibrosis progression after DAA therapy is consistent with that in a previous report [5], which showed that liver fibrosis stage (F0–F2 vs F3/4) was associated with progression of liver fibrosis after IFN-based therapy. However, compared with the previous report [5], the rate of improvement of liver fibrosis stage was high in the present study. These discrepancies might be attributable to the different liver fibrosis diagnostic methods used for pathological evaluation and LSM-based liver fibrosis staging. The LSM-based liver fibrosis stage could be affected by inflammation due to HCV infection; thus, this should be considered when comparing present with previous findings based on liver biopsy.

The multivariate analysis also revealed that baseline Ang2 levels significantly predicted regression of liver fibrosis stage after successful HCV eradication. In the subgroup analysis of patients with baseline advanced liver fibrosis (F3 and F4 stage), baseline Ang2 levels alone could predict progression of liver fibrosis stage after successful HCV eradication (Table 4). Thus, baseline serum Ang2 level might be a significant factor associated with liver fibrosis non-regression after successful HCV eradication by DAAs, especially in patients with advanced liver fibrosis. In the present study, as well as in a previous report [12], Ang2 levels were significantly correlated with LSM and spleen size, which are factors associated with portal hypertension (Figure 1A-D) [27]. This result is consistent with those in previous

reports that portal hypertension-induced slow blood flow could increase Ang2 expression [13, 14].

Recently, Seko et al. reported that the presence of varices was an independent predictor of deterioration of the FIB-4 index after successful HCV eradication by DAAs [9]. Additionally, Mauro et al. revealed that pretreatment of high hepatic venous pressure gradient and LSM are significant determinants of liver fibrosis non-regression after SVR by DAAs in liver transplantation patients [7]. The results of those previous reports and the present study support the hypothesis that baseline advanced portal hypertension causes non-regression of liver fibrosis stage after successful HCV eradication by DAAs. In the present study, although the overall Ang2 level significantly decreased after DAA therapy (Supplementary Figure 4A, P=0.01), as shown in a previous report [14], serum Ang2 levels were comparable between baseline and EOT in patients with advanced liver fibrosis (Figure S4B). However, as shown in Supplementary Figure 4C, in patients without liver fibrosis stage regression, Ang2 levels tended to increase; on the contrary, in patients with liver fibrosis stage regression, Ang2 levels tended to decrease. Because portal hypertension-induced slow blood flow can enhance Ang2 expression [13, 14], in patients with liver fibrosis stage regression, portal hypertension might be restored; on the contrary, in patients without liver fibrosis regression, portal hypertension might tend to show no change or worsen. Additionally, increased Ang2 level causes vascular leak and inflammation via the antagonistic effect of Tie2-mediated signaling [10, 11]. Thus, continuously high Ang2 levels might cause progressive focal liver inflammation, resulting in non-regression of LSM-based liver fibrosis stage. Further analysis in this regard is required.

Recently, Ang2 was reported to predict de novo and recurrent HCC after successful HCV eradication by DAAs [14]. Faillaci et al. showed that Ang2 expression in HCC or in cirrhotic liver tissue in patients with HCV infection was independently associated with risk of

HCC recurrence or occurrence, and serum Ang2 levels after DAA therapy were significantly correlated with liver tissue Ang2 expression. In the present study, although Ang2 levels were similar between baseline and EOT in patients with advanced liver fibrosis (F3/4) (Figure S4B), serum Ang2 levels tended to increase in patients with liver fibrosis non-regression after DAA therapy. As a result, compared with baseline Ang2 level, serum Ang2 level at EOT could predict liver fibrosis progression after successful HCV eradication more accurately (baseline Ang2 level, cutoff value, 354 pg/mL, sensitivity 0.882, specificity 0.733; EOT Ang2 level, cutoff value, 354 pg/mL, sensitivity 0.941, specificity 0.867) (Figure S5). Taking together the results of the present and previous studies, high serum Ang2 level might predict liver fibrosis progression after successful HCV eradication by DAA therapy. However, further analysis is required.

Recently, Lefere et al. reported that serum Ang2 levels were significantly elevated in patients with NASH than in those with simple liver steatosis [15]. Importantly, the authors revealed that inhibition of Ang2 reduced hepatocyte ballooning and fibrosis in a NASH mouse model. This report clearly revealed that Ang2-mediated signaling could be a therapeutic target; thus, in patients with increased Ang2 level and liver fibrosis non-regression after successful HCV eradication, inhibitors of Ang2 might be effective and reduce liver fibrosis progressions and HCC occurrence.

This study has several limitations. First, as this was a retrospective study, some data might be missing, including that regarding fibrosis markers, such as Mac2 binding protein glycosylation isomer and autotaxin. Second, the gold standard for liver fibrosis stage diagnosis is liver biopsy; however, because biopsy may cause various complications and prone to sampling error, we evaluated the LSM-based liver fibrosis stage, and this should be considered when interpreting the present these. Third, the number of patients analyzed and

observation period were relatively limited, especially in the analysis at EOT, SVR24, and SVR96; therefore, a longer prospective study with a larger number of patients is required.

In conclusion, advanced LSM-based liver fibrosis stage and baseline Ang2 levels were significantly associated with regression of liver fibrosis stage after DAA therapy. Additionally, in patients with baseline advanced liver fibrosis, baseline Ang2 level alone could predict non-regression of liver fibrosis stage. A baseline Ang2 cutoff value (354 pg/mL) could predict non-regression of liver fibrosis stage with high accuracy (sensitivity 0.882 and specificity 0.733) in patients with advanced liver fibrosis. Thus, the results of this study might indicate the importance of the evaluation of serum Ang2 levels before DAA therapy and provide a novel mechanistic insight into non-regression of LSM-based liver fibrosis stage after DAA therapy. A longer prospective study with a larger number of patients is required to validate these results.

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References

1. Takehara T, Sakamoto N, Nishiguchi S, et al. Efficacy and safety of sofosbuvir-velpatasvir with or without ribavirin in HCV-infected Japanese patients with decompensated cirrhosis: an open-label phase 3 trial. J Gastroenterol. 2019;54(1):87-95.
2. Suda G, Ogawa K, Morikawa K, et al. Treatment of hepatitis C in special populations. J Gastroenterol. 2018;53(5):591-605.
3. Suda G, Furusyo N, Toyoda H, et al. Daclatasvir and asunaprevir in hemodialysis patients with hepatitis C virus infection: a nationwide retrospective study in Japan. J Gastroenterol. 2018;53(1):119-28.
4. Suda G, Nakai M, Sho T, et al. The Successful Retreatment with Glecaprevir and Pibrentasvir of Genotype 1 or 2 HCV-infected Hemodialysis Patients who Failed to Respond to NS5A and Protease Inhibitor Treatment. Internal medicine (Tokyo, Japan). 2019;58(7):943-7. Epub 2018/12/21.
5. Tachi Y, Hirai T, Miyata A, et al. Progressive fibrosis significantly correlates with hepatocellular carcinoma in patients with a sustained virological response. Hepatol Res. 2015;45(2):238-46.
6. Shiratori Y, Imazeki F, Moriyama M, et al. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. Ann Intern Med. 2000;132(7):517-24.
7. Mauro E, Crespo G, Montironi C, et al. Portal pressure and liver stiffness measurements in the prediction of fibrosis regression after sustained virological response in recurrent hepatitis C. Hepatology. 2018;67(5):1683-94.
8. Curry MP, O'Leary JG, Bzowej N, et al. Sofosbuvir and Velpatasvir for HCV in Patients with Decompensated Cirrhosis. N Engl J Med. 2015;373(27):2618-28.
9. Seko Y, Moriguchi M, Hara T, et al. Presence of varices in patients after hepatitis C virus eradication predicts deterioration in the FIB-4 index. Hepatol Res. 2019;49(4):473-8.
10. Souma T, Thomson BR, Heinen S, et al. Context-dependent functions of angiopoietin 2 are determined by the endothelial phosphatase VEPTP. Proc Natl Acad Sci U S A. 2018;115(6):1298-303.
11. Benest AV, Kruse K, Savant S, et al. Angiopoietin-2 is critical for cytokine-induced vascular leakage. PLoS One. 2013;8(8):e70459.
12. Allegretti AS, Vela Parada X, Ortiz GA, et al. Serum Angiopoietin-2 Predicts Mortality and Kidney Outcomes in Decompensated Cirrhosis. Hepatology. 2019;69(2):729-41.
13. Goettsch W, Gryczka C, Korff T, et al. Flow-dependent regulation of angiopoietin-2. J Cell Physiol. 2008;214(2):491-503.
14. Faillaci F, Marzi L, Critelli R, et al. Liver Angiopoietin-2 Is a Key Predictor of De Novo or Recurrent Hepatocellular Cancer After Hepatitis C Virus Direct-Acting Antivirals. Hepatology. 2018;68(3):1010-24.

15. Lefere S, Van de Velde F, Hoorens A, et al. Angiopoietin-2 Promotes Pathological Angiogenesis and Is a Therapeutic Target in Murine Nonalcoholic Fatty Liver Disease. Hepatology. 2019;69(3):1087-104.
16. Castera L, Forns X, Alberti A. Non-invasive evaluation of liver fibrosis using transient elastography. J Hepatol. 2008;48(5):835-47.
17. Kawagishi N, Suda G, Nakamura A, et al. Liver steatosis and dyslipidemia after HCV eradication by direct acting antiviral agents are synergistic risks of atherosclerosis. PloS one. 2018;13(12):e0209615. Epub 2018/12/24.
18. Castera L, Vergniol J, Foucher J, et al. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology. 2005;128(2):343-50.
19. Koga T, Morikawa Y. Ultrasonographic determination of the splenic size and its clinical usefulness in various liver diseases. Radiology. 1975;115(1):157-61.
20. Suda G, Nagasaka A, Yamamoto Y, et al. Safety and efficacy of daclatasvir and asunaprevir in hepatitis C virus-infected patients with renal impairment. Hepatol Res. 2016.
21. Suda G, Kurosaki M, Itakura J, et al. Safety and efficacy of elbasvir and grazoprevir in Japanese hemodialysis patients with genotype 1b hepatitis C virus infection. J Gastroenterol. 2019;54(1):78-86.
22. Suda G, Nakai M, Sho T, et al. The Successful Retreatment with Glecaprevir and Pibrentasvir of Genotype 1 or 2 HCV-infected Hemodialysis Patients who Failed to Respond to NS5A and Protease Inhibitor Treatment. Intern Med. 2019;58(7):943-7.
23. Suda G, Ogawa K, Kimura M, et al. Novel Treatment of Hepatitis C Virus Infection for Patients with Renal Impairment. J Clin Transl Hepatol. 2016;4(4):320-7.
24. Takeuchi Y, Akuta N, Sezaki H, et al. Efficacy and safety of elbasvir plus grazoprevir combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. Hepatol Res. 2019;49(3):256-63.
25. Itokawa N, Atsukawa M, Tsubota A, et al. Efficacy of direct-acting antiviral treatment in patients with compensated liver cirrhosis: A multicenter study. Hepatol Res. 2019;49(2):125-35.
26. Ogawa E, Furusyo N, Nakamuta M, et al. Glecaprevir and pibrentasvir for Japanese patients with chronic hepatitis C genotype 1 or 2 infection: Results from a multicenter, real-world cohort study. Hepatol Res. 2019;49(6):617-26.
27. Berzigotti A, Zappoli P, Magalotti D, et al. Spleen enlargement on follow-up evaluation: a noninvasive predictor of complications of portal hypertension in cirrhosis. Clin Gastroenterol Hepatol. 2008;6(10):1129-34.

Table legends

Table 1 Baseline characteristics of patients

Table 2 Changes in fibrosis stage based on LSM after direct-acting antiviral agent therapy

LSM, liver stiffness measurement

Table 3 Factors associated with non-regression of liver fibrosis stage after direct-acting antiviral agent therapy

Table 4 Factors associated with non-regression of liver fibrosis stage after DAAs in patients with advanced liver fibrosis (F3/4)

Figure legends

Figure 1 Relationship among baseline Ang2 levels, LSM, spleen index, and LSM-based liver fibrosis grade

A. Baseline Ang2 level and LSM were significantly correlated in all patients (r=0.35, P =0.01).

B. Baseline Ang2 level and LSM were significantly correlated in patients with advanced liver fibrosis (r=0.56, P <0.001).

C. Baseline Ang2 level and spleen index were significantly correlated in all patients (r=0.22, P =0.02).

D. Baseline Ang2 level and spleen index were significantly correlated in patients with advanced liver fibrosis (r=0.41, P =0.03).

E. Baseline Ang2 levels were significantly different among each liver fibrosis stage based on LSM (P<0.001).

F. Baseline Ang2 levels were compared between patients with liver fibrosis stage F0–2 and F3/4.

Baseline Ang2 levels were significantly higher in patients with liver fibrosis stage F3/4 than those with F0-2 (P<0.01)

Ang2, Angiopoietin-2; LSM, liver stiffness measurement

Figure 2 Baseline Ang2 level according to liver fibrosis regression or non-regression

A. In patients with liver fibrosis stage F0–F2, baseline Ang2 levels were comparable between patients with and without regression. In patients with liver fibrosis stage F3/4, baseline Ang2 levels were significantly higher in patients without regression than in those with regression.

B. In patients with liver fibrosis stage F0/1 and 2, baseline Ang2 levels were comparable between patients with and without regression. In patients with liver fibrosis stage F3 and F4, baseline Ang2 levels were significantly higher in patients without regression than in those with regression.

Ang2, Angiopoietin-2

Figure 3 Cutoff value of baseline serum angiopoietin-2 (Ang2) level for predicting non-regression of liver fibrosis stage after direct-acting antiviral agent therapy

A. Receiver operating characteristics (ROC) curve analysis for baseline Ang2 level in patients with advanced liver fibrosis (F3/4). The cutoff baseline Ang2 level associated with non-regression of liver fibrosis stage is 354 pg/mL (ROC-AUC=0.855; sensitivity, 0.882; specificity, 0.733).

B. Rate of regression and non-regression of liver fibrosis stage in patients with baseline Ang2 with >354 pg/mL and advanced liver fibrosis.

C. Rate of regression and non-regression of liver fibrosis stage in patients with baseline Ang2 <354 pg/mL and advanced liver fibrosis. Ang2, Angiopoietin-2

Supplementary Table 1

Factors associated with non-regression of liver fibrosis stage after direct-acting antiviral agent therapy in patients with F0/1 and 2

Supplementary Table 2

Factors associated with non-regression of liver fibrosis stage after direct-acting antiviral agent therapy between end of treatment and SVR24

Supplementary Table 3

Factors associated with non-regression of liver fibrosis stage after direct-acting antiviral agent therapy between end of treatment and SVR96

Figure S1. Study design

Figure S2. Relationship between baseline serum angiopoietin-2 (Ang2) and esophageal varices in patients with advanced liver fibrosis (F3/4)

In patients in whom varices were evaluated before direct-acting antiviral agent treatment and had advanced liver fibrosis, the relationship between the presence of varices and baseline Ang2 levels were evaluated.

Figure S3 Relationship between baseline angiopoietin-2 (Ang2) levels and controlled attenuation parameter (CAP) values

A. Baseline Ang2 level and CAP were not significantly correlated in all patients

B. Baseline Ang2 level and CAP were not significantly correlated in patients with advanced liver fibrosis

Figure S4. Change in serum angiopoietin-2 (Ang2) levels after direct-acting antiviral agent therapy in patients with or without liver fibrosis regression

A. Comparison between serum Ang2 levels at baseline and at end of treatment.

B. Comparison between serum Ang2 levels at baseline and at end of treatment in patients with liver fibrosis stage F0–F2 and F3/4.

C. Comparison of changes in Ang2 level between baseline and at end of treatment in patients with liver fibrosis stage F0–F2 and F3/4

Figure S5 Cutoff value of angiopoietin-2 (Ang2) levels at end of treatment point for predictive of liver non-regression after direct-acting antiviral agent therapy

Receiver operating characteristics (ROC) curve analysis for Ang2 level at end of treatment in patients with advanced liver fibrosis (F3/4). The cutoff baseline Ang2 level associated with non-regression of liver fibrosis stage is 354 pg/mL (sensitivity, 0.941; specificity, 0.867).

Table 1 Baseline patients' characteristics

	All	F0-2	F3-4	P value
Number	116	84	32	
Age (years)[a]	66(22-87)	65(22-83)	66(33-87)	0.595
Sex (male/female)	43/73	29/55	14/18	0.358
HCV-RNA (log IU/mL)[a]	6.3(3.6-7.2)	6.3(3.6-7.2)	6.3(4.7-7.2)	0.625
DCV/ASV, SOF/LDV, SOF/RBV, OBV/PTV/r	20/49/42/5	10/39/31/4	10/10/11/1	0.09
F0-1/2/3/4	64/20/9/23	64/20/0/0	0/0/9/23	
White blood cell count (/μL)[a]	4800(1900-10800)	4800(2700-10800)	4800(1900-10000)	0.834
Hemoglobin level (g/dL)[a]	13.5(8.9-16.8)	13.5(10.1-16.8)	13.3(8.9-16.6)	0.897
Platelet count ($\times 10^4$)[a]	15.9(2.2-37.3)	17.8(2.6-37.3)	11.3(2.2-24.7)	*<0.001
Albumin (g/dL)[a]	4.3(2.7-5.0)	4.3(2.7-5)	4(3-4.7)	*<0.001
AST (IU/L)[a]	39(14-180)	34(14-180)	57(31-175)	*<0.001
ALT (IU/L)[a]	38(6-273)	31(6-273)	58(22-211)	*<0.001
γGTP (IU/L)[a]	29.5(9-559)	24(9-276)	40(14-559)	*<0.001
FIB-4 index[a]	2.83(0.54-23.55)	2.41(0.54-13.51)	5.58(0.91-23.55)	*<0.001
Angiopoietin-2 (pg/mL)[a]	305.5(131.9)	293.9(131.9)	415(155.5)	*0.002
CAP (dB/m)[a]	214(100-386)	210(100-343)	225(106-386)	0.432
Spleen index (cm^2)[a]	25.9(11.1-59.1)	23.9(11.1-56.5)	30.7(16.4-59.1)	*<0.001

Abbreviations: HCV, Hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, γ-glutamyl transpeptidase; FIB-4, fibrosis 4; CAP, Controlled Attenuation Parameter. [a]Data are shown as median (range) values. *Statistically significant difference, P <0.05

Table 2 Changes in fibrosis stage based on LSM after direct-acting antiviral agent

	At SVR 24				Disease Activity	
	F0-1	F2	F3	F4	Regressed	Non-regression
	n				n/n (%)	
Baseline						
F0-1	60	4	0	0	60/64 (93)	4/64 (6)
F2	12	5	3	0	12/20 (60)	8/20 (40)
F3	5	1	3	0	6/9 (67)	3/9 (33)
F4	3	1	5	14	9/23 (39)	14/23 (61)
Total					87/116 (75)	29/116 (25)

LSM, liver stiffness measurement, SVR, sustained viral response;

Table 3 Factors associated with non-regression of liver fibrosis stage after direct-acting antiviral agent therapy

	Regression	Non-regression	Univariate analysis	Multivariate analysis	Odds ratio
Number	**87**	**29**			
Age (years)[a]	64(22-87)	68(44-81)	0.104		
Sex (male/female)	31/56	12/17	0.579		
HCV-RNA (log IU/mL)[a]	6.3(3.6-7.2)	6.2(4.4-7.1)	0.257		
DCV/ASV, SOF/LDV, SOF/RBV, OBV/PTV/r	11/41/32/3	9/8/10/2	0.076		
F0-2/3-4	72/15	12/17	*<0.001	*0.003	4.474(1.651-12.125)
White blood cell count (/μL)[a]	4900(2700-10800)	4400(1900-10000)	0.103		
Hemoglobin level (g/dL)[a]	13.6(10.1-16.8)	13.1(8.9-16.4)	0.173		
Platelet count ($\times 10^4$)[a]	17.4(2.2-37.3)	12.9(4.7-24.7)	*<0.001	0.158	
Albumin (g/dL)[a]	4.2(2.7-5.0)	4(3-4.7)	*0.013		
AST (IU/L)[a]	35(14-180)	55(24-125)	*<0.001	0.229	
ALT (IU/L)[a]	36(11-273)	44(6-107)	0.103		
γGTP (IU/L)[a]	26(9-275)	40(11-559)	*0.012		
FIB-4 index[a]	2.42(0.54-23.55)	4.88(2.13-9.12)	*<0.001	0.257	
Angiopoietin-2 (pg/mL)[a]	274.8(131.9-864.5)	434.1(205.6-1545.6)	*<0.001	*0.006	1.004(1.001-1.007)
CAP (dB/m)[a]	215(100-386)	214(100-280)	0.747		
Spleen index (cm^2)[a]	24.8(11.1-59.1)	28.6(12.5-53.4)	*0.017		

Abbreviations: HCV, Hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, γ-glutamyl transpeptidase; FIB-4, fibrosis 4. ; CAP, Controlled Attenuation Parameter. [a]Data are shown as median (range) values. *Statistically significant difference, P <0.05.

Table 4

Factors associated with non-regression of liver fibrosis stage after DAAs in patients with advanced liver fibrosis (F3/4)

	Regression	**Non-regression**	**P value**
Number	**15**	**17**	
Age (years)[a]	64(33-87)	68(44-79)	0.35
Sex (male/female)	6/9	8/9	0.688
HCV-RNA (log IU/mL)[a]	6.5(4.9-7.2)	6.3(4.7-7.1)	0.628
DCV/ASV, SOF/LDV, SOF/RBV, OBV/PTV/r	3/5/7/0	7/5/4/1	0.347
F3/4	6/9	3/14	0.243
White blood cell count (/μL)[a]	4800(3200-7700)	4500(1900-10000)	0.794
Hemoglobin level (g/dL)[a]	13.6(11.4-16.6)	13.1(8.9-15.19	0.35
Platelet count ($\times 10^4$)[a]	12.7(2.2-19.3)	11.2(4.7-24.7)	0.455
Albumin (g/dL)[a]	4(3.6-4.5)	3.9(3-4.7)	0.246
AST (IU/L)[a]	54(31-175)	57(35-125)	0.433
ALT (IU/L)[a]	56(36-211)	58(22-101)	0.576
γGTP (IU/L)[a]	40(17-189)	63(14-559)	0.655
FIB-4 index[a]	4.36(0.91-23.55)	5.94(2.18-9.12)	0.202
Angiopoietin-2 (pg/mL)[a]	251.9(155.5-848)	502.3(284.7-1545.6)	*<0.001
CAP (dB/m)[a]	226(123-386)	224(106-280)	0.941
Spleen index (cm^2)[a]	29.7(18-59.1)	30.7(16.4-53.4)	0.683

Abbreviations: HCV, Hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γGTP, γ-glutamyl transpeptidase; FIB-4, fibrosis 4; CAP, Controlled Attenuation Parameter. [a] Data are shown as median (range) values. *Statistically significant difference, P <0.05.

Figure 1 Relationship among baseline Ang2 levels, LSM, spleen index, and LSM-based liver fibrosis grade

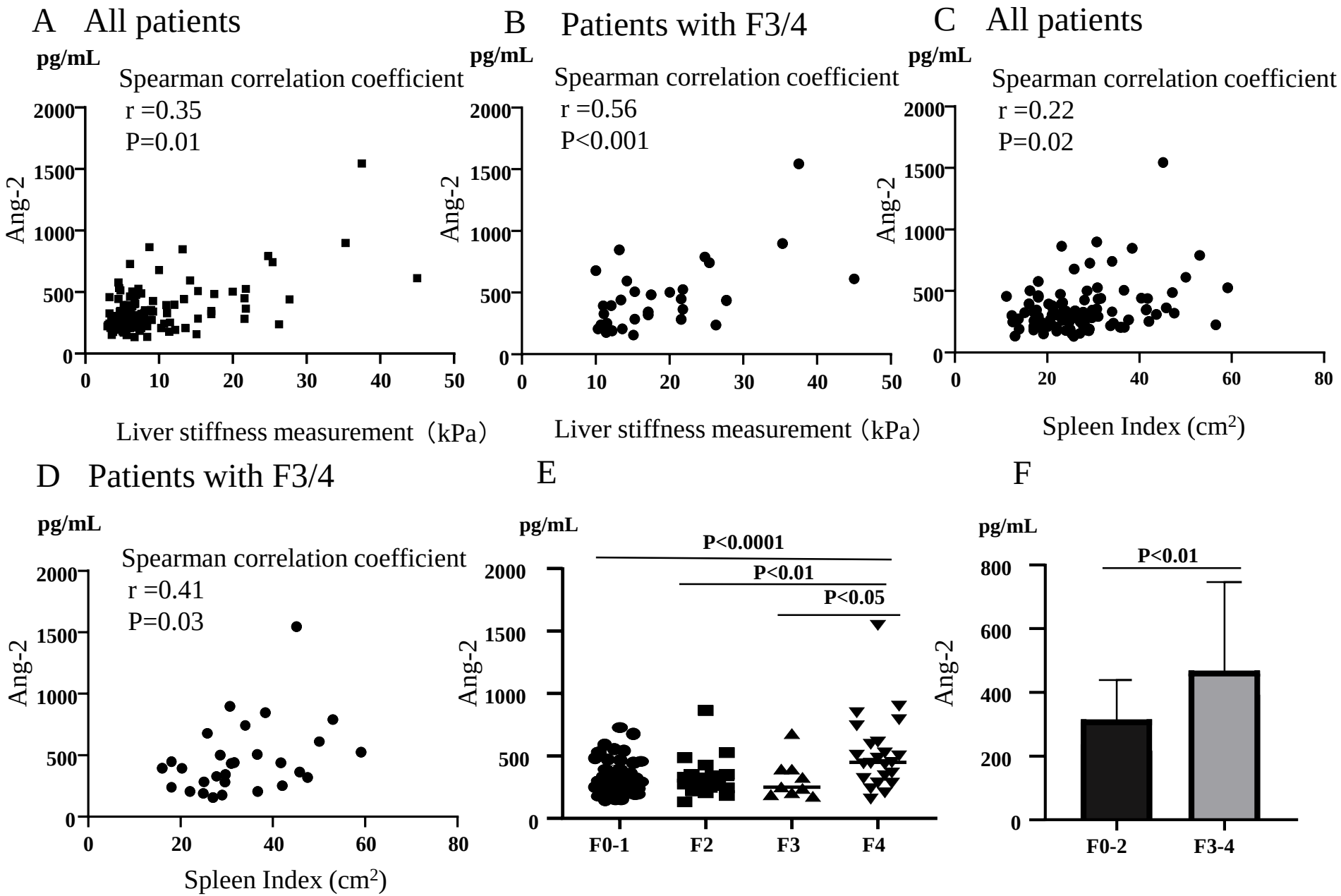


Figure 2 Baseline Ang2 level according to liver fibrosis regression or non-regression

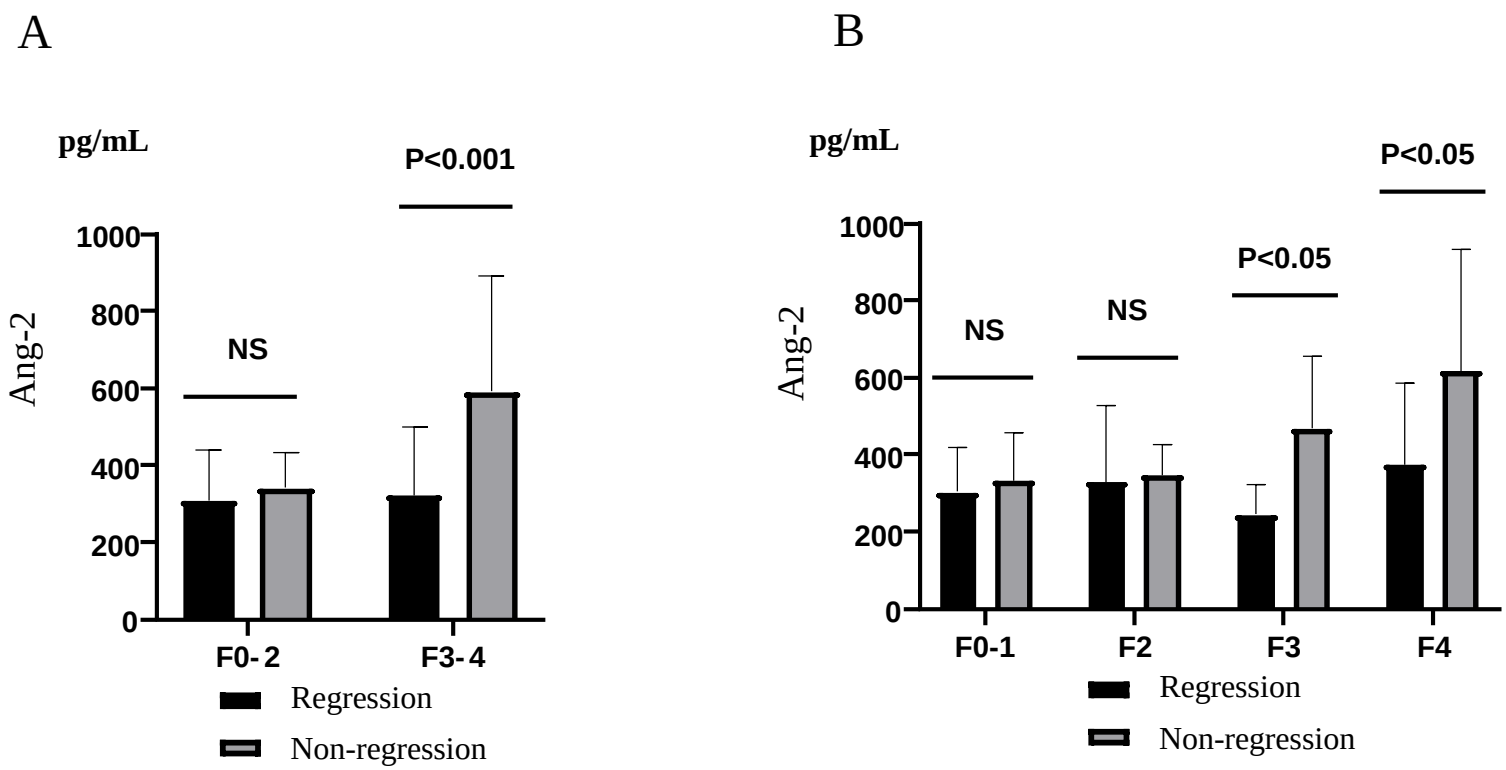
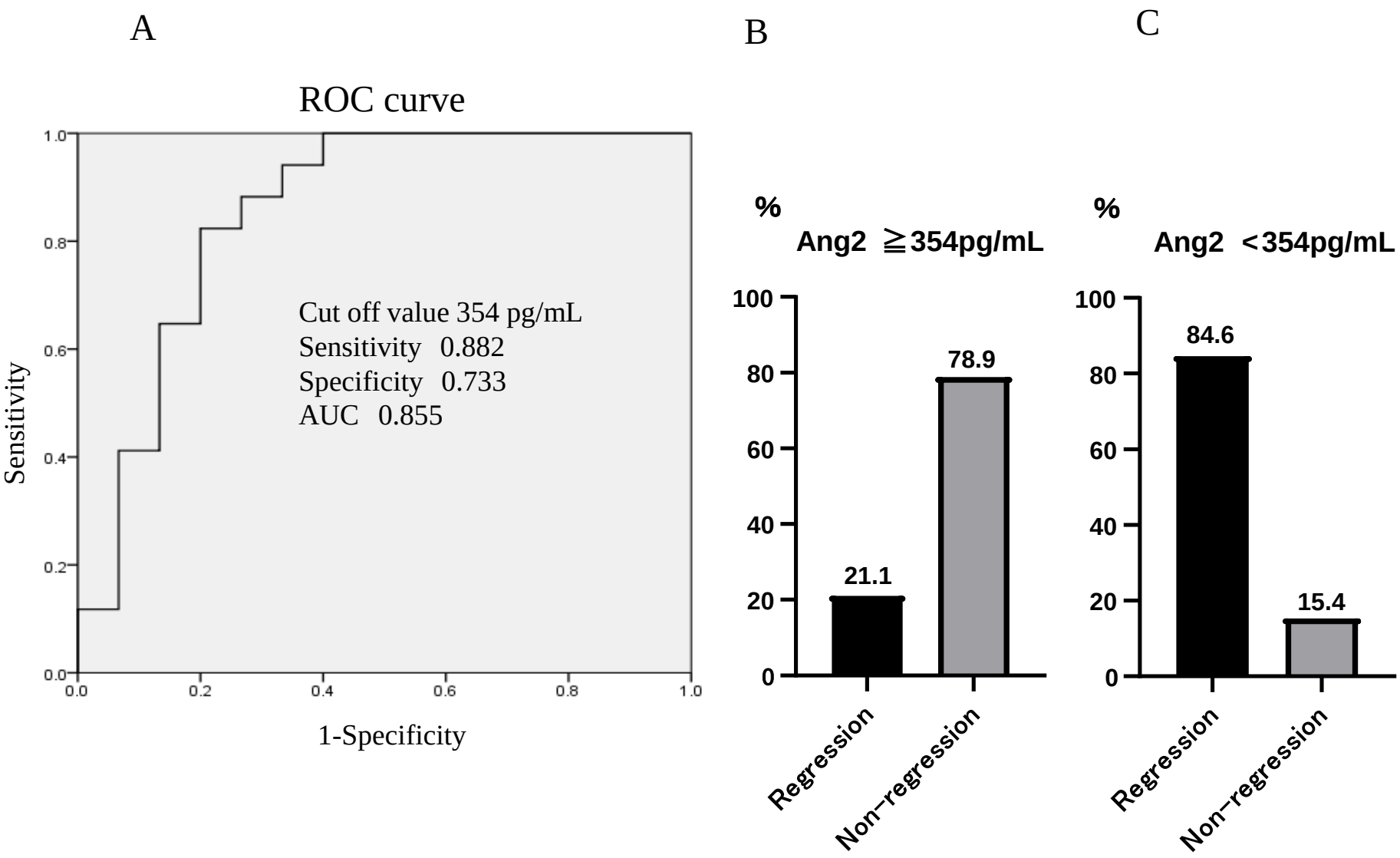
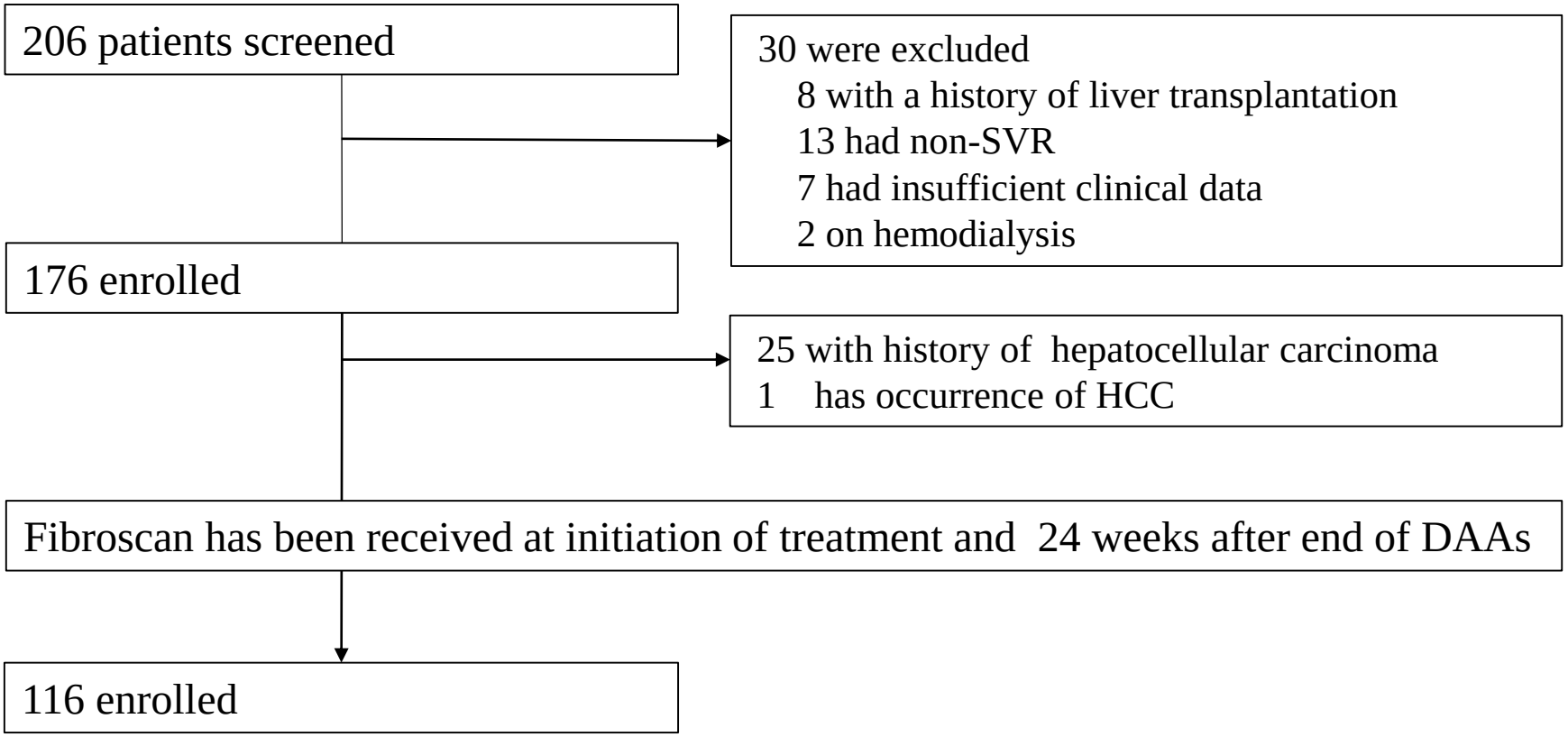


Figure 3 Cutoff value of baseline serum angiopoietin-2 (Ang2) level for predicting non-regression of liver fibrosis stage after direct-acting antiviral agent therapy

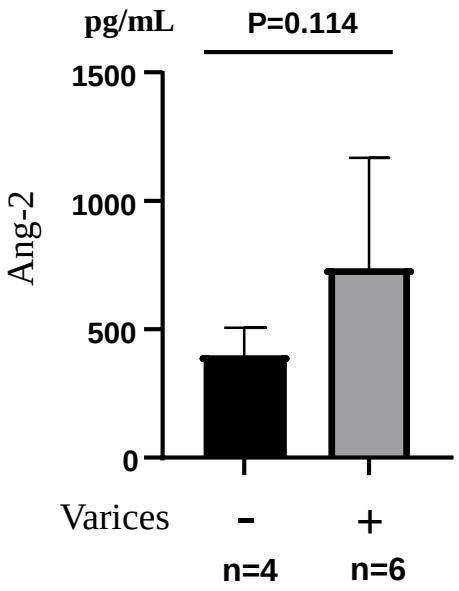


Supplementary Figure 1

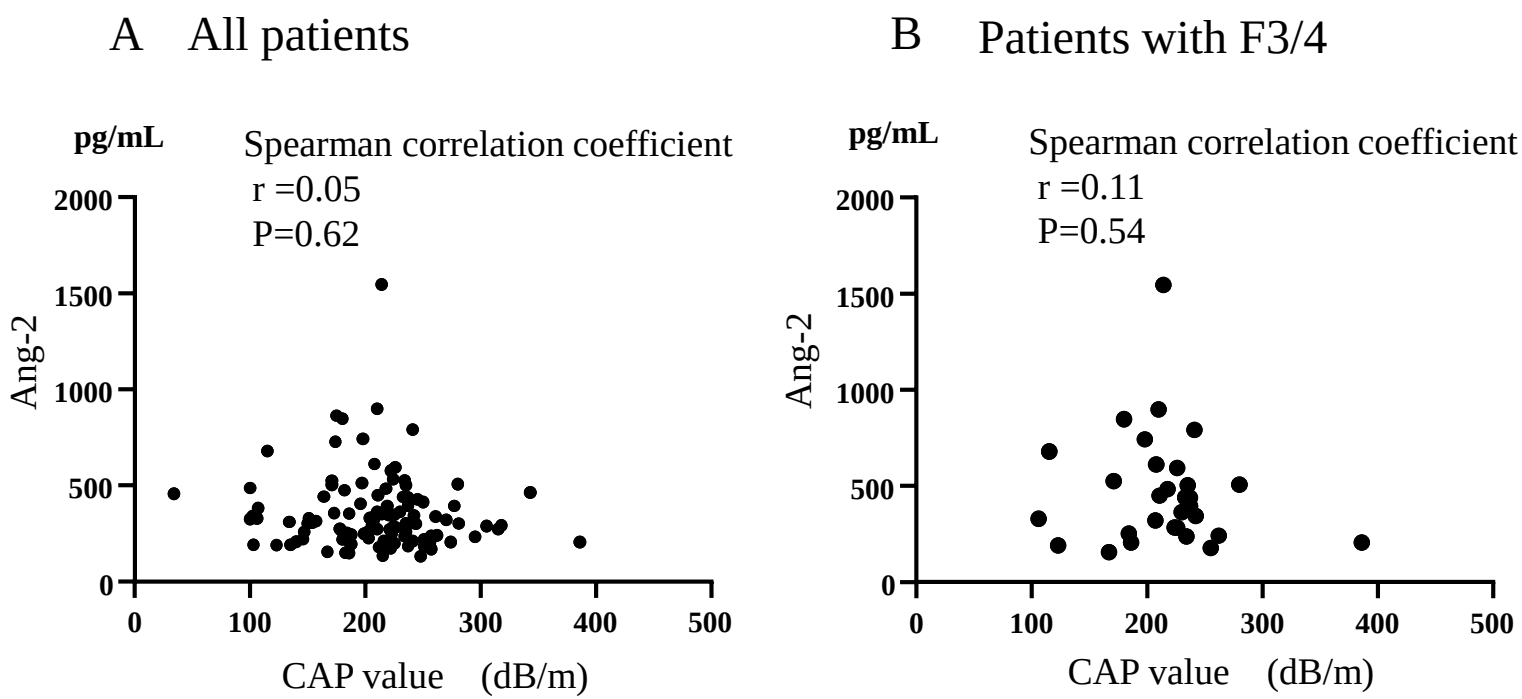
Study design



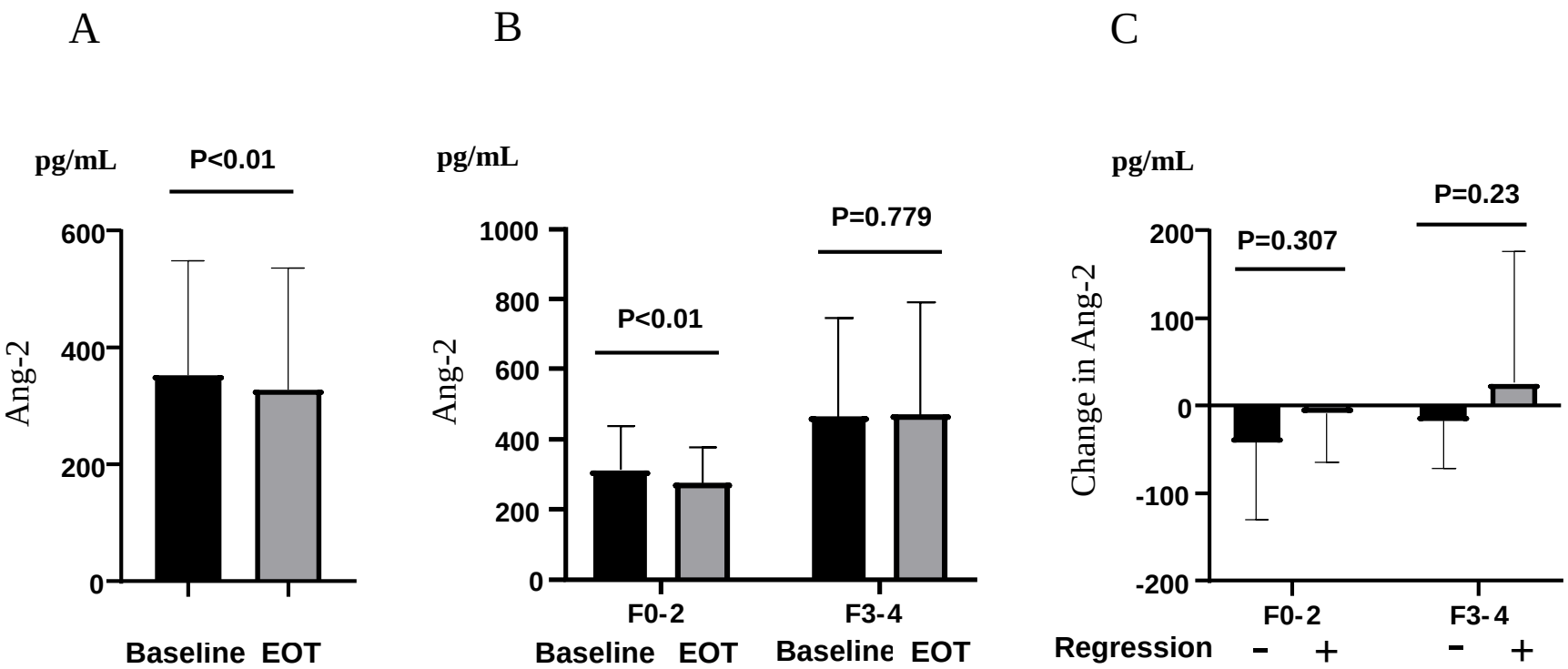
Supplementary Figure 2. Relationship between baseline serum angiopoietin-2 (Ang2) and esophageal varices in patients with advanced liver fibrosis (F3/4)



Supplementary Figure 3 Relationship between baseline angiopoietin-2 (Ang2) levels and controlled attenuation parameter (CAP) values



Supplementary Figure 4 Change in serum angiopoietin-2 (Ang2) levels after direct-acting antiviral agent therapy in patients with or without liver fibrosis regression



Supplementary Figure 5 Cutoff value of angiopoietin-2 (Ang2) levels at end of treatment point for predictive of liver non-regression after direct-acting antiviral agent therapy