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Liver collagen proportionate area predicts decompensation in patients with recurrent hepatitis C virus cirrhosis after liver transplantation

Vincenza Calvaruso,*,‡ Amar Paul Dhillon,† Emanuel Tsochatzis,* Pinelopi Manousou,* Federica Grillo,† Giacomo Germani,* David Patch,* James O'Beirne* and Andrew Kenneth Burroughs*

*The Royal Free Sheila Sherlock Liver Centre and University Department of Surgery, UCL and Royal Free Hospital, †Department of Histopathology, Royal Free Hospital, London, UK; and ‡Gastroenterology and Hepatology Unit, DIBIMIS, University of Palermo, Palermo, Italy

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Correspondence
Dr Vincenza Calvaruso, Gastroenterologia and Epatologia, DIBIMIS, Università di Palermo, Palermo 90127, Italy. Email: vcalvaruso@libero.it

Abstract

Background and Aims: Current histological scoring systems do not subclassify cirrhosis. Computer-assisted digital image analysis (DIA) of Sirius Red-stained sections measures fibrosis morphologically producing a fibrosis ratio (collagen proportionate area [CPA]). CPA could have prognostic value within a disease stage, such as cirrhosis. The aim of the present study was to evaluate CPA in patients with recurrent hepatitis C virus (HCV) allograft cirrhosis and assess its relationship with hepatic venous pressure gradient (HVPG).

Methods: In 121 consecutively-transplanted HCV patients with HVPG, measured contemporaneously with transjugular liver biopsies, 65 had Ishak stage 5 or 6 disease (43 with HVPG measurement). Biopsies were stained with Sirius Red for DIA, and the collagen content was expressed as a CPA. In three cases, a tissue for Sirius Red staining was not obtained, and the patients were excluded.

Results: Sixty-two patients were analyzed. The median HVPG was 8 mmHg (interquartile range [IQR]: 5–10). Portal hypertension (HVPG $\geq 6 < 10$ mmHg) was present in 30 (69.8%), and HVPG ≥ 10 mmHg in 13 (30.2%). The median CPA was 16% (IQR 10.75–23.25). Median Child–Pugh score and HVPG were not significantly different between Ishak fibrosis stage 5 or 6, whereas CPA was statistically different: 13% in stage 5 (IQR 8.3–12.4) versus 23% in stage 6 (IQR 17–33.7, $P < 0.001$). In the multivariate analysis, CPA was the only variable significantly associated with clinically-significant portal hypertension (HVPG ≥ 10 mmHg, odds ratio: 1.085, confidence interval: 1.004–1.172, $P = 0.040$). A CPA of 14% was the best cut-off value for clinically-significant portal hypertension (CSPH) and liver decompensation, which occurred in 24 patients. Event-free survival was significantly shorter in patients with CSPH or with a CPA value $\geq 14\%$, or with a combination of both.

Conclusion: In Ishak stages 5 and 6, CPA correlated with HVPG, but had a wider range of values, suggesting a greater sensitivity for distinguishing "early" from "late" severe fibrosis/cirrhosis. CPA was a unique, independent predictor of HVPG ≥ 10 mmHg. CPA can be used to subclassify cirrhosis and for prognostic stratification.

Introduction

Cirrhosis is defined histologically by the diffuse replacement of the normal lobular architecture of the liver by regenerative parenchymal nodules surrounded by fibrous tissue. Liver biopsy remains the gold standard for staging parenchymal liver disease based on the degree of fibrosis and architectural distortion. However, although all histological classification systems have cirrhosis as the most advanced stage, there are no accepted histological criteria to determine the severity of cirrhosis. Conversely, clinical classifications do score the severity of cirrhosis, such as Child–Pugh or model for end-stage liver disease (MELD) scores, or evaluate the occurrence of complications of cirrhosis, such as ascites, varices, or bleeding.[1] The concept of "severity of cirrhosis" has significant clinical implications. Hepatic venous pressure gradient (HVPG) has been confirmed as a good prognostic index by several groups in relation to survival and complications.[2] Complications of cirrhosis are known to develop once HVPG reaches a threshold level of 10–12 mmHg, reflecting more severe portal hypertension.[3–6] This threshold is of prognostic value, and

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is termed "clinically-significant portal hypertension" (CSPH); it is predictive of the development of cirrhosis complications[6] and allograft cirrhosis after liver transplantation for hepatitis C virus (HCV) chronic liver disease.[7]

Theoretically, specific histological findings might also be able to determine the severity of cirrhosis and predict the likelihood of developing complications. This relationship would be of practical clinical value, not only prognostically, but also in regard to assessing the progression of cirrhosis, in assessing the potential reversibility of fibrosis, and identifying patients for antifibrotic and other therapies. Only two studies have evaluated this. Nagula *et al*.[8] performed a study assessing the relationship between HVPG and specific histological features using 43 liver biopsies with a diagnosis of cirrhosis, and evaluated whether any histological features correlated with CSPH. They found that septal thickness and small nodules were the only two independent associations with CSPH. Another similar study[9] of 47 patients with biopsy-proven cirrhosis and HVPG measurements concluded that small nodularity and thick septa are independent predictors of the presence of CSPH. This was also the case in a study of 123 patients with predominantly alcoholic cirrhosis.[10] These three studies suggest that cirrhosis can be subclassified histologically, and used similar methods for evaluating nodule size and septum thickness, but is not clear how the particular nodules or septa that were measured were chosen in any histological section or microscopic field of view. Large and small nodules can be present in different parts of a single liver biopsy section; septal thickness is similarly variable and is generally inversely related to nodule size. Indeed, thick septa/small nodules and thin septa/large nodules reflect the proportion of connective tissue (much of which is collagen in advanced-stage liver disease) in the cirrhotic biopsies (i.e. collagen proportionate area).

What is needed is a histological measurement that measures the amount of fibrosis properly in advanced-stage liver disease. The traditional histological scoring systems are categorical assignments, and are neither quantitative in nature nor are they measurements. Thus, the numbers assigned to the categories should not be evaluated as continuous variables, nor be treated as numbers, as there is no arithmetical relationship between them: stage 4 does not mean twice the amount of fibrosis as stage 2.[11] A proper fibrosis measurement can be correlated with other continuous variables, such as HVPG and non-invasive indices of fibrosis, including transient elastography. Ideally, this histological fibrosis measurement should be applicable to all stages of liver disease and to monitor fibrosis progression/regression. One technique that fulfils these requirements is the quantitative measurement of liver fibrosis by a computer-assisted digital image analysis (DIA) of histological sections that are stained histochemically by the PicroSirius Red technique,[11–13] which primarily identifies tissue collagen.[14] The quantity of bound stain correlates well with biochemically-determined collagen content and morphometrically-determined hepatic fibrosis.[15,16] DIA uses segmentation of digital images to measure the area of collagen and the area of tissue. This produces a "fibrosis ratio" or collagen proportionate area (CPA). Other necessary evaluations required for routine histological diagnoses can still be made, and collagenous structures irrelevant to the disease process (and which contribute to the variability between samples) can be excluded precisely.[11] We recently showed[16] that CPA in a cohort of patients with recurrent HCV after liver transplantation had a good correlation with HVPG. For the purpose of comparison with previous papers, we also showed a progression of CPA values with increasing Ishak stages. In patients transplanted for HCV cirrhosis, rapid progression to allograft cirrhosis develops in up to 30% after 5 years from liver transplantation,[17] and the policy of scheduled biopsies[18–20] by several groups, including our own, to assess progression of recurrent HCV, provides an appropriate, if not optimal, clinical setting to evaluate the relationship between CPA and cirrhosis.

The aim of the present study was to assess the relationship between CPA with HVPG and the clinical outcomes of allograft liver biopsies in patients with Ishak stage 5 or 6 due to recurrent HCV infection, and to assess changes in CPA in these patients who had undergone more than one biopsy.

Methods

Between March 1990 and October 2008, we prospectively evaluated a consecutive series of 121 patients with recurrent hepatitis C after liver transplantation, who had serial transjugular liver biopsies (TJB); from 1999, TJB was combined with HVPG measurements. All patients had been transplanted for HCV cirrhosis, had received a cadaveric graft, and had been evaluated for at least 6 months' post-transplant.[17] We identified all patients with a histological diagnosis of Ishak 5 or 6, and used the first biopsy in which either stage was found as the "baseline" biopsy. All patients were followed up with 3–6-monthly outpatient visits, and episodes of decompensation (ascites, variceal bleeding, encephalopathy) were recorded. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki. All patients gave written, informed consent for both the procedure and histological evaluation.

TJB. All TJB procedures were performed in the X-ray suite by experienced personnel (DP, JO'B, DY), as detailed elsewhere,[21] using a 19G Tru-cut-type biopsy needle (Quick core; William Cook Europe, Bjaeverkov, Denmark). We performed three, or more recently, four passes[22] through the same hepatic vein wall (right or middle) to optimize the samples of liver tissue obtained.[21,22] We have previously shown that TJB compare well or are superior to percutaneous biopsies[23] for both the number of portal tracts and total length.[24]

Biopsy specimen study. Liver biopsy samples were formalin fixed, paraffin embedded, and stained with hematoxylin–eosin, Gordon and Sweet staining for reticulin, and chromotrope aniline blue. Another section of tissue was stained with Sirius Red for collagen quantification and determination of CPA by DIA.

Each biopsy sample was evaluated histologically, according to Ishak *et al*.,[25] for disease stage and grade of necroinflammatory activity. For each biopsy, we recorded total length (length of each fragment summed). Portal tracts were not counted, because recognition and enumeration of individual portal tracts in advanced stage liver disease is not possible. The sections of each biopsy stained with Sirius Red were used for DIA, which was performed by one author (VC). The equipment setup used consisted of a digital camera (Canon Powershot A640; Canon, Middlesex, UK; attached to a close-up copystand with backlighting) connected to a

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compatible personal computer. The calibration of the camera setup was 154×154 pixels = 23 716 = 1 mm^2. After whole-section digital image capture, CPA was measured with Zeiss KS300 image analysis software (Zeiss, Hertfordshire, UK). The CPA measurement process included a manual editing step to eliminate image artifacts, and operator-dependent thresholding to determine the stained area of the section.

In our previous study,[16] which evaluated our entire cohort of patients with HCV recurrence after liver transplantation, we had assessed intraobserver and interobserver variability by repeating the CPA assessment using two sets of 20 biopsies (10% of cohort). The concordance coefficients between the intraobserver and interobserver evaluations were 0.98 and 0.97, respectively.[16]

Hemodynamic study. Hepatic vein pressures were measured using a 5-F balloon catheter (Royal Flush plus Straight Visceral Angiographic Beacon tip catheter; William Cook Europe, Denmark), using the technique recently described.[26] Three sets of measurements were taken using monitor DATASCOPE MEDICAL 2000A 6189-L7 (London, UK), setting the external zero point at the mid-axillary line. A difference $\leq$ 1 mmHg of the free hepatic pressure gradient with the measured inferior vena cava pressure was considered acceptable. Wedged hepatic vein pressure was measured for at least 1 min each time. The trace was examined to ensure "occlusion". If there was patient movement or breathing artifacts, measurements were repeated. HVPG (the difference between the wedged and free hepatic pressure gradient) was calculated as the mean of the three measurements.

Statistical analysis. All data were analyzed using the statistical package SPSS (version 15.0; SPSS, Chicago, IL, USA). Continuous variables were summarized as median and interquartile range (IQR), and categorical variables as frequency and percentage. Correlation between variables was evaluated by Spearman correlation. Significance testing was two sided and set to ≤ 0.05.

Comparisons between unpaired samples for HVPG and CPA were made using the Mann–Whitney test, whereas paired comparisons in the same patient for HVPG and CPA were made using the Wilcoxon test. Intraobserver and interobserver variability was assessed by calculation of the concordance coefficient.

Logistic regression analysis was used to determine independent associations with the HVPG cut-offs of $\geq$ 6 mmHg and $\geq$ 10 mmHg, representing the presence of portal hypertension[26] and clinically significant portal hypertension, respectively.[5] Decompensation was defined as ascites, variceal bleeding, or hepatic encephalopathy. We evaluated the proportion of patients who suffered decompensation and the time to the event by Kaplan–Meier plots and log–rank analysis.

Results

Ishak stage score 5 or 6 was diagnosed in 65 patients at a median of 48 (range: 12–135) months after liver transplantation. In three cases, a tissue for Sirius Red staining was not obtained, and the patients were excluded. Among the 62 patients, 27 (56.5%) had a diagnosis of developing cirrhosis (Ishak 5), and 35 (43.5%) of complete cirrhosis (Ishak 6). A total of 24 patients developed decompensation (18 ascites, 2 ascites and hepatorenal syndrome, 3 variceal bleeding, 1 portosystemic encephalopathy [PSE]) at a median of 22 months (range: 4–56). HVPG at the time of histological diagnosis of developing or complete cirrhosis was measured in 43 patients (25 HVPG measurement in Ishak 5, and 18 in Ishak 6), with a median value of 8 mmHg (IQR: 5–10). Portal hypertension (HVPG $\geq$ 6 mHg) was found in 30 patients (69.8%), and CSPH (HVPG $\geq$ 10 mmHg) in 13 patients (30.2%).

Table 1 Median and IQR values of CPS, HVPG and CPA, according to Ishak stage score in all 62 patients, and in the 43 with HVPG measurement

Variable Median value (IQR)	Ishak 5 (35 points)	Ishak 6 (27 points)	*P*-value
CPS	5.0 (5.0–6.0)	6.0 (5.0–8.0)	0.132
CPS†	5.0 (5.0–6.5)	6.0 (5.0–8.0)	0.243
HVPG mmHg†	7.0 (4.0–9.50)	8.5 (5.75–12.25)	0.097
Mean CPA (%)	13.0 (8.3–16.4)	23.0 (17.0–33.7)	**< 0.001**
CPA (%)†	13.8 (8.0–19.0)	18.8 (16.8–26.1)	**0.009**

†Forty-three patients with HVPG measurement.
CPA, collagen proportionate area; CPS, Child–Pugh score; HVPG, hepatic venous pressure gradient; IQR, interquartile range.

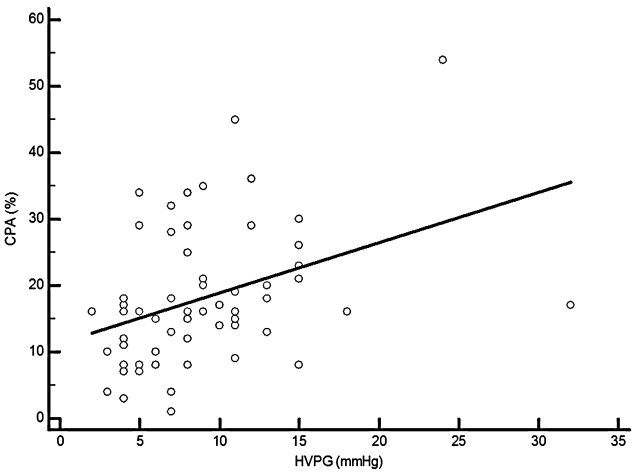

Figure 1 Correlation between collagen proportionate area (CPA) and hepatic venous pressure gradient (HVPG) in 43 patients with recurrent hepatitis C virus-related Ishak stage 5 or 6 after liver transplantation.

The median CPA value was 16% (IQR: 10.75–23.25) for all 62 patients. Analyzing only the 43 patients with HVPG measurements, the median CPA value was 16.4% (IQR: 12–22). The difference between the medians of Child–Pugh score (CPS) and HVPG for stages 5 and 6 were not statistically significant (CPS: 5 [IQR: 5–6] *vs* 6 [IQR: 5–8], $P = 0.132$; HVPG: 7 mmHg [IQR: 4–9.5] *vs* 8.5 mmHg [IQR: 5.75–12.25] $P = 0.097$), as we have noted before.[21] However, the CPA was statistically significantly different: in stage 5, the median CPA was 13% (IQR: 8.3–12.4), and in stage 6, it was 23% (IQR: 17–33.7) ($P < 0.001$; Table 1).

Overall, there was a significant correlation between CPA and HVPG (r = 0.370, $P = 0.017$; Fig. 1).

We subdivided patients with HVPG values lower or higher than 10 mmHg (CSPH) as previously published.[3]

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Table 2 Clinical and histological associations with HVPG ≥ 6 mmHg and HVPG ≥ 10 mmHg (CSPH) in 43 patients with recurrent HCV infection, and Ishak stage 5 or 6 after liver transplantation

	Univariate analysis			Multivariate analysis	
	HVPG				
	< 6 mmHg	≥ 6 mmHg	*P*-value	OR (95% CI)	*P*-value
Ishak grading score	5 (3–6)	6 (4–7)	0.188		
Ishak staging score = 6	4/14 (28.6%)	13/28 (46.4%)	0.323		
CPA (%)	12.0 (7.5–17.2)	17.4 (13.0–24.0)	0.096	1.057 (0.978–1.142)	0.165
CPS	6.0 (5–7)	5 (5–8)	0.354		
	HVPG			OR (95% CI)	*P*-value
	< 10 mmHg	≥ 10 mmHg	*P*-value		
Ishak grading score	6 (4–6)	6 (4.5–7)	0.723		
Ishak staging score = 6	11/29 (37.9%)	5/13 (38.5%)	0.823		
CPA (%)	15.7 (8.2–19.0)	19.2 (16.0–28.7)	0.054	1.085 (1.004–1.172)	**0.040**
CPS	5 (5–7)	6.0 (5–10)	0.550		

CI, confidence interval; CPA, collagen proportionate area; CPS, Child–Pugh score; HCV, hepatitis C virus; HVPG, hepatic venous pressure gradient; OR, odds ratio.

We evaluated whether three parameters, the CPS, the Ishak stage (5 or 6) or grade, and CPA, were associated with portal hypertension (HVPG ≥ 6 mmHg) or CSPH (HVPG ≥ 10 mmHg) in a multiple regression model. CPA was the only variable independently associated with clinically-significant portal hypertension (odds ratio: 1.085; confidence interval: 1.004–1.172, $P = 0.040$; Table 2).

Fourteen of the 43 patients (32.6%) with HVPG measurements suffered decompensation: 11 ascites, two variceal bleeding, and one PSE. This occurred at a median of 18.5 months (range: 4–48) after the histological diagnosis of advanced fibrosis or cirrhosis. In the 19 patients without HVPG measurements, who were followed for a median of 48 months (5–132), 10 (52.6%) decompensated (7 ascites, 2 ascites and hepatorenal syndrome, and 1 variceal bleeding) at a median time of 25 months (range: 5–56).

By Kaplan–Meier methods, the event-free survival (i.e. to first episode of decompensation) was significantly shorter in patients with CSPH (log–rank test, $P = 0.039$; Fig. 2).

For liver decompensation, the best area under receiver operating curve for CPA was 0.65 (95% CI: 0.55–0.86), and the best cut-off value was 14% CPA, with 71% sensitivity and 57% specificity.

The event-free survival was significantly shorter in patients with CPA values equal or higher than 14% (log–rank test, $P = 0.042$; Fig. 3).

Upon dividing the patients into four different groups according to CPA cut-off values, and CSPH, a marginal difference was found between the event-free survival in patients with a CPA value equal or higher than 14% and CSPH (log–rank test, $P = 0.051$; Fig. 4).

Seventeen patients of the 43 with Ishak 5 or 6 had further TJB combined with HVPG measurement. The differences between the medians of CPS, HVPG, and CPA values are shown in Table 3. Only the CPA value was found to be different between the first and subsequent biopsies (15.2% [IQR: 8.3–20.8] *vs* 17% [IQR: 10.6–21.4], $P = 0.079$). All but one patient had an increased CPA value in the second biopsy. This patient had a diagnosis of complete cirrhosis (Ishak stage 6) in the first biopsy, and Ishak stage 5 in the second biopsy, without any change in HVPG value (7 mmHg in both procedures).

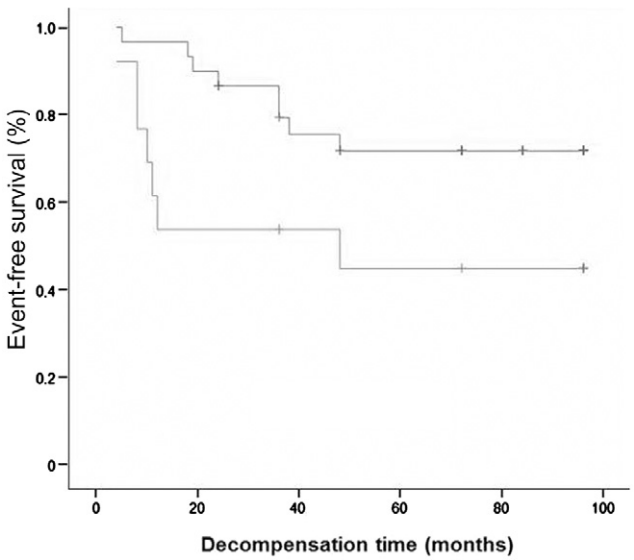

Figure 2 Event-free survival (first episode of decompensation: ascites, variceal bleeding, or encephalopathy) according to clinically-significant portal hypertension (hepatic venous pressure gradient [HVPG] ≥ 10 mmHg) in 43 patients with recurrent hepatitis C virus infection and Ishak stage 5 or 6 after liver transplantation. Log–rank = 0.039. (⌊⌉) HVPG < 10 mmHg, (⌊⌉) HVPG ≥ 10 mmHg.

Discussion

Measuring the severity of cirrhosis other than by clinical scores that incorporate complications (e.g. ascites and encephalopathy

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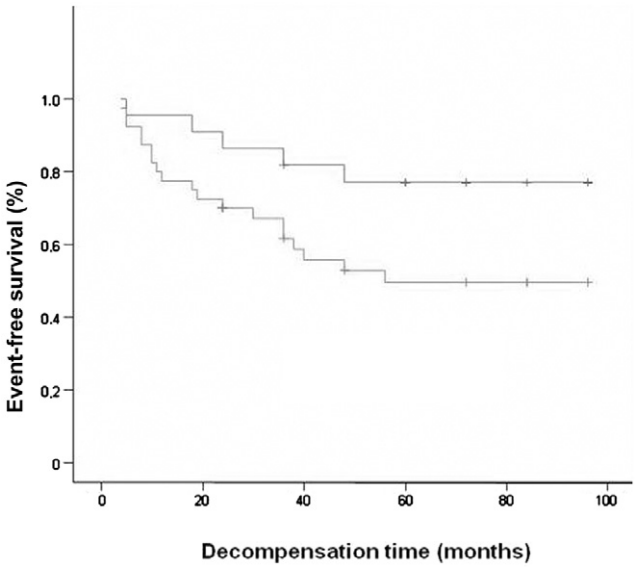

Figure 3 Kaplan–Meier plot of event-free survival (first episode of decompensation) according to collagen proportionate area (CPA) value in 62 patients with recurrent hepatitis C virus infection and Ishak stage 5 or 6 after liver transplantation. Log–rank = 0.042. (⌐¬) CPA < 14%, (⌐¬) CPA ≥ 14%.

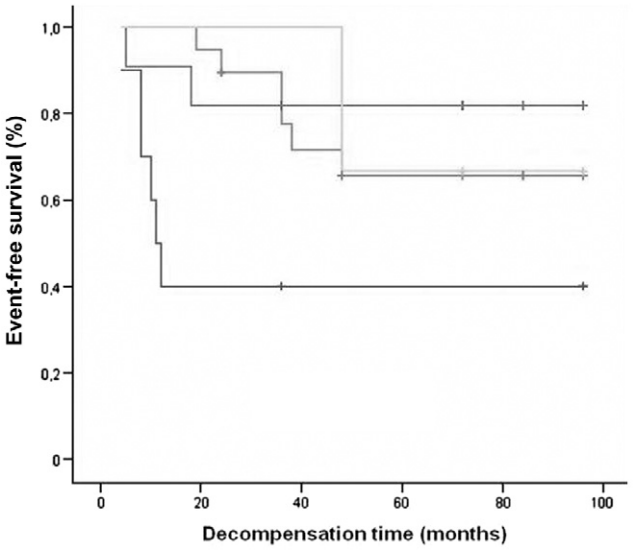

Figure 4 Event-free survival (first episode of decompensation) according to collagen proportionate area (CPA) value and clinically-significant portal hypertension (hepatic venous pressure gradient [HVPG] ≥ 10 mmHg) in 43 patients with recurrent hepatitis C virus infection and Ishak stage 5 or 6 after liver transplantation or greater/equal to 10 mmHg. Log–rank = 0.051. (⌐¬) HVPG < 10 mmHg, CPA < 14%, (⌐¬) HVPG < 10 mmHg, CPA ≥ 14%, (⌐¬) HVPG ≥ 10 mmHg, CPA < 14%, (⌐¬) HVPG ≥ 10 mmHg, CPA ≥ 14%.

[Child–Pugh) or renal function [MELD]) would be a useful clinical tool. At present, only HVPG provides prognostic information before decompensation.[6,7] However, the spread of HVPG values is low in early cirrhosis. We previously found that CPA had a greater discrimination for the severity of fibrosis at lower HVPG values.[16] In our current study, which specifically evaluated 62 patients with cirrhosis, CPA had a median value of 16% (IQR: 10.75–23.25) with Ishak stage 5 or 6 disease, with increasing values from stage 5 to 6. We again found a significant correlation with HVPG. Interestingly, while differences in HVPG were not statistically significant between stages 5 and 6, differences in CPA were significantly different, suggesting a greater sensitivity to distinguish changes in advanced stage liver disease, in the same way that CPA gave a greater spread of values for low HVPG values.[16] By logistic regression analysis, we also found that CPA had a unique and independent association with CSPH, indicating a high risk of decompensation. A CPA of 14% or more was associated with a higher rate of liver decompensation, as is the case with CSPH.[7]

Table 3 CPS, HVPG, and CPA in first and last paired biopsies in 20 patients who had baseline Ishak stage 5 or 6 in their first biopsy

Variable (median value IQR)	1st biopsy	2nd biopsy	*P*-value
CPS	5.0 (5.0–5.8)	5.0 (5.0–6.3)	0.852
CPA (%)	15.2 (8.3–20.8)	17.0 (10.6–31.4)	0.079
HVPG mmHg†	8.0 (6.8–11.0)	8.0 (5.5–12.0)	0.903

†Seventeen patients with HVPG measured with during both liver biopsies.
CPA, collagen proportionate area; CPS, Child–Pugh score; HVPG, hepatic venous pressure gradient; IQR, interquartile range.

The analysis of the 17 patients with cirrhosis, who had repeated biopsies combined with HVPG measurements, showed that only CPA could be shown to increase over time, which was not the case with HVPG over the study intervals. Thus, CPA in cirrhosis, as well as in precirrhosis,[16] has potentially greater sensitivity for measuring the progression and regression of fibrosis. CPA could provide a basis for a more refined histological subclassification of cirrhosis. Although our results show the value of CPA to further classify cirrhosis histologically, the technique must be studied in other forms of chronic liver disease, before it can be considered a "universally"-applicable tool. It also needs to be validated in other centers. A limitation of the technique is that extra time is added to the histological examination, and the need for the new equipment (16), but the current cost of this is under 10 000 pounds sterling. Similar concepts concerning histological severity, but assessing the size of nodules and thickness of septa in cohorts of 43[8] 47[9] and 123 patients,[10] have also suggested that histological features in cirrhosis have prognostic value. However, in these studies, it is not clear which nodule or septum constitutes the reference structure in any particular microscopic field.

CPA is a sensitive index for the histological assessment of fibrosis, and being a measurement expressed as a continuous variable, it is well suited to assess therapies that might affect fibrosis, including both antifibrotics and antiviral drugs, particularly in patients within the category of cirrhosis in whom current histological scoring systems cannot assess degrees of severity of cirrhosis. As previously documented,[27] with regard to the correlation of transient elastography and high HVPG, we also found that at higher values of HVPG, the correlation between CPA and HVPG was not as good. Although the dynamic component of HVPG might explain why the correlation of HVPG with CPA, or indeed

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transient elastography, is not as good at higher HVPG values,[27] another study[28] did not find as good a correlation between transient elastography and HVPG, so this issue needs further evaluation. Studies evaluating the pharmacological reduction of HVPG should be able to assess how much of the HVPG might be due to fibrosis and how much due to dynamic intrahepatic vascular changes by assessing CPA.

In the present study, we have shown that CPA as a continuous variable is a suitable histological measurement to assess the degree of fibrosis in advanced-stage liver disease, including established cirrhosis, and would be a better marker compared to histological staging systems for comparisons with direct and indirect non-invasive measures, currently advocated for the evaluation and quantitation of hepatic fibrosis.[29]

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