Research Article

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Digital image analysis of collagen assessment of progression of fibrosis in recurrent HCV after liver transplantation

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Transplantation

Background & Aims: Histological assessment of fibrosis progression is currently performed by staging systems which are not continuous quantitative measurements. We aimed at assessing a quantitative measurement of fibrosis collagen proportionate area (CPA), to evaluate fibrosis progression and compare it to Ishak stage progression.

Methods: We studied a consecutive cohort of 155 patients with recurrent HCV hepatitis after liver transplantation (LT), who had liver biopsies at one year and were subsequently evaluated for progression of fibrosis using CPA and Ishak staging, and correlated with clinical decompensation. The upper quartile of distribution of fibrosis rates (difference in CPA or Ishak stage between paired biopsies) defined fast fibrosers.

Results: Patients had 610 biopsies and a median follow-up of 116 (18–252) months. Decompensation occurred in 29 (18%) patients. Median Ishak stage progression rate was 0.42 units/year: (24 (15%) fast fibrosers). Median CPA fibrosis progression rate was 0.71%/year (36 (23%) fast fibrosers). Clinical decompensation was independently associated by Cox regression only with CPA ($p = 0.007$), with AUROCs of 0.81 (95% CI 0.71–0.91) compared to 0.68 (95% CI 0.56–0.81) for Ishak stage.

Fast fibrosis defined by CPA progression was independently associated with histological *de novo* hepatitis (OR: 3.77), older donor age (OR: 1.03) and non-use/discontinuation of azathioprine before 1 year post-LT (OR: 3.85), whereas when defined by Ishak progression, fast fibrosers was only associated with histological *de novo* hepatitis.

Conclusions: CPA fibrosis progression rate is a better predictor of clinical outcome than progression by Ishak stage. Histological *de novo* hepatitis, older donor age and non-use/discontinuation of azathioprine are associated with rapid fibrosis progression in recurrent HCV chronic hepatitis after liver transplantation.

Keywords: Digital image analysis; Collagen; Fibrosis progression; Recurrent HCV; Liver transplantation.

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Introduction

Evaluation of liver fibrosis is an important aspect of the clinical management of chronic liver disease. Histological assessment is the reference standard [1], which has been used to assess changes in fibrosis with therapy, and to validate non-invasive markers of fibrosis [2].

Currently, all histological scores for staging in chronic viral hepatitis use categorical systems, which include description of architectural changes and sites of fibrosis. They do not assess fibrosis quantitatively [1–3]. These scoring systems distinguish between the grade and stage of chronic hepatitis, with the stage used as a measure of fibrosis and architectural changes. Although, fibrosis is a very important component of stage, the two terms have been confused [3] and this has led to misinterpretation of data in the literature, as stages have been evaluated incorrectly as quantitative estimates of fibrosis and as continuous variables statistically [1,2]. Indeed, fibrosis progression or regression has most often been evaluated in terms of a one or two Ishak or Metavir stage change.

We have published a method using computer-assisted digital image analysis (DIA) using picroSirius red stained histological sections to quantify liver collagen [1,4], as the quantity of picroSirius red correlates well with morphometrically calculated hepatic fibrosis [5]. The quantitative assessment of collagen is evaluated as collagen proportionate area. In previous studies, CPA in recurrent HCV post-LT correlated with both Ishak stage scores and HVPG, with greater percentage changes in CPA than in HVPG in early portal hypertension [6]. CPA at 1-year biopsy post-LT for HCV cirrhosis was highly predictive of clinical outcome and better than Ishak stage or HVPG [7]. Lastly, CPA allows subclassification of cirrhosis [8] and this potentially increases the possibility of using histological parameters in cirrhosis to predict clinical outcomes.

Fibrosis progression before [9,10] and after transplantation has prognostic significance: rapid fibrosis and early cirrhosis are associated with hepatic decompensation [11–13] and poor

survival [14]. Therefore, determination of CPA fibrosis progression rate could be prognostically useful.

The aims of this study were, firstly, to define the best method for evaluation of fibrosis progression (Ishak stage *vs.* CPA) with decompensation being the clinical end point, and secondly to identify risk factors for fibrosis progression in this population.

Patients and methods

Between October 1988 and October 2010, 304 patients were transplanted at the Royal Free Hospital with end-stage liver disease due to HCV infection (325 transplants): 155 patients with a first transplant, who had both a biopsy at one year (performed between 12–15 months) and at least one additional subsequent follow-up biopsy, were selected for the study. In our centre, patients transplanted for HCV cirrhosis are scheduled for biopsies at yearly intervals after transplantation, as part of routine care. If 'unexplained' changes in LFTs occur, patients are also biopsied. The last biopsy during follow-up was used to compare with the first, to assess the fibrosis progression rate adjusted for the time interval between biopsies. Predictive factors were evaluated with respect to changes in fibrosis from first to last biopsy.

For each patient, the following were recorded (listed in Table 1): demographic and clinical data, donor age and gender, cold and warm ischaemia time, initial and one year post-LT immunosuppression, characteristics and reatment of rejection episodes, the year of transplantation (divided into 3 eras, n_1 = 1988–1994, n_2 = 1995–2000, n_3 = 2001–2008), cytomegalovirus (CMV) post-LT infection or any other infection, histological episodes of *de novo* hepatitis (in biopsies performed during follow-up to diagnose causes of abnormal LFTs), genotype, viral load pre and 1 year post-LT, diabetes mellitus pre and post-transplant, human leukocyte antigen (HLA), and blood group compatibility of donor and recipient.

We calculated the percentage of fibrosis change according to CPA (CPA in the latest biopsy subtracted from CPA at one year in each patient, divided by time in years between the last and one year biopsy: (CPA last – CPA at one year)/time last – time at one year: CPA%/year). Ishak stage progression was calculated as stage in the most recent biopsy subtracted from stage in the biopsy at one year, divided by time in years – stage "units"/year between the two biopsies. If SVR was achieved at any time point, we evaluated only the last biopsy before starting therapy followed by SVR. We also evaluated changes in liver fibrosis at fixed intervals, at 3 and 5 years after liver transplantation, in relation to clinical decompensation, in order to make the time frame for evaluation more homogenous. The Ishak stage change calculation was evaluated as it has been used to assess fibrosis progression in the literature and we wished to compare our results with those of others [15,16] [2]. However, we acknowledge that since Ishak stage scores are not numerical measurements, without an arithmetical relationship between them (the "scores" are merely categorical labels), this method is an approximation [1–3]. Fast fibrosers were defined as patients in the upper quartile of the distributions of fibrosis progression values for either Ishak stage or CPA. The remainder constituted 'non-fast fibrosers'.

Clinical decompensation was defined as whichever occurred first of either, ascites/hydrothorax or variceal bleeding or encephalopathy. In the evaluation of the association of indices of fibrosis progression with clinical decompensation, we only evaluated the last biopsy before decompensation.

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki. All patients gave written informed consent for biopsies, HVPG and the histological evaluation for research purposes.

The 155 patients were followed-up for a median of 116 months (18–252). The median recipient age was 53 years (21–68), 126 were male (81%); median donor age was 41 years (16–62); 46% had genotype 1, 31.5% genotype 3, 44 (28%) had concomitant ALD and 35 (22%) HCC pre-LT and 6 (4%) concomitant HBV infection (HBV DNA continuously suppressed). Antiviral therapy for HCV was given in 46 patients: 14 achieved SVR. CMV was prospectively evaluated by 3 weekly surveillance blood specimens [17]; CMV infection was treated in 28. Rejection was diagnosed by protocol biopsies as previously described [18]: 21% (33 patients) had no episodes of acute rejection in protocol biopsies, 36% (56 patients) had 1 episode (14 episodes were not treated), 21% (33 patients) had 2 (3 were not treated), 19% (30 patients) had more than 2 episodes (5 did not receive treatment). Acute rejection episodes were treated with intravenous 1 g daily methylprednisolone for 3 days. Histological *de novo* hepatitis was defined as an increase in alanine aminotransferase levels (>2 upper normal limit), together with histological changes consistent with hepatitis without diagnostic features of cellular rejection, duct loss, or any other cause of liver injury [19]; it was diagnosed in 49 (31.4%).

Liver biopsies

Liver biopsy samples were formalin fixed, paraffin-embedded, and stained with hematoxylin-eosin, Gordon and Sweet staining for reticulin, and chromotrope aniline blue. For this study all biopsies were restained with picroSirius red to ensure comparable staining technique for collagen quantification and determination of CPA by DIA. The stage of disease (fibrosis; 0–6), and the grade of necroinflammatory activity were evaluated according to Ishak *et al.* [20].

The number of liver fragments, length of biopsy, lengths of each fragment summed, and number of portal tracts per fragment and in total were recorded [21]. Liver biopsies <12 mm long were excluded. Complete portal tracts were defined according to Crawford *et al.* [22].

Histological sections stained with picroSirius red were used for DIA, performed by two authors (P.M. and G.I.) blinded to each other's results and to clinical information at that time. Interobserver error was also assessed in this way, such that CPA measurement was repeated if there was a 2% difference or more. Inadequate staining was determined by the histopathologist and restaining was performed if this was thought to be needed. The equipment used and CPA measurement were performed as previously described [6]. As described in the original method, the CPA is an edited measurement: the vascular spaces are one of the structures manually edited specifically, thus removed from the CPA measurement [6].

Acute cellular rejection was graded using the Royal Free Hospital (RFH) score [23]. Histological *de novo* hepatitis C(DNHC) was defined as above [19].

Immunosuppression regimens

Maintenance immunosuppression regimens have changed over time but have been based on cyclosporine and later tacrolimus with or without prednisolone and azathioprine. These are described in detail in a previous publication [7], but in essence comprise a period of three randomized studies; initially CYA *vs.* TAC monotherapy [24]; then the TMC study, where a cohort received either CYA or TAC-based triple drug immunosuppression [25]. After the TMC study, patients transplanted for HCV cirrhosis, in a randomized study received triple immunosuppression therapy with corticosteroids, TAC and azathioprine (AZA), or TAC monotherapy, adjusting TAC dosing as previously described [26]. Steroids were tapered and stopped between 3 and 6 months. MMF substituted AZA if there was intolerance to AZA or renal dysfunction. Following the trial [26], triple therapy for HCV transplanted cirrhosis patients became standard of care.

There were 106 patients on tacrolimus (TAC), 41 of these on TAC monotherapy, and 49 on cyclosporine (CYA) (8 as monotherapy) as maintenance calcineurin inhibitor. There were 92 patients on azathioprine (AZA) (42 eventually discontinued before reaching year 1 post-LT). Another 13 received MMF in substitution of azathioprine due to renal impairment during follow-up. There were 99 patients on steroids immediately post-LT and of these, 56 (36%) were maintained on steroids beyond 3 months. In our randomized trial in post-transplant HCV patients, 65 out of the 155 were recruited [26], 34 to tacrolimus monotherapy, and 31 to tacrolimus, azathioprine and steroids therapy. Another 61 patients received azathioprine without being randomized in the triple therapy arm, 15 of these discontinued azathioprine before year 1 post-LT.

Acute rejection episodes if histologically moderate/severe were treated with 1 g daily methylprednisolone for 3 days, intravenously. If there was no histological improvement in a biopsy 5 days after the first, and if was not satisfactorily resolved by 1 further cycle of 3 doses of methylprednisolone, rejection was treated with lymphocyte antibodies orthoclone (OKT-3) or antithymocyte globulin (ATG).

Statistical analysis

Data were analyzed using SPSS (version 20.0; SPSS IBM). The Chi squared test was used to compare frequencies. Quantitative variables, which were normally distributed, were expressed as mean values ± standard deviation and non-normally distributed as median values (range). Significance testing was set at p <0.05.

Logistic regression was used to evaluate factors associated with fast *vs.* non-fast fibrosers. The ROC analysis was used to compare the performance of CPA index *vs.* Ishak index of fibrosis progression with regard to clinical decompensation. Cox regression was used to compare the occurrence of clinical decompensation between fast fibrosers and non-fast fibrosers using CPA. Kaplan Meier-derived curves were used for each statistically significant variable in the multivariate analysis. Time to decompensation was the time from transplantation to the first episode of decompensation. For those with no decompensation, the interval to last follow-up or death was used as the observation interval. Follow-up was censored at the time of SVR achievement.

Table 1. Patients' characteristics with respect to fibrosis progression based on CPA. All these variables were included in the multivariate analysis.

			Fast fibrosers	Non-fast fibrosers
Number of patients	n	155	36	119
Recipient	Age	53	53	53
	Gender (male %)		83	80
Donor	Age	41	45	38
	Gender (male %)	73	83	62
Concomitant ALD	n (%)	44 (28)	10 (28)	34 (28.6)
HCC pre-LT	n (%)	35	9 (25)	26 (22)
Cold ischemia time	min	677	681	674
Warm ischemia time	min	43	45	43
Diabetes pre-LT	%	29	33	26
Diabetes post-LT	%	32	36	31
Viral load pre-LT	median	1.96×10^6	2.4×10^6	1.8×10^6
Genotype 1	%	46	44	47
CMV infection	%	26	6 (17)	20 (17)
Histological *de novo* hepatitis	%	48	21 (56)	28 (23.5)
ACR episodes	0/1/2/>2		30%/39%/14%	19%/35%/24%
Initial immunosuppression	TAC/CYA	106/49	26/10	80/39
	STEROIDS	99	22	77
	AZA/MMF	92/13	24/2	68/11
AZA discontinued	n (%)	42 (27)	15 (42)	27 (23)
Follow-up	months	116 (18-252)	87 (18-198)	125 (36-252)
Decompensated	n (%)	29 (12)	22 (61)	7 (5.9)

Results

There were 610 biopsies: 587 were evaluated (median number of biopsies 3/patient), as 23 were less than 12 mm long and were excluded from analysis: 36 patients had 2 biopsies only, 48 had 3 biopsies, 22 had 4 biopsies, and the remaining 49 patients had more than 4 biopsies (5–12). Median CPA fibrosis progression rate was 0.71%/year (0.0–2.6 interquartile range; 2.6%/year upper quartile). Fibrosis progression according to CPA over time (over ten years) is shown in Fig. 1. A CPA rate of increase $\geqslant$2.6%/year (upper quartile) was present in 36 (23%) patients (Fig. 2). Median progression according to Ishak stage rate of increase was 0.42/year (interquartile range 0–1; upper quartile 1/year). A stage ratio $\geqslant$1/year was found in 24 (15%) patients. Stage progression over time is shown in Fig. 1. Mean stages per year (given for comparison with previous studies by others) were: at 1 year (1.73), 2 years (2), 3 years (2.4), 4 years (2.5), 5 years (2.7), 6 years (2.8), 7 years (2.93), 8 years (3.57), 9 years (3.58), and 10 years (3.6).

During follow-up, 29 (19%) of the 155 patients decompensated at a median of 114 months (15–191) from liver transplantation: the first decompensation was ascites and/or hydrothorax in 19 patients, variceal bleeding in 3, and encephalopathy (PSE) in 5. Two patients decompensated before the second biopsy and thus were excluded from our study population. Death occurred in 33 patients (21%) at a median of 80 m (15–195): 18 were liver related (3 from recurrent HCC).

The demographic and clinical data listed in Table 1 were evaluated in the logistic and Cox regression analysis.

The ROC curve for rate of increase of fibrosis (fast *vs.* non-fast fibrosers), for the association with decompensation, is shown in Fig. 3. The AUROC for clinical decompensation was 0.81 (p <0.001, 95% CI 0.71–0.91) for CPA progression, and 0.68 (p = 0.003, 95% CI 0.56–0.81) for Ishak stage rate of increase (p = 0.67, n.s. in Cox regression).

Using logistic analysis, we studied possible factors associated with fast fibrosers *vs.* non-fast fibrosers based on CPA rate of increase. In the univariate analysis, donor age >40 years (p = 0.001), non-use of maintenance steroids post-LT (p = 0.035), non-use or discontinuation of azathioprine within 1 year post-LT (p = 0.001), and episodes of histological *de novo* hepatitis (p = 0.001) were independently associated with fast fibrosis. In the multivariate analysis, histological *de novo* hepatitis (p = 0.016, OR = 3.77, 95% CI 1.65–8.72), non-use or discontinuation of azathioprine (p = 0.004, OR = 3.85, 95% CI 1.25–11.83), and donor age >40 years (p = 0.026, OR = 1.03, 95% CI 1.006–1.061) were independently associated with fast fibrosers. With Ishak stage fibrosis progression, in the multivariate analysis, only histological *de novo* hepatitis was associated with fast fibrosers (p = 0.002, OR = 4.4, 95% CI 1.7–11.07). Comparing the patients receiving azathioprine within the randomized trial together with those patients not randomized, the continued use of azathioprine beyond one year was associated with a low rate of fast fibrosis: 2/14 *vs.* 4/30; and clinical decompensation 2/14 *vs.* 6/30, respectively, in both azathioprine groups.

The time to first clinical decompensation was associated in the univariate analysis with fibrosis rate of increase based on CPA (p <0.001), Ishak stage fibrosis rate of increase (p = 0.001), advanced donor age (p = 0.007) and histological *de novo* hepatitis C (p = 0.013). In Table 2, the details of the 29 patients who decompensated are shown. In the multivariate analysis, in Cox regression the only factor associated with clinical decompensation was rate of fibrosis increase according to CPA (p = 0.001,

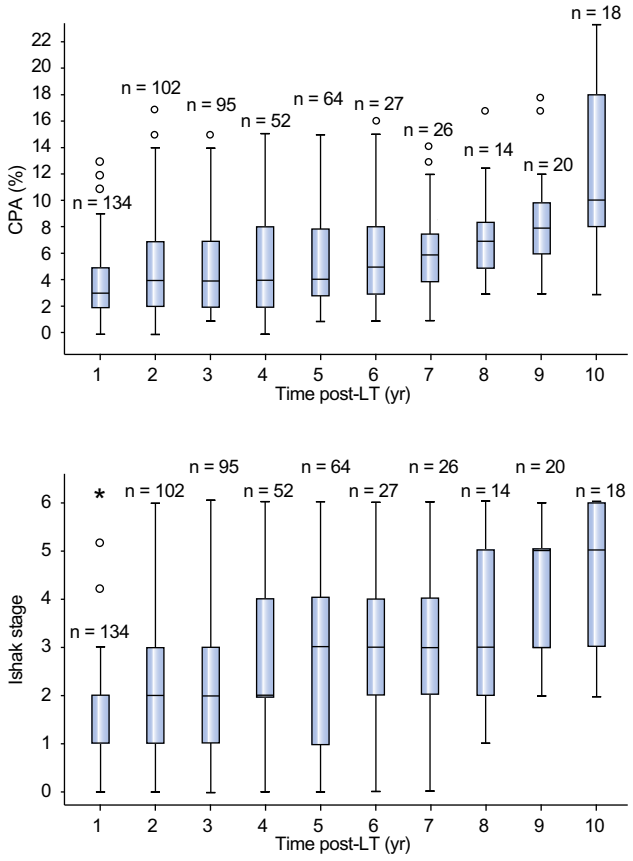


Fig. 1. Median fibrosis according to time after liver transplantation based on CPA and median Ishak stage. 587 biopsies were evaluated (median number of biopsies 3/patient): 36 patients had 2 biopsies only, 48 had 3 biopsies, 22 had 4 biopsies, and the remaining 49 patients had more than 4 biopsies (5–12). Median CPA fibrosis progression rate was 0.71%/year. Each box plot shows the median value, the interquartile range, and the range of CPA each year.

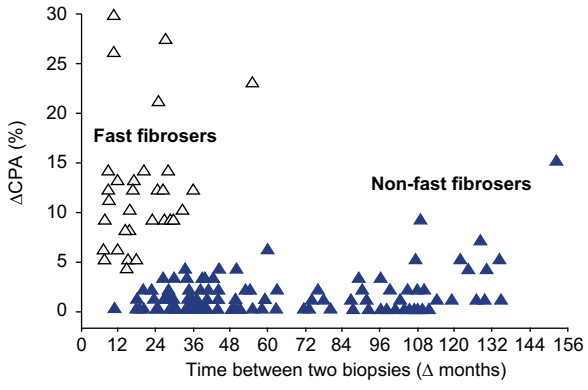


Fig. 2. CPA change (CPA at one year subtracted from CPA in the latest biopsy) in each patient *vs.* time interval between the two biopsies. The upper quartile of distribution of the fibrosis rates >2.6%/year CPA defined 'fast fibrosers'.

OR = 1.2, 95% CI 1.12–1.2). Fig. 4 shows the Kaplan–Meier curves of fast fibrosers according to CPA *vs.* 'non-fast fibrosers' (both by Mantel-Cox and Breslow, $p = 0.0001$).

Using the 3 years (95 patients) and 5-years (64 patients) fixed intervals after liver transplantation, and the upper quartile of the

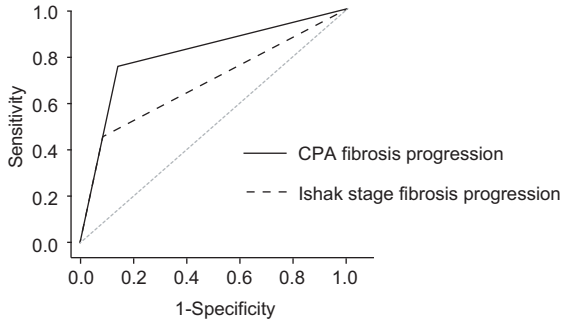


Fig. 3. ROC curves of fibrosis rate of increase according to CPA and Ishak stage for the prediction of clinical decompensation. The AUROC for clinical decompensation was 0.81 (p <0.001, 95% CI 0.71–0.91) for CPA progression and 0.68 ($p = 0.003$, 95% CI 0.56–0.81) for Ishak stage rate of increase ($p = 0.67$, n.s. in Cox regression).

distributions of CPA and Ishak for each time point *vs.* the other three quartiles, the time to clinical decompensation was statistically significant only for CPA ($p = 0.033$ and $p = 0.002$ in 3 and 5 years, respectively), but not for Ishak stage ($p = 0.16$ and $p = 0.11$ for 3 and 5 years, respectively), suggesting a more sensitive assessment of fibrosis using CPA.

There was no difference between patients with or without concomitant alcoholic liver disease pre-LT (n = 44) regarding fibrosis progression. From these 44, 7 male patients were documented as drinking more than 21 U of alcohol per week post-LT. All of these patients were fast fibrosers. However, exclusion of this group from our analysis made no difference in our results (AUROC 0.816, 95% CI 0.71–0.922).

Of the 47 patients receiving antiviral treatment, 14 achieved SVR, 33 did not. All of the 14 patients were non-fast fibrosers before achieving SVR. Patients were censored at the time of SVR. In the 33 not achieving SVR, 12 (36%) were fast and 21 (64%) non-fast fibrosers. Of the patients who did not receive antiviral treatment (108), 28 were fast fibrosers (26%).

Discussion

In this paper, we describe for the first time, the assessment of histological progression of fibrosis using collagen quantification morphometrically compared to the rate of increase of Ishak stage. We evaluated the association of these two different histological parameters with clinical decompensation as the relevant end point, to assess the potential clinical applicability of the methodology. We also performed logistic regression analysis to identify independent risk factors associated with fast fibrosers (defined by rate of CPA increase), due to recurrent HCV chronic hepatitis after liver transplantation, an evaluation not previously performed.

CPA at one time point is a histological measurement that quantifies fibrosis, and relates to clinical outcomes [6,7]. CPA also correlates with HVPG with wider range of values in patients with Ishak stage 5/6, showing that cirrhosis can be subclassified [8]. As such, it is different from the traditional histological scoring systems for fibrosis, which assign numerical symbols to descriptive categories of architectural changes in the biopsy. The numerical symbols are not quantitatively related, or are they continuous variables [1]. However, quantitative assessment of collagen is

Table 2. The 29 patients who decompensated are presented with respect to CPA and Ishak stage at year 1, CPA progression per year – defining fast fibrosers – months to decompensation post-LT, episodes of acute hepatitis (AHC), azathioprine use or discontinuation (D) before year 1 and donor age. Highlighted/in bold are those variables which are considered to be prognostic for future decompensation: CPA at year 1 >6% (7), use of azathioprine (current work), donor age >40 years, histological episodes of acute hepatitis (19) and current work.

Patients no.	Year 1 CPA	Year 1 Ishak stage	*De novo* hepatitis	AZA	Donor age	ΔCPA/year	Months to decompensation	Decompensation
1	4	2	**Yes**	**D**	**58**	**30**	17	Ascites
2	5	2	**Yes**	**D**	24	**12**	19	Ascites
3	4	2	No	**D**	**59**	**12**	22	Ascites
4	4	2	No	**D**	**69**	**17**	25	Ascites
5	4	2	No	**No**	**60**	**3**	27	Ascites
6	5	2	**Yes**	**D**	**49**	**30**	28	PSE
7	**7**	6	**Yes**	Yes	22	**30**	30	PSE
8	**16**	6	**Yes**	**No**	**59**	**9**	30	Ascites
9	**7**	4	**Yes**	**No**	**58**	**30**	35	Ascites
10	4	0	**Yes**	Yes	**55**	**3**	36	Ascites
11	3	3	**Yes**	**No**	**42**	**10**	36	PSE
12	5	3	**Yes**	**D**	**55**	**28**	39	Variceal bleeding
13	**6**	2	No	**D**	**48**	1	49	Variceal bleeding
14	**8**	3	No	**No**	22	**3**	53	Ascites
15	**9**	5	**Yes**	**No**	**45**	**18**	67	Ascites
16	**6**	4	**Yes**	Yes	**40**	**4**	73	Ascites
17	2	1	No	**D**	37	1	76	PSE
18	**8**	3	**Yes**	**D**	**45**	**10**	83	Ascites
19	3	1	No	Yes	**73**	**4**	84	Ascites
20	**13**	4	**Yes**	**No**	**65**	**7**	88	Ascites
21	4	2	**Yes**	**D**	**45**	**6**	95	Ascites
22	**12**	5	No	Yes	**61**	**3**	101	PSE
23	**7**	5	**Yes**	**No**	**42**	**11**	105	Ascites
24	1	2	**Yes**	Yes	30	1	129	Ascites
25	4	2	**Yes**	**D**	33	**3**	129	Variceal bleeding
26	2	1	No	**D**	**51**	2	132	PSE
27	1	3	No	Yes	**45**	**5**	154	Ascites
28	1	2	No	**D**	**45**	**15**	165	Ascites
29	3	1	No	Yes	**43**	1	193	Ascites

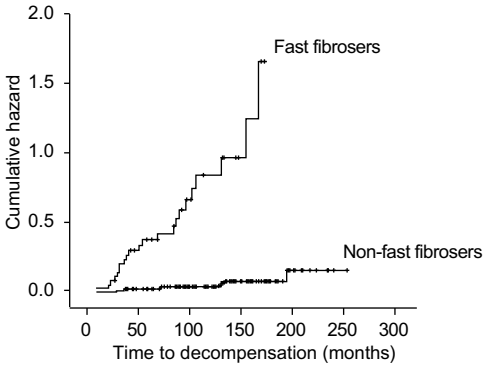
not a substitute for a descriptive evaluation of architectural changes in the liver as we have emphasized previously [2,3], but is an added evaluation.

Fibrosis progression after liver transplantation for hepatitis C related cirrhosis has been studied previously by several groups using Ishak or Metavir staging [27–30], but any definition of fast fibrosers has been arbitrary. HCV infection after liver transplantation is universal and chronic liver disease is common, leading to cirrhosis in 20% or more by 5 years after liver transplantation (LT) [31,32]. Fibrosis progression rate is rapid and is associated with older donors [19,33,34], drugs used for immunosuppression [35–37], diabetes after transplant [38], and episodes of histological *de novo* hepatitis C, defined as an increase in alanine aminotransferase levels (>2 upper normal limit), together with histological changes, consistent with hepatitis without diagnostic features of cellular rejection, duct loss, or any other cause of liver injury [19].

HCV recurrence is responsible for graft failure, which results in increased mortality. This clinical course makes the population transplanted for HCV cirrhosis an appropriate cohort to evaluate methods for the assessment of fibrosis progression with relationships with clinical outcomes. Secondly, the increased fibrosis rate allows a more accurate evaluation of risk factors associated with the progression of fibrosis.

In our cohort of 155 patients, the only factor predicting clinical decompensation using Cox regression analysis was CPA. Comparing Ishak stage and CPA progression rates by AUROC curves with respect to the first episode of clinical decompensation; this was 0.81 for CPA and 0.68 for Ishak stage fibrosis progression. This confirms the validity and better performance of using CPA to assess progression of fibrosis for recurrent HCV chronic hepatitis after liver transplantation.

The evaluation of factors by multivariate analysis associated with fast fibrosers, showed that episodes of histological *de novo* hepatitis post-LT, donor age >40 years and non-use or discontinuation of azathioprine (within 1 year of transplantation) were independently associated risk factors. Advanced donor age is well recognized to be associated with more aggressive HCV disease and liver disease progression [19,29,39–41]. However, the role of immunosuppression in HCV recurrence is still under debate. One study specifically reported no difference between cyclosporine and tacrolimus in terms of stage progression [42] without



	month:	12	48	72	108	156	180	200
Fast	Free of decompensation (%)	97	69	59	40	16		
fibrosers	Patients exposed to risk	36	23	18	9	3		
Non-fast	Free of decompensation (%)	100	98	96	96	93	87	87
fibrosers	Patients exposed to risk	119	109	96	64	34	16	8

Fig. 4. Kaplan–Meier curves of fast fibrosers defined by the upper quartile of distribution of the fibrosis rates >2.6%/year of CPA and the remainder (non-fast fibrosers) with respect to the prediction of clinical decompensation. Clinical decompensation was defined as whichever occurred first of either ascites/hydrothorax or variceal bleeding or encephalopathy, in a cohort of 155 patients with paired liver biopsies from year 1 after LT onwards.

differences in severity of HCV recurrence. In a recent review of immunosuppression in HCV transplantation, tacrolimus was shown to be better than cyclosporine with better graft and patient survival [18]. Azathioprine has been associated with reduced disease progression in observational studies compared to mycophenolate mofetil [7,19,33,43–47], but there has been no randomized comparison [18]. In our cohort, the results are in accordance with our previous published randomised trial [26], demonstrating a slower onset of histological severe fibrosis with continued use of azathioprine long term. The similar results in patients treated with azathioprine, within and without the randomized study (Supplementary Fig. 1), reinforce the general observation that azathioprine may be of benefit [2,18].

Importantly in our transplant population, the overall rate of increase of disease stage described by changes in Ishak stage is similar or lower than fibrosis rates described by others [31,40,48], with our median donor age (41) similar to others [40] (median donor age 42), or even older than those described in other studies, with a mean donor age of 34 [48]. Indeed, the fibrosis rates are as low as those published recently in an observational study in patients receiving sirolimus compared to historical controls [30]. The mean fibrosis stage in patients receiving sirolimus was 0.62 and 1.15 according to Metavir in year 1 and year 2 biopsies, respectively, while our mean fibrosis Ishak stage was 1.7, and 2, in years 1 and 2 post-LT, with the same donor age in the two populations. The relatively low overall rate of increase of disease stage in our study population most likely reflects the use of the combined therapy of TAC and AZA and/or lower trough levels of tacrolimus [18], which we found beneficial in our randomised trial [26]. Another histological feature, which has been described in a single paper as predictive for fibrosis progression, is activated stellate cells [49], but the method as yet has to be validated.

In conclusion, the rate of increase of CPA can be used as a measurement of progression of fibrosis, and is a good predictor of clinical decompensation, and is better than the rate of increase of Ishak stage. Although liver biopsy is the reference standard for assessing fibrosis and thus still needs to be done, a quantitative method of assessing fibrosis, which has clinical significance, such as CPA [7,50], will lead to comparison with non-invasive tests of fibrosis obviating the need of biopsy in the future. Although, we studied a transplant population with recurrent HCV, our results in chronic hepatitis C and B [50] suggest the method is generalisable. However, these findings need to be confirmed by others in viral and non-viral chronic liver disease before and after transplantation. Our results suggest that CPA can be considered as a potential histological index for future studies of fibrosis, including those for validation of non-invasive markers of fibrosis.

Conflicts of interest

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.12.016.

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