**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 with 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.
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 decomposition 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, n1 = 1988–1994, n2 = 1995–2000, n3 = 2001–2008), cytomegalovirus (CMV) post-LT infection or any other infection, histological episodes of acute rejection in protocol biopsies, 36% (56 patients) had 1 episode as previously described [18]: 21% (33 patients) had no episodes of acute rejection, CMV infection was treated in 28 patients. Rejection was diagnosed by protocol biopsies as previously described [17]. HBV DNA continuously suppressed. Antiviral therapy for HCV was given to 46 patients, age was 41 years (16–62); 46% had genotype 1, 31.5% genotype 3, 44 (28%) had 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 cycle further of 3 doses of methylprednisolone, rejection was treated with lymphocyte antibodies orthocline (OKT-3) or antithymocyte globulin (ATG).

Clinical definition was 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 alaminoproliferative lesions (>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%).
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 >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 >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 fibrosis. 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,
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 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
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 fibrokers 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 fibrokers, 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

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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 [51,62]. The mean fibrosis stage 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, 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
A.K. Burroughs and A.P. Dhillon have an unrestricted educational grant from Pfizer. P. Manousou is supported by a fellowship from the Hellenic Association for the Study of the Liver.

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.

References
Research Article


