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Non-invasive assessment of liver fibrosis in patients with HBV-related chronic liver disease undergoing antiviral treatment: A preliminary study

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ABSTRACT

In chronic hepatitis B (CHB) patients, fibrosis assessment during antiviral treatment is a key step in the clinical management. Aim of this study was to evaluate the performance of elastography in assessing fibrosis stage in CHB before and after two years of nucleoside/nucleotide analogues (NUC) treatment in comparison with indirect serum markers. CHB diagnosis was made according to standard criteria. A clinical and virological evaluation was performed at baseline and again at 3, 6, 9, 12, 18, and 24 months during treatment. Fibrosis was evaluated by liver biopsy, elastography and indirect serum markers. Of 75 patients, 50 had CHB, HBeAg negative and were deemed eligible for this study. Of these, 22 underwent liver biopsy. Mean histomorphometric values of fibrotic tissue differed significantly in the stage < S3 vs. stage ≥S3: 2.01 ± 2.62% vs. 12.85 ± 7.31% (p=0.03), respectively. At 18 and 24 months, stiffness values were statistically reduced from those previously observed (P=0.03 and P < 0.001). At 24 months the values of APRI, FIB-4 and LOK were not different from baseline values, while the value of FORNS score at 24 months was the only one statistically reduced. In two patients with fibrosis stage S3 and S6, respectively, fibrosis regressed to stage S2 and S5. In conclusion, the results of the present study show that liver histology, stiffness and FORNS score improve significantly during a long-term follow-up of HBV patients successfully treated with NUC. These results strongly suggest that the non-invasive evaluation of liver fibrosis represents a key step in the management and treatment of chronic HBV hepatitis.

1. Introduction

Chronic liver disease represents a major public health problem worldwide due to its morbidity, mortality, and associated economic costs (Minino et al., 2007). The introduction of an effective vaccine against the hepatitis B virus (HBV) has reduced the prevalence of hepatitis, as well as its health and economic impact in industrialised countries. In Europe, the WHO estimates that 13.3 million people are HBV infected and Italy falls among the countries with low endemicity (positivity for HBsAg < 2%) (Schweitzer et al., 2015; Stasi et al., 2015).

According to the latest recommendations, antiviral treatment for CHB needs to be considered in the presence of HBV-DNA > 2000 IU/ml, elevated ALT, and/or moderate liver fibrosis (Ishak ≥2). In HBeAg

positive patients, the primary therapeutic goal is to achieve a stable seroconversion HBeAg/anti-HBe. Patients with immune tolerance or high levels of viremia (HBV DNA > 2 × 10⁷ IU/ml) do not require treatment in the absence of hepatocellular damage, although they should still be monitored. Tenofovir, entecavir and peginterferon alfa-2a are the preferred first-line treatments for both HBeAg-positive and HBeAg-negative CHB infected patients (Carosi et al., 2011).

Some studies suggest that the complete long-term suppression of HBV replication by nucleosides/nucleotides results in a long-term improved outcome that significantly reduces the risk of developing liver cirrhosis, hepatocellular insufficiency, and, hepatocellular carcinoma (Tan and Sun, 2013). Moreover, longitudinal histopathological evaluation has demonstrated a regression of liver tissue fibrosis during

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entecavir/tenofovir therapy (Papachrysos et al., 2015).

To date, few studies have evaluated the longitudinal changes of liver fibrosis in CHB positive patients with transient elastography (TE). The aim of the current study was therefore to evaluate whether TE and indirect serum markers could represent a valuable clinical resource for monitoring tissue fibrosis during and after antiviral therapy.

2. Materials and methods

Patients with HBV referred between January 2010 and December 2015 to the Hepatology outpatient services of the Azienda Ospedaliero Universitaria Careggi (AOUC), Florence, Italy, were considered for the study.

The treatment of HBV patients was established in accordance with the Italian recommendations (Carosi and Rizzetto, 2008).

The study was clearly explained to the patients, and their written informed consent was obtained. An information form on the study design and on the treatment of clinical data collected during the same protocol was released to each patient. There was no restriction regarding current treatments for other diseases except for those therapies/diseases listed in the exclusion criteria.

Inclusion criteria were as follows: patients naïve to antiviral treatment with nucleoside/nucleotide analogues, aged between 18 and 70 years, HBsAg positive, HBV DNA > 2000 IU/ml, HBeAg negative, anti-HBe positive, with liver fibrosis assessed by liver biopsy and/or by FibroScan; patients naïve to treatment presenting clinical and biochemical diagnosis of HBV related cirrhosis (biopsy was not performed for these patients). Exclusion criteria were as follows: ALT > 5 x ULN, HBeAg positive patients, BMI > 30, coinfections (HIV, HCV, HDV), pregnancy, connective tissue diseases, psychiatric illnesses compromising compliance with therapy, presence of ascites at ultrasound, hepatocellular carcinoma (HCC), alcohol or drug related liver disease, treatment with corticosteroids and/or interferon alpha in the six months prior to enrolment in the study, resistance to antiviral treatment or the presence of side effects requiring an association or replacement with another drug.

2.1. Non-invasive assessment

2.1.1. Indirect serum markers

All patients were assessed with the following surrogate markers of liver fibrosis: APRI (Wai et al., 2003), FIB-4 (Vallet-Pichard et al., 2007), Forns score (Forns et al., 2002), Lok score (Lok et al., 2005). The above scores were calculated using biochemical tests carried out within one month before liver biopsy. The same tests were repeated and the scores calculated 24 months following the initiation of treatment.

The entire cohort of patients evaluated by Ishak score, together with the cirrhotic patients on the basis of clinical and ultrasound evaluation, was evaluated with HUI score (HUI et al., 2005), a non-invasive biomarker validated for HBV to distinguish between significant and non significant fibrosis.

2.1.2. Transient elastography

Liver stiffness was measured using FibroScan® (Echosens, Paris, France), according to the manufacturer instructions. In all patients, TE was performed after an overnight fasting (Arena et al., 2013). Procedures with 10 successful acquisitions, expressed in kilopascal (kPa), with a success rate of at least 60% and an interquartile range (IQR) lower than 30% of the median value, were considered reliable. Liver stiffness was measured at commencement, and again at 3, 6, 9, 12, 18, and 24 months during treatment.

2.2. Invasive assessment

2.2.1. Liver biopsy

On the same study day, patients underwent a measurement of liver stiffness by TE. Ultrasound-guided percutaneous liver biopsy was then performed on the right lobe of the liver with a 16-gauge semiautomatic modified Menghini needle system (BIOMOL; Hospital Service, Aprilia, Italy) under local anaesthesia. Liver specimens were formalin-fixed and paraffin-embedded for histological evaluation. Sections of liver tissue were stained with haematoxylin, eosin and Sirius red. These were then examined by an experienced pathologist who was unaware of the liver stiffness results. All liver specimens had a length > 25 mm and included at least 11 complete portal tracts, reflecting adequate standards (Guido et al., 2004). The presence of necro-inflammatory activity (grading) and fibrosis (staging) was established according to the method proposed by Ishak (Ishak et al., 1995). Each unit participating in the study, if external to AOUC, provided three unstained paraffin sections. At the end of the study, histopathological examination of all samples (pre- and post-treatment) was repeated by a pathologist. In the event of disagreement between the two pathologists, a review of the scoring by collegial observation was scheduled.

2.2.2. Morphometry

Only the sections of each biopsy stained with Sirius red were used for calculating the percentage of collagen, which was performed by one author (C.S.). The percentage of collagen content was calculated by digital image analysis (Documentation – RSB Home Page). This software enables, through a grey scale slider, to select the total tissue area of liver biopsy. Subsequently, red, green, and blue (RGB) light channels were used to select the collagen area. Before the measurement of liver fibrosis structural collagen in large portal tracts, blood vessel walls, artefacts, vascular cavities, and lymphoid aggregates were eliminated. The results of the digital analysis were compared with the standard Ishak score (Ishak et al., 1995).

2.3. Statistical analysis

All results are expressed as mean ± standard deviation. After checking similar variances within the groups using Levine's test for equality of variances, the numerical comparison of continuous data was performed using the Student's *t*-test for unpaired and for paired samples with Bonferroni correction. Statistical significance was set at $P < 0.05$.

To evaluate factors associated with hepatic fibrosis, patients were divided in 2 groups: patients with non significant (< 6 kPa) or significant fibrosis (≥ 6 kPa) along the EASL-ALEH guidelines (2015). Univariate analysis explored each variable in the data set, including red blood cells, white blood cells, platelets, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alpha-fetoprotein, gamma-glutamyltransferase (GGT), ferritin, sideremia, creatinine, glucose, cholesterol, INR, bilirubin, albumin and all non invasive serum markers.

Logistic regression models were used for multivariate analysis to identify the most significant correlation among the variables. All variables in univariate analyses with $P < 0.05$ were introduced into the multivariate analysis.

3. Results

The study evaluated 75 patients (48 males and 27 females, mean age 48.45 ± 13.98). Of these, 50 CHB, HBeAg negative patients were deemed eligible. The mean stiffness value of these patients was 10.52 ± 6.05 kPa. The biochemical parameters of the entire study population are shown in Table 1. Baseline values of APRI, FORNS, FIB-4 and LOK were 0.91 ± 1.65, 4.5 ± 1.93, 0.73 ± 0.68, 0.29 ± 0.17, respectively. In these patients, when univariate analysis was conducted to evaluate

Table 1
Clinical and laboratory parameters in the study population.

Parameter	Value	Normal Range
Age, (yrs)	48.45 ± 13.98	
BMI	25.17 ± 3.69	
Male gender, n (%)	64%	
Red Cells (10 ³)	4945.98 ± 534.16	F: 4.000 – 5.500 M: 4.500 – 5.900
White cells	5864.43 ± 1620.74	4.500 – 8.500
Cholesterol (mg/dl)	175.47 ± 35.44	< 210
γ-GT (U/L)	43.42 ± 61.03	10 – 40
AST (U/L)	45.63 ± 80.62	5 – 40
ALT (U/L)	64.76 ± 143.10	5 – 40
Sideremia (mcg/dl)	105.38 ± 44.95	M: 65 –176 F: 50 –170
Platelet count (10 ⁹ /L)	199.26 ± 61.60	140–440
HBV DNA	22869.5 ± 47449.21	< 20

Results are expressed as mean ± SD.

Abbreviations: γ-GT, gamma glutamyl-transpeptidase; AST, aspartate transaminases; ALT, alanine transaminases; BMI, Body Mass Index.

Table 2
Univariate analysis for factors associated with liver fibrosis.

Parameters	Mean ± standard deviation (Stiffness ≥6 kPa)	P value
ALT(U/L)	64.9 ± 149.42	0.0549
AST (U/L)	46.2 ± 84.47	0.0371
γ-GT (U/L)	44.61 ± 64.40	< 0.001
APRI	0.97 ± 1.73	0.0446
FORNS	4.55 ± 2.00	0.0237
LOK	0.3 ± 0.17	0.0140
Stiffness (kPa)	11.37 ± 6.13	

Abbreviations: γ-GT, gamma glutamyl-transpeptidase; AST, aspartate transaminases; ALT, alanine transaminases.

factors associated with liver fibrosis, the parameters associated with significant fibrosis (stiffness > 6 kPa) were ALT, AST,GGT, APRI, FORNS, LOK (Table 2). However, after the multivariate analysis these parameters had no relationship with hepatic fibrosis. No relationship with liver fibrosis was found for non significant fibrosis. Twenty-two patients agreed to undergo a pre-treatment liver biopsy. Out of twenty-two patients, 17 patients had Ishak score ≥S2 (3 patients with histological evaluation of liver cirrhosis). Taking into account the cohort of patients who underwent liver biopsy together with cirrhotic patients, based on clinical and ultrasound evaluation, we found that the percentage of patients with HUI > 0.15 (corresponding to Ishak score > S3) was 45%(N=10). The mean value for HUI score ≤0.15 was 0.033 ± 0.017 and the mean value for HUI score HUI > 0.15 was 0.39 ± 0.31.

All sections of liver biopsies stained with Sirius Red were analysed with image analysis. The mean histo-morphometric value of fibrotic tissue was 6.83 ± 7.03.

Patients were divided into 2 groups according to Ishak score: 12 patients were in histological stage < S3, 10 patients were in histological stage ≥S3. Mean histo-morphometric values of fibrotic tissue differed significantly in the stage < S3 vs. stage ≥S3: 2.01 ± 2.62%vs. 12.85 ± 7.31% (P=0.03), respectively (Fig. 1). A statistically significant correlation was found between morphometry and elastography (P < 0.001), morphometry and LOK (P = 0.05), and morphometry and APRI (P = 0.022).

Twenty-two patients were eligible for treatment (entecavir or tenofovir) and agreed to continue the follow-up.

3.1. Longitudinal evaluation

To avoid the potential bias related to patients lost to follow-up, the

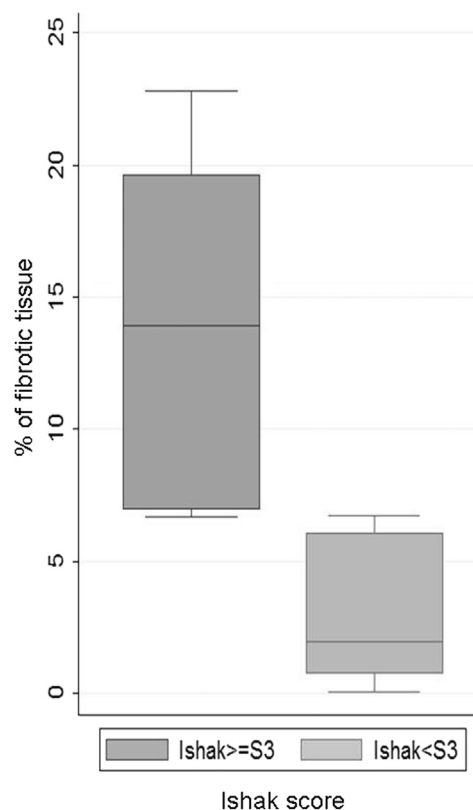


Fig. 1. Morphometry in patients who underwent liver biopsy. Liver biopsies, divided according to Ishak score, showed significantly different percentages of fibrosis at morphometry among Ishak score ≥ S3 and Ishak score < S3 (P≤0.03). *P=0.03; **P < 0.001.

difference between the mean values of basal stiffness and those of the follow-up was only calculated in 20 patients (one was lost to follow-up during therapy and one developed hepatocellular carcinoma) of the 50 who were initially enrolled. Four patients had cirrhosis based on clinical and ultrasound evaluation, and 3 patients based on histological evaluation. All patients experienced response to treatment. The difference between the mean values of the initial stiffness (12.60 kPa ± 6.31) of the patients with two years of follow-up and that of the 30 patients (6.82 kPa ± 1.07) without follow-up was significantly different (P < 0.001). Stiffness values were available for 20 patients over two years of treatment (Fig. 2). After three months of treatment, HBV DNA was still detectable in seven patients with ≥2 log reduction of viral load. Mean stiffness values (9.76 kPa ± 3.82) were not significantly different from those observed prior to therapy (12.60 kPa ± 6.31) (P = 0.15). After six months, HBV DNA was still detectable in four patients. Mean stiffness values (8.78 kPa ± 3.24) were not significantly different from those observed prior to therapy. After nine months, HBV DNA was still detectable in two patients. Mean stiffness values at nine months (8.46 kPa ± 2.94) were not significantly different from those observed previously. After 12 months, HBV DNA was not detectable in all patients (8.42 kPa ± 2.7). Mean stiffness values at 12 months were not statistically different from those observed previously. After 18 months (7.85 kPa ± 2.26) and 24 months (7.28 kPa ± 3.17) the difference between the mean values of basal stiffness and those of the follow-up were statistically different (P=0.03 and P < 0.001) (Fig. 2).

Baseline values of APRI, FIB-4 and LOK were not different from those observed at 24 months during therapy (0.36 ± 0.28; 0.74 ± 0.39; 0.22 ± 0.08, respectively), while the FORNS score at 24 months was statistically different from that observed before therapy (2.04 ± 0.34).

Mean ALT values during therapy were not significantly different from those observed before therapy. No statistically significant correlation was found between ALT and stiffness values during treatment.

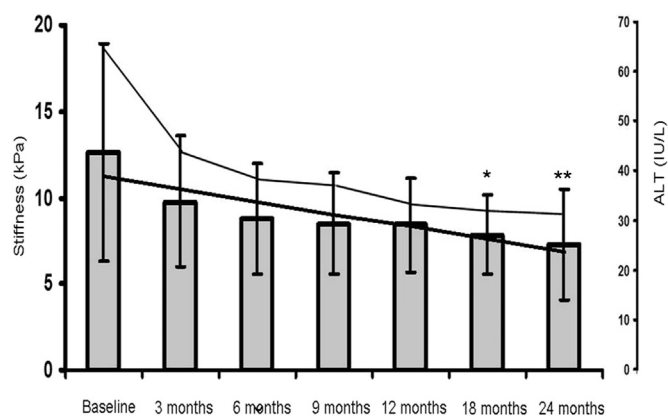


Fig. 2. Liver stiffness and ALT values at baseline and at different interval during treatment. Mean stiffness values at 3, 6, 9, and 12 months were not significantly different from those observed prior to therapy. Mean stiffness values at 18 and 24 months were statistically different from those observed prior to therapy. Mean ALT values during therapy were not significantly different from those observed before. Abbreviation: ALT, alanine aminotransferase.

In two patients, whose fibrosis stage was at a baseline of S3 and S6, respectively, fibrosis regressed to stages S2 and S5 at 24 months of therapy (Table 3). Fig. 3 shows the regression of fibrosis from stage S6 to S5.

The mean values of red blood cells, white blood cells, platelets, ALT, AST, alpha-fetoprotein, GGT, ferritin, sideremia, creatinine, glucose, cholesterol, INR, bilirubin, albumin during therapy were not significantly different from those observed before therapy.

4. Discussion

To our knowledge, this study is one of the few studies that quantitatively assesses liver fibrosis at the same time by non invasive serum markers, morphometry, Ishak score and elastography, in patients with chronic liver disease HBV-related. In our cohort the percentage of fibrotic tissue was 6.83 ± 7.03 with a significant difference between 2 groups of patients with Ishak score $< S3$ or $\geq S3$ ($2.01 \pm 2.62\%$ vs. $12.85 \pm 7.31\%$). Moreover, we found a significant association between morphometry and elastography, morphometry and LOK, and morphometry and APRI.

Hall et al. (2012) considered ten explanted patients for each aetiology of cirrhosis, including 10 HBV patients; they found a median value of fibrosis of 17%. Xie et al. (2011) analysed the collagen proportionate area by digital imaging of 53 resected liver tissue samples from HBV-related decompensated cirrhotic patients, and they found a collagen proportionate area of $35.93 \pm 14.42\%$ (11.24–63.41%) in this group of patients.

Our data underscore the relevance of histo-morphometric evaluation in the assessment of chronic HBV infection, but they also confirm that non invasive assessment by elastography, LOK, and APRI can be useful in patients with ALT levels < 5 ULN, with the best association between elastography and morphometry.

Transient elastography has been proposed for the non-invasive assessment of liver fibrosis in patients with chronic HCV (Stasi et al.,

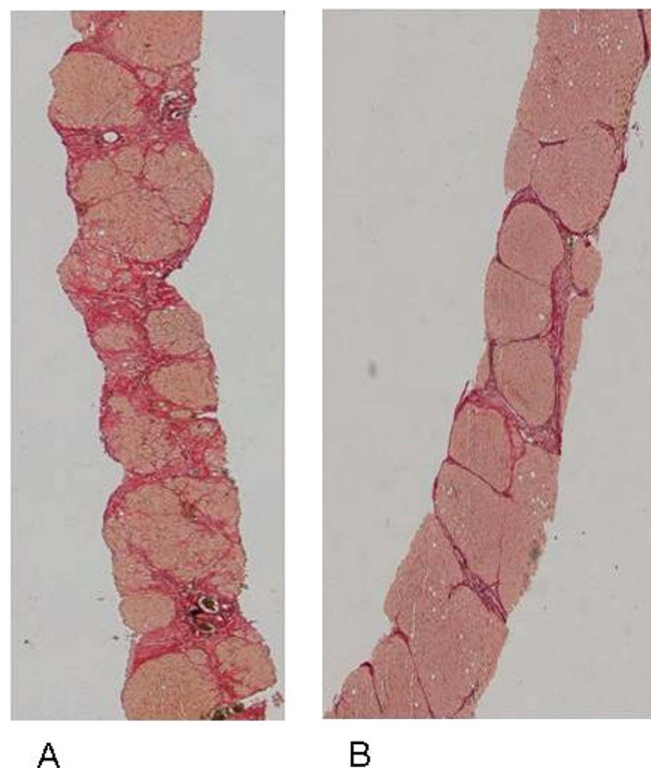


Fig. 3. Changes in liver fibrosis at baseline (panel A) and after two years of follow-up (panel B). Samples derive from the same patient.

2009; EASL-ALEH 2015; Ferraioli et al., 2015) and recommended in the longitudinal evaluation of regression or progression in patients undergoing antiviral therapy (EASL-ALEH 2015; Ferraioli et al., 2015). Different cut-offs have been proposed in HBV patients for fibrosis stage $\geq F2$ and for cirrhosis (EASL-ALEH, 2015). As recommended by EASL-ALEH Clinical Practice Guidelines (EASL-ALEH 2015), transient elastography is best used to determine liver fibrosis in HBV patients with active viraemia (HBV DNA > 2000 IU/ml) but normal ALT. Liver stiffness of 12–14 kPa often indicates liver cirrhosis in patients with high transaminase levels (liver stiffness > 9 in patients with normal ALT). However, few studies have evaluated by non invasive methodologies the fibrosis regression during treatment with entecavir or tenofovir, the most recent nucleos(t)ide analogues. In HCV patients, when compared to pre-treatment stiffness values, a significant reduction was observed at the end of treatment and at different time points thereafter for a maximum period of three years. The present study, although conducted in a limited number of patients, is the first in a European cohort to provide evidence that TE can be an effective tool to evaluate the changes in liver stiffness values during antiviral treatment in HBV patients. The results obtained in HBV patients are in substantial agreement with those reported for HCV patients undergoing treatment, and provide information on possible fibrosis regression following a successful antiviral treatment over a period of three years (Stasi et al., 2013). Accordingly, in HBV patients undergoing therapy, a significant decrease in liver stiffness was only observed at 18

Table 3

Fibrosis regression. In 2 patients the biopsy was performed at baseline and after 2 years of treatment.

	ISHAK score (Baseline)	Stiffness (baseline)	Stiffness (3 mo.)	Stiffness (6 mo.)	Stiffness (9 mo.)	Stiffness (12 mo.)	Stiffness (18 mo.)	Stiffness (24 mo.)	ISHAK score (24 mo.)
Case 1	(A:3,B:0,C:3,D:2) S:3	8,80	8,30	8,40	7,30	7,9	7,2	7	(A2, B0, C1, D2); S:2
Case 2	(A:3,B:0,C:2,D:3) S:6	28,80	17,80	11,90	8,80	8,8	10,1	8,7	(A:0,B:0,C:1, D:1); S5

and 24 months, likely due to fibrosis regression as confirmed by liver biopsy in two of such patients (Table 3). The difference in liver stiffness between baseline and month three can be viewed as a measure of treatment-induced reversal of inflammation; whereas the drop between month three and year two may reflect regression of liver fibrosis. All the serum markers of fibrosis included in our evaluation, with the exception of Forns score, did not show statistically significant differences between baseline values and values after two years of treatment.

Overall, our results are in agreement with the observations of Chang et al. (2010) and Papachrysos et al. (2015), who have reported that histologically proven necro-inflammation and fibrosis undergo significant changes during effective antiviral treatment.

Previous studies (Xu et al., 2015) in Asian cohorts have suggested that TE is a reliable technique for diagnosing liver fibrosis stages. Our results suggest that liver stiffness, although not exclusively representative of liver fibrosis, may also have value as a diagnostic discriminator for patients needing treatment. Along these lines, we concur with Xu et al. (2015) in concluding that liver stiffness measurement may be a useful alternative to liver biopsy when considering treatment.

In addition, our results provide some additional information concerning the possible antifibrotic outcome of the current more potent analogues, e.g. entecavir and tenofovir, when compared to lamivudin. Accordingly, when comparing our results with those reported by Ogawa et al. (2011) in an Asian cohort mostly treated with lamivudin (84,4% vs. entecavir 15,6%) it appears that the reduction in liver stiffness observed after 2 years of treatment was significantly higher when all the patients were treated with entecavir/tenofovir as in our study.

As outlined by Chatterjee and Mitra (2015) none of the several hundred serum markers of chronic liver disease, most of which for HCV, NAFLD, HCC available has been independently validated with optimal accuracy for early or longitudinal progression of disease (Chatterjee and Mitra, 2015).

5. Conclusion

Although the limited number of patients who underwent liver biopsy prompts caution when interpreting our results, we believe our data suggest that the measurement of liver stiffness at baseline and during therapy with nucleoside/nucleotide analogues may prove a useful methodology to assess long-term prognosis in patients undergoing treatment for HBV-related chronic liver disease. Moreover, our results strongly suggest that the evaluation of liver stiffness may significantly help in the correct management of CHB infection. Further studies including larger cohorts will further clarify the usefulness of different non-invasive methods in this clinical setting.

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