In Press, Corrected Proof, Available online 19 October 2009

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Automation of the Hepascore and validation as a biochemical index of liver fibrosis in patients with chronic hepatitis C from the ANRS HC EP 23 Fibrostar cohort

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ARTICLE INFO

Article history:
Received 5 October 2009
Received in revised form 8 October 2009
Accepted 8 October 2009
Available online xxxx

Keywords:
Liver fibrosis
Hepatitis C
Blood marker
Hepascore
Diagnostic accuracy

ABSTRACT

Background: Hepascore combining serum bilirubin, gamma glutamyl transpeptidase, hyaluronic acid (HA) and α2-macroglobulin with age and sex, was reported as relevant in predicting liver fibrosis in patients with chronic HCV infection and was proposed as an alternative to liver biopsy.
Methods: Since an automated HA assay (Latex method, Wako, Japan) became available, we investigated to automate Hepascore by simultaneous measurements of components using an OLYMPUS AU640 analyzer (Tokyo, Japan). For its clinical evaluation, we considered a cohort of chronic HCV patients included in a multicenter prospective study (ANRS HC EP 23 Fibrostar).
Results: Automated Hepascore was not significantly different than assayed as previously described. An improvement in HA variability was evidenced. In 512 chronic HCV patients, automated Hepascore, using ROC curves analysis, showed good predictive performances for significant fibrosis (AUROC = 0.81), severe fibrosis (AUROC = 0.82), and cirrhosis (AUROC = 0.88). For significant fibrosis, Hepascore (cut-off = 0.5) had a sensitivity of 0.77, a specificity of 0.70, a positive predictive value of 0.71 and a negative predictive value (NPV) of 0.77. Hepascore <0.25 could exclude significant fibrosis with a sensitivity of 0.95 and a NPV of 0.90 and Hepascore <0.75 could exclude cirrhosis with a sensitivity of 0.86 and a NPV of 0.97.
Conclusions: This study shows that Hepascore, a non-invasive index of liver fibrosis, necessitating only one serum sample, can be totally automated using a single analyzer and confirms that Hepascore accurately predicts liver fibrosis in chronic HCV. Hepascore might be largely used in assessing liver fibrosis as surrogate to the liver biopsy.

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doi:10.1016/j.cca.2009.10.011

1. Introduction

Various non-invasive markers of liver fibrosis have been recently developed and are now interesting alternative to liver biopsy in order to evaluate the severity of liver fibrosis in patients with chronic hepatitis C [1–9]. Three indices resulting of the combination of serum markers of liver fibrosis – Fibrotest®[5], Fibrometer®[6], and Hepascore [9] – have a good diagnostic accuracy in patients with chronic hepatitis C for discriminating mild fibrosis to severe fibrosis and for assessing cirrhosis. Recently, the Haute Autorité de Santé (HAS, the French National Authority for Health) has evaluated the benefit of the available methods and considered that in adult patients with chronic untreated hepatitis C without any comorbidity, these three validated biological diagnostic tests and the ultrasonic transient elastography (FibroScan®) [10] have shown sufficient interest to be proposed to health authorities for the inscription to reimbursement [11].

While Fibrotest® and Fibrometer® algorithms were patented and the calculations have to be paid, Hepascore formula that combines serum bilirubin, hyaluronic acid (HA), α2-macroglobulin (A2M) levels, gamma glutamyl transpeptidase activity (GGT) with age and sex was published [9] and can be routinely used. Furthermore an automated HA latex agglutination assay (HA detection reagent, Latex method, Wako, Osaka, Japan) became recently available which can be used instead of the manual enzyme-linked protein binding assay [12]. We chose this test for Hepascore automation by simultaneous measurements of the biochemical components. Its diagnostic accuracy was estimated in a cohort of hepatitis C virus (HCV) infected patients included in a national prospective study (ANRS HC EP 23 Fibrostar) in which Hepascore was also assessed as previously described [9] designed to evaluate and to compare the diagnosis performance of published tests for the prediction of mild, significant, severe fibrosis and cirrhosis in HCV patients using METAVIR histological fibrosis stage as reference [13].

2. Materials and methods

The study was approved by the Committee for protection of persons of Grenoble (France). Informed consent was obtained from each patient.

2.1. Patients

Between November 2007 and July 2008, 19 academic centers prospectively enrolled in a cohort study designed to compare different biological blood markers of liver fibrosis and transient elastography (FibroScan®) 590 untreated patients with chronic hepatitis C (anti-HCV antibodies positive and RNA-HCV positive) referred for evaluation, including liver biopsy.

Patients with associated coinfection, chronic viral hepatitis B (HBsAg positive) or HIV, with other liver disease (drug hepatitis, Wilson disease, hemochromatosis, autoimmune hepatitis, alcohol consumption > 30 g/day for men and > 20 g/day for women, primary biliary cirrhosis, and alpha-1 antitrypsine deficiency), or with severe systemic diseases were excluded. Patients with antiviral therapy during the six months preceding the inclusion or with immunosuppressive therapy were also excluded.

2.2. Liver pathological examination

Histological analysis was independently performed by two senior pathologists, academic experts in liver pathology, without knowledge of any clinical and biological data except that patients had chronic HCV. To be considered as adequate for scoring, the liver biopsies had to measure at least 15 mm and/or contain at least 11 portal tracts except for cirrhosis for which no limitation was required. Fibrosis was assessed on red Sirius stained sections according to the semi quantitative Metavir scoring system [13], on a five-point scale (F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = few septa, F3 = numerous septa without cirrhosis, and F4 = cirrhosis). In case of discrepancies, slides were simultaneously reviewed by the two pathologists using a multi-pipe microscope in order to reach a consensus.

2.3. Blood samples

Fasting blood samples were collected by veinipuncture at less than two months away from the liver biopsies. The same kinds of tubes from the same lots were used for all the patients (BD Vacutainer®, type Z, Becton-Dickinson, Plymouth, UK).

Each of the biological parameters included in the Hepascore was measured in a single laboratory using serum samples immediately separated and fractioned in fractions of 0.5 mL in 1.5 mL screw cap micro tubes (Sarstedt, Nümbrecht, Germany). All the fractions were immediately frozen and stored at −80 °C until assays. The transports of samples from the hepatology centers to the laboratory were achieved in carbonic ice by a specialized transporter (AreaTime Logistics, Cergy Pontoise, France).

All the biological tests were processed blindly without knowledge of the clinical and histological data.

2.4. Reference Hepascore biochemical assays

Serum hyaluronic acid (HA) was assayed using an enzyme-linked protein binding assay (HA Test Kit, Corgenix, Westminster, USA). α2-macroglobulin (A2M) was measured by immunonephelemetric methods using a BNII nephelometer (Siemens Healthcare Diagnostics, Deerfield, USA). Serum GGT activities (IFCC methods at 37 °C) and total bilirubin concentrations were assayed using a Hitachi 917 (Roche Diagnostic, Mannheim, Germany) with CFAS calibration (Calibrator For Automated Systems, Roche Diagnostic).

2.5. Automated Hepascore biochemical assays

An OLYMPUS AU640 (Olympus Diagnostic Systems, Tokyo, Japan) analyzer was used for simultaneous assay of the parameters included in the Hepascore. Serum HA was assayed using a latex agglutination method that can be applied to general clinical chemistry analyzers (HA detection reagent, Latex method, Wako, Osaka, Japan). A2M was measured using an immunoturbidimetric assay (Alpha-2-Macroglobulin kit, DakoCytomation, Glostrup, Denmark). GGT activities (IFCC method at 37 °C) and total bilirubin concentrations were assayed using Olympus reagents with CFAS calibration (Roche Diagnostic).

2.6. Hepascore calculation

The Hepascore was computed from the results by using the model previously published by Adams et al. [9]: $\text{Hepascore} = y/(1+y)$ with $y = \exp[-4.185818 - (0.0249 \text{ age (years)}) + (0.7464 \text{ sex } (M=1, F=0)) + (1.0039 \text{ A2M (g/l)}) + (0.0302 \text{ HA (μg/l)}) + (0.0691 \text{ bilirubin (μmol/l)}) - (0.0012 \text{ GGT (U/l)})]$.

2.7. Statistical analysis

GraphPad Prism® computer software was used for statistical analysis (GraphPad Software, La Jolla, CA USA). Quantitative variables are expressed as means (SD) or median (range) as specified. The Mann–Whitney and Kruskall Wallis tests were used to compare the results. A P value of < 0.05 was considered statistically significant. The Deming model was used for linear regressions.

Receiver–operator characteristic (ROC) curves were built to visualize the discriminating performance of the Hepascore considering liver biopsy as the reference. Areas under the ROC curves (AUROC) were calculated to quantify the overall ability of the test to discriminate between fibrosis grades. The optimal cut-offs were calculated by

Please cite this article as: Guéchot J, et al, Automation of the Hepascore and validation as a biochemical index of liver fibrosis in patients with chronic hepatitis C from the ANRS HC EP 23 Fibrostar cohort, Clin Chim Acta (2009), doi:10.1016/j.cca.2009.10.011

maximizing the sum of sensitivity plus specificity. For comparison with the previous studies, in order to limit the influence of differences in the prevalence of fibrosis stages on the AUROC estimates, adjusted uniform areas under ROC curves (AduAUC) were calculated using the previously described DANA method [14] giving the same weight to each fibrosis stage.

3. Results

3.1. Patient characteristics

Because of insufficient liver tissue ($n=42$), previous interferon therapy ($n=5$), coexisting liver disease due to chronic HBV infection ($n=9$), excessive alcohol consumption ($n=5$), immunosuppressive treatment ($n=1$), non-confirmed HCV positive status ($n=3$), or incomplete data ($n=13$), the final study cohort included 512 patients, 306 male (59.8%) and 206 female (40.2%). The characteristics of the patients were summarized in Table 1.

3.2. Liver histology

The length of liver biopsies was 25.1 ± 8.8 mm (mean ± SD) and longer than 25 mm in 49.8%. Metavir stages distribution was F0 in 34 (6.6%), F1 in 231 (45.1%), F2 in 92 (18.0%), F3 in 79 (15.4%) and F4 in 76 (14.8%) patients.

3.3. Automated Hepascore compared to reference method

Since the automation of the HA assay was the principal methodological difference between the two ways used to assess Hepascore, the variabilities of the manual and automated methods were studied which showed an improvement obtained with the automation. Between-run imprecision of the Corgenix HA Test was characterized by coefficients of variation 5.7%, 6.4%, and 8.0% for HA levels of 37.4, 127.9 and 423.1 µg/l, respectively in 10 assays. Between-run imprecision of the automated Wako latex agglutination method was characterized by coefficients of variation of 4.1%, 2.4%, and 2.7% for HA levels of 49.9, 171.7 and 900.7 µg/l, respectively (1 determination/day for 20 days) using serum pools.

For HA and A2M, the less standardized assays, the comparisons did not show significant differences between assays ($P=0.627$ and 0.578, Mann and Whitney U test, respectively). The results of the Deming linear regression were $y=0.953x+3.121$ for HA assays ($r=0.988$) and $y=0.964x+0.069$ for A2M assays ($r=0.951$).

Comparison between the Hepascore results (Fig. 1) did not show significant differences between the automated and reference methods for Hepascore determination (0.56 ± 0.31 vs. 0.55 ± 0.31, mean ± SD; 0.53 [0.05–1.0] vs. 0.52 [0.06–1.0], median [range]; $P=0.320$, Mann–Whitney U test).

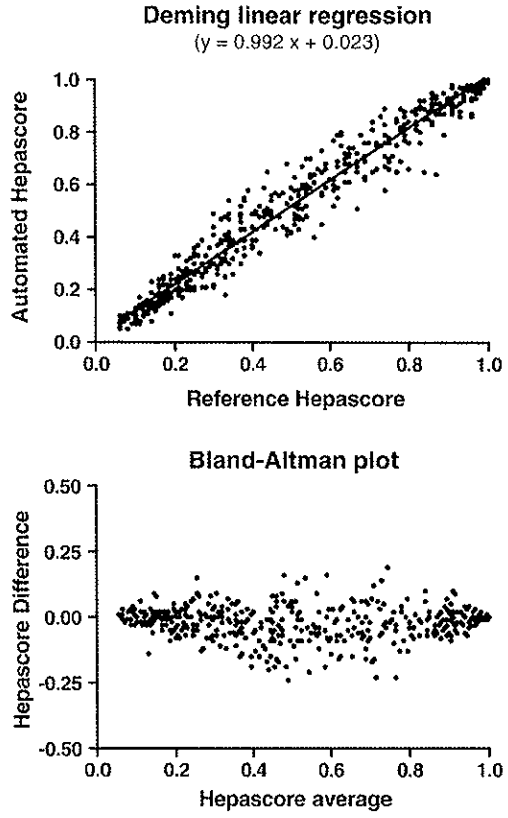

Fig. 1. Comparison of the two methods of Hepascore analysis ($r=0.976$).

3.4. Assessment of liver fibrosis using automated Hepascore

Fig. 2 shows box plot for Hepascore according to the Metavir stages of liver fibrosis. Hepascore increased with histological stage of liver fibrosis with significant differences between groups ($P<0.0001$, Kruskall Wallis test). There was a significant correlation between Metavir fibrosis stage and Hepascore ($r=0.601$, $P<0.0001$).

The ROC curve analyses showed the diagnostic performances of the automated Hepascore to discriminate significant fibrosis ($F\geq 2$), severe fibrosis ($F\geq 3$) and cirrhosis (F4) in patients with chronic VHC (Fig. 3). The areas under the ROC curves, consistently higher than 0.80, showed good performances for significant fibrosis (AUROC = 0.812; 95% Confidence Interval, 0.776–0.848) for severe fibrosis (AUROC = 0.822; 95% CI, 0.783–0.861) and for cirrhosis (AUROC = 0.876; 95% CI, 0.841–0.911).

The adjusted uniform areas under the ROC curves (AduAUC) calculated using the DANA method were 0.86, 0.84, and 0.88 for significant fibrosis, severe fibrosis and cirrhosis, respectively.

Table 1
Characteristics of the 512 studied patients.

	Median	Range
Age (years)	50	18–79
Weight (kg)	70	39–135
Height (m)	1.70	1.48–1.97
BMI (kg/m^2)	24.3	15.4–49.2
Bilirubin[a] (µmol/l)	10.5	2.4–54.8
ALT (U/l)	69	12–594
AST (U/l)	49	11–280
GGT[a] (U/l)	61	9–858
Platelet count (G/l)	213	52–474
Prothrombin time (%)	99	61–100
Hyaluronic acid[a] (µg/l)	34	5–920
α2-macroglobulin[a] (g/l)	3.27	0.98–5.73

[a] Automated assays as described in methods.

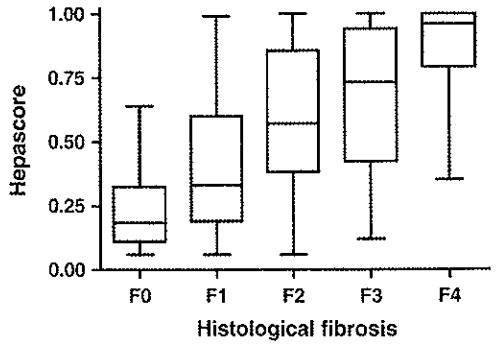

Fig. 2. Automated Hepascore (median, quartiles, and range) according to the Metavir fibrosis stages.

Please cite this article as: Guéchot J, et al, Automation of the Hepascore and validation as a biochemical index of liver fibrosis in patients with chronic hepatitis C from the ANRS HC EP 23 Fibrostar cohort, Clin Chim Acta (2009), doi:10.1016/j.cca.2009.10.011

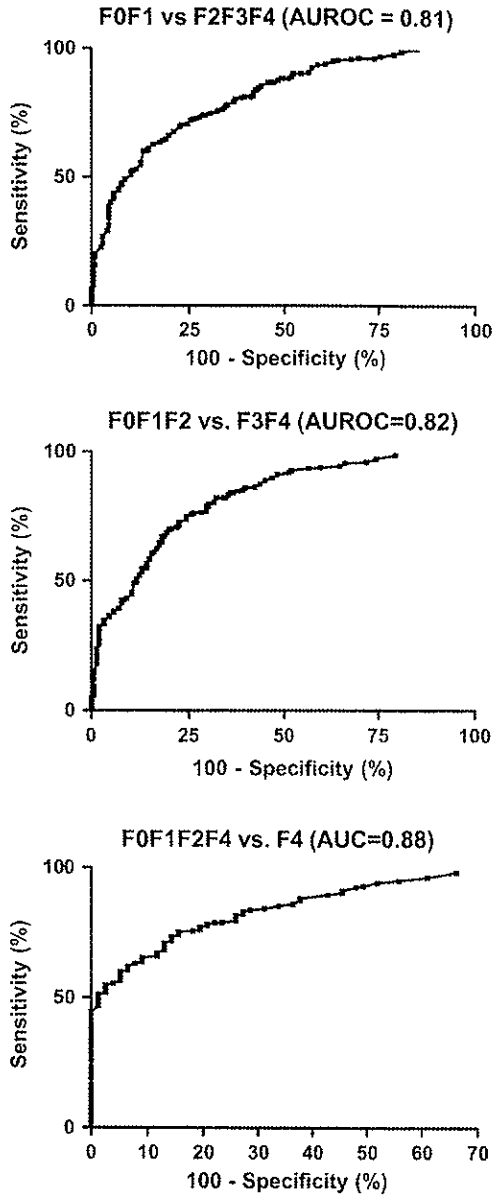

Fig. 3. ROC curves for automated Hepascore predictive value of significant fibrosis (F2–4), severe fibrosis (F3–4) and cirrhosis (F4).

For significant fibrosis (≥F2) diagnostic, the optimal cut-off was 0.5, and Hepascore had a sensitivity of 0.77 (95% CI, 0.71–0.82), a specificity of 0.70 (95% CI, 0.64–0.76), a positive predictive value (PPV) of 0.71 (95% CI, 0.65–0.76) and a negative predictive value (NPV) of 0.77 (95% CI, 0.71–0.82). Furthermore when Hepascore was <0.25, a significant fibrosis could be excluded with a sensitivity of 0.95 and a NVP of 0.90.

For severe fibrosis (≥F3) diagnostic, the optimal cut-off was 0.6, and Hepascore had a sensitivity of 0.80 (95% CI, 0.73–0.86), a specificity of 0.70 (95% CI, 0.65–0.74), a PPV of 0.54 (95% CI, 0.47–0.61) and a NPV of 0.89 (95% CI, 0.85–0.92).

For cirrhosis (F4) the optimal cut-off was 0.75, and Hepascore had a sensitivity of 0.86 (95% CI, 0.76–0.92), a specificity of 0.74 (95% CI, 0.69–0.78), a PPV of 0.37 (0.30–0.44) and a NPV of 0.97 (95% CI, 0.94–0.98). Furthermore, with the cut-off value proposed by Adams et al. [9] of 0.84, Hepascore had in our study a sensitivity of 0.73 (95% CI, 0.61–0.82), a specificity of 0.81 (95% CI, 0.77–0.85), a PPV of 0.41 (0.32–0.49) and a NPV of 0.94 (95% CI, 0.92–0.96).

4. Discussion

The prognosis of chronic liver diseases is closely related to the development of liver fibrosis which commonly occurs as the disease progresses. In chronic hepatitis C, liver fibrosis has to be evaluated both as a prognostic index and as a criterion in treatment decision. Furthermore, when cirrhosis, the end-stage consequence of progressive fibrosis, is evidenced, the assessment of the risk of severe complications occurrence, including ascites, variceal bleeding, encephalopathy, and hepatocellular carcinoma, has important clinical and therapeutical implications.

With the specific aim of replacing pathological examination of liver biopsy, various non-invasive tests of liver fibrosis have been developed for monitoring patients with chronic HCV infection [1–9]. These include routinely available laboratory tests, such as liver-associated chemistries, platelet count, and prothrombin time, as well as specific serum markers of fibrosis, such as serum hyaluronic acid.

Until now, there are no FDA approved tests and the recent American recommendations [18] considered that currently available non-invasive tests may be useful in defining the presence or absence of advanced fibrosis in persons with chronic hepatitis C infection, but should not replace the liver biopsy in routine clinical practice. The French National Authority for Health (HAS) considers that in chronic untreated hepatitis C adult patients with no co-morbidities, three biological tests have been validated as non-invasive procedures for liver fibrosis evaluation and/or cirrhosis diagnostic (Fibrotest® [5], FibroMeter® [6] and Hepascore [9]) and recommended to use one of them or liver biopsy or FibroScan® [10] as first-line test. The HAS, making an opinion based on an assessment of the benefit both for the patient and for public health, considered that the non-invasive tests have shown a clinical benefit by comparison with liver biopsy because, they are non-invasive, and their cost is lesser, but considered the benefice moderate because their diagnostic accuracies are imperfect.

Because the cost of such procedures is of importance for health authorities, we have chosen to automate the Hepascore. This way it makes possible to reduce the total cost since it needs only one serum sample, it avoids the use of time consuming manual HA assay and the formula for calculation was published.

Using automated turbidimetric HA and A2M assays on an Olympus AU640, from Wako and DakoCytomation respectively, we did not find significant differences in Hepascore when we compared the results with those obtained with method using a manual enzyme-linked protein binding HA assay and a nephelometric A2M assay as previously used by others [9,15–17]. Furthermore, as predictable, we found an improvement in the imprecision of the automated HA assay, compared with the manual assay which assumes an improvement in Hepascore analytical variability with automation.

We assessed diagnostic performance of the automated Hepascore in a large cohort ($n = 512$) of HCV patients included in a prospective controlled study. The rigorous pathological examination of liver biopsies was a criterion of the study. Indeed, it is well documented that the variability of liver biopsy is not negligible, and it was shown that the histological staging of needle biopsy specimens is impaired both by variation in the severity of the diseases in different parts of the liver and by observer variability [19–21]. All patients had liver biopsies of good sizes (> 15 mm and/or >10 portal tracts with a mean length of 25 mm) reviewed by two independent senior hepatopathologists, enough for optimizing the histopathological analysis [22]. In comparison, other studies for Hepascore validation [9,15–17] included less patients ($n = 104–391$) with smaller liver biopsies (Table 2).

In this study, the ROC analysis confirms the good diagnostic value of Hepascore as previously observed [9,15–17] for discriminating significant or extensive liver fibrosis (Table 2). The results also confirm the Hepascore cut-off value of 0.5 for discriminating patients with significant liver fibrosis selected by Adams et al. in the initial Australian study [9].

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Table 2
Comparison of Hepascore performance according to different studies.

	Adams et al. [9]	Leroy et al. [15]	Halfon et al. [16]	Becker et al. [17]	This study
Prospective study	Yes	Yes	No	Yes	Yes
Patients number	104	180	356	391	512
Reference biopsy minimal size	5 portal tracts	No	No	10 mm and/or 8 portal tracts	15 mm and/or 10 portal tracts
F0/F1/F2/F3/F4 (%)	16/27/34/7/16	8/41/22/14/14	4/55/26/11/4	16/34/15/16/19	7/45/18/15/15
For significant fibrosis					
AUROC	0.82	0.79	0.76	0.81	0.81
(AduAUC)	(0.86)	(0.84)	(0.86)	(0.82)	(0.86)
Cut-off	0.5	0.5	0.32	0.55	0.5
Sensitivity	63%	54%	77%	82%	77%
Specificity	89%	84%	63%	65%	70%
Positive predictive value	/	78%	59%	70%	71%
Negative predictive value	/	64%	80%	78%	77%
For severe fibrosis					
AUROC	0.90	0.85	0.81	0.82	0.82
(AduAUC)	(0.90)	(0.87)	(0.83)	(0.84)	(0.84)
Cut-off	/	0.84	0.53	0.8	0.6
Sensitivity	/	47%	78%	/	80%
Specificity	/	90%	72%	77%	70%
Positive predictive value	/	65%	32%	62%	54%
Negative predictive value	/	81%	95%	/	89%
For cirrhosis					
AUROC	0.89	/	0.89	0.88	0.88
(AduAUC)	(0.88)		(0.88)	(0.87)	(0.88)
Cut-off	0.84	/	0.61	/	0.75
Sensitivity	71%	/	92%	/	86%
Specificity	89%	/	72%	/	74%
Positive predictive value	/	/	11%	/	37%
Negative predictive value	/	/	100%	/	97%

Becker et al. [17] recently proposed an algorithm for managing the patients with chronic viral hepatitis C using Hepascore. Our data agree with this model, however the cut-off values that they selected are slightly larger than those we found. These authors suggested that the optimal cut-offs might differ between European, Australian, and American populations. From our data and from the previous studies that validated the Hepascore, we assume that when Hepascore is less than 0.25 (about 25% of the patients), there is no significant liver fibrosis and the treatment decision might rely on virus genotype. When Hepascore is between 0.25 and 0.5 (about 25% of the patients), another non-invasive test or liver biopsy might be used to confirm the diagnostic. When Hepascore is more than 0.5 (about 50% of the patients), there is a significant liver fibrosis, so that the antiviral treatment can be decided. When Hepascore is more than 0.75 (about 30% of the patients), the diagnostic of liver cirrhosis has to be considered, and might be confirmed either using another non-invasive test, such as transient elastography (FibroScan®) which has shown good diagnostic performances for cirrhosis [23], or using liver biopsy. From recent data in HIV/HCV and HIV/HBV patients [24,25] it is possible to deduct that this approach might be also used in co-infected patients.

In conclusion, this study shows that the Hepascore, a non-invasive index of liver fibrosis, necessitating only one serum sample, can be totally automated using a single analyzer and confirms that it accurately predicts liver fibrosis in patients with chronic hepatitis C. Hepascore might be largely available in assessing liver fibrosis as an alternative to the liver biopsy analysis.

Acknowledgments

We thank Doctor Robert Küper, Wako Pure Chemical Industries Ltd, Neuss, Germany, for kindly providing the Wako HA detection reagents and Paul Taylor for friendly English reading.

This study was funded and sponsored by the French de National Agency for Research on aids and viral hepatitis (ANRS) and received the agreements of the Société Française de Biologie Clinique (SFBC) and of the Association pour l'Étude du Foie (AFEF).

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Please cite this article as: Guéchot J, et al, Automation of the Hepascore and validation as a biochemical index of liver fibrosis in patients with chronic hepatitis C from the ANRS HC EP 23 Fibrostar cohort, Clin Chim Acta (2009), doi:10.1016/j.cca.2009.10.011