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Fibro-check: a combination of direct and indirect markers for liver fibrosis staging in chronic hepatitis C patients

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ABSTRACT

Background and rationale for the study. The assessment of liver fibrosis provides useful information not only for diagnosis but also for therapeutic decision. This study was concerned with determining the levels of collagen III and its degrading enzyme matrix metalloproteinase-1 (MMP-1) as direct and complementary markers for liver fibrosis staging. **Results.** A total of 269 chronic hepatitis C patients constituted this study. Western blotting was used for identifying collagen III and MMP-1 in serum samples. As a result, collagen III and MMP-1 were identified, respectively, at 70 and 245 kDa using their respective mono-specific antibodies. These two markers were quantified in sera of patients using ELISA. Next, Fibro-check was constructed combining collagen III and MMP-1 together with other indirect markers which reflect alteration in hepatic functions that proved useful to stage liver fibrosis. Fibro-check produced area under the receiver-operating characteristic curve (AUC) 0.91 and 0.83 for significant (F2-F4) and cirrhosis (F4), respectively. Additionally, we estimated the performance of Fibro-check in comparison with aspartate to platelet ratio index (APRI) and fibrosis index. Fibro-check seems to be more efficient than both of them. Fibro-check was then applied to the validation study to test its accuracy and reproducibility showing AUCs 0.90 for F2-F4 and 0.86 for F4. **Conclusions.** Fibro-check combining 'direct' and 'indirect' markers using a mathematical formula may improve the staging of liver fibrosis with a high degree of accuracy and seems more efficient than APRI and Fibrosis index in this group of Egyptian patients.

Key words. Collagen III. Liver biopsy. Metalloproteinases. Non-invasive.

INTRODUCTION

Hepatitis C virus (HCV) infection is a global health burden affecting approximately 160-170 million people worldwide.[1] Once chronic HCV infection is established, spontaneous HCV clearance rarely occurs. Chronic hepatitis C (CHC) can cause continuous liver damage resulting in liver cirrhosis and subsequently hepatocellular carcinoma (HCC).[2] Staging of liver fibrosis is necessary in CHC patients because it is the important factor for initiation of treatment in patients with hepatitis C infection.[3] Liver biopsy is the most commonly used reference standard for assessing liver fibrosis. However, liver biopsy is an invasive and is associated with a significant risk of complications ranged from minor complications like pain and transient hypotension to major complications like significant bleeding which may cause death.[4] These limitations have stimulated the development of non-invasive techniques for assessing the presence and the degree of liver fibrosis. The great majority of studies have investigated the diagnostic value of serum markers of liver fibrosis. These markers were divided into two categories; direct markers, which are supposed to be directly involved in the deposition and removal of extracellular matrix (ECM), i.e. in fibrogenesis and fibrolysis and indirect markers, which reflect alterations in hepatic function, but not directly ECM metabolism.[5]

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Fibrosis is characterized by the deposition of collagen and other extracellular matrix proteins and their organization in complex polymers, which are insoluble and induce loss of the liver architecture.[6] Collagen is synthesized as a procollagen by hepatic stellate cells (HSCs), later it is transformed to collagen by enzymatic splitting of the C-terminal and N-terminal end by procollagen C-proteinase and procollagen N-proteinase.[7] Matrix metalloproteinases are the main degrading enzyme of these ECM proteins, and have been implicated in the processes of liver fibrosis. Among them, the matrix metalloproteinase-1 (MMP-1) that cleaves the native helix of fibrillar collagens I, II and III.[8] Herein, we aimed to determine the expression of collagen III and its degrading enzyme MMP-1 simultaneously and then estimating their performances as surrogate markers for liver fibrosis diagnosis. Furthermore, we aimed to develop a sensitive function incorporated both collagen III and its degrading enzyme MMP-1 together with other indirect markers which reflect alteration in hepatic function for liver fibrosis staging in CHC patients and then estimated its performance in comparison with some published noninvasive tests in CHC.[9,10]

MATERIALS AND METHODS

Samples

Blood samples and liver biopsies of two hundred sixty-nine HCV infected individuals were collected from the Tropical Medicine department, Mansoura University hospitals, Mansoura, Egypt. One hundred sixty-eight patients constituted the estimation group whereas 101 patients constituted the validation group. All tissue and serum samples were obtained with informed consent. All patients were tested negative for HBsAg (Dia.Pro, Milan, Italy). Also, all patients were tested positive for anti-HCV antibodies (Biomedica, Sorin, Italy). Patients were then confirmed for the presence of HCV-RNA using quantitative polymerase chain reaction assay (COBAS Ampliprep/ COBAS TaqMan, Roche Diagnostics, Pleasanton, USA). Cirrhotic patients were compensated at the time of inclusion. Patients with an evidence of coexistent liver disease, a history of hepatocellular carcinoma, a previous interferon treatment and a decompensated liver disease were excluded from this study. Needle liver biopsy specimens were obtained with an 18-gauge or larger needle. To be considered as adequate for scoring, the liver biopsies had to measure at least 15 mm and/or contain at least five portal tracts, except for cirrhosis for which no limitation was required. Biopsies were interpreted according to METAVIR scoring system.[11] Liver function tests [albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP)] were all measured on an automated biochemistry analyzer (Hitachi 917; Roche Diagnostics, Mannheim, Germany). Complete blood count was performed using KX-21 Sysmex automated hematology analyzer (Sysmex Corporation, Kobe, Japan). Alpha fetoprotein (AFP) level was estimated by chemiluminescence, with Immulite (1000) AFP kit (Diagnostic Products Corporation; Los Angeles, CA, USA).

Western blotting and gel electroelution

First, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in 0.75 mm-thick, 12% vertical slab gels according to the method of Laemmli.[12] Next, Western electroblotting was used for transferring the separated protein bands onto a nitrocellulose membrane (0.45 mm pore size, Sigma) in a protein transfer unit according to Towbin, *et al.*[13] Then, they were immunostained using monospecific antibodies corresponding to human collagen type III and MMP-1 separately. Finally, both collagen III and MMP-1 bands were cut and electroeluted separately from preparative polyacrylamide gels at 200 V for 3 h in a dialysis bag (Sigma). The protein content of the purified bands was determined[14] and the remainder was stored at -20 °C.

Quantitation of collagen III and MMP-1 using ELISA

Diluted serum samples (1:50) in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6) were tested (50 μL/well) for collagen III bound on a 96-well microtiter plate at 4 °C overnight. After blocking with 0.5% BSA in coating buffer (200 μL/well), mono-specific antibody for collagen III at dilution 1:200 in PBS was added (50 μL/well) and incubated at 37 °C for 2 h. Goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma) 1:700 in 0.2% BSA in PBS-T20 was incubated at 37 °C for 1 h. Similarly, diluted serum samples (1:50) in coating buffer were tested (50 μL/well) for MMP-1 bound on a 96-well microtiter plate at 4 °C overnight. Likewise, after blocking with 0.6% BSA in coating buffer (200 μL/well), mono-specific antibody for MMP-1 at

dilution 1:500 in PBS was added (50 μL/well) and incubated at 37 °C for 2 h. Also, goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma) 1:500 in 0.2% BSA in PBS-T20 was incubated at 37 °C for 1 h. The plates were washed with PBS+0.5% Tween 20 after every step. The substrate was 1 mg/mL p-nitrophenyl phosphate and the intensity of the signal was determined by measuring the absorbance at 450 nm after 10 minutes using a microtiter plate reader (Σ960, Metretech Inc, Germany). The calibration curves of the serial concentrations for the purified Collagen III (0.1–30 μg/mL) and MMP-1 (0.5-24 μg/mL) were then determined.

Statistical analysis

All statistical calculations were done by SPSS software v.15.0 (SPSS Inc., Chicago, IL) and GraphPad Prism package; v.5.0 (GraphPad Software, San Diego, CA). Continuous variables were expressed as mean ± standard error of mean. A value of $P < 0.05$ was considered statistically significant. There were 2 endpoints in this study: presence of significant fibrosis and cirrhosis. The correlation was evaluated by Spearman's rank correlation coefficient. Univariate analysis identified the predictors of fibrosis by using the Student's t test. The diagnostic value was assessed by calculating the area under the receiver operating characteristic (ROC) curves. An area under the curve (AUC) of 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value. All variables with high AUCs and high significance on univariate analysis were entered in stepwise linear regression analysis to develop a model for identifying significant fibrosis. Based on the ROC analysis, the best cutoff points were selected and diagnostic performances (sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV)) were determined.

RESULTS

Patient characteristics

One hundred sixty-eight HCV-monoinfected patients fulfilled the selection criteria. The sample comprised one hundred and eleven men and fifty-seven women. Laboratory characteristics of all patients at the time of liver biopsy are summarized in table 1. Comparison of the baseline characteristics of patients according to the stage of liver fibrosis are outlined in table 2. As individuals with significant

Table 1. Comparison of patient characteristics in the estimation and validation groups.

Variable	Estimation group (n = 168)	Validation group (n = 101)	P value[c]
Age (years)	43.7 ± 0.5	45.2 ± 0.5	NS
AST (U/L)[a]	57.1 ± 2.5	55.9 ± 3.4	NS
ALT (U/L)[a]	62.0 ± 2.7	58.5 ± 3.7	NS
AST/ ALT (AAR)	0.9 ± 0.03	1.0 ± 0.04	NS
ALP (U/L)[a]	84.3 ± 4.6	87.4 ± 5.7	NS
Albumin (g/dL)[a]	4.2 ± 0.04	4.0 ± 0.05	NS
Total bilirubin (mg/dL)[a]	0.9 ± 0.04	1.0 ± 0.06	NS
Platelet count (109/L)[a]	181 ± 4.6	176 ± 0.6	NS
APRI[b]	1.0 ± 0.07	0.9 ± 0.1	NS
AFP (U/L)[a]	6.4 ± 0.8	6.8 ± 1.1	NS
Collagen III (µg/mL)	9.0 ± 0.4	8.6 ± 0.4	NS
MMP-1 (µg/mL)[b]	6.6 ± 0.6	7.2 ± 0.8	NS
Collagen III/ MMP-1 (CMR)	3.7 ± 0.4	4.2 ± 0.5	NS
Metavir fibrosis stages	n (%)	n (%)	
F1	89 (53)	51 (50.5)	
F2	31 (18.5)	13 (12.9)	
F3	14 (8.3)	14 (13.9)	
F4	34 (20.2)	23 (22.8)	

Variables were expressed as mean ± SEM. [a] Reference values: aspartate aminotransferase (AST) (male up to 37 U/L, female up to 31 U/L); alanine aminotransferase (ALT) (male up to 41 U/L, female up to 31 U/L); alkaline phosphatase (ALP) 22-92 U/L; albumin 3.8-5.4 g/dL; total bilirubin up to 1 mg/dL; platelet count 150-400 x 10^9/L; alpha fetoprotein (AFP) up to 10 U/L. [b] APRI: [(AST (U/L)/upper limits of normal)/platelet count (10^9/L)] x 100; MMP-1: matrix metalloproteinase-1.[c] $P > 0.05$ is considered non significant (NS); $P < 0.05$ is considered significant.

fibrosis (F2-F4) are at increased risk of developing cirrhosis and are usually accepted as an indication to commence treatment, the laboratory features of patients with minimal fibrosis (F1) and F2-F4 were compared by univariate analysis based on the Student's *t* test. As a result, patients with F1 were young with a mean (± SEM) of 40.2 (± 0.9) years as compared to those who developed F2-F4. Moreover, patients with F2-F4 were associated with higher AST, ALT, AAR, ALP, APRI and AFP levels than those with F1. On contrary, the mean value of albumin and platelet count decreased with the progression of liver fibrosis being lower in patients who developed F2-F4 (Table 2).

Identification and quantitation of collagen III and MMP-1

SDS-PAGE and Western blotting were used as described previously to identify the target collagen III and its degrading enzyme MMP-1. As a result, a single immunoreactive band was shown at 70 kDa and 245 kDa for collagen type III and MMP-1, respectively, due to their binding with their respective mono-specific antibodies. Patients with significant fibrosis (F2-F4) were associated with higher concentration of collagen III than those with minimal fibrosis (F1). On the other hand, patients with F2-F4 were associated with lower concentration of MMP-1 than those with F1 (as shown in table 2). The use of collagen III *per se* could discriminate F2-F4 from F1 with an AUC = 0.75 while the use of MMP-1 *per se* yielded an AUC = 0.70 for identifying F2-F4 (Figures 1A, 1B). Surprisingly, it has been observed that collagen III/MMP-1 ratio (CMR) yielded values that increased significantly in patients with F2-F4 *vs.* those with F1 (Table 2) and identified F2-F4 with a better AUC = 0.80 than each marker separately (Figure 1C). Hence, the overlap in collagen III and MMP-1 among patients with F1 and F2-F4 has been diminished and the difference in their value has been amplified. Then, based on ROC curve, a cutoff point greater than 2.5 was selected for CMR for separating patients with F2-F4 from those with F1 yielding a sensitivity of 77% and specificity of 71%.

Development of the Fibro-check

The next step was aimed to enhance the diagnostic accuracy of CMR for diagnosing F2-F4. So, it is necessary for this ratio to be combined with other routine marker. On univariate analysis, nine of the ten evaluated routine markers included in this study showed a significant association with fibrosis stages F2-F4 ($P < 0.05$). They were age, AAR, albumin, total bilirubin, platelet count, APRI, AFP, AST and ALP. Serum ALT did not differentiate between F1 and F2-F4 ($P > 0.05$) as presented in table 2. However, multivariate regression modeling demonstrated that only AAR, platelet count, and AFP together with CMR retained significance when combined with

Table 2. Variables associated with the presence of significant fibrosis (F2-F4) by univariate analysis in the estimation group (n = 168).

Variable	F1 (n = 89)	F2-F3 (n = 45)	F2-F4 (n = 79)	F4 (n = 34)	P value[c]
Age (years)	40.2 ± 0.9	44.24 ± 0.8	47.4 ± 0.7	50.2 ± 1.0	< 0.0001
AST (U/L)[a]	50.7 ± 2.4	63.51 ± 5.7	64.5 ± 4.6	65.8 ± 7.6	0.006
ALT (U/L)[a]	59.2 ± 2.2	52.2 ± 7.0	64.1 ± 5.2	73.7 ± 7.1	0.402
AAR[b]	0.8 ± 0.03	1.0 ± 0.07	1.2 ± 0.06	1.4 ± 0.1	< 0.0001
ALP (U/L)[a]	73.4 ± 6.0	79.0 ± 8.0	95.2 ± 6.6	119 ± 8.6	0.017
Albumin (g/dL)[a]	4.3 ± 0.03	4.1 ± 0.09	3.9 ± 0.08	3.7 ± 0.1	< 0.0001
T. bilirubin (mg/dL)[a]	0.7 ± 0.04	0.9 ± 0.07	1.1 ± 0.08	1.3 ± 0.1	< 0.0001
Platelet count (10^9/L)[a]	198 ± 4.8	173 ± 8.0	162 ± 7.5	149 ± 13.6	< 0.0001
APRI[b]	0.7 ± 0.04	1.0 ± 0.1	1.3 ± 0.1	1.6 ± 0.3	< 0.0001
AFP (U/L)[a]	2.6 ± 0.2	10.4 ± 1.7	10.6 ± 0.9	10.8 ± 2.7	< 0.0001
Collagen III (µg/mL)	6.9 ± 0.4	10.5 ± 0.9	11.8 ± 0.7	12.8 ± 1.0	< 0.0001
MMP-1 (µg/mL)	8.8 ± 0.9	3.4 ± 0.6	3.2 ± 0.4	3.0 ± 0.4	< 0.0001
CMR[b]	2.2 ± 0.3	5.7 ± 1.2	7.1 ± 0.9	8.2 ± 1.3	< 0.0001

Variables were expressed as mean ± SEM. [a] Reference values: aspartate aminotransferase (AST) (male up to 37 U/L, female up to 31 U/L); alanine aminotransferase (ALT) (male up to 41 U/L, female up to 31 U/L); alkaline phosphatase (ALP) 22-92 U/L; albumin 3.8-5.4 g/dL; total bilirubin (T. bilirubin) up to 1 mg/dL; platelet count 150-400 x 10^9/L; alpha fetoprotein (AFP) up to 10 U/L. [b] APRI: AST level (U/L)/40 (upper limits of normal)/platelets count (10^9/L) x 100. CMR: Collagen III/ MMP-1 ratio. [c] $P > 0.05$ is considered non significant; $P < 0.05$ is considered significant; $P < 0.001$ is considered very significant and $P < 0.0001$ is considered extremely significant.

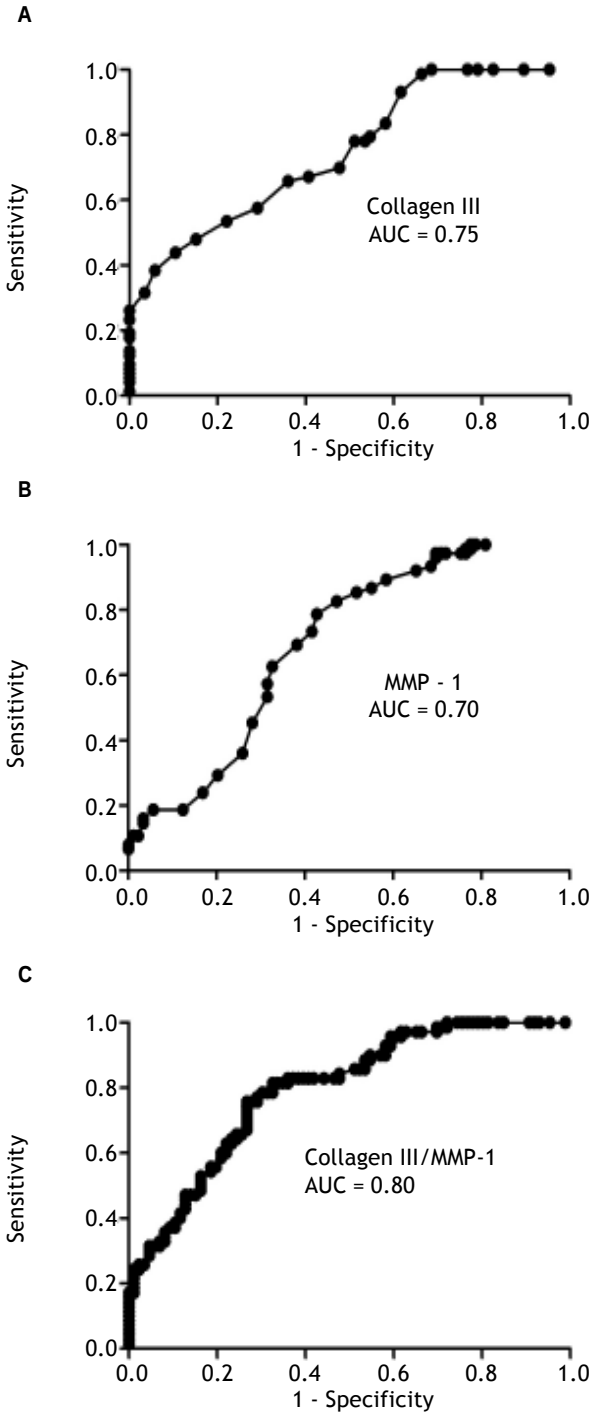

Figure 1. Areas under receiver-operating characteristic curve (AUCs) for A. Collagen III with an AUC of 0.75, B. MMP-1 with an AUC of 0.70, C. Collagen/MMP-1 ratio (CMR) with an AUC of 0.80 for predicting significant fibrosis (F2-F4) in chronic hepatitis C patients in the estimation study. Each point on the ROC plot represents a sensitivity/specificity pair corresponding to a particular decision threshold. An AUC of 1.0 is characteristic of an ideal test whereas an AUC of 0.5 or less indicates a test of no diagnostic value.

each other. Thus, CMR, AFP, AAR and platelet count were selected as the best combination for diagnosing F2-F4. Then, we put the positive correlation parameters (CMR, AAR and AFP) in the numerator and the negative correlation parameters (platelet count) in the denominator to formulate the following function (Table 3):

$$\text{Fibro-check} = \frac{\text{CMR x AAR x AFP (U/L)}}{\text{platelet count } (10^9/\text{L})}$$

Diagnostic performance of Fibro-check

The diagnostic value of Fibro-check was then assessed by calculating the area under the ROC curve that showed a superior AUC of 0.91 for identifying F2-F4. Next, an optimal cutoff point of 0.05 was selected based on the ROC curve analysis. For Fibro-check greater than 0.05, 65 out of 79 (82% PPV) would have significant fibrosis and only 14 of 89 without significant fibrosis would be classified falsely. At this cutoff point, significant fibrosis could be excluded with 84% NPV i.e., 16% of patients with Fibro-check less than 0.05 had significant fibrosis. Furthermore, the effectiveness of Fibro-check in predicting cirrhosis was then preformed using ROC curve producing an AUC of 0.83 lower than that produced in identifying significant fibrosis. An optimal cutoff point of 0.1 was selected based on the ROC curve analysis. At this cutoff point, Fibro-check had sensitivity of 74% with PPV of 45% and specificity of 77% with a NPV of 92% for predicting cirrhosis i.e. 8% of patients with Fibro-check value less than 0.1 had cirrhosis (Table 4).

Performance of Fibro-check in comparison with APRI and fibrosis index

Next, our results were compared with those of the previous reports,[9,10] ROC curves of APRI[9] and fibrosis index[10] (their calculations are shown in table 3) *vs*. Fibro-check were constructed and superimposed to determine which score would have the most clinical utility to predict significant fibrosis as displayed in figure 2A. The AUC using the procedure described by Hanley and McNeil[15] was better for Fibro-check (AUC = 0.91) than fibrosis index (AUC = 0.73) followed by APRI (AUC = 0.69). Next, ROC curves were also constructed and superimposed to determine which score would have the most clinical utility in predicting cirrhosis. The AUC was higher for Fibro-check (AUC = 0.83) than fibrosis index (AUC = 0.79) followed by APRI (AUC = 0.68) as

displayed in figure 2.B. Additionally, the sensitivity, specificity, PPV and NPV for these fibrosis tests were calculated using cutoff values exactly as originally described by their authors[9,10] which were not found to be optimal as shown in table 4. Moreover,

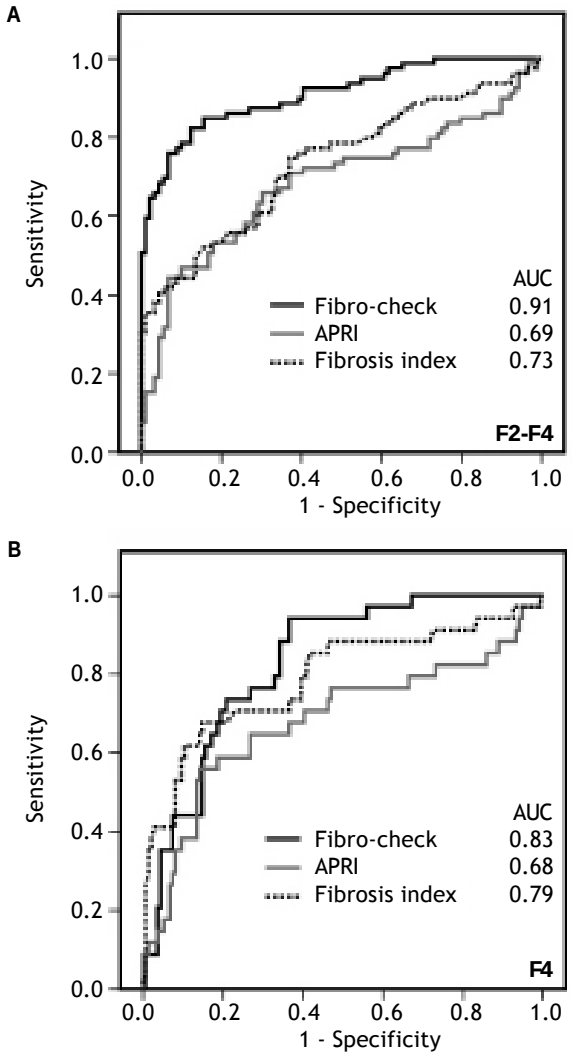

Figure 2. Area under the ROC curve for Fibro-check and comparison with APRI and Fibrosis index for predicting (A) significant fibrosis, (B) cirrhosis in chronic hepatitis C patients in the estimation study (n = 168). Calculations for Fibro-check, APRI and fibrosis index are shown in table 3.

Bivariate Spearman's rank correlation coefficient *(r)* was calculated to measure the relationship between these aforementioned tests and the METAVIR fibrosis score. As a result, Fibro-check significantly correlated with liver fibrosis stages with a better correlation coefficient ($r = 0.70$, $P < 0.0001$) than those produced by APRI ($r = 0.34$, $P < 0.0001$) and fibrosis index ($r = 0.43$, $P < 0.0001$). Then, the diagnostic performances of Fibro-check in discriminating F1 from F2-F3, F1 from F4 and F2-F3 from F4 were evaluated and presented in table 5.

Validation study

The Fibro-check was then applied to a validation cohort comprising one hundred and one patients to test its accuracy and reproducibility. The characteristics of the validation group were similar to that of the estimation group with no significant differences in any of the assessed variables (Table 1). The Fibro-check significantly correlated with liver fibrosis stages ($r = 0.60$, $P < 0.0001$). The diagnostic power of Fibro-check was assessed in the validation group by ROC curve showing AUCs of 0.90 and 0.86 for identifying significant fibrosis and cirrhosis, respectively. At cutoff point > 0.05, Fibro-check produced 80% sensitivity with 83% PPV and 86% specificity with 82% NPV for identifying significant fibrosis. At cutoff point > 0.1, Fibro-check produced 80% sensitivity with 50% PPV and 78% specificity with 93% NPV for identifying cirrhosis. Additionally, the diagnostic performances of Fibro-check in separating between F1, F2-F3 and F4 were validated as seen in table 5.

DISCUSSION

It is worthy noting that serum levels of proteins directly related to the hepatic fibrogenic process could be used as surrogate markers of liver fibrosis.[16] They reflect the greater deposition of ECM in the liver due to either increased synthesis by activated stellate cells or slow removal by Kupffer and endothelial sinusoidal cells.[17] These tests include

Table 3. Calculations of fibrosis tests in comparison with Fibro-check in the present study.

Fibrosis test[Ref.]	Calculation[a]
Fibro-check	[CMR x AFP (U/L) x AAR/platelet count (10^9/L)]
APRI[9]	[(AST/upper limit of normal)/platelet count (10^9/L)] x 100
Fibrosis index[10]	[8.0-0.01 x platelet count (10^9/L)-albumin (g/dL)]

[a] CMR: collagen III/ MMP-1 ratio; AFP: alpha fetoprotein; AAR: aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio.

Table 4. Diagnostic performances for Fibro-check in comparison with APRI and Fibrosis index for predicting significant fibrosis and cirrhosis in chronic hepatitis C patients.

Fibrosis test[Ref.]	Cutoff[a]	Sn[a]	Sp[a]	PPV[a]	NPV[a]
Significant fibrosis (F2-F4)					
Fibro-check[a]	> 0.05	82	84	82	84
APRI[9]	> 1.5	29	96	85	60
Fibrosis index[10]	≥ 2.1	57	73	65	66
Cirrhosis (F4)					
Fibro-check[a]	> 0.1	74	77	45	92
APRI[9]	> 2.0	18	94	43	82
Fibrosis index[10]	≥ 3.3	35	99	86	86

[a] Fibro-check = [CMR x AFP (U/L) x AAR/platelet count (10^9/L)]; Sn: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value.

Table 5. Performances of Fibro-check in discriminating different stages of fibrosis.

METAVIR stages	Cutoff	AUC[a]	SE[a]	P value[b]	(95% CI[a])	Sn[a]	Sp[a]	PPV[a]	NPV[a]
Estimation study (n = 168)									
F1 *vs.* F2-F3	> 0.027	0.87	0.04	< 0.0001	0.80-0.94	80	71	55	88
F1 *vs.* F4	> 0.046	0.94	0.02	< 0.0001	0.90-0.99	94	84	70	97
F2-F3 *vs.* F4	> 0.12	0.62	0.07	0.06	0.50-0.57	71	54	57	68
Validation study (n = 101)									
F1 *vs.* F2-F3	> 0.027	0.87	0.05	< 0.0001	0.78-0.96	79	78	63	88
F1 *vs.* F4	> 0.046	0.97	0.02	< 0.0001	0.93-1.00	78	86	74	93
F2-F3 *vs.* F4	> 0.12	0.69	0.09	0.03	0.53-0.84	80	50	57	75

[a] AUC: area under the receiver-operating characteristic curve; SE: standard error; CI: confidence interval; Sn: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value. [b] P > 0.05 is considered non significant; P < 0.05 is considered significant and P < 0.0001 is considered extremely significant.

measures of hepatic metabolic activity, extracellular matrix remodeling proteins, collagen synthesis and degradation products, and enzymes involved in matrix degradation. Collagen is considered to be the main component of connective tissue, and is the most abundant protein in mammals.[18] Of the many collagen subtypes described, only five have been detected in liver; they are types I, III, IV, V and VI.[19]

Extracellular degradation of matrix proteins is regulated by a family of zinc-dependent neutral proteases called the matrix metalloproteinases (MMPs). These MMPs are subdivided into 3 groups; collagenases that have a role in degrading and denaturing interstitial collagens (types I, II and III), type IV collagenases/gelatinases that degrade basement membrane (type IV) collagen and gelatins as well as stromelysins that degrade fibronectin, gelatins, proteoglycans and laminin.[20] There is a relatively large literature suggesting that MMP activity in liver decreases as fibrosis progresses. Decreased activity of ECM-removing MMPs is mainly due to an overexpression of their specific inhibitors (TIMPs) (21). Interstitial collagenase "MMP-1" is one of these MMPs which cleaves the native helix of fibrillar collagens types I, II and III, specifically at a single site into one-quarter and three-quarter products; these are susceptible to further degradation by other proteinases.[8]

Therefore, this work was concerned with the identification and quantitative determination of both collagen III and MMP-1 and then estimating their performances as surrogate markers for accurate diagnosis of liver fibrosis. Our results showed that patients with F2-F4 were associated with higher collagen III concentration than that observed in patients with F1 with extremely significant difference yielding AUC = 0.75 for diagnosing F2-F4. The latter result may be explained by the fact that ECM is a highly dynamic substratum with a precisely regulated balance between synthesis and degradation. But upon injury, the HSCs become activated and secrete large amounts of ECM. Consequently, ECM production exceeds ECM degradation, and hepatic fibrosis develops as a result of the progressive thick-

ening of fibrotic septae and chemical cross-linking of collagen.[22]

On contrary, the mean value of MMP-1 concentration decreased with the progression of liver fibrosis being lower in patients who developed F2-F4 than those with F1 with extremely significant difference yielding AUC = 0.70 for identifying F2-F4. The decreased activity of ECM-removing MMPs may be explained by the fact that TIMPs are co-expressed with MMPs and contribute to the regulation of local metalloproteinase activity. An increased hepatic TIMP expression may thus account for reduced metalloproteinase activity and is thought to be important for hepatic fibroproliferation.[23]

In order to amplify the difference in collagen III and MMP-1 values among patients with F1 and F2-F4 and increase their aptitude, collagen III/MMP-1 ratio was devised that showed a better AUC = 0.80 than that produced by each individually. Indeed, it was necessary for collagen III/MMP-1 ratio to be combined with other variables to improve its diagnostic ability for F2-F4 diagnosis. In this work, patients with F2-F4 were found to have significant lower platelet count than that observed in patients with F1. The decreased level of platelet count could be interpreted by the fact that hepatic fibrosis may cause thrombocytopenia as a consequence of impaired synthesis of thrombopoietin and/or sequestering of platelets in an enlarged spleen. Many studies supported that platelet count alone may be clinically valuable as a noninvasive marker for liver fibrosis and cirrhosis.[24,25] Ono, *et al.*[26] has reported that platelet count alone could discriminate F4 from F1-F3 in 75-80% of CHC patients. Consequently, platelet count was identified as a significant predictor of fibrosis.

On the other hand, the clinical use of AAR in the diagnostic workup of patients with chronic liver disease is supported by studies conducted in several countries.[27] AAR has been reported as a surrogate marker of liver fibrosis with values greater than one being suggestive of cirrhosis.[28] That's because of the increased release of mitochondrial AST, the decreased clearance of AST and/or impaired synthesis of ALT in advanced stages of liver disease.[29] With regard to AFP, it is an important marker in diagnosing HCC. However, elevated AFP level has also been observed in chronic hepatitis C patients.[30]

As a consequence of these analyses, a discriminant function called Fibro-check composed of collagen III, MMP-1 together with platelet count, AAR and AFP was created. The diagnostic value of Fibro-check was then assessed by the ROC curve giving 0.91 AUC for identifying F2-F4 and 0.83 AUC for identifying F4. The present work was concerned with identifying the presence of F2-F4 and F4. That is because the presence of F2-F4 is accepted as an indication to commence treatment[31,32] and the presence of F4 also has implications regarding screening for HCC.[33]

Next, we evaluated the diagnostic accuracies of some non-invasive scores for assessing the degree of liver fibrosis in comparison with our developed score in our study group. Among these, APRI[9] and fibrosis index.[10] Diagnostic accuracies for these aforementioned scores were evaluated using categories and cutoff values exactly as originally described which were not found to be optimal and had AUCs lower than those reported previously by their authors in their original study. These discrepancies may be related to differences in the prevalence of F2-F4 and F4. Also, they may be related to differences in the patient characteristics and the histopathological assessment. It is worthy noting that in Egypt, patient delay seeking medical care until symptoms get worsen that explain the lack of patient with F0 from our study population. We acknowledge that there is a limitation to the clinical use of MMP-1 and collagen III because they are not routinely available in all hospital settings.

Finally, we evaluated whether the predictive criteria identified in the estimation study were able to reproduce their predictive ability in a subsequent different, but related group of patients. In conclusion, we showed that a five-marker model combining collagen III, MMP-1 together with platelet count, AAR and AFP may improve liver fibrosis staging with a high degree of accuracy and seems more efficient than APRI and fibrosis index in our hands. Further prospective studies involving a greater number of patients are warranted to validate the usefulness of the produced score in clinical practice.

ABBREVIATIONS

- **AAR:** AST/ ALT ratio.
- **ALT:** alanine aminotransferase.
- **ALP:** alkaline phosphatase.
- **APRI:** aspartate to platelet ratio index.
- **AST:** aspartate aminotransferase.
- **AFP:** alpha fetoprotein.
- **CHC:** chronic hepatitis C.
- **CMR:** collagen III/ Matrix metalloproteinase-1 ratio.
- **ECM:** extracellular matrix.
- **HCC:** hepatocellular carcinoma.
- **HCV:** hepatitis C virus.
- **MMP-1:** matrix metalloproteinase-1.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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REFERENCES

1. Lavanchy D. Evolving epidemiology of hepatitis C virus. *Clin Microbiol Infect* 2011; 17: 107-15.
2. Maasoumy B, Wedemeyer H. Natural history of acute and chronic hepatitis C. *Best Pract Res Clin Gastroenterol* 2012; 26: 401-12.
3. Lin ZH, Xin YN, Dong QJ, Wang Q, Jiang XJ, Zhan SH, Sun Y, et al. Performance of the aspartate aminotransferase-to-platelet ratio index for the staging of hepatitis C-related fibrosis: an updated meta-analysis. *Hepatol* 2011; 53: 726-36.
4. Martínez SM, Crespo G, Navasa M, Forns X. Noninvasive assessment of liver fibrosis. *Hepatol* 2011; 53: 325-35.
5. Grigorescu M. Noninvasive Biochemical Markers of Liver Fibrosis. *J Gastrointestin Liver Dis* 2006; 15: 149-59.
6. Patrick M, Asselah T, Boyer N. Fibrosis and Disease Progression in Hepatitis C. *Hepatology* 2002; 36: S47-S56.
7. Jarčuška P, Janičko M, Veselíny E, Jarcuska P, Skladaný L. Circulating markers of liver fibrosis progression. *Clin Chim Acta* 2010; 411: 1009-17.
8. Arthur MJP. Collagenases and liver fibrosis. *J Hepatol* 1995; 22: 43-8.
9. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS, Lok AS. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatol* 2003; 38: 518-26.
10. Ohta T, Sakaguchi K, Fujiwara A, Fujioka S, Iwasaki Y, Makino Y, Araki Y, et al. Simple surrogate index of the fibrosis stage in chronic hepatitis C patients using platelet count and serum albumin level. *Acta Med Okayama* 2006; 60: 77-84.
11. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. *Lancet* 1997; 349: 825-32.
12. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-5.
13. Towbin H, Stachlin T, Gordou J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets procedure and some applications. *Proc Natl Acad Sci USA* 1979; 76: 4350-4.
14. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with folinphenol reagent. *J Biol Chem* 1951; 193: 265-75.
15. Hanley JA, McNeil BJ. A method of comparing the areas under receiver operating characteristic curves derived from the same cases. *Radiol* 1983; 148: 839-43.
16. Fontana RJ, Lok AS. Noninvasive monitoring of patients with chronic hepatitis C. *Hepatol* 2002; 36: S57-S64.
17. Ladero JM. Noninvasive evaluation of liver fibrosis in patients with chronic hepatitis C. *Hepat Mon* 2011; 11: 698-700.
18. Di Lullo GA, Sweeney SM, Korkko J, Ala-Kokko L, San Antonio JD. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. *J Biol Chem* 2002; 277: 4223-31.
19. Alcolado R, Arthur MJ, Iredale JP. Pathogenesis of liver fibrosis. *Clin Sci* 1997; 92: 103-12.
20. Arthur MJ. Degradation of Matrix Proteins in Liver Fibrosis. *Pathol Res Pract* 1994; 190: 825-33.
21. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; 115: 209-18.
22. Hernandez-Gea V, Friedman SL. Pathogenesis of liver fibrosis. *Annu Rev Pathol Mech Dis* 2011; 6: 425-56.
23. Lichtinghagen R, Bahr MJ, Wehmeier M, Michels D, Haberkorn CI, Arndt B, Flemming P, et al. Expression and coordinated regulation of matrix metalloproteinases in chronic hepatitis C and hepatitis C virus-induced liver cirrhosis. *Clin Sci* 2003; 105: 373-82.
24. Myres RP, De Torres M, Imbert-Bismut F, Ratziu V, Charlotte F, Poynard T; MULTIVIRC Group. Biochemical markers of fibrosis in patients with chronic hepatitis C: a comparison with prothrombin time, platelet count, and age-platelet index. *Dig Dis Sci* 2003; 48: 271-8.
25. Ghany MG, Lok AS, Everhart JE, Everson GT, Lee WM, Curto TM, Wright EC, et al. Predicting clinical and histologic outcomes based on standard laboratory tests in advanced chronic hepatitis C. *Gasteroenterol* 2010; 138: 136-46.
26. Ono E, Shiratori Y, Okudaira T, Imamura M, Teratani T, Kanai F, Kato N, et al. Platelet count reflects stage of chronic hepatitis C. *Hepatol Res* 1999; 15: 192-200.
27. Hsieh YY, Tung SY, Lee IL, Lee K, Shen CH, Wei KL, Chang TS, et al. FibroQ: an easy and useful noninvasive test for predicting liver fibrosis in patients with chronic viral hepatitis. *Chang Gung Med J* 2009; 32: 614-22.
28. Williams AL, Hoofnagle JH. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. Relationship to cirrhosis. *Gastroenterol* 1988; 95: 734-9.
29. Stauber RE, Lackner C. Noninvasive diagnosis of hepatic fibrosis in chronic hepatitis C. *World J Gastroenterol* 2007; 13: 4287-94.
30. Chu CW, Hwang SJ, Luo JC, et al. Clinical, virologic and pathologic significance of elevated serum alpha-fetoprotein levels in patients with chronic hepatitis C. *J Clin Gastroenterol* 2001; 32: 240-4.
31. Booth JC, O'Grady J, Neuberger J. Clinical guidelines on the management of hepatitis C. *Gut* 2001; 49: 11-21.
32. Strader DB, Wright T, Thomas DL, Seeff LB; American Association for the Study of Liver Diseases. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004; 39: 1147-71.
33. Adams LA, Bulsara M, Rossi E, DeBoer B, Speers D, George J, Kench J, et al. Hepascore: an accurate validated predictor of liver fibrosis in chronic hepatitis C infection. *Clin Chem* 2005; 51: 1867-73.