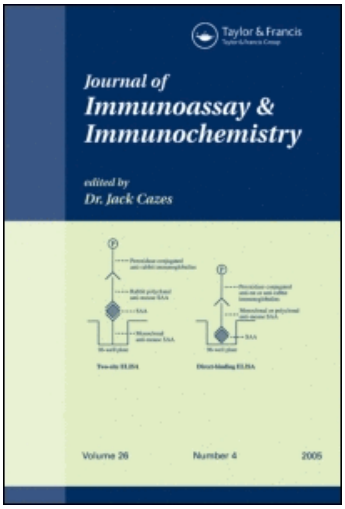

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Immunodetection of Collagen Types I, II, III, and IV for Differentiation of Liver Fibrosis Stages in Patients with Chronic HCV

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Abstract: The current study is aimed at evaluating serum collagens and other serum biochemical markers as useful, non-invasive markers of hepatic fibrosis associated with chronic hepatitis C virus (HCV). Collagen types I, II, III, and IV were detected in serum using ELISA and Western blot techniques. The ELISA levels of collagen I, II, III, and IV increased significantly with the progression of fibrosis staging. Based on receiver-operating characteristic (ROC) curve analysis, the collagen type III (70 kDa) and type IV (200 kDa) were more useful than other serum bio-markers for differentiating severe fibrosis from mild fibrosis. Multivariate discriminant analysis (MDA) selected a fibrosis discriminant score (FDS) = [2.345 + Collagen III (μg/mL) × 1.923 + Collagen IV (μg/mL) × 1.544 + ALT (U/mL) × 0.005] - [albumin(g/L) × 0.046]. The FDS correctly classified 87% of the severe fibrosis patients at a cut-off score = 2.2 (i.e., more than 2.2 indicated severe fibrotic liver and less than 2.2 indicated mild fibrotic liver) with specificity of 97%. In a validation study, the FDS was applied to the second cohort of patients and the results were reproduced without

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significant difference. In conclusion, the developed four-parameter based FDS is useful for identifying severe liver fibrosis in patients with chronic HCV infection.

Keywords: HCV, Fibrosis, Biomarkers, Collagens, Score

INTRODUCTION

HCV infection is now becoming a common health problem in both developed and developing countries.[1] The exact diagnosis is key for the understanding of the natural course and prognosis of liver disease.[2,3]

Liver fibrosis progression occurs in two-thirds of patients with initially mild chronic hepatitis C within 5–10 years and advanced fibrosis develops in one-third of those with no fibrosis.[4] The assessment of the presence and severity of liver fibrosis is of paramount importance in determining treatment strategies, response to treatment, prognosis, and the potential risk for complications in patients with chronic liver disease.[5,6] Liver biopsy is the gold standard method to assess inflammatory activity and fibrosis stage, but this is associated with morbidity and mortality.[7] Non-invasive diagnosis of hepatic fibrosis has become the focus because of limited biopsy, especially in the surveillance of treatment and in screening hepatic fibrosis.[8] Over the past decade, there has been a renewed enthusiasm to develop noninvasive serum markers or tests to assess the presence and severity of fibrosis in chronic liver disease.[6] While some investigators have focused on a combination of laboratory tests, such as reversal of aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio, or AST/platelet ratio index (APRI), others have been searching for more novel markers of fibrosis and inflammation.[9–14] The simple methods reflect the dysfunction of the liver, and do not relate to the efficacy of IFN treatment in patients with HCV-associated liver disease.[11] Methods for measuring biochemical parameters that reflect post-translation events of collagen metabolism and other matrix proteins are markers of liver fibrogenesis.[15–17] In the present study, we assessed and compared the diagnostic accuracy of collagen types as biochemical markers of liver fibrosis in chronic HCV patients. We also developed and evaluated a sensitive and specific fibrosis discriminant score (FDS) based on these blood markers to predict severe fibrosis of the liver.

EXPERIMENTAL

Samples

Blood samples and liver biopsies of 120 HCV infected individuals (69 males, 51 females; aged 21–70 years) were collected from the Gastroenterology and

Surgery Center, Mansoura University, Mansoura. The samples were obtained with informed consent. The HCV infection was diagnosed based on biochemical, serologic, and histological criteria. None of the patients had a history of habitual alcohol consumption or hepatocellular carcinoma. Moreover, all individuals were negative for hepatitis A and B viruses. Patients with chronic HCV infection (CHC) were diagnosed with a positive test for anti-HCV antibodies (Biotec Laboratories Ltd., Suffolk, UK). Liver function tests, including AST, ALT, alkaline phosphatase, albumin, bilirubin, and total protein were measured using standard methodologies (bioMérieux SA, Marcy l'Etoile, France). Anti-schistosomal antibodies were detected using an indirect hemagglutination assay (IHA) (Fumouze, Levallois-Perret, France). Needle liver biopsy specimens were examined by a pathologist who was unaware of the clinical and laboratory results of each sample examined. A METAVIR score was used to stage the fibrosis (F0 to F4).[18] The patients were pathologically classified into three groups: 20 patients with no liver fibrosis (F0, considered as controls); 40 patients with mild fibrosis liver (F1-F2); and 60 patients with severe fibrosis liver (F3-F4). All patients with no liver fibrosis (F0) were negative for anti-schistosomal antibodies; however, 100% of the patients with mild fibrosis (F1-F2) and 50% of the patients with severe fibrosis liver (F3-F4) were positive. The study protocol conforms to the ethical guideline of the 1975 Declaration of the Helsinki.

Measurement of Serum Collagen in Serum using ELISA

We have developed an ELISA to detect serum collagens I, II, III, and IV in different stages of liver fibrosis patients. After optimization of reaction conditions, the following ELISA procedure was performed. Fifty μL of serum diluted 1:10 in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6) were allowed to bind overnight to wells of ELISA plates. After five washes with phosphate buffered saline-Tween 20 (PBS-T 20), the wells were blocked with 0.5% bovine serum albumin (BSA) in coating buffer. After five washes with PBS-T20, fifty μL of mouse monoclonal antibodies to investigated human collagen types, each type tested alone (Sigma Chemical Co., St. Louis, MO, USA) were added separately per well at a dilution of 1:150 in PBS. The antigen-antibody binding was allowed to proceed for 2 hours at 37°C. The plates were washed five times with PBS-Tween 20 (0.05%) and 50 mL of goat anti-mouse IgG alkaline phosphatase conjugate (Sigma), diluted 1:400 in 0.2% BSA in PBS-T20, was added per well. After 1 hour, the plates were washed five times with PBS-T20; the amount of coupled conjugate was determined by incubation with 1 mg/mL p-nitrophenyl phosphate in substrate buffer for 30 min at 37°C. The reaction was stopped by addition of 25 μL/well of 3 M NaOH and the absorbance was read at 405 nm using an EL-311 microplate autoreader (Bio-Tek Instruments, Winooski, VT, USA).

Western Blotting

The SDS-PAGE resolved serum samples were electro-transferred onto nitrocellulose membranes (NC) (0.45 μm pore size, Sigma) in a protein transfer unit according to Towbin et al.[19] The NC membrane was blocked using 5% (w/v) BSA dissolved in 0.05% M Tris-buffered saline (TBS), containing 200 mM NaCl (pH 7.4), rinsed in TBS, and incubated with specific monoclonal antibodies to human collagen type III and type IV diluted (1:200) in 1% BSA dissolved in TBS with constant shaking. The NC membrane was washed three times (30 min each) in TBS followed by incubation separately for 2 h with anti-mouse IgG alkaline phosphatase conjugate (Sigma) diluted (1:150) in TBS. The dilutions of monoclonal antibody and conjugate were adjusted to eliminate the background, i.e., the presence of collagens in the healthy control group. After washing 3 more times with TBS (30 min each), the NC membrane was exposed to alkaline phosphatase substrate [5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) in 0.1 M Tris buffer, pH 9.6, (Sigma) for 10 min. The reaction was stopped using distilled water.

Receiver Operating Curve (ROC)

To assess the ability of the ten serum markers for differentiating chronic hepatitis (mild fibrosis score F1-F2) from severe fibrosis (F3-F4), and for differentiating liver without fibrosis (F0) from mild fibrosis (F1-F2), we calculated the sensitivity and specificity for each value of each fibrosis marker and then constructed receiver operating curves (ROC) as previously described by Attallah et al.[14,15] An area under the curve (AUC) equal to 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value. The nearer a curve shifts to the top left-hand corner of the graph, the more useful the marker is for the diagnosis. The turning point of the curve to the best cut-off value for the diagnosis was determined, and it was also a maximal value at the sum of the sensitivity and specificity. The diagnostic accuracy was calculated by sensitivity, specificity, positive and negative predictive values, considering significant fibrosis of the disease.[14,15]

Statistical Analyses

All statistical analyses were done by a statistical software package "SPSS 10.0 for Microsoft Windows, SPSS Inc.) and considered statistically significant at a two-sided $P < 0.05$. Numerical data were expressed as mean $\pm$ SD. The levels of markers were analyzed by ANOVA, but the Mann-Whitney U-test was used for comparisons between independent groups. The correlations between ten serum fibrosis markers and biochemical data were evaluated by Person's correlation coefficient. A P value < 0.05 was considered significant.

The multivariate discriminant analysis (MDA) was carried out stepwise with use of the minimum Wilks' lambda. The relative weighting of serum biochemical markers included in the discriminant model is designated by the standardized canonical discriminant coefficients.

RESULTS

Values of Collagen Types, Liver Function Tests and the Stage of Liver Fibrosis

Laboratory values of serum markers in different stages of liver fibrosis (F0-F4) are summarized in Table 1 for the 120 patients. The values of serum collagens (I, II, III, and IV), AST, ALT, alkaline phosphatase, and bilirubin increased, but albumin and total proteins decreased in the patients with HCV-associated liver disease, according to the stage of liver fibrosis (F0-F4).

Performance Characteristics of Fibrosis Discriminant Score (FDS)

The best linear combination of blood markers was selected by multivariate discriminant analysis (MDA) for construction of FDS equations based on

Table 1. Laboratory biomarkers of chronic hepatitis C patients with non fibrotic liver (F0), mild liver fibrosis (F1-F2) and severe liver fibrosis (F3-F4)

Biomarkers	F0 (n = 20)	F1-F2 (n = 40)	F3-F4 (n = 60)	*P value
Collagen I (μg/ml)[a]	0.20 ± 0.41	0.27 ± 0.08	0.30 ± 0.08	<0.0001
Collagen II (μg/ml)[a]	0.08 ± 0.04	0.12 ± 0.09	0.14 ± 0.11	<0.05
Collagen III (μg/ml)[a]	0.19 ± 0.08	0.26 ± 0.07	0.30 ± 0.09	<0.0001
Collagen IV (μg/ml)[a]	0.09 ± 0.063	0.37 ± 0.19	0.46 ± 0.22	<0.0001
ALT (U/ml)[b]	24.9 ± 10.6	63.08 ± 25.3	71.5 ± 35.3	<0.0001
AST (U/ml)[b]	28.3 ± 7.9	64.8 ± 25.4	72.4 ± 35.9	<0.0001
Alkaline phosphatase (IU/L)[b]	57.12 ± 16.3	84.49 ± 45.44	146.3 ± 73.84	<0.0001
Total proteins (g/L)[b]	7.1 ± 0.39	6.94 ± 0.61	6.30 ± 0.66	<0.001
Albumin (g/L)[b]	44.5 ± 6.6	37.4 ± 4.4	30.2 ± 7.59	<0.0001
Bilirubin (mg/dl)[b]	0.62 ± 0.15	1.25 ± 0.46	2.18 ± 1.4	<0.0001

[a]Cutoff level of collagen types for non fibrotic liver: Collagen I (<0.238 μg/ml), collagen II (<0.118 μg/ml), collagen III (<0.22 μg/ml), collagen IV (<0.24 μg/ml).

[b]Normal values for liver function tests: Alanine aminotransferase (ALT) up to 45 U/ml; aspartate aminotransferase (AST) up to 40 U/ml; alkaline phosphatase 22-92 IU/L; Total protein 6.8-8.5; Albumin 38-54 g/L; Bilirubin up to 1-mg/dl.

*$p > 0.05$ is considered not significant, $p < 0.05$ considered significant, $p < 0.001$ considered very significant and $p < 0.0001$ is considered extremely significant.

two markers (collagen III and collagen IV), three markers (collagen III, collagen IV, and albumin) and four blood markers (collagen III, collagen IV, albumin, and ALT) for discriminating patients with severe fibrosis from mild fibrosis liver. The ability of the investigated serum markers to predict severe fibrosis (F3-F4) in patients with chronic HCV was assessed using ROC. The best ROC derived from four serum fibrosis markers was applied by measuring the area under the curve (AUC). ROC curves were used to determine cut-off values with best efficiency of ALT (>45 IU/mL), albumin (<38 gm/L), collagen III (>0.22 μg/mL), collagen IV (>0.24 μg/mL); the areas under the curves and (p value) are 0.63 (0.035), 0.68 (0.025), 0.69 (0.019), and 0.76 ($p < 0.0001$), respectively (Figure 1). Serum collagen IV is the most efficient index. So, serum collagen IV was taken as the basis index to combine with other indices to discriminate between severe fibrosis and mild fibrosis liver patients. The areas under the ROC curves (p value) of two, three, and four markers were 0.80 (<0.0001), 0.83 (<0.0001) and 0.87 (<0.0001), respectively (Figure 2). Using MDA, a function selected based on absolute values of the four biochemical markers; FDS Score = [2.345 + Collagen III (μg/mL) × 1.923 + Collagen IV (μg/mL) × 1.544 + ALT (U/mL) × 0.005] − [albumin (g/L) × 0.046] was selected (Table 2). To evaluate the differential diagnostic power of the discriminant function, we constructed ROC curves for FDS and compared them with ROC curves of the other variables that we had previously found to be significantly different for liver fibrosis and no fibrotic controls. FDS, calculated for each patient on the basis of the linear combination of variables selected by MDA, was used to classify cases into one of the two groups (severe or mild

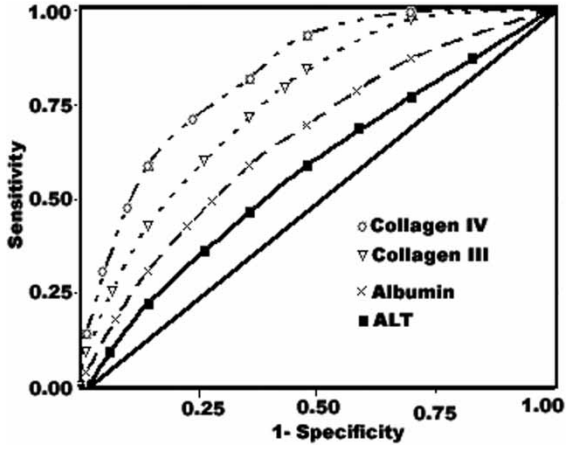

Figure 1. ROC curves for collagen IV, collagen III, albumin, and ALT for discriminating patients with severe liver fibrosis (F3-F4) from those with mild fibrosis livers (F1-F2). The areas under the curves and its significance (p value) of these markers were 0.76 ($p < 0.0001$), 0.69 (0.019), 0.68 (0.025), and 0.63 (0.035), respectively.

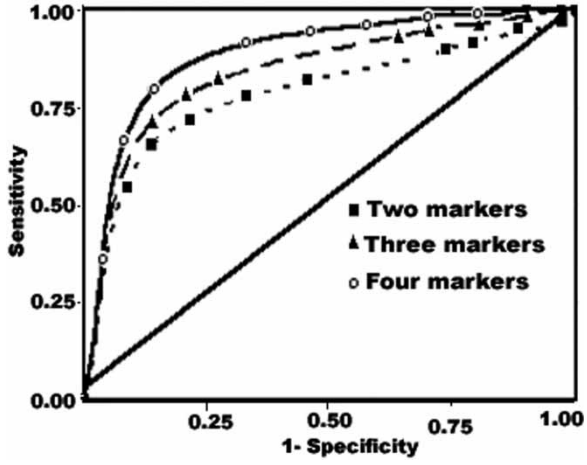

Figure 2. ROC curves for two markers (collagen IV and collagen III), three markers (collagen IV, collagen III, and albumin) and four markers (collagen IV, collagen III, albumin, and ALT) for discriminating patients with severe liver fibrosis (F3-F4) from those with mild liver fibrosis (F1-F2). The areas under the ROC curves (p value) of these combinations are 0.80 (<0.0001), 0.83 (<0.0001), and 0.87 (<0.0001) for two, three, and four markers; respectively.

fibrosis). There was highly significant correlation between the METAVIR score fibrosis stage and FDS ($r = 0.439$; $p < 0.00001$). FDS range from 0.32 to 3.9 increased significantly ($p < 0.0001$) as fibrosis stage increased (Figure 3). The median, mean and $\pm$ SD of FDS in no fibrosis (F0) were 0.89, 0.924 and $\pm$ 0.38, in mild liver fibrosis (F1-F2) patients were 1.90, 2.06 and $\pm$ 0.46; in severe liver fibrosis (F3-F4) were 2.54, 2.36 and $\pm$ 0.64 respectively. Overall significance of differences among the 3 groups was determined by ANOVA for FDS $P < 0.0001$. Significant differences ($p < 0.0001$) were shown between non fibrosis liver (F0), mild fibrosis (F1-F2), and severe fibrosis (F3-F4). Also, a significant difference ($p < 0.0001$) was shown between mild fibrosis (F1-F2) and severe fibrosis

Table 2. Multiple logistic regression model for the prediction of severe liver fibrosis

Variable	Coefficients	SE[a]	P	AUC[b]	(95 % CI[c])
Collagen III	1.923	0.810	0.019	0.69	0.61–0.79
Collagen IV	1.544	0.353	<0.0001	0.76	0.67–0.85
Albumin (g/L)	0.046	0.05	0.025	0.68	0.53–0.73
ALT (U/ml)	0.005	0.003	0.035	0.63	0.59–0.78

[a]SE = Standard error.
[b]AUC = Area under (ROC) curve.
[c]CI = Confidence interval.

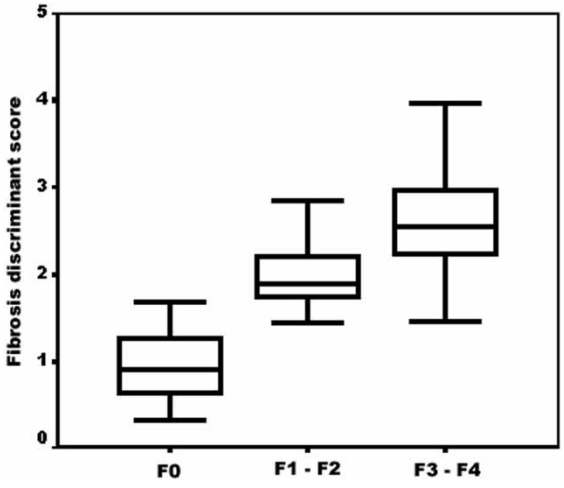

Figure 3. Box plots for fibrosis discriminant score (FDS) in patients with non-fibrotic (F0), mild fibrotic (F1-F2) and severe fibrotic liver (F3-F4). FDS with respect to stage of liver fibrosis (F0-4). The box represents the interquartile range. The whiskers indicate the highest and lowest values, and the line across the box indicates the median value. Overall significance of differences among the 3 groups was determined by ANOVA for FDS. Significance differences ($p < 0.0001$) were shown between non-fibrotic liver (F0), mild fibrosis (F1-F2), and severe fibrosis (F3-F4). Also, a significant difference ($p < 0.0001$) was shown between mild fibrosis (F1-F2) and severe fibrosis (F3-F4).

(F3-F4). When FDS was applied with a cutoff point of 2.2 for detection of severe fibrosis (F3-F4) vs mild fibrosis (F1-F2), it correctly classified 87% of the fibrosis patients at a discriminant cut-off score = 2.2 (i.e., more than 2.2 indicated severe liver fibrosis and less than 2.2 indicated liver mild fibrosis) with a sensitivity of 81% and specificity of 97 %, Table 3.

Identification of Collagen Types III and IV using Western Blot

The molecular weight of the selected collagen type III and type IV were identified in serum samples of liver diseased patients (F1-F4) at 70- and 200-kDa; respectively, (Figure 4). No reaction was observed in sera of controls.

Validation Study

In a second part of the study, we evaluated FDS identified in the first part and were able to reproduce their predictive ability in a subsequent different, but related group of patients. A total of 75 patients (43 males, 32 females; aged 27-73 year) were included. The clinical and pathological investigation, as

Table 3. Performance characteristics of different discriminant scores derived from MDA function based on absolute values of four blood markers (collagen III, collagen IV, albumin and ALT) for discriminating patients of severe liver fibrosis (F3-F4) from mild liver fibrosis (F1-F2)

MDA[a] cut-off score	Performance characteristics (%)				
	Sensitivity	Specificity	PPV[c]	NPV[d]	Efficiency
1.5	98	3	62	50	62
1.6	98	9	63	75	64
1.7	93	23	66	67	66
1.8	91	31	68	69	68
1.9	89	51	75	75	75
2.0	84	57	76	69	74
2.1	82	71	82	71	71
2.2[b]	81	97	98	76	87
2.3	71	100	100	69	82
2.4	63	100	100	63	78
2.5	52	100	100	57	70

[a]MDA= Multivariate discriminant analysis.

[b]Selected as a discriminant score at 2.2 (i.e. more than 2.2 indicated sever liver fibrosis (F3-F4).

[c]PPV= Positive predictive value.

[d] NPV= Negative predictive value.

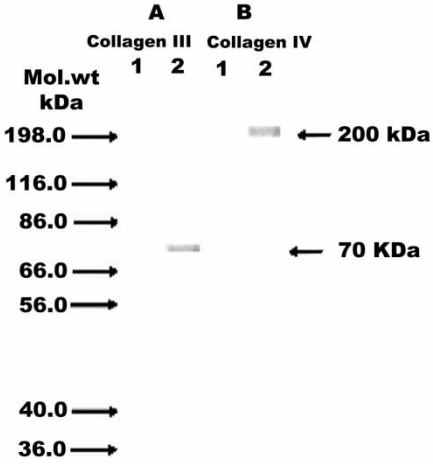

Figure 4. Immunoblots of serum samples from patients with liver diseases using mouse monoclonal antibodies to human collagen types (III and IV). A. Samples stained with anti-human collagen type III monoclonal antibody. B. Samples stained with anti-human collagen type IV monoclonal antibody. Lane 1: serum of individual with no fibrotic liver (F0), Lane 2: serum of patient with liver fibrosis (F1-F4). The molecular weights of collagen types III and IV were 70- and 200-kDa; respectively.

well as inclusion and exclusion criteria for the study, and classification adopted in this second group of patients, were the same as those used in patients in the first part. The ROC curve of the validation group using the FDS score was done, (Figure 5). The area under the ROC curve was 0.85. In practice, we applied the FDS to the second cohort of patients and found that 81% of mild fibrosis and 84% of severe fibrosis patients were correctly classified. The efficiency, and positive and negative predictive values were 83%, 86%, and 78%, respectively.

DISCUSSION

The prognosis of chronic liver disease is closely related to the development of hepatic fibrosis.[6] Fibrosis is characterized by excessive deposition of collagen and other components of the extracellular matrix, which leads to a disturbed function of the organs involved.[20–22] These parameters can be assayed in biopsy material or in biological fluids.[23] Determination in liver tissue is specific, but has the same limitations described for liver biopsy examination.[24] Serum assays of collagens are accurate, non-invasive markers of liver fibrosis and liver inflammation in chronic hepatitis C.[25–27] These markers reflected liver injury and they have high specificity and sensitivity in detecting advanced liver disease in chronic hepatitis C.[28] In our study, the molecular weights of collagen types III and IV were 70 and 200–kDa, respectively, using Western blot. Several authors identified collagen types III and IV using monoclonal antibodies at the same molecular weights.[29–31]

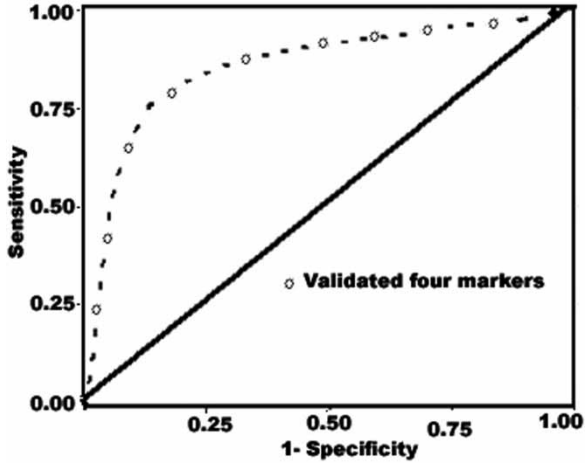

Figure 5. ROC curve of the validation group of patients using the fibrosis discriminant score (FDS) based on collagen IV, collagen III, albumin and ALT for discriminating patients with severe liver fibrosis (F3-F4) from those with mild liver fibrosis (F1-F2). The areas under the ROC curve (p value) was 0.85 (<0.0001).

It has been suggested that immunoblotting is not suitable for routine diagnosis of collagen metabolism. Certainly, for laboratories with limited resources, this could prove difficult. In the present study, the levels of collagen types I, II, III, and IV, using ELISA, increased significantly with the progression of fibrosis staging. Several authors evaluated diagnostic significance of type I, II, III, and IV collagen in patients with chronic hepatitis C for staging hepatic fibrosis using ELISA.[22,28,30,32] Fibrosis in the human liver is due to an increase in type I and type III and IV collagens; total collagen content is increased by four- to seven-fold.[33] Type II collagen is almost exclusively localized in cartilage, where it is the major structural component of the tissue, so it has no diagnostic role in differentiation between hepatic stages.[34] Once the discriminative values of each parameter were evaluated, we observed that, based on the ROC curve, the serum type IV collagen was more useful than other markers for differentiating severe fibrosis from mild fibrosis with the area under ROC curve (0.76), which is higher than the results of Cai et al.,[35] but less than the results of Walsh et al.[36] Although a single marker or test has lacked the necessary accuracy to predict severe fibrosis, different combinations of these markers or tests have shown encouraging results.[5] Serum fibrosis indices are fairly well correlated with the inflammation grade of the liver, fibrosis staging and the degree of chronic hepatitis. However, as diagnostic markers, they should be considered in combination with liver function tests, ultrasonography, and clinical manifestations.[7,15]

In our study, as an alternative to liver biopsy, an index of four markers (collagen IV, III, albumin, and ALT) has been shown to predict the severe fibrosis. The fibrosis discriminant score with efficiency, sensitivity, specificity, positive predictive, and negative predictive values were 87%, 81%, 97%, 98%, and 76%, respectively. A model published by Patel et al.[37] also incorporated hyaluronic acid and α_2-macroglobulin. This model had predictive value between 71% and 79% and, thus, may not be accurate enough to guide decision–making. Similarly, an algorithm incorporating hyaluronic acid TIMP-1, and N-terminal of propeptide of type III collagen, examined in a large number of patients with chronic liver diseased, had an AUC of 0.78 for significant fibrosis from all etiologies and an AUC of 0.77 when limited to patients with hepatitis C.[35] Of fibrosis discriminant score, ALT and albumin were measured routinely. Collagens III and IV were available to any laboratory with a micro-plate colorimetric reader.

It is, therefore, less costly and more convenient to perform these assays than a liver biopsy. In the validation study, the FDS applied to the second cohort of patients and the results were reproduced with no significant difference. Of the 75 patients of the validation study, 83% could have avoided liver biopsy, but discrepant results were recorded in 13 patients (17%). In conclusion, the results of the present study show the important contribution made by the determination of collagen components, especially III and IV, and also of ALT and albumin levels for discriminating patients with severe liver fibrosis from those with mild liver fibrosis.

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