Assessment of Hepatic Fibrosis Stages in Hepatitis C Virus Infected Patients Using Biomarkers in The Blood

Ahmed A. Attallah[*1], Sanaa O. Abdallah[2], Mohamed M. Omran[1], Khaled Farid[3], and Yehia M. Shaker[4]

[1] R & D Dept. Biotechnology Research Center, New Damietta City, Egypt.
[2] Faculty of Science, Cairo University, Giza, Egypt,
[3]Tropical Medicine Unit, Faculty of Medicine, Mansoura University, Mansoura, Egypt
[4]National Research Center, Cairo, Egypt
ahmedattallah2009@hotmail.com

Abstract: Background: An intensive research effort in the field of non-invasive evaluation of liver fibrosis has recently permitted the description of several blood markers of fibrosis, mainly in chronic hepatitis C (CHC) patients. Our aim was to evaluate the diagnostic performance of a panel of simple blood markers of liver fibrosis in CHC patients. Materials and Methods: One hundred and thirty two patients with CHC evaluated for deciding on antiviral therapy were included. We used receiver operating characteristic (ROC) curves and a stepwise combination algorithm was developed to assess and compare the diagnostic accuracy of blood markers. Results: The areas under the ROC curves of AST/ALT ratio, albumin, platelet count, APRI and fibronectin for discriminating advanced liver fibrosis (F3-F4) were 0.58, 0.73, 0.76 , 0.73 and 0.74; respectively. The AUC of combined markers score based on AST-ALT ratio, albumin, fibronectin and platelets count was 0.86 for advanced liver fibrosis patients. The combined markers correctly classified 35 positive patients from 43 patients with 81% sensitivity and classified 64 patients as negative from a total of 89 patients with 72% specificity. Discussions. we have developed multivariate discriminant analysis (MDA), a function may contribute to differentiating advanced fibrosis in patients with CHC. The MDA function is based on easily and routinely analyzed four blood markers as noninvasive, reproducible, quantitative, precise, accurate and low cost method that can be applied to patients who either have contraindications or refuse liver biopsy for the management of their HCV infection.
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1. Introduction

The world health organization has declared Hepatitis C a global health problem, with approximately 3% of the world's population infected with HCV. Egypt has one of the highest HCV prevalence in the world (15% of the population) [1]. The manifestations of chronic HCV range from an asymptomatic state to liver fibrosis, liver cirrhosis, and hepatocellular carcinoma [2]. Guidelines and recommendations indicate that staging of liver fibrosis is the most important parameter for the definition of prognosis and for the subsequent management of the patient with chronic hepatitis C [3]. Liver fibrosis is defined as the building up of excessive amount of extracellular matrix (ECM) in the liver parenchyma. During fibrosis, hepatic stellate cells (HSC) play important roles in the control of extracellular matrix synthesis and degradation in fibrotic livers [4]. The extracellular matrix remodeling markers include several glycoproteins (hyaluronan, laminin, fibronectin etc) [5]. For the past 50 years liver biopsy has been considered to be the gold standard for staging of liver fibrosis [6]. Studies reveal that the risk for hospitalization after liver biopsy is 1-5%, the risk for severe complications is 0.57%, and mortality rates vary from 0.01% to 0.12% [7]. The lack of accurate, reproducible and easily applied methods for assessment of hepatic fibrosis has been the major limitation for both the clinical management and research in liver diseases [8]. Indirect markers include molecules released into the blood due to liver inflammation, molecules synthesized or excreted by the liver, and markers of processes commonly disrupted due to liver function impairment [9-11]. In the present study, we have developed a score based on four simple blood markers that can be easily used by clinicians to predict advanced liver fibrosis in CHC patients.

2. Patients and Methods

1. Samples

Blood samples

A total of 132 consecutive Egyptian individuals (93 males, 39 females; aged 20-60 years) with clinically and laboratory confirmed CHC were included in the present study; other causes of chronic liver disease were ruled out. They were recruited from the tropical Medicine unit, Mansoura University Hospitals, Mansoura, Egypt that approved

the present study. An informed consent was obtained from each individual participated in the present study and all were fully informed concerning the nature of the disease and the diagnostic procedures involved. No patient had received interferon treatment before liver biopsy and blood collection. Patients with reduced production of platelets other than hepatic infection with HCV such as infection of typhoid, deficiency of vitamin B_{12} and leukemia were excluded from the study. The HCV infection was diagnosed based on biochemical, serologic and histological criteria. None of the patients had history of habitual alcohol consumption or hepatocellular carcinoma. Moreover, all individuals were positive for anti-HCV antibody and were negative for hepatitis A and B viruses testing. All patients were negative test for anti-HIV antibodies. Blood samples were collected from all patients by vein-puncture within 2 weeks of liver biopsy and a part of the blood was treated immediately with EDTA-K_2 for platelets count. Sera were separated from the rest of blood samples and tested fresh for liver function indexes. Routine blood pictures including platelet counting were determined by KX-21 Sysmex automated hematology analyzer (Sysmex Corporation, Hyogo, Japan). Liver function tests were measured on an automated biochemistry analyzer (Hitachi 917; Roche Diagnostics, Mannheim, Germany). The AST/ALT ratio was calculated as [AST/ ALT]. The APRI was calculated as [AST to platelet ratio index].

Liver biopsy

Needle liver biopsy specimens (n = 132) were taken from all patients and examined by a pathologist unaware of the laboratory results. Biopsies were processed for diagnostic purposes. Fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4 µm thick, routinely stained with hematoxyline and eosin. The patients were pathologically classified into different stages of fibrosis and cirrhosis classified using liver biopsy staged from F0-F4 according to Metavir staging system [12]. The patients were pathologically classified into two groups: patients with no advanced liver fibrosis (F0-F2, considered as controls) and patients with advanced liver fibrosis (F3-F4).

Detection of Fibronectin in Serum using ELISA

Quantitation of fibronectin was determined according to **Attallah *et al.*,** [13]. In brief, 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. Serial concentrations of purified fibronectin (200–1600 mg/L) were tested in parallel to establish a dose-response curve as a function of the concentration (mg/L) in serum samples. After five washes with phosphate buffered saline-Tween 20 (PBS-T 20), the wells were blocked with 0.2% BSA in coating buffer. After five washes with PBS-T20, fifty µl of mouse monoclonal antibodies to fibronectin (ABC Diagnostics) were added separately per well at dilution 1:100 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 µl /well of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma), diluted 1:500 in 0.2% BSA in PBS-T20, were added. 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 a microtiter plate reader (S960, Metretech Inc, Germany).

Statistical analysis

All statistical analyses were done by a statistical software package (SPSS 15.0 for Microsoft Windows, SPSS Inc.). Descriptive results were expressed as mean ± SD and range or number (percentage) of patients with a condition. Differences in continuous variables were assessed using student t-test or ANOVA and X^2 test for categorical variables. All tests were two-tailed and statistical significance assessed at the 0.05 level. To assess and compare the diagnostic accuracy of biomarkers for discriminating those with advanced liver fibrosis from those non advanced liver fibrosis, we plotted receiver-operating characteristic (ROC) curves. The variables with $p < 0.05$ were analyzed by multiple logistic regressions to assess independent variables for predicting advanced liver fibrosis. The MDA is carried out stepwise with use of the minimum Wilk's lambda. The relative weighting of blood markers included in the discriminant model is designated by the standardized canonical discriminant coefficients. The sign (plus or minus) of which depicts whether there is a direct or inverse relation of the independent variables with the dependent variable (advanced liver fibrosis). However, the unstandardized coefficients of the final discriminant equation obviously also depend on the measurement units in which the individual analytes are expressed. A single model with the fewest variables and the greatest area under ROC was selected. Additionally, we assessed the accuracy of the algorithm using sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

3. Results

The laboratory bio-markers of 132 patients with CHC are summarized in table 1. Overall, 20 patients (15.2 %) had no liver fibrosis (F0), 42 patients (32 %) had mild liver fibrosis (F1),10 patients (7.6 %) had moderate liver fibrosis (F2), 19 patients (14.4 %) patients had severe liver fibrosis (F3), and 41 patients (31.1 %) had liver cirrhosis (F4). Baseline characteristics of patients were comparable for all parameters considered. The ANOVA test showed that only AST, AST/ALT ratio, albumin, platelet count, APRI and fibronectin were independent variables for prediction of advanced liver fibrosis. The mean ± SD of levels, of biomarkers in patients with advanced liver fibrosis stages are listed in tables 2. The ALT was then excluded from the subsequent analysis. A strong correlation was shown between APRI and platelet count (- 0.536; P< 0.0001) and a strong correlation was shown between AST and AST/ALT (0.543; P< 0.0001). Then APRI and AST activity were excluded from subsequent analysis.

Levels of serum biomarkers of CHC patients with different stages of liver fibrosis:

Using ROC curve, we assessed and compared the diagnostic accuracy of albumin, platelet count, AST/ALT ratio and APRI and fibronectin in patients with advanced liver fibrosis. The areas under the ROC curves for AST/ALT ratio, albumin, platelet count, and fibronectin were 0.58 (P < 0.09), 0.73 (P < 0.0001), 0.76 (P < 0.0001) and 0.74 (P < 0.0001); respectively (Figure 1).

Performance characteristics of MDA function

Using multivariate discriminant analysis (MDA), a function based on two markers (Fibronectin and platelet count), three markers (Fibronectin, platelet count and albumin) and four markers (platelet count, albumin, AST/ALT ratio and fibronectin) were calculated. The best combination of blood biomarkers was selected by MDA using the minimum Wilks lambada test. The MDA selected the simplified equation: Discriminate score = 7.917 (numerical constant) + 0.002 × fibronectin + 0.426 × AST/ALT ratio - 0.01× platelet count – 0.1835 × Albumin. We constructed ROC curve of this discriminant function, the area under the ROC curve and P value were 0.86 and P<0.0001; respectively (Figure 2 A). The median, mean ± SD of the combined markers in patients with non advanced fibrotic liver were -0.46, -0.58 ± 0.99 and in patients with advanced fibrotic liver were 0.74, 0.9 ± 1.11. Overall significance of differences among non advanced fibrotic and advanced fibrotic liver patients groups was determined by *T* test for the combined markers (P<0.0001), This score correctly classified 81 % of patients with advanced liver fibrosis at a discriminant cut-off score = 0.5 (i.e. less than 0.5 indicated non advanced fibrosis (F0-F1-F2) and greater than 0.5 indicated advanced liver fibrosis (F3-F4). The sensitivity, specificity and efficiency of this score were 81%, 72% and 75%; respectively(Fig. 2B) . The positive predictive and negative predictive values were 58.3 % and 89 %; respectively.

4. Discussion

In the present study, we conducted a meta-analysis with individual data aiming primarily at comprehensively characterizing the diagnostic accuracy of four blood tests of fibrosis including Albumin, platelet count, AST/ALT ratio and fibronectin in patients with chronic viral hepatitis C. The blood markers provide useful information on alternation in fibrogenesis and liver functions. Fibronectin is multifunctional high molecular weight glycoprotein and acute phase reactant present in the blood and in the extracellular matrix proteins of tissues. Increased amounts of fibronectin are patients with 72% specificity.

Table 1 : Comparison between laboratory biomarkers of all fibrosis stages of patients with CHC

Markers	F0 N= 20	F1 N= 42	F2 N= 10	F3 N= 19	F4 N= 41	P value[b]
AST (U/ml)	34.2 ± 5.5	49.3 ± 29.9	78.6 ± 33.6	66.4± 35.5	69.1± 40.8	0.001
ALT(U/ml)	35.1± 6.1	56.7± 29.9	84.8 ± 32.4	74.9 ± 38.2	57.6 ± 23.9	0.112
AST/ALT	0.99 ± 0.19	0.93 ± 0.41	0.94 ± 0.23	0.90 ± 0.19	1.4 ±1.2	0.032
Albumin (g/L)	41±1.6	40.5±2	39±0.8	40.1±2.9	38±2.9	<0.0001
Platelet count (× 10^9/L)	264 ± 56	199 ±59	194 ± 62	163 ± 42	156 ± 34	<0.0001
APRI[a]	0.34 ± 0.1	0.7 ± 0.43	1.14 ± 0.57	0.83 ± 0.29	1.14 ± 0.68	<0.0001
Fibronectin (µg/ml)	265 ± 143	342 ± 197	520 ± 303	605 ± 375	544 ± 274	<0.0001

Normal values: aspartate aminotransferase (AST) up to 40 U/ml; alanine aminotransferase (ALT) up to 45 U/ml; albumin 38-54 g/L, platelet count 150-400(× 10^9/L); fibronectin 250-400 mg/L according to Attallah et al.[13].

[a]APRI= [AST (U/L)/(40)]/[Platelet count 10^9/L] x 100.

[b]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.

Table 2. Comparison between laboratory biomarkers of patients with non advanced liver fibrosis (F0-F2) and patients with advanced liver fibrosis (F3-F4)

Markers	Non advanced (n = 72)	Advanced (n = 60)	P value
AST (U/ml)	49.2 ± 29.2	68.6 ± 39.2	<0.0001
ALT (U/ml)	54.6 ± 30	63 ± 30.2	0.16
AST/ALT ratio	0.95 ± 0.43	1.22 ± 0.99	0.009
Albumin (g/L)	40.4 ± 2.0	39 ± 3.1	<0.0001
Platelet count ×(10^9/L)	216 ± 66	158 ± 36	<0.0001
Apri*	0.66 ± 0.6	1.1 ± 0.6	<0.0001
Fibronectin (µg/ml)	345 ± 214	562 ± 309	<0.0001

Normal values: aspartate aminotransferase (AST) up to 40 U/ml; alanine aminotransferase (ALT) up to 45 U/ml; albumin 38-54 g/L, platelet count 150-400(× 10^9/L); fibronectin 250-400 mg/L according to Attallah et al.[13].

[a]APRI= [AST (U/L)/(40)]/[Platelet count 10^9/L] x 100.

[b]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.

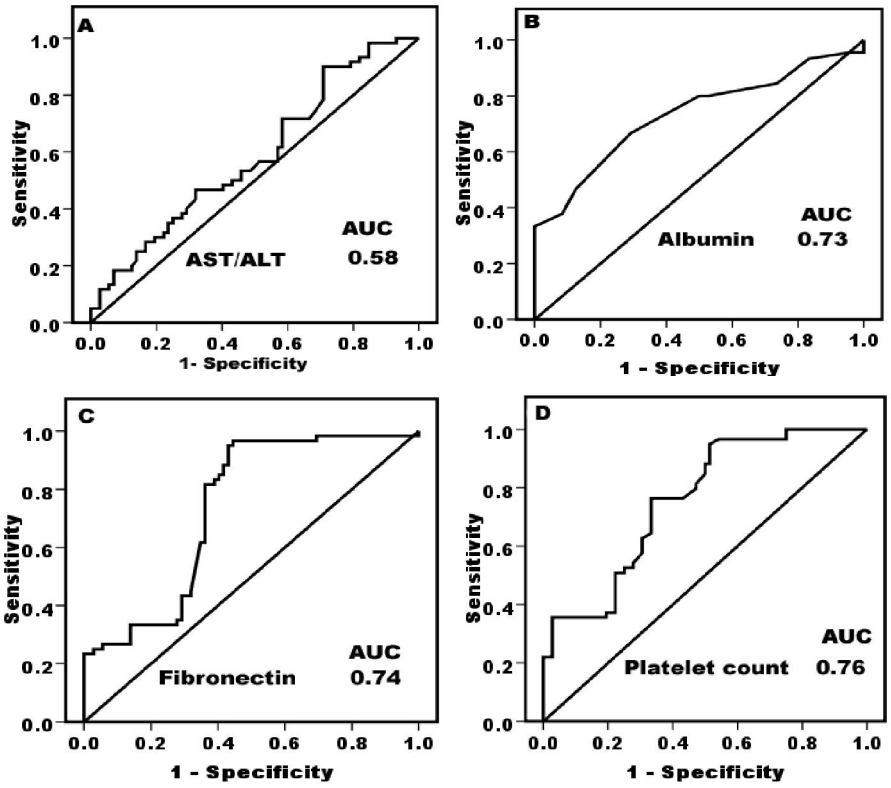

Figure 1. ROC curves of biomarkers for discriminating CHC patients with non advanced liver fibrosis (F0-F2) from those with advanced liver fibrosis (F3-F4). The area under the ROC curve (AUC) were 0.58, 0.73, 74 and 0.76 for AST/ALT, albumin, fibronectin and platelet count; respectively.

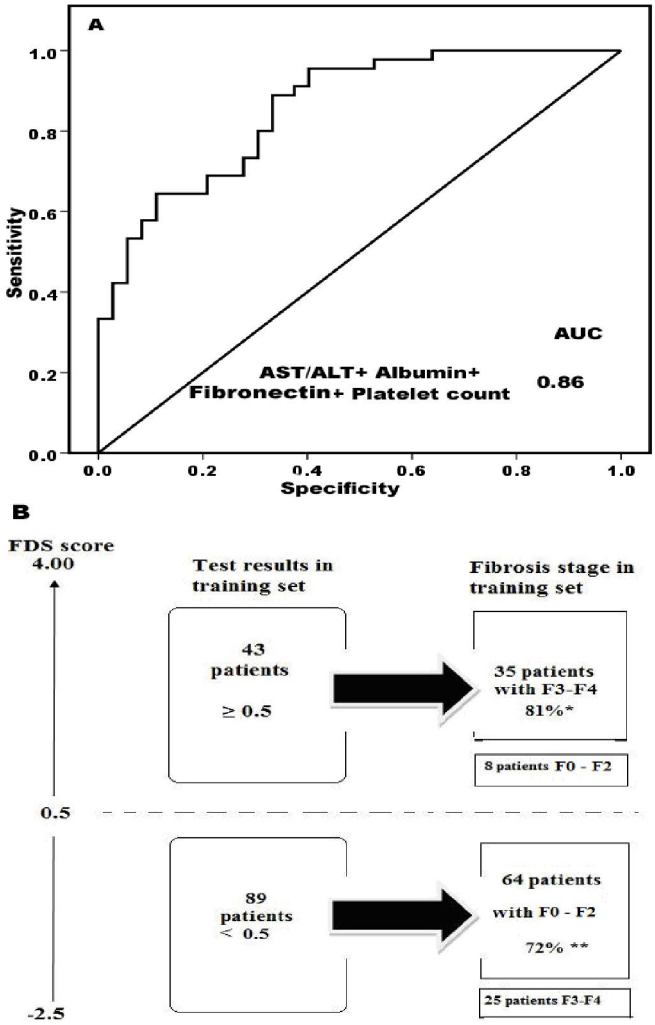

Figure 2. A. ROC curve of combined markers for discriminating CHC patients with non advanced liver fibrosis (F0-F2) from those with advanced liver fibrosis (F3-F4). The area under the ROC curve (AUC) was 0.86. **B.** Application of score to patients with CHC**.** The combined markers correctly classified 35 positive patients from 43 patients with 81% sensitivity. Also, the score classified 64 patients as negative from a total of 89

Significant in the development of early liver fibrosis and fibronectin may act as a chemotactic factor for collagen producing cells and as a skeleton for the new collagen formation [14]. Aspartate aminotransferase is a mitochondrial enzyme and HCV induced liver injury more extensively causes damage to mitochondria and increase in the AST level more than ALT and thus increasing AST/ALT ratio [15]. Also there is associated steatosis in patients with chronic hepatitis C which may raise AST levels [16]. Low platelet counts, thrombocytopenia, can be caused by a variety of reasons. In general, they can be divided into: decreased platelet production, increased platelet destruction or consumption, or increased splenic sequestration (capturing of circulating platelets in the spleen) [17]. A low serum albumin indicates poor liver function. The serum albumin concentration is usually normal in chronic liver disease until cirrhosis and significant liver damage has occurred. As the albumin level drops in liver disease, there is insufficient oncotic pressure to hold fluids within cells. Fluid moves into the interstitial spaces, causing generalized edema. The collection of fluid in the abdominal cavity, or ascites, may make it extremely difficult for the patient to breathe in a reclining position [18]. In the present study, The AUC of combined markers score based on AST-ALT ratio, albumin, fibronectin and platelets count was 0.86 for advanced liver fibrosis patients. The sensitivity, specificity, positive predictive and negative predictive values were 81%, 72%, 58.3 % and 89 %; respectively. The AUC of FibroTest for advanced fibrosis was 0.85 [19]. The FIB-4 score which

combines platelet count, ALT, AST and age, was originally developed for use in HIV-HCV co-infection. The FIB-4 score had the best diagnostic accuracy for advanced fibrosis (AUC= 0.86), followed by AST/ALT ratio (AUC= 0.83) [20]. The HepaScore based on age, gender, bilirubin, γ glutamyl transferase, hyaluronic acid, and γ2-macroglobulin. In chronic HCV patients, automated HepaScores showed good predictive performances for significant fibrosis (AUC=0.81), severe fibrosis (AUC=0.82), and cirrhosis (AUC=0.88) [21]. Serum index (HGM-3) dependent on levels of platelets, alkaline phosphatase, hepatic growth factor, tissue inhibitor of metalloproteinase-1 and hyaluronic acid. the presence of advanced liver fibrosis with 86.1% certainty [22]. The FIB-4 score which combines platelet count, ALT, AST and age, was originally developed for use in HIV-HCV co-infection. Use of this index correctly classified 87% of patients with FIB-4 values outside 1.45–3.25 and avoided biopsy in 71% of the validation set with an AUC of 0.765, sensitivity of 70% and a specificity of 97% for differentiating Ishak 0–3 from 4–6 [23]. The FibroIndex was developed by **Koda and co-authors** [24] for liver fibrosis in chronic hepatitis C. The test relies on platelet count, AST and serum IgG. FibroIndex showed high predictive values for significant fibrosis [25]. The sensitivity and specificity of FibroIndex for detecting fibrosis in patients with HCV were 78% and 74% [26]. In a comparative study, the validated AUROC of the FibroIndex for predicting significant fibrosis was found to be 0.83 and 0.82, which is better than those of the Forns index and APRI in patients with chronic hepatitis C [26, 8].

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Corresponding author
Ahmed A. Attallah
R & D Dept. Biotechnology Research Center, New Damietta City, Egypt.
ahmedattallah2009@hotmail.com

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