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ORIGINAL RESEARCH

A novel score for diagnosis of liver fibrosis based on Th17 activity and sera fibrosis biomarkers

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ABSTRACT

Background: Fibrosis is a major cause of morbidity and mortality worldwide. Biopsy is an invasive procedure. For the evaluation of liver fibrosis, an alternative, noninvasive, new, easy, and available method is determined to assess hepatic fibrosis in Egyptian patients.

Methodology: We enrolled 143 patients with fibrosis and they were divided into two groups, severe fibrosis and mild fibrosis group. Th17 cells were stimulated *in vitro with S. mansoni* soluble egg antigen (SEA), then interleukin (IL)-17, IL-22, and interferon (IFN)-γ were assessed in the culture supernatant. Serum levels of alanine aminotransferase, aspartate aminotransferase, YKL-40, and hyaluronic acid (HA) were also assessed using enzyme-linked immunosorbent assay. Analysis of individual data was conducted to characterize the diagnostic accuracy of the three highly significant fibrosis biomarkers (HA, IL-22, and IFN-γ).

Results: A highly significant difference in IL-22, IFN-γ, and HA levels between mild and severe groups was observed. Linear combination of the three biomarkers was selected by the multivariate discriminate analysis as the best combination for construction of the fibrosis discrimination equation. Application of the equation on all patients correctly classified 28.7% with severe fibrosis and 71.3% with mild fibrosis at a discriminant cut-off score (0.297) with 87.18% sensitivity and 85.57% specificity.

Conclusion: A simple fibrosis score = [0.594 (numerical constant) + 0.007 × HA (ng/ml) + 0.006 × IL-22 (ng/ml) − 0.129 × IFN-γ (pg/ml)] may be useful for discrimination of severe from mild fibrosis patients without the need of liver biopsy.

Keywords: Liver fibrosis, Th17 cells, sera fibrosis biomarkers.

Introduction

Egypt has the highest prevalence of hepatitis C virus (HCV) worldwide [1]. Coinfection of chronic HCV with schistosomiasis has been related to the rapid progress to fibrosis [2]. Fibrosis is a normal consequence of tissue injury and chronic inflammation characterized by the accumulation of excessive numbers of fibroblasts, deposition of extracellular matrix proteins such as collagen, and distortion of normal tissue architecture [3]. These changes lead to the destruction of normal tissue architecture and function loss [4]. Thus, fibrosis is a major cause of morbidity and mortality worldwide [5].

The degree of hepatic fibrosis is usually evaluated by liver biopsy [6]. However, liver biopsy is correlated with a high risk of morbidity, not accurate results, costly, invasive and requires well-trained physicians and pathologists to obtain adequate and representative results [4]. Therefore, there is a clinical need for a non-invasive method for diagnosing liver fibrosis which was our goal for this study.

A number of serological and urinary compounds such as procollagens, HA, laminin, and mediators of extracellular matrix production such as TGF-γ have been evaluated as noninvasive markers of liver fibrosis [7]. YKL-40 also known as human cartilage glycoprotein 39 or CHONDREX is an excellent marker for staging fibrosis in the liver and differentiates cirrhosis from chronic hepatitis with stages 1 and 2 fibrosis in patients with HCV [8].

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Th17 cells-associated cytokines could contribute to the hepatic granulomatous inflammation and subsequent fibrosis [9]. The functions of Th17 cells are mediated via the production of several cytokines including interleukin (IL)-17 and IL-22, and interferon-γ (IFN-γ) [10].

This study aimed to evaluate an alternative noninvasive, new, easy, and available method to assess hepatic fibrosis in Egyptian patients with *S. mansoni* infection or *S. mansoni*/HCV based on sera fibrosis biomarkers (HA and YKL-40 levels) and Th17 cells cytokines (IL17, IL22, and IFN-γ).

Subjects and Methods

In this study, 143 patients with fibrosis [91 males (mean age = 48.35 ± 12.6) and 52 females (mean age = 44.3 ± 7.5)] were enrolled between October 2012 and August 2015 from Kasr Alainy Viral Hepatitis Center, Faculty of Medicine, Cairo University, Egypt. Forty-seven healthy individuals were enrolled as controls [25 males (mean age = 43.3 ± 11.6) and 18 females (mean age = 35.8 ± 13)]. Twenty-seven patients were *S. mansoni* monoinfected and 116 were *S. mansoni*/HCV coinfected. The patients were diagnosed at Kasr Alainy Viral Hepatitis Center. The diagnosis was confirmed by the microscopic identification of *S. mansoni* ova in stool and fibrosis grade was diagnosed by biopsy specimens for HCV-infected patients or fibroscan for *S. mansoni*-infected patients. The study was approved by the ethical committee of Cairo University, Egypt, and all patients signed an informed consent.

Fifteen to twenty milliliter blood was collected from all patients and controls by vein-puncture in ethylenediaminetetraacetic acid coated vacutainers. Plasma was collected and stored at −80°C for future analysis.

Percutaneous liver biopsy specimens of *S. mansoni*/HCV patients were fixed in formalin and embedded in paraffin. Liver histopathology was assessed by two independent experienced micropathologists who were blinded to the clinical data. Histological scoring was performed according to the METAVIR scoring system.

The fibrosis is graded on a five-point scale from 0 to 4 where F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with few septa, F3 = numerous septa without cirrhosis, and F4 = cirrhosis. The amount of inflammation and the intensity of necro-inflammatory lesions is graded on a four-point scale from A0 to A3 where A0 = no activity, A1 = mild activity, A2 = moderate activity, and A3 = severe activity. According to the fibrosis stage, the enrolled subjects were subdivided into two groups: mild fibrosis group (F0–F2; $n = 79$) and severe fibrosis group (F3–F4; $n = 57$). Liver fibrosis of *S. mansoni* mono-infected patients was assessed using fibroscan.

Quantitative assessment of serum HA and YKL-40 levels was measured using human enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems; USA) and Booster Immunoleader (USA), respectively, according to the manufacturer's instructions.Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were measured using colorimetric kits (Bio-diagnostic, Egypt) according to the manufacturer's instructions.

$CD4^+$ $IL\text{-}23R^+$ cells were isolated from peripheral blood mononuclear cells using immunomagnetic beads and expanded *in vitro* as described by [10]. One hundred microliters of isolated cells (1×10^5) in complete RPMI culture medium (Lonza) with 10% fetal calf serum (Hyclone, USA), 1% of [penicillin streptomycin, L-glutamine and Sodium pyruvate (Sigma, Atdrich, USA)] were stimulated overnight with 100 ng/ml (as optimized) of *S. mansoni* SEA, (Theodore Bilharz Research Institute, Imbaba, Egypt). 240 ng/ml phytoheamaglutinine (BIOCHROM) was used as a positive control [11]. Cells were cultured at 37°C and 5% CO_2 incubator, and supernatants were collected and stored at −80°C for the future measurement of cytokine levels.

Supernatant levels of IL-17, IL-22, and IFN-γ were measured using ELISA kits (Booster Immunoleader, USA), according to the manufacturer's instructions.

Statistical analysis was performed by paired Student's *t* test using Graph Pad Prism software for analysis of Th17 cytokines and fibrosis biomarkers. MedCal version 15.2. was used to assess and compare the diagnostic accuracy of liver fibrosis biomarkers. Receiver-operating characteristic (ROC) curves were plotted for each variable. The variables within $p < 0.05$ were further analyzed by multiple logistic regressions to assess independent variables for predicting advanced liver fibrosis. Multivariate discriminate analysis (MDA) was used to construct an equation comprising the most significant biomarkers for discriminating patients with mild fibrosis from patients with severe fibrosis using the minimum Wilks lambda test.

Results

Table 1 shows the distribution of the study population according to age, sex, and fibrosis stage. Additionally, levels of ALT, AST, and ALP among controls and patients are also tabulated. ALT and ALP levels were significantly high ($p < 0.05$) in mild fibrosis females as compared to their corresponding values of control females but showed non-significant difference between males (Figure 1). A significant difference ($p < 0.05$) in ALT, AST, and ALP levels was also observed between males and females of severe and mild fibrosis patients (Figure 1).

There was a highly significant difference ($p < 0.001$) in IL-22 and IFN-γ levels secreted by Th17 cells between mild and severe groups. However, IL-17 did not show any significant difference between both groups (Figure 2).

There was a highly significant difference ($p < 0.001$) in HA level between mild and severe groups. However,

Table 1. *Demographic data and liver function tests of the enrolled fibrotic patients and control subjects.*

	Controls		Severe fibrosis		Mild fibrosis	
	Females (*n* = 18)	**Males (*n* = 25)**	**Females (*n* = 18)**	**Males (*n* = 39)**	**Females (*n* = 21)**	**Males (*n* = 58)**
Age (%)	43.3 ± 11.6 (41.8%)	35.8 ± 13 (58.2%)	47 ± 9 (31.6%)	46 ± 7.6 (68.4%)	41 ± 15 (26.6%)	42.6 ± 16 (73.4%)
ALT (U/L)	22 ± 10	22 ± 6.4	42.6 ± 20*[a]	40.5 ± 16*[a]	29.8 ± 17*	19 ± 7.4
AST (U/L)	19.7 ± 3.8	19.6 ± 8.5	35.7 ± 17*[a]	38 ± 21.7*[a]	25.57 ± 10	24.5 ± 10.7
ALP (U/L)	51.8 ± 26.5	52.3 ± 17.7	135.8 ± 85*[a]	107 ± 43*[a]	81.48 ± 38*	53.5 ± 16.6

Data are presented as mean ± standard deviation.
*Significant difference as compared to controls at $p < 0.05$.
[a]Significant difference as compared to mild fibrosis group at $p < 0.05$.

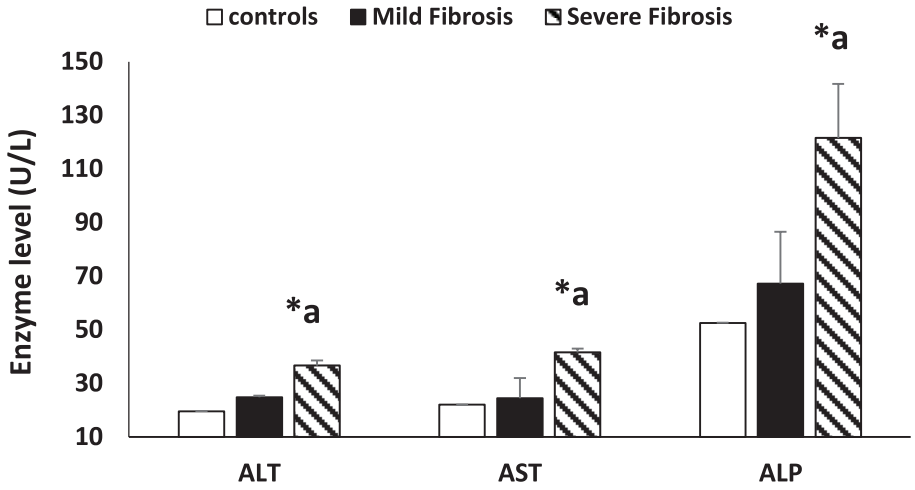

Figure 1. *Plasma levels of ALT, AST, and ALP among control subjects and fibrotic patients. *a: Significant difference as compared to controls at p < 0.05.*

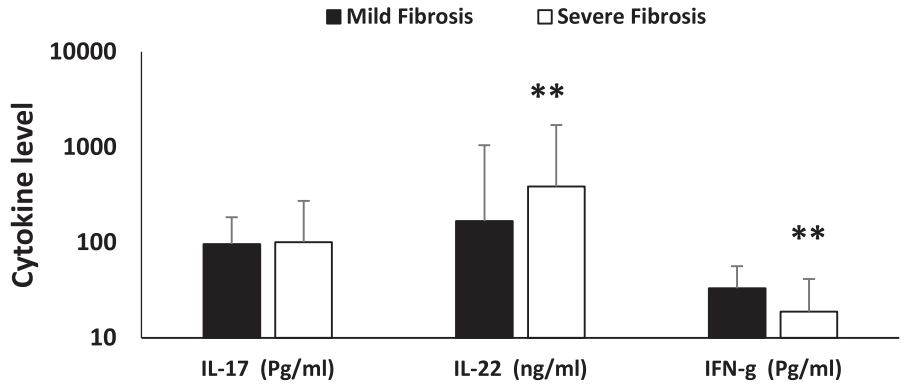

Figure 2. *Cytokines level produced by Th17 cells isolated from fibrotic patients in response to SEA stimulation in vitro. **Significant difference as compared to mild fibrosis group at p < 0.001. *Significant difference as compared to controls at p < 0.05.*

YKL-40 showed a slightly significant difference ($p < 0.05$) between both groups (Figure 3).

Patients were divided into two groups: severe fibrosis group (F3–F4) comprising 57 patients (42%) and mild fibrosis group (F0–F2) comprising 79 patients (58%). Using the ROC curve, the diagnostic accuracy of HA, IL-22, and IFN-γ was assessed individually in patients with advanced liver fibrosis and those with mild fibrosis.

The areas under the ROC curves were 0.762, 0.783, and 0.839, respectively (Figure 4).

Combinations of the significant biomarkers (IL-22 + HA, IFN-γ + HA, IL-22 + IFN-γ, and IL-22 + IFN-γ + HA) were analyzed to examine their diagnostic power. Table 2 summarizes the statistical results when the linear combination of biomarkers was analyzed by MDA using the minimum Wilks lambda test. The MDA selected a simplified equation from the three biomarkers: fibrosis discrimination score = [0.594 (numerical constant) + 0.007 × HA (ng/ml) + 0.006 × IL-22 (ng/ml) − 0.129 × IFN-γ (pg/ml)].

Linear combination of the three biomarkers (HA, IL-22, and IFN-γ) was selected by MDA as the best combination for construction of the fibrosis discrimination score equation for discriminating patients with mild liver fibrosis (F0–F2) from those with severe liver fibrosis (F3–F4). The areas under the ROC curves of all combinations (IL-22 + HA, IFN-γ + HA, IL-22 + IFN-γ and IL-22 + IFN-γ + HA) were 0.820, 0.878, 0.891, and 0.906, respectively (Figure 5).

The fibrosis discriminant equation correctly classified 28.7% of patients with severe liver fibrosis and 71.3% with mild fibrosis at a discriminant cut-off score (0.297) with 87.18% sensitivity, 85.57% specificity, and 81% efficiency. The positive and negative predictive values (PVs) were 70.8% and 94.3%, respectively, (Table 3). The ROC curve of this discriminate function gave area under the ROC curve equal 1.

Discussion

Fibrosis is the major cause of mortality and morbidity related to both schistosomiasis and HCV [12]. The current study aimed to evaluate sera fibrosis biomarkers that could be used to predict the severity of liver fibrosis in *S. mansoni* infection and *S. mansoni*/HCV co-infections. Plasma levels of ALT, AST, YKL-40, and HA were assessed.

Results of the current study revealed that ALT and ALP levels were significant in mild fibrotic females as compared to their corresponding values of control females but showed non-significant difference between males. In addition, significant differences in ALT, AST, and ALP levels of severe fibrosis patients (males and females) were observed as compared to those of control subjects or mild fibrosis patients. Previous studies

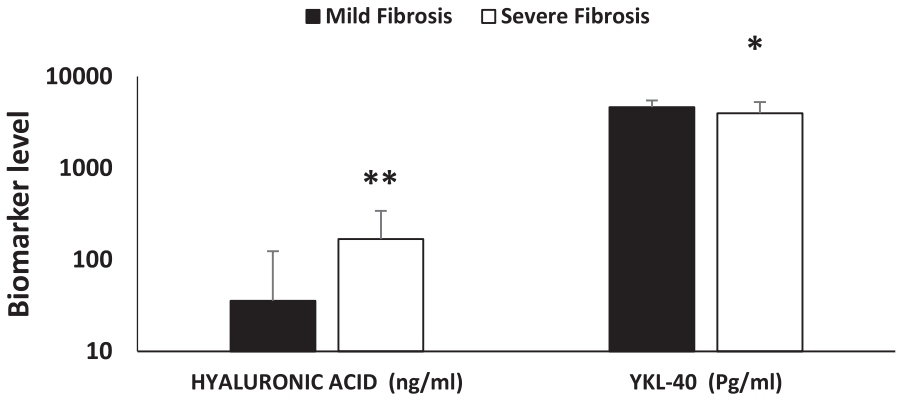

Figure 3. *Level of sera fibrosis biomarker HA and YKL-40 among control subjects and fibrotic patients. *Significant difference as compared to mild fibrosis group at p < 0.05. **Significant difference as compared to mild fibrosis group at p < 0.001.*

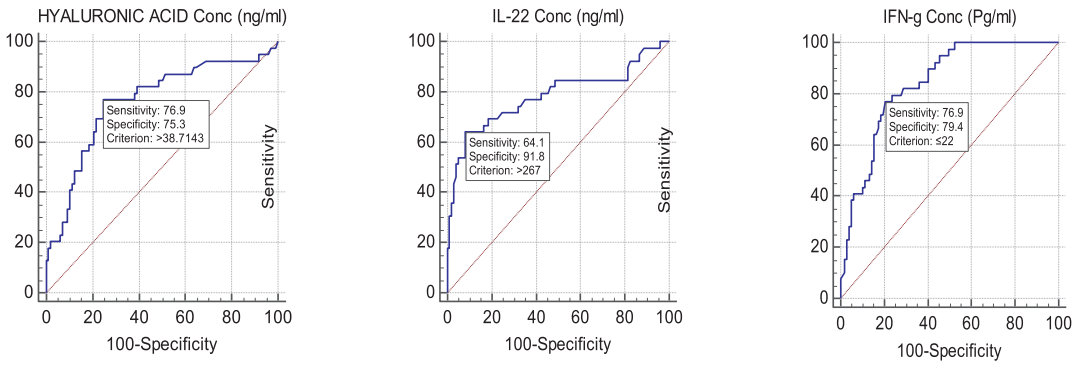

Figure 4. *ROC curves of individual biomarkers; HA, IL-22 and IFN-γ for discriminating patients with severe liver fibrosis from patients with mild liver fibrosis. The areas under curves were 0.762, 0.783, and 0.839, respectively.*

Table 2. *Multiple logistic regression model for fibrosis discrimination score based on absolute values of three biomarkers (HA, IFN-γ, and IL-22) for discriminating patients with severe liver fibrosis (F3–F4) from mild liver fibrosis (F0–F2).*

Variable	Coefficients	*P* value	SE	AUC	(95% CI)	OR
HA	0.007	0.0519	0.0034	0.762	0.681–0.831	1.0067
IFN-g	−0.129	0.0002	0.0341	0.839	0.766–0.896	0.8794
IL-22	0.006	0.0013	0.0018	0.783	0.705–0.849	1.0060

AUC, area under curve; CI, confidence interval; SE, standard error; OR, odds ratio.

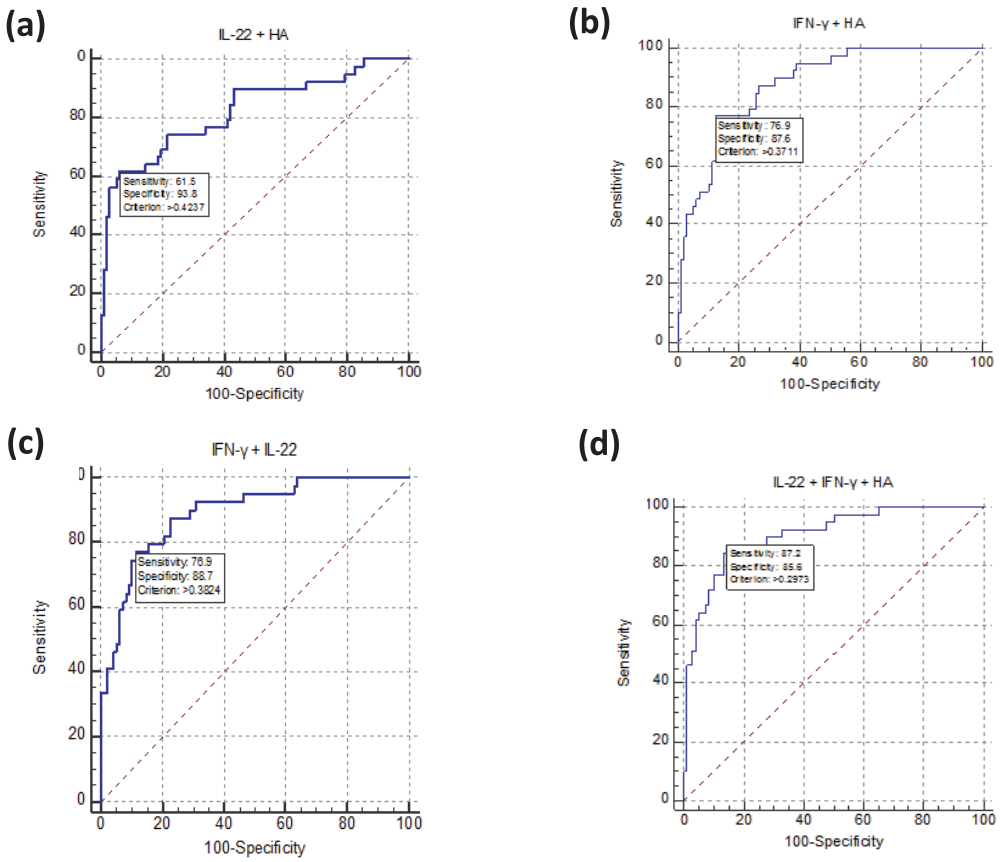

Figure 5. *ROC curves of four biomarkers combinations for discriminating patients with severe liver fibrosis from those with mild liver fibrosis; HA with IL-22 (a), HA with IFN-γ (b), IFN-γ with IL-22 (c) and HA with IFN-γ with IL-22 (d). The areas under curves were 0.820, 0.878, 0.891, and 0.906, respectively.*

Table 3. *Characteristics of the fibrosis discriminant score performance for discriminating patients with severe liver fibrosis (F3–F4) from patients with mild fibrosis (F0–F2).*

MDA	Performance characteristics				
Cut-off score	Sensitivity	Specificity	+ve PV	−ve PV	Efficiency
0.170	89.74	72.16	56.5	94.6	78.3
0.183	87.18	72.16	55.7	93.3	78.3
0.297*	87.18	85.57	70.8	94.3	81
0.298	84.62	85.57	70.2	93.3	80.4
0.303	84.62	86.60	71.7	93.3	80.4

*Selected as a discriminant score.

reported that conventional liver function tests as ALT and AST. Although reflect hepatocyte damage, they do not assess the activity of the fibrogenic process [13]. So, these enzymes were excluded from MDA analysis.

The diagnostic accuracy of serum HA level increases gradually with hepatic fibrosis and it is considered a simple non-invasive index for the diagnosis of fibrosis [14]. Current results report that there was a highly significant difference in HA level between mild and severe groups. However, YKL-40 showed a slight significant difference between both groups. So HA was included but not YKL-40 in MDA analysis. Data about the role of YKL-40 as a fibrosis biomarker is controversial [15].

HA alone is used to differentiate stages of liver fibrosis in sera of patients co-infected with HCV and human immunodeficiency virus [14]. Also, a previous study revealed that, HA, its degrading enzymes and its degradation products were scored with each other and efficiently classified patients with chronic hepatitis C into cirrhotic or non-cirrhotic liver disease using MDA score [16].

Th17 cells are increased in the liver and the serum of patients with various forms of acute and chronic liver injury [17]. In the current study, Th17 cells were isolated from patients with different degrees of fibrosis and stimulated *in vitro* with SEA, then IL-17, IL-22, and IFN-γ were assessed in the culture supernatant. Results revealed that there was a high significant difference in IL-22 and IFN-γ levels between mild and severe groups. However, IL-17 did not show any significant difference between both groups. The results of this study are in contrast to all previous data [17–19]. However, most previous data are cytokines in the plasma not in the supernatants of SEA activated Th-17 *in vitro*.

A recent study suggested that Th17 cells were involved in the pathological tissue remodeling in liver fibrosis induced by schistosomiasis [20]. IL-17 has proinflammatory activities through stimulating both keratinocytes and hematopoietic stem cells to produce IL-6, TNF-γ, and TGF-γ through activation of STAT3 in mice [19].

IL-22 has been demonstrated to protect against T-cell-mediated hepatitis and lack of Th-17 cells producing IL-22 might exacerbate liver injury [21]. It inhibits liver fibrosis by inducing hematopoietic stem cell senescence in a STAT3-p53-p21-dependent manner [18].

Several studies have investigated simple non-invasive methods for prediction of fibrosis [22] and many models constituting routine parameters were used such as HGM-1 and HGM-2 indexes [23], Forns [24], and AST-to-platelets ratio index (APRI) [25]. Those scores have been assessed as substitutes for liver biopsy. In this MDA analysis, IL-22 and IFN-γ with HA were included but not IL-17 since it was not significant.

In this study, 42% of the patients had severe fibrosis and 58% had mild fibrosis. A meta-analysis with individual data was conducted to characterize the diagnostic accuracy of HA, IL-22, and IFN-γ as fibrosis biomarkers. The ROC curve of each individual marker was analyzed and the areas under the ROC curves were 0.762, 0.783, and 0.839, respectively. So, no single biomarker has an ideal area under the curve.

To address the potential, the diagnostic powers for different combinations of significant biomarkers (IL-22 + HA, IFN-γ + HA, IL-22 + IFN-γ and IL-22 + IFN-γ + HA) were assessed using logistic regression analysis. Linear combination of the three biomarkers (HA, IL-22, and IFN-γ) was selected by the MDA as the best combination for construction of the fibrosis discrimination score equation for discriminating patients with mild liver fibrosis (F0–F2) from those with severe liver fibrosis (F3–F4). The areas under the ROC curves of all combinations were 0.820, 0.878, 0.891, and 0.906, respectively.

The score was reconstructed combining the most significant markers identified and MDA selected the simplified equation from the three biomarkers: fibrosis discrimination score = [0.594 (numerical constant) + 0.007 × HA (ng/ml) + 0.006 × IL-22 (ng/ml) − 0.129 × IFN-γ (pg/ml)].

When the fibrosis discriminant equation derived from MDA was applied to all patients, it correctly classified 28.7% of patients with severe liver fibrosis and 71.3% with mild fibrosis at a discriminant cut-off score (0.297). Less than 0.297 score indicates mild liver fibrosis and greater than this value indicates severe liver fibrosis, with 87.18% sensitivity, 85.57% specificity, and 81% efficiency. The positive and negative PVs were 70.8% and 94.3%, respectively. The ROC curve of this discriminate function gave area under the ROC curve equal 1.

Tangkijvanich et al. [26] identified a cut-off point discriminated between liver cirrhosis, chronic hepatitis, hepatocellular carcinoma, and healthy controls with sensitivity, specificity, and accuracy of 82.4%, 78.2%, and 80.2% respectively. Lackner et al. [27] validated and compared the diagnostic accuracies of the simple fibro tests including cirrhosis discriminate score and platelets count (AP) index, APRI, and platelets count. ROC curves of these simple fibro tests showed comparable diagnostic accuracies for prediction of significant fibrosis (0.71, 0.74, 0.80, and 0.71, respectively). The current MDA could enable us to accurately predict the presence of severe liver fibrosis in fibrotic patients. The area under the ROC curve of our combination was similar to other reports [28].

Conclusion

A simple fibrosis score with a high predictive identification of the progression of the fibrosis process can be useful for discrimination of severe from mild fibrosis patients without the need of liver biopsy. Three biochemical markers condensed in a single MDA value for each patient can provide sufficient information for prediction of the liver fibrosis stage.

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List of Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ELISA	Enzyme-linked immunosorbent assay
HA	Hyaluronic acid
HCV	Hepatitis C virus
IFN	Interferon
IL	Interleukin
MDA	Multivariate discriminate analysis
ROC	Receiver-operating characteristic

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Conflict of Interests
None

Ethical approval
The study was approved by the ethical committee of Cairo University, Egypt.

Consent for publication
Written consent was obtained from the patients.

Authors' contribution
All the authors have made substantial contributions to (1) the conception and design or analysis and interpretation of the data, (2) the drafting of the manuscript or critical revision for important intellectual content, and (3) final approval of the version to be published. All authors attest that they meet these authorship criteria and that none of them had received any assistance in writing this manuscript.

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