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The Use of Collagen IV for Noninvasive Follow-up of Liver Fibrosis After Successful DAA Treatment of HCV-Infected Egyptians

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Running title: Collagen IV for Follow-up of Liver Fibrosis After DAA Treatment

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Abstract

The use of noninvasive surrogate blood biomarkers for the assessment of liver fibrosis has gained increasing interest. In this study, 88 chronic hepatitis C patients (aged 21–68 years) were assessed for liver fibrosis using Collagen type IV (Col-IV), both before and one year after the end of successful DAA therapy for HCV infection. FibroScan was used to assess the degree of liver fibrosis. Using sandwich ELISA, the serum level (ng/mL) of Col-IV was determined. SPSS package was used to statistically examine the data. Before therapy, there was a strong association ($r = 0.704$; $P < 0.0001$) between the degree of fibrosis and the amount of Col IV. Furthermore, ROC curve analyses demonstrated that, when compared to a panel of routine indicators, Col-IV could distinguish individuals with significant fibrosis (F2-F4) and non-significant fibrosis (F0- F1) with a high degree of efficiency (95%). In 6% of treated patients, the degree of fibrosis regressed, remained unchanged in 75%, and progressed in 19%. It is noteworthy that patients experiencing regression of fibrosis had significantly lower mean levels of Col-IV (30.60 ± 4.23 vs. 22.40 ± 4.71; $P < 0.001$), while patients with stationary fibrosis did not significantly change (26.21 ± 1.81 vs. 26.68 ± 1.77; $P > 0.05$) and patients experiencing progression of fibrosis had significantly higher mean levels (32.35 ± 3.71 vs. 46.35 ± 3.09; $P < 0.001$). In conclusion, Col-IV has the potential to be a highly effective noninvasive marker for determining the extent of liver fibrosis both before and after the eradication of HCV infection.

Key Words: Liver Fibrosis, HCV, DAA, Biomarker, Collagen IV.

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1. Introduction:

About 71 million people worldwide suffer from chronic hepatitis, with the hepatitis C virus (HCV) being one of the main causes (1,2). Hepatic fibrosis is the final common pathway that most chronic disorders of the liver, such as hepatitis C, develop to (3,4). From 2016 to 2030, the World Health Organization's worldwide hepatitis plan seeks to lower hepatitis mortality by 65% and newly acquired infections by 90% (5,6). The field of HCV treatment has changed dramatically in the last few years, culminating in the advent of direct-acting antiviral (DAAs) therapy, which has been shown to cure over 90 percent of patients with chronic infection (7). With almost 10% of people aged 15 to 59 having persistent HCV infection, Egypt had the highest rate of HCV infection worldwide (8,9). But now, Egypt is on the verge of eradicating the infection (10). The incidence of new infections decreased from 300 per 100,000 in 2014 to 9 per 100,000 in 2022 as a result of DAAs treating over 4 million Egyptians (11–14). By 2023, Egypt has made significant strides in diagnosing and treating HCV patients, surpassing WHO targets (15, 16). The assessment of fibrosis produces a plethora of essential data for making therapeutic decisions, monitoring the course of the disease naturally, and determining a diagnosis and prognosis (17). The most widely utilized reference gold method for diagnosing liver fibrosis is Liver biopsy [18]. However, a liver biopsy is an invasive surgery with a significantly high risk of side effects, ranging from minor ones like discomfort and transient hypotension to major ones like severe hemorrhage that may be lethal (19). Owing to these limitations, non-invasive techniques have been developed to assess hepatic fibrosis severity and existence (20). The vast bulk of research has looked into the usefulness of blood markers for liver fibrosis in diagnosing cases (21). Depending on their connection to the connective tissue, direct and indirect indicators of fibrosis make up liver fibrosis (22). Indirect markers include substances released into the bloodstream owing to liver inflammation, substances produced, controlled, or expelled by the liver, and indicators of processes that are frequently disturbed by a reduction in liver function (23-27). Direct markers are molecules that show the degree of fibrogenesis or fibrinolysis and are pieces of the extracellular matrix (ECM) that are created throughout the fibrotic pathway. Direct biomarkers have developed in line with our understanding of the complicated cellular and molecular underpinnings of fibrogenesis over the past few decades (28). The current investigation sought to measure serum levels of direct biomarker Collagen type IV (Col-IV) in CHC patients with different degrees of liver fibrosis before and one year after successful DAA treatment of HCV infection.

2. Materials and Methods

2.1. Patient characteristics and DAA treatment protocol.

This study included 88 Egyptian people (45 women and 43 men, ages 21 to 68 years) with chronic HCV infection who received Sofosobuvir-based DAA treatment following Egyptian national treatment guidelines for the treatment of genotype 4 CHC infection at Viral Hepatitis Center verified by tests and clinical, One-day Surgeries Hospital, Samalout City and Bani Mazar City, Minia, Egypt. The DAA treatment regimen was a daily combination of Daclatasvir (60 mg) with Sofosobuvir (400 mg) for 12 weeks. At a 12-week follow-up, undetectable HCV-RNA using a quantitative RT-PCR assay was used to define sustained virological response (SVR12). Furthermore, as controls, 20 age-matched healthy individuals (9 men and 11 women) were used. The inclusion criteria comprised aged 20-70 years, treatment-naïve patients with CHC, HCV-RNA positivity before DAA treatment, and HCV-RNA negativity one year after the end of DAA treatment, provide blood samples at the specified date. Total bilirubin greater than 3 mg/dL, serum albumin less than 2.8 g/dL, INR less than 1.7, platelets count less than 50,000/mm^3, pregnancy, uncontrolled diabetes,

HbA1C greater than 9%, decompensated liver cirrhosis, ascites, encephalopathy, variceal hemorrhage, and patient with untreated HCC were among the exclusion criteria. The Ethics Committee of the Ministry of Health and Population in Cairo, Egypt, approved the study, which was conducted following the Declaration of Helsinki's ethical guidelines.

2.2. Abdominal Imaging and FibroScan:

Abdominal and pelvic ultrasonography was done before starting medication, to evaluate the signs of liver cirrhosis, hepatic decompensation, portal hypertension, and fatty liver. FibroScan 502 (Echosens, Paris, France) was used to do transient elastography and to identify the stage of liver fibrosis before and one year after the end of HCV therapy for all 88 CHC patients. A section of the liver devoid of major vessels and with a thickness of at least 60 mm was selected for analysis. Different stages of liver fibrosis were classified using the following FibroScan cut-off values: F0 < 5 kPa; 5 ≤ F1 ≤ 7 kPa; 7 kPa < F2 < 10.2 kPa; 10.2 kPa ≤ F3 < 16.3 kPa; F4 > 16.3 kPa (29, 30). As a result, the fibrosis stage F0 was identified in 19 patients (17.6%), F1 in 21 (19.4%), F2 in 20 (18.5%), F3 in 17 (15.7%), and F4 in 11 patients (10.2%). Following therapy, the terms "fibrosis regression" and "fibrosis stationary" were used to describe different types of fibrosis: one-stage reduction in fibrosis (such as F3 to F2 ≤ 10.2 kPa), "fibrosis stationary" (such as F3 to F3 (10.2 kPa < F3 ≤ 16.3 kPa), and "fibrosis progression" (such as F2 to F3 ≤ 16.3 kPa) (30, 31).

2.3. Blood samples and traditional hematological, biochemical, and virologic assessment:

Ten mL of venous blood was obtained from each patient or control subject and divided into three parts. For all 88 CHC patients, routine laboratory evaluations were conducted before the initiation of DAA medication, during treatment, and a year after treatment concluded. A portion of the blood sample that had been treated with EDTA was subjected to a complete blood count (CBC), which included the platelet count, using the KX-21 Sysmex Automated Hematology Analyzer (Sysmex Corporation, Kobe, Japan). Another part of the blood was treated with a citrate buffer in preparation for the prothrombin-INR (International Normalized Ratio) test. After allowing the remaining blood sample to coagulate for thirty minutes, it was centrifuged for ten to twenty minutes at room temperature. Using a pipette, the serum was extracted from the clot and transferred into a sterile tube which was centrifuged for 20 minutes at a speed of 2000–3000 rpm. Aliquoted serum was kept at -80°C until it was time for analysis, labeled, and placed in cryovial tubes. As directed by the manufacturer (BioMerieux, Marcy, France), liver enzymes (ALT and AST), alkaline phosphatase, albumin, and creatinine Following the manufacturer's instructions, the Alpha-Fetoprotein (AFP) tumor marker was examined using an ELISA kit (Chemux Bioscience Inc., USA). Every patient had a negative HBsAg test result (Dia.Pro, Milan, Italy). A positive test result for anti-HCV antibodies served as the basis for the diagnosis of HCV infection (Axiom Diagnostics, Worms, Germany). Then, as will be described below, a real-time polymerase chain reaction (RT-PCR) was used to confirm the existence of HCV-RNA.

2.4. Detection of HCV RNA using qRT-PCR:

Quantitative determination of HCV-RNA was performed before starting DAA therapy then at 12 weeks after therapy completion (SVR12) and one-year after end of treatment protocol using Cobas AmpliPrep/Cobas TaqMan HCV Test (V2.0, Roche Molecular Systems, The Netherlands) with lower detection limit of 15 IU/mL (Cutoff level) and a linear range of HCV-RNA amplification from approximately 15 to 10×10^6 IU/ mL.

2.5. Quantitative Determination of Col-IV in Human Serum using ELISA:

The concentration of Col-IV in the serum was measured quantitatively using a commercial sandwich-ELISA kit according to the manufacturer's instruction (Elabscience Biotechnology Inc.,

Houston, Texas, 77079, USA). In brief, serum samples were added to the pre-coated micro ELISA plate with an antibody specific to Human Col-IV (100 □L for each well) and incubated for 90 min at 37 °C. Next, each microplate well receives 100 □L of the biotinylated detection antibody specific for Human Col-IV and the Avidin-Horseradish Peroxidase (HRP) conjugate. Free parts are removed by washing. Substance solution (90 µL) is poured to each well and incubated at 37 °C, shielded from light, for approximately 15 minutes. The addition of 50 µL/well of stop solution ends the enzyme-substrate reaction, and the optical density (OD) is immediately measured using an automatic ELISA reader (Biochrom EZ Read 400 Microplate Reader, Cambridge, UK) set to 450 nm. A standard curve representing the relationship between the concentration (ng/mL) of serum samples and serial concentrations of Col-4 (0–50 ng/mL) was established through parallel testing. By comparing the sample's optical density (OD) to the standard curve, the concentration of circulating Col-IV in the investigated samples was determined. The mean ELISA OD ± 3 SD of 32 sera from healthy controls was used to compute the ELISA cutoff level, which was set at 10 ng/mL. Above or below this level, the tested sample is either positive or negative.

2.6. Statistical analyses

With the statistical analysis software program "SPSS 15 for Microsoft Windows, SPSS Inc.," all data were statistically examined, and a two-sided $P < 0.05$ was considered statistically significant. Using the Kolmogorov-Smirnov test, the analyzed markers' data's normality was initially evaluated. The continuous variables were presented as mean and standard errors (SE), as well as the median and interquartile range (IQR). The data was presented using box charts. Mann-Whitney for quantitative comparisons, the U test was employed, and for quantitative data, the Wilcoxon. The correlations between the biomarkers were assessed using Spearman's correlation coefficient. Receiver operating characteristic (ROC) curve analysis was employed to evaluate the diagnostic value of the markers. For any ROC curve, the probability that a randomly chosen case would have a higher marker value than a randomly chosen control ranges from 0.5 (for a non-informative marker) to 1 (for a perfect marker). This is known as the area under the ROC curve, or AUROC. Additionally, using the FibroScan as a reference for the degree of liver fibrosis in our study, the performance characteristics of the Col-IV-ELISA test, such as sensitivity, specificity, and efficiency, were computed from the 2 x 2 table.

3. Results

3.1. Clinicopathological characteristics of study patients:

Out of the 88 CHC patients, 17.6% (n = 19) had no fibrosis (F0), 19.4% (n = 21) had mild fibrosis (F1), 18.5% (n = 20) had moderate fibrosis (F2), 15.7% (n = 17) had advanced fibrosis (F3), and 10.2% (n = 11) had cirrhosis (F4), based on the FibroScan for fibrosis score classification. Then, CHC illness was divided into two collectives: The non-significant fibrosis group (F0-F1, n = 40; 17 males & 23 females) and the Significant fibrosis group (F2-F4, n = 48; 26 males & 22 females)**.** The incidence rates of liver fibrosis increase sharply with age from 35 to 58 and are rare before the age of 20 years, the incidence rates of liver fibrosis decrease by ages 60 to 70. The baseline levels of the investigated laboratory markers of 88 CHC illnesses are shown in **Table 1**. The F2-F4 patients showed significantly higher levels of ALT, AST, INR, and AFP ($P < 0.05$) and significantly lower levels of ALB, and Platelets ($P < 0.05$) than the F0-F1 patients. No significant differences were shown between levels of total Bilirubin, Creatinine, Hb, and HCV-RNA of the two groups ($P > 0.05$).

Table 1. Baseline levels of investigated traditional markers for CHC patients with non-significant fibrosis (F0-F1) and of CHC patients with significant fibrosis (F2-F4).

Indirect markers of liver fibrosis**	Marker level expressed as Median (IQR)		
	F0-F1 (n = 40)	F2-F4 (n = 48)	P value*
Biochemical markers:			
ALT (U/L)	45 (7-105)	51.5 (13-160)	0.045
AST (U/L)	47 (8-113)	51.5 (16-142)	0.043
ALB (g/dL)	4.2 (3.1-5.1)	3.75 (2.4-4.9)	0.0001
BIL-Total (mg/dL)	0.88 (0.2-1.9)	0.92 (0.45-5)	0.072
Creatinine (mg/dL)	0.9 (0.5-1.2)	0.9 (0.6-1.3)	0.052
AFP (ng/mL)	1.6 (0.9-3.17)	14.1 (1-90)	0.0001
HCV-RNA (IU/ mL) × 10^6	0.19 (0.02 - 6.2)	0.28 (0.01-32.8)	0.148
Hematological markers:			
PLt × 10^9/L	226.5 (115-337)	166 (80-240)	0.0001
INR	1.1 (0.88-1.2)	1.25 (1-1.6)	0.0001
Hb (g/dL)	13.3 (10.4-15.3)	12.6 (10.5-15.2)	0.068

* P > 0.05 is considered not significant; P < 0.05 considered significant; P < 0.001 considered very significant; P < 0.0001 is considered extremely significant.

**Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, Albumin; BIL, bilirubin; AFP, alpha fetoprotein; Plt; platelets, INR, international normalized ratio; Hb, haemoglobin.

3.2. Non-invasive assessment of Collagen IV in serum using sandwich ELISA:

The concentrations of Col-IV in sera of 88 CHC patients and 20 healthy controls were quantified by using sandwich ELISA and determined from the established standard calibration curve. The boxplot of Col-IV levels in different FibroScan degrees of fibrosis is shown in **Figure 1**. The Col-IV levels increased significantly (P < 0.001) with the increasing degree of fibrosis in the liver. A highly significant correlation was shown between Col-IV levels and degree of liver fibrosis (r = 0.704; P < 0.0001). However, no significant correlation was shown between serum Col-4 expression level and age as well as sex of study patients (P > 0.05). The AUROC curve was used to evaluate the diagnostic significance of the Col-IV concerning the other indirect markers of liver fibrosis that were tested (ALT, AST, ALB, Bil-T, Creatinine, Hb, INR, and PLt), as shown in Figure 2. An ideal test has an AUROC of 1.0, while a test with no diagnostic value has an AUROC of 0.5. A curve is a more valuable marker for a diagnosis the closer it moves to the upper left corner of the graph. As seen in **Figure 2**, the Col-IV had the highest AUROC for separating CHC patients with considerable fibrosis (F2-F4) from those with non-significant fibrosis (F0-F1). According to **Figure 3 (A, B)**, the AUROC of Col-IV was 0.981 (P < 0.0001) for differentiating CHC patients (F0-F4) from healthy individuals and was 0.889 (P < 0.0001) for differentiating patients with severe fibrosis of liver (F2-F4) from CHC patients with non-significant liver fibrosis (F0-F1). By applying the best cut-off level (10 ng/mL) above or below which the tested sample is considered positive or negative respectively, 85 out of 88 patients with different degrees of liver fibrosis showed true positive test results for Col-IV and 18 out of 20 of healthy individuals showing true negative test result. The performance characteristics of Col-IV detection using ELISA in comparison to FibroScan are shown in **Figure 4.** The detection of serum Col-IV using ELISA showed high degrees of sensitivity, specificity, and efficiency (> 90 %).

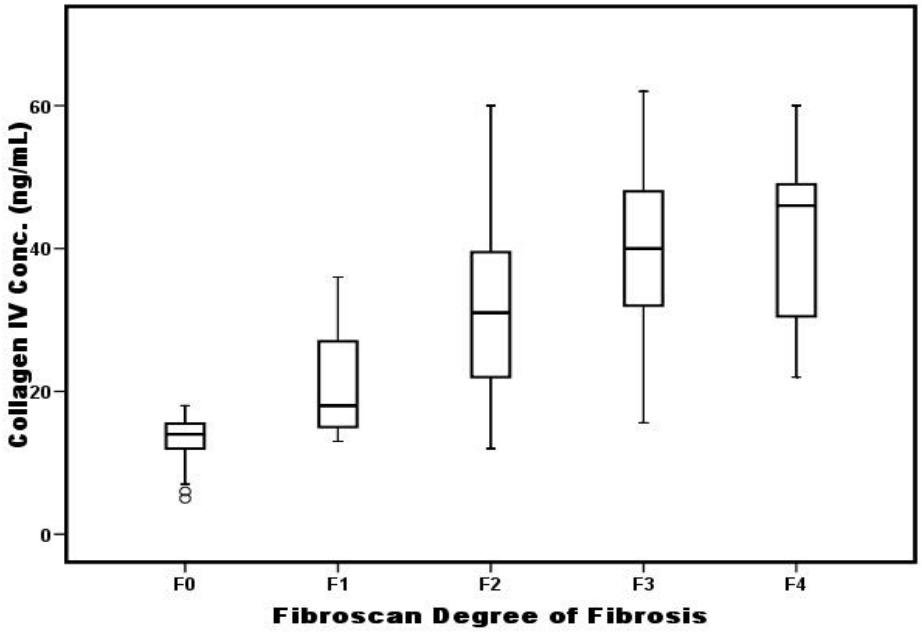

Figure 1. Boxplot of the Col-IV levels in different FibroScan degrees of liver fibrosis. The line across the box indicates the median value of Col-IV for each of the groups. The Col-IV levels increased significantly ($p < 0.001$) with the increase of the degree of liver fibrosis from F0 to F4.

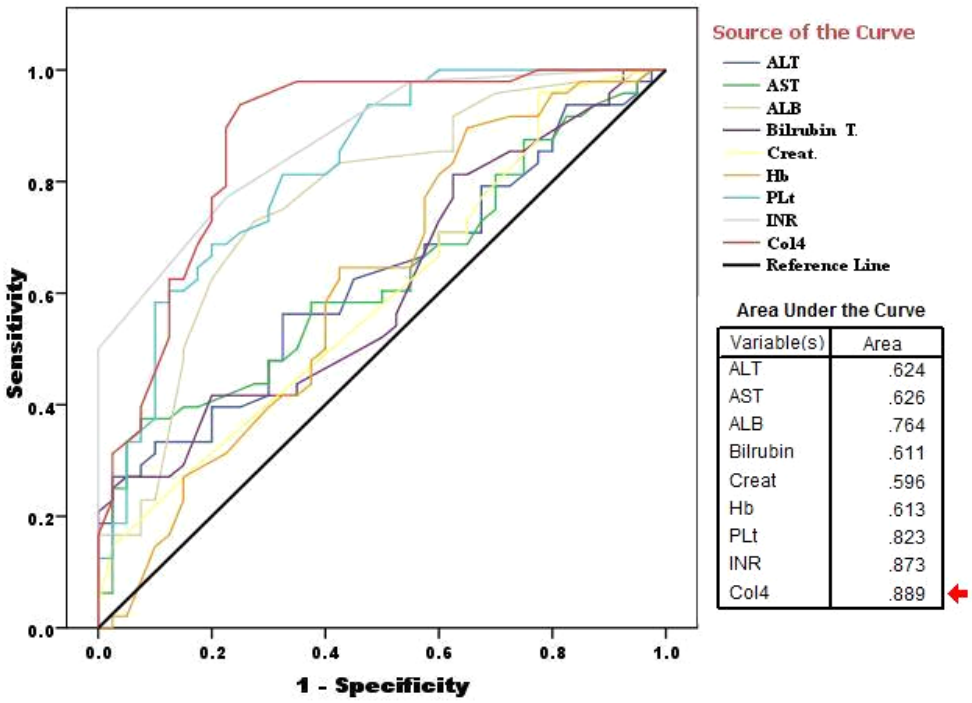

Figure 2. The AUROC curves of all investigated indirect markers of liver fibrosis (ALT, AST, ALB, Bilirubin-T, Creatinine, Hb, PLt, and INR), in comparison with Col-4 for differentiation of CHC patients with significant liver fibrosis (F2-F4, n = 48) from patients with non-significant liver fibrosis (F0-F1, n = 40).

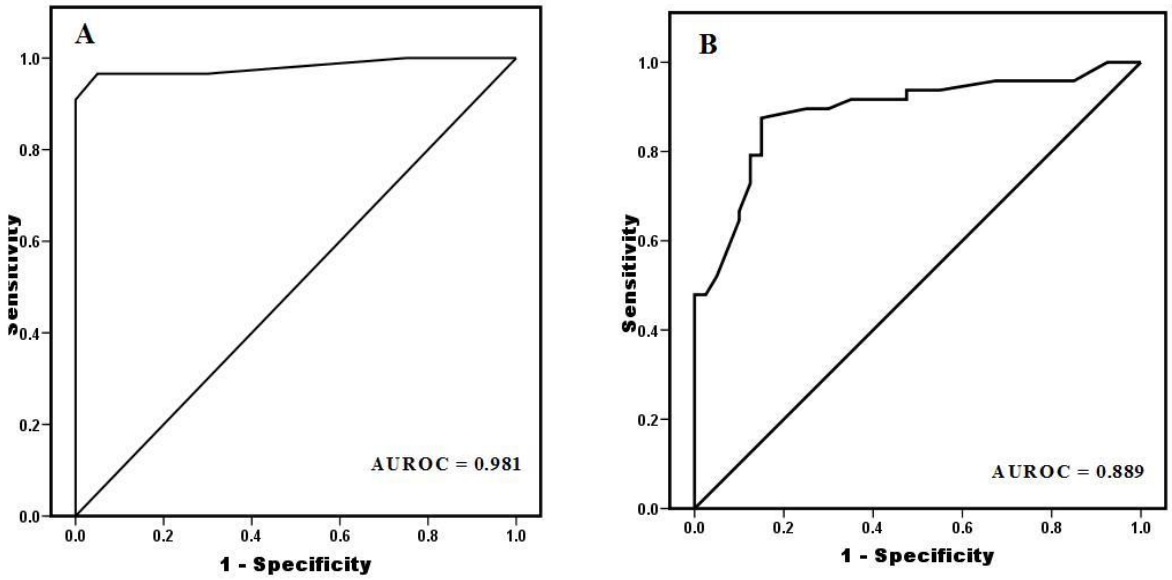

Figure 3. The use of the AUROC curve for differentiation of: **A.** CHC patients (n = 88) with different degrees of liver fibrosis (F0 to F4) from 20 healthy individuals (AUROC = 0.981, P < 0.001). **B.** Patients with significant liver fibrosis (F2-F4, n = 48) from patients with non-significant liver fibrosis (F0-F1, n = 40), (AUROC = 0.889, P < 0.001).

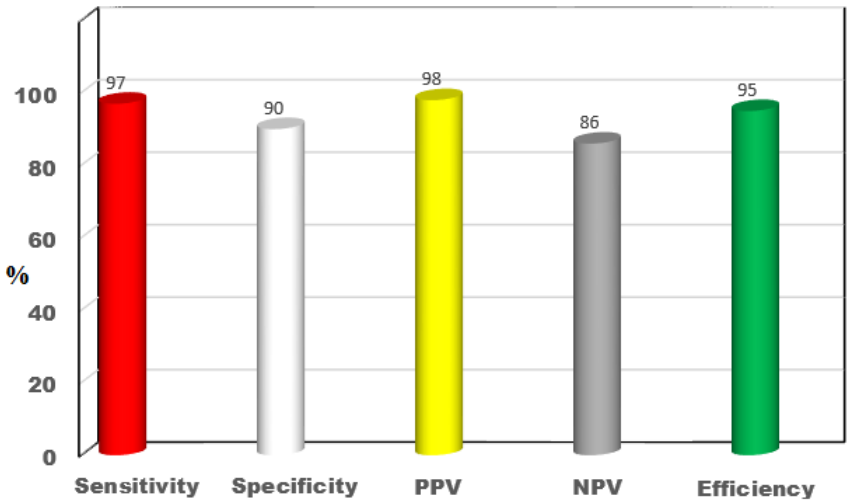

Figure 4. Performance characteristics of sandwich ELISA for the detection of serum Col-IV in sera of patients with different degrees of liver fibrosis (n=88) in comparison with healthy individuals (n = 20) at the best cutoff level 10 ng/mL. Sensitivity = 85 / (85 + 3) × 100 = 97 %, Specificity = 18 / (2 + 18) × 100 = 90 %, Positive predictive value (PPV) = 85 / (85 + 2) × 100 = 98 %, Negative predictive value (NPV) = 18 / (18 + 3) × 100 = 86 %, Efficiency = (85 + 18) / 108 × 100 = 95 %.

3.3. Follow-up of liver fibrosis after DAA treatment using FibroScan, indirect biomarkers, and Col-IV:

The FibroScan degrees of liver fibrosis after DAA treatment of 88 patients were either regressed (R), stationary (S), or progressed (P) as follows. For 19 patients with F0: 16 patients remained at F0 (84%, S) while 3 patients progressed to F1 (16%, P). For 21 patients with F1: 2 patients showed fibrosis regression to F0 (9%, R), 14 patients remained stationary at F1 (67%, S) while 5 patients progressed to F2 (24.3%, P). For 20 patients with F2: one patient showed fibrosis regression to F1 (5%, R), 14 patients remained at F2 (70%, S) while 5 patients progressed to F3 (25%, P). For 17 patients with F3: 2 patients regressed to F2 (12 %, R), 11 patients remained at F3 (65 %, S), and 4 patients progressed to F4 (23%, P). All 11 patients with F4 remained at F4 without changes in their stage (100%, S). Accordingly, most of our study patients with non-significant liver fibrosis (F0-F1; n = 40), significant liver fibrosis (F2-F3; n = 37), and liver cirrhosis (F4; n = 11) showed stationary fibrosis as shown in **Figure 5.** No reversal of fibrosis or cirrhosis (i.e. two-fibrosis stage reduction) was reported in our study patients. One year after the end of treatment, the baseline data of most investigated biochemical and hematological markers were improved in patients with non-significant fibrosis of the liver (F0-F1) and slightly increased in patients with significant fibrosis (F2-F4) but did not reach a significant level, **Table 2.** Moreover, no significant correlations were shown between most of these indirect markers of fibrosis in the liver and FibroScan degree of fibrosis after DAA treatment except Albumin ($r = 0.399$, $P < 0.001$), Platelets ($r = 0.551$, $P < 0.001$) and INR ($r = 0.413$, $P < 0.001$). A highly significant correlation was shown between levels of Col-IV and FibroScan degree of fibrosis after one year of successful DAA treatment ($r = 0.756$, $P < 0.001$). Col-IV mean levels (± SE) after DAA therapy of study patients with different FibroScan degrees of fibrosis are summarized in **Table 3.** The mean levels of Col-IV increased in sera of study patients after DAA treatment (27.64 ± 1.56 ng/mL) in comparison with its levels before DAA treatment (30.24 ± 1.69 ng/mL) but this increase did not reach a significant level except in patients with F0 degree of fibrosis ($p = 0.032$) and consequently in CHC patients with non-significant fibrosis (F0-F1; $P = 0.048$). However, the mean levels of Col-IV significantly decreased ($p < 0.001$) in sera of CHC patients showing regression of fibrosis, did not significantly change ($p > 0.05$) in patients showing stationary fibrosis, and significantly decreased ($p < 0.001$) in sera of CHC patients showing progression of liver fibrosis after successful DAA treatment of HCV infection, **Table 4.** The boxplot analysis of Col-IV levels before and after DAA treatment of patients showing regression, stationary, and progression of fibrosis in the liver are shown in **Figure 6.**

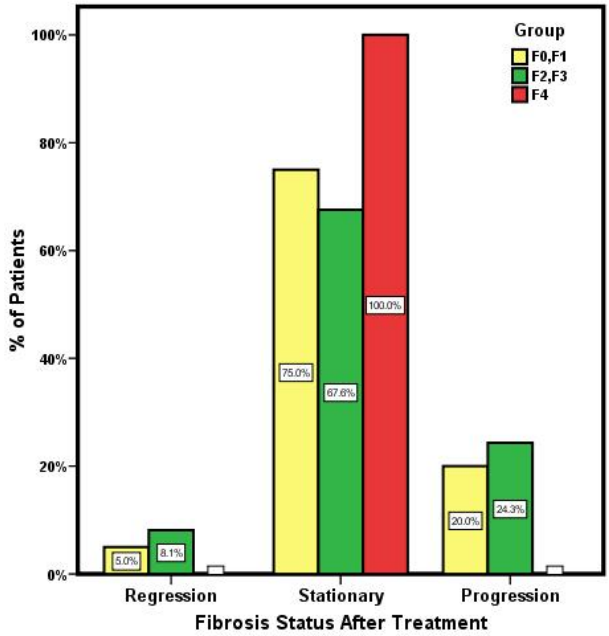

Figure 5. Status of liver fibrosis after DAA treatment of 88 CHC patients using FibroScan. The fibrosis degrees were either regressed, stationary, or progressed. Most of the patients either with non-significant fibrosis (F0-F1, n = 40), with significant fibrosis (F2-F3, n = 37), or with liver cirrhosis (F4, n = 11) showed stationary liver fibrosis after DAA treatment.

Table 2. Levels of indirect markers of patients with non-significant fibrosis (F0-F1) and patients with significant fibrosis (F2-F4) before and after treatment.

Indirect markers of liver fibrosis	**Liver Fibrosis Status of Study Patients**					
	Non-significant fibrosis (F0-F1, n= 40) Median (IQR)			**Significant fibrosis (F2- F4, n = 48) Median (IQR)**		
	Before T.	**After T.**	**P***	**Before T.**	**After T.**	**P***
Biochemical markers:						
ALT (U/L)	45 (7-105)	39.5 (23-96)	>0.05	51.5 (13-160)	50.5 (28-173)	>0.05
AST (U/L)	47 (8-113)	44.8 (9.6-138.3)	0.009	51.5 (16-142)	55.5 (16-138.3)	>0.05
ALB (g/dL)	4.2 (3.1-5.1)	4 (3-4.9)	0.001	3.75 (2.4-4.9)	3.55 (2.5-4.6)	>0.05
BIL (mg/dL)	0.88 (0.2-1.9)	1 (0.5-2)	>0.05	0.92 (0.45-5)	1 (0.5-12)	>0.05
Creatinine (mg/mL)	0.9 (0.5-1.2)	1 (0.62-1.2)	>0.05	0.9 (0.6-1.3)	1 (0.7-1.3)	0.012
AFP (ng/mL)	1.6 (0.9-3.17)	1.8 (0.8-3.3)	>0.05	14.1 (1-90)	11 (1-70)	0.002
Hematological markers:						
PLt × 10^9/L	226.5 (115-337)	210 (130-355)	0.017	166 (80-240)	162 (62-281)	>0.05
INR	1.1 (0.88-1.2)	1 (0.8-1.3)	>0.05	1.25 (1-1.6)	1.15 (0.92-1.6)	0.034
Hb (g/dL)	13.3 (10.4-15.3)	13.4 (10.4-18.5)	>0.05	12.6 (10.5-15.2)	12.1 (10.4-15.3)	0.032

* P > 0.05 is considered not significant; P < 0.05 considered significant; P < 0.001 considered very significant; P < 0.0001 is considered extremely significant.

Table 3. Levels of Collagen IV in sera of CHC patients with different FibroScan degrees of liver fibrosis (F0-F4) before and after successful DAA treatment of HCV infection.

Degree of liver fibrosis by using FibroScan	Col-IV levels (Mean ± SE, ng/mL) using ELISA		P value*
	Before DAA Treatment	After DAA Treatment	
Non-significant liver fibrosis:			
F0 (n = 19)	13.00 ± 0.82	17.16 ± 1.87	0.032
F1 (n = 21)	20.52 ± 1.72	22.05 ± 2.72	> 0.05
F0-F1 (n = 40)	**16.95 ± 1.14**	**19.73 ± 1.71**	**0.048**
Significant liver fibrosis:			
F2 (n = 20)	31.50 ± 2.89	34.10 ± 2.91	> 0.05
F3 (n = 17)	39.21 ± 3.04	42.35 ± 3.60	> 0.05
F2-F3 (n = 37)	**35.04 ± 2.16**	**37.89 ± 2.35**	**> 0.05**
Liver Cirrhosis:			
F4 (n = 11)	**41.64 ± 3.89**	**42.73 ± 4.13**	**> 0.05**
Total			
F0-F4 (n = 88)	**27.64 ± 1.**56	**30.24 ± 1.69**	**> 0.05**

* P > 0.05 is considered not significant; P < 0.05 considered significant.

Table 4. Mean levels of Collagen IV in sera of CHC patients showing regression, stationary and progression of liver fibrosis after successful DAA treatment of HCV infection.

Liver fibrosis status using FibroScan	Col-IV Concentration (Mean ± SE, ng/mL)			P value*
	N	Before DAA Treatment	After DAA Treatment	
Regression Fibrosis	5	30.6 ± 4.22	22.4 ± 4.71	< 0.001
Stationary Fibrosis**	66	26.21 ± 1.81	26.68 ± 1.77	> 0.05
Progression Fibrosis	17	32.35 ± 3.70	46.36 ± 3.09	< 0.001

* P > 0.05 is considered not significant; P < 0.05 considered significant.

****** including all 11 patients with liver cirrhosis (F4).

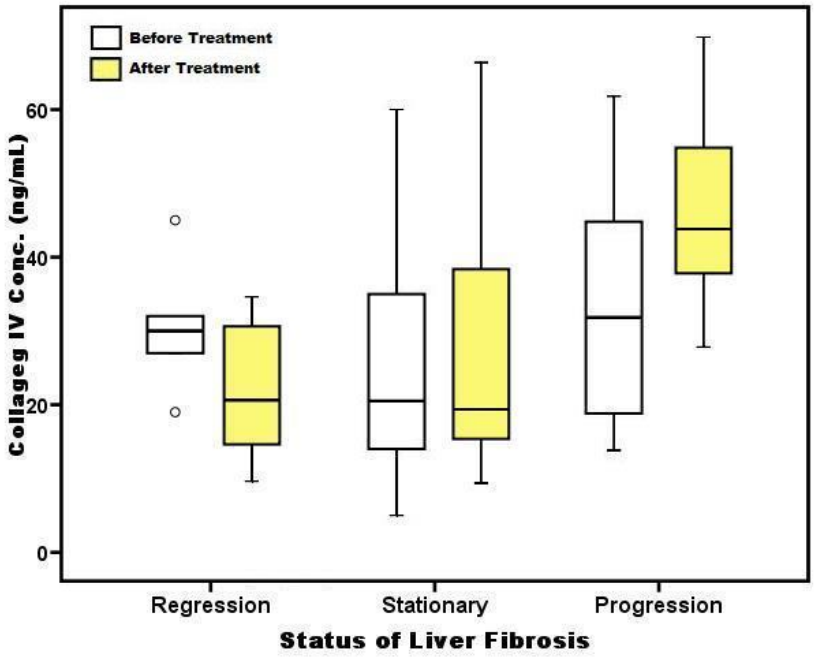

Figure 6. Box plot of Col-IV levels in sera of CHC patients showing FibroScan regression, stationary, and progression of liver fibrosis after successful DAA treatment of HCV infection. The central box represents values from the lower to upper quartile (25-75th percentile). The middle line represents a median level of Col-IV. The line extends from minimum to maximum value, excluding outside values which are displayed as separate points.

4. Discussion

Globally, chronic hepatitis C (CHC) is a significant health concern (32). Direct-acting antivirals (DAAs) therapy was recently thought to be the recommended course of treatment for individuals with CHC (33, 34). Following successful DAA treatment, the incidence of hepatic necroinflammation and liver fibrosis development will be decreased in CHC Egyptian patients due to the considerable elimination of viral infection brought about by the introduction of DAAs (35–37). Even though there is evidence for a variety of different illnesses, human liver fibrosis is at least largely reversible after the cessation of chronic hepatic insults (14). Making treatment decisions requires determining the liver fibrosis level using histological staging (38). Many noninvasive indirect and direct biomarkers, indexes, and mechanical techniques based on ultrasound have been developed to characterize the stage of liver fibrosis because liver biopsy is an invasive procedure (39). Regular medical tests can evaluate indirect markers, such as ALT, AST, and platelet counts (40,41); direct biomarkers include substances involved in the molecular pathogenesis of liver fibrosis, such as cytokines and ECM proteins (42, 43); and non-invasive mechanical methods based on ultrasound, such as acoustic radiation force impulse (ARFI) elastography (Virtual Touch) and transient elastography (FibroScan) (44). These noninvasive fibrosis markers have undergone significant development over the past 20 years, have been well-tested, and are accurate in clinical settings for predicting liver fibrosis in CHC patients before anti-HCV therapy (45). However, more research is required to determine the causes linked to such changes and to reevaluate the trustworthy noninvasive biomarkers or indicators of liver fibrosis in patients with CHC who have been successfully treated with DAAs, particularly after long-term follow-up (30, 46–48). In the present study, the direct biomarker of liver fibrosis; circulating Col-IV was detected in sera of CHC Egyptian patients associated with different degrees of liver fibrosis

(F0-F4) using sandwich ELISA. A number of groups of macromolecules, including collagens (types I, III, IV, V, and VI), non-collagenous glycoproteins (fibronectin and laminin), and proteoglycans, make up the ECM in a normal liver (49, 50). The main components of the hepatic ECM, such as collagens, are broken down by several enzyme families, such as matrix-degrading metalloproteinases (MMPs), and then released into the bloodstream when fibrosis progresses (51). The extremely dynamic character of tissue healing and remodeling in this solid organ has been underlined over the past three decades by extensive investigation of hepatic extracellular matrix formation and breakdown employing methodologies including human disease, laboratory animal models, and cell culture (51). Collagen Type IV is a crucial part of the extracellular matrix of the liver. Since type IV collagen remains intact in the matrix, in contrast to type I and III collagens, circulating components of collagen type IV are thought to largely signal matrix breakdown (42,52,53). In this instance, there were no significant associations found between the research patients' age or sex and the serum levels of Col-IV and the degree of liver fibrosis as measured by FibroScan. Furthermore, when compared to all other indirect markers of liver fibrosis that were studied, the Col-IV demonstrated the greatest AUROC for separating CHC patients with significant fibrosis (F2-F4) from CHC patients with non-significant fibrosis (F0-F1). Type IV collagen has been linked in a number of studies to the occurrence of liver fibrosis, either by itself or in conjunction with indirect indicators of fibrosis (54–56). Furthermore, it has been demonstrated that Collagen IV strongly correlates with the state of liver fibrosis before DDA antiviral medication as well as one year following the completion of effective therapy (40, 43, 57). Additionally, our research showed that the ELISA method for detecting Col-IV exhibited high levels of sensitivity, specificity, and efficiency (> 90%). As a result, it may be regarded as a helpful tool for evaluating liver fibrosis in routine clinical practice, as has been recently suggested by a number of investigators (58-60). The evaluation of the liver fibrosis stage is a critical factor in predicting liver-related adverse outcomes, including hepatic decompensation, hepatocellular carcinoma, and liver cirrhosis, in patients who receive DAA treatment and achieve SVR (48,61,62). Unfortunately, the SVR obtained does not ensure that liver-related adverse events will no longer occur, and post-SVR long-term follow-up with planned liver fibrosis status surveillance is required (30,63). This situation enhanced our efforts to measure the liver fibrosis status of our CHC illness using Collagen IV as well as FibroScan, indirect biomarkers one year after achieving SVR12 with DAA. The significant changes in Col-IV values from baseline to one year after the end of DAA may disprove the link between it and the development of fibrosis regression, which might take much longer. Moreover, the reversibility or regression of liver fibrosis was challenged especially after the successful removal of the etiology using DAA (14). Few research has looked at the temporal changes in LSMs in CHC patients receiving DAA therapy while using FibroScan. Nonetheless, a systematic review demonstrated that LSMs are a precise and trustworthy technique for liver fibrosis staging in CHC patients who have not yet received treatment (64). One year after the end of DAA treatment, the FibroScan degrees of liver fibrosis were either regressed (R), stationary (S) or progressed (P) in our treated CHC patients. For patients with F0: 84% remained at F0 (S) while 16% progressed to F1 (P). For F1 patients: 9% showed fibrosis regression to F0 (R), 67% remained stationary at F1 (S) while 24% progressed to F2 (P). For F2 patients: 5% showed fibrosis regression to F1 (R), 70% remained at F2 (S) and 25% of patients progressed to F3 (P). For F3 patients: 12% of patients regressed to F2 (R), 65% stayed at F3 (S), and 23% of patients progressed to F4 (P). All (100%) patients with F4 remained at F4 without changes in their stage (S). No reversal of fibrosis or cirrhosis (i.e. two-fibrosis stage reduction) was reported in our study patients. Accordingly, most of our study patients either with non-significant liver fibrosis (F0-

F1), significant liver fibrosis (F2-F3) or liver cirrhosis (F4) showed stationary fibrosis. These findings indicate an excellent long-term outcome of DAA-mediated SVR12 was obtained in our study patients one year later. Our results are in agreement with several studies. Shiha et al. (30). showed that in the majority of their treated patients, HCV clearance after DAA treatment is linked to hepatic fibrosis regression in CHC patients with advanced fibrosis. For example, in F3 patients, 31.5% showed fibrosis regression while 30.6% remained stationary, and in F4 patients, 27.4% showed fibrosis regression while 50.8% remained stationary. Furthermore, a noteworthy regression in fibrosis was observed in 40% of CHC patients receiving DAAs; this effect was particularly prominent in patients with severe liver fibrosis and cirrhosis at baseline. This was reported by Lledó et al. (65). Furthermore, FibroScan-derived LSMs have been demonstrated to diminish 6–12 months following viral eradication, leading researchers to conclude that the resolution of necroinflammation was likely associated with the early fall in liver stiffness (66–68). The reduction in the values of most noninvasive biomarkers of liver fibrosis e.g. AST and ALT levels and APRI after treatment was reported in several studies mostly after SVR12 due to reduction or improvement in necroinflammation rather than in fibrosis regression which may take considerably longer to develop (69). Accordingly, long-term follow-up prospective studies are required to conclude (30). In the present study, the baseline data of most investigated biochemical and hematological markers were improved after DAA treatment in patients with non-significant fibrosis (F0-F1) and slightly increased in patients with significant fibrosis (F2-F4) but did not reach a significant level. Moreover, no significant correlations were shown between most of these indirect markers of liver fibrosis and FibroScan degree of fibrosis after DAA treatment except Albumin, Platelets, and INR. In a preliminary study, we have developed a noninvasive index, based on Collagen IV, INR, Platelets and AST enabling correct identification and efficient prediction (> 90%) of different stages of liver fibrosis (unpublished data). However, the relation between indirect markers and Col-IV needs extensive evaluation and validation in a large number of patients before and after antiviral treatment. Interestingly, a highly significant correlation was shown also between levels of Col-IV and FibroScan degree of fibrosis after one year of successful DAA treatment. This confirms the reliability and validity of the pretreatment correlation between Col-IV levels and FibroScan degree of fibrosis. Tanwar et al. (70) showed that when combined with the starting point histologic staging, an alteration in the expanded liver fibrosis direct markers (hyaluronic acid, tissue inhibitor of matrix metaloproteinase-1, and terminal peptide of procollagen III) from baseline to 12 months after Peg-IFN-based treatment beginning could predict histologic fibrosis regression at 24 months after the therapy. The mean levels of Col-IV increased in sera of our study patients after DAA treatment in comparison with its levels before DAA treatment but this increase did not reach a significant level except in patients with F0 degree of fibrosis and consequently in patients with non-significant fibrosis (F0-F1). This increase in F0 patients may be due to HCV infection activating an uncontrolled inflammatory response leading to the rapid development of liver fibrosis (70). However, the mean levels of Col-IV significantly decreased in the sera of our study patients showing regression of fibrosis, did not significantly change in patients showing stationary fibrosis, and significantly decreased in sera of CHC patients showing progression of liver fibrosis after successful DAA treatment of HCV infection. These data confirm the association of collagen IV with the status of liver fibrosis after one year of successful DAA therapy (52,58). In conclusion, we have assessed collagen IV as a reliable noninvasive direct marker of liver fibrosis in sera of CHC patients who have been treated successfully with DAA before and after long-term follow-up with a high degree of efficiency. Our study's limitations include its small sample size, investigation of Collagen IV's

relationship to other trustworthy fibrosis markers, and collection of laboratory data for only a single year. Notwithstanding these drawbacks, the current research showed stationary liver fibrosis using Col-IV and Fibroscan values in CHC patients with SVR after one year of DAA therapy. Finally, future studies involving large sample size, development of Col-IV-based reliable fibrosis index, and long-term assessment of changes in Col-IV levels, related biochemical parameters, and FibroScan after DAA therapy will be performed.

Authors contributions

All authors confirm contribution to the manuscript as follows: HI and HAM analyze the idea and design when creating the first draft. ASA and AMA Methodology and collection of data. SMA, HAM, and HI analysis and interpretation of the findings. The manuscript's final form was approved by all authors.

Data accessibility

Upon request, the original data may be obtained from the corresponding author at himosman@mu.edu.eg.

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