

DETECTION OF FIBRONECTIN IN SERA OF HCV INFECTED PATIENTS WITH LIVER FIBROSIS

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ABSTRACT

An accurate assessment of liver fibrosis is very important in order to predict the prognosis and to start the appropriate therapy to prevent disease progression. Several biochemical markers have been proposed as indicators of the change from chronic hepatitis to cirrhosis. This study was carried out on 126 serum samples pathologically divided into two groups. A, the fibrotic group with different stages (F1-F3) consists of 114 patients (85 males and 29 females) with liver fibrosis associated with HCV infections (aged 50.6 ± 11.7 yrs.). B, the control group consists of 12 non-fibrotic (F0) individuals with HCV infection without fibrosis (aged 29.2 ± 5.2 yrs.), used as controls. The results revealed that the level of serum fibronectin increased in the all patients in the fibrotic group. Significant differences ($p < 0.0001$) were shown between non-fibrotic liver (F0), liver fibrosis (F1-F3). Fibronectin was positively correlated with age, ALT, AST, and bilirubin and negatively correlated with albumin. In conclusion, fibronectin fragment had been identified in serum a sample of fibrotic group at 90-kDa and its level was increased in these samples; while it was normal in non-fibrotic group. These data confirm the role of fibronectin as a pacemaker of fibrosis.

INTRODUCTION

The most common causes of chronic liver disease are chronic hepatitis C ⁽¹⁾. An estimated 170 million people worldwide are infected; most of them are chronically infected and risky for liver cirrhosis and hepatocellular carcinoma "HCC" ⁽²⁾. Egypt has possibly the highest HCV problem worldwide, where 10-20% of the general population is infected. In the majority of patients, chronic HCV infection is a silent disease until significant hepatic fibrosis has developed. Chronic HCV infection results in damage to hepatocytes and may eventually lead to liver fibrosis, cirrhosis and/or HCC ⁽³⁾. One of the most important actions to prevent the un-controlled damage is early diagnosis then effective follow-up and treatment ⁽⁴⁾. Hepatic fibrosis has been a common response to chronic liver injury and might result in potentially lethal sequelae ^(5,6).

The diagnosis of liver fibrosis and liver cirrhosis in patients is of therapeutic and prognostic importance ⁽³⁾. Liver biopsy "histological examination" is the gold criterion for the severity of fibrosis and cirrhosis ⁽³⁾. Histological examination of liver biopsy specimens is still the gold standard for the diagnosis of chronic HCV, pathogenesis of liver injury, and assessment of anti-viral treatment. However, liver biopsy is invasive, requires an experienced gastroenterologist, examination is required by a professional histopathologist, adds expense, and is associated with complications and mortality patients with chronic HCV ⁽⁷⁾. Searching for a noninvasive diagnostic approach is an interesting subject worldwide ⁽⁸⁾. Several biochemical markers of liver fibrosis, such as hyaluronic acid, fibronectin, N-acetyl- β -glucosaminidase (β -NAG), laminin, and procollagen III propeptide (PIIP), have been proposed as indicators of the change from chronic hepatitis to cirrhosis ^(9,10,11,12). These markers correlate with matrix content; secreted in large amounts as a result of activation of hepatic satellite cells ^(13,14).

Fibronectin is a large adhesive glycoprotein and a prominent constituent of the ECM. Secreted as a dimer composed of 240-kDa monomers joined by two disulfide bonds, fibronectin forms an adhesive lattice surrounding virtually every cell of the body ⁽¹⁵⁾. Fibronectin is a multifunction glycoprotein containing about 5%

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carbohydrate that bind to receptor proteins spanning the cell called integrins ⁽¹⁶⁾.

This study aimed to detect fibronectin in serum samples from pathologically diagnosed patients with liver fibrosis associated HCV infections as a simple and rapid alternative diagnostic method for liver fibrosis.

MATERIALS & METHODS

***Samples:**

Serum samples and liver biopsies were collected from 126 individuals from Internal Medicine University Hospital "IMUH", Mansoura University, Egypt. These samples were pathologically divided into two groups: fibrotic group (moderate degree of fibrosis) and non-fibrotic group. The fibrotic group consists of 114 patients with liver fibrosis associated with HCV infections (mean age 50.6 ± 11.7 years). The second group consists of 12 non-fibrotic individuals associated with HCV infections (mean age 29.2 ± 5.2 years) served as controls. Serum samples were stored at -20°C until use.

***Liver function tests:**

Serum samples were screened for different liver function tests including liver enzymes (AST, ALT), T. bilirubin and albumin according to the method of Reitman and Frankel ⁽¹⁷⁾, Dacie and Lewis, ⁽¹⁸⁾ and Doumas *et al.* ⁽¹⁹⁾ respectively.

***Detection of anti-HCV antibodies using ELISA:**

The HCV-Ab ELISA kit (Biotec Laboratories Ltd., Suffolk, USA) as an immuno-enzymatic method was performed according to manufacture instruction in which the wells of a micro-titer plate are coated with HCV-specific synthetic antigens derived from core and NS regions representing epitopes of HCV.

***SDS-PAGE and Western blot:**

Serum samples from non fibrotic individuals as controls (F0) and patients with liver fibrosis (F1-F3) were resolved on 12 % SDS-PAGE. The separation of proteins based on molecular weights as they move through a polyacrylamide gel matrix toward the anode according to Laemmli ⁽²⁰⁾. The separated proteins were electrophoretically transferred to nitrocellulose (NC) paper to identify

and determine the molecular weight of fibronectin according to the method of Towbin *et al.* ⁽²¹⁾.

The anti-fibronectin monoclonal antibody (ABC Diagnostics, New Damietta, Egypt) was used as a primary antibody, and anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO, USA) was used as a secondary antibody.

***ELISA for the detection of Fibronectin in serum**

Fibronectin was detected in serum using specific monoclonal antibody as primary antibody (ABC Diagnostics) according to the method of Engvall and Perlman⁽²²⁾ as follow: polystyrene micro-plates were coated with serum diluted 1:20 and serial of purified fibronectin in coating buffer (pH 9.6). The plates were incubated overnight at 4°C. Then washed 4 times with 0.05% PBS-T₂₀ (pH 7.2) and then incubated for 1 hr at room temperature with 200 µl/well of 0.2% BSA in coating buffer (pH 9.6). 50 µl /well of specific primary antibody diluted 1:100 in PBS-T₂₀ were added and incubated at 37 °C for 2 hr. Then washing wells 4 times with 0.05% PBS-T₂₀ (pH 7.2). After washing, 50 µl/well of anti-mouse IgG alkaline phosphatase conjugate (whole molecule, Sigma) was diluted 1:300 in 0.2% BSA PBS-T₂₀, was added and incubated at 37°C for 1 hr. excess conjugate was removed by extensive alkaline phosphatase in substrate buffer (ABC Diagnostics) for 30 min. at 37 °C. The reaction was stopped and the absorbance was read at 490 nm on Σ960 ELISA readers (Metretech Inc, USA).

***Statistical analysis:**

All statistical analyses were done by a statistical for social science package "SPSS" 10.0 for Microsoft Windows, SPSS Inc. ⁽²³⁾. P value considered statistically significant at a two-sided P < 0.05. Numerical data were expressed as mean ± SD. The levels of markers were analyzed by ANOVA but the Mann-Whitney U-test was used for comparisons between independent groups. The correlations were evaluated by Person's correlation coefficient.

RESULTS

Serum samples from non fibrotic individuals as controls (F0) and patients with liver fibrosis (F1-F3) were resolved on 12 % SDS-

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PAGE. The protein bands were identified after staining the SDS-PAGE by the Coomassie Brilliant Blue R-250 dye. The coomassie blue separated polypeptides have a wide range of molecular weights ranged from 215 kDa to 18.3 kDa. The separated proteins were electrophoretically transferred to nitrocellulose (NC) paper. The anti-fibronectin monoclonal antibody identified using western blot reactive band at 90 kDa **Fig. 1**.

The mean value of serum fibronectin in fibrotic patients (F1-F3) was increased, and reached 459.0 ± 170.3 mg/L ($p < 0.0001$) compared to its mean value in the non-fibrotic group (F0) that was found within normal level 235.0 ± 93.0 mg/L. These results are in line with **Fortunato *et al.*** ⁽⁹⁾ who reported a value of 250 mg/L. The fibronectin was higher than normal value (250 mg/L) in 86 out of 114 serum samples of liver fibrosis patients with sensitivity (75 %). 10 samples out of 12 non fibrotic liver individuals (controls) were within normal value for fibronectin with 82 % specificity. So, the efficiency of fibronectin for discriminating patients with liver fibrosis from those with no fibrosis livers was 76%. The area under ROC curve of fibronectin for discriminating patients with liver fibrosis (F1-F3) from those without liver fibrosis (F0) was 0.78 ($P < 0.0001$) **Fig. 2**. Different relations were carried out between fibronectin and some standard liver function tests to confirm the accuracy of fibronectin in prognosis of liver fibrosis **table (1)**. There was no significant difference between the value of fibronectin in female and male. Fibronectin was positively correlated with age ($r = 0.522$; $P < 0.0001$) **Fig. 3 (A)**. Also, there were significant correlations between fibronectin and some routine liver functions as ALT, AST, bilirubin and albumin.

The normal mean value of ALT and AST was found to be 26.7 ± 2.4 U/L and 29.2 ± 6.4 U/L respectively. These results are in accordance with those of **Vaubour-dolle *et al.*** ⁽²⁴⁾ who reported a value of ALT $24 \pm$

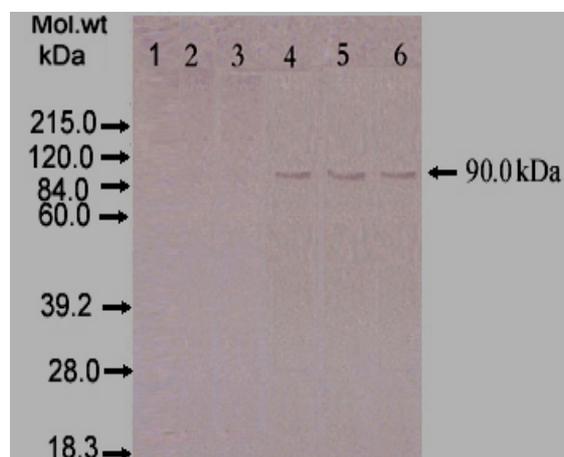


Figure 1. Western blot analysis of sera from non-fibrotic individuals and fibrotic liver diseased patients. Lanes 1-3: Sera of non fibrotic individuals (F0) as negative controls. Lanes 4-6: Sera of fibrotic patients (F1-F3) with chronic Hepatitis C. Molecular weight marker includes: Myosin (215.0 kDa), phosphorylase B, (120.0 kDa), Bovine serum albumin (84.0 kDa), Ovalbumin (60.0 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.0 kDa), and lysozyme (18.3 kDa).

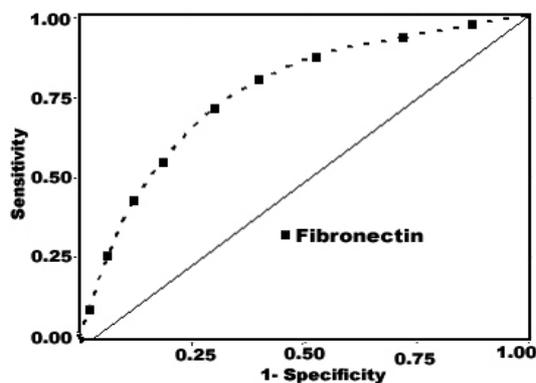


Figure 2. ROC curve of fibronectin for discriminating patients with liver fibrosis from those without fibrosis in chronic viral hepatitis C.

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9.8 U/L and AST of 22 ± 12.1 U/L. The mean value of ALT and AST was found to be 71.2 ± 51.2 U/L and 64.9 ± 35.6 U/L in fibrotic group in comparison to control group respectively. So, there was a positive significant correlation between fibronectin and ALT ($r = 0.372$; $P < 0.0001$) and a positive significant correlation between fibronectin and AST ($r = 0.312$; $P < 0.001$) **Fig. 3 (B and C)**.

Table (1): Mean \pm S.D of fibronectin, ALT, AST, Total bilirubin, and albumin in the fibrotic group in comparison with non-fibrotic group.

Variable*	Controls (n = 12)	Patients (n = 114)	P value**	% change
Male (%)	7 (58 %)	85 (75 %)	< 0.0001	-
Female (%)	5 (42%)	29 (25%)	<0.0001	-
Age (Years)	29.2 ± 5.2	50.6 ± 11.7	< 0.0001	-
ALT (U/L)	26.7 ± 2.4	71.2 ± 51.2	< 0.0001	166.66
AST (U/L)	29.2 ± 6.4	64.9 ± 35.6	< 0.0001	122.26
Bilirubin (mg/dl)	0.7 ± 0.14	2.7 ± 2.3	< 0.01	285.71
Albumin (g/dl)	4.6 ± 0.41	3.6 ± 0.83	< 0.0001	-27.77
Fibronectin (mg/L)	235.0 ± 93.0	459.0 ± 170.3	< 0.0001	95.32

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

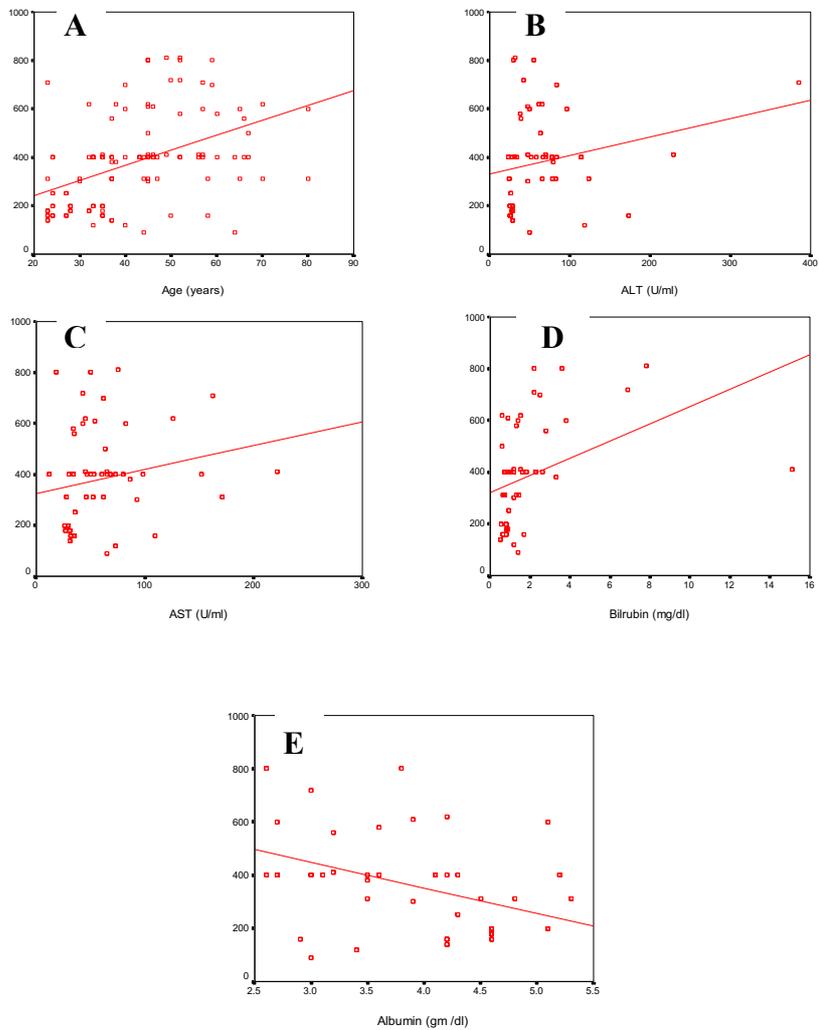


Figure 3. Correlation between serum fibronectin, age and liver function tests. A. Positive Correlation was observed between serum fibronectin and age ($r = 0.522$; $P < 0.0001$). B, C and D revealed positive correlations between serum fibronectin and the severity of liver disease as judged by the values of ALT ($r = 0.372$; $P < 0.0001$), AST ($r = 0.312$; $P < 0.001$), and bilirubin ($r = 0.554$; $P < 0.0001$) respectively. While albumin shows a negative correlation (E) ($r = -0.497$; $P < 0.0001$).

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The normal mean value of total bilirubin was found to be 0.7 ± 0.14 mg/dl. These results are in line with those of **Bishop *et al.*** ⁽²⁵⁾ who reported a value of 0.2-1 mg/dl. The mean value of total bilirubin was found to be 2.7 ± 2.3 mg/dl in fibrotic group in comparison to control group. So, there was a positive significant correlation between fibronectin and bilirubin ($r = 0.554$; $P < 0.0001$) **Fig. 3 (D)**.

The normal mean value of albumin was found to be 4.6 ± 0.41 g/dl. These results are in line with those of **Williams and Marks** ⁽²⁶⁾ who reported a value of 3.5-5 g/dl. The mean value of albumin was found to be 3.6 ± 0.83 g/dl in fibrotic group in comparison to control group. So, there was a negative significant correlation between fibronectin and albumin ($r = -0.497$; $P < 0.0001$) **Fig. 3 (E)**.

DISCUSSION

Chronic HCV infection results in damage to hepatocytes and may eventually lead to liver fibrosis, cirrhosis and/or HCC ⁽⁴⁾. Fibrosis is a primary end point of clinical trials in chronic HCV ⁽¹⁸⁾ characterized by excessive accumulation of extracellular matrix proteins ⁽¹³⁾. The diagnosis of liver fibrosis and liver cirrhosis in patients is of therapeutic and prognostic importance ^(5, 13). In the present study, western blot analysis revealed that monoclonal antibody reacted against fibronectin at an apparent molecular weight of 90 kDa in serum samples of patients with liver fibrosis. Proteolytic fragments of the cell-binding domain >75 kDa show adhesive activity equal to that of intact fibronectin **Sara *et al.*** ⁽²⁷⁾. Our results are in line with **Katnik-Prastowska *et al.*** ⁽²⁸⁾ who examined the fragmentation of fibronectin (FN) by immunoblotting with a monoclonal antibody specific to the central cellular FN domain. Nine FN fragments between 60 and 200 kDa and five fragments of 60–150 kDa were identified. The relative amounts of the 60, 90 and 100 kDa FN fragments were 2–3 times higher in the pathological samples ⁽²⁸⁾.

ELISA as a simple, rapid and very sensitive technique was carried out to determine the mean value of fibronectin in serum samples. It was found that, the concentration of serum fibronectin was increased in fibrotic group than normal value according to the severity of liver fibrosis (450.9 ± 170.3 mg/L) in comparison to control group

serum fibronectin concentration that within normal level (230.5 ± 90.3 mg/L). Hepatic fibrosis is a scarring process that is associated with an increased and altered deposition of extracellular matrix in liver. This progressive process is mainly characterized by cellular activation of hepatic stellate cells and aberrant activity of transforming growth factor- β 1 **Gressner and Weiskirchen** ⁽²⁹⁾. TGF- β 1 appears to be a key mediator in human fibrogenesis **Gressner *et al.*** ⁽³⁰⁾. TGF- β 1 is synthesized in non-parenchymal cells such as hepatic stellate cells (HSC). TGF- β 1 increases the synthesis and deposition of extracellular matrix proteins such as fibronectin by HSC and is closely associated with the progression of hepatic fibrosis **Date *et al.*** ⁽³¹⁾. Our results are in parallel to those of **Glud *et al.*** ⁽³²⁾ who found significantly ($P < 0.01$) raised plasma fibronectin concentrations (median 506 mg/l (range 339-804) in patients with viral hepatitis compared to controls (median 399 mg/l (range 304-462)). Our results also, are in accordance with **Yamauchi *et al.*** ⁽³³⁾ found that; the serum level of fibronectin receptor was significantly higher in patients with chronic hepatitis than in normal subjects. Also, they revealed that the fibronectin receptor was increased in fibrotic areas and on the plasma membrane of hepatocytes and sinusoidal lining cells of fibrotic liver. The serum level of fibronectin receptor in patients with chronic liver diseases may therefore be a useful marker of hepatic fibrosis. And with **Grieco *et al.*** ⁽³⁴⁾ who found that fibronectin levels in 101 patients with chronic liver disease were increased 426.72 mg/L in chronic active hepatitis and fibrosis in comparison to those of the control (mean 372.00 mg/L).

The foregoing data confirm our findings in the present study including the observed correlations between fibronectin and different parameters including sex, age, and some routine liver function tests as ALT, AST, bilirubin, and albumin.

There was no significant difference between the value of fibronectin in female and male. Our results are in accordance with **Gironi *et al.*** ⁽³⁵⁾ who reported that the mean value of fibronectin was not strongly influenced by sex. Most studies of hepatic fibrosis have reported that male sex is significantly associated with progression of

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fibrosis. The mechanisms by which sex affects fibrosis progression are unknown **Marcellin et al.** ⁽³⁶⁾.

Fibronectin was positively correlated with age ($r = 0.522$; $P < 0.0001$) **Fig. 3 (A)**. Our results are in a line with **Topaloglu et al.**, ⁽³⁷⁾ who found that a strong direct correlation between fibronectin level and age ($r = 0.86$; $P < 0.001$). At least 20% of patients develop cirrhosis during the first 15 to 20 years of HCV infection **Marcellin et al.** ⁽³⁶⁾. Liver fibrosis progression is linear over time **Maci'as et al.** ⁽³⁸⁾. Patients with liver fibrosis can be a-symptomatic for 15-20 years with morbidity and mortality only occurring after progression to cirrhosis, the lethal end-stage of liver fibrosis **Pineda and Maci'as** ⁽³⁹⁾. The increased fibronectin synthesis in senescent fibroblasts appeared to correlate with the general increase in rate of protein synthesis/cell. A progressive increase in the rate of fibronectin and total cellular protein synthesis per cell was observed by late passage human diploid fibroblasts **Shevitz et al.** ⁽⁴⁰⁾.

There was a positive significant correlation between fibronectin and ALT and AST ($r = 0.372$; $P < 0.0001$) ($r = 0.312$; $P < 0.001$) respectively **Fig. 3 (B and C)**. Our results are in accordance with **Topaloglu et al.** ⁽³⁷⁾ who found that a positive correlations between concentration of fibronectin and ALT and AST. **Marshall** ⁽⁴¹⁾, reported that increased aminotransferases activities (ALT and AST) reflect cell damage, plasma levels may be 20 times the upper limits of normal in patients with hepatitis. **Mark and Ira** ⁽⁴²⁾ reported that the elevations in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) indicate liver cell damage. **Mehta et al.** ⁽⁴³⁾ reported that HCV infections were recognized by elevation in liver enzymes, such as alanine amino-transferase (ALT) and aspartate amino-transferase (AST). In fibrosis, cell damage increases membrane permeability, causing AST, ALT enzymes to spill into the sinusoids and from there into the peripheral blood.

Also, there was a positive significant correlation between fibronectin and bilirubin ($r = 0.554$; $P < 0.0001$) **Fig. 3 (D)**. Our results are in a line with **Soresi et al.** ⁽⁴⁴⁾ who found that a significant correlation between fibronectin and the total bilirubin ($p < 0.005$). **Christopher and Heather** ⁽⁴⁵⁾ reported that the elevation of bilirubin

is an indicator of chronic hepatitis and severity of fibrosis. **Swan** ⁽⁴⁶⁾ reported that liver cell injury is indicated when the total bilirubin level is high while indirect bilirubin is low. Hepatitis C infection can slow the processing of bilirubin in the liver and bilirubin levels become elevated, causing Jaundice.

While, there was a negative significant correlation between fibronectin and albumin ($r = -0.497$; $P < 0.0001$) **Fig. 3 (E)**. Our results are in accordance with **de Angelis *et al.*** ⁽⁴⁷⁾ who found a significant inverse relationship between fibronectin and serum albumin. **Fontana and Lock** ⁽⁴⁸⁾ reported that a seriously damaged liver is unable to produce sufficient albumin. **Bioshop *et al.*** ⁽²⁵⁾ found that most proteins are produced by the liver. A decreased serum albumin may be as a result of decreased liver protein synthesis. The albumin level correlated well with the severity of functional impairment and is found more often in chronic rather than in acute liver disease.

CONCLUSION

In conclusion, serum fibronectin showed satisfactory reproducibility and may be simple, rapid, sensitive, and suitable for routine use to differentiate HCV infected patients with liver fibrosis from patients without fibrosis as alternative diagnostic method to liver biopsy.

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