

## ASSESSMENT OF LIVER FIBROSIS DEVELOPMENT IN CHRONIC HEPATITIS B PATIENTS BY SERUM HYALURONIC ACID AND LAMININ LEVELS

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**SUUMARY** – The aim of this work was to determine serum cut-off levels of hyaluronic acid (HA) and laminin (LN) as predicting factors for liver necro-inflammatory injuries, highlighting their diagnostic and therapeutic follow up value in chronic hepatitis B patients. Serum HA and LN were measured in chronic hepatitis B patients (n=35) by ELISA and compared with control group (n=20). Liver histopathologic parameters were evaluated according to the modified Knodell score. The mean serum HA and LN concentrations in patients (108.4±58.7 and 95.0±20.1 ng/mL, respectively) were greater than those in controls (46.6±10.6 and 46.1±10.2ng/mL, respectively;  $P<0.001$ ). Serum levels of HA and LN in all stages and grades of hepatic fibrosis and inflammation were significantly higher than those in the control group of healthy subjects ( $P<0.05$ ). Cut-off levels of 52.0 ng/mL HA and 64.0 ng/mL LN in serum for discrimination of patients with liver fibrosis from those without liver fibrosis showed acceptable AUC, sensitivity and specificity. After 6 months of treatment, a decrease was observed in serum HA and LN levels; however, the levels were still higher than those in the control group ( $P<0.05$ ). The correlation of fibrosis stages (not inflammation grades) with serum HA and LN levels was significant ( $P<0.01$ ). The findings suggested the increase in serum HA and LN concentrations above the predictive values (52.0 ng/mL HA and 64.0 ng/mL LN, respectively) to be associated with liver fibrosis. Therefore, serum HA and LN levels could be determined as an additional clinical tool for evaluation of liver fibrosis, when liver biopsy is impossible to perform.

**Key words:** *Hepatitis B, chronic – blood; Biological markers – blood; Hyaluronic acid – blood; Liver cirrhosis*

### Introduction

Staging of liver fibrosis and evaluation of inflammation grade is useful for (a) estimating the rate of liver fibrosis progression; (b) choice of treatment; and (c) monitoring disease recovery. Liver biopsy is still the reference test for staging and grading of hepatic

fibrosis and inflammation. Therefore, due to some limitations of liver biopsy, noninvasive methods are preferred<sup>1</sup>.

Liver fibrosis is a process involving production and deposition of various components that constitute the extracellular matrix. Some of these components are noncollagenous glycoproteins such as laminin, collagens, hyaluronic acid, and proteoglycans. In recent years, some studies have proposed hyaluronic acid (HA) and laminin (LN) as indices of the extent of liver fibrosis in chronic liver diseases<sup>1,2</sup>.

Hyaluronic acid is a linear polymer distributed in the extracellular spaces and built from D-glucuronic

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acid and N-acetyl-D-glucosamine. In the liver, HA is produced by the hepatic stellate cells and degraded by the sinusoidal endothelial cells. An increase in HA levels was observed in chronic hepatitis C and cirrhosis patients<sup>2</sup>.

One of the main glycoproteins of the basement membrane is LN, which is produced by hepatocytes and sinusoidal cells of the liver<sup>3</sup>. An increase in serum LN levels was observed in advanced stages of fibrosis (in patients with hepatic disease)<sup>4,7</sup>. Kropf *et al.* report that determination of LN and HA serum concentrations are sensitive tests for diagnosis of hepatic fibrotic disease and portal hypertension<sup>8,9</sup>.

In the Iranian population, there are few published studies<sup>10,11</sup> concerning the relationship of HA and LN serum levels with liver fibrosis stage or inflammation grade in chronic hepatitis B patients. The published studies used the Ishak scoring system to assess liver fibrosis in liver biopsy, and there is no report on the relationship of HA and LN serum levels with liver necro-inflammatory injuries according to the modified Knodell score system. Therefore, the main aims of this study were: (a) determination of serum HA and LN cut-off levels as predictive factors for liver fibrosis and inflammation; (b) applicability of these tests in staging of necro-inflammatory injuries as compared with the results obtained by histologic tests (modified Knodell score system); and (c) treatment follow up by determination of changes in serum levels of HA and LN during treatment.

## Materials and Methods

### *Study population*

Among subjects referred to the Liver and Gastrointestinal Diseases Research Centre of Tabriz and Gonbad (north of Iran), 35 patients (20 men and 15 women, aged 35.6±11.8 years) were selected. Patients were included in the study if they were positive for serum hepatitis B surface antigen (HBsAg) and had persistently elevated serum aminotransferases greater than 1.5 upper limit of the reference range for at least six months. Patients with a history of gastrointestinal bleeding and other chronic liver diseases (chronic hepatitis C, autoimmune hepatitis, Wilson's disease, hemochromatosis, alpha1-antitrypsin deficiency, biliary disease, hepatocellular carcinoma), active intra-

venous drug abuse, and liver transplantation were excluded. Only patients that had fibrosis stage ≥1 were treated and followed up for at least six months. Patients were treated according to the standard protocol with interferon (3 million units 3 times *per week*) or adefovir (10 mg/day) or lamivudine (100 mg/day)<sup>12</sup>. All study patients underwent liver biopsy for assessment of liver fibrosis score and were subclassified according to the histological activity index (HAI) score.

Control sera for determination of HA and LN was obtained from 20 healthy volunteers referred to the Tabriz University of Medical Sciences (10 women and 10 men, mean age ± SD 42±14.7 years). These healthy persons had normal serum levels of aminotransferases and alkaline phosphatase (ALP), were negative for HBsAg, and therefore did not require liver biopsy. These subjects had no history of gastrointestinal bleeding and chronic liver disease, smoking (never smoker), alcohol intake (never drinker), no family history of hepatitis and liver disease, and no active intravenous drug abuse and liver transplantation, according to the information gathered in the questionnaire form. The subjects that smoked (>1 cigarette/day) and took alcohol drinks (>5 g/day) were classified as smokers and alcohol drinkers.

All subjects gave their written informed consent to use these data for scientific purposes and the Tabriz University of Medical Sciences Ethics Committee approved the study.

### *Blood sampling and analysis*

Blood samples (5 mL) were collected after an overnight fast on the day before treatment initiation. In addition, fasting venous blood (5 mL) was also sampled at two-month intervals, i.e. at two, four and six months of treatment initiation. Serum was separated at 2500 g for 5 minutes. Hepatitis B serology and liver function tests (LFT), including ALP, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were performed on aliquots of each sample using commercially available kits. In brief, ALP was assayed with PNPP (para nitrophenyl phosphate) as a substrate (Ziestchem kit, Tehran, Iran) and serum aminotransferases (AST and ALT) were measured according to Reitman and Frankel by colorimetric test (Ziestchem kit, Tehran, Iran) with 2, 4 DNPH (2, 4 dinitrophenylhydrazone) on an Apel spectrophotom-

eter (PD303S, Japan). The hepatitis B serology markers were analyzed on an ELISA reader (Norahan Fajr, Iran) using the following kits: hepatitis B surface antigen (HBsAg; Diakey, Shinjin Medics Inc. ELISA kit, Korea), hepatitis B surface antibody (HBsAb; Diakey, Shinjin Medics Inc. ELISA kit, Korea) and hepatitis B core antibody (HBcAb; Dia-Pro ELISA kit, Italy).

The rest of blood samples were stored at  $-20^{\circ}\text{C}$ . Serum HA and LN levels were determined in all sera upon completion of blood sampling.

Serum HA and LN were assayed using an ELISA reader (Immunoscan, Lab System, Switzerland) and the following kits, respectively: HA-ELISA Kit (HA-test, product number: K-1200, Echelon Bioscience Inc., USA) and LN EIA Kit (Takara Bio, code number: MK107).

#### *Serum HA assay*

Serum HA levels were determined by an HA-ELISA kit. The HA-ELISA method is a competitive ELISA in which the colorimetric signal is inversely proportional to the amount of HA present in the sample. The concentration of HA in the samples was determined from a standard curve using the reagent blank (0 ng HA/mL) and HA reference solutions (50, 100, 200, 400, 800 and 1600 ng HA/mL). Serum HA concentrations were determined in one analytical batch on one working day. The intra-assay variability (coefficient of variation, CV) of the procedure according to the manufacturer's declaration was 5%.

#### *Serum LN assay*

Serum LN concentrations were assayed using an LN EIA Kit. The LN EIA kit is a solid phase EIA based on a sandwich method that utilizes two mouse monoclonal anti-LN antibodies to detect LN by a two-step procedure. The amount of LN was determined by measuring the absorbance using an EIA plate reader. A standard curve of 5, 10, 20, 40, 80, 160 and 320 ng/mL LN was used to convert sample absorbance into ng LN/mL serum. The intra-assay and inter-assay variability (CV) of the procedure according to the manufacturer's declaration was 4.0%-5.7% and 0.3%-5.0%, respectively. Again serum LN concentrations were determined in one analytical batch on one working day. Control samples were analyzed in the

same manner, except that the control group provided blood only once at entry.

#### **Histologic assessment of liver damage**

All study patients underwent percutaneous liver biopsy (with a Trucut needle number 16 guided by B type ultrasound) to confirm the presence of liver fibrosis and to assess the severity of liver disease. Biopsy fragments were fixed in 10% formalin solution for 12 hours and fixed in paraffin. Sections were stained with hematoxylin-eosin, Masson's trichrome and reticulin stain to establish the histologic diagnosis and the extent of liver lesions. Specimens were graded and staged according to the modified Knodell scoring system<sup>13</sup>.

The fibrosis scores were determined on the basis of the following instructions: stage 0, no fibrosis; stage 1, fibrous expansion of some portal areas, with or without short fibrous septa; stage 2, fibrous expansion of most portal areas, with or without short fibrous septa; stage 3, fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging; stage 4, fibrous expansion of portal areas with marked bridging [portal to portal (P-P) as well as portal to central (P-C)]; stage 5, marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis); and stage 6, probable or definitive cirrhosis.

The grading system scored 0-18 and was based on sum of four indices: piecemeal necrosis (score 0-4), confluent necrosis (score 0-6), focal lytic necrosis (score 0-4), and portal inflammation (score 0-4)<sup>13</sup>.

#### *Statistical analysis*

All statistical analyses were done by SPSS version 12.0 for Microsoft Windows (SPSS Inc.) and a *P* value less than 0.05 was considered statistically significant. Numerical data were expressed as mean  $\pm$  SD. Mean of serum HA and LN levels of the patient group as a whole and various chronic hepatitis stages and control group were compared using nonparametric test (Mann-Whitney U test) and ANOVA model. Spearman's correlation coefficients (*r*<sub>S</sub>) were calculated to assess the relationship between the histologic degree of liver fibrosis stages (or inflammation grades) with the concentrations of serum HA and LN and LFT. To assess and compare diagnostic accuracy of serum HA and LN levels for discrimination of patients with

Table 1. Comparison of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities, hyaluronic acid (HA) and laminin (LN) levels (mean  $\pm$  SD) in patient and control groups (Mann-Whitney U test)

Variable	Patient group	Control group
ALT (IU/L)	110.4 $\pm$ 72.7‡	27.3 $\pm$ 6.4
AST (IU/L)	62.0 $\pm$ 34.5†	28.3 $\pm$ 6.5
ALP (IU/L)	228.4 $\pm$ 156.6†	130.6 $\pm$ 38.1
HA (ng/mL)	108.4 $\pm$ 58.7‡	46.6 $\pm$ 10.6
LN (ng/mL)	95.0 $\pm$ 20.1‡	46.1 $\pm$ 10.2

liver fibrosis from healthy individuals, we plotted the receiver operating characteristic curves (ROC) and calculated the areas under the curves (AUC) for comparison<sup>14</sup>. The ROCs were generated by plotting the relationship of true positivity (sensitivity) and false

positivity (1- specificity) at various cut-off points of the test. An AUC of 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value<sup>15</sup>. The diagnostic sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were also calculated.

## Results

Histologic examination of the liver for fibrosis scoring revealed fibrosis stage 0 in eight patients; these patients were excluded and the rest were treated and followed up. The results of laboratory tests, presented in Table 1, showed that serum ALT, AST and ALP levels in patient group were significantly higher than those in control group ( $P < 0.05$ ), and revealed the presence of hepatocellular disorders in the former. Table 1 also shows the mean serum HA and LN concentrations ( $\pm$ SD) in patients and healthy control

Table 2. Comparison of serum hyaluronic acid (HA) and laminin (LN) concentrations between patient subgroups (stages of liver fibrosis) and control group during treatment period

Liver fibrosis stage (n)		At entry (P value)	**2 <sup>nd</sup> sampling (P value)	**3 <sup>rd</sup> sampling (P value)	**4 <sup>th</sup> sampling (P value)
1 (n=11)	HA	86.3 $\pm$ 21.8 (0.000)	74.8 $\pm$ 19.2 (0.001)	75.9 $\pm$ 20.5 (0.001)	70.0 $\pm$ 22.1 (0.006)
	LN	85.3 $\pm$ 6.0 (0.000)	80.5 $\pm$ 5.5 (0.000)	77.0 $\pm$ 5.4 (0.000)	74.7 $\pm$ 6.9 (0.000)
2 (n=6)	HA	108.8 $\pm$ 20.5 (0.000)	103.3 $\pm$ 11.9 (0.000)	92.0 $\pm$ 12.9 (0.000)	90.6 $\pm$ 11.2 (0.000)
	LN	96.8 $\pm$ 7.9 (0.000)	93.3 $\pm$ 9.9 (0.000)	89.2 $\pm$ 11.9 (0.000)	89.0 $\pm$ 8.2 (0.000)
3 (n=5)	HA	132.8 $\pm$ 21.6 (0.000)	122.0 $\pm$ 16.6 (0.000)	114.2 $\pm$ 11.8 (0.000)	104.4 $\pm$ 12.8 (0.000)
	LN	101.8 $\pm$ 8.1 (0.000)	96.6 $\pm$ 7.6 (0.000)	88.8 $\pm$ 3.9 (0.000)	87.2 $\pm$ 4.9 (0.000)
4 (n=3)	HA	194.0 $\pm$ 14.2 (0.002)	173.6 $\pm$ 12.0 (0.001)	170.3 $\pm$ 7.1 (0.000)	146.6 $\pm$ 33.4 (0.034)
	LN	118.3 $\pm$ 4.5 (0.000)	107.6 $\pm$ 14.4 (0.013)	95.6 $\pm$ 9.0 (0.004)	91.6 $\pm$ 5.8 (0.000)
*5 (n=1)	HA	251.0	230.0	220.0	210.0
	LN	149.0	141.0	122.0	115.0
*6 (n=1)	HA	285.0	249.0	232.0	215.0
	LN	143.0	138.0	127.0	115.0

\*In fibrosis stages 5 and 6, presented by only one patient each, comparison was not performed; \*\*two, four and six months of treatment initiation.

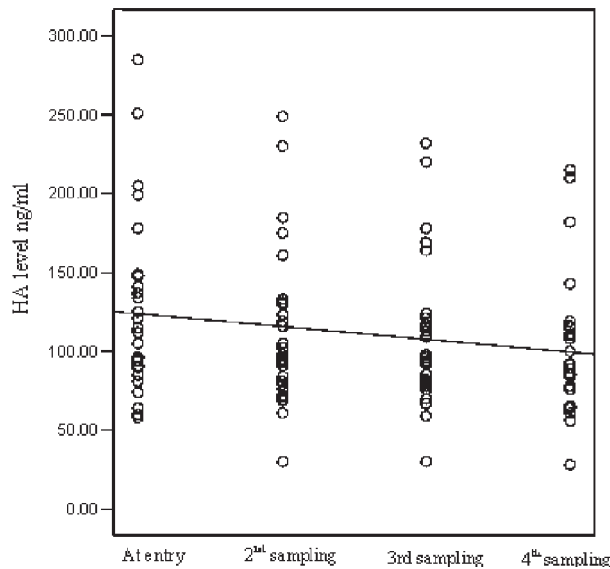


Fig. 1. Correlation analysis between mean serum hyaluronic acid (HA) concentrations at entry and two (2<sup>nd</sup>), four (3<sup>rd</sup>) and six (4<sup>th</sup> sampling) months of treatment initiation ( $rS=-0.193$ ;  $P=0.045$ ).

subjects. The results showed serum HA and LN levels to be statistically significantly increased in patients as compared with healthy controls ( $P<0.001$ ).

Tables 2 and 3 show comparison of serum HA and LN concentrations between patient subgroups (stages of fibrosis and grades of inflammation) and control group at entry and during the treatment period. As shown in these tables, serum levels of HA and LN were significantly higher in all stages of hepatic fibrosis and grades of inflammation as compared with control group ( $P<0.05$ ). The fibrosis stages 5 and 6, and inflammation grades 3, 5, 6 and 10-12 were present in only one patient each, and therefore could not be compared with the control group, thus only serum levels of HA and LN are presented. Also, there were no patients with inflammation grades 13-18. After the beginning of treatment, a gradual decrease in serum HA and LN levels was observed and was statistically significant. As shown in Figures 1 and 2, Spearman correlation coefficient ( $rS$ ) for HA and LN was  $rS=-0.193$  ( $P=0.045$ ) and  $rS=-0.326$  ( $P=0.001$ ), respectively. However, after six-month treatment, serum levels of HA and LN in these patients *versus* healthy control group were still high and the difference was statistically significant ( $P<0.05$ ).

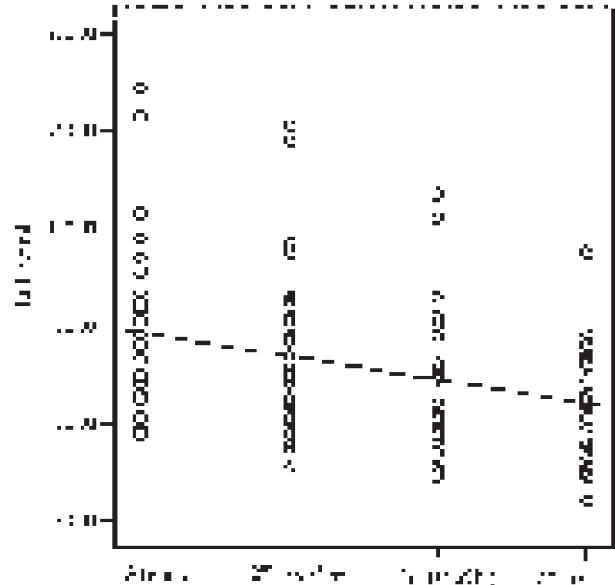


Fig. 2. Correlation analysis between mean serum laminin (LN) concentrations at entry and two (2<sup>nd</sup>), four (3<sup>rd</sup>) and six (4<sup>th</sup> sampling) months of treatment initiation ( $rS=-0.326$ ;  $P=0.001$ ).

Table 4 shows correlation analysis of the serum biochemical profile and histopathologic parameters of patients with chronic hepatitis B disease. Correlations between these parameters were calculated by the Spearman correlation coefficient.

Table 5 presents ROC data of HA and LN serum levels for discrimination of patients with liver fibrosis and control group. A cut-off level of 52.0 ng HA/mL and 64.0 ng LN/mL for discrimination of patients with liver fibrosis from those without liver fibrosis showed good sensitivity, specificity, PPV and NPV.

## Discussion

The mean serum HA and LN levels in patients were significantly higher than those in healthy controls ( $P<0.001$ ), as shown in Table 1. Our data are consistent with the work of other groups that have reported increased levels of serum HA and LN in chronic liver disease, particularly in patients with cirrhosis<sup>4-11,16</sup>.

Patients in higher stages of liver fibrosis and grades of inflammation had higher serum HA and LN concentrations (Tables 2 and 3). According to these tables, differences in serum levels of HA and LN in various sampling stages during the treatment protocol as



Table 3. Comparison of serum hyaluronic acid (HA) and laminin (LN) concentrations (mean  $\pm$  SD) between patient subgroups (grades of liver inflammation) and control group during treatment period

Liver inflammation grade (n)		At entry (P value)	** 2 <sup>nd</sup> sampling (P value)	** 3 <sup>rd</sup> sampling (P value)	** 4 <sup>th</sup> sampling (P value)
1 (n=5)	HA	127.4 $\pm$ 21.4 (0.000)	115.6 $\pm$ 16.4 (0.000)	105.4 $\pm$ 13.3 (0.000)	95.8 $\pm$ 9.5 (0.000)
	LN	99.0 $\pm$ 6.7 (0.000)	95.2 $\pm$ 6.3 (0.000)	92.0 $\pm$ 7.4 (0.000)	89.6 $\pm$ 6.0 (0.000)
2 (n=6)	HA	88.2 $\pm$ 28.3 (0.000)	78.8 $\pm$ 19.6 (0.000)	84.0 $\pm$ 22.2 (0.000)	68.3 $\pm$ 28.6 (0.000)
	LN	86.5 $\pm$ 10.2 (0.000)	82.5 $\pm$ 12.3 (0.000)	80.1 $\pm$ 11.8 (0.000)	77.5 $\pm$ 11.0 (0.000)
*3 (n=1)	HA	81.0	76.0	70.0	65.0
	LN	90.0	85.0	82.0	83.0
4 (n=8)	HA	99.3 $\pm$ 25.5 (0.000)	88.6 $\pm$ 28.9 (0.013)	81.7 $\pm$ 24.4 (0.004)	84.6 $\pm$ 18.7 (0.000)
	LN	92.2 $\pm$ 10.2 (0.000)	86.8 $\pm$ 8.7 (0.013)	80.1 $\pm$ 5.9 (0.004)	81.2 $\pm$ 9.1 (0.000)
*5 (n=1)	HA	90.0	94.0	82.0	86.0
	LN	89.0	84.0	78.0	76.0
*6 (n=1)	HA	134.0	123.0	121	119.0
	LN	103.0	101.0	92.0	83.0
8 (n=2)	HA	242.0 $\pm$ 60.8 (0.000)	217.0 $\pm$ 45.2 (0.000)	198.0 $\pm$ 48.0 (0.000)	179.0 $\pm$ 50.9 (0.000)
	LN	133.0 $\pm$ 14.1 (0.000)	127.5 $\pm$ 14.8 (0.000)	109.5 $\pm$ 24.7 (0.000)	105.5 $\pm$ 13.4 (0.000)
*10 (n=1)	HA	251.0	230.0	220.0	210.0
	LN	149.0	141.0	122.0	115.0
*11 (n=1)	HA	205.0	175.0	178.0	182.0
	LN	114.0	91.0	89.0	85.5
*12 (n=1)	HA	178.0	161.0	169.0	115.0
	LN	118.0	115.0	106.0	115.0

\*In inflammation grades 3, 5, 6 and 10-12, presented by only one patient each, comparison was not performed; also, we had no patients with inflammation grades 7, 9 and 13-18; \*\*two, four and six months of treatment initiation.

compared with healthy controls were statistically significant ( $P < 0.05$ ). In early stages of liver fibrosis (stages 0-2) and inflammation grades (grades 0-5), serum HA and LN concentrations were high and the highest levels were observed in liver fibrosis stages  $\geq 3$  and inflammation grade  $\geq 6$ . It seems that the progression of liver fibrosis and inflammation was accompanied by impairment in the liver endothelial cell function and reduced degradation of these components, eventually

resulting in elevation of serum HA and LN concentrations. Castera *et al.*<sup>7</sup> report that LN concentration increases in early stages of chronic liver disease and the highest concentrations were recorded in active cirrhosis and chronic active hepatitis. In other reports, it is proposed that determination of serum levels of LN and HA can be used for the diagnosis of hepatic fibrotic disease and portal hypertension<sup>8,9</sup>. Korner *et al.* report that among various serum parameters such as

LN, A1-apolipoprotein, prothrombin time and HA, serum HA level was most strongly correlated with the stage of liver fibrosis, and can be used for long-term monitoring of disease progression<sup>17</sup>.

After the beginning of treatment, a gradual decrease in serum HA and LN level were observed, but after six months of treatment, serum levels of HA and LN in these patients was still higher as compared with control group, were the differences being statistically significant. In a study by Guechot *et al.*<sup>18</sup>, HA serum concentrations were significantly lower after treatment in chronic hepatitis patients. In another study, HA levels were demonstrated to decrease during treatment in parallel to improvement in fibrosis staging; clinical usefulness of HA was its ability to exclude patients with significant fibrosis and cirrhosis<sup>19</sup>.

In our study, there was a significant relationship between serum levels of HA and LN and liver fibrosis stages ( $P<0.01$ ) both at entry and during the treatment, but the correlation with inflammation grades

revealed a different pattern. At entry, the correlation was statistically significant ( $P<0.01$ ), but during the treatment the correlation was not statistically significant. Maybe the treatment protocol led to a decrease in inflammation factors and caused regeneration of liver endothelial cells. Thus regenerated endothelial cells better metabolized HA and LN, and a decrease in serum levels of HA and LN occurred and caused an indefinite correlation.

As shown in Table 4, the correlations between stages of fibrosis and inflammation grades with serum aminotransferases and ALP were not statistically significant, but the grades of inflammation showed better correlation with aminotransferases. Interestingly, we also found that serum HA levels had good correlation with serum LN levels at entry and during the treatment protocol ( $P<0.01$ ). This suggested that during the liver fibrinogenesis, extracellular matrix components such as HA and LN increased simultaneously.

	Stage	Grade	HA1	HA2	HA3	HA4	LN1	LN2	LN3	LN4	AST	ALT	ALP	
Stage	-													
Grade	rS P	0.638 0.000	-											
HA1	rS P	0.916 0.000	0.632 0.000	-										
HA2	rS P	0.888 0.000	0.406 0.036	0.914 0.000	-									
HA3	rS P	0.864 0.000	0.351 0.072	0.870 0.000	0.944 0.000	-								
HA4	rS P	0.811 0.000	0.449 0.019	0.927 0.000	0.902 0.000	0.851 0.000	-							
LN1	rS P	0.903 0.000	0.544 0.002	0.891 0.000	0.918 0.000	0.812 0.000	0.856 0.000	-						
LN2	rS P	0.817 0.000	0.327 0.096	0.800 0.000	0.888 0.000	0.805 0.000	0.806 0.000	0.943 0.000	-					
LN3	rS P	0.756 0.000	0.211 0.291	0.720 0.000	0.772 0.000	0.756 0.000	0.708 0.000	0.844 0.000	0.945 0.000	-				
LN4	rS P	0.755 0.000	0.183 0.360	0.678 0.000	0.714 0.000	0.651 0.000	0.655 0.000	0.797 0.000	0.861 0.000	0.876 0.000	-			
AST	rS P	-0.110 0.596	0.326 0.104	0.036 0.860	0.112 0.610	0.029 0.097	0.090 0.689	0.094 0.617	0.135 0.549	0.011 0.962	0.037 0.069	-		
ALT	rS P	0.151 0.460	0.284 0.160	0.241 0.237	0.230 0.308	0.196 0.383	0.067 0.766	0.302 0.133	0.231 0.300	0.205 0.359	0.101 0.654	0.649 0.000	-	
ALP	rS P	0.193 0.267	0.042 0.845	0.250 0.239	0.317 0.173	0.302 0.195	0.138 0.562	0.409 0.047	0.512 0.021	0.512 0.021	0.384 0.094	0.074 0.730	0.115 0.592	-

Table 4. Correlation analysis of serum and histopathologic parameters in chronic hepatitis B patients (correlations between these parameters were calculated by Spearman correlation coefficient)

Liver biopsy evaluation: stage = stage of fibrosis; grade = grade of inflammation; laboratory parameters: HA1 and LN1 = serum HA and LN levels at entry; HA2-4 and LN2-4: serum HA and LN levels at two, four and six months of treatment initiation; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; statistics: rS = Spearman correlation coefficient; P = P value; † $P<0.05$ ; ‡ $P<0.01$ .

Table 5. ROC curve of serum hyaluronic acid (HA) and laminin (LN) levels for discrimination of patients with liver fibrosis and control group (HA cut-off level = 52.0 ng/mL and LN cut-off level = 64.0 ng/mL)

Variable	AUC (CI=95%)	P- value	Sensitivity %	Specificity %	PPV %	NPV %
HA	0.962 (0.861-0.992)	0.000	91.4	80	88.8	84.2
LN	0.986 (0.962-1.009)	0.000	94.3	90	94.3	90

AUC = area under the curve; CI = confidence interval; PPV = positive predictive value; NPV = negative predictive value.

The cut-off levels of 52.0 ng HA/mL and 64.0 ng LN/mL for discrimination of patients with liver fibrosis from those without liver fibrosis showed good sensitivity, specificity, PPV and NPV. The AUCs of serum HA and LN in ROC analysis were 0.962 and 0.986, respectively. It means that serum HA and LN can discriminate patients with liver fibrosis and healthy individuals. Comparable results were obtained for the diagnosis of liver fibrosis at the same cut-off values in other studies<sup>20,21</sup>.

Several mechanisms have been proposed for the elevation of serum HA and LN levels in chronic hepatitis patients. Increased production of these components in the liver and the lack of degradation by liver endothelial cells are the major causes of elevation of serum HA and LN levels in chronic hepatitis patients as compared with healthy individuals<sup>1</sup>.

In conclusion, the findings of this study suggest that measurement of serum HA and LN concentrations can discriminate between patients with liver fibrosis (but not inflammation grades) and healthy individuals and can give information about progressive changes in the liver with reduced function of liver endothelial cells. It seems that serum HA and LN concentrations are associated with histopathologic changes in chronic liver disease according to the modified Knodell scoring system and an increase in serum concentrations of HA and LN above the predictive value (HA cut-off level = 52.0 ng/mL and LN cut-off level = 64.0 ng/mL) is related to liver fibrosis. Therefore, we can determine serum HA and LN levels as an additional clinical tool for evaluation of liver fibrosis. In addition, it seems that measurement of serum HA and LN levels can be useful for long-term monitoring of disease progression during the treatment protocol, where liver biopsy cannot be performed.

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### Sažetak

## PROCJENA RAZVOJA JETRENE FIBROZE U BOLESNIKA S KRONIČNIM HEPATITISOM B ODREĐIVANJEM HIJALURONSKE KISELINE I LAMININA

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Cilj ove studije bio je utvrditi prijelomne vrijednosti hijaluronske kiseline (HA) i laminina (LN) u serumu kao čimbenike koji predskazuju nekrotično-upalna oštećenja jetre, te ukazati na njihovu vrijednost u dijagnosticiranju i praćenju terapijskog učinka u liječenju bolesnika s kroničnim hepatitisom B. Razine HA i LN u serumu mjerile su se kod bolesnika s kroničnim hepatitisom B (n=35) pomoću metode ELISA i usporedile s razinama u kontrolnoj skupini (n=20). Jetreni histopatološki parametri procjenjivali su se prema modificiranom Knodellovom bodovnom sustavu. Srednje serumske koncentracije HA i LN bile su više kod bolesnika (108,4±58,7 odnosno 95,0±20,1 ng/mL) nego u kontrolnoj skupini (46,6±10,6 odnosno 46,1±10,2 ng/mL;  $P<0,001$ ). U usporedbi s kontrolnom skupinom zdravih ispitanika serumske razine HA i LN bile su značajno više u svim stadijima jetrene fibroze i stupnjevima upale u skupini bolesnika ( $P<0,05$ ). Prijelomne vrijednosti od 52,0 ng/mL HA i 64,0 ng/mL LN u serumu za razlikovanje bolesnika s jetrenom fibrozom od onih bez nje pokazale su prihvatljivu vrijednost AUC, osjetljivost i specifičnost. Nakon 6 mjeseci liječenja zabilježeno je sniženje serumskih razina HA i LN, ali su te razine još uvijek bile više od onih u kontrolnoj skupini ( $P<0,05$ ). Korelacija stadija fibroze (ne i stupnjeva upale) sa serumskim razinama HA i LN bila je značajna ( $P<0,01$ ). Ovi nalazi ukazuju na to da je porast serumskih koncentracija HA i LN iznad prediktivnih vrijednosti (52,0 ng/mL HA i 64,0 ng/mL LN) udružen s jetrenom fibrozom. Stoga bi se serumske razine HA i LN mogle određivati kao dodatno kliničko sredstvo u procjenjivanju jetrene fibroze kad nije moguća biopsija jetre.

*Ključne riječi: Hepatitis B, kronični – krv; Biološki biljezi – krv; Hijaluronska kiselina – krv; Jetrena ciroza*

