Serum laminin, type IV collagen and hyaluronan as fibrosis markers in non-alcoholic fatty liver disease

V.N. dos Santos[1],
M.M.B. Leite-Mór[1],
M. Kondo[1], J.R. Martins[2],
H. Nader[2], V.P. Lanzoni[3]
and E.R. Parise[1]

[1]Seção de Gastroenterologia, Departamento de Medicina,
[2]Divisão de Biologia Molecular, Departamento de Bioquímica,
[3]Departamento de Patologia, Universidade Federal de São Paulo,
São Paulo, SP, Brasil

Abstract

Correspondence

E.R. Parise
Rua Botucatu, 740, 2º andar
03411-900 São Paulo, SP
Brasil
Fax: +55-11-5549-1711
E-mail: parise@gastro.epm.br

Hepatic fibrosis in patients with non-alcoholic fatty liver disease is associated with progression of the disease. In the present study, we analyzed the discriminative ability of serum laminin, type IV collagen and hyaluronan levels to predict the presence of fibrosis in these patients. In this preliminary report, we studied 30 overweight patients divided into two groups according to the absence (group I, N = 19) or presence (group II, N = 11) of fibrosis in a liver biopsy. Triglycerides, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl-transpeptidade, hyaluronan (noncompetitive fluoroassay), type IV collagen, and laminin (ELISA) were determined. Group II presented significantly higher mean laminin, hyaluronan, type IV collagen, and aspartate aminotransferase values, which were due to the correlation between these parameters and the stage of fibrosis in the biopsy (Spearman's correlation coefficient, rS = 0.65, 0.62, 0.53, and 0.49, respectively). Analysis of the ROC curve showed that laminin values >282 ng/ml were those with the best diagnostic performance, with 87% accuracy. Association of laminin with type IV collagen showed improvement in the positive predictive value (100%), but with reduction in diagnostic sensitivity (64%). When compared with the criteria of Ratziu et al. [Gastroenterology (2000) 118: 1117-1123] for the diagnosis of septal fibrosis, laminin values presented a better diagnostic accuracy (83 *vs* 70%). Determination of extracellular matrix components in serum, especially of laminin, may identify patients with non-alcoholic fatty liver disease and fibrosis and these components may be used as indicators for liver biopsy in these patients.

Introduction

Non-alcoholic fatty liver disease (NAFLD) affects approximately 20% of the general population (1,2). Most of these patients present steatosis alone or associated only with an inflammatory infiltrate, without degenera-tive or fibrotic alterations. The follow-up of these patients shows that the disease is stable (3,4). However, in about 2-3% of the population steatosis will be accompanied by bal-looning degeneration, Mallory's hyaline bod-ies and fibrosis, characterizing non-alcoholic steatohepatitis (NASH) which may progress

to cirrhosis and liver failure (4-6).

Differentiation between these NAFLD forms can be safely performed only by liver biopsy (4,7-9). However, due to the high prevalence of the disease, it would be necessary to find noninvasive parameters which would allow the selection of patients at a higher risk of developing cirrhosis to be submitted to a biopsy, despite the slight risk of mortality and morbidity involved.

The presence of fibrosis in the biopsy is accompanied by progression of the disease to cirrhosis and liver failure in 15 to 26% of cases (3-6), thus constituting a criterion of NAFLD severity. On the other hand, extracellular matrix components, especially type III and IV collagens (COL-IV), laminin (LM) and hyaluronic acid (HA), have been used as indirect fibrogenesis markers in many chronic liver diseases, such as hemochromatosis, viral hepatitis and alcoholic liver disease (10-13).

In the present study, we assessed the discriminative ability of serum LM and COL-IV to predict the presence of fibrosis in NAFLD in patients with compensated disease, without clinical or ultrasound signs of cirrhosis or portal hypertension.

Patients and Methods

Thirty patients with body mass index (BMI) >25, with an ultrasound diagnosis of liver steatosis and with a persistent increase of at least 1.5 times the upper limit of normality of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and/or γ-glutamyltranspeptidase (γ-GT) levels for a minimum period of three months were studied. All patients were submitted to a percutaneous liver biopsy.

Patients ingesting more than 20 g alcohol per day, with decompensated non-insulin-dependent diabetes mellitus (NIDDM), taking hepatotoxic drugs, with systemic diseases or infections, those presenting hepatitis B or C virus or other concomitant liver disease were excluded. Patients with clinical and/or ultrasound signs of liver failure and/or portal hypertension were also excluded.

Serum COL-IV and LM concentrations were determined by ELISA with mouse monoclonal antibodies against human LM and COL-IV using commercial Fuji Chemical Ind. (Takaoka, Tokyo, Japan) kits.

HA determinations were performed using a noncompetitive fluorescence-based assay with specific binding proteins from bovine cartilage which were later used in solid phase on ELISA plates and with biotinylated probes (14,15).

Biopsy fragments were stained with hematoxylin-eosin, Masson's trichrome, Prussian blue (Perls' test), and reticulum impregnation with silver (Gomori).

NAFLD grading of liver tissue samples was performed according to Lee (16), Brunt et al. (17), and Dixon et al. (18). Liver steatosis was classified according to the percentage of fatty vacuole-containing hepatocytes: grade 0 (absent), grade 1 (<5% of the hepatocytes), grade 2 (5-25% of the hepatocytes), grade 3 (25-75% of the hepatocytes), grade 4 (>75% of the hepatocytes). Lobular inflammation, portal inflammation, hepatocyte ballooning, and Mallory's hyaline bodies were also evaluated. Fibrosis was classified into 0 = absent; stage 1 = perisinusoidal/pericellular fibrosis in zone 3; stage 2 = perisinusoidal/pericellular fibrosis in zone 3, with focal or diffuse periportal fibrosis; stage 3 = perisinusoidal/pericellular fibrosis and focal portal fibrosis with focal or diffuse bridging fibrosis; stage 4 = cirrhosis. Patients with cirrhosis and portal fibrous expansion with emission of septa of any intensity were classified as patients with septal fibrosis.

NAFLD was classified on the basis of histological data using the classification of Matteoni et al. (4): type 1 (isolated liver steatosis); type 2 (liver steatosis and lobular inflammation); type 3 (presence of hepatocyte ballooning); type 4 (presence of Mallory's hyaline bodies and/or fibrosis).

After evaluation of the histological pa-

rameters, the patients included in the study were divided into 2 groups: group I (patients with type 1, 2 and 3 NAFLD) and group II (patients with type 4 NAFLD, with fibrosis).

BMI, age, ALT, and triglyceride concentrations (BAAT criteria) were considered for the evaluation of parameters indicating fibrosis in overweight patients according to the criteria proposed by Ratziu et al. (19).

The Mann-Whitney test and the Spearman nonparametric correlation coefficient were used for statistical analysis. The discriminative power of each variable was assessed on the basis of positive predicting value, negative predicting value and accuracy, with determination of the cut-off of the area under the ROC curve. The level of significance was set at 5% in all analyses (20,21).

Results

A total of 186 patients were originally evaluated, 156 of whom were excluded for various reasons, the main cause being the ingestion of more than 20 g/day alcohol. Refusal to submit to a biopsy, use of hepatotoxic drugs, and signs of advanced hepatopathy were other frequent causes of exclusion.

Of the 30 patients included, 18 were males. Mean age was 44.9 years. All patients presented BMI >25, with a mean of 31. Eight patients (26.7%) presented hepatomegaly with no signs suggestive of hepatocellular failure.

Besides obesity, 7 patients (23.3%) presented NIDDM and dyslipidemia, without identification of any other risk factor for NAFLD development.

Based on the histological data obtained, 6 patients were classified as type 1, 10 as type 2, 3 as type 3, and 11 as type 4 NAFLD according to the criteria proposed by Matteoni et al. (4).

Upon histological analysis all patients presented steatosis, with 43.3% of the cases being grades 1 and 2. No correlation be-

tween steatosis grade and severity of NAFLD classification was observed. Fibrosis was found in 11 (36.7%) of the 30 patients evaluated.

The groups, separated according to the presence or absence of fibrosis, did not differ regarding age, gender or the presence of signs and symptoms on the occasion of diagnosis. They also did not differ regarding γ-GT, ALT levels and AST/ALT ratio. Mean AST levels as well as serum COL-IV, LM and HA levels were significantly higher in group II (Table 1).

Serum LM levels correlated significantly with fibrosis grade (P = 0.65), Mallory's bodies (P = 0.51) and lobular inflammation (P = 0.50), the same being observed for HA (P = 0.62), COL-IV (0.53), and AST (0.49) levels. Figure 1 shows the correlation be-

Table 1. Biochemical (serum) and demographic parameters of patients with non-alcoholic fatty liver disease with (group I) and without (group II) fibrosis.

Parameter	Group I (N = 19)	Group II (N = 11)
Gender (male/female)	11/8	7/4
Age (years)	41.6 ± 2.90	50.5 ± 4.10
BMI	31.1 ± 1.00	31 ± 2.40
ALT (IU/l)	60.6 ± 11.5	105.8 ± 34.3
AST (IU/l)	36.4 ± 5.90	47.6 ± 3.10*
AST/ALT ratio	0.7 ± 0.1	0.7 ± 0.1
γ-GT (IU/l)	84.9 ± 12.5	118.4 ± 30.4
Type IV collagen (ng/ml)	116.7 ± 4.6	149.3 ± 12*
Laminin (ng/ml)	244.7 ± 11.9	381.4 ± 38.9*
Hyaluronan (ng/ml)	25.6 ± 7.0	73.5 ± 23.5*

Data are reported as means ± SEM. BMI = body mass index; ALT = alanine aminotransferase; AST = aspartate aminotransferase; γ-GT = γ-glutamyltranspeptidase.
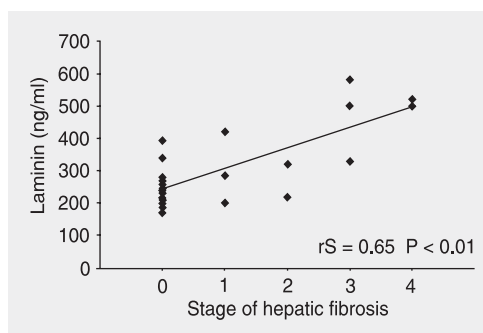*P < 0.05 compared to group I (Mann-Whitney test).

Figure 1. Correlation between serum laminin concentrations and grade of fibrosis in liver biopsies. Correlation between these parameters was calculated by the Spearman nonparametric correlation coefficient (rS).

tween serum LM values and grade of fibrosis in liver biopsy.

Once the discriminative values of each parameter were evaluated, we observed that, based on the ROC curve, the best diagnostic accuracy in the identification of patients with NAFLD and fibrosis was obtained with serum LM levels, followed by AST and HA values. Association of serum LM values with HA, COL-IV or AST determinations showed improvement in the positive predictive value (specially for the combination of LM with COL-IV values), but with an important reduction in diagnostic sensitivity (Table 2).

Serum LM values were then compared with BAAT criteria (22) considering the diagnosis of septal fibrosis (stages 2 to 4). In such situation, LM determination presented a better diagnostic performance than the BAAT score (Table 3).

Discussion

NAFLD is currently the most prevalent liver disease worldwide. Since it may be associated with different risk factors, we included in this study only overweight individuals who did not present significant variation in body weight during the last three months. These patients presented a high NAFLD prevalence (23-25), often associated with the presence of fibrosis (7).

Table 2. Discriminative serum concentrations of extracellular matrix components and of aspartate aminotransferase alone or associated with laminin values for the identification of fibrosis in patients with non-alcoholic fatty liver disease.

	SEN	SP	PPV	NPV	Accuracy
Laminin (>282 ng/ml)	82	89	82	89	87
Type IV collagen (>145 ng/ml)	64	89	78	81	80
Hyaluronan (>24.6 ng/ml)	82	68	60	87	73
AST (>44 IU/l)	64	79	70	75	73
Laminin + hyaluronan	73	95	89	86	87
Laminin + type IV collagen	64	100	100	83	87
Laminin + AST	55	95	86	78	80

Data are reported as percent. SEN = sensitivity; SP = specificity; PPV = positive predictive value; NPV = negative predictive value; AST = aspartate aminotransferase.

With the potential progress of NAFLD to cirrhosis, an adequate evaluation of these patients is required in order to define the prognosis of the disease. Retrospective studies have indicated that, if steatosis associated or not with an inflammatory infiltrate is detected in a biopsy, no progression of the disease is observed even during follow-up of the patients for a long period of time (3,4). On the other hand, in patients with associated ballooning degeneration and/or Mallory's bodies with or without fibrosis, the development of cirrhosis can be observed in up to 30% of cases (4,26,27). In addition, the presence of liver fibrosis, even in its initial form, seems to be a good marker for the evolution of the disease, as observed by Matteoni et al. (4).

Therefore, based on a liver biopsy, patients with liver fibrosis (group II) were separated from the other NAFLD patients (group I) in the expectation that this increase in fibrogenic activity could be detected by serum determination of extracellular matrix components.

No differences were observed between these groups regarding clinical or demographic characteristics (gender, age, BMI, present signs and symptoms), or ALT and γ-GT levels and the AST/ALT ratio. Several studies have shown that serum ALT and γ-GT and the AST/ALT ratio may represent indicators of advanced liver disease and fibrosis (3,8,19,26,28). In particular, an AST/ALT ratio >1 would be observed in patients with a more advanced grade of liver fibrosis, such as cirrhosis and septal fibrosis (4,7). In our patients, the AST/ALT ratio was less than 1 in both groups, certainly reflecting the selection criterion by which patients with signs of liver failure and with manifest portal hypertension were excluded from this study.

However, AST levels were significantly higher in group II patients, as also reported by others (4,7,29). Such difference seems to be due to the significant correlation observed between this parameter and the grade of

fibrosis in the biopsy. It is believed that AST clearance by endothelial liver cells is decreased in individuals with advanced fibrosis, possibly explaining the correlation observed (30).

The extracellular matrix components studied presented significantly higher values in patients with liver fibrosis than in those without this histological finding. Among the extracellular matrix components, we selected for this study those with the highest correlation with parenchymatous and perisinusoidal fibrosis as it occurs in NAFLD (31-33). LM is the largest protein of the basement membrane and in chronic liver diseases it is progressively deposited along Disse's space accompanying sinusoid capillarization (34,35). In these patients, its serum values have been related to the degree of portal hypertension (13,36,37) and, together with HA determination, to the diagnosis of fibrosis (31). COL-IV is the main collagen component of the basement membrane, being co-distributed with LM in the perisinusoidal space (32,35). Studies on patients with chronic liver disease have demonstrated a relationship between serum COL-IV values and severity of liver fibrosis (22,32). HA is a non-sulfated glycosaminoglycan synthesized in the liver by stellate cells. Its serum concentration significantly increases in chronic diseases of the liver of different etiologies, due not only to a greater hepatic production but also to a decrease in its degradation by endothelial liver cells (33,38). In these patients, especially those with disease of viral and alcoholic etiology, increased levels of this glycosaminoglycan have been detected and were shown to be related to the degree of fibrosis in liver tissue (38,39).

We confirmed this association between serum concentration of these extracellular matrix components and the degree of fibrosis in patients with NAFLD. In addition, we also observed a significant correlation of such parameters with the presence of Mallory's bodies which are associated with

a higher fibrogenic activity of the disease (4, 18). Type IV collagen, instead, was the only parameter correlated with hepatocyte ballooning and steatosis grade, suggesting a relationship between this basement membrane protein and lipid metabolism. However, the mechanism of this interaction is still unknown.

In attempts to obtain a noninvasive diagnosis of fibrosis, several parameters have been proposed (7,18,19). Since our study only included overweight patients, it was closer to that by Ratziu et al. (19) who proposed an index of fibrosis predictors based on BAAT criteria. Comparing the discriminative ability of blood LM concentrations with these BAAT criteria, it can be observed that LM values presented a better diagnostic performance for the diagnosis of septal fibrosis. It should be emphasized that the values observed here were similar to those described in the original paper by the cited authors.

It is important to note that the patients in the present study did not present any clinical or ultrasound sign of chronic liver disease, exactly because our intention was to assess patients who did not present the stigmata of liver failure or indirect signs of liver cirrhosis, in need of a biopsy to define the diagnosis and prognosis of the disease. Thus, in this type of patient, the finding of high LM values would be associated with liver fibrosis in 82% of the cases and would present a false-

Table 3. Discriminative values of laminin and of BAAT criteria (body mass index, age, alanine aminotransferase and triglyceride concentrations) for the diagnosis of septal fibrosis and cirrhosis in overweight patients with non-alcoholic fatty liver disease.

Parameter	PPV	NPV	Accuracy
Laminin	64	95	83
BAAT	47	93	70

Data are reported as percent. PPV = positive predictive value; NPV = negative predictive value.

negative result in about 10% of the cases. Therefore, LM determination could be used for the screening of patients to be submitted to a liver biopsy, since it presents a better performance than the criteria currently used to indicate a biopsy for these patients (7,19).

On the other hand, the good performance observed for serum AST levels should be emphasized. Differentiation of advanced NAFLD forms according to AST values has also been reported by other authors (4,18), who, however, did not evaluate the discriminative ability of this determination. Since the observed cut-off value represents a 1.5-fold increase in the upper limit of normality and since it is an easily obtained biochemical parameter, it is especially attractive for the selection of patients for biopsy.

The results of the present study show the important contribution made by the determination of extracellular matrix components, especially LM, and also of AST levels, to the selection of patients with NAFLD for a liver biopsy, in order to identify patients with the progressive forms of the disease. Studies involving a larger number of patients and repeated determinations of these parameters should be performed for a better evaluation of their prognostic value in NAFLD.

References

1. Clark JM, Brancati FL & Diehl AM (2002). Nonalcoholic fatty liver disease. *Gastroenterology*, 122: 1649-1657.
2. Yu AS & Keeffe EB (2002). Nonalcoholic fatty liver disease. *Reviews in Gastroenterological Disorders*, 2: 11-19.
3. Teli MR, James OFW, Burt AD, Bennett MK & Day CP (1995). The natural history of nonalcoholic fatty liver: A follow-up study. *Hepatology*, 22: 1714-1719.
4. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC & McCullough AJ (1999). Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology*, 116: 1413-1419.
5. Younossi ZM, Gramlich T, Liu YC, Matteoni C, Petrelli M, Goldblum J & Rybicki L (1998). Nonalcoholic fatty liver disease: assessment of variability in pathologic interpretations. *Modern Pathology*, 11: 560-565.
6. Falck-Ytter Y, McCullough AJ, Younossi ZM & Marchesini G (2001). Clinical features and natural history of nonalcoholic steatosis syndromes. *Seminars in Liver Disease*, 21: 17-26.
7. Angulo P, Keach JC, Batts KP & Lindor KD (1999). Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. *Hepatology*, 30: 1356-1362.
8. Neuschwander-Tetri BA (2002). Evolving pathophysiologic concepts in nonalcoholic steatohepatitis. *Liver*, 4: 31-36.
9. Garcia-Tsao G & Boyer JL (1993). Outpatient liver biopsy: how safe is it? *Annals of Internal Medicine*, 118: 150-153.
10. Luo R, Yang S, Xie J, Zhao Z, He Y & Yao J (2001). Diagnostic value of five serum markers for liver fibrosis. *Zhonghua Ganzangbing Zazhi*, 9: 148-150.
11. Gibson PR, Fraser JRE, Brown TJ, Finch CF & Jones PA (1992). Hemodynamic and liver function predictors of serum hyaluronan in alcoholic liver disease. *Hepatology*, 15: 1054-1059.
12. Niemelä O, Risteli J, Blake JE, Risteli L, Compton KV & Orrego H (1990). Markers of fibrogenesis and basement membrane formation in alcoholic liver disease. Relation to severity, presence of hepatitis and alcohol intake. *Gastroenterology*, 98: 1612-1619.
13. Kondo M, Miszputen S, Leite-Mor MMB & Parise ER (1995). The predictive value of serum laminin for the risk of variceal bleeding related to portal pressure levels. *Hepatogastroenterology*, 42: 542-545.
14. Hardingham TE & Adams P (1976). A method for the determination of hyaluronate in presence of other glycosaminoglycans and its application to human intervertebral disc. *Biochemical Journal*, 159: 143-147.
15. Martins JRM, Passerotti CC, Maciel RMB, Sampaio LO, Dietrich CP & Nader HBN (2003). Practical determination of hyaluronan by a new noncompetitive fluorescence-based assay on serum of normal and cirrhotic patients. *Analytical Biochemistry*, 319: 65-72.
16. Lee RG (1989). Nonalcoholic steatohepatitis: a study of 49 patients. *Human Pathology*, 20: 594-598.
17. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA & Bacon BR (1999). Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *American Journal of Gastroenterology*, 94: 2467-2474.
18. Dixon JB, Bhathal PS & O'Brien PE (2001). Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. *Gastroenterology*, 121: 91-100.
19. Ratziu V, Giral P, Charlotte F, Bruckert E, Thibault V, Theodorou I, Khalil L, Turpin G & Opolon P (2000). Liver fibrosis in overweight patients. *Gastroenterology*, 118: 1117-1123.
20. Zweig MH & Campbell G (1993). Receiver-operating characteristics (ROC) plots: a fundamental evaluation tool in clinical medicine (review). *Clinical Chemistry*, 39: 561-577.
21. Glantz AG (1992). *Primer of Bio-Statistics*. 4th edn. McGraw-Hill, Inc., New York.
22. Takamatsu S, Nakabayashi H, Okamoto Y & Nakano H (1997). Noninvasive determination of liver collagen content in chronic hepatitis. Multivariate regression modeling with blood chemical parameters as variables. *Journal of Gastroenterology*, 32: 355-360.
23. Andersen T, Christoffersen P & Gluud C (1984). The liver in consecutive patients with morbid obesity: a clinical, morphological, and biochemical study. *International Journal of Obesity*, 8: 107-115.
24. Hautekeete ML, Degott C & Benhamou JP (1990). Microvesicular

steatosis of the liver. *Acta Clinica Belgica*, 5: 311-326.

25. Willner IR, Waters B, Patil SR, Reuben A, Morelli J & Riely CA (2001). Ninety patients with nonalcoholic steatohepatitis: insulin resistance, familial tendency, and severity of disease. *American Journal of Gastroenterology*, 96: 2957-2961.

26. Powell EE, Cooksley WGE, Hanson R, Searle J, Halliday JW & Powell LW (1990). The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology*, 11: 74-80.

27. Pinto HC, Baptista A, Camilo ME, Valente A, Saragoça A & Moura MC (1996). Nonalcoholic steatohepatitis. Clinicopathological comparison with alcoholic hepatitis in ambulatory and hospitalized patients. *Digestive Diseases and Sciences*, 41: 172-179.

28. Marchesini G, Brizi M, Bianchi G, Tomassetti S, Bugianesi E, Lenzi M, McCulloug AJ, Natale S, Forlani G & Melchionda N (2001). Nonalcoholic fatty liver disease. A feature of the metabolic syndrome. *Diabetes*, 50: 1844-1850.

29. Sorbi D, Boynton J & Lindor KD (1999). The ratio of aspartate aminotransferase to alanine aminotransferase: potential value in differentiating nonalcoholic steatohepatitis from alcoholic liver disease. *American Journal of Gastroenterology*, 94: 1018-1022.

30. Sheth SG, Flamm SL, Gordon FD & Chopra S (1998). AST/ALT ratio predicts cirrhosis in patients with chronic hepatitis C virus infection. *American Journal of Gastroenterology*, 93: 44-48.

31. Kropf J, Gressner AM & Negwer A (1988). Efficacy of serum laminin measurement for diagnosis of fibrotic liver diseases. *Clinical Chemistry*, 34: 2026-2030.

32. Hahn E, Wick G, Pencev D & Timpl R (1980). Distribution of basement membrane proteins in normal and fibrotic human liver: collagen type IV, laminin, and fibronectin. *Gut*, 21: 63-71.

33. Fraser JRE, Laurent TC & Laurent UBG (1997). Hyaluronan: its nature, distribution, functions and turnover. *Journal of Internal Medicine*, 242: 27-33.

34. Orrego H, Medline A, Blendis LM, Rankin JG & Kreaden DA (1979). Collagenization of the Disse space in alcoholic liver disease. *Gut*, 20: 673-679.

35. Martinez-Hernandez A, Delgado FM & Amenta PS (1991). The extracellular matrix in hepatic regeneration. Localization of collagen types I, III, IV, laminin and fibronectin. *Laboratory Investigation*, 64: 157-166.

36. Parise ER & Rosa H (1992). Serum laminin in hepatic schistosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 86: 179-181.

37. Gressner AM (1991). Liver fibrosis: perspectives in pathobiochemical research and clinical outlook. *European Journal of Clinical Chemistry and Clinical Biochemistry*, 29: 293-311.

38. Engström-Laurent A, Lööf L, Nyberg A & Schröder T (1985). Increased serum levels of hyaluronate in liver disease. *Hepatology*, 5: 638-642.

39. Guéchot J, Laudt A, Loria A, Serfaty L, Poupon R & Giboudeau J (1996). Diagnostic accuracy of hyaluronan and type III procollagen amino-terminal peptide serum assays as markers of liver fibrosis in chronic viral hepatitis C evaluated by ROC curve analysis. *Clinical Chemistry*, 42: 558-563.