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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

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Diagnostic accuracy of two serum markers of liver fibrosis, hyaluronan (HA) and amino-terminal peptide of type III procollagen (P-III-P), was studied in a cohort of 326 untreated patients with chronic viral hepatitis C. Both P-III-P (RIA-gnost P-III-P, Behring Diagnostic) and HA (HA-test, Pharmacia) serum concentrations correlated with the histological grades of liver fibrosis ($P < 0.001$). Receiver-operating characteristic (ROC) curves showed that serum HA had greater diagnostic performance than P-III-P, both for discriminating patients with extensive liver fibrosis from those with no or mild fibrosis (area under the ROC curves: 0.864 vs 0.691, $P < 0.001$) or for discriminating patients with cirrhosis from those without cirrhosis (area under the ROC curves: 0.924 vs 0.734, $P < 0.001$). At cutoff values of 0.8 kU/L for serum P-III-P and 85 μg/L for serum HA, sensitivities were 70.0% and 64.5%, and specificities were 63.4% and 91.2%, respectively, for discriminating patients with extensive liver fibrosis from those with no or mild fibrosis. At the cutoff values of 1.0 kU/L for serum P-III-P and 110 μg/L for serum HA, sensitivities were 60.0% and 79.2%, and specificities were 74.0% and 89.4%, respectively, for discriminating patients with liver cirrhosis from those without cirrhosis. We conclude that, because the diagnostic accuracy of serum HA is greater than that of serum P-III-P as a marker of liver fibrosis, serum HA should be preferred when monitoring liver fibrosis in patients with chronic viral hepatitis C.

INDEXING TERMS: procollagen III propeptide • liver disease • diagnostic accuracy

Acute viral hepatitis C (VHC) is usually an asymptomatic infection, but the long-term consequences may be serious, with progression to chronic hepatitis in most cases and to liver cirrhosis and hepatocellular carcinoma in some.[3] In one study, up to 20% of patients with chronic VHC developed cirrhosis within 5 years *[1]*.

The prognosis of chronic VHC is closely related to the development of liver fibrosis, which commonly occurs, as in other chronic liver diseases, as the disease progresses. The liver parenchyma is first replaced by connective tissue, then becomes fibrotic, and finally is cirrhotic. This process needs to be assessed in monitoring the course of chronic VHC. At present, the assessment of liver fibrosis necessitates histopathological examination of percutaneous biopsy specimens. This method is both invasive and of questionable value because of the heterogeneous distribution of pathological changes in the liver. As a result, noninvasive biochemical markers for assessing liver fibrosis in chronic hepatitis are being actively sought to help evaluate histological damage and monitor the progression of fibrosis *[2]*.

Liver fibrosis is a complex process involving production and deposition of insoluble components that constitute the extracellular matrix (ECM). These components can be divided into collagens (types I and III being predominant in the liver, with lesser amounts of types IV, V, and VI), noncollagenous glycoproteins (fibronectin, laminin, undulin, entactin, vitronectin, tenascin, osteonectin, and elastin), proteoglycans (heparan, dermatan, and chondroitin sulfates), and a polysaccharide [hyaluronan (hyaluronic acid; HA)] *[3]*.

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[3] Nonstandard abbreviations: ECM, extracellular matrix; HA, hyaluronic acid or hyaluronan; P-III-P, amino-terminal peptide of type III procollagen; VHC, viral hepatitis C; HCV, hepatitis C virus; HBV, hepatitis B virus; and AUC, area under the curve.

Given the increased production of ECM components in liver fibrosis, interest has grown in recent years regarding determination of blood substances related to ECM as potential markers of liver fibrosis. HA and amino-terminal propeptide of type III procollagen (P-III-P) have been the most extensively studied serum components and have been proposed as indices of the extent of liver fibrosis in chronic liver diseases. HA, ubiquitously distributed in the extracellular spaces, is a linear polymer built from repeating disaccharide units {[D-glucuronic acid (1-β-3) *N*-acetyl-D-glucosamine (1-β-4)]$_n$} and reaching molecular masses of 10^4–10^7 Da. In the liver, HA is synthesized by the Ito cells and degraded by the sinusoidal endothelial cells *[4, 5]*. P-III-P is the N-terminal cleavage product of procollagen III into collagen III, which is synthesized during liver fibrogenesis by Ito cells *[6, 7]*.

Investigators have found that serum HA concentrations increase in chronic liver diseases; these studies suggest that progressive liver damage can be identified early and managed by serum HA assessment *[8–10]*. Like HA, serum P-III-P concentrations increase in patients with liver fibrosis *[11–13]*, and P-III-P has mainly been used to study fibrosing conditions of the liver. Several P-III-P assays have been developed, varying in their specificities for the intact aminopropeptide and the smaller peptide fragments *[14, 15]*. An extensively used IRMA (RIA-gnost P-III-P; Behring Diagnostic, Rueil-Malmaison, France) detects degradation products, particularly those of aminopropeptide incorporated into the collagen fibrils constituted with uncleaved type III procollagen and not just the aminopropeptide cleaved from the procollagen conversion into collagen *[16]*.

Serum P-III-P decreases in patients with chronic VHC treated with alpha-interferon, which has an antifibrotic effect *[17, 18]*, whereas serum HA changes in parallel with liver fibrosis *[19]*.

Because most patients with VHC develop chronic hepatitis and some of them develop cirrhosis, we decided to include serum HA and P-III-P measurements in biological assessment of the disease, using commercially available methods previously used and documented in chronic VHC *[17–20]*.

To assess and compare the diagnostic accuracy of these two tests as biochemical markers of liver fibrosis or liver cirrhosis in chronic VHC, we carried out receiver-operating characteristic (ROC) analysis of data from a large cohort of 326 untreated patients.

Patients and Methods

PATIENTS

Included in this study with their informed consent were 326 patients with chronic VHC (178 men and 148 women, mean ± SD ages 43.6 ± 13.5 years) while they were in hospital for clinical, histological, and biological evaluation before potential α-interferon therapy. All the patients had persistently high serum alanine aminotransferase (more than twice the upper limit of normal values) for at least 6 months and three determinations, had anti-hepatitis C virus (HCV) antibodies (positive by 2nd-generation ELISA), were positive for HCV RNA by polymerase chain reaction, and had liver histological findings consistent with chronic VHC. Among them, 53 had histological evidence of liver cirrhosis. None had been previously treated for VHC, and none had clinical, biological, or histological evidence of other chronic liver disease or pulmonary fibrosis or rheumatic disorders. Patients with evidence of hepatitis B virus (HBV) infection (detectable serum hepatitis B surface antigen or HBV-DNA) were excluded from the study.

Blood samples were collected between 0800 and 1000 after an overnight fast. Serum was separated without delay and used for routine liver tests or stored at −20 °C until assayed for HA and P-III-P.

PROCEDURES

Liver function tests. Total serum bilirubin, alanine aminotransferase activity, aspartate aminotransferase activity, γ-glutamyltransferase activity, alkaline phosphatase activity, and prothrombin time were assayed in all the patients. Biochemical tests were performed with a Synchron CX-4 analyzer (Beckman, Brea, CA), with Société Française de Biologie Clinique-recommended procedures for enzyme activities (performed at 30 °C).

Serum HA and serum P-III-P measurements. Serum HA was assessed with a sequential radiometric assay (HA-test; Pharmacia Diagnostics, Uppsala, Sweden) based on the use of specific HA-binding proteins isolated from bovine cartilage. Intra- and interassay CVs were <7% and <9%, respectively. The serum reference interval, determined in the laboratory from 30 healthy subjects, was 27 ± 29 μg/L (mean ± SD).

Serum P-III-P was assessed with an IRMA using monoclonal antibodies (RIA-gnost P-III-P coated tube; Behring). Intra- and interassay CVs were <5% and <7%, respectively. The serum reference interval, determined in the laboratory from 30 healthy subjects, was 0.41 ± 0.11 kU/L (mean ± SD).

Histological assessment of liver fibrosis. A percutaneous liver biopsy specimen was obtained from all patients. The histopathological changes were assessed and scored according to the numerical scoring system of Knodell et al. for assessing histological activity in asymptomatic chronic active hepatitis *[21]*. Liver fibrosis was graded as 0 for no fibrosis, 1 for fibrous portal expansion (mild fibrosis), 3 for bridging fibrosis, and 4 for cirrhosis. The degree of inflammation and necrosis was calculated as the sum of scores of periportal and bridging necrosis (piecemeal necrosis, scored 0–10), lobular degeneration and focal necrosis (scored 0–4), and portal inflammation (scored 0–4). The Knodell score (scale 0–22) is the sum of the histological scores of fibrosis, inflammation, and necrosis.

For analysis of data, patients were classified as (*a*) patients without liver fibrosis or with mild liver fibrosis (histological scores of fibrosis: 0 or 1), and (*b*) patients with extensive liver fibrosis (histological scores of fibrosis: 3 or 4); or as (*c*) patients without histological evidence of liver cirrhosis (histological scores of fibrosis: 0, 1, or 3), and (*d*) patients with biopsy-proven cirrhosis (histological score of fibrosis: 4).

Statistical analysis. Numerical data were expressed as mean ± SD. The Mann–Whitney *U*-test was used for comparisons between

independent groups. The Kruskal–Wallis analysis of variance was used to compare serum HA and serum P-III-P concentrations according to the histological score of fibrosis. Correlation coefficients were calculated to assess the relationship between the histological degree of fibrosis and the concentrations of the two serum markers.

To assess and compare the diagnostic accuracy of serum HA and serum P-III-P for discriminating in chronic VHC patients those with extensive liver fibrosis from those with no or mild fibrosis or for discriminating those with liver cirrhosis from those without cirrhosis, we plotted ROC curves *[22]* and calculated the areas under the curves (AUC) for comparison. ROC curves were generated by plotting the relationship of the true positivity (sensitivity) and the false positivity (1 − specificity) at various cutoff points of the tests. An AUC of 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value *[23]*. ROC analysis was computed by using the CLABROC-PC software kindly supplied by C. E. Metz (University of Chicago, Chicago, IL). This program is applicable to correlated, continuously distributed test-result data.

Cutoff values of HA and P-III-P serum concentrations for diagnosis of extensive liver fibrosis or for the diagnosis of liver cirrhosis were selected from experimental data as the values that maximized the sum of sensitivity and specificity in a clinical situation where false-negative classifications could be considered as harmful as false-positive classifications.

Results

CHARACTERISTICS OF THE PATIENTS

Liver biochemical and histological tests are given in Table 1.

Serum HA and serum P-III-P concentrations in liver fibrosis. The serum HA and P-III-P concentrations according to the fibrosis score are summarized in Table 2. There were significant differences between the groups of patients. Both HA and P-III-P concentrations were correlated with the Knodell scores, mainly because they were correlated with the grade of liver fibrosis (Table 3).

The patients with extensive liver fibrosis (histological grade of fibrosis = 3 or 4, n = 110) had significantly higher HA or P-III-P serum concentrations than the patients with no or mild liver fibrosis (histological grade of fibrosis = 0 or 1, n = 216): HA = 165 ± 62 μg/L vs 43 ± 21 μg/L (P <0.001); P-III-P = 1.01 ± 0.16 kU/L vs 0.77 ± 0.21 kU/L (P <0.001).

Patients with cirrhosis (n = 53) had significantly higher HA or P-III-P serum concentrations than patients without cirrhosis (n = 273): HA = 219 ± 89 μg/L vs 60 ± 72 μg/L (P <0.001); P-III-P = 1.72 ± 0.23 kU/L vs 0.80 ± 0.33 kU/L (P <0.001).

Table 1. Characteristics of the patients (n = 326).

Characteristic	Upper reference limit	Mean ± SD
Alanine aminotransferase, U/L	35	83 ± 63
Aspartate aminotransferase, U/L	35	53 ± 36
γ-Glutamyltransferase, U/L	40	48 ± 55
Alkaline phosphatases, U/L	100	54 ± 21
Bilirubin, mg/L	12	13 ± 6
Prothrombin time, %		91 ± 11
Knodell score		6.1 ± 2.9

Diagnostic performances. As shown by the ROC curves, in cases of chronic VHC, the ability of serum HA to differentiate patients with extensive liver fibrosis from those with no or mild liver fibrosis exceeded that of serum P-III-P (AUC = 0.864 vs 0.691, P <0.0001) (Fig. 1). The ability to differentiate patients with cirrhosis from those without cirrhosis was also greater for serum HA than for serum P-III-P (AUC = 0.924 vs 0.734, P <0.0001) (Fig. 2).

The selected cutoff values for diagnosing extensive liver fibrosis in patients with chronic VHC were 85 μg/L and 0.80 kU/L for serum HA and P-III-P, respectively. The selected cutoff values for diagnosing cirrhosis in patients with chronic VHC were 110 μg/L and 1.00 kU/L for serum HA and P-III-P, respectively. The sensitivity and specificity of each test are shown in Table 4 for the diagnosis of extensive liver fibrosis and for the diagnosis of cirrhosis.

Discussion

Chronic VHC is a progressive disease. Because the prognosis for patients with this disease depends largely on the development of liver fibrosis, leading to cirrhosis, liver damage has to be regularly evaluated. However, histological examination, the "gold standard" for assessing liver lesions, requires liver biopsy, which is potentially associated with severe complications *[24, 25]* and for ethical reasons cannot be repeated to monitor liver status. Furthermore, this method is not totally reliable because of the high degree of sampling variability *[26]*.

As a consequence of the difficulties linked to liver histological examination, serum markers of fibrosis are now used in therapeutic trials with patients with chronic VHC *[27]*. The results of these trials are particularly useful for comparing markers so that investigators can choose an assay suitable for routine patient monitoring. In the present study, we evaluated and compared the clinical performances of serum P-III-P and HA as markers of fibrosis in a large cohort of untreated patients. We found that both serum P-III-P and serum HA concentrations are increased in patients with liver fibrosis, the highest concentrations being seen in patients with cirrhosis.

The increase of serum concentrations of these analytes in patients with chronic VHC may reflect the stimulated production of ECM components by activated fat-storing cells, the decreased uptake and degradation by endothelial cells, or both. The ability of Ito cells to synthesize and secrete collagens and HA has been documented, and Kupffer cells may be involved in stimulating their production through secretion of various mediators *[5]*. Nevertheless, because blood HA and P-III-P are cleared and degraded by liver endothelial cells *[28, 29]*, serum concentrations might be related to pathological mechanisms that affect sinusoidal cell function and impair plasma uptake.

The serum concentrations of the two studied markers are correlated with the Knodell histological score. Among the different scoring indices of the Knodell score, fibrosis explains this relationship. The correlations between the P-III-P and HA

Table 2. Serum HA and P-III-P concentrations (mean ± SD) according to liver fibrosis histological scores.

	Ref. interval[a]	Histological fibrosis grade 0	1	3	4	P[b]
No. of patients		44	172	57	53	
HA, μg/L	27 ± 29	36 ± 15	46 ± 37	119 ± 102	219 ± 89	<0.001
P-III-P, kU/L	0.41 ± 0.11	0.75 ± 0.81	0.78 ± 0.10	0.92 ± 0.29	1.72 ± 0.23	<0.001

[a] Determined in 30 healthy subjects.
[b] Overall significance.

Table 3. Relation between HA and P-III-P serum concentrations and histological scores for chronic hepatitis.

	HA		P-III-P	
	r	P	r	P
Total Knodell score[a]	0.392	<0.001	0.290	<0.001
Fibrosis score	0.583	<0.001	0.342	<0.001
Inflammation and necrosis score[b]	0.180	<0.005	0.164	<0.005

[a] Includes score of fibrosis and score of inflammation and necrosis.
[b] Includes score of periportal and bridging necrosis (piecemeal necrosis), score of lobular degeneration and focal necrosis, and score of portal inflammation.

serum concentrations and the histologically assessed grade of liver fibrosis were strong, whereas those between the P-III-P and HA serum concentrations and the histopathological index of liver inflammation and necrosis were poor. These data, obtained from a large number of subjects, support previous results that showed increases in these two markers of fibrosis in patients with active chronic VHC and a positive correlation of the concentrations of the analytes with the histological scores of fibrosis *[12, 13, 27, 30]*. They also provide evidence that serum concentrations of HA are more strongly correlated with liver fibrosis than are serum P-III-P concentrations.

To compare the performance of the two tests, we used ROC plots, which provide pure indices of accuracy *[22]*. As shown by the relative positions of the plots in Figs. 1 and 2, the HA assay exhibits greater observed accuracy than does the P-III-P assay for diagnosis of extensive fibrosis and for diagnosis of cirrhosis. At any given sensitivity value, the HA value has higher specificity than the P-III-P value. Statistical comparison of the AUC shows that the accuracy differences are highly significant ($P < 0.0001$). The ROC plot AUC is the most convenient global way to quantify the diagnostic accuracy of a test *[23]*. The AUC values for HA mean that a randomly selected patient with extensive liver fibrosis will have a higher concentration of serum HA than will a randomly selected patient with no or mild fibrosis in 86.4% of the cases; similarly, a randomly selected patient with cirrhosis will have a concentration of serum HA greater than that in randomly selected patients without cirrhosis in 92.4% of the cases. In fact, the evaluation of the diagnostic accuracy of the serum markers of liver fibrosis is limited primarily by the sampling variability of liver biopsy, given that we used histological examination of one sample as the reference value in this study.

Using ROC curve analysis to assess the diagnostic accuracy of a biological marker for use in patient management requires the selection of a decision threshold. The large number of subjects included in this study allowed us to calculate with precision the best cutoff values of serum P-III-P and HA for

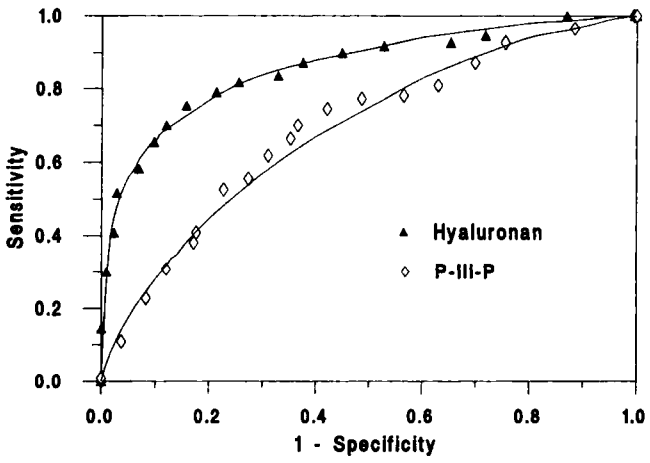

Fig. 1. ROC curves for HA and P-III-P serum concentrations for discriminating patients with extensive liver fibrosis from those without extensive liver fibrosis in chronic viral hepatitis C.

The areas under the curves are 0.864 and 0.691 ($P < 0.0001$) for HA and P-III-P, respectively.

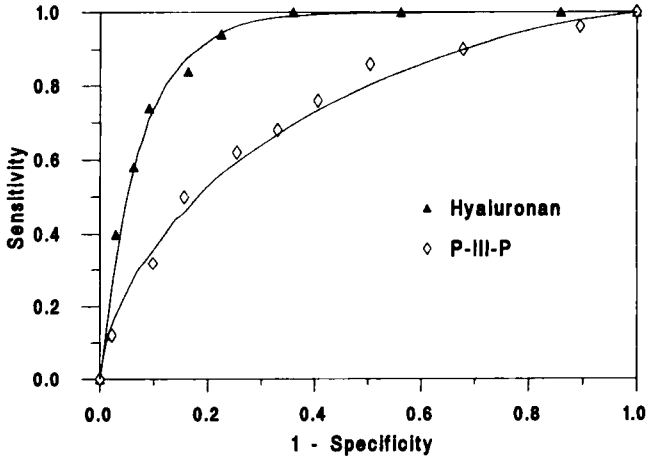

Fig. 2. ROC curves for HA and P-III-P serum concentrations in discriminating patients with liver cirrhosis from those without cirrhosis in chronic viral hepatitis C.

The areas under the curves are 0.924 and 0.734 ($P < 0.0001$) for HA and P-III-P, respectively.

Table 4. Diagnostic performance of HA and P-III-P at the selected thresholds in patients with chronic VHC.

	Extensive vs mild or no liver fibrosis		Liver cirrhosis vs no cirrhosis	
	HA	P-III-P	HA	P-III-P
Threshold	85 μg/L	0.8 kU/L	110 μg/L	1.00 kU/L
Sensitivity, %	64.5	70.0	79.2	60.0
Specificity, %	91.2	63.4	89.4	74.0

discriminating in patients with chronic VHC those with extensive liver fibrosis or those with cirrhosis. Assuming that, at present, both sensitivity and specificity are equally important in classifying patients with chronic VHC for liver fibrosis, we selected cutoff values that maximized the sum of sensitivity and specificity. However, the risks and benefits of diagnostic and therapeutic choices made from the results of the assays can be used if it becomes desirable to reoptimize the cutoff values so as to minimize false-positive or false-negative classifications.

The differences in diagnostic accuracy of these two serum markers of liver fibrosis found in this study may be related to the fact that serum P-III-P concentrations reflect more fibrogenesis and inflammation than fibrosis, as suggested by previous studies *[27, 30]*, and that serum HA depends primarily on morphological changes that accompany hepatic sinusoidal capillarization in the evolution of chronic diseases to cirrhosis *[31–33]*. Another possible reason for the less-powerful accuracy of serum P-III-P is the lack of specificity of the Behring assay for the intact P-III-P. A specific assay *[15]* should be evaluated in further studies to assess whether using an assay that does not cross-react with degradation products might be more interesting as a marker of liver fibrosis than the assay used here. However, this explanation is not supported by data in alcoholic liver diseases *[14]*.

In conclusion, in comparison with the results of histological examination, this study shows that serum HA is more accurate than serum P-III-P for evaluating liver fibrosis in patients with chronic VHC. This suggests that assay of serum HA may be preferable to assay of P-III-P as a noninvasive test for monitoring fibrotic processes in the course of chronic VHC.

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