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Serum connective tissue markers as predictors of advanced fibrosis in patients with chronic hepatitis B and D

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Background/aims: *Liver biopsy to assess fibrosis is invasive and prone to sampling error. While algorithms of serum markers to predict fibrosis stage have been described for chronic hepatitis C, these cannot be applied equally well to hepatitis B.* ***Methods:*** *We therefore determined 9 serum fibrosis markers, liver biochemical tests and ultrasound parameters in 109 consecutive adult patients with chronic hepatitis B and D. All patients had compensated liver disease. Using the METAVIR score, advanced disease was defined as fibrosis stage ≥F2, and active inflammation as grade ≥A2. A gold standard was created considering splenomegaly and/or platelets <150,000 as indicators of advanced fibrosis irrespective of histology. Area under receiver operating characteristics curves was used for assessment of single markers and odds ratio for their combinations.* ***Results:*** *Patients with advanced disease were older, had lower albumin, higher gamma glutamyl transferase and lower platelet. Levels of 6 of the 9 fibrosis markers, tissue inhibitor of metalloproteinases-1, procollagen type III aminoterminal propeptide, matrix metalloproteinase-2, laminin, hyaluronan and collagen IV correlated with advanced fibrosis. Markers useful for fibrosis prediction also predicted marked inflammation. Using the gold standard, age, prothrombin time, gamma glutamyl transferase and albumin were independent predictors of fibrosis with odds ratio's of 3.11, 4.18, 3.35 and 5.25, respectively. Their combined use predicted fibrosis with an odds ratio of 228.8. Tissue inhibitor of metalloproteinases-1 and hyaluronan were powerful predictors of fibrosis (Odds ratio's of 8.65 and 8.38). Their combined use revealed an odds ratio of 28.6, when compared with the gold standard.* ***Conclusion:*** *In conclusion, advanced liver fibrosis in chronic hepatitis B and D may be predicted with use of these two fibrosis markers.*

Key words: Tissue inhibitor of metalloproteinases-1, procollagen, matrix metalloproteinase, laminin, hyaluronan, collagen, Metavir, platelets, progression, stage, grade

Kronik hepatit B ve D hastalarında ileri fibrozis belirleyicileri olarak serum bağ doku belirteçleri

Amaç: *Karaciğer fibrozisinin biyopsi ile değerlendirilmesi invaziv bir yöntemdir ve örnekleme hatasına yol açabilir. Fibrozis evresini öngören serum fibrozis belirteç algoritmaları kronik hepatit C'de tanımlanmış olmasına rağmen, kronik hepatit B'de benzer şekilde uygulanmamıştır.* ***Yöntem:*** *Bu amaçla 9 serum fibrozis belirteci, karaciğer biyokimyasal testleri ve ultrasonografik parametreleri, ardışık olarak 109 yetişkin kronik hepatit B ve D hastasında değerlendirdik. Tüm hastalar kompanse karaciğer hastalığına sahipti. METAVİR skoru ile evre ≥2 fibrozis ve grade ≥2 inflamasyon varlığı ileri karaciğer hastalığı olarak tanımlandı. Splenomegali ve/veya 150,000 altında trombosit sayısının, histolojiden bağımsız olarak, ileri fibrozisin belirleyicisi olduğu hesaba katılarak altın standart yapılmaya çalışıldı. Belirteçlerin ve bunların kombinasyonlarının Odds oranlarının değerlendirilmesinde ROC eğrisinin altında kalan alan (AUROC) hesaplaması kullanıldı.* ***Bulgular:*** *İleri hastalığı olanlar daha ileri yaşlıydı, daha düşük albumin, daha yüksek gama glutamil transferaz ve daha düşük platelet sayısına sahipti. Dokuz fibrozis belirtecinin 6'sının seviyesi (metalloproteinazlar-1 doku inhibitörü, prokollagen tip III aminoterminal propeptide, matriks metalloproteinaz, laminin, hyaluronan and kollagen IV) ileri fibrozis ile korele idi. Bu altı belirteç ayrıca inflamasyonun tahmininde de faydalıydı. Altın standart kullanımıyla, yaş, protrombin zamanı, gama glutamil transferaz ve albumin sırasıyla 3.11, 4.18, 3.35 ve 5.25 odds oranları ile fibrozisin bağımsız belirleyicisiydi. Bunların kombinasyonu odds oranı 228.8 ile fibrozisi öngördü. Metalloproteinaz-1 doku inhibitörü ve hyaluronan fibrozisin güçlü belirleyicileriydi (odds oranı 8.65 ve 8.38, sırayla). Altın standart ile karşılaştırıldığında, bu ikisinin kombinasyonunda odds oranı 28.6 idi.* ***Sonuç:*** *Sonuç olarak, kronik hepatit B ve D'de ileri karaciğer fibrozisi, bu iki fibrozis belirtecinin kullanımı ile doğru olarak öngörülebilir.*

Anahtar kelimeler: Metalloproteinazlar-1 doku inhibitörü, prokollagen, matriks metalloproteinaz, laminin, hyaluronan, kollagen, Metavir, trombosit, progresyon, evre, derece

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INTRODUCTION

Chronic viral hepatitis is the leading cause of liver failure and hepatocellular carcinoma in most parts of the world (1,2). The need for treatment is obvious in patients with advanced or severe chronic viral hepatitis, in which the fibrosis stage is the most important predictor of morbidity and mortality. While liver biopsy is the standard procedure to assess fibrosis, it is invasive, with the potential of severe complications. Furthermore, biopsy is prone to sampling error, leading to 20-33% of false histological allocations, especially in patients with advanced liver disease (stage ≥F2 in the METAVIR scale) (3-6). In addition, the use of suction needles is widespread and often results in tissue fragmentation, further complicating a reliable assessment (7).

Transient elastography (FibroScan) and serum biomarkers are validated noninvasive methods to assess the severity of liver fibrosis. Transient elastography and serum biomarkers are the first-step tools in the evaluation of liver fibrosis in patients with chronic hepatitis C (8). In hepatitis B, these noninvasive tools remain less explored. Determining the severity of liver fibrosis is important for the decision to initiate antiviral therapy, and in the setting of chronic hepatitis B infection, in choosing the most suitable treatment regimen.

The search for noninvasive alternatives to liver biopsy for grading and staging goes back to the late 1970s when the aminoterminal propeptide of type III procollagen (PIIINP) was suggested as a serum marker of liver fibrosis and fibrogenesis, i.e., *de novo* formation of (hepatic) connective tissue (9). Since then, other extracellular matrix (ECM) polypeptides that derive from laminin, procollagen type IV, procollagen type I, procollagen type III, as well as hyaluronan were assessed with regard to their ability to predict the stage or the evolution of liver fibrosis (10). On the other hand, biochemical markers of liver function have also been evaluated as predictors of histological stage (11-17). Despite these efforts throughout 30 years, none of these serum markers has received universal acceptance, in part due to the study of heterogeneous populations and the use of insufficiently validated assays.

In this study, we assessed the predictive value of nine fibrosis markers derived from the ECM (18-21) as well as biochemical markers of liver function and inflammation in well-defined cohorts of adult patients with HBeAg-positive and -negative chronic hepatitis B and chronic hepatitis D. We could show that, compared to all other markers and their combinations, the combination of two of these markers, tissue inhibitor of metalloproteinases-1 (TIMP-1) and hyaluronan, achieved the best prediction of fibrosis stage ≥F2, when matched to histological fibrosis, which was complemented with spleen size and platelet count to minimize sampling error.

MATERIALS AND METHODS

Patients

Sera were collected from a total of 116 consecutive patients. Of these, 43 had HBeAg (+) chronic hepatitis B, 40 had HBeAg (-), anti-HBe (+) and HBV DNA (+) chronic hepatitis B, and 33 suffered from chronic delta hepatitis. Sera were either obtained at baseline from patients who had participated in various clinical trials (22-24), or were obtained during evaluation of candidates for eventual treatment. In this context, patients represented a homogeneous cohort, since all of them had clinically compensated liver disease with a serum albumin >3.5 g, a normal bilirubin, and a prothrombin time not beyond 3 seconds of the normal range. Four patients were excluded from the analysis because of lack of one or more parameters investigated in this study. In addition, 3 more patients with biopsy specimens containing less than 6 portal tracts were also excluded. Hence, the study analysis on prediction of fibrosis by fibrosis markers is based on the data of 109 patients. The diagnosis of chronic hepatitis B was based on HBsAg positivity of at least six months duration. Hepatitis serology, including HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HCV, and anti-human immunodeficiency virus (HIV), was determined by a microparticle enzyme immunoassay and anti-HDV by an enzyme immunoassay (Abbott Laboratories, Chicago, IL, USA). Exclusion criteria were co-infection with HIV, HCV and other liver diseases, such as hemochromatosis and Wilson's disease. Age and sex of the patients were recorded. Patients either drank no alcohol or consumed alcohol in amounts of <30 g/week. Information on alcohol intake was obtained during the initial assessment for treatment.

Blood Tests

The following hematological and biochemical parameters were collected from patients: platelet count, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase

(ALP), gamma glutamyl transferase (GGT), total bilirubin, albumin, and prothrombin time. A total of nine serum fibrosis markers were measured: TIMP-1, tenascin-C, collagen type IV, collagen type VI, PIIINP, matrix metalloproteinase-2 (MMP-2), MMP-9, laminin, and hyaluronan. These fibrosis markers were quantified using monoclonal antibodies in sandwich immunoassays performed in an automated analyzer employing fluorescein-labelled capture and ALP-labelled detection antibodies, except for hyaluronan, which was measured with a sandwich based on hyaluronan binding protein derived from cartilage. Immune complexes were separated from serum using magnetic particles covered with monoclonal anti-fluorescein. The assays were developed for the BAYER IMMUNO 1™ immunoassay system and validated in a large cohort of liver patients and healthy subjects (18-21). Serum samples were stored at -80°C until use.

Histological Grading and Staging

Histological evaluation of liver specimens was analyzed according to the METAVIR classification (25) by two pathologists who were unaware of patient characteristics and the results of blood tests. Only biopsies where at least 6 portal tracts were available were used in the study analysis. Liver biopsies were fixed, paraffin-embedded and stained both with hematoxylin-eosin and Masson's trichrome stain, and fibrosis was staged as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, enlarged portal tracts with rare septa; F3, numerous septa without cirrhosis; and F4, cirrhosis. Necroinflammatory activity was graded as follows: A0, no activity; and A1, A2 and A3, mild, moderate and severe activity, respectively. Patients were classified as having advanced disease when fibrosis stage was ≥2. For the activity score, patients were classified as having active disease when grading was ≥A2. In addition to histological assessment, a gold standard was created for better definition of advanced liver disease. Accordingly, patients with splenomegaly, defined as a spleen size with a vertical axis ≥120 mm and/or patients with a platelet count of <150,000 were regarded as having advanced disease even when the biopsy result was not consistent.

Statistics

Data on demographics, biochemistry and fibrosis markers are reported as means and standard deviations (SD). Comparison between two groups was performed with the unpaired Student's t test or Mann-Whitney U test as appropriate. Multiple intergroup comparisons were made using one way analysis of variance, followed by post-hoc analysis with Bonferroni's test. The area under receiver operating characteristics (AUROC) curve was used to assess the overall diagnostic value of biochemical and fibrosis markers to correctly predict advanced liver disease. Logistic regression analysis followed by stepwise multivariate logistic regression was used to assess the independent effect of quantitative variables on the diagnostic accuracy of advanced fibrosis. SPSS for Windows was used for statistical analysis.

RESULTS

Patient Groups

Three groups of patients were investigated in this study: patients with HBeAg (+) and HBeAg (-) chronic hepatitis B and patients with chronic delta hepatitis. None of them displayed signs of hepatic decompensation, and all of them had albumin levels >3.5 g, a normal bilirubin and a normal prothrombin time. However, certain differences among the three groups emerged when demographic, biochemical and fibrosis marker data were analyzed. The mean age was different among the three study groups. HBeAg (+) chronic hepatitis B represented the youngest cohort, followed by patients with chronic delta hepatitis and HBeAg (-) chronic hepatitis B (29.5±10.6 [x ± SD] vs. 36.3±10.7 [$p<0.01$] vs. 42.9±9.5 [$p<0.001$], respectively). Among biochemical and hematological markers, albumin levels and platelet counts were lower and GGT was higher in chronic delta hepatitis patients compared to HBeAg (+) chronic hepatitis B cases ($p=0.001$, $p=0.012$ and $p=0.01$, respectively; Table 1). Platelets were also lower in HBeAg (-) chronic hepatitis B patients compared to HBeAg (+) cases ($p<0.05$). Among the fibrosis markers, TIMP-1 and collagen VI levels were higher in chronic delta hepatitis cases when compared to HBeAg (+) cases ($p=0.001$ and 0.003, respectively; Table 2), and TIMP-1 was higher in HBeAg (-) chronic hepatitis B patients compared to HBeAg (+) cases ($p=0.0001$). Other fibrosis markers showed a similar trend, but differences were not significant.

Characteristics of Mild vs. Advanced and Inactive vs. Active Liver Disease

According to the METAVIR score, 70 patients had early and 39 had advanced liver disease. Patients

Table 1. Demographic, hematological and biochemical parameters in the three study groups

	HBeAg (+) patients	HBeAg (-) patients	Delta (+) patients
Age	29.5±10.6	42.9±9.5[3]	36.3±10.7[1,2]
ALT	112.6±158.3	117.7±98.2	97.5±77.3
AST	67.3±106.2	68.1±47.3	70.0±71.4
Albumin	4.77±0.49	4.65±0.35	4.40±0.48[3,4]
PT	13.0±1.80	12.99±1.02	13.04±1.66
Bilirubin	0.81±0.47	0.84±0.34	0.89±0.37
ALP	103.0±45.3	99.1±37.2	104.0±48.4
GGT	37.2±37.7	50.7±35.4	67.0±58.6[1]
WBC (x100)	6.2±1.79	6.1±1.30	5.7±1.69
Platelets (x100)	201±48	168±50[6]	162±55[5]

[1]p=0.01, [3]p=0.001, [5]p=0.012 and [6]p=0.046 vs. HBsAg (+) group; [2]p=0.027 and [4]p=0.046 vs. HBeAg (-) group.

Table 2. Fibrosis parameters in the three study groups

	HBeAg (+) patients	HBeAg (-) patients	Delta (+) patients
TIMP-1	603.7±146.8	762.5±172.7[2]	746.5±170.0[1]
Tenascin	449.9±194.8	506.6±269.8	516.0±223.3
Collagen VI	8.55±2.81	9.04±3.27	11.03±3.54[3,4]
PIIINP	11.66±5.85	12.83±7.62	13.67±5.65
Laminin	23.79±8.77	23.81±10.33	29.75±13.23
Hyaluronan	66.2±88.7	118.7±133.1	117.5±163.9
MMP-9	417.9±213.1	390.4±37.2	457.9±281.0
Collagen IV	185.4±86.9	197.4±78.0	219.6±85.6
MMP-2	787.2±202.4	878.0±375.8	808.4±142.2

TIMP: Tissue inhibitor of metalloproteinases-1. PIIINP: Aminoterminal propeptide of type III procollagen. MMP: Matrix metalloproteinase.

[1]p=0.001, [2]p=0.0001 and [3]p=0.003 vs. HBsAg (+) group; [4]p=0.03 vs. HBeAg (-) group

with advanced disease were older, had lower albumin, higher GGT levels, and lower platelet counts compared to patients with early disease, i.e., patients with no or little fibrosis METAVIR stage <2 (Table 3). Six of the 9 fibrosis markers investigated, namely TIMP-1, PIIINP, MMP-2, laminin, hyaluronan, and collagen type IV, displayed higher levels in patients with advanced (METAVIR stage ≥2) compared to patients with mild fibrosis (Table 3).

Of the 109 patients, 54 displayed mild (METAVIR <A2) and 55 marked necroinflammatory activity (METAVIR ≥A2). Patients with marked necroinflammatory activity were older and had lower platelet counts (Table 3). Patients with active disease showed a trend for higher GGT levels, but this did not reach statistical significance. Five of the 9 fibrosis markers (TIMP-1, PIIINP, laminin, hyaluronan, and collagen type IV) were higher in active compared to inactive disease (Table 3).

Diagnosis of Advanced Fibrosis with Biochemical and Fibrosis Markers

The diagnostic value of age and hematological and biochemical markers is given in Table 4 using ROC curve analysis. Age, albumin, prothrombin time, ALP, GGT, and platelet count had an independent predictive value for the diagnosis of advanced liver disease. Among them, high GGT and low platelets best predicted advanced fibrosis, with AUROC curves of 0.729 and 0.719, respectively. GGT at a cut-off value of 37 (normal values: 10-50), had a sensitivity of 67.5% and a specificity of 72.3%, and platelets at a cut-off value of 172,000 had a sensitivity of 66.2% and a specificity of 65%. The diagnostic values of fibrosis markers are given in Table 5. Of these, TIMP-1, PIIINP, MMP-2, laminin, hyaluronan, and collagen type IV were diagnostic, with TIMP-1 and hyaluronan offering the best predictive value with AUROC curves of 0.769 and 0.771, respectively. TIMP-1 at a cut off value of 711 ng/ml had a sensitivity of 75% and a specificity of 74.2%, and hyaluronan at a cut-off value of 64 ng/ml had a sensitivity of 70.7% and a specificity of 77.6%. The relative risk (odds ratio, OR) of having advanced disease using the cut-off values determined by ROC curve analyses were 5.42 for GGT, 3.63 for albumin, 3.63 for platelets, 3.55 for age, and 2.1 for prothrombin time. The OR for having advanced disease at cut-off values determined with AUROC curves for fibrosis markers were 8.65 for TIMP-1, 8.38 for hyaluronan, 4.52 for MMP-2, 3.57 for collagen IV, 3.47 for laminin, and 2.47 for PIIINP.

The diagnostic value of biochemical and fibrosis markers to diagnose patients with marked necroinflammatory activity (METAVIR ≥A2) was also explored. Many markers useful for fibrosis prediction were also useful for prediction of marked liver inflammation. GGT, platelets, age, prothrombin time, and albumin displayed AUROC curves of 0.676, 0.661, 0.653, 0.644, and 0.635, respectively. Among the fibrosis markers, TIMP-1, hyaluronan, collagen type IV, laminin, PIIINP, and MMP-2 displayed AUROC curves of 0.747, 0.733, 0.699, 0.673, 0.657, and 0.601, respectively (Table 6). Sensitivities and specificities to diagnose marked necroinflammation (METAVIR grade ≥A2) were 82.5% and 61.4% for TIMP-1 at a cut-off value of 661 ng/ml and 69% and 72% for hyaluronan at a

Table 3. Characteristics of patients with METAVIR fibrosis stage <F2 vs. ≥F2 and with a necroinflammatory score of <A2 vs. ≥A2

	< F2 (n=70)	≥ F2 (n=39)	p value	< A2 (n=54)	≥ A2 (n=55)	p value
Age	33.9±11.9	40.3±9.8	0.005	33.6±12.7	38.7±9.8	0.019
ALT (U/L)	117.8±134.6	95.9±76.3	0.344	103.1±84.9	116.7±142.2	0.543
AST (U/L)	65.7±83.3	73.3±68.8	0.622	55.1±35.3	81.2±102.9	0.074
Albumin (mg/dl)	4.71±0.44	4.47±0.48	0.009	4.70±0.43	4.55±0.49	0.084
PT (sec)	12.9±1.74	13.3±0.97	0.217	12.9±1.95	13.1±0.94	0.503
Bilirubin (mg/dl)	0.88±0.42	0.80±0.35	0.310	0.90±0.46	0.79±0.32	0.155
ALP (U/L)	96.9±43.0	110.7±42.7	0.107	98.8±46.2	104.8±40.4	0.459
GGT (U/L)	40.8±40.5	68.8±48.5	0.003	42.5±44.0	58.8±45.6	0.056
Platelets (per mm^3)	190950±50138	154625±52048	0.001	192066±530117	164421±50980	0.006
TIMP-1 (ng/ml)	649.2±170.2	788.1±155.0	0.001	633.0±169.3	762.9±161.8	0.001
Tenascin (ng/ml)	479.7±231.3	503.3±231.1	0.601	467.0±237.4	509.6±223.3	0.324
Collagen VI (ng/ml)	9.08±3.06	10.09±3.72	0.144	8.92±3.10	9.95±3.49	0.099
PIIINP (ng/ml)	11.37±5.40	14.9±7.55	0.010	10.83±4.58	14.46±7.52	0.002
MMP-2 (ng/ml)	787.4±261.1	890.4±257.7	0.044	798.3±289.9	850.4±233.2	0.290
Laminin (ng/ml)	23.5±8.90	29.3±13.3	0.015	22.1±8.44	29.0±12.1	0.001
Hyaluronan (ng/ml)	59.2±75.0	171.5±191.4	0.001	56.8±77.2	140.9±171.2	0.001
MMP-9 (ng/ml)	413.6±202.7	432.9±235.4	0.647	402.3±195.6	438.3±231.0	0.371
Collagen IV (ng/ml)	182.5±74.7	230.4±92.3	0.006	175.1±77.1	223.5±84.4	0.002

Values are x±SD.

TIMP: Tissue inhibitor of metalloproteinases-1. PIIINP: Aminoterminal propeptide of type III procollagen. MMP: Matrix metalloproteinase.

Table 4. Diagnostic value of age and hematological and biochemical markers for advanced fibrosis

Markers	Area under curve ([±SEM] ROC curve)	95% Confidence interval	Cut-off value	p (logistic regression)
Age	0.685±0.052	0.582±0.787	37.5	0.002
ALT	0.460±0.057	0.349±0.572		0.497
AST	0.592±0.057	0.480±0.703		0.115
Albumin	0.698±0.054	0.592±0.804	4.65 g/L	0.001
Prothrombin time	0.669±0.054	0.563±0.774	12.95 sec	0.004
Bilirubin	0.458±0.058	0.344±0.572		0.474
ALP	0.629±0.054	0.523±0.735	91 IU/ml	0.027
GGT	0.729±0.052	0.627±0.831	37.5 IU/ml	0.000
WBC	0.511±0.061	0.390±0.631		0.856
Platelets	0.719±0.052	0.617±0.821	172000	0.001

Advanced fibrosis is based on liver biopsy assessment and covers patients with METAVIR fibrosis stage F2-F4. Cut-off values are given for parameters with significant p values.

cut-off value of 49 ng/ml. When the gold standard, as defined by splenomegaly and/or a platelet count of <150,000, was added to differentiate patients with mild disease from those with advanced disease, the number of patients with advanced disease increased from 39 to 56. Thus, 17 patients (17/109, 15.6%) were reclassified with the gold standard from the mild or no fibrosis group to the advanced fibrosis group. Of the 17 patients, 13 had a platelet count of <150,000 and 4 had a normal platelet count but splenomegaly on abdominal ultrasound. With the application of this gold standard as the dependent variable, the area under the curve for fibrosis markers tended to increase for hyaluronan (Table 7).

When stepwise multistep logistic regression analysis was applied to age and hematological and biochemical markers, age (p=0.037), prothrombin time (p=0.05) and GGT (p=0.001) arose as the three independent markers for the prediction of an

Table 5. Diagnostic value of nine fibrosis markers for advanced fibrosis, based on liver biopsy assessment and covering patents with METAVIR fibrosis stage F2-F4

Markers	Area under curve ([±SEM] ROC curve)	95% Confidence interval	Cut-off value	p (logistic regression)
TIMP-1	0.769±0.046	0.678±0.859	711.35 ng/ml	0.000
Tenascin	0.546±0.058	0.433±0.659		0.421
Collagen VI	0.578±0.057	0.467±0.690		0.178
PIIINP	0.658±0.055	0.551±0.765	11.6 ng/ml	0.006
MMP-2	0.674±0.056	0.565±0.784	776.7 ng/ml	0.003
Laminin	0.654±0.057	0.543±0.765	22.65 ng/ml	0.008
Hyaluronan	0.771±0.047	0.678±0.863	64.35 ng/ml	0.000
MMP-9	0.529±0.060	0.412±0.647		0.613
Collagen-IV	0.682±0.055	0.575±0.789	186.4 ng/ml	0.002

Cut-off values are given for parameters with significant p values.

TIMP: Tissue inhibitor of metalloproteinases-1. PIIINP: Aminoterminal propeptide of type III procollagen. MMP: Matrix metalloproteinase.

Table 6. Diagnostic value of nine fibrosis markers for active disease as assessed by the METAVIR necroinflammatory score

Markers	Area Under Curve ([±SEM] ROC curve)	95% Confidence interval	Cut-off value	p value
TIMP-1	0.747±0.047	0.654±0.839	661.75 ng/ml	0.001
Tenascin	0.574±0.054	0.469±0.679		0.172
Collagen VI	0.588±0.053	0.483±0.693		0.105
PIIINP	0.657±0.050	0.559±0.756	10.4 ng/ml	0.004
MMP-2	0.601±0.053	0.497±0.706	754.25 ng/ml	0.061
Laminin	0.673±0.050	0.574±0.771	22.65 ng/ml	0.001
Hyaluronan	0.733±0.046	0.642±0.824	49.35 ng/ml	0.001
MMP-9	0.545±0.054	0.438±0.651		0.411
Collagen-IV	0.699±0.049	0.602±0.795	175.55 ng/ml	0.000

Cut-off values are given for parameters with significant p values.

TIMP: Tissue inhibitor of metalloproteinases-1. PIIINP: Aminoterminal propeptide of type III procollagen. MMP: Matrix metalloproteinase.

Table 7. Diagnostic value of nine fibrosis markers for advanced fibrosis as assessed by the METAVIR score or by the "gold standard", which takes into account not only METAVIR staging but also the presence of splenomegaly and/or a platelet count of <150,000 for the diagnosis of advanced liver fibrosis

	Area Under Curve	
Markers	METAVIR	Gold standard
TIMP-1	0.769	0.733
Tenascin	0.546	0.548
Collagen VI	0.578	0.622
PIIINP	0.658	0.662
MMP-2	0.674	0.640
Laminin	0.654	0.630
Hyaluronan	0.771	0.809
MMP-9	0.529	0.469
Collagen-IV	0.682	0.671

TIMP: Tissue inhibitor of metalloproteinases-1. PIIINP: Aminoterminal propeptide of type III procollagen. MMP: Matrix metalloproteinase.

advanced METAVIR fibrosis score (Table 8A). With the gold standard, the independent predictive value of these single markers for advanced liver disease rose slightly (Table 8B). When all of these four markers (age, albumin, prothrombin time, and GGT) were above the cut-off values determined by ROC curves, patients were 228.8 times more likely to have advanced disease. However, only 6 patients met these criteria.

Among the fibrosis markers, TIMP-1 and hyaluronan emerged as two independent fibrosis markers for the prediction of an advanced METAVIR stage. When both TIMP-1 and hyaluronan levels were above the cut-off values defined by ROC analyses, the relative risk of fibrosis stage ≥F2 rose from 8.65 and 8.38 for TIMP-1 or hyaluronan alone, respectively, to 18.35 (Table 9A). When the gold standard was used as the dependent variable, the rela-

Table 8. Value of age and independent biochemical parameters for the prediction of advanced fibrosis, either alone (Table 8A), or with the gold standard (Table 8B), for the diagnosis of advanced fibrosis METAVIR stage ≥F2

A

Age & Biochemical markers	Relative risk	95% Confidence interval
Age	2.55	1.06-6.12
Prothrombin time	2.41	1.00-5.82
GGT	4.46	1.85-10.74
Age + Prothrombin time + GGT	27.44	

B

Age & Biochemical markers	Relative risk	95% Confidence interval
Age	3.11	1.25-7.77
Albumin	5.25	1.91-14.40
Prothrombin time	4.18	1.53-11.45
GGT	3.35	1.34-8.39
Age + Albumin + Prothrombin time + GGT	228.8	

tive risk of advanced disease was 5.41 for TIMP-1 and 14.97 for hyaluronan, reaching 28.57 with the combined use of both parameters (Table 9B).

DISCUSSION

The current study aimed to extend our existing knowledge on several points: (i) it was performed in a large cohort of patients with chronic hepatitis B and D, whereas most other studies were performed in patients with chronic hepatitis C and alcoholic liver disease; (ii) a large spectrum of well-validated serum fibrosis markers was tested together with biochemical tests in the same sample cohort (18-21); (iii) these markers were assessed not only for their predictive value of fibrosis stage but also of necroinflammation; and (iv) a 'new gold standard' using splenomegaly and a platelet count of <150,000 as indirect evidence for advanced liver disease was created to minimize possible sampling error in advanced liver disease.

HBeAg-positive chronic hepatitis B patients were younger than HBeAg-negative patients. This is expected and fits well with the natural history of chronic hepatitis B infection (26). Delta hepatitis patients had lower platelet counts, lower albumin and higher GGT levels and thus had more advanced disease compared to HBeAg-positive patients, which again is in line with the known more aggressive course of delta hepatitis (27). Delta hepatitis patients were older than HBeAg-positive but younger than HBeAg-negative cases, which indicates that despite its decrease or even disappearance in some parts of the world (28), delta hepatitis likely continues to constitute an important health problem in Turkey (29,30). Since the patient cohort was selected among patients who were eligible for various treatment trials for compensated chronic liver disease, we consider the above drawn conclusions as valid interpretations.

Our data show that many of the tested biochemical and fibrosis markers not only predict patients with advanced fibrosis but also marked necroinflammation. Although similar findings have been previously reported for some of these parameters (10,14), other authors found only a minor correlation between fibrosis stage and necroinflammation (31,32). In addition, some fibrosis-specific markers have previously been suggested to represent surrogate markers for inflammatory activity in the liver, since they were found to correlate better with inflammation than matrix content (10,33).

Our patients with advanced fibrosis were older than those with mild or no fibrosis. This is in line with the existing literature on patients with chronic hepatitis C (13,15,34). However, in our study, age continued to be an independent predictive factor after multivariate analysis, which is in varian-

Table 9. Diagnostic value of the two independent predictors of fibrosis, TIMP-1 and hyaluronan, either alone (Table 9A), or with the gold standard (Table 9B), for the diagnosis of advanced fibrosis METAVIR stage ≥F2

A

Fibrosis markers	Relative risk	95% Confidence interval
TIMP-1	8.65	2.5-16.78
Hyaluronan	8.38	2.67-17.4
TIMP-1+Hyaluronan	18.35	

B

Fibrosis markers	Relative risk	95% Confidence interval
TIMP-1	5.41	2.11-15.11
Hyaluronan	14.97	4.55-25.88
TIMP-1+Hyaluronan	28.57	

ce with the previously reported reduced predictive value of age compared to other markers (17,35,36), but in line with a recent paper (15).

Among biochemical and hematological markers, GGT and prothrombin time were the two independent predictive parameters for advanced fibrosis by multivariate analysis. Similar results have been reported by others in alcoholic liver disease and chronic hepatitis C (11,14,15,36,37). In a larger study in patients with chronic hepatitis B, the FibroTest, which includes GGT as well as bilirubin, gamma globulin, haptoglobin, and $\alpha 2$ macroglobulin, has been reported to predict fibrosis stage ≥ 2 with an AUROC of 0.78, sparing biopsy for 46% of the patients, and with positive and negative predictive values of 92% in these 46% (38). Other studies performed by various groups have also indicated that FibroTest was a useful biomarker for predicting liver fibrosis in patients with hepatitis B infection (39,40).

Hyaluronan and TIMP-1 emerged as the two independent predictive fibrosis markers for METAVIR stage ≥ 2. Of these two markers, hyaluronan had previously been quoted as the single marker that most successfully predicts advanced fibrosis in patients with chronic hepatitis C (41,42) and various other liver diseases (37,43-45). Hyaluronan is a glycosaminoglycan produced by hepatic stellate cells among others (10). However, elevated levels appear to derive mainly from decreased uptake from the circulation by sinusoidal endothelial cells (46-48). Failure to degrade the increased interstitial matrix is a key feature of advanced fibrosis. TIMP-1 inhibits interstitial collagenases (MMP-1, -8 and -13) that degrade type I and III collagens, the major collagens in the fibrotic liver (49). Increased hepatic TIMP-1 levels are instrumental in the progression of liver fibrosis (50), and elevated serum levels correlate with hepatic TIMP-1 protein (51). With the cut-off values defined by the AUROC curves, patients with elevated levels of both of these markers were 18.4 times more likely to have advanced histological fibrosis. An algorithm named as the Enhanced Liver Function (ELF) test was developed by the European Liver Fibrosis Group in a multi-center cohort study performed with 1,021 chronic liver disease patients. The ELF test is based on the combination of the PIIINP marker with hyaluronan and TIMP-1 markers (52). The results of the study revealed high diagnostic accuracy in detection of significant fibrosis (AUROC 0.80, 95% confidence interval [CI]: 0.76 to 0.85). In a recent retrospective study comparing FibroTest and ELF, the accuracy for the diagnosis of advanced fibrosis ($F \geq 2$) was found to be 0.69 (95% CI: 0.57-0.82) and 0.78 (95% CI: 0.67-0.89), respectively (53). However, this difference was not statistically significant.

The one major drawback of this study was that liver biopsies with 6 portal tracts were allowed for assessment. This may be seen as a weakness of the study since for optimal assessment of the liver biopsy, the requirement of at least 9-11 portal tracts is suggested (54,55). However, to circumvent the problems inherent in suboptimal liver histology, we validated the biochemical and fibrosis markers not only against liver histology but also against a novel "gold standard" including clinical indices of advanced liver disease. Using this novel standard, the number of patients with advanced fibrosis increased from the biopsy-defined number of 39 to 57. Only four of the additional patients had splenomegaly as the only sign of advanced diseases (with a platelet count >150,000), while the other 13 patients had a platelet count <150,000. Since previously, only the platelet count was added to predict advanced disease (11,13,32), further addition of splenomegaly apparently provides an improved approach. In addition, it questions once more the reliability of liver histological assessment alone as the gold standard for assessment of the stage of liver disease, in view of the reported sampling error rate of up to 20-33%, especially in patients with cirrhosis (3-6). Furthermore, several liver biopsy needles with various needle diameters and differing techniques exist, such as suction, cutting and spring-loaded cutting needles, and in patients with advanced fibrosis the commonly used suction needles may not be optimal (56). With these reservations as to liver biopsy, we believe that the novel standard is reliable for the staging of liver fibrosis. When this standard was used, the predictive value for advanced liver fibrosis of the combined use of the four parameters (age, albumin, prothrombin time and GGT), all of which had an independent predictive value after multivariate analysis, was as high as 228.3. Thus, when for a given patient all four parameters were above the cut-off values defined by ROC curves, advanced liver disease was almost certain. Since only 6 out of the 109 patients met these criteria, this information is unlikely to be useful in clinical practice. On the other hand, two matrix-derived markers, TIMP-1 and hyaluronan,

remained highly predictive of advanced fibrosis after stepwise multivariate analysis when using the gold standard, correctly allocating 32 patients to the advanced fibrosis group, when using the "gold standard" as the dependent variable, with a specificity of 88.9% (32 of 36) and a sensitivity of 56.1% (32 of 57 patients). Once both of these markers were above the cut-off values in a given patient, as determined by ROC curves, this patient was 25 times more likely to have advanced disease. We think that the use of these two markers is simple and could be an important adjunct in daily clinical practice.

In summary, this study provides data that suggest that the use of two serum fibrosis markers, TIMP-1 and hyaluronan, can provide important information for correctly predicting advanced liver disease in patients with chronic hepatitis B. The validity of these tests should be tested in prospective trials. These fibrosis markers should be used to complement information obtained by liver biopsy, platelet count and spleen size. Such an approach could be relevant not only in clinical practice but also in studies assessing the natural history of various chronic liver diseases as well as their long-term treatment outcomes by minimizing misleading results obtained by liver biopsy alone.

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