Multivariate Discriminant Function Based on Six Biochemical Markers in Blood Can Predict the Cirrhotic Evolution of Chronic Hepatitis

GIULIANA FORTUNATO,1 GIUSEPPE CASTALDO,1,3 GIOVANNANGELO ORIANI,1,4 RAIMONDO CERINI,2 MARIANO INTRIERI,3 EUGENIA MOLINARO,1 IVAN GENTILE,2 GUGLIELMO BORGIA,2 MARCELLO PIAZZA,2 FRANCESCO SALVATORE,1* and LUCIA SACCHETTI1

Background: Serologic markers have been proposed for monitoring hepatic fibrosis in chronic active liver disease. Because none of these markers, when used singly, is totally satisfactory, we developed and evaluated a multivariate approach.

Methods: We studied two cohorts of chronic hepatitis (54 patients) and cirrhosis patients (49 patients) to identify a panel of biochemical markers that discriminates between the two diseases. Using multivariate discriminant analysis, we selected a function, based on the concentrations of six biochemical markers (fibronectin, prothrombin, pseudocholinesterase, alanine aminotransferase, manganese superoxide dismutase, and N-acetyl-β-glucosaminidase). We then prospectively validated this function on a second temporal cohort of patients.

Results: Multivariate discriminant analysis correctly classified 93.7% of patients (94.3% of chronic hepatitis and 92.9% of cirrhosis patients) in the first cohort and 85% of patients (89.5% of chronic hepatitis patients and 81% of cirrhosis patients) in the second cohort.

Conclusions: Discriminant analysis of results of six inexpensive biochemical markers provides a high predictive value for differentiation between liver cirrhosis and chronic hepatitis. Consequently, these biochemical markers condensed into a multivariate discriminant analysis value for each patient provide information that can be contributory for subsequent options during the evolution of the natural history of chronic hepatitis.

© 2001 American Association for Clinical Chemistry

Chronic liver diseases often begin with the anatomic-clinical state of chronic hepatitis and can evolve to liver cirrhosis and hepatocarcinoma. Various etiologic factors have been associated with chronic hepatitis: B and C hepatitis viruses, alcohol, and less frequently, such metabolic disorders as α1-antitrypsin and ceruloplasmin deficiency and cystic fibrosis (1). Among these causative factors, chronic hepatitis attributable to hepatitis virus C is the entity that most frequently evolves to liver cirrhosis (2). The cirrhotic evolution of chronic hepatitis is associated with liver fibrogenesis. The timely recognition of liver fibrogenesis can improve the choice and, therefore, the outcome of treatment (3). The reference procedure for early identification of cirrhotic evolution of chronic hepatitis is histopathology incorporating semiquantitative scores (4–7). This procedure, however, is invasive and is subject to interobserver variability (8) because the alterations in liver morphology are very heterogeneous. Imaging techniques (5) and biochemical markers of liver fibrosis, such as fibronectin, N-acetyl-β-glucosaminidase (β-NAG),5 laminin, and procollagen 3 propeptide (PIIIP), have been proposed as indicators of the change from chronic hepatitis to cirrhosis (3). However, PIIIP and laminin concentrations are increased in the plasma of alcoholic patients independent of the stage of liver disease (9). Consequently, biochemical markers at present do not

1 Dipartimento di Biochimica e Biotecnologie Mediche and CEINGE scarl, and 2 Dipartimento di Medicina Pubblica e della Sicurezza Sociale-Sezione di Malattie Infettive, Università di Napoli “Federico II”, I-80131 Naples, Italy.
3 Facoltà di Scienze Matematiche, Fisiche e Naturali, and 4 Dipartimento “SAVA”, Università del Molise, 86170 Isernia, Italy.
*Address correspondence to this author at: Dipartimento di Biochimica e Biotecnologie Mediche, via S. Pansini 5, I-80131 Naples, Italy. Fax 39-081-746-3650; e-mail salvator@unina.it.

Received March 22, 2001; accepted June 18, 2001.

5 Nonstandard abbreviations: β-NAG, N-acetyl-β-glucosaminidase; PIIIP, procollagen 3 propeptide; MDA, multivariate discriminant analysis; Mn-SOD, manganese superoxide dismutase; Apo, apolipoprotein; PCHE, pseudocho-
clearly distinguish between chronic hepatitis and cirrhosis. Our group used multivariate discriminant analysis (MDA) to select biochemical analytes that contribute to the differentiation between liver cirrhosis and hepatocarcinoma (10), between hepatocarcinoma and secondary liver neoplasia (11), and between neoplastic and nonneoplastic ascites (12).

To identify a panel of biochemical markers that differentiate between chronic hepatitis and cirrhosis, we analyzed biochemical markers related to liver function in a first temporal cohort of chronic hepatitis and cirrhosis patients, independently classified on the basis of histology. Using MDA, we selected a function, based on the concentrations of the six biochemical markers that efficiently differentiated between the two groups of patients. We then prospectively validated this function on a second temporal cohort of patients.

**Materials and Methods**

**Patients**

The patients enrolled in this study were from the Department of Infectious Diseases of our Medical School. The study was approved by the Ethics Committee of our Medical School, and informed consent was obtained from each patient. Patients with clinically and laboratory-confirmed chronic hepatitis or Child-A liver cirrhosis underwent a percutaneous liver biopsy. The Menghini needle, which yields samples of at least 2 cm, was used for this procedure. The histologic evaluation was performed by a panel of pathologists from our Medical School; at least two independent pathologists who were unaware of the clinical and laboratory results examined each sample (4, 6, 7). The first temporal cohort of patients consisted of 35 patients affected by chronic active hepatitis staged according to Desmet et al. (13) and 28 affected by liver cirrhosis subsequent to chronic hepatitis (Child A, n = 10; Child B, n = 8; Child C, n = 10). The second temporal cohort consisted of 19 patients affected by chronic active hepatitis and 21 by liver cirrhosis subsequent to chronic hepatitis (Child A, n = 10; Child B, n = 7; Child C, n = 4). In all cases the etiology was the hepatitis C virus; other causes of liver disease (e.g., other viruses, genetic, metabolic, or alcoholism) were ruled out. No patient had clinical or laboratory evidence of concomitant diseases or had received interferon treatment before liver biopsy and blood collection.

**Analytical Methods**

Blood obtained by venipuncture was processed for the analysis of fibronectin (turbidimetric; Roche), β-NAG (enzymatic; Cortecs diagnostics), PIIIP (RIA; Behring), glutathione peroxidase (enzymatic; Randox), manganese superoxide dismutase (Mn-SOD; ELISA; Bender MedSystem), ferritin and α-fetoprotein (immunoenzymatic; Boehringer), apolipoprotein (Apo) AI and Apo B (nephelometric; Behring), and prothrombin activity (clotting method; Organon Tecnika). Albumin, pseudocholinesterase (PCHE), alkaline phosphatase, γ-glutamyltransferase, cholesterol, LDL- and HDL-cholesterol, triglycerides, total and direct bilirubin, and aspartate and alanine aminotransferase (AST and ALT) were measured using standard methodologies (Boehringer). Platelet counting was performed on a Bayer-Technicon H2 analyzer (Bayer-Technicon Instruments).

**Statistical Analyses**

We used the statistical design adopted in previous studies by our group (10–12). Univariate statistical analysis was performed with the nonparametric Mann–Whitney U-test to compare the distribution of all analyte values in chronic hepatitis and cirrhosis patients from the first temporal cohort. Multivariate analysis based on stepwise multiple linear discriminant analysis with the Wilks lambda criterion was used to select the best combination of serum biochemical markers that differentiated chronic hepatitis and cirrhosis.

We preliminarily tested the differences in distribution of each analyte between the chronic hepatitis subgroups by ANOVA (13). No statistically significant differences were found across these subgroups; we therefore considered the chronic hepatitis patients as a single group. Furthermore, before the discriminant analysis, all of the analytes were checked for gaussian distribution with the Shapiro–Wilks method within the whole chronic hepatitis group. Most of the variables deviated significantly from a gaussian distribution in the group of patients with cirrhosis, whereas all analytes had a gaussian distribution in the chronic hepatitis patient group; the deviation was successfully corrected by natural logarithm (ln) transformation of the data.

The first cohort of patients was used to select the linear combination of variables that best differentiated chronic hepatitis from cirrhosis. To avoid introducing correlated variables, we tested the correlation between each pair of analytes with the Pearson correlation coefficient (r) (12). In the case of highly correlated analytes (r >0.70), the least significant markers at a univariate level were excluded from the subsequent MDA. We used the jackknife procedure to validate the results of the MDA on the first cohort of patients. We then prospectively tested the efficiency of the discriminant function on the second temporal cohort of patients. Diagnostic sensitivity and specificity were defined according to Galen and Gambino (14).

**Results**

The Mann–Whitney U-test showed that the distribution of 18 of the 24 markers analyzed differed significantly between chronic hepatitis and cirrhosis patients (Table 1) in the first cohort of patients. However, none of these analytes efficiently differentiated between the two populations because of widely overlapping values (see example in Fig. 1). We thus used the MDA.

The following variables deviated from a gaussian distribution: β-NAG, PIIIP, Mn-SOD, ferritin, Apo B,
prothrombin activity, albumin, alkaline phosphatase, γ-glutamyltransferase, LDL- and HDL-cholesterol, triglycerides, total and direct bilirubin, AST, ALT, and α-fetoprotein. The ln transformation restored the gaussian distribution. Total cholesterol and LDL-cholesterol were excluded from the subsequent MDA because they were correlated with Apo B, a more significant variable (Pearson $r > 0.70$). The best linear combination of blood mark-

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Reference interval</th>
<th>Chronic hepatitis (n = 35)</th>
<th>Cirrhosis (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>$P^*$</td>
</tr>
<tr>
<td>Albumin, g/L</td>
<td>36–52</td>
<td>43</td>
<td>0.6</td>
</tr>
<tr>
<td>Prothrombin activity, %</td>
<td>70–120</td>
<td>96.2</td>
<td>1.9</td>
</tr>
<tr>
<td>PCHE, μkat/L</td>
<td>90–220</td>
<td>155</td>
<td>9.5</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>3.10–5.17</td>
<td>4.5</td>
<td>0.2</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>&lt;3.62</td>
<td>2.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Apo A1, mg/L</td>
<td>1100–1700</td>
<td>1550</td>
<td>45</td>
</tr>
<tr>
<td>Apo B, mg/L</td>
<td>1150–2200</td>
<td>1044</td>
<td>69</td>
</tr>
<tr>
<td>Total bilirubin, μmol/L</td>
<td>3.42–17</td>
<td>19.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Direct bilirubin, μmol/L</td>
<td>0.0–3.42</td>
<td>0.34</td>
<td>0.51</td>
</tr>
<tr>
<td>ALP, μkat/L</td>
<td>1.6–4.5</td>
<td>3.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Fibronectin, mg/L</td>
<td>250–400</td>
<td>315.8</td>
<td>12</td>
</tr>
<tr>
<td>MnSOD, μg/L</td>
<td>35–114</td>
<td>121.06</td>
<td>69</td>
</tr>
<tr>
<td>AST, μkat/L</td>
<td>0–0.6</td>
<td>1.1</td>
<td>0.15</td>
</tr>
<tr>
<td>LDH, μkat/L</td>
<td>3.75–7.50</td>
<td>4.78</td>
<td>0.17</td>
</tr>
<tr>
<td>GPX, μkat/L</td>
<td>6.63–16.45</td>
<td>11.05</td>
<td>0.37</td>
</tr>
<tr>
<td>PIIIP, kilounits/L</td>
<td>0.3–0.8</td>
<td>0.91</td>
<td>0.59</td>
</tr>
<tr>
<td>Platelet count × 10⁹/L</td>
<td>150–400</td>
<td>150.8</td>
<td>8.8</td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>0.608</td>
<td>0.32</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

$^a$ Mann–Whitney $U$-test.

$^b$ ALP, alkaline phosphatase; LDH, lactate dehydrogenase; GPX, glutathione peroxidase.

Fig. 1. Scattergrams of three blood markers in patients affected by chronic hepatitis (n = 35) or cirrhosis (n = 28).
Biochemical markers have already been proposed to assess the liver fibrogenesis usually associated with cirrhosis. For example, PIIIP is a well-known marker of liver fibrogenesis; however, its plasma concentrations do not differ significantly between chronic active hepatitis and liver cirrhosis (15). Similarly, plasma concentrations of hyaluronic acid do not discriminate chronic hepatitis from cirrhosis (16).

The analytes selected by the MDA showed satisfactory efficiency. They are not related, which means that there is no redundancy and that they explore different biochemical abnormalities associated with the two conditions. Prothrombin activity and PCHE are known markers of liver protein synthetic activity, which is gradually impaired during the cirrhotic evolution of chronic hepatitis. β-NAG and fibronectin are well-known markers of fibrosis (17). The increase in serum β-NAG activity in cirrhotic patients is attributable to the increased accumulation of collagen typical of the disease. β-NAG is known to be important for the collagen pathways (18). By contrast, the reduction of circulating fibronectin in cirrhotic patients is likely related to the impaired protein synthesis that occurs in cirrhotic liver (17). Mn-SOD is located mainly in the mitochondrial matrix, a site of reactive oxygen species production; it is involved in the antioxidative pathways of human cells (19).

Mn-SOD mRNA concentrations in peripheral blood mononuclear cells and serum Mn-SOD concentrations are higher in patients with hepatitis C viral infections than in healthy controls (20). Furthermore, oxidative stress has been implicated in the pathogenesis of hepatitis C viral infection (21). Finally, serum ALT could be considered a signal of liver cytology, which differs in chronic hepatitis and cirrhosis.

The degree of hepatic fibrosis is usually evaluated by liver biopsy. However, liver biopsy is correlated with a high risk of morbidity, especially in patients with coagulative disorders such as those induced by chronic liver diseases; moreover, the accuracy of liver biopsy is questionable because of interobserver variability (8). Similarly, although quantitative image analysis has a greater diagnostic accuracy, it provides limited information, giving only a partial reflection of liver fibrosis, and it is expensive (5). The analysis of serum biochemical markers is, on the contrary, a rapid and inexpensive method, is noninvasive, and leads to high predictive identification of the progression of the fibrotic process and hence its correct treatment, e.g., antifibrotic therapy, which seems to have been successful in some cases (5).

Lastly, we show that the Bayesian probability plot can be used to plan more reliably the diagnostic and therapeutic options for each patient because the degree of fibrosis is one of the factors affecting duration of treatment (22).

In conclusion, fibronectin, ALT, PCHE, prothrombin activity, Mn-SOD, and β-NAG blood concentrations are
easily and rapidly analyzed. Moreover, the assays are quite inexpensive. The MDA score calculated from each patient according to our data may contribute to differentiating between liver cirrhosis and chronic hepatitis. Thus, these six biochemical markers condensed in the MDA value for each patient provide information that can be contributory for subsequent options during the evolution of the natural history from chronic hepatitis.

We gratefully acknowledge grants from the Ministero dell’Università Ricerca Scientifica e Tecnologica (MURST; Rome, Italy), Regione Campania (L.R. 41/96), Associazione Italiana per la Ricerca sul Cancro (AIRC; Milan, Italy), and Regione Molise (P.O.P. 94-99). We thank Jean Ann Gilder for editing the text.

References