Relationship of Serum Fibrosis Markers with Liver Fibrosis Stage and Collagen Content in Patients with Advanced Chronic Hepatitis C

Robert J. Fontana,[1] Zachary D. Goodman,[2] Jules L. Dienstag,[3] Herbert L. Bonkovsky,[4] Deepa Naishadham,[5] Richard K. Sterling,[6] Grace L. Su,[7] Mita Ghosh,[1] Elizabeth C. Wright,[8] and the HALT-C Trial Group

This study determined the utility of a panel of serum fibrosis markers along with routine laboratory tests in estimating the likelihood of histological cirrhosis in a cohort of prior nonresponders with chronic hepatitis C. The relationship between serum markers and quantitative hepatic collagen content was also determined. Liver biopsy samples from 513 subjects enrolled in the HALT-C trial were assigned Ishak fibrosis scores. The collagen content of 386 sirius-red stained, nonfragmented biopsy samples was quantified using computerized morphometry. Serum tissue inhibitor of matrix metalloproteinase-1 (TIMP-1), amino-terminal peptide of type III procollagen (PIIINP), hyaluronic acid (HA), and YKL-40 levels were determined using commercially available assays.Sixty-two percent of patients had noncirrhotic fibrosis (Ishak stage 2-4) whereas 38% had cirrhosis (Ishak stage 5,6). Multivariate analysis identified a 3-variable model (HA, TIMP-1, and platelet count) that had an area under the receiver operating curve (AUROC) of 0.81 for estimating the presence of cirrhosis. This model was significantly better than that derived from the cirrhosis discriminant score (AUROC 0.70), the AST-to-platelet ratio (AUROC 0.73), and a prior model developed in HALT-C patients (AUROC 0.79). Multivariate analysis demonstrated that the serum fibrosis markers correlated substantially better with Ishak fibrosis scores than with the log hepatic collagen content (AUROC 0.84 versus 0.72). *Conclusion:* A 3-variable model consisting of serum HA, TIMP-1, and platelet count was better than other published models in identifying cirrhosis in HALT-C Trial subjects. The stronger correlation of the serum markers with Ishak scores suggests that serum fibrosis markers reflect the pattern of fibrosis more closely than the quantity of hepatic collagen. (HEPATOLOGY 2008;47:789-798.)

Abbreviations: ALT, alanine aminotransferase; APRI, AST-to-platelet ratio index; AST, aspartate aminotransferase; BMI, body mass index; CDS, cirrhosis discriminant score; CHC, chronic hepatitis C; CV, coefficient of variation; HA, hyaluronic acid; HALT-C, Hepatitis C Antiviral Long-term Treatment against Cirrhosis; HCV, hepatitis C virus; INR, international normalized ratio; OR, odds ratio; PCR, polymerase chain reaction; PIIINP, amino-terminal peptide of type III procollagen; TIMP-1, tissue inhibitor of matrix metalloproteinase-1.

[1]Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI; [2]Armed Forces Institute of Pathology, Washington, DC; [3]Gastrointestinal Unit, Medical Services, Massachusetts General Hospital, and the Department of Medicine, Harvard Medical School, Boston, MA; [4]Departments of Medicine and Molecular and Structural Biology and The Liver-Biliary-Pancreatic Center, University of Connecticut Health Center, Farmington, CT; [5]New England Research Institutes, Watertown, MA; [6]Hepatology Section, Virginia Commonwealth University Health System, Richmond, VA; [7]Ann Arbor Veteran's Administration Health Center, Ann Arbor, MI; [8]Office of the Director, National Institutes of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, MD.

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Dr. Bonkovsky's current address is: Cannon Research Center, Suite 201, Carolinas Healthcare System, Charlotte, NC, 28203.

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Address reprint requests to: Robert Fontana, Department of Internal Medicine, University of Michigan Medical School, 3912 Taubman Center, Ann Arbor, MI 48109-0362. E-mail: rfontana@umich.edu; fax: 734-936-7392.

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The severity of hepatic fibrosis and inflammation at diagnosis correlates strongly with the likelihood of disease progression in patients with chronic hepatitis C (CHC).[1,2] In addition, histologic staging in patients with CHC influences disease monitoring as well as antiviral treatment decisions.[3,4] For example, according to treatment guidelines, antiviral therapy is a high priority for patients with moderate to severe fibrosis on liver biopsy. In the same vein, subjects with bridging fibrosis or cirrhosis should undergo surveillance for esophageal varices and hepatocellular carcinoma.[5,6] Therefore, assessment of disease stage is an integral part of the evaluation and management of patients with CHC worldwide.

Liver biopsy is currently considered the "gold standard" for assessing disease severity and stage in patients with various forms of chronic liver disease. However, limitations of liver biopsy include sampling error, understaging, and interobserver variability in interpretation.[7-9] In one study of simultaneous right and left lobe laparoscopic liver biopsies, a discordance of at least 1 fibrosis stage was identified in 24% of the biopsies and understaging of cirrhosis in 14%.[8] Furthermore, because of its risks, inconvenience, and costs, serial liver biopsy is not practical to monitor disease progression and treatment effect. Therefore, developing noninvasive tests that can accurately predict initial disease stage and fibrosis progression over time represents a high priority and growing medical need.[10] Routinely obtained blood tests such as serum aspartate aminotransferase (AST), AST/alanine aminotransferase (ALT) ratio, and platelet count levels have been proposed as indirect markers of advanced fibrosis in patients with CHC.[11-15] Although widely available, these laboratory tests do not reliably distinguish between individual stages of fibrosis nor the presence of advanced fibrosis with a high level of certainty. In addition, none of the blood tests included in these panels directly reflect the pathophysiology of hepatic fibrogenesis mediated by hepatic stellate cells.[16] Panels of serum fibrosis markers such as hyaluronic acid (HA), amino-terminal peptide of type III procollagen (PIIINP), tissue inhibitor of matrix metalloproteinase inhibitor-1 (TIMP-1), and YKL-40 are believed to more directly reflect the resorption of low-density extracellular matrix and deposition of high-density matrix in patients with chronic liver disease.[17-19] Cross-sectional studies suggest that some of these serum fibrosis markers, either individually or in combination, may provide important disease staging information such as the presence or absence of advanced fibrosis or cirrhosis.[17]

The Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) Trial is a prospective multicenter study designed to investigate the potential benefit of maintenance peginterferon therapy in reducing the rate of clinical outcomes and histological progression in prior interferon nonresponders with advanced fibrosis.[20,21] The aim of the current study was to determine the utility of a panel of serum fibrosis markers including serum PIIINP, TIMP-1, HA, and YKL-40 in estimating initial disease stage in 513 subjects enrolled in the HALT-C trial. In addition, the relationship between serum fibrosis markers and the quantitative assessment of hepatic collagen content as measured by computerized morphometry was determined.[22,23] Finally, the accuracy of models derived from our study population that incorporated serum fibrosis markers and routine laboratory tests were compared to the results obtained with other published models based solely on standard laboratory tests.

Patients and Methods

Patient Population. Inclusion criteria for the HALT-C Trial include the presence of detectable serum hepatitis C virus (HCV) RNA, a liver biopsy within 12 months of enrollment demonstrating bridging hepatic fibrosis (that is, an Ishak fibrosis score $\geq$3) or cirrhosis, and lack of a sustained response to a prior course of interferon with or without ribavirin.[20,21] All patients underwent a pretreatment liver biopsy to confirm the presence of at least bridging fibrosis (that is, an Ishak fibrosis score $\geq$3). All baseline liver biopsies were obtained at least 2 months after completing prior interferon therapy in patients that had failed to clear HCV RNA. Subjects were retreated with peginterferon alfa-2a and ribavirin for 24 weeks in the "lead-in phase" of the study. Subjects who remained viremic at week 20 were eligible for entry into the randomized phase of the HALT-C trial whereas subjects with undetectable HCV RNA by polymerase chain reaction (PCR) continued in the "responder arm" of the study and completed a 48-week course of combination antiviral treatment. All participants entering the lead-in phase of the HALT-C Trial enrolled at the University of Michigan, University of Massachusetts/University of Connecticut, Massachusetts General Hospital, and the Virginia Commonwealth University had extra serum collected for this ancillary study. Serum was isolated from whole blood samples within 1 hour of collection and frozen immediately at -80°C. Samples were batch-shipped on dry ice and stored at a central repository (Seracare, Washington, DC). The study was approved by local Institutional Review Boards, and all patients provided written informed consent.

Baseline Evaluation. Laboratory tests included complete blood counts, liver panel (AST, ALT, serum bilirubin, alkaline phosphatase, albumin), prothrombin time/

international normalized ratio (INR), and tests to exclude other causes of liver disease. HCV genotype and quantitative HCV RNA levels were performed by a central laboratory (University of Washington, Seattle, WA).[20,21] Serum AST, ALT, and alkaline phosphatase levels were expressed as a ratio of the upper limit of normal based on the reference range of each local hospital laboratory. Liver ultrasounds and upper endoscopies were performed in the participating clinical centers.[6] Lifetime alcohol consumption was determined using the modified Skinner survey.[20]

Liver Histology. Baseline liver biopsies were reviewed by a committee of hepatopathologists representing the 10 clinical centers. The modified histological activity index (also known as the Ishak score) was chosen as the principal scoring system.[24] Fibrosis scores were evaluated with the Masson trichrome stain and determined by committee consensus. Biopsies were recorded as fragmented when the specimen had broken into multiple small rounded pieces that were usually less than 2 mm in maximal diameter. A small number of patients (n = 41) with a biopsy showing stage 2 fibrosis but with a prior biopsy showing stage ≥3 were included in HALT-C. For analysis purposes, biopsies were grouped as Ishak 2, 3, or 4 (precirrhotic fibrosis) versus Ishak 5 or 6 (cirrhosis). Hepatic steatosis was graded using a semi-quantitative grading scheme as follows: 0 (<1%), 1 (1%- 5%), 2 (6%-33%), 3 (34%-67%), 4 (>67% of hepatocytes showing fat) using previously described methods.[25]

Morphometric Image Analysis of Hepatic Fibrosis. Sections of each liver biopsy stained with sirius red were used for assessment of hepatic collagen content as previously described using Image Pro Plus 6.0 imaging software (Media Cybernetics, Silver Spring, MD).[26] The method is based upon the observation that the degree of sirius red staining measured by the saturation of the red channel correlates well with chemically determined collagen content and morphometrically determined estimates of the area of hepatic fibrosis. The collagen content per unit area of the specimen is expressed in arbitrary units calculated as the sum of the pixel-wise collagen measurements divided by the number of summed pixels and is directly proportional to the thickness of the tissue section. Therefore, the collagen content per unit area was divided by the section thickness in microns measured with a confocal laser scanning microscope (Zeiss LSM 310) to yield the final result that was reported in arbitrary units per volume of tissue that can be used for comparison of individual samples and in statistical calculations. Because most liver histologic sections are 4 to 6 μm thick, the arbitrary collagen units were multiplied by 5 to create units with a range of 0 to 0.35. Because computerized morphometry of fragmented liver biopsies tends to underestimate the amount of hepatic fibrosis and provide inaccurate results, only the data obtained from the 386 patients with nonfragmented liver biopsies were included in this analysis.

Serum TIMP-1 Assay. Serum TIMP-1 was measured using a commercial ELISA kit (Quantikine, R&D Systems, Minneapolis, MN). Serum samples were diluted 100-fold and run as singlets on a 96-well microplate. The intra-assay coefficient of variation (CV) was 3.6% and the interassay CV was 5.6%. Samples below the lower limit of detection of <1.0 ng/mL were repeated and samples that were above the upper limit of quantitation of >500 ng/mL were retested using another aliquot at a dilution of 1:2.

Serum PIIINP Assay. Intact N-terminal propeptide of type III procollagen (PIIINP) levels were measured using a commercial RIA kit (UniQ, Orion Diagnostica, Espoo, Finland). Briefly, undiluted serum samples were run as singlets in glass tubes in batches of 42 samples. The intra-assay CV was 3.4% and the interassay CV was 7.3%. Samples below the lower limit of detection of < 1.0 μg/L were repeated. Samples that exceeded the upper limit of quantitation of 15 μg/L were retested using a dilution of 1:10.

Serum YKL-40 Assay. YKL-40 also known as human cartilage glycoprotein 39 is a 40-kilodalton glycoprotein that is believed to be involved in tissue remodeling.[27] Serum YKL-40 levels were determined using a commercial ELISA kit (Metra YKL-40, Quidel Corp., San Diego, CA). Undiluted serum samples were run as singlets on microplates and the intra-assay CV was 3.5% and the interassay CV was 6%. Samples that exceeded the upper limit of quantitation of 300 μg/L were retested after a 1:10 dilution.

Serum Hyaluronic Acid Assay. Serum hyaluronic acid (HA) levels were determined by an automated immunoassay, using liquid-phase binding assay technology (LBA) on the LiBASys analyzer (Wako Diagnostics, Richmond, VA). An undiluted serum sample was automatically aspirated and added to a reagent containing hyaluronic acid binding protein (HABP) and a peroxidase labeled anti-HABP monoclonal antibody.[28] The resulting immune complexes were separated from the remaining free-labeled antibody with an anion-exchange column and the peroxidase activity of the bound fraction was measured using a fluorescent substrate. The assay is linear for HA concentration of 10-1000 ng/mL; samples exceeding 1000 ng/mL were retested after a 1:10 dilution.

Statistical Methods. The individual serum fibrosis marker data were imported as whole datasets from the University of Michigan research laboratory and Wako Diagnostics into a secure internet-based website main-

tained by the data coordinating center (New England Research Institute, Watertown, MA). Non-normally distributed variables were log-transformed when necessary. Logistic regression modeling was used to identify a multivariate model that predicted the presence of cirrhosis in all 513 HALT-C Trial patients. We then compared the area under the receiver operating curve (AUROC) of our model to that derived from standard laboratory tests published in the literature including a previously reported model based on platelets, INR, and AST/ALT developed from baseline data in 1141 HALT-C Trial patients (Lok model), the APRI, and the cirrhosis discriminant score (CDS).[12,14,15] The specific equation for the Lok model is: Log odds = -5.56 -0.0089 × platelet ($\times 10^3$/mL) + 1.26 × AST/ALT + 5.27 × INR and the predicted probability of cirrhosis is: exp ((logodds)/(1 + exp (logodds)).

The CDS is a summary score of platelets (0 to 6 points), ALT/AST ratio (0 to 3 points), and INR (0 to 2 points) with scores ≥8 predicting presence of cirrhosis.[12] Logistic regression was used to compare AUROC among the different models. Because 31% of the 386 patients with computerized morphometry had histological cirrhosis, we modeled the presence of high log-hepatic collagen content by dividing this patient population into similar proportions.

Results

Overall Patient Population. A total of 515 subjects were enrolled into the lead-in phase of the HALT-C Trial at the 4 participating sites; two were excluded from this analysis because of inadequate liver biopsies. The clinical features of the 513 participants included cirrhosis (Ishak 5/6) in 193 subjects (38%), Ishak 2 in 38 subjects (7.4%), Ishak 3 in 189 subjects (37%), and Ishak 4 in 93 subjects (18%) (Table 1). The majority of subjects were male (71%), mean (standard deviation [SD]) age was 49.4 (7.1) years, and 75% were non-Hispanic Caucasians. Participants included 66% who were nonresponders to prior interferon and ribavirin combination therapy, 27% with diabetes mellitus, and 30% with a body mass index (BMI) exceeding 30 kg/m^2. The mean serum AST level was 2.25 times the upper limit of normal and the mean (SD) platelet count was 172 (65) × $10^3/\mu$L.

Serum Fibrosis Markers and Ishak Fibrosis Scores. The mean values of serum PIIINP, HA, TIMP-1, and YKL-40 were significantly higher in subjects with cirrhosis compared to those with noncirrhotic fibrosis (Table 1). On univariate analysis, nearly all of the tested variables were independent predictors of cirrhosis with log HA having the highest odds ratio (Table 2). On multivariate analysis, a model consisting of serum TIMP-1, log HA, INR, and platelet count had an AUROC of 0.83 (95% confidence interval [CI]: 0.79-0.87). When we simplified the model to 3 variables after removing INR, a 3-variable HALT-C model had an AUROC of 0.81 (0.77, 0.85). The 3-variable model was significantly better than any of the individual serum fibrosis markers alone in estimating the presence of cirrhosis (Table 2). In addition, the AUROC of our 3-variable model was significantly better at predicting the presence of cirrhosis compared to other published models that use standard laboratory tests including the APRI, CDS, and Lok model (Fig. 1). The regression formula for the new 3-variable model is as follows: logodds (cirrhosis) = $-3.66 - 0.00995 \times$ platelets (10^3/mL) + 0.008 × serum TIMP-1 + 1.42 × log (HA).

In order to optimize the estimation of the absence of cirrhosis from the AUROC, a cut-off of <0.2 leads to a sensitivity of 88% with a NPV of 86% (Table 3). Similarly, to estimate the presence of cirrhosis, a cut-off of ≥0.5 gives a specificity of 92% with a PPV of 78%. Therefore, use of the 3-variable model in the HALT-C Trial patients could have reduced the need for liver biopsy in 153 (30%) subjects who were predicted to have a low likelihood of cirrhosis with an accuracy of 86% and 146 (28%) patients with a high likelihood of cirrhosis with an accuracy of 73%.

The 3-variable model performed better in subjects with longer biopsies (that is, >1.5 cm in length compared to <1.5 cm). Similarly, the AUROC of the model was consistently higher in subjects with larger biopsy samples that contained a greater number of portal triads and in subjects without fragmented liver biopsy samples (Table 4).

Computerized Morphometry Patient Population. Computerized morphometry was performed on nonfragmented liver biopsy slides from 386 subjects. Figure 2 demonstrates the variability in hepatic collagen content between patients with differing stages of fibrosis. Because the hepatic collagen levels were skewed toward lower levels, the log-hepatic collagen value was used for analyses. The 386 subjects with computerized morphometry data tended to have less advanced fibrosis and a lower frequency of cirrhosis than the overall group of 513 patients due to the exclusion of subjects with fragmented biopsies (31% versus 38% cirrhosis) (Table 1). As expected, they also had higher platelet counts, a lower frequency of varices, and laboratory abnormalities consistent with less severe liver disease and portal hypertension. Figure 3 shows a significant relationship between the log-hepatic collagen content and the Ishak fibrosis scores ($r = 0.48$, $P < 0.001$, Spearman correlation coefficient). However, there was also substantial overlap in the values of log hepatic collagen content and Ishak fibrosis scores. When we com-

Table 1. Baseline Characteristics of the 512 HALT-C Trial Subjects and the 386 with Evaluable Computerized Morphometry

Characteristic	All subjects (n = 512)	Ishak 2-4 (n = 319)	Ishak 5-6 (n = 193)	P-value Ishak 2-4 versus 5/6	Computerized morphometry (n = 386)	*P* value Comp – morphometry (386) versus others (126)*
Age (years)	49.4	48.8	50.3	0.0157	49.16	0.25
% Female	29%	29%	28%	0.78	31%	0.0379
Ethnicity						
% Caucasian	75%	76%	75%	0.75	73%	0.0323
% African-American	19%	18%	21%	0.4	22%	0.0302
% Hispanic	4%	4%	4%	0.67	5%	0.26
Duration of infection (years)	27.0	26.6	27.8	0.11	26.8	0.17
Duration of alcohol (years)	19.5	18.5	21.2	0.0116	19	0.0745
Lifetime alcohol consumption (drinks)	18024	17812	18375	0.83	17496	0.46
Lifetime smoking (pack-years)	15.1	14.9	15.5	0.67	15.2	0.78
% Diabetes	27%	24%	33%	0.0406	28%	0.50
% BMI $>$ 30 kg/m^2	40%	38%	42%	0.38	39%	0.60
Mean BMI (kg/m^2)	29.5	29.5	29.7	0.74	29.5	0.63
% Splenomegaly on ultrasound	32%	27%	40%	0.0021	31%	0.49
% Esophageal varices	17%	7%	30%	$<$0.0001	13%	0.0054
% Prior IFN + ribavirin	66%	65%	68%	0.46	65%	0.17
Log HCV RNA (IU/mL)	6.44	6.5	6.34	0.001	6.43	0.64
% Genotype 1	89%	88%	91%	0.31	89%	0.75
AST ($\times$ ULN)	2.25	1.98	2.7	$<$0.0001	2.21	0.41
ALT ($\times$ ULN)	2.23	2.15	2.35	0.19	2.23	0.97
AST/ ALT	0.86	0.8	0.97	$<$0.0001	0.86	0.58
Alkaline phosphatase ($\times$ ULN)	0.87	0.8	0.99	$<$0.0001	0.87	0.61
T. Bilirubin (mg/dL)	0.76	0.7	0.86	$<$0.0001	0.76	0.94
INR	1.04	1.01	1.08	$<$0.0001	1.02	$<$0.0001
Albumin (g/dL)	3.85	3.94	3.7	$<$0.0001	3.86	0.27
Platelets (10^3/mL)	172	190	142	$<$0.0001	178	0.0002
White blood count (cells/mL)	6.05	6.17	5.84	0.0673	6.08	0.47
Log YKL-40 (μg/L)	2.42	2.32	2.59	$<$0.0001	2.42	0.88
Log HA (ng/mL)	1.96	1.78	2.24	$<$0.0001	1.93	0.0175
Serum PIIINP (μg/L)	6.2	5.44	7.5	$<$0.0001	6.12	0.24
Serum TIMP-1 (ng/ml)	240.8	221.7	272.8	$<$0.0001	239.2	0.37
Biopsy specimen length (cm)	1.84	1.92	1.71	0.006	1.95	$<$0.0001
Mean Ishak fibrosis score	4.04	3.18	5.46	$<$0.0001	3.88	$<$0.0001
% Hepatic steatosis $\geq$ 2	41%	38%	48%	0.0254	41%	0.70
% Fragmented biopsies	22%	14%	36%	$<$0.0001	0%	NA

Data reported as mean or %

*P value $<$ 0.05 for 126 HALT-C subjects without morphometry data versus the 386 with evaluable morphometry scores

pared the 121 subjects with cirrhosis and interpretable computerized morphometry scores to the 72 subjects with cirrhosis and fragmented liver biopsies in whom morphometry analysis was precluded, the patients in whom morphometry was done had similar platelet counts, AST levels and values for most other laboratory tests (Data not shown). Exceptions, however, were the INR (1.06 $\pm$ 0.08 versus 1.11 $\pm$ 0.11, $P < 0.0001$) and serum PIIINP levels (6.90 $\pm$ 2.62 versus 7.86 $\pm$ 2.69, $P = 0.021$) of patients with cirrhosis with morphometry which were significantly lower compared to patients with cirrhosis who were excluded from the morphometry analysis, suggesting that the liver disease in the former group was milder.

Serum Fibrosis Markers and Hepatic Collagen Content. Because the log-hepatic collagen content is reported as a continuous variable, we used correlational analyses to identify predictors of log-hepatic collagen con-

Table 2. Logistic Regression Models Predicting Cirrhosis (Ishak 5/6) in the Overall Population

Characteristic	Univariate Odds ratio * (95% CI)		*P* value
AST ratio	1.51	(1.21,1.88)	0.0003
AST/ALT	1.78	(1.45,2.20)	$<$0.0001
Alkaline phosphatase ratio	1.60	(1.31,1.95)	$<$0.0001
Total bilirubin	1.43	(1.17,1.75)	0.0005
Albumin	0.50	(0.40,0.62)	$<$0.0001
Hemoglobin	0.82	(0.68,0.99)	0.0406
White blood count	0.82	(0.67,1.00)	0.05
Log HCV RNA	0.77	(0.64,0.93)	0.0069
Spleen size	1.38	(1.15,1.67)	0.0006
PIIINP	2.34	(1.85,2.95)	$<$0.0001
YKL-40	1.82	(1.49,2.23)	$<$0.0001
TIMP-1	2.22	(1.77,2.79)	$<$0.0001
Log HA	3.21	(2.49,4.14)	$<$0.0001
INR	2.11	(1.69,2.64)	$<$0.0001
Platelets	0.40	(0.32,0.51)	$<$0.0001

*Models were developed in 471 HALT-C patients with complete data available.

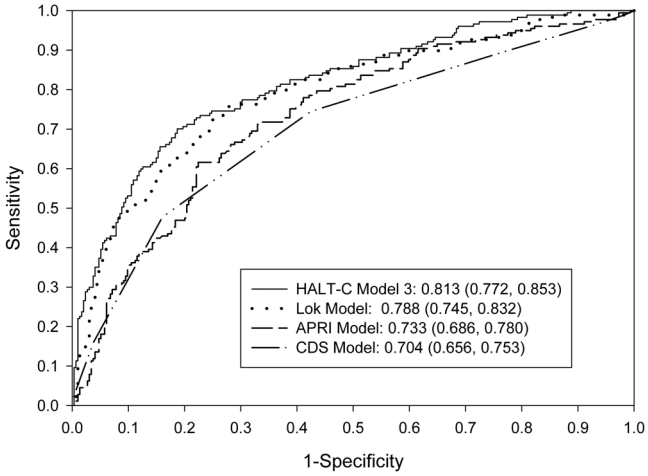

Fig. 1. AUROC for predicting the presence of cirrhosis in HALT-C patients. A 3-variable model derived from our dataset consisting of TIMP-1, log HA, and platelet count had an AUROC of 0.81 which was significantly better than that achieved with the APRI, CDS, or Lok model alone.

Table 4. Application of the 3-Variable Model to Identify Cirrhosis (Ishak 5/6) in Relationship to Liver Biopsy Size

Biopsy length	< 1.5 cm	≥ 1.5 cm
Number of patients	169	301
Number with cirrhosis (%)	67 (40%)	110 (37%)
AUROC	0.768	0.833
(95% CI)	(0.695, 0.842)	(0.785, 0.882)
No. of portal triads	**< 14**	**≥ 14**
Number of patients	216	223
Number with cirrhosis (%)	89 (41%)	77 (35%)
AUROC	0.805	0.847
(95% CI)	(0.745, 0.866)	(0.795, 0.899)
Fragmented Biopsies	**Yes**	**No**
Number of patients	109	374
Number with cirrhosis (%)	66 (61%)	117 (31%)
AUROC	0.788	0.817
(95% CI)	(0.701, 0.875)	(0.770, 0.863)

tent (Table 5). On univariate analyses, nearly all of the laboratory variables tested were significantly associated with the log-hepatic collagen content. Because 31% of the 386 patients with evaluable log-hepatic collagen were known to have cirrhosis based on histological diagnosis, we then divided the population into the upper 31% with high hepatic collagen content and the lower 69%. Using logistic regression, serum TIMP-1 (odds ratio [OR]: 1.004 (CI: 1.001, 1.008), $P = 0.02$), alkaline phosphatase ratio (OR: 3.0 (CI: 1.63, 5.53), $P = 0.0004$), and platelets (OR: 0.61 (CI: 0.47, 0.79), $P = 0.0002$) were identified as independent predictors of high log-hepatic collagen content with an overall c-statistic of 0.72 (CI: 0.67, 0.78). Interestingly, when we modeled the presence of histological cirrhosis by light microscopy, which was identified in 109 of these 386 subjects, a different model consisting of serum PIIINP, TIMP-1, log HA, AST/ALT, and platelet count was identified that had a substantially higher AUROC of 0.84 (CI: 0.80, 0.89). Therefore, the serum fibrosis markers correlated better with the presence of cirrhosis on light microscopy than log-hepatic collagen content in the same group of patients.

Discussion

In this study, a large group of well-characterized CHC patients with advanced fibrosis who were nonresponders to interferon were prospectively studied. Liver histology was scored according to the Ishak fibrosis system and hepatic collagen content was assessed using computerized morphometry. In addition to testing routine laboratory variables, the utility of serum PIIINP, YKL-40, HA, and TIMP-1 levels in estimating cirrhosis was determined. Multiple studies have demonstrated a moderate to strong correlation of serum PIIINP levels with hepatic inflammation and fibrosis stage in patients with CHC.[27] Serum HA is a glycosoaminoglycan that can increase as a result of activated hepatic stellate cell production or reduced he-

Table 3. Application of the 3-Variable Model in Predicting Cirrhosis (Ishak 5/6) in HALT-C Trial Patients

Predicted Values	Number of Patients	Number (%) with Cirrhosis	Number (%) without Cirrhosis	Sensitivity*	Specificity*	PPV*	NPV*
<0.1	64	3 (5%)	61 (95%)	98	21	43	95
0.1-0.2	89	18 (20%)	71 (80%)	88	45	49	86
0.2-0.3	77	16 (21%)	61 (79%)	79	66	58	84
0.3-0.4	55	14 (25%)	41 (75%)	71	80	68	82
0.4-0.5	40	19 (48%)	21 (53%)	60	87	73	78
0.5-0.6	38	23 (61%)	15 (39%)	47	92	78	74
0.6-0.7	33	22 (67%)	11 (33%)	35	96	83	71
0.7-0.8	39	29 (74%)	10 (26%)	19	99	92	67
0.8-0.9	23	21 (91%)	2 (9%)	7	100	92	64
>0.9	13	12 (92%)	1 (8%)	0	100	.	62

*Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for a cutoff of < compared to ≥
Data derived from 177 HALT-C Trial patients with cirrhosis and 294 HALT-C Trial patients with Ishak 2-4.

Fig. 2. Biopsies that illustrate the dissociation between fibrosis stage and hepatic collagen content. The specimen on the left is Ishak fibrosis stage 3 with some very large fibrotic portal areas and occasional bridges but not cirrhosis; its collagen content by morphometry is 0.0205 units (sirius red stain). The biopsy in the center has cirrhosis, Ishak stage 6, with thin septa and a collagen content of 0.0112 units, whereas the biopsy on the right also has stage 6 fibrosis, but much more collagen, 0.521 units.

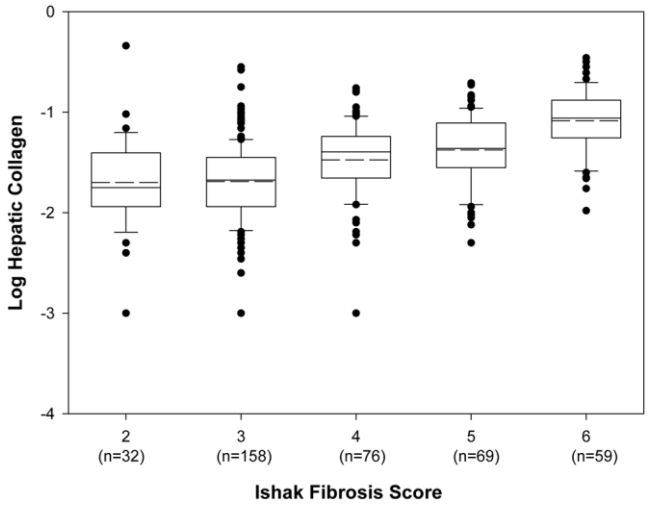

Fig. 3. Box plot of the log hepatic collagen content versus Ishak fibrosis scores in 387 HALT-C Trial patients. Outliers are indicated with circles whereas the mean is indicated with the dashed line. The median is the solid line in the box that represents the interquartile range. The correlation between these variables was significant ($R^2 = 0.22$, $P < 0.0001$). However, there was also substantial overlap in the log hepatic collagen values among patients with differing Ishak fibrosis scores.

patic sinusoidal endothelial cell clearance.[16,17,28] Serum TIMP-1 is an inhibitor of circulating matrix metalloproteinases that can increase as fibrogenesis is activated. The biology of YKL-40 is less well understood, but both cross-sectional and longitudinal studies have demonstrated a moderate to strong correlation between serum YKL-40 levels and fibrosis severity and progression over time in CHC patients.[19] More recent studies have demonstrated that a combination of serum fibrosis markers with or without other host variables may be a more accurate means of estimating fibrosis severity, although longitudinal validation studies in large groups of patients are lacking.[16,17]

On univariate analysis, nearly all of the laboratory and clinical variables that we tested were significantly associated with the presence of cirrhosis (Table 1). However, only 4 laboratory tests were independently associated with cirrhosis using logistic regression. Interestingly, this 4-variable model included 2 serum fibrosis markers (TIMP-1, log HA) as well as 2 routinely obtained laboratory tests (INR, platelets). Identification of serum HA as a strong and independent correlate of cirrhosis in CHC is consistent with prior studies.[16,17] One of the goals of this analysis was to develop a model potentially useful to clinicians; we found that a simplified 3-variable model that excluded the INR performed nearly as well. The 3-variable model was significantly better at identifying cirrhosis than previously described models that exclusively rely on routine laboratory tests (Fig. 1). Therefore, measurement of serum hyaluronic acid, TIMP-1, and platelet counts can help identify CHC patients with cirrhosis who should

Table 5. Correlational Analyses of Laboratory Parameters and Hepatic Collagen Content

Predictor variable	Log hepatic collagen Correlation coefficient (*P* value)*
AST	0.123 (0.0207)
ALT	0.017 (0.75)
AST/ ALT	0.244 (<0.0001)
Alkaline phosphatase	0.256 (<0.0001)
Total bilirubin	0.185 (0.0005)
INR	0.151 (0.0046)
Albumin	−0.117 (0.0282)
Platelets	−0.271 (<0.0001)
Log YKL-40	0.211 (<0.0001)
Log HA	0.237 (<0.0001)
PIIINP	0.237 (<0.0001)
TIMP-1	0.262 (<0.0001)
APRI	0.153 (0.0041)
Lok model	0.315 (<0.0001)
CDS model	0.213 (0.0001)

*Analyses conducted in the 351 HALT-C patients with evaluable computerized morphometry data and corresponding laboratory data.

be monitored more closely for complications of cirrhosis and liver cancer. The AUROC of our model was very good (0.81) and would allow the correct categorization of 153 HALT-C patients as having a low likelihood of cirrhosis with 86% accuracy and 146 subjects with a high likelihood of cirrhosis with 73% accuracy. However, 42% of the HALT-C Trial subjects are predicted to have an intermediate probability of cirrhosis. The AUROC could have potentially been higher had it not been for the confounding effect of previous interferon therapy on serum fibrosis marker expression.[33,34] In addition, only subjects with advanced fibrosis were allowed into the HALT-C Trial so it is possible that the serum fibrosis markers would have performed better if subjects with Ishak fibrosis scores of 0 to 6 had been included, highlighting the need for additional validation studies in broader, patient populations. Finally, individual biopsy samples may have been misclassified as a result of sampling error which can lead to understaging of fibrosis and cirrhosis. In support of this, our model performed better in subjects without fragmented biopsies and in patients with larger biopsy samples (Table 4).[35,36]

The serum fibrosis markers also correlated with the log-hepatic collagen content as determined by computerized morphometry. Prior studies have shown that morphometry is more sensitive for detecting changes in total hepatic fibrosis during antiviral therapy compared to light microscopy and strongly correlates with chemical determination of liver hydroxyproline content.[23,26,29,37,38] Morphometric analysis of hepatic collagen content, however, is also limited by potential sampling variability and morphometry is unreliable in subjects with fragmented biopsies. The correlation between Ishak fibrosis scores and log-transformed morphometry scores was significant but substantial overlap among individual fibrosis stages was apparent indicating that these measurements are not the same (Fig. 3). Nevertheless, the correlation observed between morphometry and Ishak fibrosis scores was similar to that reported in other studies of CHC patients and a broader range of fibrosis.[30-32] Thus, the information provided by computerized morphometry is likely complementary to that provided by light microscopy staging. Nonetheless, because computerized morphometry is more objective, reproducible, and quantitative than Ishak fibrosis scores, it was anticipated that the serum markers would correlate more strongly with morphometric collagen content than with Ishak fibrosis scores.[32] However, the AUROC for identifying subjects with a high log hepatic collagen content was substantially lower than that obtained based on Ishak fibrosis scores in the 386 HALT-C Trial patients (0.72 versus 0.84). Therefore, if the serum markers and laboratory tests used in our model accurately reflect hepatic fibrogenesis, our observations suggest that the pattern of collagen deposition as measured by the Ishak score is more reflective of disease severity and stage than the absolute amount of collagen in the biopsy. Potentially contributing to the discordance between fibrosis pattern and collagen content is the substantial proportion of HALT-C Trial patients who had diabetes mellitus, obesity, or high lifetime alcohol consumption (Table 1). These conditions are associated with hepatic steatosis and perisinusoidal hepatic collagen deposition which may increase the hepatic collagen content but not influence Ishak fibrosis scores. However, hepatic steatosis scores were not associated with hepatic collagen content ($P = 0.21$) even though there was a significant correlation between each of the serum fibrosis markers tested and the hepatic steatosis grade ($P < 0.0001$ for YKL-40, HA, TIMP-1, and PIIINP with hepatic steatosis grade, data not shown).

Several limitations of our study should be acknowledged. Firstly, the models were not tested in an external validation cohort to confirm and refine them. Because the HALT-C cohort is so unique, an independent cohort of CHC patients with advanced but compensated fibrosis for comparison who also had stored serum available for testing could not be identified. An alternative strategy would have been to develop a test and validation set from this subgroup of HALT-C patients. However, the limited number of patients with computerized morphometry data of 386 precluded us from doing this. Therefore, it is possible that we may have "overfit" our data and independent validation of the proposed serum marker algorithims is needed. In addition, we were not able to differentiate among the individual Ishak fibrosis stages (that is, Ishak 2 from 3, and so forth); however, we felt that grouping fibrosis scores together would help reduce sampling error. Furthermore, the serum fibrosis markers tested are produced in multiple tissues throughout the body which can lead to spurious elevations. For example, serum HA and YKL-40 levels increase in subjects with active rheumatological conditions and in subjects with renal insufficiency[39]; however, both conditions were rare in our population. Moreover, the serum markers evaluated, which reflect active fibrogenesis and/ or fibrolysis rather than the static amount of collagen or scar that has accumulated in the liver, are not as good at estimating disease stage. Nonetheless, the correlation among all of the individual markers tested with the Ishak fibrosis scores was good (Table 1). Finally, the inclusion of the serum markers improved our ability to identify subjects with cirrhosis and our 3-variable algorithim was superior to that obtained using routine laboratory tests (Fig. 1). Although statistically significant, however, the overall increment at-

tributable to the serum fibrosis markers was modest and less than anticipated. Finally, it would have been desirable to compare our algorithim to that obtained using other models that include serum fibrosis markers such as the Fibrotest, Fibrometer, and Hepascore.[40,41] However, because serum samples were not prospectively assayed for haptoglobin, apopliprotein A1 and α2-macroglobulin, these analyses were not possible.

In summary, all of the serum fibrosis markers tested correlated significantly with Ishak fibrosis stage on univariate analysis among 513 patients entering the lead-in phase of the HALT-C Trial. On multivariate analysis, a 3-variable model consisting of TIMP-1, HA, and platelet count distinguished patients with noncirrhotic CHC from those with cirrhosis. Also, this new model performed significantly better than other models based on routine laboratory tests, suggesting that serum fibrosis markers provide useful, incremental information in estimating disease stage in CHC. Contrary to expectations, the serum marker panels did not correlate better with quantitative hepatic collagen levels than with Ishak fibrosis scores. This observation may result from the limited range of morphometry scores in our patients with CHC who all had advanced fibrosis. In addition, our findings suggest that the pattern of fibrosis distribution as determined by light microscopy may be linked more faithfully to the severity of chronic liver diseases such as CHC than the hepatic collagen content determined by computerized morphometry.

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