Serum Hyaluronic Acid with Serum Ferritin Accurately Predicts Cirrhosis and Reduces the Need for Liver Biopsy in C282Y Hemochromatosis

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Diagnosing the presence of cirrhosis is crucial for the management of patients with C282Y hereditary hemochromatosis (HH). HH patients with serum ferritin >1,000 µg/L are at risk of cirrhosis; however, the majority of these patients do not have cirrhosis. Noninvasive markers of hepatic fibrosis may assist in determining which patients with a serum ferritin >1,000 µg/L have cirrhosis and require liver biopsy. This study evaluated the utility of current diagnostic algorithms for detecting cirrhosis, including serum ferritin concentration, platelet counts, and aspartate aminotransferase (AST) levels, in combination with serum markers of fibrosis, hyaluronic acid and collagen type IV (CLIV), in predicting cirrhosis in HH patients. Stage of fibrosis, serum hyaluronic acid and CLIV levels, were measured in 56 patients with HH. No patient with a serum ferritin <1,000 µg/L had cirrhosis, but only 40% of patients with serum ferritin >1,000 µg/L were cirrhotic. A combination of platelet count (<200 × 10⁹/L), elevated AST, and serum ferritin >1,000 µg/L did not detect 30% of cirrhotic subjects. Serum hyaluronic acid was increased in HH compared with controls (42.0 ± 9.8 ng/mL versus 19.3 ± 1.8 ng/mL; P = 0.02). A hyaluronic acid concentration >46.5 ng/mL was 100% sensitive and 100% specific in identifying patients with cirrhosis. In patients with serum ferritin >1,000 µg/L, hyaluronic acid levels were significantly elevated in patients with cirrhosis versus those without cirrhosis (137 ± 34.4 ng/mL versus 18.6 ± 1.5 ng/mL, respectively; P = 0.006). CLIV >113 ng/mL was 100% sensitive but only 56% specific for cirrhosis (area under the curve = 0.78; P = 0.01). Conclusion: In HH, the measurement of hyaluronic acid in patients with serum ferritin >1,000 µg/L is a noninvasive, accurate, and cost-effective method for the diagnosis of cirrhosis. (HEPATOLOGY 2009;49:418-425.)
Hereditary hemochromatosis (HH) is an autosomal recessive disorder that results in an inappropriate increase in intestinal iron absorption. Excess iron is deposited in the liver, which results in 10% to 25% of the affected male population developing fibrosis, with hepatic cirrhosis evident in 4% to 6%. Associated complications include portal hypertension, liver failure, and hepatocellular carcinoma. The development of cirrhosis is the single most important determinant of survival in affected individuals, and patients with cirrhosis should undergo regular radiological and endoscopic surveillance to reduce morbidity and mortality from hepatocellular carcinoma or complications of portal hypertension. Thus, diagnosing the presence of cirrhosis is a crucial element of the clinical management of patients with HH.

Prior to the identification of the HFE gene, most patients with suspected iron overload underwent liver biopsy to provide precise diagnostic information based on measurements of hepatic iron concentration, and to evaluate the extent of hepatic fibrosis and the presence or absence of cirrhosis. Because most patients with the typical HH phenotype are homozygous for the C282Y mutation, genetic testing has now replaced liver biopsy in diagnosis of the disease. Thus, the role of liver biopsy in affected patients is now primarily to determine the presence or absence of hepatic cirrhosis.

An important risk factor for cirrhosis is the hepatic iron concentration, and there exists a close relationship between serum ferritin concentration and liver iron stores. Previous studies have highlighted a serum ferritin concentration of 1,000 μg/L as an important threshold below which patients are at minimal risk of cirrhosis, and Beaton and colleagues included serum ferritin, platelet count less than 200 × 10⁹/L, and elevated AST in an algorithm designed to predict those HH patients with cirrhosis. A correct diagnosis of cirrhosis was made in 81% of cases. However, liver biopsy is still required in most patients with a serum ferritin greater than 1,000 μg/L, because the Beaton et al. diagnostic algorithm is not sufficiently accurate to detect all HH patients with cirrhosis.

Although liver biopsy remains the gold standard method of detecting the presence of cirrhosis, it is an invasive and expensive procedure, and in recent years there have been considerable efforts to develop noninvasive markers of hepatic fibrosis. Most of the serum markers reported to accurately detect cirrhosis include the measurement of hyaluronic acid as a component of a large panel of automated immunoassays that reflect liver function or injury, matrix constituents, and mediators of matrix remodeling. Hyaluronic acid plays a prominent role in the pathogenesis of liver fibrosis, and the elevation of serum hyaluronic acid and other noninvasive biomarkers (type IV collagen and laminin) is due to either increased synthesis or degradation by inflammatory cells and hepatic stellate cells, and in the case of hyaluronic acid, reduced clearance by sinusoidal endothelial cells. HH is characterized by a sparse inflammatory cell infiltrate suggesting that the use of biomarkers used to diagnose fibrosis or cirrhosis in HH may be quite different to more inflammatory liver diseases such as hepatitis C viral infection and alcoholic liver disease.

In this study, we evaluated the use of biomarkers of hepatic fibrosis in combination with the measurement of serum ferritin concentration to determine whether this combined strategy could improve the sensitivity and specificity in detecting cirrhosis in a well-characterized cohort of HH patients. This study demonstrates that measurement of serum hyaluronic acid in HH patients with a serum ferritin greater than 1,000 μg/L increases both the sensitivity and specificity of detecting cirrhosis and therefore reduces the requirement for liver biopsy in HH. We propose that measuring hyaluronic acid levels in HH patients with a serum ferritin greater than 1,000 μg/L is a cost-effective and noninvasive approach that may be useful in clinical practice for the detection of cirrhosis in HH.

Patients and Methods

Patients. Fifty-six previously untreated patients homozygous for the C282Y mutation in the HFE gene were studied. Analysis of the C282Y mutation was performed via polymerase chain reaction analysis as described. Each patient had undergone a complete clinical, laboratory, and histological evaluation supervised by one of two hepatologists (D. C., L. W. P.). Other causes of liver disease were excluded in each patient by testing for anti–hepatitis C virus antibody, hepatitis B surface antigen, smooth muscle antibodies, antimitochondrial antibody, copper, ceruloplasmin, and alpha-1-antitrypsin. Patients were not excluded on the basis of excess alcohol consumption. Serum hyaluronic acid concentration was measured in 20 healthy volunteers (controls) without evidence of iron overload (11 male, 9 female; age range, 21-49) to determine the effects of age and sex on hyaluronic acid measurements in our population. This cohort of patients and controls includes a subset of subjects previously used by George and colleagues for the evaluation of a number of different serum markers of the progression of hepatic fibrosis (F0-F4), including serum collagen type IV (CLIV). All patients gave informed consent, and studies were approved by the Human Research Ethics Committees of The Queensland Institute of Medical Research, the...
Princess Alexandra Hospital, and the Royal Brisbane and Women’s Hospital, Brisbane, Australia.

Biochemical and Histological Assessment. The following data were recorded on patients at the time of diagnosis and retrieved from medical records: age, sex, serum ferritin concentration, transferrin saturation, serum aspartate transaminase (AST) levels, and full blood evaluation including platelet count. Percutaneous liver biopsies were performed on each patient and quantitative measurements of hepatic iron concentration were determined using the method described by Bassett and colleagues.7 Liver biopsy tissue was stained with hematoxylin-eosin for histological analysis, and Van Gieson stain was performed on each specimen to detect collagen protein. The extent of hepatic fibrosis was graded according to the method of Scheuer by a single histopathologist (A. C.).21

Serum Assays. Serum hyaluronic acid and CLIV levels were measured using enzyme-linked immunosorbent assays (from Chugai Diagnostic Science Corporation and Fuji Chemical Industry, Tokyo, Japan) according to the manufacturer’s instructions.

Statistical Analysis. Results are expressed as the mean ± standard error of the mean, except for serum ferritin concentration where data are presented as the median and range. Data were analyzed via independent sample t test, chi-square test, and one-way analysis of variance where appropriate. Correlations between serum markers and the stage of fibrosis were determined using the Spearman rank correlation coefficient (where the stage of fibrosis is a variable with an ordinal scale). Correlations between serum markers and age, serum ferritin concentration, hepatic iron concentration, and alcohol consumption were determined using Pearson’s correlation for continuous variables. Serum ferritin concentrations were log-transformed prior to correlation with serum markers. Receiver operator characteristic (ROC) analysis was performed to assess the use of serum hyaluronic acid and CLIV in identifying HH patients with cirrhosis. Results were considered statistically significant at P < 0.05.

Results

Patients. The clinical and laboratory characteristics of the patients are presented in Table 1. The distribution of fibrosis scores was as follows: F0, 10 patients; F1, 12 patients; F2, 14 patients; F3, 9 patients; F4, 11 patients. Overall, the mean age of the patients was 41.8 + 12.1 years (range, 21-73 years). Forty-five patients were male and 11 were female. The median serum ferritin concentration was 1,160 μg/L (range, 123-6,255 μg/L) and the mean hepatic iron concentration was 242 ± 137 μmol/g dry weight of liver tissue (range, 50-675 μmol/g). HH patients with cirrhosis were older and had higher serum ferritin, AST, and hepatic iron concentrations but lower platelet counts than HH patients without cirrhosis.

Application of Beaton et al. Predictive Model of Cirrhosis. Serum ferritin concentration was available in 55 patients and was less than 1,000 μg/L in 25 subjects. Concordant with previous studies, no patient with a serum ferritin concentration less than 1,000 μg/L had cirrhosis. However, cirrhosis was present in only 11 of the 30 subjects (36.7%) with a serum ferritin concentration greater than 1,000 μg/L, similar to previously reported data.9

We applied the Beaton et al. predictive model of cirrhosis in HH10 to the cohort of 48 patients in whom data on serum ferritin, platelet count, and AST values were available and compared our findings with the French and Canadian HH populations (Fig. 1, Table 2). In this cohort of 48 patients, 26 subjects had a serum ferritin concentration greater than 1,000 μg/L, and of these, 15 had a platelet count greater than 200 × 109/L. Two of these 15 (13.3%) had cirrhosis, and both of these subjects had elevated AST. None of the eight patients with a serum ferritin concentration greater than 1,000 μg/L, platelet count greater than 200 × 109/L, and normal AST had cirrhosis, whereas two of the seven (28.6%) with elevated AST had cirrhosis. These findings are remarkably concor-
dant with the Canadian HH subjects reported in the study by Beaton et al.\textsuperscript{10}

Eight of the 11 patients with a serum ferritin concentration of greater than \(1,000 \mu g/L\) and platelet count of less than \(200 \times 10^9/L\) had cirrhosis. Seven of these eight patients with cirrhosis had an elevated AST, and AST was normal in the other subject. Thus, three of the 10 patients (30.0%) with cirrhosis did not have all of the following characteristics: a serum ferritin concentration greater than \(1,000 \mu g/L\), platelet count \(<200 \times 10^9/L\), and elevated AST. In comparison, 37% of Canadian subjects and 64% of French subjects did not have all of these three criteria as described in the Beaton et al. predictive model for cirrhosis.\textsuperscript{10}

**Measurement of Serum Hyaluronic Acid and CLIV.** In an attempt to improve the noninvasive diagnostic accuracy of cirrhosis detection in HH patients, we assessed two established markers of hepatic fibrosis, hyaluronic acid, and CLIV to investigate their use in combination with the Beaton et al. predictive model of cirrhosis.\textsuperscript{10}

**Serum Hyaluronic Acid.** The concentration of hyaluronic acid was significantly increased in HH subjects compared with control subjects (42.0 ± 9.8 ng/mL, [95% confidence interval (CI) 24-60] versus 19.3 ± 1.8 ng/mL [95% CI 16-23], respectively; \(P = 0.02\)). This difference was principally due to the marked increase in hyaluronic acid levels seen in HH subjects with grade 4 fibrosis (\(n = 11\)) compared with HH subjects with F0-F3 (\(n = 45\)) (137 ± 34.4 ng/mL [95% CI 61-214] versus 18.6 ± 1.5 ng/mL [95% CI 16-22], respectively; \(P = 0.006\) (Fig. 2, Table 3). ROC curve analysis showed that a cutoff value of 46.5 ng/mL provided an area under the

![Fig. 1. Prediction of cirrhosis in 48 Australian C282Y homozygotes using the Beaton et al.\textsuperscript{10} predictive model for the detection of cirrhosis including serum ferritin, platelet count, and AST values. ULN, upper limit of normal.](image)

**Table 2. Utility of the Beaton et al. Predictive Model of Cirrhosis in Australian versus Canadian and French Hereditary Hemochromatosis Populations**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Canadian\textsuperscript{10}</th>
<th>French\textsuperscript{10}</th>
<th>Australian</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of cohort with serum ferritin &gt;1,000 (\mu g/L) with cirrhosis</td>
<td>36%</td>
<td>39.7%</td>
<td>38.5%</td>
</tr>
<tr>
<td>Percentage of cohort with serum ferritin &gt;1,000 (\mu g/L) and platelet count &lt;200 (\times 10^9/L) with cirrhosis</td>
<td>66%</td>
<td>76%</td>
<td>72.7%</td>
</tr>
<tr>
<td>Percentage of cohort with serum ferritin &gt;1,000 (\mu g/L), platelet count &lt;200 (\times 10^9/L), and elevated AST with cirrhosis</td>
<td>77%</td>
<td>90%</td>
<td>77.8%</td>
</tr>
<tr>
<td>Number of patients with cirrhosis not having all three criteria</td>
<td>10/27 (37%)</td>
<td>16/25 (64%)</td>
<td>3/10 (30%)</td>
</tr>
</tbody>
</table>

![Fig. 2. Serum hyaluronic acid in control subjects and in patients with HH. Serum hyaluronic acid levels plotted versus the Scheuer\textsuperscript{20} stage of hepatic fibrosis. The dotted line represents the serum hyaluronic acid threshold for cirrhosis of 46.5 ng/mL, as determined by ROC curve analysis (see Fig. 3A).](image)
Table 3. Hyaluronic Acid and Collagen Type IV Concentration in Relationship to Fibrosis Grade in Hereditary Hemochromatosis Patients

<table>
<thead>
<tr>
<th>Hyaluronic Acid (ng/mL)</th>
<th>Controls (n = 10)</th>
<th>F0 (n = 12)</th>
<th>F1 (n = 14)</th>
<th>F2 (n = 9)</th>
<th>F3 (n = 11)</th>
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<tr>
<td></td>
<td>19.3 ± 1.8</td>
<td>13.8 ± 2.5</td>
<td>20.3 ± 3.3</td>
<td>17.2 ± 2.6</td>
<td>23.9 ± 2.8</td>
</tr>
<tr>
<td>Type IV collagen (ng/mL)</td>
<td>90 ± 6</td>
<td>70 ± 7</td>
<td>117 ± 17</td>
<td>110 ± 14</td>
<td>286 ± 54</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± standard error of the mean.

Hyaluronic acid concentration correlated with each of these variables (age: $r = 0.53$, $P = 0.00004$; serum ferritin: $r = 0.36$, $P < 0.01$; hepatic iron concentration: $r = 0.36$, $P = 0.01$; data not shown). There was no significant correlation between serum hyaluronic acid levels and the consumption of alcohol ($r = 0.20$, $P = 0.18$; data not shown).

In our control population there was no significant effect of sex on serum hyaluronic acid, nor was there any correlation with age (data not shown).

**Serum CLIV.** Serum CLIV was significantly correlated with the stage of hepatic fibrosis ($r = 0.65$, $P < 0.0001$; data not shown), as we have previously demonstrated. As seen with hyaluronic acid, CLIV was significantly increased in HH patients compared with controls ($149 ± 14$ ng/mL versus $90 ± 6$ ng/mL, respectively; $P = 0.0006$) (Table 3). While CLIV levels in HH subjects with cirrhosis were increased compared with controls ($209 ± 34$ ng/mL; $P = 0.008$), CLIV values were higher in F3 ($286 ± 54$ ng/mL) (Table 3) versus F4 patients with considerable overlap across all fibrosis grades as previously reported. A serum CLIV concentration of $113$ ng/mL had $100\%$ sensitivity and $56\%$ specificity in predicting cirrhosis ($AUC = 0.78$; $P = 0.01$) (Fig. 3B).

**Addition of Serum Hyaluronic Acid or CLIV to the Beaton et al. Predictive Model of Cirrhosis.** Our data confirm that a serum ferritin less than $1,000 \mu g/L$ is a highly reliable negative predictor of cirrhosis with $100\%$ accuracy (Fig. 1). However, $61.5\%$ of HH subjects with a serum ferritin concentration greater than $1,000 \mu g/L$ did not have cirrhosis. The predictive value for cirrhosis is only improved to $77.8\%$ (7/9) with the inclusion of platelet counts less than $200 \times 10^3/L$ and an elevated AST.

Therefore, we examined the use of either hyaluronic acid or CLIV in determining which patients with a serum ferritin concentration greater than $1,000 \mu g/L$ had cirrhosis. The mean hyaluronic acid concentration in HH patients with serum ferritin greater than $1,000 \mu g/L$ without cirrhosis was $21.8 ± 2.0$ ng/mL compared with $137.7 ± 34.4$ ng/mL ($P = 0.007$) in patients with cirrhosis. The highest hyaluronic acid concentration in the patient group with serum ferritin greater than $1,000 \mu g/L$ without cirrhosis was $43$ ng/mL, whereas the lowest hyaluronic acid concentration in the patients with cirrhosis was $50$ ng/mL. Therefore, the combination of serum fer-
ritin and hyaluronic acid, using cutoff values of 1,000 µg/L and 46.5 ng/mL, respectively, correctly identified all patients with cirrhosis (Fig. 4), even if their platelet count was greater than 200 × 10⁹/L and AST was normal.

We then assessed the contribution of serum CLIV to the Beaton et al. predictive model. Twenty-five subjects with a serum ferritin concentration greater than 1,000 µg/L were studied. Twenty of these had a CLIV greater than 113 ng/mL, the cutoff for cirrhosis, and of these 20, only 9 had cirrhosis. Thus, serum CLIV did not improve the diagnostic accuracy of the Beaton et al. predictive model for the detection of cirrhosis.

**Discussion**

Since the HFE gene was identified, genetic testing has largely replaced liver biopsy in the diagnosis of HH. The role of liver biopsy largely remains for prognostic implications and the assessment of possible cirrhosis to appropriately implement surveillance strategies for portal hypertension and hepatocellular carcinoma.

In recent years, there has been considerable interest in the use of noninvasive markers of hepatic fibrosis and cirrhosis in HH, because liver biopsy is an invasive and expensive procedure with potential complications. Beaton et al. studied a combination of easily measured laboratory markers to predict the risk of cirrhosis in large cohorts of French and Canadian patients. The combination of serum ferritin greater than 1,000 µg/L, platelet count less than 200 × 10⁹/L and AST level above the upper limit of normal allowed the accurate diagnosis of cirrhosis in approximately 80% of C282Y homozygotes. The concordance between French, Canadian, and Australian patients with respect to the prevalence of cirrhosis with different combinations of simple laboratory measures is remarkable and validates the findings in previous studies by Guyader et al. and Beaton et al. Whereas the findings of Guyader et al. were relevant to clinical practice, the Beaton et al. findings did not appreciably change patient management, because in that study, a significant proportion of patients with cirrhosis had normal AST and platelet counts greater than 200 × 10⁹/L. The importance of a threshold serum ferritin concentration of greater than 1,000 µg/L has been emphasized by other clinicians and forms part of the current American Association for the Study of Liver Diseases guidelines for management of HH.

In the current study, we confirmed the observation that a serum ferritin concentration of less than 1,000 µg/L is an important negative predictor of cirrhosis and that C282Y patients with serum ferritin concentration below this value need not undergo liver biopsy unless relevant cofactors are present. Importantly, we were able to refine the prediction of cirrhosis in patients with serum ferritin concentration greater than 1,000 µg/L by the addition of serum hyaluronic acid measurements to the diagnostic algorithm.

The hyaluronic acid concentration in patients with a serum ferritin greater than 1,000 µg/L was 21.8 ± 2.0 ng/mL.
in patients without cirrhosis, compared with $137.7 \pm 34.4$ ng/mL in patients with cirrhosis. There was no overlap between the highest serum hyaluronic acid in the patients with serum ferritin greater than 1,000 µg/L without cirrhosis and the lowest value in those with cirrhosis. Application of the proposed hyaluronic acid cutoff value of 46.5 ng/mL accurately identified all of the patients with serum ferritin concentration greater than 1,000 µg/L who had cirrhosis (Fig. 4), even if the platelet count was greater than 200 x 10⁸ and the AST was normal. Indeed, hyaluronic acid correctly identified all HH patients with cirrhosis irrespective of their serum ferritin concentration, with an ROC AUC of 1.0. However, we propose that it is more cost-effective to measure hyaluronic acid only in patients with a serum ferritin concentration greater than 1,000 µg/L because this is such a well-validated threshold below which cirrhosis is extremely unlikely. We believe that cirrhosis is highly unlikely in HH patients who have a serum ferritin concentration greater than 1,000 µg/L if the serum hyaluronic acid concentration is less than 46.5 ng/mL. Thus, the measurement of hyaluronic acid could obviate the need for liver biopsy in these patients. Although a formal cost analysis has not been performed, we suggest that the proposed clinical pathway to liver biopsy using a combination of serum ferritin and hyaluronic acid measurements will result in cost savings since it is well recognized that costs of liver biopsy are relatively high compared with most serum biochemical tests.

In contrast to findings in other liver diseases, we did not see an elevation of hyaluronic acid in the early stages of liver fibrosis. Rather, the serum value of hyaluronic acid was similar in each grade of fibrosis in the noncirrhotic HH population. It has been suggested that the early increase in serum hyaluronic acid is due in part to increased release from necrotic liver cells and in the latter stages is due to increased synthesis by hepatic stellate cells combined with reduced clearance by sinusoidal endothelial cells. HH is noted for a paucity of both inflammatory cells and necroinflammatory activity. These pathological characteristics may account for the absence of any increase in hyaluronic acid in the early stages of liver fibrosis in HH patients. We also examined the relationship between hyaluronic acid and patient characteristics associated with increased risk of cirrhosis and found that serum hyaluronic acid was associated with advancing age, increased serum ferritin concentration, and elevated hepatic iron concentration, all of which are features associated with increased incidence of cirrhosis. Alcohol is an important and common concomitant factor determining the development of cirrhosis in HH patients. Patients were not excluded from the study on the basis of alcohol consumption. Therefore, the patient group that formed the basis of the study are representative of the cohort of HH patients that present in clinical practice. We found no significant correlation between alcohol consumption and serum hyaluronic acid levels in these patients, suggesting that hyaluronic acid measurements can be reliably used to predict cirrhosis in HH patients, irrespective of their alcohol consumption.

In conclusion, the data presented in this study indicate that the measurement of hyaluronic acid in HH patients who have a serum ferritin concentration greater than 1,000 µg/L is an important indicator of the presence of cirrhosis. All patients with a serum ferritin greater than 1,000 µg/L and serum hyaluronic acid concentration greater than 46.5 ng/mL had cirrhosis. The measurement of serum hyaluronic acid can assist in the clinical assessment of the need for liver biopsy in HH.

References