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Original Research

Assessment of serum laminin and hyaluronic acid as markers of hepatic fibrosis in patients with chronic Hepatitis B.

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Abstract

Background: Hepatitis B virus (HBV) is a major cause of chronic liver disease worldwide. Fibrosis of hepatic parenchyma has been reported as a common pathway to complications of chronic liver disease. There is a need to monitor fibrosis in these patients to abort or delay disease progression following treatment. Liver biopsy is recognized as the gold standard for disease monitoring; however, the procedure is invasive and bedeviled with potential complications. For these reasons, non-invasive biomarkers of fibrosis are now being evaluated as alternatives to liver biopsy. The study aimed to assess the characteristics of laminin, and hyaluronic acid as markers of hepatic fibrosis in patients with chronic hepatitis B.

Methodology: One hundred participants with HBV-induced chronic liver disease (CLD) were recruited for the study. A liver biopsy was conducted, and the degree of hepatic fibrosis was scored using the Metavir scoring system. Serum levels of the biomarkers were determined using enzyme-linked immunosorbent assay (ELISA) technique. Medians and interquartile ranges were compared using the Mann-Whitney U test. The degree of correlation between continuous variables was determined using Spearman's correlation analysis. Statistical significance was set at $p \leq 0.05$.

Results: Serum laminin was significantly higher in participants with hepatic fibrosis: 39.09 (27.6-89.4) ng/ml [median (interquartile range)], vs 24.3 (21.5-31.9) ng/ml, $p = 0.001$, Hyaluronic acid was significantly higher in participants with hepatic fibrosis: 45.1 (26.9-94.4) ng/ml vs 23.1 (12.7-35.7) ng/ml, $p < 0.001$. There was a strong significant positive correlation of both serum laminin and hyaluronic acid with Metavir score in the study participants ($r=0.766$, $p<0.001$; $r=0.708$, $p<0.001$ respectively). At a serum laminin concentration of 44.6 ng/ml, sensitivity and specificity for detecting moderate to severe hepatic fibrosis were 86.8% and 88.7% respectively, with an area under the curve (AUC) of 0.943 on the Receiver Operator Characteristic (ROC) curve. The sensitivity and specificity of hyaluronic acid for detecting moderate to severe hepatic fibrosis were 81.6% and 85.5% at a serum concentration of 53.5 ng/ml. AUC was 0.930 on the ROC curve.

Conclusion: This study underscores the evidence that laminin and hyaluronic acid may be helpful clinically in identifying patients with moderate to severe hepatic fibrosis. Serum laminin had a slightly better diagnostic ability than hyaluronic acid in the study participants. Further studies are needed to elucidate our findings.

Keywords: Laminin, Hyaluronic Acid; Chronic Hepatitis B; Chronic Liver Disease; Hepatic Fibrosis.

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Introduction

Chronic liver disease (CLD) causes significant morbidity and mortality and is a source of significant economic burden globally. CLD is responsible for about 2 million deaths per year globally and is the 11th leading cause of death worldwide [1]. The 4 major causes of CLD globally are chronic hepatitis C virus (CHC), chronic hepatitis B virus (CHB), alcohol-related liver disease (ALD), and nonalcoholic fatty liver disease (NAFLD) [2]. Other causes include autoimmune hepatitis, sclerosing cholangitis, primary biliary cirrhosis, haemochromatosis, schistosomiasis, and Wilson's disease.

Global prevalence of CHB infection was estimated at about 4% in 2019 [3]. Chronic hepatitis B viral infection is the commonest cause of chronic liver disease in Nigeria with a national prevalence of 12.2% [4].

Liver fibrosis is a sort of healing by scarring characterized by hepatic stellate cell deposition of excess and abnormal extracellular basement membrane proteins is the common pathological consequence of chronic liver disease [5]. A liver biopsy is necessary for diagnosis, assessing the severity and staging of liver damage, predicting prognosis, and monitoring response to treatment [6]. Liver biopsy, due to its invasive nature, may not be suitable for all patients with chronic liver disease. Limitations of liver biopsy include risk of patient injury, sampling error, and inter- and intra-observers' variability [6].

Noninvasive markers have therefore been suggested to replace or complement liver biopsy in assessing the stage and grade of liver disease in situations where biopsy is contraindicated such as prolonged International Normalized Ratio (INR), deranged prothrombin time, or significant ascites [7]. Serum markers of hepatic fibrosis offer an attractive, cost-effective alternative to liver biopsy for both patients and clinicians. In addition to being substantially less invasive, there are practically no complications, little or no sampling errors, and small observer-related variability.

Biomarkers of hepatic fibrosis have been researched and may be classified as direct or indirect markers. The direct markers are factors localized within the liver and include: laminin, procollagen type 1 carboxy-terminal peptide, procollagen type 3 amino-terminal peptide, metalloproteinases, tissue inhibitors of matrix metalloproteinases, transforming growth factor beta 1, and hyaluronic acid among others. The indirect markers result from other systemic processes outside the liver and include: the AST/ALT ratio, AST to platelet ratio index, and 13C methacetin test. Biomarkers may also be combined to generate hepatic fibrosis scores such as the Forns index, FIB-4 score, FibroTest/FibroScore, FibroIndex, SHASTA index, and HepaScore[8].

Laminin is a major non-collagenous high molecular weight glycoprotein component of the extracellular matrix that is produced by hepatic stellate cells, hepatocytes, and sinusoidal cells of the liver [9]. It participates in the attachment of cells to the basement membrane network through the non-covalent interaction with type IV collagen, proteoglycan sulphate, entactin, and other cell surface components [10]. During fibrosis, laminin accumulates around the hepatic vessels, in the perisinusoidal spaces, and near the portal tract [9].

Hyaluronic acid is a non-sulfated glycosaminoglycan that is a component of the extracellular matrix in all vertebrates' connective tissue [11]. In the liver, it is produced by hepatic stellate cells. The endothelial cells of the hepatic sinusoids are responsible for the final degradation of hyaluronic acid reaching the liver from the circulation [12]. Defective endothelial cell degradation due to fibrosis may contribute to the rise in circulating hyaluronic acid associated with chronic liver disease [13].

Studies have been carried out by several researchers on the characteristics of laminin and hyaluronic acid as markers of fibrosis in chronic liver disease [14-19]. Some researchers have opined for various reasons that serum markers cannot yet be used as an alternative to liver biopsy in the assessment of fibrosis [20,21].

This study was an assessment of the serum markers: laminin and hyaluronic acid in chronic hepatitis B-related chronic liver disease in a population of indigenous Nigerian patients to determine their ability to serve as an alternative to liver biopsy for the assessment of fibrosis.

Methodology

This was a cross-sectional study of 100 subjects who met our final inclusion criteria. All consecutive volunteer hepatitis B virus-related-chronic liver disease patients who required liver biopsy for management of the hepatitis B virus-induced chronic liver disease were recruited as study participants. Liver biopsy is indicated in chronic HBV-related liver disease when there is a disparity between the serum HBV-DNA level and the ALT concentration (HBV-DNA above 2000 IU/ml and normal ALT or low HBV-DNA less than 2000 IU/ml and high ALT above the upper reference limit).

Patients with chronic hepatitis B infection above 20 years of age who tested positive to HBsAg at least on two occasions for more than 6 months, or in any patient who tested anti-HBcAg-IgG and has a negative test to anti-HBcAg-IgM even if less than 6 months duration of HBV infection were also selected for liver biopsy and included in the study. Those with acute wounds that could cause false elevation in the serum level of hyaluronic acid, patients with autoimmune disorders, and other types of chronic liver disease were excluded.

Liver Biopsy

The liver biopsy procedure was carried out using the Menghini needle in the one-second Menghini procedure [22]. A local anaesthetic agent, 1% or 2% xylocaine was used to infiltrate the skin up to the liver capsule. The procedure was carried out using the thoracic approach. The liver tissue obtained was fixed for 8 hours with 10% buffered formalin. Following fixation and paraffin embedding, the tissue was stained with Haematoxylin& Eosin, and Masson's Trichrome. Liver tissue was considered adequate if 11-15 portal tracts were seen under a microscope.

The degree of liver fibrosis is staged by the Metavir scoring into 5 stages: F0- no fibrosis; F1-mild fibrosis; F2-moderate fibrosis; F3-severe fibrosis and F4-cirrhosis [23].

Scoring of liver biopsies

The slides were scored for fibrosis using the Metavir Scheme. One hundred and twenty slides were assessed independently by two hepato-pathologists to reduce intra observers' errors and differences in their assessment were resolved at a joint review. One hundred biopsies were found to be adequate while twenty slides were excluded. A liver biopsy assessment was carried out blinded relative to serum biomarker levels. A total of 100 participants with adequate liver biopsy specimens were thus eventually recruited into the study.

Blood collection, processing, and storage

5mls of venous blood was drawn from an antecubital fossa vein with restricted occlusion of the arm with the tourniquet for less than a minute. The blood sample was transferred into a plain specimen bottle and allowed to clot and retract for two hours. A blood sample was spun in a centrifuge at 3000xg for five minutes. The separated serum was kept frozen at -70^{o}C until analysed.

Laboratory analysis

Serum laminin (REF UMK05302) and hyaluronic acid (REFUM-K01052) were determined by non-competitive enzyme-linked immunosorbent assay (ELISA). The reagent kits were procured from Span Biotech Limited, Hollywood Plaza, 610 Nathan Road, Mongkok, Hong Kong.

Statistical Analysis of Data

The data collected was analyzed using descriptive and inferential statistics via Statistical Package for the Social Sciences (SPSS), version 20.0 (IBM-SPSS Armonk, New York, U.S.A). Continuous variables were tested for normality using the Shapiro-Wilk test. The variables were subsequently described using medians and interquartile ranges and compared via the Mann-Whitney U test. The degree of correlation of continuous data was determined via Spearman's correlation analysis. Sensitivity and specificity and area under the curve (AUC) were determined by plotting the Receiver Operating Characteristic curve (ROC curve). Statistical significance was set at $p \leq 0.05$.

Ethical Approval

The ethical approval (No.: ERC/2017/08/04) for the study was obtained from the Research and Ethics Committee of the institution where the study was carried out before the commencement of the study.

The participants were fully informed about the study with the use of the patient's information sheet and written informed consent was obtained from each participant. All participants were identified using codes and all information gathered from them was kept confidential.

Results

The demographic characteristics of the studied population are shown in Table 1. One hundred patients (64 males, 36 females) with hepatitis B-induced chronic liver disease were recruited into the study. The participants were aged 20 to 62 years (mean 35.8±9.3). Nineteen percent of the participants had no evidence of fibrosis (F0) Metavir score, forty five percent had F1 (mild fibrosis), twenty nine percent had F2 (moderate fibrosis) and seven percent had F3 (severe fibrosis). There was no participant with F4 score (definite cirrhosis) (Figure 1).

Serum laminin was significantly higher in participants with hepatic fibrosis than in participants without fibrosis 39.0 (27.6-89.4) ng/ml vs 24.3 (21.5-31.9) ng/ml, (p=0.001). There was no significant difference in the serum laminin between participants with mild fibrosis and participants without fibrosis 29.1 (22.6-35.6) ng/ml vs 24.3 (21.5-31.9) ng/ml, (p=0.280). However, there was a statistically significant difference in the serum laminin between participants with moderate to severe fibrosis and participants without fibrosis 91.2 (65.8-108.9) ng/ml vs 24.3 (21.5-31.9)ng/ml, (p<0.001).

Serum hyaluronic acid level was significantly higher in participants with hepatic fibrosis than in participants without fibrosis 45.1 (26.9-94.4) ng/ml vs 23.1 (12.7-35.7) ng/ml, (p<0.001). There was also no significant difference in hyaluronic acid level between participants with mild fibrosis and participants without fibrosis 29.1 (19.9-42.1) ng/ml vs 23.1 (12.7-35.7) ng/ml, (p= 0.078). Similar to laminin, there was a statistically significant difference in the serum hyaluronic acid between participants with moderate to severe fibrosis and participants without fibrosis 94.4 (61.2-114.5) ng/ml vs 23.1 (12.7-35.7) ng/ml, (p-value <0.001). The concentration of biomarkers of fibrosis in different groups is shown in Tables 2, and 3.

There was a strong significant positive correlation between Metavir score and serum laminin level (r_S-0.766, p<0.001) and with serum hyaluronic acid level (r_S-0.708, p<0.001). (Table 4)

On the Receiver Operator Characteristic(ROC) curve to differentiate between patients with no fibrosis, and those with moderate to severe fibrosis; a laminin cut-off concentration of 44.6 ng/ml had sensitivity and specificity of 86.8% and 88.7% respectively, with area under curve (AUC) of 0.943, and hyaluronic acid cut-off concentration at 53.5 ng/ml had a sensitivity and specificity of81.6% and 85.5% respectively, with AUC of 0.930. (Table 5)

Table 1: Socio-demographic characteristics of Study Population N=100

Variables	Frequency	Percentage %
Sex		
Male	64	64
Female	36	36
Smoking		
Yes	6	6
No	94	94
Alcohol		
Yes	10	10
No	90	90
Risk factors for HBV		
Multiple sexual partners	4	4
Sharing of sharp objects	10	10
Risky injection exposure	4	4
No identifiable risk	82	82
Symptoms		
Loss of appetite	4	4
Upper quadrant abdominal pain	3	3
Weight loss	3	3
Asymptomatic	90	90

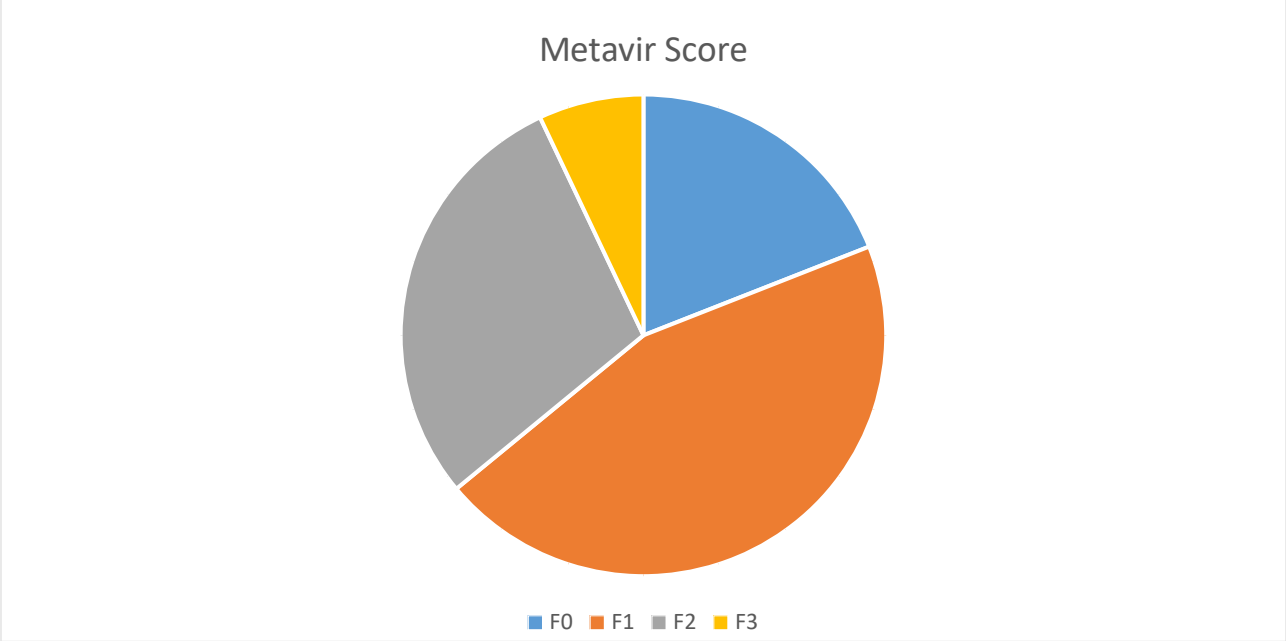

Figure 1: Distribution of hepatic fibrosis among the participants based on the Metavir Scoring System

Table 2: Fibrotic Biomarkers in Patients without Fibrosis (F0) and with Fibrosis (F1-F3)

Variable	No fibrosis (Median, Interquartile range)	Fibrosis (Median, Interquartile range)	Z score	p-value
Laminin(ng/ml)	24.3 (21.5-31.9)	39.0 (27.6-89.4)	3.471	0.001*
HA (ng/ml)	23.1(12.7-35.7)	45.1(26.9-94.4)	3.822	<0.001*

*Statistically significant, HA-hyaluronic acid

Table 3: Fibrotic Biomarkers in Patients without fibrosis (F0) and with mild fibrosis (F1)

Variable	No fibrosis (Median, Interquartile range)	Mild fibrosis (Median, Interquartile range)	p-value
Laminin(ng/ml)	24.3 (21.5-31.9)	29.1 (22.6-35.6)	0.280
HA (ng/ml)	23.1(12.7-35.7)	29.1 (19.9-42.1)	0.078

HA-hyaluronic acid

Table 4: Correlation of Fibrotic Biomarkers with Stages of Fibrosis

Metavir score	r_s	p- value
Laminin	0.77	<0.001*
Hyaluronic acid	0.71	<0.001*

*Statistically significant.

Table 5: Diagnostic performance of the biomarkers in detecting moderate to severe fibrosis

Biomarkers	Sensitivity	Specificity	PPV	NPV	AUC
Laminin (44.6ng/ml)	86.8	88.7	82.5	91.7	0.943
Hyaluronic Acid (53.5ng/ml)	81.6	85.5	77.5	88.3	0.93

PPV: Positive Predictive Value, NPV: Negative Predictive Value, AUC: Area Under Curve.

Discussion

In this study, serum laminin in the participants with moderate to severe fibrosis was higher than those with mild to no fibrosis, with serum levels showing a strong positive correlation with hepatic fibrosis Metavir scores. Laminin forms the main component of the basement membrane and interacts with type IV collagen, heparan sulfate, proteoglycan, and entactin [24]. During hepatic fibrogenesis, laminin accumulates due to increased stellate cell production and has thus reportedly been associated with hepatic fibrosis [8,20,21].

Similarly, hyaluronic acid was higher in participants with significant fibrosis, there was also a strong positive correlation between serum levels and severity of fibrosis. Hyaluronic acid confers on the structural characteristics of the extracellular matrix and is present in the connective tissue of all vertebrates [11]. Impaired hepatic sinusoidal degradation of hyaluronic acid in hepatic fibrosis causes elevated serum concentration [13].

Laminin showed a slightly stronger positive correlation with the degrees of hepatic fibrosis than hyaluronic acid and had a slightly higher area under the curve (AUC) than hyaluronic acid in differentiating between patients without fibrosis and those with moderate to severe fibrosis in the study group.

Shamkhi et al [15] studied 100 patients with chronic liver disease of various etiology, both biomarkers were higher in patients with moderate to severe fibrosis than those with no- or mild fibrosis. Hyaluronic acid was however better able to distinguish between the two groups than laminin with an AUC of 0.94 at >95ng/ml vs 0.89 at a laminin concentration of >1414pg/ml [15].

In the study of 87 patients with chronic hepatitis B by Feng Li et al [17] in a Chinese province, hyaluronic acid and laminin levels exhibited a strong positive correlation with significant fibrosis. The AUC for hyaluronic acid was 0.91 at 185.3ng/ml and 0.82 at 132.7ng/ml for laminin in predicting significant fibrosis [17]. In a comprehensive meta-analytical study, pooled AUC for laminin, hyaluronic acid, and fibronectin were 0.89, 0.82, and 0.73 respectively in diagnosing liver fibrosis.

Hafez et al [20] studied 30 subjects with chronic hepatitis B and found serum laminin to be positively correlated with the severity of hepatic fibrosis. The optimal cut-off for differentiating significant fibrosis in the study was a serum laminin of 107.5ng/ml. Sensitivity and specificity at this level were 84.2% and 63.6% at this level [20]. In contrast, a study by Tawhida et al [18] of 50 pediatric patients with chronic liver disease found no significant correlation between serum laminin and the stage of hepatic fibrosis. Laminin however showed a significant positive correlation with the hepatic histological activity index in the study [18]. These findings may have been due to the age group of the patients, or the limited sample size employed in the study.

McHutchison et al [14] assayed hyaluronic acid in 486 patients with chronic hepatitis C via a radiometric assay and found a moderate positive correlation of serum levels with the severity of hepatic fibrosis graded using the Knodell Histological Activity Index [14]. A serum hyaluronic acid level of <60µg/l was described as optimal for excluding significant fibrosis.

Conclusion

Findings from our study buttress the evidence that non-invasive blood biomarkers may be useful in the evaluation of hepatic fibrosis in patients with CLD, particularly in situations where liver biopsy may not be suitable. Laminin performed slightly better than hyaluronic acid in this regard according to our findings. The restraint of earlier researchers against adopting these markers as a gold standard for the diagnosis of hepatic fibrosis remains valid for the following reasons. The findings from this study showed that the markers could not differentiate clearly between patients with mild fibrosis and those without fibrosis. In addition, the lack of a standardized assay for the markers has resulted in various values and units being reported by different researchers thus creating an obstacle toward their definitive adoption as diagnostic markers. Lastly, more robust, adequately powered studies, including studies in the pediatric age group, are still required on the subject before a conclusive decision can be reached on the ability of the markers to serve as a practical alternative to liver biopsy in the diagnosis of liver fibrosis.

Limitations of the study

Masson's Trichrome stain was used to stain fibro-collagenous tissues to assess the degree of hepatic fibrosis in the selected participants with hepatitis B virus-induced chronic liver disease, however, it does not adequately stain newly formed collagens and could thereby lead to false negative results. This could be a cause of the inability to significantly differentiate the participants with no fibrosis from those with mild fibrosis.

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