ejpmr, 2017,4(9), 149-154

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Review Article

ISSN 2394-3211

EJPMR

A SIMPLE COLOURIMETRIC ASSAY FOR NONINVASIVE DIAGNOSIS OF LIVER FIBROSIS IN CHRONIC HEPATITIS B PATIENTS

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Article Received on 17/07/2017 **Article Revised on 07/08/2017** **Article Accepted on 27/08/2017**

ABSTRACT

Background: Chronic hepatitis B virus run the risk of developing liver fibrosis, cirrhosis and in later life hepatocellular carcinoma. **Aim**: The aim is to test the diagnostic power of N-acetyl-β-D-glucosaminidase (NAG) in assessing liver fibrosis and its ability to improve those of AAR, FIB4 and GUCI scores in the previous patients. **Methods:** Blood samples for complete blood count and serum for N-acetyl-β-D-glucosaminidase (NAG) assays, liver function tests, HBV DNA and HBV markers evaluation were taken (n=71, F0 - F4 according to liver stiffness calculated by transient elastography). The numerical values of the six scores were quantized and correlated with that of NAG. **Results:** NAG can efficiently differentiate patients with non-significant (F0, F1) from those with significant fibrosis (F2 –F4); sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 81, 91, 90, 81, respectively with AUC of 0.900. The additions of the results of NAG to those of the previous scores greatly enhance their AUCs and their diagnostic power in the majority of cases. **Conclusions:** NAG, itself, cannot only help in assessing hepatic fibrosis but can also enhance the diagnostic power of the tested scores to discriminate the fibrotic stages of HBV chronically infected Egyptian patients.

KEYWORDS: N-acetyl-β-D-glucosaminidase, Hepatitis B Virus, Liver fibrosis, transient elastography and non-invasive scores**.**

INTRODUCTION

Chronic hepatitis B virus infection (HBV) is considered a global public issue with more than 78.000 people per year dying of its evolution.[1] It is estimated that 350 million individuals are chronically infected with hepatitis B virus (HBV) and that more than 1 million die from cirrhosis and hepatocellular carcinoma (HCC) each year.[2] Approximately 5-10% of infected adults and 80-90% of children become chronic carriers of HBV.[3] According to Egyptian studies, the prevalence of HBsAg in Egypt is of intermediate endemicity (2–8%). Nearly 2-3 million Egyptians are chronic carriers of HBV.

Liver biopsy has the advantage of allowing to obtain information not only on fibrosis, but also on many useful parameters, such as inflammation, necrosis, steatosis, hepatic iron and so on.[4] Although ultrasonography, computed tomography, and magnetic resonant imaging are useful in investigation of liver disease, liver biopsy is still essential for diagnosis in the majority of patients. However, biopsy may lead to many complications and has limitations as hospitalization of the patients is required; in addition, during sampling of biopsy, ~30% of patients feel pain.[5] In addition, it is considered as a static test and does not reflect the dynamicity of all liver tissue. Because of these limitations, there is an instant need to develop and validate a novel noninvasive score for the assessment of liver fibrosis. This specific noninvasive alternatives should be cheap, simple, and easy to do, safe, and turning back.[6] Because of the complication of liver biopsy, several serum biomarkers, combined scores, and imaging techniques; including transient elastography[7] have been introduced for the assessment of liver fibrosis non-invasively.[8]

One of the main limitation to the clinical use of direct markers of liver fibrosis (e.g. serum hyaluronate, laminin and YKL-40 and NAG) is that they are not routinely available in all hospital settings. While direct markers of liver fibrosis (e.g. routine blood tests, such as the prothrombin index, platelet count and albumin) reflect the process of fibrogenesis,[9] satisfy the request for a simple and easy to perform marker.

Many score systems have been developed, but these were dependent on a combination of parameters that are not part of routine investigations and are not available in all hospitals, besides the additional costs involved.[10]

In this study, a simple and direct marker; namely N-acetyl-β-D-glucosaminidase (NAG), in which its activity participate in hyaluronic acid (HA) degradation and correlate with its level , as a backbone of extracellular matrix (ECM), and colourimetrically assayed will be tested to be a marker of hepatic fibrosis or not.

NAG is a high molecular weight (140 kDa) hydrolytic lysosomal enzyme that is found in many tissues of the body. It breaks chemical bonds of glycosides and amino sugars that form structural components in many tissues. It is necessary for the degradation and disposal of various parts of the cell, including the cell membrane as well as matrix of cell –cell interactions.[19] This enzyme catalyzes the removal of the N-acetyl glucosamine residue from the non-reducing end of the oligosaccharide chains of glycoconjugates including HA.[9] The latter was used as a marker of tissue remodeling as in inflammatory processes.[11]

As before, not only NAG will be tested to be a marker of hepatic fibrosis but diagnostic power as a simple, colorimetric and direct marker will be compared with those of 3 of the indirect and published scores [aspartate to age to AST ratio (AAR), FIB-4 index[12] and Gotebörg University Cirrhosis Index (GUCI)[13] in Egyptian CHB patients.

Secondly, whether the addition of the individual activities of such enzyme to those of the later 3 scores could enhance their diagnostic power in differentiating the fibrotic stages in HBV chronically infected patients or not will also be investigated.

PATIENTS AND METHODS

71 Egyptian patients which were positive for HBV-DNA and HBsAg and negative for hepatitis C (HCV) antibody and HCV-RNA were included in this study. All of them were randomly chosen (males and females) from Egyptian Liver Research institute and Hospital (ELRIAH), Dakahlia, Egypt. Non-of them take any anti-viral treatment before blood samples.

Transient Elasastografy

Liver stiffness was also measured by transient elastography (Fibroscan; Echosens SA, Paris, France). Ten successful acquisitions were performed on each patients. The results that obtained ten valid measurements with a success rate of at least 60% and an interquartile range under 30% were considered successful. A median of 10 valid measurement was regarded as the liver stiffness for a given subject and expressed in kilopascals (kPa).[14] The following cut off values of liver stiffness, assessed by transient elastography and previously published by **Nitta et al**.[20]: up to 7.1 KPa for F0, from 7.1 KPa to 9.6 KPa for F1, from 9.6 KPa to 11.6 KPa for F2, from 11.6 KPa – 16.9 KPa for F3 and 16.9 KPa to 75.0 KPa for F4.

Blood samples

Blood samples were drown from patients in two portions; one of them was on EDTA for platelets counting and the other was separated after clotting and was preserved at -80ºC until its use.

Biochemical, Immunological and Hematological assays

Biochemical assays

The NAG activity was calorimetrically assayed by the method of **East *et al.* (1941)** with slight modification.

Liver function tests included albumin (DIAMOND Diagnostic Company for research reagents, Egypt), total bilirubin (BioMed-Bilirubin (Total) Colorimetric method with sample blank), aspartate aminotransferase (AST, ALT, BIOMED Egy-Chem for lab technology company) and alanine aminotransferase (ALT, BIOMED Egy-Chem for lab technology company) were measured.

Immunological assays

HBV-DNA and HBV markers; namely, hepatitis B surface antigen (HBsAg) quantization, hepatitis B envelope Ag (HBeAg) and HBsAb, HBeAb and HBcAb (IgM - IgG) measured by using ARCHITECT HBeAg assay (CMIA). Also, HCV infection was excluded. N-acetyl- β -D-glucosaminidase enzyme activity was determined by basing on the hydrolysis of p-nitrophenyl-N-acetyle-β-D-glucosaminidase by the enzyme.

Hematological assays

A D-cell 60 automated Hematology analyzer (Diagon Ltd, Budapest, Hungary) used for calculating complete blood pictures including platelets counting.

Statistical analyses

All statistical analyses were carried out using MedCal software (version 15; Medcalc Software Bvba, Mariakerke, Belgium). The main endpoint was the identification of patients and their classification to (F0-F1) named as non-significant group and those (F2-F4) are significant group. Second classification that (F0-F2) are non-sever group and (F3-F4) are sever group. Multiple logistic regression analysis was done using variables found to show significant differences between the two groups by using (ROC) receiver operating characteristic analysis. Markers with high (AUC)area under the curve or a high significance on univariant analysis were well for using to create multivariable models. For figuration of a well score we choose the most significant variable or that with highly AUC for differentiation between patients with significant and sever fibrosis versus to non-significant and non-sever fibrosis.

RESULTS

NAG activity

According to liver stiffness, assessment by transient elastography as shown in table 1, 13 patients were included in F0 (18.3%), 21 patients with F1 (29.6%), 17

patients with F2 (23.9%), 13 patients with F3 (18.3%) and 8 patients with F4 (9.8%).

N-acetyl-β-D-glucosaminidase (NAG) activities versus the levels of the routine liver function tests and platelets counts

Table 2 showed that the mean values of NAG, GOT and INR were significantly increased in the blood of patients with significant fibrosis compared with those with non-significant as well as those of the control group (P<0.044 or less). On the other hand, serum albumin levels and platelets count were decreased in the former compared those of patients with non-significant fibrosis (P<0.001 or less) or those of the control group.

Discriminating power of NAG versus those of the selected 3 non-invasive scores in hepatic fibrosis

The ability of NAG and the numerical values of the selected 3 non-invasive scores to stage liver fibrosis were listed in table 3. The activity of NAG at a cutoff value more than 17.0 can able to discriminate patients with significant from those with non-significant fibrosis with sensitivity of 81%, specificity 91%, PPV 90% and NPV 81% with an area under curve of 0.900 and p<0.001 (Table 3 and Figure 1).

The latter performance characteristics were much higher than those which were listed in the same table for the 3 selected non- scores for differentiating patients with non-significant fibrosis from those with significant one.

Role of serum NAG activities in the improvement of the diagnostic power of the 3 non-invasive fibrogenic scores

The results of the ability of NAG to enhance the diagnostic power of the selected 3 non-invasive scores listed in table 4. In fact, the performance characteristics of each score in discriminating patients with significant from those with non-significant fibrosis were highly elevated with AUCs values ranging between 0.901 and 0.928, sensitivity (75 - 91%), specificity (79 - 97 %), PPV (83 – 96 %) and NPV (80 - 90%) but with new cutoff values shown in the same table.

Correlation coefficients

Table 5 list the correlation coefficients between serum NAG activities and that of age, blood picture, liver function tests and the numerical values of the 3 selected scores for patients with non-significant versus those of significant fibrosis. The r values which were higher than 0.5 were found to be with Age, direct bilirubin, albumin, FIB4 and GUCI score.

Table 1: Frequency distribution of different patients groups using liver stiffness by assessment by transient elastography (Median).

Median	Pathological groups	Frequency (n)	Percent (%)
Up to 7.1 KPa	F0	13	18.3%
7.1 – 9.6 KPa	F1	21	29.6%
9.6 – 11.6 KPa	F2	17	23.9%
11.6 – 16.9 KPa	F3	13	18.3%
16.9 – 75.0 KPa	F4	7	9.8%
Total	Total	71	100%

n= number

Table 2: The mean values and standard deviations (SD) of NAG, alanine amino transeferase (ALT) and aspartate amino-transeferase (AST), albumin and the international normalization ratio (INR) as well as platelets count of patients with chronic hepatitis B virus infection (n=71).

	Non-significant (F0,1) n=35	Significant (F2,3,4) n=36	P-value
NAG	13.2±3.12	21.6±5.6	0.0001
Age	35.1±10.6	47.6±11.1	0.0001
GOT	29.9±24.3	41.6±23.0 [141%]$	0.044
Albumin	4.6±0.3	4.1±0.7 [112%]$	0.001
T. Bil	1.12±1.72	1.04±0.52	NS
Platelets	247.6±84.9[a]	175.3±58.5[a] [141%]$	0.0001
INR	1.04±0.04	2.9±4.6 [207%]$	0.017

[$]: Percent of change and degree of significancy (P>0.05 is non-significant; P<0.05 is significant; P<0.001 is very significant and P<0.0001 is considered extremely significant) compared to Non- significant fibrosis [*].

Table 3: Diagnostic values of N-acetyl-β-d glucosaminidase (NAG) versus those of the selected 3 non-invasive scores (APRI, AAR, FI, FIB-4, GUCI and KING Score) with optimal cut-off for discriminating between non-significant and significant fibrosis.

Group	Non-significant versus significant						
Variable	**Cut-off**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**AUC**	**P value**
AAR	≤0.91	62	61	63	60	0.615	0.089
FIB4	>2.67	70	85	83	72	0.838	<0.001
GUCI	>1.0	64	82	80	68	0.797	<0.001
NAG	>17.0	81	91	90	81	0.900	<0.001

AUC: area under the receiver-operating characteristic curve; PPV: positive predictive value; NPV: negative predictive value. P>0.05 is non-significant; P<0.05 is significant and P<0.001 is considered very significant.

Table 4: Combination between N-acetyl-β-d glucosaminidase (NAG) and the other 3 non-invasive scores (AAR, FIB-4 and GUCI score) in non-significant versus significant fibrosis.

Group	Non-significant ¥ significant						
Variable	**Cut-off**	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**AUC**	**P value**
AAR	>0.735	78	94	93	80	0.901	<0.0001
FIB4	>-0.453	89	88	89	88	0.924	<0.0001
GUCI	>-0.844	75	97	96	79	0.902	<0.0001

AUC: area under the receiver-operating characteristic curve; PPV: positive predictive value; NPV: negative predictive value. P>0.05 is considered non-significant; P<0.05 is considered significant and P<0.001 is considered very significant.

Table 5: The selected functions of multivariate discriminant analysis (MDA) after addition of the individual results of N-acetyl-β-d glucosaminidase (NAG) to those of 3 non-invasive scores (AAR, FIB4 and GUCI).

Parameters	Multivariate discriminant analysis (MDA) functions
AAR	-6.73 + 0.412 x NAG - 0.0358 x AAR
FIB4	-7.55 + 0.391 x NAG + 1.069 x FIB4
GUCI	-7.16 + 0.406 x NAG + 0.0262 x GUCI

Table 6: Correlation coefficient between N-acetyl-β-d-glucosaminidase (NAG) expression levels and studied parameter: non-significant versus significant.

Variable	NAG	
	r	**P value**
Age	0.550	<0.0001*
Hb	0.158	NS
RBCs	- 0.380	0.001*
WBCs	0.088	NS
Platelets	- 0.478	0.001*
T,Bil	0.048	NS
D.Bil	0.512	<0.0001**
SGOT	0.430	0.0002**
SGPT	0.149	NS
Albumin	0.676	< 0.0001**
INR	0.389	0.008*
AAR	0.321	0.006*
FIB-4	0.638	<0.0001**
GUCI	0.563	<0.0001**

*: statistically significant, ** highly significant, NS: not significant and r: correlation coefficient.
Grade of r: 0.00-0.24 = weak or no correlation; 0.25-0.49 = fair correlation; 0.50-0.74= moderate correlation and ≥0.75 = strong correlation.

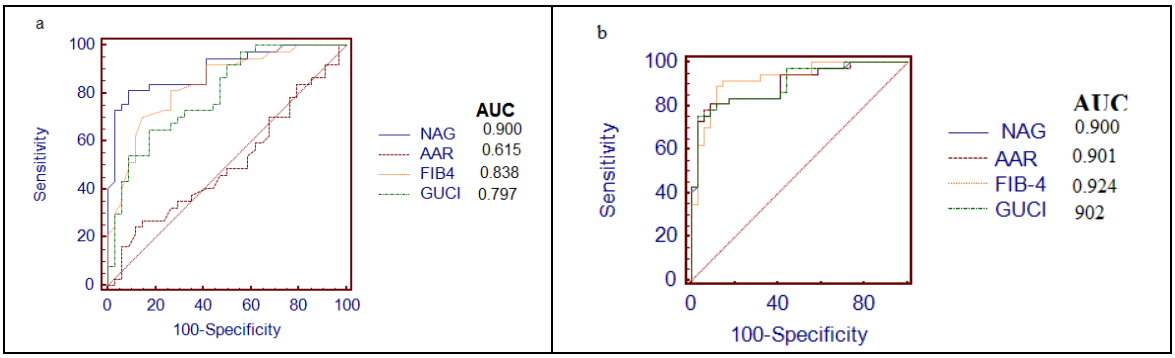

Figure 1: ROC curve of NAG versus those of the selected 7non-invasive scores (AAR, FIB-4 and GUCI) a and ROC curve of NAG in combination with those 3 scores for discriminating between non-significant via significant fibrosis b.

DISCUSSION

Hyaluronic acid (HA) is the backbone of extracellular matrix aggregates.[15] Many enzymes; including N-acetyl-β-D-glucosaminidase (NAG) participate in its degradation.[17] Thus, HA is considered a direct marker of liver disorders, and thus NAG as well. For these reasons, the ability of the results of NAG as well as those of the 3 of the published and indirect scores; namely, AAR, FIB4 and GUCI were tested to discriminate between patients with non-significant from those with significant fibrosis in patients with chronic HBV infection. **Fortunato et *al.*[16]** attributed the increase in the activity of NAG to the increase in the accumulation of ECM; including HA, in sera of patients with liver disorders. The enzyme belongs to the group of the enzymes which participate in the degradation of such

backbone molecule. The increase in the activity of NAG in the sera of our patients with the increase in the severity of the disease (i.e.in sera of patients with significant than those with non-significant fibrosis) confirm that of **Fortunato et al.,**[16] **Motoshi *et al*., 2012**[17] **and that of Toson *et al*., 2014**[18] who suggested that NAG may be a valuable procedure to ascertain and monitor liver cirrhosis and fibrosis. The present study confirm the criteria of the diagnostic reliability of this enzyme.

In recent study, AAR differentiates patients with non-significant from those with significant fibrosis. The diagnostic power including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 62, 61, 63, 60, respectively and the AUC was 0.615.

Fib-4 index was able to differentiate between patients with non-significant from those with significant one with sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 70, 85, 83, 72, respectively and AUC of 0.838 , respectively.

In addition GUCI was able to differentiate between patients with non-significant from those with significant one with sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of 64, 82, 80, 86 and AUC of 0.797, respectively.

After addition of the individual activities of NAG to those of the 3 specified scores, it was found that the sensitivity of AAR became 78, specificity = 94, PPV = 93 and NPV was 80 with AUC of 0.901 for differentiating patients with non-significant from those with significant fibrosis. Also, those of FIB4 were 89, 88, 88 and 0.924, respectively. Moreover, for GUCI these values became 75, 97, 96, 79 and 0.902, respectively (Table 4).

Also, the individual activities of NAG were weakly correlated with those of AAR but were good correlated with both FIB4 and GUCI with r values of 0.321, 0.638 and 0.563, table 5).

From these results one can conclude that, the colourimetric assay of NAG activity is of value in assessing hepatic fibrosis, especially if the individual values of such activities were added to those of AAR, Fib-4 index and GUCI; at least in part on the levels of both specificity and PPV as were described.

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