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EVALUATION OF NON-INVASIVE MARKERS OF FIBROSIS AND THEIR CORRELATION WITH THE VIRAL LOAD IN SUBJECTS WITH HEPATITIS B VIRUS IN NNEWI

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ABSTRACT

Hepatitis B is an infectious disease of great public health importance. Nigeria is one of the countries with the highest incidence of Hepatitis B Virus (HBV) infection worldwide. However, the accessibility and affordability of HBV DNA quantification (viral load) assay which is the key laboratory test for therapy initiation, and monitoring is a challenge to HBV management. This study was designed to evaluate the non-invasive markers of fibrosis and their correlation with the viral load in subjects with hepatis B virus in Nnewi. Cross sectional study design was used with a total of 264 subjects comprising of 88 HBsAg seropositive treatment naïve subjects, 88 HBsAg seropositive subjects on antiviral therapy as case subjects and 88 age-matched apparently healthy HBsAg seronegative individuals were recruited as control subjects. Hepatitis B virus DNA assay was performed using real time PCR technique, Hepatitis C Virus assay, Human Immunodeficiency Virus testing, Gamma glutamyl transferase, Aspartate transaminase, Alanine transaminase tests were determined using spectrophotometric methods. Non-invasive markers of fibrosis - RDW-Platelet Ratio (RPR), Neutrophil to Lymphocyte ratio, Platelet to Lymphocyte ratio, GGT to Platelet ratio, AST-Platelet Ratio index and AST-ALT Ratio were calculated. The noninvasive markers of fibrosis –GGT to Platelet ratio, AST to platelet ratio and AST to ALT ratio were significantly higher in test subjects (0.06±0.11; 0.44±0.87; 1.49±0.86) compared with control subjects (0.02±0.01;0.11± 0.69;0.89±0.59 respectively, $p<0.001$). Some Liver function tests studied –gamma glutamyl transferase, alkaline phosphatase aspartate transaminase and alanine transaminase were all significantly higher in test subjects 4.60±1.66U/L, 40.30±14.50U/L, 11.61±5.13U/L and 9.10±2.94U/L) compared with the control group(9.15±1.75µmol/l, 2.33±0.78U/L,28.89±5.17U/L, 5.98±0.11U/L and 7.07±1.49U/L). This study showed that noninvasive markers of fibrosis play significant role in HBV management but should not be used as its alternative.

KEYWORDS: Non-invasive markers, fibrosis, viral load, hepatitis B virus, Nnewi.

INTRODUCTION

Hepatitis B is an infectious disease of great public health importance caused by hepatitis B virus (HBV). It is an enveloped DNA virus that infects the liver and causes hepatocellular necrosis and inflammation (Iroegbu and Nwajiobi, 2016). This virus belongs to the family hepadnaviridae and genius orthohepatodnavirus and it is the only hepadnavirus that causes infection in humans (Fairley and Read, 2012). It is 100 times more infectious than the most dreaded human immunodeficiency virus Infection and 10 times more infectious than hepatitis C virus (CDC, 2015). Hepatitis B virus is one of several viruses known to cause viral hepatitis and continues to be the major cause of viral hepatitis in the developing and underdeveloped world. In addition to causing chronic liver disease and cirrhosis, it has a formidable track record of being linked to primary hepatocellular carcinoma. It is estimated that HBV and HCV is the root cause of about 80% of all hepatocellular carcinomas (HCC) by promoting cirrhosis which significantly

reduced the life expectancy of the infected patients (Crossan *et al*, 2015).

About 257 million people are chronically infected annually and about 2 in 3 people with Hepatitis B do not know they are infected(CDC, 2018). Recent statistics indicate that not less than 23 million Nigerians are estimated to be infected with the HBV, making Nigeria one of the countries with the highest incidence of HBV infection in the world (WHO, 2017). A national study done in Nigeria in 2016 shows a prevalence rate of 12.4 percent (Olayinka *et al*., 2016). This worldwide burden of hepatitis B mandates accurate and timely diagnosis of patients infected with HBV and the use of treatment strategies derived from evidence-based guidelines. Most hepatitis B patients are asymptomatic in the early stage as specific clinical symptoms often occur at advanced disease stages, which are usually irreversible. Hence, the prognosis of the infection to liver disease is very crucial. The presence of derangement in specific laboratory analytes at the early stage of infection may signal a risk of fibrosis, cirrhosis and ultimately HCC.

The diagnosis of HBV is not only imperative but also complex because of different viral antigens, which bring about varying serological profiles in different stages of the disease (Nwokediuko, 2011). The ability of HBV to induce chronic hepatic inflammation gives rise to these intricate serological profiles. Serological markers are used routinely as diagnostic and prognostic indicators of acute and chronic HBV infection(Amorocho *et al*, 2012). Abnormal liver enzyme levels (Alanine aminotransferase, ALT) may signal liver damage due to cirrhosis, fibrosis or alteration in bile flow. Other investigations include staging of liver fibrosis by non-invasive tests (NITs) such as aspartate aminotransferase (AST)-to-platelet ratio index (APRI), transient elastography (FibroScan) or FibroTest (Lok and McMahon, 2009).

The detection and quantification of liver fibrosis is a key factor for disease management and prognostication for an individual with HBV. Reliance on invasive liver biopsy to stage disease is diminishing with the advent of robust non-invasive blood- and imaging-based algorithms which can reliably stage disease in many cases(Xie *et al.*, 2017; Erdogan *et al.*, 2013). Also, inactive HBV carriers constitute most chronic hepatitis B cases and they have a low risk of hepatocellular carcinoma (HCC) or cirrhosis. Therefore, close follow-up for inactive carriers is important in terms of development of liver cirrhosis and HCC(Zeng *et al.*, 2016). The relatively low risk of HCC limits the usage of invasive methods such as biopsy. Consequently, the use of non-invasive methods like RDW-Platelet ratio, Mean Platelet Volume, AST/Platelet index, GGT-Platelet ratio and other non invasive markers of fibrosis is important to determine the level of inflammation and chronicity in inactive HBV carriers(Zeng *et al*, 2016; Chen *et al*, 2017).

Among all the several clinical diagnostic tests which have been developed for the detection of HBV infections, the serum HBV DNA level is a key factor affecting the initiation of antiviral therapy and evaluation of its efficacy(Demiroren *et al.*,2015). Evaluation of the relationship between the serum HBV DNA levels and hepatic pathology is a current hotspot in the diagnosis and treatment of CHB(Cornberg *et al.*, 2017).

Also non invasive Hepatitis B Indices could show some relationship with HBV DNA quantification. Establishing this relationship might simplify and reduce the cost of hepatitis B diagnosis and management.

This study was designed to evaluate the non-invasive markers of fibrosis and their correlation with the viral load in subjects with hepatis B virus in Nnewi.

MATERIALS AND METHODS

Study Area

The study was carried out at the Gastroenterology unit of Nnamdi Azikiwe University Teaching Hospital (NAUTH), a tertiary institution in South Eastern Nigeria which is a referral center for Hepatitis B care in Nigeria.

Study Design

Cross sectional study design was used in the study. A total of 264 subjects were recruited which comprised of 88 HBsAg seropositive treatment naïve subjects, 88 HBsAg seropositive subjects on antiviral therapy (Tenofovir 300mg daily/Entecavir 0.5mg daily or Pegylated interferons 180µg weekly) as case subjects and 88 age-matched apparently healthy HBsAg seronegative individuals were recruited as control subjects.

Sample Size Determination

Power analysis for a one-way ANOVA with three groups was conducted in G Power to determine a sufficient sample size using an alpha of 0.05, a power of 0.96, and a medium effect size(f=0.25). Based on the aforementioned assumptions, the total sample size is 264, with 88 subjects per group (Faul *et al*., 2013).

Sampling Technique

Purposive sampling technique was employed in selecting the participants based on the inclusion criteria. Patients that gave their consent who also met the selection criteria were recruited as they come to the clinic until the sample size was completed.

Sample Collection

After obtaining informed consent, 10ml of venous blood was collected from the fore arm of each subjects using a disposable syringe; 7ml was dispensed to a sterile K_2-EDTA vacutainer(1.2mg/ml concentration) for hepatitis B DNA Viral load, hepatitis B panel, complete blood count, retroviral screening (RVS) and anti HCV screening while 3ml was added to a sterile plain container for HBsAg quantitation, HBcAb IgM titre quantitation, HDV IgG assay, Gamma Glutamyl

Transferase(GGT) and liver function tests. The plasma and serum samples were separated into sterile plastic containers and preserved at –86°C.

Ethical Consideration
Ethical Approval
Ethical approval was sought and obtained from the Ethics Committee of Nnamdi Azikiwe University Teaching Hospital Nnewi before the commencement of this study (Reference: NAUTH/CS/66/VOL.10 /187/ 2017/096).

Inclusion Criteria
Participants included in this study were hepatitis B seropositive subjects attending Gastroenterology Clinic of NAUTH, Nnewi and apparently healthy HBsAg seronegative individuals (NAUTH staff, students and others) who gave their consent.

Exclusion Criteria
Those patients who were co-infected with HIV, HDV and HCV including HBsAg seronegative subjects who have received HBV vaccination were excluded from the study.

Laboratory Diagnosis
HBV DNA Viral Load using CobasAmpliprep/Taqman Real Time PCR Technique (Roche Molecular System Inc, USAas described by Iregbu and Nwajiobi-Princewill (2016)

Procedure
The frozen plasma was placed at room temperature until completely thawed before use. The High Positive, Low Positive and Negative controls were removed from 2-8°C storage and brought to room temperature before use. All reagent cassettes were removed from 2-8°C storage and loaded immediately onto the COBAS AmpliPrep Instrument and allowed to equilibrate to ambient temperature on the instrument for at least 30 minutes before the first specimen was processed. The appropriate number of reagent cassette racks, sample racks with Input S-tubes, SPU racks, K-tip racks, K-tube racks and K-carriers on K-carrier racks was loaded onto the respective rack positions of the COBAS AmpliPrep Instrument. Sample rack was prepared by attaching a barcode label clip to each sample rack position where a specimen (S-tube) was to be placed. The specific barcode label clips for the controls were attached to each sample rack position where the controls (S-tube) were to be placed. One Input S-tube was placed into each position containing a barcode label clip.

The Amplilink software was used to create specimen orders for each specimen and control in the Orders window Samplefolder and HBV test definition file was selected and saved. The specimen and controls were vortexed for 5 seconds and 650 µL added to the S-tubes. The tubes were then inserted in the sample rack and loaded to the Ampliprep. The Amplilink software was used to start the COBAS Ampliprep. At the completion of the COBAS TaqMan Analyzer run, the Results Report was validated and printed.

Full Blood Count Determination Using Mythic 22 Al Haematology Auto-Analyser (Diamond Diagnostics, Switzerland as described).
Procedure
Blood specimens collected in EDTA containers was mixed using mechanical haematology blood mixer for 5 minutes. When properly mixed, the sample was brought to the aspiration chamber of the analyser and the 'Aspirate' button pressed while the specimen container is held to ensure that the correct volume is properly aspirated before removing. Then, it was allowed to run and displayed results printed from the analyzer.

Anti-Hepatitis C Virus (HCV) Serological Assay (Chembio Diagnostic Systems, Inc. Medford, Newyork as described by CDC, 2003)
Procedure
The test kit was brought to room temperature, removed from the foil and placed on a flat surface. It was labelled with the specimen's identification number, and approximately 50µl of the serum sample was applied onto the sample pad. The test was allowed to run for 15 minutes after which the result was read.

HumanImmunodefiency Virus (HIV) Screening by Serial Algorithm
Determine HIV 1/2 Assay (Abbort Rapid Diagnostics CA, USA as described by Delaney and Branson, 2011)
Test procedure
The protective foil was removed, and the test device was kept on a flat surface. The device was labeled with the specimen's identification number and 50µl of the serum sample was applied to the sample pad. The result was read after 15 minutes.

Unigold HIV 1/2 Rapid Test (Trinity Biotech PLC, Ireland as described by CDC, 2001)
Procedure
The test device was removed from the protective wrapper and kept on a flat surface. On the sample port, 60µl of serum sample was added also, two drops of wash buffer reagent. Result was read after 10 minutes.

Chembio Stat-Pak HIV 1/2 Assay (Chembio Diagnostic Systems, Inc. Medford, Newyork as described by Kenealy *et al.*, 1987)
Procedure
The test kit was removed from its pouch and placed on a flat surface. The test device was labelled with specimen's identification number. The 5µl sample loop was touched on the specimen allowing the opening of the loop to be filled. The sample loop was then held vertically to touch the centre of the sample (S) well of the device to dispense approximately 5µl of sample onto the sample pad. The Running Buffer bottle was held vertically and three drops (approximately 105µl) of the Buffer was

added slowly. The test result was read after 10 minutes of adding the Running Buffer.

Determination of Gamma Glutamyl Transferase (GGT) Activity using the spectrophotometric method of Persijn and van der Slik, (1978)

Procedure

One (1.0) ml of γ-GT reagent was added into a test tube and allowed to equilibrate to 37°C for 3 minutes. The spectrophotometer was blanked with water at 405 nm. 0.10 ml of specimen was added to the γ-GT reagent and mixed gently and the solutionwas still maintained at 37°C. Then, the absorbance was read three times at 60 seconds interval at 405 nm.

Calculation

The mean change in absorbance readings were calculated thus (ΔA/min). Therefore, γ-GT activity (U/L) =

$$\frac{\Delta A/min. \times TV \times 1000}{Є \times SV}$$

Where: ΔA/min = Average absorbance change per minute
TV=Total reaction volume (ml)
1000 = Conversion of IU/mL to IU/L
Є= Millimolar absorptivity of 5-amino-2-nitrobenzoate at 405 (0.0095) SV= sample volume

Determination of Aspartate Transaminase (AST) and Alanine Transaminase (ALT)

Procedure

One (1.0) ml of ALT or AST reagent was added into a test tube and allowed stand for 3 minutes to equilibrate to 37°C. 0.10 ml of specimen was added to the ALT or AST reagent and mixed gently, and the solution was still maintained at 37°C. Then, the absorbance was read three times at 60 seconds interval at 340 nm.

Calculation

The mean change in absorbance readings were calculated thus (ΔA/min). Therefore, ALT orAST activity (IU/L) =

$$\frac{\Delta A/min. \times TV \times 1000}{Є \times SV \times LP}$$

Where: ΔA/min = Average absorbance change per minute
TV = Total reaction volume (ml)
1000 = Conversion of IU/mL to IU/L
Є = Millimolar absorptivity of NADH (6.22)
LP = Light path (cm)

Note: samples with values above 500 IU/L were diluted 1:1 with normal saline, and re-assayed and the results mulplied by two.

Non-Invasive Markers of Fibrosis were calculated as follows

GPR, RPR, NPR and APRI are calculated thus:
GPR=[GGT/ULN]/PLT [10^9 L^{-1}])×100;
RPR=RDW (%)/PLT (10^9 L^{-1})
NPR=Neut x 10^3 L/ PLT (10^9 L^{-1});
APRI = AST (/ULN) ×100/PLT (10^9 L^{-1})

Statistical Analysis

Data obtained were analyzed using Statistical Package for Social Sciences (SPSS) version 20) software. Data were expressed as mean ± SDand median. The significance of differences in mean values among groups were analyzed using one-wayAnova for normally distributed variables, while Kruskall Wallis was used to analyze the significant differences in median values among different groups for variables not normally distributed. Mann-Whitney was also used to analyse significant differences between groups. Spearman's correlation coefficient was used to assess the levels of relationship between two variables. Regression analysis and receiver Operators curve were also used appropriately. The level of significance was considered at $p < 0.05$.

RESULTS

Levels of Some Non invasive Markers of fibrosis in Hepatitis B Subjects and control Group (mean±SD)

Table 4.13 shows the mean levels of Some Non invasive Markers of fibrosis in HBV Seropositive Subjects and control Group. The mean levels of GPR, APRI and AAR (0.06±0.11, 0.44±0.87 and 1.49 ± 0.86 respectively) were significantly higher in test subjects compared with control group. There was no significant difference in mean levels of RPR, NLR and PLR ($P > 0.005$).

Table 1: Level of Some Non invasive Markers of fibrosis in Hepatitis B Subjects and control Group (mean±SD).

Parameters	Test group	Control group	t-test	*P-Value*
RPR	0.38± 0.11	0.14±.02	1.654	0.099
GPR	0.06±0.11	0.02±0.01	4.000	<0.001
APRI	0.44±0.87	0.11±0.69	3.658	<0.001
NLR	1.32 ± 1.90	1.22 ± 0.34	0.492	0.502
PLR	109.71 ±85.91	130.51± 30.94	0.062.245	0.004
AAR	1.49± 0.86	0.89 ± 0.59	5.810	<0.001

Key: RPR= Red cell distribution width to Platelet ratio, GPR = GGT to Platelet ratio, APRI= AST to Platelet ratio, NLR= Neutrophil to Lymphocyte Ratio, PLR= Platelet to Lymphocyte ratio, AAR= AST to ALT ratio.

Correlation of HBV DNA viral load with RPR, GPR, NLR, PLR, APRI, AAR in Hepatitis B subjects

Table 4.18 shows the Correlation of Hepatitis B viral load with RPR, GPR, NLR, PLR and AAR in HBV Seropositive subjects. Correlation between HBV viral load and AAR showed a significant negative correlation (r= -.153, p< 0.045). RPR, GPR, NLR and APRI did not show any significant correlation with HBV viral load (P > 0.05).

Table 2: Correlation of HBV DNA viral load with HBsAg Quantification, RPR, GPR, NLR, PLR, APRI, AAR in Hepatitis B subjects.

Parameters	HBV load (rho)	P-Value
RPR	-0.005	0.949
GPR	-0.021	0.789
APRI	0.052	0.504
NLR	0.032	0.680
PLR	-0.018	0.817
AAR	-0.153	0.045

RPR= Red cell distribution width to Platelet ratio, GPR = GGT to Platelet ratio,
APRI= AST to Platelet ratio, NLR= Neutrophil to Lymphocyte Ratio,
PLR= Platelet to Lymphocyte ratio, AAR= AST to ALT ratio.

Mean Age and Sex Distribution of Test Subjects

Figure 1 shows the total number of female test subjects was 76(42.7%) while their male counterparts were 100(57.3%). The female subjects with ages less than 20 had the lowest frequency of 3(3.9%), this was followed by those female subjects greater than 50 years of age. Those female within the age bracket of 21-30 had the highest frequency of 32(42.9). Those females within the ages of 31-40 were second to the highest with the frequency of 20(26.9%) while those within ages 41-50 were 13(16.9%). The male subjects with ages less than 20 also had the lowest frequency of 4(3.9%), this was followed by those male subjects greater than 50 years of age with frequency of 12(11.8%). Those male subjects within the age bracket of 31-40 had the highest frequency of 42(43.1%). Those within the ages of 21-30 were second to the highest with the frequency of 26(25.5%) while those within ages 41-50 were 16(15.7%).

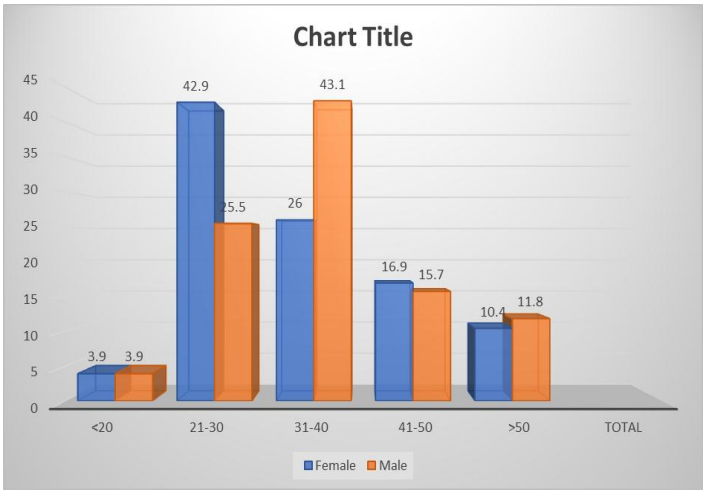

Figure 1: Mean Age and Sex Distribution of Test Subjects (N=176).

DISCUSSION

Infection with HBV most times is asymptomatic, hence timely and accurate laboratory diagnosis is essential to make therapeutic decisions and to monitor the patients for the development of advanced liver disease. In HBV management, viral load (HBV DNA quantification) has long been used as a major diagnostic tool for management. The HBV DNA provides a marker of active HBV replication, monitors response to antiviral therapy and identifies the development of resistance. However, HBV DNA has limitations of not being affordable, not routinely done, high turnaround time and being labour intensive which constitutes a barrier to the management.

Raised serum ALT and AST are indicators of hepatocellular damage and raised ALP and GGT which are indicators of hepatobilliary damage. ALT and AST—are two of the most useful measures of liver cell injury, although the AST is less liver specific than is ALT level. Elevations of the AST level may also be seen in acute injury to cardiac or skeletal muscle. Thus, in clinical practice, it is not uncommon to see elevations of AST, ALT or both in common non-hepatic conditions such as myocardial infarction and rhabdomyolysis. Diseases that primarily affect hepatocytes, such as viral hepatitis, will cause disproportionate elevations of the AST and ALT levels. This may be as a result of liver inflammation caused by HBV infection. However, the study shows no correlation between these biochemical parameters and HBV DNA levels. This finding is in consonance with the report of Demiroren *et al.* (2015) but contrasts that of

Ganji *et al*, 2011 who reported that no correlation exists between HBV DNA levels and HBsAg Quantification.

Changes in platelet count accompany the progression of various forms of liver disease. This explains the use of platelet count as an indirect marker in most of the non invasive assessment of hepatic fibrosis. This work revealed significant increase in the values of non invasive markers of fibrosis. Platelet to lymphocyte ratio (PLR), AST-ALT ratio (AAR), AST-platelet ratio (APRI) and gamma glutamyl transferase to Platelet ratio (GPR) were all elevated among hepatitis B subjects. The finding agrees with that of Lemoine *et al*. (2016) and Li *et al*. (2016) among Chinese HBV population. It is also in agreement with the findings of Boyd *et al*. (2016) who recorded significant increase among patients with HBV-HIV co infection. The findings also support the reports of Xing *et al.* (2018) that non invasive markers of fibrosis play significant roles in HBV infection. They are novel inflammatory markers which may be used in many diseases for predicting inflammation and mortality. The Neutrophil to lymphocyte ratio (NLR) and red cell distribution width to platelet ratio (RPR) did not show any significant difference among the two groups. This contrasts the findings of (Tae and Ji, 2017) which observed significant increase among HBV seropositive subjects. This difference may be related differences in prevalence of liver fibrosis in the studied populations.

This study also hypothesied the relationship between non invasive markers of fibrosis and viral load. Although the study observed significant increase in the markers among hepatitis B subjects, there was no significant correlation between them.

The sex distribution of the subjects in this study showed that there were more males 100(57.2%) males and 76(42.8%) females. This shows that HBV infection is more prevalent in males than females and the finding is in keeping with the findings of Yewande *et al.* (2018) which reported males are 2.8 times more likely to get infected with HBV compared to their female counterparts (Nwokediuko *et al.*, 2011). This could be due to the fact that males are more prone to risk factors and behaviors sexual intercourse, injection drug use, barbing et ce tera which predispose them to HBV infection. Regarding the age distribution, highest frequency of HBsAg seropositivity was found among the younger age group 21-30 followed by 31-40 group for both males and females. A work done by Gheorghe *et al*. (2013) gave a similar report. Considering the modes of transmission of HBV, the high sexual activity of individuals within these age brackets might explain this. The age group <20 had the least frequency both among males and females (3.9 and 3.9) respectively. The >50 age group were the second to the least of the subjects (9% females and 12% for females). This finding is in agreement with that of Yewande *et al.* (2018). The children (<20) and the aged (>50) have less exposure to risky behaviours than the middle aged (21-30, 31-40).

CONCLUSION

Haematological parameters, biochemical and non invasive markers of fibrosis play significant but independent role in HBV management hence should not be used as its alternative.

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