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Original article

Liver histopathology detects more chronic hepatitis B virus genotype D patients who need to be treated

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Abstract:

Patients with chronic hepatitis-B-virus (HVB) infection may exhibit significant liver pathology despite alanine aminotransferase (ALT) and HBV-DNA levels below current treatment guideline's cut-off values. This study evaluated the candidacy for HBV therapy if baseline histopathological changes were considered. Clinical, biochemical, serological, virological, and histopathological (Metavir Score) data of a cohort of 161 consecutive patients [129 (80.1 %) males, mean ± SD age 35.2 ±11.2 years, 130 (80.7% HBeAg-negative, 149 (90.1 %) genotype D] were collected and analyzed. Our results showed that significant pathology (F≥2 and/or A≥2) and significant fibrosis (F≥2 ± A≥2) were found in 98/161(60.9 %) and 81/161(50.3 %) patients respectively. Based on HBV-DNA (>2000 iu/mL or >20000 iu/mL according to HBeAg status) and ALT level >2x40 u/L (the standard cut-off value), only 36/161(22.4 %) patients were candidate for therapy. This increased to 71/161(44.1 %) patients when the new ALT cut-off values (30 u/L for males, and 19 u/L for females) were applied. Relying on either (F≥2 and/or A≥2) or (F≥2±A≥2) increases the treatment candidacy by 62/161(38.5 %) or 45/161(28 %), and further increases the candidacy for treatment by 27/161(16.8 %) or 10/161(6.2 %) patients when standard and new ALT cut-off values are applied respectively. ***Finally,*** *liver histopathology is more reliable than ALT and HBV-DNA levels in the decision to treat patients with chronic HBV infection.*

Keywords:
HBV genotype D
Pathology
Fibrosis
Viral load
Transaminases
Therapy

Running Head: *Liver histopathology and hepatitis B therapy*

Abbreviations: *HBV; hepatitis B virus. PCR; polymerase chain reaction. KFSH&RC; King Faisal Specialist Hospital & Research Centre. ALT; alanine aminotransferase. AST; aspartate aminotransferase. HBeAg; hepatitis B envelop antigen. ULN; upper limit of normal. AASLD; American Association for the Study of Liver disease.*

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1. Introduction

Hepatitis B virus (HBV) infection is a major cause of morbidity and mortality with an estimated 350-400 million chronically infected person worldwide [1]. Chronic HBV infection in Mediterranean and Middle East countries including Saudi Arabia, is characterized epidemiologically by: First, an intermediate endemicity (an overall prevalence of HBsAg 6.7 %) [1,2]. Second, prevalence of HBeAg-negative variant in> 80 % of patients [2,3] that represents the late phase of infection where HBV pre/basic core promoter variants are predominant with reduced or abolished HBeAg expression [4,5]. Third, predominant HBV genotype D [3], which is more often associated with HBeAg-negative variants [6] and more severe liver disease [7]. This HBeAg negative disease is characterized by persistent viral replication, progression of liver disease, and early development of cirrhosis [8,9]. The available HBV treatment guidelines are almost similar [10-13], despite geographical differences in epidemiology of the disease [14]. According to the American Association for Study of Liver Diseases (AASLD) 2007 guidelines, the parameter used to determine candidacy for treatment of HBeAg-positive patients is HBV-DNA is ≥ 20,000 IU/ml and alanine aminotransferase (ALT) ≥2×upper limit of normal (ULN), whereas HBeAg-negative patients should be considered for treatment if HBV-DNA is ≥2,000 IU/ml and ALT is ≥2×ULN. However, patients with lower ALT and/or HBV-DNA levels may have abnormal histology and are consequently at increased risk of chronic liver disease complications [13]. Therefore, the new guidelines recommended a new lower ULN ALT and aspartate aminotransferase (AST) of 30 U/l for men and 19 U/l for women [11,13]. ALT and HBV-DNA levels do fluctuate necessitating their frequent monitoring or performing liver biopsy to assess disease activity, grade of fibrosis, and the eligibility for treatment [13]. Additionally, the REVEAL study has indicated that patients with HBVDNA<2,000 IU/ml are also at risk of disease progression [15]. Moreover, assessing liver histopathology was advised by the AASLD for evaluation of histologic disease in chronic HBV patients presenting with normal ALT and low HBV-DNA levels [11]. Therefore, the main objective of this study in patients with chronic HBV infection were to evaluate whether incorporation of liver histopathological changes and

HBV genotype affects the decision to treat both HBeAg-negative and HBeAg positive patients and influence the candidacy to treat such patients compared to the current strategies that rely mainly on both serum ALT and HBV-DNA levels.

2. Patients and Methods

2.1. *Patients*

A total of 592 patients with chronic HBV infection attending our hepatology outpatient clinic from May 2011 to May 2015 were included.

2.2. *Exclusion criteria*

After exclusion of cases with concomitant HIV, hepatitis C virus (HCV), hepatitis delta infections, and those with hepatocellular carcinoma (HCC), alcoholic liver disease, diabetes mellitus and hyperlipidemia, we were left with 256 patients with isolated HBV infection. Only 161 patients remained after further exclusion of patients with: **1)** No liver biopsy (not done because of failure to obtain consent or clinical contraindication), **2)** Non-typable HBV DNA, **3)** Previous anti-HBV therapy, **4)** Decompensated liver disease.

2.3. *Inclusion criteria*

The 161 patients enrolled in this study fulfilled the following inclusion criteria after obtaining institutional approval and written informed consent: HBsAg-positive test for >6months, HBeAg-negative or negative, detectable HBV-DNA by PCR, recently done liver biopsy, determined HBV genotype, no prior treatment with interferon or neucleos(t)ide analogues, and absence of the above exclusion criteria.

2.4. *Laboratory tests*

Routine liver biochemical tests were performed using commercially available auto-analyzers and hepatitis serological markers were assayed using commercially available enzyme-linked immunoassays (Clinotech Diagnostics). According to the instructions of the manufacturer, the ULN for ALT was determined to be 40 U⁄ml (Bicon Diagnostik, Germany). Quantitative HBV DNA levels were measured by a COBAS TaqMan System (Roche Diagnostics Corporation, Indianapolis, IN, USA), which has a lower detection limit of 30 IU⁄ml. Genotyping was performed using INNO-LiPA HBV Genotyping assay (Innogenetics N.V., Ghent, Belgium).

2.5. *Assessment of liver histopathology*

All patients (n=161) included in this study had ultrasound-guided liver biopsies (Hematoxylin and Eosin stains for morphological evaluation and Masson's trichrome stain for fibrosis assessment). Liver specimens were adequate in size (at least 15 mm long) and >6 portal tracts were assessed. All liver biopsy specimens were assessed and scored according to the METAVIR scoring system [16] by a single experienced hepatopathologist who was blinded to all clinical and virological results. Patients with inflammatory grade ≥A2 and/or those with fibrosis stage ≥F2 were considered to have significant pathology.

2.6. *Statistical analyses:*

Data were collected initially in a data collection Form, transferred to the Statistical Package for Social Sciences *version* 15.0 (SPSS, Chicago, IL, USA). Means of continuous variables were compared using Student's t-tests, non-parametric tests (Wilcoxon's & Mann Whitney) or one-way analysis of variance (ANOVA) with Post-Hoc test (Turkey's) as appropriate. The Chi-Square or Fisher's exact test was used to compare ratios and proportions. A p-value of <0.05 was taken as statistically significant. Correlation between continuous variables with skewed distribution was tested by Spearman'srank correlation.

3. Results

3.1. *Baseline patients' characteristics*

As shown in tab. (1) a total of 161Saudi patients with treatment-naïve chronic HBV infection, 129 (80.1 %) were male and 32 were female, aged 17-70 years (median = 36) were included in the study. Of them, 131 (80.7 %) were HBeAg-negative, and 145 (90.1 %) were infected with isolated genotype D. We could not accurately calculate the duration of the disease. The HBV-DNA level ranged from 31-11x10^8 IU/ml (median =5.3x10^5 IU/ml) and the ALT level ranged from 9-919 U/ml (median= 40.0 U/l) were found, while the range of AST was 13-344 U/l (median = 30). Moderate-to-severe inflammation (≥A2) was detected in 74 (46.0 %) patients and significant fibrosis (≥F2) was found in 81 (50.3 %).

Table (1) Subjects characteristics (n=161).

Variable	Categories & Expression	Result
Age (years)	Mean±SD Median(Range) ≤40 : >40 years	35.2±11.2 35 (18-70) 118 (73.3) : 43(26.7)
Sex	Male : Female	129 (80.1) : 32(19.1)
HBeAg status	Negative Positive	130 (80.7) 31 (19.3)
ALT	u/l Median(Range)	75.6±113.9 40 (9-919)
AST	u/l Median(Range)	44.6±46.3 30 (13-344)
HBV DNA	IU/ml Median(Range)	1E+008±3E+007 $5.3x10^5$ ($31-11x10^8$)
HBV genotype	D Others	145 (90.1) 16 (9.9)
Inflammatory grade	0 1 2 3 4 2-4	10 (6.2) 77 (47.8) 59 (36.6) 13 (8.1) 2 (1.2) 74 (46.0)
Fibrosis stage	0 1 2 3 4 2-4	33 (20.5) 47 (29.2) 53 (32.9) 19 (11.8) 09 (5.6) 81 (50.3)

Data expressed as mean ± SD or n (%) and/or median (range) as appropriate. HBV, hepatitis B virus. SD, standard deviation. n, number. ALT, alanine aminotransferase. AST, aspartate aminotransferase. HBeAg; hepatitis B envelop antigen.

3.2. *Comparison between HBeAg negative and HBeAg positive patients*

As shown in tab. (2), and compared to HBeAg positive patients, HBeAg negative subjects were significantly older (p= 0.003), had significantly lower HBV DNA levels (p= 0.000), but similar ALT (p= 0.31), AST levels (p= 0.44), and HBV genotype distribution (p= 0.96). Significant fibrosis (≥F2) was similar in both groups and was detected in 65/130 (50 %) and 16/31 (51.6 %); p= 0.87. The same applies to significant inflammation (A≥2) that was detected in

62/130 (47.7 %) and 11/31 (38.7 %); p= 0.43; of both HBeAg negative and HBeAg positive patients respectively. Table 2 also shows that the proportion of patients who are candidates to therapy is similar between HBeAg negative and HBeAg positive cases irrespective of the strategy used to decide it.

Table (2) Comparison between HBeAg negative and HBeAg positive patients.

Variable	HBeAg negative (n=130)	HBeAg positive (n=31)	P value
Age (years)	36 (18-70)	29 (17-53)	0.003
>40years	40 (30.8)	3 (9.7)	0.02
Male sex	106 (81.5)	23 (74.2)	0.26
ALT (u/l)	46.5 (9-925)	38 (19-170)	0.31
AST (u/l)	30 (13-357)	30.5 (17-226)	0.44
HBV DNA (IU/ml)	$2x10^5$ ($37-1x10^8$)	$6.6x10^7$ ($31-1x10^8$)	0.00
Genotype D	117 (90.0)	28 (90.5)	0.96
Inflammation grade ≥2	62 (47.7)	12 (38.7)	0.43
Fibrosis stage ≥2	65 (50.0)	16 (51.6)	0.87
Candidate for therapy (DNA& standard ALT)	33 (25.4)	3 (9.7)	0.06
Candidate for therapy (DNA& new ALT)*	61 (46.9)	10 (32.3)	0.16
Biopsy based therapy	80 (61.5)	18 (58.1)	0.84

*Data expressed as median (range) or n (%) as appropriate. ALT, alanine transaminase. AST, aspartate transaminase. HBV; hepatitis B virus. *, New upper limit f normal cut-off values of 19 and 30 U/l.*

3.3. *Comparison between genotype D and non-genotype D patients*

As expected in our geographic locality, genotype D predominated in our patients [145/161(90.1 %)]. Genotypes B, A, E, and mixed genotypes were detected only in 1, 4, 5, and 6 patients respectively. The mixed genotypes were as follows: E/D in 4 patients, A/C/D in 1 patient, and C/D in 1 patient. When compared to non-genotype D patients [n= 16/161(9.9 %)], genotype D cases had had similar median age, sex distribution, HBeAg status, liver pathology, but with significantly higher viral load (p= 0.03, tab. (3). Of note that the percentage of patients who are candidates to therapy was significantly higher in genotype D patients; 68/145 (46.95) than non-genotype D patients; 3/16 (18.8 %) when serum HBV-DNA (>2000 iu/mL or >20000 iu/mL according to HBeAg status) and the new ALT (2x the upper limit of the new cut-

off values (30 u/l for males, and 19 u/l for females) were applied (p= 0.03). The same is not true when the standard ALT cut-off value (40 u/l) was used.

Table (3) Comparison between HBV genotype D and HBV non-genotype D patients.

Variable	Genotype D patients (n = 145)	Non-genotype D patients (n= 16)#	P value
Age (years)	35 (17-70)	36 (19-53)	0.67
>40years	39 (26.9)	4 (25.0)	0.87
Male sex	118 (81.4)	11 (66.8)	0.23
ALT (u/l)	40 (9-925)	36 (11-187)	0.39
AST (u/l)	31 (13-357)	25 (15-226)	0.12
HBV DNA (IU/ml)	115773 (31-1x10^8)	3109 (118-1x10^8)	0.03
HBeAg status	117 (80.7)	13 (81.3)	0.96
Inflammation grade ≥2	68 (64.9)	6 (37.5)	0.47
Fibrosis stage ≥2	72 (49.7)	9 (56.3)	0.62
Candidate for therapy (DNA& standard ALT)*	34 (23.4)	2 (12.5)	0.32
Candidate for therapy (DNA& new ALT)**	68 (46.9)	3 (18.8)	0.03
Biopsy based therapy	88 (66.7)	10 (62.5)	0.89

Data expressed as mean ± SD or n (%) as appropriate. ALT, alanine transaminase. AST, aspartate transaminase. HBV; hepatitis B virus. #, Genotypes B, A, E, and mixed genotype were detected in 1, 4, 5, and 6 patients respectively. The mixed cases were: E/D in 4, A/C/D in 1, and C/D in 1.

3.4. *Relation between the HBV-DNA and liver histopathology*

There was a significant weak positive correlation between the HBVDNA level and the grade of inflammation (r = 0.20, p= 0.01). Similar significant positive correlation was detected between the HBV-DNA level and the stage of fibrosis (r = 0.22, p = 0.005). HBV DNA level correlated positively with ALT level (r = 0.38, p = 0.00). According to AASLD guidelines 2007 standards, taking HBVDNA level of 2,000 IU/ml as a cut-off value for HBeAg-negative patients, our results have shown that 11 of 33 patients (33.3 %) with serum HBV-DNA level below 2,000 IU/ml had stage 2 or higher fibrosis (≥F2), and 16 of them (48.5 %) had fibrosis ≥F2 and/or inflammation grade 2 or more (A≥2). Patients with HBV DNA >20,000 IU/ml showed significantly more hepatic pathology (≥F2) and (≥F2 and/or ≥A2) compared to patients with lower HBV DNA; p = 0.017 and p = 0.011 respectively, tab. (4).

Table 4: Significant liver pathology by viral load and ALT level.

Variable	Level	n(%)	F≥2	F≥2 and/or A≥2
HBV DNA	<2000 IU/ml	33 (20.5)	11 (33.3)	16 (48.5)
	2000-<20000 IU/ml	38 (23.6)	16 (42.1)	18 (47.4)
	≥20000 IU/ml	90 (55.9)	54 (60.0)#	64 (71.1)$
ALT *	≤1xULN	82 (50.9)	27 (32.9)##	36 (43.9)##
	>1x-<2xULN	39 (24.2)	27 (69.2)	30 (76.9)
	≥2xULN	40 (24.8)	27 (67.5)	32 (80.0)
ALT**	≤1xULN	25 (15.5)	5 (20.0)	9 (36.0)
	>1x-<2xULN	54 (33.5)	18 (33.3)	22 (40.7)
	≥2xULN	82 (50.9)	58 (70.7)##	67 (81.7) ##
Total	N	161	81	98

Data expressed as n (%). n, number. ALT, alanine aminotransferase. AST. aspartate aminotransferase. HBV, hepatitis B virus. ULN, upper limit of normal. using the standard cut-off value of 40 u/l. ** using the new cut-off values of 30/19 u/l. F≥2, fibrosis stage 2 or more by Metavir score. A≥2, inflammation grade 2 or more by Metavir score. #: p=0.017. ##, p=0.000. $, p=0.011.*

3.5. *Relation between the ALT level and liver histopathology*

There was a weak positive correlation between the ALT level and each of the grade of inflammation (r = 0.27, p = 0.00) and the stage of fibrosis (r = 0.36, p = 0.00). According to AASLD guidelines 2007 standards, taking ALT of as 40 ULN, our results show that 27 of 82 patients (32.9 %) with serum ALT level ≤1xULN (40 U/l) had fibrosis stage ≥F2, and 36 of them (43.9 %) had (≥F2 and/or A≥2). However, patients with ALT ≤1xULN showed significantly less hepatic pathology; (≥F2) and (≥F2 and/or ≥A2) compared to those with higher ALT levels; p = 0.00 for both, tab. 4. Even with the new reduced ALT cut-off values, 5 of 25 patients (20.0 %) with serum ALT level ≤1xULN (19/30 U/l) had stage ≥F2), and 9 of them (36.0 %) had fibrosis ≥F2 and/or A≥2, tab. 4. Patients with ALT levels ≥2x new ULN (19/30 U/l) have significantly more hepatic pathology; (≥F2) and (≥F2 and/or ≥A2) compared to those with lower ALT levels; p=0.00 for both, tab. (4).

3.6. *Candidacy to treatment decision*

Based on serum HBV-DNA (>2000 iu/mL or >20000 iu/mL according to HBeAg status) and ALT level >2x40 u/L (the standard cut-off value), only 36/161(22.4 %) patients were candidates for therapy. This increases to 71/161(44.1 %) patients when the new ALT cut-off values (30

u/L for males, and 19 u/L for females) were applied. For the start of therapy, implementation of significant liver pathology (F≥2 and/or A≥2), or significant fibrosis (F≥2±A≥2) increases the patients eligible for therapy to 81/161 (50.3 %) and 98/161(60.9 %) respectively, tab (5) & fig. (1). This increases the candidacy to therapy by 62/161(38.5 %) and 45/161(28.0 %) patients respectively. With application of the new lower ALT cut-off values, application of (F≥2 and/or A≥2) and (F≥2±A≥2) still increases the candidacy for treatment by 27/161(16.8 %) and 10/161(6.2 %) patients respectively.

Table (5) Comparison between the number and percentage of patients who need to be treated according to used strategies: Pathology, ALT and HBV DNA levels.

Question?	Standard ALT cut-off value# & HBV DNA (Strategy 1)	New cut-off ALT values## & HBV DNA (Strategy 2)	F≥2±A≥2 (Strategy 3)	F≥2 and/or A≥2 (Strategy 4)
Decision to treat:				
Candidate for therapy?	36/161 (22.4)	71/161 (44.1)	81/161 (50.3)	98/161 (60.8)
Not candidate?	125/161 (77.6)	90/161 (55.9)	80 (49.7)	63 (39.2)
Cases missed:	-	-	-	-
Compared to strategy 2?	35/161(21.7)	-	-	-
Compared to strategy 3?	46/161(28.6)	10/161(6.2)	-	-
Compared to strategy 4?	62/161(38.5)	27/161(16.8)	17/161(10.6)	-

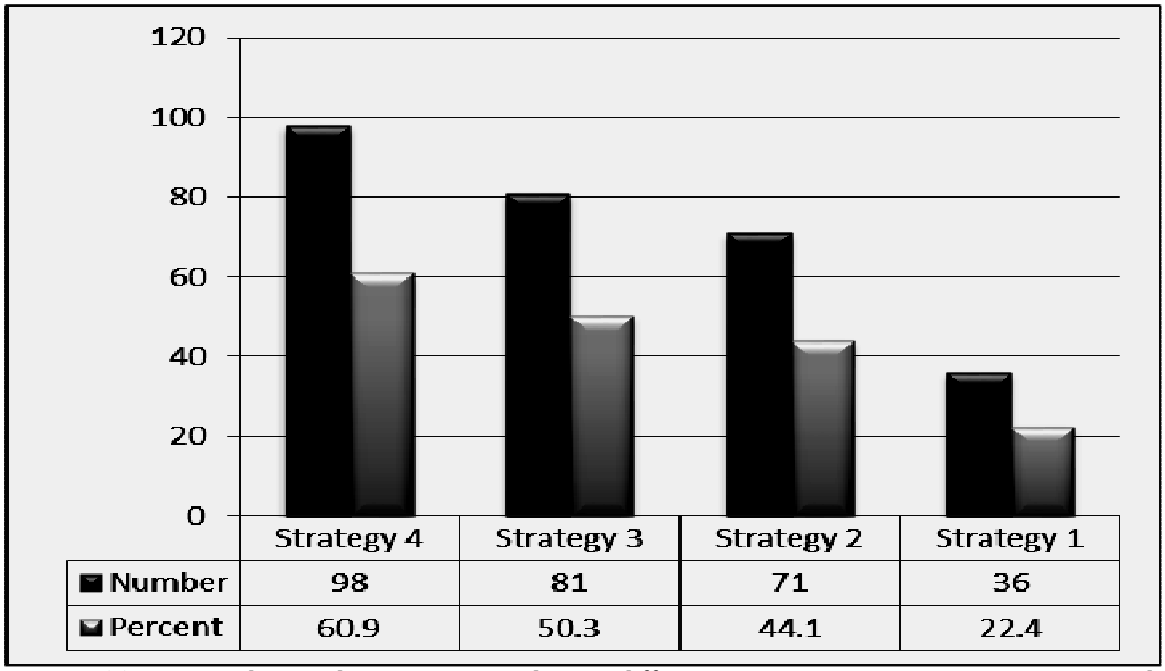

Figure (1) Patients that need treatment according to different strategies. Strategy 1: HBV DNA and standard ALT value (40 u/l). Strategy 2: HBV DNA and new ALT cut-off values (19/30 u/l). Strategy 3: Fibrosis stage F≥2. Strategy 4: Fibrosis stage F≥2 and/or inflammation grade A≥2.

4. Discussion

We believe that the current international guidelines that relay mainly on HBV DNA and ALT levels in the decision to treat patients with chronic HBV infection require regular re-evaluation. Based on our results and those of others [17] liver histopathology, but not HBV genotype needs to be more incorporated in any future practice guidelines. Currently available guidelines recommend observation of HBV infected patients especially those with viral load above the threshold and normal ALT [10,12,13] whereas Keeffe algorithm considered liver biopsy and treatment if biopsy reveals fibrosis [11] particularly inpatients above the age of 40 years. Recent reports revealed that 12-45.5 % of patients with chronic HBV infection and persistently normal ALT levels has fibrosis stage F≥2 [18-20] and that age >40 years is an independent predictor of significant pathology. This together with our results suggests that the future management guidelines should recommend more liver biopsies in this patient group. The majority of our patients have HBeAg negative disease (130/161; 80.7 %). These cases usually require closer follow up and frequent monitoring [21]. This requires not only extra costs, but also patient's awareness and education as most of these patients are asymptomatic. In addition, many of these patients present at an older age(median: 35 versus 29 years for HBeAg positive patients in this study, p= 0.003) with significant hepatic pathology and persistent viral replication [7-9] In the present study, there was no significant difference in the severity of liver pathology based on the HBeAg status. The small number of the HBeAg positive group (n= 31) may be responsible, at least in part, for this unexpected result. The predominant genotype in our patients is genotype D (145/161; 90.1 %), and is associated with other genotypes in 3 of the 6 cases (50 %) of the mixed infection. This finding is consistent with other earlier reports from Saudi Arabia and other Middle Eastern countries like Egypt [3,22]. Unfortunately, this genotype is known to be associated with the HBeAg-negative variant [6], and with more severe liver disease [7]. Therefore, patients with HBV infection with the above characteristics (genotype D and HBeAg negative variant) may need early liver biopsy rather than frequent laboratory follow-up with ALT and HBV DNA evaluations, which are often fluctuating, particularly HBVDNA levels [23]. This view is supported by Keeffe

algorithm that considers therapy in patients with known significant histologic disease even if ALT is within normal and the HBV DNA is low [11]. We did not find any significant difference in hepatic fibrosis stage or inflammation grade between genotype D cases and non-genotype D ones. This can probably be attributed to the small number (n=16) of patients infected with non-D genotypes included in this study, and to the fact that genotype D coexists in 3 of them. For the same reasons, HBV genotype was not shown to affect candidacy for treatment. The role of genotype in this matter requires future studies with more patients in the non-D genotype group for better comparison. In this study, 50 % of HBeAg-negative patients exhibited significant liver pathology and were associated with normal or slightly elevated ALT level, tab. (2). This supports the concept that lower HBV-DNA levels (3-5 $\log_{10}$ IU/ml) may be associated with progressive liver disease and therefore warrant treatment [13]. Serum ALT levels correlated positively with the grade of inflammation, and the stage of fibrosis. The more recent guidelines recommended reducing the ULN ALT and AST to 30 U/l for men and 19 U/l for women [11,13]. Applying these new values in the present study increased the patients eligible for treatment by 21.7 % (35 patients), tab. (5). We have also shown significant more pathology (60-71.1 %) in patients with higher HBV DNA levels, tab. (4). This is consistent with previous reports in HBeAg-negative patients [17,24,25], but not universal [26]. The low HBV-DNA level among Saudi patients is multi-factorial and is probably related to viral factors (HBeAg-negative variants predominance [27,28], genotype D prevalence) and other hitherto (duration of infection, racial and geographic differences). Our results show clearly the importance of liver biopsy findings in saving many patients the consequences of missing the chance of treatment. Based on serum HBV-DNA (>2000 iu/ml or >20000 iu/ml according to HBeAg status) and ALT level >2x40 u/l (the standard cut-off value), only 36/161(22.4 %) patients were candidates for therapy. This increases to 71/161(44.1 %) patients when the new ALT cut-off values (30 u/l for males, and 19 u/l for females) were applied. Significant liver pathology (F≥2 and/or A≥2), or significant fibrosis (F≥2±A≥2) increased the treatment-eligible cases to 81/161(50.3 %) and 98/161(60.9 %) respectively. This increases the candidacy

to therapy by 62/161(38.5 %) and 45/161(28.0 %) patients respectively. Compared to the new ALT cut-off levels recommended by AASLD (30 U/l for male and 19 U/l for female patients), application of (F≥2 and/or A≥2) and (F≥2±A≥2) still increases the candidacy for treatment by 27/161(16.8 %) and 10/161(6.2 %) patients respectively.

5. Conclusion

Liver biopsy is more reliable than both ALT and HBV-DNA levels in the decision to treat patients with chronic HBV even with implementation of the recommended lower ALT levels, irrespective of the HBV genotype and the HBeAg status. Whether to replace liver biopsy with the currently evolving noninvasive measure is a debatable issue that warrants future investigations.

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