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Main mutations in the hepatitis B virus basic core promoter (A1762T/G1764A) before HBeAg loss are markers that identify patients who will require long-term treatment

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Title: Main mutations in the hepatitis B virus basic core promoter (A1762T/G1764A) before HBeAg loss are markers that identify patients who will require long-term treatment

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Running head: HEPATITIS B MUTATIONS AND THERAPY RESPONSE

Keywords: HBeAg, long-term treatment, HBV-DNA levels, basic core promoter

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3 **Background:** Some patients continue to have detectable HBV-DNA levels with liver
4 disease progression after hepatitis B e antigen (HBeAg) loss. It is important to identify
5 these patients, **candidates for long-term treatment.**
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10 **Aims:** To evaluate hepatitis B virus (HBV) genotype and the main **mutations** in the basic
11 core promoter (BCP, **A1762T/G1764A**) and precore (**G1896A**) sequences as markers of
12 persistent HBV-DNA after HBeAg loss.
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17 **Methods:** We analyzed 60 serum samples from 20 Caucasian, HBeAg-positive, chronic
18 hepatitis B patients, who lost HBeAg and were followed-up longitudinally. HBV
19 genotype and precore and BCP mutations were determined before, at the time of, and
20 after HBeAg loss.
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27 **Results:** After HBeAg loss, 8 (40%) patients continued to have undetectable HBV-DNA
28 and 12 (60%) had persistent HBV-DNA (median level 4.7 log₁₀ copies/mL). The
29 presence of BCP mutations prior to therapy was the only variable associated with
30 persistently detectable viremia ($P=0.017$). Four patients with genotype A and no
31 mutations in the BCP region experienced HBsAg loss after a mean **period** of 35 months
32 **from baseline.**
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41 **Conclusions:** **Main BCP mutations in HBeAg-positive patients are useful markers to**
42 **identify patients who will not have sustained virologic suppression after HBeAg loss and**
43 **therapy discontinuation, and could benefit from long-term treatment.**
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Introduction

More than 350 million people worldwide are infected with the hepatitis B virus (HBV), the majority of whom reside in Asia, Africa and Western Europe. The natural history of HBV infection represents a continuum of different phases, including the immune tolerance phase, immune reactive phase, inactive HBV carrier state, and low replicative phase [1]. It is important to distinguish the early, initial replicative phase, which consists of viral replication defined by the presence of hepatitis e antigen (HBeAg), high serum HBV-DNA levels, and active liver disease (elevated serum alanine aminotransferase (ALT), and chronic hepatitis on liver biopsy), from the late, low replicative phase with remission of liver disease, which is usually associated with undetectable HBeAg serology and HBV-DNA levels. The transition between the two phases is marked by spontaneous HBeAg clearance, which occurs at an annual rate of 10% to 20% [2-3].

Despite HBeAg loss and seroconversion, some patients continue to have moderate levels of HBV replication and active liver disease (elevated serum ALT and chronic inflammation on liver biopsy), but remain HBeAg negative [4]. Such patients are described as having HBeAg-negative chronic hepatitis, in which variants with null or reduced HBeAg production predominate in the HBV quasispecies because of precore and basic core promoter (BCP) genetic variations; in addition, the latter are related to more severe liver disease [5-7]. The percentage of patients who achieve seroconversion and continue to have detectable HBV-DNA levels after seroconversion has not been well established among Caucasian patients, and could differ from that of Asian patients [8-9]. In a study performed in Asia, 33% of 283 patients who underwent spontaneous HBeAg

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3 seroconversion had virological breakthrough after a median follow-up of 8.6 years and
4
5 showed a significantly higher risk of developing cirrhosis and hepatocellular carcinoma
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7 (HCC) compared to those with sustained remission [8]. With the introduction of sensitive
8
9 polymerase chain reaction (PCR)-based assays, varying degrees of viremia have been
10
11 detected after HBeAg loss, which raises the question as to whether HBeAg loss and
12
13 seroconversion alone are appropriate endpoints of HBV therapy or if they should be
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15 accompanied by sustained viral suppression to prevent disease progression and the
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17 development of HCC [10-12].
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22 Few studies have investigated the long-term outcome of Caucasian patients after
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24 HBeAg loss or the possible role in predicting persistent viral replication of certain factors
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26 associated with a reduction or suppression of HBeAg expression, such as HBV genotype
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28 and the main mutations in the precore region (nucleotide (nt) substitution G1896A) and
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30 BCP region (nt substitutions A1762T and G1764A) [13-15]. In this study, serum samples
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32 from a cohort of patients with HBeAg-positive chronic hepatitis B who lost HBeAg have
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34 been longitudinally followed-up to determine whether HBV genotype or the main precore
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36 and BCP mutations could be useful as markers for predicting persistent viremia after
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38 HBeAg loss.
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46 **Patients and Methods**

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48 This is a retrospective study of 60 serum samples selected from 20 patients with
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50 chronic HBeAg-positive HBV infection who experienced HBeAg loss, either
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52 spontaneously or with antiviral treatment, and who had a period of follow-up without
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54 treatment. The medical records of 272 patients with chronic HBV infection treated in our
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3 hospital from January to December 2008 were reviewed. Those who met the following
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5 criteria were selected: an initial visit to the hospital when they were HBeAg-positive with
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7 stored serum samples, HBeAg loss that was either spontaneous or during antiviral
8
9 treatment, and a follow-up period of more than 12 months after HBeAg loss. **According**
10
11 **with current clinical practice guidelines [1], patients who received nucleos(t)ide analogs**
12
13 **(NUCs) had a 6-month period of consolidation therapy after losing HBeAg, the follow-up**
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15 **period in these patients started after finishing this timeframe.** Patients had periodic visits
16
17 at least every 3 to 6 months and laboratory studies, including complete biochemical
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19 determinations, HBV serology, and HBV-DNA levels. Patients coinfecting with human
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21 immunodeficiency virus, hepatitis C virus, or hepatitis D virus were excluded. Three
22
23 stored serum samples from each patient were selected. The baseline sample was obtained
24
25 prior to starting the last treatment before HBeAg loss, or at the first visit for those who
26
27 cleared HBeAg spontaneously. The second serum sample coincided with HBeAg loss,
28
29 and the third was taken at the last follow-up visit in order to have the maximum follow-
30
31 up after HBeAg loss **and therapy discontinuation.** In addition to the biochemical studies,
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33 HBV genotype, HBV-DNA levels, and the main precore and BCP mutations were
34
35 determined in the samples. In addition, information about previous treatment, time
36
37 elapsed since the previous treatment, type of antiviral treatment, follow-up time after
38
39 completing treatment, clinical outcome, and the need for retreatment in patients with
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41 persistent viremia after HBeAg loss was recorded. HBeAg loss was defined as a lack of
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43 HBeAg in patients previously diagnosed as HBeAg positive, confirmed in at least 2
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45 separate serum samples obtained at an interval of 3 months. Complete suppression of
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47 viral replication was defined by HBV-DNA values below 2.5 log₁₀ copies/mL, the lower
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3 limit of detection of the technique. Persistent complete suppression was established when
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5 HBV-DNA values were undetectable for a minimum follow-up of 12 months. HBV
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8 treatment was initiated when ALT values were greater than twice the upper normal limit
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10 and HBV-DNA values were higher than 1×10^5 copies/mL in 2 determinations, separated
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12 by an interval of 6 months.
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15 16 17 18 *Serological and Virological Tests*

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20 HBsAg, HBeAg, and antibodies to HBeAg (anti-HBe) were determined by EIA
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22 (Ortho Clinical Diagnostics, Amersham, Bucks, UK).
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25 HBV-DNA levels were quantified by real-time PCR (Light-Cycler-FastStart DNA
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27 Master; Roche Diagnostics, Mannheim, Germany), targeting a conserved region of the
28
29 precore-core of the HBV genome, as previously described [16]. The detection limit of the
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31 PCR technique was 500 HBV copies/mL ($2.5 \log_{10}$ copies/mL). HBV-DNA was
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33 expressed in a \log_{10} scale.
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37 Main HBV precore and BCP variants were detected using a line-probe assay
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39 (INNO-LiPA HBV PreCore assay; Innogenetics NV, Ghent, Belgium) according to the
40
41 manufacturer's instructions. Briefly, viral DNA was isolated from 200 μ L of serum and a
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43 fragment of the precore-core region of the HBV (outer primers: nt 1652 to nt 1977 and
44
45 nested nt 1704 to nt 1942), was amplified by nested PCR. The amplified biotinylated
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47 product obtained in the second-round amplification was used for reverse hybridization
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49 with specific oligonucleotide probes immobilized on nitrocellulose strips. The line probe
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51 assay contained specific probes covering the following motifs: 1) BCP nt 1762/1764 wild
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53 type (A/G), 2) BCP nt 1762/1764 mutation (A/A), 3) BCP nt 1762/1764 mutation (A/T),
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3 4) BCP nt 1762/1764 mutation (T/A), 5) precore codon 28 (nt 1896) wild type (codon
4 TGG → amino acid W), and (6) precore codon 28 (nt 1896) mutation (codon TAG →
5 STOP codon). The INNO-LiPA assay can detect a variant that accounts for at least 5% of
6 the total population [17]. Nucleotides are numbered according to Okamoto et al [18].
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12 HBV genotyping was performed with a line-probe assay (INNO-LiPA HBV
13 Genotyping Assay; Innogenetics NV, Ghent, Belgium), according to the manufacturer's
14 instructions. This assay is designed to identify HBV genotypes A to H by nested PCR
15 amplification of a fragment of the S region of the HBV genome, obtaining a biotinylated
16 product required for hybridization. The amplified region extends from nt 415 to 842 for
17 the outer primers and from nt 456 to 798 for the nested inner primer.
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29 *Statistical Analysis*

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32 Results are expressed as median and range, and percentages. Comparisons
33 between quantitative variables were performed using the Student *t* test. Qualitative
34 variables were compared with the Fisher test and Pearson chi-square test. **Variables that**
35 **were significant in the univariate analysis were included in a logistic regression model.**
36 Differences were considered statistically significant at a *P* value of <0.05. All statistical
37 analyses were performed with the Statistical Program for Social Sciences (SPSS 15.0 for
38 Windows; SPSS Inc., Chicago, IL).
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50 **Results**

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53 **Baseline characteristics of patients who met the inclusion criteria are summarized in**
54 **Table 1.**
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3 All patients cleared HBeAg after a median of 13.5 months (4-94) after the baseline
4 sample was obtained. No differences were observed in the time of HBeAg loss between
5 treated and untreated patients. Sixteen patients achieved sustained anti-HBe
6 seroconversion, 2 had transient anti-HBe seroconversion with posterior loss of these
7 antibodies and persistence of HBeAg negativity, and 2 never developed anti-HBe
8 antibodies.

9
10 Patients were categorized into 2 groups depending on HBV-DNA detection at the end of
11 follow-up (Table 2). Overall, 8 (40%) patients had persistently undetectable HBV-DNA
12 levels and were classified as sustained virologic responders, and 12 (60%) had persistent
13 viral replication despite HBeAg loss. Patients with persistent viral replication after
14 HBeAg loss had main BCP mutations ($P=0.017$) and a fibrosis degree on liver biopsy
15 greater than 2 ($P=0.018$). An association of female gender with persistent viral replication
16 was also found ($P=0.035$, Table 2). Multivariate analysis was performed with these
17 variables to confirm their association with persistent viral replication. The statistical
18 results confirmed the relationship of persistent viral replication with main BCP mutations
19 ($P=0.015$) and female gender ($P=0.036$), but not with fibrosis >2 ($P=0.226$). There were
20 no differences at baseline in HBV genotype distribution, median ALT levels, or median
21 HBV-DNA values between the 2 patient groups. At the time of HBeAg loss, 3 of the 8
22 patients with sustained virologic response achieved undetectable HBV-DNA levels,
23 whereas all the patients with persistent viral replication continued with detectable levels.
24 Then median HBV-DNA levels were significantly lower ($3.5 \log_{10}$ copies/mL) in patients
25 with sustained virologic response relative to those with persistent viremia ($4.6 \log_{10}$
26 copies/mL) ($P=0.020$, Figure 1). No differences were observed in sustained anti-HBe
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3 seroconversion between patients with a sustained virologic response or persistent viral
4 replication ($P=0.166$). Transient anti-HBe seroconversion was observed in 1 case in each
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8 group. There was no relationship between the type of treatment (interferon-based or
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10 NUCs) and sustained virologic response (Table 2). The characteristics of patients with
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12 persistent viral replication after HBeAg loss at baseline, at the time of HBeAg loss, and
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14 during follow-up, are shown in Table 3. It is important to highlight that 6 of these patients
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16 had main BCP mutations at baseline ($P=0.017$, Table 2) and 2 had mutations that
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18 emerged during follow-up.
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22 HBsAg loss occurred in 4 patients after a mean period of 35 months from baseline. The
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24 characteristics at baseline of patients who cleared HBsAg and those who did not are
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26 shown in Table 4.
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32 Discussion

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34 A variable percentage of patients continue to show detectable HBV-DNA after
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36 HBeAg seroconversion. In Asian patients the reported rate is around 30% [8]; less
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38 information is available for Caucasian patients: In a long-term follow-up study of 61
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40 Caucasian patients who underwent spontaneous HBeAg seroconversion, 9 (15%)
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42 continued to have detectable serum HBV-DNA determined by assays with relatively low
43
44 sensitivity [9]. In our study, which used a sensitive PCR, 60% of patients had detectable
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46 HBV-DNA after a median follow-up of 71.5 month after HBeAg loss, and 5 (42%) of
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48 them had HBV-DNA levels higher than 10^4 copies/mL and elevated ALT values, an
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50 indication for HBV treatment. A study by Wu et al in 45 Asian patients, described a
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3 similar percentage (50%) of persistent viremia in patients who experienced sustained
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5 HBeAg seroconversion after adefovir therapy [13].
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8 In the light of this high percentage of patients with persistent viremia after HBeAg
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10 loss and/or seroconversion and therapy discontinuation, is important to identify baseline
11
12 and on-treatment factors that enable selection of patients who would benefit from
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14 extended treatment. Very few studies have addressed this issue; the results from some of
15
16 them seem to be consistent with our findings. Wu et al, detected BCP and precore
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18 mutations in 11 of 13 patients with persistent viremia after HBeAg loss, however,
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20 samples prior to adefovir were not available in all patients and it is unclear whether they
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22 harbored mutations prior to or during therapy. Ferns et al, analyzing serum samples from
23
24 21 untreated patients before HBeAg loss, detected BCP mutations by direct sequencing
25
26 more often in patients with persistently high HBV-DNA levels ($>10^5$ copies/mL) [15].
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28 Pichoud et al, showed that persistence of significant viremia levels, which were not
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30 detected by a branched DNA assay, may be observed after anti-HBe seroconversion [19].
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32 In contrast, the results from a recent study involving 50 Asian patients treated with
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34 pegylated-interferon alpha-2b, found that in HBeAg-positive patients the presence of both
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36 main BCP mutations at the start of treatment was associated with a higher response rate
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38 [20]. Nonetheless, the ethnicity and HBV genotypes (B and C) of the patients included
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40 differed from those of our patients (Caucasian and genotypes A and D), and it is known
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42 that the probability of response to pegylated-interferon alpha-2b is related to the viral
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44 genotype [21]. Moreover, the follow-up period is longer in our study and the criteria to
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46 establish inhibition of viral replication are different.
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3 HBeAg is translated from pre-core mRNA. The transcription of this mRNA is
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5 under the control of several regulatory elements, including the BCP [22]. The presence of
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7 main BCP mutations, which are detected in around 50% of HBeAg-positive patients in
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9 our area [23], results in reduced binding of liver-specific transcription factors, a
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11 concomitant reduction in precore mRNA transcription, less precore protein formation (the
12
13 precursor of HBeAg), and ultimately, a decrease in HBeAg production of [23-24]. The
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15 role of main BCP mutations in seroconversion to anti-HBe and in persistent HBV-DNA
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17 replication after that event has not been well established. In a study performed among
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19 Asian patients, BCP mutations were associated with later seroconversion in patients
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21 infected with genotype C, despite a decrease in HBeAg levels [25]. In our study, HBeAg-
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23 positive patients viral replication after HBeAg loss was related to the presence of BCP
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25 mutations before therapy and emergence during therapy, whereas there was no
26
27 relationship with the type of antiviral drug. Antiviral treatments usually reduce or
28
29 suppress viral replication, or increase immunologic pressure against the HBV, but they do
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31 not affect mRNA transcription. This could explain why viral antigen expression, such as
32
33 core protein and HBeAg, persist despite decreases in HBV-DNA levels. In addition,
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35 HBeAg shares some epitopes with the core protein; therefore, immunological stimulation
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37 by core expression would increase the probability of achieving HBeAg seroconversion
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39 [26-28]. HBeAg expression is lower in the presence of BCP mutations; hence, an intense
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41 viral load reduction, such as that needed in wild-type cases, would not necessarily be
42
43 required to achieve HBeAg loss. Instead, HBeAg loss may occur with higher levels of
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45 HBV-DNA, and this could justify the persistent viral replication after seroconversion in
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47 patients with BCP mutations [29].
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3 Previous studies have suggested that HBeAg-negative disease allows selection of
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5 “e” minus variants [26]. However, 30% of our patients had precore mutants at the time of
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7 HBeAg positive, suggesting these mutations preceded HBeAg-negative disease and their
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9 detection may depend on the sensitivity of the assays used.
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13 This study has some limitations. The number of patients included is relatively
14
15 small and heterogeneous regarding the type of treatment. **This small number of patients**
16
17 **could be the reason why statistical significance in multivariate analysis was not achieved**
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19 **when certain variables were compared, such as persistent viral replication after HBeAg**
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21 **loss and HBV-related histological complications, as was found in another study [14].**
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23 Another limitation is that only HBV genotypes A and D, the most frequent genotypes
24
25 detected in Caucasian patients, are represented.
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29 The advantages of the study include a long follow-up after HBeAg
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31 seroconversion, which allowed detection of viremia and use of the LiPA assay, a
32
33 commercial test that can detect the main precore and BCP mutations even when they are
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35 present in low percentages. LiPA detects mutations that comprise at least 5% of a mixed
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37 population of wild-type and mutated HBV variants, while detection with direct
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39 sequencing techniques requires a proportion higher than 10% [17]. Nevertheless, some
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41 less frequent BCP or precore mutations may also have a role in persistence of viremia
42
43 after HBeAg loss, and these are not included in the LiPA assay and were not investigated
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45 in this study. Among these, it might be interesting to study the role of the T1753C
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47 substitution, which appears together with the double substitution A1762T/G1764A in a
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49 high percentage of HBeAg-negative patients who experience clinical reactivation during
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51 lamivudine treatment [30].
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3 In summary, current guidelines recommend stopping antiviral treatment at 6 to 12
4 months following seroconversion, but complete virologic response is not always attained
5 and some patients require repeated treatment when developing HBeAg-negative disease.
6 Markers to identify patients at risk for virologic rebound after HBeAg loss and therapy
7 discontinuation would be helpful, so that treatment could be prolonged in these patients.
8 As was seen in this study, the presence of main HBV basic core promoter mutations in
9 HBeAg-positive patients prior to treatment appears to predict persistent viremia and
10 could be useful for guiding therapy decisions. These findings merit further evaluation in
11 studies with a larger number of patients treated with different drugs.
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3 **Statement of Interests:**
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Table 1. Characteristics of the patients at the time of the inclusion in the study.

Baseline characteristics	No of patients = 20
Age, median years (range)	37 (18-75)
Gender, male	15 (75%)
ALT* level, median IU/mL (range)	112 (31–761)
HBV DNA level, median log ₁₀ copies/mL (range)	7.7 (5.7–10)
HBV Genotype	
A	13 (65%)
D	7 (35%)
Ethnicity	
Caucasians	20 (100%)
Main Precore Changes	
G1896A	6 (30%)
BCP† Changes	
A1762T	6 (30%)
G1764A	6 (30%)
Both Precore and BCP Changes	2
Type of treatment	
Untreated	2
Interferon based regimens	8
Nucleos(t)ides analogs‡	10
Liver biopsy	15 (75%)
Fibrosis stage	
<2	3 (20%)
≥2	12 (80%)

* ALT : alanine aminotransferase

† BCP: Basic Core Promoter

‡ Of the 10 patients treated with Nucleos(t)ides analogs, 5 received lamivudine, 4 adefovir, and 1 tenofovir.

Table 2. Baseline characteristics of patients, depending on the type of virologic response at the end of follow-up.

Variables	Sustained Virologic Response	Persistent Viral Replication	p-value
	HBV DNA < 2.5 log ₁₀ copies/mL No=8	HBV DNA > 2.5 log ₁₀ copies/mL No=12	
Age, median years (range)	35 (18-52)	41 (28-75)	0.129
Gender, male	8 (100%)	7 (58%)	0.035
ALT level, median IU/mL (range)	125 (40-761)	112 (31-355)	0.346
HBV DNA level, median log ₁₀ copies/mL (range)	8 (7-9.6)	7.5 (5.7-10)	0.414
HBV Genotype			
A	5 (62%)	8 (67%)	0.848
D	3 (38%)	4 (33%)	
Main Precore Changes			
G1896A	2 (25%)	4 (33%)	0.690
Basic Core Promoter Changes			
A1762T	0	6 (50%)	0.017
G1764A	0	6 (50%)	0.017
Both Precore and BCP changes	0	2	0.22
Type of treatment			
Untreated	2	-	0.18
Interferon based regimens	3	5	
Nucleos(t)ides Analogues	3	7	
Median follow-up after HBeAg loss and therapy discontinuation [§] , months (range)	38 (14-233)	71.5 (27-134)	0.862
Liver biopsy	6	9	
Fibrosis score			
<2	3 (50%)	0	0.018
≥2	3 (50%)	9 (100%)	

[§] Patients who received nucleos(t)ide analogs had a 6-month period of consolidation therapy after losing HBeAg. The follow-up period started after finishing this timeframe.

Table 3. Biochemical and Virologic characteristics of the 12 patients with persistent viral replication at the different time points.

	Baseline	HBeAg loss	Follow-up
ALT level, median IU/mL (range)	112 (31-355)	28 (18-163)	62 (16-963)
HBV DNA level, median log ₁₀ copies/mL (range)	7.5 (5.7-10)	4.6 (3.2-7.5)	4.7 (3.6-7.9)
Genotype			
A	8 (67%)	6 (50%)	7 (58%)
D	4 (33%)	4 (33%)	3 (25%)
A/D	-	2 (17%)	1 (8%)
A/F	-	-	1 (8%)
Main Precore Changes			
G1896A	4 (33%)	5 (42%)	5 (42%)
Basic Core Promoter Changes			
A1762T	6 (50%)	7 (58%)	8 (67%)
G1764A	6 (50%)	6 (50%)	7 (58%)
Both Precore and BCP changes	2 (16%)	2 (16%)	1 (8%)

Table 4. Baseline characteristics of patients who lost HBsAg compared with those without HBsAg loss.

Patients characteristics	HBsAg negative No=4	HBsAg positive No=16	p-value
Age, median years (range)	38 (23-52)	37 (18-75)	0.61
Gender, male	4 (100%)	11 (69%)	0.19
ALT level, median IU/mL (range)	161 (70-400)	104 (31-761)	0.78
HBV DNA level, median log ₁₀ copies/mL (range)	8.6 (7.4-9.6)	6.7 (5.7-10)	0.18
Genotype			
A	4 (100%)	9 (56%)	0.10
D	0	7 (44%)	
Precore Changes			
A1896G	1 (25%)	5 (31%)	0.81
Basic Core Promoter Changes			
A1762T	0	6 (40%)	0.14
G1764A	0	6 (40%)	0.14
Both Precore and BCP changes	0	2 (12%)	0.45
Type of treatment			
Untreated	-	2	0.26
IFN base regimens	3	5	
Nucleos(t)ides Analogues	1**	9	
Liver Biopsy	3 (75%)	12 (75%)	
Fibrosis score			
<2	2 (67%)	1 (8%)	0.024
≥2	1 (33%)	11 (92%)	

** This patient received a combined therapy consisting in lamivudine and adefovir.

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3 Figure 1. Comparison between the three time points of HBV-DNA and ALT levels in
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6 relation to the type of virologic response at the end of treatment.
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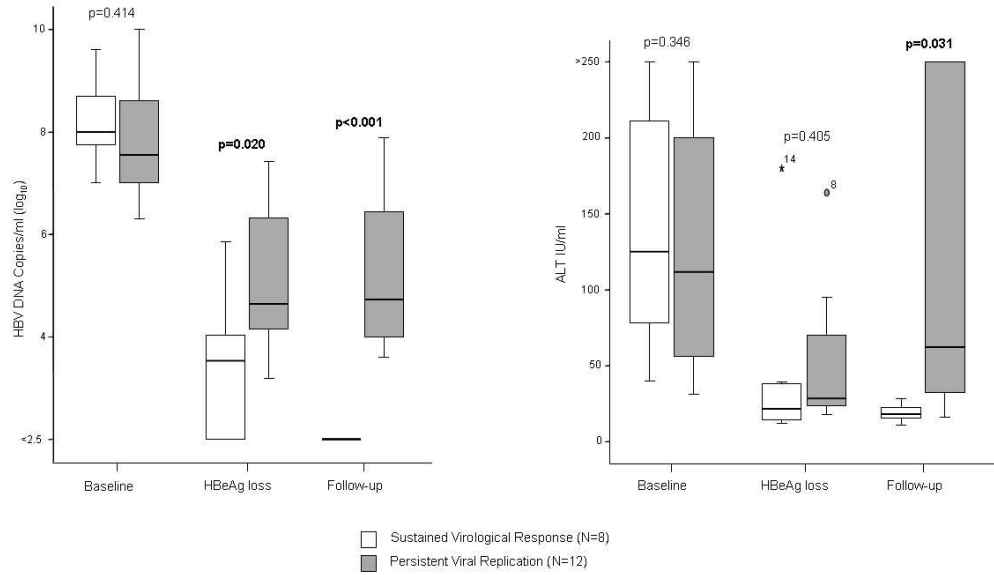


Figure 1. Comparison between the three time points of HBV-DNA and ALT levels in relation to the type of virologic response at the end of treatment.
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Review

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