



## Research paper

## Role of viral load in Hepatitis B virus evolution in persistently normal ALT chronically infected patients

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## ABSTRACT

Chronic HBV infection has been associated with severe liver disease although most of them do not progress to this stage. Even though low replicative carriers form the largest group of HBV chronically infected patients, there is a paucity of longitudinal studies to evaluate the molecular evolution of the whole genome in this subset of patients.

In this study, longitudinal samples from 10 patients with persistently normal ALT levels were collected. HBV full-length genome sequences were obtained from 3 samples per patient (baseline, 5 and 10-years of follow-up). Patients were grouped according to HBV-DNA level into  $< 10^3$  IU/ml (group A) or  $> 10^3$  IU/ml (group B). The substitution rate was inversely related with HBV-DNA levels. Moreover, the rate in the 10-year follow-up was significantly higher in group A ( $6.9 \times 10^{-4} \pm 1.3 \times 10^{-4}$ ) than group B ( $2.7 \times 10^{-4} \pm 7.4 \times 10^{-5}$  substitution/site/year,  $p < .001$ ). Most of the substitutions were in the Core region and the majority were non-synonymous changes.

The rate of nucleotide substitution was inversely related to HBV-DNA levels, highlighting the role of viral load in the HBV intra-host dynamics, even in low replicative state patients. Moreover, the difference in the substitution rate between the analysed groups was mainly consequence of substitutions restricted to the Core region, particularly in the simple coding region and antigenic epitopes, which suggest that the immune pressure drives the different evolutionary behaviour of groups.

## 1. Introduction

Hepatitis B virus (HBV) infection is a major public health problem worldwide (Lavanchy, 2004; Schilsky, 2013). The natural course of HBV chronic infection is a dynamic process including high and low replicative phases. Moreover, a wide spectrum of clinical manifestations is observed, ranging from an inactive HBsAg carrier state to a progressive chronic hepatitis, potentially evolving to cirrhosis and hepatocellular carcinoma (HCC) (Busch and Thimme, 2015).

The onset of the infection is characterised by the presence of circulating HBsAg, HBeAg and high level of serum HBV-DNA. For unknown reasons, during chronic HBV infection, the tolerogenic effect of HBeAg is lost and patients may enter into the immune clearance phase. This phase is characterised by a decrease in HBV-DNA concentrations

and an increase in ALT levels and histologic activity, reflecting immune-mediated lysis of infected hepatocytes. In this context, HBeAg seroconversion is usually verified, leading in most of the cases to a low replicative phase, otherwise known as “inactive HBsAg carrier state” (Gish et al., 2015; Shi and Shi, 2009). This phase, constitutes a late stage in the natural history of the infection and is characterised by HBeAg negativity and Anti-HBe positivity, undetectable or low HBV-DNA levels [suggested levels  $< 2 \times 10^3$  IU/ml ( $10^4$  copies/ml)], persistently normal ALT levels and inactive liver histology, usually with a minimal amount of fibrosis (Shi and Shi, 2009; Sharma et al., 2005; EASL, 2017; Pungpapong et al., 2007). The significant reduction in viral load associated with HBeAg clearance suggests a control of viral replication by the immune system.

The characterization of the molecular evolution of HBV and the

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reliable estimation of substitution rates contribute to a better understanding of the interplay between the virus and the host (Vrancken et al., 2017). Nonetheless, the estimation of HBV substitution rate has been a challenge, due to structural and functional characteristics of the virus, in addition to the complex dynamics of the chronic infection. The overlapping reading frames (ORFs) in the HBV genome causes constraints in the evolution of the virus since a synonymous mutation at a given ORF might involve an amino acid change in the overlapping ORF, increasing their impact on viral biology. On the other hand, unlike other DNA viruses, HBV replication includes an inherently error-prone reverse transcriptase without 3'-5' proofreading exonuclease activity (Seeger and Mason, 2015; Karayiannis, 2017).

The HBV intra-host substitution rate has been calculated in a wide range ( $\sim 10^{-4}$ – $10^{-5}$  substitutions per site per year, s/s/y) and correlated with the HBeAg status. Several authors conclude that viruses that do not express the HBeAg (HBeAg-negative) would evolve faster than those that do express it (HBeAg-positive) (Harrison et al., 2011; Hannoun et al., 2000). Because the HBeAg has a primary immunological function, it has been suggested that the host immune response might be the most relevant driving force in the evolution of HBV (Wang et al., 2010).

Even though low replicative carriers form the largest group of HBV chronically infected patients, there is a paucity of longitudinal studies to evaluate the molecular evolution of the whole genome in this subset of patients. A few studies have addressed the substitution rates issue, most of them based on the analysis of sub-genomic regions, and none included a close monitoring of patients during follow-up. This could be because of blood samples that span many years of HBV infection are rare, as well as the technical difficulty of analyse samples with low viral loads. In fact, there is only one study analysing two samples 25 years apart from eight inactive carriers, in which the mean nucleotide substitution rate of the full-length genome was  $1.9 \times 10^{-4}$  s/s/y (Osioy et al., 2006). Consequently, the HBV evolution in HBeAg-negative with persistently normal ALT patients remains poorly understood.

The aim of this study was to estimate the molecular evolutionary rate and distribution of mutations occurring throughout the HBV genome in the low replicative phase. To this purpose, we analyse HBV full-length sequences obtained from sequential samples in a 10-year follow-up from 10 HBV chronically infected patients with persistently normal ALT levels.

## 2. Materials and methods

### 2.1. Patients and samples

This study included 10 unrelated patients with HBV chronic infection who attended to the Hepatology Unit of the Hospital F.J. Muñiz, Buenos Aires City, Argentina. All patients were HBeAg-negative and had persistently normal ALT levels (PNALT) over the analysed period. Additional inclusion criteria were (i) no evidence of cirrhosis or HCC until the last sampling time; (ii) a minimum of eight stored serum specimens tested for ALT and HBV-DNA assay during the follow-up period, (iii) no evidence of other liver disease (autoimmune, alcohol or drug-induced) or concurrent infection with hepatitis C virus, hepatitis delta virus or human immunodeficiency virus infection; and (iv) patients who have not undergone antiviral therapy.

Serum samples were collected between 4 January 2004 and 20 May 2016; a median of 12.5 (range 8 to 14) samples per patient was available. In order to characterize HBV evolution, the full-length genome of three representative samples from each patient: baseline, intermediate ( $5.4 \pm 0.8$  years) and final ( $10.5 \pm 1.3$  years), were analysed (Supplementary Fig. S1).

Informed consent was obtained from each subject. The study protocol was approved by the Bioethical Committee of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires EXP-UBA: 0069893/14.

### 2.2. Laboratory testing

HBV serological markers were analysed with the Architect Abbott system (HBsAg, Anti-HBc, HBeAg, and Anti-HBe; Abbott Diagnostics, Wiesbaden, Germany). Liver function was evaluated by alanine aminotransferase test (ULN = 40 UI/l). Serum HBV DNA level was quantified by COBAS TaqMan HBV test (Roche Diagnostic Systems Inc., Mannheim, Germany). The dynamic range of quantification is  $10^1$  to  $10^8$  IU/ml.

### 2.3. HBV-DNA extraction, amplification and sequencing

DNA was extracted from 200  $\mu$ l of serum using the High Pure Viral Nucleic acid kit (Roche Diagnostics, Germany). Full-length HBV genomes were amplified by six overlapping nested PCR protocols described elsewhere (Mojsiejczuk et al., 2016). For the first round of PCR amplification, 3  $\mu$ l of extracted DNA and 0.25  $\mu$ M of each primer were added to AmpliTaq Gold® 360 Master Mix in a final volume of 25  $\mu$ l. For the second round, 2  $\mu$ l of the first-round product were added to 40  $\mu$ l final volume of PCR mix.

PCR products were purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and submitted to direct nucleotide sequencing reaction in both directions (Unidad de Genómica, INTA, Castelar, Buenos Aires, Argentina) with the same primers used in amplification stage.

### 2.4. Sequence assembly and characterization

Chromatograms were individually examined to confirm the quality of sequences using the MEGA6 software; complete genomes were assembled with BioEdit v7.1.3.0 and sequences were aligned with ClustalX v2.1 with default parameters (Hall, 1999; Larkin et al., 2007).

Genotype or subgenotype determination was based on the phylogenetic analysis of sequences obtained in this study and references retrieved from GenBank. The analysis was performed with the MEGA6 package (Tamura et al., 2013). Evolutionary models were inferred according to the Bayesian Information Criterion statistics, and phylogenetic trees were constructed using the neighbor-joining method. The robustness of the reconstructed phylogeny was evaluated by bootstrap analysis (1000 pseudoreplicates).

The monophyletic origin of the three sequences corresponding to each patient was confirmed by a supplementary phylogenetic analysis, in order to discard co-infection and/or methodological errors (such as misidentification or cross-contamination). To this end, additional sequences were selected from GenBank as follows: BLAST searches (nucleotide database) were performed using as “query” each one of the three complete or partial genomic sequences obtained from a patient; the five most related sequences to each query were retrieved, duplicated sequences were discarded; the dataset was split by genotypes and neighbor-joining phylogenetic analyses were performed as described above.

### 2.5. Evolutionary rates

The number of nucleotide substitutions per site per year (s/s/y) was calculated by direct comparison between sample pairs of sequences. Complete genome sequences and individual genes corresponding to each patient were confronted; genetic distances and the standard error were calculated with the MEGA6. Due to the low genetic divergence between the sequences of the same patient, molecular evolutionary models in order to correct the observed genetic distance were not implemented.

### 2.6. Selection pressures analyses

In order to determine if there were any selection pressures acting on

the lineages during the follow-up period, gene and site-specific selection pressures were analysed by direct comparison of baseline (t1) and final (t3) sequences of each patient. The ratio of non-synonymous ( $d_N$ ) and synonymous ( $d_S$ ) nucleotide substitutions per site for HBV protein-coding regions (Core, Surface, X, and Polymerase genes) among samples was calculated using the method of Nei and Gojobori (Nei and Gojobori, 1986), utilizing SNAP software (Korber, 2000).

### 2.7. GENBANK accession numbers

Nucleotide sequences reported in this work were submitted to GenBank under accession numbers MG877700 to MG877728.

## 3. Results

### 3.1. Patient clinical and VIROLOGICAL data

Serum samples from 10 HBV chronically infected individuals were included. During the 10-year follow-up, 8 to 14 samples per patient were collected and tested for ALT and viral load. All patients were HBeAg-negative, AntiHBe-positive and showed ALT levels persistently below the normal threshold throughout the follow-up. Based on the HBV-DNA, patients were grouped according to a viral load limit of  $2 \times 10^3$  IU/ml in group A (patients A to E, lower the limit) and group B (patients F to J, upper the limit) (Table 1).

The full-length genome sequence was obtained in 26 out of 30 samples. Partial genomes were successfully sequenced in three intermediate samples (2739, 2739 and 2167 nucleotides in length for samples I\_02, D\_02, and E\_02, respectively) while the intermediate sample of patient H (H\_02) could not be sequenced. Genotypes and subgenotypes were assessed by phylogenetic analysis (Supplementary Fig. S2). The following subgenotypes were identified: A1 (n = 1), D3 (n = 3); F1b (n = 1) and F4 (n = 5).

To rule out the presence of unrelated viral sequences among samples of each patient, additional phylogenetic analyses were performed. In all cases, nucleotide sequences from the same patient formed a monophyletic cluster in the phylogenetic tree when they were analysed together with the most similar GenBank sequences, indicating a close evolutionary relationship (Supplementary Fig. S3). Intra-host sequence similarity was  $99.3 \pm 0.4\%$ , which falls within the expected range for intra-patient isolates.

### 3.2. Evolutionary rate dynamics

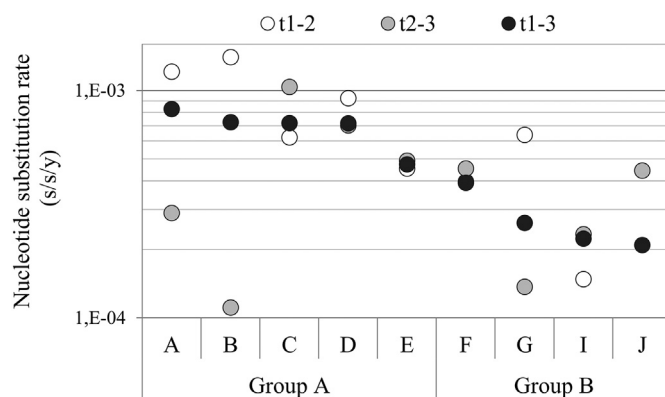
To determine the impact of the follow-up length in the accuracy of

**Table 1**

Demographic, biochemical and virological characteristics of patients.

Patients (n)	Total	Group A	Group B	p
	10	5	5	
Age at enrollment (years) Median	37.3	46.7	36.7	ns
Mean $\pm$ SD	37.5 $\pm$ 12.3	36.5 $\pm$ 8.4	38.5 $\pm$ 16.3	
Gender (M/F)	8/2	4/1	4/1	ns
Follow-up (years) Median	10.7	10.4	10.6	ns
Mean $\pm$ SD	10.3 $\pm$ 1.5	10.6 $\pm$ 1.7	9.9 $\pm$ 1.3	
Sampling/case (n) Median	12.5	11	14	ns
Mean $\pm$ SD	12.3 $\pm$ 3.5	10.4 $\pm$ 2.3	14.2 $\pm$ 3.6	
ALT (UI/l) Median	25	29.5	20.0	ns
Mean $\pm$ SD	27.1 $\pm$ 7.0	23.8 $\pm$ 5.9	30.4 $\pm$ 6.9	
HBV-DNA (IU/ml) Median	$1.2 \times 10^3$	$3.9 \times 10^2$	$2.7 \times 10^3$	0.012
Mean $\pm$ SD	$2.6 \times 10^3 \pm 2.9 \times 10^3$	$4.6 \times 10^2 \pm 2.6 \times 10^2$	$4.7 \times 10^3 \pm 2.9 \times 10^3$	
Genotype A1/D3/F1b/F4	1/3/1/5	0/1/1/3	1/2/0/2	ns

Patients were grouped according to HBV-DNA levels lower than  $10^3$  IU/ml (group A) or higher than  $10^3$  IU/ml (group B) during the follow-up. ALT and HBV-DNA were determined in 8 to 14 samples per patient. All patients showed normal ALT levels below the ULN during follow-up (40 UI/l). Samples with HBV-DNA below the detectable threshold (10 IU/ml) were not included in the statistical calculations.



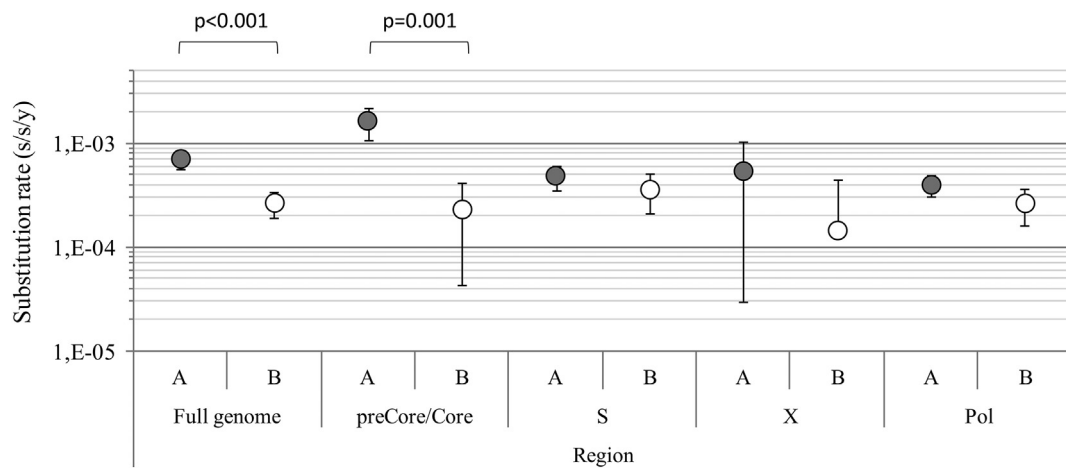
**Fig. 1.** Nucleotide substitution in HBV full genome sequences over five years follow-up. Nucleotide substitution rates by comparing baseline and intermediate sample (○), intermediate and final sample (●) and baseline and final sample (●). Patient J showed no changes between samples t1 and t2.

evolutionary rate estimation, the nucleotide substitution rates between the baseline (t1) and the intermediate (t2) sample (separated by  $5.47 \pm 0.86$  years) and the intermediate (t2) and final (t3) sample ( $5.14 \pm 1.26$  years) were compared. Substitution across the genome in both analysed periods (t1-t2 and t2-t3) were observed in all cases, except for t1-t2 in the patient J. In three cases of group A (patients C, D, and E) and one case of group B (patient F) the nucleotide substitution rates between t1-t2 and t2-t3 were similar (Fig. 1). Conversely, a considerable difference was observed in the remaining five cases: the rate was higher in t1-t2 in three cases (A, B and G) whereas in two cases (I and J) the fastest rates were detected between t2-t3.

### 3.3. Molecular evolutionary rate

The overall mean nucleotide substitution rate estimated at the 10-year follow-up was  $4.8 \times 10^{-4} \pm 2.5 \times 10^{-4}$  s/s/y (range  $2.1 \times 10^{-4}$  to  $8.3 \times 10^{-4}$  s/s/y) and double coding regions showed lower substitution rates than single coding regions ( $3.4 \times 10^{-4} \pm 1.2 \times 10^{-4}$  vs  $6.1 \times 10^{-4} \pm 4.5 \times 10^{-4}$  s/s/y respectively).

The nucleotide substitution rate was significantly higher for group A than group B ( $6.9 \times 10^{-4} \pm 1.3 \times 10^{-4}$  vs  $2.7 \times 10^{-4} \pm 7.4 \times 10^{-5}$  s/s/y,  $p < .001$ ) (Fig. 2). This difference mainly proceeds from substitutions in the Core region, where the nucleotide substitution rate in group A was significantly higher than in group B ( $1.6 \times 10^{-3} \pm 5.6 \times 10^{-4}$  vs  $5.6 \times 10^{-4} \pm 1.9 \times 10^{-4}$  s/s/y,  $p = .001$ ).



**Fig. 2.** Nucleotide substitution rates by group over ten years follow-up in the full-length genome and subgenomic regions. Mean nucleotide substitution rates were obtained by comparing baseline and final sample (t1-t3) of each patient for the full-length genome and for each ORF. Significant differences between the groups were only observed in the rates for the full-length genome and the Core region.

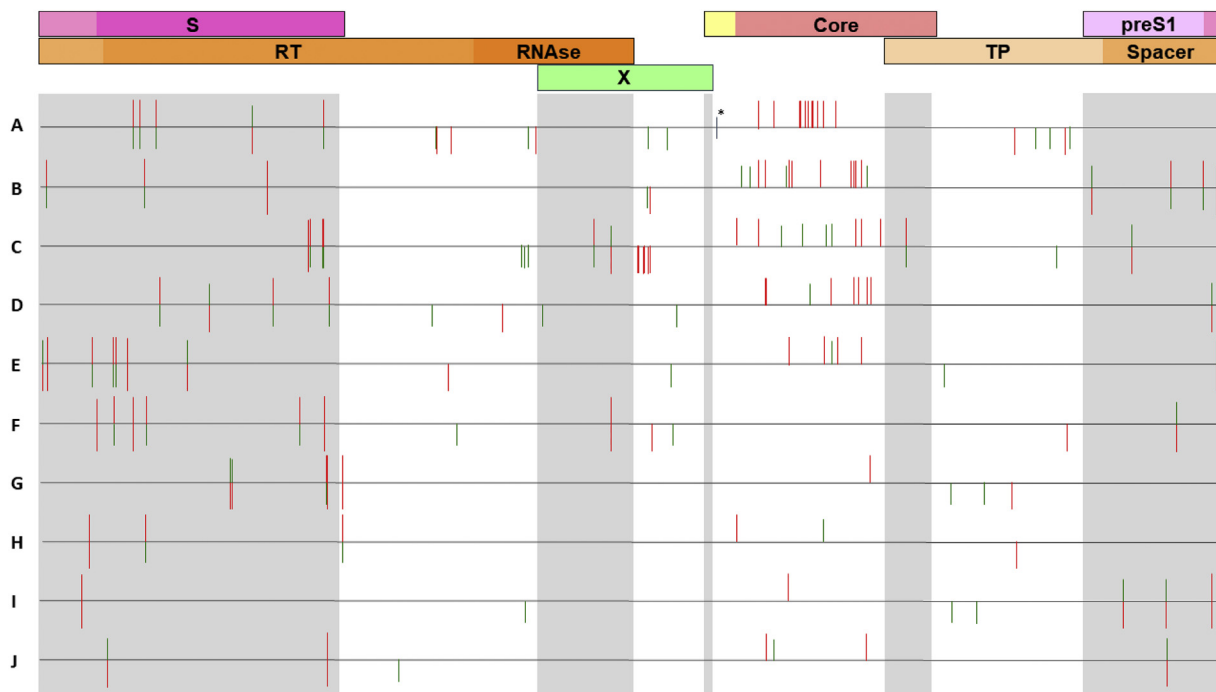
Moreover, the nucleotide substitution rate observed in the Core region was significantly higher than that observed in the other ORFs in group A, while no significant differences were observed among other genes either between or within groups.

### 3.4. Mutation pattern

Intra-patient sequences were compared to visualize nucleotide changes occurring throughout the entire genome. Overall 160 substitutions were observed when baseline (t1) and final (t3) sequences were compared (118 in group A and 42 in group B) (Fig. 3). An average of  $23.8 \pm 6.7$  (range of 16 to 32) and  $3.8 \pm 0.4$  (range of 3 to 4) was observed in group A and B respectively.

Most of the substitutions (39.4%,  $n = 63$ ) were in the Core region. Remarkably, 51 out of 63 were non-synonymous (Table 2), 45 of which occurred in group A and only 6 in group B. Although substitutions were observed throughout the entire Core region, most of them occurred in the simple coding region (nt1901–2307). In particular, substitution in the residue 21, included in the FLPSPDFPVSV HLA-A2.01class I T cell epitope (aa 18–27), occurred in 5 out of 10 cases (S21A/H/V).

Additionally, fifty substitutions were observed in the Pol/S overlapped region (nt 2854–835): 44% were synonymous in the Pol ORF and non-synonymous in the S ORF; 32% were non-synonymous in the Pol ORF and synonymous in the S ORF and remarkably, 24% were non-synonymous in both ORFs. Nonetheless, no significant differences between group A and B were observed in this region.



**Fig. 3.** Distribution of substitution along the HBV genome. Nucleotide sequences of baseline (t1) and final (t3) samples were compared. HBV reading frames are shown at the top of the figure and shaded regions indicate frames overlapping. Horizontal lines represent the genome of each patient, vertical lines show the position of the synonymous (green) and non-synonymous (red) substitutions observed across the genome between the samples of each patient. The vertical line next to an asterisk represents one nucleotide insertion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**  
Non-synonymous and Synonymous Substitutions by ORF.

Region	Group A				Group B			
	Substitutions			$d_N/d_S$ <sup>†</sup>	Substitutions			$d_N/d_S$ <sup>†</sup>
	Total	NS	S		Total	NS	S	
PreCore/Core	55 (6–16)	45 (5–15)	10 (0–4)	1.53 (0.63–3.00)	8 (0–3)	6 (0–2)	2 (0–1)	NA
S	29 (5–8)	21 (3–6)	8 (1–2)	0.26 (0.21–0.31)	21 (3–7)	14 (1–6)	7 (0–2)	NA
X	15 (1–8)	8 (0–7)	7 (1–2)	NA	3 (0–3)	2 (0–2)	1 (0–1)	NA
Pol	51 (4–16)	21 (2–7)	30 (2–9)	0.28 (0.08–0.64)	33 (3–13)	20 (2–7)	13 (1–6)	0.30 (0.29–0.31)

Nucleotide substitutions (median) by comparing baseline (t1) and final (t3) sequences in each ORF. The range of substitution is indicated in brackets. NS: non-synonymous, S: synonymous. <sup>†</sup> $d_N/d_S$  are expressed as the median value of all patients. For individual ratios, see Supplementary Table S1. NA, not available ( $d_N/d_S$  was not calculated if either  $d_N$  or  $d_S$  were 0).

#### 4. Discussion

In this study, we described the evolutionary rate and distribution of substitutions in the HBV genome in sequential samples from a cohort of low replicative patients with persistently normal ALT levels. Although this stage represents most of HBV chronic infections, there is a paucity of data regarding the rate of nucleotide substitution in this subset of patients. In the analysed cohort, five patients showed viral load persistently below  $2 \times 10^3$  IU/ml (group A) and the remaining five had viral loads above the threshold (group B). Interestingly, the substitution rate was inversely related to HBV-DNA level. Most of the nucleotide changes were in the Core region and the majority were non-synonymous.

The HBV chronic infection is a dynamic process whose outcome relies on a complex interplay between the virus and the host immune system (Wang et al., 2010). It has been suggested that the immune selective pressure has a significant role on the HBV evolution. Most of the studies attempting to estimate the intra-host HBV evolutionary dynamics have been carried out in patients with chronic active hepatitis. Unlike, there are scant studies in HBV chronically infected patients with persistently normal ALT levels, a surrogate marker of hepatic injury. These patients are usually characterised by minimal or no necroinflammation, slight fibrosis, or even normal histology on biopsy (Gish et al., 2015). Consequently, this might be understood as a state of balance in the virus-host interplay, with a limited selective pressure exerted by the immune system.

One of the drawbacks of the study of the intra-host evolution of HBV is the availability of samples collected over long observation periods (Hannoun et al., 2000). Additionally, it is hard to assert which is the suitable sampling period in order to obtain a reliable rate that could reflect the HBV intra-host evolutionary process. To shed light on this issue, we compared the rates obtained between the first half (t1-t2) and the second half (t2-t3) of the follow-up. Although the substitution rate in both periods was similar in four cases, significant differences were observed in the remaining five patients. This suggests, in line with previous studies, that a relaxed molecular clock model fits better than the strict molecular clock, even when evaluating the HBV evolutionary process at intra-host levels, in a short period and in a relatively stable environment (Torres et al., 2013).

Besides, the mean rate obtained in the present study after the 10-year follow-up was similar in magnitude to that observed in sample pairs from HBeAg-negative patients obtained over 25 years (Osioy et al., 2006). This suggests that a decade of follow-up might be enough to obtain a reliable estimation of the intra-host substitution rate and that these values may be compared with those obtained from studies covering longer periods. Because of this, henceforth we will use the rate obtained in t1-t3 to discuss the HBV intra-host evolution in this cohort

of patients.

In the present study, the overall mean nucleotide substitution rate was  $4.8 \times 10^{-4}$  s/s/y. This rate is in line with substitution rates reported previously in HBeAg-negative patients (Harrison et al., 2011). Particularly, it is similar to the rate obtained by Osioy et al. (2006) in the only study performed on full-length genomes of HBeAg-negative asymptomatic carriers and close in magnitude to that observed in HBeAg-negative patients with active chronic hepatitis (Zhang et al., 2007). Whereas, the rate estimated in our HBeAg-negative cohort was significantly higher than those reported in the HBeAg-positive stage, which is in accordance with previous studies (Harrison et al., 2011; Hannoun et al., 2000; Wang et al., 2010;). Based on these results, the substitution seems to be more related to the HBeAg status than the ALT levels. In fact, in individuals with normal ALT levels, it has been shown that the frequency of mutations is significantly lower in HBeAg-positive than HBeAg-negative patients; this suggests a strong immune pressure during the HBeAg-negative stage (Fujiwara et al., 1998).

In the cohort included in this study, the substitution rate in the HBV genome was inversely related to the level of HBV viremia. Analogous results were obtained in other studies based on the analysis of partial genomic sequences (van de Klundert et al., 2012; Zaaijer et al., 2008). Likewise, in patients with chronic active hepatitis, viremia levels in the HBeAg positive stage are significantly higher than in the HBeAg-negative stage and nevertheless, the observed nucleotide substitution rates are lower (Zhang et al., 2007). This difference has been mostly attributed to the strongest immune pressure exerted by the host in the HBeAg-negative stage. In fact, viral genetic diversity was positively correlated with host immunity, represented by levels of alanine aminotransferase and negatively correlated with the viral load during the immunotolerant and early immunoclearance phases (Wang et al., 2010). Overall, the evolutionary rate seems to inversely correlate with the viral load in all the stages of HBV infection.

It is well known that overlapping genes and secondary structures involved in regulation of replication impose a constraint on amount and nature of substitutions occurring in the HBV genome. In addition, it cannot be ignored that there are numerous regulatory regions of the expression of proteins (promoters, enhancers) in the HBV genome; therefore, the implications of a synonymous change in these regions on the biology of the virus could have been overlooked. In this study, the difference in the substitution rate between groups was mainly consequence and restricted to substitutions in the Core gene. The accumulation of substitution in the Core region may be due to two factors: it is a simple coding region (more likely to fix substitutions) and includes several antigenic epitopes that could be subject of selective pressures to evade immune detection (Belnap et al., 2003). Therefore, the differential substitution rates in Core between Group A and B suggest that the immune pressure drives the differential behavior of the groups,



although it cannot be inferred through transaminase levels. This is in line with the finding that asymptomatic HBeAg-positive carriers showed a high degree of conservation in the Core gene, despite the high levels of viremia, while in asymptomatic HBeAg-negative carriers, with low or undetectable levels of HBV-DNA, showed a greater number of mutations (Bozkaya et al., 1997; Fujiwara et al., 1998; Seo et al., 2003). On the other hand, Pol and X genes showed lower nucleotide substitution rates, emphasizing the critical function performed by this region in HBV biology. These findings are in line with previous studies demonstrating that the genetic diversity in a cross-sectional set of samples is larger in the Core region than in the S-gene (Boot et al., 2008; van de Klundert et al., 2012; Zaaizer et al., 2008).

The differential distribution of changes throughout the genome was also observed when considering whether these were synonymous or non-synonymous. The ratio of non-synonymous to synonymous substitutions ( $d_N/d_S$ ) is widely used to study the strength and mode of natural selection acting on protein-coding genes. Unfortunately, it was not possible to make an accurate estimation of the selective pressures by this method in several patients because the number of synonymous or non-synonymous substitutions in several genes was equal to zero. Therefore, the results represent the sum of substitutions found in all patients and the average  $d_N/d_S$ , only considering those cases where the ratio could be calculated; consequently, they have limitations and should be taken with caution. An increase in the  $d_N/d_S$  ratio was detected in the Core gene. Typically, a ratio  $> 1$  could be qualified as “positive selection” and, due to the biological function of this region, could represent evidence of preferential selection of variants with changes in T cell-directed epitopes to evade immune detection or either due to replication advantages. Alternatively, they could represent transient deleterious mutations that are tolerated by this simple coding region in a low selective pressure environment, rather than a real positive selection (Domingo, 2015; Pybus et al., 2007; Torres et al., 2013).

Finally, it is generally assumed that the low-replicative phase constitutes a state of balance in the virus-host interplay, with a limited selective pressure on viral populations exerted by the immune system. However, the inverse relationship between evolutionary rates and the viral load found in this subset of patients suggests a selective environment. The increased number of substitutions observed in T cell-directed epitopes of the Core region could represent the selection of variants that escape the immune response. Furthermore, the viral load decrease might be the result of non-cytolytic mechanism to control the viral replication and/or the elimination of infected hepatocytes, even though it cannot be displayed through transaminases levels (Fujiwara et al., 1998; Guidotti and Chisari, 2001; Phillips et al., 2010; Suri et al., 2001; Wang et al., 2018).

In summary, we show that the rate of nucleotide substitution was inversely related to HBV-DNA levels, highlighting the role of viral load in the HBV intra-host dynamics, even in low replicative state patients. Moreover, the difference in the substitution rate between the analysed groups was mainly consequence and restricted to substitutions in the Core region, particularly in the simple coding region and antigenic epitopes, which suggest that the immune pressure drives the different evolutionary behavior of groups.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.meegid.2018.10.017>.

## Declarations of interest

None.

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