UNIVERSITY OF TURIN

PhD in
EXPERIMENTAL MEDICINE AND THERAPY

Cycle XXIX

Title: Different HBsAg decline during 3 years of treatment with entecavir in patients affected by chronic hepatitis B HBeAg-negative and HBV genotypes A, D and E

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1. INTRODUCTION

1.1 Epidemiology of hepatitis B infection

Infection with the hepatitis B virus (HBV) is a major problem worldwide with an estimated 248 million chronically infected individuals. The HBV surface antigen (HBsAg) seroprevalence is 3.61% globally with the highest prevalence in Africa at 8.83% and in the western Pacific at 5.26%[1]. The HBV infection is transmitted through blood or sexual contacts, and the chronic hepatitis B infection (CHB) is most likely to occur during exposure in infancy (vertical transmission) or early childhood (horizontal transmission) when the immune system is not yet mature[2]. With the onset of screening, vaccination, and treatment efforts, the overall HBsAg seroprevalence has decreased since 2000, but the magnitude of this reduction has been heterogeneous and a few countries in Africa have actually demonstrated an increase and approximately 686,000 HBV related deaths occur each year[3]. Chronic HBV infection is highly endemic in Africa with some countries experiencing prevalences greater than 8% and the coinfection with human immunodeficiency virus (HIV) has been reported in up to 28.4% of individuals with chronic HBV infection[4]. Coinfection rates are highest in Western African countries (median: 11.5%) and southern African countries (median 5.4%) and lowest in eastern African countries (median 4.1%).

The global annual incidence of hepatocellular carcinoma (HCC) has increased from 626,000 in 2002 to 748,000 in 2008, of which approximately half is attributed to chronic HBV infection[5]; up to 40% of individuals with chronic HBV infection progress to cirrhosis, liver failure, and HCC[6].

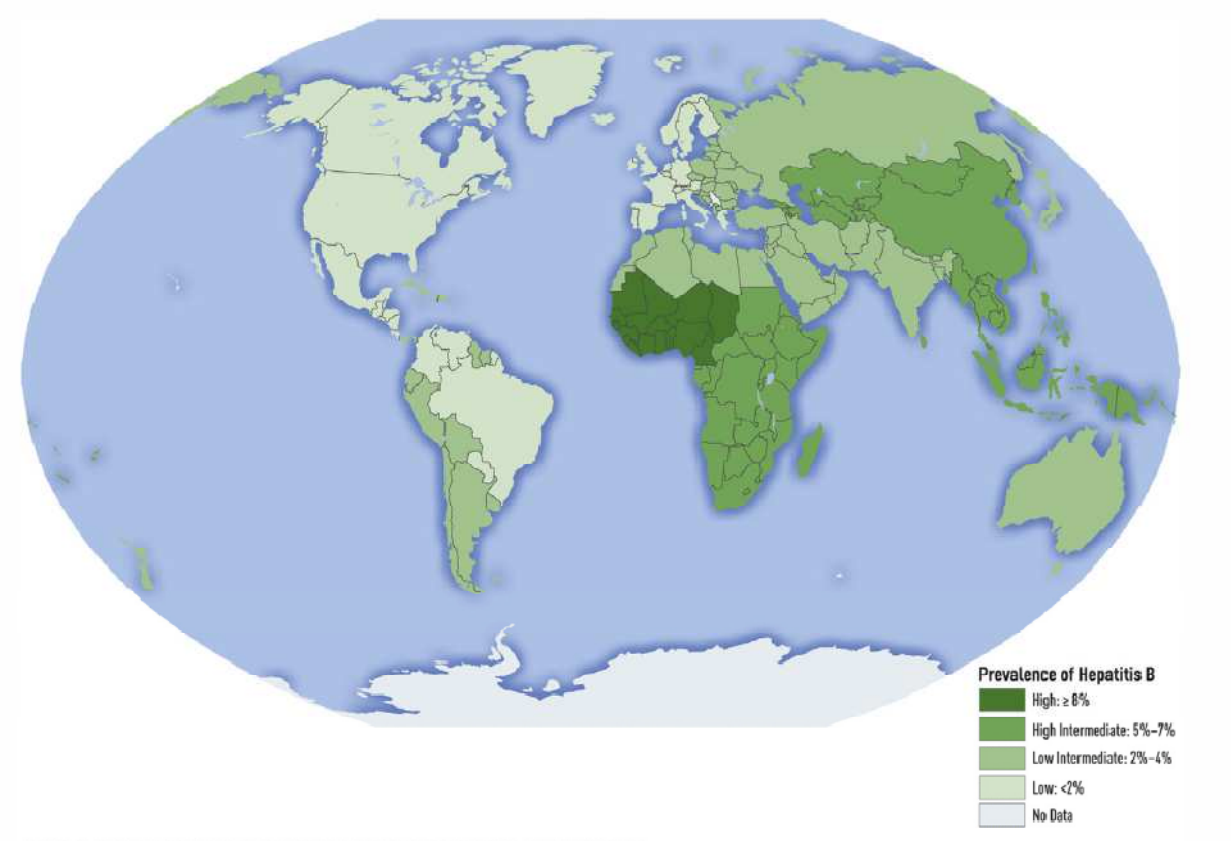

Figure 1. Global prevalence of chronic hepatitis B infection in adults

The vaccination is indicated in unvaccinated adults who are at increased risk for acquiring HBV infection due to the potential for frequent exposure to HBV-infected individuals;. HBV vaccination, with or without HBV immune globulin, is recommended for post-exposure prophylaxis to prevent HBV infection[7].

1.2 HBV genotypes and geographical distribution

HBV is differentiated into many genotypes, according to genome sequence. To date, eight well-known genotypes (A-H) of the HBV genome have been defined. Moreover, two new genotypes, I and J, have also been identified. Some HBV genotypes are further classified as sub-genotypes. HBV sequence is characterized by > 8% nucleotide differences for genotype,

and 4%-8% nucleotide differences for sub-genotype[8]. Over 30 related sub-genotypes belonging to HBV genotypes have been determined to date, but the mechanisms of different pathogenic characteristics of HBV genotypes are not known for certain. Many studies have reported that different genotypes and sub-genotypes show different geographical distribution, and are related to disease progression, clinical progression, response to antiviral treatment, and prognosis. A-D and F genotypes are divided into various sub-genotypes; no sub-genotypes have been defined for E, G and H genotypes.

The HBV subgenotypes are also described with a divergence of > 4% (but less than 7.5%) of the nucleotide sequence in the complete genomic sequence. There have been numerous (up to 40) subgenotypes reported amongst genotypes A-D and F[9]. There are a number of studies documenting the effect of genotype on various clinical outcomes. Many of these provide comparisons of 2 prevalent genotypes in a region, *e.g.,* A *vs* D, or B *vs* C. Table 3 sets out some of what is known about the different genotypes and clinical associations. There is a paucity of information about genotype E and its associations with clinical outcome.

Genotype A appears to have the highest risk of progression to chronicity following acute acquired hepatitis B infection[10], while genotype C2 was independently associated with progression to chronicity, compared to genotype B[11]. Acute infection with genotype D appears to be more commonly associated with acute liver failure than other genotypes[12].

Rates of spontaneous HBsAg clearance are higher in genotype B compared to C. Sustained remission following HBeAg seroconversion has been reported to be more commonly seen in genotype A than D as was HBsAg clearance[13].

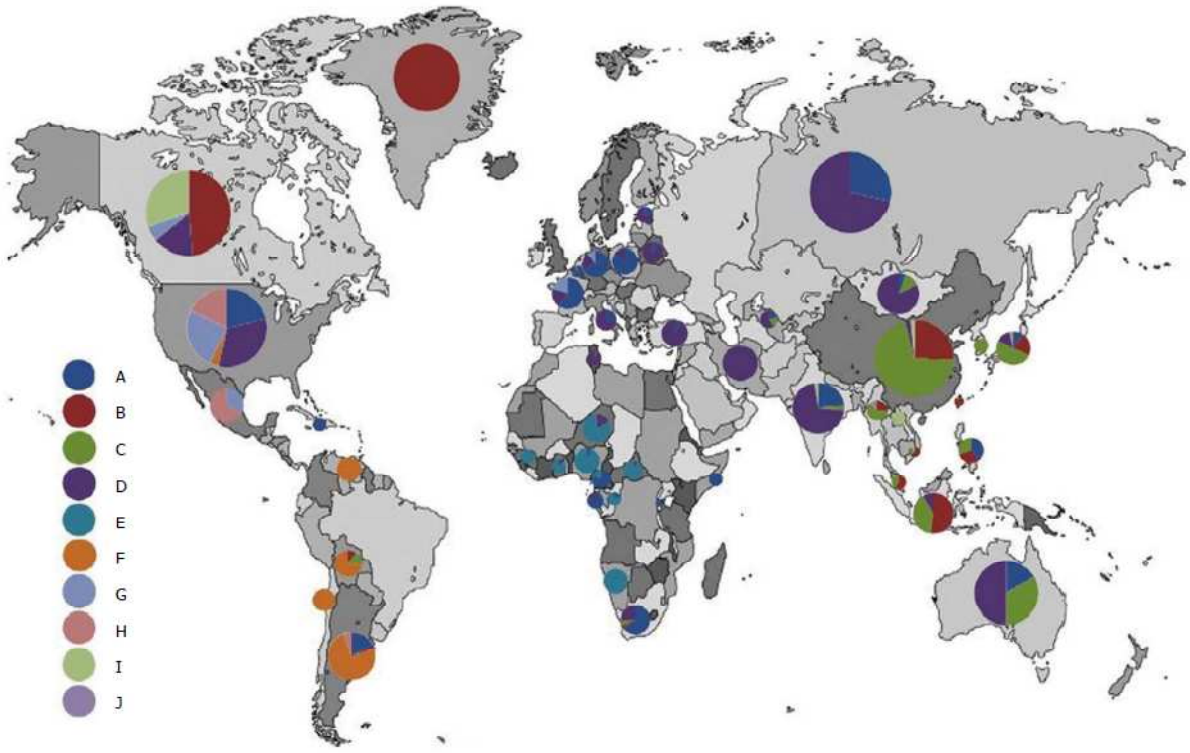

Figure 2. Geographical distribution of HBV genotypes

Genotype C patients are more prone to the complications of advanced fibrosis and cirrhosis than Genotype B patients. Genotype A appears to have a more favorable prognosis than genotype D; the genotype D is associated with HBeAg negative CHB and reports from Mediterranean countries of high rates of cirrhosis associated with HBeAg negative disease are now thought to possibly be attributable to genotype D[14]. Genotype C has been shown to carry an increased risk for the development of HCC in the REVEAL study cohort, with an adjusted hazard ratio of 2.35[15], while the genotype B may be more likely to be associated with HCC in non cirrhotic patients and has been reported to have higher rates of solitary tumour and more satellite nodules than genotype C[16].

Response rates to treatment with PEG-IFN differ by genotype. In HBeAg positive patients treated with 52 wk of Peg IFN α-2b, HBeAg loss varied with genotype, being 47% in genotype A, 44% in Genotype B, 28% in genotype C and 25% in D[17].

1.3 Vaccine escape mutants

Variations in the HBsAg protein can result in viral infection developing in a vaccinated subject. Anti- HBs is directed towards a highly conserved region of the surface protein (amino acids 99-160) which includes the major "a" determinant of this protein. HBsAg mutations resulting in amino acid substitutions in the region 137-147 of the surface protein can change the conformational epitope in the "a" determinant so that it is not recognized by the neutralizing anti-HBs antibodies. In particular the G145R vaccine escape mutant is known to be stable and replication competent[18]. The infectivity of HBsAg mutants is currently thought to be low, however another problem is their lack of detectability by serological tests as was reported in a recent case of HBV with a 4 amino acid repeat insertion at position 115 in the surface protein. The development of vaccine escape mutants has been thought in some parts to be related to the emergence of anti-viral drug resistant mutants because of the overlap of the polymerase gene (where nucleot(s)ide analogue associated resistant mutations occur) with the surface antigen domains recognized by anti-HBs. There is evidence however that emergence of the vaccine escape mutants predates mass vaccination programs[19].

1.4 Natural history of HBV infection

A characteristic feature of chronic HBV infection is that can be divided into phases reflecting the dynamic relationship between the host's immune system and the virus. Generally, HBV comprises two phases with active hepatitis and two phases with low disease activity (inflammatory vs. non-inflammatory). The four phases do not always occur in a sequence, and include:

High replicative phase with HBeAg positivity (previously: immune-tolerant phase). In addition to HBsAg, HBeAg is detected in the serum, and HBV-DNA reaches high

values (> 106 IU/ml) with normal or slightly elevated ALT levels (> 19 IU/ml in women and > 30 IU/ml in men). Signs of inflammation, necrosis and fibrosis determined in liver biopsies are minimal or nonexistent. The phase, which is known to be highly infectious, may be of short duration in patients infected during late childhood and in adults. With increasing age, there is an increased likelihood of transitioning to the immune-reactive phase.

HBeAg-positive immune-reactive phase. The phase is claimed to be caused by changes in the expression of HBV antigens and increased anti-HBV immune responses associated with the inflammatory response. The serum levels of HBV-DNA are variable, but lower than in the previous phase. The ALT levels periodically exceed the values listed in item a) above. Necroinflammatory changes in the hepatic tissue are moderate or severe, with different degrees of fibrosis (potentially progressive). The stage has a variable duration from months to years, and may culminate in the loss of HBeAg and the development of anti-HBe (2-15%). In approximately 1-4% of patients, reverse-seroconversion and re-emergence of HBsAg are observed. The higher the frequency of exacerbations, the greater the severity of liver fibrosis.

Inactive HBV carrier phase. Anti-HBe antibodies are present, and HBV-DNA levels are low (typically below 2,000 IU/ml), but occasionally higher or, conversely, undetectable. The ALT levels remain within the range specified in item a). Histopathological changes are variable, reflecting the incidence and severity of lesions during the previous phase of the disease. Minimal inflammatory changes and variable degrees of fibrosis are noted. There is a risk of cirrhosis and HCC. The rate of spontaneous loss of HBs and emergence of anti-HBs is estimated at 1-3%/year. The concentration of HBsAg (hereinafter in the text − quantification of HBsAg : qHBs) is

below 1,000 IU/ml in genotype D infection, however it is usually higher in patients infected with genotype A.

HBeAg-negative chronic hepatitis. Following seroconversion from HBeAg to anti-HBe, active inflammation in the liver is observed in 10-30% of patients. Anti-HBe antibodies are present, and considerable variation in HBV-DNA and ALT levels as well as necroinflammatory changes in the liver are noted. The key features of this phase of infection are intermittent disease exacerbations with intervening periods of remission. Most patients in this phase have detectable mutations in the HBV precore/core promoter gene, which is associated with the inability to synthesize the HBe antigen.

Occult infection (HBsAg-negative) is most commonly associated with undetectable or periodically very low serum concentrations of HBV-DNA accompanied by its presence in the liver. Anti-HBc with or without anti-HBs are present in the serum. The loss of HBsAg is associated with a reduced risk of cirrhosis and liver failure, though the risk of HCC continues to be higher than in the general population. The state of immune-suppression may lead to the reactivation of the virus due to its episomal DNA form − HBV cccDNA.

1.5 Goals of therapy in CHB

The ultimate goal of antiviral therapy is HBV eradication. At the current stage of knowledge and therapeutic opportunities, the goal is unattainable due to the episomal DNA form of HBV (covalently closed circular DNA, cccDNA), which is a structure showing very high resistance to the activity of currently available antiviral drugs. The persistence of this form of HBV-DNA is responsible for recurrences of the infection. Since HBV eradication is, for the time being, unattainable, the primary goal of therapy is complete suppression of HBV

replication: sustained loss of HBV-DNA in the serum confirmed by a highly sensitive real-time PCR test together with elimination of the HBs antigen and formation of anti-HBs antibodies. In consideration of the above, the therapeutic goal both in HBeAg-positive and HBeAg-negative patients is sustained undetectability of HBsAg combined with seroconversion to anti-HBs.

In the majority of patients, the loss or significant suppression of HBV replication which precede the attainment of that goal, leads to the following effects: inhibition of the progression of liver fibrosis and reversal of the process in the majority of patients, as demonstrated by long-term follow-up of patients treated successfully with entecavir and tenofovir;

b) normalization of biochemical indicators of liver damage; in a proportion of cases they may continue to be elevated for reasons other than HBV infection (e.g. non-alcoholic fatty liver disease, NAFLD);

c) reduction in the risk of progression to HCC; a number of studies indicate that HBV replication, particularly at a high level, is a factor contributing to the development of HCC; the incidence of HCC is reported to be lower in patients treated successfully with antiviral drugs, however the effect is not observed until four years after achieving stable suppression of viremia, and does not occur in patients with cirrhosis

d) prolongation of survival; sustained reduction of HBVDNA viremia in patients with advanced disease or cirrhosis slows down the progression of the disease/ fibrosis, increasing survival, lowering the risk of liver failure and reducing the need for liver transplantation

e) prevention of HBV infection in the transplanted organ in post-transplant patients; antiviral drugs have documented efficacy in promoting transplant survival

f) enhancement of the quality of life through improved liver function which contributes to achieving a better mental state and cognitive functions in patients

g) inhibition or reversal of extrahepatic changes associated with HBV infection

h) halting of the spread of HBV infections; the loss or marked inhibition of HBV replication reduces the infectiousness of HBsAg-positive individuals.

1.6 Current treatment available in CHB

Drugs approved in the European Union for the therapy of HBV infections are:

· **interferons (IFN):**

− natural interferons,

− a-2a and a-2b (IFNa-2a and IFNa-2b),

− pegylated a-2a (PegIFNa-2a);

· **analogues (NA):**

− nucleoside analogues: lamivudine (LMV), telbivudine (LdT) and entecavir (ETV), − nucleotide analogues: adefovir (ADV), tenofovir disoproxil (TDF) and tenofovir alafenamide (TAF). PegIFNa-2a is the preferred choice among IFN, offering a clear advantage in terms of the highest efficacy, convenience of use and treatment regimen (once a week). The preferred NA drugs include ETV, TDF and TAF owing to the most potent antiviral activity and a high genetic barrier.

To assess treatment eligibility both in HBeAg-positive and HBeAg-negative patients, HBsAg must be consistently detectable for at least six months, and at least two out of the three criteria below (evaluated concurrently)must be met:

1) HBV-DNA > 2,000 IU/ml;

2) ALT level exceeding the upper limit of normal;

3) signs of liver inflammation or fibrosis. Inflammation should be evaluated by histological examination of liver biopsy specimens, and fibrosis − by share wave elastography or transient elastography to measure the stiffness of the liver tissue expressed in kPa. However, attention should be given to different cutoff points compared to other liver diseases

including those induced by HCV infection. If coexisting liver diseases of different aetiology are suspected, elastography results are inconsistent with the clinical state of the patient or discrepancies are observed between results obtained by various noninvasive examination methods, liver biopsy is recommended (unless contraindications are present). In such cases biopsy results are regarded as conclusive. Patients in the high replicative HBeAg(+) phase, particularly younger (aged less than 30 years), without clinical features of liver disease and without family history of HCC, do not require liver biopsy and should not be treated. In such patients ALT levels should be determined at three-monthly intervals. In addition, fibrosis should be evaluated periodically using noninvasive methods. If elevated ALT levels or signs of liver fibrosis are found, antiviral therapy should be initiated. Patients with positive family history of HCC and/ or cirrhosis of unknown etiology should be assessed for liver inflammation and fibrosis. If characteristic features of chronic hepatitis are found, the patient should be immediately referred for treatment. Also, immediate therapy should be initiated in patients with cirrhosis, regardless of their HBV-DNA level. Before selecting the first-line drug, patients should be evaluated for HCV and HIV coinfection. During therapy, patients should be tested for anti-HDV IgG antibodies when their ALT levels rise or persist at an elevated level.

Regardless of the patient's HBeAg status, a drug with the highest proven efficacy and safety of use in a given patient group should be chosen as first-line therapy in treatment-naive patients with chronic HBV infection. The preferred IFN is PegIFNa-2a, and the preferred NA include ETV, TDF and TAF.

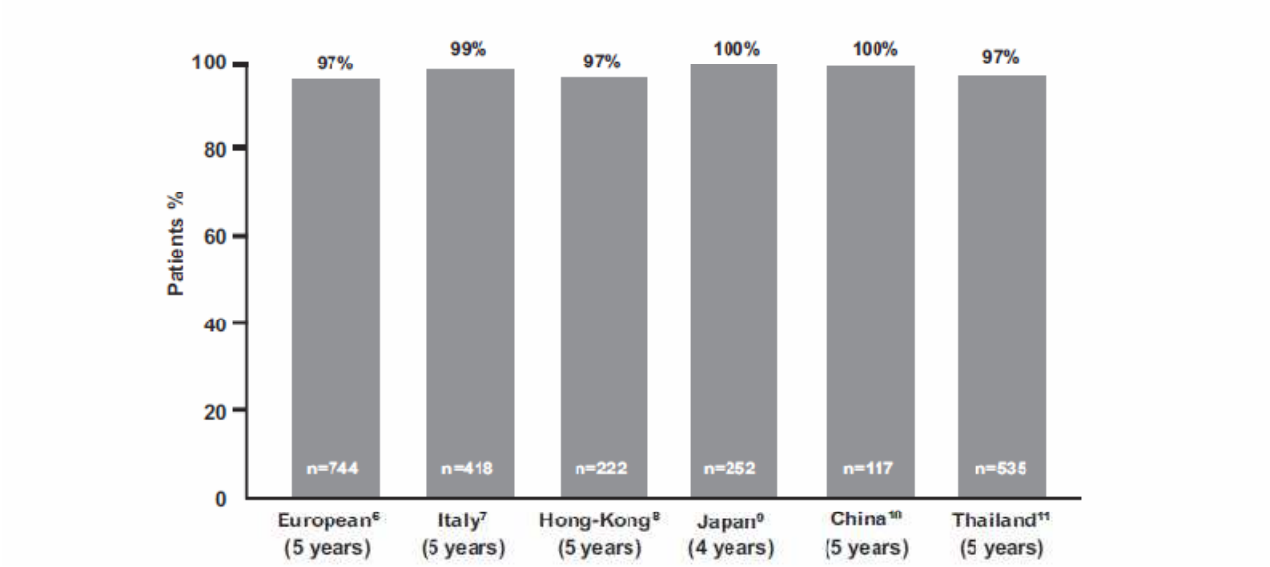

Figure 3. Rates of virological suppression in patients treated with ETV in clinical practice

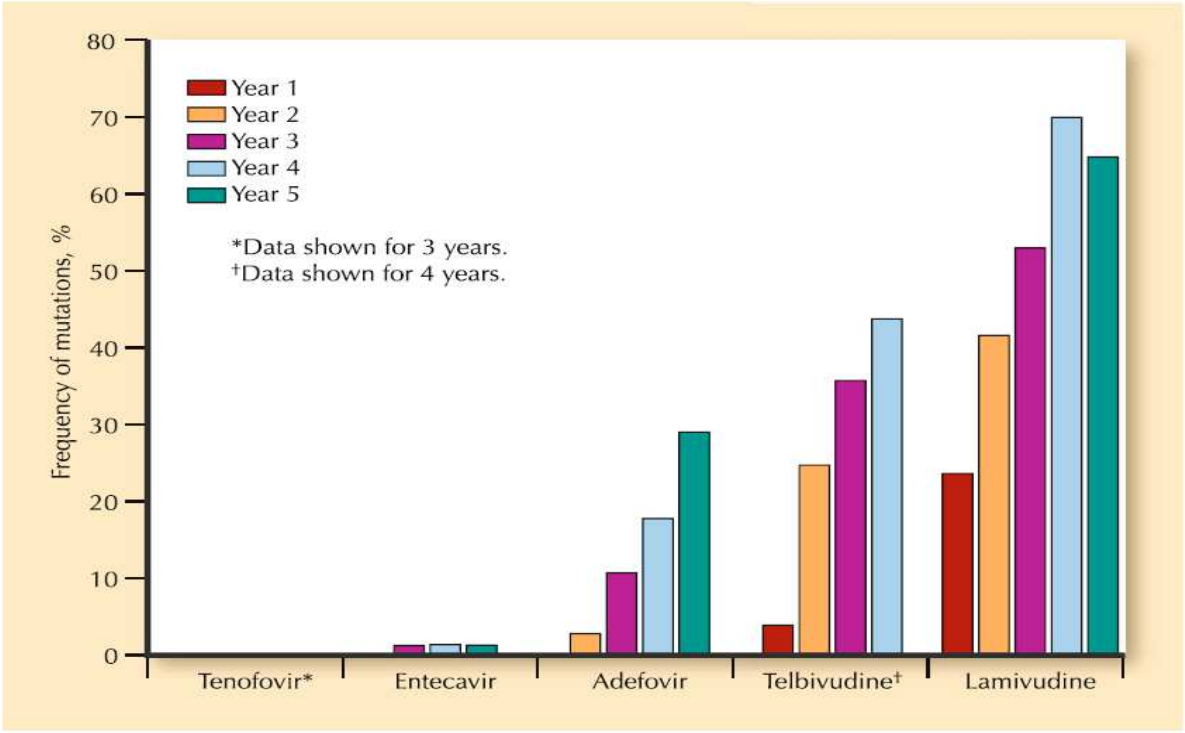

Figure 4. Rates of virological failure during the treatment with NAs

1.7 Role of quantitative HBsAg during the natural history of hepatitis B infection

In the first 20–30 years of life, HBV infection is characterized by an immune tolerance phase in which patients usually have positive HBeAg, very high HBV DNA, normal ALT levels and minimal histological damage. This is followed by an immune clearance phase, which may lead to HBeAg seroconversion. Two studies, one European and one Asian, reported that HBsAg levels varied during the natural history of the disease[20 21]. They showed that the HBsAg value was higher during the immune tolerance phase than during the immune clearance phase as well as being higher in HBeAg-positive than in HBeAg-negative patients. The European study reported an HBsAg level of 4.96 vs 4.37 log10 IU/ml in patients during the immune tolerance phase vs the immune clearance phase, respectively, while the Asian study reported an HBsAg level of 4.53 vs 4.03 log10 IU/ml in patients during the immune tolerance phase vs immune clearance phase respectively.

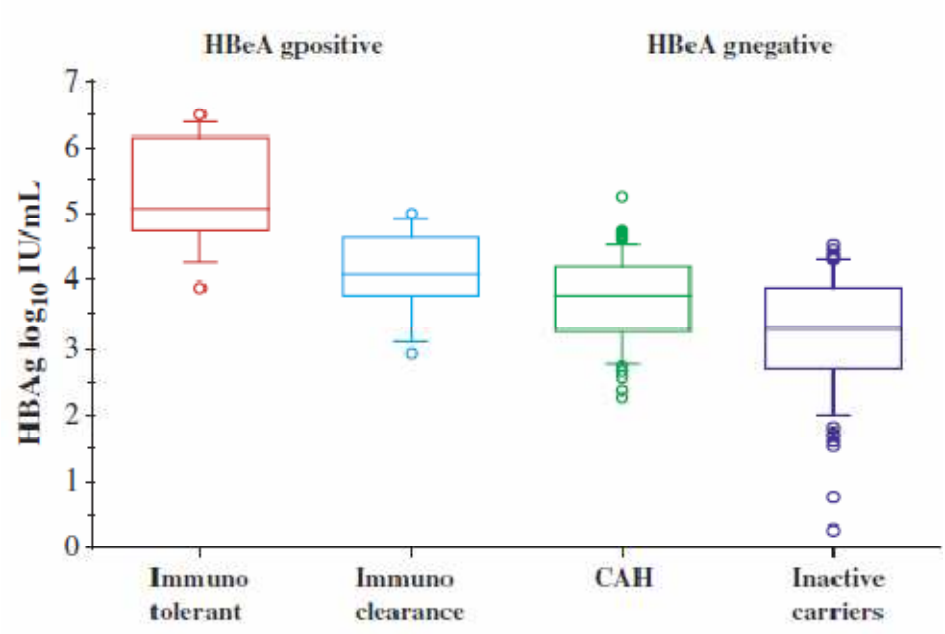

Figure 5. Quantification of HBsAg during the different phases of CHB infection

After HBeAg seroconversion, the clinical spectrum of HBeAg-negative patients, CHB ranges from 'inactive carrier' status to aggressive HBeAg-negative CHB. The latter is

generally differentiated from ‘inactive carriers’ by serial serum ALT and HBV DNA level determinations. However, in 45–65% of cases ALT activity can fluctuate with long periods of normal ALT levels resulting in misclassification. Inactive carriers have no or mild histological lesions in the liver with an excellent prognosis for survival and a low incidence of cirrhosis and HCC, while patients with HBeAg-negative CHB with fluctuating activity have a more severe disease progression with frequent cirrhosis[22].

1.8 Role of quantitative HBsAg during the treatment

HBsAg synthesis during the HBV viral life cycle is complex and usually occurs in the endoplasmic reticulum. Upon entry into the hepatocyte, the virion sheds its protein coat and its genome is transported into the nucleus where it resides as stable fully double stranded covalently closed circular HBV DNA (cccDNA) and acts as a template for the transcription of the viral gene[23]. HBsAg is translated from mRNA with the transcriptional template-active cccDNA, which is the reflection of the number of infected hepatocytes. The clinical relevance of HBsAg levels is inferred from the relationship of this marker to the intrahepatic amount of cccDNA. There is a correlation between serum HBsAg concentrations and the intrahepatic levels of cccDNA, with the highest levels occurring in HBeAg positive hepatitis B and the lowest in patients with resolved hepatitis[24].

Several automated assays have been developed for the quantitative measurement of HBsAg. The most widely used assays are the Architect HBsAg QT assay (Abbott Diagnostics, Abbott Park, IL, USA) and the Elecsys HBsAg II assay (Roche Diagnostics, Indianapolis, IN, USA). (14). The range of the Architects Assay is 0.05– 250 IU/ml and a manual or automatic 1/500 dilution is needed for higher levels. The Elecsys Assay has an automatic on board dilution with range of quantification from 0.05 to 52 000 IU/ml. The assays express results in international units per millilitre (IU/ml), based on the WHO reference

standard; 1 IU/ml is equivalent to 1– 10 ng/ml of HBsAg or 5 9 107 virions[25]. Results with both assays are highly correlated and in close agreement for each HBV genotype.

Although HBV-DNA becomes rapidly undetectable, studies have clearly shown that the decline in HBsAg during NAs therapy is less pronounced than that during PEG-IFN treatment[26]. The decline appears more marked in HBeAg-positive patients[27].

In our previous study, we reported a significant difference in the decline in HBsAg levels at 2 years of therapy depending on the NAs administered[28]. Several studies have reported that baseline HBsAg levels and on treatment HBsAg quantification are good pre-dictors of end of treatment response and SVR (60–67). Zoutendijk et al.[29] investigated HBsAg kinetics in patients who were successfully treated with long-term ETV or TDF. The authors used linear mixed regression analysis of individual HBsAg declines to estimate the duration of therapy required to achieve an HBsAg decline of 1 log10 IU/ml from baseline and HBsAg clearance. They showed that the median durations of therapy to achieve a 1 log10 IU/ml decrease were: 6.6 [1.7–18] years and 8 [0.5–15] years in HBeAg-positive and HBeAg-negative patients respectively.

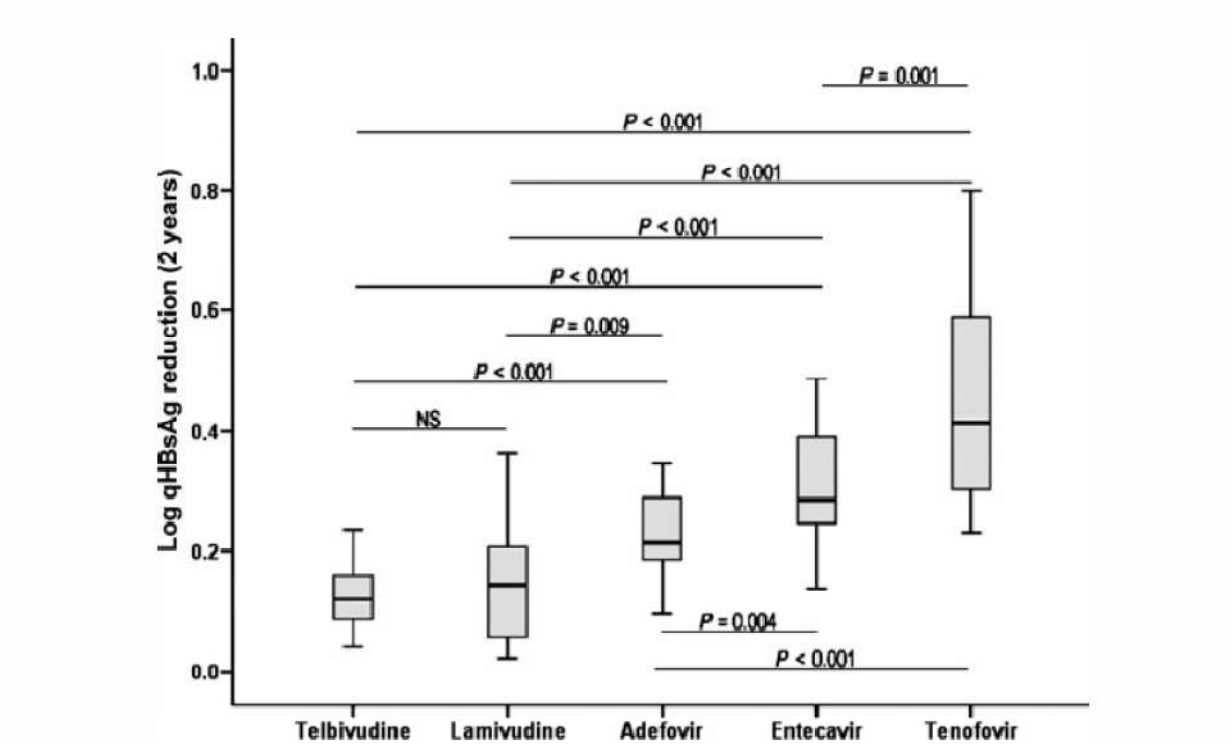

Figure 6. Decline of qHBsAg during the treatment with NAs in patients with CHB HBeAg-negative

2. AIMS OF THIS STUDY

The role of hepatitis B “s” antigen (HBsAg) during ETV administration is still debatable. No data are available about the role of HBV genotype in qHBsAg decline during ETV treatment. This study analyzed the qHBsAg decline during the first 3 years of treatment with ETV in a population of chronic hepatitis B HBeAg-negative patients, with HBV genotypes A, D, E. Nevertheless, the role of therapeutic drug monitoring (TDM) of NAs in HBV treatment is still undefined. In this study we also evaluated the role of ETV plasma concentration in the treatment naïve patients.

3. METHODS

Patient population

This is a prospective cohort observational study and all patients were consecutively enrolled at our Centre of Infectious Diseases Amedeo di Savoia Hospital in Turin from April 2007 to May 2010. Inclusion criteria were: adult age (>18 years); chronic hepatitis B with a documented HBsAg- positive for at least 6 months; baseline HBV-DNA level > 2000 IU/mL and ALT > 40 IU/mL; HBeAg-negative/antiHBe-positive, naive for previous treatment with IFN or nucleos(t)ide analogues. Only the patients which gained the virological response (HBV-DNA undetectable after 24 weeks of therapy) were included into the analysis. Exclusion criteria were: co-infection with hepatitis C or D virus or the immunodeficiency virus; decompensated cirrhosis; HCC; previous treatment with IFN or nucleos(t)ide analogues .

All drop-out patients for any reason (lost at follow-up, virological failure or without good compliance) were excluded from this analysis. Baseline fibrosis stage was determined with Fibroscan® using the Metavir score. All patients received a treatment with ETV 0.5 mg/day.

The evaluation of qHBsAg, HBV-DNA, HBV genotype and ALT were performed before starting the treatment; qHBsAg level, HBV-DNA and ALT were tested every 3 months during the first year of treatment, then every 6 months for a time of at least 3 years.

The study was conducted in compliance with the Declaration of Helsinki and in accordance with local Health Authority regulations: all patients gave written informed consent according to standards of the local ethics committee.

Study end points

Primary end-point of the study was the comparison of qHBsAg kinetics among patients with different HBV genotypes. Secondary end-point was the evaluation of the expected time of HBsAg loss according to the different viral genotypes.

Assays

Serum HBV-DNA levels were quantified by the Real Time PCR COBAS AmpliPrep/COBAS TaqMan HBV Test 2.0 (Roche Molecular Systems, NJ, USA). HBV genotypes were determined using the INNOLIPA reverse hybridization assay (Innogenetics, Belgium). HBsAg, HBeAg and anti-HBe were detected by the Elecsys instrumental platform (Roche Diagnostics, Italy); qHBsAg test was performed with ARCHITECT HBsAg (Abbott Diagnostics, Ireland) with a dynamic range of 0.05–250.0 IU/mL; qHBsAg values above 250.0 IU/mL were subsequently 1:100 serially diluted and retested until falling within the dynamic range. Liver fibrosis stage was expressed in kPa using the Fibroscan®.

Statistical analysis

For descriptive statistics, continuous variables were summarized as median and inter-quartile range (IQR: 25th to 75th percentiles) and ranges. Categorical variables were described as frequencies and percentages. All data were tested for normality using a Shapiro-Wilk test. Differences in categorical data between groups were analyzed using Kruskal-Wallis and Mann Whitney tests. To investigate continuous data, a Spearman Rank correlation was used. The association was calculated using the χ^2-test. Univariate and multivariate analyses were performed using a linear regression model. The estimated time to HBsAg loss was evaluated through a linear equation calculated by

interpolating the median logarithmic decline over time for each single genotype, considering the first determination (baseline) as starting point.

Statistical analyses were conducted by SPSS software package ver. 20.0 (Chicago, IL, USA).

RESULTS

Baseline characteristics of the population

Data from 123 patients enrolled from April 2007 with at least 3 years of treatment with ETV, have been collected. Baseline characteristics of these patients are reported in Table 1. Patients were infected by three HBV genotypes: A, 44 (35%); D, 45 (36%); E, 34 (27%). The median age was 39 years, 104 patients (83%) were male; the HBV A genotype was prevalent in patients from East-Europe (65%), the D genotype in Italian patients (80%), the E genotype only in patients from Africa (central and west) (100%). Median values of log qHBsAg was 3.88 IU/mL and ALT was 87 IU/mL.

Characteristics	All patients	Genotype A	Genotype D	Genotype E
Number of patients n (%)	123 (100)	44 (35.7)	45 (36.6)	34 (27.6)
Age (yr) median[IQR]	39 [34-52]	37 [33-41]	54 [39-61]	37 [33-42]
Route of transmission n (%)				
Intravenous drug use	9 (7.3)	5 (11.3)	4 (8.8)	0 (0)
Transfusion	12 (9.7)	2 (4.5)	10 (22.2)	0 (0)
Sexual	17 (13.9)	15 (34)	2 (4.4)	0 (0)
Family history of HBV	44 (35.8)	8 (18)	12 (26.6)	24 (70.5)
Unknown	41 (33.3)	14 (31.8)	17 (37.7)	10 (29.4)
Male sex n (%)	104 (83.2)	33 (75)	37 (82.2)	34 (100)
Geographical origin n (%)	Italy: 47 (38.2) East-Europe: 37 (30) Africa: 38 (30.8) South America: 1 (0.8)	Italy: 11 (25) East-Europe: 29 (65.9) Africa: 3 (6.8) South America: 1 (2.3)	Italy: 36 (80) East-Europe: 8 (17.8) Africa: 1 (2.2)	Africa: 34 (100)
Liver stiffness (kPa) median [IQR]	10.3 [8.4-16.7]	9.6 [8.3-13.2]	11.4 [9.6-17.6]	10.1 [7.8-13.4]
qHBsAg (log IU/mL) median [IQR]	3.88 [3.74-3.95]	3.89 [3.75-3.96]	3.85 [3.70-3.91]	3.92 [3.74-3.99]
HBV-DNA (log IU/mL) median [IQR]	5.72 [5.61-6.02]	5.88 [5.74-6.14]	5.32 [4.96-5.71]	5.87 [5.77-6.09]
Albumin (g/dL) median [IQR] (	42 [38-44]	43 [39-45]	41 [37-42]	42 [36-43]
ALT (IU/L) median [IQR]	87 [74-112]	84 [74-103]	90 [73-135]	84 [74-105]
AST (IU/L) median [IQR]	78 [71-104]	74 [66-100]	77 [74-112]	71 [67-101]

Table 1. Baseline characteristics of study population

Response to antiviral treatment and qHBsAg decline

The qHBsAg (log IU/mL) decline measured after 6 months, 1 year, 2 years and 3 years, divided for viral genotype, resulted as following: after 6 months, it was 0.10 log IU/mL for A genotype (IQR: 0.06-0.16), 0.05 log IU/mL for D genotype (IQR: 0.03-0.08) and 0.04 log IU/mL for E genotype (IQR: 0.02-0.10).

The median qHBsAg drop resulted significantly different in A vs D genotype ($p<0.001$) and A vs E genotype ($p=0.002$). The median decrease after 1 year was 0.25 log IU/mL for A genotype (IQR: 0.17-0.33), 0.12 log IU/mL for D genotype (IQR: 0.09-0.16), 0.12 log IU/mL for E genotype (IQR: 0.08-0.16). After 2 years it was 0.47 log IU/mL for A genotype (IQR: 0.35-0.74), 0.43 log IU/mL (IQR: 0.27-0.54), 0.28 log IU/mL for E genotype (IQR: 0.18-0.35), with significant difference between A and E genotype ($p<0.001$) and D and E genotype ($p<0.001$). Finally, after 3 years of therapy with ETV, a decrease of 0.77 log IU/mL was observed for A genotype (IQR: 0.58-0.77), 0.65 log IU/mL for D genotype (IQR: 0.52-0.76), 0.45 for E genotype (0.39-0.51), with significant differences between A and D genotype ($p=0.012$), A and E genotype ($p<0.001$), D and E genotype ($p<0.001$) (Figure 7-11).

A significant correlation between the qHBsAg decrease after 3 years, the geographical origin of the patients ($p=1.24\text{x}10^{-4}$) and the HBV genotype ($p=7.93\text{x}10^{-8}$) was observed.

In the univariate analysis (Table 2) the following factors resulted significantly predictive for the qHBsAg decline: geographical origin (β=0.142 DS=0.036 $p<0.001$) and HBV genotype (β=-0.116 DS=0.020 $p<0.001$). In the multivariate analysis only the HBV genotype resulted significantly predictive for the qHBsAg decline after 3 years of treatment with ETV (β=-1.133 DS=0.033 $p<0.001$).

Characteristics	Univariate	Multivariate
Age	β = 0.002 DS=0.001 *p*= 0.198	β = 0.002 DS=0.001 *p*= 0.271
Sex	β = -0.057 DS=0.052 *p*= 0.283	
Geographical origin	β = 0.142 DS=0.036 *p*< 0.001	β = -0.042 DS=0.060 *p*= 0.485
HBV genotype	β = -0.116 DS=0.020 *p*< 0.001	β = -1.133 DS=0.033 *p*<0.001
Liver stiffness	β = -0.011 DS=0.017 *p*= 0.520	
Log qHBsAg baseline	β = 0.101 DS=0.107 *p*= 0.346	
Log HBV-DNA baseline	β = -0.151 DS=0.075 *p*= 0.456	
ALT baseline	β = 0.131 DS=0.098 *p*= 0.336	
AST baseline	β = 0.173 DS=0.124 *p*= 0.477	

Table 2. Univariate and multivariate analyses of baseline characteristics predicting qHBsAg decline after 3 years of treatment with entecavir.

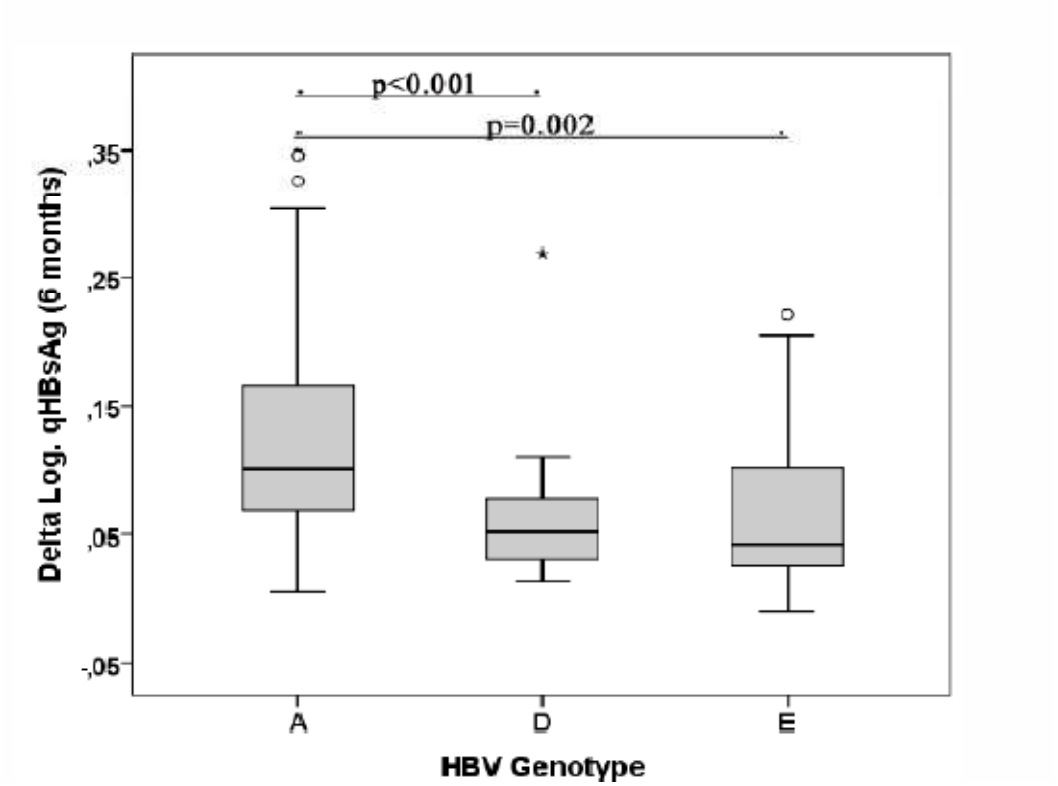

Figure 7. Decline of qHBsAg (log IU/mL) decline after 6 months of treatment with ETV and statistical differences.

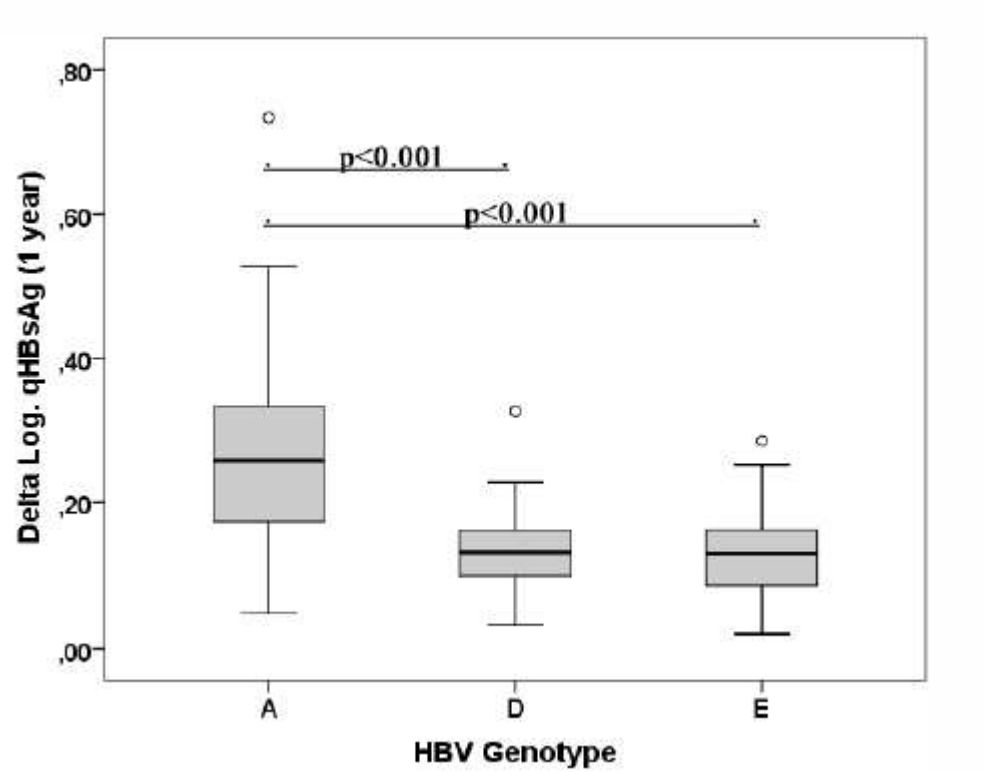

Figure 8. Decline of qHBsAg (log IU/mL) decline after 1 year of treatment with ETV and statistical differences.

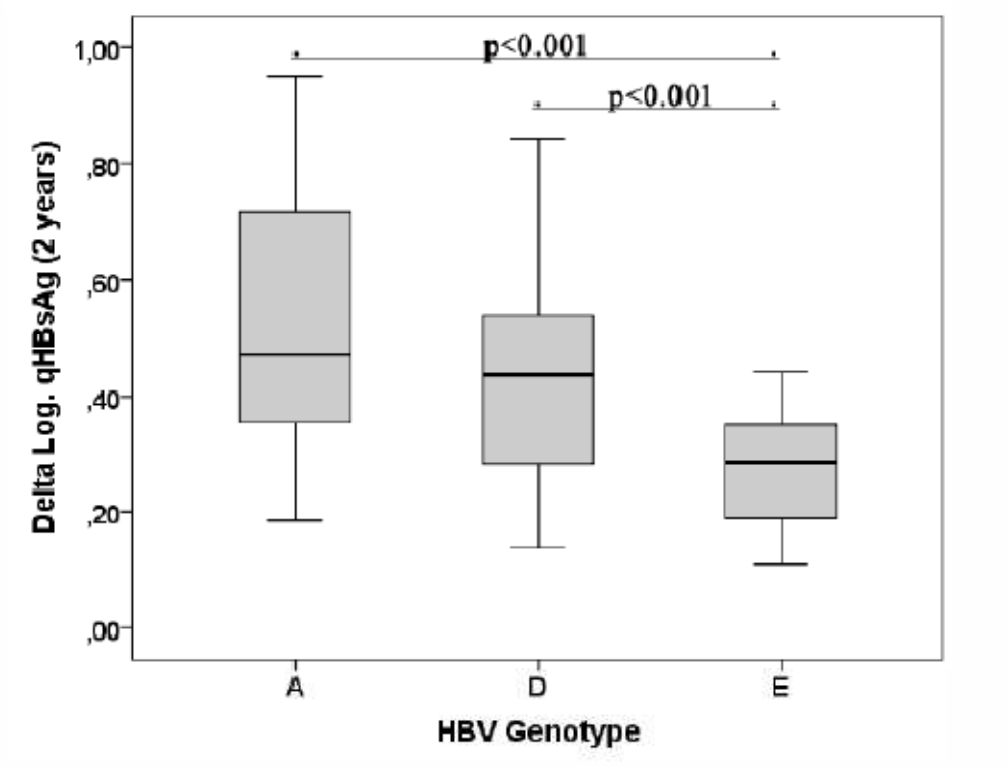

Figure 9. Decline of qHBsAg (log IU/mL) decline after 2 year of treatment with ETV and statistical differences.

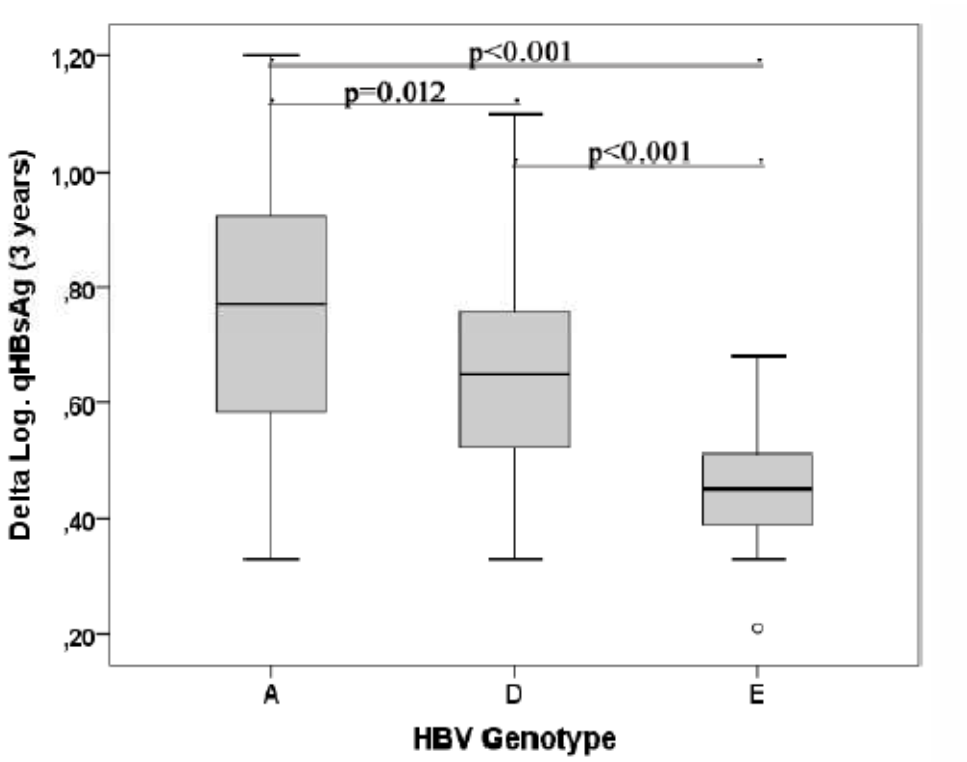

Figure 10. Decline of qHBsAg (log IU/mL) decline after 2 year of treatment with ETV and statistical differences.

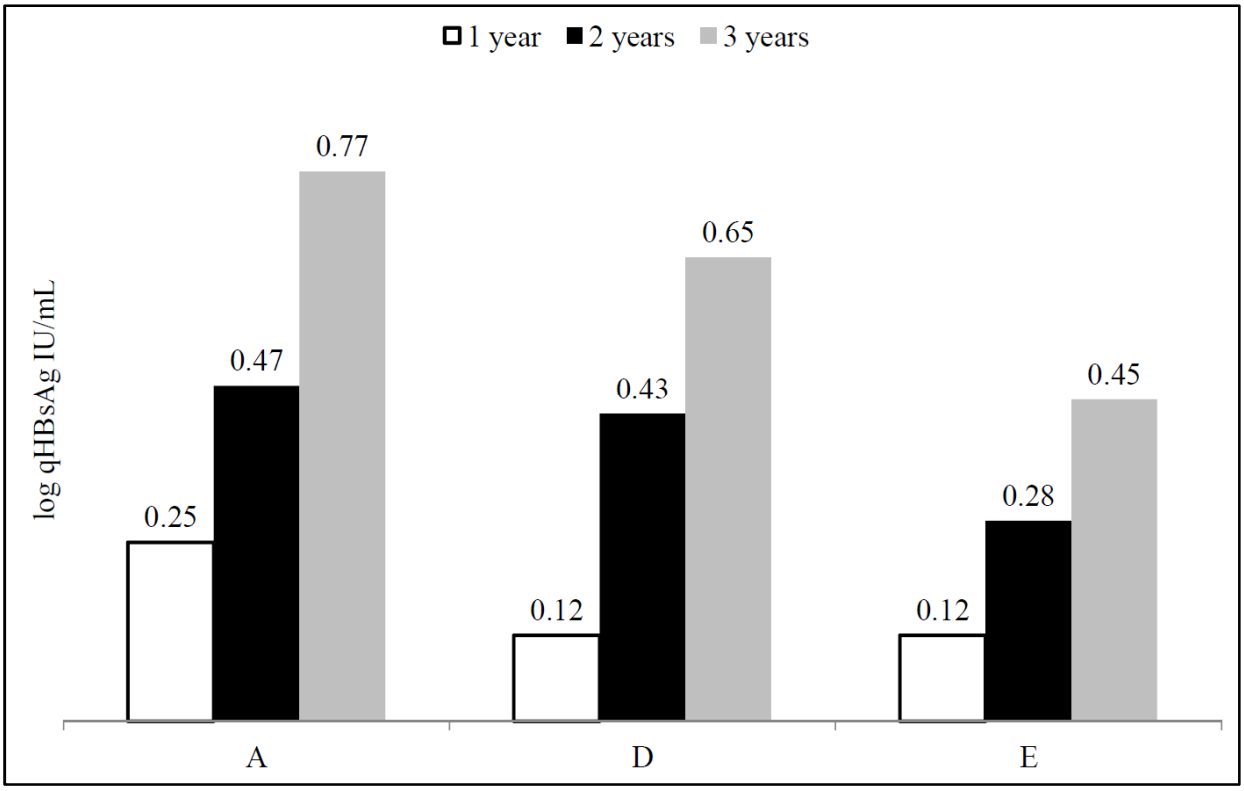

Figure 11. Overall decline of qHBsAg (log IU/mL) during the treatment with ETV in the study population.

Expected time of HBsAg loss

The obtained linear regression models describing the changes in qHBsAg (in logarithmic scale, Y) over the time (X) were: Y= -0.129X + 4.025 (r^2=0.9819) for A genotype, Y= -0.118X + 4.014 (r^2=0.9089) for D genotype and Y= -0.081X + 3.987 (r^2=0.9863) for E genotype.

According to these models, the expected time for HBsAg loss was 15.6 years for the A genotype, 17 years for D genotype, 24.6 years for E genotype (p<0.001) (Figure 12).

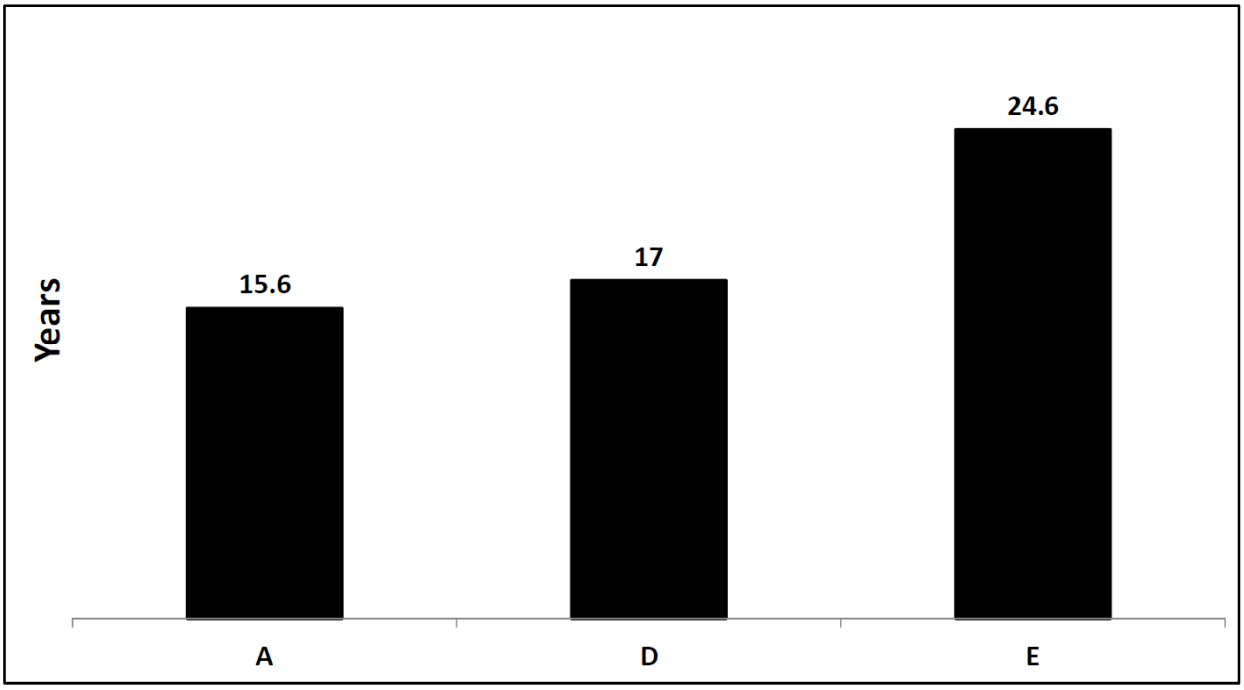

Figure 12. Expected time to HBsAg loss (years) during ETV treatment in different HBV genotypes

DISCUSSION

The use of the nucleos(t)ide-based therapy is increasing for the treatment of patients with chronic hepatitis B and HBeAg-negative. However, it seems difficult to identify reliable end-points, because the HBsAg loss is a very rare occurrence in these patients, and suppression of HBV-DNA levels and ALT normalization are not useful to predict the long-term outcome of treatment. The measurement of viral load is useful to assess the antiviral efficacy and observe viral breakthrough, while the monitoring of qHBsAg drop during therapy with nucleos(t)ide analogues should be used to predict the HBsAg loss. Previous studies examined the kinetics of qHBsAg during the treatment with ETV: Fung et al.[30] observed that, despite the HBV-DNA suppression, qHBsAg decline was not significant in HBeAg-positive patients and the decrease at 12 or 24 weeks was not predictive of HBeAg seroconversion; however, in this study the analysis of HBV genotype was not performed. Lee et al.[31] described a significant decrease of qHBsAg with ETV and a correlation with seroconversion in 57 patients with HBeAg; the mean drop of qHBsAg after 2 years of therapy was 0.24 log IU/mL in HBeAg-positive, 0.21 log IU/mL in 38 HBeAg-negative patients; however, all patients were infected by genotype C.

Recently, in a study performed in this hospital, including 48 HBeAg-negative patients with chronic hepatitis B and D genotype, it was observed that the median qHBsAg declines after 1 year and 2 years of treatment with ETV were 0.12 and 0.38 log IU/mL, respectively[28]. A different decline of qHBsAg between various HBV genotypes during the therapy with ETV has not yet been described; though it seems possible that certain genotypes may differently behave regarding to qHBsAg drop.

In this prospective study it was found for the first time a significant difference among three HBV genotypes concerning the qHBsAg decline during the ETV treatment. All patients were HBeAg-negative, with homogeneous baseline characteristics and virological response after 6 months of therapy, therefore the observed difference of qHBsAg kinetics may depend, in our opinion, on the different HBV genotypes. The finding about the D genotype was similar to the

previous data of decline in our retrospective study: 0.12 and 0.38 log IU/mL after 1 and 2 years vs 0.12, 0.43 and 0.65 log IU/mL after 1, 2 and 3 years, respectively. The HBV A genotype showed in our patients the greatest qHBsAg decrease, that was most evident after 3 years of treatment with 0.77 log IU/mL. This is an interesting novelty, because in the previous studies it has been described that A genotype showed a significantly better response to IFN treatment according to HBeAg, HBsAg loss and seroconversion compared to B, C and D genotype[32 33], and it is currently considered a predictor of response to IFN in the HBeAg-positive patients. Regarding the virological response, no evidences have been found about an hypothetical impact of HBV genotype in the HBV-DNA suppression and viral resistance to nucleos(t)ide analogues, but otherwise different kinetics of qHBsAg were not still reported.

The D genotype showed in our study a dual trend of qHBsAg decline: after 6 months and 1 year the qHBsAg drop was poor and similar to E genotype, but after 2 and 3 years it tended to reach the A genotype with 0.43 and 0.65 log IU/mL, respectively.

In the patients with E genotype the qHBsAg kinetic was always lower than in A genotype, but initially was close to D genotype; after 3 years of ETV treatment however, the E genotype showed the smaller qHBsAg decline, despite the HBV-DNA was persistently undetectable. This is, to our knowledge, the first report of qHBsAg kinetic in this genotype.

Up to now, we previously reported a poor response to PEG-IFN in a small population of patients with E genotype, that was one of the less investigated, leading to the hypothesis of a possible effect of viral genetics on the treatment response, probably related to the recent appearance of this genotype (derived from the A) into the general African population[34] (all patients with E genotype were immigrant from central and west Africa).

The different kinetics of decline in qHBsAg determined symmetric prediction times of HBsAg loss, resulting in 15.6 and 17 years for the A and D genotypes, 24.6 years for the E genotype, which requires the longer treatment period to achieve HBsAg loss. The lack of patients with B

and C genotype in our population did not allow a complete comparison, which will be the aim of further studies.

These data may be useful to clarify the perspective of long-term treatment with ETV in patients with HBeAg-negative: in A and D genotypes the goal of achieving qHBsAg loss and HBV-DNA undetectable, and therefore the possibility of suspension of treatment, requires approximately 15-17 years, while in the E genotype the treatment duration has to be much longer in order to obtain the same goal and, consequently, the clinician should also evaluate the adherence to the therapy and the risk of selection of viral resistances.

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