The decrease of HBsAg during nucleos(t)ide analogues (NUC) therapy in Bulgarian patients

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(Received 22 December 2014; accepted 30 March 2015)

Serum surface antigen of the hepatitis B virus (HBsAg) level has been recently introduced and studied during the last years as a simple and non-invasive serum surrogate marker for covalently closed circular DNA (cccDNA). The aim of the present study was to evaluate the serum HBsAg levels in Bulgarian patients during the first 12 months of therapy with nucleoside/nucleotide analogues (NUC). Twenty patients with chronic hepatitis B (CHB) infection were studied. Initially, 9 out of the 20 subjects were Hepatitis B ‘e’ antigen (HBeAg)-positive and 11 were HBeAg-negative. Serum hepatitis B virus DNA (HBV DNA), as well as serum HBsAg levels were measured prior therapy and after 3 and 12 months of treatment. The baseline HBsAg levels in HBeAg-positive patients were more than three times higher, compared to HBeAg-negative subjects. We found an initial slight increase of HBsAg in 2/9 HBeAg-positive patients and in 3/11 HBeAg-negative subjects. A positive correlation was found between baseline HBsAg and HBV DNA. A rapid decline of HBsAg levels was observed in one-third of the HBeAg-positive patients at the 12th month of treatment. In the HBeAg-negative patients, HBsAg levels remained relatively unchanged. Measurement of HBsAg levels during NUC therapy is not used routinely. After achieving a persistently undetectable serum HBV DNA and normal aminotransferase levels, HBsAg level remains an important surrogate marker for the course of the liver disease. The HBsAg levels, together with HBV DNA, may provide essential additional information and may help to define and evaluate the upcoming new therapeutic strategies.

Keywords: HBsAg; nucleos(t)ide analogues; HBeAg; HBV DNA

Introduction
Despite the significant advances in the antiviral therapies and vaccination programmes, hepatitis B virus (HBV) infection remains a major health problem. It has affected about two billion people worldwide, causing chronic hepatitis in up to 400 million individuals.[1,2] In Bulgaria, HBV is with intermediate prevalence of 3.9%. [3,4] Similarly to other South European and Mediterranean countries, about 85% of the chronic hepatitis B (CHB) patients are hepatitis B “e” antigen (HBeAg)-negative and genotype D was found in almost 100% of them.[5,6]

Current treatment strategies for CHB include attempts for achieving a sustained immune control by a finite treatment course with pegylated interferon (Peg-IFN), or attaining a maintained on-treatment viral control by suppression of HBV replication with nucleoside/nucleotide analogues (NUC). The therapeutic endpoints include a sustained suppression of HBV DNA to undetectable serum levels, normalization of aminotransferase levels and histological improvement and, ideally, serum surface antigen of the hepatitis B virus (HBsAg) loss or seroconversion.[7] The final CHB treatment goal is HBsAg clearance by a spontaneous or treatment-induced loss of HBsAg, which is associated with sustained disease remission and improved survival.[8–14]

Persistence of chronic HBV infection is associated with covalently closed circular DNA (cccDNA) presence in the nuclei of the infected hepatocytes that are less affected by the current therapy. The measurement of cccDNA requires a liver biopsy. There is a need of simple and non-invasive serum surrogate marker that correlates well with the cccDNA in the liver. Quantification of the serum HBsAg levels (qHBsAg) has been recently introduced and intensively studied in the clinical practice during the last years.

Evaluation of the serum HBsAg titter during therapy with Peg-IFN may be useful in predicting the likelihood of HBsAg loss.[11] Sustained responders to Peg-IFN treatment tend to show a greater qHBsAg decline, and reduction of cccDNA, than non-responders.[15–17] Prolongation of Peg-IFN therapy in HBeAg-negative genotype D patients is associated with continuous HBsAg decline and increased rates of HBsAg loss.[18,19] The optimal on-treatment HBsAg cut off level to predict response in HBeAg-positive patients is probably 1,500 IU/mL at the 12th or the 24th week of treatment.

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Absence of any HBsAg decline, together with <2 log reduction in HBV DNA at the 12th week was proposed as a stopping rule in HBeAg-negative patients. NUC inhibit the HBV polymerase with no direct effect on HBsAg transcription and translation. Long-term therapy with NUC leads to HBV replication management, which results in functional and morphological improvement of the liver. Unfortunately, this effect lasts only during the NUC therapy. HBV reactivation occurs within several weeks after the treatment discontinuation. NUC may also suppress cccDNA, but this effect seems not as strong as during the IFN-based therapy. Thus, the reduction of HBsAg from the NUC therapy is less pronounced than the HBsAg reduction during Peg-IFN therapy. Furthermore, in patients with NUC therapy, the decline of HBsAg appears more significant in HBeAg-positive than in HBeAg-negative patients. A rapid on-treatment HBsAg decline of ≥1 log IU/mL by the 6th month and the 12th month is a predictor for consecutive HBsAg loss in HBeAg-positive patients treated with tenofovir or telbivudine. The stable HBsAg levels during the first 24 weeks of therapy are an early indication that there is a little chance (0%–5.9%) of treatment-induced HBsAg decrease. In HBeAg-negative patients HBsAg loss occurs only in few patients even after a long-term NUC therapy. Thus, after achieving a durable on-treatment virological response with NUC, the HBsAg level remains an important marker for the course of a chronic liver disease.

The aim of the present study was to evaluate serum HBsAg levels in both HBeAg-positive and HBeAg-negative Bulgarian patients during the first 12 months of therapy with NUC.

Materials and methods

Patients and treatment

Twenty Caucasian patients (16 males and 4 females) were studied. The median age was 42.5 years, ranging between 29 and 63 years of age. All of them were with chronic HBV infection, defined as the presence of serum HBsAg for more than six months and with serum alanine aminotransferase values above the upper limit of normal within six months prior to treatment initiation. Hepatitis D virus (HDV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) co-infections were excluded in all patients. Initially, 9 out of the 20 subjects were HBeAg-positive and 11 were HBeAg-negative. All subjects were treated with NUC (Table 1).

HBsAg quantification

Serum HBsAg levels were measured at baseline, as well as at the 3rd and the 12th month of treatment by electrochemiluminescence immunoassay (Roche Diagnostics). The results were expressed as IU/mL.

HBV DNA measurement

Serum HBV DNA levels were measured at baseline and on a three month interval thereafter by using LightCycler real time polymerase chain reaction assay (PCR) (Roche Diagnostics). The results were expressed as IU/mL.

| Table 1. Baseline characteristics of the studied patients. |
|---------------------------------------------------------|
| HBeAg-positive | HBeAg-negative |
| Number of patients | 9 | 11 |
| Age – median (range) | 37 (29–60) | 47 (34–63) |
| Male/female (n) | 6/3 | 10/1 |
| ALT (IU/L) – median (range) | 40 (25–186) | 79 (17–482) |
| HBV DNA (IU/mL) – median (range) | 11,842,000 (6654–496,000,000) | 1,110,000 (279–32,115,000) |
| HBsAg (IU/mL) – median (range) | 9319 (1721–311,150) | 2683 (174–12,140) |
| Liver histology /METAVIR/ | | |
| Chronic hepatitis /fibrosis 0–3 (n) | 5 | 7 |
| Without liver biopsy /liver cirrhosis/(n) | 3 | 2 |
| Without liver biopsy /chronic hepatitis/(n) | 1 | 2 |
| NUC therapy | | |
| Tenofovir (n) | 5 | 3 |
| Entecavir (n) | 3 | 7 |
| Telbivudine (n) | 1 | 1 |

Note: n – number of patients; alanine aminotransferase (ALT).
Standard laboratory methods were used for the assessment of the blood chemistry parameters. Alanine aminotransferase (ALT) was tested at baseline and at every three months during the therapy. ALT < 40 U/mL was accepted for normal, according to the laboratory report.

Liver biopsies
Pretreatment liver biopsy, according to Mengini’s method, was performed on 12 out of the 20 patients and was evaluated by using the METAVIR system. Five subjects without biopsy were with clinical and ultrasound signs of liver cirrhosis (F4 – stage of fibrosis), while the rest three subjects without biopsy were with chronic hepatitis.

Statistical methods
Standard statistical analyses were performed using SPSS® v. 17.0. Individual characteristics between groups were evaluated by means of the Wilcoxon and Mann–Whitney. Nonparametric correlation methods like Kendall and Spearman were used. All reported P values were two-sided, and P values less than 0.05 were considered significant.

Informed consent
The study was approved by relevant Institutional Ethics Committee. Written informed consent was obtained from each of the participating patients.

Results and discussion
The baseline HBsAg levels in HBeAg-positive patients were more than three times higher, compared to the levels in HBeAg-negative subjects. Viral load also tended to be higher in HBeAg-positive than in HBeAg-negative patients, but the difference was not significant (Table 2).

Results from HBeAg-positive patients
Serum HBsAg, HBV DNA and ALT levels during NUC therapy in HBeAg-positive patients are presented in Table 3.

A significant correlation was found between baseline level of HBsAg and HBV DNA ($r = 0.683; P = 0.042$, Spearman), but there was no correlation between baseline HBsAg and ALT levels ($P > 0.05$).

During the therapy, the serum HBV DNA levels progressively decreased in all subjects and dropped to undetectable levels at the 3rd, 6th, 9th and 12th treatment months in four, six, eight and eight out of the nine HBeAg-positive patients, respectively. Neither HBeAg-loss nor HBsAg-loss or seroconversion was observed.

The kinetics of serum HBsAg differed from the rapid kinetics of serum HBV DNA during NUC therapy. Three months after the initiation of NUC therapy, the HBsAg levels increased with approximately 20% in two patients and remained relatively unchanged in three subjects. There was at least 20% reduction of the HBsAg levels in four out of the nine HBeAg-positive patients.

After 12 months of NUC therapy, seven of the HBeAg-positive patients were with at least 20% reduction of the HBsAg level compared to the baseline level. The following patterns of changes in the HBsAg level were identified:

- **Rapid decline** (>20% at the 3rd month and >0.5 log 10 IU/mL at the 12th month) was observed in three patients (numbers 1, 4 and 5) (Table 3). The decline of qHBsAg in two of them was >1 log10 IU/mL. The baseline characteristics in both subjects (number 4 and 5) were - male gander, age under 40 years old, mild liver injury, high viral load (approximately 8.5 log10 IU/mL), baseline HBsAg level >4.5 log10 IU/mL and elevated ALT (2.3 to 4.6 times above the upper limit of normal). The third patient (number 1) was with HBsAg reduction >0.5 log10 IU/mL, but <1 log10 IU/mL - a 60 years old female with advanced fibrosis (F3), high viral load (about 8.5 log10), HBsAg > 4.5 log10 IU/mL and normal ALT.

- **Slow decline** (a decrease of >20% at the 12th month, but <0.5 log10 IU/mL) was observed in two patients (numbers 3 and 6) (Table 3).

- **Slow increase** (continuous slight increase >20% (between 23% and 31%) at the 12th month) was observed in two patients (numbers 2 and 8) (Table 3). One of them was with early elevation of HBsAg during the 3rd month (number 8).

### Table 2. Median baseline levels of HBV DNA, ALT and qHBsAg in HBeAg-positive and HBeAg-negative patients.

|                  | HBeAg-positive | HBeAg-negative | $P^*$ |
|------------------|----------------|----------------|------|
| HBV DNA (IU/mL)  | 11,842,000 (6,654–496,000,000) | 1,110,000 (279–32,115,000) | 0.152 |
| HBsAg (IU/mL)    | 9319 (1721–311,150) | 2683 (174–12,140) | 0.002 |
| ALT (IU/L)       | 40 (25–186) | 79 (17–482) | 0.552 |

Note: *Mann–Whitney nonparametric analysis; alanine aminotransferase (ALT).
Fluctuation of qHBsAg was observed in two patients (numbers 7 and 9) (Table 3). In the first one, qHBsAg initially decreased during the 3rd month and then an elevation was observed during the 12th month. In the second case, the HBsAg level increased during the 3rd month and dropped down during the 12th month. In both patients, irrespectively of fluctuations, there was at least 20% reduction of HBsAg levels during the 12th treatment month, compared to the baseline levels.

**Results from HBeAg-negative patients**

Serum HBsAg, HBV DNA and ALT levels during NUC therapy in HBeAg-negative patients are presented in Table 4.

In HBeAg-negative patients there was no significant correlation between baseline qHBsAg and HBV DNA, as well as between baseline HBsAg and ALT.

During therapy, the serum HBV DNA levels progressively decreased in all subjects and became undetectable at the 3rd, 6th, 9th and 12th treatment months in 5, 9, 10 and 11 of the 11 HBeAg-negative patients, respectively. HbsAg loss was not observed.

At the 3rd month, the HBsAg levels increased from 10% to 280% in three of the HBeAg-negative patients and remained similar during the 12th month (patient numbers 1, 3 and 8 from Table 4).

At the 12th month only one subject (number 7) was with slow HBsAg decline, but reduction \(0.5\) log10 IU/mL from the baseline level was not achieved. The rest 10 HbeAg-negative patients remained with steady HBsAg levels, compared to the baseline or the third month levels (Table 4).

In the whole group of 20 patients (HBeAg-positive and HBeAg-negative), there were no significant differences found between the baseline HBsAg levels and those of month 12 in HBeAg-negative and in HBeAg-positive patients during NUC therapy.
Only one of our HBeAg-positive patients achieved a marked HBsAg drop to 100 IU/mL. In this particular subject, HBsAg loss may occur during further years of NUC therapy, as the rates of HBsAg clearance in HBeAg-positive patients may progressively increase from 3% to 8% after prolongation of tenofovir therapy from one to three years, respectively.[19] On the other hand, HBsAg levels in the vast majority of HBsAg-positive patients in the present study remained >1000 IU/mL after 12 months of treatment. Previous studies in HBeAg-positive CHB have shown that the reduction of HBsAg is pronounced within the first year of NUC treatment and that HBsAg level remains relatively stable thereafter.[11,16,26].

We did not observe any rapid HBsAg decline among the HBeAg-negative subjects. This was consistent with findings in most studies in CHB, HBeAg-negative. [26,27,32] Together, all these data clearly suggested that HBsAg levels remained steady or decreased very slowly even after a long-term NUC therapy, so HBsAg loss rarely occurs. Mathematical models showed that the majority of patients will need more than three decades of NUC therapy for HBsAg seroclearance.[27] From a conceptual viewpoint, it is known that NUC only blocks the reverse transcriptase that diminishes the HBV DNA synthesis, but has a weak effect on cccDNA, which is the limiting factor for the complete clearance of chronic HBV infection.[16]

Although the mechanism of HBsAg decline during NUC therapy is unclear, it may be hypothesized that the reduction of HBsAg levels reflects a better degree of host immune control against HBV.[16] HBsAg decline may occur only in patients, who have some level of immune response, which is generally associated with higher ALT levels. The role of the host immune response for HBsAg clearance was recently reported by Jaroszewicz et al. [33] who showed that high baseline interferon-inducible protein 10 (IP-10) levels predict HBsAg loss during NUC therapy.

As HBsAg seroclearance remains a rare event with NUC, adding of immunomodulators to NUC is an essential therapeutic strategy to increase this important event. We recently reported that adding of inosine pranobex to NUC, after achieving a durable on-treatment virological response to NUC, may lead to additional decrease in HBsAg levels.[34] The add-on of IFN to NUC treatment is currently under investigation. First reports in small patient groups showed encouraging results.[35,36] The interim data of a larger study with similar design are also promising, but this trail is still ongoing.[37] Currently, several novel immune modulatory agents, like therapeutic vaccines or toll-like-receptor agonists, are in clinical development.[38]
remains an important surrogate marker for the course of a liver disease. Monitoring of HBsAg levels together with HBV DNA may provide essential additional information. It may help to define and evaluate the upcoming new therapeutic strategies.

Conclusions
NUC strongly inhibit HBV replication, but the knowledge of their effect on HBsAg levels is limited, especially in patients with HBV genotype D. This is the first report on quantitative HBsAg in Bulgarian subjects during NUC therapy. The baseline HBsAg levels in HBeAg-positive patients were more than three times higher compared to HBeAg-negative subjects ($P = 0.002$). There was a positive correlation between baseline HBsAg and HBV DNA. We found an initial slight increase of HBsAg in 5 out of the 20 subjects with chronic HBV infection. A rapid decline of HBsAg levels was observed in one-third of the HBeAg-positive patients at the 12th month of treatment, and in the HBeAg-negative patients, HBsAg levels remained relatively unchanged.

After achieving a persistently undetectable serum HBV DNA and normal aminotranferase levels, HBsAg level remains an important surrogate marker for the course of the liver disease, may provide essential additional information, and may help to define and evaluate the upcoming new therapeutic strategies.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This research was supported by the Medical University of Sofia [grant number Nr.20-D/2013]: dynamics of quantitative HBsAg in patients with chronic HBV infection during the course of standard antiviral therapy with nucleos(t)ide analogues.

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