Medical University

"Prof. Dr. Paraskev Stoyanov"-Varna

Second Department of Internal Medicine

ES Gastroenterology, Hepatology and Nutrition

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ANTIVIRAL THERAPY IN CHRONIC HEPATITIS B-VIRAL MARKERS DYNAMICS AND LONG-TERM OUTCOMES

AUTOREFERAT

OF DISSERTATION FOR OBTAINING THE DEGREE OF DOCTOR OF SCIENCE

Supervisor:

Assoc. Prof. Dr. Irina Ivanova, PhD

Varna, 2024

The dissertation contains 106 pages and is illustrated with 47 figures and 15 tables. The cited literature includes 176 sources, of which 2 in Cyrillic and 174 in Latin.

The dissertation was discussed and directed for defense by the Departmental Council of the Second Department of Internal Medicine at the Medical University of Varna on 14.02.2024.

The formal defense of the dissertation will take place on 15.05.2024 at an open meeting of the Scientific Jury:
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Abbreviations used:

HBV- hepatitis B virus
CHB- chronic hepatitis B
HDV- hepatitis D virus
HCV- hepatitis C virus
HCC- hepatocellular carcinoma
HBsAg- HB surface antigen
qHBsAg- quantitative HBsAg
HBeAg- HB "envelope" antigen
HBcrAg- HB "core" antigen
Anti HBs- antibodies against HBsAg
Anti HBe- antibodies against HBeAg
Anti HBc- antibodies against HBcrAg
NA- nucleoside/nucleotide analogues
IFN-alpha- interferon alpha
PEG-IFN-alpha- pegylated interferon alpha
ALT-alanine aminotransferase
AST- aspartate aminotransferase
GGT-gamma glutamyl transferase
ULN- upper normal limit
cccDNA- covalently closed circular DNA
rcDNA- relaxed circular DNA
pgRNA- pregenomic RNA
mRNA- messenger RNA
NITs- non-invasive tests for assessment of fibrosis
TE- transient elastography
2D- SWE- 2-D shear wave elastography/ ultrasoound elastography
EASL- European Association for the Study of the Liver
LAM- lamivudine
TBV- telbivudine
ETV-entecavir
ADF- adefovir dipivoxil
TDF- tenofovir disoproxil fumarate
TAF- tenofovir alafenamide
NAFLD- non-alcoholic fatty liver disease
MAFLD- metabolic-associated fatty liver disease
LS- liver stiffness
FGDS - fibrogastroduodenoscopy

PHG- portal hypertensive gastropathy
DM- diabetes mellitus

Introduction

According to WHO data from 2019, 296 million are living with chronic hepatitis B virus (HBV) infection and are at risk of complications, mainly liver cirrhosis and hepatocellular carcinoma (HCC), driving an annual death toll of 820 000. Despite the 2016 strategy adopted to reduce the impact of HBV worldwide, only 10% of chronically infected people are diagnosed and only 2% of them receive antiviral treatment. Modern therapy with HBV replication inhibitors improves patient survival and prevents progression of chronic liver disease. On the other hand, spontaneous or therapeutically induced clearance of HB surface antigen (HBsAg) occurs rarely, about 1% per year in chronic HBV infection. Thus, patients with viral suppression cumulatively increase in the course of long-term nucleoside/nucleotide analogue (NA) treatment. Prolonged therapy leads to a burden in the healthcare system, a risk of HBV resistance to NA with loss of efficacy, the so-called virological breakthrough. Although NA are well tolerated medications, associated side effects are likely to occur, such as decreased bone density and renal tubular toxicity with tenofovir treatment. It is difficult to motivate patients to take medication regularly and they often report negative psychological impact and impaired well-being. In follow-up of antiviral treatment, transaminase activity, liver synthetic parameters and creatinine clearance are standard are performed together with abdominal ultrasound for early detection of liver lesions and HCC, respectively. Of interest are the new possibilities of multiparametric ultrasound for the evaluation of traditional histopathological concepts such as inflammatory activity, fibrosis and steatosis. In the course of NA admission, an undetectable viral load (HBV DNA) with a sensitive polymerase chain reaction assay is expected. This raises the need for searching of new markers to monitor the effect of antiviral drugs on the HBV life cycle, such as viral protein synthesis (HBsAg, HB core-bound antigens). This dissertation attempts to address some of the current challenges in the clinical management of patients with chronic HBV infection during the course of antiviral treatment.

Aim:

To provide a contemporary assessment of the effectiveness of antiviral treatment and activity of chronic hepatitis B virus infection in patients with long-term nucleotide/nucleoside analogues.

Objectives:

1. To characterize patients with chronic HBV infection with indications for antiviral treatment.
2. To investigate virological response to long-term NA treatment by monitoring viral load (HBV DNA).
3. To assess the biochemical response to NA therapy by monitoring transaminase activity.
4. To track the dynamics of quantitative HBsAg and assess the relationship between HBsAg level and viral load, baseline HBeAg status, duration of treatment and stage of liver disease.
5. To investigate the level of HBcrAg and to evaluate the role of this marker in monitoring antiviral treatment against HBV.
6. To compare treatment outcomes and viral marker dynamics between HBeAg(-) and HBeAg(+) CHB.
7. To monitor liver disease during the course of long-term NA treatment by laboratory indicators of liver synthetic function, indirect serum markers of fibrosis, abdominal ultrasound, US elastography and upper endoscopy for endoscopic criteria of portal hypertension.
8. To assess the role of co-factors such as metabolic disease in the course of liver disease in patients with hepatitis B receiving NA therapy.

Patients and methods

Patients

Patients with chronic HBV infection receiving antiviral treatment at the Clinic of Gastroenterology at University Hospital "St. Marina", Varna who met the following criteria:

Inclusion criteria:

- Diagnosed chronic HBV infection at the stage of chronic hepatitis or cirrhosis (based on a positive HBsAg for at least 6 months);
- Baseline assessment of liver disease by morphologic examination and/or criteria of liver disease from physical examination, laboratory studies, abdominal ultrasound and upper endoscopy in case of liver cirrhosis;
- Baseline evidence of transaminase activity, significant HBV replication, or indications related to prevention of HBV activation in the course of immunosuppressive or biologic treatment of a concomitant disease;
- NA therapy for at least 6 months;
- Follow-up of antiviral treatment in the Clinic of Gastroenterology at University Hospital "St. Marina".

Exclusion Criteria:

- Co-infection with HCV, HDV, HIV;
- Poor patient cooperation with antiviral treatment interruptions;
- Patient disagreement for analysis of laboratory test data.

Patients were studied in a prospective observational study from May 2022 to November 2023. During this period, enrolled patients were at different time points from NA initiation. Data collected on baseline, prior to initiation of therapy included: age; sex; duration of HBV infection and possible risk route of infection; family history for HBV; risk factors; comorbidities and medications; past antiviral treatment with IFN-alpha or NA; baseline transaminases (mean), HBV DNA, HBeAg status, and stage of liver disease. Factors related to the current treatment plan were analyzed, such as: duration of NA treatment; dynamics of liver disease assessed by abdominal ultrasound, TE elastography, endoscopic criteria for portal hypertension, indirect serum markers of fibrosis, monitoring of liver enzymes, HBV DNA, HBsAg, HBcrAg, HBeAg status changes during treatment.

The baseline indications for treatment as well as the rules for monitoring of antiviral therapy as outlined in the Bulgarian association of gastroenterology consensus on the treatment of chronic viral hepatitis and the current criteria for antiviral treatment were followed. Thus, treatment was initiated in cases with: HBeAg (-) or HBeAg (+) CHB with HBV DNA level > 2000 IU/ml, ALT > ULN (upper normal limit) and/or evidence of at least moderate liver inflammation or fibrosis; patients with compensated or decompensated liver cirrhosis, regardless of HBV DNA and ALT level; patients with HBV DNA > 20 000 IU/ml and ALT 2xULN, regardless of the degree of fibrosis; HBsAg(+) patients who are on immunosuppressive therapy, regardless of HBV DNA level, and are at high risk of HBV reactivation. The phase of chronic HBV infection was assessed according to the EASL guidelines for the management of hepatitis B (9). Therapy was administered with lamivudine (LAM), entecavir (ETV) or tenofovir (TDF) with reimbursement protocol.

The study was approved by the Research Ethics Committee of MU-Varna. All patients signed informed consent to participate in the study. In order to optimize the processing, the results of the observations and clinical indicators were systematized in in Microsoft Excel.

Methods

1. History and physical examination

A history was taken from all patients regarding current complaints, the period from HBV diagnosis to registration in the gastroenterology clinic, comorbidities, medications taken, including immunosuppressive therapy, family history of HBV, risk factors, co-infections with HCV, HDV, HIV, and presence of oncological disease.
Physical examination was performed in order to look for signs of chronic advanced liver disease, such as icterus of the skin and sclerae, hyperpigmentation of the skin, scratch marks on the skin, reduced hairiness of the skin, telangiectasias on the face and upper body with the character of "vascular stars", palmar erythema, gynaecomastia, splenomegaly, hepatomegaly with hypertrophy of the left hepatic lobe, thick liver on palpation, sharp edge of the liver on palpation, sign of ascites, presence of colaterals on the abdominal wall, hypo or atrophy of skeletal muscles, oedema of the lower limbs.

2. Laboratory, imaging and invasive tests

2.1.Routine laboratory tests

As an integral part of the baseline evaluation of a patient with chronic liver disease and during the course of antiviral treatment (at an interval of 6 months on

average), the following laboratory tests were performed and recorded: Hematological parameters- complete blood count, biochemical parameters- AST, ALT, GGT, AP, fasting glucose, total and direct bilirubin, total protein and albumin, creatinine, urea, ionogram, and coagulation status- prothrombine index %, INR and fibrinogen. Alpha-fetoprotein (AFP) was tested in patients with suspected or proven HCC.

2.2 Viral markers and viral load

- **HBeAg and anti HBe Ab** - at baseline and at 6-12 months in the course of therapy, in the Laboratory of Virology at University Hospital "St. Marina", by semi-quantitative ELISA analysis of HBe Ag&Ab DIA.PRO in serum or plasma.
- **HBV DNA** - at baseline and at 6 months in the course of therapy by PCR method in Laboratory of Virology of University Hospital "St. Marina". The viral load test has a detection level of 7 IU/ml. Viral suppression is defined as HBV DNA≤ 10 IU/ml, according to EASL recommendations. (4) Undetectable HBV DNA is accepted in cases with persistent, confirmed with at least 2 measurements (at least 6 months between them), testing in which no viral load is detected by a highly sensitive PCR method.
- **HBsAg quantification testing** - baseline (in some patients) and during the course of NA treatment, using the LIAISON® XL MUREX HBsAg Quant kit, which applies chemiluminescent immunoassay (CLIA) technology and the result is shown in IU/mL with a threshold value distinguishing the presence from absence of HBsAg at a cutoff of 0.05 IU/mL. Patients' sera were analyzed on the day of sample collection. In all enrolled patients, at least 2 HBsAg concentration values were prospectively recorded at 6- to 12-month intervals. The tests were performed at the Laboratory of Virology at "St. Marina" with the assistance of Assoc. Prof. Dr. Zhivka Kalcheva Stoykova and under the guidance of Prof. Dr. Temenuga Stoeva.
- **HBcrAg assay** - using the Lumipulse G HBcrAg IRC kit (FujiRebio) with the principle of the chemiluminescent enzyme immunoassay method, where the measurement range is from 2.0_{log} U/ml to 7.0_{log} U/ml, with a lower detection limit of 2.0_{log} U/ml. In patients in the course of antiviral treatment with NA, a single blood sample was obtained, with sequential centrifugation (EBA 200 centrifuge (Hettich) at 1500 rpm for 15 minutes) and serum separation of 5-10 ml. According to the manufacturer's recommendations, sera were frozen on the same day and stored at -20º C until the day of transport. The final HBcrAg testing was performed in the Clinical Laboratory of the University Hospital "Prof. Dr. Al. Chirkov", Sofia, under the guidance and with the assistance of Prof. Dr. Margaritka Boncheva. The tests for quantitative analysis of HBsAg and HBcrAg were purchased with the

financial support and in the course of a scientific project at the Science Fund of MU-Varna, under number 21014: "Monitoring the etiological therapy of hepatitis B".

- Anti HCV Ab, anti HDV Ab, HIV were tested on baseline and if suspected new co-infection in the Laboratory of Virology of the University Hospital "St. Marina".

2.3Indirect serum markers of fibrosis

Two serum markers were used to assess fibrosis: APRI and FIB-4. APRI is a combined laboratory index including AST, ULN of AST and platelet count, while FIB-4 includes the parameters age, platelet count, AST and ALT (Figure 1). Calculations were performed with the online calculator MDCalc (https://www.mdcalc.com). The markers were informative of disease stage at the following accepted threshold values: APRI <0.5 rejected fibrosis; >0.7 corresponded to significant fibrosis (F≥ 2), and >1 to cirrhosis(F4); FIB-4 <1.3 in patients 36 to 64 years of age rules out advanced fibrosis, and <2.0 rules out advanced fibrosis in patients≥ 65 years of age; FIB-4 between 1.3 and 2.67 is a gray area for patients 36 to 64 years of age, and between 2.0 and 2.67 is a gray area for patients≥ 65 years of age (118).

$$\text{FIB-4} = \frac{\text{Age (years)} \times \text{AST (U/L)}}{\text{Platelet Count } (10^9/\text{L}) \times \sqrt{\text{ALT (U/L)}}} \qquad \text{APRI} = \frac{\dfrac{\text{AST Level}}{\text{AST (Upper Limit of Normal)}}}{\text{Platelet Count } (10^9/\text{L})} \times 100$$

Figure 1. Formula for determining FIB-4 and APRI in a patient with CHB. Source: MDCalc (https://www.mdcalc.com)

2.4. Abdominal ultrasound

Abdominal ultrasound was performed in all patients with Prosound alfa7 (Aloka) ultrasound devices and after August 2023 with Aplio i800 (Canon) at baseline, before initiation and at 6-month intervals during the course of treatment, with the use of B-mode and Doppler ultrasound (US) tools. Left and right hepatic lobe dimensions (with the categories of normal size, hepatomegaly, left lobe disproportionate enlargement/hypertrophy), liver surface area (in the categories of smooth/nodular), liver structure (homogeneity, echogenicity), spleen dimensions (longitudinal, transverse, surface area), portal blood flow (cm/sec) were recorded. Figure 2 shows ultrasonographic images of a patient with CHB and baseline findings of initial fibrosis.

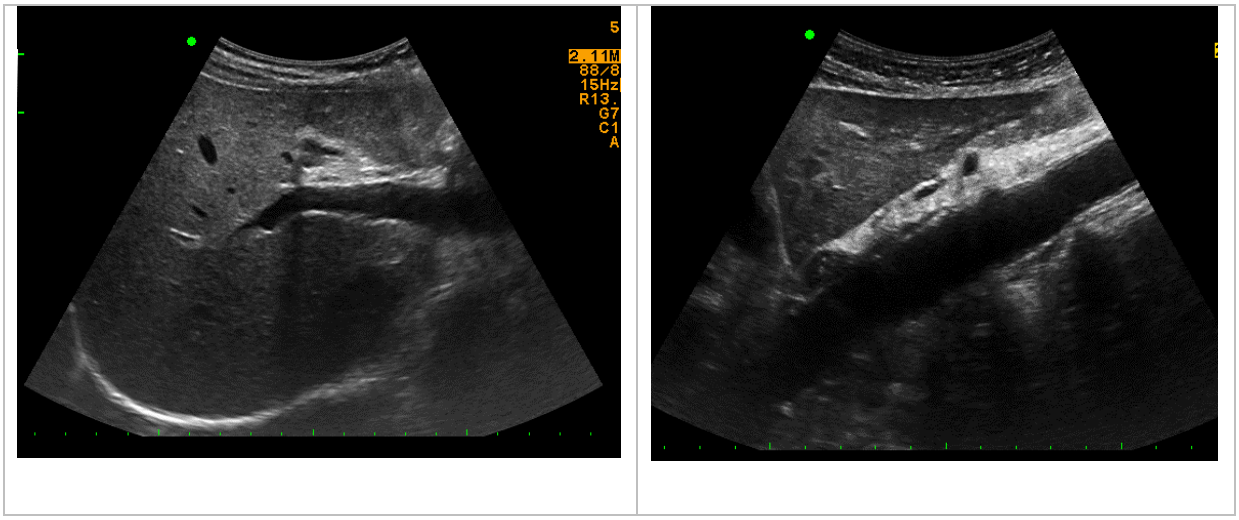


Figure 2. B-mode imaging of a patient with early-stage CHB.

Figure 3 illustrates patients with compensated cirrhosis with HBV etiology.

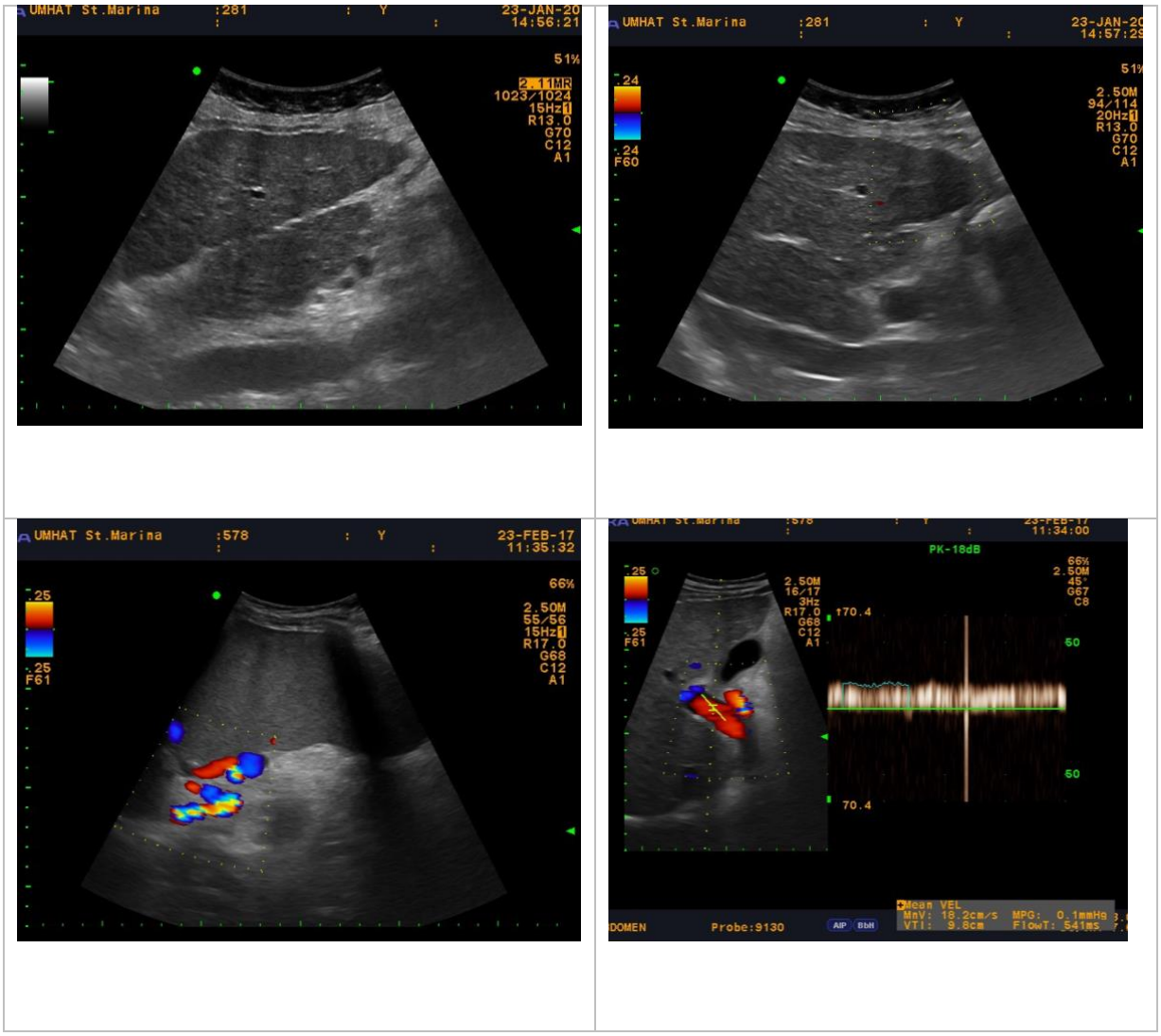
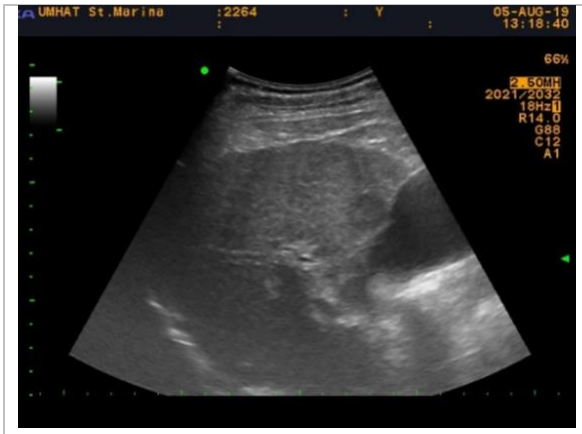


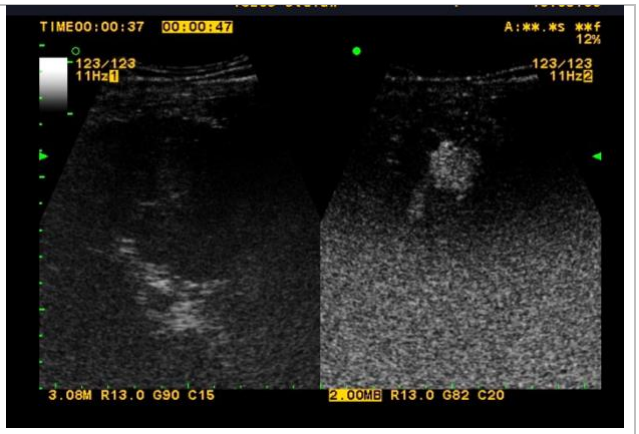
Figure 3. B-mode and Doppler ultrasonography in a patient with HBV cirrhosis

All patients underwent US analysis of hepatic steatosis in a 4-grade scale (absent/mild/moderate/severe), following the criteria and comparing hepatic with renal parenchyma, where: grade 0 is normal homogeneity (no steatosis), grade 1 is mild increase in echogenicity of hepatic parenchyma compared to right renal parenchyma on clear imaging of diaphragm and portal vein walls (mild steatosis); grade 2 is a moderate increase in hepatic parenchyma echogenicity, with mildly impaired imaging of the diaphragm and intrahepatic hepatic vessel walls (moderate steatosis); and grade 3 is a significant increase in hepatic parenchyma echogenicity with poor to absent visualization of the diaphragm and portal vein walls, as well as the posterior subdiaphragmatic areas of the hepatic parenchyma due to marked attenuation of the ultrasound wave in depth (severe steatosis).

In the presence of collaterals/portosystemic abdominal shunts and ascites, the information is further noted. The finding of focal liver lesions placed the patient as suspicious for HCC, with subsequent clarification by contrast-enhanced imaging- CEU (contrast-enhanced ultrasonography), CT (contrast-enhanced computed tomography) of the abdomen, MRI (contrast-enhanced magnetic resonance imaging) of the abdomen.

Patients with de novo focal liver lesions defined as benign (e.g., regenerative nodules) or less than 1 cm in size were followed up at 3-6 months with contrast-enhanced imaging and additionally with tumor markers (AFP, CA 19-9). Figure 4 shows ultrasonographic images of a patient with compensated HBV cirrhosis with a solitary focal lesion near the gallbladder detected during lamivudine treatment; contrast-enhanced ultrasonography revealed hyperenhancement in the arterial phase followed by delayed and gradual partial wash-out.





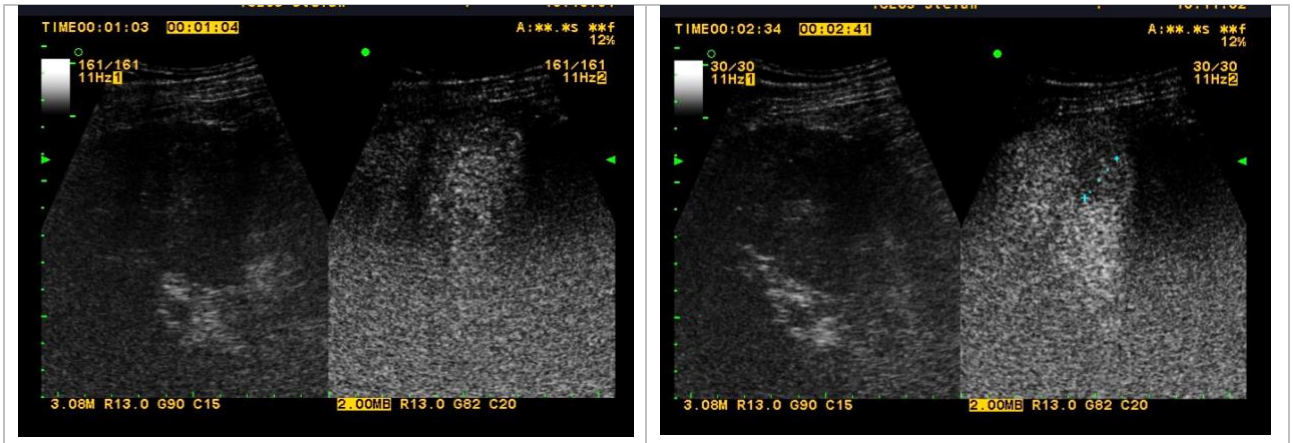


Figure 4. Ultrasonography including contrast enhancement (arterial, portal and late venous phase after SonoVue application) in a patient with cirrhosis and focal lesion.

2.5 Liver biopsy

Prior to initiation of antiviral treatment, some patients (n=53) underwent a liver biopsy assisted by ultrasound, using the aspiration biopsy technique (Menghini) or a cutting needle (Tru-cut type). Materials were analyzed in the presence of at least 1 cm of biopsy material. The evaluation of the stage of activity and fibrosis and the presence of steatosis was carried out in the Clinic of General and Clinical Pathology at "St. Marina". The degree of inflammation was assessed in a 4-grade scale as absent (A0), mild (A1), moderate (A2) and marked (A3) histological activity, according to the combined assessment of portal and periportal inflammation (interface hepatitis) and foci of lobular necrosis. The fibrotic stage was investigated according to the METAVIR 5-grade scale by a pathologist from "St. Marina". According to the METAVIR grading system, fibrosis was graded from F0 to F4, respectively, according to the criteria in Table 1.

F	Description
0	No fibrosis (F0)
1	Stellar expansion of portal spaces without septa formation (F1)
2	Extended portal spaces with single septa (F2)
3	Multiple fibrous septa without cirrhosis (F3)
4	Cirrhosis (F4)

Table 1. Assessment of fibrosis stage by METAVIR.

In patients with definite evidence of liver cirrhosis (clinical, laboratory, ultrasound data, endoscopic criteria for portal hypertension) liver biopsy was not performed. Liver morphological examination was also not performed in patients at high risk of complications on invasive testing (mainly taking anti-aggregants/anticoagulants) and in indication for antiviral treatment: taking immunosuppressants for underlying disease.

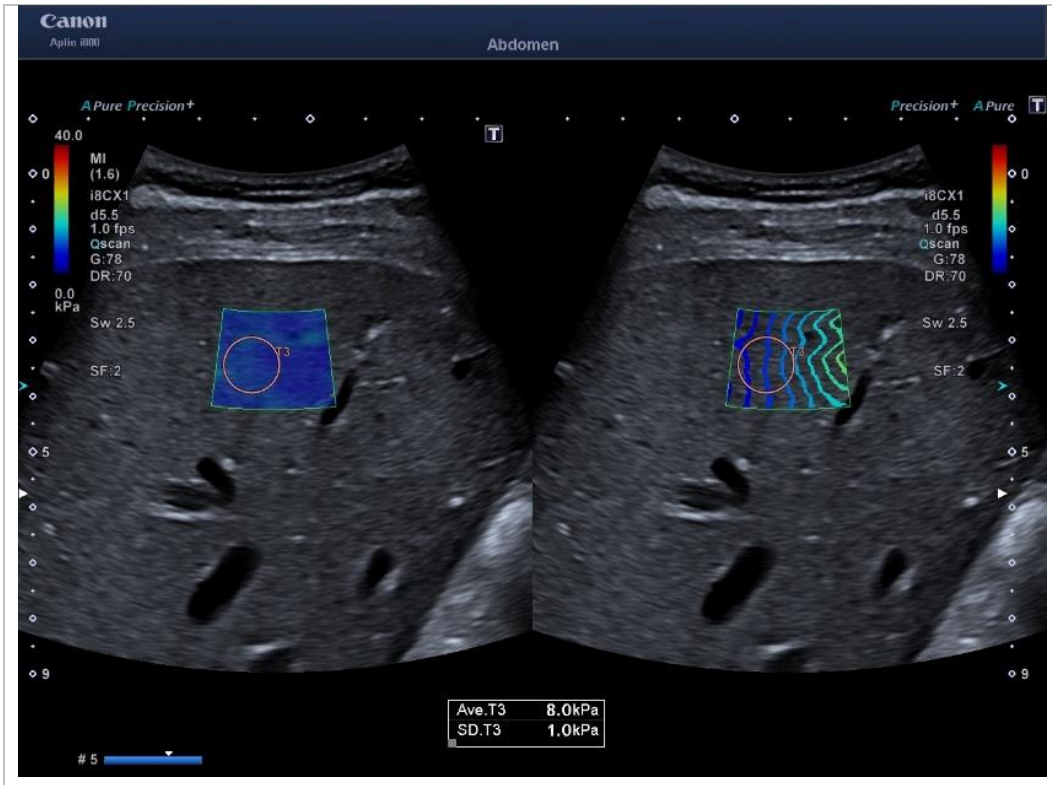
2.6. Transient elastography and ultrasound shear-wave elastography

Transient elastography (vibration-controlled transient elastography, VCTE) data before initiation of antiviral treatment were available in 8 patients.

The method was performed in the gastroenterology clinic of "St. Marina" hospital using the FibroScan® 402 or 502 device with two M and XL probes, according to the patient's habitus, mainly with the M-probe. Liver stiffness (LS) was defined as the mean (median) of at least 10 valid measurements in kPa units. Study quality rules were IQR < 30% or 1/3 of the mean. FibroScan® results range from 2.5 kPa to 75 kPa. Between 90-95% of healthy people without liver disease have a liver density <7.0 kPa (median is 5.3 kPa). The assessment of fibrosis stage F1-F4 is according to accepted cut-offs and normal elasticity is assumed at values of 5-5.5 kPa, F1 up to 7 kPa, F2 up to 10 kPa, F3 up to 14 kPa, and cirrhosis (F4) above 14 kPa (119). Estimation of steatosis was performed with the Controlled Attenuation Parameter (CAD) measured in dB/m with a measurement range between 100 and 400 dB/m. Estimation of the degree of steatosis S0-S3 was according to the cut-off values and S0 was confirmed at values < 238 dB/m, S1 at values from 238 to 259 dB/m, S2 at values from 260 to 290 dB/m and S3 at values > 290 dB/m (120). Figure 5 shows the protocol of TE in a patient with CHB and in a patient with HBV-related compensated cirrhosis.

Figure 5. Transient elastography (FibroScan) with assessment of liver stiffness and attenuation parameter, respectively steatosis.

Ultrasound 2D shear-wave elastography (2D-SWE) was performed in 68 patients using the Aplio i800 ultrasound system (Canon). Intercostally, when an optimal window and good B-mode imaging was achieved, ultrasound modality was initiated and, with breath-holding by at least 3 measurements, liver stiffness in kPa was calculated, again as mean ("median") and confidence interval, with IQR variation up to 30%. To control the measurement quality, a secondary, shear-wave propagation scheme was used to select the most optimal measurement region at parenchyma depths up to 5 cm from the transducer surface. Figure 6 shows the measurement of liver stiffness using the 2D-SWE method. The best LS cutoff values evaluated with 2D-SWE to assess the stage of liver fibrosis were F≥1 for LS >7.1 kPa; F≥2 for LS >7.8 kPa; F≥3 for LS >8 kPa and for F=4 for LS >11.5 kPa (121). Additionally, the Aplio i800 was used to assess the attenuation parameter of the US wave in depth (attenuation index, ATI) in order to assess the degree of hepatic steatosis. In accordance with the manufacturer's recommendations, the ATI was obtained in numerical value (units dB/cm/MHz) and as a degree of steatosis with ranges up to 0.63 for absent steatosis, up to 0.72 for mild steatosis, up to 0.82 for moderate steatosis and above 0.82 for severe steatosis.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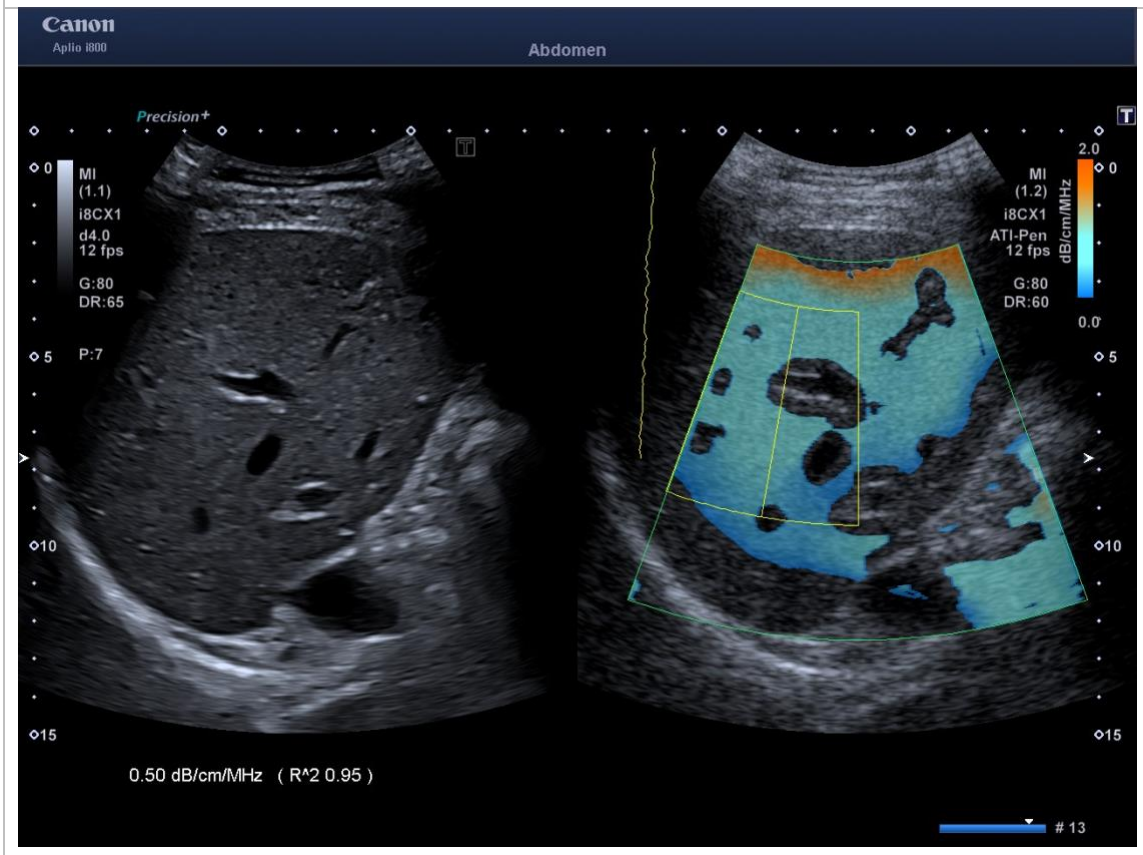
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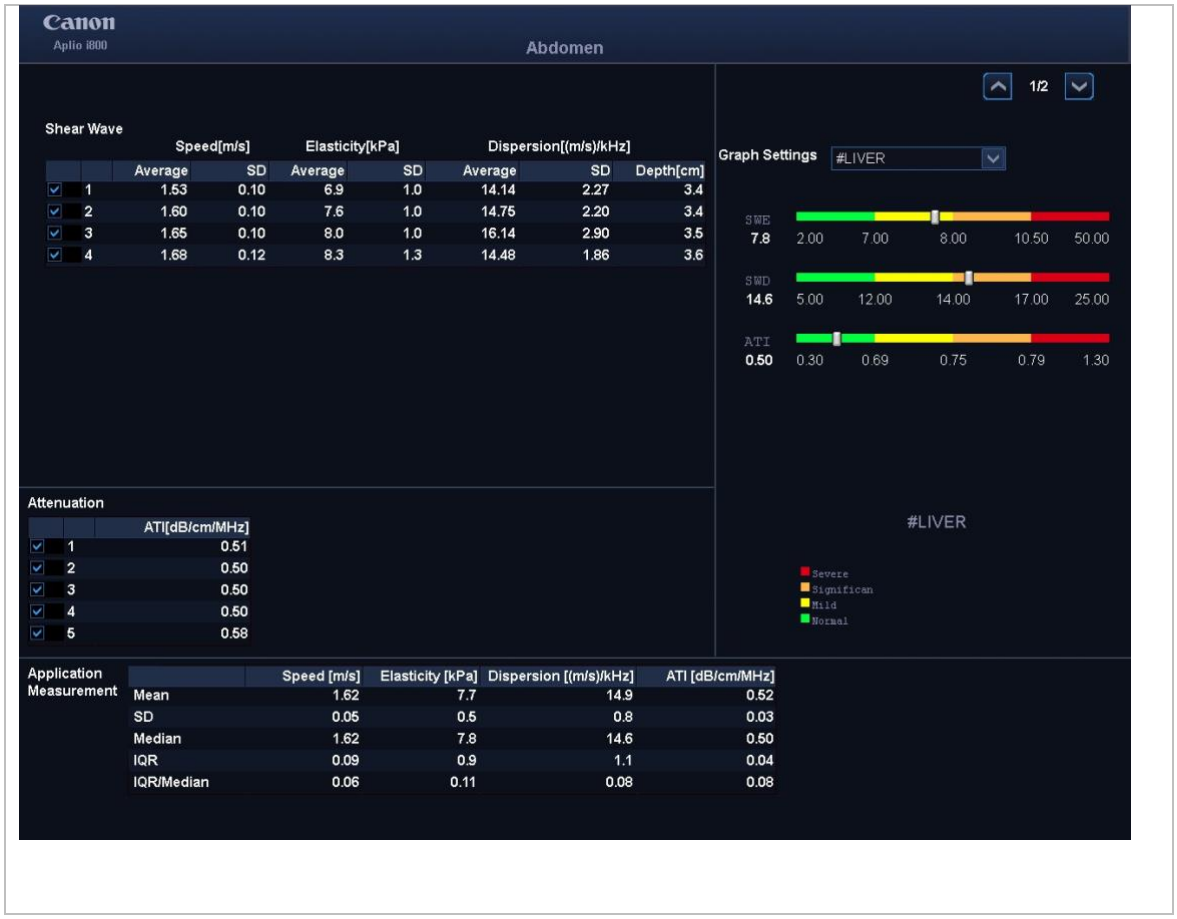

Figure 6. 2D-SWE elastography in a patient with CHB with calculation of liver stiffness (LS, kPa) and additional calculation of a steatosis parameter (ATI) as part of the multiparametric ultrasonography of patients with CHB.

Liver stiffness dynamics were assessed in patients with at least 2 LS measurements (n=39).

2.7. Fibrogastroduodenoscopy

Fibrogastroduodenoscopy (FGDS) was performed in patients with clinical, laboratory and imaging evidence of liver cirrhosis to evaluate the features of portal hypertension- presence of esophageal and gastric varices, presence of portal hypertensive gastro and duodenopathy (PHG, PHD). The examinations were performed with Fujifilm ED-580XT and Olympus EXERA II CV-180 endoscopic systems. Figure 7 shows images from the endoscopic protocol of a patient with HBV cirrhosis and evidence of moderate esophageal varices and PHG.

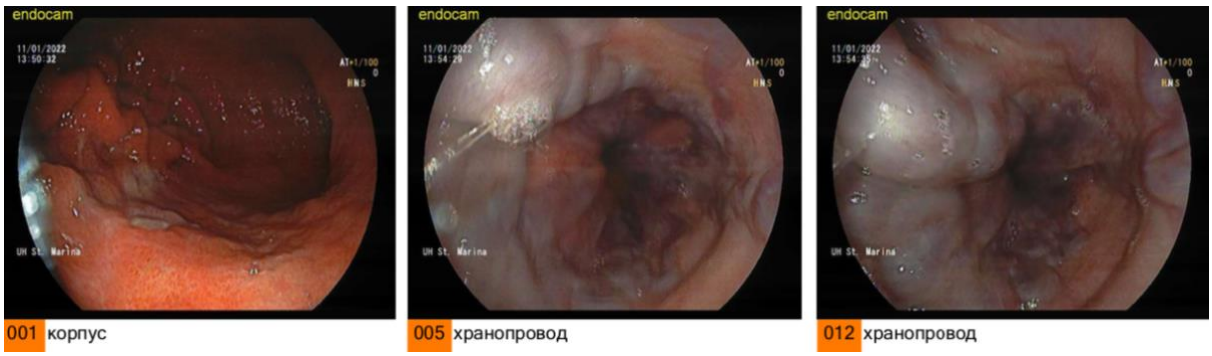

Figure 7: Endoscopic criteria for portal hypertension in a patient with HBV-cirrhosis.

3.Statistical methods

Data from the baseline characteristics of the included group of patients and their follow-up during the course of treatment were recorded in .xls format, where the statistical analytical methods and the presentation of the data in graphs were also performed using Microsoft Excel software. The following statistical analysis methods were applied:

3.1 Determination of statistical quantities: mean; minimum (min) and maximum (max) values; standard deviation (SD) in data classification, presentation of their summary characteristics, and their frequency distribution.

3.2 Statistical evaluation (statistical inferences and conclusions):

- Independent t-test under normal data distribution for pairwise parameter estimation of dynamics in a continuous variable and when comparing quantitative means of two samples;
- Non-parametric methods - in cases where the significance of the trait is represented in the nominal, rank or ordinal scale: Mann-Whitney and Wilcoxon test;
- One-factor analysis of variance (ANOVA) to compare means of more than two samples; Tukey's post-hoc test was used.

3.3 Statistical study of dependencies and hypothesis testing:

- Chi-square (χ^2 -) method - when both variables are on the nominal or ordinal scale with contingency coefficients calculated and to look for significant differences in the frequency representation of categorical values.

- correlation analysis to examine the strength of the relationship between two variables by calculating the correlation coefficient, (r, Pearson's coefficient); single and multiple correlations;

We interpreted the resulting correlation coefficients based on: r=0 (no association); r=0.0-0.1 (very weak association); r=0.1-0.3 (weak association); r=0.3-0.5 (moderate association); r=0.5-0.7 (significant association); r=0.7-0.9 (strong association); r=0.9-1.0 (very strong association).

- Non-parametric (biserial) correlation analysis for variables with characteristic categories;
- Multivariate (multiple) regression analysis - when examining the joint influence of 2 or more indicators on an outcome; calculating regression coefficients (β_1); coefficient of multiple determination (R^2); modelling relationships by creating a regression model.

3.4. Graphical analysis - graphical display of statistical data

A statistically significant difference was reported at the standard significance level, i.e. at $p \leq 0.05$. All analyses were performed by the PhD student.

Results and discussion

1. Baseline characteristics of patients with chronic HBV infection

1.1 General characteristics

The study included 84 consecutive patients with chronic HBV infection from the Gastroenterology Clinic registry: 29 women and 55 men, mean age 57 years (min 29, max 81 years, SD 12.8 years). The **prevalence of HBeAg-negative CHB was dominant, n=75 (89.3%), with the remaining 9 patients (10.7%) having baseline HBeAg-positive CHB.**

The mean age of HBeAg-positive patients was 43.1 years (min 29, max 62, SD 12.1). The age distribution of HBeAg(+) CHB was: 1 patient in the 20-29 years group, 3 patients in the 30-39 years group, 2 patients in the 40-49 years group, 2 patients in the 50-59 years group, and 1 patient in the 60-69 years group (Figure 8).

The mean age of HBeAg-negative patients was 58.1 years (min 33, max 81, SD 12.04). The age distribution of HBeAg (-) CHB was as follows: 5 patients in the 30-39 years group, 13 patients in the 40-49 years group, 21 patients in the 50-59 years group, 22 patients in the 60-69 years group, 11 patients in the 70-79 years group, and 3 patients in the 80+ years group (Figure 9). **There was a statistically significant difference in the age of the patients, compared to baseline HBeAg, in favor of younger age of HBeAg (+) CHB patients (p=0.00003).**

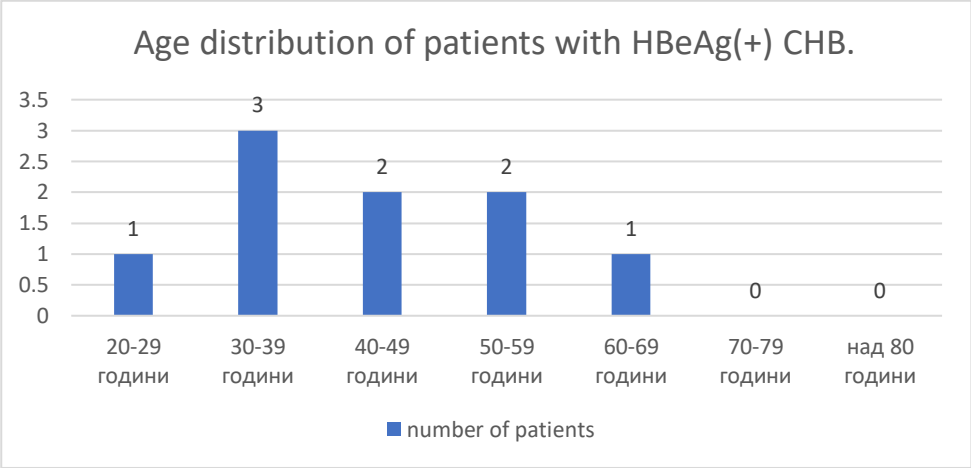

Figure 8: Age distribution of patients with HBeAg (+) CHB.

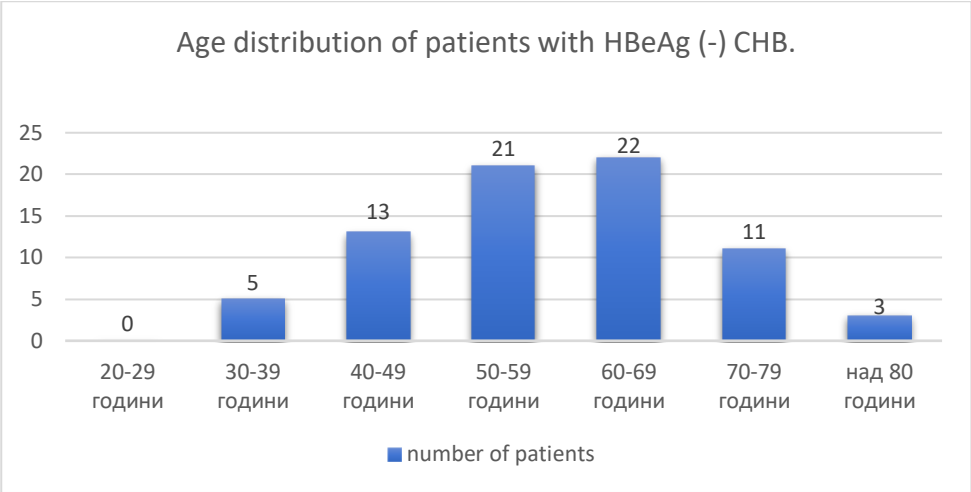

Figure 9. Age distribution of patients with HBeAg (-) CHB.

Data on prevalent acute hepatitis B were available for 5 patients (5.8%) and all of them were in the HBeAg (-) CHB group. Thus, the age of infection in most patients is unknown, but the time since diagnosis of HBsAg (+) is on average 12.5 years (between 1 and 25 years). The triggers for HBsAg(+) detection are most commonly elevated AST and ALT values on routine or prophylactic laboratory tests ordered by a general practitioner, screening for viral hepatitis prior to initiation of immunosuppressive therapy, or a history of acute viral hepatitis B.

Perinatal infection could be assumed in 4 patients (4.7%).

Familial HBV infection (close family members with HBV) was present in 9 patients (10.7%).

Four patients (4.7%) reported systemic consumption of more than 1 or 2 drinks/day during antiviral treatment, including 2 men and 2 women.

Baseline evidence of overweight or obesity was present in 13 patients (15.4%).

1.2.History and physical examination data

Most patients (n=60, 71.4%) had asymptomatic course of CHB. In 28.5% of cases, patients described intermittent, episodic right upper abdominal pain. We also included 1 patient who was hospitalized for a severe onset of acute hepatic decompensation, with icterus, dense significant hepatomegaly, and large ascites, who subsequently died from the complications of liver cirrhosis: acute thrombosis of the v. portae, spontaneous bacterial peritonitis, hepatic encephalopathy, and acute kidney injury. Repeated hospitalizations in the GE clinic in this patient excluded virological breakthrough in the course of lamivudine therapy and new viral co-infection and malignization of cirrhosis.

1.3.Comorbidities

Regarding comorbidities, these were recorded in 44.4% of patients with HBeAg (+) CHB and in 73.6% of patients with HBeAg (-) CHB. In the HBeAg(+) CHB group, the following diseases were described: gastroesophageal reflux disease (n=1); peptic ulcer disease (n=1); type 2 DM (n=1); arterial hypetension (n=1). Of interest was the combination of HBeAg(+) CHB and autoimmune pathology: 1 patient had Hashimoto's thyroiditis and another had autoimmune vasculitis with a course similar to Schönlein-Henoch purpura and was on maintenance immunosuppressive steroid therapy. Patients with HBeAg(+) CHB lacked a history of cancer.

In the group of patients with HBeAg (-) CHB: 37.3% had arterial hypertension (n=28); 10.6% had dyslipidemia (n=8); 1.3% had peptic ulcer disease (n=1), 13.3% had rheumatoid arthritis (n=10), 2.6% had psoriasis (n=2), 1.3% had diffuse membranous glomerulonephritis (n=1), 1.3% had Hashimoto's thyroiditis (n=1), 1.3% had Crohn's disease (n=1), 1.3% were with benign prostatic hyperplasia (n=1), 1.3% with gout (n=1), 1.3% with Parkinson's disease (n=1), 1.3% with moderate mental retardation (n=1), 1.3% with paranoid schizophrenia (n=1), 1.3% with epilepsy (n=1), 1.3% with bronchial asthma (n=1), and 1.3% with terminal renal failure undergoing chronic dialysis (n=1).

5 patients (6.6%) with HBeAg (-) CHB had a history of cancer:

Endometrial carcinoma in combination with Non-Hodgkin B-cell lymphoma (n=1); breast cancer (n=1); colon cancer (n=1); sinonasal carcinoma (n=1); pheochromocytoma (n=1). Three neoplasms with extrahepatic localization were recorded during treatment- Non-Hodgkin's B-cell lymphoma, pheochromocytoma and sinonasal carcinoma. Additionally, a patient with hepatocellular carcinoma diagnosed prior to antiviral treatment participated in the study.

The prevalence of DM in the observed group of 84 patients was 15.4% (n=13), and all patients had DM type 2.

1.4.Indications for antiviral treatment

In the patient group studied, indications for antiviral treatment included HBeAg (-) active CHB, HBeAg (+) active CHB, baseline liver cirrhosis, and immunosuppressive or biologic therapy for chronic HBV infection. Correspondingly, HBeAg (+) active CHB was present in n=8 (9.5%); HBeAg (-) active CHB in n=41 (48.8%); liver cirrhosis in n=24 (28.5%) and immunosuppressive or biological treatment for chronic

HBV infection in n=11 (13%). Patients on preventive NA had HBeAg (-) chronic HBV infection. There were no cases with baseline positive HBeAg in the cirrhosis group.

1.5.Baseline viral load

The mean baseline HBV DNA was 55 427 912 IU/ml (min 7 IU/ml, max 955 112 640 IU/ml, SD 191 415 874 IU/ml). Patients with baseline HBV DNA < 2,000 IU/ml accounted for 18 (21.4%), those with HBV DNA between 2,000-20,000 IU/ml accounted for 10 (12%), and those with **HBV DNA > 20,000 IU/ml** accounted for **the largest proportion of those treated, 56 (66.6%)** (Figure 10).

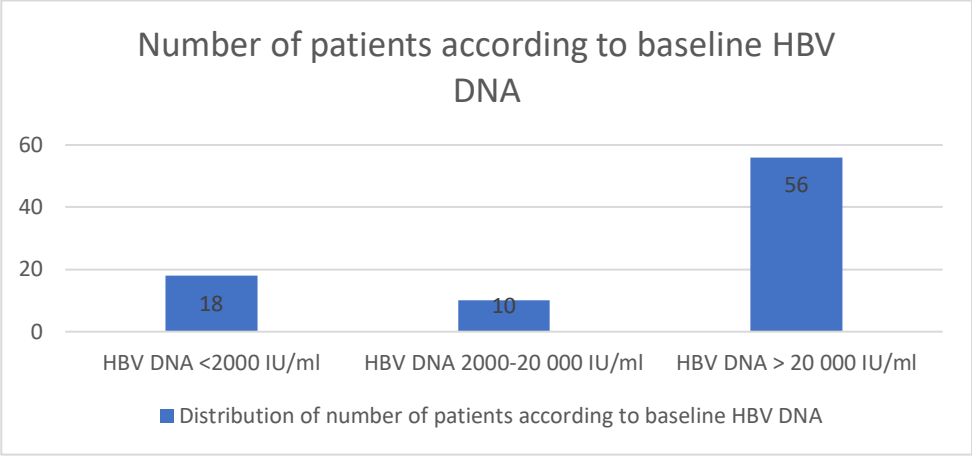

Figure 10. Distribution of number of patients according to viral load categories.

The mean baseline HBV DNA level in patients with HBeAg (+) was 240 135 294 IU/ml (min 267 IU/ml, max 955 112 640 IU/ml, SD=397 152 669 IU/ml) and in patients with HBeAg (-) was 33 554 670 IU/ml (min 7 IU/ml, max 867 000 000 IU/ml, SD=140 119 400 IU/ml). **There was a statistically significant difference in baseline HBV DNA according to baseline HBeAg, with a higher viral load detected before initiation of therapy in the HBeAg (+) CHB group (p=0.0008)** (Table 2).

1.6.Baseline biochemical activity

Mean baseline ALT was 67 U/L (SD 158.4 U/L, min 10 U/L, max 958 U/L). ALT was ≤ of 40 U/L in 32% (n=27) and 60% (n=50) had a biochemical activity, meaning ALT between 40 and 400 U/L. The proportion of patients with marked transaminase activity, ALT > 400 U/L (10xULN) was 8% (n=7) (Figure 11). Severe cytolysis was not associated with icterus in any of the patients. **Correlation analysis showed no association between baseline ALT level and baseline HBV DNA (r=0.02).**

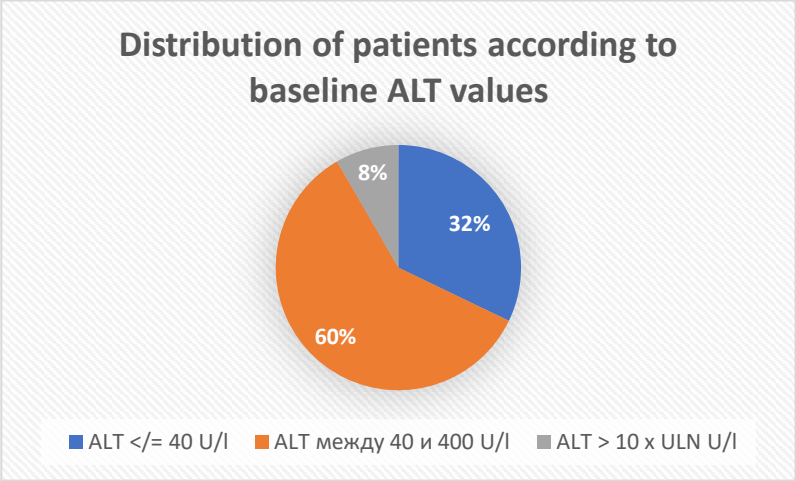

Figure 11. Distribution of patients according to baseline TA activity.

The mean baseline ALT in patients with HBeAg (+) was 189 U/l (min 56 U/l, max 530 U/l, SD 154 U/l) and in patients with HBeAg (-) was 128 U/l (min 10.3, max 958 U/l, SD 174 U/l). Despite the trend for higher biochemical activity in HBeAg (+) CHB, statistical analysis revealed no significant difference in baseline ALT value relative to HBeAg status (p=0.15) (Table 7).

Table 2 summarizes the baseline characteristics of the enrolled patients, including the type of current NA treatment, with comparisons according to HBeAg status. Our data are consistent with those reported by other Bulgarian collectives. Zhelev D. reported a higher proportion of HBeAg (-) patients, lower viremia in HBeAg, younger age of HBeAg (+) patients, but similar transaminase activity in HBeAg (+) and HBeAg (-). Patients with HBeAg (-) had more advanced fibrosis, supporting the notion that HBeAg (-) CHB occurs later in the course of chronic HBV infection, after HBeAg seroconversion (122). Gerdzhikova K. also reported a lower rate of HBeAg (+) among the group of patients with CHB-associated cirrhosis she examined (123).

Parameter	HBeAg (+) status	HBeAg (-) status	p value
Number of patients included, n (%)	9 (10.7%)	75 (89.3%)	
Age (years)*	43.1 +/- 12.1	58.1 +/- 12.04	p=0.00003
Baseline HBV DNA (IU/ml)*	240 135 294 +/- 397 152 669	33 554 670 +/- 140 119 400	p=0.0008
Baseline ALT (U/l)*	189 +/- 154	128 +/- 174	p=0.15
Phase of chronic HBV infection, n (%) **-chronic active hepatitis** **-chronic HBV infection** **-liver cirrhosis**	 8 (89%) 1 (11%)	 41 (54.7%) 10 (13.3%) 24 (32%)	
Baseline stage of liver disease, n (%) **F<2** **F 2≥** **F4**	 5 (56%) 4 (44%)	 24 (32%) 27 (36%) 24 (32%)	
Type of NA, n (%) **LAM** **ETV** **TDF**	 1 (11%) 8 (89%)	 21 (28%) 2 (3%) 52 (69%)	

Table 2. Baseline characteristics of patients with HBeAg (+) and HBeAg (-) CHB

*Data presented as **mean +/- standard deviation**

1.7.Baseline stage of liver disease

Patients with early stage of liver disease (absent or insignificant fibrosis) (F<2) were 29 (34%), those with significant/septal or bridging fibrosis (F≥ 2) were 31 (37%), and those with cirrhosis (F4) were 24 (29%) (Figure 12). All patients with cirrhosis had HBeAg (-) status.

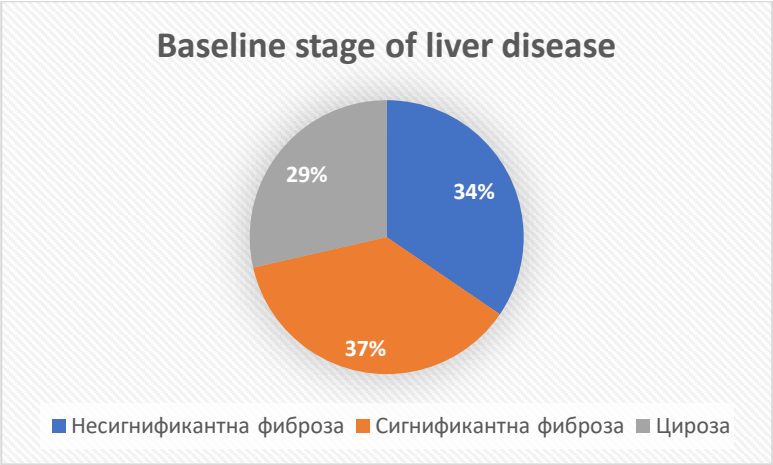

Figure 12. Baseline stage of liver disease.

At baseline, the class of cirrhosis was calculated according to the Child-Turcotte-Pugh system. Most patients were found to have compensated, class "A" cirrhosis (n=16, 67%). Decompensated cirrhosis at baseline, prior to initiation of NA treatment with class B on CTB was present in 21% (n=5) and class "C" in 12% (n=3). For better prognostic assessment, MELD Na scoring was also calculated using an online calculator (MDCalc). In half of the cirrhosis patients (n=12) the MELD Na score was≤ 9 points, in 42% (n=10) the MELD Na score was between 10 and 19 points and in 2 patients (8%) the MELD-Na score was high, between 20 and 29 points, respectively.

A comparative analysis of baseline viral load was performed according to disease stage. Thus, the mean baseline HBV DNA in patients with nonsignificant fibrosis was 91 732 344 IU/ml (min 7 IU/ml, max 955 112 640 IU/ml, SD 273 721 340 IU/ml), in patients with significant fibrosis was 51 020 951 IU/ml (min 169 IU/ml, max 777 622 784 IU/ml, SD 152 509 205 IU/ml) and in patients with cirrhosis was 15 739 695 IU/ml (min 7 IU/ml, max 135 969 024 IU/ml, SD 35 989 024 IU/ml). **Therefore, patients with early stage liver disease had a statistically significant higher viral load. In contrast, patients with cirrhosis had the lowest baseline HBV DNA level** (p=0.04, ANOVA test) (Figure 13).

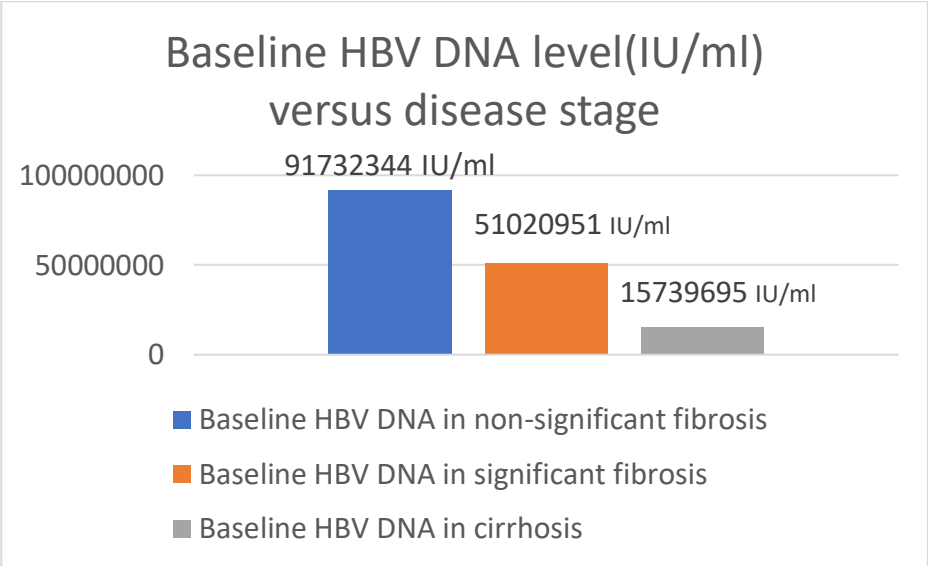

Figure 13. Baseline HBV DNA according to the stage of liver disease.

The level of HBV DNA is very different in the phases of chronic HBV infection. On the other hand, data in the literature are conflicting regarding the relationship between HBV replication rate and disease stage. HBV DNA level is not associated with histologically proven stage of fibrosis, including in HBeAg(-)CHB (124). In HBeAg(-) CHB, there is also evidence of an increase in viral load with increasing of fibrosis degree (125). High viral load is one of the strongest predictors of liver disease progression and the development of complications, including HCC. HBV DNA level has been assumed to be a factor in HCC, independent of HBeAg status, ALT activity, and evidence of cirrhosis (126). On the other hand, it is possible that as liver cirrhosis progresses with the development of decompensation, HBV DNA levels may decrease due to insufficient resources to support HBV replication or increased clearance of HBV into the circulation. In our study, we found a significantly lower baseline viral load in the cirrhosis group, which may be due to the small number of patients with advanced liver disease enrolled, as well as due to the initiation of antiviral treatment in every patient with cirrhosis regardless of HBV DNA level. In comparison, in the study by Gerdzhikova K., the mean HBV DNA level in patients with cirrhosis, mostly compensated, was 7 489 304 IU/ml and the incidence of cases with HBV DNA < 2000 IU/ml was only 25%, but again the analysis was performed in a small group of patients (123). Assessment of the dynamics of HBV DNA levels in the spontaneous evolution of chronic HBV infection is difficult because of the heterogeneity of patient populations in different dynamic phases of infection, the influence of HBV genotype, and the need to initiate antiviral treatment.

Biochemical activity was assessed in patients with different stages of the disease. Thus, the mean baseline ALT was 132 U/L in patients with F<2 (min 14 U/L, max 958

U/L, SD 202 U/L), 153 U/L in the F≥ 2 group (min 12 U/L, max 615 U/L, SD 134 U/L), and 117 U/L (min 10.3 U/L, max 559 U/L, SD 115 U/L) in patients with cirrhosis. Therefore, there was no statistically significant difference between baseline biochemical activity values in patients with early, intermediate and advanced disease (f=0.4, p=0.6, ANOVA analysis with Tukey's post hoc test) (Figure 14). Overall, those indicated for antiviral treatment had moderate transaminase activity.

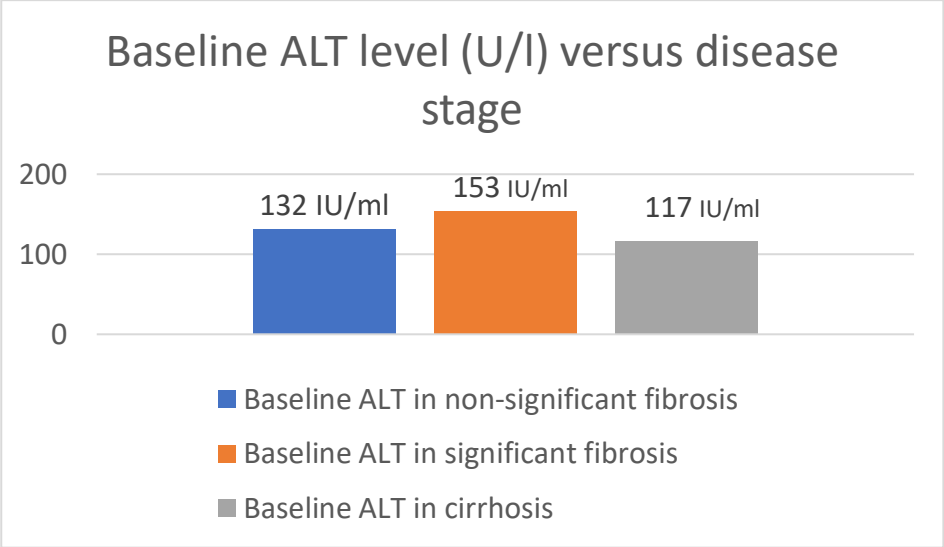

Figure 14. Baseline ALT level, according to disease stage

1.8.Non-invasive, indirect markers of fibrosis (NITs) before treatment

At baseline, prior to initiation of antiviral therapy, APRI and FIB-4. The mean APRI for the entire group (n=85) was 1.49 (min 0.10, max 13.5, SD 2.3). The mean FIB-4 was 2.54 (min 0.4, max 14.6, SD 3.02). **Disease stage (F0-F4) was associated with APRI value (moderate positive association, r=0.5) and FIB-4 (moderate positive association, r=0.6,** nonparametric correlation analysis). Additionally, in the fibrosis categories, patients had a statistically significant difference in indirect serum markers: the mean APRI value in patients with F<2 was 0.56 (min 0.2, max 4.1, SD 0.69), at F≥ 2 e 1.43 (min 0.2, max 9, SD 1.9) and at F4 e 2.73 (min 0.1, max 13.5, SD 3.32); the mean value of FIB-4 in patients with F<2 was 1.05 (min 0.4, max 2.15, SD 0.57), at F≥ 2 e 1.78 (min 0.46, max 10.5, SD 1.79) and at F4 e 5.3 (min 0.52, max 14.8, SD 3.9). Figure 15 shows the level of biomarkers of fibrosis, according to the baseline invasive assessment of patients with HBV infection by liver biopsy or with definite evidence of portal hypertension/cirrhosis. Although in a small observational sample, this analysis suggests that the **widely available and easily calculable APRI and FIB-4 indices are useful for baseline assessment of patients with chronic disease in HBV infection, with better informativeness for the FIB-4 marker.**

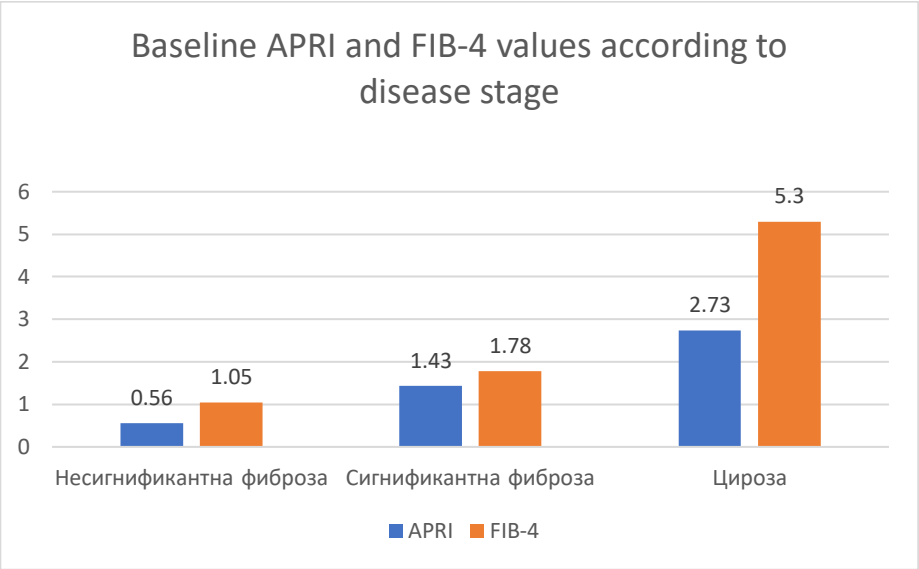

Figure 15. Baseline APRI and FIB-4 values according to disease stage.

The use of indirect serum markers of fibrosis (NITs) APRI or FIB-4 in the evaluation of hepatitis B is controversial, in contrast to the extensive data in patients with HCV infection. Overall, threshold values of APRI >2.0 and FIB-4 >3.25 can be used to confirm cirrhosis, and APRI <1.0 and FIB-4 <1.45, respectively, to rule out cirrhosis. In HBV infection, however, the indicated values for FIB-4 and APRI placed 23% and 24% of patients, respectively, in the so-called "gray zone" in which they could not be classified: 41% and 45% of cases with cirrhosis were incorrectly defined as "no evidence of cirrhosis" by application of the routine cutoff values of FIB-4 and APRI (127, 128). The dissertation of Gerdzhikova K. indicated that the FIB-4 value in patients with HBV cirrhosis in more than 70% was less than 3.25 (123). Therefore, other rules for the use of NITs should be developed in hepatitis B, and one suggestion is to use a FIB-4 value ≤ 0.70 to exclude cirrhosis in patients with HBV infection and age over 30 years, according to data from the SONIC-B study (127). The reason for the suboptimal diagnostic accuracy of APRI and FIB-4 for classifying patients with septic, advanced fibrosis or cirrhosis is the baseline high transaminase activity and AST, respectively. Further studies are needed to define threshold values for NITs in different phases of HBV chronic infection.

1.9. Antiviral treatment

The current NA therapy is the first course of treatment in 54 patients (63.5%) - "NA naive".

Prior antiviral treatment was reported in:

- 14 patients (16.6%) with conventional or PEG-IFN-alpha, within 6 months (n=2), with discontinuation due to lack of biochemical and/or virological response) or 12 months (n=12), achieving remission over a period of 3 months to 10 years.
- 4 patients with TBV as first course of antiviral therapy, the drug was discontinued due to unavailability in pharmacy network in 3 patients and due to virological breakthrough and proven drug resistance to nucleoside analogues in 1 patient. In 3 patients, TBV was a second course of antiviral treatment after a failed PEG-IFN-alpha regimen or after LAM replacement; again, TBV was discontinued in these cases because of drug unavailability (n=2) or resistance (n=1). In summary, virologic breakthrough with prior TBV treatment was recorded in 2 of 7 patients in the study (28%).
- 12 patients on lamivudine (LAM) discontinued due to virological breakthrough and proven resistance to nucleoside analogues by molecular biology testing (most common L180M, M204V, I80L, I204M mutations) in n=11 or in the course of a physician panel discussion to replace the drug with TBV in n=1.

Thus, 16 patients (19%) had prior NA exposure, 13 of whom had evidence of genetic resistance to nucleoside analogues.

The current course of treatment was Lamivudine (LAM) 100 mg in 26% (n=22), Entecavir (ETV) 0.5 mg in 2% (n=2) and Tenofovir (TDF) 245 mg in 72% (n=60). Figure 16 shows the type of NA treatment administered. Motivation for initiation of first-generation NA-LAM antiviral treatment was the need for rapid inhibition of viral replication in patients with advanced liver disease and low viral load, and for prophylaxis of hepatitis B flare during the course of immunosuppressive, biologic, or cytostatic therapy.

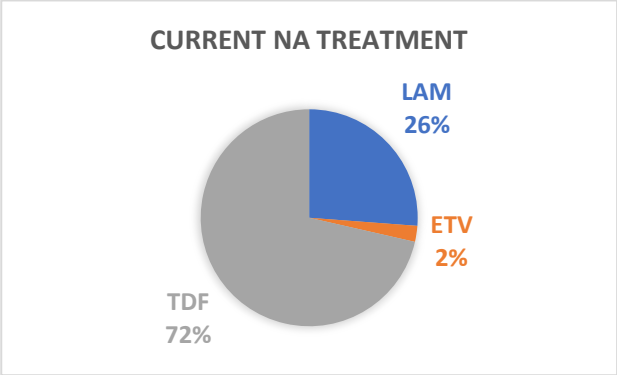

Figure 16. Current NA treatment

The Clinic of Gastroenterology at the University Hospital "St. Marina" is a referral center for follow-up and treatment of patients with chronic viral hepatitis with traditions since the beginning of antiviral treatment in the 1990s. A well-established registry is the basis for accumulated experience and a prerequisite for correct decisions. The present sample of 84 consecutive patients undergoing NA therapy is a small

fraction of the total number of cases in the centre. The lack of clinical experience in our country with the application of TAF should be noted.

The duration of NA therapy in the included patients varied, ranging between 1 and 20 years, with an average of 8.2 years. To systematize the patients according to the duration of NA admission, they were categorized into groups:

- NA therapy of 1 to 3 years duration: 11% (n=9);
- NA therapy over 3 years: 89% (n=75); of these, 39% (n=29) had NA therapy≥ 10 years.

Therefore, the present study included patients with long-term NA intake, with a high proportion, over 1/3 cases with NA suppression for more than 10 years.

At this stage, in Bulgaria there is a lack of observation of patients with NA long-term therapy over 5 years.

We do not yet have sufficient literature data regarding the effect of long-term NA intake and so-called "lifetime" NA therapy has not been well studied. It is possible that prolongation of NA therapy beyond the 10th year does not result in a significant increase in HBsAg negativity rates. Thus, Yao-Chun et al. reported a 2.1% loss of HBsAg with 10 years of high-barrier NA therapy, compared with an incidence of 1.2% at year 5 of NA treatment. On the other hand, as treatment is prolonged, patient compliance issues arise, loss to follow-up is described, and not least, financial costs cumulate causing a burden on health systems (39, 41, 129). Regarding that, there is a necessity for the development of criteria for safe NA cessation. Many reports have emerged favoring short-term NA therapy and evidence of an increase in HBsAg seroclearance rates with short-term NA administration. However, the risk of virologic and biochemical breakthrough with NA cessation remains high (108, 109, 110, 111). With this rationale, hepatologists are united in the concept of prolonged, lifelong treatment for advanced hepatitis B and cirrhosis, and scientific debate continues as to whether safe discontinuation of NA is feasible in selected patients (9).

2. Analysis of virological response regarding HBV DNA dynamics in patients with HBeAg (-) CHB

2.1. Initial virological response, at 6-12 months from the initiation of antiviral treatment

Viral suppression (defined as HBV DNA < 10 IU/ml, according to EASL recommendations) at months 6-12 of ongoing treatment was reported in 68% (n=51) of patients with HBeAg(-) CHB. According to the type of drug administered, the "early" virological response differed, with viral suppression achieved by 80.9% (n=17) of LMV-treated and 63.0% of NA-treated patients with a high barrier to resistance-

TDF/ETV (n=34). Importantly, 76.1% (n=16) of patients on LAM treatment had baseline HBV DNA < 20,000 IU/ml, compared with 20.3% (n=11) of patients on TDF or ETV treatment. Table 3 shows the incidence of baseline viral suppression, defined as HBV DNA < 10 IU/ml, according to the type of drug administered.

Viral suppression at 6-12 months of NA: 51/75 (68%)	
LAM (n=21)	80.9% (n=17)
TDF/ETV (n=54)	63% (n=34)

Table 3. Viral suppression at 6-12 months, according to NA administered

Undetectable HBV DNA at month 6-12 of treatment was recorded in 65% (n=49) of patients with HBeAg (-) CHB. Again, a higher proportion of HBV DNA negativity was detected in the group receiving LAM: 76.1% (n=16) versus 61.1% (n=33) of those treated with the potent TDF or ETV, data shown in Table 4.

Undetectable HBV DNA at 6-12 months, according to NA administered. 49/75 (65%)	
LAM (n=21)	76.1% (n=16)
172	61.1 % (n=33)

Table 4. Undetectable HBV DNA at month 6-12, according to NA administered

In summary, data from the literature show an initial virologic response in 72-73% and 90-93% of patients with HBeAg(-) CHB after 52 weeks of treatment with LAM or TDF/ETV (130-148). The higher initial virologic response observed with LAM in our study is associated with the significantly lower baseline viral load and the inclusion of preventive LAM in patients with chronic inactive hepatitis B and in the treatment of patients with liver cirrhosis and minimal HBV replication.

2.2. Virological response during follow-up with prolonged NA intake

Evaluation of patients during the course of treatment revealed viral suppression (HBV DNA < 10 IU/ml) in a total of 92% (n=69), 85.7% (n=18) and 94.4% (n=51) of those treated with LAM or TDF/ETV, respectively. The data on viral suppression with continuous therapy are summarized in Table 5.

Viral suppression at the end of follow-up, according to NA administered. 69/75 (92%)	
LAM (n=21)	85.7% (n=18)
TDF/ETV (n=54)	94.4 % (n=51)

Table 5. Viral suppression at the end of follow-up, according to NA administered.

The rate of HBV DNA negativity at the last visit in the GE clinic for antiviral treatment evaluation was 87% (n=65). Undetectable (below detection level) HBV DNA was found, with a higher frequency in patients taking TDF/ETV compared to patients taking LAM, 89% vs. 80.9%, respectively. Therefore, the initial favorable dynamics in terms of viral replication inhibition by LAM is lost with prolonged therapy and becomes a prerequisite for the development of drug resistance. A number of authors report the advantage of high-barrier NAs over low-barrier NAs in the direction of higher virological response, given the increasing drug resistance. Gerdzhikova K. also reported the lowest antiviral activity of LAM (70.7%), compared to the optimal of ETV/TDF (80-100%). A number of investigators have highlighted the advantage of high-barrier NAs, namely that treatment with TDF for 3-4 years results in viral suppression in 92-100% of patients, and 99% have viral suppression after 8 years of TDF treatment (135-139).

Table 6 shows the virological response assessed as sustained HBV DNA negativity over the course of NA therapy.

Undetectable HBV DNA at the end of follow-up, according to NA 65/75 (87%)	
LAM (n=21)	80.9% (n=17)
TDF/ETV (n=54)	89 % (n=48)

Table 6: Undetectable HBV DNA at the end of follow-up, according to NA applied.

3. Analysis of biochemical response (ALT activity) in patients with HBeAg(-) CHB

3.1. Biochemical response at 6th -12th month from the initiation of antiviral treatment

At 6th -12th month of NA treatment, 87% of patients had an initial biochemical response (assessed as ALT≤ 1.25 xULN, according to EASL recommendations), figure 26. The difference in mean ALT at baseline and at month 6-12 of treatment initiation was statistically significant (p=0.00001). The remaining 13% of treated subjects had

persistent biochemical activity (ALT > 1.25 xULN) in the first year of NA treatment (Figure 17).

Pearson correlation analysis showed a moderate association between HBV DNA level and ALT at 6-12 months (r=0.54, p<0.00001).

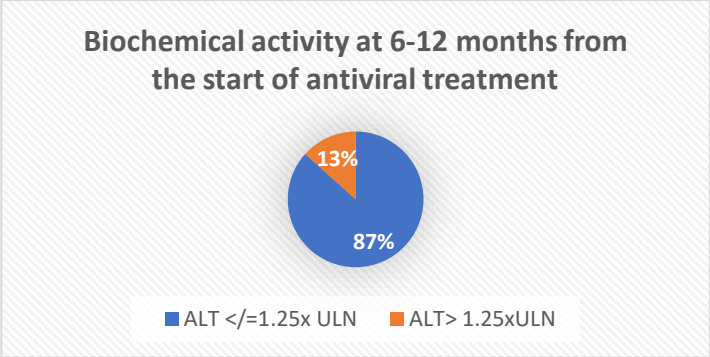

Figure 17. Biochemical activity at 6-12 month from the start of antiviral treatment

The analysis according to the drug administered showed that 90.4% (n=19) and 85.1% (n=46) of patients on LAM or TDF/ETV treatment achieved an initial biochemical response, respectively, and the results are shown in Table 7.

Biochemical response at 6-12 month, according to NA administered. 65/75 (87%)	
LAM (n=21)	90.4% (n=19)
TDF/ETV (n=54)	85.1% (n=46)

Table 7: Biochemical response (ALT < 1.25xULN) at month 6-12, according to NA administered

Our data show a high initial biochemical response rate that is higher in the LAM group, which we again attribute to the fact that most patients receiving LAM have chronic inactive hepatitis or liver cirrhosis, and their baseline biochemical activity is significantly lower than that of patients with chronic active hepatitis who predominantly start with high-barrier NA. Data from leading investigators show similar biochemical responses regardless of NA type: 71-79% and 76-78% of patients with HBeAg(-)CHB had an initial virologic response, on a 52-week treatment with LAM or TDF/ETV, respectively (130-148).

3.2. Biochemical response in the course of long-term NA therapy in (-) HBeAg CHB

Over the course of antiviral treatment with NA for an average of 8 years, the biochemical response rate increased slightly from an initial rate of 87%, reaching 91%, figure 18. The dynamics of ALT activity at baseline and at the end of follow-up was statistically significant in the direction of reduction (p=0.00001). There was no association between ALT level and that of viral load at the patients' last visit during the course of therapy (Pearson r=-0.04, p=0.7). Analyzing the reasons for lack of biochemical response, we found metabolic associated fatty liver disease (MAFLD) in 7 patients and hepatic infiltration in the presence of hepatocellular carcinoma in 1 patient.

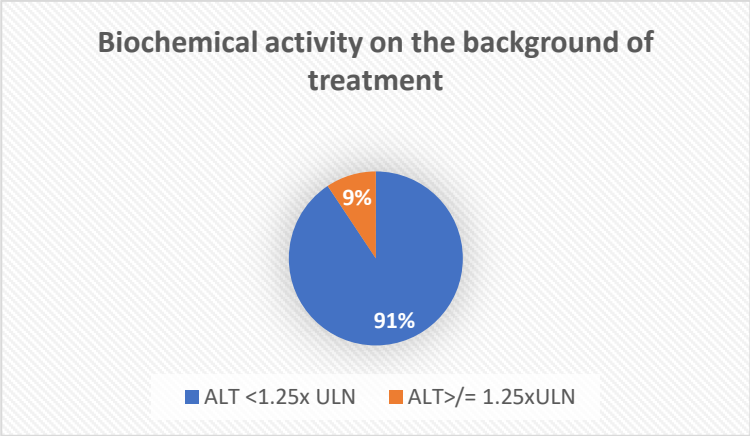

Figure 18. Biochemical activity on treatment background

In the included group of patients, the biochemical response rates for lamivudine and TDF/ETV treatment were 95% (n=20) and 89% (n=48), respectively (Table 8).

Biochemical response in the course of follow-up, according to NA administered 68/75 (91%)	
LAM (n=21)	95% (n=20)
TDF/ETV (n=54)	89% (n=48)

Table 8. Biochemical response during follow-up, according to the NA administered.

The advantage of a higher biochemical response over the course of prolonged therapy was maintained in the LAM group, but the biochemical response rate in the TDF/ETV group also rose. We report a biochemical response rate at the end of follow-up similar to data reported in the literature. Authors report that long-term treatment

with TDF results in ALT normalization in 88% of cases (134). In cases of persistent biochemical activity against a background of viral suppression, cofactors of liver injury, such as coinfection with another hepatotropic virus, concomitant MAFLD, and alcohol consumption, should be actively sought. Patients who do not normalize ALT are at higher risk of liver complications and increased risk of HCC. Authors report a cumulative incidence of liver complications after six years of 3.51% in patients with normal ALT and 5.70% in the group without normal ALT (35).

4. Analysis of virological response by HBV DNA in patients with HBeAg (+) CHB

4.1. Virological response at 6-12 months after initiation of antiviral treatment and during long-term therapy

Viral suppression (HBV DNA < 10 IU/ml) at months 6-12 of treatment was reported in only 22% (n=2) of patients with HBeAg (+) CHB. HBV DNA was undetectable at months 6-12 similarly in the same 2 patients, 22%.

Continuation of NA therapy increased the incidence of viral suppression to 78% (n=7), and HBV DNA was below the level of detection in 6 patients (66%) at the last visit during the course of treatment. Figure 19 compares the early virological response with viral replication inhibition with continued treatment.

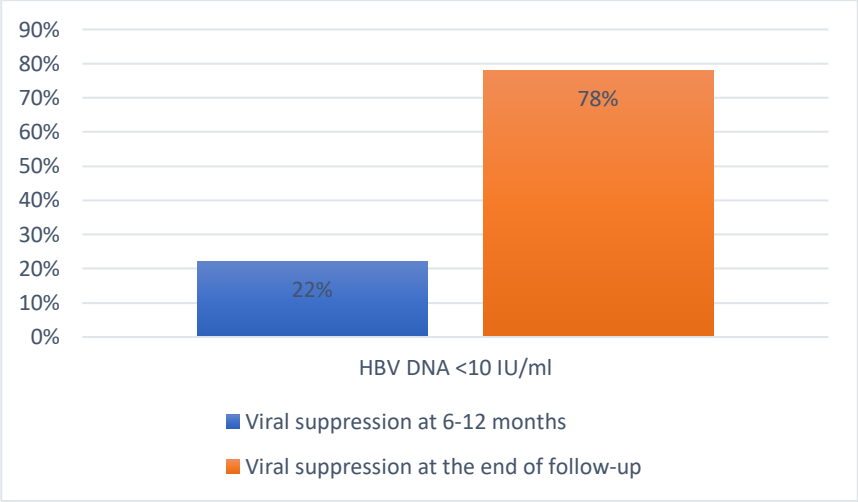

Figure 19. Viral suppression at 6-12 months and at the end of follow-up in patients with HBeAg(+) CHB.

We report a significant increase in the proportion of patients with viral suppression on a background of long-term NA treatment. Authors report 36-44% and 76% of patients with HBeAg(+) CHB with HBV DNA <60-80 IU/ml after 52 weeks of treatment with LAM or TDF, respectively (140, 149-155). 97% of patients with HBeAg(+) CHB achieve viral suppression (HBV DNA <10 IU/ml) during 5 years of

TDF treatment (140, 141, 156). The initial virological response is lower in patients with HBeAg (+) CHB, given the higher baseline viral load, compared with HBeAg (-) CHB, which was confirmed in our study group. Prolonged treatment with high-barrier NA shows optimal viral suppression in both HBeAg(-) and HBeAg(+) CHB. Here, the significantly smaller number of patients with HBeAg(+) CHB that we examined should be considered.

5. Analysis of biochemical response in patients with HBeAg(+) CHB

5.1. Biochemical response at 6-12 months after initiation of antiviral treatment in HBeAg(+) CHB and over the course of continued NA intake

All patients with HBeAg(+) status had transaminase activity (ALT >1.25x ULN) before initiation of antiviral treatment. Early biochemical response was recorded in 89% (n=8). There was a statistically significant difference in the dynamics of ALT activity at baseline and at 6-12 months of treatment initiation towards improvement (p=0.004). **There was a moderate correlation between HBV DNA level and ALT activity at 6-12 months of treatment (Pearson r=0.56). Over the course of continued treatment, all patients normalized ALT.** There was a statistically significant difference in mean ALT at baseline and at the end of follow-up (p=0.003) (n=9). Figure 20 summarizes the difference of proportion of biochemical response in the first year compared with continued NA therapy.

Figure 20. Difference in the proportion of patients with biochemical activity at month 6-12 and at the end of follow-up in HBeAg(+) CHB

We report an increase in the proportion of patients without biochemical activity during NA treatment. Leading investigators report that 68% of patients with HBeAg(+)

CHB normalize ALT after 52 weeks of TDF treatment, and 73% are without biochemical activity on year 5 of TDF treatment (140, 141, 156).

6. Analysis of virological response in HBeAg(+) CHB by HBeAg dynamics

The incidence of complete HBeAg seroconversion (sustained HBeAg negativity with the appearance of anti HBe Ab) was recorded in 67% (n=6) of those treated. This favorable outcome was reported after a **mean of 4.3 years of NA administration** (min 1 year, max 12 years, SD 4.17 years). The remaining 3 patients did not achieve HBeAg negativity and this necessitated continuation of NA treatment, which appeared to be long term, over 3 years. Figure 21 shows the incidence of HBeAg seroconversion in NA-treated patients.

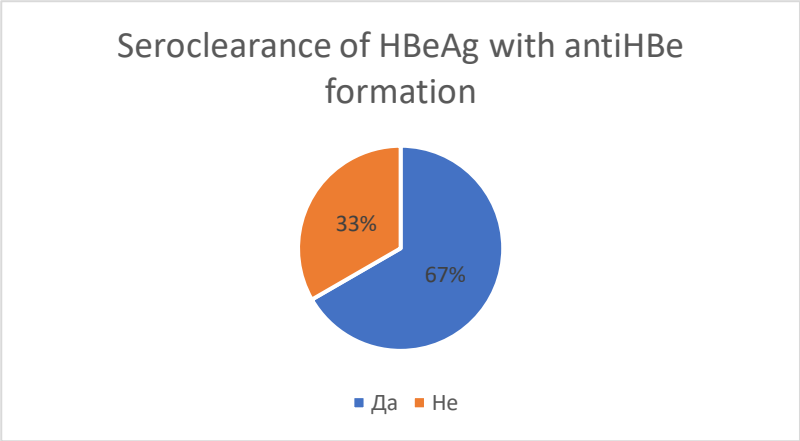

Figure 21. Seroclearance of HBeAg with formation of antiHBe, on the background of NA treatment.

HBeAg seroclearance is one of the goals of NA therapy in patients with HBeAg(+) CHB. The percentage of HBeAg seroclearance during administration of high-barrier NA is low during the first year but increases over the course of treatment. In summary, according to literature data, 5 years of TDF treatment results in loss of HBeAg in nearly half of patients and anti-HBe antibody formation in 40% (138, 156).

7. Analysis of virological response by qHBsAg level

In a prospective observational study, dynamics in qHBsAg concentration over the course of NA administration were assessed by at least two measurements, 6 months apart. **Overall, continuous NA therapy for an average of 8 years achieved complete HBsAg seroconversion (with the appearance of anti-HBs Ab) in 4.8% of patients (n=4). Loss of HBsAg is an optimal endpoint of NA treatment, but this occurs rarely-10% and 1% of patients with HBeAg(+) and HBeAg(-) CHB lose HBsAg after 5 years of TDF treatment (9).**

A trend towards a decrease in HBsAg with an average of 397 IU/ml was recorded in the majority of those treated, 90%. A trend towards an increase (with an average of 430 IU/ml) of qHBsAg was reported in only 9 patients. NA therapy resulted in a decrease in qHBsAg in most patients. Nikolova et al. reported data regarding HBsAg dynamics at baseline and on treatment and reported a decrease in HBsAg levels in 70% of the patients observed (157).

To look for determinants of the change in HBsAg concentration, the relationship between qHBsAg and duration of treatment, baseline liver disease stage, HBeAg status and HBV DNA level were evaluated.

7.1. Analysis of qHBsAg level according to the duration of NA treatment

The mean qHBsAg level receiving NA between 1 and 3 years was 2364 IU/ml (min 250 IU/ml, max 6400 IU/ml, SD 2217 IU/ml), in those on treatment for more than 3 but not more than 9 years was 2414 IU/ml (min 0.04 IU/ml, max 16,000 IU/ml, SD 3682 IU/ml) and 898 IU/ml (min 0.2 IU/ml, max 3700 IU/ml, SD 1093 IU/ml) in patients receiving NA treatment≥ 10 years (Figure 22). A statistically significant difference in mean values and level of qHBsAg was found in patients receiving NA≥ 10 years (ANOVA analysis, p=0.002). Pearson correlation analysis showed a moderate negative association between duration of treatment and HBsAg levels (r=-0.3, p=0.01). **Therefore, a large proportion of NA-treated (HBeAg (+) and HBeAg (-)) patients showed favorable dynamics in HBsAg concentration with the lowest qHBsAg values after 10 years of NA intake.**

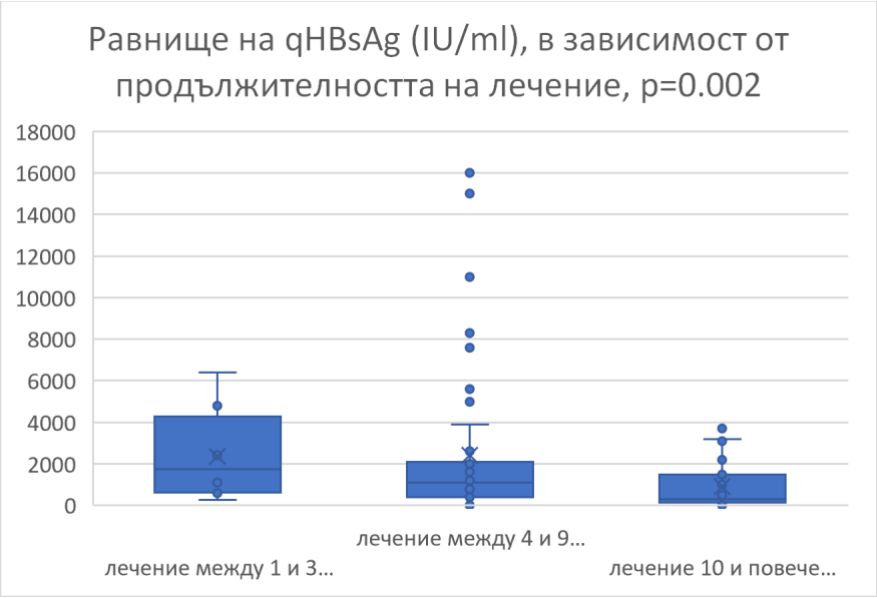

Figure 22. Level of qHBsAg depending on the duration of treatment.

Given the fact that viral replication is inhibited in most patients receiving NA, the level of HBV DNA does not reflect and is not informative about the amount of viral ccc DNA, RNA, or HBV antigen production in hepatocytes. One of the best-studied markers of HBV activity in NA-suppressed patients is qHBsAg. Prolonged NA treatment results in a gradual and slow decline in qHBsAg levels in some patients. Mathematical models have estimated that it would take 30-40 years of NA treatment for loss of HBsAg to occur (75). Wang et al. reported a mean qHBsAg level of 2.72 log10 IU/ml, compared with 3.8 log10 IU/ml at baseline, with NA treatment over an 8-year period. The percentage of patients with qHBsAg <1000 IU/ml increased from 14.9% at baseline to 55.3% at year eight of treatment. The same collective reported no association between qHBsAg decline in patients with different baseline viral loads or type of NA administered (158). There is also a notion that the NA-induced therapeutic clearance of HBsAg in HBeAg(-)CHB does not exceed the spontaneous frequency of HBsAg seroconversion, with the main reasons being the resistance of cccDNA to the effect of NA and the lack of inhibitory effect of NA on integrated HBV sequences in the hepatocyte genome (159).

7.2. Analysis of qHBsAg according to the stage of liver disease

The mean qHBsAg value at the last visit in the clinic during NA treatment in patients without cirrhosis was 2520 IU/ml (min 0.05 IU/ml, max 16 000 IU/ml, SD 3571 IU/ml) and in those with HBV-associated cirrhosis was 686 IU/ml (min 0.04 IU/ml, max 2900 IU/ml, SD 672 IU/ml). **Thus, patients with CHB had significantly higher HBsAg concentrations compared to cases staged at baseline with liver cirrhosis (p=0.0001).** Figure 23 shows the mean and distribution of qHBsAg according to the initial stage of the disease. Biserial correlation analysis revealed a moderate negative association between liver disease stage and qHBsAg level (r= -0.31, p=0.02).

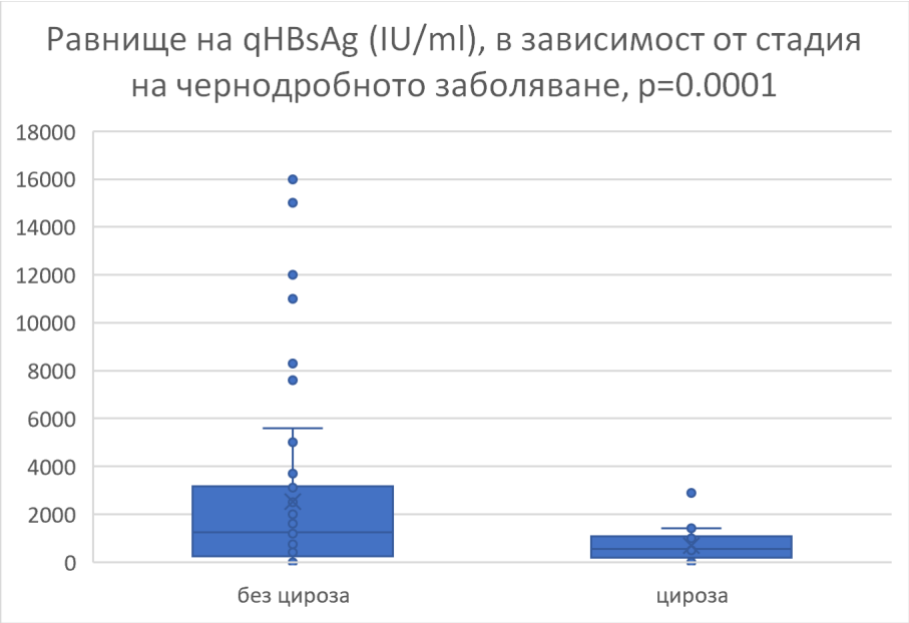

Figure 23. Level of qHBsAg according to the stage of liver disease.

The serum HBsAg level decreases due to immune activity and immune-mediated death of infected hepatocytes (160). This immune response, in turn, is associated with a higher degree of liver inflammation and fibrosis and is a possible explanation for why the lower HBsAg level is seen in advanced fibrosis and cirrhosis. The inversely proportional relationship between qHBsAg and fibrosis stage was not confirmed by an European collective in a study of patients with HBeAg(-)CHB. Results from the SONIC-B study, published in 2022, showed that low HBsAg was associated with cirrhosis only in HBeAg(+) patients, but not in HBeAg(-) CHB. HBsAg production in HBeAg (-) CHB patients is mainly from integrated HBV sequences in the hepatocyte genome and not so much from ccc DNA. The integrative stage of HBV infection is associated with the absence of an active HBV-specific immune response, respectively no necroinflammatory changes and fibrogenesis are induced (160, 161, 162).

7.3. Analysis of qHBsAg level in relation to baseline HBeAg

We found that the mean qHBsAg value in HBeAg (+) patients was 4302 IU/ml (min 56 IU/ml, max, 12 000 IU/ml, SD 4328 IU/ml) and in HBeAg (-) patients was 1685 IU/ml (min 0.04, max 16 000 IU/ml, SD 2847 IU/ml) (Figure 24). The qHBsAg was higher in patients with baseline HBeAg (+) and the difference in level was statistically significant (p=0.04).

Using biserial correlation analysis, a moderate positive association was found between baseline HBeAg status and HBsAg level (r=0.30, p=0.01).

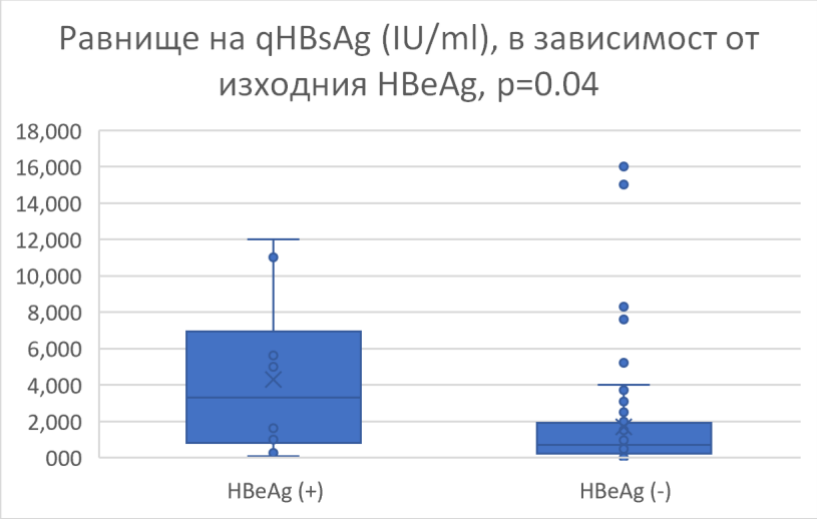

Figure 24. Level of qHBsAg depending on baseline HBeAg

The kinetics of qHBsAg reported from a number of studies in Asia and Europe show high levels during phase 1 of chronic HBV infection, a decrease during the immune clearance phase (HBeAg(+)CHB), the lowest levels during phase 3, and some increase in the HBeAg(-)CHB phase (163).

7.4. Analysis of the level of qHBsAg according to HBV DNA at the end of follow-up

HBV DNA was below the detection level in 86% of the entire study group at the end of follow-up. The mean HBsAg was 1600 IU/ml in patients with viral suppression (min 0.04 IU/ml, max 16 000 IU/ml, SD 2773 IU/ml), Figure 25. Conversely, a mean HBsAg e of 4448 IU/ml (min 100 IU/ml, max 12 000 IU/ml, SD 4084 IU/ml) was found in 14% of treated patients without evidence of viral suppression. **Therefore, patients who did not achieve HBV replication inhibition during the course of NA therapy had a significantly higher HBsAg level (p=0.01).**

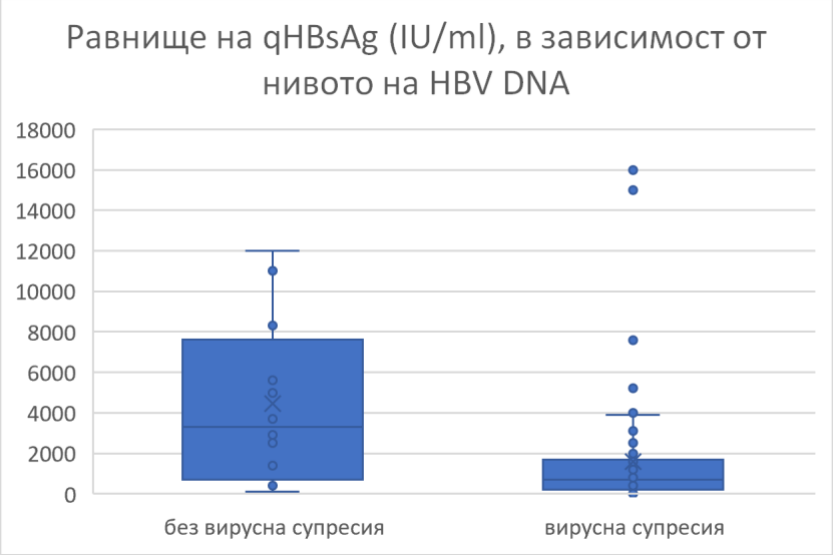

Figure 25. Level of qHBsAg according to the registration of viral suppression (HBV DNA < 10 IU/ml).

Pearson correlation analysis showed a moderate positive association between HBV DNA level at the end of follow-up and HBsAg level (r=0.43, p<0.05).

Several authors have reported a positive association between HBsAg level and HBV DNA, both in untreated patients and in patients on long-term NA treatment. Zhu et al. reported a strong association between qHBsAg and HBV DNA level in patients on NA treatment (r=0.65, p<0.05), further highlighting the role of qHBsAg as a marker of virological response (164).

We performed a regression analysis regarding the effect of HBV DNA on treatment background on HBsAg levels and concluded that **a 1 IU/ml increase in HBV DNA resulted in a 30.5 IU/ml increase in qHBsAg** (Table 9).

Models	1	2	3	4	5	6
$Y_i = b_0 + b_1 X_i$	1875.806 30.478	5.419 3.271	27.14	5.763	1,193	1,78
$\hat{Y}_i = b_0 + b_1 \ln X_i$	1635.281 25.257	4.257 2.875	24.14	5.257	1.216	1.75
$\hat{Y}_i = b_0 + b_1 \frac{1}{X_i}$	1456.203 12.231	3.245 1.205	19.25	3.014	2.562	1.69
$\ln \hat{Y}_i = b_0 + b_1 X_i$	256.231 17.154	5.968 1.541	22.05	3.587	1.421	1.71
$\ln \hat{Y}_i = b_0 + b_1 \ln X_i$	234.21 16.258	4.968 1.758	20.96	3,639	1.599	1.72

Table 9. Regression model for the relationship between HBV DNA and qHBsAg levels at the end of follow-up.
Legend:
1 - Ratios (b_0 , b)$_1$
2 - Empirical t-criterion values (t)$_{емп.}$
3 - Coefficient of determination (R^2 .100) - %
4 - Empirical values of the F-criterion
5 - Standard model error ( )σ
6 - Empirical values of the Durbin-Watson criterion (d)

The theoretical values of F, t and DW criterion are:
1) $F_{teop.} = 3.92$
2) $t_{teop.} = 1.66$
3) $d_L = 1.62$; $d_U = 1.67$

8. Analysis of virological response by testing HBcrAg levels

HBcrAg levels were tested in all 84 patients at the last visit to the Gastroenterology Clinic. Over the course of long-term, on average 8 years of NA treatment, HBcrAg was detected and measured in serum in the entire group of patients (100%). **HBcrAg values ranged between 2.5 log_{10} U/ml and 5.6 log_{10} U/ml (mean 3.9 log_{10} U/ml).** In 9.5% of cases (n=8) the HBcrAg concentration was between 2.5 and 3.1 log_{10} U/ml, 38.1% (n=32) had HBcrAg between 3.2 and 3.8 log_{10} U/ml, 48.8% (n=41) between 3.9 and 4.5 log_{10} U/ml, 2.4% (n=2) between 4.6 and 5.2 log_{10} U/ml, and 1.2% (n=1) between 5.3 and 5.9 log_{10} U/ml (Figure 26). **In summary, two-thirds of those treated (67%) had a detectable HBcrAg level between 3.0 and 4.0 log_{10} U/ml.**

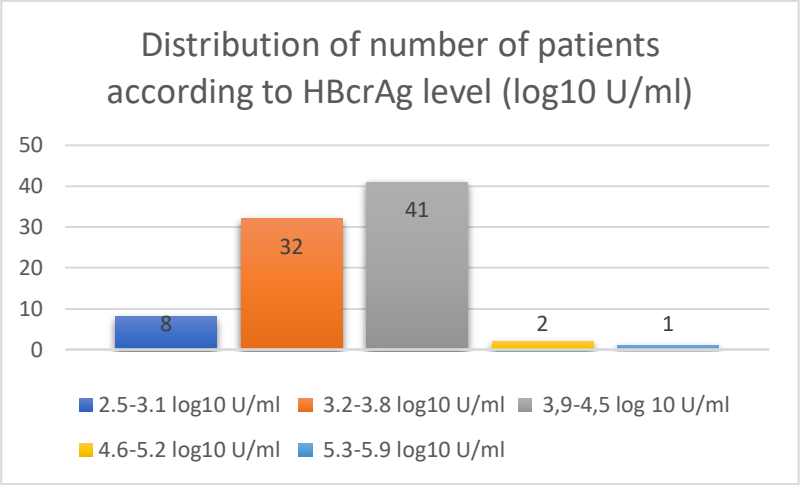

Figure 26. Distribution of the number of patients according to the level of HBcrAg on the background of NA treatment

Similar data were reported by a collective from Japan, who examined HBcrAg in HBeAg(-) CHB in the course of long-term suppression with NA and negative HBV DNA, using a chemiluminescent immunoenzymatic assay (iTACT-HBcrAg; Fujirebio, Inc., with lower limit of detection < 2.1 LogU/ml) (165). **Despite persistent non-detectable HBV DNA by sensitive RT-PCR during the course of NA treatment, HBcrAg levels were detectable in most subjects (97%). Experimental and clinical studies have shown that the HBcrAg level reflects the concentration of intrahepatic ccc DNA, with the highest correlation coefficient of 0.7**. No significant dynamics in HBcrAg levels were found during the course of NA therapy. Furthermore, HBcrAg > 3.4 LogU/ml may predict virological relapse after NA cessation. Importantly, HBcrAg > 3 LogU/ml in NA-suppressed patients is a predictor of HCC development (166).

Correlation analysis showed a very weak association between HBsAg and HBcrAg levels, and between HBV DNA and HBcrAg levels at the end of follow-up (r =0.09 and r=0.05, respectively). **There was no association between HBcrAg level and duration of treatment, stage of liver disease, and baseline HBeAg status.** A possible explanation for the lack of correlation between other markers of HBV activity and HBcrAg is the small number of patients studied with insufficient power of the statistical analysis (especially in the HBeAg(+) CHB group), and the retrospective nature of the results for baseline viral load (before initiation of NA), making comparison with HBcrAg level before antiviral treatment impossible.

Of interest was the observation that patients with HBsAg seroconversion also had detectable serum HBcrAg levels with an average of 3.8 log 10 IU/ml. Undoubtedly, this fact known from previous publications is striking and is again explained by the life cycle of HBV. HB core-associated antigens are a complex of HBeAg, HBcAg, and the 22-kDa precore protein p22cr, encoded by the precore/core region of the viral genome, which can be jointly quantified in the blood of patients with HBV infection by a serological method. **These 3 different types of proteins are translational products of mRNA, in turn transcribed from ccc** DNA during the HBV life cycle in the hepatocyte. HBcoreAg cannot be determined alone in the serum of patients with HBV infection, but only as a complex within HBcrAg. Serological measurement of HBcrAg was first introduced by Kimura et al. in 2002 as a method examining the products of the precore-core region of HBV (167). The current method for HBcrAg detection is ICT - CLEIA ("Immune complex transfer - Chemiluminescence enzyme immunoassay) with ultra-high sensitivity for detection of viral proteins. Multiple recent studies have shown that HBcrAg is detectable > 2 LogU/ml in the serum of patients at different phases of HBV chronic infection, including phase 5, in HBV DNA negative patients, in HBeAg (-) including complete HBeAg seroconversion, and in the rare cases of mutations in the HBs region causing lack of HBsAg secretion. Thus, HBcrAg is indicative of ongoing antigen expression and HBV replication (with transcription of the precore/core region of the viral genome) and can be a reliable indicator for the evaluation of chronic HBV infection and future antiviral therapy (168, 169, 170).

9. Analysis of abdominal ultrasonography results

During follow-up with abdominal ultrasound every 6 months, no patients with initial or absent fibrosis (F<2) were suspected of malignancy. In the group of F≥ 2, 1 patient (3.2%) developed hepatocellular carcinoma after 1 year of NA treatment. **In the patients with baseline evidence of cirrhosis, a total of 3 cases (12.5%)**

developed HCC, with one case of initial HCC, before antiviral treatment. A χ^2 test was performed to test for significance between the distribution of HCC among patients with or without cirrhosis, finding a significantly higher risk of malignancy in patients with cirrhosis (χ^2 =4.5, p=0.03) (Figure 27).

	HCC	no-HCC	*Marginal Row Totals*
без цироза	1 (2.87) [1.22]	60 (58.13) [0.06]	61
цироза	3 (1.13) [3.1]	21 (22.87) [0.15]	24
Marginal Column Totals	4	81	85 (Grand Total)

Figure 27.χ^2 test to test for significance between HCC distribution among patients with or without cirrhosis.

It is generally accepted that long-term NA therapy reduces but does not eliminate the risk of developing HCC, and it remains more significant in patients with CHB-associated cirrhosis than in those with CHB. The annual risk of HCC during TDF/ETV therapy is between 0.01% and 1.4% in patients without cirrhosis and between 0.9% and 5.4% in those with cirrhosis. No lower incidence of HCC was found in patients receiving NA with low barrier to viral resistance mutations, such as LAM, compared with high barrier TDF/ETV. Some risk factors for HCC are known to be unaffected by antiviral therapy, and in other cases, carcinogenesis has begun before treatment initiation (87, 88).

Dynamics in the degree of steatosis were assessed in 61 patients by abdominal ultrasound using standard criteria in the course of B-mode imaging. On treatment background, 49% (n=30) had no dynamics in steatosis grade, 21% (n=13) had improvement in steatosis grade, and **approximately one-third of treated patients (30%) showed worsening of steatosis grade (Figure 28)**.

Figure 28. Dynamics in the degree of steatosis on the background of treatment.

MAFLD (or according to the updated terminology MASLD, metabolic-dysfunction associated steatotic liver disease) affects 1/3 of the world's population and in combination with CHB are one of the leading causes of liver disease progression and HCC development. Of particular interest in the last few years has been how HBV affects MASLD and how steatosis in turn affects the HBV life cycle. HBx protein is known to activate genes associated with lipid accumulation in hepatocytes, which promote lipogenesis, hepatocyte proliferation, inhibit apoptosis and promote HCC development. Under steatosis conditions, the level of methylated DNA is reduced, which leads to chromosomal instability and is a prerequisite for HCC. HBV suppresses NCTP function, which increases bile salt secretion, cholesterol accumulation, and leads to the development of steatosis. On the other hand, in the presence of MASLD, HBV replication is suppressed by modulation of the immune response, activation of NK and CD8 T-Ly via the TLR signaling pathway, by induction of apoptosis and inhibition of autophagy in HBV-infected hepatocytes, and on the basis of suppressed PPAR-gamma activity in the presence of insulin resistance. **Therefore, initial data suggest that the combination of MASLD with HBV infection may facilitate clearance of HBsAg** (171). This is another interesting difference in the biology of HBV infection when compared with HCV, where the viral life cycle requires and is facilitated by steatotic liver injury.

At this stage, there is no convincing evidence in the published literature that prolonged treatment with NA induces or enhances hepatic steatosis as a side effect of this class of drugs. However, in clinical practice, we have observed the occurrence or enhancement of hepatic steatosis as assessed by abdominal ultrasound in patients on long-term NA therapy. **In the present study, steatosis increased by at least one grade in 1/3 of the treated subjects over the course of long-term NA intake. In addition to potential drug toxicity, the co-factors of increasing age and body weight associated with cumulative metabolic disorders and, last but not least, non-compliance with recommendations for complete abstinence from alcohol use are likely to play a role.** In all patients with the onset or worsening of steatosis in the course of NA therapy, metabolic disorders and other causes should be sought, including a previously undetected etiology of chronic liver disease, such as Wilson's disease and alpha-1 antitrypsin deficiency. New methods of quantitative analysis of hepatic steatosis in the course of multiparametric ultrasonography will make the follow-up of this type of chronic liver injury more objective and accurate. It is undeniable that because of the synergistic effect of SLD-HBV in the development of HCC, this combination should be actively sought and ways to influence it should be further developed.

In the group of patients with cirrhosis, baseline data for ascites were found in 4 cases: of these, on the background of NA treatment**, 3 patients (75%) had compensation of cirrhosis, with permanent disappearance of ascites, and 1 patient continued to have minimal ascites.**

In the context of the recent concept of liver cirrhosis compensation, the published data on the success of NA therapy, which can improve the decompensation rate in more than 50% of those treated, should be noted. Thus, Li et al. reported a proportion of patients with ascites regression of 77.6%, 81.4%, 70.5%, 93.8%, and 80.8, respectively, at the 12th, 24th, 36th, 48th, and 60th months from the start of antiviral therapy. Consequently, the severity of ascites on background treatment improved, with an increase in the proportion of patients with no or minimal ascites (86). **In the present study, the proportion of patients with decompensated cirrhosis was too small to warrant reliable conclusions about NA therapy at this stage of the disease.**

10.Analysis of serum markers of fibrosis- APRI and FIB-4 during NA treatment

APRI and FIB-4 were calculated at baseline and at patients' last visit during the course of NA therapy. The mean APRI at the end of follow-up was 0.52 (min 0.10, max 7.2, SD 0.81). There was a statistically significant positive trend in the reduction of APRI during NA treatment (p=0.0002) and this is shown in Figure 29.

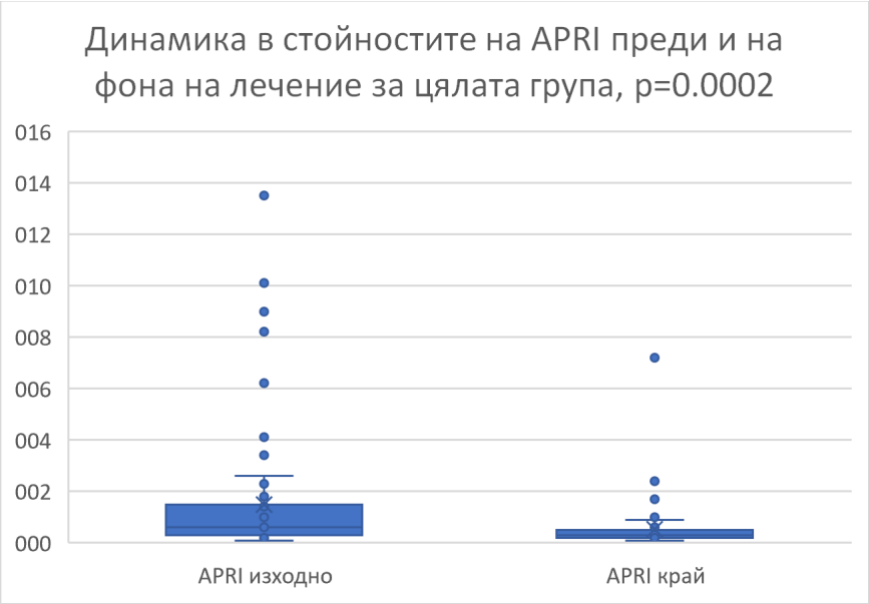

Figure 29. Dynamics in APRI before and during treatment for the whole group.

In patients with baseline data for F<2, the mean APRI was 0.34 (min 0.1, max 1.3, SD 0.21), in F≥ 2 was 0.36 (min 0.2, max 0.9, SD 0.19) and 0.96 (min 0.2, max 7.2,

SD 1.39) in F4. Thus, **the main categories of patients with baseline non-significant fibrosis, significant fibrosis and cirrhosis had statistically significant differences in APRI values both at baseline and during the course of NA treatment** (with significance level p=0.05 for F<2, p=0.001 for F≥ 2 and p=0.01 for F4, respectively). Figures 30, 31 and 32 illustrate the dynamics in APRI over the course of continuous NA treatment, according to disease stage.

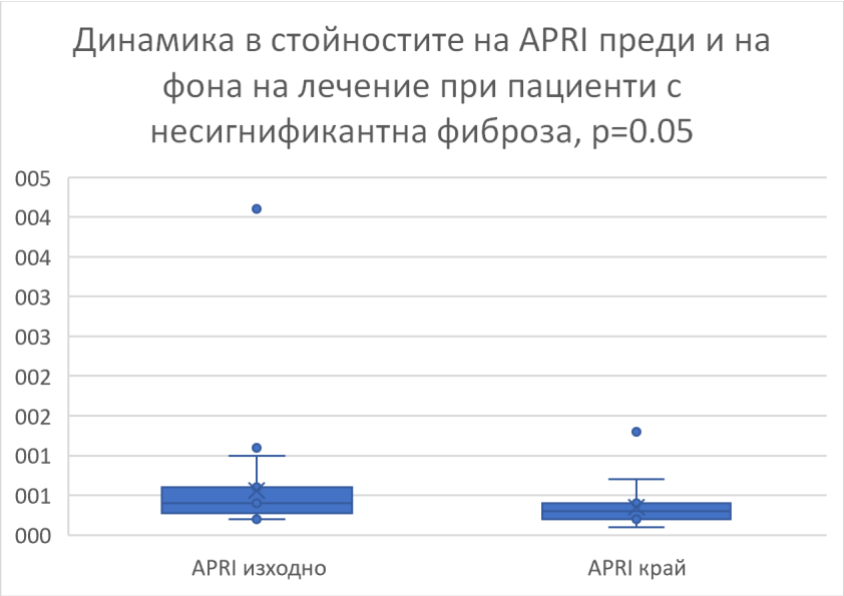

Figure 30. Dynamics of APRI before and during treatment in patients with non-significant fibrosis

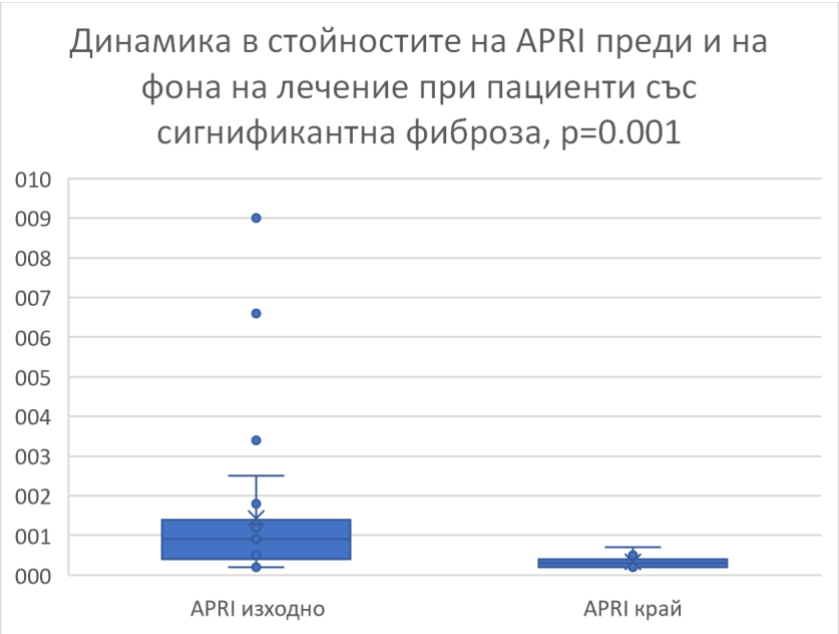

Figure 31. Dynamics of APRI before and during treatment in patients with significant fibrosis

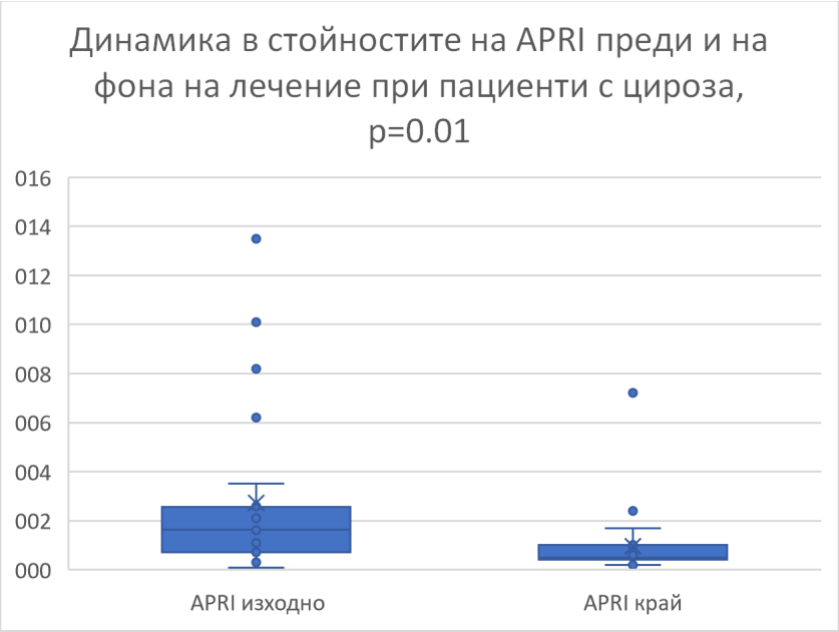

Figure 32. Dynamics of APRI before and during treatment in patients with cirrhosis

The mean FIB-4 at the end of follow-up was 2.12 for the entire treatment group (min 0.56, max 18.2, SD 2.72). **In contrast to the change in APRI, no significant difference was observed for FIB-4 at baseline and at the final follow-up visit during NA treatment (p=0.1) (Figure 33).**

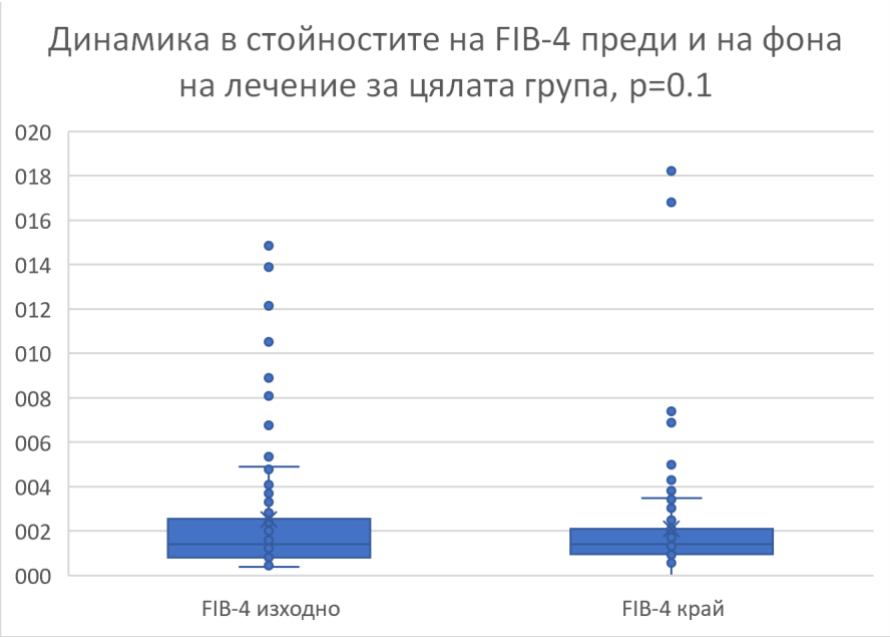

Figure 33. Dynamics of FIB-4 at baseline and during treatment for the whole group

Further analysis of FIB-4 levels in patients with different stages of the disease showed that in the group with non-significant fibrosis the mean FIB-4 level was 1.21 (min 0.56, max 2.21, SD 0.43), in the group with significant fibrosis the mean FIB-4 level did not differ - 1.41 (min 0.57, max 3.83, SD 0.67). **Cases with baseline evidence**

of cirrhosis during the course of NA intake again had FIB-4 values of 4.16 on average (min 0.8, max 18.2, SD 4.3), indicative of advanced disease. There was no statistically significant difference in FIB-4 values at baseline and on treatment in the different patient groups, according to disease stage (significance levels p=0.13 for F<2, p=0.14 for F≥ 2 and p=0.17 for F4, respectively). The dynamics of FIB-4 at baseline and during NA treatment according to baseline fibrosis grade is illustrated in Figures 34, 35, 36.

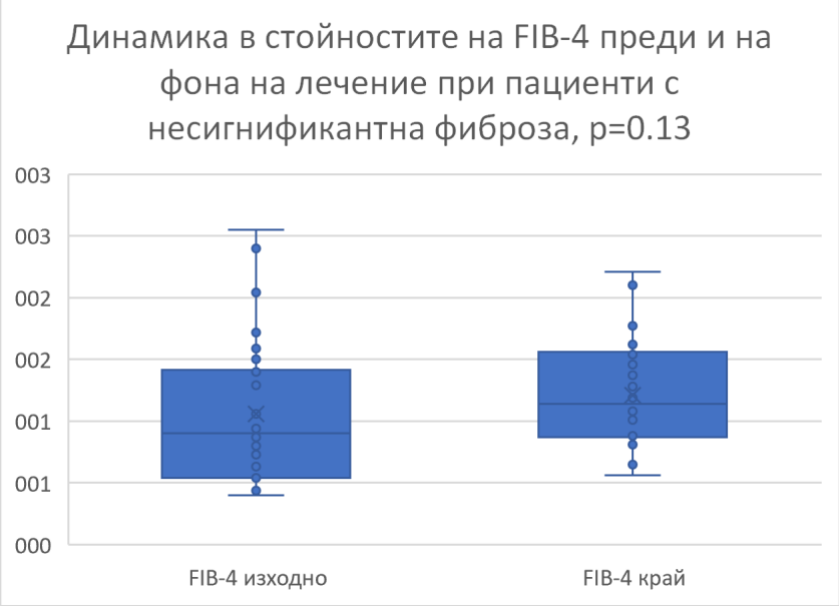

Figure 34. Dynamics of FIB-4 before and during treatment in patients with non-significant fibrosis

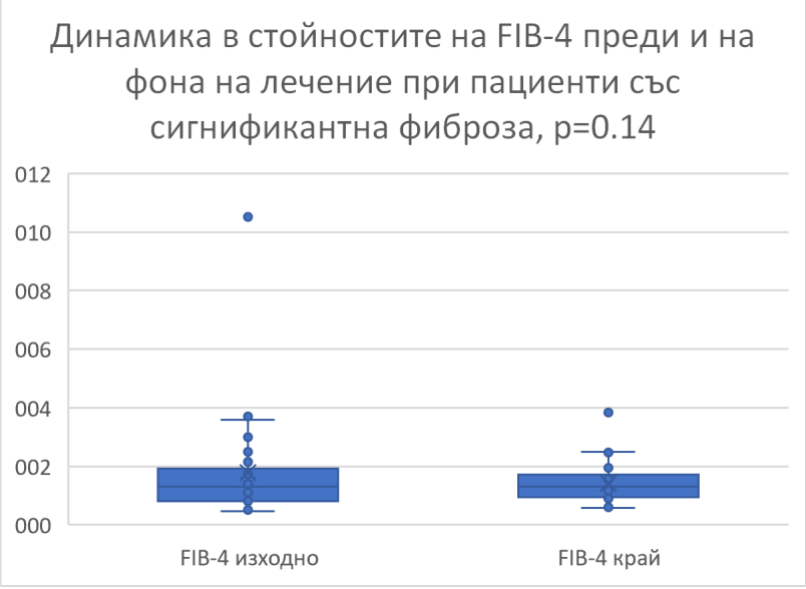

Figure 35. Dynamics of FIB-4 before and during treatment in patients with significant fibrosis

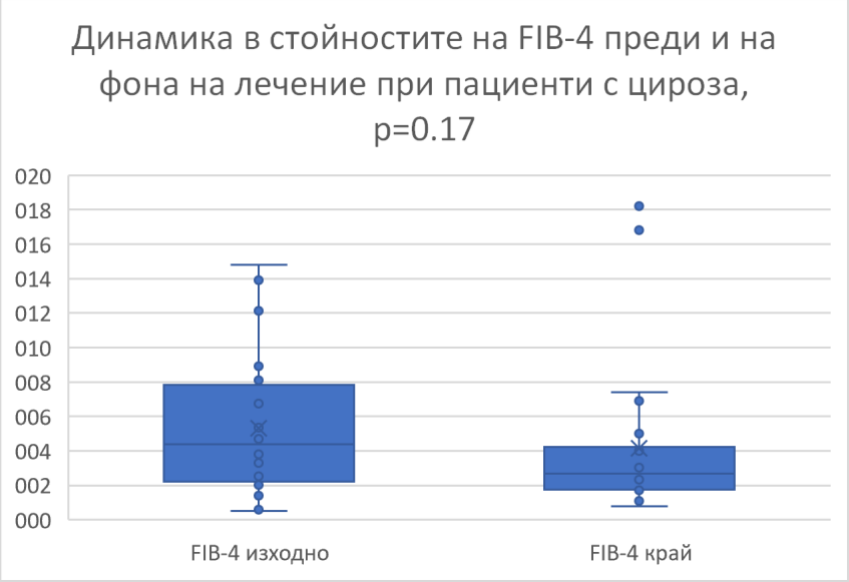

Figure 36. Dynamics of FIB-4 before and during treatment in patients with cirrhosis

A number of collectives have reported that during treatment with potent NA, APRI and FIB-4 values improve and these, together with liver stiffness assessment, are suitable non-invasive methods to assess and monitor fibrosis during treatment. It is discussed that the use of serum markers of fibrosis is more applicable in patients with significant fibrosis and those with cirrhosis. Liu et al. found that APRI values decreased more rapidly than FIB-4 values after 5-year ETV treatment. The same collective reported that the dynamics in FIB-4 after 3 and 5 years of NA treatment in patients with baseline evidence of advanced liver disease, F3 and F4, were significant (172). Sonneveld et al. proposed optimization of cut-off values of APRI and FIB-4 to assess CHB-associated cirrhosis when comparing liver biopsy results and noninvasive markers of fibrosis. A new cut-off value to exclude cirrhosis has been proposed for FIB-4 $\leq$ 0.70 in patients with HBV infection and age older than 30 years (127). Moosavy et al. reported the strongest correlation between FibroScan and APRI values for assessing fibrosis in patients with CHB and proposed APRI as the best surrogate for FibroScan (173).

We calculated FIB-4 in patients undergoing NA≥ treatment for 10 years (n=29), who had a mean age of 60.3 years (min 35, max 81, SD 13.7). Thus, FIB-4 in this group of patients averaged 2.45 (min 0.56, max 16.8, SD 3.09). The observed high FIB-4 value, despite years of NA treatment, can be commented in several ways. First, age is included in the calculation of the FIB-4 score and in elderly patients, it could be discussed that FIB-4 overestimates liver fibrosis. Long-term NA therapy normalized transaminases in more than 90% of the patients we observed, and in cases of normal transaminases, their ratio does not play a role in the assessment of fibrosis. Considering these facts, FIB-4 does not appear to be the most appropriate serum marker to assess fibrosis dynamics in patients over 65 years and with normal transaminases.

Graupera et al. also reported the low informative value of FIB-4 for fibrosis assessment in hepatitis B. In more than a third of the 5129 patients they observed, FIB-4 overestimated fibrosis, and the same authors again implicated the role of age in the calculation of FIB-4 (174).

11. Dynamics in liver stiffness (LS) during NA treatment assessed by ultrasound elastography (2D-SWE)

The dynamics of liver stiffness were evaluated in 39 patients **with at least two LS measurements during NA treatment by 2D SWE, with an interval between measurements of 1 to 6 years**. In summary, there was no significant difference in liver stiffness at follow-up SWE elastography: the mean LS at the first measurement was 10.02 kPa (min 3.7 kPa, max 34 kPa, SD 7.6 kPa), compared to 8.54 kPa (min 2.3 kPa, max 26.3 kPa, SD 5.6 kPa) at the follow-up measurement (p=0.1) (Figure 37). **On the other hand, 64% of the treated subjects (n=25) showed evidence of improvement in LS by a mean of 4 kPa (min 0.1 kPa, max 21.8 kPa, SD 5.8 kPa). In the remaining 36% (n=14) there was a worsening of LS with a mean of 3.2 kPa (min 0.3 kPa, max 8.9 kPa, SD 2.7 kPa).**

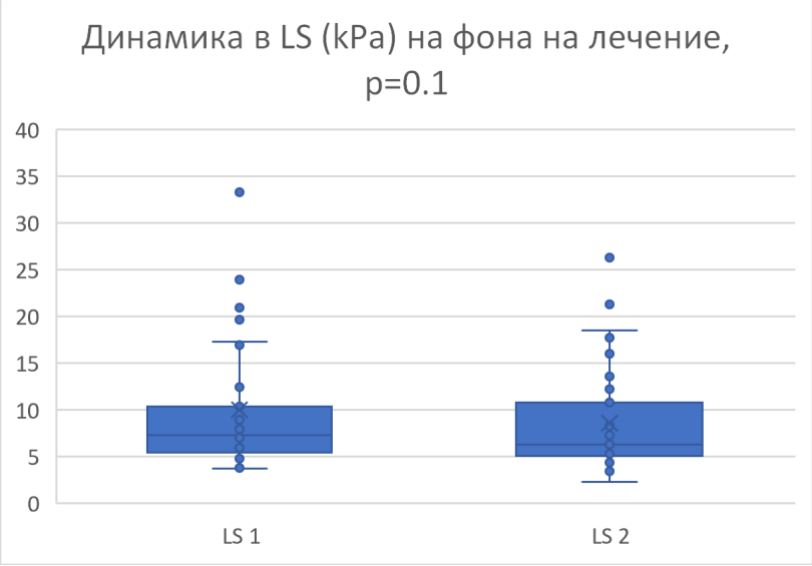

Figure 37. Dynamics of LS values during treatment, with interval between two measurements from 1 to 6 years

Long-term treatment with NA resulted in improvement of liver fibrosis and consequently LS values in the majority of the observed patients. The dynamics of liver stiffness reported by a number of authors was biphasic, with a faster reduction in the first 6 months of NA treatment, including at baseline cirrhosis. The closer to the exponent is the individual LS change curve, the more this may predict a good disease course (in terms of histological fibrosis regression and long-term risk of complications

and death). Of note, this favorable outcome is not the rule, occurring in 1/3 of those treated with ETV at year 5 (82, 83).

Longitudinal studies with LS monitoring compared with follow-up results and with repeat liver biopsy showed that the reduction in liver stiffness measured by TE at week 78 of NA treatment mainly reflected improvement in liver inflammation and edema (175).

Ramji et al. followed 465 patients on long-term NA therapy and reported improvement in LS in a majority of patients with an average of 4.2 kPa in TDF and 1.6 kPa in LAM (86). Therefore, the improvement in liver stiffness was higher in patients receiving high-barrier NA.

New prospective studies are needed to assess the role of LS monitoring in the course of hepatitis B antiviral therapy.

12. Analysis of FGDS results in patients with cirrhosis

Of the 24 patients with baseline evidence of cirrhosis, FGDS prior to initiation of NA was performed in 22. Of these, 59% had esophageal varices (n=13) in the following grades: small in 84.6% (n=11), moderate in 7.7% (n=1), and large in 7.7% (n=1). Additionally, a total of 11 patients had endoscopic criteria for both esophageal varices and portal hypertensive gastropathy (PHG), 2 patients had esophageal varices alone, and 1 patient had PHG alone.

Follow-up upper endoscopy was performed in a small proportion of the included patients with cirrhosis - 5 cases. Endoscopic criteria for portal hypertension showed persistence of esophageal varices in 40% (n=2). **Regression of portal hypertension could be commented in 3 patients (60%) due to absence of esophageal varices and PCG in control upper endoscopy.**

Wang et al. reported a 40.4% regression in the rate of esophageal varices during NA treatment with an average duration of about 3 years. The same collective reported only a 13% progression of esophageal varices, which was seen predominantly in patients with moderate-to-severe varices (85). Again, it should be noted that because of the small proportion of patients with cirrhosis analyzed in the present study, it is difficult to achieve good results on the role of NA therapy in clinically significant portal hypertension. However, although small, the empirical experience is important to improve the monitoring of patients with advanced liver disease during the course of antiviral treatment and not to compromise the upper endoscopy control protocol, especially in proven baseline esophageal varices.

13. Dynamics in the stage of liver cirrhosis during long-term NA therapy, according to CTP and MELD-Na scores

At the last visit, a reduction in the stage of liver cirrhosis was found in the majority of NA-treated patients: 96% (n=23) patients had an improvement in the CTP score of at least 1 unit and only 1 patient showed no improvement in CTP on NA treatment. **Regarding MELD-Na dynamics, improvement was found in 54% (n=13) of treated patients with a mean of 5.9 points (min 1, max 16, SD 5.04).** On the other hand, despite prolonged antiviral treatment, 46% (n=11) of those treated showed no improvement in the prognostic MELD-Na score, with progression in cirrhosis severity with a mean of 4.2 points (min 1, max 12, SD 4.46). In our study group, during long-term NA treatment, we observed CTP A in 95.8% (n=23) and MELD-Na < 10 points in 70.8% (n=17).

Wang et al. proposed a threshold for MELD-Na < 10 points and CTP A, as criteria for a stable course of liver cirrhosis, in patients receiving high-barrier NA (ETV). They observed compensation of CHB-associated cirrhosis in more than 50% of patients who were decompensated at baseline; improvement in laboratory indices of liver function, as assessed by CTP and MELD-Na, was found on a background of ETV treatment (176). **Thus, it can be reemphasized that the administration of NA at the stage of decompensated HBV cirrhosis exemplifies the therapeutic options in achieving recompensation and reversibility of advanced liver disease.**

Conclusions:

1. Administration of nucleotide/nucleoside analogues in hepatitis B was highly effective in inhibiting HBV replication, providing an initial virological response in 63% and maintaining viral suppression, respectively HBV DNA <10 IU/ml in 90.4% and undetectable HBV DNA in 84.5% of those treated.
2. The virological response assessed by sustained HBV DNA negativity was different in patients with baseline HBeAg-negative versus HBeAg-positive chronic hepatitis B: 87% versus 66%.
3. A biochemical response assessed by sustained normalization of transaminase activity was achieved in 91.6% of those treated with nucleoside/nucleotide analogues.
4. The administration of lamivudine is suboptimal because it does not provide complete viral suppression in 19% of those treated, which becomes a prerequisite for drug resistance.
5. Patients with liver cirrhosis had significantly lower concentrations of HBsAg but not of HB core-related antigens.
6. Monitoring hepatitis B therapy requires monitoring HBsAg levels.
7. Long-term therapy with nucleoside/nucleotide analogues progressively reduced HBsAg levels, with favourable dynamics in 90% of those treated and the lowest HBsAg concentration of 898 IU/ml on average when the drugs were administered for more than 10 years.
8. Negation of HBsAg with the appearance of anti HBs antibodies, respectively functional cure, was recorded in 4 patients during the course of therapy (4.8%).
9. HB core-bound proteins are detectable in the blood of patients with long-term antiviral therapy and suppression of viral replication; 67% of patients studied had a level between 3 and 4 $\log_{10}$; patients with clearance of HBsAg had a detectable level for HBcrAg.
10. Long-term therapy resulted in an improvement in indirect serum markers of fibrosis, with a significant improvement in APRI in most cases and a reduction in FIB-4 in 56% of those treated.
11. Prolonged treatment with nucleoside/nucleotide analogues reduced liver density assessed by US elastography by a mean of 4 kPa in 64% of patients.
12. In cases where progression of liver disease can be assumed, co-factors such as alcohol consumption and metabolic disorders should be sought based on the dynamics in indirect markers of fibrosis and liver density.
13. The follow-up of patients with hepatitis B during long-term inhibition of viral replication should include a comprehensive evaluation of the interpretation of

new viral markers, noninvasive indicators for the assessment of liver disease, and abdominal ultrasonography data.

Contributions:

A comprehensive evaluation of the results of nucleoside/nucleotide analogues administration was performed over an average period of 8.2 years, with 37% of the patients studied receiving the drug for more than 10 years.
The analysis of HB core-associated HBV antigens is original and for the first time in Bulgaria.
HBsAg levels were evaluated dynamically and in terms of disease stage and viral load (HBV DNA).
Initial clinical experience with the use of novel viral markers of HBV activity in patients with drug-inhibited viral replication has been collected.
The analysis of the long-term results of the current treatment of hepatitis B is a prerequisite for setting new goals to optimize therapeutic management.

Publications related to the thesis:

- Monitoring the effectiveness of treatment of hepatocellular carcinoma-are there other useful biomarkers besides serum alpha-fetoprotein (clinical case)- J.Stoyanova, I.Ivanova, S.Banova, M.Mirchev, Y.Bocheva- VI National Congress for Young Gastroenterologists, 03.2021.
- Is liver biopsy necessary for monitoring antiviral treatment in hepatitis B- analysis of a clinical case-J.Stoyanova, I.Ivanova, Z.Kostadinova, K.Kalchev- VIII National Conference on Hepatology, 11.2022.
- New serum biomarkers for response to treatment with nucleoside/nucleotide analogues in patients with chronic hepatitis B-literature review- Y. Stoyanova, Varna Medical Forum, 12.2023.

I would like to thank

To my supervisor Assoc. Prof. Irina Ivanova for her ideas, critical comments, support and help in the preparation of my thesis.

To all my colleagues from the Gastroenterology Clinic at St. Marina University Hospital for their support and courage.

To Prof. Dr. Yana Bocheva, Head of Clinical Laboratory, Prof. Dr. Temenuga Stoeva, Head of Laboratory of Virology and Assoc. Prof. Dr. Zhivka Stoykova at University Hospital "St. Marina" for the precision of the laboratory tests.

To all the colleagues at the Clinic of General and Clinical Pathology at St. Marina University Hospital for their help in the invasive evaluation of liver disease.

To Prof. Dr. Margaritka Boncheva, Head of the Clinical Laboratory at the University Hospital "Prof. Dr. Al. Chirkov" for her assistance in the study of HB-core-associated antigens.

To my family and all my loved ones for their patience, support and belief in me.