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FULL PAPER

Evaluation of pregenomic HBV RNA in HBeAg-negative patients

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SUMMARY

The distinction between chronic HBeAg-negative hepatitis (CHB) and chronic HBeAg-negative infection (CIB) can be challenging and important for providing advice on prognosis, as well as determining need for treatment.

The aim of the present study was to evaluate pgRNA levels in treatment-naïve HBeAg-negative chronic HBV-infected patients. In addition, pgRNA levels were compared to traditional markers in order to assess their clinical utility.

A retrospective study was carried out, including 85 cases of CHBs and 74 CIBs.

Globally, when the virological markers (pgRNA, qHBsAg, and HBV DNA) were analyzed, significant differences were found between the CHB and CIB groups (P<0.001). Overall, positive correlations were demonstrated, as follows: between pgRNA levels and qHBsAg (Spearman r=0.30, P<0.001), between pgRNA and HBV DNA (Spearman r=0.73, P<0.001), and between pgRNA and ALT (Spearman r=0.67, P<0.001).

Out of the 85 CHB patients, 82 (96.5%) agreed to start treatment. At baseline, 38/82 patients, as well as the 3 untreated CHB patients, had undetectable pgRNA levels. The 74 CIB carriers also had undetectable pgRNA levels. During the follow-up period, no patients experienced viral reactivation or progression of liver disease.

These results suggest that the addition of plasmatic HBV-pgRNA levels to the traditional diagnostic flowchart of HBeAg-negative patients may improve the correct identification of cases at risk, especially patients with occasional increases in HBV viremia.

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INTRODUCTION

Chronic hepatitis B is a dynamic process with different phases reflecting the complex interaction between infection and immune response. The natural course of chronic HBV infection has been divided into five phases and HBeAg-negative patients' clinical performance is divided in two forms: CIB (HBeAg-negative chronic HBV infection), previously termed 'inactive carrier' phase, and CHB (HBeAg-negative hepatitis) (EASL, 2017).

CIB carriers have no or only mild liver tissue damage and show a low incidence of cirrhosis and hepatocellular carcinoma (HCC), in contrast to CHB patients, who have severe disease progression and a high incidence of cirrhosis (Brunetto *et al*., 2002; Papatheodoridis *et al*., 2008; Martinot-Peignoux *et al*., 2002). Therefore, their distinction is fundamental for disease management, including the decision of initiating antiviral therapy (EASL, 2017). Currently, differentiation of these two stages is based on quantification of HBV DNA (cut-off 2000 IU/mL) and ALT (alanine aminotransferase) levels (normal or elevated) (EASL, 2017), but these criteria often may not correctly identify these two stages.

Brunetto *et al*. proposed a threshold qHBsAg (quantitative hepatitis B surface antigen) level in order to characterize the patients with genotype D and an "inactive carrier" state, where the combination between the level of qHBsAg <1000 IU/mL and HBV DNA <2000 IU/mL identifies the CIB carrier with high diagnostic accuracy (94.3%) and a positive predictive value of 87.9% (Brunetto *et al*., 2010). In Italy, this evaluation has become a useful tool to classify and correctly manage CIB carriers (Marzano *et al.,* 2017), genotype D being the most common (Velkov *et al*., 2018).

Recently, pregenomic HBV RNA (pgRNA) has been investigated as surrogate of intrahepatic replicative activity of cccDNA (covalently-closed circular DNA) (Wang *et al*., 2016).

Key words:
Pregenomic HBV RNA (pgRNA), Hepatitis B core-related antigen (HBcrAg), Hepatitis B virus (HBV), Chronic HBeAg-negative infection, biomarker.

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Despite the usefulness of HBV RNA quantification in the management of chronic HBV infection, especially in predicting antiviral efficacy (Jansen *et al*., 2016; van Bömmel *et al*., 2015) and viral rebounding after drug withdrawal (Wang *et al.*, 2016; Perrillo *et al*., 2002; Liu *et al*., 2019), there are no unequivocal data on baseline HBV RNA levels in the various stages of chronic hepatitis.

This study aimed to compare plasma HBV-pgRNA levels with traditional diagnostic biomarkers and to assess whether testing pgRNA levels could help to correctly identify clinical profiles in a population of HBeAg-negative patients. In particular, we studied patients with occasional increases in HBV viremia >2000 IU/mL, which are difficult to categorize.

MATERIALS AND METHODS

Study population

A retrospective study was carried out, analyzing plasma samples from patients (n=159) who attended the hepatology clinics of St. Orsola-Malpighi Hospital (Bologna). All patients enrolled were HBsAg-seropositive for at least 6 months and HBeAg-seronegative. Exclusion criteria were the following:

1) age <18 years;
2) pregnancy;
3) concomitant hepatitis C or D virus;
4) other causes of liver disease, such as alcoholism or autoimmune or metabolic liver disease.

In accordance with the clinical and laboratory criteria proposed by EASL guidelines (EASL 2017), HBV infections were classified as follows: chronic HBeAg-negative hepatitis (CHB) and chronic HBeAg-negative infection (CIB), previously defined as "inactive disease".

As shown in *Figure 1*, patients underwent a follow-up with different time intervals (ranging from three to twelve months), according to both clinical and virological assessment, as described in EASL guidelines (EASL 2017). During each clinical appointment, the following markers were analyzed: qHBsAg, HBV DNA, and ALT levels. The CHB group was observed for a median time of 48 (interquartile range: 12-120) months, while the CIB subjects were followed for a median time of 36 (interquartile range: 12-96) months. Written informed consent was obtained from all subjects and the study protocol was approved by the Ethics Committee (Comitato Etico AVEC, n. 239 of 8th November 2016). This study was carried out in accordance with the Declaration of Helsinki, following the recommendations of the Ethics Committee.

Laboratory testing

pgRNA values were obtained at the time of enrollment (T0), whereas qualitative HBsAg (qHBsAg), HBV DNA levels were also analyzed during the follow-up, in addition to biochemical tests (alanine aminotransferase, aspartate aminotransferase, phosphatase alkaline, albumin, total protein, bilirubin, gamma glutamyl transferase, lactate dehydrogenase, coagulation panel, alpha fetoprotein) (*Figure 1*).

pgRNA was isolated from 500 µL plasma using the DSP Virus/Pathogen Midi commercial extraction kit (QIAsymphony, Qiagen) according to the manufacturer's protocol and treated with DNase I (Promega). Detection was performed by homemade RT-qPCR. To detect HBV RNA in plasma, we used the primers previously described by Laras *et al*. (2006), whereas the

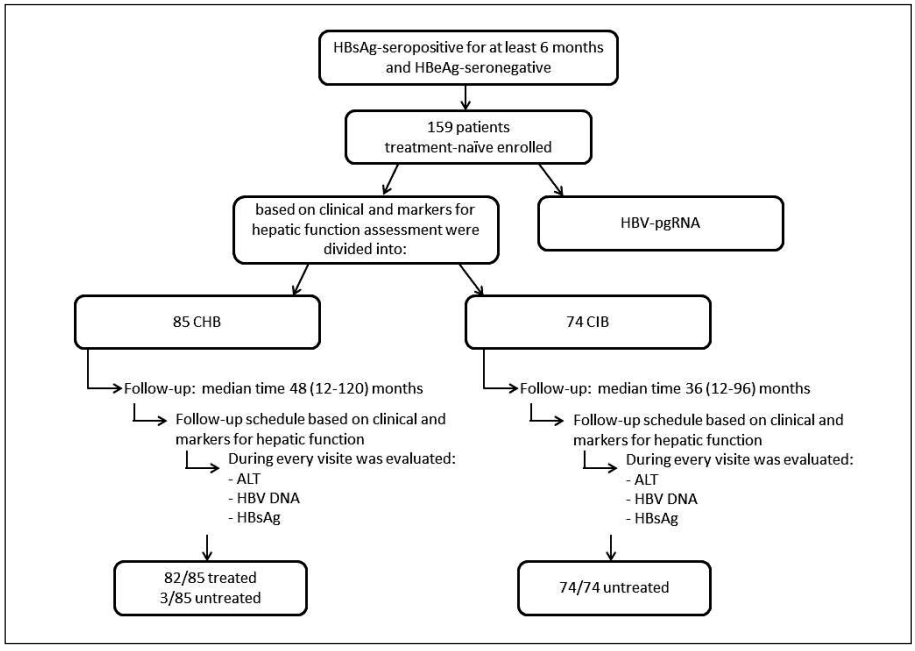

Figure 1 - *Study design.*

probe was designed by us (5'-CAAGCCTCCAAGCTGTGCCTTGGGTGGC, nucleotide [nt] 1866-1894).
Briefly, the PCR reaction mixture (SuperScript III Platinum One-Step qRT-PCR, Invitrogen) contained 0.4 mmol/L of both primers and 0.2 mmol/L of probe. Retrotranscription was performed at 50°C for 15 min, followed by 40 cycles at 95°C for 10s and 60°C for 30s. Assay specificity and LoD (limit of detection) were evaluated prior to sample processing. The LoD was determined by 10-fold serial dilution of the standard from 1.0 Log copies/mL to 10.0 Log copies/ml. Each dilution was tested in triplicate. A recombinant plasmid was used as a standard.
Briefly, DNA was extracted from 500 µL of plasma from viremic HBV-positive patients, by using the DSP Virus/Pathogen Midi commercial extraction kit (QIAsymphony, Qiagen) according to the manufacturer's protocol. A DNA fragment corresponding to 841 bp (nt 1546-2387) was amplified using the primers previously described by Wang *et al*. (2017). Sanger sequencing was used to confirm that the amplified fragment comprised the target sequence. PCR products were cloned in a pGEM-T-Easy vector (Promega, Madison, WI), according to the manufacturer's protocol. Recombinant plasmids were purified and HBV DNA concentration was determined by using a spectrophotometer. RNA transcripts were produced by using RNA polymerase SP6 according to the manufacturer's protocol (Roche Diagnostics, Germany) and used as standards, after evaluating the concentration. Assay specificity was evaluated by testing specimens from 30 HBV-negative blood donors and 50 HCV-positive patients.
The results were expressed as Log copies/mL.
The serology for traditional markers, qHBsAg and HBeAg, was performed by chemiluminescent microparticle immunoassay (CMIA) on the Architect platform (Abbott). The qHBsAg limit of detection was 0.05 IU/mL.
Finally, HBV DNA was quantified by real-time qPCR by Cobas AmpliPrep/Cobas TaqMan (Roche Diagnostics, Italy). This assay had an LoD of 20 IU/mL, with primers and probes targeting the highly conserved pre-core and core regions.

Statistical analysis

A descriptive analysis was performed to assess the main characteristics of all the subjects enrolled in the study (number of subjects, proportion of males and females, mean age).
The D'Agostino-Pearson omnibus normality test and Shapiro-Wilk tests were used to investigate the normality of data distribution. To evaluate statistically significant differences, t-test was used for quantitative data, whereas categorical data were analyzed with Chi-square test or Fisher's exact test. Differences between the clinical groups were analyzed using nonparametric Mann-Whitney U test. Correlations between variables were performed using the nonparametric Spearman rank correlation.
Data were analyzed with GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). A P value <0.05 was considered statistically significant.

RESULTS

Study population

A total of 159 HBeAg-negative patients were enrolled, including 85 cases of CHBs and 74 CIBs. The

Table 1 - *Baseline characteristics of patients.*

	CHB (n=85)	*CIB (n=74)*	*P-value*
Age	45 [18-71]	47 [25-83]	0,475
Male (%)	53 (62,4%)	36 (48,6%)	0,083
Ethnicity			
Caucasian	71 (83,5%)	70 (94,6%)	
African	4 (4,7%)	3 (4,1%)	
Asian	10 (11,8)	1 (1,3%)	
Genotype[a]			
A	8 (10,1%)		
B	3 (3,8%)		
C	6 (7,6%)		
D	62 (78,5%)	4 (100%)	
pgRNA (Log copies/mL)	5,62 (UD-6.60)	UD	<0.0001
HBV DNA (Log IU/mL)	5.33 (3.87-6.38)	2.66 (1.98-2.95)	<0.0001
qHBsAg (Log IU/mL)	3.58 (3.32-3.97)	2.66 (1.61-3.30)	<0.0001
ALT (U/L)[b]	48 (34-94)	21 (17-28)	<0.0001
Liver stiffness[c] *(kPa)*	6.35 (3.3-68)	4.65 (1.8-8)	<0.0001

qHBsAg: quantitative hepatitis B surface antigen, ALT: alanine aminotransferase, UD: undetectable [range]; Median value (interquartile range).
[a]Available in 83 patients, [b]normal value (<35 IU/L), [c]Available in 118 patients.

patients included in the study were 89 males (56.0%) and 70 females (44.0%). Overall, the mean age was 47.7±13.0 (± standard deviation, SD), ranging between 18 and 83, with no statistically significant differences between females and males (P<0.98).
Viral genotype information was available for only 83 patients (52.2%), genotype-D being the most prevalent (*Table 1*).
In the CIB group, 90.5% of patients (67/74) had HBV DNA levels under 2000 IU/mL. The remaining 7 had a viremia level >2000 IU/mL: they were classified as CIB cases since they showed both normal ALT values and absent or low fibrosis levels (fibroscan values F0-F1).

Comparison of viral biomarker levels in the two clinical categories

The excellent specificity of pgRNA assay was confirmed by the negative results from 30 HBV-negative blood donors and 50 HCV-positive patients. The LoD of the pgRNA assay was 10^2 copies/mL of plasma.
When analyzing the pgRNA levels, a significant difference was found between the two CHB and CIB groups (median 5.62 Log copies/mL *vs* undetectable copies/mL, respectively, *P*<0.001; *Figure 2a*). When analyzing CHB and CIB cases, similar results were obtained for qHBsAg (3.58±0.5 SD Log IU/mL *vs* 2.39±1.22 SD Log IU/mL, respectively, *P*<0.001; *Figure 2b*). Finally, HBV DNA viremia also showed a significant difference between CHB and CIB cases (median 5.33 Log IU/mL *vs* 2.66 Log IU/mL, respectively, *P*<0.001; *Figure 2c*). When analyzing the correlation between different serological indicators, pgRNA and qHBsAg showed a weak correlation (Spearman r=0.30, *P*<0.001), whereas close correlations were found both between pgRNA and HBV DNA (Spearman r=0.73, *P*<0.001), and between pgRNA and ALT (Spearman r=0.67, *P*<0.001).
When stratifying HBeAg-negative groups into two different clinical categories, we found a positive correlation between pgRNA and HBV DNA or ALT in CHB patients (Spearman r=0.77, *P*<0.001, *Figure 3a*; Spearman r=0.42, *P*<0.001, *Figure 3b*; respectively).

Follow-up

Eighty-two CHB patients (96.5%) gave their consent to start treatment. 52.4% of the patients were treated with Pegylated-Interferon (Peg-IFN) while the rest received nucleos(t)ide analogues (NAs). Out of these 82, 38 subjects already had an undetectable pgRNA level at T0. The remaining 44 patients with positive pgRNA level at T0 had a mean level of 6.66 Log copies/mL (ranging between 5.14 to 8.39 Log copies/mL). The three CHB patients refusing the antiviral treatment had negative pgRNA results at T0.
The 41 CHB patients with negative pgRNA results showed median HBV DNA levels lower than CHB patients with positive pgRNA (3.90 Log IU/mL *vs* 6.25 Log IU/mL, respectively). On the contrary, median HBsAg levels were similar in the two groups (3.59 Log IU/mL *vs* 3.58 Log IU/mL, respectively).
At the end of the follow-up period, 51 CHB patients had HBV DNA levels ≤20 IU/mL. Out of the 41 patients with negative pgRNA levels at T0, 5 seroconverted to anti-HBs, whereas 30 showed HBV DNA levels ≤20 IU/mL (range: from undetectable to 20 IU/mL). Time to seroconversion averaged 163.2 months. Out of the 74 CIB patients, 8 lost HBsAg, and 4 of them seroconverted to anti-HBs. All of these patients had an undetectable pgRNA result at T0.
At the end of the follow-up period, 42 patients (56.8%) had HBV DNA levels ≤20 IU/mL while 26 showed HBV DNA levels >20 IU/mL and ≤2000 IU/mL.
During follow-up, no patient displayed viral reactivation or liver disease progression.
For the assessment of liver disease progression, in addition to biochemical and virological testing, both abdominal ultrasound and transient elastography (fibroscan) were performed.
In patients with advanced disease, an abdominal ultrasound was performed every six months; once a year in cases with minor risks.

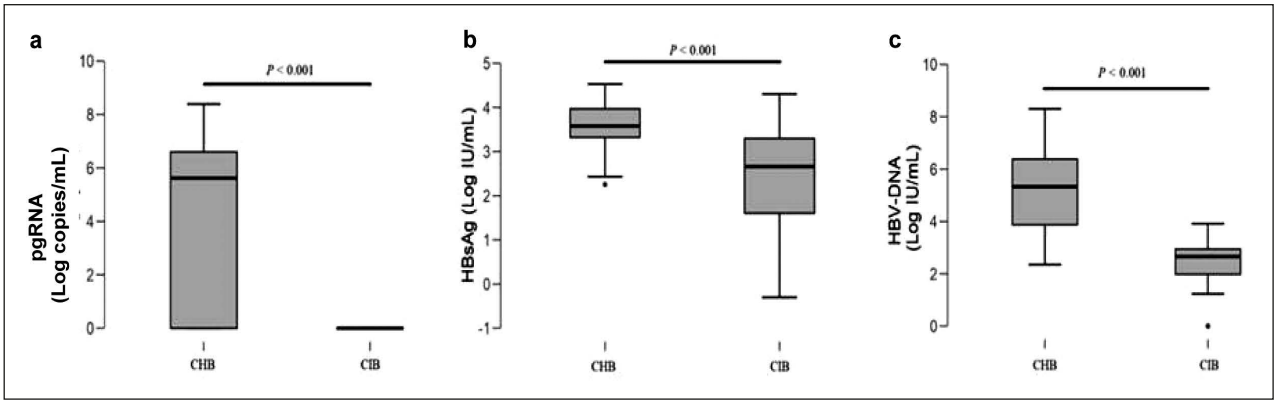

Figure 2 - *Distribution of different virological markers between the two clinical categories. (a) pgRNA (log copies/mL); (b) HBsAg (log IU/mL); (c) HBV DNA (log IU/mL).*

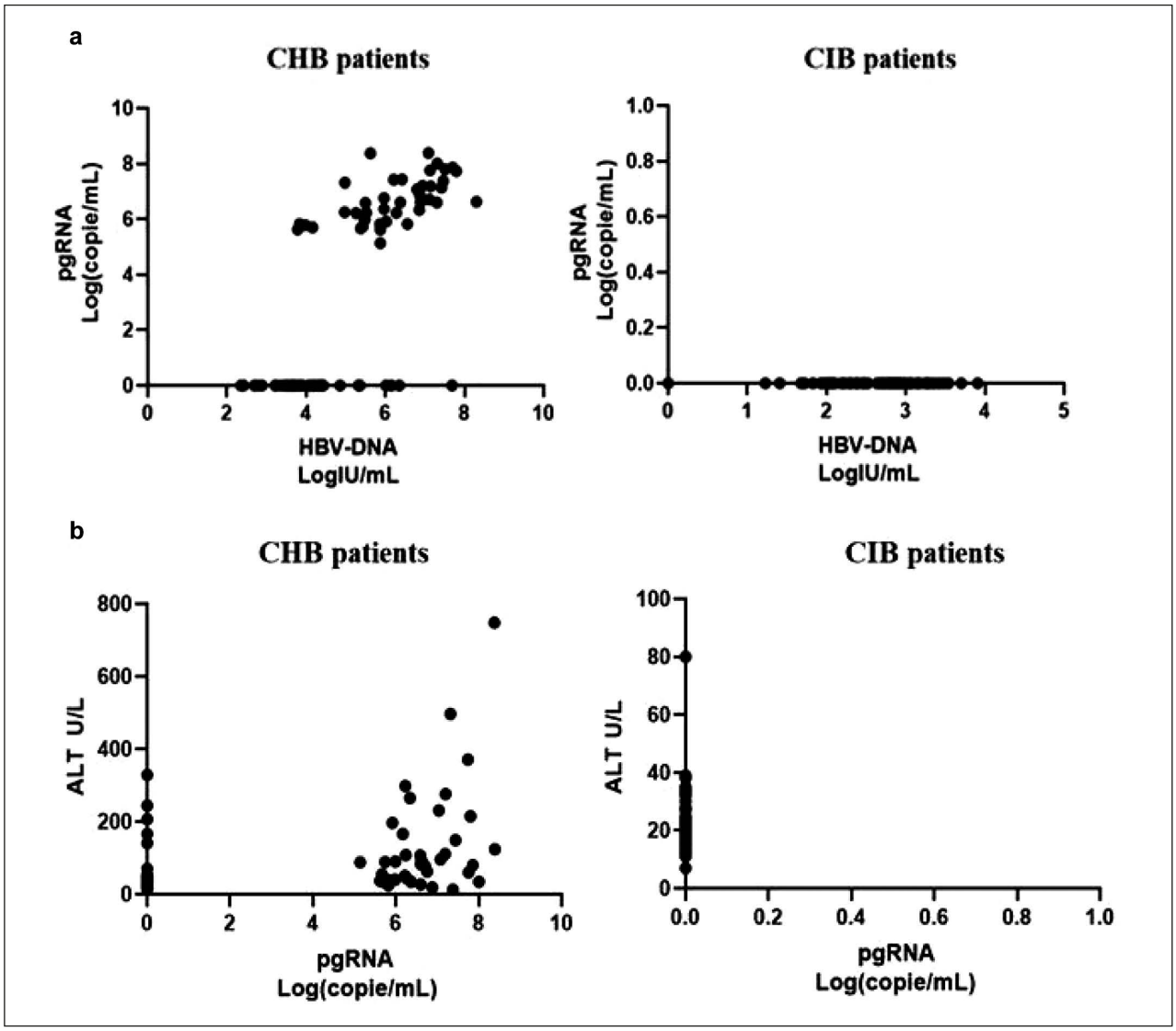

Figure 3 - *Correlation between (a) pgRNA and HBV DNA, (b) pgRNA and ALT in CHB and CIB groups.*

DISCUSSION

In this study, we detected HBV-pgRNA levels in the plasma of HBeAg-negative patients by RT-qPCR method. The correlation between pgRNA and traditional markers of HBV-infection was analyzed to determine the diagnostic performance of the proposed biomarker.

As expected, levels of pgRNA, HBV DNA and qHBsAg were higher in the CHB group than in CIB patients.

Previous studies showed a positive correlation between pgRNA and HBV DNA levels in chronic HBV patients (Wang *et al*., 2018; Mak *et al*., 2021). Our results confirm the presence of this correlation in the CHB group, as already reported in another study (Liu *et al*., 2019). Our findings are not unexpected, based on the higher viral replication activity observed in CHB patients in comparison with inactive carriers.

It is worth noting that despite other positive correlations among the various HBV serologic and molecular markers, the correlation between pgRNA and qHBsAg was not excellent. This weaker value might be attributed to the low capacity of conventional markers to correctly detect residual transcriptional activity (Seeger *et al*., 2015; Warner *et al.*, 2009; Liu *et al*., 2019). Indeed, HBsAg can be synthesized through multiple pathways, as opposed to pgRNA, which is produced only in the presence of cccDNA (Glebe *et al*., 2013; Cornberg *et al*., 2020; Wang *et al*., 2017). Moreover, during the low replicative phase, the production of defective particles exceeds that of virions (Dienes et al., 1990), leading to HBsAg levels higher than those of cccDNA. In addition, it has been hypothesized that serum HBsAg could be considered an indirect expression of transcriptionally active cccDNA rather than total intrahepatic cccDNA, thus leading to su-

per-production of HBsAg and lower cccDNA levels (Brunetto, 2010).

Other interesting aspects of the present study emerged when the follow-up was considered. First, during the follow-up, no subjects displayed viral reactivation or liver disease progression. Antiviral therapy with NAs can efficiently inhibit reverse transcriptase activity, thus suppressing the production of HBV DNA. On the contrary, NAs have no effect on pgRNA levels (Butler *et al.*, 2018). In this study, 72.3% of patients (115/159) had undetectable pgRNA levels at T0. In these cases, pgRNA levels correlated positively with the data observed during the follow-up.

Second, we observed the potential utility of pgRNA in discriminating between the two groups. On the basis of pgRNA levels and the data obtained throughout the follow-up, the 41 CHB patients with negative pgRNA levels would be reclassified as CIBs. These patients with occasional increases in HBV viremia >2000 IU/mL can hardly be categorized. Previous studies showed that basal pgRNA levels differed significantly in the natural course of chronic HBV infection. In particular, pgRNA levels were lower in CIB patients, approaching the lower limit of detection (Wang *et al*., 2017; Liu *et al.*, 2019). Furthermore, as shown by Limothai *et al.* (2019), low pgRNA levels before therapy can identify patients with high probability of virologic response. Two limitations of the study need to be mentioned. First, pgRNA levels were not monitored during the follow-up to assess a clearance or the stability of the data. Second, there were no kPa values of fibroscan for all of the patients (this data was available for only 118 patients). It would be interesting to evaluate whether there is a correlation between kPa values and pgRNA values. Further studies are needed to confirm these results.

In conclusion, in this retrospective study of HBeAg-negative patients, we demonstrated that pgRNA levels correlated with traditional markers. Our data showed that detection of pgRNA is a valuable marker for correct management of HBeAg-negative patients. Moreover, low levels of pgRNA at baseline can identify patients with a high probability of virological response. For this reason, pgRNA could be an alternative tool for detecting residual transcriptional activity without invasive investigation.

These results suggest that the detection of HBV pgRNA levels in sera of HBV-infected patients is comparable with the detection of other conventional diagnostic markers.

However, further studies are needed to understand and identify the most useful timeline for pgRNA testing.

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Conflicts of interest

None.

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