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Hepatitis B Virus RNA as Early Predictor for Response to Pegylated Interferon Alpha in HBeAg-Negative Chronic Hepatitis B

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Background. Hepatitis B virus RNA (HBV-RNA) is a novel serum biomarker that correlates with transcription of intrahepatic covalently closed circular (cccDNA), which is an important target for pegylated interferon (PEG-IFN) and novel therapies for functional cure. We studied HBV-RNA kinetics following PEG-IFN treatment and its potential role as a predictor to response in HBeAg-negative chronic hepatitis B (CHB) patients.

Methods. HBV-RNA levels were measured in 133 HBeAg-negative CHB patients treated in an international randomized controlled trial (PARC study). Patients received PEG-IFN α-2a for 48 weeks. HBV-RNA was measured from baseline through week 144. Response was defined as HBV-DNA <2000 IU/mL and ALT normalization at week 72. Kinetics of HBV-RNA were compared with HBV-DNA, HBsAg, and HBcrAg.

Results. Mean HBV-RNA at baseline was 4.4 (standard deviation [SD] 1.2) $\log_{10}$ c/mL. At week 12, HBV-RNA declined by −1.6 (1.1) $\log_{10}$ c/mL. HBV-RNA showed a greater decline in responders compared to nonresponders early at week 12 (−2.0 [1.2] vs −1.5 [1.1] $\log_{10}$ c/mL, $P = .04$). HBV-RNA level above 1700 c/mL (3.2 $\log_{10}$ c/mL) had a negative predictive value of 91% at week 12 and 93% at week 24 ($P = .01$) for response. Overall, HBV-RNA showed a stronger correlation with HBV-DNA and HBcrAg (.82 and .80, $P < .001$) and a weak correlation with HBsAg (.25). At week 12, HBV-RNA was significantly lower among patients with lower HBsAg (<100 IU/mL) or HBsAg loss at week 144.

Conclusions. During PEG-IFN treatment for HBeAg-negative CHB, HBV-RNA showed a fast and significant decline that correlates with treatment response and HBsAg loss at long-term follow-up.

Clinical Trials Registration. NCT00114361

Keywords. Serum biomarkers; cccDNA; treatment response; finite treatments.

Response to treatment of chronic hepatitis B virus (HBV) infections with pegylated interferon alpha (PEG-IFN) is a stable remission through the establishment of sustained immune control. This stage is characterized by low HBV replication and normal alanine aminotransferase (ALT) levels and is ideally followed by hepatitis B surface antigen (HBsAg) loss, often referred to as a functional cure. Sustained immune control is associated with reduced risk of liver disease progression and the development of hepatocellular carcinoma (HCC) [1, 2]. Although of finite duration, PEG-IFN treatment is associated with significant side effects. Thus, the ability to predict treatment response would optimize PEG-IFN treatment by minimizing treatment exposure in those patients unlikely to respond [3].

The main transcriptional template of HBV is the intrahepatic covalently closed circular DNA (cccDNA) in the hepatocyte nuclei, and immune control of HBV infection is believed to be associated with reduced copy numbers or transcriptional activity of the cccDNA [2]. As a liver biopsy is invasive and potentially dangerous and cccDNA quantification is not well established, serum markers are required that are a reliable surrogate of cccDNA activity, and that may act as an early predictor for sustained immune control following treatment [4–6].

HBV-RNA can be quantified using polymerase chain reaction (PCR) techniques and is one of the serum markers that is considered a direct transcriptional product of cccDNA [7–9]. The nature of HBV-RNA in serum has not been thoroughly investigated, but it most likely includes the 3.5 kb pregenomic RNA (pgRNA), which is the template for reverse transcription to

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HBV-DNA [9–11]. Serum HBV-RNA has shown a strong correlation with the transcriptional activity of cccDNA during treatment with nucleos(t)ide analogues (NA) and PEG-IFN [12, 13]. Additionally, HBV-RNA levels were found to be predictive for response to NA and PEG-IFN treatment in HBeAg-positive individuals [2, 13–16]. However, to date, it is unclear what the role is of serum HBV-RNA in HBeAg-negative chronic hepatitis B (CHB) to predict response and functional cure by PEG-IFN or any of the novel compounds in development [15, 17, 18]. Due to the existence of integrated viral DNA, which makes HBsAg a less reliable marker, reliable response markers for those patients represent a significant unmet medical need. Therefore, we have assessed the association of serum HBV-RNA levels with response to PEG-IFN in a large and well-characterized HBeAg-negative population, and we compared its performance to other circulating HBV biomarkers.

PATIENTS AND METHODS

HBV-RNA levels were measured in stored serum samples of 133 HBeAg-negative CHB patients of Caucasian descent who participated in a previously conducted international randomized controlled multicenter trial (PARC study) [19]. All patients had given consent for biomarker studies. Details on the PARC study has been published previously [19]. Patients were treated with PEG-IFN alpha-2a (180 µg/week) in 1:1 randomization with ribavirin (RBV) for 48 weeks. Groups with or without RBV were similar with respect to baseline patient characteristics, and because no difference in response was found between the treatment groups, patients were pooled for analysis of this study (Supplemental Figure 1). After week 72, all sites were given the option to enroll patients into a long-term follow-up (LTFU) study that extended follow-up until week 144, and 72 patients were enrolled. All authors had access to the study data and reviewed and approved the final paper.

Measurements and Endpoints

Levels of HBV-RNA were measured at baseline, weeks 12, 24, 48 (end of treatment [EOT]), 72 (follow-up [FU]) and 96–144 (long-term follow-up [LTFU]). HBV-RNA samples during LTFU were analyzed based on the available samples during this time period and the latest visit occurring between weeks 96 and 144. The definition of response included HBV-DNA level below 2000 IU/mL and normalization of ALT at month 6 after the end of PEG-IFN treatment (corresponding to week 72 from baseline) as determined by current treatment guidelines [20, 21]. Also, we studied HBV-RNA kinetics and compared those with the kinetics of other viral biomarkers (HBV-DNA, qHBsAg, and HBcrAg) in relation to response.

We studied both the correlation between HBV-RNA and response as well as the actual significance of HBV-RNA to predict response versus nonresponse. Because the study follow-up was officially completed by week 72 and not all patients were followed afterward for LTFU, we used week 72 as our endpoint for response prediction. Also, we studied long-term response, HBsAg changes, and HBsAg loss, 12 through 24 months posttreatment, (corresponding to weeks 96–144 from baseline) [22]. Those with data for LTFU were similar to the total study population (data not shown).

HBV-RNA Quantification in Serum

HBV-RNA was quantified from serum samples using a rapid amplification of complementary DNA (cDNA)-ends (RACE)-based real-time polymerase chain reaction (PCR) technique (Section of Hepatology, University Hospital Leipzig, Germany) that has been previously described [23, 24]. Quantification of polyadenylated HBV-RNA was performed using specific primers (including HBV-RNA RT primer 5′-ACC ACG CTA TCG CTA CTC AC (dT17) GWA GCT C). To increase the assay's sensitivity in comparison to previously used methods, nucleic acids that were extracted from 50 µL of serum were eluted in a smaller volume of 20 µL (Qiaamp minelute virus kit, Quiagen, Hilden, Germany) prior to reverse transcription. As a result, the assay's lower limit of quantification (LoQ) was 640 (2.8 $\log_{10}$) copies/mL, and the lower limit of detection (LoD) was 80 (1.9 $\log_{10}$) copies/mL. HBV-RNA values between LoQ and LoD were recorded in order to describe the changes among the large subgroup of patients in our cohort with a very low on-treatment HBV-RNA level.

Other Laboratory Measurements

Routine biochemical and hematological tests were measured at the participating centers. Serum ALT levels were standardized by calculating the ratio to the local upper limit of normal per center and according to sex. HBV-DNA was measured using TaqMan-based PCR assays (Roche Diagnostics, Basel, Switzerland; lower limit of detection 35 copies/mL [6 IU / mL]). Serum qHBsAg levels were measured using the Architect HBsAg assay (Abbott Laboratories, Santa Clara, CA, USA; range .05–250 IU/mL). Serum HBcrAg was measured using the Lumipulse® G HBcrAg assay (Fujirebio Europe, Belgium; linear range 3.0–7.0 $\log_{10}$ U/mL). HBV genotype analysis was performed using the INNO-LiPA HBV genotype assay (Fujirebio Europe, Ghent, Belgium). All these virological tests were performed at the central laboratory of the Erasmus Medical Center, Rotterdam, The Netherlands.

Statistical Analysis

IBM SPSS version 24.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analyses. Skewed laboratory values were log-transformed before analyses and were expressed as mean (standard deviation [SD]). Associations between variables were tested using Student *t*-test, χ^2 test, Pearson correlation, or their nonparametric equivalents where appropriate. For

linear correlations, Pearson correlation coefficients (r) and the correlation coefficients through multivariate repeated measurement models with a Kronecker product covariance were provided. The performances of the retrieved prediction models were tested with receiver operating characteristic (ROC) curve analysis. All analyses were performed 2-sided at the .05 level of significance.

For the analyses of response prediction, HBV-RNA values were grouped based on of .2 $\log_{10}$ c/mL increments at a range 1.0–5.0 $\log_{10}$ copies/mL. Choosing the optimal cutoff value was performed through a grid search based on a minimum of 15 patients per subgroup, the highest specificity, significant χ^2 test, significant likelihood-ratio test, and the lowest sum of log-likelihood ratios. In order to analyze HBV-RNA in relation to late HBsAg changes and HBsAg loss, last HBsAg results were carried forward from week 72 to 144.

RESULTS

Study Cohort

Study population characteristics are shown in Table 1. Mean age was 42 (SD 11) years, and 96% of the patients were Caucasians. HBV genotype distribution was 17/1/3/80% for genotype A/B/C/D. In our population, 21.9% had a response, and 23.6% had a long-term response. Five patients (9%) had HBsAg loss through week 144.

Table 1. Patients Characteristics at Baseline

Characteristics	N = 133
Demographics	
Age, years	42 (11; 19–67)
Male, n (%)	98 (74)
Race, n (%)	
Caucasian	127 (96)
Asian	4 (3)
Other	2 (2)
HBV genotype, n (%)	
A	16 (13)
B	1 (1)
C	3 (2)
D	97 (80)
Other	5 (4)
Laboratory results	
HBV-DNA[a]	6.1 (1.2; 3.5–9.1)
qHBsAg[a]	3.8 (.6; 1.1–5.0)
ALT (× ULN)[b]	3.2 (2.6; .7–17.2)
HBcrAg[c]	5.0 (1.2; 2.0–7.8)
HBV RNA characteristics at baseline	
HBV-RNA[d]	4.4 (1.2; 1.9–7.7)
Peg-IFN monotherapy	4.4 (1.0; 1.9–7.7)
Peg-IFN + RBV	4.2 (1.3; 1.9–7.0)
HBV-RNA LoQ, n (%)	13 (10)

Continuous variables are expressed as mean (SD), categorical variables as n (%).

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; HBcrAg, hepatitis B core-related antigen; LoQ, lower limit of quantification; PEG-IFN, pegylated interferon; qHBsAg, quantitative hepatitis B surface antigen; RBV, ribavirin; SD, standard deviation; ULN, upper limit of normal.

[a]Logarithmic scale, IU/mL.

[b]Multiples of upper limit of the normal range.

[c]Logarithmic scale, U/mL.

[d]Logarithmic scale, c/mL.

Serum HBV-RNA During PEG-IFN Treatment and Follow-up

HBV-RNA was quantified in a total of 563 serum samples (Figure 1). At baseline, the mean serum HBV-RNA level was 4.4(SD 1.2; 1.9–7.7)$\log_{10}$c/mL.

During PEG-IFN treatment and follow-up, HBV-RNA declined significantly over time: week 12: −1.57[1.1] $\log_{10}$c/mL, week 24: −1.63[1.2] $\log_{10}$c/mL, week 48: −1.72[1.3] $\log_{10}$c/mL, and week 72: −.99(1.6)$\log_{10}$ c/mL (all $P < .001$). After stopping therapy, from week 48 to week 72, HBV-RNA levels showed a mean increase of .7 (1.4; −2.4–4.6) $\log_{10}$c/mL ($P < .0001$) (Figure 1A).

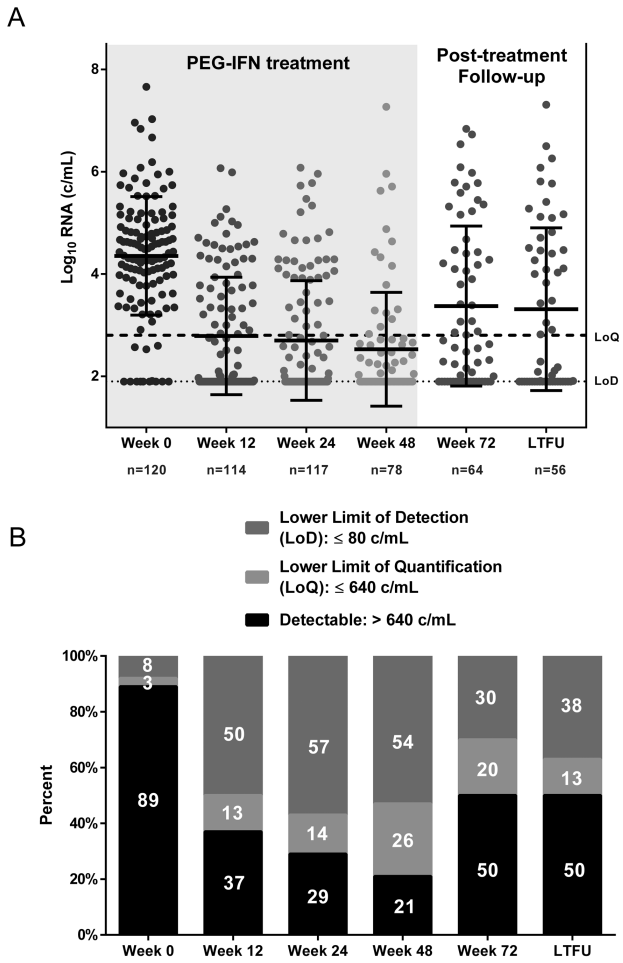

Figure 1. *A*, Individual HBV-RNA measurements according to different time points during and after treatment. Dots represent individual HBV-RNA measurements from baseline to week 144. Vertical lines represent the standard deviation and horizontal lines represent mean level of HBV-RNA (log c/mL) at each time point. LoQ ≤ 640 c/mL and LoD ≤ 80 c/mL. (*n = 56). *B*, HBV-RNA detectability in the total study population (N = 133).The bars represent the proportion of patients with detectable, below LoQ or LoD of HBV-RNA. Abbreviations: HBV-RNA, hepatitis B virus RNA; LoD, lower limit of detection; LoQ, lower limit of quantification; LTFU, long-term follow-up; PEG-IFN, pegylated interferon.

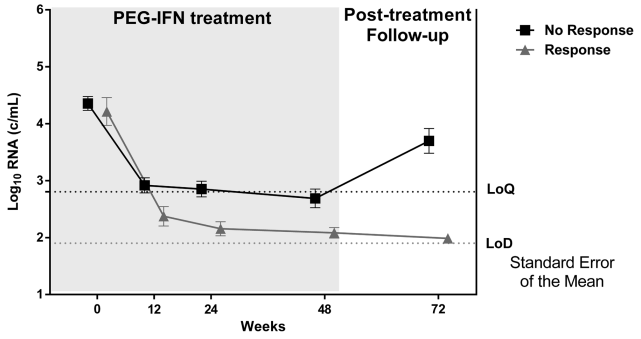

Figure 2. HBV-RNA dynamics according to treatment response. Lines represent mean levels of HBV-RNA (log c/mL) from baseline to week 72 according to treatment response at week 72. Vertical lines represent the standard deviation of HBV-RNA (log c/mL) at each time point. LoQ ≤ 640 c/mL and LoD ≤ 80 c/mL. Abbreviations: HBV-RNA, hepatitis B virus RNA; LoD, lower limit of detection; LoQ, lower limit of quantification; PEG-IFN, pegylated interferon.

Among the total study population, 13(11%) had HBV-RNA levels below LoQ ($P < .001$) and 8%(n = 9) were below LoD at baseline ($P < .001$). At EOT, 62(80%) had HBV-RNA levels below LoQ. However, 32 (50%) had HBV-RNA below LoQ at week 72. (Figure 1B).

HBV-RNA Levels During Treatment Correlate With Response

At baseline, there was no significant difference in HBV-RNA levels between responders and nonresponders (4.2 [1.2] vs 4.4 [1.1] $\log_{10}$c/mL, $P = .6$, respectively; Figure 2). However, during treatment, HBV-RNA declined more among responders than in nonresponders (week 12: −2.0[1.2] vs −1.5[1.1] $\log_{10}$c/mL, $P = .04$; week 24: −2.1[1.2] vs −−1.5[1.3] $\log_{10}$c/mL, $P = .04$; week 48: −2.2[1.2] vs −1.6[1.3] $\log_{10}$c/mL, $P = .09$). During posttreatment follow-up, HBV-RNA increased significantly among nonresponders but not in responders (−.6[1.5] vs −2.5[1.1] $\log_{10}$c/mL) (Supplemental Figure 2).

Relation of HBV-RNA to Other HBV Serum Biomarkers

HBV-RNA, HBcrAg, and HBV-DNA all declined significantly after starting treatment, whereas there was a more limited decline in HBsAg levels (Figure 3). All biomarkers showed more decline among responders at week 12 from baseline compared with nonresponders. During treatment, HBV-RNA was undetectable in more individuals than other biomarkers at all time points (Supplemental Table 1). Regarding correlation between different biomarkers, overall, HBV-RNA showed a stronger correlation with HBV-DNA and with HBcrAg (r = .69 and r = .74, respectively; $P < .001$) and a weak correlation with qHBsAg (r = .17, $P < .001$) (Figure 4A). Before treatment, HBV-RNA showed a strong correlation with HBV-DNA and HBcrAg (ρ = .80 and ρ = .81, respectively; $P < .001$) and a weak correlation with quantitative HBsAg (ρ = .17) (Figure 4B). When comparing the performance of the different biomarkers and their combinations to predict response, adding HBV-RNA showed the best predictive ability (Supplemental Table 2). HBV-RNA and HBcrAg appear to show the same pattern of decline/nondecline by response/nonresponse (Figure 3). However, HBV-RNA discriminate better between response and nonresponse as compared to HBcrAg (Figure 5; note that HBcrAg has a higher variation than HBV-RNA).

Response Prediction by HBV-RNA Levels

Various cutoffs were selected to identify prediction groups with the highest specificity and sensitivity. HBV-RNA level above 1700 (3.2 $\log_{10}$)c/mL was selected as the best cutoff for predicting nonresponse. Using this cutoff, nonresponse to PEG-IFN could be identified with a high accuracy. Thus, 30 out of 33 patients at weeks 12 and 27 out of 29 patients at week 24 who were above the cutoff were found to be nonresponders, implying a 91% and 93% negative predictive value for both time points ($P = .01$ and .01, respectively) (Figure 6). However, the positive predictive value of HBV-RNA levels <1700 c/mL (3.2 $\log_{10}$ c/mL) at weeks 12 and 24 were only 30% and 29%, respectively.

HBV RNA to Optimize PEG-IFN Treatment Stopping Rule

We assessed the role of adding the proposed HBV-RNA cutoff (3.2 $\log_{10}$c/mL) at week 12 to the current stopping rule (no HBsAg decline and < 2 $\log_{10}$IU/mL HBV DNA decline at week 12). The response was determined at week 72 by achieving ALT normalization and HBV-DNA < 2000 or < 200 IU/mL. In response with HBV-DNA < 2000, stopping rule showed 100% NPV and 27% specificity while adding HBV-RNA showed 91% NPV and 37% specificity. In response with HBV-DNA < 200, stopping rule showed 100% NPV and 23% specificity and adding HBV RNA showed 100% NPV and 35% specificity (Supplemental Figure 3).

Serum HBV-RNA According to Long-term Response, HBsAg Changes and HBsAg Loss

HBV-RNA dynamics were assessed according to the long-term response (weeks 96–144). Long-term responders showed lower levels of HBV-RNA during treatment and follow-up periods compared to nonresponders (week 12: 2.5 [1.0] vs 2.9 [1.2] $\log_{10}$c/mL, $P = .1$; week 24: 2.2 [.8] vs 2.9 [1.3] $\log_{10}$c/mL, $P = .02$; week 48: 2.2 [.7] vs. 2.7 [1.4] $\log_{10}$c/mL, $P = .2$; week 72: 2.5 [1.6] vs. 3.6 [1.6] $\log_{10}$c/mL, $P = .09$; LTFU: 2.1 [.8] vs. 3.7 [1.6] $\log_{10}$c/mL, $P < .001$).

In order to correlate early HBV-RNA kinetics with long-term HBsAg outcome for PEG-IFN and potentially novel treatment options, patients were divided into 4 groups (>1000 IU/mL, 100–1000 IU/mL, <100 IU/mL, and HBsAg loss) according to their last available HBsAg level. During the overall follow-up period, 5 patients achieved HBsAg loss. HBV-RNA mean

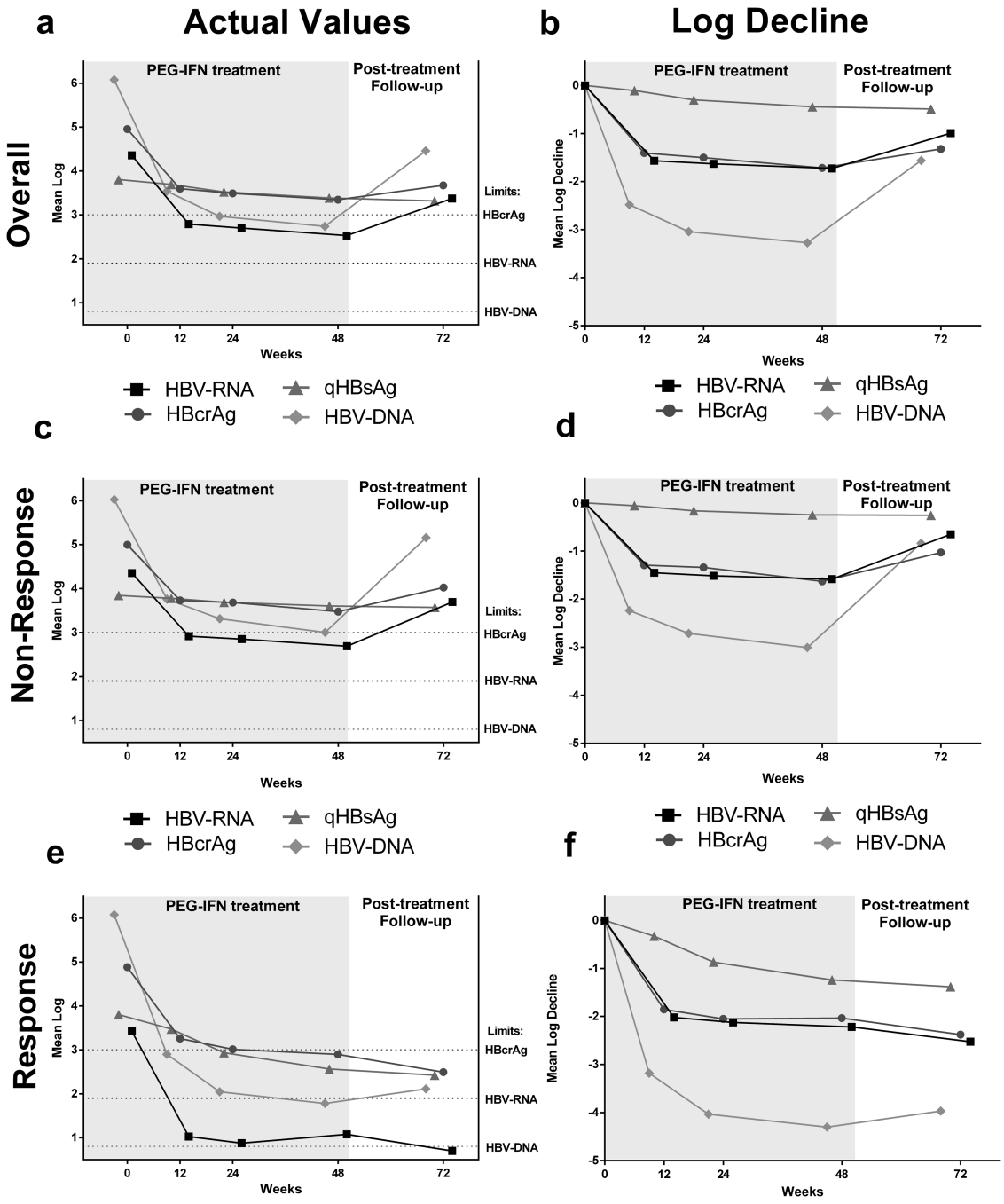

Figure 3. Patterns of HBV-RNA, HBcrAg, HBV-DNA and qHBsAg biomarkers during and after treatment. *Left*, actual log values, *right*, log decline. Lines represent mean levels of HBV-RNA, HBcrAg, HBV-DNA, and qHBsAg from baseline to week 72. (*A* and *B*) represent overall changes; (*C* and *D*) represent changes among nonresponders; and (*E* and *F*) represent changes among responders. Horizontal dotted lines LoD of each biomarker. Abbreviations: HBcrAg, hepatitis B core-related antigen; HBV-DNA, hepatitis B virus DNA; HBV-RNA, hepatitis B virus RNA; LoD, lower limit of detection; PEG-IFN, pegylated interferon; qHBsAg, quantitative hepatitis B surface antigen.

values were significantly lower among patients with HBsAg loss and < 100 IU/mL early at weeks 12 and 24 compared with HBsAg 100–1000 IU/mL and > 1000 IU/ml (Figure 7). HBV-RNA at week 24 showed higher diagnostic accuracy in area under the curve (AUC) analysis compared to week 12 among the different HBsAg groups (HBsAg loss: .72 vs .69; <100 IU/mL: .73 vs .70; 100–1000 IU/mL: .68 vs .62).

DISCUSSION

Our study is the first to our knowledge to analyze HBV-RNA dynamics in a large randomized controlled trial of HBeAg-negative CHB patients treated with PEG-IFN, enabling us to clarify the role of HBV-RNA as an indicator for predicting treatment outcome, including HBsAg loss. HBV-RNA showed a significantly stronger decline in responders compared to nonresponders as

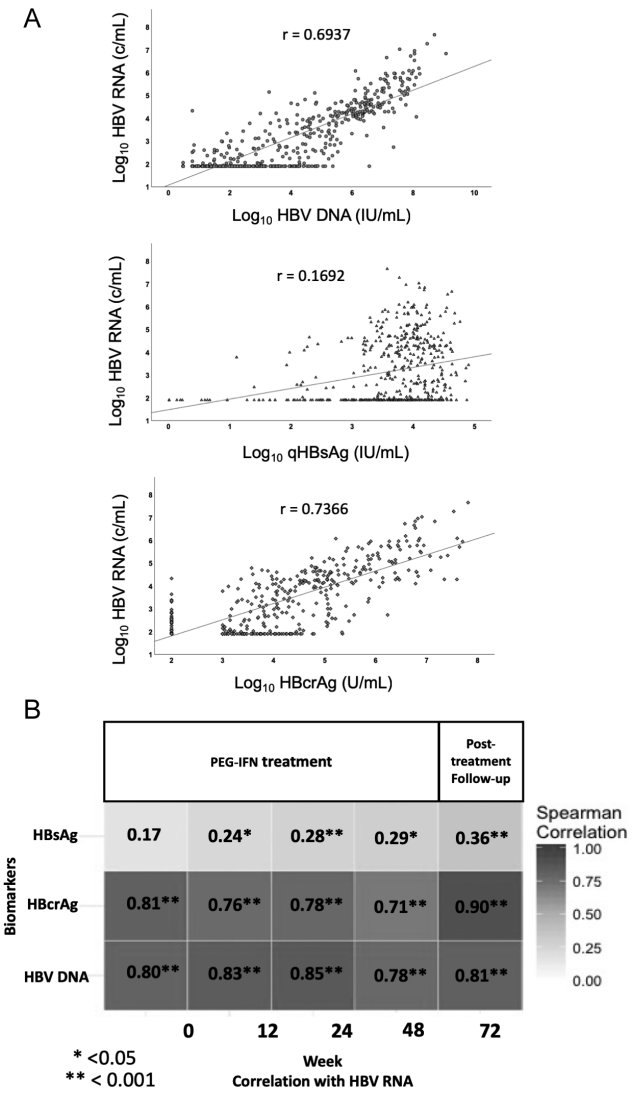

Figure 4. *A*, Correlation coefficient of serum levels of HBV-RNA with HBV-DNA, qHBsAg, and HBcrAg. Overall correlation of serum levels of HBV RNA with serum levels of HBV-DNA, qHBsAg, and HBcrAg. For repeated measurements correlation Kronecker product covariance were provided. *B*, Correlation of serum levels of HBV-RNA with HBcrAg, HBV-DNA, and qHBsAg before, during, and after PEG-IFN treatment. Heat map represent correlation of HBV-DNA, HBcrAg, and HBsAg with HBV-RNA from baseline to week 72. Abbreviations: HBsAg, hepatitis B surface antigen; HBcrAg, hepatitis B core-related antigen; HBV-DNA, hepatitis B virus DNA; HBV-RNA, hepatitis B virus RNA; LoD, lower limit of detection; PEG-IFN, pegylated interferon; qHBsAg, quantitative hepatitis B surface antigen.

early as treatment week 12. An HBV-RNA level of ≥1700 c/mL (3.2 $\log_{10}$ c/mL) at week 12 treatment was highly predictive for nonresponse at week 72 with a 91% negative predictive value. HBV RNA level of ≥1700 c/mL lead to 10% additional inclusion to the current stopping rule. At EOT (week 48), HBV-RNA was under LoQ in almost all responders in contrast to nonresponders (P = .01). HBV-RNA decline at weeks 12 and 24 also correlated well with HBsAg levels < 100 IU/mL and HBsAg loss at longer-term follow-up.

The biological role of HBV-RNA in serum is still unknown. However, it has recently been considered a biomarker for transcriptionally active cccDNA [5]. Previous studies have demonstrated that HBV cell cultures secrete a large amount of nonenveloped capsids which—among other HBV-DNA replicative intermediates—contain HBV-RNA [25–27]. However, it has not been proven whether the detected serum HBV-RNA is covered by capsid in vivo.

Several studies have inferred that serum HBV-RNA can be a useful marker for monitoring the efficacy of NA and PEG-IFN [10, 15, 18, 28, 29]. However, limited data have been published on HBV-RNA levels in patients with HBeAg-negative CHB who were treated with PEG-IFN, which leads to substantial HBsAg decline and even functional cure in a minority [30]. In our HBeAg-negative population, HBV-RNA levels were lower at baseline compared to the HBeAg-positive patients in other studies [18, 24]. HBV-RNA became subsequently undetectable in almost all responders to PEG-IFN during the treatment period. However, patients who had higher levels had a high probability of having nonresponse (P = .01) and to have HBsAg levels that remain high after treatment. The pronounced observed early HBV-RNA decline may be explained by the substantial inhibitory effect of interferon on HBV-RNA production itself [9, 15, 16]. Also, PEG-IFN has shown to act on the posttranscriptional steps of the HBV life cycle and at the levels of the capsids containing pregenomic RNA either to accelerate their degradation or to prevent their assembly [17]. We feel that the strong suppression of this molecule in responders may be an expression of silencing of cccDNA activity, which might be a prerequisite for the establishment of durable immune control over the infection.

Both HBV-DNA and HBcrAg showed a stronger correlation with HBV-RNA in contrast to HBsAg that showed a week correlation. We observed highly similar kinetics in HBV-RNA and HBcrAg levels at all time points in responders as well as in nonresponders following the initiation of PEG-IFN treatment. The correlation between HBV-RNA and HBcrAg is not well established. However, a similar correlation between both markers was previously found by Rokuhara et al in untreated patients and may suggest that both markers reflect cccDNA transcription [10].

We used the currently applicable definition for response and serum HBsAg levels as endpoints in our analysis [20, 21]. In contrast to the other HBV biomarkers included in our study, the levels of HBsAg showed no decrease in nonresponders and only a mild decrease in responders (Figure 3), an observation that has been made in previous studies. It is known that HBsAg production is mainly driven from the cccDNA. However, HBsAg may also be produced from HBV-DNA integrated into the host genome, particularly in HBeAg negative CHB [31]. Therefore, the weaker correlation of HBV-RNA with HBsAg as compared to HBcrAg and HBV-DNA might be caused by the different

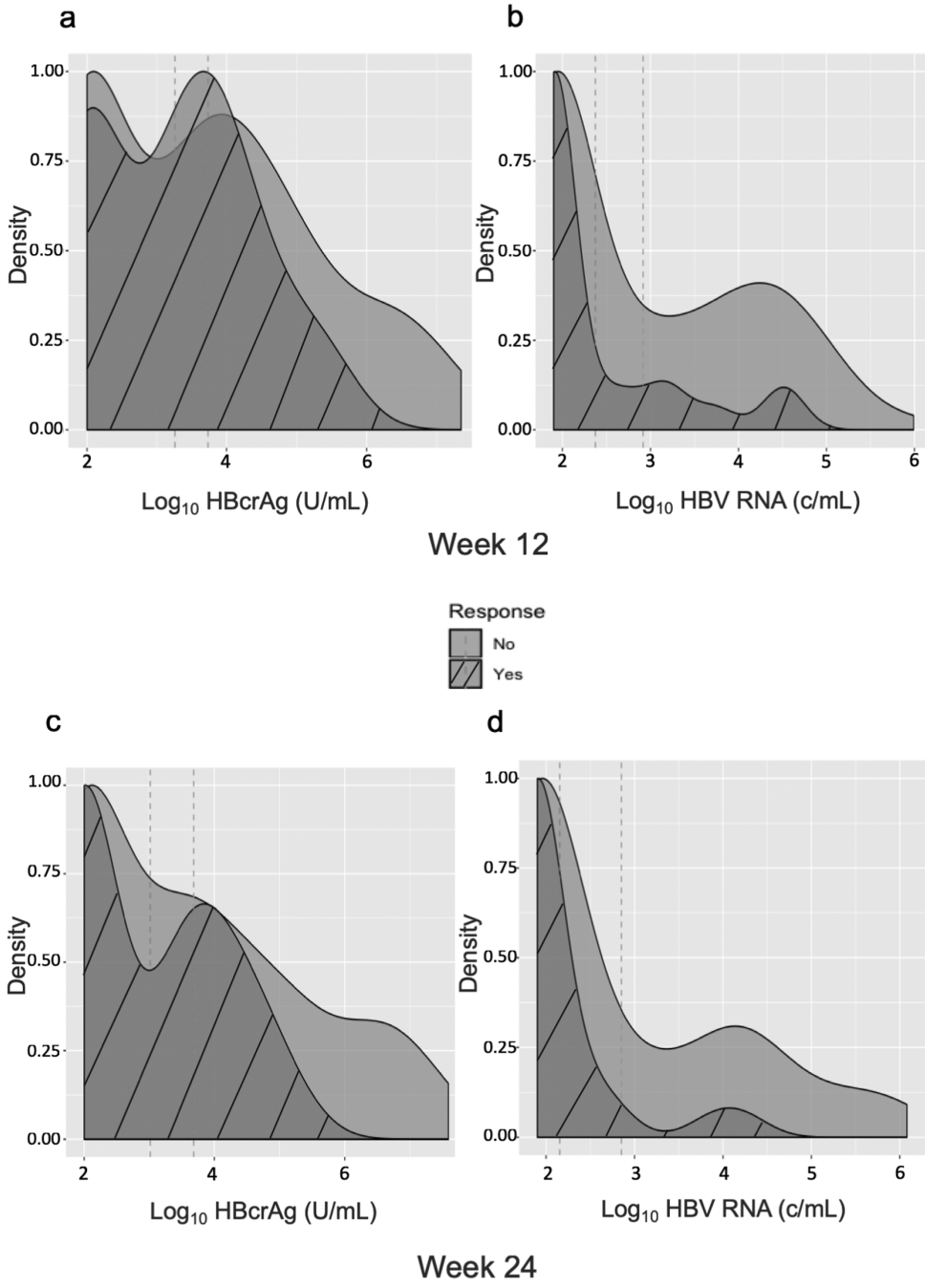

Figure 5. Kernel smoothed density estimate for response prediction comparing HBV-RNA and HBcrAg at weeks 12 (*A*, *B*) and 24 (*C*, *D*). The mapping shows a distinctive difference between response and no response in HBV-RNA. However, HBcrAg did not exhibit the same pattern as an overlap is observed between responders and nonresponders. Abbreviations: HBcrAg, hepatitis B core-related antigen; HBV-RNA, hepatitis B virus RNA.

sources of HBsAg. Indeed, it was demonstrated in chronically HBV-infected chimpanzees that the frequency of HBV integration increases during the HBeAg negative stage of the disease, leading to a rate of > 90% of the messenger RNA derived from integrated HBV sequences and only 10% from the cccDNA [32, 33]. In contrast, the common decrease of HBV-RNA, HBcrAg, and HBV-DNA observed in our study suggests a different source of these markers than HBsAg, most likely cccDNA.

To improve the HBV-RNA test performance, we modified our assay toward a higher sensitivity by the concentration of nucleic acids before reverse transcription. Additionally, we report HBV-RNA values under the LoQ, which give additional information. Nonetheless, it may be necessary to assess whether even lower limits can increase the clinical value of HBV-RNA quantification and if such a marker can help define a "para-functional cure" [34] as quantitative HBsAg may be a suboptimal marker

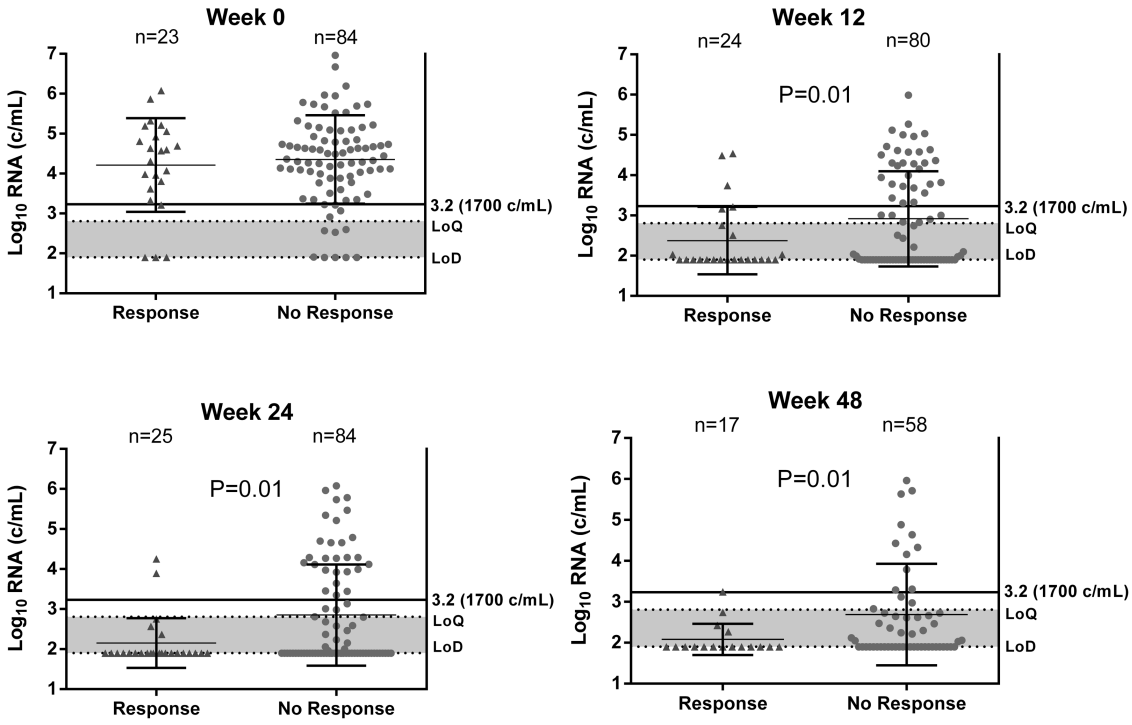

Figure 6. HBV-RNA proposed cutoff in predicting response vs nonresponse. Dots represent individual HBV-RNA measurements during treatment period according to treatment response at week 72. Vertical lines represent the standard deviation and horizontal line represents mean level of HBV-RNA (log c/mL) at each time point. HBV-RNA above 3.2 log c/mL predicts nonresponse to PEG-IFN treatment in HBeAg-negative patients according to current assay (net present value 91–93%, $P = .01$). Abbreviations: HBe-Ag, hepatitis B e antigen; HBV-RNA, hepatitis B virus RNA; LoD, lower limit of detection; LoQ, lower limit of quantification; PEG-IFN, pegylated interferon.

to assess of functional cure among HBeAg-negative patients [31].

The strength of our study is the large, well-documented, and prospectively investigated HBeAg negative population including underrepresented HBV genotypes (mainly genotype A and D) and the availability of all relevant other HBV biomarkers (HBV-DNA, HBcrAg, and HBsAg). Also, we were able to study HBV-RNA in HBeAg negative patients who developed HBsAg loss as a result of their finite PEG-IFN treatment. This is important for the development of biomarkers and endpoints in ongoing studies investigating new compounds aiming for a functional cure. In addition, this is the first study to our knowledge to use a more sensitive HBV RNA assay applied to a clinically relevant population. We could not further assess the prediction of HBV-RNA levels for HBsAg loss following PEG-IFN treatment due to the small number of patients achieving this endpoint.

In conclusion, our study shows that HBV-RNA levels decrease rapidly and profoundly in HBeAg-negative patients treated with PEG-IFN. HBV-RNA levels can predict nonresponse early at week 12 of treatment and also showed much lower levels among patients who achieved HBsAg loss. Overall, HBV-RNA showed a stronger correlation with HBV-DNA and HBcrAg and weak correlation with HBsAg. Although the role of HBV-RNA in clinical use is still in development, this is of particular interest in novel treatment approaches, and its biological role needs to be further researched as the newly developed therapeutic agents aim for a sustained off-treatment response or even functional cure, a goal similar for finite PEG-IFN treatment.

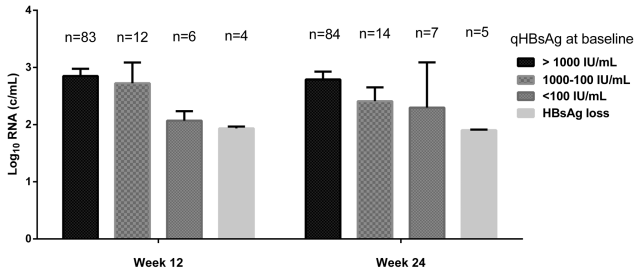

Figure 7. Mean HBV-RNA values according to HBsAg levels during long-term follow-up. Graph represent mean HBV-RNA levels at weeks 12 and 24 of treatment period according to different HBsAg groups (vertical bars) during follow-up (week 72–144). Abbreviations: HBsAg, hepatitis B surface antigen; HBV-RNA, hepatitis B virus RNA; qHBsAg, quantitative hepatitis B surface antigen.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the

posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. Study coordination and design, data collection, data analysis, writing of the paper, approval of final version: M. S. F. Laboratory work, data analysis, critical review of the paper, approval of final version: M. P., J. F., D. D. Study coordination and design, data collection, critical review of the paper, approval of final version: H. L. A. J., T. B., J. J. F. Study coordination and design, data collection and analysis, draft of the paper: F. v. B. Data collection, critical review of the paper, approval of final version: M. J. H. v. C., A. B., A. J. v. V., P. F. Study design, statistical analysis, critical review of the paper, approval of final version: B. E. H. All authors had access to the study data and have reviewed and approved the final paper.

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