Quantitation of large, middle and small hepatitis B surface proteins in HBeAg-positive patients treated with peginterferon alfa-2a

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Abstract

Background & Aims: Hepatitis B virus (HBV) contains three viral surface proteins, large, middle and small hepatitis B surface protein (LHBs, MHBs, SHBs). Proportions of LHBs and MHBs are lower in patients with inactive vs active chronic infection. Interferon alfa may convert hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB) to an inactive carrier state, but prediction of sustained response is unsatisfactory. The aim of this study was to test the hypothesis that quantification of MHBs and LHBs may allow for a better prognosis of therapeutic response than total hepatitis B surface antigen (HBsAg) concentration.

Methods: Hepatitis B surface proteins were measured before and during peginterferon alfa-2a therapy in serum from 127 Asian patients with HBeAg-positive CHB. Sustained response was defined as HBeAg seroconversion 24 weeks post-treatment.

Results: Mean total HBs levels were significantly lower in responders vs nonresponders at all time points (P < .05) and decreased steadily during the initial 24 weeks treatment (by 1.16 vs 0.86 ng/mL in responders/nonresponders respectively) with unchanged relative proportions. Genotype B had a two-fold higher proportion of LHBs than genotype C (13% vs 6%). HBV DNA, HBeAg, HBsAg and HBs protein levels predicted response equally well but not optimally (area under the receiver operating characteristic curve values >0.70).

Conclusions: Hepatitis B surface protein levels differ by HBV genotype. However, quantification of HBs proteins has no advantage over the already established HBsAg assays to predict response to peginterferon alfa-2a therapy in HBeAg-positive patients.

KEYWORDS
HBs proteins, HBsAg, peginterferon alfa-2a, predictors of response, subviral particles
1 | INTRODUCTION

Hepatitis B virus (HBV) surface antigen (HBsAg) is essential for diagnosis of HBV infection. Monitoring of HBsAg levels is recommended in chronic hepatitis B (CHB) treatment guidelines, with HBsAg loss considered the ideal treatment outcome, commensurate to a functional cure.1-3

HBsAg consists of three co-carboxyterminal proteins: large (L), middle (M) and small (S) HBs proteins (Figure S1A). The 226 amino acid (aa)-long S domain comprises the SHBs protein and forms the carboxyterminus in LHBs and MHBs proteins. The preS2 is present in LHBs and MHBs, while the preS1 domain is present only in LHBs.4,5 HBs proteins form the virion envelope, but also assemble into noninfectious subviral particles (SVPs) (Figure S1B) that are secreted in approximately 3000-fold excess relative to virions.6 Most SVPs are pleomorphic 20 nm spheres with a smaller proportion of LHBs than virions.

SHBs is the major component of the virion envelope and SVPs.5 The exterior hydrophilic loop of the multiple membrane-spanning S domain forms the major antigenic determinants of HBsAg. MHBs is nonessential for replication,7 but is conserved in all known orthohepadnaviruses, the genus of HBV.7 MHBs has been detected in hepatitis B e antigen (HBeAg)-positive chronic HBV carriers but was not as readily detectable in HBeAg-negative carriers,8,9 suggesting a correlation and a potential synergism between MHBs and HBeAg expression. However, Pfefferkorn et al10 recently reported that HBsAg carriers with long-term stable inactive HBV infection have significantly lower LHBs and MHBs levels compared with CHB patients, irrespective of HBeAg status.

Treatment with pegylated interferon alfa (PegIFN) aims to convert active chronic HBV infection to the inactive carrier state. Given the long duration and the side effects of PegIFN, an early predictor of the highly uncertain therapy outcome is desirable. Quantification of total HBsAg has been established to individualize treatment with PegIFN, particularly when deciding early termination of PegIFN in presumed nonresponders.3 However, the established stopping rules are suboptimal, as they only identify approximately 23% of nonresponders when applied at Week 24 of treatment.11

Standard quantitative HBsAg assays use antibodies against the S domain and do not differentiate the three HBs proteins.12,13 The relative abundance of these proteins was originally studied in purified SVPs and virions using gel electrophoresis.4 Later, enzyme immune assays were used to quantify LHBs levels in a limited number of sera from HBV carriers,8 but the true abundance of LHBs and MHBs in patients with CHB remained unclear. Recently, MHBs and LHBs were described as better predictors for a stable inactive HBV carrier state in HBeAg-negative infection than total HBsAg levels.10 It is currently unknown whether LHBs and MHBs are also useful to more reliably predict a stable inactive HBV carrier state as sustained response to PegIFN.

The objectives of this retrospective study were to determine the levels of all three HBs proteins in a large group of HBeAg-positive patients before and during PegIFN therapy, and to assess the performance of the LHBs-, MHBs- and SHBs-specific assays in predicting HBeAg seroconversion as response to treatment with PegIFN.

2 | MATERIALS AND METHODS

2.1 | Patients

Stored serum samples from patients enrolled in two large, international, randomized phase III/IV studies (WV16240 [NCT00048945] and WV19432 [NCT00435825]) were retrospectively analysed. The design and primary results of the two studies have been published previously.14,15 Briefly, patients had confirmed CHB, were HBeAg-positive and treatment naive (no antiviral therapy for CHB in the previous 6 months) at screening and had elevated serum alanine aminotransferase (ALT) (>1-10 × upper limit of normal) and HBV DNA levels (>500 000 copies/mL14 or >100 000 IU/mL).15 Patients with decompensated liver disease, serious co-existing medical conditions or co-infection with hepatitis C or D virus or HIV were excluded. Participants provided written informed consent including permission for future analyses of samples.

Only HBeAg-positive patients who had been randomized to PegIFN 180 μg/wk with or without lamivudine (LAM) for 48 weeks, and had available baseline and on-treatment (Weeks 12 and 24) samples were considered for this retrospective analysis. All patients had completed 24 weeks post-treatment follow-up. This study is registered at ClinicalTrials.gov: NCT01705704.

2.2 | Laboratory assessments

Clinical and laboratory data were obtained from the study sponsor (F. Hoffmann-La Roche Ltd). Quantitative HBV DNA levels were measured using a COBAS® Amplicor HBV Monitor (Roche Molecular Diagnostics, Pleasanton, CA; lower limit of quantification [LLOQ] 200 copies/mL, or 38 IU/mL) in Study WV16240 and a COBAS® TaqMan® HBV Test (Roche Molecular Diagnostics; LLOQ 29 IU/mL) in Study WV19432. HBeAg was quantified using a custom assay (LLOQ 0.15 PEIU/mL) based on calibrating the AxSYM® HBe 2.0 (Abbott)
HBeAg and HBV DNA (\(r_5 = 0.94-0.97, P < .0001\)) and moderately with HBeAg and HBV DNA (\(r_5 = 0.53-0.74, P < .0001\)), but not with age or ALT levels (\(r_5 < 0.25;\) Figure 2A). Overall, similar correlations were observed for total HBsAg and HBs protein proportions (Figure 2B).

Genotype-specific analyses were explored only for genotypes B (n = 45) and C (n = 77), as genotypes A (n = 3) and D (n = 2) were too infrequent. Compared with genotype C, genotype B had significantly higher mean HBsAg (18 645 vs 7865 IU/mL, \(P < .01\)) and LHBs levels (1558 vs 369 ng/mL, \(P < .0001\)). Levels of all three HBs proteins correlated strongly with quantitative HBsAg (\(r_5 = 0.94-0.97, P < .0001\)) and moderately with HBeAg and HBV DNA (\(r_5 = 0.53-0.74, P < .0001\)), but not with age or ALT levels (\(r_5 < 0.25;\) Figure 2A). Overall, similar correlations were observed for total HBsAg and HBs protein proportions (Figure 2B).

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### Table 1 Summary of patient characteristics

| Characteristic    | PegIFN n = 74 | PegIFN + LAM n = 53 | P-value |
|-------------------|---------------|---------------------|---------|
| Gender, male      | 51 (69)       | 39 (74)             | ns      |
| Race, Asian       | 70 (85)       | 52 (78)             | ns      |
| Age, y            | 31 ± 7.5      | 30 ± 8.2            | ns      |
| HBV genotype      |               |                     |         |
| A                 | 2 (3)         | 1 (2)               | ns      |
| B                 | 21 (28)       | 24 (45)             |         |
| C                 | 49 (66)       | 28 (53)             |         |
| D                 | 2 (3)         |                     |         |
| HBV DNA, log_{10} IU/mL | 8.6 ± 1.8 | 9.2 ± 1.8           | ns      |
| ALT, log_{10} U/L | 2.0 ± 0.3    | 2.0 ± 0.3           | ns      |
| HBeAg, log_{10} PEIU/mL | 2.3 ± 0.9 | 2.3 ± 0.9           | ns      |
| HBsAg, log_{10} IU/mL | 4.1 ± 0.8 | 4.1 ± 0.7           | ns      |
| HBeAg seroconversion\(^a\) | 38 (51) | 26 (49) | ns |

Note: Data are n (%) or mean ± SD.

Abbreviations: ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; LAM, lamivudine; ns, nonsignificant (\(P > .05\)); PegIFN, peginterferon alfa-2a.

\(^a\)At 24 wk post-treatment.
Remarkably, genotype B infection was associated with an approximately two-fold higher proportion of LHBs (13% vs 6%, \( P < .0001 \)), in contrast to similar proportions of SHBs (82% vs 91%, \( P < .0001 \)) and MHBs (4% vs 3%, \( P = .0428 \)) when compared with genotype C (Figure 3B). The higher proportion of LHBs was confirmed by Western blot analysis conducted in a randomly selected subset of patients (\( n = 41 \) genotype B, \( n = 21 \) genotype C), using a mab against a linear epitope of the S domain. The Western blot analyses revealed much higher MHBs levels (21% genotype B, 17% genotype C) than seen in ELISA leading to a slightly lower proportion of SHBs (72% and 80%; Figure 3C). ELISA results for the subset whose samples were subjected to Western blot analysis were comparable with the overall population. The difference in HBsAg composition between genotype B and C subgroups persisted during PegIFN \( \pm \) LAM treatment (Figure S2B).

### 3.3 | Hepatitis B surface protein levels during peginterferon alfa-2a therapy

Individual HBs protein levels decreased steadily during the initial 24 weeks of treatment (~1 log decline in mean levels by Week 24) (Figure 4A). A moderate change was observed in the composition of HBs protein proportions; by Week 24, MHBs and LHBs had slightly decreased (from 4% to 2%, and from 8% to 6% respectively), while the proportion of SHBs had slightly increased (from 88% to 92%; Figure 4B).

Concomitant administration of LAM did not significantly affect individual HBs protein levels during treatment (Figure 4C; \( P > .05 \) for between-group comparisons at each time point, unpaired t test), and this was also the case for ALT, HBsAg and HBeAg levels (Figure S3A). As expected, HBV DNA levels were much lower in patients treated with PegIFN plus LAM vs PegIFN monotherapy at both Week 12 (1.96 vs 5.53 log_{10} IU/mL, \( P < .0001 \)) and Week 24 (0.91 vs 4.57 log_{10} IU/mL, \( P < .0001 \)).

In responders with HBeAg seroconversion at 24 weeks post-treatment, mean HBs protein levels were significantly lower than in nonresponders at all time points (Figure 4D; \( P < .05 \) for every comparison). Likewise, the mean HBeAg, HBsAg and HBV DNA levels, but not ALT levels, were lower in responders than nonresponders at all time points (Figure S3B).

By Week 24 of treatment, mean HBs protein levels decreased by approximately 1.16 (range 1.04-1.23) log_{10} ng/mL in responders, with a smaller decrease of 0.86 (range 0.68-1.03) log_{10} ng/mL in nonresponders. Interestingly, MHBs and LHBs became undetectable in more responders (from 9% to 57%, and 8% to 26% respectively) compared to nonresponders (from 3% to 23%, and 0% to 6%).
HBsAg, with comparable AUC values at each time point (ie AUC values at Week 24 > Week 12 > Baseline). Better predictors than change from baseline (consistently higher values <0.55 throughout) (Table 2). Absolute biomarker levels were values consistently exceeding 0.70, while ALT was nonpredictive (AUC s <0.55 throughout) (Figures S4 and S5) demonstrated that HBV DNA, HBeAg, HBsAg and SHBs/MHBs/LHBs proteins predicted treatment response moderately well, with area under the ROC curve (AUC) values >0.70. However, the PegIFN-associated decrease in HBsAg levels during PegIFN treatment and their utility in predicting treatment response.

SHBs protein showed a highly significant, close linear correlation with quantitative HBsAg levels as measured by a commercial assay, thus validating the accuracy of our custom HBs assay for total HBs proteins. The very close correlation of Western blot data and HBs ELISA or quantitative HBsAg suggests that immune complexes of anti-HBs and HBsAg were not present in significant amounts in this patient cohort, although HBs immune complexes have been frequently detected in HBV carriers. Surprisingly, patients with genotype B infection had significantly higher mean quantitative HBsAg, MHBs and LHBs protein levels than those with genotype C. Furthermore, the proportion of LHBs was significantly higher in genotype B as reported by Pfefferkorn et al. These differences are unlikely to be caused by differential binding of antibodies, but may result from different relative amounts of virions, spheres and filaments. Interestingly, Pfefferkorn et al observed differences, also, in the HBs protein composition between genotypes, with genotype A showing lower MHBs and LHBs levels than genotype D. The differences in HBs proteins by genotype merit further investigation, given the known differences in interferon alpha treatment response and outcome associated with different genotypes.

Hepatitis B surface protein levels measured by ELISA decreased after the initiation of PegIFN treatment, with or without LAM, in a monophasic manner throughout the first 24 weeks of therapy. The decrease in HBsAg levels during PegIFN therapy may be mainly caused by a moderate suppression of replication and a subsequently reduced renewal of the cccDNA pool, as suggested by Chuaypen et al. The PegIFN-associated decrease was apparent for each of the three HBs proteins, but – in contrast to LAM – it was more pronounced for HBsAg.

#### 4 | DISCUSSION

This study provides insight into the proportions of HBs proteins in the serum of patients with HBeAg-positive CHB, their kinetics during PegIFN treatment and their utility in predicting treatment response.

Given that both treatment subgroups had comparable response rates and on-treatment biomarker kinetics (apart from HBV DNA), biomarker data were pooled for further analysis. Pooling of treatment subgroup data was not deemed appropriate for HBV DNA; thus, further analyses were restricted to the PegIFN monotherapy subgroup. In addition, individual biomarker cut-offs were further explored to assess their utility for use as stopping rules during treatment.

Receiver operating characteristic curve analyses of biomarker data (Figures S4 and S5) demonstrated that HBV DNA, HBeAg, HBsAg and SHBs/MHBs/LHBs proteins predicted treatment response moderately well, with area under the ROC curve (AUC) values consistently exceeding 0.70, while ALT was nonpredictive (AUC values <0.55 throughout) (Table 2). Absolute biomarker levels were better predictors than change from baseline (consistently higher AUC values), and biomarkers became more predictive at later treatment time points (ie AUC values at Week 24 > Week 12 > Baseline).

The ROC curves of the three HBs proteins were similar to total HBsAg, with comparable AUC values at each time point (P = nonsignificant for each pair-wise AUC comparison). Furthermore, AUCs of HBs protein proportions were inferior to absolute levels at each time point (data not shown).

Individual biomarker cut-offs were explored for absolute biomarker levels (higher ROC AUCs than change from baseline), that were associated with high negative predictive values (90%) at Week 12 of treatment. Cut-offs were identified for HBV DNA, HBeAg and HBsAg, as well as for the S/M/LHBs proteins, but not for ALT levels. The LHBs (960 ng/mL) and quantitative HBsAg (27 000 IU/mL) cut-offs identified the largest proportions of nonresponders (32% and 31% respectively), followed by the HBV DNA (8.9 log10 IU/mL, 26%), SHBs (27 000 ng/mL, 18%), MHBs (1500 ng/mL, 16%) and HBeAg (1700 IU/mL, 12%) cut-offs.
to spontaneous HBeAg seroconversion – the relative proportions of MHBs and LHBs remained unchanged during the initial 24 weeks of treatment. HBs protein levels in responders were significantly lower at baseline, and remained significantly lower after 12 and 24 weeks of treatment; furthermore, the decline in HBs proteins was greater in responders after 24 weeks of treatment, and MHBs/LHBs proteins became undetectable in a larger proportion of responders than in nonresponders.

Similar to the results of Wang et al.\textsuperscript{25} and Zhu et al.,\textsuperscript{26} we found that HBs levels correlated with those of other viral markers, but not with ALT levels. While a weak correlation between LHBs and HBsAg (r = 0.46-0.59) was reported by these authors, the correlation between HBs proteins (including LHBS) and HBsAg in our study was particularly high (r = 0.94-0.97). The divergence may be a result of suboptimal HBsAg quantitation, because Zhu et al.\textsuperscript{26} found a better correlation of HBV DNA to LHBs than to HBsAg. Furthermore, the HBs levels measured in our study were approximately 10-20 times higher than those found by Zhu et al.\textsuperscript{26} Such large divergences highlight the need for international standardization, as exists for total HBsAg. Consistent with Zhu et al.,\textsuperscript{26} we showed that absolute biomarker levels are more predictive of treatment response than change from baseline levels.

Current CHB treatment guidelines recommend monitoring HBsAg levels during PegIFN therapy.\textsuperscript{1-3} In HBeAg-positive patients, a decrease in HBsAg to <1500 IU/mL at Week 12 is a strong predictor of HBeAg seroconversion and an indication that treatment should continue. In contrast, treatment cessation is recommended in patients who fail to achieve a serum HBsAg level <20 000 IU/mL or any decline in HBsAg because of the low probability of achieving HBeAg seroconversion. The current study demonstrates that cut-offs for LHBs, and to a lesser degree those for SHBs and MHBs, have comparable performance with HBsAg cut-offs in accurately identifying nonresponders after 12 weeks of PegIFN therapy. However, the single HBs protein assays have no advantage over the already established HBsAg assays in HBeAg-positive patients.

It is noteworthy that the ELISA and Western blot results for LHBs – in contrast to those for MHBs – agreed very well. This suggests that the ratio of internal and external preS1 domains in virions or HBsAg filaments is not highly variable, and that external preS1
**FIGURE 4**  HBs protein kinetics during PegIFN ± LAM therapy. A, Mean ± 95% CI and (B) relative proportions of HBs protein levels (+SD) in the overall cohort (n = 127). C, Mean ± 95% CI levels during PegIFN (n = 74) vs PegIFN + LAM therapy (n = 53), and (D) in responders (n = 64) vs nonresponders (n = 63). Abbreviations: CI, confidence interval; HBeAg, hepatitis B e antigen; HBs, hepatitis B surface (protein); HBsAg, hepatitis B surface antigen; LAM, lamivudine; PegIFN, peginterferon alfa-2a; SHBs/MHBs/LHBs, small/middle/large hepatitis B surface (protein); SC, seroconversion; SD, standard deviation

**TABLE 2**  Summary of area under the receiver operating characteristics curve scores

|               | ALT    | HBV DNA^a | HBeAg  | HBsAg  | SHBs   | MHBs   | LHBs   |
|---------------|--------|-----------|--------|--------|--------|--------|--------|
| **Absolute biomarker levels** |        |           |        |        |        |        |        |
| Baseline      | 0.54(0.45-0.63) | 0.69(0.58-0.80) | 0.62(0.52-0.71) | 0.70(0.61-0.78) | 0.71(0.62-0.79) | 0.71(0.63-0.79) | 0.70(0.61-0.78) |
| Week 12       | 0.53(0.44-0.61) | 0.82(0.71-0.90) | 0.72(0.63-0.81) | 0.69(0.60-0.77) | 0.70(0.61-0.78) | 0.71(0.62-0.78) | 0.68(0.60-0.76) |
| Week 24       | 0.51(0.42-0.60) | 0.85(0.75-0.93) | 0.77(0.68-0.85) | 0.73(0.64-0.80) | 0.72(0.63-0.80) | 0.73(0.65-0.81) | 0.71(0.62-0.79) |
| **Change from baseline in biomarker levels** |        |           |        |        |        |        |        |
| Week 12       | 0.51(0.42-0.60) | 0.75(0.64-0.85) | 0.61(0.51-0.71) | 0.51(0.42-0.60) | 0.53(0.44-0.62) | 0.52(0.43-0.61) | 0.52(0.43-0.61) |
| Week 24       | 0.52(0.43-0.61) | 0.78(0.67-0.87) | 0.66(0.56-0.75) | 0.58(0.49-0.67) | 0.58(0.49-0.67) | 0.54(0.45-0.63) | 0.50(0.41-0.59) |

**Note:** 95% confidence intervals shown in brackets.  
Abbreviations: ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; SHBs/MHBs/LHBs, small/middle/large hepatitis B surface (protein).  
^aRestricted to the PegIFN group.
domains were not masked by antibody or other serum components in the patients of our study. The preS2 domain of MHBs and possibly also of LHBs is masked to a large degree and, thus, undetectable by the ELISA, as has been shown previously.27 Deepen et al8 found high levels of free MHBs in HBeAg-positive carriers only if the HBsAg concentration was >20,000 ng/mL. However, in our study, quantitative Western blot revealed a relatively high proportion of MHBs in samples from all HBeAg-positive CHB patients (21% and 17% in the examples shown in Figure 3C).

mRNAs encoding HBs proteins may be transcribed from either viral cccDNA or HBV DNA fragments integrated into host chromosomes. Transcription of LHBs mRNA is regulated by the liver-specific preS1 promoter, while MHBs and SHBs mRNAs are controlled by the same preS2/S promoter. The relatively constant proportions of the three HBs proteins suggest that there is no differential effect of PegIFN on the transcription and expression of the two groups of mRNA.

The low levels of LHBs and MHBs protein in the sera of asymptomatic HBeAg-negative HBV carriers10 suggest that the still-abundant SHBs secretion occurs via expression of integrated HBV DNA that often lacks a complete preS region because of preferential integration of HBV DNA at that site.28 For this reason, monitoring LHBs and MHBs proteins may provide insight into the presence and expression activity of integrated HBV DNA in patients with CHB. The absence of a selective effect of interferon therapy on either LHBs or MHBs levels during the initial 24 weeks of therapy is somewhat surprising because the expression pattern of HBs proteins differs following spontaneous HBeAg seroconversion. Thus, the mechanisms of interferon-induced HBeAg seroconversion may be different from those leading to spontaneous HBeAg seroconversion in untreated patients. Further studies are warranted during the post-treatment follow-up of seroconverted HBeAg-positive CHB patients and in treated HBeAg-negative CHB patients, who were not included in our study.

This study has certain limitations. We evaluated HBs levels retrospectively using stored serum samples from a subset of patients enrolled in a randomized controlled trial. Thus, it is possible that the results have been affected by sample deterioration or selection bias. Our study cohort was found to be responder-enriched, indicating some degree of selection bias; thus, the results may not be representative of all patients undergoing PegIFN therapy. Our analysis showed that the results from patients treated with PegIFN alone and in combination with LAM could be combined; in the future, it is preferable to evaluate larger cohorts of patients treated with PegIFN monotherapy. Considering that the ideal treatment outcome of HBsAg loss is rarely observed during treatment, we evaluated associations between HBs protein kinetics and HBeAg seroconversion after 24 weeks of untreated follow-up. The lack of longer follow-up is a limitation of our study. Thus, future studies should attempt to evaluate responses after longer durations of follow-up and explore other endpoints. To our knowledge, this is the largest cohort of CHB patients in which HBs protein levels have been evaluated during treatment with PegIFN; however, our analysis was restricted, initially, to HBeAg-positive patients and most patients had genotype B or C. There were insufficient patients with HBV genotype A or D to allow for a more complete genotype-specific analysis, and it is difficult to relate results to other studies in this area such as that by Pfefferkorn et al,10 where genotype B and C were not the most common. The population was also predominantly of East Asian origin; future analyses should strive to include a more ethnically diverse population. Finally, the underestimation of MHBs in ELISA is an additional limitation of this analysis.

In conclusion, the results of this retrospective analysis show that SHBs levels are strongly correlated with quantitative HBsAg levels; and that HBs protein levels differ by HBV genotype. SHBs/ MHBs/LHBs protein levels were found to be significantly lower in responders than in nonresponders during PegIFN treatment, and to predict PegIFN response in a manner comparable to quantitative HBsAg levels. Although HBs proteins were not superior to total HBsAg for predicting response to PegIFN, LHBs and MHBs represent novel biomarkers that warrant further investigation.

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CONFLICT OF INTEREST
MC: consultant and speaker – Roche, BMS, Gilead; research funding – Roche. MPM: consultant – Roche, BMS, Gilead, Novartis, GlaxoSmithKline, Medigenics, Enyo Pharma, Curevac; travel grants – Roche, BMS, Gilead, Novartis; research grants – Gilead, Novartis. HW: consultant/ speaker – Roche, Abbott, AbbVie, BMS, Boehringer Ingelheim, Merck, Gilead, Novartis, Siemens, Transgene, Viiv; research grants – Roche, Abbott, AbbVie, BMS, Gilead, Novartis, Siemens. CW: employment, stockholding – Roche. LY: employment – Roche. VP was employed by Roche when this work was carried out. All authors disclose medical writing support from F. Hoffmann-La Roche Ltd.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.