Host and Viral Predictors of Response to Antiviral Therapy in Chronic Hepatitis B

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Colofon

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INTRODUCTION

Chronic hepatitis B (CHB) is a major cause of liver disease worldwide despite the availability of effective vaccination. There are still more than 350 million people chronically infected with the hepatitis B virus (HBV)[1] and progression of HBV-related liver inflammation to cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC) is estimated to result in up to 1.2 million deaths annually.[1]

PHASES OF INFECTION

Patients with CHB may present in any one of four, not necessarily sequential, phases of infection.[2] During the immunotolerant phase, hepatitis B e antigen (HBeAg) is detectable and serum HBV DNA level is high (generally >20,000 IU/mL), while serum alanine aminotransferase (ALT) is within the normal range and liver histology shows minimal inflammation. In the immune clearance phase, the host immune response results in a decline in HBV DNA levels, elevated ALT levels and hepatic necroinflammation. During this phase HBeAg loss and seroconversion to anti-HBe can occur. HBeAg seroconversion is often followed by an inactive carrier state characterized by a low serum HBV DNA level (<2,000 IU/mL) and normalization of ALT. However, in a significant proportion of HBeAg-negative patients viral replication recurs or persists at higher levels, resulting in active HBeAg-negative CHB. This phase of the infection develops through presence of viral strains harbouring mutations in the precore or basal core promoter region that reduce or abolish the expression of HBeAg.[3] Serum levels of hepatitis B surface antigen (HBsAg) also vary across the 4 phases of infection, with the highest levels observed in immunotolerant patients, a significant decrease in patients progressing to immune clearance, and the lowest levels in patients achieving sustained immune control.[4]

TREATMENT OF CHRONIC HEPATITIS B: PARADIGMS AND RATIONALE

Complete eradication of HBV from host hepatocytes cannot be achieved with currently available agents, due to the persistence of HBV covalently closed circular DNA (cccDNA) in host hepatocytes. The main goal of treatment of CHB is therefore to halt the progression of liver inflammation to fibrosis, cirrhosis or hepatocellular carcinoma (HCC).[5] Because these outcomes typically do not occur until after decades of active infection, surrogate outcomes are used as measures of therapy efficacy and success.

Several independent studies have shown that lower levels of HBV DNA, as well as clearance of HBeAg and HBsAg, are associated with a lower risk of cirrhosis, HCC and death.[6-8] The major endpoints of treatment are therefore a reduction of HBV DNA to undetectable levels (virological response), loss of HBeAg with or without the appearance of antibodies to HBeAg (anti-HBe, serological response), a reduction of ALT to levels considered normal (biochemical response) and a reduction in liver necroinflammation with or without an improvement of liver histology.[9] Loss of HBsAg from serum, accompanied by appearance of anti-HBs, is currently considered the optimal surrogate endpoint, although it is rarely achieved.[10]

TREATMENT OF CHRONIC HEPATITIS B

Current guidelines recommend both peginterferon (PEG-IFN), an immune modulator, as well as the viral polymerase inhibiting nucleo(s)tide analogues (NA) entecavir and tenofovir as first-line treatment options of CHB. The efficacy of treatment is evaluated differently for the two treatment modalities, based on their different modes of action. In patients treated with NA, *on-treatment* maintained undetectable HBV DNA level is the most important endpoint, because persistently detectable HBV DNA during treatment is a major risk factor for the development of viral breakthrough, antiviral resistance and progression of liver disease.[11-13] While HBeAg seroconversion may herald immune control in untreated patients, discontinuation of treatment after NA induced HBeAg seroconversion is associated with a high probability of post-treatment relapse.[14-19] Similarly, discontinuation of NA in patients with HBeAg-negative CHB will result in relapse in nearly all patients.[20] Continuation of NA therapy until HBsAg loss or seroconversion therefore appears to be the most prudent.[21]

In contrast, PEG-IFN is typically administered for a finite one-year course, after which therapy is discontinued. After discontinuation, a subset of patients will develop an *off-treatment* sustained response, assessed at six months post-treatment, and require no further therapy. In HBeAg-positive CHB patients treated with PEG-IFN, off-treatment sustained loss of HBeAg accompanied by the appearance of anti-HBe (HBeAg seroconversion) is frequently used as a definition of sustained response, since it is associated with a reduced mortality rate.[8,21] However, a subset of patients will have persistently high HBV DNA levels after HBeAg seroconversion, i.e. progress to HBeAg-negative CHB, and thus require retreatment.[22,23] The use of a combined serological *and* virological endpoint (HBeAg loss or seroconversion with concomitant HBV DNA <2,000 IU/mL) therefore seems preferable, since this endpoint is associated with a low probability of relapse[24], a reduction in the risk of development of HCC[7,25] and a high probability of subsequent HBsAg loss and seroconversion.[26] In HBeAg-negative

CHB patients, prolonged suppression of HBV DNA to low levels (typically below 2,000 IU/mL), combined with normalization of ALT is currently considered the optimal definition of response to PEG-IFN[27], although late relapse beyond 6 months post-treatment has been described.[21]

CHOOSING THE APPROPRIATE FIRST-LINE THERAPY

Both NA and PEG-IFN have substantial advantages and important limitations.[28] The potent NA entecavir and tenofovir are generally recognised as first line treatment options, and may induce and maintain undetectable levels of HBV DNA in nearly all patients with limited safety concerns through up to 5 years of treatment.[29-31] Nevertheless, the high rates of relapse observed after discontinuation of NA based treatment in both HBeAg-positive and HBeAg-negative CHB patients suggests that decades if not lifelong therapy will be necessary in most patients.[14,20] Since a one year course of PEG-IFN may induce an off-treatment sustained response in about 25% of HBeAg-positive patients,[28,32] PEG-IFN may be a valuable treatment option for those patients with a high likelihood of response. However, the limited number of patients who achieve a response, and the high costs and side-effects associated with PEG-IFN treatment constrain its clinical use. Selection of patients with the highest probability of response is therefore essential for effective use of PEG-IFN in clinical practice. Recent studies have shown that response to PEG-IFN depends upon the infecting HBV genotype, baseline levels of HBV DNA and ALT, and possibly also patient age and sex and previous failure to respond to IFN therapy. However, a prediction model incorporating these variables provides only limited discrimination, and reliable prediction of response probabilities for individual patients remains a challenge.

ON-TREATMENT PREDICTION OF RESPONSE TO PEG-IFN

Possibly, on-treatment monitoring of viral replication may help identify patients with a very high or a very low probability during the first months of treatment. Currently, three quantitative markers are available for monitoring HBV replication: serum HBV DNA, HBeAg and HBsAg levels. Using data from the PEG-IFN alpha-2a trial, Fried *et al* identified a relationship between on-treatment HBV DNA levels and treatment response.[33] Patients with HBV DNA levels below 5 log copies/mL at week 24 had a 53% probability of response, as opposed to a probability of only 14% in patients with HBV DNA >9 log copies/mL.[33] Nevertheless, the clinical utility of these findings is

limited, since a considerable number of future responders would be lost if all patients with HBV DNA >9 log copies/mL discontinued therapy.[33] Since baseline HBV DNA levels are associated with treatment response, combining baseline and on-treatment HBV DNA levels may offer advantages. Nevertheless, discrimination between (non-) responders using modeling of HBV DNA kinetics during PEG-IFN therapy remains difficult.[34,35]

Other studies have reported on the predictive capabilities of HBeAg levels at week 24 of therapy.[33,36] Patients who achieved a sustained HBeAg seroconversion show a considerably more pronounced on-treatment decline in serum HBeAg levels. Patients with the lowest HBeAg levels at week 24 have a 52% probability of sustained HBeAg seroconversion, as opposed to a probability of only 4% in patients with the highest levels.[33] Unfortunately, prediction of response with HBeAg levels must be postponed to week 24 of therapy, and reliable assays for quantification of HBeAg are not widely available.

Recently, studies with newly available automated quantitative assays showed that serum HBsAg levels vary significantly during the different phases of chronic HBV infection and are inversely correlated with the immune control of HBV: the higher control, the lower HBsAg level.[37-40] These findings are consistent with the hypothesis that HBsAg serum levels reflect the complex interplay between virus and immune system and provide complementary information to viral load as measured using HBV DNA levels. Importantly, serum HBsAg levels comprise not only virions (42 nm, Dane particles), but mainly the non-infectious HBsAg particles (20 nm diameter filaments of variable length and 20-22 nm spheres) which do not contain viral nucleic acids and exceed virions by a factor ranging between 10^2 and 10^5. HBsAg is secreted from the hepatocyte during viral replication as part of the HBV nucleocapsid, or as part of noninfectious viral particles.[41] Several studies have reported that serum HBsAg levels correlate with intrahepatic cccDNA levels in HBeAg-positive patients.[42,43] On-treatment HBsAg decline may therefore reflect the efficacy of PEG-IFN in decreasing intrahepatic cccDNA and consequently predict a sustained response.[42,43]

The aims of the current thesis were therefore to (1) identify baseline factors associated with an increased likelihood of response to PEG-IFN and (2) to investigate whether on-treatment monitoring of HBV replication using HBsAg levels can help identify (non-)responders to PEG-IFN therapy.

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Polymorphisms near *IL28B* and serologic response to peginterferon in HBeAg-positive patients with chronic hepatitis B

1

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ABSTRACT

Background & Aims

A limited number of HBeAg-positive patients with chronic hepatitis B respond to treatment with peginterferon-alfa (PEG-IFN). We investigated whether *IL28B* genotypes are associated with response.

Methods

We studied 205 HBeAg-positive patients who were treated with PEG-IFN (some were also treated with lamivudine) at 11 European and Asian hospitals; genotype analysis was performed for *IL28B* rs12980275 and rs12979860. Response was defined as HBeAg loss with the appearance of anti-HBe at the end of PEG-IFN therapy (HBeAg seroconversion), along with HBeAg seroconversion and HBsAg clearance during long-term follow-up.

Results

The patients were infected with hepatitis B virus (HBV) genotypes A (13%), B (20%), C (47%), and D (13%). The proportions of *IL28B* genotypes were 77%, 19%, and 5%, for AA/AG/GG at rs12980275 and also for CC/CT/TT at rs12979860, respectively. *IL28B* genotype was significantly associated with HBeAg seroconversion at end of treatment ($p<0.001$); the adjusted odds ratio for seroconversion was 3.16 (95% confidence interval [CI], 1.26–8.52; $p=0.013$) for AA vs AG/GG at rs12980275, after adjustment for HBV genotype, age, levels of HBV DNA and alanine aminotransferase, and combination therapy. *IL28B* genotype was independently associated with an increased probability of HBeAg seroconversion during long-term follow up (adjusted hazard ratio [HR], 2.14; 95% CI, 1.14–4.31; $p=0.018$ for AA vs AG/GG by Cox regression analysis). Similar results were obtained for rs12979860. *IL28B* genotype was also associated with HBsAg clearance (HR 3.47 for AA vs AG/GG; 95% CI, 1.04–13.48; $p=0.042$).

Conclusions

Polymorphisms near *IL28B* are independently associated with serological response to PEG-IFN in HBeAg-positive patients with chronic hepatitis B.

INTRODUCTION

Chronic hepatitis B affects over 350 million people worldwide and is one of the leading causes of cirrhosis and hepatocellular carcinoma.[1] Antiviral therapy with peginterferon-alfa (PEG-IFN) results in viral suppression, hepatitis B e antigen (HBeAg) seroconversion, hepatitis B surface antigen (HBsAg) clearance, normalization of alanine aminotransferase (ALT) and histological improvement.[2-4] The long-term therapeutic effect of PEG-IFN is durable,[5-6] and patients who achieve an IFN induced HBeAg seroconversion have a reduced risk of hepatocellular carcinoma and cirrhosis.[7-9]

However, only 30% to 40% of HBeAg-positive chronic hepatitis B patients treated with PEG-IFN achieve HBeAg seroconversion.[2-4] Since PEG-IFN treatment is expensive and associated with considerable side effects, selection of patients with the highest probability of response is essential for optimal utilization of this agent in clinical practice.

Recently, a number of baseline and on-treatment parameters were shown to be associated with response to PEG-IFN.[10-11] However, a multivariate prediction model incorporating these predictors provides only limited discrimination, and considerable uncertainty therefore remains on the individual level. It is important to note that most described predictors are either virological, or related to the phase of hepatitis B virus (HBV) infection.[10-11] The influence of host factors on treatment response to PEG-IFN in chronic hepatitis B is currently unclear.

Recently, several independent genome-wide association studies have shown that genetic polymorphisms at or near the interleukin 28B gene (*IL28B*; also known as IFN-λ-3), including rs12980275 and rs12979860, are associated with higher rates of sustained virological response in chronic hepatitis C patients treated with PEG-IFN and ribavirin.[12-15] The findings have been confirmed in other populations, including chronic hepatitis C patients who have undergone liver transplantation.[16] While the mechanism by which *IL28B* influences response to PEG-IFN therapy remains elusive, it is likely that the relationship is not specific to hepatitis C virus infection. The type of IFN coded for by *IL28B*, IFN-λ, has been shown previously to be active against several other viruses, including DNA viruses such as HBV.[17-18] Whether *IL28B* polymorphisms are related to response to PEG-IFN in patients with chronic hepatitis B is currently unknown.

In this study, we aimed to investigate the effect of *IL28B* gene polymorphisms on PEG-IFN induced serological response at the end of treatment and through long-term follow-up in a large global cohort of HBeAg-positive chronic hepatitis B patients.

METHODS

Patients

Consecutive patients with HBeAg-positive chronic hepatitis B who received PEG-IFN alfa-2a or PEG-IFN alfa-2b at the liver clinics of 11 European and Asian hospitals were enrolled. Patients were treated during various studies from 1988 through 2008 and subsequently enrolled in long-term follow-up (LTFU) studies.[2-3,5-6,9] Host DNA was prospectively collected during LTFU. DNA from patients who previously participated in the HBV 99-01 study[3] was collected during a scheduled long-term follow-up visit at selected centers. This LTFU cohort was previously shown to be representative of the overall cohort.[5] DNA from patients treated with PEG-IFN at the Prince of Wales Hospital[2,6] was collected during LTFU visits. Patients previously treated with conventional IFN[9] at the Erasmus MC University Medical Center were contacted specifically for this study. All patients had positive HBsAg for at least 6 months and were positive for HBeAg and negative for antibodies to HBeAg (anti-HBe) at baseline. Short-term (maximum two years) concomitant use of nucleos(t)ide analogues was allowed. We excluded patients co-infected with hepatitis C virus, delta virus, or human immunodeficiency virus and those who received immunosuppressants, chemotherapy or systemic corticosteroids during the study period. Race was determined by the local investigator. The study protocol was approved by the ethics committees of the participating centres and was consistent with the principles of the declaration of Helsinki. All patients provided written informed consent for participation in the long-term follow-up study, and for use of genetic material for this study.

Follow-up assessments

The follow-up time was calculated from the starting date of PEG-IFN. For the current study, liver biochemistry and hepatitis B virus serology measurements were performed at the start of PEG-IFN, during treatment, at the end of treatment and 6 months after PEG-IFN discontinuation. Patients were subsequently followed at the outpatient clinic every 3-6 months. HBV DNA levels were assessed at therapy initiation, end of treatment and at last follow-up date.

Endpoints

The primary endpoint was HBeAg seroconversion, defined as negative HBeAg and positive anti-HBe in serum. HBeAg seroconversion was assessed at the end of PEG-IFN treatment to account for differences in post-IFN treatment with nucleo(s)tide analogues within the study cohort, and was also investigated through long-term post-treatment follow-up. At long-term follow-up, HBeAg seroconversion was defined as HBeAg seroconversion that was sustained to end of follow-up whether or not followed

by HBsAg clearance. In patients retreated with other agents after discontinuation of the initial therapy regimen, follow-up was terminated at the start of retreatment, with (anti-)HBeAg status before retreatment used as outcome parameter. Other endpoints assessed were a combination of HBeAg seroconversion with HBV DNA <2,000 IU/mL, and HBsAg clearance. Patients who were retreated were considered non-responders for both endpoints.

Laboratory tests

Enzyme-linked immunosorbant assay kits were used to test for HBsAg (Roche Diagnostics Corp., Indianapolis, IN, USA or AxSYM, Abbott, Abbott Park, IL, USA), HBeAg and anti-HBe (Sanofi Diagnostics, Pasteur, France or AxSYM, Abbott, Abbott Park, IL, USA). TaqMan real-time polymerase chain reaction assays were used to measure HBV DNA levels. HBV genotypes were determined by restriction fragment length polymorphism or by INNO-LiPA line-probe assay (Innogenetics, Gent, Belgium).[19]

IL28B genotyping

TaqMan SNP Genotyping Assays were employed for the detection of the reference single nucleotide polymorphisms (SNPs) near the *IL28B* gene on chromosome 19, rs12979860 and rs12980275 (Applied Biosystems, Foster City, CA).[12,20] These SNPs were chosen because they were previously reported in two independent studies, including mostly Caucasian[12] and Asian[14] hepatitis C virus infected patients. The laboratory procedure was carried out locally in the Erasmus MC University Medical Center and the Prince of Wales Hospital following the manufacturer's instructions. Purified genomic DNA of 10 to 20 ng was used for genotyping. After amplification by polymerase chain reaction, endpoint plate read was performed using Real-Time PCR System (Applied Biosystems). Fluorescence values in each well were read using the Sequence Detection System Software.

Statistical analyses

All statistical analysis was performed using SPSS version 16.0 software (SPSS, Chicago, IL) and SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Continuous variables were expressed as mean ± standard deviation or median (interquartile range (IQR)). Group comparison was performed using the Student *t* test, Mann-Whitney U test, chi-square test, or Fisher's exact test as appropriate. Binary logistic regression modelling was used to identify independent factors associated with HBeAg seroconversion ± HBV DNA <2,000 IU/mL at end of treatment and long-term follow-up. Kaplan-Meier analysis and Cox proportional hazard models were used to estimate the association between *IL28B* genotypes and HBeAg seroconversion and HBsAg clearance during follow-up. For logistic and Cox regression modelling, the Likelihood Ratio test was

used. Statistical significance was taken as a two-sided *P* value of less than 0.05. The proportional hazards assumption was verified using log-minus-log plots and by the addition of an interaction term between *IL28B* genotype and time.

RESULTS

Patient characteristics

A total of 205 patients, comprising all those with available host DNA, were enrolled in the study cohort. All were successfully genotyped at rs12980275 and rs12979860 near *IL28B* (table 1). Patients were predominantly male and of either Asian or

Table 1: Baseline patient characteristics

Characteristics	Study Population (n=205)
Demography	
Mean (SD) age, years	35.0 (11.4)
Male	149 (72.7%)
Route of transmission (%)	
Vertical	141 (68.8%)
Other	64 (31.2%)
Race	
Caucasian	59 (28.8%)
Asian	133 (64.9%)
African/other/unknown	13 (6.3%)
Treatment	
PEG-IFN monotherapy	85 (41.5%)
PEG-IFN + lamivudine	106 (51.7%)
Conventional IFN	14 (6.8%)
Laboratory results	
Mean (SD) ALT*	3.49 (2.74)
Mean (SD) HBV DNA, logIU/mL	7.70 (1.07)
HBV Genotype	
A	26 (12.7%)
B	41 (20.0%)
C	96 (46.8%)
D	27 (13.2%)
Other/mixed	9 (4.4%)
Unknown	6 (2.9%)

*multiples of the upper limit of the normal range

Caucasian origin. All major HBV genotypes were present in the study cohort, with HBV genotypes B and C being the most prevalent. The patients enrolled into the study were comparable to patients treated at the participating centres who were not enrolled with regard to important baseline characteristics. Patients were treated with either PEG-IFN monotherapy for one year (n=85)[3], PEG-IFN 32 or 52 weeks + lamivudine for 52 weeks (n=83)[2-3,6], PEG-IFN for 32 weeks + lamivudine for 96 or 104 weeks (n=23)[6] or conventional IFN for 16-42 weeks (n=14)[9]. A total of 76 (37%) patients were retreated after PEG-IFN therapy. Overall follow-up lasted a median of 173 (IQR 108 – 356) weeks.

Prevalence of *IL28B* genotypes

rs12980275 and rs12979860 were in linkage disequilibrium. Only 6 patients were classified discordantly. *IL28B* genotype frequencies were 77%/19%/5%, both for AA/AG/GG genotypes at rs12980275 and CC/CT/TT genotypes at rs12979860, respectively. Considerable differences were observed concerning the distribution of the rs12980275 genotypes for Asians when compared to non-Asians in the study cohort ($p<0.001$, figure 1A), and across HBV genotypes A through D (figure 1B). Similar distributions were observed for rs12979860.

Relationship between *IL28B* genotypes and HBeAg seroconversion at end-of-treatment

At the end of PEG-IFN treatment, a total of 90 (44%) patients achieved HBeAg seroconversion. HBeAg seroconversion rates varied significantly by *IL28B* genotype ($p<0.001$, figure 2A). Figures 2B and 2C show the HBeAg seroconversion rates at end of treatment by *IL28B* genotype across HBV genotypes A through D. The relationship between *IL28B* polymorphisms and HBeAg seroconversion at the end of PEG-IFN treatment remained statistically significant after adjustment for other baseline predictors, including age, HBV genotype, combination treatment, HBV DNA level and ALT level. The adjusted odds ratio (OR) for HBeAg seroconversion of patients with rs12980275 genotype AA versus AG/GG was 3.16 ($p=0.013$, 95% CI: 1.26-8.52, table 2). Similar data were observed for rs12979860 genotype CC versus CT/TT; the adjusted OR was 2.89 ($p=0.024$, 95% CI: 1.15-7.80, table 2). Importantly, the relationship between *IL28B* genotype and HBeAg seroconversion was similar when analysed separately for Asians and non-Asians. Combining the genotyping results for rs12979860 and rs12980275 into haplotypes was not superior to use of rs12980275 genotype alone. Since HBV genotype and route of transmission were highly collinear, we chose to build the models using HBV genotypes.

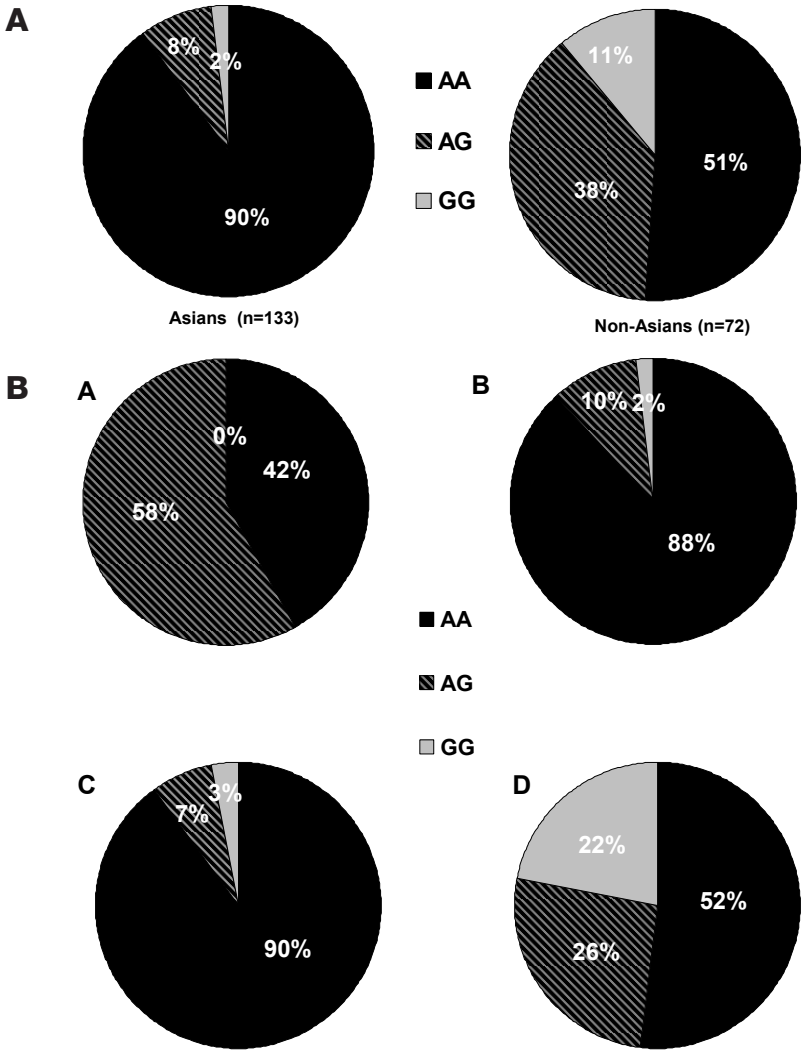

Figure 1: Distribution of rs12980275 genotypes in Asians versus non-Asians (A, p<0.001), and across HBV genotypes A through D (B). Similar frequencies were observed for rs12979860; only 6 (3%) patients were classified discordantly.

Relationship between *IL28B* genotype and HBeAg loss with or without HBV DNA <2,000 IU/mL at 6 months post-treatment

To allow for comparison with previously published prediction models that defined response as HBeAg clearance[3] or HBeAg clearance with HBV DNA <2,000 IU/mL at 6 months post-treatment[10], we also considered these endpoints. The analyses at 6 months post-treatment were limited to the 182 (89%) patients who received no LAM or LAM for ≤52 weeks. In this population, *IL28B* genotype was independently

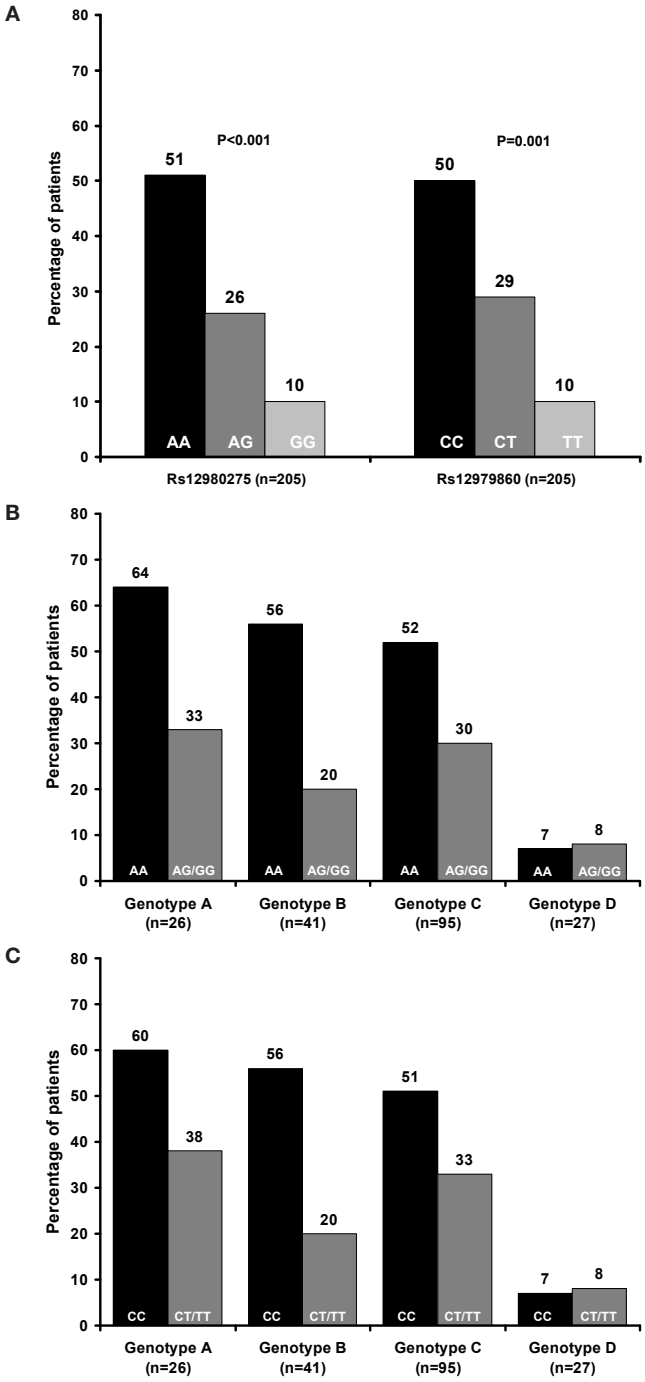

Figure 2: Relationship between rs12980275 and rs12979860 genotype and HBeAg seroconversion at end of PEG-IFN treatment, for the overall cohort (A) and across HBV genotype A through D (B and C).

Table 2. Odds ratios for HBeAg seroconversion at end of treatment

rs12980275			**rs12979860**		
Variable	**OR (95% CI)**	**p**	**Variable**	**OR (95% CI)**	**p**
AA genotype	3.16 (1.26 – 8.52)	0.013	CC genotype	2.89 (1.15 – 7.80)	0.024
HBV DNA*	0.58 (0.40 – 0.83)	0.003	HBV DNA*	0.57 (0.39 – 0.81)	0.002
HBV Genotype		0.050	HBV Genotype		0.044
A	Reference		A	Reference	
B	0.57 (0.15 – 2.05)		B	0.55 (0.15 – 2.02)	
C	0.50 (0.15 – 1.58)		C	0.47 (0.14 – 1.54)	
D	0.15 (0.02 – 0.79)		D	0.14 (0.02 – 0.77)	
ALT#	1.06 (0.93 – 1.21)	0.400	ALT#	1.05 (0.93 – 1.20)	0.453
Age	1.04 (1.01 – 1.07)	0.025	Age	1.04 (1.00 – 1.07)	0.026
Monotherapy	0.49 (0.24 – 0.97)	0.040	Monotherapy	0.47 (0.23 – 0.93)	0.030

*as log IU/mL; #as multiples of the upper limit of the normal range.

associated with HBeAg clearance at six months post-treatment (OR for AA vs AG/GG 3.54 [95% CI: 1.33 – 9.41, p=0.008]; OR for CC vs CT/TT 3.24 [95% CI: 1.21 – 8.69, p=0.016]) when adjusting for HBV genotype, baseline levels of HBV DNA and ALT, baseline age and previous IFN exposure[3]. In a model with the same variables, *IL28B* genotype AA (for rs12980275) and CC (for rs12979860) appeared to be associated with a higher probability of HBeAg clearance with HBV DNA <2,000 IU/mL, although associations were not significant (OR 2.09 [95% CI: 0.76 – 5.75, p=0.139] for AA vs AG/GG and OR 1.92 [95% CI: 0.70 – 5.26, p=0.188] for CC vs CT/TT). The relationship between *IL28B* genotype and response was not significant when *IL28B* genotype was added to the previously calibrated PEG-IFN treatment index[10]: OR for AA vs AG/GG 1.28 (p=0.588), and OR for CC vs CT/TT 1.03 (p=0.948).

Relationship between *IL28B* genotypes and HBeAg seroconversion through long-term follow-up

A total of 100 (49%) patients achieved HBeAg seroconversion in a median of 93 (IQR 49 – 192) weeks. Only 15 of 90 (17%) patients with a HBeAg seroconversion at end-of-treatment experienced a relapse during follow-up, whereas an additional 25 achieved HBeAg seroconversion after treatment discontinuation. HBeAg seroconversion at long-term follow-up was associated with *IL28B* genotype; HBeAg seroconversion rates were 54%/35%/20% in patients with AA/AG/GG genotypes (p=0.005). By Kaplan-Meier analysis, AA genotype (versus AG/GG, p=0.006) was associated with a higher cumulative probability of HBeAg seroconversion. In a Cox proportional hazards model, rs12980275 genotype AA was associated with a higher probability of HBeAg seroconversion by Cox regression analysis, when adjusting for HBV genotype, and baseline ALT and HBV DNA levels (HR 2.14, 95% CI: 1.14 – 4.31, p=0.018, figure 3A).

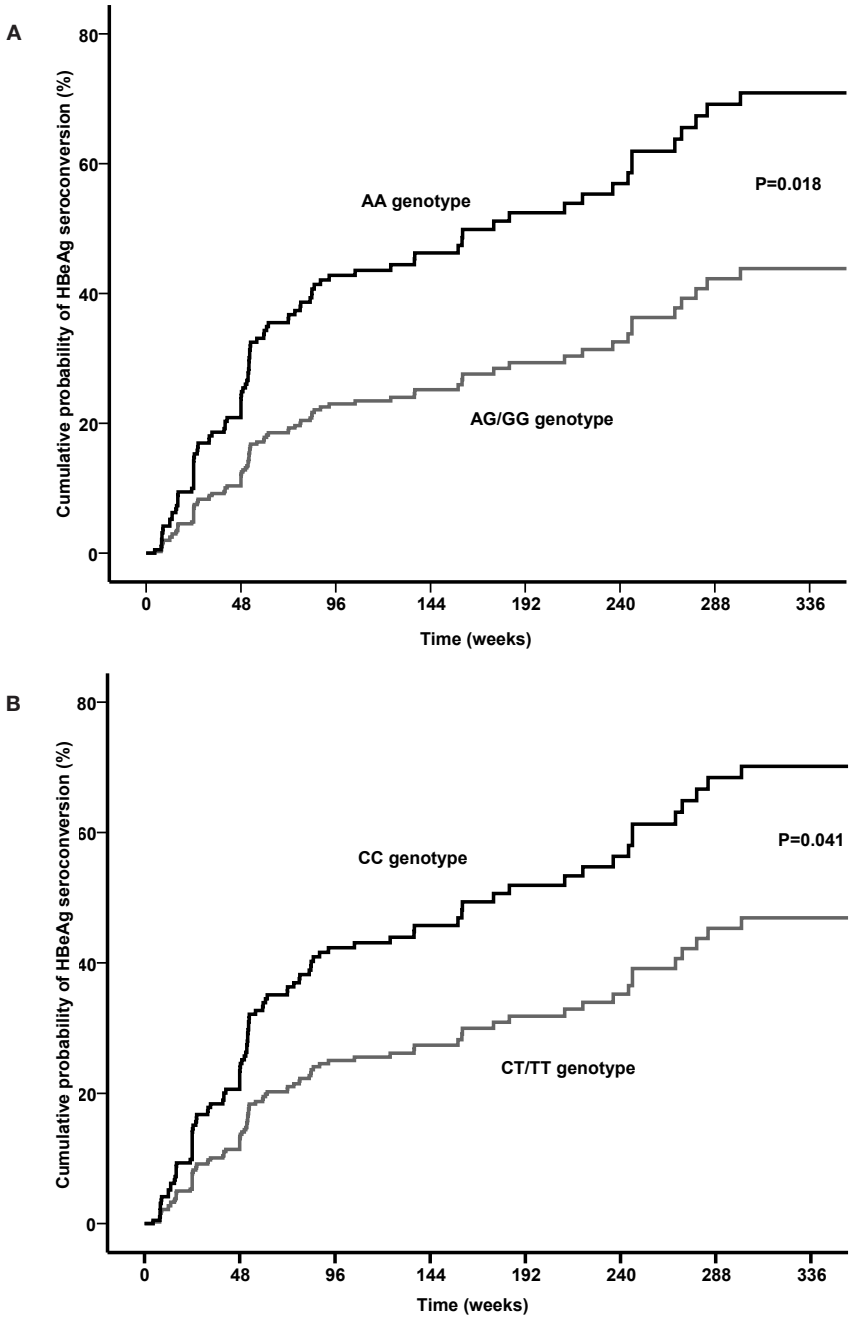

Figure 3: Cox proportional hazard model survival function of the relationship between rs12980275 (AA versus AG/GG) (A), rs12979860 (CC versus CT/TT) (B) and HBeAg seroconversion through long term follow-up, plotted for the mean values of baseline HBV DNA and ALT and weighted by HBV genotype distribution. In retreated patients, follow-up was terminated and (anti)HBeAg status before retreatment was used.

Similar data were observed for rs12979860. CC genotype was associated with a higher probability of HBeAg seroconversion when adjusting for the same parameters (adjusted hazard ratio (HR) 1.91, 95% CI: 1.03 – 3.78, p=0.041, figure 3B). For both analyses, combination treatment (p>0.76) was not a significant predictor of response. Stratification by ethnicity revealed no significant differences in the effect estimates for Asians versus non-Asians.

Relationship between *IL28B* genotypes and combined HBeAg seroconversion and low HBV DNA levels at long-term follow-up

A total of 57 patients (28%) achieved both sustained HBeAg seroconversion and HBV DNA <2,000 IU/mL at long-term follow-up. The differences in the probability of combined HBeAg and HBV DNA response across *IL28B* genotypes was not statistically significant. By multivariate regression analysis, patients with *IL28B* genotype AA (versus AG/GG) at rs12980275 tended to be more likely to achieve HBeAg seroconversion with HBV DNA <2,000 IU/ml (OR 2.12, 95% CI: 0.86 – 5.72, p=0.11), when adjusting for the only other significant predictor at baseline, HBV genotype. Similar data was observed for patients with CC (versus CT/TT) (OR 1.79, 95% CI: 0.73 – 4.78, p=0.21).

Relationship between *IL28B* genotypes and HBsAg clearance through long-term follow-up

Overall, 18 (9%) achieved HBsAg clearance through a median of 173 (IQR 108 – 356) weeks of follow-up. By univariate analysis, HBsAg clearance was shown to occur in patients with all *IL28B* genotypes. After adjustment for HBV genotype A and ethnicity, rs12980275 AA genotype was associated with a higher probability of HBsAg clearance (AA versus AG/GG, HR 3.47, 95% CI 1.04 – 13.48, p=0.042, figure 4). The HR for CC versus CT/TT was 2.57 (95% CI: 0.78 – 9.08, p=0.12).

DISCUSSION

In this large international collaborative study, we have shown that polymorphisms near *IL28B* are strong independent predictors of serological response to PEG-IFN in HBeAg-positive chronic hepatitis B. Patients with favourable *IL28B* genotypes have an increased probability of achieving HBeAg seroconversion both at the end of PEG-IFN treatment and through long-term follow-up, illustrating the importance of host factors when aiming to achieve a PEG-IFN induced immune response in HBeAg-positive chronic hepatitis B.

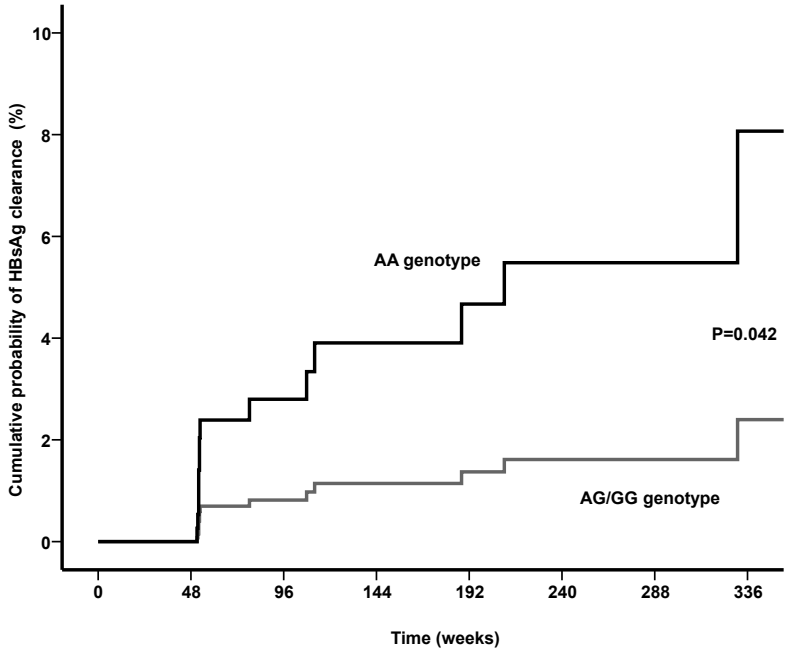

Figure 4: Cox proportional hazard model survival function of the relationship between rs12980275 (AA versus AG/GG) and HBsAg clearance through long-term follow-up, weighted by HBV genotype and ethnicity. Retreated patients were classified as non-responders.

PEG-IFN based treatment can induce a durable response in a substantial proportion of HBeAg-positive chronic hepatitis B patients,[3-5,21] but the clinical use of PEG-IFN is compromised by side-effects,[22] costs and the considerable uncertainty as to whether an individual patient will achieve such a sustained response. Selection of patients with a high probability of response is therefore essential for successful use of this agent in clinical practice. We have recently published a baseline prediction model based upon a pooled dataset from studies of PEG-IFN for HBeAg-positive chronic hepatitis B.[10] Unfortunately, discrimination was limited, leaving substantial uncertainty on the individual level. Several studies have therefore focused on on-treatment monitoring of viral replication, and the formulation of stopping-rules.[11,23-24]

Recently, large genome-wide association studies in hepatitis C virus infected patients have revealed an association between sustained virological response to PEG-IFN and ribavirin treatment and several single nucleotide polymorphisms on chromosome 19, located near the *IL28B* gene.[12-14,20] These studies have convincingly shown that favorable *IL28B* genotypes, such as AA for rs12980275, confer increased probabilities of sustained virological response, independent of other predictors.[20]

The exact biological mechanism or pathway of the association between these favorable genotypes and response to PEG-IFN remains unclear. *IL28B* itself is a member

of the IFN-λ family, and IFN-λ was recently shown to be active against both RNA and DNA viruses.[17-18,25] Indeed, preliminary data from a phase 2 study show that treatment with PEG-IFN-λ is effective in HCV infected patients.[26] Furthermore, in-vitro studies have shown that IFN-α, such as administered in the form of PEG-IFN in our study, may induce IFN-λ production. Since the two types of IFN, IFN-α and IFN-λ, have common downstream signaling pathways, the two types of IFN may act synergistically in patients treated with PEG-IFN.[27-30]

The current study shows that *IL28B* genotypes also play an important role in responsiveness to the immunomodulatory effects of PEG-IFN in chronic hepatitis B. Within this global cohort of 205 HBeAg-positive chronic hepatitis B patients, comprising both Caucasian and Asian patients infected with all major HBV genotypes, *IL28B* genotype was strongly associated with PEG-IFN induced HBeAg seroconversion, the first step towards sustained immune control over HBV. Importantly, the relationship between favorable *IL28B* genotypes, AA for rs12980275 and CC for rs12979860, and HBeAg seroconversion was apparent both at the end of PEG-IFN treatment and through prolonged off-treatment follow-up. Additionally, the relationship between the favorable *IL28B* genotypes and HBeAg seroconversion was independent of other recently published predictors of response.[10] It is important to note that viral genotype remains a very important determinant for response to PEG-IFN, in addition to *IL28B* genotype. In the current cohort, HBV genotype D was associated with a very low probability of response, suggesting that these patients with HBeAg-positive disease are poor candidates for PEG-IFN therapy.[10] Similarly, the level of HBV DNA at baseline is very strongly related to the probability of response. Taken together, these findings suggest that *IL28B* can be a valuable addition when estimating a patient's probability of response, but should be used together with, rather than instead of, previously described predictors of response.[10]

Our findings may have important clinical implications, because sustained HBeAg clearance induced by IFN has been proven to reduce the risk of hepatocellular carcinoma and to prolong survival.[8-9,31-32] Our finding that host genetic factors are strongly related to the occurrence of such a PEG-IFN induced immune response to HBeAg-positive HBV provides new insight into the mechanism of action of PEG-IFN in chronic hepatitis B, and may aid future selection of patients with a high probability of achieving a PEG-IFN induced immune response.

However, recent reports on the long-term prognosis of patients with PEG-IFN induced HBeAg clearance have revealed that some patients maintain detectable HBV DNA levels and elevated ALT.[5-6] A possible mechanism for these observations is the selection of viral strains that have mutations in the pre-core and basal core promoter regions that prohibit the synthesis of HBeAg, allowing for persistence of viral replication in the face of anti-HBe. [33-34] The data from the current study support the importance of

virological factors in the clearance of HBV after a host immune response to HBeAg. Despite the overt differences in HBeAg seroconversion rates across *IL28B* genotypes, HBsAg clearance occurs both in patients with favourable and unfavourable *IL28B* genotypes. These findings may be partly explained by HBV genotype distribution in this cohort, since HBV genotype A, associated with an increased probability of HBsAg clearance[5], was also associated with unfavourable *IL28B* genotypes. In our cohort, patients with genotype A had the highest probabilities of response, both overall and after stratification for *IL28B* genotype. This is in line with previous reports,[5-6] and is despite the relatively low frequency of favourable *IL28B* genotypes in these patients when compared to patients infected with genotype B and C. It should therefore be appreciated that HBV genotype remains a very important determinant of the probability of response to PEG-IFN, irrespective of *IL28B* genotype. Furthermore, the interplay between host factors such as *IL28B* genotype, and viral factors such as HBV genotype, in influencing the outcome of PEG-IFN therapy requires more extensive and prospective investigation. This is especially relevant since *IL28B* genotype appears to be associated with serological responses, whereas a decrease of HBV DNA after HBeAg clearance or seroconversion appears to depend on other factors beside *IL28B* genotype. The poor performance of *IL28B* genotype in predicting a combined response of HBeAg clearance with HBV DNA <2,000 IU/mL when added to a previously calibrated model suggests that re-establishment of prediction models is necessary when *IL28B* genotype is used to estimate a probability of response.

Possible caveats of the current study relate to the heterogeneity of the study cohort. Patients in this study were treated with different types and durations of PEG-IFN with or without lamivudine. However, several independent long-term follow-up studies have shown that the combination of PEG-IFN with lamivudine provides no advantage [3,5-6], and we adjusted for combination therapy in multivariate analysis when necessary. Furthermore, we enrolled a limited number of patients with genotypes other than A through D, and only one African patient, and it is therefore unclear whether our findings can be extended to these patients. Additionally, DNA samples for this study were prospectively collected during follow-up, but not all patients participating in the original studies on treatment effects could be enrolled.

Concluding, we have shown that polymorphisms near *IL28B*, at rs12979860 and rs12980275, are strong predictors of PEG-IFN induced HBeAg seroconversion. The association is independent of other important predictors, such as HBV genotype and baseline HBV DNA and ALT levels, and is apparent both at the end of treatment and through long-term post-treatment follow-up. The current study therefore shows that host factors are important for the induction of an immune response during antiviral therapy of HBeAg-positive chronic hepatitis B.

APPENDIX

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Presence of precore and core promoter mutants limits the probability of response to peginterferon in HBeAg-positive chronic hepatitis B

2

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ABSTRACT

Background & Aims

Peginterferon (PEG-IFN) treatment of HBeAg-positive chronic hepatitis B (CHB) results in HBeAg loss in 30% of patients, but clearance of HBV DNA and HBsAg from serum is less often achieved. We aimed to investigate whether the presence of precore (PC) and basal core promoter (BCP) mutants before PEG-IFN treatment affects serological and virological response.

Methods

A total of 214 HBeAg-positive CHB patients treated with PEG-IFN±lamivudine for 52 weeks in a global randomized trial were classified at baseline as wildtype (WT) or non-WT (detectable mutants at PC/BCP) by line-probe assay. Response was assessed at 6 months post-treatment and through long-term follow-up(LTFU).

Results

Mutants were detected in 64% of patients, in varying frequencies across HBV genotypes A through D. Patients with WT had higher baseline HBV DNA, HBeAg and HBsAg levels than patients with non-WT. Patients with WT were more likely to achieve HBeAg loss with HBV DNA<10,000 copies/mL (response, 34 versus 11%, p<0.001) and HBsAg clearance (18 versus 2%, p<0.001) at week 78 than non-WT patients. Among WT patients who achieved HBeAg clearance at week 78, 78% had undetectable HBV DNA and 61% achieved HBsAg clearance at LTFU (versus 26% and 15% in non-WT patients, p<0.001 for both). Presence of WT virus at baseline was an independent predictor of response (OR 2.90, 95%CI: 1.15–7.31, p=0.023) and HBsAg clearance (OR 5.58, 95%CI: 1.26–24.63, p=0.013) and patients with non-A genotypes with detectable mutants had a low probability of response.

Conclusions

Presence of only WT virus at baseline is a strong predictor of response (HBeAg loss with HBV DNA <10,000 copies/mL) to PEG-IFN for HBeAg-positive CHB. Patients with detectable PC and/or BCP mutants have a lower probability of response and are less optimal candidates for PEG-IFN therapy.

INTRODUCTION

Hepatitis B e Antigen (HBeAg)-positive chronic hepatitis B (CHB) is generally regarded as the earliest stage of a four stage disease continuum.[1-3] HBeAg does not appear to be required for infection with hepatitis B virus (HBV), nor for viral replication, but the presence of HBeAg in serum is associated with higher HBV DNA levels and was also recently shown to be an independent risk factor for the development of hepatocellular carcinoma (HCC).[4-5] Clearance of HBeAg (with or without appearance of anti-HBe) has therefore been adopted as a primary treatment endpoint for HBeAg-positive CHB.[1,3]

Current treatment options for CHB result in increased rates of HBeAg clearance when compared to placebo treated patients. A one year course of peginterferon (PEG-IFN) results in HBeAg clearance in 34% of patients, compared to about 20% in patients treated with lamivudine (LAM), entecavir or tenofovir.[1,6] However, emerging data show that mere loss of HBeAg from serum may be insufficient to induce disease remission. Indeed, among patients who experience HBeAg clearance during nucleo(s)tide analogue (NA) based therapy, a considerable number experience HBeAg reversion or have persistently detectable HBV DNA levels after discontinuation of therapy.[7] Similarly, while HBeAg loss induced by IFN therapy seems to be more durable, a proportion of patients continues to have detectable HBV DNA during long-term follow-up.[8-11] A possible explanation for these observations is the presence of viral strains that have mutations in the precore (PC) and basal core promoter (BCP) regions that prohibit the synthesis of HBeAg. Presence of these mutants before treatment initiation has been shown to predict HBeAg loss after IFN treatment,[12] possibly through positive selection during antiviral therapy[13], but may predispose patients to persistence of HBV DNA after HBeAg clearance. [14-15]

The aims of the current study were therefore to study the presence and frequency of PC and BCP mutants within a cohort of HBeAg-positive CHB patients and to relate presence of PC and BCP mutant strains to serological and virological response to PEG-IFN therapy.

PATIENTS AND METHODS

Patients

In this study, the presence of PC and BCP mutants was assessed in a cohort of HBeAg-positive CHB patients who were previously enrolled in an investigator-initiated multicenter randomized trial and had available HBsAg quantification measurements.[6,8,16] The inclusion and exclusion criteria for the original study have previously

been described elsewhere.[6] In summary, patients were eligible if they had been HBsAg positive for at least 6 months before randomization, were HBeAg positive, had elevated serum alanine aminotransferase (ALT) levels of >2, but <10 times the upper limit of normal (ULN), and had a serum HBV DNA concentration of more than 1.0 x 10^5 copies/mL. Patients were treated with PEG-IFN alfa-2b 100 µg weekly (PegIntron, Schering-Plough, Kenilworth, NJ, USA) in combination with placebo or LAM 100 mg (Zeffix, GlaxoSmithKline, Greenford, UK) daily for 52 weeks. The dose of PEG-IFN was reduced to 50 µg per week after 32 weeks of treatment to limit the probability of treatment discontinuation.

Inclusion criteria for the present analysis were completion of the 26-week follow-up phase of the main study, and availability of a baseline serum sample for PC / BCP mutation assessment. Of the 266 patients in the initial study, 214 fulfilled these criteria. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. All patients gave written informed consent according to standards of the local ethics committees.

Laboratory measurements

The presence of PC and BCP mutants was assessed using the INNO-LiPA HBV PreCore assay (Innogenetics, Ghent, Belgium). This very sensitive line probe assay allows for easy detection of PC (at nucleotide position G1896) and BCP (at nucleotide positions A1762 and G1764) mutants, even when only present as minority species.[17] Patients were subsequently classified as wildtype (*WT*, only WT virus detectable), *PC* (only PC or both PC and WT detectable), *BCP* (either or both BCP mutations detected, with or without WT), or as *both* when PC and BCP mutants were found. Serum HBV DNA, HBeAg and HBsAg was quantified in samples taken at baseline, during the treatment period (weeks 4, 8, 12, 24 and 52) and during follow-up (week 78). HBV DNA quantification was performed using an in-house developed TaqMan polymerase chain reaction (PCR) assay (lower limit of quantification 400 copies/mL) based on the EuroHep standard.[18] HBsAg was measured using the Abbott ARCHITECT HBsAg assay (Abbott laboratories; range 0.05 - 250 IU/mL) and HBeAg with the Roche ELECSYS HBeAg assay using a quantitative protocol (Roche Diagnostics, range 0.2 – 100 IU/ml).

Statistical analysis

For the current study a composite endpoint of HBeAg loss and HBV DNA level <10,000 copies/mL (~2,000 IU/mL) was chosen for definition of response at week 78, since this endpoint is associated with a low probability of relapse and low risk of disease progression.[5, 19-20] At long-term follow-up (LTFU), retreated patients were classified as non-responders. For multivariate analyses, patients were classified as WT (only

WT virus detectable) or non-WT (detectable PC and/or BCP mutants). Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. To investigate the value of WT/non-WT status in addition to our previously published PEG-IFN Treatment Index prediction model[19], we used a multivariable logistic regression model with the predicted probability calculated using the PEG-IFN Treatment Index as a fixed value. The PEG-IFN treatment index was built using data on HBV genotype, baseline levels of ALT and HBV DNA, as well as age, patient gender and previous IFN therapy. The gain of adding WT/non-WT status was quantified using the net reclassification improvement (NRI), which is the sum of reclassification of subjects with a response and without a response, where reclassification is the percentage of patients with an improved prediction minus the percentage of patients with a worse prediction.[21] SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.2 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

Role of the funding source

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RESULTS

Patient characteristics

Since treatment outcome did not differ between patients treated with PEG-IFN ± LAM, the data of the two treatment arms were pooled for the current analysis. The characteristics of the 214 patients are shown in table 1.

Prevalence of PC and BCP mutants at baseline

Within the total cohort, a minority (36%) of patients had only wildtype virus (WT) detectable. PC and BCP mutants were detected in 138 (64%) of patients, either alone or in combination (table 1). When stratified by HBV genotype, considerable differences were observed with regard to the frequency and type of detected mutant virus ($p<0.001$). A majority of patients with genotype A harboured only WT virus, whereas mutants were often detected in patients with genotypes B, C and D (figure 1).

Table 1: Characteristics of the study cohort

Characteristics	Study population (n=214)
Demography	
Mean (SD) age, years	33.8 (13)
Male	167 (78%)
PEG-IFN Monotherapy	104 (49%)
Race	
Caucasian	157 (73%)
Asian	40 (19%)
Other	17 (8%)
Laboratory results	
Mean (SD) ALT*	4.3 (3.0)
Mean (SD) HBV DNA, log c/mL	9.1 (0.90)
Mean (SD) HBsAg, log IU/mL	4.4 (0.60)
Mean (SD) HBeAg, log IU/mL	2.5 (0.70)
HBV Genotype	
A	74 (35%)
B	19 (9%)
C	29 (14%)
D	85 (40%)
Other/mixed	7 (3%)
INNO-LiPA result	
Wildtype	76 (36%)
Precore	56 (26%)
Basal core promoter	47 (22%)
Precore and basal core	35 (16%)
Response at week 78	
Response#	41 (19%)
HBeAg loss	77 (36%)
HBV DNA <10,000 c/mL	43 (20%)
HBsAg loss	17 (8%)

*Multiples of upper limit of the normal range
#HBeAg loss and HBV DNA <10,000 copies/mL at week 78

Relationship between PC and BCP mutants and HBV serum markers at baseline

Considerable differences were observed with regard to baseline HBV DNA, HBeAg and HBsAg levels in patients with WT or detectable PC or BCP mutants. After adjustment for HBV genotype distribution, patients with WT had higher baseline HBV DNA levels (9.20 versus 8.86 log copies/mL, p=0.015), higher HBeAg levels (2.81 versus 2.33 log

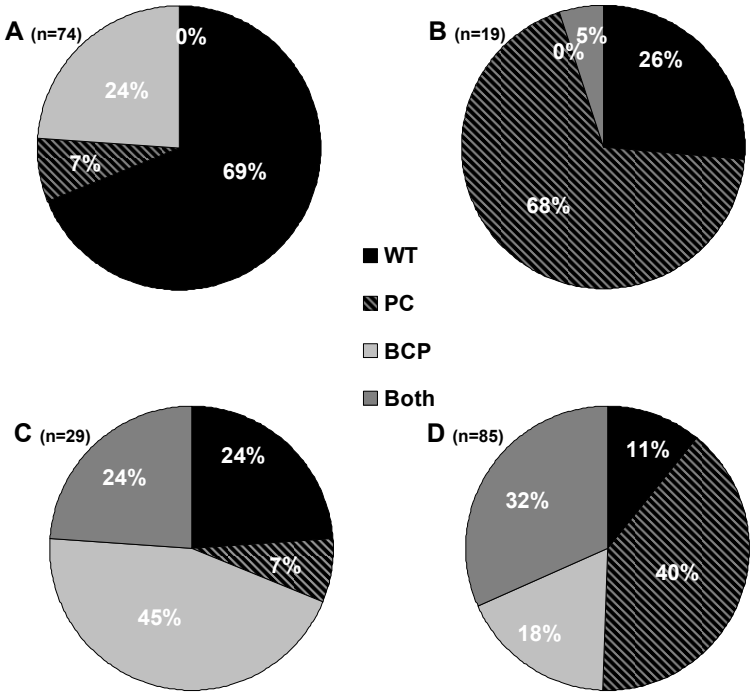

Figure 1: Frequency of PC and BCP mutants in the cohort by HBV genotype.

IU/mL, p<0.001) and higher HBsAg levels (4.53 versus 4.28 log IU/mL, p=0.007) than patients with detectable mutants.

Relationship between PC and BCP mutants and treatment response at week 78

Among the total population, rates of HBeAg loss were not significantly different between patients with WT virus or PC/BCP mutants (figure 2A). However, patients with only WT virus were considerably more likely to achieve a response (HBeAg loss and HBV DNA <10,000 copies/mL; 34% versus 11%, p<0.001), HBV DNA <10,000 copies/mL (34% versus 12%, p<0.001), HBV DNA < 400 copies/mL (20% versus 2%, p<0.001) and HBsAg clearance (18% versus 2%, p<0.001) by week 78, when compared to patients with PC/BCP mutants. Patients with both PC and BCP mutants had both a very low probability of response and no chance of HBsAg loss.

Long-term follow-up of patients with negative HBeAg at week 78

Long-term follow-up (mean 3.0 years, range 1.9 – 5.0) data were available in 50 of 77 (65%) patients who were HBeAg negative at week 78. The majority of patients had sustained HBeAg loss through prolonged follow-up, irrespective of the presence of WT or PC/BCP mutants at baseline (Figure 2B). However, patients with only WT

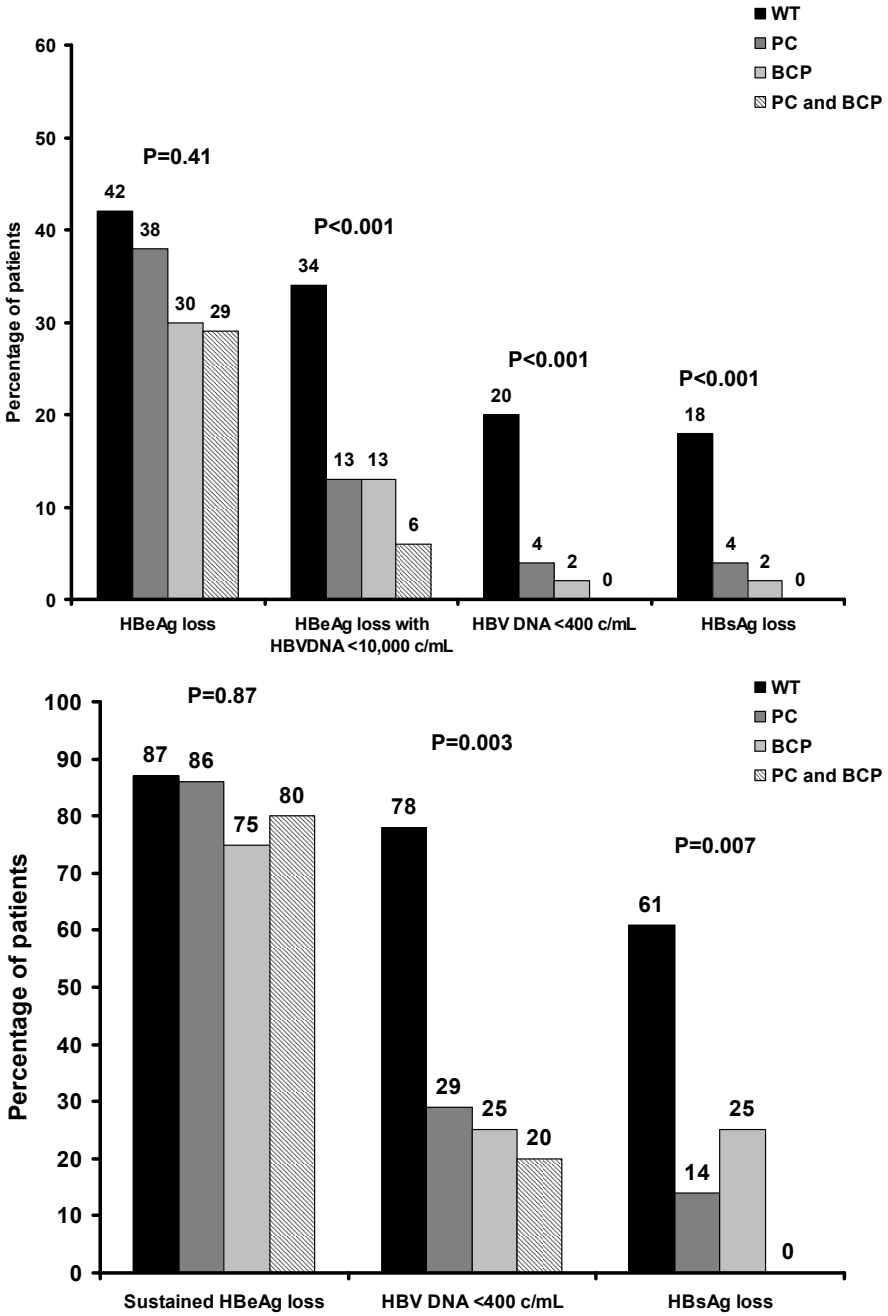

Figure 2: Relationship between presence of PC and BCP mutants at baseline and response at week 78 in the total cohort (n=214) (A) and response at long-term (mean 3.0 years) follow-up among patients who were HBeAg negative at week 78 (n=50) (B). Response was defined as HBeAg clearance and HBV DNA <10,000 copies/mL at week 78. WT, wildtype; PC, precore; BCP, basal core promoter.

virus at baseline who cleared HBeAg by week 78 had a much higher probability of achieving undetectable (<400 copies/mL) HBV DNA levels at long-term follow-up when compared to those with mutants (p=0.003, figure 2B). Additionally, patients who achieved HBeAg loss at week 78 and had WT virus at baseline had a 61% probability being HBsAg negative at long-term follow-up.

Presence of PC and BCP mutants in patients with HBeAg loss with HBV DNA >10,000 copies/mL at week 78

Of the 36 patients who cleared HBeAg but had HBV DNA >10,000 copies/mL, serum samples and PC/BCP data at week 78 were available in a subset of 29 (81%). All of the patients with detectable mutants at baseline had detectable mutants at week 78, and 5 of 6 patients with WT at baseline had detectable mutants at week 78 (figure 3).

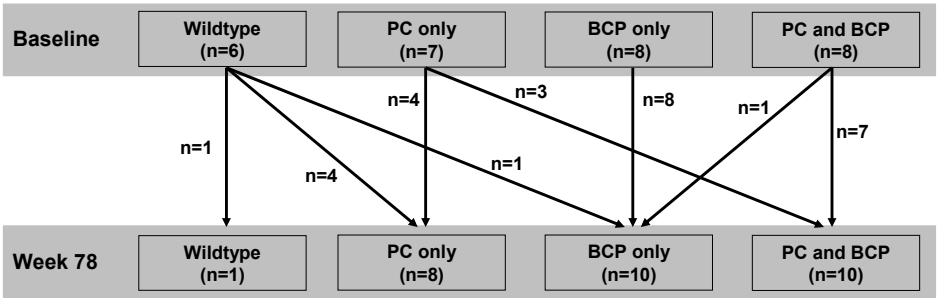

Figure 3: Baseline and week 78 INNO-LiPA test results in patients with HBeAg loss and HBV DNA >10,000 copies/mL (n=29). WT, wildtype; PC, precore; BCP, basal core promoter.

Relationship between PC/BCP mutants and response; multivariate analysis

To account for the differences in PC and BCP mutant distributions across HBV genotypes, presence of WT/non-WT status was added as an independent determinant of response to other previously described predictors, HBV genotype, baseline HBV DNA level, ALT and age. Presence of only WT virus remained a strong predictor of response (odds ratio (OR) WT versus non-WT: 2.60, 95% CI: 1.05 – 6.42, p=0.037). Combination therapy (PEG-IFN + LAM) was not associated with response. Similarly, presence of only WT virus at baseline was a significant predictor of HBsAg loss at week 78 (OR for WT versus non-WT: 5.58, 95% CI: 1.26 – 24.63, p=0.013), after adjustment for other predictors, HBV genotype and age. Flowcharts for treatment response for WT versus non-WT by HBV genotype and baseline HBV DNA and ALT levels are depicted in Figure 4A and 4B.

We added WT/non-WT status to a model including the previously published PEG-IFN treatment index[19], and found that it significantly improved prediction of response (WT

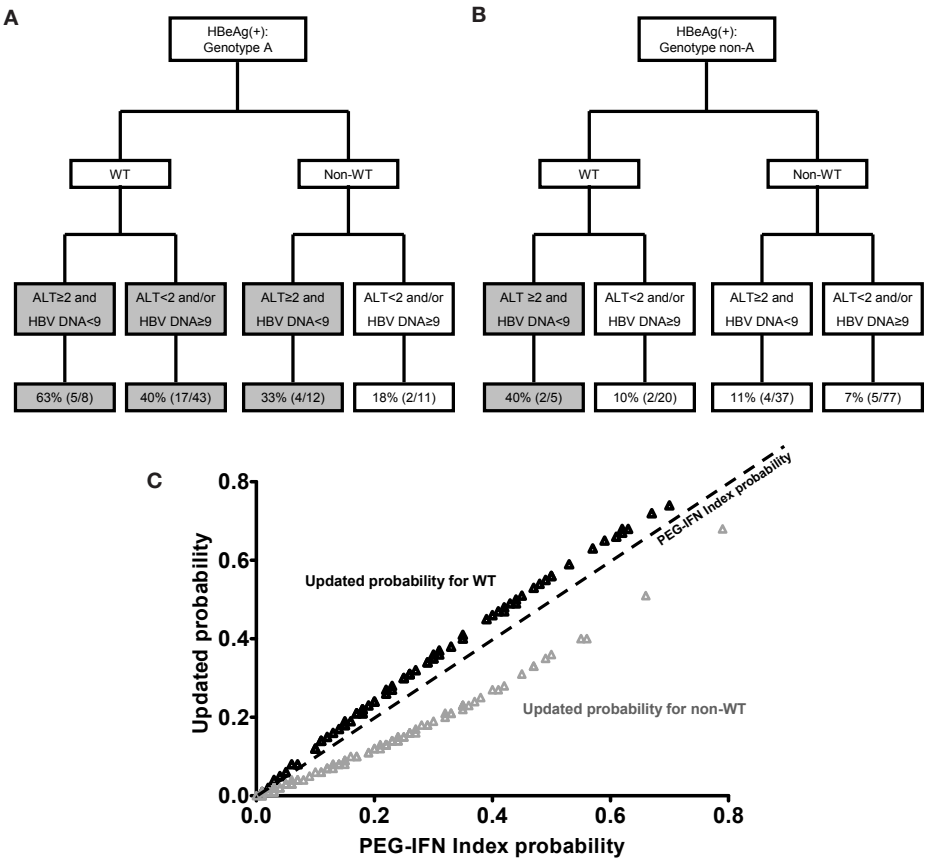

Figure 4: Probability of response (HBeAg loss with HBV DNA <10,000 copies/mL at week 78) according to presence of WT or detectable mutants (non-WT) and baseline HBV DNA and ALT levels, stratified by genotype A (A) or non-A (B). Grey boxes mark patients groups where peginterferon should be considered based on a probability of more than 30% (19). Panel C shows the individual probability of response as estimated by extending the previously published PEG-IFN Treatment Index (19) with data on WT/non-WT. WT, wildtype.

versus non-WT OR 2.35, 95% CI: 1.06 – 5.19, p=0.033, NRI=0.66). The individual patients' estimated probabilities for the PEG-IFN Treatment Index extended with WT/non-WT status are depicted in figure 4C. Re-calibration of the model showed that WT/non-WT significantly predicts response to PEG-IFN, independently of HBV genotype, baseline ALT and HBV DNA, patient age, and previous IFN therapy failure (table 2). An interaction term between HBV genotype and WT/non-WT was not significant (p=0.954). Figure 5A shows the estimated probabilities of response from this model for WT/non-WT, stratified by HBV genotype. Figure 5B shows the estimated probabilities of HBsAg clearance for patients with WT or non-WT, stratified by HBV genotype and adjusted for patient age.

Table 2. Logistic regression model of probability of response to peginterferon

Variable	OR (95% CI)	p
WT	2.90 (1.15 – 7.31)	0.023
HBV Genotype		0.043
A	ref	
B	0.56 (0.14 – 2.21)	
C	0.11 (0.02 – 0.59)	
D	0.35 (0.11 – 1.14)	
HBV DNA*	0.58 (0.35 – 0.97)	0.038
ALT#	1.10 (0.95 – 1.26)	0.210
Age	1.04 (1.01 – 1.08)	0.014
No previous IFN	5.20 (1.55 – 17.4)	0.003

*HBV DNA in log copies/mL, #ALT in x ULN.

PC and BCP mutants and on-treatment HBV DNA, HBeAg and HBsAg levels

To explore the relationship between presence of PC/BCP mutants and on-treatment kinetics of HBV DNA, HBeAg and HBsAg, only data from the PEG-IFN monotherapy group were analysed, because HBV DNA and HBsAg kinetics differ considerably between patients treated with PEG-IFN monotherapy and PEG-IFN + LAM combination therapy.[6,16] Within the monotherapy cohort, 40 (38%) patients harboured only WT virus (WT group), and 64 (62%) had evidence of either PC mutants, BCP mutants or both (non-WT group).

HBV DNA

After 52 weeks of treatment, HBV DNA decline from baseline was 2.49 log copies/mL in the WT group, compared to 2.22 log copies/mL in patients with non-WT (p=0.60). After treatment discontinuation, relapse was observed in patients with mutants, while patients with only WT virus at baseline continued to decline. Mean HBV DNA declines at week 78 were 2.82 versus 1.77 log copies/mL (p=0.05, figure 6A).

Quantitative HBeAg

HBeAg decline was somewhat more pronounced during the first 8 weeks of therapy in patients with PC or BCP mutants, but end of treatment HBeAg declines were similar in both groups: 1.07 versus 1.04 log IU/mL in patients with only WT or detectable mutants, respectively (p=0.93), as were HBeAg declines at week 78 (p=0.94, figure 6B).

Quantitative HBsAg

Patients with only WT virus detectable before therapy had a distinctly steeper HBsAg decline than did patients with PC or BCP mutants. End of treatment declines were 1.46 and 0.43 log IU/mL (p=0.007), and the difference increased through off-treatment follow-up to 1.55 versus 0.38 log IU/mL at week 78 (p=0.003, figure 6C).

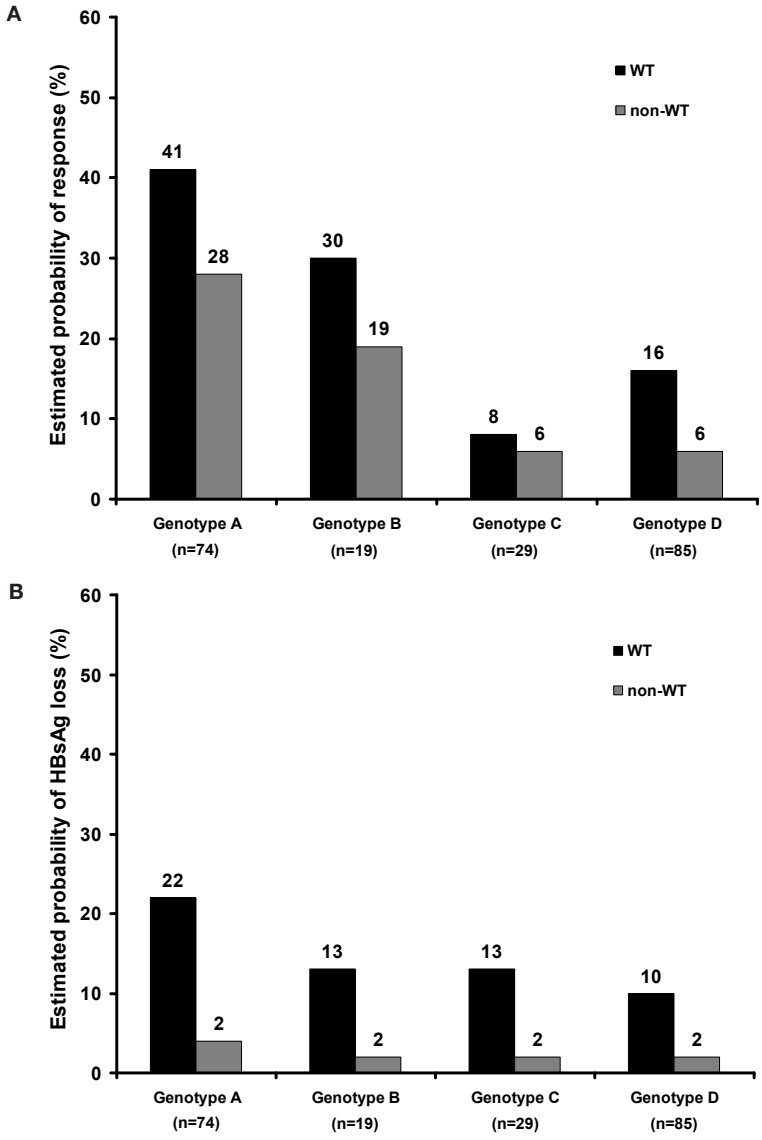

Figure 5: The estimated probability of response from the model shown in table 2 for WT versus non-WT, stratified by HBV genotype (A), and the estimated probability of HBsAg loss at week 78 by WT/non-WT, adjusted for patient age and stratified by HBV genotype (B).

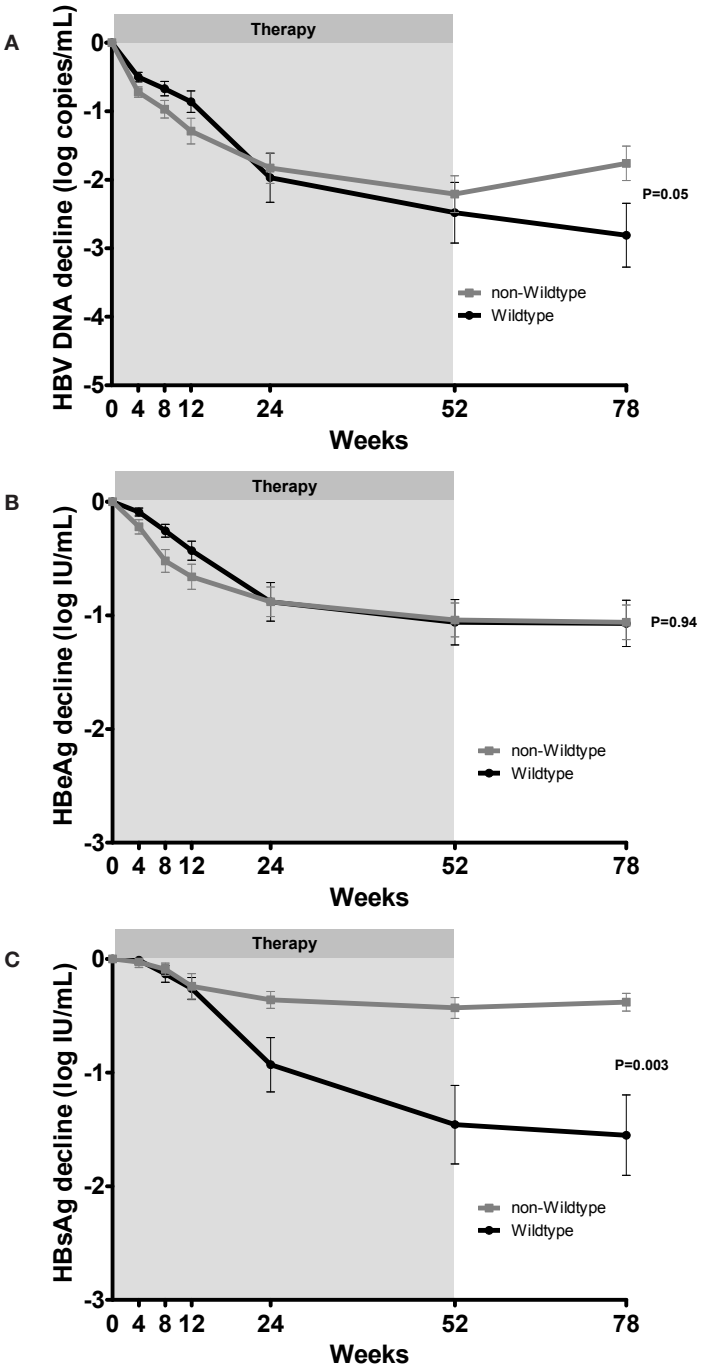

Figure 6: HBV DNA (A), HBeAg (B) and HBsAg (C) declines among patients with only WT virus, or with detectable PC or BCP mutants (non-WT). Analyses were performed in patients treated with PEG-IFN monotherapy (n=104). WT, wildtype.

DISCUSSION

This is the largest study to date investigating the relationship between presence of PC and BCP mutants and response to PEG-IFN in HBeAg-positive CHB patients. We found that achievement of combined serological and virological response, as well as HBsAg seroclearance, is largely confined to patients without detectable PC and BCP mutants at baseline. Assessment of WT/non-WT status before therapy thus provides valuable additional insight into an individual patient's probability of achieving a response to PEG-IFN, and can help optimize patient selection for this burdensome treatment modality.

Prolonged presence of HBeAg in serum of CHB patients was recently shown to be an independent predictor of emergence of HCC, and clearance of HBeAg, whether spontaneous or treatment induced, is therefore considered an important event.[1,3] However, it has become increasingly clear that a considerable number of patients with CHB have persistently detectable HBV DNA levels after HBeAg clearance, predisposing these patients to progression of their liver disease to cirrhosis, HCC and premature death.[5,22-23] A possible mechanism for this phenomenon is the selection for HBV mutants with mutations in the PC or BCP region, both of which reduce or abolish the production of HBeAg and are able to maintain replication despite seroclearance of HBeAg.[14,24] Data from the current study now show that these PC and BCP mutants may be detected in the majority of HBeAg-positive patients, confirming data from a US survey.[25] The clinical relevance of these findings, especially in relation to response to immunomodulatory treatment of CHB, was however unclear. In patients treated with NA, presence of PC or BCP mutants confers an increased probability of HBeAg clearance.[26] Similarly, several older studies have reported that presence of PC or BCP mutants is associated with a higher probability of achieving response to standard IFN treatment. [12,27-28] Response in these studies was commonly defined as HBeAg clearance, with or without an HBV DNA undetectability criterion. However, most of these studies used HBV DNA assays that were not sensitive enough to detect HBV DNA at levels that are currently considered to confer substantial risk for progression of liver disease.[23] In concurrence with these studies, we found that patients with PC or BCP mutants have a good probability of HBeAg clearance, and of remaining HBeAg negative through prolonged follow-up. However, the current study also shows that the majority of patients with detectable PC or BCP mutants who achieve HBeAg clearance do not achieve HBV DNA levels <10,000 copies/mL, necessitating further therapy in these patients according to recent guidelines.[1,3] Furthermore, it is important to note that HBeAg clearance alone does not appear to be an appropriate marker for immune control in patients with detectable PC or BCP mutants at baseline, since HBsAg loss after HBeAg clearance is extremely rare in this group of patients.

These observations, along with the high frequency (>60%) of PC and BCP mutant strains in our HBeAg-positive cohort, suggest that HBeAg clearance alone is not a suitable marker for response to PEG-IFN therapy in the general HBeAg-positive patient population, but perhaps only in those with confirmed WT virus at baseline.

We observed considerable differences in the frequency of PC and BCP mutants across HBV genotypes A through D. These observations are in line with other reports, and may be accounted for by differences in genetic make-up of the respective genotypes.[25] Several recent reports, also from our group, have shown that HBV genotype is an important predictor of response to (PEG-)IFN based therapy of CHB.[19] The current study sheds considerable new light on these observations, since the advantages of patients with genotype A may be partly due to the relatively high frequency of WT virus, accounting for the high rates of HBV DNA undetectability and HBsAg loss through long-term follow-up in patients with HBV genotype A.[8] Nevertheless, the advantages of patients with only WT virus over those with detectable PC or BCP mutants holds true independent of HBV genotype. Presence of only WT virus is a very strong independent predictor of response and HBsAg clearance, when adjusting for the other established predictors HBV genotype, baseline HBV DNA level and ALT level and age. Importantly, WT/non-WT status adds significantly to our previously published PEG-IFN Treatment Index prediction model.[19] Extension of this model with data on detection of mutants may help optimize prediction of response at baseline and help select only those patients who have a very high probability of response for PEG-IFN therapy. Moreover, detection of PC or BCP mutants in patients with non-A genotypes confers such a low probability of a combined HBeAg and HBV DNA response, that in our view PEG-IFN should not be used as a first-line treatment option in these patients.

Another important observation is the relationship between the presence of PC and BCP mutants and established markers of HBV infection such as HBV DNA, HBeAg and HBsAg. We found comparable on-treatment HBV DNA and HBeAg declines in patients with only WT or with mutant virus. These findings show the inherent limitations of HBeAg levels in monitoring PEG-IFN therapy efficacy, for they are highly influenced by the presence of PC and BCP mutants, and underscore the limitations of recent prediction rules for PEG-IFN therapy based on HBeAg thresholds.[29] In contrast, the current study confirms recent data on the relationship between HBsAg decline during PEG-IFN therapy and sustained off-treatment response, for HBsAg decline was largely confined to patients with WT virus. [16,30]

Possible limitations of the current study are related to the assay used to classify patients as having mutant virus. The INNO-LiPA assay is more sensitive than conventional sequencing technology, and can detect mutant virus at very low levels.[17] However, the prevalence of mutants at baseline may have been underestimated if

mutants were only present as minority species (<5%).[31] Additionally, the assay can only detect known mutations and ignores others. Possible misclassification introduced by this limitation may have influenced the results. The current study enrolled patients infected with all major HBV genotypes, but only a limited subgroup of patients was infected with genotypes B and C. Possibly, this may have resulted in a non-significant test for interaction of WT with HBV genotype. Confirmation of our findings in Asian patients is therefore required.

Concluding, patients with only WT virus have the highest probability of achieving a combined HBeAg and HBV DNA response and HBsAg loss, whereas patients with PC or BCP mutants are predisposed to persistent viral replication after HBeAg clearance. This limits the use of HBeAg clearance alone when assessing therapy efficacy of PEG-IFN, and potentially other treatment options as well. Patients with WT virus appear to be the most suitable candidates for PEG-IFN therapy, irrespective of HBV genotype.

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Precore and core promoter mutants are associated with higher HBeAg seroconversion but low disease remission rates in HBV patients treated with nucleos(t)ide analogues

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ABSTRACT

Background & Aims

HBeAg seroconversion in chronic hepatitis B (CHB) patients is considered an important event as it is associated with better clinical outcome. However, a proportion might not achieve immune control after HBeAg seroconversion.

Methods

We determined presence at baseline of precore (PC) and basal core promoter (BCP) mutants in 137 consecutive HBeAg-positive patients treated with nucleos(t)ide analogues (NA) by INNO-LiPA HBV PreCore assay (Innogenetics). Baseline HBeAg and HBsAg levels were measured using the ELECSYS platform.

Results

The majority (>60%) of patients with genotype B, C and D had PC/BCP mutants present at baseline, compared to only 40% of patients with HBV genotype A (p=0.02). Patients with mutants had lower HBV DNA (p=0.006), HBeAg (p<0.001) and HBsAg (p=0.04) levels compared to patients with wildtype HBV. During a median treatment duration of 29 (IQR 18-56) months, 45 patients achieved HBeAg seroconversion. The probability of HBeAg seroconversion during therapy was higher in patients with PC/BCP mutants (HR 2.2, 95% CI: 1.13 - 4.44, p=0.01). After HBeAg seroconversion, patients with BCP mutants tended to have a higher probability of HBeAg relapse (p=0.07), and detectable PC mutants tended to be associated with a lower probability of achieving HBV DNA <2000 IU/mL 1 year after HBeAg seroconversion (p=0.07).

Conclusions

The presence of PC and/or BCP mutants in NA-treated HBeAg-positive patients appears an independent predictor of HBeAg seroconversion. However, presence of BCP mutants was associated with a higher risk of serological relapse, patients with detectable PC mutants tended to have a lower probability of achieving virological remission the first year after HBeAg seroconversion.

INTRODUCTION

Hepatitis B e Antigen (HBeAg)-positive chronic hepatitis B (CHB) is an early stage of the disease continuum.[1-3] HBeAg does not appear to be required for infection with hepatitis B virus (HBV), nor for viral replication, but the presence of HBeAg in serum is associated with higher levels of HBV DNA and was shown to be an risk factor for the development of hepatocellular carcinoma.[4-6] HBeAg clearance has therefore been adopted as a treatment endpoint for HBeAg-positive CHB in all major HBV guidelines.[3,7]

About 20% of patients treated with nucleos(t)ide analogues achieve HBeAg seroconversion after one year of treatment.[7-8] However, emerging data show that mere loss of HBeAg from serum may be insufficient to induce disease remission. Indeed, reversal to HBeAg positivity is frequently observed after NA-induced HBeAg seroconversion, as is persistence of detectable HBV DNA levels after discontinuation of therapy.[9] Similarly, while HBeAg negativity induced by peginterferon (PEG-IFN) therapy seems to be more durable, a proportion of patients fail to achieve low levels of HBV DNA and HBsAg clearance.[10-12] A possible explanation for these observations is the presence of viral strains with mutations in the precore (PC) and basal core promoter (BCP) regions that prohibit the synthesis of HBeAg.[13] In patients treated with PEG-IFN, presence of these mutants predisposes to persistent replication after HBeAg clearance, possibly through positive selection during antiviral therapy,[14] and these mutants may therefore also predict persistence of substantial HBV DNA replication after HBeAg clearance in patients treated with NA.[14-15]

The aim of the current study was therefore to investigate the relationship between presence of PC or BCP mutants and the probability of NA-induced HBeAg seroconversion. Secondly, we assessed the relation between these mutants and true disease remission after HBeAg seroconversion in patients treated with NA.

PATIENTS AND METHODS

Study population

All consecutive patients who were positive for HBeAg, negative anti-HBe and treated with NA therapy for at least 6 months from January 1st 1996 onwards at the Erasmus MC University Medical Center in Rotterdam were enrolled. Patients were excluded when no stored baseline sample was available for retesting, when co-infected with hepatitis C virus, hepatitis D virus or human immunodeficiency virus, when treated with peginterferon within 6 months before start of NA therapy or when peginterferon was (temporarily) added to the NA regimen.

Follow up evaluation

ALT and virological parameters were assessed at least every 3-6 months. HBeAg seroconversion was defined as loss of HBeAg with concurrent appearance of anti-HBe. HBsAg seroconversion was defined as loss of HBsAg with appearance of anti-HBs. Serological recurrence was defined as a confirmed reappearance of HBeAg in at least 2 consecutive samples after HBeAg seroconversion. Virological remission was defined as achieving HBV DNA <80 IU/ml within one year after achieving HBeAg seroconversion. In patients in whom treatment was stopped after HBeAg seroconversion accompanied by an HBV DNA <80 IU/mL, relapse was defined as confirmed HBV DNA level above 2000 IU/mL and/or restart of antiviral therapy.

Laboratory testing

ALT level was measured using automated techniques. The presence of PC and/or BCP mutants was assessed at baseline by Innolipa HBV Precore assay (Innogenetics, Ghent, Belgium). This sensitive probe assay allows for detection of PC mutants (at nucleotide position G1896) and BCP mutants (at nucleotide positions A1762 and G1764), even when a minority species is present.[16] Patients were thus classified as having wildtype virus (WT, no PC or BCP mutants were detected), PC (PC mutants detected with or without WT), BCP (BCP mutants detected with or without WT), or as both (both PC and BCP mutants were found). HBV DNA quantification was performed using a TaqMan polymerase chain reaction (PCR) assay (lower limit of quantification 373 copies/mL) based on the EuroHep standard[17] or by using the AmpliPrep-COBAS TaqMan HBV test (CAP-CTM v 2.0; Roche Molecular Systems, Inc., Branchburg, NJ, USA, lower limit of detection 20 IU/mL). HBeAg and HBsAg were quantified at baseline using the Roche ELECSYS assay (Roche Diagnostics, Indianapolis, IN, USA) using a standardised protocol.

Statistical analysis

Data are presented as either mean (SD) or median (interquartile range, IQR) where appropriate. Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. Time-dependent analyses (HBeAg seroconversion, HBeAg seroreversion and virological relapse) were performed by Kaplan-Meier survival analysis and Cox' regression analysis. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.2 program (SAS Institute Inc., Cary, NC, USA) were used. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

Role of the funding source

The sponsor of this study was Foundation for Liver and Gastrointestinal Research (SLO) in Rotterdam, the Netherlands. Financial support and test kits were provided by Innogenetics, Ghent, Belgium. The funding source did not have influence on study design, data collection, analysis and interpretation of the data, writing of the report nor the decision to submit for publication.

RESULTS

Patient characteristics

The study population consisted of 137 HBeAg-positive, anti-HBe-negative CHB patients (table 1). Median follow up of the study population was 29 (IQR 18-56) months from start of NA therapy.

Prevalence of PC and BCP mutants at baseline

Within the total cohort 39% of patients had only WT detectable. PC and/or BCP mutants were detected in 84 (61%) of patients (table). After stratification by HBV

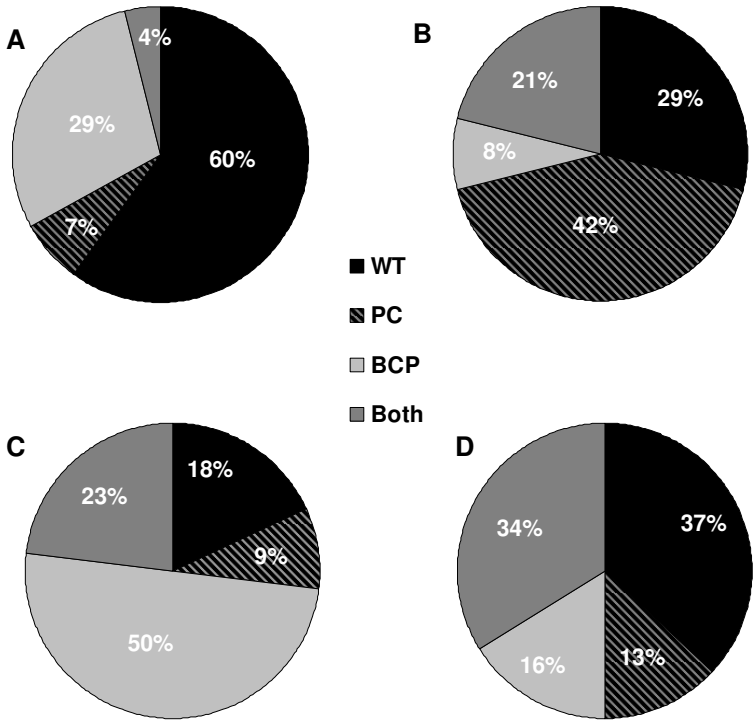


Figure 1: Frequency of PC and BCP mutants at baseline in the study cohort by HBV genotype.

Table 1. Characteristics of the study cohort

Characteristics	Study population (n=137)
Demography	
Mean (SD) age, years	36.5 (14.5)
Male	105 (77%)
Race	
Caucasian	84 (61%)
Asian	40 (29%)
Other	13 (9%)
Laboratory results	
Median (IQR) ALT*	2.3 (1.3-4.5)
Mean (SD) HBV DNA, log IU/mL	7.4 (1.6)
Mean (SD) HBsAg, log IU/mL	4.1 (1.1)
Mean (SD) HBeAg, log IU/mL	2.1 (1.0)
HBV Genotype	
A	45 (33%)
B	24 (18%)
C	22 (16%)
D	38 (28%)
Other/mixed	8 (6%)
INNO-LiPA result	
Wildtype	53 (39%)
Precore	24 (18%)
Basal core promoter	33 (24%)
Precore and basal core	27 (20%)
Nucleos(t)ide analogue regimen	
Lamivudine	52 (38%)
Entecavir	41 (30%)
Adefovir**	32 (23%)
Tenofovir**	12 (9%)

*Multiples of upper limit of the normal range. **Adefovir+lamivudine in 2 and tenofovir+lamivudine in 3.

genotype, the frequency of PC and/or BCP mutants differed widely (p=0.02, figure 1). The majority of patients with genotype A had only WT virus (60%) present at start of NA therapy. In contrast, patients infected with HBV genotypes B, C and D had mutants present in 63 to 82% of patients. Patients with only WT virus at baseline had higher levels of HBV DNA, HBsAg and HBeAg compared to patients with PC and/or BCP mutants (Figure 2). In contrast, ALT levels were comparable between both groups.

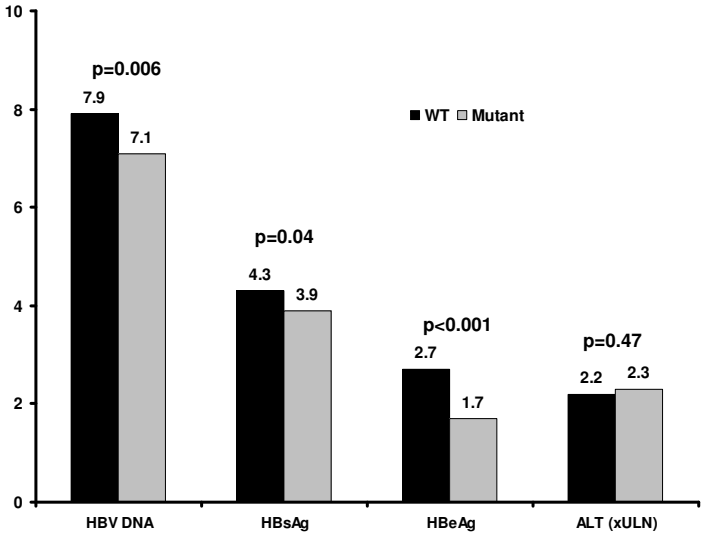


Figure 2 Relationship between presence of PC and/ or BCP mutants at baseline and virological and biochemical parameters of CHB infection. The y-axis represents mean values for: HBV DNA log IU/mL, HBsAg log IU/mL, HBeAg log IU/mL, and median for ALT xULN.

Relationship between PC and BCP mutants and serological response

A total of 45 (33%) patients achieved HBeAg seroconversion after a median of 76 (28-137) weeks (Figure 3). Patients with PC/BCP mutants present at baseline had a higher probability of achieving HBeAg seroconversion compared with WT patients (HR 2.20, 95% CI: 1.13 - 4.44, p=0.01, figure 4). In univariate analysis, higher baseline ALT was also associated with a higher probability of achieving HBeAg seroconversion (HR 1.05, 95% CI 1.02 – 1.07, p=0.002). In contrast, higher levels of HBV DNA (HR 0.87, 95% CI: 0.74 – 1.01, p=0.08) and HBsAg (HR 0.84, 95% CI 0.71 - 1.00, p=0.05) were associated with a lower probability of HBeAg seroconversion. Baseline HBeAg (HR 0.93, 95% CI 0.68 – 1.26, p=0.62) levels were not associated with probability of achieving HBeAg seroconversion. After adjustment for baseline HBV DNA, HBsAg, HBeAg, HBV genotype and ALT within a Cox regression model, the presence of PC/ BCP mutants at baseline remained independently associated with a higher probability of achieving HBeAg seroconversion (95% CI 1.11-6.15, p=0.03). Only 7 (16%) patients achieved HBV DNA negativity before achieving HBeAg seroconversion. Probability of achieving HBV DNA negativity did not differ between patients with or without HBeAg seroconversion (p=0.29 by log rank). HBsAg seroconversion was achieved in only 6 (4%) patients and did not differ between patients with WT and PC/ BCP mutants (p=0.99).

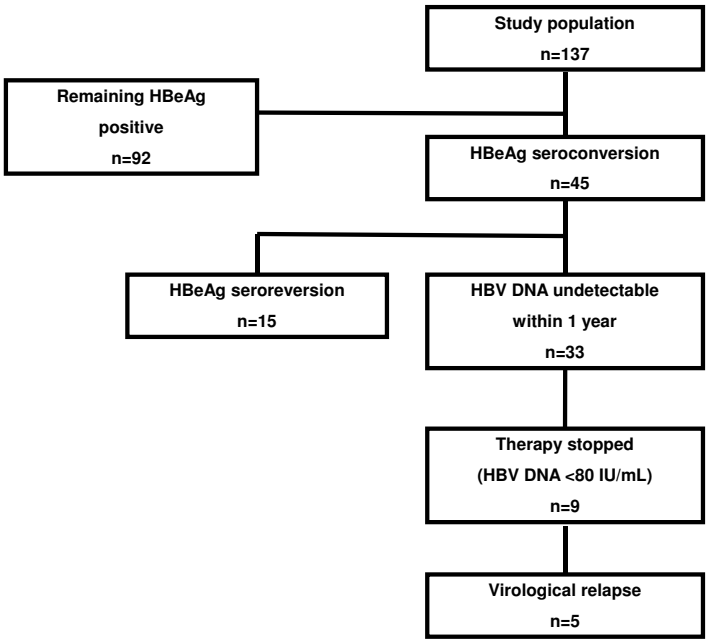
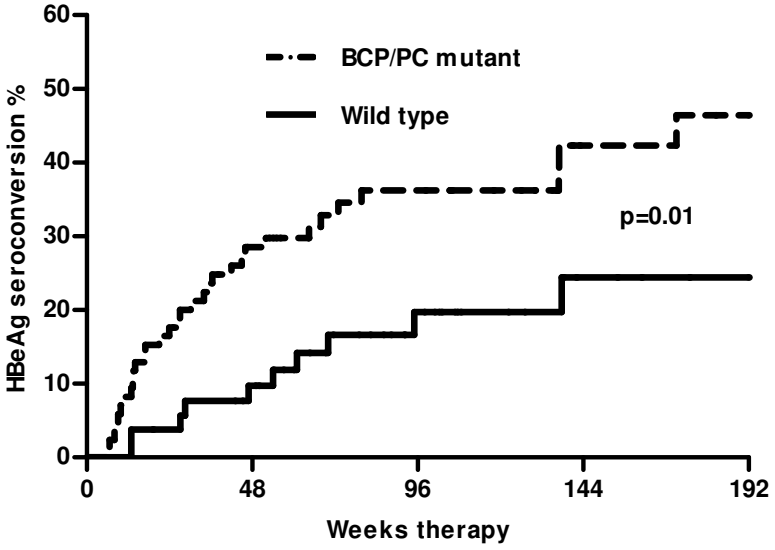


Figure 3 Study flow chart of 137 HBeAg-positive patients.



At risk					
BCP/PC mutant	84	56	36	16	12
Wild type	53	44	26	14	11

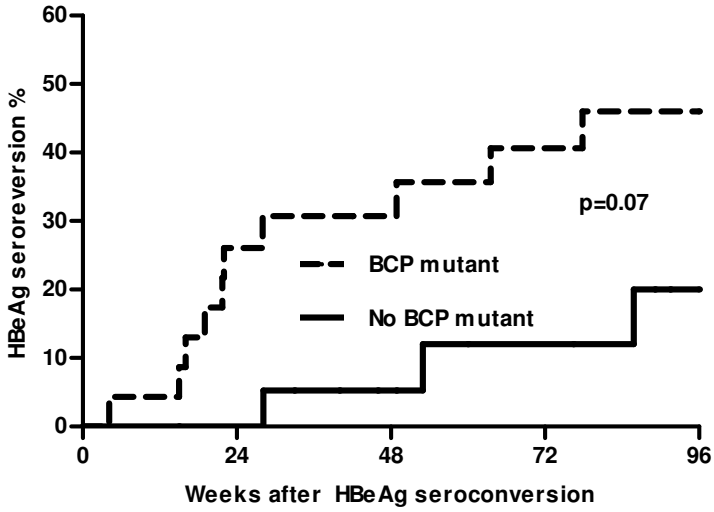
Figure 4 Cumulative probability of achieving HBeAg seroconversion during NA therapy up to 192 weeks of continuous therapy. Comparison between patients with WT virus and patients with detectable PC/BCP mutants at baseline.

Follow-up after HBeAg seroconversion

After achieving HBeAg seroconversion, 15 of 45 (33%) patients had HBeAg seroreversion (HBeAg reappearance confirmed in a consecutive sample). In 11 of these 15 (73%) patients HBeAg relapse occurred before cessation of therapy. HBeAg seroreversion tended to occur more frequently in patients with BCP mutants (HR 2.69, 95% CI 0.86-8.49, p=0.07, figure 5). Eight of these 11patients (73%) remained HBeAg-positive during prolonged follow up. Fifteen (33%) patients experienced confirmed anti-HBe relapse after achieving HBeAg seroconversion, but this was not related to the presence of mutants.

A total of 33 (73%) patients (17 LAM, 6 ADV, 9 ETV and 1 TDF) achieved virological remission (HBV DNA <80 IU) within one year after HBeAg seroconversion. After exclusion of 7 patients with documented genotypic resistance to their current NA regimen (6 LAM and 1 ADV), the presence of PC mutants at baseline remained associated with a lower probability of achieving virological remission within one year after HBeAg seroconversion compared with patients without PC mutants (73% versus 96%, p=0.07). Importantly, type of therapy was not a significant predictor for achieving this endpoint (p=0.39).

NA therapy was stopped after at least 6 (median 18, IQR 10-48) months of consolidation therapy in 9 patients (7 LAM, 1 ETV and 1 ADV) who achieved HBeAg-seroconversion



At risk					
BCP mutant	24	17	14	11	10
No BCP mutant	21	18	15	12	8

Figure 5 Comparison between patients with BCP mutants and patients without BCP mutants at baseline for confirmed reappearance of HBeAg after achieving HBeAg seroconversion (n=45).

and HBV DNA <80 IU/mL. Two of these 9 (22%) patients had WT virus at baseline. However, after cessation of therapy within this group, 5 (56%) patients had virological relapse (confirmed HBV DNA >2000 IU/mL or restart of NA therapy) after a median follow up of 25 (15-157) weeks. Probability of virological relapse was not significantly influenced by the presence of PC and/or BCP mutants (p=0.63 by log rank), by type of therapy (p=0.55 by log rank) or by length of consolidation therapy (p=0.43 by log rank).

DISCUSSION

This study shows that the presence of PC/BCP mutants in HBeAg-positive patients is HBV genotype dependent and associated with a higher rate of HBeAg-seroconversion during NA therapy. However, after HBeAg seroconversion there was a trend towards a higher rate of HBeAg relapse in patients with BCP mutants and a lower rate of virological remission in patients with PC mutants.

HBeAg seroconversion during the natural course of CHB is associated with disease remission. However, it has become increasingly clear that a substantial proportion of patients develops active HBeAg-negative CHB after HBeAg seroconversion and thus remains at risk for clinical progression of liver disease.[5,18-19] Current data indicate a possible role for mutations within the PC or BCP region for developing active HBeAg-negative CHB. Mutations in both of these regions are associated with higher levels of viral replication after achieving HBeAg seroconversion.[14,20-21] In this study we showed that these mutants are already detectable in a considerable number of HBeAg-positive patients at initiation of therapy, particularly in non-genotype A patients.

In addition, we showed that the presence of these mutants is predictive for achieving HBeAg seroconversion during NA-therapy. This is supported by previous studies showing that a PC mutant at position 1896 results in a stopcodon and thus cannot produce HBeAg and that BCP mutants show a diminished precore mRNA and HBeAg production.[22-23] Despite this higher rate of serological response, patients with BCP mutants have a higher likelihood of HBeAg seroreversion, and those with PC mutants of maintaining high serum HBV DNA levels.

BCP mutants are still able to produce HBeAg at a lower level and it was suggested that an accumulation of additional mutations could cause relapse of HBV DNA.[15,21] Further evidence that achieving HBeAg seroconversion in these patients does not confer real immune control over the virus is supported by our finding that they have a low probability of achieving HBsAg seroconversion. These findings are important because stopping of NA therapy is still a major point of discussion in current HBV treatment guidelines.[7,24]

Possible limitations of our study are the retrospective design and the heterogeneity of the cohort. However, the rate of HBeAg seroconversion does not vary across the different NA, and the current design enabled us to include a substantial number of HBeAg positive patients with long-term follow up.[25-26] Furthermore, the INNO-LiPA assay can only detect mutations at positions 1896 (PC), 1762 and 1764 (BCP), which are the most established mutated positions.[13] However, this assay is more sensitive than conventional sequencing, and can thus detect mutant virus at lower levels.[16]

In conclusion, the presence of PC and/or BCP mutants in HBeAg-positive patients was HBV genotype dependent, and independently associated with a higher probability of achieving HBeAg seroconversion during long-term NA therapy. However, after HBeAg-seroconversion patients with BCP mutants frequently have serological relapse while patients with PC mutants often do not achieve a complete virological response. HBsAg seroconversion was rare and not affected by the presence of PC and/or BCP mutants.

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Serum levels of interferon gamma-inducible protein-10 and response to peginterferon therapy in HBeAg-positive chronic hepatitis B

4

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ABSTRACT

Background & Aims

Serum levels of interferon-gamma inducible protein 10 (IP-10) are a marker for immune activity, and may predict response to peginterferon (PEG-IFN) therapy in chronic hepatitis B.

Methods

IP-10 was measured at baseline and on-treatment week 12 in 210 HBeAg-positive patients treated with PEG-IFN for 52 weeks. Response to treatment was assessed at 6 months post-treatment and defined as HBeAg loss, combined response (HBeAg loss with HBV DNA <10,000c/mL) or HBsAg loss.

Results

Median baseline IP-10 levels were 158.2 pg/mL. Higher baseline IP-10 was associated with more HBV DNA, HBeAg and HBsAg decline from week 4 onwards, and IP-10 was higher in patients who achieved HBeAg loss (p=0.001) and combined response (p=0.052). A combination of high IP-10 (>150 pg/mL) with absence of precore (PC) and core promoter (BCP) mutants strongly predicted combined response and HBsAg loss: 48% of patients with high IP-10 and no detectable mutants achieved a combined response (p<0.001). IP-10 decline from baseline to week 12 was very limited, but more pronounced in patients who achieved HBeAg loss (0.05 log pg/mL, versus an increase of 0.05 in patients without HBeAg loss, p=0.04).

Conclusions

Higher pre-treatment IP-10 levels are associated with an increased probability of HBeAg loss after PEG-IFN therapy. A combination of high baseline IP-10 and absence of PC and BCP mutants identified patients with the highest probability of combined response and HBsAg loss.

INTRODUCTION

Peginterferon (PEG-IFN) is a first-line treatment option for chronic hepatitis B (CHB), because a finite treatment course may result in a sustained response in about 25% of patients.[1-3] In HBeAg-positive patients, HBV genotype, patient age, low baseline HBV DNA and high baseline ALT are independent predictors of response to PEG-IFN therapy.[4] Another recent study suggests that host *IL28B* genotype may also influence the probability of serological response to PEG-IFN,[5] while absence of precore (PC) and basal core promoter (BCP) mutants may predict virological response after HBeAg clearance.[6] The association of both high ALT and *IL28B* genotype with response to PEG-IFN suggests that successful induction of an immune response with PEG-IFN depends upon a susceptible host in combination with an active immune response, and biomarkers of immune activity may therefore predict response to treatment.

The interferon-gamma inducible protein 10 (IP-10), also known as chemokine C-X-C motif ligand (CXCL-)10, targets the CXC3 receptor, attracts T-lymphocytes and influences T-cell as well as natural killer cell adhesion.[7-9] Therefore, serum levels of IP-10 may be a marker for immune activity.[10] Pre-treatment IP-10 levels appear to predict response to PEG-IFN therapy in chronic hepatitis C patients,[10-13] independent of other known predictors, such as viral load, HCV genotype and stage of liver disease.[10,12,13] Moreover, recent studies have shown that quantification of IP-10 may add substantially to *IL28B* genotyping when aiming to predict a sustained response in hepatitis C patients, possibly through an association with interferon stimulated gene expression.[14-16]

Although the precise role of IP-10 in CHB remains unclear, one previous study showed that IP-10 kinetics are associated with the occurrence of flares in CHB patients. These findings suggest that serum levels of IP-10 could reflect the immune activity of patients, and consequently predict response to PEG-IFN therapy.[17]

The aim of the current study was therefore to investigate the relationship between serum levels of IP-10 and response to PEG-IFN in HBeAg-positive CHB patients.

PATIENTS AND METHODS

Patients

In this study, serum levels of IP-10 were measured before treatment initiation and at week 12 of treatment in 210 HBeAg-positive CHB patients treated with PEG-IFN alfa-2b within an investigator-initiated multicenter randomized trial.[1,18,19] The inclusion and exclusion criteria for this study have previously been described elsewhere.[1] In summary, patients were eligible if they had been HBsAg positive for at least 6 months before randomization, were HBeAg positive, had elevated serum alanine

aminotransferase (ALT) levels of >2, but <10 times the upper limit of normal (ULN), and had a serum HBV DNA concentration of more than 100,000 copies/mL. Patients were treated with PEG-IFN alfa-2b 100 µg weekly (PegIntron, Schering-Plough, Kenilworth, NJ, USA) in combination with placebo or lamivudine (LAM) 100 mg (Zeffix, GlaxoSmithKline, Greenford, UK) daily for 52 weeks. Inclusion criteria for the present analysis were completion of the 26-week post-treatment follow-up phase of the main study, data on PC / BCP mutants at baseline, and available serum for IP-10 assessment at baseline. Of the 266 patients in the initial study, 210 fulfilled these criteria.
The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. All patients gave written informed consent according to standards of the local ethics committees.

Laboratory measurements

Serum IP-10 was assessed at baseline and at week 12 of treatment using a commercially available ELI+SA kit (Alta Analytical Laboratory, San Diego, USA) in samples that were stored at -80° Celcius since the original studies. The presence of PC and BCP mutants was assessed using the INNO-LiPA HBV PreCore assay (Innogenetics, Ghent, Belgium). This very sensitive line probe assay allows for easy detection of PC (at nucleotide position G1896) and BCP (at nucleotide positions A1762 and G1764) mutants.[20] Patients were classified as wildtype (*WT*, only WT virus detectable), or as *non-WT* (when PC, BCP or both mutants were detected). Serum HBV DNA, HBeAg and HBsAg were quantified in samples taken at baseline, during the treatment period and at 6 months post-treatment. HBV DNA was measured using an in-house developed TaqMan polymerase chain reaction (PCR) assay (lower limit of quantification 400 copies/mL).[21] HBsAg was measured using the Abbott ARCHITECT HBsAg assay (Abbott laboratories; range 0.05 - 250 IU/mL) and HBeAg with the Roche ELECSYS HBeAg assay using a quantitative protocol (Roche Diagnostics, range 0.2 – 100 IU/ml).

Statistical analysis

Response was assessed at 6 months post-treatment (week 78) and was defined as either HBeAg loss, HBeAg loss with HBV DNA <10,000 copies/mL (combined response) or HBsAg loss. Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.2 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

Role of the funding source

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Research (SLO) in Rotterdam, the Netherlands. The funding source did not have influence on study design, data collection, analysis and interpretation of the data, writing of the report nor the decision to submit for publication.

RESULTS

Patient characteristics

The characteristics of the enrolled patients are shown in table 1. Overall, 75 (36%) cleared HBeAg, 39 (19%) achieved a combined response and 17 (8%) cleared HBsAg.

Table 1: Characteristics of the study cohort

Characteristics	
Demography	
Mean (SD) age, years	33.7 (12)
Male	164 (78%)
Previous IFN therapy	38 (18%)
PEG-IFN Monotherapy	102 (49%)
Race	
Caucasian	153 (73%)
Asian	40 (19%)
Other	17 (8%)
Laboratory results	
Mean (SD) ALT*	4.3 (3.0)
Mean (SD) HBV DNA, log c/mL	9.1 (0.89)
Mean (SD) HBsAg, log IU/mL	4.4 (0.60)
Mean (SD) HBeAg, log IU/mL	2.5 (0.70)
Median (range) IP-10, pg/mL	158.2 (6.6-1500)
HBV Genotype	
A	73 (35%)
B	19 (9%)
C	29 (14%)
D	82 (39%)
Other/mixed	7 (3%)
INNO-LiPA result	
Wildtype	76 (36%)
Precore	54 (26%)
Basal core promoter	45 (21%)
Precore and basal core	35 (17%)

*Multiples of upper limit of the normal range

Since combination treatment with lamivudine did not influence response rates[1], data from the monotherapy and combination arms were pooled for the current analysis. Treatment allocation was controlled for in multivariate analyses whenever applicable. Median baseline level of IP-10 was 158.2 pg/mL (range: 6.6 – 1500 pg/mL). IP-10 levels were logarithmically transformed for further analysis, and also divided into quartiles: quartile 1 (<2.02 log pg/mL), quartile 2 (2.02 – 2.20 log pg/mL), quartile 3 (2.20 – 2.42 log pg/mL) and quartile 4 (>2.42 log pg/mL).

Relationship between IP-10 levels and baseline characteristics

Baseline IP-10 levels did not significantly differ across the HBV genotypes A through D, across patients with different ethnicities, nor among patients with only WT virus versus those with detectable PC and/or BCP mutants. Baseline IP-10 level did not correlate with baseline HBV DNA, HBeAg, or HBsAg levels, but was significantly associated with patient age ($r=0.23$, $p=0.001$) and correlated strongly with baseline ALT ($r=0.45$, $p<0.001$).

Association of baseline IP-10 with on-treatment decline of HBV DNA, HBeAg and HBsAg

Higher baseline IP-10 level was associated with more HBV DNA ($p=0.001$), HBeAg ($p<0.001$) and HBsAg decline ($p=0.028$) at 6 months after PEG-IFN discontinuation (week 78). Figure 1A-C shows the on-treatment declines in HBV DNA, HBeAg and HBsAg stratified by a baseline IP-10 level of 150 pg/mL (~median). Importantly, a baseline IP-10 level >150 pg/mL independently predicted HBsAg decline at week 78 when adjusted for HBV genotype, presence of only WT virus and baseline HBsAg. Adjusted HBsAg decline for patients with an IP-10 >150 pg/mL was 1.38 log IU/mL, compared to 0.89 for those with an IP-10 <150 pg/mL ($p=0.034$). In similar models, baseline IP-10 level > 150 pg/mL was associated with more HBeAg decline (1.35 vs. 0.83 log IU/mL, $p=0.002$) and HBV DNA decline (3.00 vs. 1.96 log c/mL, $p=0.002$). Combination therapy did not predict HBsAg, HBeAg or HBV DNA decline at week 78 in these models ($p\geq0.370$). Importantly, the association between high IP-10 level at baseline (>150 pg/mL) and more pronounced on-treatment decline was apparent as soon as week 4 of treatment for HBsAg ($p<0.001$), HBeAg ($p=0.002$) and HBV DNA ($p<0.001$), when adjusting for HBV genotype, presence of only WT virus, baseline level and combination therapy.

Baseline IP-10 levels and response at 6 months post-treatment

Baseline IP-10 level was higher in patients who cleared HBeAg by week 78 when compared to those who did not (2.34 vs. 2.17 pg/mL, $p=0.001$), as was the case for patients who achieved a combined response versus those who did not (2.32 vs. 2.21

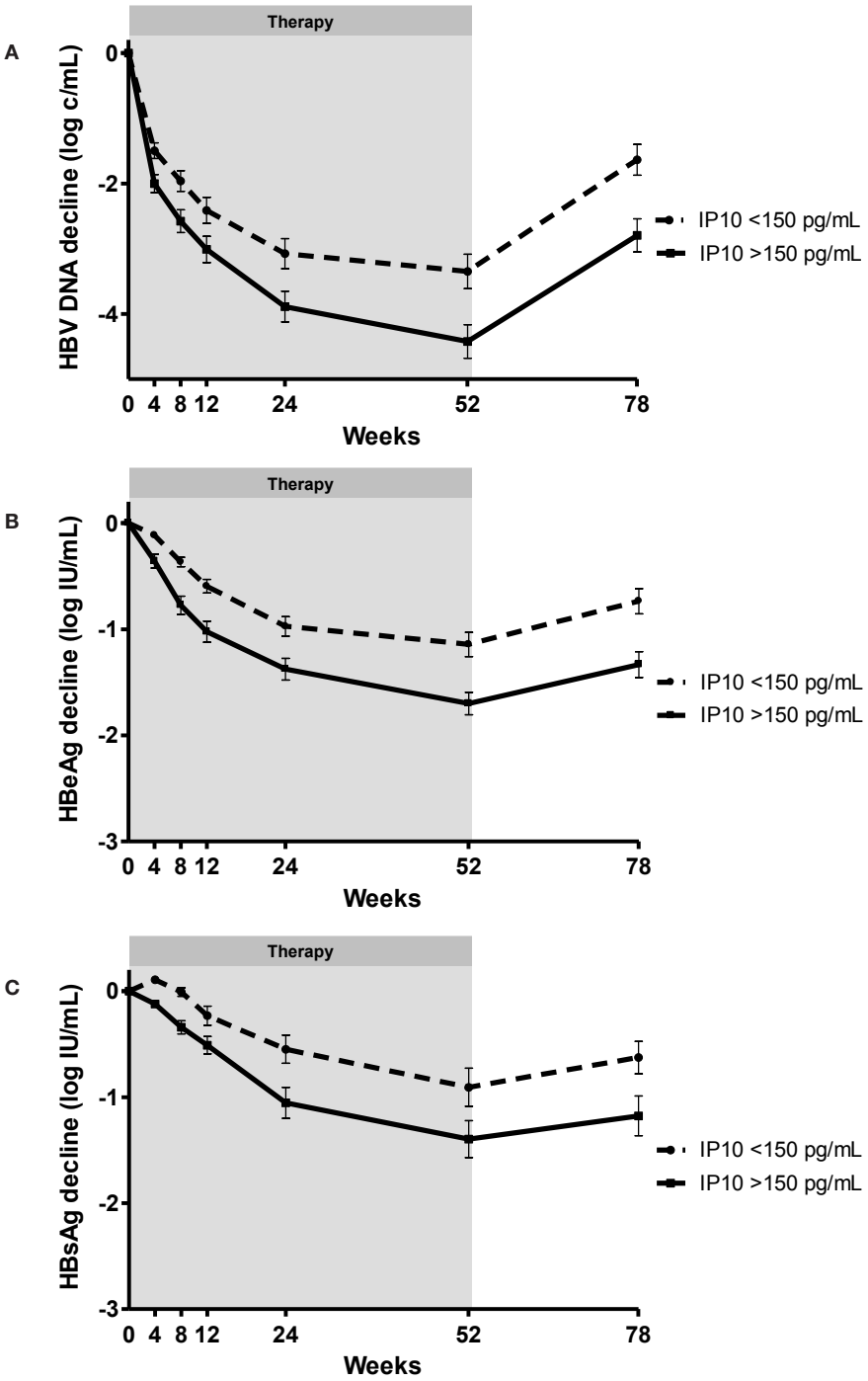

Figure 1. Relationship between baseline IP-10 level and on-treatment viral decline. Decline of serum HBV DNA (A), HBeAg (B) and HBsAg (C) during treatment by baseline IP-10 level (n=210).

log pg/mL, p=0.052). The association between baseline IP-10 level (in quartiles) and probability of response at 6 months post-treatment is shown in figure 2. In multivariate analysis, IP-10 levels at baseline were significantly associated with the occurrence of HBeAg clearance (adjusted OR: 3.60, 95% CI: 1.15 – 11.22, p=0.024, table 2) when adjusting for HBV genotype, presence of only WT virus, baseline age, HBV DNA and ALT and previous IFN treatment failure. Of note, presence of PC and/or BCP mutants was not an independent predictor of HBeAg loss after PEG-IFN therapy, nor was combination therapy. Interestingly, serum IP-10 at baseline was not significantly associated with the occurrence of a combined response (adjusted OR: 2.48, 95% CI: 0.59 – 10.48, p=0.21, table 2).

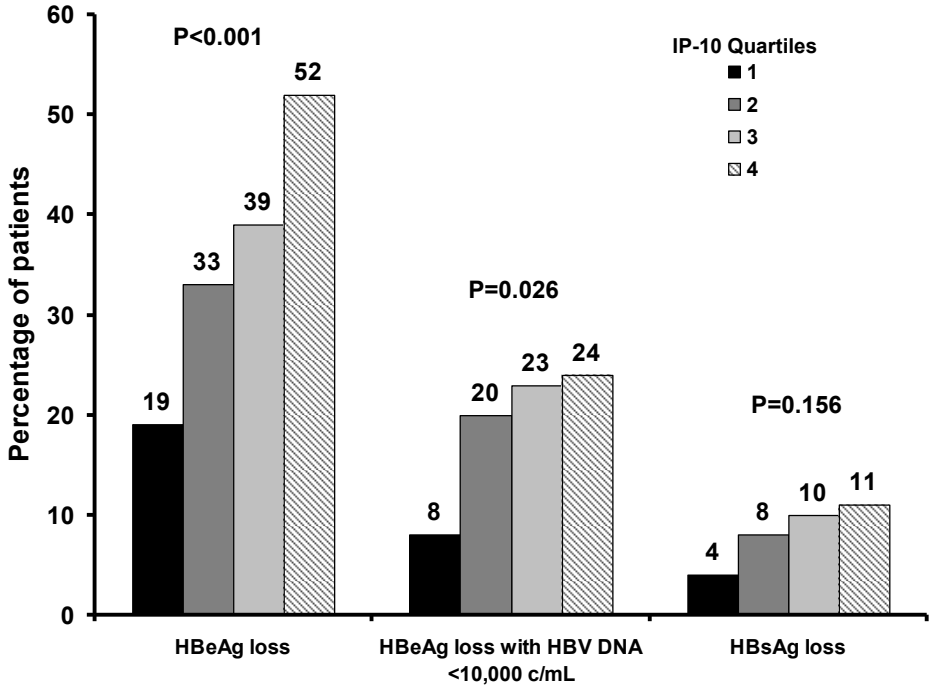

Figure 2. Relationship between baseline IP-10 and response to treatment in the HBeAg-positive population in the overall cohort

A combination of IP-10 and presence of only WT virus identifies patients with a high likelihood of response

Since baseline levels of IP-10 was associated with HBeAg loss, but not combined response, whereas presence of only WT virus was previously shown to be associated with achievement of low HBV DNA levels after HBeAg clearance[6], we explored the interplay between these two variables. In a model for combined response, an

Table 2. Logistic regression model of probability of response to peginterferon in HBeAg-positive patients

HBeAg loss week 78			Combined Response week 78		
Variable	**OR (95% CI)**	**p**	**Variable**	**OR (95% CI)**	**p**
IP-10*	3.60 (1.15 – 11.2)	0.024	IP-10*	2.48 (0.59 – 10.5)	0.209
Wildtype	1.11 (0.50 – 2.46)	0.799	Wildtype	3.45 (1.30 – 9.18)	0.011
HBV Genotype		0.001	HBV Genotype		0.048
A	Reference		A	Reference	
B	0.62 (0.18 – 2.13)		B	0.52 (0.12 – 2.19)	
C	0.10 (0.03 – 0.37)		C	0.11 (0.02 – 0.61)	
D	0.45 (0.18 – 1.14)		D	0.33 (0.10 – 1.12)	
ALT#	1.11 (0.99 – 1.25)	0.081	ALT#	1.06 (0.90 – 1.25)	0.528
Age	1.02 (0.99 – 1.05)	0.280	Age	1.04 (1.00 – 1.07)	0.036
HBV DNA**	0.58 (0.38 – 0.88)	0.010	HBV DNA**	0.55 (0.32 – 0.94)	0.031
No previous IFN	3.74 (1.50 – 9.32)	0.003	No previous IFN	5.07 (1.49 – 17.3)	0.004

Combined response was defined as HBeAg loss with HBV DNA <10,000 c/mL at 6 months post-treatment. *IP-10 in log pg/mL, **HBV DNA in log copies/mL, #ALT in x ULN.

interaction term between WT virus and baseline IP-10 level was highly significant (p=0.002), indicating that the association of IP-10 levels with response is not the same for patients with WT virus compared to those with detectable mutants at baseline. A similar interaction was also found for WT virus and baseline ALT (p=0.030). Such an interaction was not found when HBeAg loss was considered (p=0.15 for interaction of IP-10 and WT, p=1.0 for ALT and WT). Figure 3 shows the estimated probability of combined response as predicted by the prediction model shown in table 2, with addition of an interaction term of WT with IP-10. The probability of response for patients with only WT virus strongly improved with increasing IP-10 level, and similar findings were obtained with ALT. In contrast, patients with detectable PC and/or BCP mutants did not benefit from higher IP-10 or ALT levels. A combination of baseline IP-10 >150 pg/mL and absence of PC and BCP mutants could identify patients with a very high likelihood of response (figure 4). Furthermore, it can also be inferred from figure 4 that high IP-10 level at baseline predisposes to HBeAg clearance after PEG-IFN therapy, but that this did not translate to increased combined response rates or HBsAg loss if PC and/or BCP mutants were present.

On-treatment IP-10 and response to treatment

IP-10 levels remained stable from baseline to week 12; a minimal non-significant decline was observed of 0.015 log pg/mL (p=0.52 compared to baseline). IP-10 decline was more pronounced in patients who achieved HBeAg loss (0.05 log pg/mL decline, versus an increase of 0.05 in patients without HBeAg loss, p=0.04), and

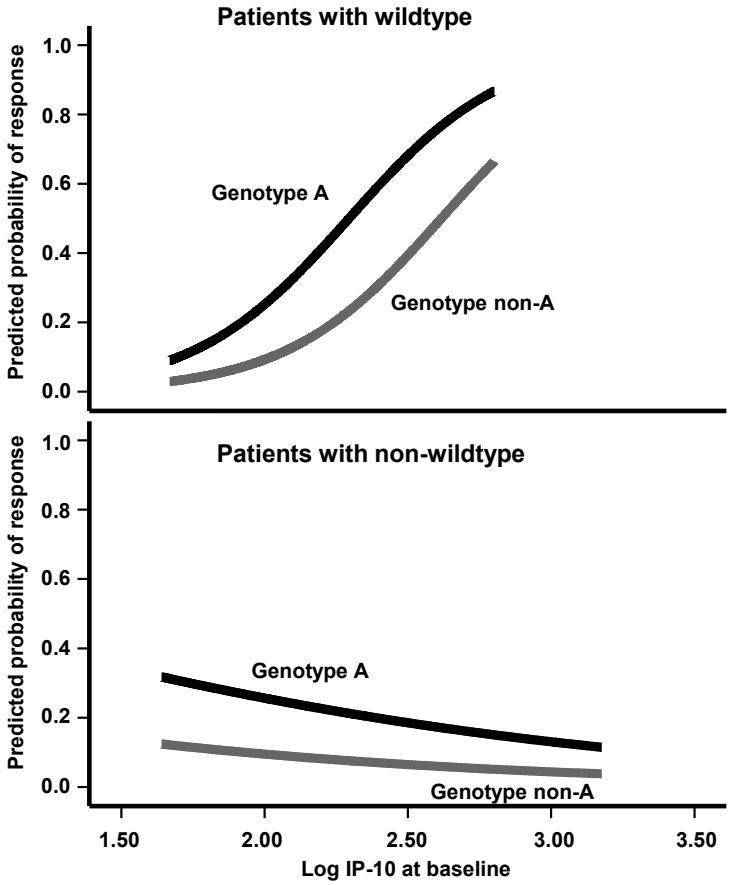

Figure 3. Estimated probability of combined response (HBeAg loss and HBV DNA <10,000 c/mL) stratified by presence of precore and/or core promoter mutants

the proportion of patients who achieved a decline of IP-10 from baseline was higher among patients with HBeAg loss (59 versus 42%, p=0.024) and combined responders (60 versus 45%, p=0.11). Patients with an IP-10 decrease at week 12 achieved more HBV DNA decline (2.55 versus 1.85 log c/mL, p=0.06), HBeAg decline (1.24 versus 0.84 log IU/mL, p=0.029) but not HBsAg decline (1.04 versus 0.75, p=0.26).

DISCUSSION

This is the first study to describe the association of IP-10 level and response to PEG-IFN therapy in CHB. In our study, higher baseline level of IP-10 strongly predicted HBeAg loss after PEG-IFN therapy, and a combination of high IP-10 and presence of

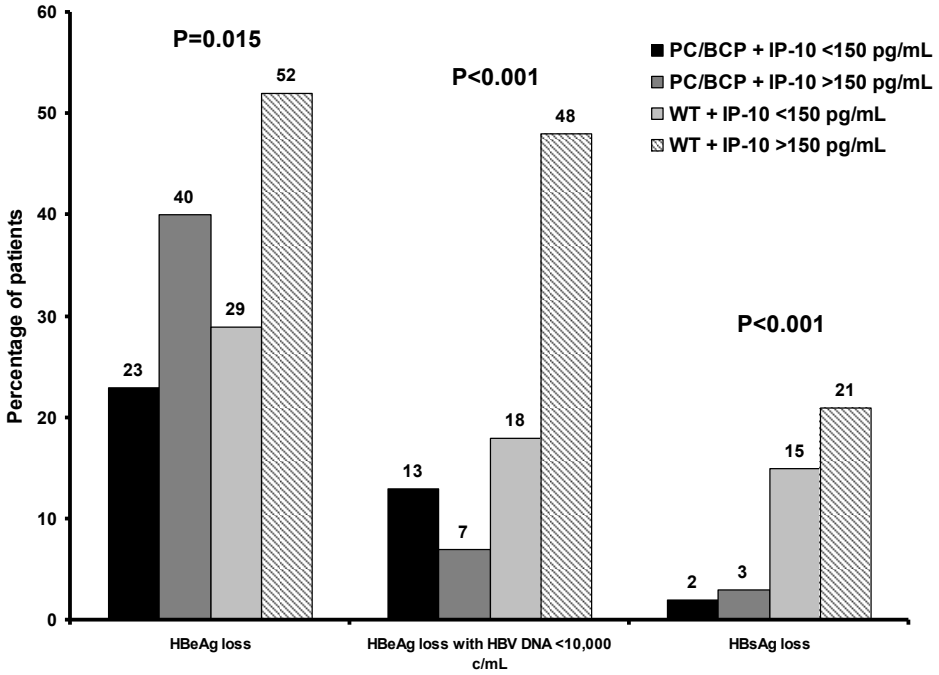

Figure 4. The probability of combined response according to a baseline IP-10 in combination with presence or absence of precore and/or core promoter mutants.

only WT virus identified patients with a high likelihood of combined serological and virological response.

PEG-IFN is a valuable treatment option for CHB, for it is the only agent that can be expected to induce a sustained off-treatment response after a finite treatment course.[22] However, the limited response rates observed in the general CHB patient population necessitate careful selection of patients.[23] Our group recently published a baseline prediction model that can help clinicians identify HBeAg-positive patients with a high likelihood of response[4], and extensions with host and viral factors have been proposed.[5,6,24] The current study shows that response to PEG-IFN in HBeAg-positive patients also depends upon pre-treatment serum level of IP-10. Importantly, the association of IP-10 with response to treatment is already apparent from week 4 of therapy, as shown by the more pronounced HBV DNA, HBeAg and HBsAg decline observed in patients with higher levels of IP-10 at baseline. Given the strong association of IP-10 with ALT, high levels of IP-10 may be a proxy for an active host immune response, resulting in more active liver inflammation.[25] Importantly, blocking the effects of IP-10 may reduce liver damage in mice,[26] further supporting the association of IP-10 with immune activity. These findings are corroborated by recent data from Cornberg et al, showing more pronounced HBsAg decline in nucleo(s)tide analogue

treated patients with high IP-10 levels.[27] The association with immune activity is further strengthened by the observation that IP-10 levels decline during PEG-IFN therapy in patients who achieve a response, mimicking reductions in intrahepatic inflammation previously observed in responders to PEG-IFN.[1] Previous studies have shown that a pre-existing immune response may be a pre-requisite for response to PEG-IFN therapy,[4,28] and the current study shows that serum levels of IP-10 may help identify patients with such favourable characteristics. Nevertheless, the observed IP-10 decline in responders is very limited, restraining the use of IP-10 as an on-treatment predictor of response to PEG-IFN therapy in HBeAg-positive CHB. Furthermore, recent studies in HCV infected patients treated with PEG-IFN have shown that a slight increase in IP-10 levels may be observed after PEG-IFN dosing, which may also reduce the reliability of IP-10 quantification during treatment. The current study therefore does not support the use of IP-10 for on-treatment decision-making in HBeAg-positive CHB patients treated with PEG-IFN.[12]

It should be appreciated that baseline and on-treatment IP-10 levels appear to be mainly associated with the probability of HBeAg clearance after PEG-IFN therapy, and less so with a combined serological and virological response. Persistence of viral replication after HBeAg loss may be accounted for by the presence PC and BCP mutants, which can be detected in a considerable proportion of HBeAg-positive patients.[6] Combining levels of IP-10 and presence of PC and BCP mutants showed that both contribute to the achievement of a combined serological and virological response; patients with both a high baseline level of IP-10 and absent mutants achieved high rates of combined response, whereas patients with a high IP-10 level with detectable mutants progressed to active HBeAg-negative CHB. A similar association was found if a combination of baseline ALT and presence of mutants was explored, further illustrating the importance of active inflammation. Based on these findings, we propose that combined serological and virological response to PEG-IFN in HBeAg-positive CHB requires both a susceptible host (high IP-10, high ALT), as well as a susceptible virus (absence of PC and BCP mutants).

Concluding, high levels of IP-10 predict HBeAg loss, and a combination of high IP-10 and absent PC and BCP mutants predicts combined serological and virological response to PEG-IFN in HBeAg-positive CHB. There appears little use for on-treatment quantification of IP-10 for prediction of response to PEG-IFN in CHB.

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Peginterferon results in higher serological, but not virological, response rates when compared to continuous entecavir

5

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ABSTRACT

Background & Aims

HBeAg and HBsAg clearance are associated with an improved prognosis in chronic hepatitis B patients (CHB). These endpoints are more often achieved with a one year course of peginterferon (PEG-IFN) compared with one year of nucleos(t)ide analogue (NA) therapy. However, prolonged NA therapy may result in comparable serological response rates as with PEG-IFN.

Methods

We compared serological and virological response rates among HBeAg-positive CHB patients treated with long-term continuous entecavir (ETV) (n=91) for a median of 92 (IQR 50-132) weeks or one year of PEG-IFN (n=266) with comparable follow up.

Results

Finite PEG-IFN therapy resulted in significantly higher rates of HBeAg seroconversion (adjusted hazard ratio (HR): 3.16, $p<0.001$) and HBsAg clearance (HR 5.66, $p=0.027$) when compared to prolonged ETV treatment, whereas ETV resulted in higher rates of HBV DNA undetectability (OR 31.14, $p<0.001$) also after adjustment for HBV genotype and other relevant baseline factors.

Conclusions

Our study shows that finite PEG-IFN is associated with a higher probability of serological, but not virological, response for HBeAg-positive chronic hepatitis B patients when compared to prolonged ETV, even after correction for baseline differences.

INTRODUCTION

Prolonged infection with the hepatitis B virus (HBV) may ultimately result in severe liver-related morbidity and mortality, and treatment of CHB is therefore indicated in patients with persistent liver inflammation.[1-4] Hepatitis B e Antigen (HBeAg)-positive CHB is regarded as the earliest stage of a four stage disease continuum.[1,3-4] HBeAg seroconversion (HBeAg clearance with positive anti-HBe) and Hepatitis B surface Antigen (HBsAg) seroclearance are important treatment end-points in HBeAg-positive CHB,[3-4] since they are associated with disease remission, a reduced risk of hepatocellular carcinoma and an improved prognosis.[5-6] These serological endpoints are more often achieved with a one year course of peginterferon (PEG-IFN) when compared to one year of nucleo(s)tide analogues (NA), but prolonged NA therapy may result in serological response rates approximating those achieved with PEG-IFN.[7-8] Current treatment guidelines recommend both treatment modalities as first-line therapy options in HBeAg-positive CHB.[3-4] However, head-to-head comparisons of finite PEG-IFN versus long-term NA therapy have not been performed, and differences in baseline characteristics prohibit direct comparison of previously published study results. We therefore aimed to compare rates of HBeAg seroconversion and HBsAg clearance, as well as HBV DNA undetectability, in HBeAg-positive CHB patients treated with continuous entecavir (ETV) monotherapy or one year of PEG-IFN with subsequent off-treatment follow-up.

METHODS

Patients

A total of 266 HBeAg-positive CHB patients were treated with PEG-IFN alfa-2b (PegIntron, Schering-Plough, Kenilworth, NJ, USA) daily for 52 weeks ± lamivudine (LAM, Zeffix, GlaxoSmithKline, Greenford, UK) for one year.[8] Patients were subsequently followed-up for another 6 months off-treatment and were enrolled in a long-term follow-up study.[9] Key inclusion criteria for this study were: HBsAg positive for at least 6 months before randomization, HBeAg positivity, elevated serum alanine aminotransferase (ALT) levels <10 times the upper limit of normal (ULN), and serum HBV DNA of more than 1.0×10^5 copies/mL. Another 91 consecutive NA-naïve patients were treated with ETV 0.5 mg daily as recommended by current treatment guidelines and were followed-up at the outpatient clinic at least every 3-6 months. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. All patients gave written informed consent according to standards of the local ethics committees.

Laboratory assays

(anti-)HBeAg and HBsAg tests were performed using commercially available ELISA kits. HBV DNA was measured at baseline using real-time Taqman based methods.[8] ALT was measured locally and was expressed as multiples of the upper limit of normal (ULN). HBV genotype was assessed by line-probe assay (Innogenetics, Ghent, Belgium).

Statistical analysis

HBeAg seroconversion (HBeAg negativity with anti-HBe) and HBsAg clearance rates were compared by Kaplan-Meier and Cox-proportional hazard analyses. Rates of HBV DNA undetectability were evaluated at week 78 (i.e. 6 months post-treatment for patients treated with PEG-IFN) and at last follow-up evaluation. The analysis at last follow-up was limited to patients treated with ETV for ≥78 weeks. Follow-up time was calculated from start of treatment, and is expressed as median with the interquartile range (IQR). Follow-up was terminated in patients retreated after PEG-IFN and serological status before retreatment used as outcome parameter. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.2 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

RESULTS

Study Cohort

Treatment outcomes did not differ between patients treated with PEG-IFN ± LAM, and patients were therefore pooled for this analysis.[8-9] Median follow-up was 92 weeks (IQR 78–198) for patients treated with PEG-IFN, and 92 (IQR 50-132) weeks for patients treated with ETV. HBV genotype distributions among patients treated with PEG-IFN were A/B/C/D/other in 34%, 9%, 15%, 39% and 4%, compared to 27%, 8%, 21%, 33% and 11% in the ETV group (p=0.11). The PEG-IFN and ETV groups were also well-balanced with regard to age (34.95 versus 36.93 years, p=0.21), sex (78% male versus 75%, p=0.55) and previous IFN therapy (both 21%, p=0.97). Baseline HBV DNA levels were higher in patients treated with PEG-IFN (9.06 versus 7.98 log copies/mL, p<0.001) as were ALT levels (4.30 versus 3.05 times the upper limit of normal, p=0.004).

HBeAg seroconversion

A total of 114 (32%) patients achieved HBeAg seroconversion in a median of 78 weeks (IQR 52–120). By Kaplan-Meier analysis, the cumulative probability of HBeAg

seroconversion was higher in patients treated with PEG-IFN for one year versus those treated with ETV (p=0.007). PEG-IFN therapy remained an independent determinant of HBeAg seroconversion in a Cox proportional hazard model; the hazard rate (HR) for PEG-IFN versus ETV was 3.16 (95% CI: 1.64 – 6.75, p<0.001), after adjustment for HBV genotype, baseline ALT and baseline HBV DNA (figure 1A).

HBsAg loss

A total of 30 (8%) patients cleared HBsAg in 92 (IQR 78–170) weeks. By Kaplan-Meier analysis, cumulative HBsAg clearance rates were higher in patients receiving PEG-IFN therapy compared to patients treated with ETV (p=0.032). In a Cox proportional hazards model, PEG-IFN therapy was independently associated with HBsAg clearance, with a HR of 5.66 (95% CI: 1.17 – 101.80, p=0.027) after adjustment for HBV genotype, age and previous IFN exposure (figure 1B).

HBV DNA undetectability

At week 78, a total of 69 (21%) patients had undetectable HBV DNA. HBV DNA undetectability was achieved in 77% of ETV treated patients, compared to only 8% of patients treated with PEG-IFN (p<0.001). At last follow-up evaluation, 90 (39%) of patients achieved undetectable HBV DNA, comprising 92% of patients on ETV, and 19% of PEG-IFN treated patients (p<0.001). In a logistic regression model, ETV therapy (OR 31.14, 95% CI: 13.30 – 72.90, p<0.001), HBV genotype A (OR 3.54, 95% CI: 1.58 – 7.95, p=0.002) and log HBV DNA (OR 0.65, 95% CI: 0.48 – 0.89, p=0.007) but not ALT (p=0.74) were independently associated with HBV DNA undetectability at week 78. An interaction term of HBV genotype and therapy was non-significant (p=0.26).

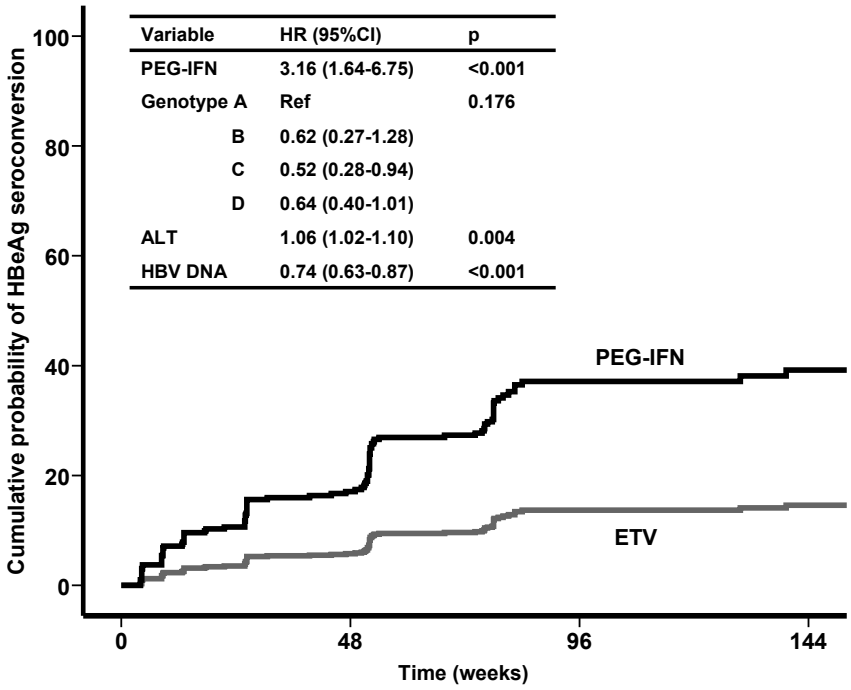
DISCUSSION

This is the first study to compare a finite course of PEG-IFN to prolonged potent NA therapy for serological and virological response rates. We found that PEG-IFN results in higher rates of HBeAg seroconversion and HBsAg seroclearance than continuous ETV therapy, and this difference remained after adjustment for baseline factors. Nevertheless, HBV DNA undetectability was achieved in only a minority of PEG-IFN treated patients, whereas most patients on ETV achieved this endpoint.

PEG-IFN and the NA have distinctly different modes of action in CHB.[10] PEG-IFN is an immunomodulator with limited direct antiviral efficacy that is able to induce a host immune response in a subset of patients.[11] NA competitively inhibit HBV polymerases and thus HBV DNA production. Several studies have shown that currently approved

A

Variable	HR (95%CI)	p
PEG-IFN	3.16 (1.64-6.75)	<0.001
Genotype A	Ref	0.176
B	0.62 (0.27-1.28)	
C	0.52 (0.28-0.94)	
D	0.64 (0.40-1.01)	
ALT	1.06 (1.02-1.10)	0.004
HBV DNA	0.74 (0.63-0.87)	<0.001



B

Variable	HR (95%CI)	p
PEG-IFN	5.66 (1.17-101.80)	0.027
Genotype A	Ref	0.002
B	0.42 (0.07-1.56)	
C	0.10 (0.01-0.48)	
D	0.12 (0.02-0.46)	
Age	1.03 (1.00-1.06)	0.037
Previous IFN	0.29 (0.07-0.83)	0.019

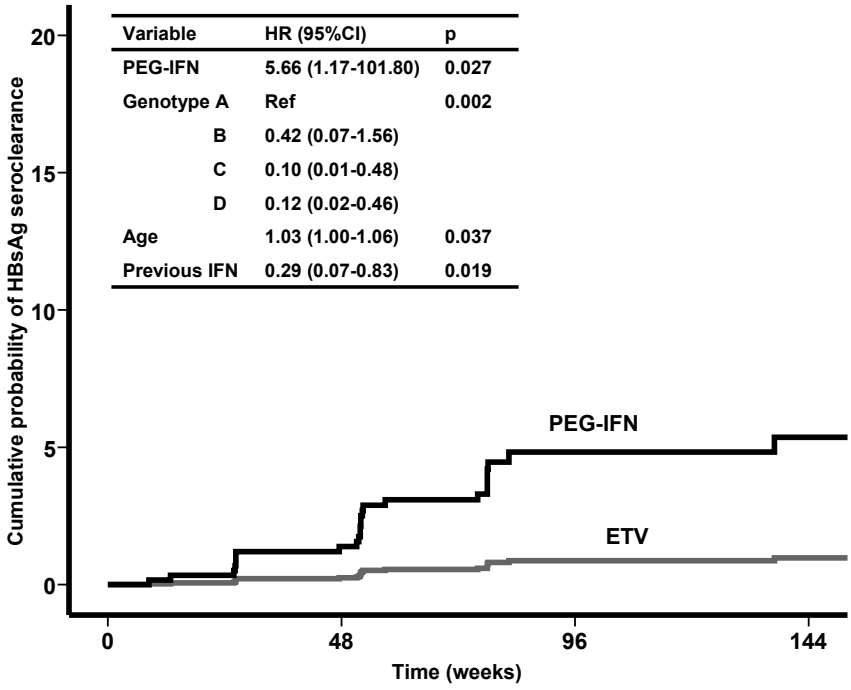


Figure 1: Cox-proportional hazard plots of the probability of HBeAg seroconversion (A) and HBsAg clearance (B) in patients treated with PEG-IFN (± LAM) or ETV.

potent NA ETV and TDF can induce and maintain undetectable HBV DNA levels for prolonged therapy duration with a low risk of viral resistance or complications.[7,12] However, relapse is common after discontinuation of therapy.[13] HBeAg seroconversion has previously been shown to be associated with an improved prognosis [5-6], and is considered a first step towards immune control over HBV. The current study shows that this endpoint is more often achieved with a finite course of PEG-IFN than with prolonged ETV therapy, suggesting that immune control can more often be achieved with PEG-IFN. However, long-term follow-up studies of patients treated with PEG-IFN have revealed that some patients maintain elevated HBV DNA levels after HBeAg seroconversion.[9,14] Similarly, in patients treated with NA, viral rebound is frequently observed when therapy is discontinued after HBeAg seroconversion.[15]

Furthermore, our study also shows that a finite course of PEG-IFN results in superior rates of HBsAg seroclearance, the closest outcome to clinical cure one can hope to achieve in CHB.[3-4] HBsAg clearance is highly durable, confers an excellent long-tem prognosis and is associated with a low probability of HBV reactivation in immune competent patients [5,16]. Although HBsAg clearance is only rarely achieved with currently available agents, particularly in patients treated with NA, we showed that a substantial proportion of patients treated with PEG-IFN may achieve this end-point during treatment and long-term off-treatment follow-up. This is in line with recent studies showing increased decline of HBsAg levels in patients treated with PEG-IFN, particularly in responders, when compared to patients treated with ETV.[17-18]

Nevertheless, serological response is only achieved in a limited subset of patients treated with PEG-IFN, and persistence of HBV DNA after PEG-IFN is a reality in the majority of patients.[9,14] In contrast, nearly all patients on ETV achieved HBV DNA undetectability, and thus disease remission, while on-treatment. Taking into consideration the considerable side-effects of PEG-IFN and the limited response rates, PEG-IFN therapy should be limited to those patients with the highest chances of achieving both serological and virological response, whereas ETV is a powerful treatment option for the vast majority of patients.

Although the current study was not randomized, we conducted a thorough multivariate analysis including all major determinants of serological response.[19] Of note, the HBV genotype distribution was comparable in the two cohorts, and we adjusted for baseline ALT and HBV DNA levels, age, sex, presence of cirrhosis and previous IFN exposure when necessary. We also pooled the results of patients treated with PEG-IFN ± LAM to increase power. Importantly, several independent randomized studies and a meta-analysis have revealed no benefits of combination therapy.[8,13-14,19]

In conclusion, our study shows that a finite course of one year PEG-IFN results in superior rates of HBeAg seroconversion and HBsAg seroclearance, but not HBV DNA undetectability, when compared to prolonged ETV monotherapy.

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Prediction of sustained response to peginterferon alfa-2b for hepatitis B e antigen-positive chronic hepatitis B using on-treatment hepatitis B surface antigen decline

6

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ABSTRACT

Background & Aims

Serum hepatitis B surface antigen (HBsAg) decline during treatment may reflect the immunomodulatory efficacy of peginterferon (PEG-IFN). We aimed to investigate within a large randomised trial whether quantitative HBsAg levels predict response to PEG-IFN in HBeAg-positive chronic hepatitis B patients.

Methods

Serum HBsAg was measured in samples taken at baseline, week 4, 8, 12, 24, 52 and 78 of 221 patients treated with PEG-IFN alfa-2b ± lamivudine for 52 weeks. HBsAg decline was compared between treatment arms and between responders and non-responders. Response was defined as HBeAg loss with HBV DNA <10,000 copies/mL at 26 weeks post-treatment (week 78).

Results

Forty-three of 221 (19%) patients achieved a response. One year of PEG-IFN ± lamivudine resulted in a significant decline in serum HBsAg, which was sustained post-treatment (decline 0.9 log IU/mL at week 78, $P<0.001$). Patients treated with combination therapy experienced a more pronounced on-treatment decline, but relapsed subsequently. Responders experienced a significantly more pronounced decline in serum HBsAg compared to non-responders (decline at week 52: 3.3 versus 0.7 log IU/mL, $P<0.001$). Patients who achieved no decline at week 12 had a 97% probability of non-response through post-treatment follow-up and no chance of HBsAg loss. In a representative subset of 149 patients similar results were found for prediction through long-term (mean 3.0 years) follow-up.

Conclusions

PEG-IFN induces a significant decline in serum HBsAg in HBeAg-positive patients. Patients who experience no decline from baseline at week 12 have little chance of achieving a sustained response and no chance of HBsAg loss and should be advised to discontinue therapy with PEG-IFN.

INTRODUCTION

Chronic hepatitis B (CHB) is a major health problem, affecting more than 350 million people worldwide. Prolonged infection with the hepatitis B virus (HBV) may ultimately result in severe liver-related morbidity and mortality, and treatment of CHB is therefore indicated in patients with persistent liver inflammation.[1-4] The ideal outcome of treatment of CHB would be complete eradication of HBV, but this is only scarcely, if ever, achieved, for HBV covalently closed circular DNA (cccDNA) persists in host hepatocytes.[5] Therefore, the main goal of therapy is to halt the progression of liver inflammation to fibrosis, cirrhosis or hepatocellular carcinoma.[6-7]

Current treatment options for CHB consist of nucleo(s)tide analogues (NA) and (pegylated) interferons (PEG-IFN). Antiviral treatment with NA aims at inhibiting viral polymerase activity,[8] and the most recently approved NA can effectively maintain suppression of HBV DNA levels for prolonged periods of time in the vast majority of patients.[9-11] Nevertheless, PEG-IFN remains an important first-line treatment option for CHB, especially in hepatitis B e antigen (HBeAg)-positive disease, since a long-term off-treatment sustained response can be achieved in about 25% of patients after a finite treatment course[12-14]. Response to IFN-based therapy in these patients is accompanied by high rates of hepatitis B surface antigen (HBsAg) seroconversion,[15] a reduced incidence of hepatocellular carcinoma and prolonged survival.[16-17]

The development of a durable off-treatment response is attributed to the immunomodulatory effect of PEG-IFN,[18] which results in a decrease in intrahepatic cccDNA.[19] CccDNA levels at the end of therapy are indeed predictive of a sustained off-treatment response,[20] but since these can only be assessed invasively the clinical utility is limited. Recent studies report an excellent correlation between decline in intrahepatic cccDNA and serum HBsAg levels in HBeAg-positive patients.[5,21] A decline in serum HBsAg levels may therefore reflect the efficacy of PEG-IFN in decreasing intrahepatic cccDNA and consequently predict a sustained off-treatment response.

The aims of our study were to investigate the effects of one year of PEG-IFN ± lamivudine (LAM) therapy on serum HBsAg levels in HBeAg-positive CHB patients, and to describe the relationship between on-treatment HBsAg decline and a sustained off-treatment response.

METHODS

Patients

In this study serum HBsAg levels were assessed in HBeAg-positive CHB patients who were previously enrolled in an investigator-initiated multicenter randomized controlled

trial and a subsequent long-term follow-up (LTFU) study.[12-13] Patients were eligible for the initial study if they had been HBsAg positive for at least 6 months prior to randomization, were HBeAg positive on two occasions within 8 weeks prior to randomization, had elevated serum alanine aminotransferase (ALT) levels of 2 - 10 times the upper limit of normal (ULN), and had a serum HBV DNA concentration above 1.0 x 10^5 copies/mL. Key exclusion criteria were: antiviral therapy within 6 months prior to randomization, presence of viral co-infections, pre-existing cytopenia or decompensated liver disease. Treatment comprised of PEG-IFN alfa-2b 100 µg weekly (PegIntron, Schering-Plough, Kenilworth, NJ, USA) in combination with placebo or LAM (Zeffix, GlaxoSmithKline, Greenford, UK) 100 mg daily for 52 weeks. To limit the probability of early treatment discontinuation, the dose of PEG-IFN was reduced to 50 µg per week after 32 weeks of treatment. Patients attended the outpatient clinic at least every 4 weeks for routine examinations and laboratory assessments during both the treatment and the post-treatment follow-up phase of the initial study. For the LTFU study, patients were re-evaluated at one additional visit at the local participating center. The mean duration of follow-up was 3 years.[12]

Inclusion criteria for the present analysis were completion of the 26-week follow-up phase of the main study and availability of a baseline serum sample for HBsAg quantification. Of the 266 patients in the initial study, 221 fulfilled these criteria. Of these patients, 149 participated in the associated LTFU study.[12] The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. All patients gave written informed consent according to standards of the local ethics committees.

Laboratory measurements

Serum HBsAg was quantified in samples taken at baseline, during the treatment period (weeks 4, 8, 12, 24 and 52) and during follow-up (week 78) using the ARCHITECT HBsAg assay (Abbott laboratories; range 0.05 - 250 IU/mL).[22] HBV DNA quantification for the initial study was performed with 4-week intervals using an in-house developed TaqMan polymerase chain reaction (PCR) assay (lower limit of quantification 400 copies/mL) based on the EuroHep standard.[23] For the LTFU study, HBV DNA was measured with the Cobas TaqMan HBV assay (Roche Molecular Systems, Branchburg, NJ, USA), with a dynamic range of quantification of 174 - 6.4 x 10^8 copies/mL (30 - 1.1 x 10^8 IU/mL). It has previously been demonstrated that there is an excellent correlation between the two assays.[12] HBeAg was assessed using EIA (AxSYM, Abbott, Abbott Park, IL, USA) or ELISA (DiaSorin SpA, Saluggia, Italy). ALT was measured locally in accordance with standard procedures and is presented as multiples of the upper limit of normal (ULN). HBV genotype was assessed using the INNO-LiPA assay (Innogenetics).

Statistical analysis

For the current study a composite endpoint of HBeAg loss and HBV DNA level <10,000 copies/mL was chosen for definition of response.[24] Patients who were retreated after the initial study were considered non-responders at LTFU. Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. The differences in HBsAg decline between treatment arms and (non-)responders were analysed using repeated measurement models with an unstructured covariance allowing heterogeneity across compared groups. Discrimination, or the ability of HBsAg concentration and decline at various time-points to distinguish patients who will develop a response from those who will not, was quantified by the area under the receiver-operating characteristic curve (AUC). Our aim was to use on-treatment HBsAg levels to identify a stopping rule that would enable a clinician to discontinue patients who had a very low chance of response as early as possible, while maintaining >90% of responders on treatment. The optimal cut-off in HBsAg decline was identified using a grid-search of possible cut-off points at weeks 4, 8, 12, and 24. For each cut-off point the chi-square test was calculated together with the sensitivity and the negative predictive value (NPV). The highest chi-square identified the optimal cut-off point. [25] SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.2 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

RESULTS

Patient characteristics

The characteristics of the 221 patients are shown in table 1 according to assigned treatment regimen. Patients were comparable across both groups with regard to age, race, HBV genotype distribution, baseline prevalence of cirrhosis and ALT and HBV DNA levels. Overall, 43 (19%) patients had a response at week 78, and these patients were distributed equally across the two study arms. Baseline mean serum HBsAg was 4.4 log IU/mL in both treatment groups. Serum HBsAg was positively correlated with HBV DNA ($r = 0.66$, $p<0.01$) and inversely correlated with age ($r = -0.16$, $P = 0.02$) but did not correlate with ALT. Variation was observed in pre-treatment HBsAg levels between genotypes, with the highest baseline levels in genotypes A and D (mean 4.5 log IU/mL for both) and lower levels in genotypes B (mean 4.3 log IU/mL) and C (mean 3.8 IU/mL) ($P<0.001$ for genotype C versus other genotypes with Bonferroni correction).

Table 1: Patient characteristics according to treatment regimen

Characteristics	PEG-IFN and placebo (n=111)	PEG-IFN and lamivudine (n=110)	P
Demography			
Mean (SD) age, years	34 (13)	33 (12)	0.52
Male	90 (81%)	83 (75%)	0.33
BMI	24.2 (3.4)	25.1 (4.5)	0.10
Race			0.31
Caucasian	80 (72%)	80 (73%)	
Asian	25 (23%)	19 (17%)	
Other	6 (5%)	11 (10%)	
Laboratory results			
Mean (SD) ALT*	4.3 (3.0)	4.2 (3.0)	0.75
Mean (SD) HBV DNA, log copies/mL	9.1 (0.8)	9.1 (1.0)	0.87
Mean (SD) HBsAg, log IU/mL	4.4 (0.6)	4.4 (0.7)	0.74
HBV Genotype			0.52
A	40 (36%)	34 (31%)	
B	10 (9.0%)	10 (9.1%)	
C	18 (16%)	14 (13%)	
D	41 (37%)	46 (42%)	
Other/mixed	2 (1.8%)	6 (5.5%)	
Response at week 78			
Response#	20 (18 %)	23 (21%)	0.61
HBV DNA <400 copies/mL	9 (8.1%)	13 (12%)	0.38
HBeAg loss	43 (39%)	41 (37%)	0.89
HBsAg loss	8 (7.3%)	11 (10%)	0.48

*Multiples of upper limit of the normal range
#HBeAg loss and HBV DNA <10,000 copies/mL

On-treatment HBsAg decline according to treatment regimen

Overall, HBsAg levels decreased significantly through 52 weeks of therapy (mean decline 1.2 log IU/mL, P <0.001), and the decrease was sustained after 26 weeks of follow-up (mean decline compared to baseline 0.9 IU/mL, P<0.001). Patterns of HBsAg decline for both treatment groups are depicted in figure 1. Declines were similar in both treatment arms at weeks 4, 8 and 12, but slightly more pronounced in the combination (PEG-IFN + LAM) compared to the monotherapy group (PEG-IFN + placebo) at week 24 (mean decline 1.0 log IU/mL versus 0.6 log IU/mL, P = 0.04) and at week 52 (mean decline 1.46 and 0.87 log IU/mL for combination therapy and monotherapy, respectively, P = 0.04). This difference was not sustained through post-treatment follow-up (mean decline of 0.98 and 0.86 log IU/mL for combination and

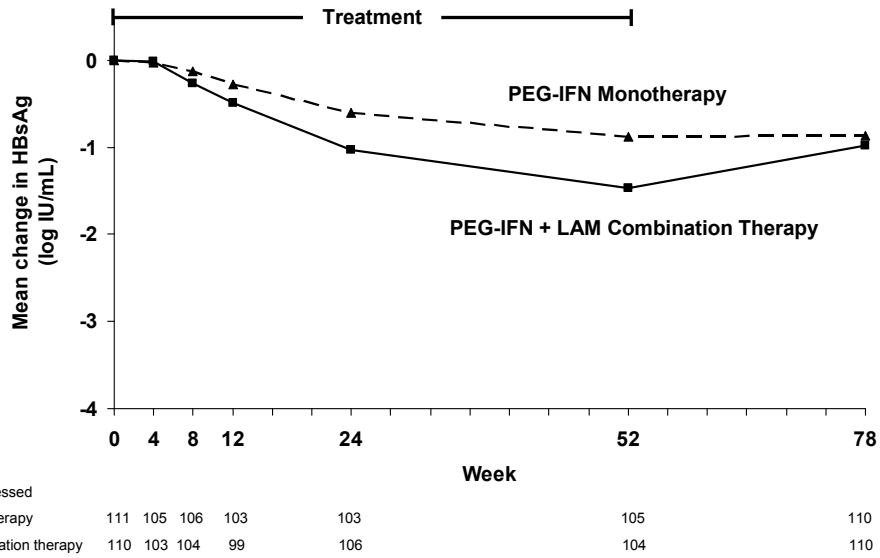

Figure 1: Mean change in serum HBsAg from baseline in patients treated with monotherapy (PEG-IFN + placebo) and combination therapy (PEG-IFN + LAM).

monotherapy at week 78, respectively, P = 0.63). Considering the equal response rates and HBsAg levels at week 78 in the two treatment groups, we analysed the relationship between HBsAg decline and treatment response in all 221 patients.

HBsAg decline according to treatment response at week 78

Baseline mean HBsAg levels were comparable in the 43 patients who achieved a response at week 78 and those who did not; 4.4 versus 4.3 log IU/mL in non-responders and responders, respectively (P = 0.19). Mean HBsAg declines from baseline for responders and non-responders at week 78 are shown in figure 2. Non-responders showed a modest decline through 52 weeks of therapy (0.69 log IU/mL, P <0.001), and relapsed during follow-up (decline from baseline at week 78 was 0.35 log IU/mL, P <0.001 compared to week 52). Mean decline from baseline in responders was 3.3 log IU/mL at week 52 and 3.4 at week 78 (P<0.001 for both when compared to baseline). Responders thus showed a more vigorous decline in HBsAg starting at week 4, and this difference increased through 52 weeks of therapy and was sustained during post-treatment follow-up (P<0.005 for week 4 and P≤0.001 for all other time-points compared to non-responders).

Prediction of response

Since HBsAg decline patterns differed depending on treatment response, we investigated the discriminatory capabilities of HBsAg decline at weeks 4, 8, 12 and 24 for

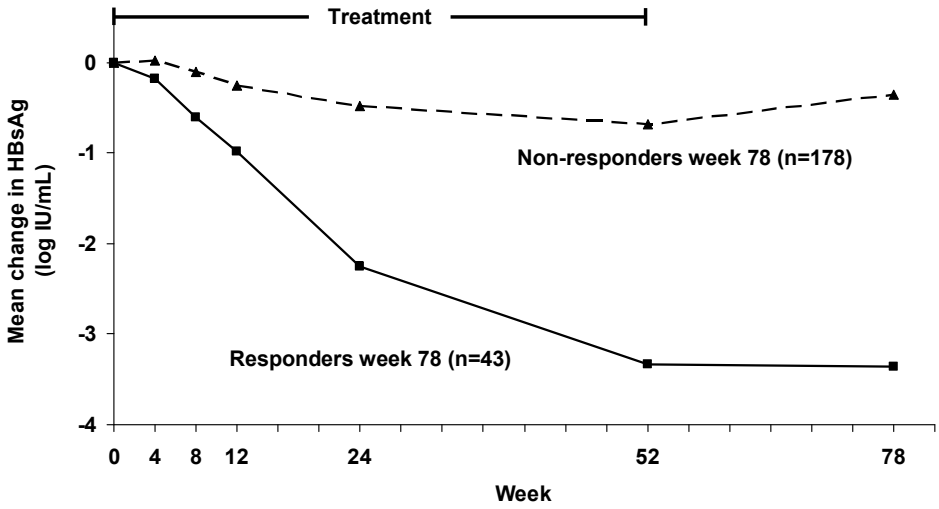

Figure 2: Mean change in serum HBsAg from baseline in patients who achieved a response (HBeAg loss and HBV DNA <10,000 copies/mL) at week 78 and those who did not.

predicting response. Using ROC analysis, areas under the curve (AUC) were 0.70, 0.76, 0.75 and 0.78 for decline at week 4, 8, 12 and 24, respectively, for predicting response at week 78. We also investigated the discriminatory values of absolute HBsAg levels (in log IU/mL) and HBV DNA decline, but these proved inferior to HBsAg declines.

Next, we proceeded to investigate the optimal cut-off point, according to our preset criteria, in HBsAg decline at week 4, 8, 12 and 24 for prediction of response. A cut-off of any decline in serum HBsAg level from baseline (i.e. the HBsAg level on-treatment was lower than the level measured at baseline: $\log(HBsAg_{on\text{-}treatment}) - \log(HBsAg_{baseline}) < 0$) proved superior. Subsequently, prediction of response at weeks 12 and 24 was superior to weeks 4 and 8 since it allowed for more patients to be stopped, while maintaining >90% of responders on-treatment (Figure 3). In addition, week 12 was superior to week 24 because it allowed for earlier discontinuation of therapy, while maintaining high predictive values for both response and HBsAg loss (Table 2).

At week 12, 69% of patients achieved a decline in HBsAg when compared to baseline. Of the 31% who did not only 3% achieved a response at week 78. Consequently, the negative predictive value (NPV) of the presence of any decline in HBsAg at week 12 is 97% for prediction of response at week 78. Comparable NPVs were found for prediction of response at week 24 (Table 2, figure 4). Of those patients that achieved a decline at week 12, 25% achieved a response at week 78, and 12% achieved HBsAg loss.

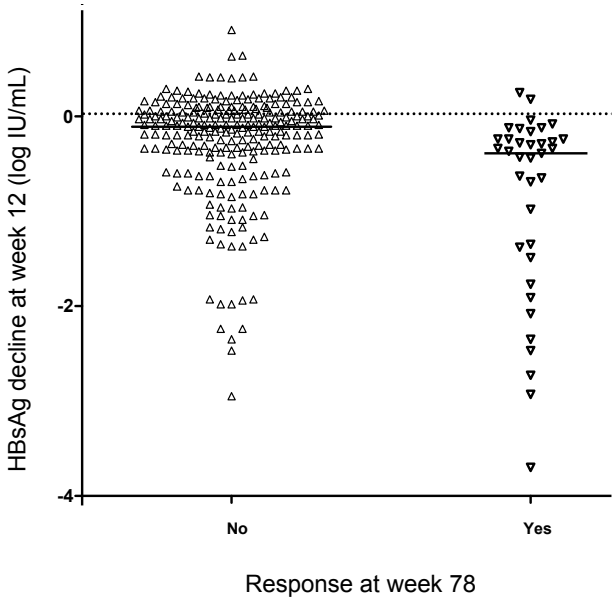

Figure 3: Individual HBsAg declines at week 12, stratified by response at week 78. All but two patients who achieved a response at week 78 experienced a decline in HBsAg from baseline to week 12. Lines represent median decline.

Table 2: Positive and negative predictive values for any HBsAg decline at week 12 and 24 for prediction of response and HBsAg loss at week 78

		Response week 78*				HBsAg loss week 78			
		No	**Yes**	**PPV**	**NPV**	**No**	**Yes**	**PPV**	**NPV**
Any decline week 12	Yes	104	35	25%	–	122	17	12%	–
	No	61	2	–	97%	63	0	–	100%
Any decline week 24	Yes	122	36	23%	–	140	18	11%	–
	No	47	4	–	92%	51	0	–	100%

PPV, positive predictive value; NPV negative predictive value;
* Response is defined as HBeAg loss and HBV DNA < 10,000 copies/mL

Prediction of response through LTFU

Of the 149 patients with LTFU data available, 36 (24%) had a response at LTFU. Similar decline patterns were observed for responders and non-responders at LTFU when compared to (non-)responders at week 78; responders showed a steeper on-treatment decline. Declines were 0.53 log IU/mL versus 2.76 log IU/mL at week 52, for (non-)responders, respectively (P=0.007 for weeks 4 and 8, $P \leq 0.002$ for all other time-points), and the difference was sustained post-treatment. Furthermore, of the patients who did not achieve a decline through 12 weeks of therapy, only 5% achieved a sustained response through LTFU and none lost HBsAg (table 3).

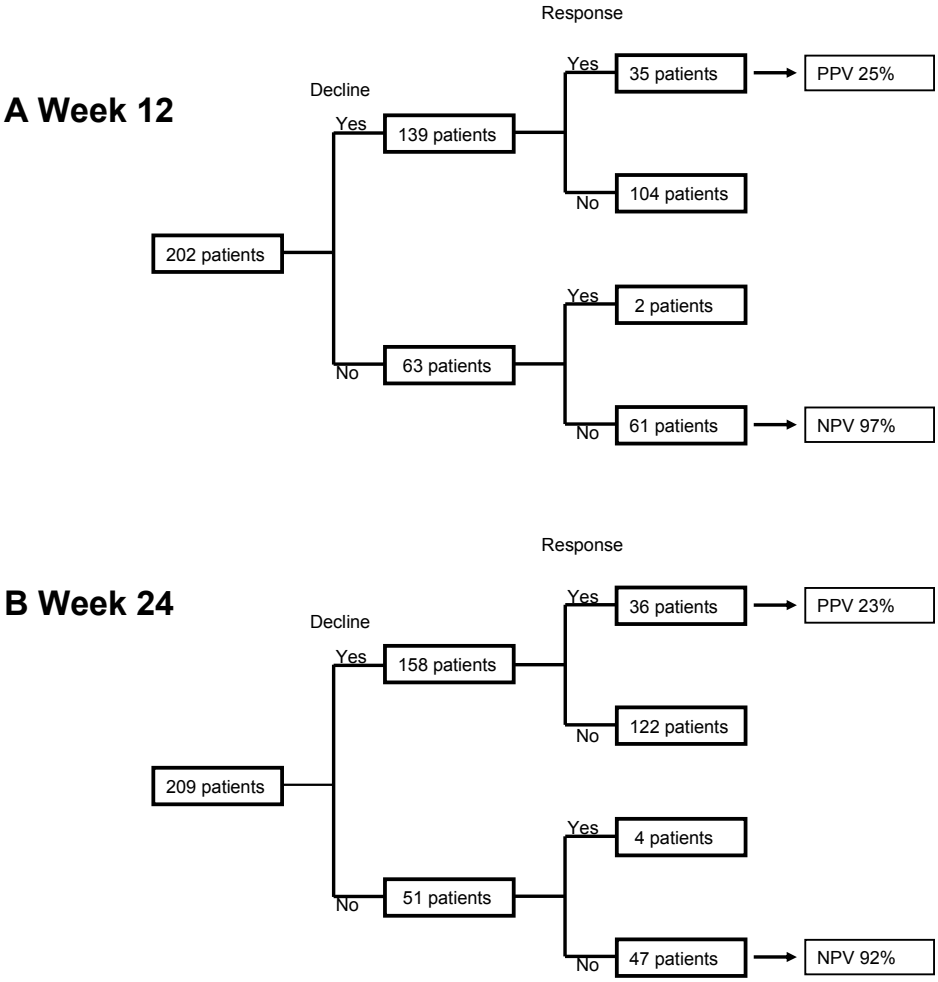

Figure 4: Flowcharts for any decline in serum HBsAg levels from baseline at weeks 12 (A) or 24 (B) in relation to sustained off-treatment response at week 78.

Table 3: Positive and negative predictive values for any HBsAg decline at week 12 and 24 for prediction of response and HBsAg loss at LTFU

		Response LTFU *				HBsAg loss LTFU			
		No	**Yes**	**PPV**	**NPV**	**No**	**Yes**	**PPV**	**NPV**
Any decline week 12	Yes	65	29	31%	–	80	14	15%	–
	No	42	2	–	95%	44	0	–	100%
Any decline week 24	Yes	76	30	28%	–	90	16	15%	–
	No	32	3	–	91%	35	0	–	100%

PPV, positive predictive value; NPV negative predictive value;
* Response is defined as HBeAg loss and HBV DNA < 10,000 copies/mL

DISCUSSION

We report the first large study on serum HBsAg decline during PEG-IFN treatment for HBeAg-positive CHB in relation to a sustained off-treatment response. One year of therapy with PEG-IFN significantly reduced serum HBsAg levels, and the decrease was sustained through post-treatment follow-up. HBsAg decline was significantly more pronounced in patients who achieved a response (HBeAg loss and HBV DNA <10,000 copies/mL). Furthermore, we found that reliable prediction of non-response to PEG-IFN is possible as early as week 12 of therapy, based on the absence of a decline in serum HBsAg. Patients who do not experience a decline in serum HBsAg from baseline to week 12, comprising 31% of our study population, have a minimal chance of achieving a sustained off-treatment response. Our results can help clinicians in their decision of whether to continue PEG-IFN therapy based on an individual patient's probability of non-response.

PEG-IFN can induce an off-treatment sustained response in a substantial proportion of HBeAg-positive CHB patients,[12-15] but its clinical use is compromised by the frequent occurrence of side-effects[26] and the uncertainty as to whether a patient will actually benefit from this therapy. Reliable prediction of non-response at baseline or during the first weeks of therapy is therefore essential to optimal utilization of this agent. Recently, a baseline prediction model has been published, based upon data from the two largest studies involving PEG-IFN in HBeAg-positive chronic hepatitis B.[24] The model enables the clinician to predict response (HBeAg loss and HBV DNA < 2,000 IU/mL (~10,000 copies/mL)) of HBeAg-positive patients to PEG-IFN, based on readily available data, such as HBV genotype, HBV DNA and ALT levels, age and sex. While the model provides considerable support when considering a patient for PEG-IFN therapy, substantial uncertainty remains as to whether an individual patient will respond to a one year course of PEG-IFN. On-treatment monitoring of viral replication using HBV DNA, HBeAg and HBsAg levels may aid decision-making and frequent HBV DNA monitoring is therefore recommended in treatment guidelines[3]. However, modeling of HBV DNA kinetics during PEG-IFN therapy has shown only limited clinical utility,[27-28] and reliable prediction of non-response is only possible at week 24 of therapy (negative predictive value (NPV) 86%).[29]

Recent technical advances have allowed for the quantitative assessment of HBsAg in serum. HBsAg is secreted from the hepatocyte during viral replication as part of the HBV nucleocapsid, or as part of noninfectious viral particles.[30] Several studies have reported that serum HBsAg levels correlate with intrahepatic cccDNA levels in HBeAg-positive patients.[21,31] On-treatment HBsAg decline may therefore reflect the efficacy of PEG-IFN in decreasing intrahepatic cccDNA and consequently predict a sustained response.[21,31] This hypothesis was first tested in HBeAg-negative patients,

and it was found that patients with low HBsAg levels at the end of treatment had the highest probability of achieving a sustained off-treatment response.[32] Furthermore, another study showed that patients who did not achieve a 0.5 log decline in serum HBsAg from baseline to week 12 of therapy had only 10% probability of achieving a response (NPV 90%).[33]

Our observations in HBeAg-positive patients corroborate these results on the excellent predictive capabilities of on-treatment HBsAg decline. In our study population, patients who did not achieve a decline in serum HBsAg concentration from baseline to week 12 of therapy had only 3% chance of achieving a sustained off-treatment response. The resulting NPV of 97% is superior to that achieved using HBV DNA and comparable to HBeAg monitoring.[29] Furthermore, our findings indicate that prediction of non-response to PEG-IFN is possible as early as week 12, as opposed to week 24 when using serum HBV DNA or HBeAg levels[29] and that prediction of non-response using HBsAg decline can accurately indentify those patients with a low probability of sustained response through 3 years of post-treatment follow-up. Furthermore, if our on-treatment stopping-rule was applied combined with the baseline prediction model.[24] the AUC increased from 0.75 for the stopping-rule alone to 0.79 for the combination, showing that application of both two models to guide therapy decisions may be beneficial.

Other studies have reported that HBsAg levels of <1500 IU/mL at week 12 or week 24 of therapy were highly predictive of sustained HBeAg seroconversion 6 months post-treatment[34]. We found comparable positive predictive values (PPVs) for HBsAg levels <1500 IU/mL at week 12 for response at LTFU (PPV: 55%) and for loss of HBsAg at LTFU (PPV: 35%). Prediction did not improve at week 24, with PPVs of 53% for response at LTFU, and 41% for HBsAg loss at LTFU. Anyhow, these results have limited clinical significance, since even patients with HBsAg levels >1500 IU/mL at either of these time-points have a considerable probability of response. If one were to discontinue therapy all patients with HBsAg >1500 IU/mL at week 24, one would miss out on 48% of patients with a response at LTFU in our study population.

A possible caveat of our study is that we pooled data from the two treatment arms for the formulation of our stopping-rule. Patients who received combination therapy experienced a somewhat larger decline from week 24 to week 52. To account for this, we validated our stopping-rule in both treatment groups, and found that it performed equally well in both populations. Sensitivity analysis confirmed that a cut-off of any decline was superior in both groups. Additionally, our LTFU population comprised only a subgroup of the total study group (149 out of 221). However, it was previously shown that the LTFU group was representative of the entire study cohort [12], and we confirmed these findings (data not shown). Also, the cut-off of any decline performed well in both groups (tables 2 and 3). Furthermore, one could argue that we should

have chosen a different definition of response. In this study, we defined response as off-treatment sustained HBeAg loss combined with HBV DNA <10,000 copies/mL (~2000 IU/mL), since HBeAg loss 6 months post-treatment has been reported to be highly durable[12] and since patients with low HBV DNA levels are less likely to develop HBV related liver complications or require antiviral therapy according to recent guidelines.[3,35-37] Moreover, this end-point is in line with other recently published papers on response to PEG-IFN in HBeAg-positive CHB,[24] and the high negative predictive values were maintained if HBeAg seroconversion combined with HBV DNA <10,000 copies/mL was applied as end-point.

Concluding, a one year course of PEG-IFN results in a significant decline in serum HBsAg in HBeAg-positive CHB patients. The decline is considerably more pronounced in patients who achieve a response (HBeAg loss and HBV DNA <10,000 copies/mL) when compared to non-responders. Patients who do not experience a decline in HBsAg levels through 12 weeks of therapy have a low chance of achieving a sustained off-treatment response (<5%) and no chance of HBsAg loss, and should therefore be considered for treatment discontinuation.

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Durable hepatitis B surface antigen decline in hepatitis B e antigen-positive chronic hepatitis B patients treated with peginterferon alfa-2b: relation to response and HBV genotype

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ABSTRACT

Background & Aims

On-treatment decline of serum Hepatitis B surface Antigen (HBsAg) may reflect the immunomodulatory effect of peginterferon (PEG-IFN) for HBeAg-positive chronic hepatitis B (CHB). We aimed to compare HBsAg decline across HBV genotypes between combined responders (HBeAg loss and HBV DNA <10,000 copies/mL at week 78), HBeAg responders (HBeAg loss with HBV DNA >10,000 copies/mL) and nonresponders.

Methods

HBsAg was measured at baseline, on-treatment and 6 months post-treatment in 221 HBeAg-positive CHB patients treated with PEG-IFN±lamivudine for 52 weeks, and in a representative subgroup of 142 patients at long-term follow-up (LTFU; mean follow-up 3.0 years).

Results

On-treatment HBsAg decline significantly varied according to HBV genotype (A and B more than C and D, $p<0.001$). On-treatment HBsAg decline also differed between patients with a combined response (n=43) and those without (n=178, 3.34 versus 0.69 logIU/mL decline at week 52; $p<0.001$). Among patients without a combined response, no difference was observed between HBeAg responders (n=41) versus nonresponders (n=137). HBsAg decline was sustained in combined responders and progressed to 3.75 logIU/mL at LTFU. Patients with a combined response achieved pronounced HBsAg declines, irrespective of HBV genotype, and those who achieved HBsAg levels <1,000 IU/mL at week 78 had a high probability of a sustained response and HBsAg clearance through LTFU.

Conclusions

On-treatment HBsAg decline during PEG-IFN therapy for HBeAg-positive CHB depends upon HBV genotype. Patients with a combined response to PEG-IFN achieve a pronounced HBsAg decline, irrespective of HBV genotype, which is sustained through 3 years of off-treatment follow-up.

INTRODUCTION

Chronic hepatitis B (CHB) is an important global health problem, with over 350 million people being chronically infected.[1] Prolonged liver inflammation due to infection with the hepatitis B virus (HBV) may progress to cirrhosis, liver failure and hepatocellular carcinoma (HCC).[1-2] Hepatitis B e Antigen (HBeAg)-positive CHB is generally regarded as the earliest phase of infection in what is essentially a four phase disease continuum.[2] Current treatment guidelines recommend both pegylated interferon (PEG-IFN) and nucleos(t)ide analogues (NA) for the treatment of HBeAg-positive patients.[2-3] A one year course of PEG-IFN results in an off-treatment sustained response, defined as HBeAg loss and HBV DNA <10,000 copies/mL at 6 months post-treatment, in around 25 percent of patients. [4-5] Response to IFN-based therapy has been reported to be associated with a lower incidence of HCC and prolonged survival. [6-8]

Covalently closed circular DNA (cccDNA) is the main replication template of HBV [9] and low cccDNA levels following antiviral therapy have been shown to be predictive of a sustained response.[10] Intrahepatic cccDNA can only be assessed invasively, but it has recently been demonstrated that serum levels of hepatitis B surface antigen (HBsAg) reflect intrahepatic cccDNA levels in HBeAg-positive CHB patients and may consequently predict a sustained response.[11-13]

PEG-IFN induces a strong decline in serum HBsAg levels in both HBeAg-positive and HBeAg-negative patients.[14-18] Patients who achieve a sustained response to PEG-IFN exhibit a steeper HBsAg decline compared with non-responders. A recent study among HBeAg-negative patients suggested that the degree of HBsAg decline may be influenced by the infecting HBV genotype as well.[19] Given these findings, a durable suppression of HBsAg may reflect immunological control over the virus. The long-term sustainability of PEG-IFN induced HBsAg decline is however currently unknown. The aims of our study were therefore to investigate (1) which factors are associated with HBsAg decline induced by PEG-IFN for HBeAg-positive CHB and (2) whether HBsAg decline is durable through long-term follow-up.

METHODS

Patients

Serum HBsAg levels were assessed in 221 HBeAg-positive CHB patients who were previously enrolled in an investigator-initiated multicenter randomized controlled trial and a subsequent long-term follow-up (LTFU) study.[4, 20] The in- and exclusion criteria for this study have been described elsewhere. In short, patients were eligible if they had been HBsAg positive for at least 6 months prior to randomization, were HBeAg

positive twice within 8 weeks prior to randomization, had elevated serum alanine aminotransferase (ALT) levels of 2 - 10 times the upper limit of normal, and had a serum HBV DNA level above 1.0 x 10^5 copies/mL. Exclusion criteria were antiviral therapy within 6 months prior to randomization, viral co-infections, pre-existing cytopenia and/or decompensated liver disease. Patients were treated with PEG-IFN alfa-2b 100 µg weekly (PegIntron, Merck, Whitehouse Station, NJ, USA) in combination with a placebo or lamivudine (LAM) 100 mg (Zeffix, GlaxoSmithKline, Greenford, UK) daily for 52 weeks. The PEG-IFN dose was reduced to 50 µg per week after 32 weeks of therapy to limit the probability of early treatment discontinuation. Patients attended the outpatient clinic every 4 weeks for routine examinations and laboratory assessments during both the treatment and the post-treatment follow-up phase of the study. For the LTFU study, patients were re-evaluated at one additional visit at the local participating center. The mean duration of follow-up was 3.0 years.[20]

Inclusion criteria for the present study were: completion of the 26-week follow-up phase of the main study and availability of a baseline serum sample for HBsAg quantification. Of the 266 patients in the initial study, 221 fulfilled these criteria. Of these patients, 142 participated in the associated LTFU study and had LTFU samples available for HBsAg quantification.[20]

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. All patients gave written informed consent according to standards of the local ethics committees.

Laboratory measurements

Serum HBsAg was quantified in samples taken at baseline, during the treatment period (weeks 4, 8, 12, 24 and 52), during follow-up (week 78) and at LTFU using the ARCHITECT HBsAg assay (Abbott laboratories, Abbott Park, IL; range 0.05 - 250 IU/mL). HBV DNA quantification was performed using an in-house developed TaqMan polymerase chain reaction (PCR) assay (lower limit of quantification 400 copies/mL) based on the EuroHep standard.[21] For the LTFU study, HBV DNA was measured with the Cobas TaqMan HBV assay (Roche Molecular Systems, Branchburg, NJ, USA), with a dynamic range of quantification of 174 - 6.4 x 10^8 copies/mL (30 - 1.1 x 10^8 IU/mL). There is an excellent correlation between the two assays.[20] ALT was measured locally in accordance with standard procedures and is presented as multiples of the upper limit of normal (ULN). HBV genotype was assessed using the INNO-LiPA assay (Innogenetics, Gent, Belgium).

Statistical analysis

Response to treatment was assessed at week 78 in all patients. A composite endpoint of HBeAg loss and HBV DNA level <10,000 copies/mL was chosen for definition of

combined response, [5] and patients who achieved HBeAg loss but failed to achieve HBV DNA <10,000 copies/mL were considered HBeAg responders. All others were non-responders. Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.2 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

RESULTS

Patient characteristics

A total of 221 patients were included in this study. The mean age of the total study population was 34 years at the start of therapy, patients were predominantly male (78%), and of Caucasian origin (72%). HBV genotypes A and D were most prevalent in the study cohort (34% and 39%), followed by C (15%) and B (9%). Mean baseline ALT levels were 4.2 times ULN, HBV DNA levels were 9.1 log copies/mL and HBsAg levels were 4.4 log IU/mL (Table 1). Of the 221 patients, 43 (19%) achieved a combined response (HBeAg negativity and HBV DNA <10,000 copies/mL at week 78). An additional 41 patients were HBeAg responders (HBeAg loss with HBV DNA levels >10,000 copies/mL), and 19 patients (9%) lost HBsAg.

A total of 142 patients had serum available at LTFU (mean follow-up 3.0 years). The characteristics of the main study population and the LTFU cohort were fully comparable with regard to baseline HBV DNA, ALT and HBsAg levels, and HBV genotype distribution (table 1). Follow-up duration was similar in responders and non-responders. Importantly, only one patient with a combined response at week 78 experienced HBeAg relapse, and 54% of combined responders at week 78 was HBsAg negative at LTFU.

HBsAg decline on-treatment

One year of treatment with PEG-IFN resulted in a mean decline in serum HBsAg levels of 1.17 log IU/mL. This decline was sustained through 6 months of post-treatment follow-up: the mean decline from baseline was 0.92 log IU/mL at week 78. At baseline, only age ($p<0.01$) and HBV genotype ($p<0.01$) were related to HBsAg decline at week 78 by univariate analysis. Combination therapy, ALT, log HBV DNA, sex and race were all not associated with HBsAg decline at week 78. By multivariate analysis, HBV genotype, age and log HBV DNA level at baseline were related to HBsAg decline at week 78.

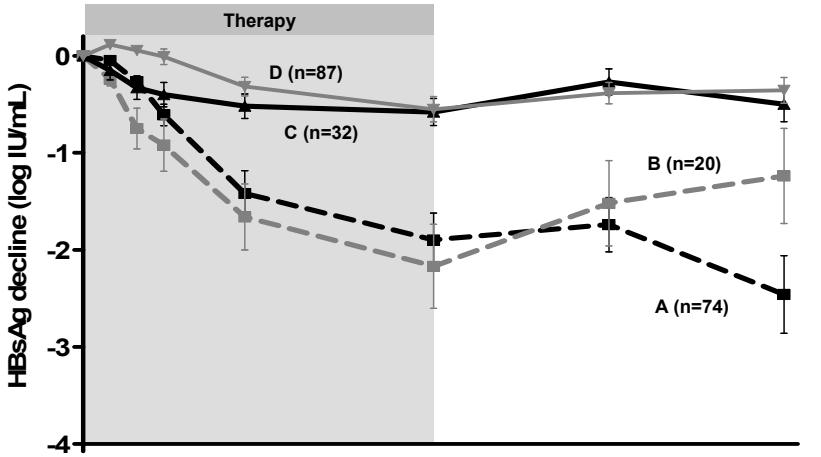
Table 1: Patient characteristics

Characteristics	Main study population (n=221)	LTFU study population (n=142)	P
Demography			
Mean (SD) age, years	34 (12)	34 (12)	1.0
Male	173 (78%)	115 (81%)	0.60
BMI	25 (4.0)	25 (4.2)	0.66
Monotherapy	111 (50%)	75 (53%)	0.63
Race			0.83
Caucasian	160 (72%)	99 (70%)	
Asian	44 (20%)	30 (21%)	
Other	17 (8%)	13 (9%)	
Laboratory results			
Mean (SD) ALT*	4.2 (3.0)	4.6 (3.4)	0.31
Mean (SD) HBV DNA, log copies/mL	9.1 (0.89)	9.1 (0.80)	1.0
Mean (SD) HBsAg, log IU/mL	4.4 (0.64)	4.3 (0.69)	0.26
HBV Genotype			0.78
A	74 (34%)	41 (29%)	
B	20 (9%)	12 (9%)	
C	32 (15%)	27 (19%)	
D	87 (39%)	56 (39%)	
Other/mixed	8 (4%)	6 (4%)	
Response at week 78			
Combined response#	43 (19%)	24 (17%)	0.58
HBeAg loss	84 (38%)	49 (35%)	0.51
HBsAg loss	19 (9%)	10 (7%)	0.69

*Multiples of upper limit of the normal range
#HBeAg loss and HBV DNA <10,000 copies/mL

HBsAg decline according to HBV genotypes

Baseline HBsAg levels were significantly different in genotypes A through D. Mean HBsAg levels were 4.53 log IU/mL in patients with genotype A, 4.33 in genotype B, 3.79 in genotype C, and 4.51 in genotype D ($p<0.001$ for genotype C versus other genotypes). Furthermore, considerably different HBsAg decline patterns were observed in the respective genotypes. While patients with genotype D experienced a slight increase during the first 12 weeks of therapy, patients with genotypes A and B showed a strong initial decline (figure 1). At the end of treatment, patients infected with genotypes A and B had a significantly more pronounced HBsAg decline (mean 1.90 and 2.17 log IU/mL, respectively), when compared to patients harbouring genotypes C or D (0.59 and 0.55, respectively; $p<0.001$ for A and B versus C and D). Through



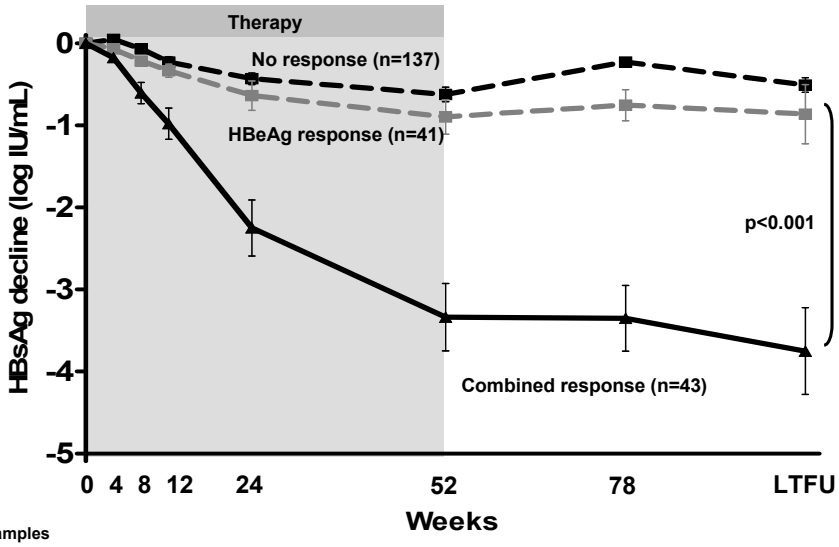
Available samples					
A	74	65	68	73	41
B	20	19	20	20	12
C	32	32	32	32	27
D	87	86	81	87	56

Figure 1: HBsAg decline in all patients according to HBV genotype. Differences between patients with genotype A or B versus C or D were statistically significant from week 24. Error bars represent the standard error of the mean.

post-treatment LTFU, considerable relapse was observed in patients with genotype B (decline at LTFU 1.24 log IU/mL), whereas HBsAg levels in patients with genotype A continued to decrease (decline at LTFU 2.46 log IU/mL). HBsAg levels remained stable through LTFU in patients infected with genotypes C and D.

HBsAg decline according to treatment response

Pre-treatment HBsAg levels were comparable in patients who achieved a combined response and in those who did not; 4.29 versus 4.43 log IU/mL (p=0.19). However, on-treatment HBsAg kinetics clearly differed between patients with a combined response and those without. Combined responders exhibited a decline of 3.34 log IU/mL, compared to 0.69 in all other patients ($p<0.001$, figure 2). Within the group who failed to achieve a combined response, no significant difference was observed between patients who achieved a HBeAg response (n=41) versus those who remained HBeAg positive (n=137); mean declines were 0.90 and 0.62 log IU/mL at week 52, respectively (p=0.17). The HBsAg decline induced by PEG-IFN was sustained in patients with a combined response, and declined further through LTFU to 3.75 log IU/mL (p=0.27 versus end of treatment). In those who were HBeAg positive at week 78, mean HBsAg decline at LTFU was 0.51 log IU/mL, (figure 2, $p<0.001$ compared to combined responders), while the HBeAg responders achieved a decline of 0.86 log IU/mL at LTFU (p=0.35 versus those with positive HBeAg).



Available samples					
No response	137	129	132	137	93
HBeAg response	41	40	39	41	25
Combined Response	43	40	38	42	24

Figure 2: HBsAg decline in patients with a combined response (HBeAg loss and HBV DNA <10,000 copies/mL) versus patients with an HBeAg response (HBeAg loss but HBV DNA >10,000 copies/mL) or no response. Response was assessed at week 78. Differences between patients with a combined response versus those with no response or HBeAg response were significant from week 8 of treatment. Error bars represent the standard error of the mean.

HBsAg decline according to treatment response and HBV genotype

Of the 43 combined responders, 42 were infected with genotype A through D (28 (65%) genotype A, 5 (12%) genotype B, 3 (7%) C and 6 (14%) D). Of the 41 HBeAg responders, 9 (22%) were infected with genotype A, 5 (12%) with B, 5 (12%) with C and 18 (44%) with D. Similar to the overall population, combined responders infected with genotypes A through D achieved more HBsAg decline than did patients with the same genotype who were HBeAg responders or who remained HBeAg positive (figure 3A-D).

Combined responders experienced marked declines in HBsAg levels from baseline to end of treatment (genotype A 3.81, genotype B 2.98, genotype C 1.47 and genotype D 2.68 log IU/ml; p=0.59). At LTFU, declines were 4.18, 2.38, 1.87 and 3.09 log IU/mL in combined responders with genotypes A, B, C and D, respectively (p=0.56).

In contrast, on-treatment decline in the HBeAg responders (patients who cleared HBeAg but did not achieve HBV DNA <10,000 copies/mL) significantly varied according to HBV genotype; patients with genotype A and B achieved declines of 2.02 and 1.56 log IU/mL at week 52, compared to 0.50 and 0.43 log IU/mL in patients with C and D (P=0.02 for A and B versus C and D). At LTFU, declines were 3.33, 0.92, 0.09 and 0.22 in HBeAg responders with genotypes A through D, respectively (p=0.03).

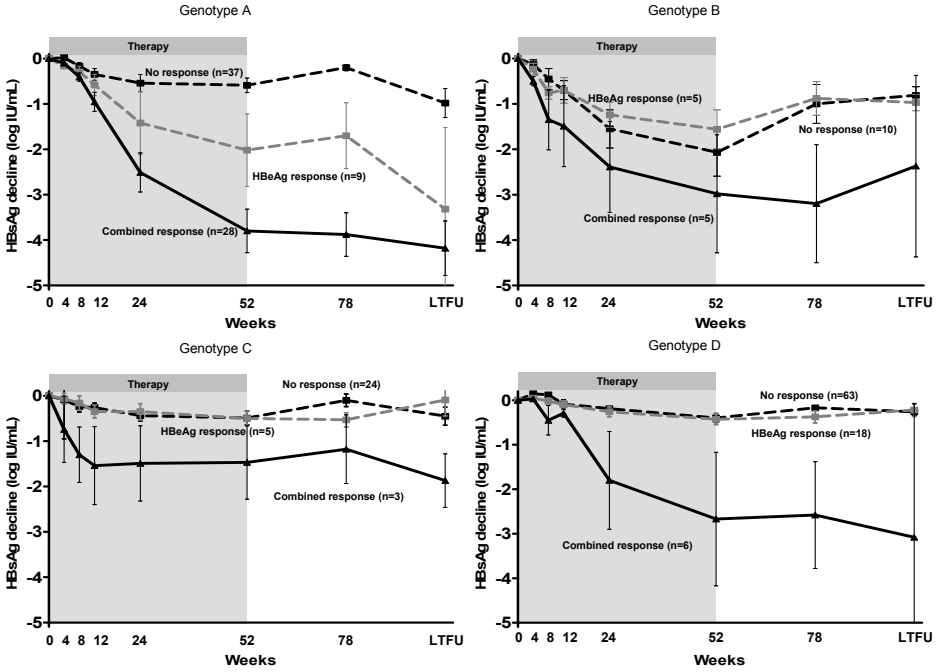


Figure 3: HBsAg decline in patients with a combined response (HBeAg loss and HBV DNA <10,000 copies/mL at week 78) versus patients with an HBeAg response (HBeAg loss but HBV DNA >10,000 copies/mL at week 78) or no response for genotypes A (A), B (B), C (C) and D (D). Error bars represent the standard error of the mean.

Relationship between HBsAg levels at week 78 and response at LTFU

Thirty-three of 142 (23%) patients with HBsAg data at LTFU achieved a combined response at LTFU. Analysis was limited to the 141 with available HBsAg levels at week 78. Probabilities of achieving a combined response at LTFU in relation to HBsAg levels and decline at week 78 are shown in table 2, both for the overall population (n=141) and for patients with a combined response at week 78 with available LTFU data (n=23). Importantly, no patient without a decline at week 78 achieved HBsAg clearance by LTFU, whereas patients with an HBsAg level <1,000 IU/mL at week 78 (n=28) had a probability of HBsAg clearance of 46% (13/28). Only 2 of 113 (2%) patients with HBsAg ≥1,000 IU/mL achieved HBsAg clearance at LTFU. Among patients with a combined response, those with HBsAg <1,000 IU/mL achieved HBsAg negativity at LTFU in 73% (11/15), compared to 25% (2/8) in those with HBsAg ≥1,000 IU/mL.

Table 2. HBsAg level and decline at week 78 and response at LTFU.

		All patients* (n=141)				Combined response 78# (n=23)			
Combined response	**LTFU**	**No**	**Yes**	**NPV**	**PPV**	**No**	**Yes**	**NPV**	**PPV**
HBsAg level week 78	<1,000	11	17	–	61%	0	15	–	100%
	≧1,000	98	15	87%	–	4	4	50%	–
Any decline week 78	Yes	71	31	–	30%	3	19	–	86%
	No	38	1	97%	–	1	0	100%	–

* All patients with HBsAg levels at week 78 and at LTFU. #Patients with a combined response at week 78. Combined response was defined as HBeAg negativity with HBV DNA <10,000 copies/mL. NPV, negative predictive value; PPV, positive predictive value.

DISCUSSION

In this large study we show that on-treatment HBsAg decline is a sensitive marker for response to PEG-IFN, showing a steep decline in patients who achieved a combined response (defined as HBeAg loss and HBV DNA <10,000 copies/mL), irrespective of HBV genotype. A very limited decline was observed in patients who remained HBeAg positive or those who cleared HBeAg but progressed to active HBeAg-negative CHB, and patients without an HBsAg decline at week 78 have a very limited probability of response at LTFU. Furthermore, HBsAg decline was sustained in patients with a combined response through 3 years of follow-up, reflecting a long-term sustained suppression after therapy discontinuation.

The HBV replication pathway that produces HBsAg is separate from the pathway that produces HBV DNA. HBsAg is transcribed and subsequently translated from the HBV envelope gene, and produced as small, medium or large HBsAg proteins.[22-23] Synthesized HBsAg proteins may then be incorporated into mature HBV nucleocapsids, and subsequently secreted from the hepatocyte. However, HBsAg production far exceeds that required for the production of HBV virions and HBsAg is therefore also secreted in the form of non-infectious particles. Commercially available HBsAg quantification assays probably detect all forms of HBsAg, [24] but the clinical relevance of the different HBsAg forms is so far unclear.

HBsAg levels in the sera of patients with CHB depend on the phase of infection. Patients classified as being in the immune tolerant and immune clearance phases of the disease have the highest HBsAg levels, whereas HBsAg is lowest in inactive carriers. [24-26] Furthermore, patients classified as inactive carriers who experienced a subsequent HBV DNA increase (reactivation) had higher HBsAg levels compared to those who did not.[25] A post-treatment sustained reduction in HBsAg levels achieved with PEG-IFN therapy may therefore signify an immunological response, resulting in

transition to the inactive carrier state. [26] In our study, one year of PEG-IFN therapy induced a pronounced decline in serum HBsAg levels, particularly in patients who achieved HBeAg loss and HBV DNA <10,000 copies/mL at week 78. Importantly, the decline achieved in these combined responders was durable through LTFU. This implies not only long-term sustained disease remission with a very low probability of relapse,[25] but also reflects the high probability of subsequent HBsAg loss as described previously in HBeAg-positive responders to (PEG-)IFN.[20,27] Furthermore, we found that patients who achieved a combined response (HBeAg clearance with HBV DNA <10,000 copies/mL at week 78) with concomitant HBsAg levels <1,000 IU/mL did not experience relapse during off-treatment follow-up and were very likely to clear HBsAg. Conversely, patients who failed to achieve an HBsAg decline from baseline by week 78 had little chance of combined response, and no chance of HBsAg loss, suggesting the necessity of retreatment with other agents.

The current study also shows that baseline HBsAg levels and on-treatment HBsAg decline in HBeAg-positive patients are dependent upon HBV genotype. These findings are in line with a report on HBeAg-negative subjects,[19] and may reflect a difference in transcription efficacy between respective genotypes. [28] However, the differences observed in HBsAg decline according to genotype may also be a reflection of the variance in the efficacy of PEG-IFN across the genotypes. Among HBeAg responders, those with genotypes C and D experienced only a limited reduction in HBsAg levels, and these patients were reported to have a high probability of HBeAg seroreversion and persistently detectable HBV DNA through LTFU.[20,29] Conversely, HBeAg responders infected with genotype A experienced a pronounced decline in HBsAg levels, and also had the highest probability of losing HBsAg through LTFU. [20] Interestingly, we found detectable precore and/or core promoter mutants by INNO-LiPA line-probe assay in all but one of the HBeAg responders with available serum, irrespective of HBV genotype (n=29, data not shown). This suggests that the differences in HBsAg decline across genotypes are not due to the presence or absence of these mutants.

In contrast to the genotype specific differences among HBeAg responders, all combined responders experienced pronounced HBsAg declines. This shows that, irrespective of HBV genotype, a combined response is associated with a sustained reduction in HBsAg levels and a high probability of HBsAg loss through long-term follow-up.[27] Taken together, these observations corroborate recent data highlighting the influence of HBV genotype on response to PEG-IFN, and show that a combined response of HBeAg loss and HBV DNA < 10,000 copies/mL is the most appropriate marker for response to PEG-IFN, especially in patients with non-A genotypes.[5,20]

Recent studies among HBeAg-positive[18] and HBeAg-negative[14,30] patients treated with PEG-IFN have shown that HBsAg levels during therapy may be used to predict response to treatment. However, the current study now shows that HBsAg decline

during PEG-IFN treatment for HBeAg-positive CHB depends upon HBV genotype as well, and the differences in HBsAg decline suggest that genotype specific thresholds may be required when using HBsAg to guide PEG-IFN based therapy.

A possible caveat of our study is that we pooled data from the two treatment arms of the original trial. Combination therapy of PEG-IFN and LAM is known to cause a slightly steeper on-treatment HBsAg decline. However, this effect of LAM was the same regardless of treatment response or HBV genotype, and was not sustained post-treatment. Furthermore, we found no difference in any of the outcomes when we analyzed the treatment arms separately. Response rates were similar in patients treated with PEG-IFN monotherapy versus the combination with LAM (18 versus 21%, p=0.61), as were HBsAg decline at week 78 (0.86 versus 0.98 log IU/mL, p=0.63) and at LTFU (0.93 versus 1.32, p=0.22). A subgroup of non-responders was retreated after the initial study. Retreatment did not affect HBsAg kinetics during LTFU.

In conclusion, HBeAg-positive CHB patients who achieve a combined response (HBeAg loss and HBV DNA <10,000 copies/ml) to PEG-IFN therapy achieve a pronounced decline in HBsAg levels, irrespective of HBV genotype. In contrast, patients who clear HBeAg but have HBV DNA levels >10,000 copies/mL experience a limited decline, showing that HBeAg loss alone may be a suboptimal marker for response to PEG-IFN in patients with non-A genotypes. The HBsAg decline achieved in patients with a combined response is sustained through 3 years of post-treatment follow-up reflecting a durable response with a low chance of relapse and a high probability of subsequent HBsAg loss.

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Response-guided peginterferon therapy in HBeAg-positive chronic hepatitis B using serum hepatitis B surface antigen levels

8

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Submitted

ABSTRACT

Background & Aims

On-treatment levels of hepatitis B surface antigen (HBsAg) may predict response to peginterferon (PEG-IFN) therapy in chronic hepatitis B (CHB), but previously proposed prediction rules have shown limited external validity.

Methods

We analysed 803 HBeAg-positive patients treated with PEG-IFN in 3 global studies with available HBsAg measurements. A stopping-rule based on absence of a decline from baseline was compared to a prediction-rule that uses HBsAg levels of <1500 IU/mL and >20,000 IU/mL to identify patients with high and low probabilities of response.

Results

Patients with an HBsAg level <1,500 IU/mL at week 12 achieved response (HBeAg loss with HBV DNA <2,000 IU/mL at 6 months post-treatment) in 45%. At week 12, patients without a decline in HBsAg achieved a response in 14%, compared to only 6% of patients with HBsAg >20,000 IU/mL, but performance varied across HBV genotype. In patients treated with PEG-IFN monotherapy (n=465), response rates were low in patients with genotypes A or D if there was no decline of HBsAg by week 12 (negative predictive value [NPV]: 97-100%), and in patients with genotypes B or C if HBsAg at week 12 was >20,000 IU/mL (NPV: 92-98%). At week 24, nearly all patients with HBsAg >20,000 IU/mL failed to achieve a response, irrespective of HBV genotype (NPV for response and HBsAg loss 99% and 100%).

Conclusions

HBsAg is a strong predictor of response to PEG-IFN in HBeAg-positive CHB. HBV genotype specific stopping-rules may be considered at week 12, but treatment discontinuation should be considered in all patients with HBsAg >20,000 IU/mL at week 24, irrespective of HBV genotype.

INTRODUCTION

Chronic hepatitis B (CHB) affects over 350 million people and is one of the leading causes of cirrhosis and hepatocellular carcinoma.[1] Antiviral treatment with peginterferon-alfa (PEG-IFN) may result in suppression of HBV DNA, hepatitis B e antigen (HBeAg) loss and hepatitis B surface antigen (HBsAg) clearance.[2-5] Response to PEG-IFN therapy is durable, and patients with a sustained response have a reduced risk of developing hepatocellular carcinoma.[6-8]

However, clinical application of PEG-IFN is compromised by the limited response rates and the occurrence of side-effects.[3-5] Careful selection of patients with the highest probabilities of response to PEG-IFN therapy is therefore essential. Several studies have shown that response rates are higher in patients with HBV genotypes A or B versus C or D,[3,5,9] and in patients with higher levels of ALT[5,9] and lower levels of HBV DNA.[9] Recent studies also suggest that host factors such as *IL28B* genotype, as well as viral characteristics such as absence of precore and/or core promoter mutants also influence response probabilities.[10,11]

Nevertheless, prediction models incorporating these variables have only limited discriminatory capabilities. Recent studies have shown that serum levels of HBsAg correlate with intrahepatic cccDNA concentrations, and that achievement of a decline in HBsAg may herald induction of immune-control.[12,13] HBsAg levels during treatment with PEG-IFN can be used to identify patients with very high or very low probability of response,[5,14] but interpretation of the findings is hampered by the use of different definitions of response across the studies. Furthermore, the external validity of proposed stopping-rules was shown to be limited,[15] which may be accounted for by the influence of HBV genotype on HBsAg levels and kinetics.[16] Since HBV genotype distribution differed considerably across the different study cohorts, only a combined analysis of individual patient data would allow for adequate assessment of the performance of the prediction rules across patients with different HBV genotypes.

The aim of the current study was therefore to evaluate the performance of two recently proposed prediction rules for HBeAg-positive CHB patients treated with PEG-IFN in a pooled dataset of patients participating in 3 of the largest randomized studies conducted worldwide.[3-5]

PATIENTS AND METHODS

Patients

In this study serum HBsAg levels were assessed in HBeAg-positive CHB patients who were previously enrolled in 3 separate pivotal multicenter randomized controlled trials

on PEG-IFN therapy: the PEG-IFN alfa-2a Phase 3 study,[4] the HBV 99-01 study,[3,14] and the Neptune study.[5] The PEG-IFN alfa-2a Phase 3 study compared PEG-IFN alfa-2a alone, lamivudine alone, or the two combined for a treatment duration of 48 weeks.[4] The HBV 99-01 study compared PEG-IFN alfa-2b alone with PEG-IFN alfa-2b combined with lamivudine for 52 weeks.[3] The Neptune study compared 48 weeks of PEG-IFN alfa-2a at the full dose of 180ug/week for 48 weeks or 24 weeks, with a reduced dose of 90 ug/week for 48 or 24 weeks.[5] Only patients from the Neptune study randomized to the full dose for 48 weeks of PEG-IFN alfa-2a were eligible for participation in the current study. Response to treatment was assessed at 6 months post-treatment in all 3 studies, corresponding to study week 72 for the PEG-IFN alfa-2a Phase 3 and Neptune studies, and week 78 for the HBV 99-01 study. Inclusion criteria for these studies have been published previously, but in short: patients were positive for HBsAg for at least six months, positive for HBeAg, had an elevated ALT between 1 and 10 times the upper limit of normal (ULN) and HBV DNA levels exceeding 1.0×10^5 copies/mL. Exclusion criteria included co-infection with hepatitis C virus, hepatitis delta virus or human immunodeficiency virus, decompensated liver disease, previous antiviral therapy within six months and pre-existing neutropenia or thrombocytopenia.

Patients were eligible for the current analysis if they were infected with HBV genotypes A through D, had available HBsAg measurements at baseline, available HBsAg measurements at week 12 and/or week 24, and available data on treatment outcome at 6 months post-treatment. Out of a total of 899 patients with available data (PEG-IFN alfa-2a Phase 3: n=542; HBV 99-01: n=221; Neptune: n=136), 803 patients complied with these criteria. Of the excluded 96 patients, 17 were infected with HBV genotypes other than A through D, 38 patients did not have available HBsAg levels at baseline and week 12 and/or 24, and 41 did not have available outcome data on (anti-)HBe, HBV DNA levels or HBsAg at 6 months post-treatment.

Laboratory measurements

Serum HBsAg was quantified in samples taken at baseline, during the treatment period and during follow-up. HBsAg was measured using the Architect (Abbott, Abbott Park, IL, USA[17]) in patients from the PEG-IFN alfa-2a Phase 3 and the HBV 99-01 studies, and using the Elecsys HBsAg II (Roche Diagnostics, Indianapolis, IN, USA) for patients enrolled in the Neptune study. A large previous study has shown a high correlation and close agreement between the two assay and demonstrated that prediction rules derived from measurements conducted with one platform may be confidently used on the other.[18] HBV DNA quantification was performed on Taqman based PCR assays with a lower limit of detection <400 copies/mL. ALT was measured locally in accordance with standard procedures and is presented as multiples of the

upper limit of normal (ULN). HBV genotype was assessed using the INNO-LiPA line probe assay (Innogenetics, Ghent, Belgium).

Statistical analysis

Response to treatment was defined as a composite endpoint of HBeAg loss with an HBV DNA level <2,000 IU/mL (~10,000 copies/mL)[9] or HBsAg loss. The prediction rules evaluated in the current analysis included the stopping-rule proposed by Sonneveld *et al.*, which recommended treatment discontinuation if there is no decline of serum HBsAg levels from baseline to weeks 12 or 24,[14] and a prediction-rule identified previously by Piratvisuth *et al.* on the PEG-IFN alfa-2a Phase 3 dataset, which used HBsAg levels of <1500 IU/mL and >20,000 IU/mL at weeks 12 and 24 to identify patients with a high and low probability of response, respectively.[19] The validity of these cut-offs was confirmed in the pooled dataset using logistic regression analysis fitting a spline with 5 knots. The optimal cut-point was chosen based on a sensitivity of at least 95% and the highest negative predictive value (but always >90%) for response and HBsAg loss. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.2 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

RESULTS

Patient characteristics

A total of 803 patients were analysed, 104 (13%) treated with PEG-IFN alfa-2b alone, 100 (13%) treated with PEG-IFN alfa-2b with LAM, 361 (45%) treated with PEG-IFN alfa-2a alone and 238 (30%) treated with PEG-IFN alfa-2a with LAM. The characteristics of the 803 enrolled patients are shown in table 1 by assigned therapy regimen. Overall, 182 (23%) achieved a response (HBeAg loss with HBV DNA <2,000 IU/mL) and 39 (5%) cleared HBsAg by 6 months after PEG-IFN discontinuation.

HBsAg decline according to therapy regimen and HBV genotype

Baseline HBsAg levels significantly varied across HBV genotype, baseline levels were 4.59, 4.23, 3.91, and 4.53 log IU/mL for patients with genotypes A, B, C, and D($p<0.001$ by ANOVA). Mean HBsAg decline at six months post-therapy was 0.73 log IU/mL. HBsAg decline during treatment varied significantly by therapy regimen; patients treated with combination therapy (n=338) achieved an end of treatment decline of 1.37 log IU/mL, compared to 0.92 in patients treated with PEG-IFN monotherapy($p<0.001$). However, HBsAg declines at 6 months post-treatment did not

Table 1: Characteristics of the study cohort

Characteristics	PEG-IFN alone (n=465)	PEG-IFN + LAM (n=338)	p
Demography			
Mean (SD) age, years	33.0 (10.5)	32.0 (10.6)	0.161
Male	349 (75%)	255 (75%)	0.899
Race			0.202
Caucasian	100 (22%)	88 (26%)	
Asian	347 (75%)	233 (69%)	
Other	18 (4%)	17 (5%)	
Previous IFN	47 (10%)	46 (14%)	0.126
Laboratory results			
Mean (SD) ALT*	3.76 (3.5)	4.01 (3.2)	0.294
Mean (SD) HBV DNA, log c/mL	9.35 (1.8)	9.84 (1.7)	<0.001
Mean (SD) HBsAg, log IU/mL	4.11 (0.73)	4.23 (0.66)	0.019
HBV Genotype			0.299
A	58 (13%)	45 (13%)	
B	122 (26%)	82 (24%)	
C	230 (50%)	156 (46%)	
D	55 (12%)	55 (13%)	
Response at week 78			
Response#	109 (23%)	73 (22%)	0.538
HBsAg loss	18 (4%)	21 (6%)	0.127

*Multiples of upper limit of the normal range
#HBeAg loss and HBV DNA <2,000 IU/mL at 6 months post-treatment

differ: declines were 0.68 and 0.80 log IU/mL for patients treated with PEG-IFN alone versus PEG-IFN with LAM(p=0.293). HBsAg decline during treatment also varied across the HBV genotypes (figure 1). At six months post-treatment, mean declines were 1.60 and 0.96 log IU/mL for patients with genotypes A or B, versus 0.46 and 0.39 log IU/mL for patients infected with genotypes C or D(p<0.001).

HBsAg decline according to response: overall and by HBV genotype

A decline of HBsAg levels was most pronounced in patients who achieved a response (figure 2A). HBsAg declines at end of treatment and at six months post-treatment were 2.39 and 1.98 log IU/mL in responders, compared to 0.73 and 0.34 log IU/mL in non-responders (p<0.001 for responders versus non-responders). Similar patterns were observed across the HBV genotypes (figures 2B-E). Responders achieved more HBsAg decline by 6 months post-treatment than non-responders, also when adjusting for combination therapy and HBV genotype: 2.05 versus 0.50 log IU/mL (p<0.001).

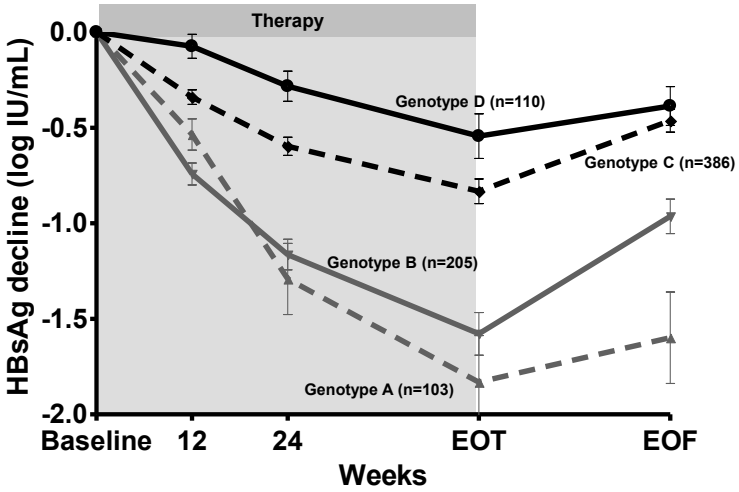

Figure 1: Change in serum HBsAg from baseline during treatment and 6 months of off-treatment follow-up across HBV genotypes A through D.

On-treatment prediction of response using HBsAg levels at weeks 12 and 24

Of the 803 enrolled patients, 779 (97%) had available HBsAg levels at week 12, and 788 (98%) had HBsAg levels at week 24. Analysis of the association between HBsAg levels and declines at weeks 12 and 24 and response to treatment showed that the previously identified cut-offs from the respective studies (<1,500 for identification of patients with a high likelihood of response, >20,000 IU/mL or absence of a decline for identification of non-responders) were valid also in the pooled dataset.

At week 12, patients with HBsAg levels <1500 IU/mL had a probability of response of 45%, compared to 6% in patients with HBsAg >20,000 IU/mL (NPV: 94%, p<0.001, figure 3A). The probability of HBsAg loss was 15% for patients with an HBsAg level <1500 IU/mL at weeks 12 or 24. However, 6 patients with HBsAg >20,000 IU/mL at week 12 achieved HBsAg loss by 6 months post-treatment (6 out of 38 with HBsAg loss, or 16%). At week 24, only 4 of 162 patients with HBsAg >20,000 IU/mL achieved a response, and none cleared HBsAg (NPVs 98% and 100%, figure 3B).

Of patients who did not achieve a decline in HBsAg levels from baseline to week 12, 14% achieved a response (NPV 86%, p=0.001, figure 3C) and 2 cleared HBsAg (5% of all patients with HBsAg loss). Similar observations were made when decline was assessed at week 24 (Figure 3D).

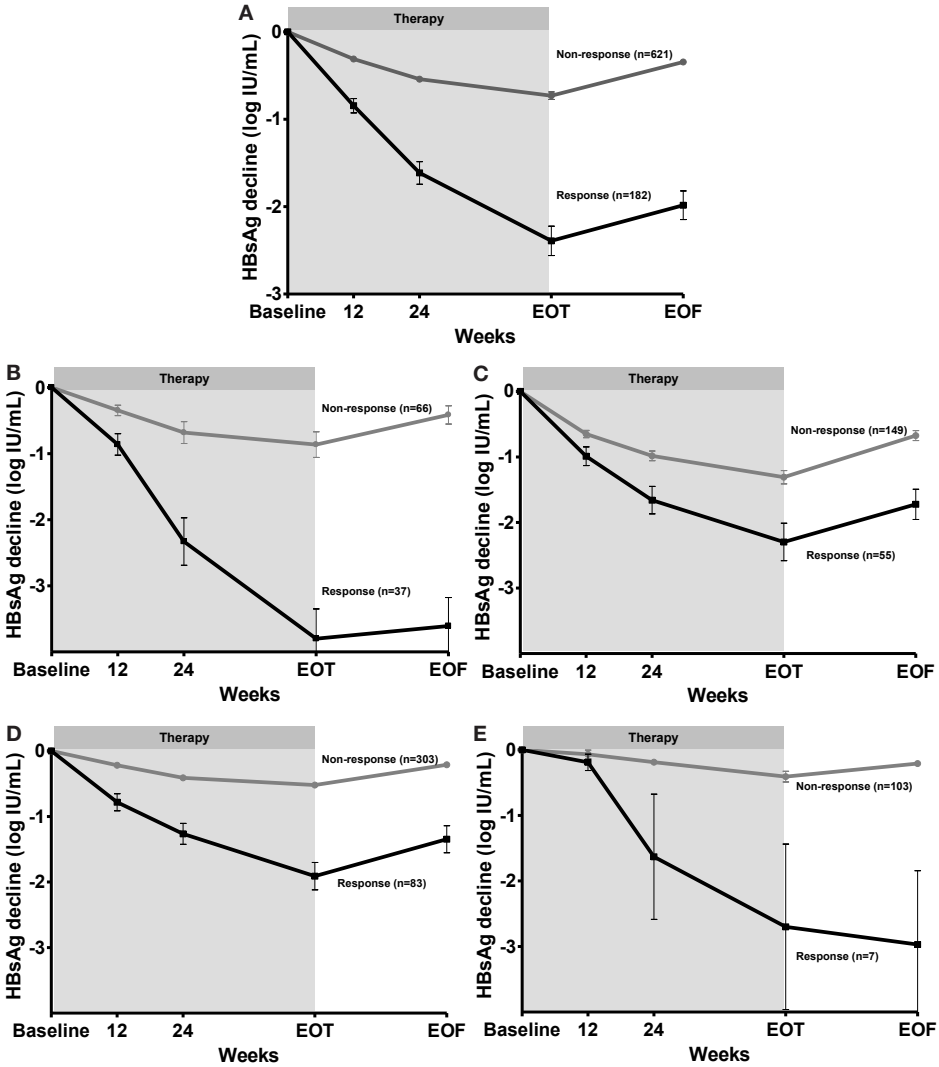

Figure 2: Mean change in serum HBsAg from baseline in patients with a response (HBeAg loss with HBV DNA <2,000 IU/mL) in the overall cohort (A) and by HBV genotype A through D (B-E).

On-treatment prediction of response at week 12 or 24 across HBV genotypes

The performance of the prediction rules varied across HBV genotypes A through D (table 2A and table 2B). At week 12, patients with HBV genotypes A, B or C with HBsAg levels <1500 IU/mL had a high probability of response (42% - 86%), whereas such low HBsAg levels were hardly ever achieved in genotype D patients. Furthermore, application of the two stopping-rules (absence of a decline from baseline or an HBsAg level >20,000 IU/mL) yielded varying results across the HBV genotypes. In

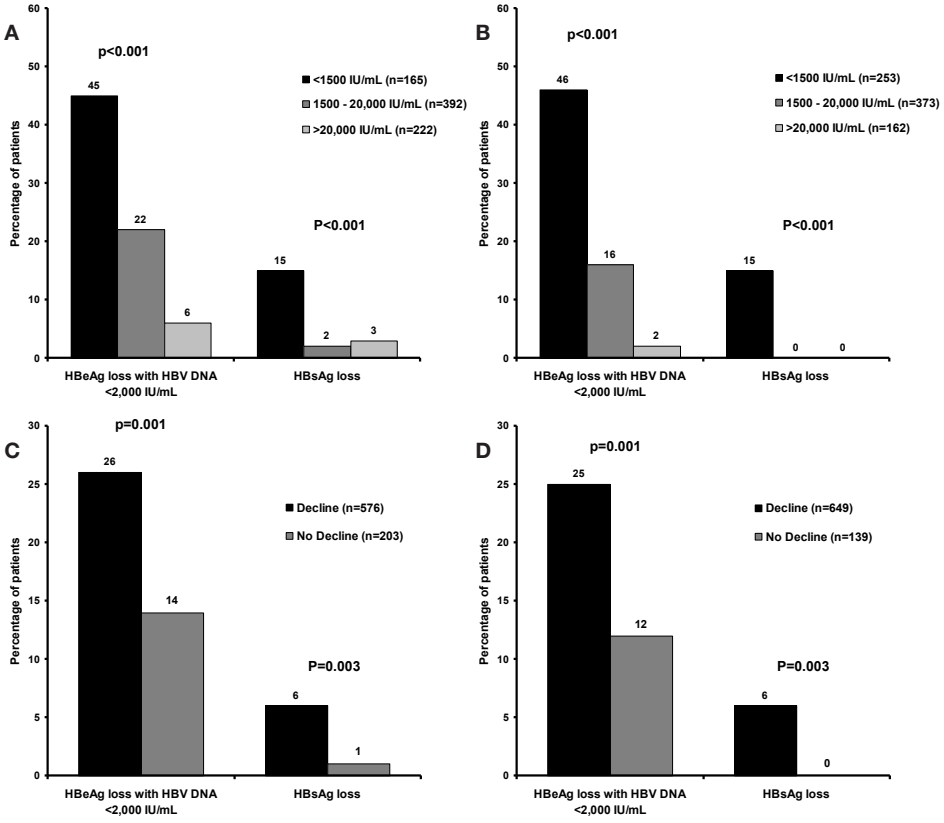

Figure 3: Application of the prediction rules based on HBsAg levels at week 12 (A) and 24 (B) and HBsAg declines at weeks 12 (C) and 24 (D).

patients with genotype A, relatively high negative predictive values for response (83 and 88%) were achieved with both stopping-rules. However, 4 of 38 (10%) genotype A patients with an HBsAg >20,000 IU/mL would subsequently achieve HBsAg loss (20% of all genotype A patients with HBsAg loss), compared to none of the patients without a HBsAg decline at week 12. Discontinuation of PEG-IFN in genotype A patients with HBsAg >20,000 IU/mL is therefore not indicated.

In patients with genotypes B and C, an HBsAg level >20,000 IU/mL at week 12 accurately identified patients with a low likelihood of response and HBsAg loss. In patients with HBV genotype D, very few patients achieved a response, and absence of a decline at week 12 best identified non-responders.

At week 24, an HBsAg level of >20,000 IU/mL accurately identified patients with a low likelihood of response and HBsAg loss (figure 3B) across all genotypes (NPVs for genotype A, B, C and D were 94%, 100%, 100% and 97% for response, respectively, and 100% for HBsAg loss among all HBV genotypes).

Table 2A. Observed response rates according to HBsAg level at week 12 stratified by HBV genotype. Response was defined as HBeAg loss with HBV DNA <2,000 IU/mL.

	Genotype A (n=98)			Genotype B (n=199)		
Response	**<1500**	**1500-20000**	**>20000**	**<1500**	**1500-20000**	**>20000**
No	2 (14%)	25 (60%)	35 (83%)	35 (58%)	75 (74%)	35 (92%)
Yes	12 (86%)	17 (41%)	7 (17%)	25 (42%)	26 (26%)	3 (8%)
HBsAg loss	**<1500**	**1500-20000**	**>20000**	**<1500**	**1500-20000**	**>20000**
No	3 (21%)	38 (91%)	38 (91%)	57 (95%)	100 (99%)	38 (100%)
Yes	11 (79%)	4 (10%)	4 (10%)	3 (5%)	1 (1%)	0 (0%)
	Genotype C (n=377)			**Genotype D (n=105)**		
Response	**<1500**	**1500-20000**	**>20000**	**<1500**	**1500-20000**	**>20000**
No	52 (58%)	178 (81%)	66 (99%)	2 (100%)	26 (93%)	72 (96%)
Yes	37 (42%)	43 (20%)	1 (2%)	0 (0%)	2 (7%)	3 (4%)
HBsAg loss	**<1500**	**1500-20000**	**>20000**	**<1500**	**1500-20000**	**>20000**
No	78 (87%)	219 (99%)	67 (100%)	2 (100%)	28 (100%)	73 (97%)
Yes	11(12%)	2 (1%)	0 (0%)	0 (0%)	0 (0%)	2 (3%)

Table 2B. Observed response rates according to HBsAg decline at week 12 stratified by HBV genotype. Response was defined as HBeAg loss with HBV DNA <2,000 IU/mL.

	Genotype A (n=98)		Genotype B (n=199)	
Response	**Decline**	**No decline**	**Decline**	**No decline**
No	47 (58%)	15 (88%)	130 (73%)	15 (68%)
Yes	34 (42%)	2 (12%)	47 (27%)	7 (32%)
HBsAg loss	**Decline**	**No decline**	**Decline**	**No decline**
No	62 (76%)	17 (100%)	173 (98%)	22 (100%)
Yes	19 (24%)	0 (0%)	4 (2%)	0 (0%)
	Genotype C (n=377)		**Genotype D (n=105)**	
Response	**Decline**	**No decline**	**Decline**	**No decline**
No	203 (77%)	93 (83%)	49 (93%)	51 (98%)
Yes	62 (23%)	19 (17%)	4 (8%)	1 (2%)
HBsAg loss	**Decline**	**No decline**	**Decline**	**No decline**
No	253 (96)	111 (99%)	52 (98%)	51 (98%)
Yes	12 (5%)	1 (1%)	1 (2%)	1 (2%)

Performance of the stopping-rules in patients treated with PEG-IFN monotherapy

Based on the varying performance of the stopping-rules across the HBV genotypes when applied at week 12, we compared the use of a stopping-rule based on an HBsAg level >20,000 IU/mL with a genotype specific approach (application of no decline for genotypes A and D and >20,000 IU/mL for genotypes B and C). A grid-search of cut-off

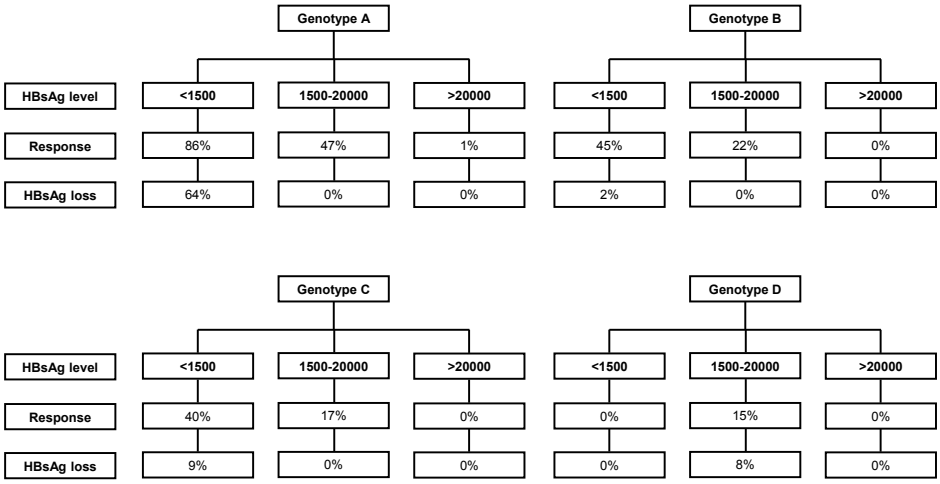

Figure 4: Relationship between HBsAg level (in IU/mL) at week 24 of treatment and response (HBeAg loss with HBV DNA <2,000 IU/mL) and HBsAg loss at 6 months off-treatment.

Table 3. Performance of proposed stopping-rules at week 12 and 24 for response (HBeAg loss with HBV DNA <2,000 IU/mL) and HBsAg loss across the HBV genotypes in patients treated with peginterferon monotherapy for one year.

Week	HBV genotype	Applied rule	N identified	NPV response	NPV HBsAg loss
Week 12	A (n=55)	No decline	13 (24%)	100%	100%
	B (n=120)	>20,000 IU/mL	24 (20%)	92%	100%
	C (n=225)	>20,000 IU/mL	45 (20%)	98%	100%
	D (n=54)	No decline	33 (61%)	97%	97%
Week 24	A (n=55)	>20,000 IU/mL	24 (44%)	96%	100%
	B (n=122)	>20,000 IU/mL	16 (13%)	100%	100%
	C (n=224)	>20,000 IU/mL	27 (12%)	100%	100%
	D (n=53)	>20,000 IU/mL	36 (68%)	100%	100%

points showed that the genotype specific approach at week 12 was superior to the use of an HBsAg >20,000 for all patients. At week 24, all patients with an HBsAg level >20,000 had a very low probability of response, irrespective of HBV genotype and it was therefore applied to all patients. The proposed algorithm performed excellently when applied on the patients treated with PEG-IFN monotherapy (table 3 and figure 4). Figure 4 shows the probability of response according to HBsAg level at week 24, stratified by HBV genotype.

DISCUSSION

This study shows that quantification of HBsAg in HBeAg-positive patients receiving PEG-IFN may help individualise on-treatment decision-making. At week 24, all patients with HBsAg levels >20,000 IU/mL have a low probability of response, irrespective of HBV genotype, and PEG-IFN discontinuation is indicated. Use of HBV genotype specific stopping-rules may also be considered at week 12.

PEG-IFN is a powerful treatment option for HBeAg-positive CHB, but the limited response rates achieved in the general patient population, as well as the frequent side-effects, prohibit wide-spread use.[20] Previous studies have used serum levels of HBV DNA and HBeAg during PEG-IFN therapy to identify patients with a low probability of response.[21-23] HBeAg levels yielded higher negative predictive values than did HBV DNA levels, but both could only be confidently used after at least 24 weeks of therapy.[24] Unfortunately, HBeAg levels in serum are also influenced by presence of precore and core promoter mutants, which may impair the reliability of prediction.[10] Recent studies have therefore focussed on the use of serum HBsAg levels for monitoring of PEG-IFN efficacy. The current study, a pooled analysis of 803 patients from 3 of the largest global cohorts and treated with both formulations of PEG-IFN alfa, shows that HBsAg decline during PEG-IFN therapy is strongly associated with the occurrence of a response to treatment. Importantly, the pronounced HBsAg decline observed in responders was apparent across all major HBV genotypes. Given the association of HBsAg kinetics with response, several of us have attempted to use HBsAg levels at weeks 12 and 24 of treatment to estimate the probability of response. Sonneveld *et al.* showed that in a cohort of predominantly Caucasian patients, absence of a HBsAg decline from baseline at week 12 identified patients with a low likelihood of response. Conversely, Piratvisuth *et al*. found that only few patients with HBsAg >20,000 IU/mL at the same time-point achieved a response.[19] Subsequent studies have shown the suboptimal external validity of these prediction rules.[15,16] Interpretation was further hampered by the use of different definitions of response; HBeAg loss with HBV DNA <10,000 copies/mL in one study and HBeAg seroconversion in others.[14,19]

The current study finally resolves these issues by providing a pooled analysis of the patients enrolled in the previous studies, allowing for careful stratified analysis across HBV genotypes and using a clinically relevant definition of response. We defined response as HBeAg loss with HBV DNA <2,000 IU/mL, since this endpoint is highly durable[7,16] and since patients with low HBV DNA levels are less likely to develop HBV related liver complications or require antiviral therapy.[25-29] Our results indicate that, when assessed at week 12, both an HBsAg level >20,000 IU/mL as well as absence of a decline from baseline may identify non-responders to PEG-IFN, but the differences in performance across HBV genotypes warrant careful application. The requirement

for different HBsAg cut-offs across HBV genotypes at week 12 of treatment may partly reflect the differences in baseline HBsAg levels; patients with HBV genotypes A had substantially higher levels than those with genotype B or C, which may account for the observation that patients with genotypes A with HBsAg levels >20,000 IU/mL at week 12 may still achieve a response and HBsAg loss. Furthermore, a recent study in a cohort of mostly patients with genotypes A and D showed that HBeAg-positive patients with only detectable wildtype virus (ie. no detectable precore and/or core promoter mutants) have both higher baseline levels of HBsAg and a higher probability of HBsAg loss after PEG-IFN therapy.[10] The high rate of response and HBsAg loss observed in genotype A patients with HBsAg >20,000 IU/mL at week 12 (17% and 10%, respectively) is an important finding, and shows that HBV genotyping is essential if a week 12 prediction-rule is to be used in areas where HBV genotype A is prevalent.

Fortunately, an HBsAg level >20,000 IU/mL at week 24 may be confidently used as a stopping-rule for all patients with high NPVs for response and HBsAg loss, irrespective of HBV genotype. Given the wide availability of HBsAg quantification platforms, the low cost of the test, and the excellent predictive performance observed in the current study, assessment of the HBsAg concentration at week 24 should be considered a vital part of optimal PEG-IFN therapy.

Side-effects and patient preferences should also be taken into consideration, and the performance of the prediction algorithm should be re-evaluated when data becomes available on extension of PEG-IFN therapy beyond 48 weeks or when a combination with nucleo(s)tide analogues other than LAM is used. Nevertheless, HBsAg based response-guided therapy is a valuable tool for optimization of PEG-IFN therapy, and can help with achieving higher response rates for every therapy course completed. Early identification of non-responders may help make this treatment modality more acceptable to patients, physicians and healthcare policy makers and possibly increase the cost-effectiveness of PEG-IFN in HBeAg-positive CHB.

In conclusion, the current study shows that HBsAg levels can be confidently used to guide therapy decisions in HBeAg-positive patients treated with PEG-IFN. Discontinuation of PEG-IFN treatment is indicated in all patients with HBsAg levels >20,000 IU/mL after 24 weeks of PEG-IFN therapy.

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Close monitoring of HBsAg levels helps classify flares during peginterferon therapy and predicts treatment response

9

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ABSTRACT

Background & Aims

ALT flares occur frequently during peginterferon(PEG-IFN) therapy. We related occurrence of flares to presence of precore(PC) and/or core promoter(BCP) HBV mutants and studied kinetics of HBeAg and HBsAg levels during flares.

Methods

Fifty of 214(23%) patients treated with PEG-IFN±lamivudine for 52 weeks experienced flares during treatment or 6 months off-treatment follow-up. Flares were host-induced (ALT elevation followed by HBV DNA decline,n=19), virus-induced (HBV DNA increase with subsequent ALT elevation,n=17) or indeterminate(n=14). Presence of wildtype (WT) or non-WT (detectable PC/BCP mutants) was studied by lineprobe assay.

Results

Fifty-eight percent of host-induced flares occurred in WT HBV patients, whereas 94% virus-induced flares occurred in patients with PC and/or BCP mutants(p=0.003). HBsAg loss was only achieved in patients with a host-induced flare, and WT patients with a host-induced flare cleared HBsAg in 64%. Analysis of individual patient data revealed that serum HBsAg levels only declined after a host-induced flare, whereas virus-induced flares were accompanied by stable or increasing levels of HBsAg. Patients with a host-induced flare achieved a mean HBsAg reduction of 3.24 logIU/mL by 6 months post-treatment, compared to 0.25 logIU/mL in virus-induced flares ($p<0.001$). Patients who achieved a decline in HBsAg of more than 0.5 logIU/mL within four weeks after the flare cleared HBsAg in 64% (7/11), increasing to 75% (6/8) in patients with a decline >1 logIU/mL ($p<0.001$).

Conclusions

Host-induced flares are associated with WT virus and may result in decline and clearance of HBV DNA, HBeAg and HBsAg. Monitoring of HBsAg levels during and after flares may help predict a favourable treatment outcome.

INTRODUCTION

Pegylated interferon (PEG-IFN) is a first-line treatment option for HBeAg-positive chronic hepatitis B (CHB), but results in a response in only a limited number of patients.[1-3] Spontaneous elevations of ALT, or flares, are a well-recognised phenomenon in patients treated with PEG-IFN, although the pathogenesis is not well understood.[4] Flares may occur in up to 25% of patients treated with a one year course of PEG-IFN, and have been associated with an increased probability of serological response.[5,6] However, flares can also have considerable detrimental effects, and may result in hepatic decompensation and death in patients with advanced cirrhosis.[7] We previously recognised different types of flares during PEG-IFN therapy of HBeAg-positive CHB.[5,8] In that study, host-induced flares, characterised by an ALT flare followed by a decline in HBV DNA, were associated with response to treatment, whereas virus-induced flares, ALT elevations that were preceded by an increase in HBV DNA, were not.[5] Why some patients develop host-induced flares where others experience virus-induced flares is currently unclear. Acute flares in untreated CHB patients were recently shown to be associated with presence of precore (PC) and basal core promoter (BCP) mutant virus.[9] These mutants are associated with impaired or absent production of HBeAg, and may be less susceptible to an immune response against HBeAg. Since ALT flares that are preceded by an increase in HBV DNA levels do not trigger an effective immune response,[10] it is possible that these virus-induced flares during PEG-IFN therapy are associated with presence of PC and BCP mutant virus.

It has recently become clear that serum levels of HBsAg reflect intrahepatic cccDNA and may be a marker for response to PEG-IFN.[11-14] Possibly, monitoring of serum HBsAg levels may therefore provide additional insight into the differences of host versus virus-induced flares and may help identify patients with a high likelihood of a favourable outcome after flares.

The aims of the current study were therefore to (1) relate presence of PC and BCP mutants before PEG-IFN therapy to occurrence of host or virus-induced flares, (2) to study the kinetics of HBeAg and HBsAg levels during these flares, and (3) investigate whether ALT and HBsAg monitoring can help predict therapeutic outcome.

PATIENTS AND METHODS

Patients

Patients treated with PEG-IFN alfa-2b alone or in combination with lamivudine (LAM) in an investigator-initiated multicenter randomized trial were enrolled into the current

study.[1,14,15] The inclusion and exclusion criteria for the original trial have previously been described elsewhere.[1] Patients were HBsAg positive for at least 6 months before randomization, were HBeAg positive, had elevated serum alanine aminotransferase (ALT) levels of >2, but <10 times the upper limit of normal (ULN), and had a serum HBV DNA concentration of more than 1.0 x 10^5 copies/mL. Patients were treated with PEG-IFN alfa-2b 100 µg weekly (PegIntron, Schering-Plough, Kenilworth, NJ, USA) in combination with placebo or LAM 100 mg (Zeffix, GlaxoSmithKline, Greenford, UK) daily for 52 weeks. Of the 266 patients in the original study, 214 had available data on baseline presence of PC and/or BCP mutants. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. All patients gave written informed consent according to the standards of the local ethics committees.

Laboratory measurements

Presence of PC and BCP mutants was assessed using the INNO-LiPA HBV PreCore assay (Innogenetics, Ghent, Belgium). This line probe assay allows for easy detection of PC (at nucleotide position G1896) and BCP (at nucleotide positions A1762 and G1764) mutants, even when only present as minority species.[16] Patients were subsequently classified as wildtype (WT, only WT virus detectable), or non-WT (PC, BCP or both mutants detectable). Serum HBV DNA, HBeAg and HBsAg were quantified in samples taken at baseline, during treatment, and during 6 months of off-treatment follow-up. Patients were seen at the outpatient clinic at least every 4 weeks during the study. HBV DNA quantification was performed using an in-house developed TaqMan polymerase chain reaction (PCR) assay (lower limit of quantification 400 copies/mL) based on the EuroHep standard.[17] HBsAg and HBeAg were measured using the Roche ELECSYS assay using a quantitative protocol (Roche Diagnostics).

Classification of flares

A flare was defined as a threefold increase in serum ALT compared with baseline levels.[5] The time point of the flare was defined as the time of the peak level of serum ALT. If a patient experienced multiple flares, the first was used for classification. Two types of flares were recognized, as described previously by Flink et al.[5] A flare was designated as *virus-induced* when the ALT peak was preceded by at least a 1 log increase in HBV DNA levels within four months. *Host-induced* flares were characterized as an ALT peak, without a preceding increase in HBV DNA, and were typically followed by a subsequent decrease in HBV DNA of at least 1 log within the next 4 months. Flares that could not be classified were designated *indeterminate*.[5]

Statistical analysis

Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.2 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

Role of the funding source

Financial support was provided by the Foundation for Liver and Gastrointestinal Research (SLO) in Rotterdam, the Netherlands. The funding source did not have influence on study design, data collection, analysis and interpretation of the data, writing of the report nor the decision to submit for publication.

RESULTS

Patient characteristics

Flares occurred in 50 of 214 patients with available PC/BCP data (23%). The characteristics of patients with a host-induced, virus-induced or indeterminate flare are shown in table 1. The frequency of flares was similar for patients with WT when compared to those with PC and/or BCP mutants (25 versus 23%, p=0.675).

Relationship between type and timing of flare and presence of PC and BCP mutants

The type of flare differed according to presence of mutants; host flares occurred predominantly in patients with WT virus (58%), whereas virus-induced flares almost always occurred in patients with non-WT (16/17, or 94%). Of the 76 patients with WT, 11 (14%) experienced a host-induced flare, compared to 6% of patients with non-WT (p=0.033). Conversely, 12% of non-WT patients experienced a virus-induced flare, versus only 1 of 76 (1%) of WT patients (p=0.008). Of the 25 (50%) flares that occurred during treatment, 15 (60%) occurred in patients with WT virus. Interestingly, the timing of the flare was also associated with presence of WT or non-WT (p=0.004, figure 1). Presence of PC and/or BCP mutants at baseline was independently associated with the occurrence of a virus induced flare, also after adjustment for HBV genotype, patient age and use of combination therapy (adjusted odds ratio for virus induced flare: 7.04, p=0.024).

Table 1: Characteristics of the study cohort

Characteristics	Flare			No flare (n=164)	p
	Host (n=19)	Virus (n=17)	Indeterminate (n=14)		
Demography					
Mean (SD) age, years	42 (12.8)	27 (9.8)	34 (11.4)	33.5 (12.4)	0.005
Male	15 (79%)	14 (82%)	12 (86%)	126 (77%)	0.846
PEG-IFN Monotherapy	7 (37%)	3 (18%)	14 (100%)	80 (49%)	<0.001
Race					0.221
Caucasian	15 (79%)	14 (82%)	8 (57%)	120 (73%)	
Asian	1 (5%)	1 (6%)	4 (29%)	34 (21%)	
Other	3 (16%)	2 (12%)	2 (14%)	10 (6%)	
Laboratory results					
Mean (SD) ALT*	3.1 (1.3)	3.2 (1.7)	2.6 (1.0)	4.7 (3.3)	0.005
Mean (SD) HBV DNA, log c/mL	9.3 (0.5)	9.2 (1.1)	9.3 (0.6)	9.1 (0.9)	0.505
Mean (SD) HBsAg, log IU/mL	4.6 (0.4)	4.4 (0.7)	4.4 (0.5)	4.4 (0.6)	0.375
Mean (SD) HBeAg, log IU/mL	2.5 (0.9)	2.2 (0.8)	2.6 (0.6)	2.5 (0.7)	0.546
HBV Genotype					0.038
A	12 (63%)	2 (12%)	5 (36%)	55 (34%)	
B	2 (11%)	2 (12%)	0 (0%)	15 (9%)	
C	0 (0%)	1 (6%)	4 (29%)	24 (15%)	
D	4 (21%)	12 (71%)	4 (29%)	65 (40%)	
Other/mixed	1 (5%)	0 (0%)	1 (7%)	5 (3%)	
Previous IFN therapy	3 (16%)	5 (29%)	2 (14%)	28 (17%)	0.615
INNO-LiPA result					0.007
Wildtype	11 (58%)	1 (6%)	7 (50%)	57 (35%)	
Non-wildtype	8 (42%)	16 (94%)	7 (50%)	107 (65%)	
Response					
HBeAg loss	11 (58%)	2 (12%)	1 (7%)	63 (38%)	0.003
Combined response	8 (42%)	0 (0%)	0 (0%)	33 (20%)	0.003
HBsAg loss	8 (42%)	0 (0%)	0 (0%)	9 (6%)	<0.001

*Multiples of upper limit of the normal range
#HBeAg loss and HBV DNA <10,000 copies/mL at week 78

Relationship between type of flare, presence of mutants and response at week 78

Host-induced flares resulted more frequently in HBeAg loss than virus induced flares (58% versus 12%, p=0.001). HBeAg loss with HBV DNA levels <10,000 copies/mL and HBsAg clearance were achieved in 42% of patients with a host-induced flare, and in none of the patients with virus-induced or indeterminate flares (p<0.001). HBV DNA undetectability was observed in 5 (26%) of patients with a host induced flare,

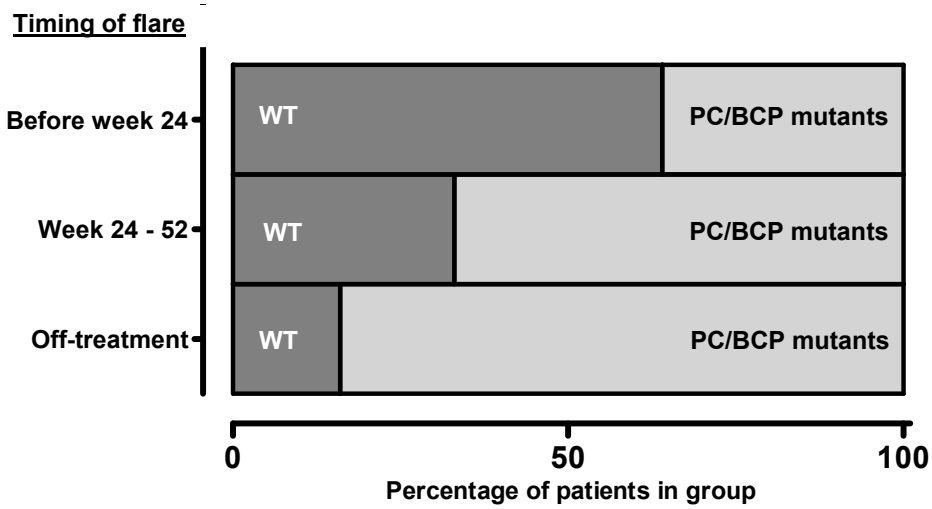

Figure 1: Proportion of patients with wildtype or precore and/or core promoter mutants according to the timing of the flare. Twenty-one patients experienced a flare before week 24, 4 between week 24 and 52, and 25 between week 52 and the end of the study.

compared to none of patients with other types of flares (p=0.011). Importantly, in the subgroup of WT patients with a host-induced flare (n=11), HBsAg clearance was achieved in 64% (7 / 11, p<0.001).

Monitoring of HBeAg and HBsAg levels during flares in individual patients

We analysed individual patients' changes in ALT, HBV DNA, HBeAg and HBsAg monthly during PEG-IFN therapy and up to 6 months post-treatment. As shown in figure 2A and 2B, host-induced flares were characterised by a steep ALT flare, followed by pronounced declines of HBV DNA, HBeAg and HBsAg. In contrast, virus-induced flares typically showed an increase in HBV DNA before ALT elevation, and serum levels of HBeAg and HBsAg remained either stable (figure 2C) or temporarily increased (Figure 2D).

Pronounced HBsAg decline in patients with a host induced flare

Only patients with a host-induced flare achieved substantial HBV DNA decline by week 78 (HBV DNA decline from baseline was 4.21, 1.51 and 0.58 log copies/mL, in patients with host, virus or indeterminate flares, respectively [p<0.001]). Importantly, similar HBeAg reductions were achieved in patients with host and virus-induced flares, whereas only patients with a host-induced flare achieved a pronounced HBsAg decline at 6 months post-treatment; HBeAg declines were 0.84, 1.02 and 0.25 log IU/mL at week 78 (p=0.309) in patients with a host-induced, virus-induced

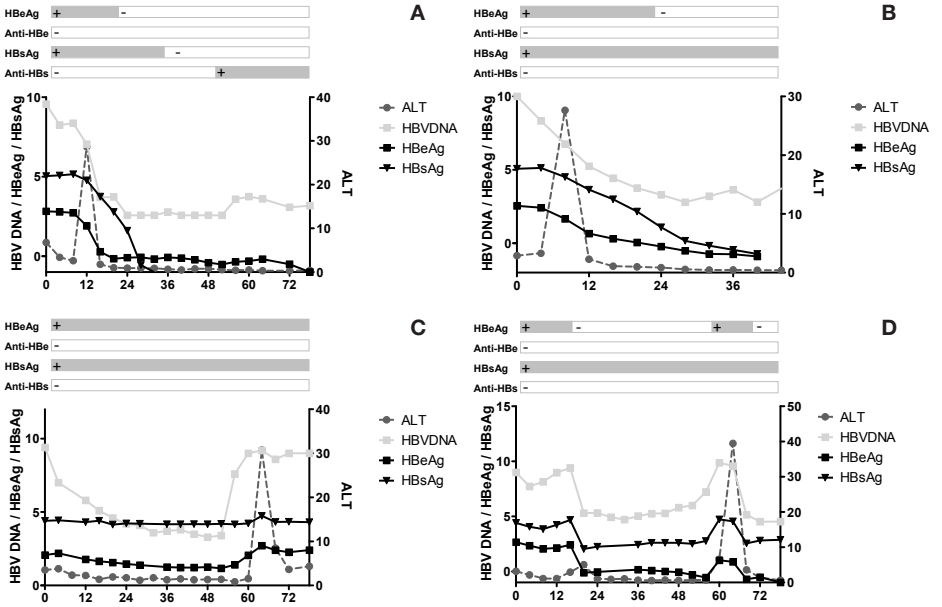

Figure 2: Kinetics of ALT, HBV DNA, HBeAg and HBsAg in two patients with a host-induced flare (A and B) and in two patients with a virus-induced flare (C and D). Time in weeks, HBV DNA in log copies/mL, HBeAg and HBsAg in log IU/mL and ALT in times upper limit of normal

or indeterminate flare, compared to HBsAg declines of 3.24, 0.25 and 0.31 log IU/mL, respectively ($p<0.001$).

HBsAg decline after flare predicts HBsAg clearance

Mean HBsAg levels at the peak of ALT during the flare were simialr in patients with a host-induced, virus-induced or indeterminate flare ($p=0.562$). However, pronounced HBsAg declines immediately after the flare were achieved only in patients with a host-induced flare: mean declines were 0.78 and 1.29 log IU/mL at 1 and 2 months after the ALT peak in patients with a host-induced flare. This contrasted strongly with the stable levels of HBsAg observed in patients with a virus-induced or an indeterminate flare (mean increase two months after ALT peak 0.02 and 0.16 log IU/mL, respectively, $p=0.002$). The probability of an HBsAg decline of at least 0.5 log IU/mL was 53% in patients with a host induced flare, 8% in patients with a virus induced flare and 7% in patients with an indeterminate flare ($p=0.003$). Patients who achieved a decline of more than 0.5 log IU/mL during the first month after the peak of the flare had a probability of HBsAg clearance of 64% (7 / 11), increasing to 75% (6 / 8) in patients with a decline > 1 log IU/mL ($p<0.001$ for both).

DISCUSSION

The current study shows that host-induced flares occur more frequently in patients with only WT virus, and result in pronounced declines of HBsAg levels. Frequent monitoring of serum HBsAg levels during flares may help predict a favourable outcome.

Spontaneous elevations of ALT are frequently encountered in the natural history of CHB, and may herald progression from the immunotolerant phase of HBV infection to immune clearance and eventual HBeAg seroconversion.[18] However, flares that occur during or after discontinuation of nucleo(s)tide analogues (NA) therapy hardly ever lead to virological remission, and may result in hepatic decompensation.[19,20] Flares that occur after NA discontinuation do not trigger an adequate immune response[10], suggesting these flares are different from flares that induce disease remission in untreated patients. Flink *et al*. showed that different types of flares occur during PEG-IFN therapy, and showed that where host-induced flares frequently resulted in therapy response, virus-induced flares did not.[5] The current study shows that these virus-induced flares occur more frequently in patients where PC and BCP mutants are present before PEG-IFN initiation. Since these mutants have a reduced production of HBeAg, they are less susceptible to a PEG-IFN induced immune response against HBeAg, which could result in positive selection and a subsequent virus-induced flare not unlike those observed in patients with viral breakthrough during NA based therapy.[19] A relationship between type of flare and the host immune system should also be investigated. It has been shown that flares that do not result in viral clearance may result in hepatic decompensation or death, and that presence of PC and BCP mutants may more frequently be found in patients suffering from such acute exacerbations.[4,19,21] Given that patients with detectable PC and BCP mutants before PEG-IFN initiation are unlikely to achieve a virological response [22] and are more likely to experience virus-induced flares, it appears that patients with PC and BCP mutants are less suitable candidates for PEG-IFN therapy.

Several studies have shown that the serum level of HBsAg is a good marker for immune control, both in the natural history and during PEG-IFN therapy.[12] Levels of HBsAg are a proxy for intrahepatic transcriptionally active cccDNA, and a reduction in HBsAg levels may signify a decrease in intrahepatic cccDNA and induction of immune control over HBV.[12] In the current study, host-induced flares resulted in pronounced HBsAg declines that were sustained during post-treatment follow-up. Importantly, close monitoring of serum HBsAg levels during flares revealed that the effect of host-induced flares in reducing serum HBsAg levels was apparent even on the individual patient level. It was also clear that virus-induced flares do not result in a reduction of serum HBsAg levels, which is in line with the low response rates observed in these

patients. Furthermore, the induction of a decline in serum HBsAg levels during a flare appears to herald HBsAg clearance, suggesting that monitoring of HBsAg levels in patients experiencing flares during PEG-IFN therapy may give valuable information on a patient's probability of treatment response. We propose to use frequent monitoring of serum HBsAg, since it is relatively cheap, may provide information on response to PEG-IFN therapy [3,14] and can help predict outcome of flares. A decline in HBsAg levels during the first months after the flare may identify patients with a very high likelihood of HBsAg loss.

Importantly, our findings were also valid when extending the window for HBV DNA increase or decline from 4 to 6 months before or after the flare, and when increasing the ALT level from 3 times to more than 4 times the level at baseline.

Concluding, host-induced flares during PEG-IFN therapy may result in HBsAg decline and subsequent clearance, whereas virus-induced flares do not. Monitoring of serum HBsAg levels in patients with flares may help identify patients with a high likelihood of subsequent HBsAg clearance.

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A comparison of two assays for quantification of hepatitis B surface antigen in patients with chronic hepatitis B

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ABSTRACT

Background & Aims

Serum Hepatitis B surface Antigen (HBsAg) levels correlate with hepatitis B virus intrahepatic covalently closed circular DNA and may predict response to treatment. Currently, 2 commercial platforms are available for HBsAg quantification in clinical practice, the Architect HBsAg QT and the Elecsys HBsAg. We aimed to directly compare the results of these assays.

Methods

HBsAg levels were measured in 1427 serum samples from HBeAg-positive chronic hepatitis B patients who participated in a randomized trial of peginterferon alfa-2b ± lamivudine. Samples were extracted from our serum bank, thawed, and subsequently analysed for HBsAg levels using both assays.

Results

Of 1427 samples, 242 (17%) were taken before and 1185 during the treatment phase of the study. Distribution of HBV genotypes was 447 (31%) genotype A, 125 (9%) B, 210 (15%) C and 534 (37%) D. Correlation between Architect and Elecsys results was high ($r=0.96$, $p<0.001$). By Bland-Altman analysis, agreement between the two assays was close (mean difference between Architect and Elecsys: -0.01 logIU/mL, 95%CI: -0.55 - 0.52 logIU/mL), also when analysed separately for HBV genotypes A-D. Additionally, the performance of our recently published stopping rule for HBeAg-positive patients treated with peginterferon was comparable: the negative predictive values were 96% and 98% for Elecsys and Architect, respectively.

Conclusions

There is a high correlation and close agreement between quantitative HBsAg measurements conducted with the Architect and the Elecsys. Clinical prediction rules derived from data from one platform can be applied on the other; both can therefore be used in clinical practice.

INTRODUCTION

Chronic hepatitis B (CHB) is an important global health problem, with over 350 million people being chronically infected.[1] Hepatitis B surface Antigen (HBsAg) is an established marker of infection with the hepatitis B virus (HBV), and is therefore often used as a screening tool.[1] In addition to its use as a qualitative marker, recent innovations have allowed for the quantitative assessment of HBsAg in serum. The clinical relevance of HBsAg levels is derived from its correlation with intrahepatic HBV covalently closed circular (ccc)DNA, the main replicative template of HBV.[2-3] Through this association, serum HBsAg is hypothesized to be a marker for immunological response to therapy, independent of virological response as measured using HBV DNA levels.

Several clinical applications of HBsAg levels have been described. For example, HBsAg levels appear to differentiate patients in the inactive carrier state (persistent HBeAg negativity, with HBV DNA <2,000 IU/mL, normal ALT[4]) from those with active disease or from inactive carriers with a high probability of subsequent relapse. [5-7] Furthermore, recent studies have shown that on-treatment HBsAg levels are predictive of a durable off-treatment response to peginterferon (PEG-IFN), and can accurately identify non-responders early during therapy, both in HBeAg-positive,[8-10] and HBeAg-negative patients.[11-12]

METHODS

Assays

HBsAg was quantified using the Architect platform (Abbott Laboratories, Abbott Park, IL, USA) and Elecsys HBsAg (Roche Diagnostics, Indianapolis, IN, USA). HBsAg testing was performed according to the manufacturer's package insert and with test kits from a single lot. For the Architect, sample material and anti-HBs-coated paramagnetic microparticles are combined. After a washing step, acridinium labelled anti-HBs conjugate is added, and after another washing cycle, pre-trigger and trigger solutions are added. The subsequent chemiluminescent reaction is measured in relative light units, which can be converted directly to HBsAg units. The range of the assay is 0.05 – 250 IU/mL, and if results were outside of this range, material was diluted according to the manufacturer's recommendations (package insert HBsAg, Abbott Laboratories 2008). The Elecsys HBsAg assay is an immunoassay that measures HBsAg using a sandwich principle: first a complex is formed with two monoclonal HBsAg-specific antibodies, one of which is biotinylated, the other labelled with a ruthenium complex. After addition of streptavidin-coated microparticles, the complexes bind to the solid phase through interaction of biotyn and streptavidin. The mixture is subsequently

aspirated into a measuring cell, where application of a voltage then induces chemiluminescent emission, which is measured by a photomultiplier. The measured results are compared to a cut-off value obtained through HBsAg calibration. The obtained signal to cut-off index may then be converted to IU/mL using a WHO standard conversion factor of 0.055 IU/mL (all methodology as per Elecsys package inserts for quantitative HBsAg measurement, Roche Diagnostics 2011). All assays were performed at the Erasmus MC University Medical Center in Rotterdam, the Netherlands.

Samples

Samples for this study were derived form a multicenter randomized trial investigating the efficacy of 52 weeks of PEG-IFN alfa-2b ± lamivudine for the treatment of HBeAg-positive CHB.[14] In- and exclusion criteria for this study have been previously published. [14] Samples were stored after the study in a large serum bank. For this study, samples were thawed and serum was extracted for quantitative HBsAg measurements using the two assays. HBV genotype was assessed using the INNO-LiPA line-probe assay.[15]

Statistical analysis

After transformation to log(10) IU/mL, the results of the two assays were compared using correlation (Pearson) and Bland-Altman analyses. A p value of <0.05 was considered statistically significant. Additionally, the results of a previously reported stopping rule for PEG-IFN therapy, established using the Architect assay[10], were applied on the Elecsys data for comparison.

RESULTS

Samples

A total of 1427 samples were measured using both assays, of which 242 (17%) were samples taken before treatment and 1185 (83%) were taken during the treatment phase. The samples retested for this study represent a random subset of the original study population.[10]

Comparison between Architect and Elecsys measurements

A correlation of measurements conducted with the two assays is shown in Figure 1; correlation between the assays was excellent ($r=0.96$, $p<0.001$). Using regression analysis, it was determined that $HBsAg_{Architect} = 0.979 * (HBsAg_{Elecsys}) + 0.074$. Bland-Altman analysis (depicted in Figure 2) revealed a close agreement between the two tests: the results of the Elecsys were on average only 0.01 log IU/mL higher than

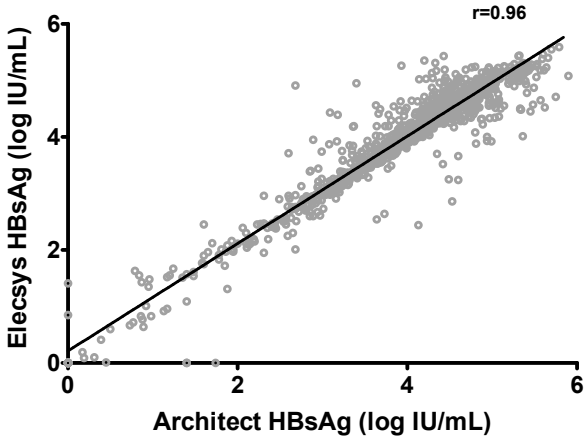

Figure 1: Correlation between HBsAg measurements using the Architect HBsAg QT assay and the Elecsys HBsAg.

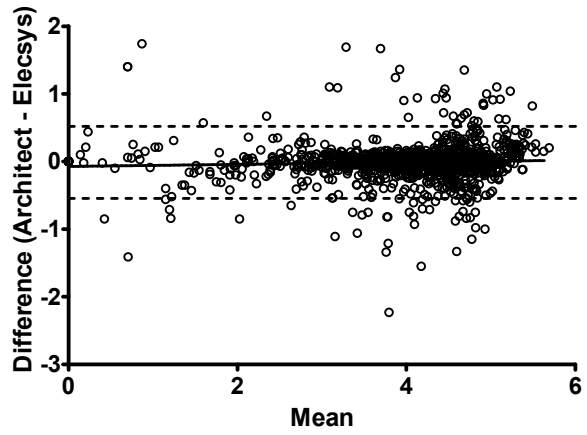

Figure 2: Bland-Altman plot of HBsAg measurements using the Architect HBsAg QT assay and the Elecsys HBsAg. Dashed lines represent 95% confidence limits.

the Architect assay. Overall, the range for the mean difference between the two test results (± 2 standard deviations) was -0.55 through 0.52 log IU/mL. Additionally, in 83.4% of paired samples the difference between the two assays was less than 0.25 log IU/mL, in 93.6% less than 0.5 log IU/mL and in 98.2% the difference between the two test results was less then 1 log IU/mL.

Comparison according to HBV genotype

Of the 1427 samples, 447 (31%) were taken from patients with HBV genotype A, 125 (9%) from patients with genotype B, and 210 (15%) and 534 (37%) from patients with genotypes C and D, respectively. The remaining samples (n=111, 8%) were from patients who harboured different or mixed HBV genotypes, and were not considered

Table 1: Bland-Altman analysis per genotype

Genotype	Bias	95% limits of agreement	
		- 2SD	+ 2SD
A (n=447)	-0.051	-0.555	0.454
B (n=125)	0.063	-0.414	0.540
C (n=210)	-0.053	-0.387	0.281
D (n=534)	0.011	-0.610	0.631

Overview of the results of the Bland-Altman comparison of the Architect versus the Elecsys HBsAg data in samples from patients with HBV genotypes A, B, C and D. SD, Standard deviation.

for this part of the analysis. The results of the two assays were comparable, within narrow confidence limits, irrespective of HBV genotype (table 1).

Retesting of samples with high divergence

A divergence of >1 log IU/mL was found in 25 samples (1.8%). High divergence was not related to HBV genotype or treatment week. Sufficient serum for retesting on both platforms was available in 20 (80%). The test results observed in the retested samples were highly correlated (0.99, $p<0.001$), and the mean difference between the results for the Architect when compared to the Elecsys was -0.10 log IU/mL, with none of the samples with detected HBsAg diverting >0.5 log IU/mL. The mean differences between first and second measurement were -0.02 log IU/mL for the Architect and 0.02 log IU/mL for the Elecsys.

Comparison of the performance of a threshold based stopping-rule

Recently, we have reported a stopping-rule for HBeAg-positive patients treated with PEG-IFN, based on the presence or absence of a decline in serum HBsAg levels from baseline. Patients without a decline from baseline to week 12 of treatment (log ($HBsAg_{week12}$) – log ($HBsAg_{baseline}$)>0) had a very low probability of response (<5%), and we advised to discontinue therapy in these patients.[10] For the current study, we applied this threshold based rule on the subset of patients that had both Architect and Elecsys data available. For this analysis, a representative subset of 181 patients of the original study (out of 221, 82%) were included. As shown in table 2, the stopping

Table 2. Positive and negative predictive values of the stopping-rule.

		Architect				Elecsys			
	Response	**No**	**Yes**	**PPV**	**NPV**	**No**	**Yes**	**PPV**	**NPV**
Any decline week 12	Yes	97	30	24%	–	101	29	22%	–
	No	53	1	–	98%	49	2	–	96%

Response was defined as HBeAg loss and HBV DNA<10,000 copies/mL 6 months after discontinuation of treatment. NPV, negative predictive value; PPV, positive predictive value.

rule performed very well within the subset of patients included in this analysis, and the positive predictive values (PPVs) and negative predictive values (NPVs) obtained with the two assays were very similar (NPVs: 96% for the Elecsys, compared to 98% for the Architect).

DISCUSSION

This is the first large study comparing the two major commercial platforms, Architect and Elecsys, for HBsAg quantification in patient sera. We found a very close agreement between the assays, irrespective of HBV genotype. Both assays can therefore be used for HBsAg quantification in clinical practice in HBeAg-positive patients.

Presence of HBsAg is commonly used as a screening tool for infection with HBV, and is often detectable in serum before evidence of liver inflammation is present.[16] However, recent technological advancements have allowed for the quantification of HBsAg in serum. The clinical relevance of these HBsAg levels is derived from the apparent correlation with intrahepatic cccDNA levels, which are strong predictors of sustained response to treatment, but can only be assessed invasively through a liver biopsy.[3,17]

Recent studies have shown that HBsAg levels may be used in the monitoring of HBV therapy. During therapy with nucleos(t)ide analogues (NA), HBV DNA is potently suppressed to undetectable levels in a majority of patients after 48 weeks of treatment, but only moderate declines in HBsAg levels are achieved.[8,11,18] HBsAg and HBV DNA therefore seem largely uncorrelated during antiviral therapy, and HBsAg decline may signify an immunological response that is independent of HBV DNA suppression as achieved with NA based treatment. Monitoring of HBsAg levels during antiviral therapy for CHB may therefore provide additional information when compared to HBV DNA levels alone. One year of PEG-IFN therapy induces a pronounced decline in HBsAg levels, especially in patients who achieve an off-treatment sustained response.[8,11-12,19-20] The differences in HBsAg decline between responders and non-responders can be used to predict success of therapy.[10,12,20] Additionally, HBsAg decline during therapy with NA may be used to predict subsequent loss of HBsAg through prolonged follow-up.[21-22]

Most of the recent studies on HBsAg quantification were conducted using the Architect platform, but the manual for HBsAg quantification on the Elecsys was recently released (HBsAg Quant, Roche Diagnostics 2011).[23] The comparability of the results of the two assays was however still unknown, and the limited insight into the agreement between the two assays prohibited extrapolation of data and findings from studies using either platform to studies or clinics that use the other. The current study

was designed to address these issues, and we have now shown that the two assays provide comparable results. Importantly, there is a very close agreement between the two platforms, within ± 0.5 log IU/mL in 94% of the samples that were tested, and this close agreement is independent of HBV genotype.

In addition to the use of HBsAg in monitoring therapy efficacy, several reports have now provided stopping-rules for PEG-IFN therapy for CHB. A post-hoc analysis of the phase 3 study of PEG-IFN alfa-2a showed that lower HBsAg levels during treatment were associated with a sustained off-treatment response.[8] Similarly, we showed that HBeAg-positive patients without a decline at week 12 of therapy had a low probability of sustained response, and no chance of HBsAg loss, even through long term follow-up.[10] The current study shows that our findings reported from data gathered using the Architect can be extrapolated to measurements conducted with the Elecsys. This implies that published threshold or decline based prediction rules established using either platform may be used on the other as well, without losing predictive accuracy. Furthermore, our findings can also be extended to measurements conducted with other Cobas serology systems, which share characteristics with the Elecsys. This should make quantitative HBsAg diagnostics more easily accessible around the globe.

In summary, the current study shows a high correlation and agreement between quantitative HBsAg measurement conducted with the Architect and Elecsys assays, irrespective of HBV genotype. The performance of a clinical stopping rule established using Architect measurements was excellent when applied on Elecsys data. Both assays can therefore be applied for HBsAg quantification in clinical practice

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Hepatitis B e antigen levels and response to peginterferon: influence of precore and basal core promoter mutants

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ABSTRACT

Background

Hepatitis B e antigen (HBeAg) levels may predict response to peginterferon (PEG-IFN) but are also influenced by presence of precore(PC) and core promoter(BCP) mutants.

Methods

HBeAg was measured in 214 patients treated with PEG-IFN±lamivudine for 52 weeks. Patients were classified at baseline as wildtype (WT) or non-WT (detectable PC/BCP mutants). Combined response (HBeAg loss with HBV DNA <2,000 IU/mL), HBeAg response (HBeAg loss with HBV DNA >2,000 IU/mL) or non-response was assessed at week 78.

Results

Mean baseline HBeAg levels were 2.65 log IU/mL in combined responders, 2.48 in non-responders and 2.24 in HBeAg responders (p=0.034). Baseline HBeAg levels were not associated with combined response after stratification by WT/non-WT. Within the PEG-IFN monotherapy group (n=104), patients with HBeAg <1 log IU/mL at week 24 had a higher probability of combined response (29% versus 12%, p=0.041). After stratification by WT/non-WT, WT patients with HBeAg <1logIU/mL at week 24 had a probability of combined response of 78% (versus 19% in patients with >1log IU/mL, p<0.001), whereas no difference in response rates was observed in non-WT patients (p=0.848).

Conclusions

The relationship between HBeAg levels and response to PEG-IFN depends upon the presence of PC/BCP mutants. HBeAg levels should therefore not be routinely used to select patients for PEG-IFN, nor for monitoring of therapy.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection may ultimately result in severe liver-related morbidity and mortality, and treatment of chronic hepatitis B (CHB) is therefore indicated in patients with signs of persistent liver inflammation.[1] Current treatment options for CHB consist of nucleo(s)tide analogues (NA) and (pegylated) interferons (PEG-IFN). Despite the recent registration of potent NA that are able to maintain undetectable HBV DNA levels through prolonged therapy, PEG-IFN remains an important first-line treatment option, especially in hepatitis B e antigen (HBeAg)-positive disease, as finite PEG-IFN therapy results in an off-treatment sustained response in about 25-30% of patients.[2-5] Response to PEG-IFN therapy in these patients is accompanied by high rates of hepatitis B surface antigen (HBsAg) seroclearance, a reduced incidence of hepatocellular carcinoma (HCC) and prolonged survival.[6,7]

The clinical application of PEG-IFN is limited by high costs, frequent occurrence of side-effects, and relatively low probability of response.[5] Selecting patients for PEG-IFN based on an individual's probability of response would help optimize application of this agent, but published prediction models, incorporating host and viral factors, provide only limited discrimination.[8,9] Recent studies have therefore focused on on-treatment predictors of response, including serum HBV DNA and HBsAg levels.[10-15] Yet another study focused on the use of HBeAg levels to predict response to PEG-IFN for HBeAg-positive CHB, and showed that patients with low HBeAg levels at baseline had a higher probability of HBeAg seroconversion 6 months after treatment discontinuation.[10] Unfortunately, several independent studies have shown that a considerable number of patients who achieve clearance of HBeAg have persistently high HBV DNA levels.[2,16] We recently showed that presence of viral mutants harboring mutations in the precore (PC) and basal core promoter (BCP) regions may prohibit achievement of virological response after HBeAg clearance.[17] Presence of these mutants may also influence HBeAg production and thus HBeAg levels in serum.[18] We therefore hypothesized that presence of PC and BCP mutants influences the association between HBeAg levels and response to PEG-IFN therapy.

The aim of the current study was therefore to investigate the association between HBeAg levels and response to PEG-IFN in relation to the presence of PC and BCP mutants.

PATIENT AND METHODS

Patients

HBeAg levels were assessed in HBeAg-positive CHB patients who were previously enrolled in an investigator-initiated international multicenter randomized controlled trial.[2,3] In- and exclusion criteria for this study have previously been described elsewhere.[3] Patients were treated with PEG-IFN alfa-2b 100 µg weekly (PegIntron, Schering-Plough, Kenilworth, NJ, USA) in combination with placebo or lamivudine (LAM) 100 mg (Zeffix, GlaxoSmithKline, Greenford, UK) daily for 52 weeks. Inclusion criteria for the present analysis were completion of the 26-week follow-up phase of the main study, availability of data on presence of PC or BCP mutants at baseline and availability of a baseline serum sample for HBeAg measurements. Of the 266 patients in the initial study, 214 fulfilled these criteria.

The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. All patients gave written informed consent according to standards of the local ethics committees.

Laboratory measurements

Serum HBeAg was quantified in samples taken at baseline, during the treatment period (weeks 4, 8, 12, 24 and 52) and during follow-up (week 78) using the ELECSYS HBeAg assay (Roche Diagnostics, range 0.2 – 100 IU/ml). HBV DNA quantification was performed using an in-house developed TaqMan polymerase chain reaction (PCR) assay (lower limit of quantification 400 copies/mL) based on the EuroHep standard. HBsAg was measured using the ARCHITECT HBsAg assay (Abbott laboratories; range 0.05 - 250 IU/mL). [11,19] The presence of PC (at nucleotide position G1896) and BCP (at nucleotide positions A1762 and G1764) mutants was assessed using the INNO-LiPA HBV PreCore assay (Innogenetics, Ghent, Belgium).[17]

Statistical analysis

Response was defined as HBeAg loss with HBV DNA <2,000 IU/mL (~10,000 copies/mL; combined response), HBeAg loss with HBV DNA >2,000 IU/mL at week 78 (HBeAg response) or non-response.[8] Because therapy outcomes did not differ across the treatment arms, associations between baseline HBeAg levels and response were analysed for the pooled cohort. For on-treatment analyses only the patients treated with PEG-IFN monotherapy were analysed. Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.2 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All statistical tests were two-sided and were evaluated at the 0.05 level

of significance. The ability of HBeAg levels to discriminate between patients with and without a response was evaluated by Radio Operator Charateristic (ROC) analyses, and quantified using the area under the ROC curve (AUROC).

RESULTS

Baseline characteristics

Patients were predominantly male (78%), of Caucasian (73%) or Asian origin (19%), and harboured HBV genotypes A (35%), B (9%), C (14%) or D (40%). Other characteristics are shown in table 1. Median baseline HBeAg levels were 501.8 IU/mL (IQR 125 – 924) in the 214 patients, and HBeAg levels were log transformed for further analysis. Mean HBeAg levels significantly varied by HBV genotype; 2.59 log IU/mL for genotype A, 2.68 for B, 2.56 for C and 2.30 log IU/mL for genotype D (p=0.021 by ANOVA). Baseline HBeAg levels did not correlate with age (r=0.11, p=0.10), or ALT (r=-0.01, p=0.95), but did correlate with baseline HBV DNA (r=0.39, P<0.001) and HBsAg levels (r=0.31, P<0.001).

Relationship between presence of PC and/or BCP mutants and HBeAg levels

Baseline HBeAg levels were highest in patients with WT (2.77 log IU/mL), and 2.56 log IU/mL in patients with PC mutants, 2.04 log IU/mL in those with only BCP mutants, and 2.26 in patients with both PC and BCP mutants (p<0.001 by ANOVA, p<0.001 for WT versus non-WT). When adjusted for HBV genotype distribution, HBeAg levels were still higher in patients with WT versus those with PC and/or BCP mutants (2.81 versus 2.33 log IU/mL, p<0.001). To further explore the relationship between baseline HBeAg levels and presence of PC and/or BCP mutants, patients were allocated into groups according to baseline HBeAg levels: quartile 1 (baseline HBeAg <2.098 log IU/mL), quartile 2 (>2.098, <2.70 log IU/mL), quartile 3 (>2.70, <2.97 log IU/mL) and quartile 4 (>2.97 log IU/mL). Patients were also classified as WT only (n=76), or non-WT (PC and/or BCP mutants, n=138). As shown in figure 1A, the proportion of patients harboring only WT virus increased as baseline HBeAg level was higher (figure 1A).

Baseline HBeAg levels in relation to response

Among the total population of 214 patients 77 (36%) cleared HBeAg, 41 (19%) achieved a combined response of HBeAg loss with HBV DNA <2,000 IU/mL, and 17 (8%) cleared HBsAg at week 78. Baseline HBeAg levels were highest in patients who achieved a combined HBeAg and HBV DNA response (2.65 log IU/mL), and lowest in patients who cleared HBeAg but did not achieve HBV DNA <2,000 IU/mL

Table 1: Characteristics of the study cohort

Characteristics	PEG-IFN (n=104)	PEG-IFN + LAM (n=110)	Overall (n=214)
Demography			
Mean (SD) age, years	34.3 (13)	33.4 (12)	33.8 (13)
Male	84 (81%)	83 (76%)	167 (78%)
Race			
Caucasian	77 (74%)	80 (73%)	157 (73%)
Asian	21 (20%)	19 (17%)	40 (19%)
Other	6 (6%)	11 (10%)	17 (8%)
Laboratory results			
Mean (SD) ALT*	4.4 (3.1)	4.2 (3.0)	4.3 (3.0)
Mean (SD) HBV DNA, log c/mL	9.2 (0.80)	9.1 (0.98)	9.1 (0.90)
Mean (SD) HBsAg, log IU/mL	4.4 (0.54)	4.4 (0.65)	4.4 (0.60)
Mean (SD) HBeAg, log IU/mL	2.5 (0.70)	2.4 (0.70)	2.5 (0.70)
HBV Genotype			
A	40 (39%)	34 (31%)	74 (35%)
B	9 (9%)	10 (9%)	19 (9%)
C	15 (14%)	14 (13%)	29 (14%)
D	39 (38%)	46 (42%)	85 (40%)
Other/mixed	1 (1%)	6 (6%)	7 (3%)
INNO-LiPA result			
Wildtype	40 (39%)	36 (33%)	76 (36%)
Precore	25 (24%)	31 (28%)	56 (26%)
Core promoter	20 (19%)	27 (25%)	47 (22%)
Precore and core	19 (18%)	16 (15%)	35 (16%)
Response at week 78			
Combined response#	18 (17%)	23 (21%)	41 (19%)
HBeAg loss	36 (35%)	41 (37%)	77 (36%)
HBsAg loss	6 (6%)	11 (10%)	17 (8%)

*Multiples of upper limit of the normal range P<0.231
#HBeAg loss and HBV DNA <2,000 IU/mL at week 78
All comparisons of peginterferon alone versus peginterferon with lamivudine p > 0.23

(HBeAg responders, 2.24 log IU/mL). Non-responders had intermediate levels of 2.48 log IU/mL (p=0.034 by ANOVA). Analysis of the probability of response across HBeAg quartiles revealed that patients with the lowest (quartile 1) and the highest (quartile 4) HBeAg levels at baseline had a somewhat higher probability of HBeAg clearance at week 78 (42% and 43%, respectively), compared to 32% and 28% for patients in quartiles 2 and 3 (figure 2, p=0.259 by Chi-square). However, combined HBeAg and HBV DNA response was most frequently achieved in patients in the highest quartile of

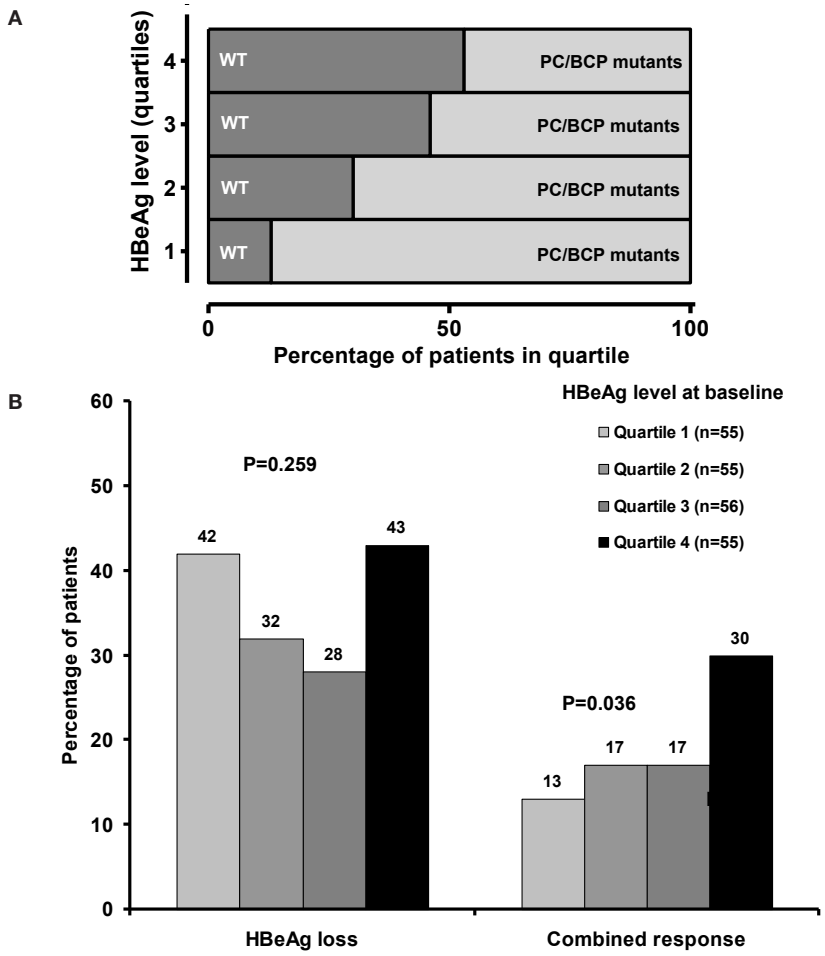

Figure 1: Baseline HBeAg levels; relation to presence of mutants and probability of response. The proportion of wildtype (WT) and non-WT (precore and/or core promoter mutants) by baseline HBeAg level (quartile 1 (baseline HBeAg <2.098 log IU/mL), quartile 2 (>2.098, <2.701 log IU/mL), quartile 3 (>2.701, <2.966 log IU/mL) and quartile 4 (>2.966 log IU/mL)) (figure 1A) and relationship between baseline HBeAg levels and response to PEG-IFN at 6 months post-treatment (figure 1B). Combined response was defined as HBeAg loss with HBV DNA <2,000 IU/mL at 6 months post-treatment.

HBeAg levels at baseline (30%, p=0.036, figure 1B). Similarly, patients in the highest quartile of HBeAg levels tended to have a higher probability of HBsAg clearance; probabilities were 4%, 7%, 9% and 11% across quartiles 1 through 4, respectively (p=0.14). Stratification by WT versus non-WT showed that within these groups, baseline HBeAg levels were not associated with combined HBeAg and HBV DNA response to PEG-IFN (figure 2).

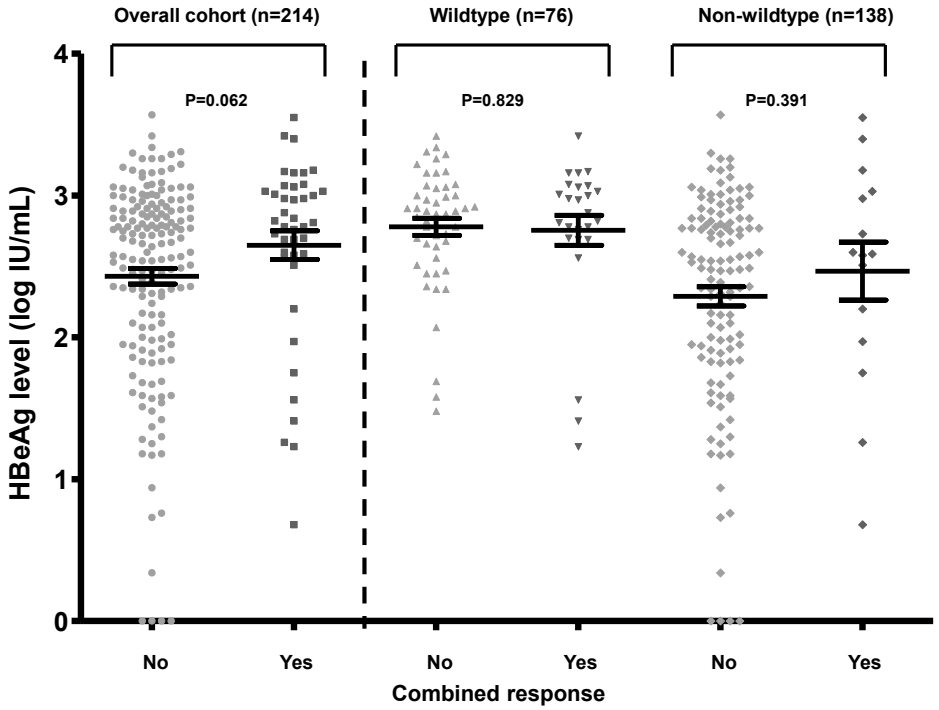

Figure 2: Baseline HBeAg levels and combined response. Relationship between baseline HBeAg levels and combined response (HBeAg loss with HBV DNA <2,000 IU/mL) at week 78, stratified by presence of wildtype virus or non-wildtype (precore and/or core promoter mutants). Combined response was defined as HBeAg loss with HBV DNA <2,000 IU/mL at 6 months post-treatment. WT, wildtype.

HBeAg levels during therapy

Mean baseline HBeAg levels were 2.53 and 2.42 log IU/mL in the monotherapy and combination therapy groups (p=0.23). HBeAg levels declined in both treatment groups, and end of treatment levels were 1.45 and 0.57 log IU/mL in the monotherapy and combination therapy groups, respectively (p<0.001, figure 3A). The more pronounced reduction in the patients receiving combination therapy was not sustained post-treatment; week 78 levels were 1.46 and 1.37 log IU/mL in the monotherapy and combination therapy groups, respectively (p=0.61).

On-treatment changes in HBeAg level according to response: comparison with HBsAg levels

The relationship between on-treatment changes in HBeAg level and off-treatment response at week 78 was analysed only in patients treated with PEG-IFN monotherapy (n=104). Baseline HBeAg levels were highest in patients who achieved a combined response, and declined progressively through 52 weeks of therapy and during the

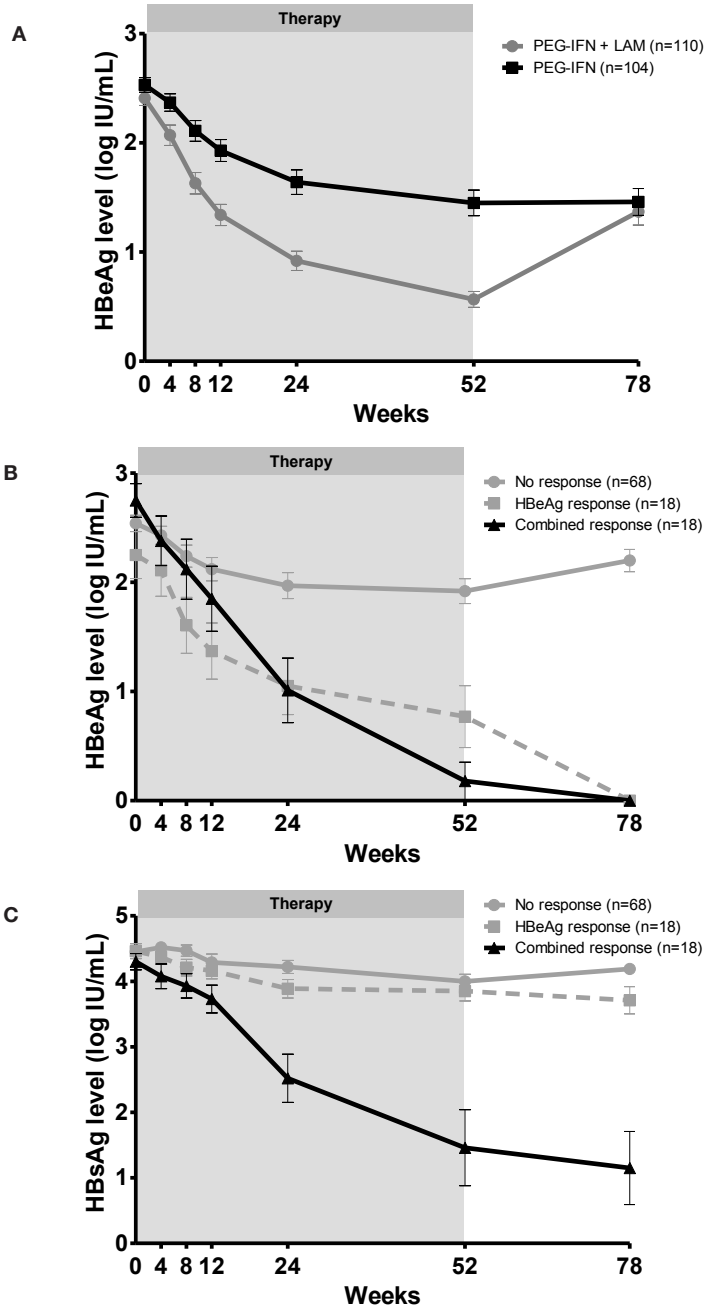

Figure 3: On-treatment HBeAg and HBsAg levels by treatment and response. HBeAg levels at baseline and during therapy across treatment arms (A), and levels of HBeAg (B) and HBsAg (C) in combined responders (HBeAg clearance with HBV DNA <2,000 IU/mL at week 78), HBeAg responders (HBeAg loss with HBV DNA >2,000 IU/mL) and nonresponders for patients treated with PEG-IFN monotherapy.

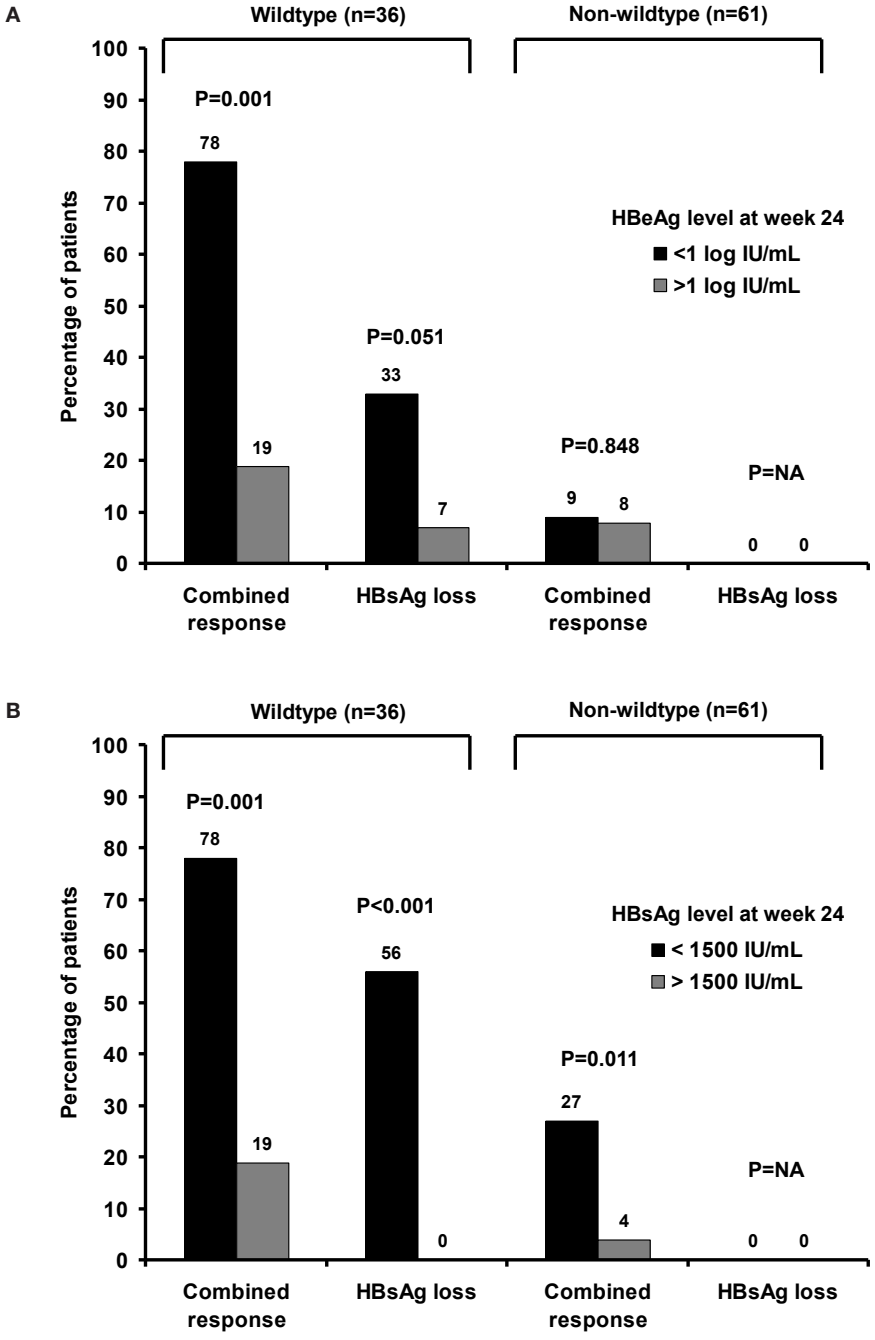

Figure 4: HBeAg and HBsAg levels at week 24 and response to treatment. Probability of response at week 78 by HBeAg (A) or HBsAg (B) level at week 24 stratified by presence of wildtype or non-wildtype (precore and/or core promoter mutants) at baseline.

off-treatment follow-up phase. Importantly, HBeAg levels remained higher in combined responders versus non-responders during the first 3 months and HBeAg levels were lower in combined responders versus HBeAg responders only after week 24 of treatment (figure 3B). HBeAg levels could therefore not be used to differentiate between HBeAg responders versus those with a combined response. For comparison, the HBsAg levels at the same time-points[14] are shown in figure 3C. Serum levels of HBsAg only showed a pronounced decline in patients with a combined response, whereas no or only a limited decline was observed in HBeAg responders or non-responders.[14]

Predicting response using on-treatment HBeAg or HBsAg levels

The use of on-treatment HBeAg levels for prediction of response at week 78 was again only evaluated in patients treated with PEG-IFN monotherapy. By ROC analysis, week 24 HBeAg levels were superior to week 12 levels (AUC 0.694 versus 0.503). Of the 97 patients with available serum at week 24, 31 (32%) had an HBeAg level less than 1 log IU/mL. Patients with an HBeAg level less than 1 log IU/mL at week 24 had a higher probability of HBeAg clearance (65% versus 23%, $p<0.001$), HBeAg seroconversion (58% versus 18%, $p<0.001$), combined response (29% versus 12%, $p=0.041$) and HBsAg clearance at week 78 (10% versus 3%, $p=0.167$). However, stratification by WT/non-WT status at baseline revealed that the higher probability of combined response in patients with HBeAg levels under 1 log IU/mL at week 24 was confined to those with WT virus at baseline, and absent in those with PC and/or BCP mutant virus (figure 4A). In contrast, low levels of HBsAg at week 24 (< 1500 IU/mL [20,21]) were associated with a higher rate of combined response, irrespective of the presence of mutants (figure 4B).

DISCUSSION

In this investigator initiated study, we have shown that the relationship between HBeAg levels and response to PEG-IFN therapy is highly dependent upon the presence of viral strains with mutations in the PC and BCP domains. HBeAg levels should therefore not be routinely used to monitor PEG-IFN therapy.

PEG-IFN is an effective treatment option for HBeAg-positive CHB, but the limited probability of response to this agent necessitates careful selection of patients.[1,5] Baseline serum HBeAg levels were previously shown to be associated with HBeAg seroconversion after PEG-IFN therapy, suggesting they may be used to select patients for PEG-IFN treatment.[10] Several long-term follow-up studies of patients treated with PEG-IFN have however shown that a considerable number of patients do not achieve low (< 2000 IU/ml) HBV DNA levels after HBeAg seroconversion.[2,16] These patients

are considered to have progressed to HBeAg-negative CHB,[22,23] and are thus at risk for the development of cirrhosis, liver cancer and death.[24-28]

For this reason we investigated whether serum HBeAg levels predict achievement of a combined HBeAg and HBV DNA response to PEG-IFN. Our study shows that patients who will achieve such a combined response have higher baseline HBeAg levels than patients who fail to clear HBeAg or those who achieve HBeAg loss but have persistent HBV DNA more than 2000 IU/mL. These somewhat counter-intuitive findings may be explained by the influence of PC and BCP mutant strains. We have previously reported that these mutants can be detected in a considerable number of HBeAg-positive patients[17], and the current study shows that patients with detectable mutants have lower HBeAg levels than those who do not. Since patients without detectable mutants at baseline have a high probability of HBV DNA and HBsAg clearance after HBeAg loss[17], absence of these mutants may explain the association between high HBeAg levels and a high probability of a response to PEG-IFN. Furthermore, we also hypothesize that the patients with the lowest HBeAg levels were already experiencing spontaneous transition of HBeAg-positive to HBeAg-negative CHB, with selection for PC and BCP mutants. This translated to the high rate of HBeAg loss, but low rate of subsequent HBV DNA clearance, in these patients. Our findings therefore show that HBeAg levels cannot be used to select patients for PEG-IFN therapy without considering the presence of viral mutants.

Considering the difficulty of selecting patients with a high probability of response, several recent studies have focussed on on-treatment predictors. We have previously reported that patients who achieve a combined response to PEG-IFN achieve strong HBsAg declines during treatment, and that patients who fail to achieve a decline have a low probability of response.[11,13-15] Fried *et al* reported a very strong association between low HBeAg levels at week 24 of treatment and HBeAg seroconversion at 6 months post-treatment. In concurrence with that study, we showed that a reduction of HBeAg levels to less than 1 log IU/mL by week 24 confers an increased probability of HBeAg clearance or seroconversion, as well as combined response and HBsAg clearance. These findings are in line with a previous report that suggested that early HBeAg clearance during PEG-IFN therapy predicts subsequent HBsAg clearance.[29] However, the current study also points out that the relationship between low HBeAg levels at week 24 of therapy and subsequent off-treatment response is confined to patients with WT virus, and virtually absent in those with PC and/or BCP mutants at baseline. Importantly, low serum levels of HBsAg remained a strong predictor of response, even among patients with PC and BCP mutants. Taken together, our findings do not support the routine use of HBeAg levels when monitoring response to PEG-IFN therapy, whereas they provide further evidence for the excellent predictive capabilities of HBsAg levels.

A possible caveat of our study pertains to the method we used to classify patients as WT/non-WT. The INNO-LiPA line probe assay is a sensitive method, but can only detect a few often encountered and widely acknowledged mutations, while others less frequently observed in previous studies are ignored.

In conclusion, we have shown that HBeAg levels do not adequately predict a combined HBeAg and HBV DNA response to PEG-IFN for HBeAg-positive CHB. Our findings are probably explained by the influence of PC and BCP mutant strains which are associated with both low HBeAg levels and failure to achieve a combined response. The relationship between HBeAg levels and response to PEG-IFN is therefore unpredictable, limiting the use of HBeAg levels as a sole predictor of treatment response in CHB. In contrast, serum levels of HBsAg were able to predict response in patients with and without detectable mutants.

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Quantification of serum hepatitis B surface antigen: is it useful for the management of chronic hepatitis B?

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INTRODUCTION

Chronic hepatitis B infection is a global health problem affecting more than 350 million people worldwide.[1] Prolonged liver inflammation caused by active infection with the hepatitis B virus (HBV) may result in progression to liver fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC) and death.[1]

Hepatitis B Virus Surface Antigen (HBsAg) represents, since the '60s, the hallmark of HBV infection because its concentrations can reach several hundred micrograms per milliliter.[2]. Recently, studies with newly available automated quantitative assays showed that serum HBsAg levels vary significantly during the different phases of chronic HBV infection and are inversely correlated with the immune control of HBV: the higher control, the lower HBsAg level.[3-6] These findings are consistent with the hypothesis that HBsAg serum levels reflect the complex interplay between virus and immune system and provide complementary information to viral load as measured using HBV DNA levels. Actually, serum HBsAg levels result not only from virions (42 nm, Dane particles), but mainly from non-infectious HBsAg particles (20 nm diameter filaments of variable length and 20-22 nm spheres) which do not contain viral nucleic acids and exceed virions by a factor ranging between 10^2 and 10^5. The ratios between defective HBsAg particles and virions are not stable, but change over time. Filaments and spherical particles are produced in large excess in highly viremic HBeAg positive carriers. While filaments decline in parallel with virions, spherical particles remain in moderate excess in low viremic HBeAg negative carriers. [7,8] Thus, HBsAg production varies both quantitatively and qualitatively over time and appears to be dynamically regulated during different phases of the infection. HBsAg derives mainly from the intrahepatic viral minichromosome (cccDNA) by translation of specific RNAs, that are distinct from pregenomic RNA (pgRNA), and HBsAg synthesis follows a pathway that is distinct from viral replication [9]. Accordingly, the correlation between HBsAg serum levels and HBV replication (serum and liver HBV DNA) is mostly but not always present in HBeAg-positive carriers, in whom HBsAg serum levels correlate with the amount of intrahepatic cccDNA and reflect the overall burden of HBV infection.[10] It is however absent in HBeAg-negative patients, in whom serum HBsAg circulates mainly as defective particles, reflecting the residual transcriptional activity of cccDNA, that is differently regulated in this phase of HBV infection.[10-12]

Thus, in clinical practice HBsAg quantification cannot be proposed as a substitute for viral load: on the contrary the combined use of the two markers may be very useful to define the specific condition of the single HBV carrier throughout the highly dynamic phases of chronic HBV infection.

MEASUREMENT OF SERUM HBSAG LEVELS

Several automated assays have been developed for the qualitative assessment of HBsAg and 2 are commercially available for HBsAg quantification: the Architect HBsAg assay (Abbott Diagnostics)[13] and the Elecsys HBsAg II quant assay (Roche Diagnostics) [14-16] Both are fully automated chemiluminescent immunoassays and, using manual or on board pre-dilution steps, provide a wide dynamic range of quantification of HBsAg levels in International Units (IU)/mL. Quantification in IU/mL is recommended by World Health Organization and uses as reference the Second International Standard, that is produced from heat inactivated HBsAg plasma.[17] Importantly, the results acquired with both platforms are very highly correlated ($r \geq 0.96$[14]) and in close agreement across all major HBV genotypes. The performance of a threshold based prediction rule for patients treated with peginterferon (PEG-IFN) established using Architect measurements was excellent when applied on Elecsys data, suggesting that prediction rules derived from studies conducted with one platform may be used confidently on the other. The choice of platform should therefore primarily be based on availability and local costs.

HBSAG IN THE NATURAL HISTORY OF HBV INFECTION

During the natural history of chronic HBV infection HBsAg serum levels decline progressively from the immune-tolerant to the low-replicative phase ([3,4,6], Figure 1).
Concordantly, all studies have reported higher and more stable HBsAg levels in HBeAg-positive immune-tolerant carriers, with values about half a log higher than those observed in HBeAg positive patients in the immune-clearance phase. Nevertheless, in different studies among immune tolerant patients the median values show some variability with levels of about 5 log IU/ml in cohorts from Europe (4.9 log IU/ml) and Hong Kong (4.9 log IU/ml), but only 4.5 log IU/ml in another study from Asia [3,4,6]. Different criteria in the definition of the infection phases, immune-tolerance and immune-clearance, may account for such a discrepancy. In addition, the influence of HBV genotype may be of significant importance.
In HBeAg-positive patients who are in the immune clearance phase median HBsAg levels are about half a log lower, ranging from 3.7 to 4.3 log IU/ml. However, significant differences may be found across individual patients in the same phase of infection.[3,4,6]. Nevertheless, the combined presence of HBsAg $\geq$ 5 log IU/ml with HBV DNA $\geq$ 8 log and normal or minimally elevated ALT values may be suggestive of sustained immune tolerance. The available data suggest that HBsAg serum levels are similar between patients who achieve spontaneous HBeAg seroconversion and

those who fail to clear HBeAg during the immune clearance phase.[6] Once HBeAg to anti-HBe seroconversion occurs, HBsAg levels begin to decline, usually with very slow kinetics as compared to the sharp and pronounced reduction of HBV DNA levels.[6] A recent study from Hong Kong reported that a 1 log decline of HBsAg was only achieved in a subset of patients, and then only after several years of follow-up [6]. However, a recent Taiwanese study reports that a rapid HBsAg decline below 1000 IU/ml within one year from spontaneous HBeAg to anti-HBe seroconversion could predict HBsAg clearance within 6 years, with hazard ratios of 4.4 and 24.3 for patients who achieve HBsAg levels of 100-999 or ≤100 IU/ml, respectively [18]. It remains to be clarified whether a less pronounced HBsAg decline (about 1 log) after spontaneous HBeAg to anti-HBe seroconversion might also indicate the achievement of an effective immune control of HBV infection, and could help distinguish HBsAg carriers who will become inactive from those who will develop HBeAg negative CHB. In fact, HBsAg serum levels are significantly lower, as compared to HBeAg positive patients, not only in the inactive carriers but also in HBeAg negative CHB patients, who show median levels ranging from 2.5 to 3.8 log IU/ml [3-6, 19]. In low replicative, inactive carriers HBsAg serum levels are even lower, but vary in the different cohorts ranging from 1.4-1.7 log IU/ml to 2.8-3.3 log IU/ml [3-6]. Such variability depends, at least in part, on HBV genotypes and stringency of criteria classifying the inactive carriers. A major influence of HBV genotypes on HBsAg secretion is suggested by several reports both *in vivo* and *in vitro*, where the highest levels has been observed for genotype A[20]. Accordingly, in a cohort of 375 patients with HBeAg negative CHB, HBsAg levels were higher in genotype A and D than genotype B and C patients, with values (4.1 log IU/ml for genotype A and 3.8 log IU/ml for genotype D) similar to those reported by Jaroszewicz *et al*.[19]. Moreover, Nguyen *et al*. found lower levels in genotype B than in genotype C inactive carriers (2.2 log IU/ml vs 3.3 log IU/ml) [4]. In addition to this virological explanation, the high variability could be caused by the difficulty to identify at a single time point true inactive carriers from HBeAg negative patients in temporary replicative remission [21]. Jaroszewicz *at al.* indeed showed that individuals classified as inactive at baseline, but with evidence of active infection during follow-up had HBsAg serum levels three-fold higher than those who remained inactive [3]. In a cohort of 209 genotype D HBeAg negative HBV carriers, the individuals with true inactive infection after one year of monthly follow-up of HBV DNA levels had baseline HBsAg levels significantly lower than patients who had baseline HBV DNA < 2000 IU/ml, but showed fluctuations of viremia above 2000 IU/ml thereafter. In the same cohort the diagnostic accuracy and positive predictive value to identify inactive carriers of single point measurement of quantitative HBsAg was 92 % and 93% when using 650 IU/ml as cut-off and 88% and 75% with 1000 IU/ml, as compared to 89% and 70% for a baseline HBV DNA level < 2000 IU/ml. By combining HBsAg (<1000

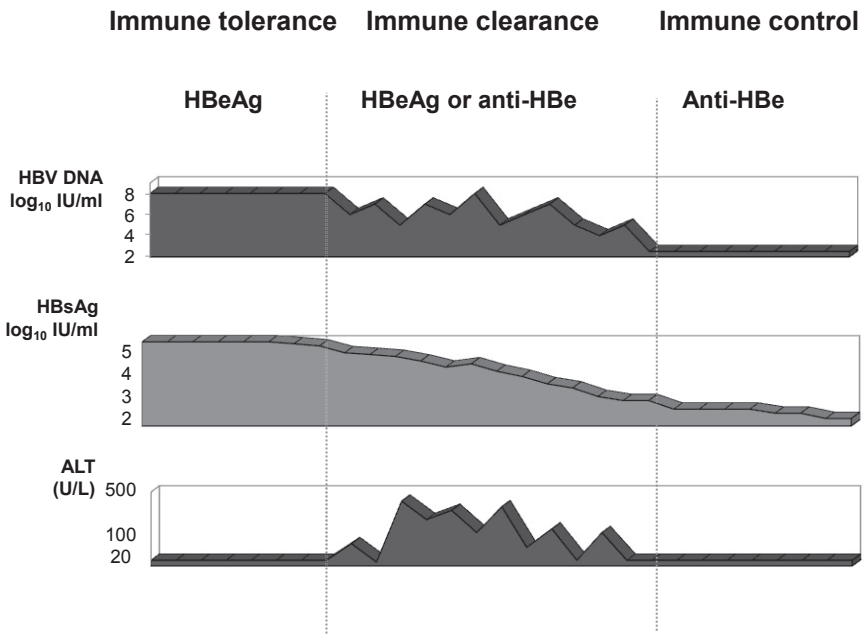

Figure 1. Serum levels of HBV DNA, HBsAg and ALT during the different phases of hepatitis B virus infection.

IU/ml) and HBV DNA (< 2000 IU/ml) the diagnostic performance was optimized with a diagnostic accuracy of 94% and a positive predictive value of 87% [5]. A preliminary report on a cohort of 165 HBeAg negative patients infected by different genotypes (A to E) indicates a positive predictive value of 100% for cut-offs of 2000 IU/ml for both HBsAg and HBV DNA [22].

Overall, these findings suggest that the diagnostic accuracy of single point observation can be ameliorated only by combining the quantification of HBsAg and HBV DNA, where the significant improvement stems from the additional information on the status of immune control of HBV infection provided by HBsAg serum levels (Figure 1).

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HBSAG IN PEGINTERFERON TREATMENT

PEG-IFN has limited direct antiviral efficacy, but stimulates the induction of a host immune response against HBV. A successful immune response causes the destruction of infected liver cells, resulting in a decline of intrahepatic HBV DNA and cccDNA.[23,24] Importantly, the concentration of infected cells at the end of treatment predicts the risk of relapse after therapy discontinuation.[24] However, the intrahepatic HBV DNA and cccDNA concentrations can only be assessed invasively with liver biopsy, limiting

their clinical use. Several independent studies have shown that the decline of serum HBsAg levels during PEG-IFN therapy mimics that of intrahepatic cccDNA, suggesting that a decline in serum HBsAg levels is associated with the induction of an effective anti-HBV immune response.[23,25,26]

HBeAg-positive patients

In a study of 221 HBeAg-positive patients treated with PEG-IFN alfa-2b ± lamivudine (LAM), one year of PEG-IFN induced a pronounced decline in serum HBsAg levels.[27] Patients treated with a combination of PEG-IFN and LAM achieved a somewhat more pronounced on-treatment decline, but the difference was not sustained post-treatment.[27] As expected through the association with intrahepatic markers of HBV replication, the decline in serum levels of HBsAg was strongly associated with the occurrence of a response to PEG-IFN.[27-29] Patients who achieved a combined serological and virological response to PEG-IFN (HBeAg clearance and HBV DNA levels <2,000 IU/mL at 6 months post-treatment) experienced the most pronounced declines, whereas non-responders achieved little to no decline in serum HBsAg levels.[27] Importantly, serum HBsAg decline in responders to PEG-IFN appears to be durable, signifying true off-treatment sustained immune control with a low probability of relapse. The relationship between achievement of immune control and on-treatment HBsAg decline during PEG-IFN therapy is illustrated by the limited difference in HBsAg decline between patients who achieved HBeAg clearance but failed to achieve HBV DNA levels below 2.000 IU/mL, i.e. those patients who progressed to active HBeAg-negative CHB, and those who failed to clear HBeAg altogether.[30]

Since decline in serum HBsAg during PEG-IFN therapy is largely confined to patients who achieve a response to PEG-IFN, monitoring of HBsAg levels may help distinguish patients likely to achieve a response from those who will not. Lau *et al.* showed that patients with low HBsAg levels (<1,500 IU/mL) at week 12 or 24 of therapy have an increased likelihood of HBeAg seroconversion, whereas patients with levels >20,000 IU/mL have a very limited probability of success.[28] These findings were recently confirmed in other studies of Asian patients treated with PEG-IFN (table).[31,32] However, an HBsAg level <1,500 IU/mL can only be used to motivate patients to continue therapy, and it remains to be determined whether HBsAg levels >20,000 IU/mL can be used as a stopping-rule in non-Asian patients.[28] Sonneveld *et al.* used HBsAg decline at week 12 of therapy to identify patients with a limited likelihood of HBeAg clearance with HBV DNA levels <2,000 IU/mL at 6 months post-treatment in a predominantly Caucasian population, and showed that failure to achieve a decline in serum HBsAg from baseline to week 12 reduced the probability of response to <5% and the probability of HBsAg clearance through 3 years of post-treatment follow-up to 0%.[27] Importantly, HBsAg kinetics appear to be a more reliable predictor of response than

Table: Prediction of response to peginterferon therapy using HBsAg levels and declines at *week 12* of treatment.

Patient population	Cut-off	Accuracy	N	Most common HBV genotypes	Ref.
HBeAg-positive					
Treatment failure	No decline	NPV: 97%	202	A and D	[27]
	No decline	NPV: 71-82%	526	B and C	[34]
	>20,000 IU/mL	NPV: 84%	399	B and C	[28]
	>20,000 IU/mL	NPV: 100%	114	B and C	[31]
Treatment success#	<1,500	PPV: 57%	399	B and C	[28]
	<1,500	PPV: 55%	202	A and D	[27]
	<1,500	PPV: 46%	92	B and C	[32]
	<1,500	PPV: 58%	114	B and C	[31]
HBeAg-negative					
Treatment failure	No decline and HBVDNA decline <2 log	NPV: 100%	102	A and D	[36]
	No decline and HBVDNA decline <2 log	NPV: 95%	160	A and D	[39]
	<0.5 log decline	NPV: 90%	48	A, B and D	[38]
	<10% decline	NPV: 84%	120	B, C and D	[37]
Treatment success#	>0.5 log decline	PPV: 89%	48	A, B and D	[38]
	>10% decline	PPV: 47%	120	B, C and D	[37]

Proposed cut-offs for prediction of response for HBeAg-positive and HBeAg-negative patients treated with peginterferon using HBsAg levels at week 12 of treatment. *>70% of cohort. #defined differently in the various studies. For HBeAg-positive patients, response was defined as HBeAg seroconversion in [28,31], as HBeAg clearance with HBV DNA <2,000 IU/mL in references [27,34] and as HBeAg seroconversion with HBV DNA <2,000 IU/mL in [32] . In HBeAg-negative patients, response was defined as HBV DNA <2,000 IU/mL with normal ALT at 6 months post-treatment in [36,39], as HBV DNA <2,000 IU/mL at one year post-treatment in [37] and as HBV DNA clearance in [38]. PPV, positive predictive value; NPV, negative predictive value.

HBV DNA kinetics in the same study cohorts.[33] A combination of the two markers has so far not been investigated in HBeAg-positive patients.

It has become increasingly clear that proposed cut-offs from one study cannot be reliably extrapolated to other study populations.[34] A possible explanation for the reduced performance of prediction rules in other cohorts may be the influence of HBV genotype on HBsAg kinetics. Although patients with a combined serological and virological response to PEG-IFN achieve substantial HBsAg decline, irrespective of HBV genotype, considerable differences in HBsAg kinetics were reported in non-responders across HBV genotypes A through D.[30] The performance of any

prediction rule incorporating HBsAg levels in HBeAg-positive disease therefore probably depends upon the HBV genotype distribution and HBV genotype specific prediction rules appear to be required.[30]

HBeAg-negative patients

Treatment with PEG-IFN alfa-2a with or without LAM induces a decline in serum HBsAg levels of 0.7 log IU/mL after one year of treatment [19] and similar declines were reported in another study.[35] The potential use of HBsAg levels in the management of HBeAg-negative patients treated with PEG-IFN is appealing, because only a minority of patients achieves a sustained response and many of them achieve undetectable HBV DNA during therapy but relapse post-treatment. This leaves quantitative HBsAg the only detectable marker in serum during treatment.[19,36]

HBsAg decline during PEG-IFN therapy for HBeAg-negative CHB is associated with an off-treatment sustained response in a manner similar to that observed in HBeAg-positive patients. HBeAg-negative patients who achieve a response to therapy achieve more HBsAg decline than do non-responders.[19,36,37] Several groups have attempted to formulate prediction rules using HBsAg levels or declines. Moucari *et al.* showed that patients who failed to achieve a decline of 0.5 logIU/mL by week 12 of therapy had a low probability of HBV DNA clearance after PEG-IFN therapy. This study, however, lacked sufficient power for a reliable algorithm and results could not be confirmed in another PEG-IFN alfa-2a study.[36] A post-hoc analysis of the PEG-IFN alfa-2a registration trial reported that achievement of at least a 10% decline in HBsAg concentration by week 12 predicts response (HBV DNA <2,000 IU/mL at one year post-treatment).[19,38] Rijckborst *et al.* have subsequently shown that prediction of response in HBeAg-negative patients may be optimized by combining HBsAg decline and HBV DNA decline.[36] In their study of HBeAg-negative patients treated with PEG-IFN alfa-2a, mostly infected with HBV genotype D (85%), the investigators showed that patients who failed to achieve a decline in HBsAg levels, and who did not achieve an HBV DNA decline >2 log IU/mL, had no chance of achieving a sustained response (HBV DNA <2,000 IU/mL and normal ALT at 6 months after treatment).[36] Importantly, this stopping-rule was subsequently validated in two large independent cohorts of HBeAg-negative patients treated with 48 or 96 weeks of PEG-IFN alfa-2a.[39] Its performance was best among patients infected with genotype D, with a negative predictive value of 100%, although non-responders with other genotypes could also be identified. It is not clear yet whether the limited differences in HBsAg kinetics across HBV genotype in HBeAg negative disease warrant exploration of genotype specific prediction rules in these CHB patients as well [40].

Another recent study on patients participating in the PEG-IFN alfa-2a phase 3 trials investigated the relationship between end-of-treatment HBsAg levels and a sustained

off-treatment response.[19] Brunetto *et al.* showed that patients with HBsAg levels <10 IU/mL (23 of 194 patients) at the end of PEG-IFN therapy had a 52% probability of HBsAg clearance through 3 years of post-treatment follow-up, compared to only 2% in patients with higher levels. Interestingly, the predictive capabilities of HBsAg levels at week 48 were higher than that of HBV DNA levels. Of those patients with undetectable HBV DNA at week 48 (n=161), only 15% achieved HBsAg clearance. Furthermore, patients with a HBsAg level more than 19 IU/mL at week 48 (or a decline from baseline less than 0.46 log IU/mL) had such a low probability of an off-treatment sustained response that retreatment seems necessary.[19]

HBSAG IN NUCLEO(S)TIDE ANALOGUE TREATMENT

The most recently approved NA, entecavir and tenofovir, can induce and maintain undetectable levels of HBV DNA through prolonged therapy. Treatment with NA aims at competitively inhibiting HBV polymerase activity, which is part of a replication pathway that is separate from HBsAg production.[4] Consequently, while treatment with NA may induce pronounced HBV DNA declines, the effect of NA therapy on serum HBsAg levels is very limited and HBsAg decline during treatment with NA is considerably slower than that observed for HBV DNA.[19,41,42] Several independent studies have assessed HBsAg kinetics in patients treated with NA, and these studies, including patients treated with lamivudine, adefovir, telbivudine, entecavir and tenofovir, have shown that a reduction in HBsAg levels is largely limited to HBeAg-positive patients.[19,41,43] This is in line with the absence of HBsAg clearance during short term treatment of HBeAg-negative patients with NA.[43] HBsAg decline was also shown to be more pronounced in patients with HBV genotype A and is related to a patient's age.[41,44] In addition, Zoutendijk *et al.* described that in HBeAg-positive patients treated with entecavir, HBsAg decline is limited to patients with an elevated ALT (>2 times the upper limit of normal) before therapy initiation. Taking into consideration the limited direct effect of NA on HBsAg production and the absence of HBsAg decline in patients with low levels of ALT, it is likely that a host immune response against HBV is required to induce a decline in HBsAg levels in HBeAg-positive patients treated with NA.[41] This concept is supported by the finding that patients with a successful immune response against HBV, namely those who clear HBeAg, achieve HBsAg declines that are similar to those found in patients with HBeAg clearance during PEG-IFN therapy[41]. While fascinating from a research standpoint, the clinical applications of HBsAg levels in patients treated with NA are limited thus far. In the registration study of tenofovir, Heathcote *et al.* found that HBeAg-positive patients who achieved HBsAg clearance through 144 weeks of treatment experienced steeper HBsAg declines during

the first 6 months of treatment, suggesting that early decline of HBsAg levels may predict immune control in patients treated with NA. [43] One small study suggested a relationship between HBsAg levels at the end of telbivudine therapy and a sustained off-treatment response.[45] However, the proposed cut-off of <2 logIU/mL is achieved in a very small number of patients, limiting the clinical use. It is therefore likely that the majority of patients treated with NA will require long-term therapy at least until HBsAg clearance

Several groups have investigated the use of HBsAg levels to approximate the duration of continuous therapy required to clear HBsAg. The estimated duration until HBsAg clearance spanned several decades, and in concordance with the proposed requirement for a host immune response, was reduced only in patients who were HBeAg-positive, and shortest in those with high ALT values.

CONCLUSIONS

Serum HBsAg levels probably reflect intrahepatic transcriptionally active cccDNA and provide complementary information to HBV DNA monitoring alone.

Within the natural history of the disease high levels of HBsAg and HBV DNA are indicative of a highly productive phase of HBV infection, namely HBeAg positive immune-tolerance. On the other hand very low levels of both markers are indicative of inactive HBV infection. In between, during immune activation where serum HBV DNA fluctuates fast and widely any significant and steady decrease of HBsAg serum levels is associated with a stepwise increase of the host immune control of HBV infection that may lead to the switch from active disease to the inactive carrier status and eventually to HBsAg loss and anti-HBs seroconversion.

Within the setting of HBV therapy a decline in serum HBsAg levels in patients treated with PEG-IFN may also herald the successful induction of immune control over HBV and can consequently be used to predict response. However, HBsAg kinetics are also influenced by HBV genotype, suggesting the requirement for HBV genotype specific stopping-rules. The first stopping-rule for HBeAg-negative patients has recently been validated in several independent cohorts and appears ready for clinical use. HBsAg decline in patients treated with NA is largely confined to HBeAg-positive patients with an elevated ALT, suggesting the requirement for a pre-existing immune response to HBV in achieving HBsAg decline and subsequent clearance. Current clinical applications for HBsAg monitoring in patients treated with NA are limited.

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13 Correspondence

Studies of *IL28B* genotype and response to peginterferon in chronic hepatitis B should be stratified by HBV genotype

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Dear editor,

In a recent issue of *Hepatology*, Lampertico *et al.* present a study of mostly HBV genotype D HBeAg-negative chronic hepatitis B (CHB) patients treated with (PEG-) IFN, and show that HBsAg loss was significantly associated with *IL28B* genotype.[1] Our group recently published a study on the association of *IL28B* genotype with response to PEG-IFN in HBeAg-positive CHB patients. Favourable *IL28B* genotypes, CC for rs12979860 and AA for rs12980275, were associated with higher rates of HBeAg seroconversion and HBsAg loss.[2] Taken together, these findings provide mounting evidence for the importance of *IL28B* genotype for prediction of response to PEG-IFN in CHB, although these findings require further confirmation.

There is however an important pitfall that should be taken into consideration. In our study, *IL28B* genotype distribution varied across ethnicity; 90% of Asian patients were genotyped CC, compared to 50% of non-Asians.[2] Response to PEG-IFN in CHB also depends upon HBV genotype: patients with HBV genotype A achieve higher rates of response than those with HBV genotypes B, C or D.[3] Importantly, HBV genotypes A and D predominate in Caucasians, and nearly all Asian patients are infected with HBV genotypes B or C. Since *IL28B* genotype is associated with ethnicity, it is also associated with HBV genotype. In our study of HBeAg-positive patients, the favourable *IL28B* genotype was present in 42% of HBV genotype A patients, in 88-90% of patients with HBV genotypes B or C, and in 52% of HBV genotype D patients.[2] If differences in HBV genotype distribution are ignored, analyses of the association between *IL28B* genotype and HBsAg loss in a cohort of patients with mixed ethnicities could result in an overrepresentation of Asian patients (with “poor response” HBV genotypes B or C) in the favourable CC group, and an overrepresentation of Caucasians and black Africans (with “good response” HBV genotype A) in the unfavourable CT/TT groups. This could result in a biased estimate of association, or failure to detect one. This issue is particularly relevant for studies conducted in countries with mixed ethnicities, such as those in Western Europe and the United States, where the HBV infected population comprises Caucasians, Asians, and black Africans.

In conclusion, the study by Lampertico *et al.* provides fascinating new data and urges further studies of *IL28B* genotype and response to PEG-IFN in CHB. However, the association of *IL28B* genotype distribution with that of HBV genotype may introduce an important pitfall. Therefore, we strongly recommend that future studies of *IL28B* in CHB be stratified by, or adjusted for, HBV genotype.

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Reply to: Further analysis is required to identify an early stopping rule for peginterferon that is valid for all HBeAg-positive patients

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Dear editor,

We thank Dr. Piratvisuth and Dr. Marcellin for their valuable contribution to the debate. First of all, it is important to note that our findings of a more pronounced HBsAg decline in HBeAg-positive patients with a response to peginterferon were confirmed in their study, reflecting the induction of an immune response in these patients.[1]

Their analysis shows that failure to achieve a decline in HBsAg levels through 12 weeks of therapy does not predict non-response as well in their cohort as it did in our study. Possible explanations for these discrepant findings could be the type of peginterferon or duration of therapy, as suggested by Piratvisuth and Marcellin. However, the most probable explanation is the difference in HBV genotype distribution between the study cohorts. Preliminary data from our group show that HBsAg decline among HBeAg-positive patients treated with peginterferon is strongly related to HBV genotype.[2] These differences across genotypes, particularly among patients who fail to achieve a response, may be an important determinant of the performance of a threshold based stopping-rule. Consequently, the performance of any threshold is primarily dependent upon the distribution of HBV genotypes in the study cohort.

The importance of HBV genotype when applying stopping-rules for peginterferon therapy in chronic hepatitis B was recently illustrated by a validation study of our stopping-rule for HBeAg-negative patients. This stopping-rule, recommending discontinuation of peginterferon in patients who fail to achieve a decline in HBsAg and a decline in HBV DNA of >2log at week 12, was based on a cohort of mostly genotype D patients.[3] When validated in two independent study cohorts, performance was best in genotype D patients treated with either 48 or 96 weeks of peginterferon.[4]

Concluding, monitoring of HBsAg levels during peginterferon therapy of chronic hepatitis B may provide valuable insight into a patient's probability of achieving a response. However, it appears that differences in HBsAg decline across HBV genotypes have to be taken into consideration. A pooled analysis of the data from our respective studies, stratified by HBV genotype and possibly incorporating HBV DNA levels, appears to be a crucial next step.

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Summary

Current treatment options for CHB consist of the nucleo(s)tide analogues (NA) and (pegylated) interferons (PEG-IFN). Antiviral treatment with NA aims at competitively inhibiting viral polymerase activity[1], and the most recently approved NA entecavir and tenofovir can effectively maintain suppression of HBV DNA levels for prolonged periods of time in the vast majority of patients.[2-5] Nevertheless, PEG-IFN remains an important first-line treatment option for CHB, since an off-treatment sustained response can be achieved in a considerable number of patients after a finite treatment course.[6-10] Response to (PEG-)IFN-based therapy is accompanied by high rates of hepatitis B surface antigen (HBsAg) seroconversion,[11] a reduced incidence of hepatocellular carcinoma and prolonged survival.[12,13] However, the clinical use of PEG-IFN is compromised by the frequent occurrence of side-effects;[14] the most frequently reported side-effects are a flu-like syndrome, headache, myalgia, fatigue and local reactions at the site of injection.[7,10,15] Selection of patients with the highest probability of achieving a response to PEG-IFN is therefore essential to successful application of this agent in clinical practice and may help reduce therapy associated side-effects and costs. Ideally, patients should be selected for PEG-IFN therapy based on their individual probability of response; this requires a procedure that can accurately predict response using readily available baseline parameters. Once therapy is initiated, an individual patient's probability of response can be updated using on-treatment parameters that can accurately identify those patients who are likely to become non-responders, while maintaining all future responders on therapy. Our group has recently published a baseline prediction model based upon a pooled dataset from studies of PEG-IFN for HBeAg-positive chronic hepatitis B.[16] This study identified HBV genotype A, low baseline HBV DNA, high baseline ALT, older age, female sex and no previous IFN therapy as factors that predicted a favorable therapy response.[16] A prediction model based on these variables may help select patients for PEG-IFN therapy. However, discrimination was limited, leaving substantial uncertainty on the individual level. Identification of other baseline or on-treatment factors that influence the probability of response is therefore required.

RESPONSE TO PEGINTERFERON REQUIRES A SUSCEPTIBLE HOST AND A SUSCEPTIBLE VIRUS

Recently, large genome-wide association studies in hepatitis C virus infected patients have revealed an association between sustained virological response to PEG-IFN and ribavirin treatment and several single nucleotide polymorphisms on chromosome 19, located near the *IL28B* gene.[17-20] These studies have convincingly shown that favorable *IL28B* genotypes, such as AA at rs12980275, confer increased probabilities

of sustained virological response, independently of other predictors.[20] The exact biological mechanism or pathway by which these genotypes influence response to PEG-IFN is currently unclear. *IL28B* itself is a member of the IFN-λ family, and IFN-λ was recently shown to be active against both RNA and DNA viruses.[21-23] Indeed, preliminary data from a phase 2 study show that treatment with PEG-IFN-λ is effective in HCV infected patients.[24] Furthermore, in-vitro studies have shown that IFN-α, such as administered in the form of PEG-IFN alfa, may induce IFN-λ production. Since the two types of IFN, IFN-α and IFN-λ, have common downstream signaling pathways, the two types of IFN may act synergistically in patients treated with PEG-IFN.[25-28] In **Chapter 1**, we present the results of a large multicenter study of 205 HBeAg-positive patients treated with PEG-IFN in 11 hospitals across the globe.[7,29,30] All patients were genotyped at rs12980275 and rs12979860 near the *IL28B*. In this study, we found that *IL28B* genotypes were independently associated with the occurrence of HBeAg seroconversion after PEG-IFN therapy, and also with HBsAg clearance through 7 years of follow-up. However, this study also showed that the majority of patients with a successful immune response to PEG-IFN (ie. who achieved HBeAg seroconversion) did not achieve low HBV DNA levels (<2,000 IU/mL), nor HBsAg clearance. A possible explanation for these observations is the presence of viral strains that have mutations in the precore and basal core promoter regions that prohibit the synthesis of HBeAg. Presence of these mutants before treatment initiation has been shown to predict HBeAg loss after IFN treatment,[31] possibly through positive selection during antiviral therapy,[32] but may predispose patients to persistence of HBV DNA after HBeAg clearance.[33,34] In **Chapter 2**, we therefore investigated whether presence of precore and/or core promoter mutants in HBeAg-positive patients who were treated with PEG-IFN influenced the probability of response. In this study of 214 patients treated with PEG-IFN within a global randomized study[7], we found that precore and core promoter mutants were most frequently detected in patients with HBV genotypes B, C, and D, and in only a subset of patients with the favorable HBV genotype A. Patients with detectable mutants had lower levels of HBeAg, and frequently achieved HBeAg clearance and seroconversion after PEG-IFN therapy. However, the majority of patients with detectable precore and core promoter mutants who achieved HBeAg clearance did not achieve low levels of HBV DNA, nor did they clear HBsAg. In contrast, patients with only wildtype virus at baseline had a very high probability of achieving either of these endpoints. The lower baseline levels of HBeAg, high rates of HBeAg seroconversion but low rates of subsequent disease remission in patients with detectable mutants was also apparent in a large cohort of patients treated with NA, as shown in **Chapter 3**.

Given the association of *IL28B* genotype with serological response and the association between precore and core promoter mutants and failure to achieve low HBV

DNA levels after HBeAg clearance, we hypothesized that response to PEG-IFN therapy requires both a susceptible host (susceptible to IFN alfa, or an active immune response) as well as a susceptible virus (absence of detectable precore and/or core promoter mutants). In **Chapter 4** we therefore investigated whether a combination of markers of an active immune response, serum levels of interferon gamma-inducible protein (IP)-10 and ALT, together with confirmed absence of precore and core promoter mutants could identify patients with a very high likelihood of response to PEG-IFN therapy. The results of this study showed that a combination of high baseline levels of IP-10 or high baseline levels of ALT (ie. an active immune response), together with presence of only wildtype HBV identified patients with high *a priori* response chances. Possibly, combined use of the described predictors of response to PEG-IFN may help select patients with the most favourable characteristics for PEG-IFN therapy, resulting in higher overall response rates and a more attractive cost-benefit ratio for this treatment option.

RESPONSE-GUIDED PEGINTERFERON THERAPY

While use of baseline predictors may help identify the best candidates for PEG-IFN efficacy, the individual probability of response remains limited. Early on-treatment identification of non-responders to therapy may help optimize the use of PEG-IFN. On-treatment monitoring of viral replication using HBV DNA, HBeAg and HBsAg levels may aid decision-making and frequent HBV DNA monitoring is therefore recommended in treatment guidelines.[35] However, modeling of HBV DNA kinetics during PEG-IFN therapy has shown only limited clinical utility,[36,37] and reliable prediction of non-response is only possible at week 24 of therapy (negative predictive value (NPV) 86%).[38] Recent technical advances have allowed for the quantitative assessment of HBsAg in serum. HBsAg is secreted from the hepatocyte during viral replication as part of the HBV nucleocapsid, or as part of noninfectious viral particles.[39,40] Several studies have reported that serum HBsAg levels correlate with intrahepatic cccDNA levels in HBeAg-positive patients.[41,42] Importantly, the concentration of infected cells at the end of treatment predicts the risk of relapse after therapy discontinuation.[43] On-treatment HBsAg decline may therefore reflect the efficacy of PEG-IFN in decreasing intrahepatic cccDNA and consequently predict a sustained response.

In **Chapter 6**, we present the results of a study that investigated the kinetics of serum HBsAg levels during treatment with PEG-IFN alone or in combination with lamivudine. PEG-IFN treatment induced a significant decline in serum HBsAg levels, which was sustained post-treatment. Importantly, on-treatment HBsAg decline was limited to patients who achieved a sustained response to therapy, suggesting that

the on-treatment kinetics of serum HBsAg can be used to monitor therapy efficacy. In this study, patients who failed to achieve a decline of serum HBsAg levels during the first 3 months of treatment had a probability of response of <5%, suggesting that PEG-IFN is not effective and therapy discontinuation can be considered. However, patients in this cohort were predominantly of Caucasian origin, and infected with HBV genotype A or D. In **Chapter 7**, we report that HBsAg kinetics are also influenced by HBV genotype, suggesting that one prediction rule may not be applicable across all major HBV genotypes. Indeed, another study that comprised mostly Asian patients infected with HBV genotypes B or C reported that a considerable number of patients without a HBsAg decline by month 3 of treatment went on to achieve a response. This study proposed a prediction-rule that used an HBsAg levels >20,000 IU/mL at week 12 or 24 to identify patients with a limited probability of response.[44] We were unable to confirm the use of this cut-off in our dataset, which may be accounted for by the differences in HBV genotype distribution across the study cohorts. In **Chapter 8**, we finally resolved this issue by evaluating the performance of our stopping-rule, as well as the stopping-rule based on an HBsAg level >20,000 IU/mL, in a pooled dataset of patients participating in 3 of the largest randomized studies of PEG-IFN for HBeAg-positive CHB conducted worldwide.[7,8,45] We were able to analyse the individual patient data of 803 patients, and found that on-treatment changes in HBsAg levels strongly predicted response to treatment across all major HBV genotypes. At week 24 of treatment, all patients with HBsAg levels >20,000 IU/mL have a low probability of response, irrespective of HBV genotype, and PEG-IFN discontinuation is indicated. Use of HBV genotype specific stopping-rules may be considered at week 12; patients infected with genotypes A or D who do not achieve a decline in HBsAg by week 12 have a very low probability of response, and for patients with genotypes B or C, those with HBsAg levels >20,000 IU/mL at week 12 could be eligible for therapy discontinuation. Our findings may help make PEG-IFN more acceptable to patients, physicians and healthcare policy makers and could possibly increase the cost-effectiveness of PEG-IFN in HBeAg-positive CHB.

Spontaneous elevations of ALT, or flares, are a well-recognised phenomenon in patients treated with PEG-IFN, although the pathogenesis in unclear.[46] Flares may occur in a considerable number, up to 25%, of patients treated with a one year course of PEG-IFN, and have been associated with an increased probability of serological response.[47,48] However, flares can also have considerable detrimental effects, and may result in hepatic decompensation and death in patients with advanced cirrhosis.[49] Flink *et al*. recognised different types of flares during PEG-IFN therapy of HBeAg-positive CHB. In this study, host induced flares, characterised by an ALT flare followed by a decline in HBV DNA, were associated with response to treatment, whereas virus induced flares, or ALT elevations that were preceded by an increase

in HBV DNA, were not.[47] Since a decline of HBsAg levels may signify induction of immune control, close monitoring of HBsAg levels in patients who experience flares may provide further insight into the nature of the flare. In **Chapter 9**, we studied 50 patients who experienced flares during PEG-IFN therapy, and found that patients who experience a host induced flare achieve pronounced declines in HBsAg levels, whereas stable or even increasing HBsAg levels were observed in patients with virus induced flares. Furthermore, a rapid HBsAg decline during the first weeks after the peak of the flare predicts the occurrence of HBsAg loss. Close monitoring of HBsAg levels during flares may therefore help classify flares and predict subsequent treatment response.

Several automated assays have recently been developed for the quantitative assessment of HBsAg and the most commonly used are the Architect HBsAg assay (Abbott Diagnostics)[50] and the Elecsys HBsAg II quant assay (Roche Diagnostics). To assess whether results obtained using these platforms yield comparable results, we measured HBsAg in 1427 samples using both platforms, as reported in **Chapter 10**. We found that the results acquired with both platforms are very highly correlated ($r \geq 0.96$) and in close agreement across all major HBV genotypes. The performance of a threshold based prediction rule (as discussed above) for patients treated with PEG-IFN established using Architect measurements was excellent when applied on Elecsys data, suggesting that prediction rules derived from studies conducted with one platform may be used confidently on the other. The choice of platform should therefore primarily be based on availability and local costs.

Previous studies have also shown that monitoring of HBeAg levels during PEG-IFN therapy may help predict HBeAg seroconversion. Fried *et al.* showed that achievement of low HBeAg levels at week 24 of PEG-IFN therapy accurately identified patients with a high probability of HBeAg seroconversion.[38] However, as described above, HBeAg seroconversion does not result in disease remission in a substantial proportion of patients. We therefore investigated whether HBeAg levels, both before and during PEG-IFN therapy, could help predict a combined response of HBeAg loss with HBV DNA <2,000 IU/mL. In **chapter 11**, we show that the association between HBeAg levels and such a combined response to therapy is unreliable, due to the influence of precore and core promoter mutants which are both associated with lower levels of HBeAg as well as a reduced probability of combined response to PEG-IFN. Serum HBeAg levels should therefore not be used to select patients for PEG-IFN therapy, nor for monitoring of treatment efficacy, if presence of precore and core promoter mutants is not taken into consideration. In contrast, serum levels of HBsAg were able to predict response to PEG-IFN irrespective of presence of precore and core promoter mutants.

CONCLUSIONS

PEG-IFN is an effective treatment option for a selected group of patients with favourable characteristics. Response to PEG-IFN requires both a susceptible host (favourable *IL28B* genotype, high ALT, high IP-10), as well as a susceptible virus (absence of precore and core promoter mutants). On-treatment monitoring of viral replication using serum HBsAg levels may help optimize decision-making, whereas use of HBeAg levels to monitor therapy efficacy is not recommended. Prediction rules based on HBsAg levels at week 12 of PEG-IFN therapy require HBV genotyping, but discontinuation should be considered in all patients with an HBsAg >20,000 IU/mL at week 24.

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Samenvatting

De huidige behandelopties voor chronische hepatitis B (CHB) bestaan uit de nucleo(s)tide analogen (NA) en (gepegyleerd) interferon (PEG-IFN). Antivirale behandeling met NA remt het virale polymerase,[1] en behandeling met de meest recent ontwikkelde NA, entecavir en tenofovir, resulteert in een ondetecteerbaar HBV DNA in het overgrote deel van de patiënten.[2-5] Desondanks blijft PEG-IFN een belangrijke eerstelijns behandeloptie, omdat bij een deel van de patiënten een respons optreedt die ook duurzaam is na het staken van de behandeling.[6-10] Patiënten die een respons hebben op PEG-IFN hebben een hoge kans op hepatitis B surface antigen (HBsAg) seroconversie,[11] een lagere kans op het krijgen van een hepatocellulair carcinoom en een gunstige prognose.[12,13] Het klinisch gebruik van PEG-IFN wordt echter bemoeilijkt door de vaak optredende bijwerkingen;[14] de meest voorkomende zijn algehele malaise, hoofdpijn, myalgie, vermoeidheid en huidreacties rond de injectieplaats.[7,10,15] PEG-IFN dient daarom alleen te worden gebruikt bij patiënten met een hoge kans op respons. Idealiter zouden patiënten moeten worden geselecteerd op basis van de individuele kansen; hiervoor is echter een methode nodig die de kans op respons voldoende betrouwbaar kan vaststellen aan de hand van gemakkelijk beschikbare factoren. Daarnaast kan therapie worden bijgestuurd door veranderingen in de virale replicatie te monitoren gedurende de behandeling. Voor dit laatste is het belangrijk patiënten met een lage kans op succes zo vroeg mogelijk te identificeren, zonder de behandeling te staken bij patiënten die toch nog zouden reageren.

Onze groep heeft recent een predictiemodel gepubliceerd dat is gebaseerd op de data van patiënten die deel hebben genomen aan 2 grote internationale studies naar de effectiviteit van PEG-IFN in HBeAg-positieve CHB.[16] Deze studie laat zien dat HBV genotype A, lage HBV DNA concentraties, hoge ALT waarden, een hogere leeftijd, vrouwelijk geslacht en niet eerder behandeld zijn met IFN de kans op succes vergroten.[16] Helaas is de betrouwbaarheid op individueel niveau beperkt, en er is dan ook nog een grote onzekerheid of de patiënt wel of niet succesvol met PEG-IFN kan worden behandeld. Identificatie van andere factoren die het behandelresultaat beïnvloeden is daarom essentieel.

DE KANS OP EEN SUCCESVOLLE BEHANDELING MET PEGINTERFERON HANGT AF VAN DE GEVOELIGHEID VAN DE DRAGER EN VAN HET VIRUS

Recent hebben enkele grote genoomwijde associatie studies laten zien dat de kans op een succesvolle behandeling van een hepatitis C infectie met PEG-IFN en ribavirine beïnvloed wordt door enkele *single nucleotide polymorphisms* op chromosoom 19, nabij het *IL28B* gen.[17-20] Patiënten met een gunstig genotype, zoals AA bij rs12980275,

hebben een grotere kans de behandeling succesvol af te sluiten.[20] Het biologische mechanisme achter de associatie is op dit moment nog onduidelijk. *IL28B* is een lid van de IFN-λ familie, en IFN-λ zelf is effectief tegen zowel RNA als DNA virussen.[21-23] De eerste studie naar de klinische toepassing van PEG-IFN-λ bij patiënten met hepatitis C laat veelbelovende resultaten zien.[24] Daarnaast hebben *in vitro* studies laten zien dat IFN-α, bijvoorbeeld toegediend in de vorm van PEG-IFN-α, de IFN-λ productie kan stimuleren. Omdat de twee typen IFN, IFN-α en IFN-λ, dezelfde reactiecascades hebben kunnen ze synergistisch werken bij een behandeling met PEG-IFN-α.[25-28]

In **hoofdstuk 1** beschrijven we de resultaten van een grote multicenter studie waarin 205 HBeAg-positieve hepatitis B patiënten werden geïncludeerd die behandeld waren met PEG-IFN in 11 ziekenhuizen in Europa, Canada en zuidoost Azie.[7, 29, 30] Alle patiënten werden gegenotypeerd voor rs12980275 en rs12979860 nabij het *IL28B* gen. We vonden dat het *IL28B* genotype van de patiënt een onafhankelijke voorspeller was voor de kans op HBeAg seroconversie na de PEG-IFN behandeling, en ook samenhing met de kans op HBsAg verlies gedurende een follow-up duur van ruim 7 jaar. De resultaten laten echter ook zien dat er bij een aanzienlijk deel van de patiënten met een succesvolle immuunrespons (het optreden van HBeAg seroconversie) daarna niet een laag HBV DNA (<2,000 IU/mL) of HBsAg verlies gevonden werd. Een mogelijke verklaring hiervoor is de aanwezigheid van virale subpopulaties met mutaties in het *precore* of *core promoter* gebied. Deze virussen maken minder of geen HBeAg aan, en de aanwezigheid van dergelijke subpopulaties is mogelijk geassocieerd met HBeAg verlies na de behandeling[31] ten gevolge van positieve selectie.[32] Echter, aanwezigheid van deze mutanten is ook in verband gebracht met een blijvend hoog HBV DNA na HBeAg seroconversie.[33, 34]

In **hoofdstuk 2** hebben we daarom onderzocht of aanwezigheid van *precore* en *core promoter* mutanten bij HBeAg-positieve patiënten die met PEG-IFN behandeld werden samenhing met de kans op respons. Met dit onderzoek, dat werd uitgevoerd binnen een grote international gerandomiseerde studie,[7] konden wij aantonen dat de *precore* en *core promoter* mutanten vaak al aanwezig waren bij onze patiënten, en dan vooral bij patiënten met HBV genotypes B, C en D. Patiënten met detecteerbare mutanten hadden lagere HBeAg concentraties voor de behandeling en hadden een goede kans op HBeAg verlies en seroconversie. Echter, het overgrote deel van de patiënten met detecteerbare mutanten die HBeAg negatief werden slaagde er niet in daarna ook een ondetecteerbaar HBV DNA te krijgen of HBsAg negatief te worden. Daarentegen hadden patiënten met alleen wildtype virus juist een hele hoge kans op het behalen van deze eindpunten. De lagere HBeAg concentratie bij patiënten met detecteerbare mutanten, alsook de hoge kans op HBeAg verlies, hebben we vervolgens bevestigd in een cohort patiënten die met NA werden behandeld, zoals gerapporteerd in **hoofdstuk 3**.

Omdat het *IL28B* genotype samenhangt met serologische respons (HBeAg seroconversie) en aanwezigheid van *precore* en *core promoter* mutanten juist iets zegt over de kans een blijvend hoog HBV DNA na HBeAg verlies, stelden wij de hypothese op dat een succesvolle behandeling met PEG-IFN afhangt van de gevoeligheid van de drager en de gevoeligheid van het virus. In **hoofdstuk 4** hebben we daarom onderzocht of een combinatie van immuunactivatie, gemeten middels de serum concentratie van het *interferon gamma inducible protein-10* (IP-10) en het ALT, samen met de afwezigheid van *precore* en *core promoter* mutanten kan helpen patiënten met een grote kans op succesvolle PEG-IFN behandeling te identificeren. De resultaten laten zien dat vooral patiënten met een hoog IP-10 en een hoog ALT (activatie van het immuunsysteem) en met alleen wildtype HBV goede kandidaten zijn voor een behandeling met PEG-IFN. De combinatie van bovenstaande factoren kan daarom helpen alleen patiënten voor PEG-IFN behandeling te selecteren als zij een hoge kans hebben op succes.

RESPONS GESTUURDE PEGINTERFERON BEHANDELING

Naast het gebruik van baseline factoren is het ook mogelijk de behandeling bij te sturen aan de hand van veranderingen in de virale replicatie. Het identificeren van non-responders gedurende de eerste behandelweken kan helpen het gebruik van PEG-IFN verder te optimaliseren. Er zijn 3 belangrijke markers voor virale replicatie: HBV DNA, HBeAg en HBsAg.[35]
De veranderingen van de HBV DNA concentratie tijdens de behandeling heeft maar weinig klinische toepasbaarheid,[36,37] omdat betrouwbare responspredictie pas mogelijk is na 24 weken behandeling (negatief voorspellende waarde 86%).[38] Recent is het ook mogelijk geworden het HBsAg kwantitatief te meten in serum. Het HBsAg wordt in de hepatocyt geproduceerd gedurende de virale replicatie en wordt uitgescheiden als onderdeel van het virale deeltje, maar ook als niet-infectieuze partikels.[39,40] Enkele studies hebben laten zien dat de HBsAg concentratie in serum samenhangt met de intrahepatische cccDNA concentratie in HBeAg-positieve patiënten.[41,42] Een hoge cccDNA concentratie is voorspellend voor het optreden van een terugval na het staken van de behandeling.[43] Het optreden van een daling van het HBsAg is mogelijk een weergave van een daling van de intrahepatische cccDNA concentratie en kan daarom het succes van de behandeling voorspellen.
In **hoofdstuk 6** beschrijven we de resultaten van een onderzoek naar de kinetiek van het HBsAg gedurende een behandeling met PEG-IFN. Een daling van de HBsAg concentratie trad vooral op bij patiënten met een respons, hetgeen suggereert dat de HBsAg concentratie kan worden gebruikt om het succes van de behandeling te monitoren. In dit onderzoek hadden patiënten die na 12 weken behandeling geen daling

hadden van de HBsAg concentratie een kans op succes van minder dan 5%, zodat de behandeling beter gestopt kan worden. Het is echter belangrijk dat dit cohort voornamelijk bestond uit Europese patiënten, geïnfecteerd met HBV genotypes A of D. In **hoofdstuk 7** laten we zien dat de HBsAg kinetiek ook wordt beïnvloed door het HBV genotype, en mogelijk moeten er daarom verschillende predictieregels worden opgesteld voor patiënten met verschillende HBV genotypes. In een andere studie waarin vooral Aziatische patiënten werden bestudeerd, meestal geïnfecteerd met genotypes B of C, werd duidelijk dat patiënten met een HBsAg concentratie van meer dan 20,000 IU/mL op week 12 een lage kans hadden op succes.[44] Wij konden deze bevindingen niet bevestigen in ons cohort, wat mogelijk kan worden verklaard door de verschillen in HBV genotypes tussen de cohorten.

In **hoofdstuk 8** hebben we getracht dit probleem op te lossen door patiënten te onderzoeken die behandeld zijn met PEG-IFN in de 3 grootste gerandomiseerde studies naar de effectiviteit van PEG-IFN in HBeAg-positieve patiënten.[7,8,45] In dit onderzoek, waarin 803 patiënten vanuit de hele wereld werden geïncludeerd, konden we wederom laten zien dat de HBsAg concentratie een sterke voorspeller is van respons op PEG-IFN ongeacht het HBV genotype. Uit deze studie bleek dat de kans op succes erg laag werd als de patiënt een HBsAg concentratie had van meer dan 20,000 IU/mL na 24 weken behandeling, en dat het HBV genotype hierbij niet van belang is. Wil men echter na 12 weken behandeling al iets zeggen over de kansen, dan moet een HBV genotype specifieke stopregel worden gekozen. Deze bevindingen kunnen helpen op de PEG-IFN behandeling verder te optimaliseren, de kosten te verlagen en PEG-IFN acceptabeler te maken voor patiënten, artsen en beleidsmakers.

ALT stijgingen (*flares*) komen vaak voor tijdens een behandeling met PEG-IFN, tot bij 25% van de patiënten, al is de oorzaak hiervan onduidelijk.[46] Patiënten die een ALT flare doormaken hebben mogelijk een hogere kans op respons,[47,48] maar de flare kan ook resulteren in leverdecompensatie en de dood in patiënten met cirrose.[49] Flink c.s. hebben verschillende typen flares beschreven: drager geïnduceerd (eerst een ALT stijging, gevolgd door een HBV DNA daling) of virus geïnduceerd (eerst een HBV DNA stijging, dan een ALT stijging). Drager geïnduceerde flares gingen gepaard met een hogere kans op respons, maar dit was niet het geval bij virus geïnduceerde ALT stijgingen.[47] Omdat de HBsAg concentratie tijdens de behandeling iets kan zeggen over de mate van immuuncontrole hebben wij onderzocht of de kinetiek van het HBsAg verschilde tussen patiënten met drager geïnduceerde ALT flares en patiënten met virus geïnduceerde flares. In **hoofdstuk 9** onderzochten wij 50 patiënten die een flare doormaakten en vonden dat patiënt geïnduceerde flares een sterke HBsAg daling tot gevolg hadden. De HBsAg concentratie bleef stabiel of steeg juist bij patiënten met een virus geïnduceerde flare. Verder vonden wij ook dat patiënten met een daling van de HBsAg concentratie na een ALT piek een hoge kans hadden om ook HBsAg

negatief te worden. Het monitoren van de HBsAg concentratie tijdens flares kan dus helpen flares te karakteriseren en behandelsucces te voorspellen.
Recent zijn verschillende geautomatiseerde assays ontwikkeld om het HBsAg te kwantificeren. De twee meest gebruikte zijn de Architect HBsAg assay (Abbott Diagnostics) en de Elecsys HBsAg II quant assay (Roche Diagnostics). Om te onderzoeken of de twee apparaten vergelijkbare resultaten geven hebben we HBsAg gemeten met beide testen in 1427 bloedmonsters. De resultaten hiervan staan beschreven in **hoofdstuk 10**. We vonden dat de resultaten van de twee testmethoden zeer vergelijkbaar waren onafhankelijk van het HBV genotype. Verder werkte een predictieregel die was ontwikkeld met data van de Architect goed toen hij werd toegepast op de meetresultaten van de Elecsys. Beide testmethoden kunnen daarom in de klinische praktijk worden gebruikt en de keuze moet vooral afhangen van beschikbaarheid en lokale kosten.
Eerdere studies hebben laten zien dat ook het HBeAg een goede voorspeller is van respons op PEG-IFN. Fried c.s. publiceerden dat een lage HBeAg concentratie na 24 weken behandeling een goede voorspeller is voor het doormaken van een HBeAg seroconversie. Echter, zoals boven beschreven, lang niet alle patiënten hebben na de HBeAg seroconversie een laag HBV DNA. Wij hebben daarom onderzocht of de HBeAg concentratie ook samenhangt met het optreden van een lage HBV DNA concentratie na de PEG-IFN behandeling. In **hoofdstuk 11** laten we zien dat de relatie tussen de concentratie HBeAg en respons op PEG-IFN zeer onbetrouwbaar is. Dit komt mogelijk door de associatie met aanwezigheid van *precore* and *core promoter* mutanten, welke samenhangt met zowel lage HBeAg levels alsook een verhoogde kans op het falen van de behandeling. De concentratie van HBeAg lijkt daarom afgedaan te hebben als marker voor het monitoren van de patiënt die behandeld wordt met PEG-IFN.

CONCLUSIE

PEG-IFN is een effectieve behandeling voor een selecte groep hepatitis B patiënten met goede karakteristieken. Een succesvolle behandeling met PEG-IFN hangt af van de gevoeligheid van zowel de drager (gunstig *IL28B* genotype, hoog IP-10) als van het virus (afwezigheid van *precore* en *core promoter* mutanten). Het beloop van de concentratie van het HBsAg tijdens de behandeling kan informatie geven over het optreden van een respons, en patiënten die een HBsAg concentratie hebben van meer dan 20,000 IU/mL na 24 weken dienen de behandeling te staken.

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Dankwoord

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De promotieperiode bij de afdeling leverziekten wordt voornamelijk doorgebracht in een houten keet die al decennia "tijdelijk" op een van de bijgebouwen van het Erasmus MC is geplaatst. Toegang: via een brandtrap. Temperatuur in de zomer: 30 plus. Mogelijkheden om post te ontvangen: nihil. Toch blijkt het ophokken van een groep jonge gemotiveerde onderzoekers een succesformule. Tijdens de afgelopen jaren had ik het genoegen om hoogte- en dieptepunten te mogen delen met: Robert, Jurriën, Edith, Desiree, Vera, Aria, Celine, Nicoline, Renate, Margot, Aafke, Jorie, Edmee, Susanne, Atija, Wouter, Veerle, Wim, Jildou, Leonie, Caroline, Ludi, Femme, Lisanne, Wouter en natuurlijk Jeoffrey.

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Curriculum Vitae

Milan Johan Sonneveld werd op 12 januari 1987 geboren te Rotterdam. In 2005 behaalde hij het atheneum diploma aan de tweetalige VWO opleiding van het Wolfert van Borselen Lyceum te Rotterdam. Daarnaast slaagde hij voor het International Baccalaureate examen Engels op A1 Standard Level (near-native speaker) en werd hij geselecteerd voor deelname aan het Leiden Advanced Pre-University Programme for Top-students aan de Universiteit van Leiden in 2004. In 2005 begon hij met de opleiding Geneeskunde aan de Erasmus Universiteit Rotterdam, waar hij in 2010 het doctoraal examen behaalde. Tijdens zijn studie, van 2006 tot 2009, werkte hij als student assistent en later als studententeamleider op de Post Anesthesia Care Unit (PACU) van het Erasmus MC. Simultaan aan de Geneeskunde studie startte hij in 2007 de Research Master opleiding Clinical Research aan het Netherlands Institute for Health Sciences (NIHES). Onder begeleiding van dr. M. Klimek en dr. F. Grüne (afdeling Anesthesiologie), dr. G. Visser (afdeling Neurologie) en drs. Ingrid Brussé (afdeling Verloskunde en Gynaecologie) deed hij onderzoek naar de doorbloeding van het brein van zwangere vrouwen met pre-eclampsie, en in 2010 verwierf hij voor zijn dissertatie *Cerebral Perfusion Pressure remains elevated in pre-eclamptic patients with normalized blood pressure* de Master of Science titel. Daarnaast nam hij gedurende de zomer van 2009 deel aan het Summer Program van de Harvard School of Public Health in Boston, Massachusetts, USA. In 2009 startte hij onder begeleiding van prof. dr. H.L.A. Janssen aan een promotietraject op de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC met als onderzoeksonderwerp de behandeling van patiënten met chronische hepatitis B. Hij woont samen met Paula Clarijs in Rotterdam.

PhD Portfolio

Summary of PhD training and teaching

Name PhD student: Milan J. Sonneveld

PhD period: 2009-2013

Erasmus MC Department: Gastroenterology and Hepatology

Promotor: Prof. Dr. H.L.A. Janssen

1. Research training

Courses in methodology and biostatistics	**Year**	**Workload**
Study design	2007	104 hours
Principals of Research in Medicine and Epidemiology	2007	15 hours
Methods of clinical research	2007	15 hours
Clinical trials	2007	15 hours
Pharmaco-epidemiology	2007	15 hours
Case-control studies	2007	15 hours
Introduction to decision-making in medicine	2007	15 hours
Introduction to data analysis	2008	25 hours
Regression analysis	2008	30 hours
Topics in meta-analysis	2008	15 hours
Survival analysis	2008	30 hours
Modern statistical methods	2008	104 hours
Advanced topics in decision-making in medicine	2008	15 hours
Intervention research and clinical trials	2008	15 hours
Diagnostic research	2008	15 hours
Prognosis research	2008	15 hours
Ethnicity, health and healthcare	2008	15 hours
Scientific writing in English for publication	2009	30 hours
Pharmaco-epidemiology and drug safety	2009	15 hours
Advanced topics in clinical trials	2009	15 hours
Advanced analysis of prognosis studies	2009	15 hours
Principles of epidemiologic data analysis	2009	15 hours

Oral presentations at (inter)national conferences		
Prediction of sustained response to peginterferon alfa-2b for HBeAg-positive chronic hepatitis B using on-treatment HBsAg decline. Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands.	2010	12 hours
Polymorphisms at rs12979860 and rs12980275 near *IL28B* predict serological response to peginterferon in HBeAg-positive chronic hepatitis B. 46th Annual meeting of the European Association of the Study of the Liver, Berlin, Germany.	2011	36 hours

Presence of precore and core promoter mutants limits the probability of achieving a sustained virological response to peginterferon in HBeAg-positive chronic hepatitis B. Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands.	2011	12 hours
HBsAg levels at six months post-treatment predict sustained response through long-term follow-up in HBeAg-positive patients treated with peginterferon alfa-2b.Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands.	2011	12 hours
Presence of only wildtype virus is the strongest determinant of HBV DNA undetectability and HBsAg clearance in HBeAg-positive chronic hepatitis B patients treated with peginterferon. 47th Annual meeting of the European Association of the Study of the Liver, Barcelona, Spain	2012	36 hours
Adding peginterferon alfa-2a to entecavir increases HBsAg decline and HBeAg clearance – first results from a global randomized trial (ARES study). Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands..	2012	12 hours
Response-guided peginterferon therapy in HBeAg-positive chronic hepatitis B using serum hepatitis B surface antigen levels: a pooled analysis 803 patients. Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands.	2012	12 hours
Adding peginterferon alfa-2a to entecavir increases HBsAg decline and HBeAg clearance – first results from a global randomized trial (ARES study). 63rd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America.	2012	36 hours
Response-guided peginterferon therapy in HBeAg-positive chronic hepatitis B using serum hepatitis B surface antigen levels: a pooled analysis 803 patients. 63rd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America.	2012	36 hours

Poster presentations

HBsAg decline during peginterferon alfa-2b therapy for HBeAg-positive chronic hepatitis B depends on HBV genotype: relation to sustained response. 61st Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America.	2010	32 hours
Prediction of sustained response to peginterferon alfa-2b for HBeAg-positive chronic hepatitis B using on-treatment HBsAg decline. 61st Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America.	2010	32 hours
Peginterferon alfa-2b induced HBsAg decline is durable through long-term follow-up in HBeAg-positive patients. 61st Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America.	2010	32 hours
A comparison of two assays for quantification of hepatitis B surface antigen in patients with chronic hepatitis B. 46th Annual meeting of the European Association of the Study of the Liver, Berlin, Germany.	2011	32 hours
The performance of HBsAg based stopping-rules for HBeAg-positive chronic hepatitis B patients treated with peginterferon depends upon HBV genotype. 62nd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, CA, United States of America.	2011	32 hours

HBsAg levels at six months post-treatment predict sustained response through long-term follow-up in HBeAg-positive patients treated with peginterferon alfa-2b. 62nd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, CA, United States of America.	2011	32 hours
Influence of the presence of precore and core promoter mutants on the relationship between hepatitis B e antigen levels and response to peginterferon therapy. 62nd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, CA, United States of America.	2011	32 hours
Presence of precore and core promoter mutants limits the probability of achieving a sustained virological response to peginterferon in HBeAg-positive chronic hepatitis B. 62nd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, CA, United States of America.	2011	32 hours
Peginterferon is superior to prolonger entecavir therapy for serological response in HBeAg-positive chronic hepatitis B. 62nd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, CA, United States of America.	2011	32 hours
Relationship between precore / basal core promoter mutants and serological or virological response in HBeAg-positice chronic hepatitis B patients treated with nucleo(s)tide analogues. 62nd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, CA, United States of America.	2011	32 hours
Elevated ALT is only predictive for a sustained virological response to peginterferon in HBeAg-positive chronic hepatitis B patients with wildtype virus. 62nd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, CA, United States of America.	2011	32 hours
Serum levels of interferon-gamma inducible protein (IP-)10 are associated with response to peginterferon treatment in genotype D HBeAg-negative chronic hepatitis B. 62nd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, CA, United States of America.	2011	32 hours
Prediction of response to peginterferon for HBeAg-positive chronic hepatitis B using quantitative serology: HBeAg versus HBsAg. 47th Annual meeting of the European Association of the Study of the Liver, Barcelona, Spain	2012	32 hours
Pre-treatment levels of IP-10 predict response to peginterferon in HBeAg-positive chronic hepatitis B patients. 63rd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America.	2012	32 hours

Attended (inter)national conferences

44th Annual Meeting of the European Association for the Study of the Liver (EASL). Copenhagen, Denmark.	2009	28 hours
The Liver Meeting 2009, 60th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). Boston, MA, United States of America.	2009	28 hours
45th Annual Meeting of the European Association for the Study of the Liver (EASL). Vienna, Austria.	2010	28 hours
Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands.	2010	12 hours
The Liver Meeting 2010, 61st Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). Boston, MA, United States of America.	2010	28 hours

46th Annual Meeting of the European Association for the Study of the Liver (EASL). Berlin, Germany.	2011	28 hours
Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands.	2011	12 hours
The Liver Meeting 2011, 62nd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). San Francisco, CA, United States of America.	2011	28 hours
10th European meeting on HIV and hepatitis. Barcelona, Spain.	2012	14 hours
47th Annual Meeting of the European Association for the Study of the Liver (EASL). Barcelona, Spain.	2012	28 hours
63rd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD), Boston, MA, United States of America.	2012	28 hours

Awards

Full bursary from the European Association for the Study of the Liver (EASL) awarded for the best abstracts by young investigators	2011
Full bursary from the European Association for the Study of the Liver (EASL) awarded for the best abstracts by young investigators	2012

Attended seminars and workshops

Tweede Lagerhuisdebat Hepatitis B en C. Amsterdam, the Netherlands	2009	2 hours
7th Post-AASLD symposium. Rotterdam, the Netherlands	2009	2 hours
Derde Lagerhuisdebat Hepatitis B en C. Utrecht, the Netherlands	2010	2 hours
8th Post-AASLD symposium. Rotterdam, the Netherlands	2010	2 hours
9th Post-AASLD symposium. Rotterdam, the Netherlands	2011	2 hours
Het infectieziekten laboratorium van de toekomst. Amsterdam, the Netherlands	2011	8 hours
10th Post-AASLD symposium. Rotterdam, the Netherlands	2012	2 hours

Reviewer for scientific journals

Including Gastroenterology, Hepatology, Gut, Journal of Hepatology

2. Teaching

Lecturing

Baseline predictors of response of chronic hepatitis B patients to peginterferon: HBV genotype. HBV Preceptorship Program. Rotterdam, the Netherlands	2010	6 hours
Een nieuwe toekomst voor oude markers bij de behandeling van hepatitis B. Het infectieziektenlaboratorium van de toekomst. Amsterdam, the Netherlands	2011	6 hours
Management of chronic hepatitis B: immune modulation or antiviral action? Lecture for second year student participating in a MSc programme in Infection and Immunity.	2012	6 hours
Debate: Interferons for treatment of hepatitis B. Pro standpoint. 10th European meeting on HIV & Hepatitis. Barcelona, Spain.	2012	14 hours

Behandeling van chronische hepatitis B en de rol van kwantitatief HBsAg. Landelijke Hepatitis Dag 2012.	2012	14 hours
What's new in chronic hepatitis B? Post-AASLD symposium 2012. WTC, Rotterdam	2012	6 hours

Abbreviations

ALT	Alanine aminotranferase
anti-HBe	Antibody against HBeAg
Anti-HBs	Antibody against HBsAg
BCP	Basal core promoter
cccDNA	Covalently closed circular DNA
CHB	Chronic hepatitis B
CI	Confidence interval
ETV	Entecavir
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HBV DNA	Hepatitis B virus DNA
HCC	Hepatocellullar carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HR	Hazard ratio
IL28B	Interleukin 28-B
LAM	Lamivudine
NA	Nucleo(s)tide analogues
PC	Precore
PCR	Polymerase chain reaction
PEG-IFN	Pegylated interferon
TDF	Tenofovir disoproxil fumarate
ULN	Upper limit of normal
WT	Wildtype