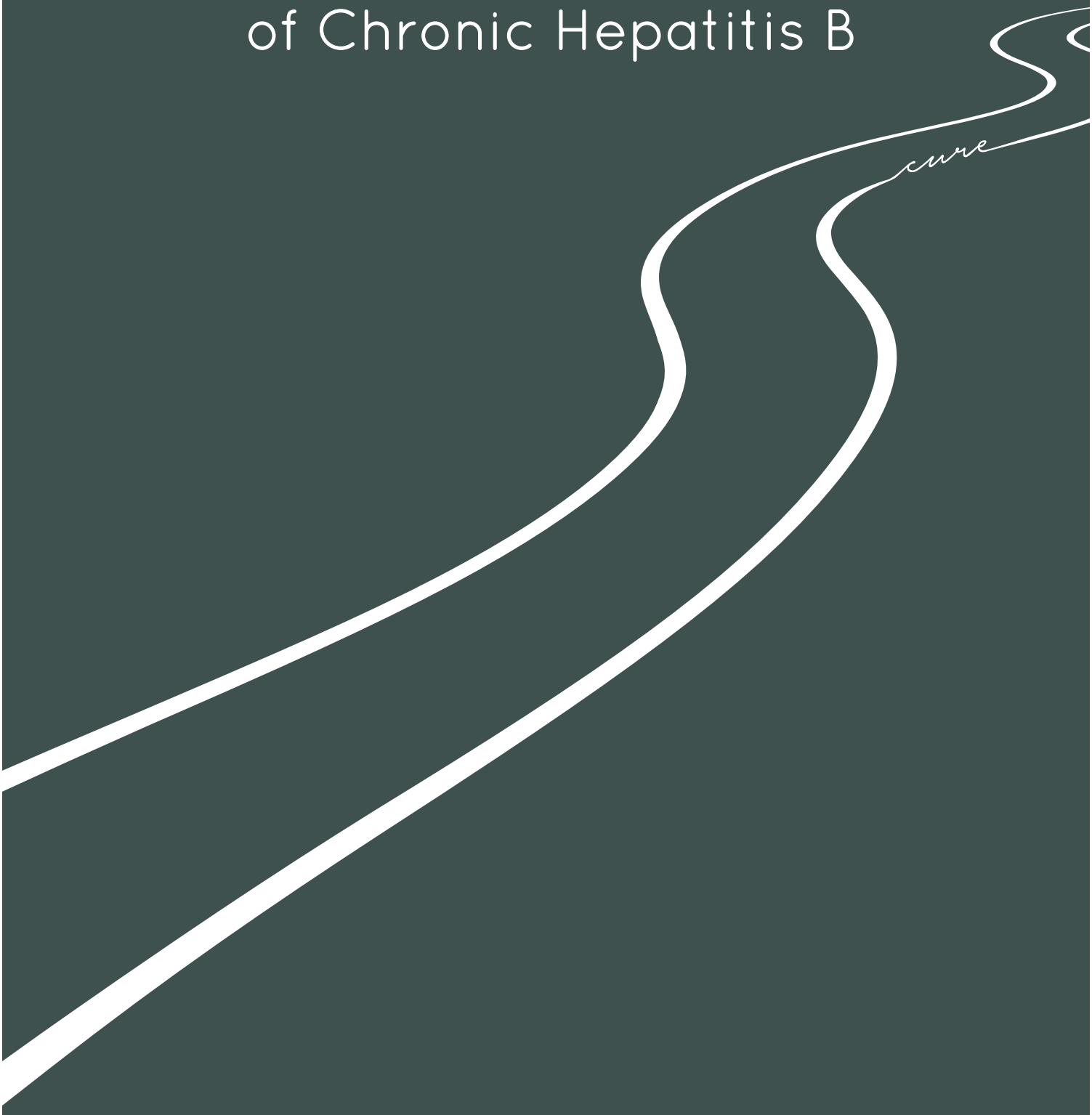
Response Prediction in Modified Treatment of Chronic Hepatitis B



Margo J.H. van Campenhout

Response Prediction in Modified Treatment of Chronic Hepatitis B

Colofon

Cover design by Ilse Modder (www.ilsemodder.nl).
Lay-out by Everdina Meilink, XML 2 Publish.
Printing by Gildeprint, Enschede, The Netherlands.

The work presented in this thesis was conducted at the department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam, The Netherlands.

ISBN: 978-94-6323-407-8

Financial support for printing this thesis was kindly provided by Erasmus Universiteit Rotterdam, the Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam, Nederlandse Vereniging voor Hepatologie, Gilead Sciences Netherlands Inc., Tramedico B.V., Dr. Falk Pharma Benelux B.V., Fujirebio Europe, Chipsoft B.V., Zambon Nederland B.V., and Norgine B.V.

Response Prediction in
Modified Treatment of Chronic Hepatitis B

Voorspellen van respons op
aangepaste behandeling voor chronische hepatitis B

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. R.C.M.E. Engels

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
vrijdag 30 november 2018 om 9.30 uur

door

Margo Jacqueline Harry van Campenhout

geboren te Hulst

Erasmus University Rotterdam

PROMOTIECOMMISSIE:

Promotoren:	Prof.dr. H.L.A. Janssen Prof.dr. H.J. Metselaar
Overige leden:	Prof.dr. R.A. de Man Prof.dr. A. Verbon Prof.dr. I.M. Hoepelman
Copromotor:	dr. B.E. Hansen

TABLE OF CONTENTS

General introduction

Based on How to achieve immune control in chronic hepatitis B? Hepatol Int. 2015 Jan;9(1):9-16

HEPATITIS B INFECTION

Hepatitis B virus (HBV) is a virus that primarily affects the human liver. Infection occurs through vertical transmission (mother-to-child) or horizontal transmission (for example sexual transmission or blood contact) [1]. Chronic HBV infection (defined as an HBV infection that lasts longer than 6 months) is one of the most prevalent infectious diseases worldwide, affecting 257 million patients [2]. In the Netherlands, the incidence of acute HBV infection is 0.7 per 100.000 persons per year, and an estimated 30.000-60.000 persons (0.2-0.4% of people) have chronic HBV infection [3]. Despite development and implementation of an effective and safe vaccine, chronic HBV leads worldwide to approximately 900,000 deaths every year because of complications of cirrhosis , hepatic failure or development of hepatocellular carcinoma (HCC, liver cancer) [2]. Most of these complications occur predominantly through chronic inflammation of the liver caused by the host immune response [1]. Because of the severity of the complications, patients who develop chronic infection need to be routinely monitored, and patients with a high risk of complications require treatment. For both monitoring and treating chronic HBV infection, insights in the complex interplay between the host immune system and the virus are essential.

LEVELS OF IMMUNE CONTROL

In order to control infection with the hepatitis B virus (HBV), both innate and adaptive immune responses play an important role [4]. At the time of infection, natural killer (NK) cells and dendritic cells (antigen-presenting) cells are activated, leading to the production of interferons and other cytokines stimulating the initiation of the adaptive immune response. HBV-specific T-cell responses eventually lead to control of infection in case of self-limiting HBV infection [5, 6]. In chronic HBV, the immune responses are inadequate, resulting in viral persistence with either high or low replication activity depending on the disease phase. The reason that chronicity can occur is that HBV, after entering the hepatocyte, forms a mini-chromosome called covalently closed circular DNA (cccDNA) that is the main transcription template for the virus [7]. During the two initial phases of chronic HBV, formerly known as the immune-tolerant phase (IT) and immune clearance or immune active phase (IA), HBeAg is an important marker of HBV replication. These phases were recently renamed HBeAg-positive chronic HBV infection and HBeAg-positive chronic hepatitis, respectively [8]. HBeAg-positive patients have a high level of viral replication illustrated by high serum levels of

HBV DNA. Classically in the first phase, alanine transaminase (ALT) level is not elevated whereas in the second phase, ALT level is elevated reflecting significant liver inflammation. Loss of HBeAg is often followed by an inactive carrier state of infection, which is characterized by normal ALT levels and suppression of viral replication. This stage, now called HBeAg-negative chronic infection, can be considered as a first step in immune control of chronic HBV, as loss of HBeAg is generally associated with lower risk of liver-related complications and improved survival [9]. However, in the years following spontaneous HBeAg-seroconversion, up to 33% of patients develop HBeAg-negative hepatitis with active inflammation and viral replication [10]. This can be partially explained by mutations in the precore and core promotor region of the HBV genome, causing viral inability to produce HBeAg with maintenance of the capacity to produce virus and induce active inflammation. However, since HBeAg-negativity does not guarantee stable disease, a higher level of immune control should be pursued. At present, seroclearance of the Hepatitis B surface Antigen (HBsAg) with appearance of antibodies (anti-HBs positivity is regarded as the highest degree of immune control and the clinical endpoint closest to cure. Although HBV DNA is often not detectable in the blood of HBsAg-negative individuals and reactivation does not occur in an immunocompetent state, a risk of reactivation does exist at the time of immunosuppression due to persistence of the cccDNA [11, 12].

TREATMENT FOR CHRONIC HEPATITIS B INFECTION

According to current treatment guidelines, treatment of chronic HBV is required in case of severe or persistent liver inflammation, in case of a high risk of HBV-related complications (such as in patients with liver cirrhosis), or in case of a high risk of transmission or reinfection (such as pregnancy or liver transplantation) [8, 13]. The two available classes of treatment are nucleos(t)ide analogue (NA) therapy and interferon-alpha (IFN)-based therapy.

NAs are potent inhibitors of viral replication that are to be taken orally once daily. They interfere with viral replication, and therefore NA treatment leads to a strong decline of infectious HBV particles in serum and durable suppression while remaining on treatment. One year after treatment is started, HBV DNA levels are suppressed in approximately two third of HBeAg-positive patients and more than 90% of HBeAg-negative patients [8]. Lower HBV DNA levels are associated with less inflammation activity, reversal of liver fibrosis, and lower incidence of HBV-related complications [14, 15]. However once started, lifelong antiviral therapy is required in most of chronic HBV patients. This is due to the high probability

of relapse after treatment discontinuation and low rates of functional cure, as HBsAg loss on the long-term is achieved in only 10-12% of HBeAg-positive patients and 1-2% of HBeAg-negative patients [16, 17]. The long therapy duration leads to high healthcare costs and to potential antiviral resistance due to adherence problems. In addition, long-term viral suppression decreases but does not eliminate the risk of HCC and may be inferior to viral suppression maintained by the host immune system [18].

IFN treatment acts on different steps in the viral life cycle, but the exact mechanisms are not fully understood [19]. It has mainly immunomodulatory effects, but has also effects on epigenetic regulation of the cccDNA [20, 21]. The advantages of PEG-IFN treatment are that it has a finite course of 48 weeks, and that the serological response rates are higher and more durable than achieved by NA therapy. In patients who lost HBeAg during IFN-a or PEG-IFNa treatment, HBeAg loss is sustained in around 85% of patients when assessed 6 months after treatment discontinuation [22-25]. In contrast, HBeAg-seroconversion induced by NA therapy is sustained in a minority of cases [26-28]. HBsAg loss at one year of treatment is achieved in 3-7% in HBeAg-positive patients and in 4% in HBeAg-negative patients [8]. However, PEG-IFN side-effects like flu-like symptoms or myelosuppressive effects are common, PEG-IFN administration requires subcutaneous injection, only a subset of patients responds to therapy, and PEG-IFN is contraindicated in patients with liver cirrhosis. This restricts the use of IFN and worldwide only a small minority of patients are currently treated with this compound.

As clearly both treatment modalities have advantages and disadvantages and functional cure of chronic HBV is hardly achieved, an need for progress in chronic HBV therapeutic options still exists. Current research not only focusses on achieving HBsAg loss, but also on silencing or eradicating the cccDNA (which would imply complete cure), or on disrupting the viral life cycle [29]. While awaiting new drugs that either increase the probability of functional cure or are even able to achieve a complete cure, optimization of current treatment options is warranted. This thesis will focus on 2 different optimization strategies, namely on combining currently available treatment modalities PEG-IFN and NA, and on response prediction.

OPTIMIZING HBV TREATMENT BY COMBINING TREATMENT MODALITIES

Since PEG-IFN and NAs are both potent in targeting HBV but with different modes of action, modified treatment strategies with both PEG-IFN and potent

NAs have unsatisfying success rates in the achievement of sustainable immune control. Although it does not seem beneficial to simultaneously start PEG-IFN and NAs [30, 31], observations of the effects of HBV on the immune system have led to new hypotheses for other combination regimens. HBV is known to negatively impact NK-cell function, IFN-mediated cell signalling and T-cell function [32-34]. Lowering HBV DNA is therefore thought to restore immune cell function. Indeed, in vitro and in vivo studies showed that NAs are able to improve HBV-specific T-cell responses [35], leading to the hypothesis that the immunomodulatory effect of PEG-IFN may be stronger when PEG-IFN is added at the time of NA-induced viral suppression. In a randomized controlled trial in HBeAg-positive patients, patients were randomized after 24 weeks of ETV treatment to either 24 weeks of PEG-IFN addition or continuation of ETV monotherapy [36]. Rates of HBeAg loss were higher in patients who had PEG-IFN addition compared to patients who continued monotherapy. In addition, sustainability of off-treatment response was better in patients who received PEG-IFN addition, which was also illustrated by a significantly stronger HBsAg decline in the PEG-IFN add-on group [36]. In patients who had received a longer duration of NA therapy before adding PEG-IFN, it was reported PEG-IFN did not lead to a significant increase of HBeAg loss rates in the overall study population, but did significantly improve response in IFN-naïve patients [37].

Because the add-on strategy is relatively new, the long term effects have not been addressed and few analyses have yet been performed to identify factors associated with response. Balancing the add-on induced gain in response rates versus the well-known PEG-IFN side-effects, in our opinion the response rates to add-on that were found earlier were not high enough to treat every patient this way. **Chapter 1** of this thesis therefore aimed to identify those patients who are likely to benefit from this modified treatment strategy. We have learned from earlier studies in PEG-IFN monotherapy that factors such as HBV genotype and quantitative HBsAg and HBV DNA levels in serum are of major importance in the pre-treatment and on-treatment identification of patients who are less likely to respond [38-40]. We expected that these factors would also be relevant to PEG-IFN add-on therapy. Additionally we aimed to investigate the long-term effects of the PEG-IFN strategy in **Chapter 2**. If the add-on induced improvement in response rate is maintained or increases even more beyond week 96 of follow-up, this would underline the potency of a short-term add-on course.

OPTIMIZING HBV TREATMENT BY BIOMARKER MONITORING

Ideally, HBV replication activity would be measured by a single, non-invasive test, which not only reflects the current replication activity but also the degree of immune control related to the future replication activity. As written before, detectability of HBsAg in serum quite meets these needs, since it implies functional cure and HBV reactivation only occurs in an immune incompetent state. Sustained HBsAg undetectabilty in serum therefore is a highly desired treatment outcome from a clinical point of view. However even more desired these days is a biomarker that reflects activity of the intrahepatic cccDNA, as HBV treatment that would be able to eliminate cccDNA would theoretically also prevent HBV reactivation [41]. CccDNA itself can be measured in liver biopsies, but as quantification is difficult and no international reference standard is available, studies describing cccDNA quantities and cccDNA activity cannot be compared one on one. Also several assays lack specificity because of the risk of simultaneous detection of relaxed circular DNA (rcDNA), and cccDNA itself is probably not spread throughout the liver in a homogeneous way. These limitations complicate interpretation of cccDNA quantities [41, 42]. To avoid the need for a biopsy for estimation of HBV replication activity, serum markers correlating with cccDNA replication activity could be the solution. Serum markers that have been extensively studied include quantitative HBV DNA, HBsAg, and HBeAg, of which especially the first two are commonly used in clinical practice [8, 13]. However since these biomarkers cannot be used in all patients and all treatment settings, novel serum markers are still under investigation, especially now new compounds are in development.

The second part of this thesis will therefore focus on the question if measuring serum levels of two novel serum biomarkers hepatitis B core-related antigen (HBcrAg, **Chapters 3 & 4**) and HBV RNA (**Chapters 5, 6, 7 & 8**) can be used for treatment monitoring and help to identify the best treatment strategy for individual HBV patients. The general design of these studies is shown in Figure 1.

HBcrAg is a biomarker that simultaneously measures hepatitis B core antigen (HBcAg), HBeAg and a 22-kDa precore protein called p22cr. The luminescent antibodies used in this CLEIA-based test are directed against the amino acid sequence that these proteins have in common. HBV RNA on the other hand is detected by RACE-PCR. Both markers were reported to correlate to intrahepatic cccDNA and to treatment response in small study populations [43-48], but in order to determine their value for clinical and research purposes, we were interested in the dynamics of these biomarkers in larger and more heterogenic

patient groups. We therefore explored the relation between serum levels of these biomarkers and levels of biomarkers that are already used in daily practice. Because studies on HBV RNA levels in untreated patients were lacking because of the recent development of the test, we first aimed to do so in untreated patients who require treatment according to current treatment guidelines (**Chapter 5**). Next, we studied the relation of both biomarkers to treatment response in PEG-IFN based-therapy for HBeAg-positive hepatitis (**Chapter 3** for HBcrAg and **Chapters 6 & 8** for HBV RNA), and HBeAg-negative hepatitis (**Chapter 4** for HBcrAg, **Chapter 7** for HBV RNA).

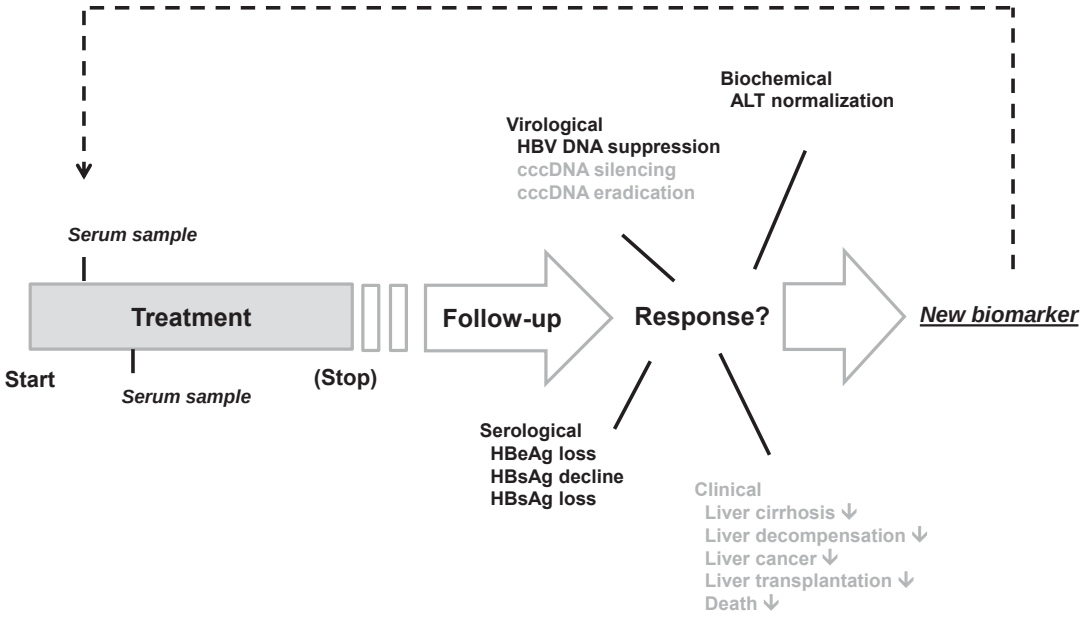


Figure 1. General design for biomarker studies

1

Low Hepatitis B surface Antigen and HBV DNA levels predict response of pegylated interferon addition to entecavir in Hepatitis B e Antigen-positive chronic hepatitis B

Kin Seng Liem[1,2], Margo J.H. van Campenhout[2], Qing Xie[3], Willem Pieter Brouwer[2], Heng Chi[2,] Xun Qi[4], Liang Chen[4], Fehmi Tabak[5], Bettina E. Hansen[1,2,6], Harry L.A. Janssen[1]

[1]*Toronto Centre for Liver Disease, Toronto General Hospital, University Health Network, Toronto, Canada.* [2]*Department of Gastroenterology and Hepatology, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands.* [3]*Department of Infectious Diseases, Ruijin Hospital, Jiaotong University, Shanghai, China.* [4]*Department of Hepatitis Disease, Shanghai Public Health Clinical Center, Fudan University, Shanghai, China.* [5]*Çerrahpasa Medical Faculty, Istanbul, Turkey.* [6]*Institute of Health Policy, Management and Evaluation, University of Toronto, Toronto, Canada.*

Alimentary Pharmacology & Therapeutics, 2018

ABSTRACT

Background & aims. Various treatment combinations of peginterferon (PEG-IFN) and nucleos(t)ide analogues have been evaluated for chronic hepatitis B (CHB), but the optimal regimen remains unclear.

Methods. We studied whether PEG-IFN add-on increases response compared to entecavir (ETV) monotherapy, and whether the duration of ETV pre-treatment influences response. Response was evaluated in HBeAg positive patients previously treated in two randomized controlled trials. Patients received ETV pre-treatment for at least 24 weeks and were then allocated to 24-48 weeks of ETV + PEG-IFN add-on, or continued ETV monotherapy. Response was defined as HBeAg loss combined with HBV DNA <200 IU/mL 48 weeks after discontinuing PEG-IFN.

Results. Of 234 patients, 118 were assigned PEG-IFN add-on and 116 continued ETV monotherapy. Response was observed in 38/118 (33%) patients treated with add-on therapy and in 23/116 (20%) with monotherapy (p=0.03). The highest response to add-on therapy compared to monotherapy was observed in PEG-IFN naïve patients with HBsAg levels below 4,000 IU/mL and HBV DNA levels below 50 IU/mL at randomization (70% vs. 34%; p=0.01). Above the cut-off levels, response was low and not significantly different between treatment groups. Duration of ETV pre-treatment was associated with HBsAg and HBV DNA levels (both p<0.005), but not with response (p=0.82).

Conclusions. PEG-IFN add-on to ETV therapy was associated with higher response compared to ETV monotherapy in patients with HBeAg positive CHB. Response doubled in PEG-IFN naïve patients with HBsAg below 4,000 IU/mL and HBV DNA below 50 IU/mL, and therefore identifies these as the best candidates for PEG-IFN add-on.

INTRODUCTION

The achievement of functional cure for chronic hepatitis B infection (CHB) remains difficult due to a persistent infection of hepatocytes with covalently closed circular DNA (cccDNA).1,2 CccDNA is a minichromosome that serves as a transcription template for hepatitis B virus (HBV) antigen and virion production. Nucleos(t)ide analogue (NA) therapy only marginally reduces levels of cccDNA such that cccDNA depletion would require years of NA treatment.3,4

NA therapy effectively suppresses the hepatitis B virus (HBV) up to eight years with few side-effects, but serological response rates remain low. The discontinuation of NA therapy leads to frequent virological relapse and patients therefore require long-term, if not indefinite NA therapy.5–10 In contrast, a finite course of pegylated interferon (PEG-IFN) achieves more sustained immune response than NA therapy.9,11,12 PEG-IFN is also able to directly target cccDNA and induce cccDNA decline in combination with NA therapy.13,14 PEG-IFN monotherapy however induces sustained response in only 30-40% of patients and has limited tolerability.15,16

These limitations of CHB therapy have led to the evaluation of various treatment combinations of NAs and PEG-IFN to maximize response rates, among which is the strategy of adding PEG-IFN to NA treatment (PEG-IFN add-on). One of the rationales for the PEG-IFN add-on strategy is that long-term NA treatment enables partial restoration of the liver-specific immunology of both the adaptive (T-cells) and innate immune system (natural killer cells).17–20 Viral load suppression could thus increase the immunomodulatory effect of PEG-IFN therapy resulting in increased HBsAg loss and HBeAg loss or accelerated HBsAg decline rates.11

Several randomized controlled trials (RCT) employed a PEG-IFN add-on strategy in HBeAg positive and negative patients on long-term NA monotherapy.21–23 PEG-IFN add-on increased HBeAg seroconversion and viral antigen decline, but primary efficacy endpoints were not reached, possibly because of insufficient power or because the effect was limited to a subgroup of patients only. Clinical practice could benefit substantially if these responsive patients can be identified at the start of PEG-IFN therapy with readily available laboratory markers. Other remaining issues concern the optimal duration of PEG-IFN add-on and of NA pre-treatment.

We therefore evaluated whether PEG-IFN add-on to ETV treatment increases serological response compared to ETV monotherapy in CHB, and whether the duration of ETV pre-treatment or the length of PEG-IFN addition therapy influenced response. To this purpose, we performed an analysis in a large HBeAg positive CHB population that was previously treated in two global RCTs.

PATIENTS AND METHODS

Combined study design

We conducted a post-hoc analysis of two international RCTs (ARES and PEGON; registered at ClinicalTrials.gov, Identifier: NCT00877760, NCT01532843).[21,23] Detailed inclusion and exclusion criteria have been previously described. In short, patients with CHB were eligible if they were HBeAg positive at randomization (baseline) and had a serum alanine aminotransferase (ALT) between 1.3 and 5 times the upper limit of normal (ULN). Patients had received pre-treatment with ETV for at least 6 months. The main exclusion criteria were history of decompensated liver disease, co-infection with hepatitis C virus or HIV, other concomitant liver disease, and any contra-indication for interferon therapy.

After initial treatment with ETV (Baraclude, 0.5 mg once-daily), patients were randomized to either 6-12 months of PEG-IFN addition or of continued ETV monotherapy (Figure 1). Patients treated within the ARES trial received PEG-IFN a2a (Pegasys, 180 µg once-weekly) and patients in the PEGON study PEG-IFN a2b (PegIntron, 1.5 µg/kg once-weekly). If patients achieved HBeAg seroclearance in combination with an HBV DNA level below 200 IU/mL at the end of PEG-IFN treatment (EOT) or at the corresponding time point for patients allocated to ETV monotherapy, ETV was discontinued after a minimum of 24 weeks consolidation therapy. Otherwise, ETV was continued until the end of follow-up (EOF), which was 48 weeks after EOT for all patients regardless of treatment response.

Several patients within the ARES study did not reach the designated primary endpoint at the end of treatment. These patients were allowed to enroll in the subsequent PEGON trial and were then randomized again to PEG-IFN add-on or ETV monotherapy. This study was approved by local ethics boards of all centers and performed in concordance with Good Clinical Practice guidelines and the Declaration of Helsinki. All patients provided written consent.

Study endpoints

Response was defined as combined HBeAg loss with HBV DNA <200 IU/mL at EOF. We analyzed the modified intention-to-treat population, which includes all patients who received at least one dose of the allocated treatment after baseline. Patients were considered non-responders in case of missing HBeAg status or HBV DNA at EOF. To assess the potential for functional cure, as studied with therapeutic compounds now in development, we also investigated specific other virological and serological outcomes (Table 2).

Study follow-up and measurements

During PEG-IFN treatment, routine examination and laboratory testing were performed every 4 weeks. After PEG-IFN treatment was stopped, patients visited the outpatient clinic every 12 weeks until EOF. Patients on ETV monotherapy had study visits every 12 weeks throughout the entire study period. Routine biochemical and hematological tests were assessed locally at every visit. Serum ALT levels were standardized according to the ULN per center and gender. Serum HBV DNA was measured with the Cobas TaqMan 48 polymerase chain reaction assay (lower limit of detection: 20 IU/mL; Roche Diagnostics, Basel, Switzerland). Serum HBeAg, anti-HBe and HBsAg were evaluated with Architect (Abbott Laboratories, North Chicago, IL, USA) or Cobas Elecsys 411 (lower limit of detection 0.30 IU/L and 0.05 IU/mL, respectively; Roche Diagnostics). HBV genotyping was performed with the INNO-LiPA HBV genotype assay (Fujirebio Europe, Ghent, Belgium). If HBV genotype could not be assessed due to undetectable HBV DNA levels at baseline, we reviewed HBV genotype data in medical charts where possible. The presence of cirrhosis was defined by Ishak stage 6 on liver biopsy, or an aspartate aminotransferase to platelet ratio index (APRI) score >1.0.[24]

Statistical analysis

Variables are summarized with mean ± SD or frequency (percentage). Non-normally distributed variables were log-transformed. Differences in outcomes were evaluated by chi-squared test, Student's *t*-test or Mann-Whitney test, where appropriate. To study the influence of PEG-IFN addition on response and adjust for confounders, we performed logistic regression analysis. Pre-defined covariates included age, gender, HBV genotype, cirrhosis, previous use of PEG-IFN, duration of ETV pre-treatment, ALT, HBV DNA and HBsAg. The duration of ETV pre-treatment and HBV DNA were categorized due to a skewed distribution. Predictors that were significantly associated with response in univariable regression (*p*-value <0.10) were further evaluated in multivariable regression (backward

stepwise selection). Interactions between response and baseline variables included in the final model were explored.

Cut-off values for HBV DNA and HBsAg at baseline were evaluated to find clinically useful starting rules for PEG-IFN add-on. HBsAg levels were dichotomized at thresholds between 2.7 and 5.0 log IU/mL in steps of 0.1. HBV DNA was categorized at 50, 100, 500 and 1,000 IU/mL. The likelihood-ratio test and sum of log-likelihood ratios of the two treatment groups were calculated. We selected optimal cut-off values based on a minimum response difference of 15% between add-on and monotherapy; a significant likelihood ratio test of add-on vs. monotherapy below the cut-offs, but not above; and the lowest sum of likelihood ratios. For each threshold Receiver Operating Characteristic (ROC) curves were constructed and AUCs were calculated and compared to each other. Furthermore, a sensitivity analysis was performed among non-responding patients within the ARES study who subsequently received retreatment in the PEGON study by modeling the correlated data in a generalized estimating equation.[25] Analyses were performed in SPSS (v. 22.0, Chicago, IL) and SAS v. 11.2 (SAS Institute Inc., Cary, NC). Two-sided p-values <0.05 were considered significant.

RESULTS

Patient population

A total of 234 patients met the inclusion criteria. Excluded were 5 patients assigned PEG-IFN add-on and 10 assigned ETV monotherapy who had achieved HBeAg loss at baseline (during ETV pre-treatment). At baseline, 118 patients were allocated to PEG-IFN add-on and 116 patients continued ETV monotherapy. Baseline characteristics were comparable between the two groups (Table 1). The mean age was 33 (SD 9) years, the majority of patients were male and of Asian ethnicity. HBV genotypes A/B/C/D/other were present in 4%, 17%, 41%, 24% and 1% of patients, respectively. In total, 80/118 (68%) patients received PEG-IFN add-on for 24 weeks and 38/118 (32%) patients received PEG-IFN add-on for 48 weeks. Among patients included in the ARES study, 36 non-responders were re-included in the subsequent PEGON trial. The baseline characteristics per trial are shown in Supplementary Table 1.

Table 1. Characteristics of the modified intention-to-treat population at randomization.

		PEG-IFN add-on (n=118)	ETV monotherapy (n=116)
Age, years (SD)		33 (10)	33 (9)
Male gender		87 (74%)	83 (72%)
Ethnicity	Asian	85 (72%)	84 (72%)
	Caucasian	31 (26%)	31 (27%)
	Other	2 (1.7%)	1 (0.9%)
HBV genotype†	A	3 (2.5%)	6 (5.2%)
	B	22 (19%)	17 (15%)
	C	45 (38%)	51 (44%)
	D	30 (25%)	26 (22%)
	Other/unknown†	18 (14%)	16 (14%)
Cirrhosis‡		3 (2.5%)	5 (4.3%)
Previous (PEG-)IFN therapy		16 (14%)	20 (17%)
ETV pre-treatment	6-12 months	80 (68%)	79 (68%)
	1-2 years	12 (10.2%)	9 (7.9%)
	2-3 years	16 (22%)	28 (24%)
Alanine aminotransferase, ULN (IQR)		0.5 (0.3-0.9)	0.5 (0.4-0.9)
HBV DNA, IU/mL	Undetectable§	38 (32%)	42 (36%)
	20-100	16 (14%)	27 (23%)
	100-1,000	27 (23%)	18 (16%)
	>1,000	37 (31%)	29 (25%)
Quantitative HBsAg, log IU/mL (SD)		3.7 (0.7)	3.6 (0.7)
Quantitative HBeAg, log IU/mL (IQR)		1.1 (0.5-2.0)	1.0 (0.4-1.9)
PEG-IFN duration	24 weeks	80 (68%)	-
	48 weeks	38 (32%)	-

† HBV genotyping was not possible for 32 patients (all Asian) due to undetectable HBV DNA at randomization.
‡ Cirrhosis was defined as Ishak stage 6 on liver biopsy; all 81 patients with unavailable biopsy data had an APRI score <1.0, which suggests absence of cirrhosis.
§ <20 IU/mL.
IQR: interquartile range; SD: standard deviation; ULN: upper limit of normal.

Response

Response was reached in 38/118 (33%) patients allocated to add-on therapy and in 23/116 (20%) patients with ETV monotherapy (p=0.03; Figure 1 and Table 2).Other serological, virological and biochemical outcomes are reported in Table 2. HBeAg seroconversion rates at EOF were also significantly higher in PEG-IFN add-on patients. The response group comprised significantly more males (84 vs 69%, p=0.03), and had a higher frequency of genotype B (26% vs 13%) and fewer genotype D (12% vs 28%) compared to non-responders. Furthermore,

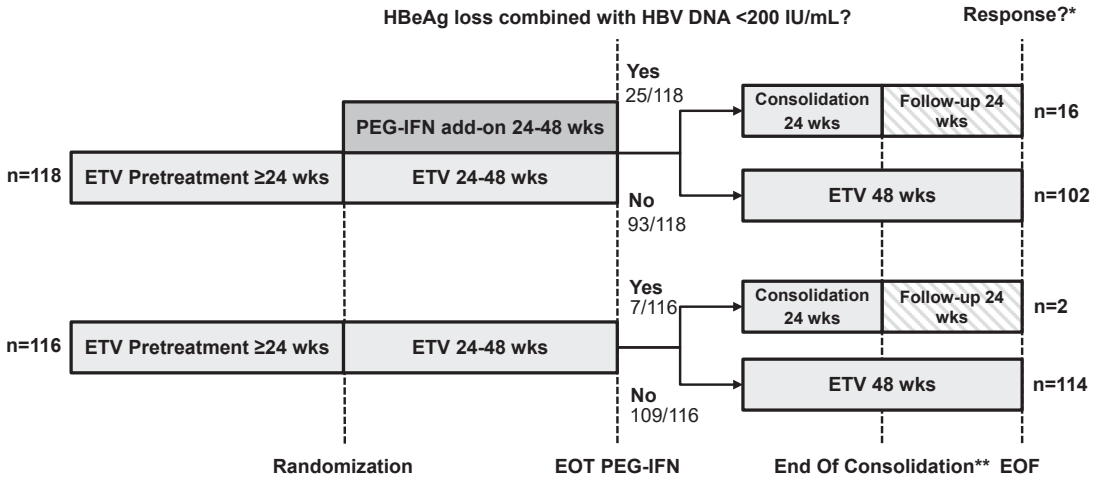


Figure 1. Combined study design

* Response: HBeAg loss in combination with HBV DNA <200 IU/mL at end of follow-up.

** Only for responders. Non-responders were treated with ETV until EOF.

responders had significantly lower ALT (0.4 vs. 0.6 x ULN, *p*=0.01), HBsAg (3.3 vs. 3.8, *p*<0.005) and HBeAg (0.5 vs. 1.4, *p*<0.005) levels at baseline, and a higher frequency of undetectable HBV DNA at baseline (53% vs. 28 %, *p*<0.005) than non-responders. Other baseline characteristics were comparable between patients with and without a response. Response occurred in 12/16 patients assigned to PEG-IFN add-on vs. 2/2 assigned to ETV monotherapy (*p*=0.42) in the subgroup that achieved HBeAg loss in combination with HBV DNA <200 IU/mL at EOT.

The two sensitivity analyses (cohort without 36 retreated non-responders and whole cohort with adjustment for correlated data) were consistent with our findings indicating that PEG-IFN add-on significantly increased response to ETV monotherapy (Supplementary table 1).

HBsAg decline and loss

HBsAg decline >0.5 log IU/mL occurred more often in the PEG-IFN add-on group compared to the ETV monotherapy group at EOF (25 [23%] vs. 11 [9.6%]; *p*=0.01). HBsAg <1,000 IU/mL was reached by 35/118 (30%) patients with PEG-IFN add-on and by 25/116 (22%) with ETV monotherapy (*p*=0.32) at EOT, which increased to 27% at EOF in both groups (*p*=0.97). The proportions of patients with HBsAg <100 IU/mL in PEG-IFN add-on vs. ETV monotherapy were 1 (1%) vs. 5 (4%) at baseline (*p*=0.09), and 6 (5%) vs. 5 (4%) at EOF (*p*=0.77). The proportion of patients in the add-on group with HBsAg <100 IU/

Table 2. Outcome over time in 234 HBeAg positive patients.

	Baseline Randomization			End of PEG-IFN Week 24-48			End of consolidation Week 72			End of follow-up Week 96		
	Add-on	ETV Mono	P	Add-on	ETV Mono	P	Add-on	ETV Mono	P	Add-on	ETV Mono	P
n (%)	n=118	n=116		n=118	n=116		n=118	n=116		n=118	n=116	
Response												
HBeAg loss + HBV DNA <200 IU/mL	–	–	–	25 (21)	7 (6.0)	<0.005*	35 (30)	15 (13)	<0.005*	38 (33)	23 (20)	0.03*
Virological outcomes												
HBV DNA <2,000 IU/mL	89 (75)	92 (79)	0.48	111 (95)	100 (86)	0.02*	104 (89)	102 (88)	0.82	99 (85)	104 (90)	0.24
HBV DNA <200 IU/mL	64 (54)	75 (65)	0.11	102 (87)	88 (76)	0.03*	93 (80)	92 (79)	0.97	95 (82)	97 (84)	0.62
HBV DNA undetectable†	38 (32)	41 (35)	0.61	37 (32)	41 (35)	0.55	37 (32)	41 (35)	0.55	36 (31)	40 (35)	0.54
Serological outcomes												
HBeAg loss	–	–	–	25 (22)	7 (6.0)	<0.005*	36 (32)	15 (13)	<0.005*	40 (36)	23 (20)	0.01*
HBeAg seroconversion	–	–	–	19 (16)	2 (1.7)	<0.005*	26 (22)	5 (4.3)	<0.005*	28 (24)	11 (9.6)	<0.005*
HBsAg loss	0 (0.0)	0 (0.0)	NS	1 (0.8)	0 (0.0)	NS	1 (0.8)	0 (0.0)	NS	1 (0.8)	0 (0.0)	NS
HBsAg <1,000 IU/mL	22 (19)	22 (19)	0.95	35 (30.0)	28 (24)	0.32	33 (28)	32 (28)	0.92	31 (27)	31 (27)	0.97
HBsAg <100 IU/mL	1 (0.8)	5 (4.3)	0.09	10 (8.5)	4 (3.4)	0.10	6 (5.1)	4 (3.4)	0.53	6 (5.2)	5 (4.3)	0.77
HBsAg decline >0.5 log IU/mL	–	–	–	30 (26)	2 (1.7)	<0.005*	30 (26)	6 (5)	<0.005*	25 (23)	11 (9.6)	0.01*
Biochemical outcome												
ALT normalization	96 (81)	90 (78)	0.56	73 (63)	94 (82)	<0.005*	104 (91)	98 (86)	0.21	103 (92)	99 (86)	0.16

** P <0.05.*
† <20 IU/mL.
NS: not significant.

mL increased from baseline to EOF (p=0.06). HBsAg loss was observed in one patient assigned to PEG-IFN add-on.

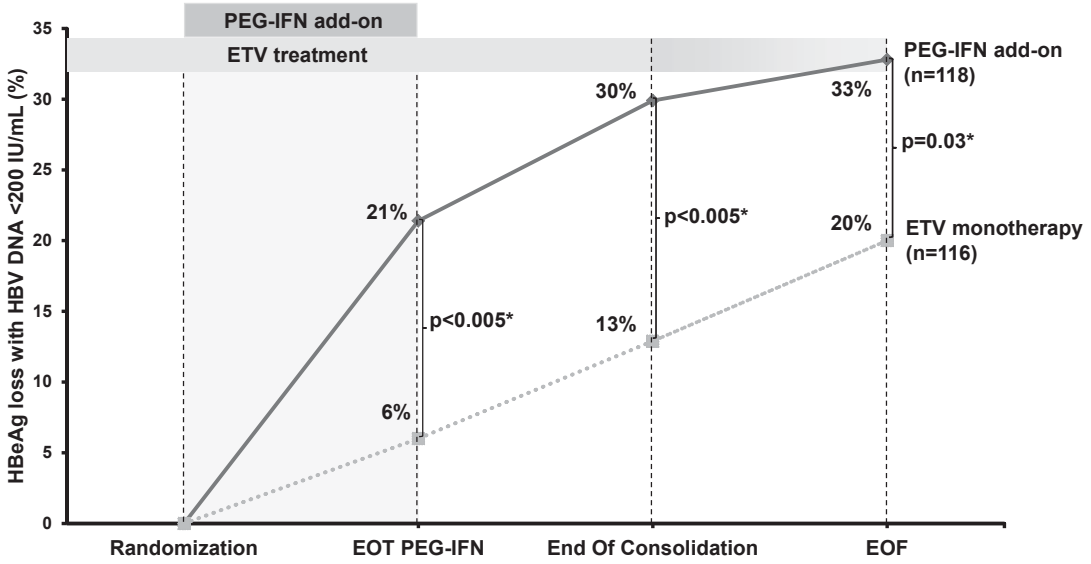


Figure 2. Response

Out of 32 patients who reached combined HBeAg loss and HBV DNA <200 IU/mL at week 48, 18 discontinued treatment after ETV consolidation therapy.

* P <0.05.

Response prediction

By univariable analysis, response was associated with PEG-IFN add-on (odds ratio [OR]: 1.9; 95% confidence interval [CI]: 1.1-3.5; p=0.03), male sex (OR: 2.3; 95%CI: 1.1-4.9; p=0.03), HBV genotype (p=0.02), lower ALT (OR: 0.3; 95%CI: 0.1-0.7; p=0.01), lower HBV DNA level (OR: 0.5; 95%CI: 0.3-0.7; p<0.005) and lower HBsAg level at baseline (OR: 0.4; 95%CI: 0.2-0.6; p<0.005; Table 3). The duration of ETV pre-treatment was associated with HBsAg and HBV DNA at baseline (both p<0.005), but not with response (1-3 years vs. 0-1 year, OR: 1.1; 95%CI: 0.6-2.2; p=0.76), nor was duration of the PEG-IFN add-on regimen (p=0.92). In multivariable analysis, PEG-IFN add-on remained independently associated with response (OR: 2.5; 95%CI: 1.3-4.8; p=0.01, when adjusted for HBV DNA and HBsAg level at baseline). Response rates to PEG-IFN add-on compared to ETV monotherapy increased especially in PEG-IFN naïve patients with lower serum HBV DNA and HBsAg at baseline (Supplementary figure 1).

Table 3. Logistic regression on response at end of follow-up.

	Univariable regression			Multivariable regression		
Variable	**OR**	**95%CI**	**p-value**	**OR**	**95%CI**	**p-value**
Age, years	1.02	0.99-1.05	0.24			
Gender, male vs. female	2.31	1.09-4.90	0.03*			NS
HBV genotype‡			0.02*			NS
- C	Reference					
- A vs. C	1.50	0.35-6.47	0.59			
- B vs. C	2.09	0.95-4.59	0.07			
- D vs. C	0.43	0.17-1.07	0.07			
- Other vs. C	1.44	0.61-3.37	0.41			
Cirrhosis	1.76	0.41-7.59	0.45			
Duration of ETV, months			0.79			
- 0-1 yr	Reference					
- 1-3 yrs vs. 0-1 yr	1.12	0.56-2.23	0.76			
- >3 yrs vs. 0-1 yr	1.28	0.46-3.54	0.64			
PEG-IFN experienced vs. naïve	0.64	0.27-1.56	0.33			
PEG-IFN duration, 12 vs. 6 mo	0.96	0.41-2.20	0.92			
PEG-IFN add-on, compared to ETV monotherapy	1.92	1.06-3.49	0.03*			
- within PEG-IFN naïve				3.72	1.76-7.87	<0.005*
- within PEG-IFN experienced				0.24	0.04-1.66	0.15
ALT, x ULN	0.32	0.14-0.74	0.01*			NS
HBV DNA, IU/mL†			<0.005*			0.02*
- Undetectable	Reference			1.00		
- 20-100 vs. undetectable	0.67	0.30-1.49	0.33	0.62	0.26-1.47	
- 100-1,000 vs. undetectable	0.53	0.24-1.17	0.12	0.47	0.19-1.16	
- >1,000 vs. undetectable	0.10	0.03-0.29	<0.005*	0.12	0.04-0.42	
HBsAg, log IU/mL	0.38	0.24-0.60	<0.005*	0.51	0.29-0.89	0.02*

** P <0.05.*
† HBV DNA groups: < lower limit of detection (<20 IU/mL); 20-100 IU/mL; 100-1,000 IU/mL; ≥1,000 IU/mL
NS: not significant; ULN: upper limit of normal.

Response-guided therapy using HBV DNA and HBsAg

To establish clinical starting rules for PEG-IFN add-on, the relationship between different cut-off values of HBsAg and HBV DNA at baseline and likelihood of response was evaluated. As previous use of PEG-IFN was strongly associated with a lack of response, we evaluated all PEG-IFN naïve patients (n=198/234, 85%). Based on this analysis, PEG-IFN naïve patients with an HBsAg level below 4,000 IU/mL (3.6 log) and HBV DNA level below 50 IU/mL (1.7 log) at base-

line achieved the largest gain in probability of response with PEG-IFN add-on compared to ETV monotherapy (70% vs. 34%, *p*=0.01; Figure 3). Patients who met one of the above criteria achieved a moderate gain in response from PEG-IFN add-on, compared to ETV monotherapy (44% vs. 17%; *p*=0.02). Above the proposed HBsAg and HBV DNA cut-off levels, response was very low and not significantly different between treatment groups (PEG-IFN add-on vs. ETV monotherapy: 9.3% vs. 5.9%; *p*=0.58). The cut-off values combined had an AUC of 0.79 (95%CI: 0.72-0.86) for probability of response.

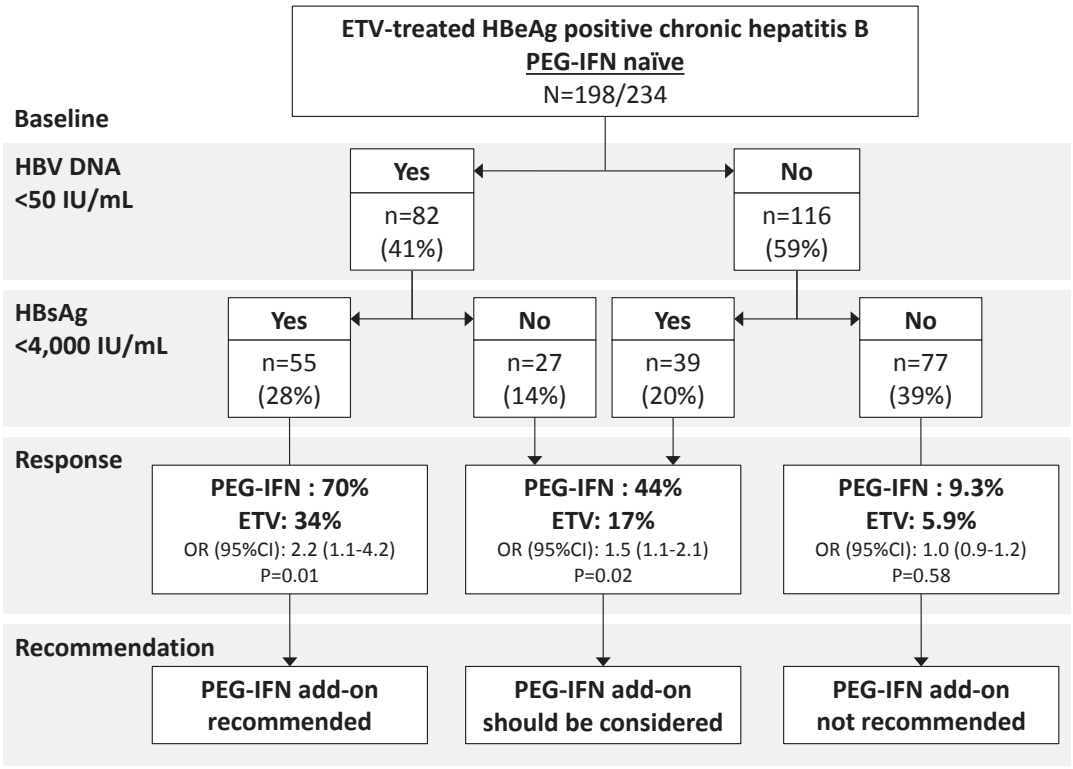


Figure 3. Algorithm for probability of response at end of follow-up based on HBV DNA and HBsAg at baseline.
The algorithm stratifies patients on ETV treatment by using HBV level and HBsAg level at baseline.

DISCUSSION

In this combined analysis of two global RCTs, PEG-IFN add-on to ETV increased response compared to ETV monotherapy in HBeAg positive patients with CHB. Response was 33% for add-on patients versus 20% for ETV monotherapy. HBeAg seroconversion rates at EOF were also significantly higher in add-on patients. The response to PEG-IFN add-on was especially high (up to 70%) among patients

who were naïve to PEG-IFN therapy and had low HBV DNA (< 50 IU/ml) and HBsAg levels (< 4000 IU/ml) at the start of PEG-IFN therapy.

This is the first study demonstrating a higher response in patients allocated to PEG-IFN add-on compared to ETV monotherapy. The strengths of this study are inclusion of a large multi-ethnic cohort of patients comprising treatment naïve and experienced patients who after ETV treatment did not reach HBeAg seroconversion. These patients are representative of the majority of treatment eligible patients seen in clinical practice who would otherwise continue NA therapy for longer duration. A finite PEG-IFN add-on regimen offers disease remission and discontinuation of treatment, thereby preventing additional costs and the potential of non-adherence and resistance associated with long-term or indefinite NA therapy.

To avoid unnecessary side-effects and costs of PEG-IFN it is essential to identify the optimal candidates for add-on therapy as only a subset will respond. The current HBV clinical practice guidelines only broadly mention the usefulness of quantifying HBV DNA and HBsAg to decide when and in whom to start PEG-IFN. Evidence to support one cut-off value over another is limited.[26,27] We established clinical starting rules for PEG-IFN add-on based on widely available biomarkers. Based on results from this study, we recommend starting PEG-IFN add-on in PEG-IFN naïve patients with an HBsAg level below 4,000 IU/mL (3.6 logs) and HBV DNA below 50 IU/mL (1.7 log) at randomization. A sufficiently large subgroup (28% of PEG-IFN naïve patients) had laboratory levels below these thresholds. PEG-IFN add-on response rates were nearly twice as high as the average PEG-IFN response in previous studies.[15,16] In patients with values below either of the cut-off values, PEG-IFN add-on should be considered, as these patients have a moderately high response to PEG-IFN. PEG-IFN add-on is not recommended in patients with both HBsAg and HBV DNA levels above the cut-off values, because of the low probability of response. Our HBsAg threshold is concordant with a threshold found in another study which showed that HBsAg <1500 IU/mL predicted response.[28] Moreover, the higher and thus more lenient HBsAg cut-off value established in this study would allow practitioners to identify even more candidates for PEG-IFN add-on at an earlier stage in their disease course. None of the previous add-on studies provided a comprehensive grid search to establish response-guided therapy. Apart from response, the side effects and cost-effectiveness should to be taken into consideration when deciding on a treatment strategy.

In recent RCTs that compared PEG-IFN add-on to continuing NA monotherapy, HBsAg decline rates were significantly higher in the add-on group, yet the primary endpoints (HBsAg loss at week 96; combined HBeAg loss with HBV DNA

<200 IU/mL at week 96) were not reached, potentially due to a type II error.[21–23] In the ARES study response was achieved in 19% of patients in the add-on arm vs. 10% in the monotherapy arm (p=0.095); declines in HBsAg, HBeAg and HBV DNA were also larger in the add-on group (all p<0.001).[21] Uncontrolled studies in HBeAg positive and negative patients reported similar findings.[29,30]

The PEGAN study in HBeAg negative patients did not find a significant effect of PEG-IFN add-on on HBsAg loss at week 96, but was possibly underpowered and included older-generation NAs.[22] This study showed that PEG-IFN add-on treatment resulted in significantly greater HBsAg declines and, within patients who received a full 48 week course, larger proportions of HBsAg loss and seroconversion. Within patients with an HBsAg titre below 3 log IU/mL at baseline, 6/26 (23%) achieved HBsAg loss (full dose analysis). The PEGAN study suggested using add-on only in patients with baseline HBsAg levels of less than 3 log IU/mL. Other regimens of PEG-IFN and NA therapy, such as sequential or combination therapy have been evaluated in CHB, but the optimal strategy remains unclear.[28,31]

The optimal duration of ETV pre-treatment or PEG-IFN add-on therapy has not yet been established. Prolonged NA pre-treatment partially restores immune function (NK and T cells).[17–20] In our study the duration of ETV pre-treatment correlated to baseline HBV DNA and HBsAg, but not to response. This suggests that levels of HBsAg and HBV DNA at the start of PEG-IFN therapy are more important in considering which patients to treat than the actual duration of ETV pre-treatment. The duration of PEG-IFN add-on treatment did not correlate with response. A post-hoc analysis in a previous study revealed larger HBsAg decline after 24 weeks of PEG-IFN add-on to ETV therapy compared to 52 weeks of combined PEG-IFN and LAM therapy.[32] This suggests that a PEG-IFN course of 24 weeks is at least as effective as 52 weeks, while the shorter regimen would reduce the risk of IFN-related adverse events and treatment costs. Our analysis lacked a comparison to PEG-IFN monotherapy. However, the focus of this study was to investigate PEG-IFN add-on in the large population of patients currently on NAs, and not treatment naïve patients.

The endpoint of HBeAg seroclearance is clinically relevant because it is associated with a lower risk of HCC and improved survival.[9] Since only a subset of patients stopped ETV therapy after receiving consolidation therapy the durability of sustained response after treatment discontinuation could not be studied in further detail. Long-term follow-up studies could focus on the effect on HBsAg loss or development of important clinical outcomes (decompensation, HCC and death), although such studies will be difficult to perform. Due to the fact that part of the patients had received long-term HBV suppressive therapy HBV genotype

and cirrhosis status was not known for some patients. Nevertheless, the sensitivity analyses performed to adjust for these partially missing baseline characteristics also showed higher response and HBsAg decline achieved by PEG-IFN add-on compared to ETV monotherapy. It is important that our findings will be validated in new PEG-IFN add-on studies.

In conclusion, PEG-IFN add-on to ETV therapy was associated with a higher probability of response and HBeAg seroconversion compared to ETV monotherapy in HBeAg-positive CHB. Response was highest in patients who were naïve to PEG-IFN therapy with levels of HBsAg below 4000 IU/ml and HBV DNA below 50 IU/ml. In particular these patients should be offered PEG-IFN add-on therapy.

SUPPLEMENTARY TABLES

Supplementary table 1: Patient characteristics of ARES and PEGON trial patients at randomization.

		ARES (n=159)	PEGON (n=75)
Age, years (SD)		32 (9)	35 (9)
Male gender		114 (72)	56 (75%)
Ethnicity	Caucasian	59 (37%)	3 (4.0%)
	Asian	97 (61%)	72 (96%)
	Other	3 (1.9%)	0 (0.0%)
HBV genotype	A	9 (5.7)	0 (0.0%)
	B	30 (19%)	9 (12%)
	C	67 (42%)	29 (39%)
	D	53 (33%)	3 (4.0%)
	Other	0 (0.0%)	34 (45%)
Cirrhosis		8 (5.2%)	-
PEG-IFN naive		141 (89%)	57 (76%)
ETV pre-treatment	6-12 months	159 (100%)	-
	1-3 years	-	55 (73%)
	>3 years	-	20 (27%)
Alanine aminotransferase, x ULN (IQR)		0.7 (0.4-1.0)	0.4 (0.3-0.5)
HBV DNA, IU/mL	Undetectable§	25 (16%)	55 (73%)
	20-100	29 (18%)	13 (17%)
	100-1,000	44 (28%)	2 (2.7%)
	>1,000	61 (38%)	5 (6.7%)
Quantitative HBsAg, log IU/mL (SD)		3.9 (0.7)	3.3 (0.7)
Quantitative HBeAg, log IU/mL (SD)		1.2 (1.0)	1.1 (0.8)
Therapy arm	PEG-IFN add-on	80 (50%)	38 (51%)
	ETV monotherapy	79 (50%)	37 (49%)
PEG-IFN duration	24 weeks	80 (50%)	-
	48 weeks	-	38 (51%)

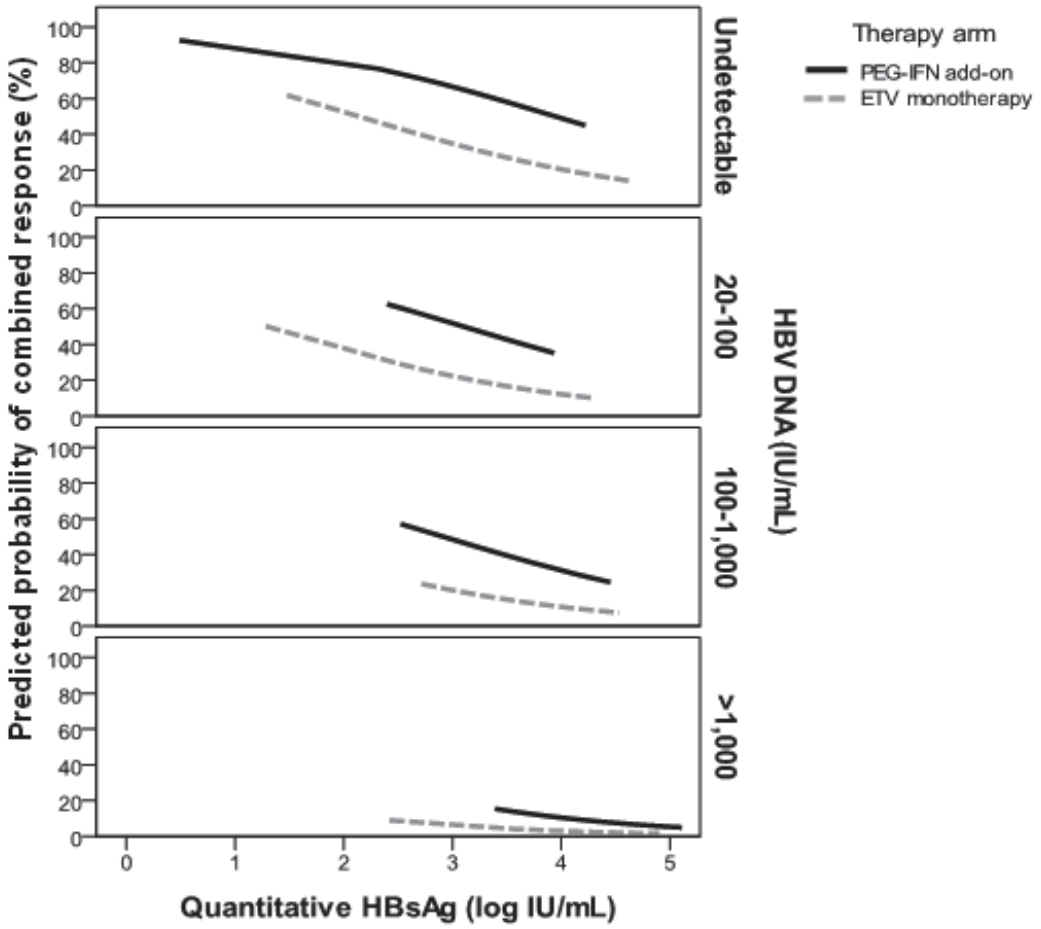
§ <20 IU/mL
IQR: interquartile range; SD: standard deviation; ULN: upper limit of normal.

Supplementary table 2: Outcome over time excluding initial non-responders who received retreatment.

	Baseline (Randomization)			End of PEG-IFN Week 24-48			End of consolidation Week 72			EOF Week 96		
n (%)	Add-on (n=100)	ETV Mono (n=98)	P	Add-on (n=100)	ETV Mono (n=98)	P	Add-on (n=100)	ETV Mono (n=98)	P	Add-on (n=100)	ETV Mono (n=98)	P
Response												
HBeAg loss + HBV DNA <200 IU/mL	–	–	–	19 (19)	5 (5.1)	<0.005*	29 (29)	14 (14)	0.01*	31 (32)	22 (22)	0.15
Virological outcomes												
HBV DNA <2,000 IU/mL	72 (72)	74 (76)	0.58	93 (94)	82 (84)	0.02*	87 (88)	85 (87)	0.81	83 (85)	87 (89)	0.40
HBV DNA <200 IU/mL	47 (47)	57 (58)	0.12	84 (85)	71 (72)	0.03*	77 (78)	75 (77)	0.84	79 (81)	80 (82)	0.86
HBV DNA undetectable†	9 (9.0)	16 (16)	0.12	46 (47)	36 (37)	0.17	42 (42)	40 (41)	0.82	46 (47)	46 (47)	0.99
Serological outcomes												
HBeAg loss	–	–	–	19 (19)	5 (5.1)	<0.005*	29 (30)	14 (14)	0.01*	33 (35)	22 (23)	0.06
HBeAg seroconversion	–	–	–	15 (15)	2 (2.0)	<0.005*	21 (21)	5 (5.1)	<0.005*	22 (22)	11 (11)	0.04*
HBsAg loss	1 (1.3)	0 (0.0)	0.32	1 (1.3)	0 (0.0)	0.32	1 (1.3)	0 (0.0)	0.32	1 (1.3)	0 (0.0)	0.32
HBsAg <1,000 IU/mL	18 (18)	17 (17)	0.90	30 (30)	21 (21)	0.16	27 (27)	22 (22)	0.43	27 (28)	22 (22)	0.41
HBsAg <100 IU/mL	0 (0.0)	0 (0.0)	0.30	9 (9.1)	2 (2.0)	0.03*	6 (6.1)	2 (2.0)	0.15	6 (6.1)	3 (3.1)	0.31
HBsAg decline >1 log IU/mL	–	–	–	9 (9.2)	0 (0.0)	<0.005*	7 (7.2)	0 (0.0)	0.01*	6 (6.4)	2 (2.1)	0.14
HBsAg decline >0.5 log IU/mL	–	–	–	24 (25)	2 (2.0)	<0.005*	26 (27)	5 (5.2)	<0.005*	23 (25)	10 (10)	0.01*
Biochemical outcome												
ALT normalization	80 (80)	72 (74)	0.28	57 (58)	76 (78)	<0.005*	87 (88)	80 (82)	0.22	88 (90)	84 (86)	0.38

* p<0.05; † <20 IU/mL
EOF, end of follow-up; NA: Not applicable.

SUPPLEMENTARY FIGURES



Supplementary figure 1. Predicted probability of response at end of follow-up according to treatment, serum HBV DNA and HBsAg levels at baseline in PEG-IFN naïve patients.
On the y-axis, black lines represent the predicted probability of response for patients treated with PEG-IFN add-on for the baseline level of HBsAg level shown on the x-axis. Grey dotted lines represent the predicted probability for patients treated with ETV monotherapy. The 4 boxes represent patients categories stratisfied by HBV DNA level at baseline.

2

Long-term follow-up of patients treated with entecavir and peginterferon add-on therapy for HBeAg-positive chronic hepatitis B infection: ARES long-term follow-up

M.J.H. van Campenhout[1], W.P. Brouwer[1], Q. Xie[2], S. Guo[2], H. Chi[1], X. Qi[3], F. Tabak[4], A. Streinu-Cercel[5], J-Y Wang[6], N. Zhang[6], R. Idilman[7], H.W. Reesink[8], M. Diculescu[9], K. Simon[10], M. Akdogan[12], W. Mazur[12], R.J. de Knegt[1], E. Verhey[1], B.E. Hansen[1,14,15], and H.L.A. Janssen[15], for the ARES Study Group.

[1]Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands; [2]Infectious Diseases, Ruijin Hospital, Jiaotong University, Shanghai, China; [3]Gastroenterology and Hepatology, Shanghai Public Health Center, Fu Dan University, Shanghai, China; [4]Cerrahpasa Medical Faculty, .Istanbul, Turkey; [5] Carol Davila University of Medicine and Pharmacy, National Institute for Infectious Diseases "Prof. Dr. Matei Balș", Bucharest , Romania; [6]Gastroenterology and Hepatology, Zhong Shan Hospital, Fu Dan University, Shanghai, China; [7]University of Ankara, Medical School, Ankara, Turkey; [8]Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, The Netherlands; [9]Department of Gastroenterology, Fundeni Cinical Institute, Bucharest, Romania; [10]Division of Infectious Diseases and Hepatology, Wroclaw Medical University, Wroclaw, Poland; [11]Department of Gastroenterology, Yuksek Ihsitas Hospital, Ankara, Turkey; [12]Department of Infectious Diseases, Silesian Medical University, Katowice, Poland; [14]Institute of Health Policy, Management and Evaluation, University of Toronto; [15]Toronto Center for Liver Disease, Toronto Western and General Hospital, University Health Network, Toronto, Canada.

Journal of Viral Hepatitis, 2018

ABSTRACT

Background & aims. Addition of peginterferon alpha (PEG-IFN add-on) to entecavir (ETV) treatment after a short lead-in phase results in more response than ETV monotherapy in HBeAg positive chronic hepatitis B infection (CHB). This study is the first to assess long-term efficacy of this treatment strategy.

Methods. Patients who received ETV +/- 24 weeks of PEG-IFN add-on in a global trial (ARES study) and completed follow-up were eligible to participate in this observational LTFU study if they had at least one combined HBeAg and HBV DNA measurement beyond week 96 of the ARES study. The primary endpoint was combined response (HBeAg loss and HBV DNA <200 IU/mL) at LTFU.

Results. In total, 48 patients treated with PEG-IFN add-on and 48 patients treated with ETV monotherapy were included. The median follow-up duration was 226 (IQR 51) weeks, and 86/96 (90%) patients were initial non-responders. At LTFU combined response was present in 13 (27%) vs. 11 (23%) patients (p=0.81), and 1 $\log_{10}$ HBsAg decline in 59% vs. 28% (p=0.02) for PEG-IFN add-on and ETV monotherapy, respectively. In 41 initial non-responders who continued ETV therapy, combined response at LTFU was present in 9 patients (PEG-IFN add-on: 5/22 [23%]; ETV monotherapy: 4/19 [21%]).

Conclusions. Beyond week 96 of follow-up, rates of serological response became comparable between PEG-IFN add-on and ETV monotherapy. Although in this LTFU study initial non-responders were overrepresented in the add-on arm, PEG-IFN add-on possibly leads rather to accelerated HBeAg loss than to increased long-term HBeAg loss rates.

INTRODUCTION

Chronic hepatitis B (CHB) infection is difficult to cure due to intrahepatic persistence of the main viral replication template cccDNA and due to complex host-virus interactions. At present it is not only debated how to achieve cure, but also which event is the best surrogate endpoint indicating cure of disease [49]. Functional cure, often reflected by HBsAg seroconversion, is the most favorable outcome that currenty available treatment options can establish, but it is achieved in only a minority of patients with HBeAg-positive CHB [30, 50-52]. Therefore sustainable disease remission remains one of the major aims in current clinical practice. HBeAg loss induced by (peg)interferon alpha (PEG-IFN) treatment, which mainly has immunomodulating effects, occurs in approximately 30% of patients and is associated with an increased probabilty of HBsAg loss and a reduced incidence of liver-related complications [22, 24, 30]. In contrast, during one year of antiviral therapy with potent nucleo(s)tide analogues (NA) entecavir (ETV) or tenofovir (TDF) HBeAg loss is achieved in only 20% of patients and is less durable [27, 53, 54]. Although treatment guidelines suggest that NA therapy may be discontinued in non-cirrhotic patients when HBeAg loss is achieved [13, 55, 56], clinical relapse occurs in around 50% of patients and virological relapse in over 90% [28, 57]. Consequently, the majority of patients on NA treatment require long-term or even lifelong therapy.

Long-term NA therapy may not be desired for several reasons such as high costs, potential non-adherence and side-effects. Another disadvantage is that decline in serum level of HBsAg, which is presumed to partly reflect intrahepatic functionally active cccDNA, is very slow, resulting in low rates of on-treatment HBsAg clearance [58, 59]. On the other side, long-term HBV suppression can improve both innate and adaptive immunity probably creating a window of opportunity for immunomodulatory treatment such as PEG-IFN to improve response [33, 60]. Indeed, the first studies in small patient groups reported that addition of PEG-IFN (PEG-IFN add-on strategy) in patients with completely suppressed HBV DNA by NA therapy increased responses rates and even leads to HBsAg loss [61, 62].

Our group recently reported the results of a randomized controlled trial comparing ETV monotherapy to ETV + 24 weeks of PEG-IFN add-on. We observed higher rates of HBeAg loss and sustained off-treatment disease remission in the PEG-IFN add-on arm, and PEG-IFN add-on led to a significantly stronger decline in serum HBsAg level [36, 63]. However, at present it is not known whether these benefits last over time. We therefore aimed to investigate the long-term effects of the PEG-IFN add-on strategy in comparison to ETV monotherapy.

PATIENTS AND METHODS

Patients

Patients were eligible for participation in this observational long-term follow-up study if they completed follow-up of a global randomized controlled trial comparing ETV + PEG-IFN add-on therapy to ETV monotherapy (ARES study), had at least one simultaneously obtained serum HBeAg and HBV DNA result after study completion at week 96, and were not (re)treated with PEG-IFN before start of the LTFU study. The inclusion and exclusion criteria of the initial study are described elsewhere [36]. In short, patients with HBeAg positive, anti-HBe negative chronic hepatitis B infection (HBsAg positive >6 months) and serum ALT level >1.3 times the upper limit of normal (ULN) were included, who had not received antiviral therapy against HBV in the 6 months prior to screening and who had no contraindication for PEG-IFN therapy. In the initial study, 175 patients started ETV treatment, and were randomized after 24 weeks of ETV treatment to either receive PEG-IFN add-on therapy from week 24 to 48, or to continue ETV monotherapy (Figure 1). At week 48, the primary endpoint of HBeAg loss in combination with HBV DNA level <200 IU/mL (initial response was assessed. Patients who had achieved combined response at week 48 stopped ETV at week 72 (PEG-IFN add-on: n=14, ETV mono: n=8), after at least 24 weeks of consolidation therapy. Patients without an initial response continued ETV through the end of the study (week 96). In the long-term follow-up study, all patients were followed and treated according to the protocols of the local study sites. The study was performed in accordance with the declaration of Helsinki and the Good Clinical Practice guidelines and were approved by the ethics committee of each participating centre. All subjects gave written informed consent.

Endpoints & definitions

The primary endpoint of this LTFU study was loss of HBeAg in combination with HBV DNA level <200 IU/mL (combined response). Secondary endpoints were HBeAg loss, HBeAg seroconversion (HBeAg loss + detectable anti-HBe), reversion to HBeAg positivity after initial HBeAg loss (sustainability of response), HBV DNA negativity (below lower limit of detection), HBsAg loss +/- seroconversion, HBsAg level <1,000 IU/mL and <100 IU/mL, HBsAg decline of 1 $\log_{10}$ or more. End of long-term follow-up was defined as the last moment at which both serum HBeAg and HBV DNA results were available. Retreatment was regarded as relapse for the assesment of off-treatment response.

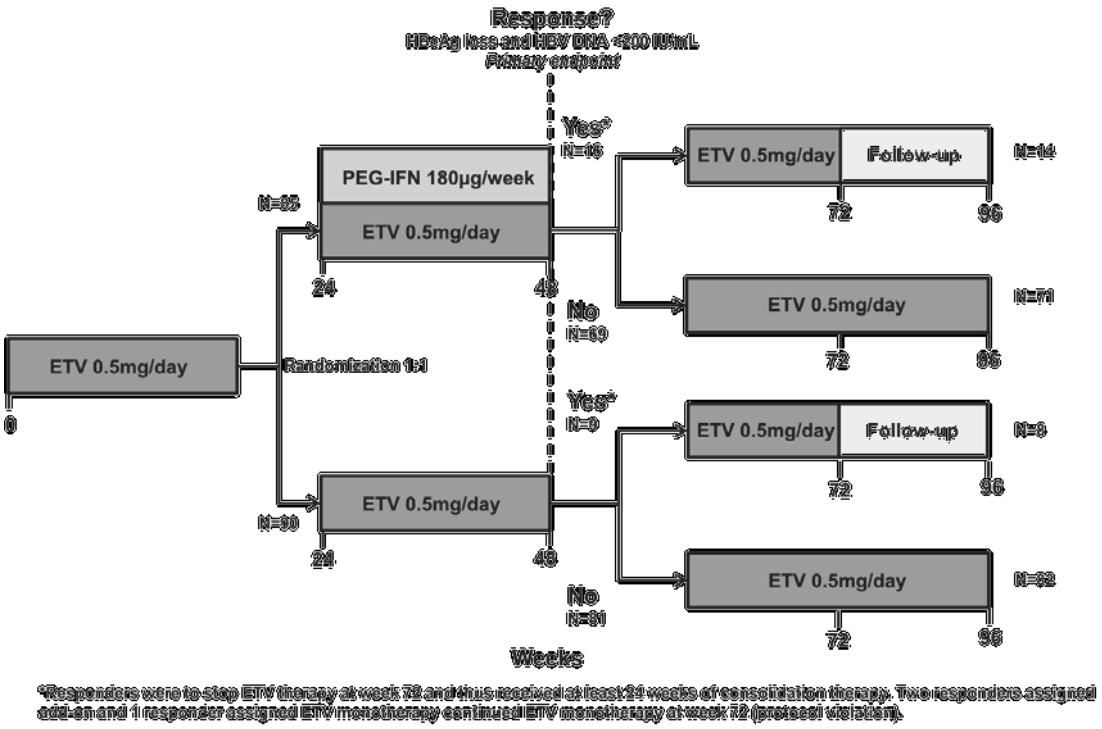


Figure 1. Trial design of the initial study.
Trial design of the ARES study.

Laboratory test procedures

For the LTFU study, all biochemical, hematological and virological tests were performed at laboratories of the local centers. For HBV DNA quantification, Abbott Realtime HBV or Taqman-based polymerase chain reaction assays were used with lower limits of quantification varying from 15-500 IU/mL. Serum HBeAg and HBsAg levels were measured using the Cobas Elecsys 411 (Roche Diagnostics, Basel, Switzerland, lower limit of detection 0.30 IU/mL and 0.05 IU/mL, respectively). For the initial study, all test procedures were described earlier [36].

Statistical analysis

Skewed laboratory values were log-transformed prior to analyses. Continuous variables were expressed as mean (standard deviation [SD]), categorical variables as n (%). Associations between variables were tested using Student's t-test, Fisher's exact test, or their non-parametric equivalents when appropriate. For the assessment of treatment response, follow-up time was censored when treatment with PEG-IFN based treatment was started during LTFU. In addition, a subgroup analysis was performed for patients who continued ETV at week 72 and at the end of the initial study. For the endpoint of HBeAg seroconversion, patients

without an anti-HBe measurement were considered non-responders. For HBsAg related endpoints, only patients with at least one available HBsAg level were analysed. All statistical tests were two-sided and were evaluated at the 0.05 level of significance. Analyses were performed using SPSS version 24.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patients

Of 175 patients who started treatment in the initial study, 169 (97%) patients completed the 96-week follow-up. Of these patients, 96 (57%) were enrolled in the LTFU study (Figure 2). The most common reason for non-participation was that patients could not be reached and were lost to follow-up (n=34). Out of 48 PEG-IFN add-on treated patients included in the LTFU study, 45 (94%) were week 48 non-responders in the initial study and out of 48 ETV monotherapy treated patients, 41 (85%) were week 48 non-responders. Out of the 11 initial responders who stopped ETV treatment according to protocol at week 72 of the original study and had sustained off-treatment response through week 96 (end of the initial study), 3 patients were included (all add-on treated). Among the other 8, 4 could not be reached by the local physician, and 4 were reached but had no available HBeAg or HBV DNA measurement. Most of these patients were from rural areas of China and therefore difficult to reach. Table 1 shows patient characteristics at baseline (start of ETV treatment) and at week 24 (randomization) of patients who participated in the initial study and of patients who participated in the LTFU study. The median follow-up duration was 226 (IQR 51) weeks from baseline. The median follow-up time did not differ for add-on versus ETV treated patients [p=0.26].

HBeAg response and combined response

Overall, 43/48 (90%) patients treated with PEG-IFN add-on vs. 45/48 (94%) patients treated with ETV monotherapy in the initial study received any type of antiviral treatment during LTFU (p=0.71, Figure 2). At the end of follow-up, HBeAg was negative in 18/48 (38%) patients treated with PEG-IFN add-on and 19/48 (40%) treated with ETV monotherapy (p=1.00, Figure 3). HBeAg seroconversion had occurred in 14/48 (29%) vs. 10/48 (21%) patients, respectively (p=0.48). Combined response was present in 13/48 patients (27%) allocated to PEG-IFN add-on vs. in 11/48 (23%) patients allocated to ETV monotherapy (p=0.81).

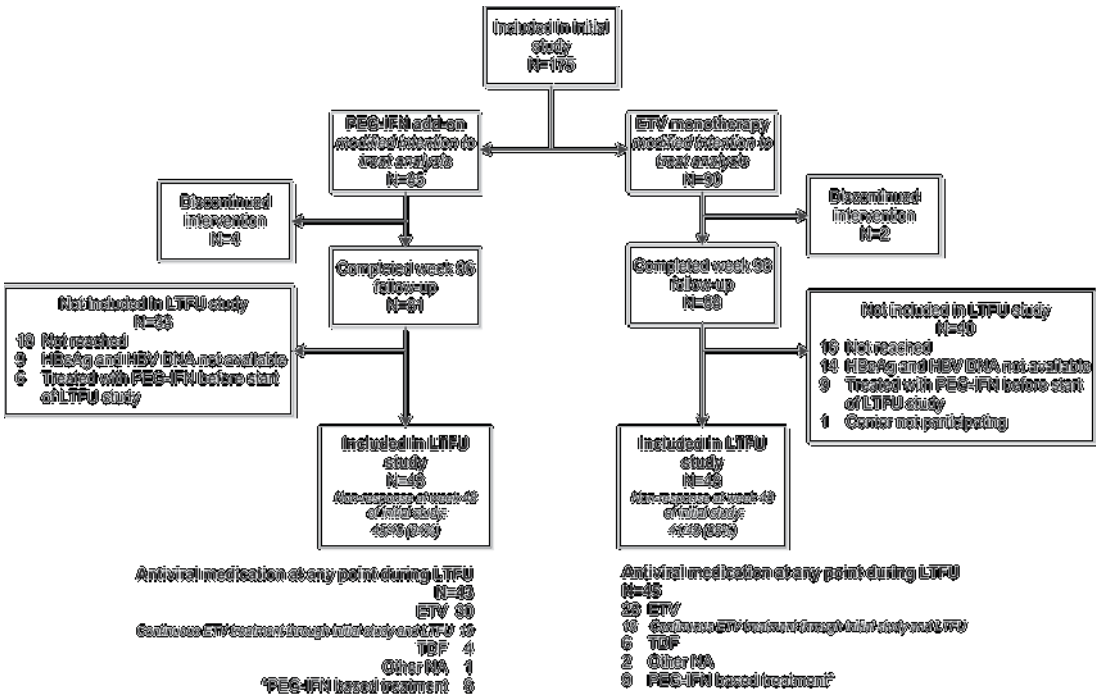


Figure 2. Flowchart of inclusion.

Overview of patients included and not included for this long-term follow-up study.

ETV, entecavir; LTFU, long-term follow-up; NA, nucleos(t)ide analogue; PEG-IFN add-on, peginterferon addition; TDF, tenofovir.

Among the 3 included patients whose initial response at week 48 had persisted through week 96, documented relapse was observed in 2 patients (week 111 and week 151). In the last patient, HBeAg was negative and HBV DNA level was <500 IU/mL. Among the remaining 93 included patients with non-response at week 96, 41 patients continued ETV at both week 72 and at the end of the initial study. In these 41 patients, HBeAg loss at end of LTFU was observed in 7/22 (32%) patients previously treated with add-on and 7/19 (37%) patients treated with ETV monotherapy (p=0.75), and late HBeAg seroconversion in 6/22 (27%) vs. 3/19 (16%), respectively (p=0.47). Combined response at the last moment of follow-up was present in 9 patients (add-on: 5/22 [23%]; ETV monotherapy: 4/19 [21%]). In 3/9 patients, ETV had been successfully discontinued 5-11 months prior to the end-of-follow-up visit (all add-on treated). In one patient treated with ETV monotherapy who did not fulfill criteria of combined response but had achieved HBeAg loss with an HBV DNA level below 500 IU/mL, ETV was succesfully discontinued (week 120).

Table 1. Patient characteristics

	Initial study (n=175)		LTFU study (n=96)	
Characteristics	**ETV monotherapy (n=90)**	**PEG-IFN add-on (n=85)**	**ETV monotherapy (n=48)**	**PEG-IFN add-on (n=48)**
Demography				
Age, years	31 (9)	32 (10)	32 (9)	33 (11)
Male, n (%)	62 (69)	63 (74)	34 (71)	35 (73)
Race, n (%)				
Caucasian	35 (39)	30 (35)	23 (48)	20 (42)
Asian	54 (60)	53 (63)	24 (50)	26 (54)
Other	1 (1)	2 (2)	1 (2)	2 (4)
HBV Genotype: A/B/C/D (%)	10/14/46/30	5/23/39/33	10/8/42/40	8/23/31/38
INNO-LiPA result, n (%)				
Wildtype virus	7 (8)	9 (12)	4 (9)	5 (11)
PC mutation	13 (16)	21 (27)	5 (11)	13 (29)
BCP mutation	16 (19)	7 (9)	7 (16)	7 (16)
PC & BCP mutation	48 (57)	40 (52)	28 (64)	20 (44)
Histology				
Cirrhosis, n (%)	5 (6)	3 (4)	1 (2)	3 (7)
Week 0 laboratory results *				
ALT (x ULN) †	2.7 (2.1)	3.1 (3.3)	2.7 (2.1)	3.4 (3.1)
HBV DNA ‡	7.8 (1.1)	7.8 (1.3)	7.8 (1.1)	7.7 (1.4)
qHBsAg ‡	4.1 (0.8)	4.2 (0.8)	4.1 (0.9)	4.2 (0.9)
qHBeAg ‡	2.3 (1.0)	2.3 (1.0)	2.4 (1.0)	2.3 (1.1)
Week 24 laboratory results				
ALT (x ULN) †	0.8 (0.6)	0.8 (0.4)	0.8 (0.7)	0.8 (0.5)
HBV DNA ‡ ¤	2.3 (1.4)	2.8 (1.5)	2.4 (1.3)	3.1 (1.3)
qHBsAg ‡	3.7 (0.8)	3.7 (0.7)	3.8 (0.8)	3.7 (0.7)
qHBeAg ‡	0.9 (1.2)	1.1 (1.1)	0.9 (1.2)	1.2 (1.0)

ALT, alanine aminotransferase; BCP, basal core promoter; ETV, entecavir; HBV, hepatitis B virus; qHBeAg, quantitative hepatitis B e antigen; qHBsAg, quantitative hepatitis B surface antigen; PEG-IFN, peginterferon; PC, Precore; SD, standard deviation; ULN, upper limit of normal.

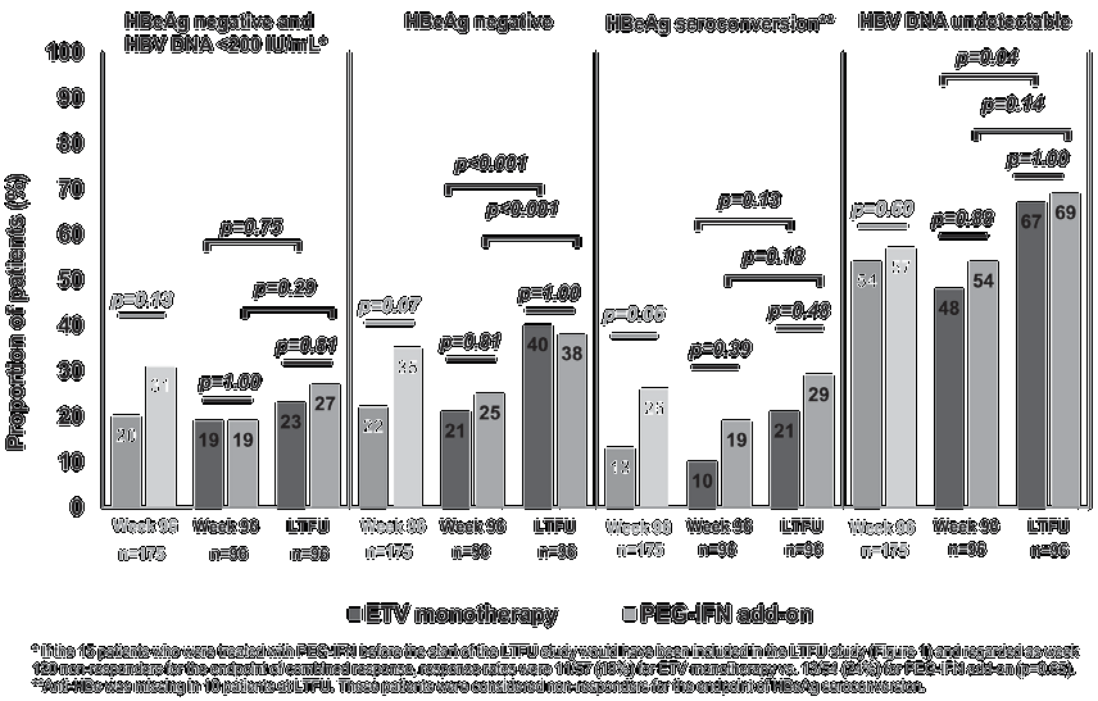
Continuous variables are expressed as mean (SD), categorical variables as n (%).

** Patients were randomised at week 24 and thus not yet allocated at week 0.*

† *Multiples of upper limit of the normal range*

‡ *Logarithmic scale, IU/mL*

¤ *HBV DNA levels differed at week 24 (p=0.021 for n=175, p=0.01 for n=96)*



*This 15 patients who were treated with PEG-IFN before the start of the LTFU study would have been included in the LTFU study (Figure 1) and regarded as week 120 non-responders for the endpoint of combined response, response rates would be 19/57 (18%) for ETV monotherapy vs. 12/57 (21%) for PEG-IFN add-on (p=0.63).
**Anti-HBe was missing in 10 patients at LTFU. These patients were considered non-responders for the endpoint of HBeAg seroconversion.

Figure 3. HBeAg response and virological response.

Rates of combined response (HBeAg loss and HBV DNA <200 IU/mL), HBeAg loss, and HBV DNA undetectability at week 96 and the end of LTFU. Bars in watermark represent the original response rates of the total ARES study population (n=175), bars not in watermark represent the patients included in this LTFU study (n=96). In each set of bars, bars in dark grey represent patients treated with ETV monotherapy, bars in lighter grey represent patients treated with PEG-IFN add-on. P-values with lines represent differences between ETV monotherapy and PEG-IFN add-on, p-values with brackets represent differences between week 96 and LTFU within each treatment arm. For the endpoint of HBeAg seroconversion, patients without an anti-HBe measurement (n=10) were considered non-responders.

ETV, entecavir; LTFU, long-term follow-up; PEG-IFN add-on, peginterferon addition.

Virological response

At the end of follow-up, HBV DNA level was undetectable (<20 IU/mL in most participating centers) in 33/48 (69%) add-on treated patients vs. in 32/48 (67%) ETV monotherapy treated patients (p=1.00). At this visit, 38/48 (79%) add-on treated patients and 39/48 (81%) were receiving NA treatment. In the subgroup of patients (n=37) who continued ETV throughout the entire initial study and LTFU period, the proportions of patients with undetectable HBV DNA level were 18/19 (95%) and 16/18 (89%), for add-on vs. ETV monotherapy, respectively.

HBsAg response

Serum qHBsAg level was available for 32 add-on treated patients and 29 ETV monotherapy treated patients. The mean qHBsAg level at the end of follow-up was 3.0 (SD 0.5) log IU/mL for PEG-IFN add-on and 3.1 (1.0) log IU/mL for ETV monotherapy (p=0.65), and the mean declines from baseline were -1.3 (SD

1.0) vs. -0.7 (SD 1.3) IU/mL, respectively (p=0.06). When compared to ETV monotherapy, PEG-IFN add-on treatment resulted in a twofold higher proportion of patients with a qHBsAg decline of ≥1 log (add-on: 19/32 (59%), ETV monotherapy: 8/29 (28%); p=0.02; Figure 4). An HBsAg level below 1000 IU/mL was achieved in 14/32 (44%) add-on treated patients and in 6/29 (21%) patients treated with ETV monotherapy (p=0.06); an HBsAg level below 100 IU/mL in 2/32 (6%) vs. 3/29 (10%) patients (p=0.66). One patient treated with add-on lost HBsAg at week 96 (45-year-old male, genotype A). During LTFU, HBsAg reappeared at week 110, but became negative again at week 151 and remained negative through the end of follow-up (week 239). Anti-HBs was positive and HBV DNA remained undetectable. HBsAg loss did not occur in other patients.

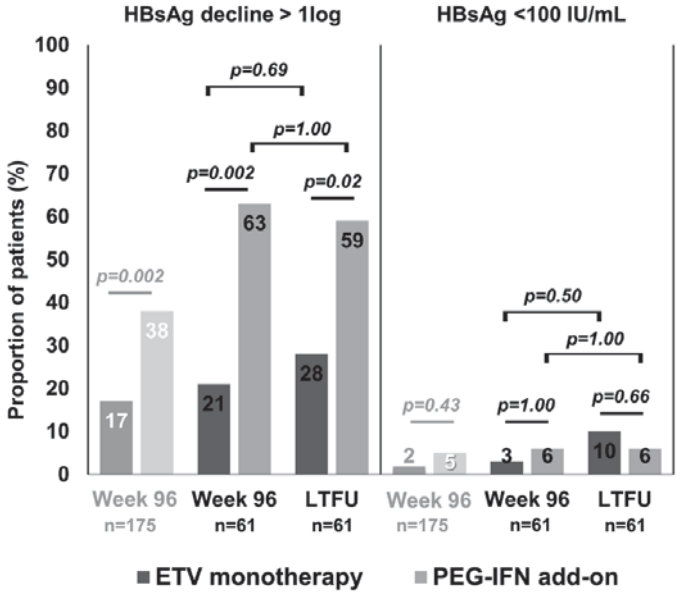


Figure 4. HBsAg response.

Rates of HBsAg decline 1 $\log_{10}$ or more and HBsAg level <100 IU/mL at week 96 and the end of LTFU. Bars in watermark represent the original response rates of the total ARES study population (n=175), bars not in watermark represent the patients included in this LTFU study who had at least 1 quantitative HBsAg (qHBsAg) measurement (n=61). In each set of bars, bars in dark grey represent patients treated with ETV monotherapy, bars in lighter grey represent patients treated with PEG-IFN add-on. P-values with lines represent differences between ETV monotherapy and PEG-IFN add-on, p-values with brackets represent differences between week 96 and LTFU within each treatment arm.
ETV, entecavir; LTFU, long-term follow-up; PEG-IFN add-on, peginterferon addition

DISCUSSION

This study is the first to describe the long-term effects of a PEG-IFN add-on strategy for the treatment of HBeAg positive CHB in comparison to ETV monotherapy. Although we showed earlier that response rates 24 weeks after PEG-

IFN discontinuation were higher in PEG-IFN add-on treated patients than in ETV treated patients, we were not able to demonstrate that PEG-IFN add-on treatment has late beneficial effects over ETV monotherapy with respect to HBeAg loss or serum HBV DNA level undetectabilty. However, the strong add-on induced serum HBsAg decline was sustained through LTFU.

These findings provide valuable information in the search for the optimal use of both currently approved treatment options which is essential as long as treatment leading to higher rates of functional cure or to complete cure is not available. Strategies that combine PEG-IFN and NA therapy simultaneously for the treatment of HBeAg-positive CHB have been studied for over a decade but no or limited clinical benefit could be demonstrated [25, 30, 64]. The observations of partially restored immune reactivation in patients on long-term NA therapy have led to the investigation of strategies in which PEG-IFN is added to NA therapy or in which NA therapy is switched to PEG-IFN.

In the OSST study, switching from long-term ETV to 48 weeks of PEG-IFN resulted in a similar rate of on-treatment HBeAg loss as in our study, which was also higher when compared to ETV continuation [65]. However in the OSST study, in which only patients with low HBeAg level were included, follow-up data has only been reported for the switch arm so the long-term added value in comparison to NA continuation could not be determined. Also, end-of-follow-up HBsAg level was not reported [66]. More recently, our group reported the results of the PEGON study that compared 48 weeks of PEG-IFN add-on to continuation of longterm NA therapy, which demonstrated an increased response but only in patients naïve to PEG-IFN [37]. Nonetheless, no follow-up data beyond week 96 is available yet. The current study is the first to report that PEG-IFN add-on does not appear to have late beneficial effects over continued ETV monotherapy with regard to HBeAg loss and HBV DNA undetectability in initial non-responders, in a setting were PEG-IFN add-on is added for 24 weeks after 24 weeks of ETV lead-in.

A possible explanation for the lack of long-term benefit could be that IFN therapy only accelerates HBeAg loss but eventually does not lead to higher rates of HBeAg loss later during follow-up when compared to natural history or NA therapy [67]. Another option is, because duration of NA therapy appears to be associated with functional T cell restoration, and it still has not been established what the optimal durations for NA pretreatment and PEG-IFN add-on are, that ETV pretreatment duration or the add-on duration in the initial study were too short to establish a durable clinical benefit of PEG-IFN add-on over ETV monotherapy in terms of an increased rate of HBeAg loss or HBV DNA undetectability over time [5].

Alternatively, the lack of significant benefit as observed in this LTFU study may represent the late effects of PEG-IFN add-on only, and not the durability of the early effects due to the fact that more initial non-responders were included in this retrospective LTFU study, primarily in the add-on arm. Particularly in our Chinese sites it was common that patients who achieved treatment response did not remain in follow-up at the same site which logically leads to an overrepresentation of patients without an early treatment success. Therefore unfortunately, we were not able draw a conclusion on the off-treatment sustainability of response. Results of an additional post-hoc analysis, extrapolating the current percentage of response sustainability to the ARES participants who were not included in this LTFU study, indeed suggest that when all original ARES participants would have been included in the LTFU study, the response rates achieved by PEG-IFN add-on remained higher than those of ETV monotherapy even at LTFU (data not shown). Further, the number of patients included in this LTFU study may be too low to demonstrate a clinical significant benefit.

We observed that PEG-IFN add-on led to an HBsAg decline of at least 1 $\log_{10}$ in 58% of patients, which was double the rate of ETV monotherapy treated patients. This was stable over time, which is probably due to the fact that most patients remained on NA therapy, and it may indicate improved immune control [63, 68]. This same phenomenon has been shown earlier by our group comparing trials with combination of PEG-IFN and ETV or Lamivudine [23]. Lower end-of-treatment HBsAg level is associated with sustained off-treatment response after both NA and PEG-IFN discontinuation [69-71], but because almost all patients included in this LTFU study received treatment after the initial study, we have not been able to assess this. Future follow-up of these patients could reveal if indeed off-treatment sustainability is better in those who have achieved a low HBsAg level.

In conclusion, although early response was stronger in PEG-IFN add-on treated patients, rates of HBeAg loss and combined response became comparable between the treatment arms beyond week 96 of follow-up. We hypothesize that PEG-IFN add-on accelerates serological response without eventually leading to higher absolute response rates than ETV on the long-term. The data we presented here provide unique insights in the long-term outcome of a PEG-IFN add-on strategy as compared to ETV monotherapy. Although no recommendation towards the exact clinical application of PEG-IFN add-on as a strategy to increase HBeAg response or virological response can be made yet, this study may serve as a lead for exploring other pretreatment and add-on durations. We additionally suggest that the PEG-IFN add-on strategy may be used in future treatment settings when low HBsAg levels or HBeAg loss would be indicated to start a novel treatment therapeutic agent.

3

Hepatitis B core-related antigen levels are associated with response to entecavir and peginterferon add-on therapy in HBeAg-positive chronic hepatitis b patients

M.J.H. van Campenhout[1], W.P. Brouwer[1], G.W. van Oord[1], Q. Xie[2], Q. Zhang[3], N. Zhang[6], S. Guo[2], F. Tabak[4], A. Streinu-Cercel[5], J. Wang[6], S.D. Pas[7], M.J. Sonneveld[1], R.J. de Knegt[1], A. Boonstra[1], B.E. Hansen[1,8], and H.L.A. Janssen,[1,9]

[1]Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands; [2]Infectious Diseases, Ruijin Hospital, Jiaotong University, Shanghai, China; [3]Gastroenterology and Hepatology, Shanghai Public Health Center, Fu Dan University, Shanghai, China; [4]Cerrahpasa Medical Faculty, .Istanbul, Turkey; [5] Carol Davila University of Medicine and Pharmacy, National Institute for Infectious Diseases "Prof. Dr. Matei Balş", Bucharest , Romania; [6]Gastroenterology and Hepatology, Zhong Shan Hospital, Fu Dan University, Shanghai, China; [7]Department of Virology, Erasmus MC University Medical Center, Rotterdam, The Netherlands; [8]Department of Public Health, Erasmus MC University Medical Center, Rotterdam, The Netherlands; [9]Toronto Center for Liver Disease, Toronto Western and General Hospital, University Health Network, Toronto, Canada

Clinical Microbiology and Infection, 2016

ABSTRACT

Background & aims. Hepatitis B core-related antigen (HBcrAg) is a new serum marker for the combined measure of HBeAg, HBcAg and p22cr. HBcrAg correlates with intrahepatic cccDNA, which is the main template for viral protein synthesis. HBcrAg levels may therefore be associated with viral replication activity, response to antiviral therapy, and immune control in chronic hepatitis B infection (CHB).

Methods. Serum HBcrAg levels were measured in 175 HBeAg-positive patients treated with entecavir (ETV) ± peginterferon (PEG-IFN) add-on therapy within a randomised trial. To study HBcrAg dynamics, and associations between HBcrAg and treatment response, we evaluated combined response (CR; HBeAg loss and HBV DNA<200 IU/mL) and HBsAg response (qHBsAg <1000 IU/mL and/or qHBsAg decline ≥1 log IU/mL).

Results. At baseline, the mean HBcrAg was 8.1 (SD 0.8) log U/mL. HBcrAg declined during therapy (ETV vs. PEG-IFN add-on: -2.10 vs. -1.96 log U/mL, p=0.12), with stronger declines in patients who achieved CR than in patients without CR (ETV: -3.22 vs. -1.71 log U/mL, p<0.001; PEG-IFN add-on: -3.16 vs. -1.83 IU/mL, p<0.001). Similarly, HBcrAg decline was more prominent in patients with HBsAg response compared with those without (ETV: -2.60 vs. -1.74 log U/mL, p<0.001; PEG-IFN add-on: -2.38 vs. -2.15 log U/mL, p=0.31). HBcrAg levels at randomisation were associated with CR (adjusted OR 0.3, CI-95% 0.2-0.5, p<0.001), but were not better than serum quantitative HBsAg levels (qHBsAg) in response prediction.

Conclusions. HBcrAg levels were associated with response to ETV monotherapy and ETV with PEG-IFN add-on therapy for HBeAg-positive CHB, but were not superior to qHBsAg in response prediction.

INTRODUCTION

In the management of chronic hepatitis B (CHB) infection, adequate assessment of viral replication activity is warranted in order to predict disease outcome and risk of reactivation. In addition, it is important for predicting response to nucleos(t)ide analogue (NA) and peginterferon (PEG-IFN) therapy, the two available treatment modalities for CHB.

Measurement of intrahepatic covalently closed circular DNA (cccDNA) is thought to provide information on the replication activity of the virus, because it is the main template for synthesis of viral proteins [42, 52]. PEG-IFN treatment induces a reduction of intrahepatic cccDNA [72], and Sung et al. reported the potential value of cccDNA measurement in post-treatment liver biopsies for the prediction of sustained response to PEG-IFN treatment [73]. To assess functional cure of CHB with current or future therapies, it is probably essential to assess the cccDNA levels in a reliable way. A major limitation for the clinical use of cccDNA measurements is the need for a liver biopsy. Availability of a serum surrogate marker could be a solution to this problem.

A possible marker associated with cccDNA is the Hepatitis B core-related antigen (HBcrAg), a combined measure of three proteins coded by the precore/core region of the cccDNA: Hepatitis B core antigen (HBcAg), Hepatitis B e antigen (HBeAg) and a 22-kDa precore protein (p22cr). It was recently demonstrated that HBcrAg correlates with intrahepatic cccDNA in CHB patients [74, 75]. Moreover, HBcrAg could be detected in patients with loss of serum Hepatitis B surface antigen (HBsAg), which is nowadays regarded to as the most favorable clinical outcome, or in patients with undetectable serum HBV DNA levels [74-76]. HBcrAg dynamics have been described in natural history and during NA therapy [77-79], but it is unknown how HBcrAg levels are influenced by PEG-IFN therapy.

The purpose of this study was to describe serum HBcrAg dynamics in HBeAg-positive CHB patients treated within an international multicenter randomised controlled trial comparing ETV monotherapy with ETV + PEG-IFN add-on therapy, and to assess the role of HBcrAg in (sustainable) treatment response in addition to HBV DNA and quantitative HBeAg (qHBeAg) and HBsAg (qHBsAg) levels.

PATIENTS AND METHODS

Study population.

HBcrAg levels were studied in 175 HBeAg-positive CHB patients treated within an international randomised trial in which patients were treated with ETV from

week 0 – 24 and were allocated at week 24 to either receive ETV with PEG-IFN add-on therapy (n=85) up to week 48, or to continue ETV monotherapy (n=90) [80]. Patients who achieved a combination of HBeAg loss and HBV DNA levels <200 IU/mL (combined response, CR) at week 48 discontinued ETV at week 72, while non-responders continued ETV for the complete study period up to week 96. All patients were followed through week 96 (Supplementary figure 1). During PEG-IFN treatment, study visits were performed every 4 weeks for routine examination and laboratory tests, while patients on ETV monotherapy visited the clinic every 12 weeks. The inclusion and exclusion criteria for this trial are described elsewhere [80]. The study was conducted in agreement 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.

Endpoints

HBcrAg level dynamics are described both during and after treatment. Also, the association between serum HBcrAg levels and combined response (CR; HBeAg loss and HBV DNA <200 IU/mL) or HBsAg response (qHBsAg decline of ≥1 $\log_{10}$ IU/mL and/or a level of <1000 IU/mL) were assessed at week 72 (24 weeks after PEG-IFN cessation), as these endpoints are associated with long-term disease remission [81]. We specifically chose week 72 as the time-point for assessment of on-treatment response, because 22 patients stopped ETV after week 72 [80]. Finally, we assessed the association between HBcrAg levels and week 96 off-treatment response. Relapse was defined as HBeAg seroreversion and/or an HBV DNA >200 IU/mL after stopping ETV.

Laboratory measurements

Serum samples were taken at baseline and during treatment. HBcrAg was measured using the Lumipulse® *G* HBcrAg assay (Fujirebio Europe, Belgium) in serum samples stored at -20^0 Celsius. In the present study units were expressed as U/mL. A detailed description of the test procedure is provided as a supplementary file. Serum ALT levels were standardized by calculating the value times the ULN per centre and gender. HBV DNA was measured using the Cobas TaqMan 48 polymerase chain reaction assay (Roche Diagnostics, Basel, Switzerland, lower limit of quantification 20 IU/mL). Serum qHBeAg and qHBsAg levels were measured using the Cobas Elecsys 411 (Roche Diagnostics, Basel, Switzerland, lower limit of detection 0.30 IU/mL and 0.05 IU/mL, respectively). HBV genotype analysis was performed using the INNO-LiPA HBV genotype assay (Innogenetics, Ghent, Belgium). The presence of precore (PC, nucleotide position G1896) and/or

basal core promoter (BCP, nucleotide positions A1762 and G1764) was assessed using the INNO-LiPA HBV PreCore assay (Innogenetics, Ghent, Belgium).

Statistical analysis

SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.3 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. Skewed laboratory values were log-transformed prior to analyses and were expressed as mean (standard deviation [SD]). Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. Subgroup analysis for mean HBcrAg levels at baseline was performed using ANOVA with Bonferroni correction for intergroup comparison. HBcrAg, qHBsAg, qHBeAg and HBV DNA declines were analyzed with repeated measurement models adjusting for baseline values. We performed logistic regression analysis to determine factors associated with CR. The performance of the retrieved models was tested with Receiver Operating Characteristic (ROC) Curve Analysis. All analyses were performed two-sided at the 0.05 level of significance.

RESULTS

Study cohort

Of the 175 patients included, 85 were treated with PEG-IFN add-on and 90 patients with ETV monotherapy. The patient characteristics are shown in Table 1. In total 107 (61%) Asian and 65 (37%) Caucasian patients were included, which was reflected by the distribution of HBV genotype A/B/C/D, found in 5/23/39/33% and 10/14/46/30% for add-on vs. ETV monotherapy, respectively.

Serum HBcrAg levels at baseline

For the total cohort, the mean of baseline serum HBcrAg levels was 8.1 (0.8) log U/mL. Mean HBcrAg levels did not significantly differ across HBV genotypes at baseline (p=0.12, Supplementary figure 2). Among the 16 patients infected with wildtype HBV only, HBcrAg levels ranged from 6.4 to 9.0 log U/mL. Mean serum HBcrAg levels were lower in the presence of BCP mutation than PC mutation (8.0 (0.8) log U/mL vs. 7.7 (0.9) log U/mL, p=0.034, Supplementary figure 2). Baseline HBcrAg levels correlated with baseline qHBeAg (r=0.9, p<0.001), qHBsAg (r=0.4 p<0.001) and HBV DNA (r=0.7, p<0.001) (Supplementary figure 3). By Bland-Altman analysis, standardized HBcrAg and qHBeAg measurements showed close agreement at baseline (Supplementary figure 4).

Table 1. Patient characteristics

Characteristics	ETV monotherapy (n=90)	PEG-IFN add-on (n=85)
Demography		
Age, years	31 (9)	32 (10)
Male, n (%)	62 (69)	63 (74)
Race, n (%)		
Caucasian	35 (39)	30 (35)
Asian	54 (60)	53 (63)
Other	1 (1)	2 (2)
HBV Genotype: A/B/C/D (%)	10/14/46/30	5/23/39/33
INNO-LiPA result, n (%)		
Wildtype virus	7 (8)	9 (12)
Precore mutations	13 (16)	21 (27)
Basal core promoter mutations	16 (19)	7 (9)
Precore & Basal core promoter mutations	48 (57)	40 (52)
Histology		
Ishak fibrosis		
0-2	47 (54)	53 (65)
3-4	35 (40)	25 (31)
5-6	5 (6)	3 (4)
Cirrhosis, n (%)	5 (6)	3 (4)
Week 0 laboratory results *		
ALT (x ULN) †	2.7 (2.1)	3.1 (3.3)
HBV DNA ‡	7.8 (1.1)	7.8 (1.3)
HBcrAg §	8.0 (0.9)	8.1 (0.8)
qHBsAg ‡	4.1 (0.8)	4.2 (0.8)
qHBeAg ‡	2.3 (1.0)	2.3 (1.0)
Week 24 laboratory results		
ALT (x ULN) †	0.8 (0.6)	0.8 (0.4)
HBV DNA ‡ [a]	2.3 (1.4)	2.8 (1.5)
HBcrAg §	6.8 (1.1)	7.0 (1.0)
qHBsAg ‡	3.7 (0.8)	3.7 (0.7)
qHBeAg ‡	0.9 (1.2)	1.1 (1.1)

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

** Patients were randomised at week 24 and thus not yet allocated at week 0.*

† *Multiples of upper limit of the normal range*

‡ *Logarithmic scale, IU/mL*

§ *Logarithmic scale, U/mL*

[a] *HBV DNA levels differed at week 24 (p=0.021)*

On-treatment serum HBcrAg dynamics

At week 72, CR had been achieved in 27/85 (32%) of patients assigned PEG-IFN add-on and 16/90 (18%) of patients assigned ETV monotherapy (p=0.032). Furthermore, HBsAg response was achieved in 39/85 (46%) vs. 25/90 (28%) for PEG-IFN add-on vs. monotherapy (p=0.013). HBcrAg decline at week 72 did not differ between patients treated with PEG-IFN add-on and patients treated with ETV monotherapy (-2.10 vs. -1.96 log U/mL, p=0.12).

ETV monotherapy

For patients allocated to the ETV monotherapy arm, significantly more decline was observed at week 72 for patients with CR than for those without CR (-3.22 vs. -1.71 log U/mL, p<0.001, Figure 1). During the first 24 weeks of treatment, this distinction between responders and non-responders was already apparent with a mean HBcrAg decline at week 24 of -2.19 vs. -1.04 log U/mL, p<0.001, respectively. This was also observed from week 48 to week 72: HBcrAg decline in responders was stronger than in non-responders (-0.59 vs. -0.28 log U/mL, p=0.05). Likewise, in those patients who achieved HBsAg response, mean declines were stronger than in non-responders (week 24: -1.64 vs. -1.10 log U/mL, p=0.03; week 72: -2.60 vs. -1.74 log U/mL, p<0.001). HBcrAg decline differed across HBV genotype, with strongest decline in genotype A and weakest decline in genotype D (A: -2.62 log U/mL; B: -2.16 log U/mL; C: -1.90 log U/mL; D: -1.74 log U/mL; A vs. D: p=0.02; other comparisons p>0.10).

Peginterferon add-on therapy

Within the group of PEG-IFN add-on treated patients, there was also a difference in HBcrAg decline between patients who achieved CR and those who did not (-3.16 vs. -1.83 log U/mL, p<0.001). Between week 48 (the moment of PEG-IFN cessation) and week 72, HBcrAg decline in responders was -0.49 log U/mL, whereas it was -0.15 log U/mL in non-responders (p=0.04). Patients in the add-on arm with HBsAg response at week 72 showed more HBcrAg decline than HBsAg non-responders (-2.51 vs. -2.04 log U/mL, p=0.05). Like in ETV monotherapy, HBcrAg decline differed across genotype with strongest decline in genotype A and weakest decline in genotype D (A: -3.16 log U/mL; B: -2.61 log U/mL; C: -2.37 log U/mL; D: -1.76 log U/mL; A vs. D: p=0.01; B vs. D: p=0.002; C vs. D: p=0.013).

One patient lost HBsAg at week 48, and still had detectable HBcrAg (5.33 log U/mL). In this patient, HBsAg had reappeared at week 72. At week 72, no patient was HBsAg negative. At week 96, another patient had confirmed HBsAg loss, who also had detectable HBcrAg (3.30 log U/mL).

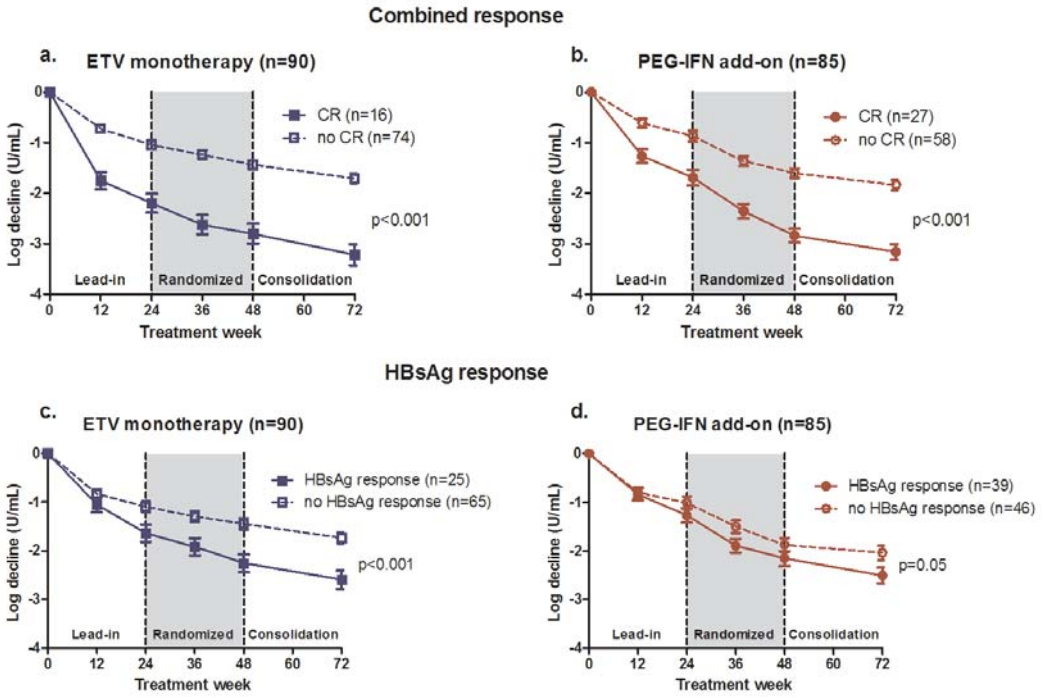


Figure 1a. HBcrAg decline according to week 72 response

a. HBcrAg decline in the ETV monotherapy arm between week 0 and 72 according to combined response at week 72.

b. HBcrAg decline in the PEG-IFN add-on arm between week 0 and 72 according to combined response at week 72.

c. HBcrAg decline in the ETV monotherapy arm between week 0 and 72 according to HBsAg response at week 72.

d. HBcrAg decline in the PEG-IFN add-on arm between week 0 and 72 according to HBsAg response at week 72.

Declines are expressed as mean log U/mL with error bars representing standard errors of mean.

On-treatment serum HBcrAg in relation to serum HBV DNA, qHBsAg and qHBeAg

Serum qHBsAg, qHBeAg, and HBV DNA, also declined significantly stronger in patients who achieved combined response than in patients who did not (qHBsAg decline -0.80 vs. -0.50 IU/mL, p=0.03, figure 1b; qHBeAg decline -3.46 vs. -1.63 log IU/mL, p<0.001; and HBV DNA decline -7.13 vs. -5.98 log IU/mL, p<0.001). In patients treated with PEG-IFN add-on, both HBcrAg and qHBsAg decline continued after HBeAg loss. In patients treated with ETV monotherapy, HBcrAg decline also continued after HBeAg loss, whereas qHBsAg levels did not. From week 48 to 72, HBcrAg and qHBeAg declined further (HBcrAg: -0.29 log U/mL, p<0.001; qHBeAg: -0.34 log IU/mL, p<0.001). In contrast, qHBsAg levels remained stable within this time interval (-0.02 IU/mL, p=0.244). No difference in decline between treatment arms was observed within this time interval (HBcrAg: p=0.55; qHBeAg: p=0.36, qHBsAg: p=0.50).

Figure 2 shows dynamics of all markers in 6 example patients, illustrating differences between these markers for different treatment and response combinations.

Serum HBcrAg as a predictor of week 72 combined response

Adjusted for therapy allocation, HBV genotype and week 24 serum ALT, lower HBcrAg at week 24 was independently associated with increased probability

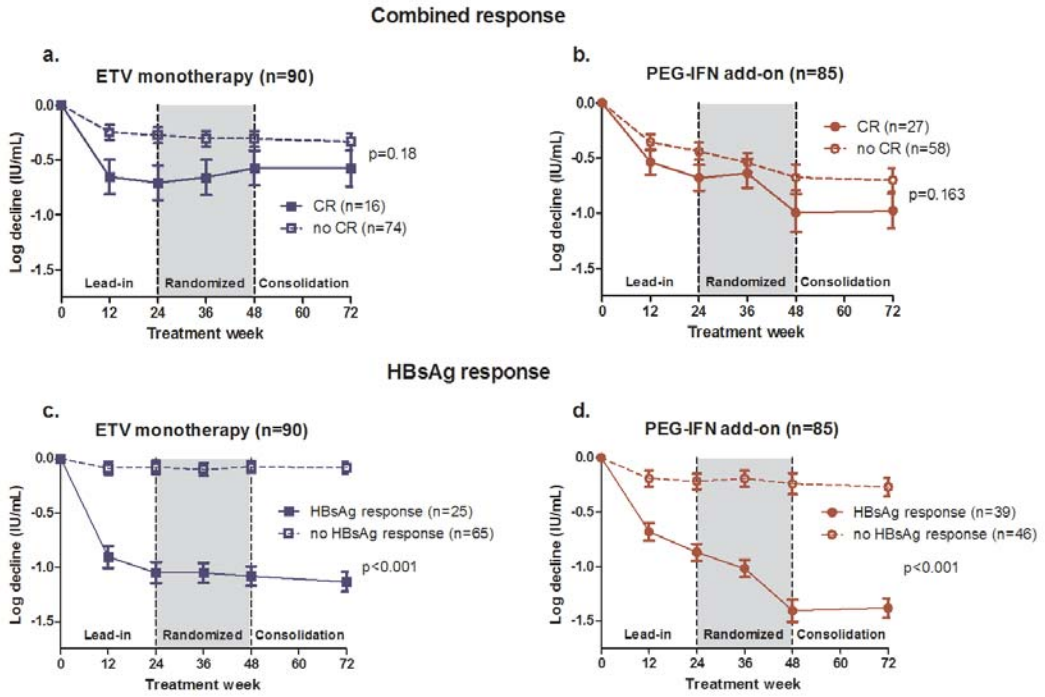


Figure 1b. qHBsAg decline according to week 72 response

a. qHBsAg decline in the ETV monotherapy arm between week 0 and 72 according to combined response at week 72.

b. qHBsAg decline in the PEG-IFN add-on arm between week 0 and 72 according to combined response at week 72.

c. qHBsAg decline in the ETV monotherapy arm between week 0 and 72 according to HBsAg response at week 72.

d. qHBsAg decline in the PEG-IFN add-on arm between week 0 and 72 according to HBsAg response at week 72.

Declines are expressed as mean log IU/mL with error bars representing standard errors of mean.

of achieving CR (OR 0.3, 95% confidence interval [CI-95%] 0.2-0.5, $p<0.001$, Table 2). There were no significant interactions with therapy allocation. There was a good predictive ability for this model (AUC 0.86, CI-95% 0.80-0.91, $p<0.001$). In a sensitivity analysis, in which 13 patients who were already HBeAg-negative at week 24 had been excluded, performance of the HBcrAg model was comparable (AUC 0.86, CI-95% 0.80-0.92, $p<0.001$). To demonstrate the performance of week 24 qHBsAg, qHBeAg, or HBV DNA levels in a similar prediction model, we substituted HBcrAg by each of these markers separately: qHBeAg AUC 0.89 (CI-95% 0.84-0.94); qHBsAg AUC 0.81 (CI-95% 0.75-0.88); HBV DNA AUC 0.86 (CI-95% 0.80-0.91) (all $p<0.001$).

Lower HBcrAg at week 36 (12 weeks after randomization) was also associated with increased probability of achieving CR (OR 0.1, CI-95% 0.04-0.2,

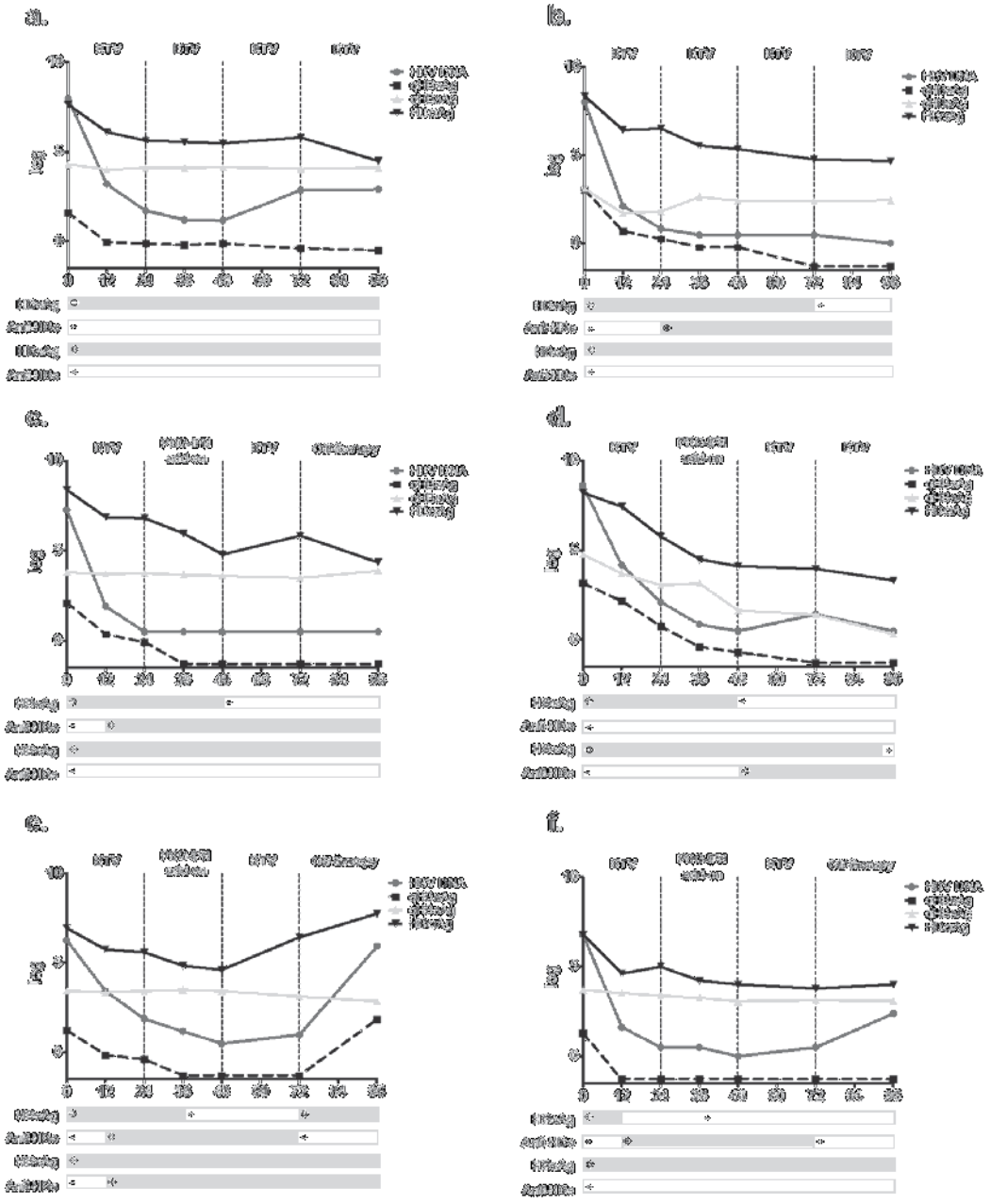


Figure 2. Dynamics of HBcrAg, qHBeAg, qHBsAg and HBV DNA in 6 example patients.

a. Patient with 96 weeks of ETV therapy, no CR.

b. Patient with 96 weeks of ETV therapy, CR.

c. Patient with 72 weeks of ETV therapy + PEG-IFN add-on from week 24-48. CR at week 48, sustained off-treatment CR.

d. Patient with 96 weeks of ETV therapy + PEG-IFN add-on from week 24-48. CR and HBsAg loss.

e. Patient with 72 weeks of ETV therapy + PEG-IFN add-on from week 24-48. CR at week 48, but off-treatment HBeAg seroreversion and HBV DNA >200 IU/mL.

f. Patient with 72 weeks of ETV therapy + PEG-IFN add-on from week 24-48. CR at week 48, off-treatment sustained HBeAg negativity, but HBV DNA >200 IU/mL at week 96.

Table 2. Univariable and multivariable analysis for combined response at week 72

Week 72	**Univariable**		**Multivariable**		**Multivariable**	
HBeAg loss &			(Full model week 24)		(Final model week 24)	
HBV DNA <200 IU/mL	**OR (CI-95%)**	**p-value**	**OR (CI-95%)**	**p-value**	**OR (CI-95%)**	**p-value**
PEG-IFN add-on	2.2 (1.1-4.4)	0.03	4.9 (1.9-12.7)	0.001	4.2 (1.7-10.3)	0.002
HBV genotype						
A	4.4 (1.2-16.1)	0.03	2.8 (0.5-17.1)	0.27	3.3 (0.6-19.9)	0.19
B	2.6 (0.9-7.1)	0.07	0.7 (0.2-2.7)	0.63	1.0 (0.3-3.5)	0.99
C	1.5 (0.6-3.7)	0.36	0.6 (0.2-1.9)	0.35	1.1 (0.4-3.1)	0.91
D	Reference		Reference			
Mutation						
No mutations (wildtype virus)	Reference					
Precore and/or basal core promoter muations	5.7 (0.7-44.7)	0.10				
Gender						
Male	Reference					
Female	1.2 (0.6-2.7)	0.62				
Asian ethnicity	1.2 (0.6-2.4)	0.65				
Age	1.0 (1.0-1.0)	0.80				
Week 24 lab values						
ALT (x ULN)	0.1 (0.03-0.3)	<0.001	0.2 (0.0-1.0)	0.05	0.1 (0.03-0.6)	0.009
HBV DNA <200 IU/mL	0.1 (0.1-0.3)	<0.001	**	**		
HBV DNA <20 IU/mL	0.2 (0.1-0.4)	<0.001	**	**		
HBV DNA (log IU/mL)*	0.4 (0.3-0.6)	<0.001	0.5 (0.2-0.9)	0.02		
qHBsAg (log IU/mL)	0.4 (0.3-0.7)	0.001	**	**		
qHBeAg (log IU/mL)	0.3 (0.2-0.4)	<0.001	**	**		
HBcrAg (log U/mL)	0.3 (0.2-0.5)	<0.001	0.5 (0.2-1.1)	0.09	0.3 (0.2-0.5)*	<0.001

** HBV DNA levels at week 24 (randomization) differed among the therapy allocation arms (p=0.021) and showed a strong correlation with qHBeAg (r=0.9), qHBsAg (r=0.4) and log HBV DNA (r=0.7) (all p<0.001). Therefore only HBcrAg was added to the final model.*

*** When HBcrAg levels were substituted by week 24 qHBsAg, qHBeAg, or HBV DNA levels, the model performance was as follows: qHBeAg AUC 0.89 (CI-95% 0.84-0.94); qHBsAg AUC 0.81 (CI-95% 0.75-0.88); HBV DNA AUC 0.86 (CI-95% 0.80-0.91) (all p<0.001).*

$p<0.001$, adjusted for therapy allocation, HBV genotype and week 36 serum ALT), and was good at predicting CR (AUC 0.88, CI-95% 0.83-93, $p<0.001$). Substitution of HBcrAg by week 36 qHBeAg, qHBsAg, or HBV DNA levels led to AUCs of 0.91 (CI-95% 0.86-0.96), 0.76 (CI-95% 0.68-0.83) and HBV DNA AUC 0.83 (CI-95% 0.76-0.89), respectively (all $p<0.001$).

Serum HBcrAg in relation to relapse

At week 72, 14/85 (17%) assigned to PEG-IFN add-on and 8/90 (9%) assigned to ETV monotherapy stopped treatment because of CR achievement at week 48. CR was maintained through week 96 in 9/14 (64%) and 2/8 (25%) patients, respectively. Regardless of therapy allocation, HBcrAg declined off-treatment in 9 out of these 11 patients, and in 1 patient HBcrAg remained stable. HBcrAg was missing at week 96 in 1 patient. In contrast, in 11 patients who did not maintain CR after ETV cessation, HBcrAg decline was seen in 2 patients only (both allocated to PEG-IFN add-on). The individual patterns of HBcrAg after cessation of ETV are shown in Figure 3.

DISCUSSION

In this study of HBeAg-positive patients treated with ETV monotherapy or ETV + PEG-IFN add-on therapy, we have shown that HBcrAg levels decline during treatment. HBcrAg dynamics strongly resembled those of qHBeAg, but HBcrAg

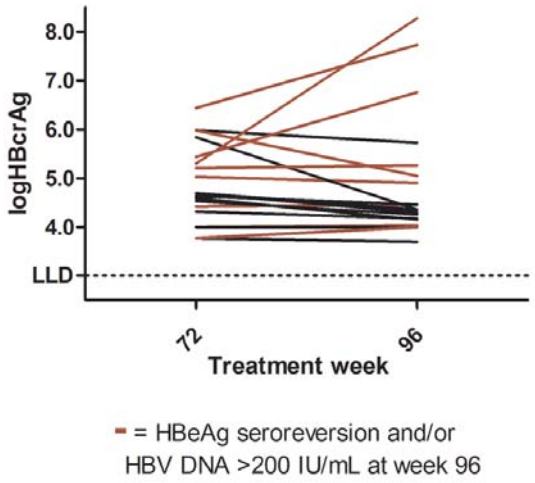


Figure 3. Individual HBcrAg dynamics in patients who stopped ETV at week 72.
Twenty-two patients stopped ETV at week 72 based on achievement of CR at week 48.
Week 72 and 96 HBcrAg levels available at both time points in 20/22 patients. Red lines represent patients who did not maintain CR through week 96 (HBeAg seroreversion and/or HBV DNA >200 IU/mL after stopping ETV).

levels declined even after HBeAg loss. Although HBcrAg levels were not superior to qHBsAg, qHBeAg, or HBV DNA levels in response prediction, we demonstrated that HBcrAg levels at randomization were independently associated with response to both ETV monotherapy and PEG-IFN add-on therapy. Interestingly, HBcrAg patterns were predictive for sustained off-treatment response. HBcrAg strongly declined prior to HBsAg loss, but could still be detected at time of confirmed HBsAg negativity. HBcrAg may thus be useful as a new serum marker for CHB treatment, particularly now that our next challenge in therapy is to achieve functional cure with HBsAg seroconversion.

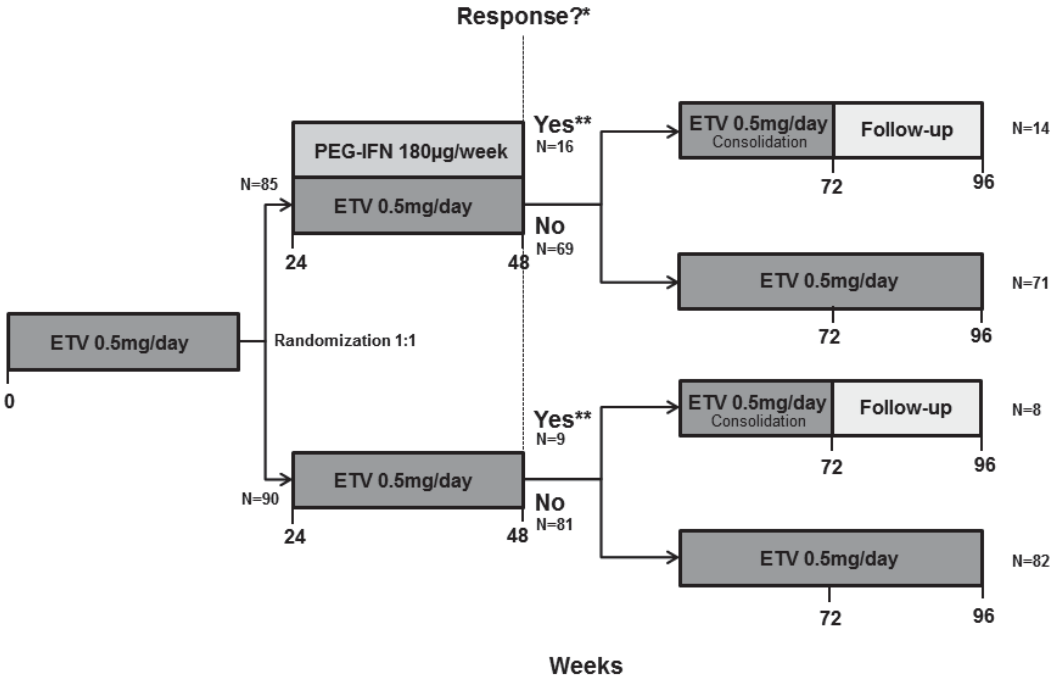
The findings of our study are important, because few serum markers are considered to be accurate to assess immune control and to predict the sustainability of treatment response in CHB. Measurement of intrahepatic cccDNA is superior to serum qHBsAg or HBV DNA in the prediction of sustained response to antiviral treatment [73]. In addition, cccDNA can still be detected after loss of HBsAg, acting as a substrate for viral reactivation during immunosuppressive states [82]. There is a need for serum makers that correlate with cccDNA and HBcrAg has been shown to do that in both HBeAg-positive and HBeAg-negative patients [74, 75]. To our knowledge, we are the first to describe on-treatment HBcrAg measurements for NA and PEG-IFN combination therapy, a treatment strategy that has been shown to induce increased immune control over NA alone [61, 80]. Our data on the comparison with other serum markers provides important information for future studies on HBcrAg, and gives a better understanding of the clinical applicability of HBcrAg in HBeAg-positive CHB.

Mean HBcrAg levels at baseline in our study population were in the same range as the mean levels reported for HBeAg-positive patients in other studies [74, 77, 78]. Previously, it has been described that the correlation of HBcrAg to serum HBV DNA and HBsAg is strongest for HBeAg-positive CHB [74, 83, 84]. Our results were similar, and showed comparable correlation coefficients. In natural history studies, the correlation of HBcrAg with HBV DNA and HBsAg in the HBeAg-negative low replicative phase was either week or absent [83, 84]. This may explain why the differences in decline between HBsAg responders and HBsAg non-responders were less apparent than those between combined responders and non-responders. HBcrAg was still detected in patients with undetectable HBsAg, which is also in line with observations by others [74, 76]. In addition to possibilities for monitoring treatment response and relapse in CHB, it would therefore be interesting to validate this finding, and to explore HBcrAg as a marker for reinfection after liver transplantation, or for reactivation during immunosuppression [76].

The close agreement of the results of HBcrAg and qHBeAg measurements in HBeAg-positive CHB illustrates that HBeAg comprises a considerable fraction of HBcrAg [85]. For HBeAg-positive disease, we did not find HBcrAg measurements to be superior to qHBsAg, qHBeAg, or HBV DNA levels in response prediction. Therefore, HBcrAg levels may not provide additional benefit in clinical practice for this patient group. However, as serum markers for immune control in HBeAg-negative CHB are lacking, new markers for response prediction in this group are in demand. The observation of continuous decline of HBcrAg after HBeAg loss suggests that the levels of at least one out of three HBcrAg components further decreases. This finding therefore highlights the importance of investigating HBcrAg measurements in HBeAg-negative disease, preferably in PEG-IFN monotherapy as we may not be able to directly extrapolate our findings towards this treatment strategy. The predictive ability of HBcrAg levels for relapse after NA cessation has previously been described by a Japanese group [86]. Due to the limited number of patients that stopped treatment in our study, we could not confirm this specific role of HBcrAg. By multivariable analysis, genotype and presence of PC and/or BCP mutations were not associated with response, but his may be explained by the small subgroups. No interaction was found between HBV genotype and HBcrAg levels.

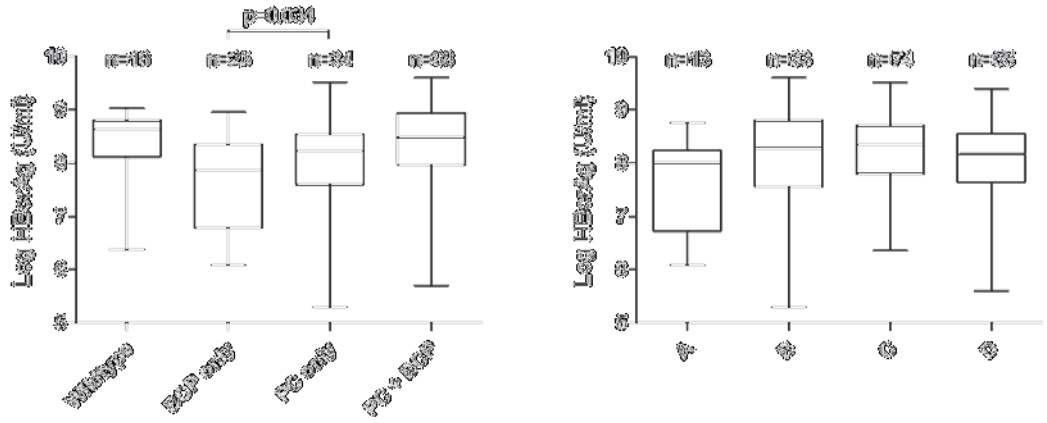
In conclusion, we have shown that serum HBcrAg levels were associated with on-treatment and off-treatment response in HBeAg-positive CHB. For better assessment of the role of this marker in off-treatment response, longer follow-up after treatment cessation is needed. Although HBcrAg was not superior to qHBsAg and HBV DNA, which are the commonly used markers for response prediction in HBeAg-positive patients,HBcrAg performed well in the prediction of response. This finding, and our findings of continuing HBcrAg decline after HBeAg loss and the detectibility of HBcrAg after HBsAg loss are an important step towards future studies of this marker in HBeAg-negative patients and HBsAg-negative patients.

SUPPLEMENTARY FIGURES

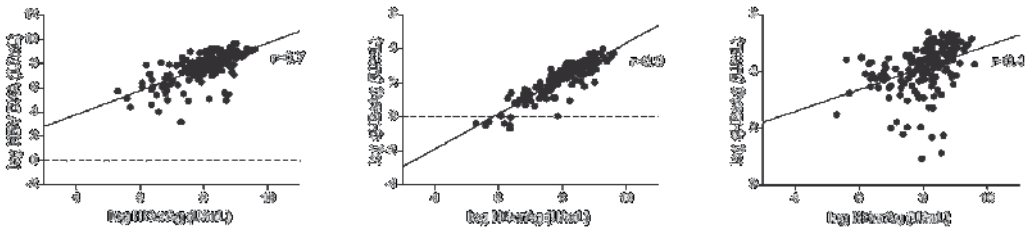


*Response was defined as HBeAg loss with a HBV DNA <200 IU/mL (primary endpoint at week 48)
**Responders were to stop ETV therapy at week 72 and thus received at least 24 weeks of consolidation therapy. Two responders assigned add-on and 1 responder assigned monotherapy continued ETV monotherapy (protocol violation)

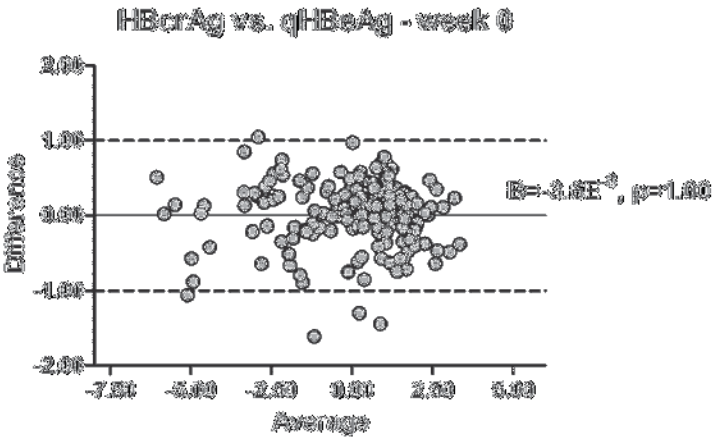
Supplementary figure 1. Original study design.
* Response was defined as HBeAg loss with HBV DNA <200 IU/mL
**Responders were to stop ETV therapy at week 72 and thus received at least 24 weeks of consolidation therapy. Two responders assigned to add-on and 1 responder assigned monotherapy continued ETV monotherapy (protocol violation).



Supplementary figure 2. Baseline HBcrAg according to precore/core mutations and genotype.
HBcrAg expressed as mean (SD) log U/mL. Mean HBcrAg levels did not significantly differ across HBV genotypes at baseline (p=0.12). Mean serum HBcrAg levels were lower in the presence of BCP mutation than PC mutation.



Supplementary figure 3. Correlation of HBcrAg to HBV DNA, qHBsAg and qHBeAg at baseline.
At baseline, HBcrAg was correlated to HBV DNA, qHBeAg and qHBsAg (r=0.7, r=0.9, r=0.4, all *p*<0.001).



Supplementary figure 4. Standardized comparison of HBcrAg and qHBeAg.
Graph markers represent standardized comparison of HBcrAg and qHBeAg per patient. Tests are considered comparable if the slope of the regression line that can be fit is significantly different from 0. HBcrAg and qHBeAg measurements showed close agreement at baseline.

4

Hepatitis B core-related antigen monitoring during peginterferon alfa treatment for HBeAg-negative chronic hepatitis B

Margo J.H. van Campenhout[1], Vincent Rijckborst[1], Willem Pieter Brouwer[1], Gertine W. van Oord[1], Peter Ferenci[2], Fehmi Tabak[3], Meral Akdogan[4], Binnur Pinarbasi[5], Krzysztof Simon[6], Robert J. de Knegt[1], André Boonstra[1], Harry L.A. Janssen[7], and Bettina E. Hansen[1,7,8]

[1]Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands; [2]Department of Internal Medicine, Gastroenterology and Hepatology, Medical University of Vienna, Vienna, Austria; [3]Cerrahpasa Medical Faculty, Istanbul, Turkey; [4]Department of Gastroenterology, Yuksek Ihtisas Hospital, Ankara, Turkey; [5]Division of Gastroenterohepatology, Department of Internal Medicine, Istanbul Faculty of Medicine, Istanbul University, Turkey; [6]Division of Infectious Diseases and Hepatology, Wroclaw Medical University, Wroclaw, Poland; [7]Toronto Center for Liver Disease, Toronto Western and General Hospital, University Health Network, Toronto, Canada; [8] Institute of Health Policy, Management and Evaluation, University of Toronto, Toronto, Canada.

Journal of Viral Hepatitis

ABSTRACT

Background & aims. Serum Hepatitis B core-related antigen (HBcrAg) level moderately correlates with cccDNA. We examined whether HBcrAg can add value in monitoring the effect of peginterferon (PEG-IFN) therapy for HBeAg-negative chronic hepatitis B (CHB) infection.

Methods. Serum HBcrAg level was measured in 133 HBeAg-negative, mainly Caucasian CHB patients, treated with 48 weeks of PEG-IFN alfa-2a. We assessed its association with response (ALT normalization & HBV DNA <2,000 IU/mL) at week 72.

Results. HBcrAg level strongly correlated with HBV DNA level (r=0.8, p<0.001) and weakly with qHBsAg and ALT (both r=0.2, p=0.01). At week 48, mean HBcrAg decline was -3.3 log U/mL. Baseline levels were comparable for patients with and without response at week 72 (5.0 vs. 4.9 log U/mL, p=0.59). HBcrAg decline at week 72 differed between patients with and without response (-2.4 vs. -1.0 log U/mL, p=0.001), but no cut-off could be determined. The pattern of decline in responders resembled that of HBV DNA, but HBcrAg decline was weaker (HBcrAg -2.5 log U/mL; HBV DNA: -4.0 log IU/mL, p<0.001). For early identification of nonresponse, diagnostic accuracy of HBV DNA and qHBsAg decline at week 12 (AUC 0.742, CI-95% [0.0.629-0.855], p<0.001) did not improve by adding HBcrAg decline (AUC 0.747, CI-95% [0.629-0.855] p<0.001), nor by replacing HBV DNA decline by HBcrAg decline (AUC 0.754, CI-95% [0.641-0.867], p<0.001).

Conclusions. In Caucasian patients with HBeAg-negative CHB, decline of HBcrAg during PEG-IFN treatment was stronger in patients with treatment response. However, HBcrAg was not superior to HBV DNA and qHBsAg to predict response in PEG-IFN treatment.

BACKGROUND

While awaiting new therapeutic agents for chronic hepatitis B (CHB) that induce a high rate of functional cure, peginterferon (PEG-IFN) treatment remains an important therapeutic strategy. In order to avoid unnecessary exposure to the common side-effects, early identification of non-responders is warranted. In patients with HBeAg-negative CHB, on-treatment monitoring of serum hepatitis B virus (HBV) DNA levels and serum quantitative hepatitis B surface antigen (qHBsAg) levels allows premature treatment cessation in those who have a low probability of achieving response. Unfortunately, identification of these patients before treatment is limited by the lack of patient and viral characteristics that accurately predict therapy response, particularly in HBeAg negative disease. [87] In addition, it is difficult to predict relapse after stopping PEG-IFN which necessitates long-term monitoring of patients after the treatment course.

The preferential method to predict and assess the effect of therapy in CHB would be to directly assess the levels of intrahepatic covalently closed circular DNA (cccDNA), as this is the main template for viral protein synthesis and viral replication. [42, 52] However, cccDNA is difficult to quantify and its measurement requires a liver biopsy, which is not a desired procedure to routinely perform due to its invasiveness. Monitoring levels of a surrogate serum marker correlating with cccDNA may be an alternative option.

A possible surrogate marker is hepatitis B core-related antigen (HBcrAg), which is a serum marker for the combined measure of 3 proteins coded by the precore/core region of the cccDNA: hepatitis B core antigen (HBcAg), hepatitis B e antigen (HBeAg) and a 22-kD precore protein (p22cr). [85] HBcrAg levels moderately correlate with cccDNA [74, 76, 88], are associated with relapse after cessation of nucleos(t)ide analogue (NA) therapy [86, 89-91], and can still be detected after HBsAg loss. [74, 92] In addition, HBcrAg levels at baseline predict response to PEG-IFN treatment in HBeAg-positive CHB. [93] It is yet unknown if HBcrAg can be used to monitor PEG-IFN treatment and to predict response and relapse in HBeAg-negative disease. Our aims were therefore to describe the dynamics of serum HBcrAg before, during and after PEG-IFN treatment in HBeAg-negative CHB patients, and to investigate the association between HBcrAg levels and sustained treatment response.

PATIENTS AND METHODS

Study population

Serum HBcrAg levels were measured in available serum samples of 133 HBeAg-negative CHB patients who were treated with PEG-IFN alpha-2a ± ribavirin within an international, double-blind randomised controlled trial. The inclusion criteria and exclusion criteria of the initial study have been described elsewhere. [94] Patients were treated for 48 weeks and were followed for an additional 24 weeks. The treatment arms were pooled for all analyses, as the response rates between the treatment groups did not differ. Serum samples were taken at baseline, during treatment at week 4, 8, 12, 24, 36, and week 48, and during post-treatment follow-up at week 60 and 72. Of 79 patients long-term follow-up (LTFU) data regarding treatment response was available. The mean interval between week 48 and this LTFU visit was 2.1 (SD 0.2) years. [95]

Endpoints

Serum HBcrAg dynamics were evaluated during treatment and during post-treatment follow-up of the initial study. We primarily assessed the correlation of HBcrAg levels with combined response (response; ALT normalization and HBV DNA <2,000 IU/mL) at week 72. Secondary endpoints were response at LTFU, and relapse (defined as not fulfilling criteria of response) at week 72 and at LTFU for patients with week 48 response.

Serum HBcrAg measurements

HBcrAg was measured using the Lumipulse® *G* HBcrAg assay (Fujirebio Europe, Belgium) in serum samples stored at -80^0 Celsius. A pre-heat treatment in presence of a provided detergent solution was applied for extracting denatured precore/core proteins and for the inactivation of circulating antibodies to HBcAg and HBeAg. The monoclonal antibodies used in the subsequent two-step immunoassay simultaneously detect all 3 HBV core-related proteins (HBeAg, HBcAg, p22cr), also when HBeAg is bound in HBeAg/anti-HBe immunecomplexes. This fully-automated assay uses ferrite particle suspension as solid phase in an immunoreaction cartridge and the relative luminescence intensity reflects the amount of HBV core-related proteins. HBcrAg concentration is calculated by a standard curve generated using recombinant pro-HBeAg (aa -10 to 183) and is expressed in unit of kU/mL by the Lumipulse® *G* system. The analytical sensitivity is 1 kU/mL and the measurement range is from 1 to 10,000 kU/mL. [85] In the present study units were expressed as U/mL. For statistical analysis, the value of 100 U/mL (2 log U/mL) was used for results below the lower limit of quantification.

Other laboratory measurements

Routine biochemical and hematological tests were performed locally. Serum ALT levels were standardized by calculating the value times for the upper limit of normal (ULN) per centre and gender. For all visits, virological tests were performed at one central laboratory (Erasmus Medical Center, Rotterdam, The Netherlands). HBV DNA was measured using the Taqman HBV assay (Roche Diagnostics, lower limit of quantification 35 copies/mL [6 IU/mL]). Serum qHBsAg levels were measured using the Architect HBsAg assay (Abbott Laboratories; range 0.05-250 IU/mL) or the Elecsys Assay (Roche Diagnostics, Basel, Switzerland, lower limit of detection 0.05 IU/mL). HBV genotype analysis was performed using the INNO-LiPA HBV genotype assay (Fujirebio Europe, Ghent, Belgium). The presence of PC and BCP mutants was assessed using the INNO-LiPA HBV PreCore assay (Fujirebio Belgium, Ghent, Belgium), which detects precore (PC) mutations at nucleotide position 1896 and basal core promoter (BCP) mutations at nucleotide positions 1762 and 1764.

Statistical analysis

SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.3 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. Skewed laboratory values were log-transformed prior to analyses and were expressed as mean (standard deviation [SD]). Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. Subgroup analysis for mean HBcrAg levels at baseline was performed using ANOVA with Bonferroni correction for intergroup comparison. We performed logistic regression analysis to determine factors associated with response. The performances of the retrieved prediction models were tested with Receiver Operating Characteristic (ROC) Curve Analysis. All analyses were performed two-sided at the 0.05 level of significance.

RESULTS

Study cohort

Patient characteristics are shown in Table 1. The majority of patients were of Caucasian ethnicity, which is reflected by the high proportion of patients with HBV genotype D and A. At baseline, mean HBV DNA level was log 6.1 (1.2) log IU/mL and mean qHBsAg level was 3.8 (0.6) log IU/mL. In 6 patients (5%) no mutations in the basal core promoter (BCP) regions 1762 and 1764 or the precore (PC) region could be detected. In the other 127 (95%) patients,

mutations were detected in one or both of the BCP regions (n=14, 10%), the PC region (n=24, 18%), or in BCP and PC regions (n=89, 67%). In 93 patients (70%) a mutation in the HBV precore region was detected without the simultaneous presence of HBV PC wildtype virus, leading to disabled HBeAg and p22cr production (non-sense G-A change in nucleotide 1896). In 20 patients (15%), both PC mutant and wildtype were detected.

Table 1. Patient characteristics

Characteristics	All patients (n=133)
Demography	
Age, years	42 (11)
Male, n(%)	98 (74)
Caucasian race, n (%)	127 (95)
HBV genotype	
A/B/C/D/other (%)	13/1/2/81/3
HBV basal core promoter and precore mutations	
Wildtype / any BCP / any PC / any BCP or PC (%)	5/10/18/67
A1762: mutant / mixed / wildtype only (%)	43/10/47
G1764: mutant / mixed / wildtype only (%)	65/12/23
A1896: mutant / mixed / wildtype only (%)	70/15/15
History	
Previous interferon therapy (≥4 weeks)	24 (18)
Cirrhosis, n (%)	4(3)
Laboratory results*	
ALT (x ULN) †	3.2 (2.6)
HBV DNA ‡	6.1 (1.2)
HBcrAg §	5.0 (1.2)
qHBsAg ‡	3.8 (0.6)

ALT, alanine aminotransferase; HBcrAg, hepatitis B core-related antigen; HBV, hepatitis B virus; qHBsAg, quantitative hepatitis B surface antigen

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

† Multiples of upper limit of the normal range

‡ Logarithmic scale, IU/mL

§ Logarithmic scale, U/mL

Legends to figures

HBcrAg levels at baseline

At baseline, mean serum HBcrAg level was 5.0 (1.2) log U/mL. Correlation of HBcrAg levels with levels of HBV DNA was strong (r=0.8, $p<0.001$) and correlations with qHBsAg and ALT were weak (both r=0.2, $p=0.01$). Mean HBcrAg

was lowest in patients with mutations in both BCP and PC regions (4.8 log U/mL), which was significantly lower than in patients with PC mutation only (5.6 log U/mL, p=0.03), but did not significantly differ to HBcrAg level in patients with BCP mutations only (5.3 log U/mL) or in patients infected with wildtype HBV (4.7 log U/mL). In the 6 patients infected with wildtype virus only, HBcrAg levels ranged from undetectable to 6.11 log U/mL. To assess the correlation of HBcrAg with HBV DNA, qHBsAg and ALT when HBeAg and p22cr production are disabled by PC mutation, subgroup analysis was performed in 93 patients with a mutation in the HBV precore region without the simultaneous presence of HBV precore wildtype. For these 93 patients, the correlations of HBcrAg with HBV DNA and ALT were similar as the correlations for the entire cohort (HBV DNA: r=0.8, p<0.001; qHBsAg: r=0.2, p=0.11; ALT: r=0.3, p=0.11).

HBcrAg levels at baseline in relation to combined response

Combined response (response) was achieved in 25/133 (19%) patients at week 72, and in 16/79 (20%) patients at long-term follow-up (LTFU). HBcrAg levels at baseline were comparable for patients with vs. without response at week 72 (5.0 vs. 4.9 log U/mL, p=0.59, Figure 1a) and with vs. without response at LTFU (5.1 vs. 4.6 log U/mL, p=0.18, Figure 1b). The mean levels of HBV DNA and qHBsAg were also not discriminative for response at week 72 (HBV DNA: p=1.00, qHBsAg: 0.99). In 5 patients HBcrAg was undetectable already at baseline. Among these 5 patients, 2 patients achieved response at both week 72 and LTFU.

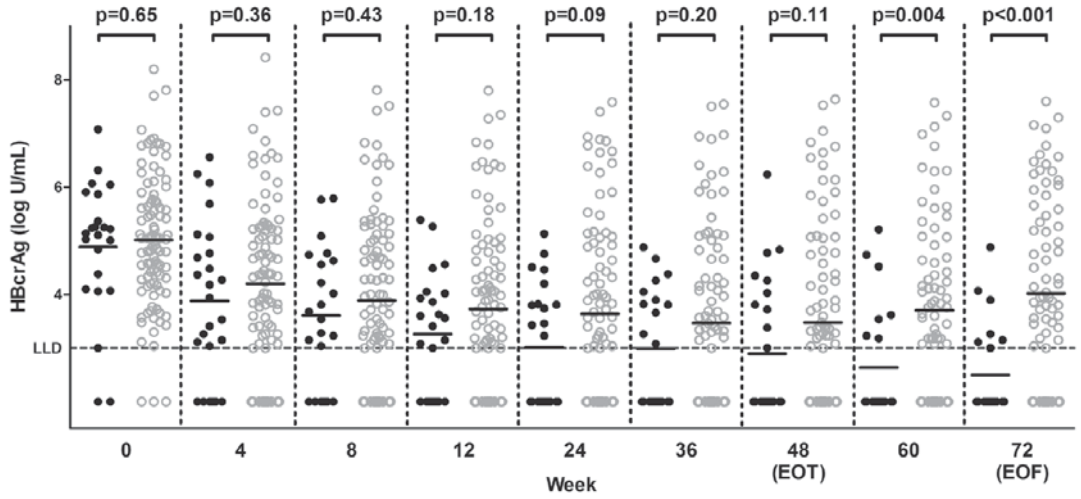


Figure 1a. Absolute serum HBcrAg levels in according to response at week 72.
Black dots represent single HBcrAg measurements, with black dots representing patients who achieved response (HBV DNA <2,000 IU/mL and normal ALT) at week 72, and grey circles representing patients who did not achieve response at week 72. HBcrAg is expressed as log U/mL. For results below the lower limit of quantification (1000 U/mL, 3 log U/mL), the value of 100 U/mL (2 log U/mL) was used.

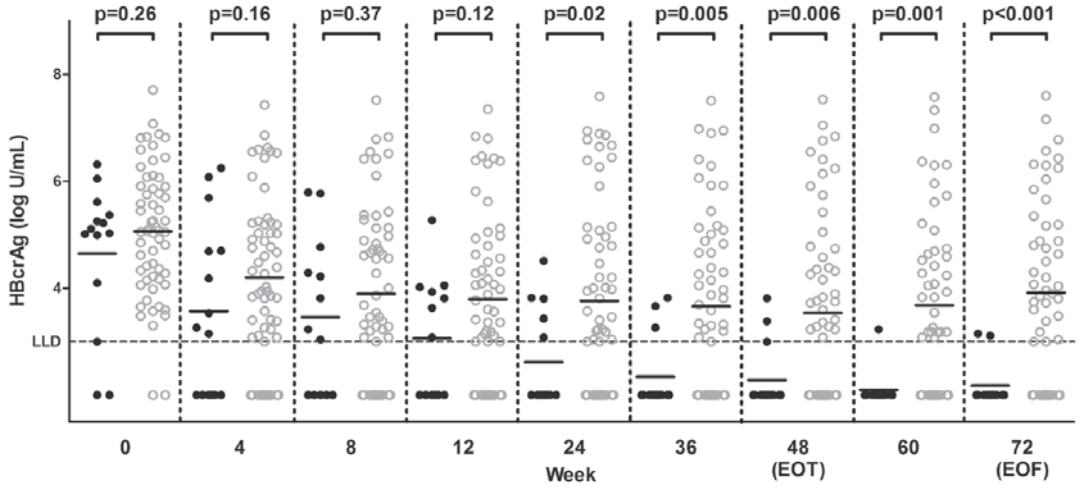
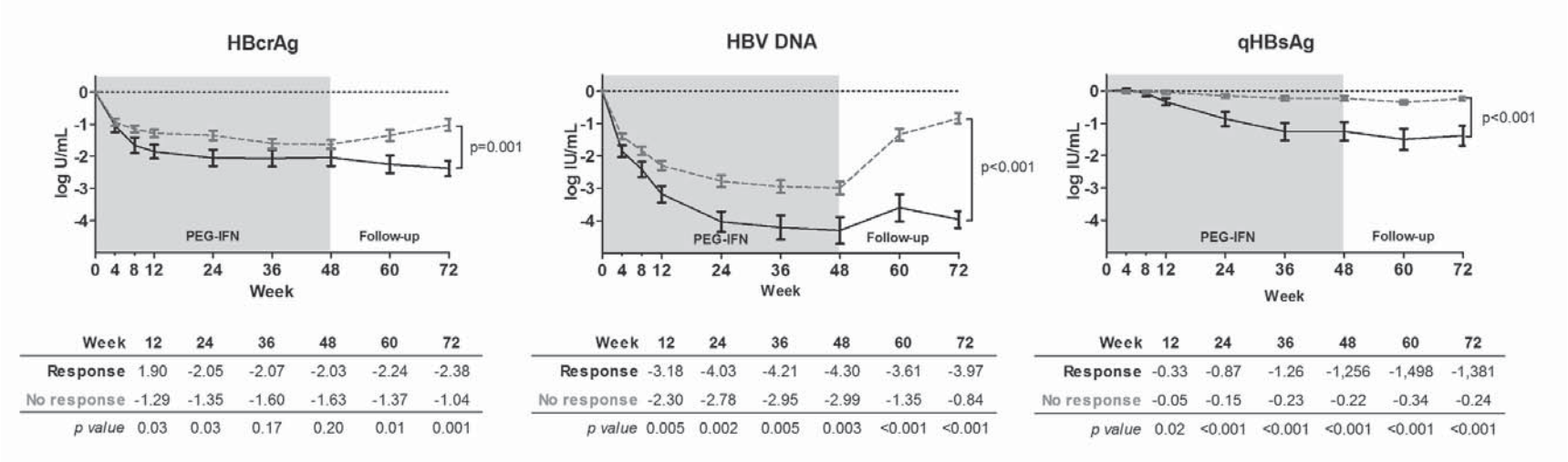


Figure 1b. Absolute serum HBcrAg levels in according to response at long-term follow-up.
In a subset of 79/133 patients, long-term follow-up (LTFU) data regarding treatment response was available. Black dots represent single HBcrAg measurements, with black dots representing patients who achieved response (HBV DNA <2,000 IU/mL and normal ALT) at long-term follow-up, and grey circles representing patients who did not achieve response at long-term follow-up. HBcrAg is expressed as log U/mL. For results below the lower limit of quantification (1000 U/mL, 3 log U/mL), the value of 100 U/mL (2 log U/mL) was used.

HBcrAg levels during and after treatment in relation to combined response at week 72 and LTFU

On-treatment HBcrAg dynamics.

During one year of PEG-IFN therapy a mean decline in HBcrAg of 3.3 log U/mL was reached, which did not differ between patients treated with ribavirin and patients treated with placebo (p=0.62). During treatment, absolute values of HBcrAg did not differ significantly between patients with and without response at week 72. (Figure 1a; week 12: 3.7 vs. 3.3 log U/mL, p=0.18; week 24: 3.6 vs. 3.0 log U/mL, p=0.09, week 36: 3.5 vs. 3.0 log U/mL, p=0.20). In contrast, absolute HBV DNA levels and qHBsAg levels already differed from week 24 onward (week 24: HBV DNA: 2.0 vs. 3.2 log IU/mL, p=0.02; qHBsAg: 2.9 vs. 3.7 log IU/mL, p<0.001). For response at LTFU, absolute HBcrAg levels differed significantly from week 24 onward (Figure 1b). When considering decline in HBcrAg from baseline (Figure 2), no significant difference between patients with and without response at week 72 was observed early on-treatment (week 4: 1.00 vs. 0.94 log U/mL, p=0.65; week 8: 1.66 vs. 1.15 log U/mL, p=0.05), At week 12, 24, 60 and 72 however, HBcrAg decline was stronger in patients with response than in patients without response at week 72. In comparison, decline of HBV DNA differed significantly between patients with and without week 72 response already from week 4 onward (week 4: -1.9 vs. -1.4 IU/mL, p=0.03), and qHBsAg decline differed from week 12 onward (week 12: -0.33



Week	12	24	36	48	60	72
Response	1.90	-2.05	-2.07	-2.03	-2.24	-2.38
No response	-1.29	-1.35	-1.60	-1.63	-1.37	-1.04
p value	0.03	0.03	0.17	0.20	0.01	0.001

Week	12	24	36	48	60	72
Response	-3.18	-4.03	-4.21	-4.30	-3.61	-3.97
No response	-2.30	-2.78	-2.95	-2.99	-1.35	-0.84
p value	0.005	0.002	0.005	0.003	<0.001	<0.001

Week	12	24	36	48	60	72
Response	-0.33	-0.87	-1.26	-1,256	-1,498	-1,381
No response	-0.05	-0.15	-0.23	-0.22	-0.34	-0.24
p value	0.02	<0.001	<0.001	<0.001	<0.001	<0.001

Figure 2. HBcrAg, HBV DNA, and qHBsAg decline according to response at week 72.
This figure displays serum marker declines compared to baseline levels. HBcrAg decline is expressed as mean log U/mL, HBV DNA decline is expressed as mean log IU/mL, and quantitative HBsAg (qHBsAg) decline is expressed as mean log IU/mL. Error bars represent standard errors of mean. Declines are stratisfied according response (HBV DNA <2,000 IU/mL and normal ALT) at week 72, with continuous lines representing patients with response, and dotted lines representing patients without response.

vs. -0.05 IU/mL, *p*=0.02). In all patients with response at week 72, HBcrAg had declined from baseline (Figure 3). For early identification of non-responders at week 72, the diagnostic accuracy based on HBV DNA and qHBsAg decline at week 12 (Supplementary table 1; area under the curve [AUC] 0.742, CI-95% [0.629-0.855], *p*<0.001) remained comparable when adding week 12 or week 24 HBcrAg decline (AUC 0.747, CI-95% [0.629-0.855]; AUC 0.747, CI-95% [0.634-0.859]; both *p*<0.001), or replacing week 12 HBV DNA decline by week 12 HBcrAg decline (AUC 0.754, CI-95% [0.641-0.867], *p*<0.001).

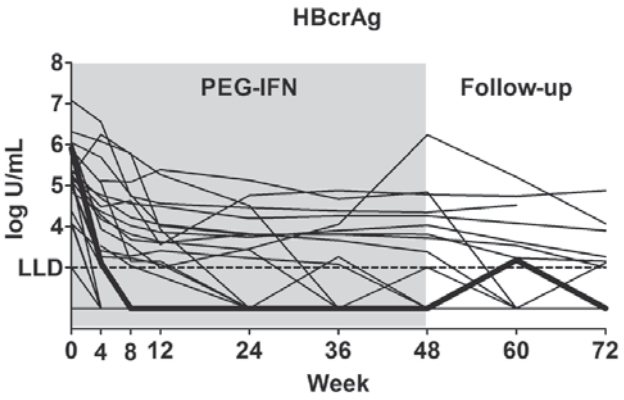


Figure 3. Individual serum HBcrAg dynamics in patients with response at week 72 (n=25).
Lines represent individual HBcrAg dynamics in the 25 patients who achieved response (HBV DNA <2,000 IU/mL and normal ALT) at week 72. HBcrAg is expressed as log U/mL. For results below the lower limit of quantification, the value of 100 U/mL (2 log U/mL) was used. The bold line represents the patient with negative HBsAg from week 60 onward, which was a 24-year-old female infected with genotype D.

End-of-treatment HBcrAg levels and relapse prediction

At week 48, the proportion of patients with undetectable HBcrAg was 60% vs. 44% (*p*=0.18) for patients with vs. without response at week 72, and 80% vs. 39% for patients with vs. without response at LTFU (*p*=0.008). Within the subgroup of patients who had response at week 48 (n=51), no significant association of week 48 levels with relapse at week 72 could be demonstrated for HBcrAg (OR 2.5, CI-95% [0.7-6.8], *p*=0.16). In comparison, qHBsAg levels at week 48 were associated with relapse at week 72 (OR 1.8, CI-95% [1.1-3.0], *p*=0.02). This was similar for relapse at LTFU (week 48 HBcrAg: OR 2.0, CI-95% [0.6-7.0], *p*=0.27; week 48 qHBsAg: OR 3.1, CI-95% [1.2-8.1], *p*=0.02).

Off-treatment HBcrAg levels

Between week 48 and week 72, the overall mean of HBcrAg additionally declined -0.33 log U/mL (*p*=0.02), whereas qHBsAg remained stable (-0.05 IU/mL, *p*=0.34). HBcrAg and qHBsAg dynamics from week 48-72 were not significantly associated with relapse (OR 0.3, CI-95% [0.07-1.1], *p*=0.07). At

week 72, HBcrAg was undetectable in 70% of patients with response vs. in 30% of patients without response (p=0.001), and mean HBcrAg levels were lower in patients with response compared to patients without response (2.5 vs. 4.0 log U/mL, p<0.001). In 36/51 patients who had a response at week 48 LTFU data regarding response was available. Ten of these 36 patients sustained response through LTFU. Neither the degree of HBcrAg decline from week 48 to 60, nor from week 48 to week 72 could identify patients with relapse at LTFU (p=0.18 and p=0.14).

HBcrAg levels during and after treatment in patients with HBsAg loss

HBsAg became negative in 1 patient at week 60, and in 4 patients during LTFU. HBcrAg at baseline in these 5 patients ranged from undetectable to 6.32 log U/mL. Figure 4 shows individual dynamics of HBcrAg, HBV DNA and qHBsAg

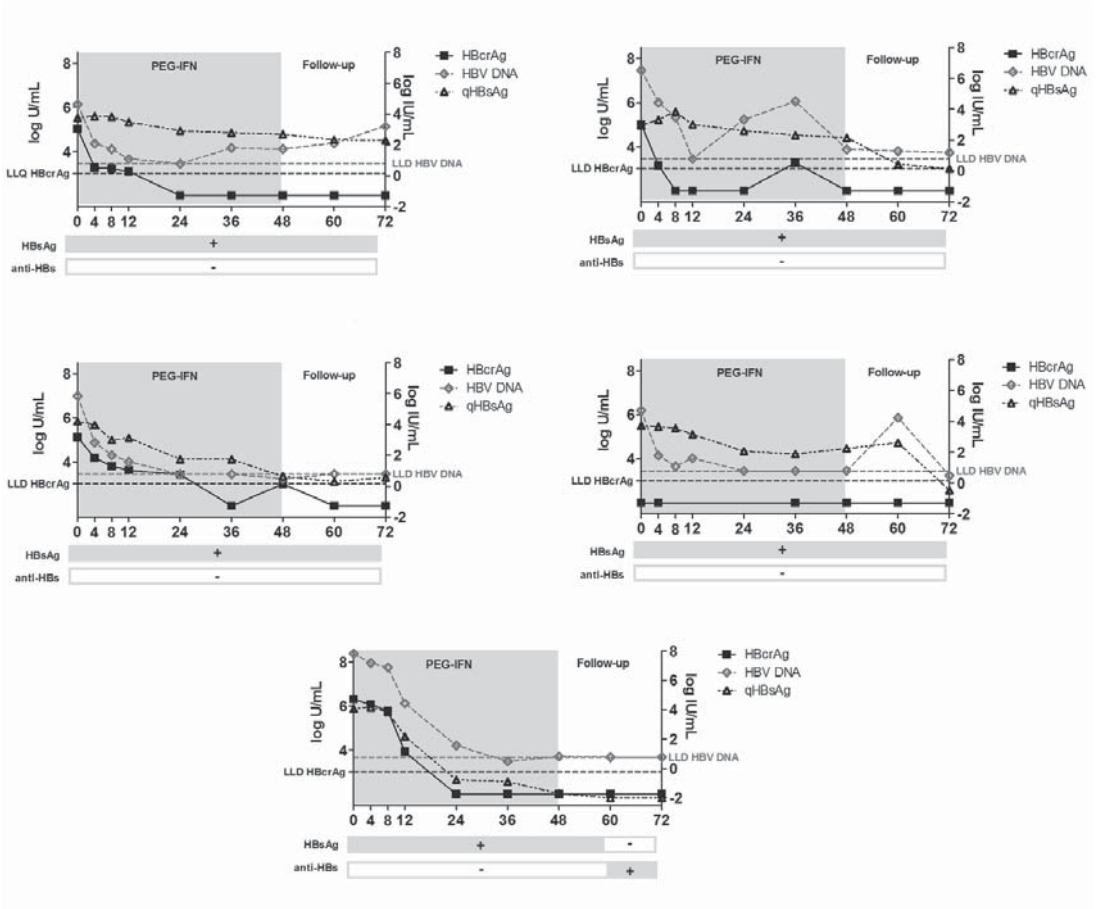


Figure 4. Individual marker dynamics in patients with HBsAg loss at long-term follow-up
Each panel represents an individual patient with HBsAg loss at LTFU. Dynamics of HBcrAg, HBV DNA and qHBsAg are shown. In all 5 patients, HBcrAg was the first virological marker to become undetectable. For HBcrAg results below the lower limit of quantification (1000 U/mL, 3 log U/mL), the value of 100 U/mL (2 log U/mL) was used.
Supplementary tables

during treatment and 24 weeks after treatment. In all 5 patients, HBcrAg became undetectable prior to HBV DNA and qHBsAg negativity.

DISCUSSION

HBcrAg is a serum marker that measures 3 precore/core HBV proteins. Whether it can be used in clinical practice of HBV treatment is largely unknown. We measured serum HBcrAg levels during and after PEG-IFN treatment in 133 patients with HBeAg-negative chronic hepatitis B who were previously treated within a multicentre randomised controlled trial. [94] HBcrAg levels strongly correlated with HBV DNA levels during and after treatment. Although the degree of HBcrAg decline was larger in patients who achieved a sustained off-treatment response than in patients who did not, the difference in HBV DNA decline appeared earlier during the treatment course than the difference in HBcrAg. For the prediction of combined response, relapse and nonresponse, we could not demonstrate superiority of either absolute levels or decline of HBcrAg over serum HBV DNA or qHBsAg.

The findings regarding clinical applicability of HBcrAg for PEG-IFN treatment are comparable to the findings we published for HBeAg-positive CHB. In HBeAg-positive CHB, HBcrAg was also associated with response but was not superior to qHBsAg and HBV DNA in response prediction. [93] In the current study, a previously described and validated stopping rule based on HBV DNA and qHBsAg decline at week 12 could not be improved by adding HBcrAg or by replacing qHBsAg or HBV DNA by HBcrAg. [39] This will also be related to the fact that HBV DNA is part of the endpoint definition of combined response, which logically makes HBV DNA one of the strongest predictors for therapy response. Our results are in line with a recent single-centre study of 62 HBeAg-negative patients focussing on baseline laboratory parameters, in which HBcrAg could also not improve prediction of response using qHBsAg. [96]

In 70% of patients with treatment response HBcrAg levels became undetectable, but this was also the case in 30% of patients without a response. In addition, we were not able to demonstrate that end-of-treatment and post-treatment HBcrAg levels predict relapse, which is in contrast to other studies in which HBcrAg levels predicted relapse after nucleos(t)ide analogue cessation. [86, 89-91] This may not only be explained by the difference in treatment modality and definitions of relapse but also by the limited number of patients that we could assess for

this endpoint as well as by differences in HBeAg and HBV genotype status. In addition, it was previously reported that the correlation of HBcrAg with cccDNA is weaker in HBeAg negative CHB than in HBeAg positive CHB. [74]

We found a strong correlation of HBcrAg levels with HBV DNA, a weak correlation with qHBsAg, and a less apparent on-treatment decline of HBcrAg levels compared to HBV DNA levels, which all corresponds to results that have been described earlier for natural history cohorts and during NA treatment. [75, 85, 86, 88-91, 97-101] The weak correlation between HBcrAg and qHBsAg may be explained by the recent observation in chimpanzees that qHBsAg in HBeAg negative CHB is predominantly produced by HBV DNA integrated in the host genome, in contrast to HBcrAg which is a likely product of the cccDNA. [102] One could therefore hypothesize that HBcrAg would be a better marker of treatment response in HBeAg-negative patients, but we were not able to demonstrate this for PEG-IFN treatment in this Caucasian population. Further, as HBV DNA replication and HBV precore/core protein synthesis are separate processes in the hepatocyte nucleus, the different kinetics of serum HBV DNA and HBcrAg decline might be explained by a difference in the degree of IFN-induced inhibition of these distinct pathways. [103] However, to our knowledge specific effects of IFN on certain transcriptional or translational pathways have not been described. It was hypothesized earlier that HBcrAg would not reflect HBV DNA levels if only precore mutant virus is present, but in our study and in a previous Japanese study, the correlation between HBV DNA and HBcrAg remained strong. [99] Thus, HBcAg may well be the major component of HBcrAg, since it correlates with HBV DNA, while HBeAg and p22cr cannot be synthesized in presence of precore mutations. This is in line with the important role that HBcAg plays in HBV replication. [104]

Our study is unique because it describes HBcrAg levels during PEG-IFN treatment for HBeAg-negative chronic hepatitis B infection in a large and well-defined group of mainly Caucasian patients of whom 19 percent had developed disease remission or functional cure after 24-weeks of off-treatment follow-up. Although recently a Thai study reported that HBcrAg levels in patients infected with HBV genotypes B and C were able to predict virological response when combined with HBsAg levels, the predictive value of HBcrAg was not compared to the predictive value of HBV DNA [105]. Future studies will therefore still have to elucidate whether our results can be extrapolated to Asian patients with HBV genotype B and C, and whether the limited clinical value also applies when response is assessed at a later stage during off-treatment follow-up.

In conclusion, during one year of PEG-IFN therapy a mean decline in HBcrAg of 3.3 log U/mL was reached in this population of Caucasian HBeAg-negative CHB patients. Although decline of HBcrAg during PEG-IFN treatment in HBeAg-negative CHB was stronger in patients with treatment response than in patients without, HBcrAg did not add clinical value to the current response prediction strategies that are based on monitoring HBV DNA and qHBsAg early on-treatment. Additional studies should be performed in genotype B and C infected patients.

SUPPLEMENTARY TABLES

Supplementary table 1. HBcrAg in comparison to other serum markers in the early on-treatment prediction of response

Model	AUC	CI-95%	p-value	p-value for comparison to reference model
HBsAg decline week 12 HBV DNA decline week 12 *(PARC stopping rule)*	0.742	0.629-0.855	p<0.001	reference
HBsAg decline week 12 HBV DNA decline week 12 HBcrAg decline week 12	0.747	0.629-0.855	p<0.001	0.460
HBsAg decline week 12 HBV DNA decline week 12 HBcrAg decline week 24	0.747	0.634-0.859	p<0.001	0.919
HBsAg decline week 12 HBcrAg decline week 12	0.754	0.641-0.867	p<0.001	0.786

AUC, area under the curve; CI-95%, 95% confidence interval alanine aminotransferase; HBcrAg, hepatitis B core-related antigen; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen

5

Host and viral factors associated with serum hepatitis B virus RNA levels among patients in need for treatment

Margo J.H. van Campenhout[1*], Florian van Bömmel[2*], Maria Pfefferkorn[2], Janett Fischer[2], Danilo Deichsel[2], André Boonstra[1], Anneke J. van Vuuren[1], Thomas Berg[2], Bettina E. Hansen[1,3,4], and Harry L.A. Janssen[1,4]

[1]*Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands;* [2]*University Hospital Leipzig, Department of Gastroenterology and Rheumatology, Section of Hepatology, Leipzig, Germany;* [3]*Institute of Health Policy, Management and Evaluation, University of Toronto;* [4]*Toronto Center for Liver Disease, Toronto Western and General Hospital, University Health Network, Toronto, Canada*

* Margo J.H. van Campenhout and Florian van Bömmel contributed equally to this manuscript.

Hepatology, 2018

ABSTRACT

Background & aims. Hepatitis B virus (HBV) RNA in serum is a novel biomarker for intrahepatic HBV replication and treatment response. For its proper use it is essential to identify factors influencing serum HBV RNA level.

Methods. Using a RACE PCR technique (lower limit of detection 800 copies/mL [c/mL]), serum HBV RNA levels were measured in samples of 488 untreated individuals with chronic HBV infection who were eligible to treatment according to currently used recommendations. We explored the association of serum levels of HBV RNA with patient and virus associated factors.

Results. HBV genotype distribution was 21/10/20/46/3% for A/B/C/D/other. Mean HBV RNA serum level was 5.9 (1.6) $\log_{10}$ c/mL (HBeAg-positive CHB: 6.5 [1.2] log c/mL, HBeAg-negative CHB: 4.1 [1.2] log c/mL; $p<0.001$). By multivariable linear regression, factors associated with lower HBV RNA level were HBeAg-negativity (β=-0.69, $p<0.001$), HBV genotypes A (β=-0.13, $p=0.002$), B (β=-0.07, $p=0.049$) and C (β=-0.61, $p<0.001$) in comparison to D, and presence of HBV basal core promoter mutation either alone (β=-0.14, $p=0.001$) or in combination with precore mutation (β=-0.22, $p<0.001$). Higher serum ALT was associated with higher HBV RNA (β=0.23, $p<0.001$). HBV RNA correlated strongly with HBV DNA (HBeAg-pos: r=0.72, $p<0.001$; HBeAg-neg: r=0.78, $p<0.001$), and moderately with qHBsAg (HBeAg-pos: r=0.54, $p<0.001$; HBeAg-neg: r=0.19, $p=0.04$) and qHBeAg (r=0.41, $p<0.001$).

Conclusion. In this multi-ethnic cohort of 488 untreated individuals with chronic hepatitis B, factors associated with serum HBV RNA level were HBeAg status, serum ALT, HBV genotype, and presence of basal core promotor mutations. For the future use of serum HBV RNA as a clinical marker it seems mandatory to take these factors in consideration.

INTRODUCTION

Currently available treatment strategies for chronic hepatitis B virus (HBV) infection can inhibit viral replication to a great extent as reflected by suppression of HBV DNA in serum. However loss of HBsAg, which is regarded as functional cure, is achieved in only a minority of patients, and treatment does not eliminate the intrahepatic covalently closed circular DNA (cccDNA), which is the main transcriptional template of the HBV. Also, HBV genome that is integrated in the host genome remains largely unaffected. Currently investigated therapeutic agents may have stronger effect on cccDNA and are potentially more effective, and therefore, serum markers which reflect the cccDNA activity are needed. These markers may allow a reliable prediction of response, ideally before treatment initiation or early during treatment, and can also help to individualize the use of already available treatments[42].

HBV RNA in serum could fulfill criteria of such a biomarker, as the 4 major types of HBV RNAs are direct transcriptional products of the cccDNA. HBV RNA can be detected in serum and can be quantified by using PCR techniques [106]. The nature of HBV RNA in serum has not fully been researched, but it most likely includes the 3.5 kb pregenomic RNA (pgRNA), which is the template for reverse transcription to HBV DNA and for translation of core protein and polymerase. It is probably released from infected hepatocytes in virion-like capsids [46, 107, 108].

The potential of serum HBV RNA as a baseline marker for treatment response was recently demonstrated by studies revealing its strong correlation with cccDNA activity on the one hand and its response to currently available treatments on the other hand. Thus, before and during treatment with nucleos(t)ide analogue (NA) or pegylated interferon alfa (PEG-IFN), a correlation between serum HBV RNA and the transcriptional activity of cccDNA was demonstrated[47]. In addition, serum HBV RNA levels were found to be associated with the probability of response to treatment with the either NAs or PEG-IFN [45, 46, 106, 109, 110]. However, prior to the further development of serum HBV RNA as a response-predicting marker it is mandatory to evaluate which factors which may influence HBV RNA levels in individuals who are eligible for treatment. To study this, we measured serum HBV RNA level in a large multi-ethnic population of well-characterized individuals with chronic HBV infection, who are in need for treatment according to recent guidelines [56]. We assessed the association with between HBV RNA level and host and viral factors, including levels of other biomarkers.

PATIENTS AND METHODS

Study population.

Full-length poly-adenylated HBV RNA levels were measured in available baseline serum samples of 441 HBeAg-positive and 133 HBeAg-negative CHB patients who participated in 3 previously conducted global randomized controlled trials (99-01 study, ARES study, PARC study) and had not received antiviral treatment in the preceding 6 months [30, 36, 94]. Specific inclusion and exclusion criteria have been described earlier. Serum samples were stored in -20 or -80 degrees Celsius since the original studies. Serum samples were available for 366 HBeAg-positive patients and 122 HBeAg-negative patients. We specifically chose to combine these three cohorts in order to create a large population and to ensure a better generalizability of HBV RNA level in serum as a biomarker for patients with different characteristics.

Endpoints

We explored factors associated with serum level of HBV RNA, and the correlation of HBV RNA to other serum markers.

Serum HBV RNA quantification

HBV RNA was quantified from serum samples using a rapid amplification of complimentary DNA (cDNA)-ends (RACE)-based real-time polymerase chain reaction (PCR) technique that has been previously described. Quantification of polyadenylated HBV RNA was performed using specific primers (including HBV RNA RT primer 5'-acc acg cta tcg cta ctc ac (dT17)gwa gct c) designed according to van Bömmel et al [45]. For the current study the assay's lower limit of detection (LOD) for HBV RNA was 800 (2.9 $\log_{10}$) copies/mL (c/mL), with a corresponding linear range of 800 to 10^6 c/mL. HBV RNA levels measured below the LOD were set to 450 c/mL prior to statistical analysis.

Other laboratory measurements

Routine biochemical and hematological tests were performed locally. Serum ALT levels were standardized by calculating the value times for the ULN per centre and gender. Virological tests were performed at one central laboratory (Erasmus Medical Center, Rotterdam, The Netherlands). HBV DNA was measured using TaqMan-based PCR assays (Roche Diagnostics, Basel, Switzerland; lower limit of detection of 400 copies/mL, 35 copies/mL, or 20 IU/mL depending on the study cohort). HBV DNA results in copies/mL were converted into IU/mL using a conversion factor of 5.8 copies per IU. The comparabiltiy of the assays was as-

sessed previously (Bland-Altman test, r=0.12, *p*=0.49*)* [23]. Serum qHBsAg levels were measured using the Architect HBsAg assay (Abbott Laboratories; range 0.05-250 IU/mL) or the Elecsys Assay (Roche Diagnostics, Basel, Switzerland, lower limit of detection 0.05 IU/mL). Serum HBeAg levels were quantified using the Cobas Elecsys HBeAg assay (Roche Diagnostics, Basel, Switzerland, measurement range 0.2 – 100 IU/mL). HBV genotype analysis was performed using the INNO-LiPA HBV genotype assay (Fujirebio Europe, Ghent, Belgium). The presence of PC and BCP mutants was assessed using the INNO-LiPA HBV PreCore assay (Fujirebio Belgium, Ghent, Belgium), which detects precore (PC) mutations at nucleotide position 1896 and basal core promoter (BCP) mutations at nucleotide positions 1762 and 1764. Results were classified into 4 groups: 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 PC + BCP when both types of mutants were found.

Statistical analysis

SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analyses. Skewed laboratory values were log-transformed prior to analyses and were expressed as mean (standard deviation [SD]). After log transformation, HBV RNA level showed a near to normal distribution in both the HBeAg positive population (skewness: -0.59 [SE 0.13]; kurtosis: 0.11 [SE 0.25]), and the HBeAg negative population (skewness: 0.29 [SE 0.24]; kurtosis: 0.03 [SE 0.47]). Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. Subgroup analysis of mean HBV RNA levels was performed using ANOVA with Bonferroni correction for intergroup comparison. We performed linear regression analysis to determine factors associated with HBV RNA level. R^2 was calculated as a measure of goodness-of-fit for the linear model. For linear correlations Pearson correlation coefficients (r) were provided, and for skewed distributions the Spearman rank coefficients (ρ) were provided. Logistic regression analysis was performed to determine factors associated with HBV RNA level below LOD. Genotypes other than A, B, C and D were not analyzed in linear regression. All analyses were performed two-sided at the 0.05 level of significance.

RESULTS

Study cohort

Study population characteristics are shown in Table 1.The mean age was 33 (SD 11) years for HBeAg-positive patients and 42 (SD 11) for HBeAg-negative pa-

Table 1. Patient characteristics

Characteristics	All patients n=488	HBeAg-pos n=366	HBeAg-neg n=122	*p*-value
Demography				
Age, years	35 (12)	33 (11)	42 (11)	<0.001
Male, n (%)	362 (74)	274 (74)	88 (72)	0.55
Race, n (%)				<0.001
Caucasian	313 (64)	197 (54)	122 (95)	
Asian	153 (31)	149 (41)	4 (3)	
Other	22 (5)	20 (5)	2 (2)	
HBV genotype, n (%)				<0.001
A	91 (19)	75 (21)	16 (13)	
B	50 (10)	49 (13)	1 (1)	
C	113 (23)	110 (30)	3 (2)	
D	222 (46)	125 (34)	97 (80)	
Other	12 (2)	11 (2)	5 (4)	
INNO-LiPA result, n (%)				<0.001
Wildtype virus	81 (17)	75 (21)	6 (5)	
PC mutation	52 (11)	38 (11)	14 (11)	
BCP mutation	102 (21)	78 (21)	24 (20)	
PC & BCP mutation	203 (42)	125 (34)	78 (64)	
Histology				
Cirrhosis, n (%)	31 (6)	27 (7)	4 (3)	0.13
Treatment history				
Previous NA	58 (12)	36 (10)	22 (18)	0.02
Previous (PEG-)IFN	86 (18)	64 (18)	22 (18)	0.89
Laboratory results				
HBV RNA §	5.9 (1.6)	6.5 (1.2)	4.1 (1.2)	<0.001
HBV RNA undetectable, n(%)	30 (6)	2 (0.5)	28 (23)	<0.001
HBV DNA ‡	7.5 (1.4)	8.1 (1.1)	6.0 (1.2)	<0.001
qHBsAg ‡	4.2 (0.7)	4.3 (0.7)	3.8 (0.6)	<0.001
qHBeAg ‡	n.a.	2.4 (0.9)	n.a.	n.a.
ALT (x ULN) †	3.7 (3.2)	3.8 (3.4)	3.2 (2.5)	0.07

ALT, alanine aminotransferase; BCP, basal core promoter; HBV, hepatitis B virus; NA, nucleos(t)ide analogue; (PEG-) IFN, (peg)interferon; PC, precore; qHBeAg, quantitative hepatitis B e antigen; qHBsAg, quantitative hepatitis B surface antigen; SD, standard deviation; ULN, upper limit of normal.

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

† Multiples of upper limit of the normal range

‡ Logarithmic scale, IU/mL

§ Logarithmic scale, c/mL

tients (p<0.001), and the majority of patients were male (n=362, 74%). Patients were mostly of Caucasian (n=313, 64%) or Asian (n=153, 31%) origin. All main HBV genotypes were represented. Mean serum HBV DNA levels were 8.1 (1.1) log IU/mL for HBeAg-positive patients and 6.0 (1.2) for HBeAg-negative patients (p<0.001), and mean serum quantitative HBsAg (qHBsAg) levels were 4.3 (0.7) log IU/mL vs. 3.8 (0.6) IU/mL (p<0.001), respectively.

Serum HBV RNA level in relation to viral and clinical characteristics.

The mean HBV RNA level was 5.9 (1.6) c/mL, and differed by HBeAg status (HBeAg-positive CHB: 6.5 [1.2] log c/mL, HBeAg-negative CHB: 4.1 [1.2] log c/mL; p<0.001; Figure 1a). HBV RNA levels were below LLD in 2 (0.5%) HBeAg-positive patients and in 28 (23%) HBeAg-negative patients (p<0.001). In these 30 patients, mean HBV DNA level was 4.7 (SD 0.7) log IU/mL. HBV RNA levels were associated with the degree of ALT elevation (r=0.29, p<0.001; Figure 1b), and HBV RNA level varied by HBV genotype (p<0.001), with mean unadjusted HBV RNA levels of 6.0 / 6.5 / 6.0 / 5.6 log c/mL for genotypes A/B/C/D (p=0.003, Figure 2a). In patients infected with wildtype HBV, highest mean unadjusted HBV RNA levels were found (6.7 log c/mL), and presence of BCP mutation either alone or in combination with PC mutation led to lower HBV RNA levels (Figure 2b, 5.5 log and 5.3 log c/mL, both p<0.001 in comparison to wildtype HBV). HBV RNA level did not differ by sex (p=0.26), presence of cirrhosis (p=0.55), or treatment history (previous IFN: p=0.96, previous NA: 0.38), but did negatively correlate with age (r=-0.23, p<0.001).

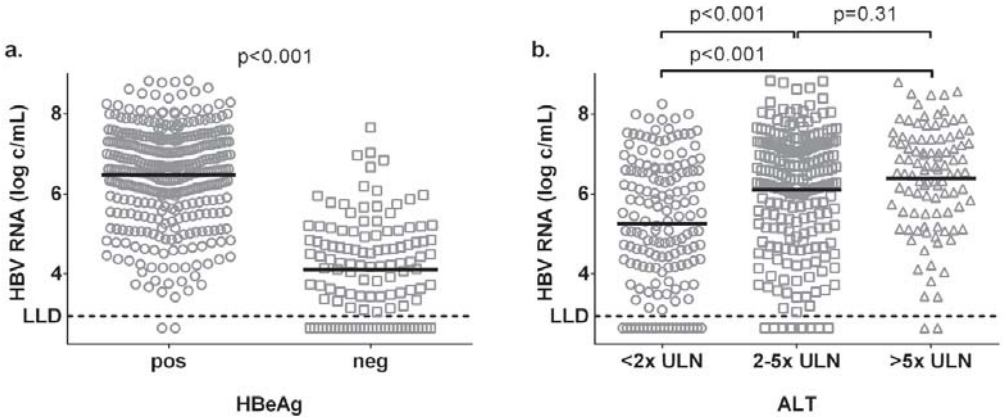


Figure 1. HBV RNA level according to HBeAg status and ALT level

Dots represent individual HBV RNA measurements, with lines representing the unadjusted mean level of HBV RNA (log c/mL) according to HBeAg status (**a**) and serum ALT level (**b**) expressed in times the upper limit of normal (xULN). The lower limit of detection (LLD) for the HBV RNA PCR is 800 c/mL (2.90 log c/mL).

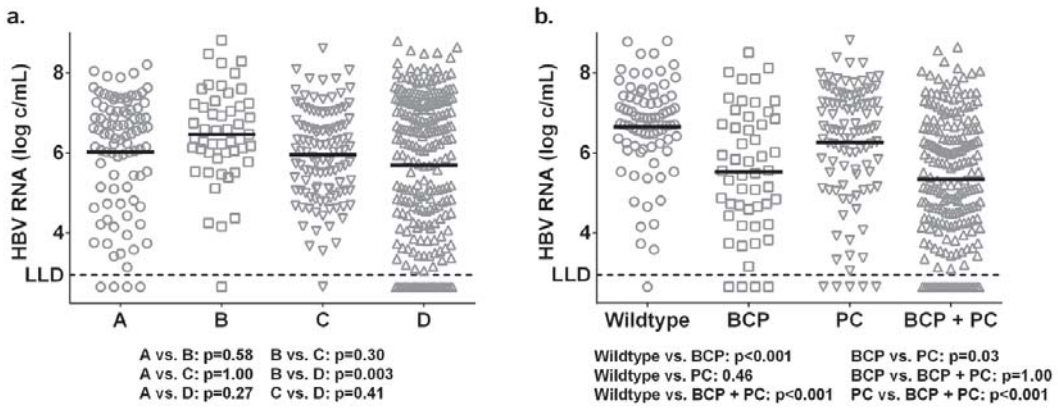


Figure 2. HBV RNA level according to HBV genotype and presence of precore and basal core promoter mutations

Dots represent individual HBV RNA measurements, with lines representing the unadjusted mean level of HBV RNA (log c/mL) according to HBV genotype (**a)** and presence of precore (PC) and basal core promoter (BCP) mutations (**b)**. The lower limit of detection (LLD) for the HBV RNA PCR is 800 c/mL (2.90 log c/mL).

Regression analysis of factors associated with HBV RNA level

By univariable linear regression, factors associated with HBV RNA level were age, HBeAg status, HBV genotype and presence of any PC or BCP mutation (Table 2). By multivariable linear regression, factors associated with lower HBV RNA level were HBeAg-negative CHB (β=–0.69, p<0.001), HBV genotypes A, B and C in comparison to genotype D (genotype A: β=-0.13, p=0.002; genotype B: β=-0.07, p=0.049 ; genotype C: β=-0.61, p<0.001), presence of any BCP mutation either alone (β=-0.14, p=0.001) or in combination with PC mutation (β=-0.22, p<0.001). Higher serum ALT was associated with higher HBV RNA level (β=0.23, p<0.001). This particular linear model explains 56.2% of the variance of the HBV RNA level (r^2 = 0.562), which diminishes to 25.3% if HBeAg is excluded from the model. When studying the factors associated with HBV RNA level below LLD using logistic regression analysis, the only factors independently associated with HBV RNA level below LLD were HBeAg-negative CHB (OR 86 CI-95% [9.1-811], p<0.001) and ALT (OR 0.07 CI-95% [0.01-0.40], p<0.001). As HBeAg was negative in 28/30 patients with HBV RNA below LLD, analysis was repeated in HBeAg-negative patients, which did not result in any additional factors independently associated with HBV RNA below LLD.

Table 2. Univariable and multivariable linear regression analysis of factors associated with serum HBV RNA level

	Univariable			Multivariable (Full model)			
	B	**CI-95%**	***p*-value**	***B***	**CI-95%**	***β***	***p*-value**
Age, years	-0.03	-0.04 ; -0.02	<0.001	0.003	-0.007 ; 0.01	0.02	0.59
Sex			0.26				
Male	*reference*						
Female	-0.19	-0.51 ; 0.14	0.26				
HBeAg			<0.001				<0.001
Pos	*reference*			*reference*			
Neg	-2.37	-2.62 ; -2.13	<0.001	-2.46	-2.74 ; -2.18	-0.69	<0.001
HBV Genotype			0.003				<0.001
A	0.39	0.01 ; 0.76	0.04	-0.53	-0.86 ; -0.19	-0.13	0.002
B	0.84	0.36 ; 1.31	0.001	-0.36	-0.72 ; -0.001	-0.07	0.049
C	0.32	-0.03 '; 0.67	0.07	-0.61	-0.91 ; -0.32	-0.16	<0.001
D	*reference*			*reference*			
PC/BCP variants			<0.001				<0.001
Wildtype HBV	*reference*			*reference*			
PC mutation	-0.38	-1.68 ; -0.90	<0.001	-0.19	-0.56 ; 0.18	-0.05	0.44
BCP mutation	-1.12	-1.66 ; -0.59	<0.001	-0.70	-1.09 ; -0.30	-0.14	0.001
PC & BCP mutation	-1.29	-0.82 ; 0.07	0.10	-0.68	-1.02 ; 0.35	-0.22	<0.001
ALT (log xULN)	1.46	1.02 ; 1.90	<0.001	1.18	0.85 ; 1.51	0.23	<0.001

BCP, basal core promoter; HBV, hepatitis B virus; CI-95%, 95% confidence interval; PC, precore; qHBeAg, quantitative hepatitis B e antigen. B, unstandardized regression coefficient; β, standardized regression coefficient.
Patients with genotype other than one of the main genotypes genotypes A, B, C, D were excluded for this analysis. When a significance level of $p<0.20$ was reached In univariable analysis, the factor was assessed in multivariable analysis. No interactions were found between HBeAg status and genotype ($p=0.14$), HBeAg status and INNO-LiPA result ($p=0.81$), genotype and INNO-LiPA result ($p=0.12$), HBeAg status and ALT ($p=0.19$), genotype and ALT ($p=0.64$), or INNO-LiPA result and ALT ($p=0.42$).

Serum HBV RNA level in relation to serum HBV DNA, qHBsAg and qHBeAg

Overall, HBV RNA correlated highly with HBV DNA (r=0.85 / ρ=0.84, $p<0.001$), moderately with qHBsAg (r=0.52 / ρ=0.60, $p<0.001$), and with qHBeAg (r=0.41 / ρ=0.34, $p<0.001$). Because HBeAg status is the strongest factor independently associated with HBV RNA level in linear regression, we also studied correlations of HBV RNA to other markers in HBeAg-positive and HBeAg-negative patients separately.

In HBeAg-positive patients, levels of HBV RNA correlated strongly correlated with HBV DNA (r=0.72, $p<0.001$), and moderately correlated with qHBsAg

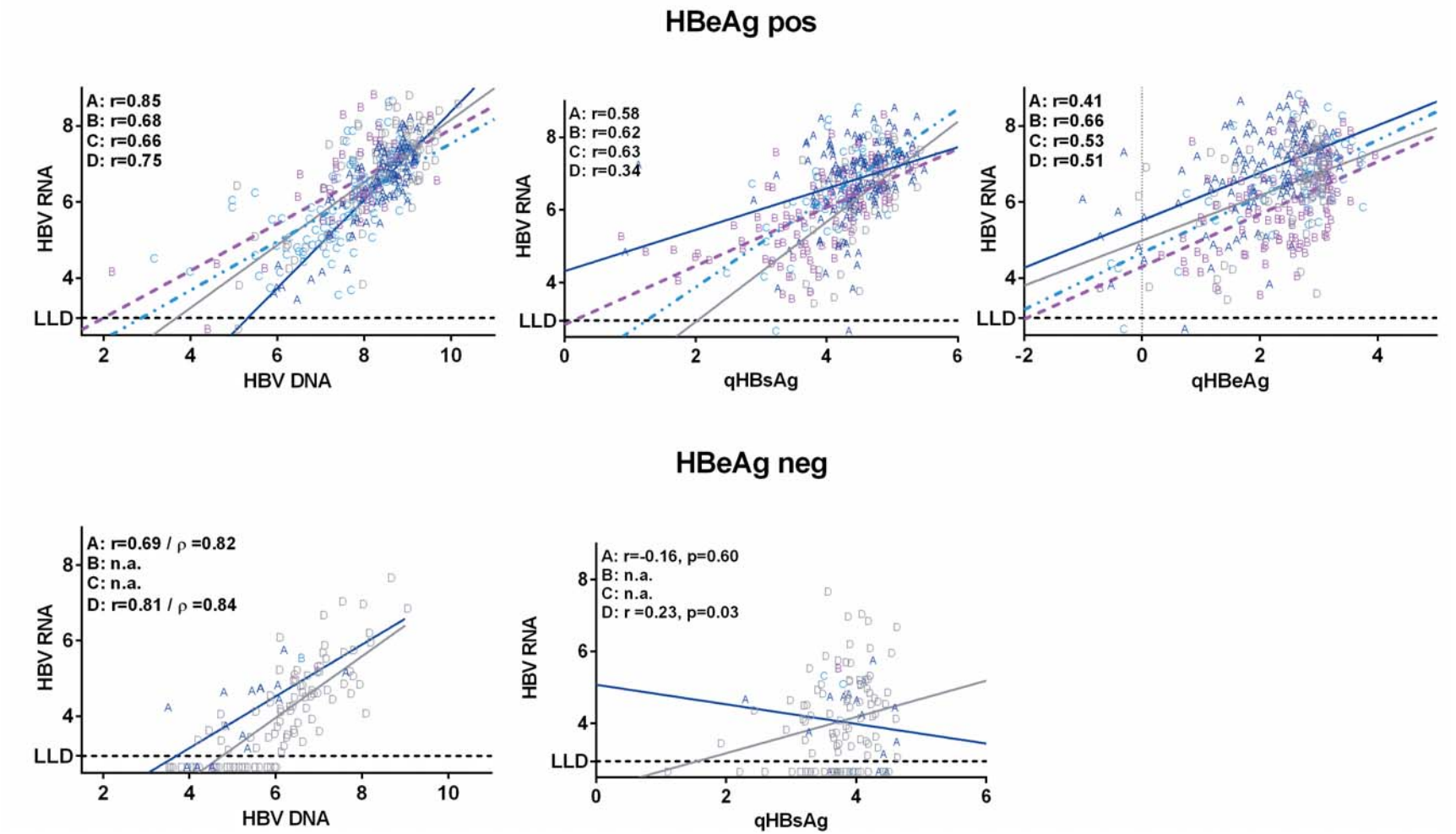


Figure 3. Correlations of HBV RNA with serum levels of HBV DNA, qHBsAg and qHBeAg

Correlations of serum levels of HBV RNA (log c/mL) with serum levels of HBV DNA, qHBsAg and qHBeAg (log IU/mL). Letters represent individual serum marker measurements according to HBV genotype. For linear correlations Pearson correlation coefficients (r) were provided, and for skewed distributions the Spearman rank coefficients (ρ) were provided. The lower limit of detection (LLD) for the HBV RNA PCR is 800 c/mL (2.90 log c/mL).

Supplementary tables

(r=0.54, p<0.001) and qHBeAg (r=0.41, p<0.001). Correlations of HBV RNA to other HBV markers, however, were HBV genotype dependent, with strongest correlation with HBV DNA observed for genotype A, strongest correlation with qHBsAg for genotype B and C, and a weakest correlation with qHBsAg for genotype D (Figure 3).

In HBeAg-negative patients, overall correlation of HBV RNA to HBV DNA was comparable to HBeAg-positive CHB (r=0.78 / ρ=0.80, p<0.001). Unlike in HBeAg-positive CHB, the overall correlation of HBV RNA to qHBsAg was weak (r=0.19, p=0.04; ρ=0.17, p=0.07). Genotype-specific correlations of HBV RNA levels could only be determined for HBV genotypes A and D. The correlation of HBV RNA with HBV DNA was comparable for these genotypes (genotype A: r=0.69 / ρ=0.82, p=0.003; genotype D: r=0.81 / ρ=0.84, p<0.001), but the correlation of HBV DNA with qHBsAg was genotype-dependent, indicated by a weak correlation within genotype D infected patients (r=0.23 / ρ=0.22, p=0.03), but no significant correlation within genotype A infected patients (r=-0.16 / ρ=-0.14, p=0.60; Figure 3).

DISCUSSION

HBV RNA in serum is a novel and promising marker for cccDNA transcriptional activity and could thus be used to monitor response for currently available treatment, but in particular for future HBV treatments targeting the HBV life cycle. However, its interactions with host factors and viral factors, such as markers of HBV transcription have not yet been systematically studied. In this study we investigate the association of HBV RNA serum levels with currently used HBV markers and patient characteristics in a large multi-ethnic cohort including 441 HBeAg-positive and 133 HBeAg-negative individuals who were candidates for antiviral treatment according to current guidelines [56]. We found that HBV RNA levels are independently associated with HBeAg status, ALT levels, BCP variants and the HBV genotype. Our results indicate that these factors have to be taken in consideration for a correct interpretation of HBV RNA serum levels.

From recent studies there is evidence that the level of HBV RNA in serum reflects cccDNA transcriptional activity [47, 48]. We identified the HBeAg status as the strongest factor associated with serum HBV RNA levels, which is likely an expression of the higher transcriptionally activity of cccDNA in HBeAg-positive patients as compared to HBeAg-negative patients. The mean HBV RNA level was 2.4 $\log_{10}$ lower in HBeAg-negative as compared to HBeAg-positive patients, and the

proportion of patients with undetectable HBV RNA serum level 23% in HBeAg-negative as compared to only 0.5% in HBeAg-positive patients (p<0.001). This is in line with previous findings in smaller patient cohorts [45, 46, 111]. The correlation of HBV RNA with HBV DNA levels was similar in HBeAg-positive and HBeAg-negative individuals. Interestingly, however, the correlation between HBV RNA and HBsAg was moderate in HBeAg-positive (r=0.54) but only weak in HBeAg-negative patients (r=0.19) (Figure 3). A possible explanation for this difference is that in HBeAg-negative patients HBsAg may be derived not only from cccDNA but in a profound number from integrated HBV genome. This hypothesis is supported by data of a recent study assessing the effect of small interfering RNA based inhibition of messenger HBV RNAs, where a significant decrease in HBsAg levels was found in HBeAg-positive but not in HBeAg-negative patients[102]. Applying this hypothesis to our observation could imply that in HBeAg-negative patients HBV RNA and HBsAg are partially derived from different matrices and that HBV RNA (and possibly also HBV DNA) might be a better marker of for remaining cccDNA in HBeAg-negative disease.

Another factor we found to be associated with lower HBV RNA serum levels was the presence of BCP variants. In presence of these variants, either alone or in combination with PC variants, HBV RNA levels were significantly lower than in wildtype virus (p<0.002). We also found lower HBV DNA and HBsAg levels in patients with BCP and/or PC variants (data not shown). Other studies, however, have reported inconclusive results regarding the effect of BCP mutations on HBV replication [112-115]. These heterogenic results may be explained by testing patients with different disease phases, genotype distributions, and frequency of BCP mutants [116]. It is yet unclear which mechanisms would explain lower HBV DNA and HBV RNA level in presence of BCP mutations, but the key may in HBV RNA packaging. Recently, virus-like particles have been proposed as medium for the transport of HBV RNA in serum [107, 117]. Therefore lower HBV RNA levels in patients bearing these variants may be a result of decreased availability of those carriers of HBV RNA instead of a direct effect of BCP mutation on HBV replication [115, 118]. Further studies are required to elucidate how BCP and PC variants truly affect serum HBV RNA levels.

HBV genotype was associated with both absolute levels of HBV RNA and correlations of HBV RNA with other serum markers. In multivariable regression analysis, genotype D was associated with the highest HBV RNA level, followed by genotypes B, A, and C. The HBV genotype mainly influenced the correlation between HBV RNA and qHBsAg in our cohort. This might be of relevance for

the use of serum HBV RNA as a clinical marker. HBV RNA levels were significantly higher in patients with ALT level >2x ULN compared to patients with ALT level <2X ULN, which was similar for HBV DNA and qHBsAg. Age and sex of individuals with chronic HBV infection did not influence HBV RNA serum levels.

In conclusion, our study is the first to show that HBeAg status, serum ALT, HBV genotype, and presence of BCP variants are independently associated with serum HBV RNA level in a multi-ethnic cohort of patients currently considered eligible for treatment. These factors should be taken into consideration for the interpretation and comparison of HBV RNA serum levels across different infected individuals and in the development of HBV RNA as a potential serum marker for cccDNA transcriptional activity. This is in particular relevant for future studies with novel agents aiming targeting the HBV live cycle for functional cure of CHB.

SUPPLEMENTARY TABLE

Supplementary table 1. Univariable and multivariable logistic regression analysis of factors associated with undetectable level of serum HBV RNA (n=34)

	Univariable			Multivariable (Full model)		
	OR	**CI-95%**	***p*-value**	***OR***	**CI-95%**	***p*-value**
Age, years	1.04	1.01 ; 1.06	**0.02**	0.97	0.93 ; 1.01	**0.16**
Sex						
Male	*reference*			*reference*		
Female	1.65	0.79 ; 3.44	**0.18**	1.69	0.66 ; 4.30	**0.59**
HBeAg						
Pos	*reference*			*reference*		
Neg	67.2	15.8 ; 286	**<0.001**	91.1	13.7 ; 606	<0.001
HBV Genotype			**<0.001**			**0.59**
A	0.32	0.11 ; 0.94	0.04	0.55	0.12 ; 2.54	0.45
B	0.14	0.02 ; 1.07	0.06	3.16	0.24 ; 42.1	0.38
C	0.06	0.01 ; 0.46	0.007	0.48	0.04 ; 5.21	0.54
D	*reference*			*reference*		
PC/BCP variants			**0.02**			
Wildtype HBV	*reference*			*reference*		
PC mutation	4.05	0.46 ; 35.4	0.21	1.35	0.10 ; 18.9	0.82
BCP mutation	6.70	0.73 ; 61.7	0.15	3.72	0.27 ; 51.2	0.33
PC & BCP mutation	10.5	1.40 ; 79.0	0.02	3.42	0.30 ; 38.7	0.29
ALT (log xULN)	0.10	0.03 ; 0.35	**<0.001**	0.04	0.01 ; 0.27	**<0.001**

BCP, basal core promoter; HBV, hepatitis B virus; CI-95%, 95% confidence interval; PC, precore; qHBeAg, quantitative hepatitis B e antigen; OR; odds ratio.

Patients with genotype other than one of the main genotypes genotypes A, B, C, D were excluded for this analysis. When a significance level of p<0.20 was reached In univariable analysis, the factor was assessed in multivariable analysis.

Summary and discussion

Treatment of chronic HBV infection leads to functional cure in only a minority of patients. Over the last decades, multiple strategies aiming to optimize the effect of the available therapeutic agents have been examined. To improve response to PEG-IFN monotherapy, response-guided treatment algorithms have developed for both HBeAg-positive and HBeAg-negative chronic hepatitis [38, 39]. For nucleo(s)tide analogue (NA) monotherapy, one of the major hurdles has been to establish finite treatment either by selection of patients whose HBV characteristics are associated with sustained off-treatment response, or by adding PEG-IFN. Many modified treatment strategies combining NA and PEG-IFN have been studied [30, 37, 64, 65, 131, 133, 138]. For all of these studies, biomarkers have played an important role as they allow disease profiling and therefore treatment individualization. The aims of this thesis are to examine whether one of the latest researched combination strategies, the PEG-IFN add-on strategy, contributes to improved response rates, and whether novel serum biomarkers HBcrAg and HBV RNA can further individualize treatment for chronic HBV.

RESPONSE TO PEGINTERFERON ADD-ON THERAPY

In **Chapter 1**, we studied virus and host characteristics associated with response to PEG-IFN add-on therapy. The reason to perform this study was an earlier observation of heterogeneity across different patient groups with regard to the effect of PEG-IFN add-on [37]. We clustered two previously conducted randomized controlled trials studying the effect of PEG-IFN add-on therapy in HBeAg-positive patients. In the ARES trial patients were treated with 24 weeks of entecavir treatment prior to 24 weeks of PEG-IFN initiation, whereas in the PEGON trial, initial NA treatment was given for at least 1 year and the duration of PEG-IFN add-on was 48 weeks [37, 138]. This combination of study populations enabled us to also study the effect of different durations of initial NA treatment and different durations of PEG-IFN add-on on the probability of treatment response. However, the main finding of our study was that neither duration of NA therapy nor duration of PEG-IFN add-on were associated with the probability of response. The factors that were independently associated with treatment response (in this study defined as HBeAg loss in combination with an HBV DNA level <200 IU/mL) were absence of prior PEG-IFN treatment, and lower HBV DNA and HBsAg levels upon PEG-IFN add-on initiation. These findings are in line with the hypothesis that HBV DNA suppression leads to immune restoration, and with earlier observations that lower serum HBsAg level may reflect better immune control and is associated with a higher chance of response to PEG-IFN monotherapy [139]. The observation

that PEG-IFN treatment appears to be less successful after the first course was already described in conventional IFN treatment for HBeAg-positive hepatitis [140]. This may be related to IFN-induced mutations decreasing IFN susceptibility, such as precore or basal core promoter mutations, or polymorphisms of HLA-DP or IL28B [141-143].

Because the study endpoint in Chapter 1 was chosen 24 weeks after discontinuation of PEG-IFN add-on, conclusions on the long-term effect of PEG-IFN therapy could not be drawn. In **Chapter 2** we studied the long-term effect of the PEG-IFN add-on strategy, specifically for the ARES approach that includes 24 weeks of initial entecavir treatment followed by 24 weeks of PEG-IFN add-on. In this study we mainly focused on the additional occurrence of HBeAg loss after the follow-up of the initial ARES study ended and the durability of add-on induced HBsAg decline. Among initial non-responders, HBeAg loss beyond week 96 of follow-up did not differ between patients allocated to PEG-IFN add-on or entecavir monotherapy continuation, suggesting that the gained increase in HBeAg loss by PEG-IFN add-on is mainly achieved in the first 2 years of treatment initiation. Interestingly, the significantly steeper decline in quantitative HBsAg level induced by PEG-IFN that was observed already in the first year was sustained across long-term follow-up. We therefore hypothesize that there may be a role for PEG-IFN add-on when HBeAg loss or a certain HBsAg level would be indicated to start a new therapeutic compound.

SERUM HBCRAG AND HBV RNA LEVELS IN RELATION TO TREATMENT RESPONSE

HBcrAg is a combined biomarker for the simultaneous detection of three proteins coded by the precore/core region of the cccDNA: HBcAg, HBeAg and a 22-kDa precore protein (p22cr) [85]. These proteins share a 149 amino acid sequence to which the labelled antibodies of the CLEIA-based assay are directed. Although it is well known that HBcAg can be found in infectious HBV particles and HBeAg is important for viral replication, the function of p22cr is unknown. HBcrAg was first described in 2002. The correlation coefficients described in literature for HBcrAg with intrahepatic cccDNA vary from 0.66 to 0.70 [74, 88, 144]. In **Chapters 3 & 4**, we studied on-treatment HBcrAg dynamics in patients with HBeAg-positive chronic hepatitis treated with entecavir with or without 24 weeks of PEG-IFN add-on, and in patients with HBeAg-negative patients treated with 48 weeks of PEG-IFN with or without ribavirin.

In the HBeAg-positive add-on study (**Chapter 3**), we found baseline correlations of HBcrAg with other known biomarkers qHBsAg (r=0.4 p<0.001) and HBV DNA (r=0.7, p<0.001) that were comparable to correlations found by others, and Bland-Altman analysis showed close agreement for HBcrAg and qHBeAg measurements. Basal core promoter mutation was associated with lower baseline levels of HBcrAg. On-treatment, we observed only a trend for stronger HBcrAg decline in patients treated with PEG-IFN add-on when compared to entecavir monotherapy. However, HBcrAg decline was HBV genotype dependent. We found that lower HBcrAg was independently associated with response although unfortunately, we could not demonstrated superiority to qHBsAg level. Our findings were replicated in other PEG-IFN based treatment strategies for this is also what an Asian study found [145]. We underline that in our study, we chose an combined endpoint based on HBV DNA level because of the fact that an endpoint based on HBeAg loss only would logically correlate to HBcrAg level because HBeAg is part of the biomarker itself, and that we did not have enough patients with HBsAg loss in this study to examine the relation of HBcrAg with HBsAg loss. In the HBeAg-negative population (**Chapter 4**), correlations between HBcrAg and HBV DNA were comparable to those in the HBeAg-positive populations, but a significant correlation of HBcrAg with qHBsAg was absent qHBsAg (r=0.2, p=0.11). This may be due to HBsAg production from integrated DNA [108]. In line with our observations in HBeAg-positive patients, HBcrAg was associated with treatment response (in this study defined as ALT normalizaton and an HBV DNA level <2000 IU/mL), but again superiority over previously validated decision rules could not be demonstrated. Almost simultaneously to our publication, a French group published comparable results [96]. We therefore recommend for this specific patient category to choose HBV DNA and qHBsAg monitoring rather than HBcrAg monitoring in clinical settings, in particular because these biomarkers have been extensively validated and have been related to solid clinical outcomes, such as cirrhosis and liver cancer .

Based on these studies, we believe that HBcrAg may well be used together with other biomarkers to assess treatment effect, but in our opinion, some crucial aspects of this test have not been fully examined which leads us to be cautious in recommending it as a single biomarker for treatment monitoring. Our most important point of discussion for research involving this new biomarker is that several studies give interpretation to results that are below the analytical sensitivity / lower limit of quantification of 3.0 log U/mL (1000 kU/mL) that is reported by the manufacturer. This may result from the fact that the analyser that is used for testing displays values down to the lower limit of detection of 2

log U/mL (100 U/mL), so below the limit of 3.0 log U/mL. However in the first studies reporting on this biomarker, HBcrAg levels between 2.0 and 3.0 log U/mL were also found in healthy controls and patients with hepatitis C infection [85]. Therefore it seems not appropriate to deviate from the manufacturer's manual in order to retrieve a broader spectrum of HBcrAg results in those with estimated low levels of replication. As to our knowledge no other studies exist that examine the specificity of HBcrAg, it is important to cautiously interpret studies that claim that HBcrAg may be a more sensitive biomarker than HBsAg just because it is also detected in patients who cleared HBsAg.

HBV RNA is a biomarker that can be detected by using RACE-PCR. The method we used for HBV RNA quantification was designed a few years ago [45], but others have designed comparable probes and primers [46, 146]. The nature of HBV RNA has not been fully examined, but several studies suggest that it predominantly concerns encapsidated pregenomic RNA but also comprises a minimum amount (<1%) of precore messenger RNA [46, 107, 146]. Reports regarding correlation of HBV RNA in serum with cccDNA are interesting. It was reported to correlate with quantitative cccDNA level in untreated HBeAg-positive patients (r=0.39) but not in untreated HBeAg-negative patients [47]. However HBV RNA detectability did seem to correlate with cccDNA transcriptional activity, and therefore undetectable HBV RNA may indicate silenced cccDNA. In **Chapters 5, 6, 7 and 8**, we studied HBV RNA levels in untreated patients and different treatment settings for both HBeAg-positive and HBeAg-negative patients.

For every virologic biomarker, it is essential how it relates to different host and viral factors in order to avoid misinterpretation of results. In **Chapter 5**, we examined factors associated with HBV RNA level in a multi-ethnic group of untreated patients with chronic HBV hepatitis. The strongest factor associated with HBV RNA level was HBeAg status, which again emphasizes the influence of HBeAg on viral replication. basal core promoter mutation was independently associated with lower levels of HBV RNA, and HBV RNA level was clearly highest in patients infected with HBV genotype D. We additionally observed a clear positive correlation between HBV RNA level and and ALT level. We took these factors in consideration in our on-treatment studies.

First, we examined HBV RNA dynamics in HBeAg-positive patients treated with PEG-IFN +/- lamivudine in **Chapter 6**. We observed that HBV RNA decline was stronger in combination therapy than in PEG-IFN monotherapy but rebounds to comparable levels after treatment discontinuation, which is a pattern already known for HBV DNA dynamics [30]. Also we observed a trend towards lower HBV RNA levels in patients infected with wildtype virus when compared non-

wildtype virus. Furthermore, we were able to validate the previously suggested HBV RNA level of ≥ 5.5 log c/mL for the prediction of non-response (defined as no HBeAg loss) for HBV genotypes A and C [44]. However we also observed that the specificity of HBV RNA as a marker for HBeAg loss was limited, as HBV RNA decline was also significant in patients without HBeAg loss. This may mean that HBV RNA is a marker of current replication cccDNA activity but not so much for immune control.

We secondly examined HBV RNA levels in patients with HBeAg-negative hepatitis treated with PEG-IFN +/- ribavirin in **Chapter 7**. Based on our prior findings in untreated patients, we expected to see lower HBV RNA levels. Indeed, HBV RNA levels were not only lower at baseline but also became undetectable already early during treatment in the vast majority of patients, regardless of allocation to monotherapy or combination therapy. Improving the sensitivity of the PCR method would therefore be very interesting in these patients. Nevertheless with the current lower limit of detection of 800 c/mL, we could find that an HBV RNA value above 1,500 c/mL (3.2 log c/mL) at week 12 predicts non-response to PEG-IFN treatment. Moreover, adding an HBV RNA cut-off of 3.18 log c/mL to the validated PARC stopping rule (which is based on HBV DNA and qHBsAg monitoring) could lead to early PEG-IFN discontinuation for non-responders who would have continued therapy based on the prior stopping rule alone [39].

Finally in **Chapter 8**, we report the interim results of our study on HBV RNA monitoring in PEG-IFN add-on therapy. Because we already found in **Chapter 1** that host and viral factors can help to select those patients with the highest probability of response to PEG-IFN add-on, we were interested to see if HBV RNA monitoring is useful for this purpose. Additionally the ARES study design allowed us to also study HBV RNA levels in the first weeks of entecavir treatment. After 24 weeks of initial entecavir treatment, PEG-IFN add-on accelerated HBV RNA decline when compared to entecavir monotherapy, and HBV RNA level after 24 weeks of initial entecavir treatment allowed us to select patients in which adding PEG-IFN is expected to double the probability of achieving HBeAg loss. This may provide a valuable tool to individualize treatment.

CONCLUSION AND RECOMMENDATIONS

In order to establish higher response rates to PEG-IFN monotherapy, both serum biomarkers HBV RNA and HBcrAg could be used for early on-treatment identification of non-responders so that exposure to PEG-IFN side effects can be limited for these patients by premature discontinuation of the treatment course.

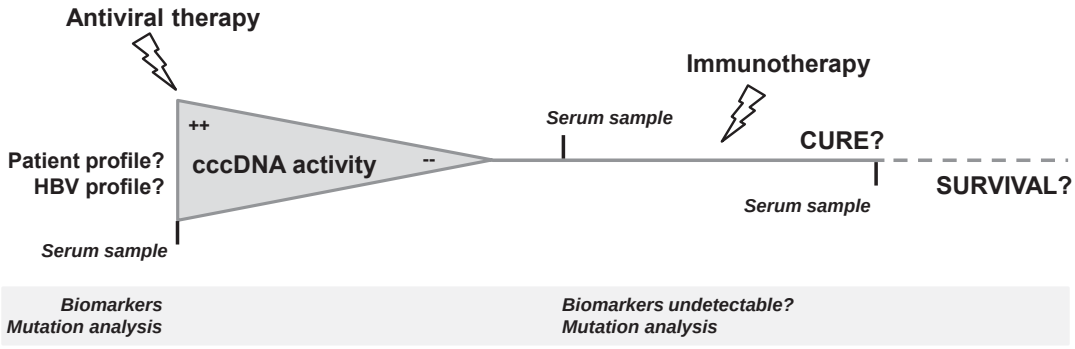
However, we could not demonstrate superiority of these markers over qHBsAg monitoring for HBeAg-positive patients, nor over previously validated stopping rules in our Caucasian HBeAg-negative population (former PARC study), especially for HBcrAg. Therefore we recommend to continue using already validated decision rules for PEG-IFN treatment unless more supportive data regarding the role of HBcrAg and HBV RNA becomes available.

Future perspectives

We see a future opportunity for PEG-IFN add-on when acceleration of HBeAg loss of HBsAg decline would be required prior to treatment with a new compounds to establish functional cure (HBsAg loss). The effect of the PEG-IFN add-on strategy depends on patient and virus characteristics. When an attempt to discontinue NA treatment by adding PEG-IF add-on is desired, we advise to select patients with the highest probability of response, preferably by choosing patients with low levels of HBV DNA, HBV RNA and quantitative HBsAg who have not been treated with PEG-IFN before. For example, by adding PEG-IFN in patients with an HBV RNA level below <5.5 log c/mL HBeAg loss rates were doubled when compared to patients with similar levels who continued of entecavir monotherapy. Validation for different treatment populations of this cut-off and other biomarker cut-offs described in this thesis needs be performed before applying them in clinical practice or for research purposes.

Lastly, one should always be aware of the possible effect of HBeAg status, HBV genotype, and baseline presence of precore and basal core promoter mutations on biomarkers levels, and perhaps also other factors that were not studied here. Newly developed HBV disease models may provide more information on how these factors exactly influence the HBV life cycle. Also on-treatment assessment of HBV mutation variants may provide additional insights. As the viral life cycle and immune response are very complex, future therapeutic strategies with improved response rates will probably require multiple virological and/or immunological approaches, and monitoring of multiple biomarkers in both treatment development as treatment monitoring. Promising therapeutic agents that are now under development include HBV entry inhibitors preventing hepatocyte (re)infection, RNA interfering agents aiming to block protein synthesis and capsid inhibitors. We hypothesize that minimizing viral replication activity and risk for reinfection by using NA therapy and a combination of these modalities for a longer period of time, followed by a non-cytolytic elimination of cccDNA achieved by an IFN-related or nonIFN-related immunological approach would provide the highest and safest chance of a durable cure of HBV. Assuming an 100% specificity of

HBV RNA and HBcrAg, undetectability of these biomarkers and other known virological biomarkers in serum could indicate silenced cccDNA, which could be sign to initiate immunomodulatory treatment. This would be in line with the observations made in this thesis that patients low levels of virological markers have a higher probability to respond to PEG-IFN based treatment. It is also likely that different HBV genetic profiles and different immunologic profiles will require different therapeutic strategies. To create such profiles, a possible but yet not clinically feasible approach could be to combine of HBV whole genome sequencing and human immune profiling by using multiplex immunoassays.



To conclude, as much as this thesis was focused on treatment optimization, it should not be forgotten that the elimination of HBV not only depends on successful treatment, but just as much on finding those who are infected and who are in need for treatment. The worldwide prevalence is probably an underestimation, and part of HBV infected people does not know to be infected [147]. Retrieval of these patients and offer them appropriate treatment together with vaccination of risk groups would not only reduce HBV-related morbidity and mortality but also reduce transmission rates. Only when our current or future improved treatment options will actually reach the ones who require treatment, a significantly decrease the worldwide HBV-related disease burden can be achieved

Samenvatting en discussie

Met de huidige behandelmogelijkheden voor een chronische hepatitis B (HBV) infectie kan het virus helaas nooit volledig uit het lichaam verdwijnen, en leidt behandeling bij slechts een klein deel van de behandelde patiënten tot controle van het afweersysteem over het virus. Die controle wordt ook wel HBsAg verlies of functionele genezing genoemd [38, 39]. Patiënten die behandeld worden met virusremmers, nucleo(s)tide analogen (NA), zijn vaak jarenlang en meestal zelfs levenslang gebonden aan deze behandeling vanwege een grote kans op opvlamming van de virusactiviteit bij het staken van de behandeling. Om de behandeling toch te kunnen staken is de afgelopen jaren gezocht naar virus en patiënt gerelateerde factoren die kunnen voorspellen of het behandeleffect blijvend is, maar ook naar manieren om de duurzaamheid van het behandeleffect te verbeteren. Daartoe heeft men onder meer nieuwe combinatiebehandelingen van NA met peginterferon (PEG-IFN) onderzocht. PEG-IFN is een middel is dat meer probeert de afweerreactie van de patiënt tegen het virus te versterken maar daarnaast ook deels het virus remt. Een van de meest recent onderzochte combinatiestrategieën is het toevoegen van PEG-IFN aan een reeds langere tijd lopende behandeling met een NA [30, 37, 64, 65, 131, 133, 138]. Deze strategie wordt de PEG-IFN add-on strategie genoemd. In alle onderzoeken naar het verbeteren van de behandeling hebben biomarkers een grote rol gespeeld. Dit zijn moleculen geproduceerd door het virus of door de patiënt die vaak worden gemeten in een onderdeel van het bloed van de patiënt, het serum. Door de gemeten waarden van deze biomarkers te combineren kan een ziekte- en patiëntprofiel gemaakt kan worden zodat de behandeling 'op maat' aangeboden kan worden. De doelen van dit proefschrift zijn gebaseerd op de onderzoeken van de afgelopen jaren, en zijn daarom enerzijds te bepalen of de PEG-IFN add-on strategie het effect van NA behandeling kan verbeteren, en anderzijds of de nieuwe biomarkers HBcrAg en HBV RNA bij kunnen dragen aan geïndividualiseerde behandeling voor chronische HBV-infecties.

EFFECT VAN PEGINTERFERON ADD-ON

In **Hoofdstuk 1** hebben we gekeken naar welke virus- en patiëntkenmerken gerelateerd zijn aan de kans op behandelsucces van de PEG-IFN add-on behandeling. De reden om dit onderzoek uit te voeren was dat in recent onderzoek aanwijzingen werden gevonden dat de kans op succes van de behandeling niet hetzelfde was voor alle patiënten [37]. Daarom voegden wij twee eerder uitgevoerde onderzoeken (de ARES en de PEGON studie) samen die allebei gekeken hebben naar het effect van PEG-IFN add-on bij HBeAg-positieve ziekte. In de

ARES studie werden patiënten eerst 24 weken behandeld met alleen entecavir (een van de meest voorgeschreven NA), waarna de ene helft van de patiënten daarna ook 24 weken PEG-IFN kreeg en de andere helft doorging met alleen entecavir. In de PEGON studie waren patiënten niet 24 weken behandeld met een NA maar gebruikten ze dit al voor minimaal een jaar, en kreeg de helft van de patiënten niet 24 weken maar 48 weken PEG-IFN erbij [37, 138]. Door deze verschillen in studieopzet konden we ook kijken naar of de duur van de aanvankelijke NA behandeling en de duur van de PEG-IFN toevoeging uitmaken voor de kans op succes van de behandeling. We konden echter geen relatie ontdekken tussen deze factoren en de kans op succes van de behandeling. De factoren die in onze studie wel gerelateerd waren met het succes van de behandeling waren het nooit eerder behandeld zijn met PEG-IFN, en lagere hoeveelheden van de biomarkers HBV DNA en HBsAg in het serum op het moment van het toevoegen van PEG-IFN. Deze bevindingen klopten met de hypothese die we voorafgaand aan deze studie hadden, namelijk dat het onderdrukken van het virus leidt tot herstel van de afweersysteem. Ook kwamen de bevindingen overeen met resultaten van eerdere onderzoeken die aantoonden dat een lage HBsAg hoeveelheid in het serum waarschijnlijk geassocieerd is met een sterkere mate van controle van het afweersysteem over het virus, en ook met een hogere kans op succes van behandeling met PEG-IFN alleen [139]. Onze bevinding dat de kans op succes van een PEG-IFN behandeling kleiner is als het niet de eerste kuur is, is ook eerder beschreven bij behandeling met conventionele interferon (IFN), wat gebruikt werd voordat PEG-IFN werd ontwikkeld [140]. Dat het effect minder is kan te maken hebben met mutaties in het virus die ontstaan door de behandeling zelf waardoor het virus minder gevoelig wordt voor PEG-IFN, zoals mutaties in de zogeheten precore en basal core promoter regio van het virus, of door mutaties bij de patiënt zelf in de HLA-DP of de IL28B regio [141-143].

Omdat Hoofdstuk 1 specifiek keek naar het behandeleffect 24 weken na het staken van PEG-IFN add-on, kon geen uitspraak worden gedaan over het effect van de PEG-IFN add-on behandeling op langere termijn. Daarom keken we in **Hoofdstuk 2** naar het lange termijneffect van de PEG-IFN behandeling bij patiënten die in de ARES studie waren behandeld, dus bij patiënten die waren behandeld met entecavir gedurende 24 weken voordat ze 24 weken PEG-IFN erbij kregen. We hebben ons met name gericht op het optreden van HBeAg verlies, wat een behandeluitkomst is die de kans vergroot op stabiele ziekte maar die ook later kan leiden tot functionele genezing. Ook keken we of de door PEG-IFN bereikte daling in de hoeveelheid HBsAg in het serum blijvend was, wat ook een uitkomst is die geassocieerd is met stabiele ziekte en een

grotere kans op functionele genezing. Bij patiënten die geen succes van de behandeling hadden bereikt in de eerste 96 weken, werd na week 96 geen verschil in aantallen patiënten met extra HBeAg verlies gezien als we de twee behandelingen vergelijken. Dit suggereert dat de toename van HBeAg verlies met name bereikt wordt in de eerste twee jaar. We zagen daarnaast dat de duidelijk sterkere HBsAg daling die optrad na het toevoegen van PEG-IFN ook lang na het staken van PEG-IFN behouden bleef. Daarom denken wij dat de PEG-IFN add-on strategie later gebruikt zou kunnen worden om HBeAg verlies of HBsAg daling te induceren voorafgaand aan een behandeling met nieuwe, potentere middelen.

SERUM HBCRAG EN HBV RNA IN RELATIE TOT BEHANDELEFFECT

HBcrAg is biomarker die drie virale eiwitten tegelijkertijd meet: HBcAg, HBeAg en een 22-kDa groot precore eiwit genaamd p22cr. Deze drie eiwitten worden allen afgeschreven van de precore/core regio van het cccDNA, een vorm van DNA die een sjabloon is voor bijna alle eiwitten die het virus produceert evenals voor het maken van volledige kopieën van het virus voor verdere verspreiding en infectie. De HBcrAg test is gebaseerd op de CLEIA-methode, wat inhoudt dat de lichtgevende antistoffen van de test zich binden zich aan de 149 aminozuren die de drie eiwitten gemeenschappelijk hebben, waarna het uitgestraalde licht kan worden gemeten [85]. Hoewel van HBcAg bekend is dat het belangrijk onderdeel is van de virusdeeltjes die voor infectie moeten zorgen en van HBeAg dat het een belangrijke rol speelt in het de virusreplicatie, is de rol van het derde eiwit p22cr niet bekend. HBcrAg werd voor het eerst beschreven in 2002. De in eerdere onderzoeken beschreven correlatiecoëfficiënten (getallen die uitdrukken hoe sterk twee factoren met elkaar samenhangen) tussen HBcrAg en cccDNA variëren van 0.66 tot 0.70, wat overeenkomt met een matige tot redelijke samenhang [74, 88, 144]. In **Hoofdstuk 3 & 4** hebben we tijdens behandeling gekeken naar de veranderingen in de hoeveelheid HBcrAg in het serum van patiënten. Bij patiënten met HBeAg-positieve ziekte was dit tijdens behandeling met entecavir met of zonder 24 weken PEG-IFN add-on, en bij patiënten met HBeAg-negatieve ziekte patiënten was dit tijdens behandeling met PEG-IFN gedurende 48 weken met of zonder ribavirine (een oude NA).
In de studie in HBeAg-positieve patiënten (**Hoofdstuk 3**), vonden we voor de start van de behandeling correlaties van HBcrAg met andere biomarkers die vergelijkbaar waren met correlaties die in voorgaande onderzoeken waren gevonden, namelijk r=0.4 voor qHBsAg en r=0.7 voor HBV DNA. In een analyse die

de vergelijkbaarheid van twee metingen test, de Bland-Altman analyse, zagen we zoals verwacht een sterke overeenkomst tussen HBcrAg en HBeAg metingen. Een mutatie in de basal core promoter regio van het cccDNA was geassocieerd met lagere hoeveelheden HBcrAg voorafgaand aan de behandeling. Tijdens de behandelingsperiode zelf leek HBcrAg iets sterker te dalen bij patiënten die PEG-IFN kregen dan bij patiënten die doorgingen met alleen entecavir, maar het verschil was niet statistisch significant. De daling in HBcrAg verschilde wel tussen de verschillende HBV genotypes. Ook zagen we dat er een relatie bestond tussen HBcrAg en succesvolle behandeling, maar als we dit vergeleken de een reeds veelgebruikte biomarker HBsAg konden we niet aantonen dat HBcrAg het beter deed in onze groep met patiënten. Een Aziatische studie vond vergelijkbare resultaten voor andere PEG-IFN en NA combinatiestrategieën [145]. Wat belangrijk te weten is bij het interpreteren van onze studieresultaten is dat we expliciet hebben gekozen voor een uitkomstmaat van behandeling waar niet alleen verlies van HBeAg is opgenomen maar ook een bepaalde hoogte van HBV DNA in het serum. Dit is gedaan omdat we vooraf reeds wisten dat HBcrAg logischerwijs sterk gecorreleerd is met HBeAg omdat HBeAg een van de drie componenten is van HBcrAg, en daarmee hoe dan ook een relatie tussen HBcrAg en therapiesucces zouden vinden als we de definite van succes alleen zouden baseren op HBeAg. In de HBeAg-negatieve patiënten (**Hoofdstuk 4**) vonden we ongeveer dezelfde correlatie tussen HBcrAg en HBV DNA als in de HBeAg-positieve patiënten, maar we vonden geen correlatie met de hoeveelheid HBsAg (r=0.2, p=0.11). Dit zou kunnen komen doordat HBsAg in HBeAg-negatieve patiënten met name afkomstig is van HBV DNA geïntegreerd in het DNA van de levercel dan van het cccDNA [108]. Zoals we eerder ook zagen in HBeAg-positieve patiënten was ook in HBeAg-negatieve patiënten HBcrAg geassocieerd met de kans op succesvolle behandeling, maar opnieuw konden we geen toegevoegde waarde aantonen van HBcrAg boven eerder gevalideerde klinische beslisregels. Een Franse studie die ongeveer tegelijkertijd verscheen met de onze liet vergelijkbare resultaten zien [96]. Voor PEG-IFN behandeling van Westerse HBeAg-negatieve patiënten adviseren we daarom om in ieder geval voorlopig de eerder gemaakte 'PARC stopping rule' te blijven gebruiken die gebaseerd is op het monitoren van veranderingen in hoeveelheden HBV DNA en HBsAg. Dit is met name omdat deze biomarkers uitgebreid gevalideerd zijn en herhaaldelijk in relatie zijn gebracht met de klinische uitkomstmaten waarin we het uiteindelijke effect van behandeling willen zien, zoals levercirrose en leverkanker.

Gebaseerd op de onderzoeken die wij hebben gedaan naar HBcrAg, zijn wij van mening dat HBcrAg zeker gebruikt zou kunnen worden om het effect van een behandeling te vervolgen indien dit in combinatie is met andere biomarkers. Voor het gebruik van HBcrAg als enige biomarker om behandeling te vervolgen zijn ons inziens nog te veel vragen onbeantwoord. Een van de belangrijkste punten die we hierbij willen noemen dat een deel van de gepubliceerde studies over deze biomarker ook HBcrAg waarden meeneemt die onder de gevoeligheidsdrempel van de test liggen. Het gaat hierbij om waarden tussen de 100 U/mL en 1000 U/mL (2-3 $\log_{10}$ U/mL). De onduidelijkheid over het wel of niet gebruiken van waarden tussen deze grenzen zou ontstaan kunnen zijn door het feit dat de handleiding van de test weliswaar weergeeft dat waarden onder 3 log U/mL niet kunnen worden gebruikt, maar dat het apparaat wat voor de test gebruikt wordt toch ook waarden geeft onder deze grens. Wat echter in de eerste studie over HBcrAg beschreven worden, is dat de ondergrens van 3.0 log U/mL is gekozen omdat waarden tussen de 2.0 en 3.0 log U/mL ook werden gemeten bij enkele gezonde patiënten en patiënten met hepatitis C [85]. Om die reden lijkt het ons belangrijk om de ondergrens van 3.0 log U/mL aan te houden om de specificiteit van de test te waarborgen in plaats van een breder spectrum aan HBcrAg metingen te gebruiken in analyses. Omdat er zover wij weten geen andere studies zijn die de specificiteit van HBcrAg beschrijven, raden we aan om dit gegeven mee te nemen in de interpretatie van studies die een potentiële rol beschrijven voor HBV-activiteit in patiënten die functionele genezing hebben bereikt. Enkel en alleen op basis van het detecteerbaar zijn van HBcrAg in HBsAg-negatieve patiënten kan ons inziens niet gezegd worden dat HBcrAg een sensitievere marker is dan HBsAg omdat we de specificiteit onvoldoende in beeld hebben.

HBV RNA is een biomarker die gemeten kan worden met de RACE-PCR techniek. De specifieke methode die wij gebruikt hebben is een aantal jaar geleden ontwikkeld [45], maar anderen hebben vergelijkbare primers en probes gemaakt [46, 146]. Het is niet geheel duidelijk welke type RNA we precies detecteren in serum, maar er zijn meerdere aanwijzingen dat het grotendeels gaat om ingekapseld pregenomic RNA en een minimale hoeveelheid (<1%) precore messenger RNA [46, 107, 146]. De correlaties die beschreven worden tussen HBV RNA in het serum en het cccDNA in de lever zijn opvallend. In onbehandelde HBeAg-positieve patiënten werd een correlatie gevonden (r=0.39) met de absolute hoeveelheid cccDNA, maar dit werd niet gezien in HBeAg-negatieve patiënten [47]. Daarentegen lijkt de aanwezigheid van HBV RNA wel te corresponderen met de aanwezigheid van transcriptieactiviteit van het cccDNA, wat zou kunnen bete-

kenen dat de afwezigheid van HBV RNA in serum duidt op inactief cccDNA. In de **Hoofdstukken 5, 6, 7 & 8**, hebben we gekeken naar HBV RNA levels in patiënten die nog niet worden behandeld en in op verschillende manieren behandelde patiënten, zowel bij HBeAg-positieve als HBeAg-negatieve patiënten. Om verkeerde interpretatie van de gemeten waarden van een nieuwe biomarker te voorkomen is het erg belangrijk om te kijken naar welke factoren van invloed kunnen zijn op de hoeveelheid van de biomarker die gemeten wordt. Daarom hebben we in **Hoofdstuk 5** gekeken naar factoren die invloed kunnen hebben op de hoeveelheid HBV RNA binnen een grote groep patiënten die wel actieve ziekte hebben maar hiervoor nog niet worden behandeld. De factor die het sterkst geassocieerd met de hoeveelheid HBV RNA was de HBeAg-status, wat opnieuw benadrukt hoe belangrijk HBeAg is voor de virale replicatie. Daarnaast was de aanwezigheid van een basal core promoter mutatie onafhankelijk geassocieerd met lagere HBV RNA hoeveelheden, en HBV RNA level was duidelijk het hoogst in patiënten met HBV-genotype D. Ook zagen we dat grotere hoeveelheden HBV RNA ook samengingen met hogere ALAT-waarden in het bloed, dus met meer ontstekingsactiviteit. Al deze factoren hebben we meegenomen in de onderzoeken die we daarna hebben gedaan in groepen patiënten die wel werden behandeld.

We hebben allereerst in **Hoofdstuk 6** gekeken naar veranderingen in de HBV RNA hoeveelheid tijdens PEG-IFN behandeling met of zonder lamivudine bij patiënten met HBeAg-positieve hepatitis. We zagen bij deze patiënten dat de daling in het HBV RNA duidelijker sterker was bij patiënten die met PEG-IFN en lamivudine werden behandeld dan bij patiënten die alleen met PEG-IFN werden behandeld. Na het staken van de behandeling zagen we echter dat de waarden niet meer verschilden, wat hetzelfde patroon is wat HBV DNA laat zien [30]. Ook leek het HBV RNA iets lager te zijn bij patiënten met wildtype virus dan bij patiënten waarbij het virus voor start van de behandeling een mutatie had in de precore en/of de basal core promoter regio, maar dit was niet statistisch significant. In onze studie vonden we verder dat de door anderen voorgestelde HBV RNA afkapwaarde van ≥ 5.5 log kopieën per mL op week 12 goed kon voorspellen dat een patiënt geen HBeAg verlies zou behalen door de behandeling. Deze afkapwaarde was in onze studie echter alleen goed genoeg voor HBV genotypes A en C, omdat bij de genotypes B en D de negatief voorspellende waarde niet boven de 90% uit kwam [44]. Ondanks onze bevindingen dat HBV RNA een sensitieve marker is voor HBeAg verlies, zagen we een beperkte specificiteit, aangezien HBV RNA ook significant daalde bij patiënten waar geen HBeAg-verlies optrad. Dit zou kunnen betekenen dat de hoeveelheid HBV

RNA in het serum meer de actuele replicatieactiviteit weerspiegelt dan de mate van controle van het afweersysteem over het virus.
In Hoofdstuk **7** voerden we een soortgelijke studie uit, maar bekeken we nu HBV RNA tijdens PEG-IFN behandeling met of zonder ribavirine voor HBeAg-negatieve hepatitis. Op basis van onze eerste studie verwachtten we in deze groep lagere HBV RNA hoeveelheden, wat inderdaad niet alleen terugzagen voor de start van behandeling maar ook tijdens de behandeling. Al na de eerste 12 weken van behandeling was het HBV RNA ondetecteerbaar in het grootste deel van de patiënten, onafhankelijk van het type behandeling (combinatie-therapie of alleen PEG-IFN). Omdat de laagste waarde die gedetecteerd kan worden 800 kopieën per mL is zou het verbeteren van de gevoeligheid van de test zeer interessant zijn voor deze groep patiënten. Met de huidige test vonden we dat het hebben van een HBV RNA waarde >1,500 kopieën/mL (3.2 log c/mL) op week 12 van de behandeling voorspelt dat de behandeling niet gaat slagen. Bij het toevoegen van een HBV RNA afkapwaarde van 3.18 log c/mL aan de eerder gevalideerde PARC stopping rule (die gebaseerd is op HBV DNA en qHBsAg monitoring) konden extra patiënten die geen respons zouden gaan behalen geïdentificeerd worden, en daarmee zouden deze patiënten niet de gehele kuur af moeten maken [39].
Tot slot keken we in **Hoofdstuk 8** weer naar HBV RNA levels bij HBeAg-positieve hepatitis, maar nu tijdens de PEG-IFN add-on strategie. Dit hoofdstuk beschrijft interim-resultaten omdat de HBV RNA waarden na week 36 op dit moment nog niet zijn gemeten. Omdat we in **Hoofdstuk 1** al zagen dat patiënt- en viruskenmerken kunnen helpen bij het selecteren van patiënten met de hoogste kans op behandelsucces door PEG-IFN add-on wilden we graag onderzoeken of HBV RNA hieraan zou kunnen bijdragen. De studieopzet van de ARES studie maakte het ons ook mogelijk om naar HBV RNA veranderingen tijdens de eerste 24 weken entecavir behandeling. Na 24 weken entecavir was een duidelijke knik in de daling van HBV RNA in het serum gezien en versnelde de HBV RNA daling ten opzichte van HBV RNA van patiënten die doorgingen met alleen entecavir. De hoeveelheid HBV RNA die gemeten werd na 24 weken entecavir behandeling was een goede voorspeller voor het wel of niet behalen van HBeAg verlies op week 72. Indien alleen patiënten PEG-IFN add-on zouden krijgen waarbij de HBV RNA hoeveelheid onder een bepaalde afkapwaarde komt na 24 weken entecavir, dan kan voor deze groep patiënten PEG-IFN add-on de kans op het verliezen van HBeAg verdubbeld worden. Hiermee zou behandeling verder kunnen worden geïndividualiseerd.

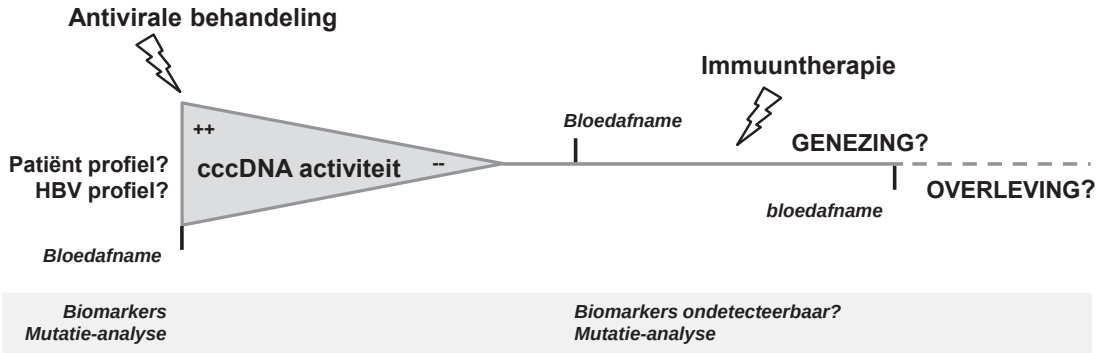
CONCLUSIE EN AANBEVELINGEN

De nieuwe biomarkers HBV RNA en HBcrAg kunnen beiden gebruikt worden om patiënten met een lage kans op therapiesucces al vroeg tijdens PEG-IFN behandeling te kunnen identificeren. Op deze manier kan de behandeling eventueel voortijdig worden gestaakt zodat deze patiënten niet onnodig worden blootgesteld aan de bijwerkingen van PEG-IFN. We konden voor beide biomarkers, maar met name voor HBcrAg, echter niet aantonen dat ze deze inschatting beter kunnen maken dan HBsAg bij HBeAg-positieve patiënten of de PARC stopping rule bij onze westerse patiëntenpopulatie met HBeAg-negatieve hepatitis. We adviseren daarom op basis van deze data op dit moment alleen de reeds gevalideerde klinische beslisregels te gebruiken, in ieder geval zo lang geen data over HBcrAg en HBV RNA verschijnt die aantoont dat deze markers superieur zijn.

Toekomst

We zien een mogelijke rol voor de PEG-IFN add-on strategie indien een bepaalde hoeveelheid HBsAg in serum of HBeAg-negativiteit een vereiste is voor de behandeling met een nieuw middel dat wel tot functionele genezing (verlies van HBsAg) kan leiden. Er moet wel rekening gehouden worden met onze bevinding dat het effect van PEG-IFN add-on afhankelijk is van meerdere patiënt- en viruskenmerken. Om die reden adviseren we om, als deze behandeling overwogen wordt in een poging een patiënt met NA behandeling te laten stoppen, vooraf patiënten te selecteren met de grootste kans van slagen op basis van de hoeveelheden HBV DNA, HBV RNA en HBsAg in het serum, die liefst ook niet eerder behandeld zijn met PEG-IFN. Bijvoorbeeld, als PEG-IFN alleen toegevoegd wordt bij patiënten waarbij HBV RNA level lager is dan 5.5 log c/mL, dan verdubbelt het toevoegen van PEG-IFN de kans op HBeAg verlies. Deze afkapwaarde en de andere afkapwaardes die voorgesteld worden in dit proefschrift moeten nog wel in andere patiëntpopulaties worden gevalideerd voordat deze daadwerkelijk gebruikt kunnen worden in de kliniek of voor onderzoek. Tot slot moet men altijd bedacht zijn op de mogelijke effecten van HBeAg status, HBV-genotype, de aanwezigheid van mutaties in de precore en basal core promoter regio van het cccDNA voordat de behandeling, of misschien van nog andere factoren die niet in dit proefschrift zijn onderzocht. Recent ontwikkelde nieuwe ziektemodellen kunnen mogelijk meer inzicht geven in hoe deze factoren precies de levenscyclus van het virus beïnvloeden. Ook zou het bepalen van verschillende mutaties tijdens behandeling hierbij kunnen helpen. Omdat zowel de levenscyclus van het virus als de menselijke afweerreactie tegen het virus extreem complex zijn, is het erg waarschijnlijk dat nieuwe behandelingen

met betere effecten op meerdere punten zullen moeten aangrijpen, en dat bij het ontwikkelen en monitoren van behandeling ook meerdere biomarkers nodig zullen zijn. Veelbelovende middelen die nu ontwikkeld worden zijn bijvoorbeeld HBV entry inhibitors die zorgen dat HBV een hepatocyt niet kan infecteren, RNA interference leidend tot blokkade van eiwitproductie en capsid inhibitors die de het inkapselen van HBV DNA en RNA tegengaat. Wij denken dat de veiligste en meest succesvolle manier om duurzame genezing te bereiken zou zijn om eerst de transcriptieactiviteit van het cccDNA volledig te blokkeren voor langere tijd evenals de mogelijkheid tot re-infectie, om daarna een immunologisch middel in te zetten om cccDNA te elimineren zonder de hepatocyt zelf ten gronde te laten gaan. Dit zou in het verlengde liggen van wat wij in dit proefschrift hebben gevonden, namelijk dat bij lage hoeveelheden van virologische biomarkers in het serum de kans op succes met PEG-IFN groter is.
Wij verwachten verder dat voor verschillende genetische en immunologische profielen ook verschillende behandelstrategieën nodig zullen zijn. Dergelijke profielen zouden gemaakt kunnen worden door technieken die wel al in onderzoek worden gebruikt maar nog niet geschikt zijn voor klinische toepassing, zoals een combinatie van HBV whole-genome sequencing en het bepalen van de immuunstatus van de patiënt met multiplex immuno-assays.



Tot slot willen we benadrukken dat, hoe zeer dit proefschrift ook gericht is op het optimaliseren van de behandelmogelijkheden voor HBV, het wereldwijd elimineren van HBV-infectie minstens zo sterk afhangt van het opsporen van patiënten en diegenen die behandeling nodig hebben. De wereldwijde prevalentie is waarschijnlijk een onderschatting, en een deel van de geïnfecteerde mensen weet niet dat zij geïnfecteerd is [147]. Vaccinatie in bevolkingsgroepen met een hoog risico op HBV, in combinatie met het vinden van deze mensen én zorgen dat zij de juiste behandeling krijgen zal morbiditeit, mortaliteit en transmissie verminderen. Alleen als huidige of toekomstige behandelingen aankomen bij degenen die het nodig hebben is een significante afname van de wereldwijde ziektelast veroorzaakt door HBV mogelijk.

References

1. Trepo C, Chan HL, Lok A. Hepatitis B virus infection. Lancet 2014.
2. World Health Organization. Hepatitis B. World Health Organization Fact Sheet 204 (Updated July 2017). 2017.
3. Hepatitis B - Rijksinstituut voor Volksgezondheid en Milieu - https://www.rivm.nl/Onderwerpen/H/Hepatitis_B. 2018.
4. Bertoletti A, Ferrari C. Innate and adaptive immune responses in chronic hepatitis B virus infections: towards restoration of immune control of viral infection. Gut 2012;61:1754-1764.
5. Bertoletti A, Ferrari C. Adaptive immunity in HBV infection. J Hepatol 2016;64:S71-S83.
6. Maini MK, Gehring AJ. The role of innate immunity in the immunopathology and treatment of HBV infection. Journal of Hepatology;64:S60-S70.
7. Tuttleman JS, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell 1986;47:451-460.
8. Liver. EAftSot. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol 2017;67:370-398.
9. Chen YC, Sheen IS, Chu CM, Liaw YF. Prognosis following spontaneous HBsAg seroclearance in chronic hepatitis B patients with or without concurrent infection. Gastroenterology 2002;123:1084-1089.
10. Hsu YS, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, Liaw YF. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. Hepatology 2002;35:1522-1527.
11. Dhedin N, Douvin C, Kuentz M, Saint Marc MF, Reman O, Rieux C, Bernaudin F, et al. Reverse seroconversion of hepatitis B after allogeneic bone marrow transplantation: a retrospective study of 37 patients with pretransplant anti-HBs and anti-HBc. Transplantation 1998;66:616-619.
12. Knoll A, Boehm S, Hahn J, Holler E, Jilg W. Reactivation of resolved hepatitis B virus infection after allogeneic haematopoietic stem cell transplantation. Bone Marrow Transplant 2004;33:925-929.
13. Terrault NA, Bzowej NH, Chang KM, Hwang JP, Jonas MM, Murad MH, American Association for the Study of Liver D. AASLD guidelines for treatment of chronic hepatitis B. Hepatology 2016;63:261-283.
14. Wong GL, Chan HL, Mak CW, Lee SK, Ip ZM, Lam AT, Iu HW, et al. Entecavir treatment reduces hepatic events and deaths in chronic hepatitis B patients with liver cirrhosis. Hepatology 2013;58:1537-1547.
15. Papatheodoridis GV, Chan HL, Hansen BE, Janssen HL, Lampertico P. Risk of hepatocellular carcinoma in chronic hepatitis B: assessment and modification with current antiviral therapy. J Hepatol 2015;62:956-967.
16. Papatheodoridis G, Vlachogiannakos I, Cholongitas E, Wursthorn K, Thomadakis C, Touloumi G, Petersen J. Discontinuation of oral antivirals in chronic hepatitis B: A systematic review. Hepatology 2016;63:1481-1492.
17. Buti M, Tsai N, Petersen J, Flisiak R, Gurel S, Krastev Z, Aguilar Schall R, et al. Seven-year efficacy and safety of treatment with tenofovir disoproxil fumarate for chronic hepatitis B virus infection. Dig Dis Sci 2015;60:1457-1464.

18. Yapali S, Lok AS. Does suppression of HBV replication by antiviral therapy confer the same benefit as host immune control of HBV? Gut 2014.

19. Liang TJ, Block TM, McMahon BJ, Ghany MG, Urban S, Guo JT, Locarnini S, et al. Present and future therapies of hepatitis B: From discovery to cure. Hepatology 2015;62:1893-1908.

20. Belloni L, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, Petersen J, et al. IFN-alpha inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. J Clin Invest 2012;122:529-537.

21. Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, Sprinzl MF, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science 2014;343:1221-1228.

22. van Zonneveld M, Honkoop P, Hansen BE, Niesters HG, Darwish Murad S, de Man RA, Schalm SW, et al. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. Hepatology 2004;39:804-810.

23. Buster EH, Flink HJ, Cakaloglu Y, Simon K, Trojan J, Tabak F, So TM, et al. Sustained HBeAg and HBsAg loss after long-term follow-up of HBeAg-positive patients treated with peginterferon alpha-2b. Gastroenterology 2008;135:459-467.

24. Chan HL, Leung NW, Hui AY, Wong VW, Liew CT, Chim AM, Chan FK, et al. A randomized, controlled trial of combination therapy for chronic hepatitis B: comparing pegylated interferon-alpha2b and lamivudine with lamivudine alone. Ann Intern Med 2005;142:240-250.

25. Lau GK, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, Cooksley G, Gane E, et al. Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. N Engl J Med 2005;352:2682-2695.

26. Song BC, Suh DJ, Lee HC, Chung YH, Lee YS. Hepatitis B e antigen seroconversion after lamivudine therapy is not durable in patients with chronic hepatitis B in Korea. Hepatology 2000;32:803-806.

27. Reijnders JG, Perquin MJ, Zhang N, Hansen BE, Janssen HL. Nucleos(t)ide analogues only induce temporary hepatitis B e antigen seroconversion in most patients with chronic hepatitis B. Gastroenterology 2010;139:491-498.

28. Jeng WJ, Sheen IS, Chen YC, Hsu CW, Chien RN, Chu CM, Liaw YF. Off-therapy durability of response to entecavir therapy in hepatitis B e antigen-negative chronic hepatitis B patients. Hepatology 2013;58:1888-1896.

29. Alter H, Block T, Brown N, Brownstein A, Brosgart C, Chang KM, Chen PJ, et al. A research agenda for curing chronic hepatitis B virus infection. Hepatology 2018;67:1127-1131.

30. Janssen HL, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y, Simon C, et al. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. Lancet 2005;365:123-129.

31. Marcellin P, Lau GK, Bonino F, Farci P, Hadziyannis S, Jin R, Lu ZM, et al. Peginterferon alfa-2a alone, lamivudine alone, and the two in combination in patients with HBeAg-negative chronic hepatitis B. N Engl J Med 2004;351:1206-1217.

32. Christen V, Duong F, Bernsmeier C, Sun D, Nassal M, Heim MH. Inhibition of alpha interferon signaling by hepatitis B virus. J Virol 2007;81:159-165.

33. Tjwa ET, van Oord GW, Hegmans JP, Janssen HL, Woltman AM. Viral load reduction improves activation and function of natural killer cells in patients with chronic hepatitis B. J Hepatol 2011;54:209-218.

34. Op den Brouw ML, Binda RS, van Roosmalen MH, Protzer U, Janssen HL, van der Molen RG, Woltman AM. Hepatitis B virus surface antigen impairs myeloid dendritic cell function: a possible immune escape mechanism of hepatitis B virus. Immunology 2009;126:280-289.

35. Boni C, Laccabue D, Lampertico P, Giuberti T, Vigano M, Schivazappa S, Alfieri A, et al. Restored function of HBV-specific T cells after long-term effective therapy with nucleos(t)ide analogues. Gastroenterology 2012;143:963-973 e969.

36. Brouwer WP, Xie Q, Sonneveld MJ, Zhang N, Zhang Q, Tabak F, Streinu-Cercel A, et al. Adding pegylated interferon to entecavir for hepatitis B e antigen-positive chronic hepatitis B: A multicenter randomized trial (ARES study). Hepatology 2015;61:1512-1522.

37. Chi H, Hansen BE, Guo S, Zhang NP, Qi X, Chen L, Guo Q, et al. Pegylated Interferon Alfa-2b Add-on Treatment in Hepatitis B Virus Envelope Antigen-Positive Chronic Hepatitis B Patients Treated with Nucleos(t)ide Analogue: A Randomized, Controlled Trial (PEGON). J Infect Dis 2017;215:1085-1093.

38. Sonneveld MJ, Hansen BE, Piratvisuth T, Jia JD, Zeuzem S, Gane E, Liaw YF, et al. Response-guided peginterferon therapy in hepatitis B e antigen-positive chronic hepatitis B using serum hepatitis B surface antigen levels. Hepatology 2013;58:872-880.

39. Rijckborst V, Hansen BE, Ferenci P, Brunetto MR, Tabak F, Cakaloglu Y, Lanza AG, et al. Validation of a stopping rule at week 12 using HBsAg and HBV DNA for HBeAg-negative patients treated with peginterferon alfa-2a. J Hepatol 2012;56:1006-1011.

40. Buster EH, Hansen BE, Lau GK, Piratvisuth T, Zeuzem S, Steyerberg EW, Janssen HL. Factors that predict response of patients with hepatitis B e antigen-positive chronic hepatitis B to peginterferon-alfa. Gastroenterology 2009;137:2002-2009.

41. Lucifora J, Protzer U. Attacking hepatitis B virus cccDNA–The holy grail to hepatitis B cure. J Hepatol 2016;64:S41-48.

42. Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 2015;64:1972-1984.

43. Mak LY, Wong DK, Cheung KS, Seto WK, Lai CL, Yuen MF. Review article: hepatitis B core-related antigen (HBcrAg): an emerging marker for chronic hepatitis B virus infection. Aliment Pharmacol Ther 2018;47:43-54.

44. van Bommel F, van Bommel A, Krauel A, Wat C, Pavlovic V, Yang L, Deichsel D, et al. Serum HBV RNA as a Predictor of Peginterferon Alfa-2a (40KD) Response in Patients With HBeAg-Positive Chronic Hepatitis B. J Infect Dis 2018.

45. van Bommel F, Bartens A, Mysickova A, Hofmann J, Kruger DH, Berg T, Edelmann A. Serum hepatitis B virus RNA levels as an early predictor of hepatitis B envelope antigen seroconversion during treatment with polymerase inhibitors. Hepatology 2015;61:66-76.

46. Jansen L, Kootstra NA, van Dort KA, Takkenberg RB, Reesink HW, Zaaijer HL. Hepatitis B Virus Pregenomic RNA Is Present in Virions in Plasma and Is Associated With a Response to Pegylated Interferon Alfa-2a and Nucleos(t)ide Analogues. J Infect Dis 2016;213:224-232.

47. Wang J, Du M, Huang H, Chen R, Niu J, Jiang J, Zhuang H, et al. Reply to: "Serum HBV pgRNA as a clinical marker for cccDNA activity": Consistent loss of serum HBV RNA might predict the "para-functional cure" of chronic hepatitis B. J Hepatol 2017;66:462-463.

48. Giersch K, Allweiss L, Volz T, Dandri M, Lutgehetmann M. Serum HBV pgRNA as a clinical marker for cccDNA activity. J Hepatol 2017;66:460-462.

49. Durantel D, Zoulim F. New antiviral targets for innovative treatment concepts for hepatitis B virus and hepatitis delta virus. J Hepatol 2016;64:S117-S131.

50. Ahn J, Lee HM, Lim JK, Pan CQ, Nguyen MH, Ray Kim W, Mannalithara A, et al. Entecavir safety and effectiveness in a national cohort of treatment-naive chronic hepatitis B patients in the US - the ENUMERATE study. Aliment Pharmacol Ther 2016;43:134-144.

51. Marcellin P, Gane E, Buti M, Afdhal N, Sievert W, Jacobson IM, Washington MK, et al. Regression of cirrhosis during treatment with tenofovir disoproxil fumarate for chronic hepatitis B: a 5-year open-label follow-up study. Lancet 2013;381:468-475.

52. Zeisel MB, Lucifora J, Mason WS, Sureau C, Beck J, Levrero M, Kann M, et al. Towards an HBV cure: state-of-the-art and unresolved questions–report of the ANRS workshop on HBV cure. Gut 2015;64:1314-1326.

53. Chang TT, Gish RG, de Man R, Gadano A, Sollano J, Chao YC, Lok AS, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. N Engl J Med 2006;354:1001-1010.

54. Marcellin P, Heathcote EJ, Buti M, Gane E, de Man RA, Krastev Z, Germanidis G, et al. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. N Engl J Med 2008;359:2442-2455.

55. Sarin SK, Kumar M, Lau GK, Abbas Z, Chan HL, Chen CJ, Chen DS, et al. Asian-Pacific clinical practice guidelines on the management of hepatitis B: a 2015 update. Hepatol Int 2016;10:1-98.

56. European Association for the Study of the Liver. Electronic address eee, European Association for the Study of the L. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol 2017;67:370-398.

57. Seto WK, Hui AJ, Wong VW, Wong GL, Liu KS, Lai CL, Yuen MF, et al. Treatment cessation of entecavir in Asian patients with hepatitis B e antigen negative chronic hepatitis B: a multicentre prospective study. Gut 2015;64:667-672.

58. Chevaliez S, Hezode C, Bahrami S, Grare M, Pawlotsky JM. Long-term hepatitis B surface antigen (HBsAg) kinetics during nucleoside/nucleotide analogue therapy: finite treatment duration unlikely. J Hepatol 2013;58:676-683.

59. Zoutendijk R, Hansen BE, van Vuuren AJ, Boucher CA, Janssen HL. Serum HBsAg decline during long-term potent nucleos(t)ide analogue therapy for chronic hepatitis B and prediction of HBsAg loss. J Infect Dis 2011;204:415-418.

60. Boni C, Penna A, Bertoletti A, Lamonaca V, Rapti I, Missale G, Pilli M, et al. Transient restoration of anti-viral T cell responses induced by lamivudine therapy in chronic hepatitis B. J Hepatol 2003;39:595-605.

61. Ouzan D, Penaranda G, Joly H, Khiri H, Pironti A, Halfon P. Add-on peg-interferon leads to loss of HBsAg in patients with HBeAg-negative chronic hepatitis and HBV DNA fully suppressed by long-term nucleotide analogs. J Clin Virol 2013;58:713-717.

62. Sarin SK, Sood A, Kumar M, Arora A, Amrapurkar D, Sharma BC, Konar A, et al. Effect of lowering HBV DNA levels by initial antiviral therapy before adding immunomodulator on treatment of chronic hepatitis B. Am J Gastroenterol 2007;102:96-104.

63. Brouwer WP, Sonneveld MJ, Xie Q, Guo S, Zhang N, Zeuzem S, Tabak F, et al. Peginterferon add-on results in more HBsAg decline compared to monotherapy in HBeAg-positive chronic hepatitis B patients. J Viral Hepat 2016;23:419-426.

64. Marcellin P, Ahn SH, Ma X, Caruntu FA, Tak WY, Elkashab M, Chuang WL, et al. Combination of Tenofovir Disoproxil Fumarate and Peginterferon alpha-2a Increases Loss of Hepatitis B Surface Antigen in Patients With Chronic Hepatitis B. Gastroenterology 2016;150:134-144 e110.

65. Ning Q, Han M, Sun Y, Jiang J, Tan D, Hou J, Tang H, et al. Switching from entecavir to PegIFN alfa-2a in patients with HBeAg-positive chronic hepatitis B: a randomised open-label trial (OSST trial). J Hepatol 2014;61:777-784.

66. Han M, Jiang J, Hou J, Tan D, Sun Y, Zhao M, Ning Q. Sustained immune control in HBeAg-positive patients who switched from entecavir therapy to pegylated interferon-alpha2a: 1 year follow-up of the OSST study. Antivir Ther 2016;21:337-344.

67. Fattovich G, Giustina G, Realdi G, Corrocher R, Schalm SW. Long-term outcome of hepatitis B e antigen-positive patients with compensated cirrhosis treated with interferon alfa. European Concerted Action on Viral Hepatitis (EUROHEP). Hepatology 1997;26:1338-1342.

68. Chan HL, Wong VW, Wong GL, Tse CH, Chan HY, Sung JJ. A longitudinal study on the natural history of serum hepatitis B surface antigen changes in chronic hepatitis B. Hepatology 2010;52:1232-1241.

69. Brunetto MR, Marcellin P, Cherubini B, Yurdaydin C, Farci P, Hadziyannis SJ, Rothe V, et al. Response to peginterferon alfa-2a (40KD) in HBeAg-negative CHB: on-treatment kinetics of HBsAg serum levels vary by HBV genotype. J Hepatol 2013;59:1153-1159.

70. Chen CH, Lu SN, Hung CH, Wang JH, Hu TH, Changchien CS, Lee CM. The role of hepatitis B surface antigen quantification in predicting HBsAg loss and HBV relapse after discontinuation of lamivudine treatment. J Hepatol 2014;61:515-522.

71. Cao J, Chi H, Yu T, Li Z, Hansen BE, Zhang X, Zhong C, et al. Off-Treatment Hepatitis B Virus (HBV) DNA Levels and the Prediction of Relapse After Discontinuation of Nucleos(t)ide Analogue Therapy in Patients With Chronic Hepatitis B: A Prospective Stop Study. J Infect Dis 2017;215:581-589.

72. Wursthorn K, Lutgehetmann M, Dandri M, Volz T, Buggisch P, Zollner B, Longerich T, et al. Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. Hepatology 2006;44:675-684.

73. Sung JJ, Wong ML, Bowden S, Liew CT, Hui AY, Wong VW, Leung NW, et al. Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. Gastroenterology 2005;128:1890-1897.

74. Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. J Med Virol 2009;81:27-33.

75. Hige S, Kobayashi T, Sho T, Nakanishi M, Chuma M, Asaka M. Clinical usefulness of hepatitis B core-related antigen for patients undergoing nucleoside analog administration. Hepatology Research 2010;40:754-755.

76. Matsuzaki T, Tatsuki I, Otani M, Akiyama M, Ozawa E, Miuma S, Miyaaki H, et al. Significance of hepatitis B virus core-related antigen and covalently closed circular DNA levels as markers of hepatitis B virus re-infection after liver transplantation. Journal of Gastroenterology and Hepatology (Australia) 2013;28:1217-1222.

77. Maasoumy B, Wiegand S, Jaroszewicz J, Bremer B, Lehmann P, Deterding K, Taranta A, et al. Hepatitis B core-related antigen (HBcrAg) levels in the natural history of hepatitis B virus infection in a large European cohort predominantly infected with genotypes A and D. Clin Microbiol Infect 2015.

78. Seto WK, Wong DK, Fung J, Huang FY, Liu KS, Lai CL, Yuen MF. Linearized hepatitis B surface antigen and hepatitis B core-related antigen in the natural history of chronic hepatitis B. Clin Microbiol Infect 2014.

79. Seo Y, Yano Y, Miki A, Saito M, Hirano H, Koyama S, Nishikawa M, et al. Quantitative HBsAg and HBcrAg provide viral markers which are independent of HBV DNA for monitoring nucleoside analogues treatment. Journal of Gastroenterology and Hepatology 2012;27:255.

80. Sung JJ, Tsoi KK, Wong VW, Li KC, Chan HL. Meta-analysis: Treatment of hepatitis B infection reduces risk of hepatocellular carcinoma. Aliment Pharmacol Ther 2008;28:1067-1077.

81. Chan HL, Thompson A, Martinot-Peignoux M, Piratvisuth T, Cornberg M, Brunetto MR, Tillmann HL, et al. Hepatitis B surface antigen quantification: why and how to use it in 2011 - a core group report. J Hepatol 2011;55:1121-1131.

82. Locarnini S, Zoulim F. Molecular genetics of HBV infection. Antivir Ther 2010;15 Suppl 3:3-14.

83. Maasoumy B, Wiegand S, Jaroszewicz J, Deterding K, Bremer B, Lehmann P, Manns MP, et al. Hepatitis b core-related antigen (HBcrAg) levels in the natural history of hepatitis b infection in a large european cohort. Journal of Hepatology 2014;60:S291.

84. Brouwer WP, Van Oord G, Sonneveld MJ, Vanwolleghem T, De Knegt RJ, Hansen BE, Boonstra A, et al. Hepatitis B core related antigen levels differ during the natural history of chronic hepatitis B infection. Hepatology 2014;60:1005A.

85. Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, Maki N. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J Clin Microbiol 2002;40:439-445.

86. Matsumoto A, Tanaka E, Suzuki Y, Kobayashi M, Tanaka Y, Shinkai N, Hige S, et al. Combination of hepatitis B viral antigens and DNA for prediction of relapse after discontinuation of nucleos(t)ide analogs in patients with chronic hepatitis B. Hepatol Res 2012;42:139-149.

87. Rijckborst V, Hansen BE, Cakaloglu Y, Ferenci P, Tabak F, Akdogan M, Simon K, et al. Early on-treatment prediction of response to peginterferon alfa-2a for HBeAg-negative chronic hepatitis B using HBsAg and HBV DNA levels. Hepatology 2010;52:454-461.

88. Wong DK, Tanaka Y, Lai CL, Mizokami M, Fung J, Yuen MF. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. J Clin Microbiol 2007;45:3942-3947.

89. Jung KS, Park JY, Chon YE, Kim HS, Kang W, Kim BK, Kim SU, et al. Clinical outcomes and predictors for relapse after cessation of oral antiviral treatment in chronic hepatitis B patients. J Gastroenterol 2016;51:830-839.

90. Shinkai N, Tanaka Y, Orito E, Ito K, Ohno T, Hirashima N, Hasegawa I, et al. Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. Hepatology Research 2006;36:272-276.

91. Tanaka E, Matsumoto A, Yoshizawa K, Maki N. Hepatitis B core-related antigen assay is useful for monitoring the antiviral effects of nucleoside analogue therapy. Intervirology 2008;51 Suppl 1:3-6.

92. Wong D, Kopaniszen M, Seto WK, Fung J, Hung I, Young J, Yuen J, et al. Reduction of hepatitis B core related antigen by long term nucleoside nucleotide analogue therapy and its correlation with intrahepatic HBV DNA reduction. Hepatology International 2015;9:S202.

93. van Campenhout MJ, Brouwer WP, van Oord GW, Xie Q, Zhang Q, Zhang N, Guo S, et al. Hepatitis B core-related Antigen levels are associated with response to entecavir and Peginterferon add-on therapy in Hbeag-positive chronic hepatitis B patients. Clin Microbiol Infect 2016.

94. Rijckborst V, ter Borg MJ, Cakaloglu Y, Ferenci P, Tabak F, Akdogan M, Simon K, et al. A randomized trial of peginterferon alpha-2a with or without ribavirin for HBeAg-negative chronic hepatitis B. Am J Gastroenterol 2010;105:1762-1769.

95. Rijckborst V, Ferenci P, Akdogan M, Pinarbasi B, ter Borg MJ, Simon K, Flisiak R, et al. Long-term follow-up of hepatitis B e antigen-negative patients treated with peginterferon alpha-2a: progressive decrease in hepatitis B surface antigen in responders. Eur J Gastroenterol Hepatol 2012;24:1012-1019.

96. Martinot-Peignoux M, Lapalus M, Maylin S, Boyer N, Castelnau C, Giuily N, Pouteau M, et al. Baseline HBsAg and HBcrAg titres allow peginterferon-based 'precision medicine' in HBeAg-negative chronic hepatitis B patients. J Viral Hepat 2016.

97. Matsumoto A, Maki N, Yoneda T, Kamijo A, Joshita S, Komatsu M, Tanaka N, et al. Viral dynamics for predicting hepatitis flares during or after antiviral treatment in patients with chronic hepatitis B. Hepatology International 2009;3:131-132.

98. Rokuhara A, Sun X, Tanaka E, Kimura T, Matsumoto A, Yao D, Yin L, et al. Hepatitis B virus core and core-related antigen quantitation in Chinese patients with chronic genotype B and C hepatitis B virus infection. J Gastroenterol Hepatol 2005;20:1726-1730.

99. Rokuhara A, Tanaka E, Matsumoto A, Kimura T, Yamaura T, Orii K, Sun X, et al. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. J Viral Hepat 2003;10:324-330.

100. Matsumoto A, Tanaka E, Minami M, Okanoue T, Yatsuhashi H, Nagaoka S, Suzuki F, et al. Low serum level of hepatitis B core-related antigen indicates unlikely reactivation of hepatitis after cessation of lamivudine therapy. Hepatol Res 2007;37:661-666.

101. Seo Y, Yano Y, Miki A, Saito M, Azuma T. Serum HBV DNA and hepatitis B core-related antigen in Japanese patients with chronic hepatitis B during nucleoside analog therapy. Hepatology International 2011;5:145.

102. Wooddell CI, Chavez D, Goetzmann JE, Guerra B, Peterson RM, Lee H, Hegge JO, et al. Reductions in cccDNA under NUC and ARC-520 therapy in chimpanzees with chronic hepatitis B virus infection implicate integrated DNA in maintaining circulating HBSAG. Hepatology 2015;62:222A-223A.

103. Cornberg M, Wong VW, Locarnini S, Brunetto M, Janssen HL, Chan HL. The role of quantitative hepatitis b surface antigen revisited. J Hepatol 2016.

104. Zlotnick A, Venkatakrishnan B, Tan Z, Lewellyn E, Turner W, Francis S. Core protein: A pleiotropic keystone in the HBV lifecycle. Antiviral Res 2015;121:82-93.

105. Chuaypen N, Posuwan N, Chittmittraprap S, Hirankarn N, Treeprasertsuk S, Tanaka Y, Shinkai N, et al. Predictive role of serum HBsAg and HBcrAg kinetics in patients with HBeAg-negative chronic hepatitis B receiving pegylated interferon-based therapy. Clin Microbiol Infect 2017.

106. van Bömmel F, van Bömmel A, Krauel A, He H, Wat C, Pavlovic V, Yang L, et al. Serum HBV RNA is an early predictor of HBeAg seroconversion in patients with chronic Hepatitis B (CHB) treated with pegylated interferon alfa-2a (40KD). Hepatology 2015;62:336A.

107. Wang J, Shen T, Huang X, Kumar GR, Chen X, Zeng Z, Zhang R, et al. Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. J Hepatol 2016;65:700-710.

108. Wooddell CI, Yuen MF, Chan HL, Gish RG, Locarnini SA, Chavez D, Ferrari C, et al. RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg. Sci Transl Med 2017;9.

109. Huang YW, Takahashi S, Tsuge M, Chen CL, Wang TC, Abe H, Hu JT, et al. On-treatment low serum HBV RNA level predicts initial virological response in chronic hepatitis B patients receiving nucleoside analogue therapy. Antivir Ther 2015;20:369-375.

110. Tsuge M, Murakami E, Imamura M, Abe H, Miki D, Hiraga N, Takahashi S, et al. Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleotide analogue treatments in chronic hepatitis B patients. J Gastroenterol 2013;48:1188-1204.

111. Krauel A, Deichsel D, Böhm S, Grossmann M, Berg T, Van Bömmel F. The level of HBV RNA in serum as a novel marker for the phase of chronic hepatitis B virus (HBV) infections. Hepatology 2016;63:895A.

112. Homs M, Caballero A, Gregori J, Tabernero D, Quer J, Nieto L, Esteban R, et al. Clinical application of estimating hepatitis B virus quasispecies complexity by massive sequencing: correlation between natural evolution and on-treatment evolution. PLoS One 2014;9:e112306.

113. Yoo BC, Park JW, Kim HJ, Lee DH, Cha YJ, Park SM. Precore and core promoter mutations of hepatitis B virus and hepatitis B e antigen-negative chronic hepatitis B in Korea. J Hepatol 2003;38:98-103.

114. Laras A, Koskinas J, Hadziyannis SJ. In vivo suppression of precore mRNA synthesis is associated with mutations in the hepatitis B virus core promoter. Virology 2002;295:86-96.

115. Li J, Buckwold VE, Hon MW, Ou JH. Mechanism of suppression of hepatitis B virus precore RNA transcription by a frequent double mutation. J Virol 1999;73:1239-1244.

116. Yan L, Zhang H, Ma H, Liu D, Li W, Kang Y, Yang R, et al. Deep sequencing of hepatitis B virus basal core promoter and precore mutants in HBeAg-positive chronic hepatitis B patients. Sci Rep 2015;5:17950.

117. Revill PA, Locarnini SA. New perspectives on the hepatitis B virus life cycle in the human liver. J Clin Invest 2016;126:833-836.

118. Chun YK, Kim JY, Woo HJ, Oh SM, Kang I, Ha J, Kim SS. No significant correlation exists between core promoter mutations, viral replication, and liver damage in chronic hepatitis B infection. Hepatology 2000;32:1154-1162.

119. De Clercq E, Li G. Approved Antiviral Drugs over the Past 50 Years. Clin Microbiol Rev 2016;29:695-747.

120. Su TH, Kao JH. Unmet Needs in Clinical and Basic Hepatitis B Virus Research. J Infect Dis 2017;216:S750-S756.

121. Wang J, Niu J, Jiang J, Lu F. Serum HBV RNA can reflect the activity of intrahepatic cccDNA. Hepatology International 2017;11:S6-S7.

122. Lam AM, Ren S, Espiritu C, Kelly M, Lau V, Zheng L, Hartman GD, et al. Hepatitis B Virus Capsid Assembly Modulators, but Not Nucleoside Analogs, Inhibit the Production of Extracellular Pregenomic RNA and Spliced RNA Variants. Antimicrob Agents Chemother 2017;61.

123. Agarwal K, Gane EJ, Cheng W, Sievert W, Roberts SK, Ahn SH, Kim YJ, et al. HBcrAg, HBV-RNA declines in A phase 2a study evaluating the multi-dose activity of ARB-1467 in HBeAg-positive and negative virally suppressed subjects with hepatitis B. Hepatology 2017;66:22A-23A.

124. Jia W, Zhu MQ, Zhang JM. Serum hepatitis B virus RNA levels as a predictor of HBeAg seroconversion during treatment with peginterferon alfa-2a in hepatitis B e antigen-positive patients. Hepatology International 2017;11:S702.

125. van Campenhout MJH, van Bommel F, Pfefferkorn M, Fischer J, Deichsel D, Boonstra A, van Vuuren AJ, et al. Host and viral factors associated with serum hepatitis B virus RNA levels among patients in need for treatment. Hepatology 2018.

126. Uprichard SL, Wieland SF, Althage A, Chisari FV. Transcriptional and posttranscriptional control of hepatitis B virus gene expression. Proc Natl Acad Sci U S A 2003;100:1310-1315.

127. Pasquetto V, Wieland SF, Uprichard SL, Tripodi M, Chisari FV. Cytokine-sensitive replication of hepatitis B virus in immortalized mouse hepatocyte cultures. J Virol 2002;76:5646-5653.

128. Biron CA. Interferons alpha and beta as immune regulators–a new look. Immunity 2001;14:661-664.

129. Wieland SF, Guidotti LG, Chisari FV. Intrahepatic induction of alpha/beta interferon eliminates viral RNA-containing capsids in hepatitis B virus transgenic mice. J Virol 2000;74:4165-4173.

130. Rang A, Gunther S, Will H. Effect of interferon alpha on hepatitis B virus replication and gene expression in transiently transfected human hepatoma cells. J Hepatol 1999;31:791-799.

131. Xie Q, Zhou H, Bai X, Wu S, Chen JJ, Sheng J, Xie Y, et al. A randomized, open-label clinical study of combined pegylated interferon Alfa-2a (40KD) and entecavir treatment for hepatitis B "e" antigen-positive chronic hepatitis B. Clin Infect Dis 2014;59:1714-1723.

132. Lok AS, Zoulim F, Dusheiko G, Ghany MG. Hepatitis B cure: From discovery to regulatory approval. J Hepatol 2017;67:847-861.

133. Bourlière M, Rabiega P, Ganne-Carrié N, Serfaty L, Marcellin P, Pouget N, Guyader D, et al. HBsAg clearance after addition of 48 weeks of PEGIFN in HBeAg negative CHB patients on Nucleos(t)ide therapy with undetectable HBV DNA for at least one year: A multicenter randomized controlled phase III trial ANRS-HB06 PEGAN study: Preliminary findings. Hepatology 2014;60:1094A-1095A.

134. Liem KS, van Campenhout MJH, Xie Q, Brouwer WP, Chi H, Qi X, Liang C, et al. Low Hepatitis B surface Antigen and HBV DNA levels predict response of pegylated interferon addition to entecavir in Hepatitis B e Antigen positive chronic hepatitis B. Submitted 2018.

135. van Campenhout MJH, van Bommel F, Pfefferkorn M, Fischer J, Deichsel D, Boonstra A, van Vuuren AJ, et al. Serum hepatitis B virus RNA predicts response to peginterferon treatment in HBeAg positive chronic hepatitis B. Submitted 2018.

136. Yuen MF, Chen DS, Dusheiko GM, Janssen HLA, Lau DTY, Locarnini SA, Peters MG, et al. Hepatitis B virus infection. Nat Rev Dis Primers 2018;4:18035.

137. Tong S, Revill P. Overview of hepatitis B viral replication and genetic variability. J Hepatol 2016;64:S4-S16.

138. Brouwer WP, Xie Q, Sonneveld MJ, Zhang NP, Zhang Q, Tabak F, Streinu A, et al. Adding peginterferon to entecavir increases response rates in HBEAG-positive chronic hepatitis B patients: Week 96 results of a global multicenter randomised trial (ares study). Journal of Hepatology 2014;60:S2.

139. Chan HL, Wong VW, Tse AM, Tse CH, Chim AM, Chan HY, Wong GL, et al. Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response. Clin Gastroenterol Hepatol 2007;5:1462-1468.

140. Janssen HL, Schalm SW, Berk L, de Man RA, Heijtink RA. Repeated courses of alpha-interferon for treatment of chronic hepatitis type B. J Hepatol 1993;17 Suppl 3:S47-51.

141. Sonneveld MJ, Rijckborst V, Zwang L, Zeuzem S, Jenny Heathcote E, Simon K, Zoutendijk R, et al. Hepatitis B e antigen levels and response to peginterferon: influence of precore and basal core promoter mutants. Antiviral Res 2013;97:312-317.

142. Sonneveld MJ, Wong VW, Woltman AM, Wong GL, Cakaloglu Y, Zeuzem S, Buster EH, et al. Polymorphisms near IL28B and serologic response to peginterferon in HBeAg-positive patients with chronic hepatitis B. Gastroenterology 2012;142:513-520 e511.

143. Brouwer WP, Sonneveld MJ, Tabak F, Simon K, Cakaloglu Y, Akarca US, Zeuzem S, et al. Polymorphisms of HLA-DP are associated with response to peginterferon in Caucasian patients with chronic hepatitis B. Aliment Pharmacol Ther 2014;40:811-818.

144. Wong DK, Seto WK, Cheung KS, Chong CK, Huang FY, Fung J, Lai CL, et al. Hepatitis B virus core-related antigen as a surrogate marker for covalently closed circular DNA. Liver Int 2016.

145. Chuaypen N, Posuwan N, Payungporn S, Tanaka Y, Shinkai N, Poovorawan Y, Tangkijvanich P. Serum hepatitis B core-related antigen as a treatment predictor of pegylated interferon in patients with HBeAg-positive chronic hepatitis B. Liver Int 2015.

146. Butler EK, Gersch J, McNamara A, Luk KC, Holzmayer V, de Medina M, Schiff E, et al. HBV serum DNA and RNA levels in nucleos(t)ide analogue-treated or untreated patients during chronic and acute infection. Hepatology 2018.

147. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet 2015;386:1546-1555.

Addendum to references

ADDITIONAL REFERENCES CHAPTER 2

1. Petersen J, Thompson AJ, Levrero M. Aiming for cure in HBV and HDV infection. J Hepatol 2016;65:835–48.
2. Lucifora J, Protzer U. Attacking hepatitis B virus cccDNA–The holy grail to hepatitis B cure. J Hepatol 2016;64:S41–8.
3. Wong DK-H, Yuen M-F, Ngai VW-S, Fung J, Lai C-L. One-year entecavir or lamivudine therapy results in reduction of hepatitis B virus intrahepatic covalently closed circular DNA levels. Antivir Ther 2006;11:909–16.
4. Dandri M, Locarnini S. New insight in the pathobiology of hepatitis B virus infection. Gut 2012;61:i6–17.
5. Buti M, Tsai N, Petersen J, Flisiak R, Gurel S, Krastev Z, et al. Seven-year efficacy and safety of treatment with tenofovir disoproxil fumarate for chronic hepatitis B virus infection. Dig Dis Sci 2015;60:1457–64.
6. Marcellin P, Lau GKK, Bonino F, Farci P, Hadziyannis S, Jin R, et al. Peginterferon Alfa-2a Alone, Lamivudine Alone, and the Two in Combination in Patients with HBeAg-Negative Chronic Hepatitis B. N Engl J Med 2004;351:1206–17.
7. Chi H, Hansen BE, Yim C, Arends P, Abu-Amara M, van der Eijk AA, et al. Reduced risk of relapse after long-term nucleos(t)ide analogue consolidation therapy for chronic hepatitis B. Aliment Pharmacol Ther 2015;41:867–76.
8. Jeng W-J, Sheen I-S, Chen Y-C, Hsu C-W, Chien R-N, Chu C-M, et al. Off-therapy durability of response to entecavir therapy in hepatitis B e antigen-negative chronic hepatitis B patients. Hepatology 2013;58:1888–96.
9. van Zonneveld M, Honkoop P, Hansen BE, Niesters HGM, Murad SD, de Man RA, et al. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. Hepatology 2004;39:804–10.
10. Reijnders JGP, Perquin MJ, Zhang N, Hansen BE, Janssen HLA. Nucleos(t)ide analogues only induce temporary hepatitis B e antigen seroconversion in most patients with chronic hepatitis B. Gastroenterology 2010;139:491–8.
11. Buster EHCJ, Flink HJ, Cakaloglu Y, Simon K, Trojan J, Tabak F, et al. Sustained HBeAg and HBsAg loss after long-term follow-up of HBeAg-positive patients treated with peginterferon alpha-2b. Gastroenterology 2008;135:459–67.
12. de Niet A, Stelma F, Jansen L, Sinnige MJ, Remmerswaal EBM, Takkenberg RB, et al. Restoration of T cell function in chronic hepatitis B patients upon treatment with interferon based combination therapy. J Hepatol 2016;64:539–46.
13. Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and Nonhepatotoxic Degradation of Nuclear Hepatitis B Virus cccDNA. Science 2014;343:1221–8.
14. Wursthorn K, Lutgehetmann M, Dandri M, Volz T, Buggisch P, Zollner B, et al. Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. Hepatology 2006;44:675–84.

15. Janssen H LA, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y, et al. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. Lancet 2005;365:123–9.

16. Lau GKK, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, Cooksley G, et al. Peginterferon Alfa-2a, Lamivudine, and the Combination for HBeAg-Positive Chronic Hepatitis B. N Engl J Med 2005;352:2682–95.

17. Boni C, Laccabue D, Lampertico P, Giuberti T, Viganò M, Schivazappa S, et al. Restored Function of HBV-Specific T Cells After Long-term Effective Therapy With Nucleos(t)ide Analogues. Gastroenterology 2012;143:963–973.e9.

18. Boni C, Lampertico P, Talamona L, Giuberti T, Invernizzi F, Barili V, et al. Natural killer cell phenotype modulation and natural killer/T-cell interplay in nucleos(t)ide analogue-treated hepatitis e antigen-negative patients with chronic hepatitis B. Hepatology 2015;62:1697–709.

19. Tjwa ETTL, van Oord GW, Hegmans JP, Janssen HLA, Woltman AM. Viral load reduction improves activation and function of natural killer cells in patients with chronic hepatitis B. J Hepatol 2011;54:209–18.

20. Sonneveld MJ, Zoutendijk R, Hansen BE, Janssen H LA. Peginterferon results in higher serological, but not virological, response rates when compared to continuous entecavir. Antivir Ther 2012;17:1605–8.

21. Brouwer WP, Xie Q, Sonneveld MJ, Zhang N, Zhang Q, Tabak F, et al. Adding pegylated interferon to entecavir for hepatitis B e antigen-positive chronic hepatitis B: A multicenter randomized trial (ARES study). Hepatology 2015;61:1512–22.

22. Bourlière M, Rabiega P, Ganne-Carrie N, Serfaty L, Marcellin P, Barthe Y, et al. Effect on HBs antigen clearance of addition of pegylated interferon alfa-2a to nucleos(t)ide analogue therapy versus nucleos(t)ide analogue therapy alone in patients with HBe antigen-negative chronic hepatitis B and sustained undetectable plasma hepatitis. Lancet Gastroenterol Hepatol 2017;2:177–88.

23. Chi H, Hansen BE, Guo S, Zhang NP, Qi X, Chen L, et al. Pegylated Interferon Alfa-2b Add-on Treatment in Hepatitis B Virus Envelope Antigen-Positive Chronic Hepatitis B Patients Treated with Nucleos(t)ide Analogue: A Randomized, Controlled Trial (PEGON). J Infect Dis 2017;215:1085–93.

24. Lin Z-H, Xin Y-N, Dong Q-J, Wang Q, Jiang X-J, Zhan S-H, et al. Performance of the aspartate aminotransferase-to-platelet ratio index for the staging of hepatitis C-related fibrosis: an updated meta-analysis. Hepatology 2011;53:726–36.

25. Generalized Estimating Equations 2015. https://support.sas.com/rnd/app/stat/topics/gee/gee.pdf (accessed March 22, 2017).

26. Terrault NA, Bzowej NH, Chang K-M, Hwang JP, Jonas MM, Murad MH, et al. AASLD guidelines for treatment of chronic hepatitis B. Hepatology 2016;63:261–83.

ADDITIONAL REFERENCES CHAPTER 7

1. Lai C-L, Yuen M-F. Prevention of hepatitis B virus-related hepatocellular carcinoma with antiviral therapy. Hepatology. 2013 Jan;57(1):399–408.
2. Su T-H, Kao J-H. Unmet Needs in Clinical and Basic Hepatitis B Virus Research. J Infect Dis Internet.. 2017 Nov 16;216(suppl_8):S750–6. Available from: http://dx.doi.org/10.1093/infdis/jix382
3. Lok AS, Zoulim F, Dusheiko G, Ghany MG. Hepatitis B cure: From discovery to regulatory approval. J Hepatol. 2017 Oct;67(4):847–61.
4. Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut. 2015 Dec;64(12):1972–84.
5. Allweiss L, Giersch K, Nuurei T, Polywka S, Volz T, Lohse AW, et al. Serum HBV pgRNA can serve as clinical marker for cccDNA activity in patients with HBV mono and HBV/HDV co-infection. J Hepatol Internet.. 2017 Jan 1;66(1):S697. Available from: http://dx.doi.org/10.1016/S0168-8278(17)31870-6
6. Giersch K, Allweiss L, Volz T, Dandri M, Lutgehetmann M. Serum HBV pgRNA as a clinical marker for cccDNA activity. Vol. 66, Journal of hepatology. Netherlands; 2017. p. 460–2.
7. van Bommel F, Bartens A, Mysickova A, Hofmann J, Kruger DH, Berg T, et al. Serum hepatitis B virus RNA levels as an early predictor of hepatitis B envelope antigen seroconversion during treatment with polymerase inhibitors. Hepatology. 2015 Jan;61(1):66–76.
8. Su Q, Wang SF, Chang TE, Breitkreutz R, Hennig H, Takegoshi K, et al. Circulating hepatitis B virus nucleic acids in chronic infection : representation of differently polyadenylated viral transcripts during progression to nonreplicative stages. Clin Cancer Res. 2001 Jul;7(7):2005–15.
9. Rokuhara A, Matsumoto A, Tanaka E, Umemura T, Yoshizawa K, Kimura T, et al. Hepatitis B virus RNA is measurable in serum and can be a new marker for monitoring lamivudine therapy. J Gastroenterol. 2006 Aug;41(8):785–90.
10. Hatakeyama T, Noguchi C, Hiraga N, Mori N, Tsuge M, Imamura M, et al. Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. Hepatology. 2007 May;45(5):1179–86.
11. Huang YW, Chayama K, Tsuge M, Takahashi S, Hatakeyama T, Abe H, et al. Differential effects of interferon and lamivudine on serum HBV RNA inhibition in patients with chronic hepatitis B. Antivir Ther. 2010;15(2):177–84.
12. Wang J, Yu Y, Li G, Shen C, Meng Z, Zheng J, et al. Relationship between serum HBV-RNA levels and intrahepatic viral as well as histologic activity markers in entecavir-treated patients. J Hepatol. 2017 Sep;
13. Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, Chang T-T, Kitis G, Rizzetto M, et al. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B. N Engl J Med. 2005 Jun;352(26):2673–81.
14. Lok AS, Zoulim F, Dusheiko G, Ghany MG. Review Hepatitis B cure : From discovery to regulatory approval. J Hepatol Internet.. 2017;67(4):847–61. Available from: http://dx.doi.org/10.1016/j.jhep.2017.05.008

15. van Bommel F, van Bommel A, Krauel A, Wat C, Pavlovic V, Yang L, et al. Serum HBV RNA as a Predictor of Peginterferon Alfa-2a (40KD) Response in Patients With HBeAg-Positive Chronic Hepatitis B. J Infect Dis. 2018 May; Epub ahead of printing.

16. van Campenhout MJH, van Bömmel F, Pfefferkorn M, Fischer J, Deichsel D, Boonstra A, et al. Serum hepatitis B virus RNA predicts response to peginterferon treatment in HBeAg positive chronic hepatitis B. submitted. 2018.

17. Wieland SF, Guidotti LG, Chisari F V. Intrahepatic induction of alpha/beta interferon eliminates viral RNA-containing capsids in hepatitis B virus transgenic mice. J Virol. 2000 May;74(9):4165–73.

18. Jansen L, Kootstra NA, van Dort KA, Takkenberg RB, Reesink HW, Zaaijer HL. Hepatitis B Virus Pregenomic RNA Is Present in Virions in Plasma and Is Associated With a Response to Pegylated Interferon Alfa-2a and Nucleos(t)ide Analogues. J Infect Dis. 2016 Jan;213(2):224–32.

19. Rijckborst V, ter Borg MJ, Cakaloglu Y, Ferenci P, Tabak F, Akdogan M, et al. A randomized trial of peginterferon alpha-2a with or without ribavirin for HBeAg-negative chronic hepatitis B. Am J Gastroenterol Internet.. 2010;105(8):1762–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20461068

20. Vincent R, E. HB, Yilmaz C, Peter F, Fehmi T, Meral A, et al. Early on-treatment prediction of response to peginterferon alfa-2a for HBeAg-negative chronic hepatitis B using HBsAg and HBV DNA levels. Hepatology Internet.. 2010 Jul 23;52(2):454–61. Available from: https://doi.org/10.1002/hep.23722

21. Yapali S, Talaat N, Lok AS. Management of hepatitis B: our practice and how it relates to the guidelines. Clin Gastroenterol Hepatol. 2014 Jan;12(1):16–26.

22. van Campenhout MJH, van Bommel F, Pfefferkorn M, Fischer J, Deichsel D, Boonstra A, et al. Host and viral factors associated with serum hepatitis B virus RNA levels among patients in need for treatment. Hepatology. 2018 Mar; Epub ahead of printing.

23. Belloni L, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, et al. IFN-alpha inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. J Clin Invest. 2012 Feb;122(2):529–37.

24. Liu F, Campagna M, Qi Y, Zhao X, Guo F, Xu C, et al. Alpha-interferon suppresses hepadnavirus transcription by altering epigenetic modification of cccDNA minichromosomes. PLoS Pathog. 2013 Sep;9(9):e1003613.

25. Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science. 2014 Mar;343(6176):1221–8.

26. Wang J, Shen T, Huang X, Kumar GR, Chen X, Zeng Z, et al. Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. J Hepatol. 2016 Oct;65(4):700–10.

Abbreviations

ALT	alanine aminotransferase
AUC	area under the curve
BCP	basal core promoter
cccDNA	covalently closed circular DNA
CHB	chronic hepatitis B infection
c/mL	copies per milliliter
CR	combined response
CI-95%	95% confidence interval
ETV	entecavir
HAI	hepatic activity index
HBV	hepatitis B virus
HBcAg	hepatitis B core antigen
HBcrAg	hepatitis B core-related antigen
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
IU/mL	international units per milliliter
IQR	interquartile range
LAM	lamuvidine
LLD	lower limit of detection
LLQ	lower limit of quantification
LTFU	long-term follow-up
MDQ	minimal detectable quantity
MITT	modified intention-to-treat
NA	nucleos(t)ide analogue
p22cr	22-kD precore protein
PEG-IFN	peginterferon
PC	precore
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RBV	ribavirin
qHBsAg	quantitative hepatitis B surface antigen
qHBeAg	quantitative hepatitis B e antigen
ROC	receiver operating characteristic
SD	standard deviation
TDF	tenofovir disoproxil fumarate
ULN	upper limit of normal
U/mL	units per milliliter
WT	wildtype

Dankwoord

Eigenlijk had het mij mooi geleken om als enige twee woorden hier 'iedereen bedankt' op te schrijven, maar dan ga ik toch te veel voorbij aan de grote moeite die veel mensen voor mij hebben gedaan in de afgelopen 5 jaar. Sorry allemaal, het is algemeen bekend hoe goed ik ben in dingen beknopt houden: het wordt een lang verhaal.

Allereerst wil ik mijn promotoren **prof.dr. Harry Janssen** en **prof.dr. Herold Metselaar** bedanken.
Herold, tijdens het laatste jaar van mijn studie kwam ik bij jou om afstudeeronderzoek te doen. Dit werd uiteindelijk een onderzoek naar CMV infecties bij levertransplantatiepatiënten, en van daaruit kwam de combinatie hepatologie/infectiologie voor het eerst op mijn pad. Bedankt voor het vertrouwen om mij ook als promovendus aan de slag te laten gaan, en bedankt voor de begeleiding en nuchtere kijk van de afgelopen jaren.
Harry, na enkele maanden maakte ik kennis met jou en aan jou was de soms logistiek ingewikkelde taak om mij op afstand te begeleiden. Onze Skype-afspraken begonnen met de PAS studie, maar als snel kwamen daar meer en meer projecten bij die uiteindelijk samen tot dit proefschrift hebben geleid. Bedankt voor alle goede ideeën, tips en je alle mogelijkheden die ik heb gekregen tijdens mijn promotietraject.
Na mijn promotoren komt natuurlijk meteen mijn copromotor, **dr. Bettina Hansen**. Beste Bettina, ik heb er nog even over getwijfeld of ik jouw one-liner 'a plot says more than a thousand words' zou opnemen in mijn stellingen, maar helaas, je moet het toch doen met het dankwoord. Ik had nooit gedacht dat iemand het ooit voor elkaar zou krijgen om mijn statistische skills op dit niveau te krijgen. We hebben hard gewerkt en veel gelachen tijdens mijn promotie, maar ik heb je ook meer dan eens achter het behang willen plakken als je weer met een nieuwe analyse kwam wanneer ik dacht dat ik al klaar was haha! Dank je wel voor al je tijd en geduld, en ik kan met recht zeggen dat zonder jou dit proefschrift er niet was geweest.
Verder wil ik graag **dr. Rob de Knegt** bedanken. Beste Rob, onze wekelijke overlegmomenten over de hepatitis B poli en onderzoek waren altijd enorm gezellig, en van begin af aan was je erg geïnteresseerd en betrokken. Als je mij ergens mee kon helpen deed je dat meteen, of zorgde je dat iemand anders dat kon doen. Dank je wel voor alle begeleiding.
Dan kom ik hierna bij **prof.dr. Janneke van der Woude**, huidige opleider MDL, en **prof.dr. Rob de Man,** oud-opleider MDL. Ik ben heel erg blij dat ik de kans heb gekregen om de opleiding tot MDL-arts te starten, bedankt! **Dr. Henk Boom**, vooropleider interne geneeskunde, bedankt voor alle ruimte en

begeleiding die ik in Delft heb gekregen. Ik voel me nog steeds erg welkom. Lieve **collega-arts assistenten uit Delft**, bedankt voor alles wat ik van jullie leer en voor de gezellige werksfeer. Vanaf nu ben ik wat vaker bij de borrels!

Hepatitis B is niet te onderzoeken als je alleen maar op klinisch niveau kijkt. Allereerst wil ik daarom **dr. Annemiek van der Eik**, viroloog, bedanken. Annemiek, ook jij bent ooit gepromoveerd op hepatitis B, en toen ik bij jou kwam voor mijn CMV-projecten en ik aankaartte dat ik promotieonderzoek wilde doen, zei jij: 'Pauline gaat weg, jij moet NU naar Bettina Hansen om te zeggen dat je die plek wil'. Zo geschiedde, en hier ligt nu het resultaat. We bleven ook samenwerken tijdens mijn promotie, en ik wil je bedanken voor je enthousiasme en inspiratie in deze tijd.
Ook wil ik natuurlijk **dr. André Boonstra**, immunoloog, bedanken. André, door onze regelmatige besprekingen heb ik nu toch het idee dat ik eindelijk wat van immunologie snap. Dank je wel voor alles wat je hebt gedaan voor de projecten en bedankt dat je vandaag plaats wilt nemen in de commissie. Dankzij jouw immunologische blik heeft menig discussie uit mijn proefschrift meer diepgang gekregen.
Dr. Hanneke van Vuuren, hoofd MDL diagnostiek. Beste Hanneke, waar in mijn proefschrift komt geen diagnostiek voor? Dank je wel voor alle uitleg over testen waarvan ik eerst geen idee had hoe ze werkten, en voor alle moeite die je hebt gedaan voor mijn projecten. Tegelijkertijd met André en Hanneke wil ik ook **Gertine van Oord, Anthonie Groothuismink, Buddy Roovers, en Frances van Deurzen** bedanken. Jullie hebben enorme bergen verzet om alle biomarker-resultaten te genereren. Ook zonder jullie was er geen proefschrift geweest. Heel erg bedankt!
En last maar zeker not least: dames van het CRB en de poli! **Heleen, Melek, Esther, Irene, Elke en Judith**, bedankt voor al jullie hulp, tips en gezelligheid. **Wilma**, dank je wel voor je hulp aan mij als beginnende dokter op de poli. We hebben wat af gelachen!

Lieve **daklabduifjes**, wat bof ik om jullie als collega's (gehad) te hebben. Niet alleen heb ik ontzettend veel van jullie geleerd, maar vooral waren de gezelligheid van alle borrels, koffiemomentjes, uitjes etc. onmisbaar. Ook daarom weer een hoop mensen te bedanken.

Allereerst mijn mede-HBV collega's **Heng, Willem-Pieter, Seng, Mina, Hannah en Lauke**. **Heng**(ster), tijdens mijn afstudeeronderzoek was jij de eerste die op mijn toenmalige plekje in het Ha-gebouw kennis kwam maken.

Toen je hoorde dat ik jouw nieuwe collega zou worden was je meteen heel behulpzaam. Ik heb heel veel met je gelachen op het dak en bij de congressen, en ik kijk er nu al naar uit om later terug collega's zijn in het EMC. **WP**, op mijn eerste dag stuurde je me vanuit Canada een hele lange welkomstmail met allemaal tips & tricks voor mij als beginnende collega. Ook in de jaren erna heb je me vaak geholpen, maar heb je ook daarbij nodige eigenwijsheid van mij mogen verduren. Bedankt! **Seng**, toen je net begon leek het alsof we beiden hele andere projecten zouden gaan doen, maar uiteindelijk hebben we heel wat samengewerkt. Jammer dat dit grotendeels op afstand was, maar ik hoop je snel weer terug in NL te zien. **Mina and Hannah**, the Toronto crew! It's been a pleasure working with you both. Thank you for all your help. Hopefully we will continue working together in the future! **Lauke**, als nieuwe translationele onderzoeker viel je meteen met je neus in de boter met de trialpoli die je van mij moest overnemen. Je enthousiasme om te beginnen met iets nieuws werkte erg aanstekelijk, en je vrolijke cynisme kan ik nog steeds erg waarderen. Bedankt, en veel succes met het afronden van je promotie. **Pauline, Milan, en Vincent**, aan mij was de taak om in jullie voetsporen te treden. Bedankt voor alle opstarthulp.

Ik heb de eer gehad om op twee kamers op het befaamde dak te mogen zitten. Toen ik net begon op het dak wisselde ik zo'n beetje wekelijks van werkplek, totdat de vraag kwam of ik interesse had in een plekje op de toffe-peren kamer. **Raoel en Eline**, ik vraag me weleens af of jullie daar qua rust weleens spijt van hebben gehad. Lieve **Eline**, ik heb het meer dan eens tegen je gezegd, maar het was ontzettend fijn om jou als kamergenootje te hebben. Dankjewel voor je betrokkenheid, kletsuurtjes, werktips en ontbijtdates. Je wordt een supergoede huisarts! Lieve **Raoel**, je hebt je rol als buddy meer dan waar gemaakt. Naast dat ik oprecht SPSS en Graphpad tips heb gekregen vanuit je 'officiële rol' maakte jij het ook erg gezellig op kamer 419. Ik herken nog steeds in 'Raoel-nummers' als er ze voorbijkomen over de speakers. Bedankt!
Toen ik na 2,5 jaar alleen achterbleef op kamer 419 dacht ik dat ik het staartje van mijn promotie zonder kamergezelligheid zou gaan afronden. Ik had alleen niet bedacht dat ik ook zelf naar een andere kamer kon verhuizen, maar gelukkig hadden **Floor en Priscilla** wel door dat dit kon. Lieve **Sil**, al voordat ik je dakroomie werd hadden we al veel mooie momentjes achter de rug. Congres, risotto-dates, feestjes, borrels, we hebben wat meegemaakt! Ik heb je leren kennen als iemand die heel erg sociaal en zorgzaam is, maar ook heel erg gedisciplineerd en georganiseerd. Ik ben hartstikke blij dat we tegelijkertijd in het EMC zijn begonnen, en dat we daardoor veel leuks hebben gedeeld en nog

zullen delen! Lieve **Floor**, wat ben jij een topper. Je staat altijd voor iedereen klaar, werkt superhard, en bent denk ik de meest optimistische persoon die ik ken. Gelukkig voor mij ben je in Delft komen werken en in Rotterdam komen wonen! Dankjewel voor al je steun en betrokkenheid.

Lieve **Esmée, Els, Joany, Louisa, Shannon, Loes, Maren, Jorn, Wim, Sophia, Anniek, Fanny, Kasper, Mitchel, Marjolein, Ingrid, Vincent, Wesley, Michelle, Elmer, Rik, Yingying, Fanny, Evelien, Renske, Arjan, Rosalie, Alison, Carlijn, Julia en Michiel**. Bedankt dat jullie mijn tijd op het dak zo leuk hebben gemaakt!

Lieve **Kèren, Annabel, Jiska, Emily, Bas, Margreet en Tinus**. Dank jullie wel dat jullie altijd begrip hebben gehad voor de vele uren die ik moest werken om dit voor elkaar te krijgen en me altijd hebben gesteund. Ik bof maar met jullie, en ik ben heel blij dat jullie er vandaag bij zijn (al dan niet in gedachten vanwege verre reizen). Ik beloof dat ik hierna mijn leven beter en meer aanwezig ben bij alle leuke dingen die gepland worden!

Lieve, **Roger, Eline, Cyril en Tea**, ik had me geen lievere en leukere schoonfamilie kunnen wensen. Dank jullie wel voor jullie interesse en humor, en ik ben heel erg blij dat ik dit moment vandaag met jullie mag delen.

Allerbeste paranimfen van de hele wereld, **Jihan en Lisanne**.

Lieve **Lies**, van begin af aan waren wij kamergenootjes bij congressen en al snel werden we ook vriendinnen. Onze gedeelde liefde voor muziek, eten en wijn heeft voor veel mooie herinneringen gezorgd waaronder onze vakantie. Meer dan eens hebben we zo hard gelachen dat we niet meer konden praten (en dat kunnen we normaal gesproken allebei toch wel goed). Ik baal er nog steeds van dat ik niet bij jouw promotie kon zijn, maar gelukkig jij wel bij de mijne, op een voor mij hele bijzondere plaats. Dankjewel voor alle mooie jaren, en we gaan er nog een hoop tegemoet!

Lieve **Ji**, ik heb het je al eens verteld, maar toen ik je net ontmoette dacht ik 'wie ís dit?'. Roze kerstboom, kauwgom, cola light, en een hele duidelijke eigen mening en wil. Toch hadden we al heel snel veel lol samen, met jouw eerste kampeerervaring op Rock Werchter toch wel als een van de hoogtepunten (first of many). Je bent niet alleen regelmatig ontzettend grappig, maar je staat ook

altijd voor me klaar. Dankjewel dat ook jij vandaag naast me staat, ook zeker niet voor de laatste keer.

Lieve **Jolien**, ondanks het feit dat je zo'n zwaar jaar hebt gehad heb je altijd tijd voor mij gemaakt. Ook al wonen we niet bij elkaar om de hoek, je hebt me er met de vele telefoontjes toch veel geholpen. Dankjewel dat je hier vandaag bent. Ik ben trots op je!

Lieve **papa en mama**, hier is het langverwachte boekje dan. Ik heb van jullie altijd alle mogelijkheden en onvoorwaardelijke steun gekregen om te doen wat ik graag wilde, ook al betekende dat soms dat jullie mij langere tijd niet zagen of weinig spraken. Ik heb van jullie geleerd om positief in het leven te staan, anderen te helpen, en hard te werken voor wat je wilt bereiken. Al die dingen hebben ervoor gezorgd dat ik hier vandaag sta, ik kan jullie niet genoeg bedanken.

Lieve **Mies**, als geen ander weet jij hoeveel tijd en energie in dit boekje zit, maar ook als geen ander heb jij achter me gestaan. In alle hectiek van een nieuwe baan in de kliniek en het afronden van mijn promotie heb jij me met beide benen op de grond gehouden; zonder de vrijheid en steun die je me heb gegeven was het me niet gelukt. Dank je wel voor alles.

Curriculum vitae

Margo van Campenhout werd op 14 juni 1988 geboren te Hulst. In 2006 behaalde zij haar gymnasiumdiploma aan het Reynaertcollege aldaar, waarna zij een jaar Biomedische Wetenschappen studeerde aan de Universiteit Gent. Vanaf 2007 studeerde zij geneeskunde aan de Erasmus Universiteit Rotterdam. Tijdens de studieperiode gaf zij als student-assistent les in lichamelijk onderzoek, werkte ze als teamleider van het studententeam Vaat- en Transplantatiechirurgie, en volgde zij keuzecoschappen in West-Pokot, Kenia. In 2013 werd het doctoraalexamen behaald met het afstudeeronderzoek 'Cytomegalovirus infection in liver transplantation recipients', begeleid door Prof. Dr. Herold Metselaar. Aansluitend werd na een oudste coschap Maag-Darm-Leverziekten in het Erasmus MC het artsexamen cum laude behaald. In november 2013 startte zij in het Erasmus MC onder begeleiding van Prof. Dr. Herold Metselaar en Prof. Dr. Harry Janssen promotieonderzoek naar het optimaliseren van hepatitis B behandeling. In deze periode heeft zij ook het deelcertificaat Basiskwalificatie Onderwijs 'Begeleiden van studenten' behaald. Per 1 mei 2017 is zij in opleiding tot Maag-Darm-Leverarts (opleider Prof. Dr. Janneke van der Woude), waarbij zij tot 2021 zal werken in het Reinier de Graaf Gasthuis in Delft en de laatste twee jaar van de opleiding zal volgen in het Erasmus MC. Ze woont in Rotterdam, samen met Michel Ruijters.

Bibliography

Serum hepatitis B virus RNA level may help to select patients for peginterferon add-on therapy in HBeAg-positive chronic hepatitis B infection
van Campenhout MJH, van Bömmel F, Pfefferkorn M, Fischer J, Deichsel D, Brouwer WP, Qi X, Zhang Q, Tabak F, Streinu-Cercel A, Wang JY, Boonstra A, van Vuuren AJ, Berg T, Hansen BE, Janssen HLA.
In preparation

HBV RNA in serum as early predictor for outcome of pegylated interferon alfa treatment in HBeAg-negative chronic hepatitis B
Farag MS, van Campenhout MJH, Pfefferkorn M, Fischer J, Deichsel D, Boonstra A, van Vuuren AJ, Ferenci P, Feld JJ, Berg T, Hansen BE, van Bömmel F, Janssen HLA.
In preparation

Serum hepatitis B virus RNA predicts response to peginterferon treatment in HBeAg-positive chronic hepatitis B
van Campenhout MJH, van Bömmel F, Pfefferkorn M, Fischer J, Deichsel D, Boonstra A, van Vuuren AJ, Berg T, Hansen BE, Janssen HLA.
Submitted

Serum hepatitis B virus RNA predicts response to peginterferon treatment in HBeAg-positive chronic hepatitis B
van Campenhout MJH, van Bömmel F, Pfefferkorn M, Fischer J, Deichsel D, Boonstra A, van Vuuren AJ, Berg T, Hansen BE, Janssen HLA.
Submitted

Diagnostic and analytical performance of the hepatitis B core-related antigen immunoassay in hepatitis B patients
van Halewijn GJ, Geurts van Kessel CH, Klaasse J, van Oord GW, de Knegt RJ, van Campenhout MJH, Boonstra A, van der Eijk AA.
Submitted

Hepatitis B Core-related Antigen Monitoring during Peginterferon Alfa Treatment for HBeAg-negative Chronic Hepatitis B
van Campenhout MJH, Rijckborst V, Brouwer WP, van Oord GW, Ferenci P, Tabak F, Akdogan M, Pinarbasi B, Simon K, de Knegt RJ, Boonstra A, Janssen HLA, Hansen BE.
Accepted J Viral Hepat. 2018

Long-term follow-up of patients treated with entecavir and peginterferon add-on therapy for HBeAg positive chronic hepatitis B infection: ARES long-term follow-up.
van Campenhout MJH, Brouwer WP, Xie Q, Guo S, Chi H, Qi X, Tabak F, Streinu-Cercel A, Wang JY, Zhang N, Idilman R, Reesink HW, Diculescu M, Simon K, Akdogan M, Mazur W, de Knegt RJ, Verhey E, Hansen BE, Janssen HLA; ARES Study Group.
J Viral Hepat. 2018 Sep 5.. [Epub ahead of print]

Host and viral factors associated with serum hepatitis B virus RNA levels among patients in need for treatment.
van Campenhout MJH, van Bömmel F, Pfefferkorn M, Fischer J, Deichsel D, Boonstra A, van Vuuren AJ, Berg T, Hansen BE, Janssen HLA.
Hepatology. 2018 Sep;68(3):839-847.

Similar frequencies, phenotype and activation status of intrahepatic NK cells in chronic HBV patients after long-term treatment with tenofovir disoproxil fumarate (TDF).
Tjwa ET, Zoutendijk R, van Oord GW, Boeijen LL, Reijnders JG, van Campenhout MJH, de Knegt RJ, Janssen HL, Woltman AM, Boonstra A.
Antiviral Res. 2016 Aug;132:70-5.

Hepatitis B core-related antigen levels are associated with response to entecavir and peginterferon add-on therapy in hepatitis B e antigen-positive chronic hepatitis B patients.
van Campenhout MJH, Brouwer WP, van Oord GW, Xie Q, Zhang Q, Zhang N, Guo S, Tabak F, Streinu-Cercel A, Wang J, Pas SD, Sonneveld MJ, de Knegt RJ, Boonstra A, Hansen BE, Janssen HL.
Clin Microbiol Infect. 2016 Jun;22(6):571.e5-9.

CMV Primary Infection Is Associated With Donor-Specific T Cell Hyporesponsiveness and Fewer Late Acute Rejections After Liver Transplantation.
Shi XL, de Mare-Bredemeijer EL, Tapirdamaz Ö, Hansen BE, van Gent R, van Campenhout MJH, Mancham S, Litjens NH, Betjes MG, van der Eijk AA, Xia Q, van der Laan LJ, de Jonge J, Metselaar HJ, Kwekkeboom J.
Am J Transplant. 2015 Sep;15(9):2431-42. doi: 10.1111/ajt.13288. Epub 2015 May 5.

How to achieve immune control in chronic hepatitis B?
van Campenhout MJ, Janssen HL.
Hepatol Int. 2015 Jan;9(1):9-16.

Bachmann's bundle: a key player in the development of atrial fibrillation?
van Campenhout MJH, Yaksh A, Kik C, de Jaegere PP, Ho SY, Allessie MA, de Groot NM.
Circ Arrhythm Electrophysiol. 2013 Oct;6(5):1041-6.

The best way to prevent cytomegalovirus infection after liver transplantation: the debate goes on.
Metselaar HJ, van Campenhout MJH, van der Eijk AA.
Transpl Int. 2013 Jun;26(6):590-1.

PhD Portfolio

SUMMARY OF PHD TRAINING AND TEACHING

Name PhD student: Margo J.H. van Campenhout

Erasmus MC Department: Gastroenterology and Hepatology

PhD period: 2013-2018

Promotor(s): Prof. Dr. H.J. Metselaar, Prof. dr. H.L.A. Janssen

Supervisor: Dr. B.E. Hansen

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
- Systematic Literature Searching in Pubmed	2013	1 ECTS
- Systematic Literature Searching in Embase, Medline, Cochrane	2013	12 hours
- Database and Web-of-Science		
- Endnote	2013	3 hours
- BROK ('Basiscursus Regelgeving Klinisch Onderzoek', GCP course)	2014	4 hours
- Research Integrity	2015	0.3 ECTS
- Regression analysis	2014	1.4 ECTS
- Biomedical English Writing and Communication	2015	3 ECTS
Specific courses (e.g. Research school, Medical Training)		
Research management for PhD students	2014	8 hours
Awards		
Full bursary from the European Association for the Study of the Liver (EASL) awarded to the best scored abstracts at EASL Special Conference: Optimal Management of Hepatitis B Virus Infection.	2014	
Registration bursary from the European Association for the Study of the Liver (EASL) awarded to high-level abstracts at 50th EASL International Liver Congress.	2015	
Young Investigator Travel Award, American Association for the Study of Liver Diseases (AASLD) Foundation, awarded to promising early-career investigators	2017	

Poster presentations		
Determinants of HBsAg response induced by addition of peginterferon to entecavir in HBeAg-positive chronic hepatitis B. EASL Special Conference: Optimal Management of Hepatitis B Virus Infection	2014	32 hours
Hepatitis B core-related antigen may be a marker for immune control in HBeAg-negative chronic hepatitis B infection. 50th EASL International Liver Congress	2015	32 hours
Hepatitis B Core-Related Antigen Levels are Inversely Correlated With Fibrosis Stage in HBeAg-Positive Chronic Hepatitis B. The Liver Meeting 2015. 66th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). San Francisco, United States of America	2015	32 hours
Hepatitis B Core-Related Antigen Level Decline in the First 12 Weeks of Peginterferon Treatment is Associated with Response in HBeAg-Negative Chronic Hepatitis B. The Liver Meeting 2015. 66th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). San Francisco, United States of America.	2015	32 hours
Clinical evaluation of hepatitis B core-related antigen monitoring during peginterferon alfa treatment for HBeAg-negative chronic hepatitis B 52th EASL International Liver Congress, Amsterdam, The Netherlands.	2017	32 hours
Serum HBV RNA level is associated with HBV genotype and BCP mutations in untreated patients with HBeAg positive chronic hepatitis B 52th EASL International Liver Congress, Amsterdam, The Netherlands.	2017	32 hours
Serum HBV RNA level monitoring for response prediction in HBeAg positive chronic hepatitis B infection 52th EASL International Liver Congress, Amsterdam, The Netherlands.	2017	32 hours
Oral presentations		
Hepatitis B core related antigen levels are associated with response to ETV and PEG-IFN treatment in HbeAg-positive chronic hepatitis B patients. Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands	2014	12 hours
Hepatitis B Core-Related Antigen Level Decline in the First 12 Weeks of Peginterferon Treatment is Associated with Response in HBeAg-Negative Chronic Hepatitis B. Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands	2015	12 hours
Addition of (pegylated) interferon to entecavir increases serological response in treatment naïve, Hepatitis B e Antigen positive patients with chronic hepatitis B Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands	2016	12 hours
Serum HBV RNA level predicts response to peginterferon add-on therapy for HBeAg-positive chronic hepatitis B The Liver Meeting 2017. 68th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). Washington D.C,, United States of America.	2017	12 hours

(Inter)national conferences		
7th Paris Hepatitis Conference	2013	12 hours
49th EASL International Liver Congress, London, UK	2014	28 hours
Twice annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands	2014	12 hours
EASL Special Conference: Optimal Management of Hepatitis B Virus Infection	2014	12 hours
The Liver Meeting 2014. 65th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). Boston, MA, United States of America	2014	28 hours
50th EASL International Liver Congress, Vienna, Austria	2015	28 hours
The Liver Meeting 2015. 66th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). San Francisco, USA	2015	28 hours
Nationale Hepatitis Dag. Amsterdam, The Netherlands.	2015	8 hours
51th EASL International Liver Congress, Barcelona, Spain	2016	28 hours
52th EASL International Liver Congress, Amsterdam, The Netherlands.	2017	28 hours
Attended seminars and workshops		
5e Lagerhuisdebat Hepatitis B en C. Utrecht, The Netherlands	2013	2 hours
1th Post-EASL symposium. Amsterdam, The Netherlands	2014	2 hours
12th Post-AASLD symposium. Rotterdam, The Netherlands	2014	2 hours
6e Lagerhuisdebat Hepatitis B en C. Utrecht, The Netherlands	2014	2 hours
2nd Post-EASL symposium. Amsterdam, The Netherlands	2015	2 hours
Other		
-		
2. Teaching		
Deelcertificaat Basiskwalificatie onderwijs (BKO)		
- Training 'Basisdidactiek'	2009	4 hours
- Training 'Omgaan met groepen'	2014	4 hours
- Training 'Individuele begeleiding'	2014	1 ECTS
- Training 'Individuele voortgangsgesprekken'	2016	3 hours
Lecturing		
A case with hepatitis B Erasmus Liverday 2016	2016	12 hours
Hepatitis B Minor 'Infectieziekten in de grote stad', Bachelor Geneeskunde (Faculty of Medicine, Erasmus University)	2016	12 hours
Supervising practicals and excursions, Tutoring		
Tutoring first-year bachelor students (Faculty of Medicine, Erasmus University)	2014	1.5 ECTS
Tutoring 'Kennismaking met de Beroepspraktijk' (Faculty of Medicine, Erasmus University)	2015	5 hours
Tutoring first-year bachelor students (Faculty of Medicine, Erasmus University)	2015	1.5 ECTS
Tutoring 'Kennismaking met de Beroepspraktijk' (Faculty of Medicine, Erasmus University)	2016	5 hours
Supervising Master's theses		
- *Long-term follow-up of patients treated with interferon alpha* - Cedric de Jong, Master Geneeskunde		
Other educational activities		
Assessing physical examination exams in third-year bachelor students (Faculty of Medicine, Erasmus University)	2014	6 hours
Shared coordination of the third-year minor program in Gastroenterology & Hepatology (Faculty of Medicine, Erasmus University)\	2015	12 hours