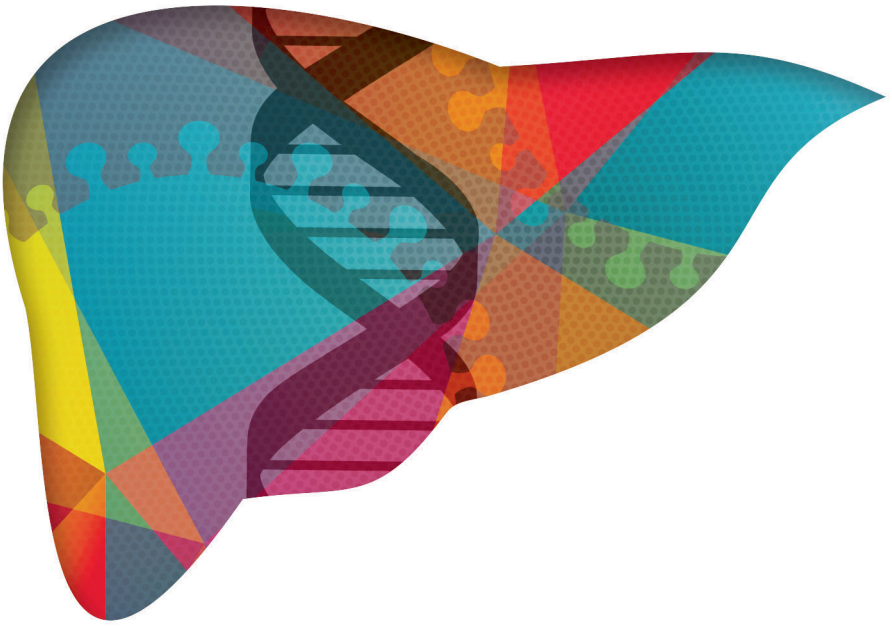
Individualized management of patients with chronic hepatitis B

Willem Pieter Brouwer



INDIVIDUALIZED MANAGEMENT OF PATIENTS WITH CHRONIC HEPATITIS B

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met een chronische hepatitis B infectie

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CHAPTER 1

General introduction

Based on:

1. Brouwer WP, Janssen HLA. New strategies for finite antiviral therapy in chronic hepatitis B patients: EASL Postgraduate Course 2014, EASL International Liver Congress, London, April 2014

2. Sonneveld MJ, Brouwer WP, Janssen HLA. Response-guided Peginterferon Therapy for HBeAg-positive and HBeAg-negative Chronic Hepatitis B using Hepatitis B Surface Antigen Levels: A Review. *Curr Hepatology Rep (2014) 13:250–255. DOI 10.1007/s11901-014-0239-1*

CLINICAL SIGNIFICANCE OF A CHRONIC HEPATITIS B INFECTION

The hepatitis B virus (HBV) is one of the smallest enveloped double-stranded DNA viruses, belonging to the family of *hepadnaviridae*. HBV replicates via an RNA intermediate and causes both acute and chronic hepatitis. The *Australia antigen*, now known as the hepatitis B surface antigen (HBsAg) – which is the hallmark of a chronic hepatitis B (CHB) infection – was discovered by Dr. Baruch Samuel Blumberg, for which he received the Nobel prize in Medicine in 1976.

Approximately 2 billion people worldwide have been in contact with HBV. Of these, circa 240 – 400 million people are chronically infected. The prevalence of CHB infection is high (> 8%) in resource poor countries, such as in East Asia, sub-Saharan Africa, and in the Amazon, and intermediate (2 – 7%) in the Mediterranean basin, Eastern Europe and Russia. The prevalence in Western countries is low (less than 2%).[1] HBV can survive outside the body for at least 7 days. During this period, it may still cause an acute or chronic infection to people who are not protected by vaccination.[2] HBV is transmitted by percutaneous and mucous membrane exposure to infected body fluids such as serum, saliva and semen.[1, 2] The major route of transmission is thought to be vertical, especially in high-endemic areas. Also horizontal infections occur during early childhood. Among adults, high-risk sexual behavior is the most important risk factor for HBV infection.[1, 2] HBV is predominantly found in the liver as it has a strong preference for infecting hepatocytes.[3] It is thought that HBV is not directly cytopathic, while the proteins produced by the virus and the viral mini-chromosome – also known as covalently closed circular DNA (cccDNA) – may have carcinogenic effects. Moreover, the host immune response directed at infected hepatocytes leads to inflammation of the liver, which results in liver damage, fibrosis, and the development of liver cirrhosis, decompensation and hepatocellular carcinoma (HCC).[1, 4] Overall, 30% of cirrhosis and 53% of all HCC is attributable to CHB infection.[4] Worldwide, approximately 780.000 patients die each year due to an HBV infection: 650.000 due to the sequelae of CHB (cirrhosis, HCC) and another 130.000 from acute HBV.[5]

NATURAL HISTORY OF CHRONIC HEPATITIS B AND MARKERS OF INFECTION

After entry of HBV into the hepatocyte, cores of the virus are presented to the cytosol and transported to the nucleus where DNA genomes are converted to a covalently closed circular

DNA (cccDNA) form.[3, 6] This cccDNA found in the nuclei of hepatocytes serves as a template for viral protein production and replication. The cccDNA utilizes the hosts cellular transcriptional machinery for further replication and production of viral proteins. Because cccDNA is camouflaged as a mini-chromosome, it remains undetectable for the host immune system. Next to the formation of this mini-chromosome, integrations of HBV DNA sequences into the host DNA also occur, especially in the presence of cell turnover or DNA damage.[7] These HBV DNA sequences are generally truncated. cccDNA consists of 4 overlapping genes encoding the viral envelope (pre-S and S), nucleocapsid (precore and core), polymerase with reverse transcriptase activity and the hepatitis B X protein.[3, 7, 8] From cccDNA 4 major viral RNA species are produced, which subsequently lead to the formation of the hepatitis B core antigen (HBcAg), hepatitis B envelope antigen (HBeAg), the Pol protein, the viral envelope proteins which express HBsAg, and the hepatitis B X antigen (HBxAg), which is thought to modify the epigenetic regulation of cccDNA function.[9, 10] The HBV circulates in the blood of infected patients as the 42 nanometer Dane particle, which comprises of an outer envelope which contains HBsAg, and an inner nucleocapsid of HBcAg, containing the viral genome.[3] Also non-infectious filamentous and spherical subviral fragments are found in serum, of which the biological function is unclear.[7] There is a high spontaneous error rate of the viral reverse transcriptase, which leads to further evolution of the virus with a rate of 1.4 – 3.2 x 10^{-5} nucleotide substitutions per site per year.[8] This has resulted in a diverse HBV genome. The HBV has been classified into 8 genotypes A – H, of which most have been further divided into sub-genotypes. These HBV genotypes are thought to have significant implications for response to antiviral therapy, and may influence the natural history and clinical outcome of CHB patients.[3, 8]

HBV DNA and the viral proteins found in serum are generally used to classify different phases of infection as they reflect the viral replication activity and the occurrence of common viral escape mutants, such as mutations in the precore (PC) and basal core promotor (BCP) regions. CHB infection is defined as serum HBsAg positivity for more than 6 months,[11-13] and is generally regarded as a 5 phase disease continuum.[12, 13] The immunopathogenesis depends on a complex interplay between host and viral factors. When infected in early childhood, over 90% of patients become chronically infected, while less than 5% of adults who contract the virus become chronically infected.[1, 12] The first phase of HBV infection is defined as the *immune tolerance (IT)* phase. Characteristic for this phase are the very high levels of serum HBV DNA, HBeAg seropositivity and normal levels of ALT, indicating no inflammation of the liver, and probably no immune pressure directed at the virus.(Figure 1) Patients in the IT phase typically do not develop liver fibrosis or symptoms. Most patients will progress to the next phase, which is predominantly observed in young adults (20 – 30 years of age), and is referred to as the

HBeAg-positive *immune active (IA)* phase. In this phase, high and fluctuating levels of HBV DNA and serum ALT are observed as an indication for liver inflammation. As a result, patients generally develop the first signs and symptoms of infection, of which fatigue is the most frequent complaint.[12, 13] These patients are prone to develop liver fibrosis and, when the immune response remains inefficient, develop cirrhosis, liver decompensation, HCC and eventually decease due to the complications of CHB. Current treatment guidelines therefore recommend to start antiviral therapy in this phase to halt the progression of liver disease.[12, 13] Patients enter the *inactive carrier (IC)* phase when an effective immune response leads to immunological control of the virus. This phase is defined by HBeAg negativity, low levels of HBV DNA, and normal serum ALT levels. Patients in the IC phase have a favourable long-term outcome and a minimal risk of developing HCC, and a higher probability of achieving serum HBsAg loss over time.[14, 15] However, these patients may also progress to have chronic HBeAg-negative hepatitis, referred to as the *reactivation phase.* This phase is associated with an escape of the immunological control, most likely due to the immune pressure which may lead to a diverse subpopulation of viral escape mutations.[12, 13] Again high levels of HBV DNA and ALT are observed as a reflection of viral replication and liver inflammation. In this phase antiviral therapy is indicated as well, as this is associated with further deterioration to liver cirrhosis and subsequent risk of decompensation and HCC development.[12-14, 16] It is of vital importance to accurately identify putative "inactive carriers" who remain at risk of developing reactivation and hepatitis. The last phase of CHB infection would be the loss of serum HBsAg. This is considered the closest to eradication of HBV and is associated with disease remission and an improved prognosis.[17]

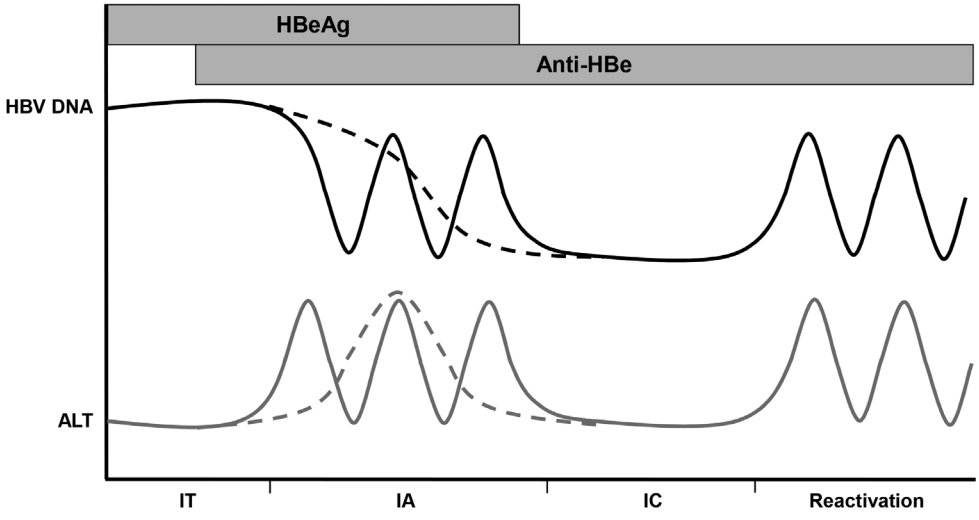

Figure 1 Clinical phases of a chronic hepatitis B infection

HOST GENETICS AND VIRAL MARKERS IN THE NATURAL HISTORY OF CHB INFECTION

Viral markers associated with the prognosis of CHB patients

For decades the gold standard to assess the prognosis of CHB patients has been liver biopsy. However, this modality is associated with several major limitations. Because of the invasive nature, there is a potential of severe complications, such as bleeding, hospitalization and death. Other examples of limitations are the inter-observer and intra-observer variability, and sampling error. It is therefore that researchers have been looking for other ways to assess the degree of liver fibrosis, inflammation and the related prognosis in a non-invasive manner. This is clinically important as patients with a high risk of disease progression may need more intensive follow-up and may require antiviral therapy.

Currently, there are several non-invasive host and viral biomarkers that have been linked with a worse prognosis. Viral markers which are associated with a worse prognosis are serum HBeAg positivity and higher levels of HBV DNA.[14, 18, 19] Moreover, among HBeAg-negative patients, it has been shown that compared to patients with low HBV DNA levels, patients with HBV DNA >2,000 IU/mL have a higher risk of HCC development.[14, 18] Quantitative HBsAg levels have been shown to vary significantly during the different phases of chronic HBV infection and appear to correlate with the state of immune control: lower levels are associated with a higher level of immune control. These findings suggest that HBsAg quantification may provide insight into the complex interplay between the host immune system and the HBV.[20-23] HBsAg levels in serum reflect not only HBsAg present in complete viral particles, but mainly comprise defective HBsAg particles which do not contain HBV DNA. These non-infectious sub-viral particles are produced in excess of the complete Dane particles. As a result, serum HBsAg levels are considered to better reflect the total transcriptionally active HBV in the liver than do serum HBV DNA levels.[24] HBsAg levels may thus better reflect the HBV phase and the level of immune control.[25-28] Moreover, several studies attributed HBsAg levels to disease progression and development of HCC in patients with low viral loads.[29-31] Therefore, previous studies have attempted to use HBsAg to predict the probability of hepatitis reactivation for IC patients. It was shown that a single-point measurement of HBsAg <1,000 IU/mL combined with an HBV DNA level <2,000 IU/mL could be used as a marker to accurately identify IC patients one year later with a high specificity and sensitivity in HBV genotype D patients.[32] However, HBsAg levels at a single-point were shown to have a poor diagnostic performance to identify maintained IC over 13 years of follow-up in a large Taiwanese cohort.[30] One of the reasons for this observation may be the remittent pattern of HBV fluctuations followed by long-term

remissions in patients with HBeAg-negative CHB. Therefore it seems that the risk prediction should be updated over time, at least every few years.[33] Longitudinal studies to assess the long-term individual risk of progression to active disease in relation to repeated HBsAg level measurements are highly warranted.

Host factors and genetics associated with the prognosis of CHB patients

Only recently improved techniques have enabled researchers to study common variations in the human genome, known as single-nucleotide polymorphisms (SNPs). This has opened avenues to study the impact of host genetics on many complex human traits and diseases. Many such studies have shown associations between SNPs and liver disease, such as a predisposition for alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD) and progression of liver fibrosis.[34-39] The I148M SNP in the *PNPLA3* gene was found to predispose patients to steatosis and steatohepatitis.[40] These conditions may lead to cirrhosis in 10-29% of individuals within 10 years. Moreover, 4-27% of patients with steatohepatitis associated cirrhosis develop HCC.[41] The 148M variation was also associated with the progression of liver disease, and with the development of HCC in patients with a chronic hepatitis C infection.[34-38] The impact of NAFLD on the progression of CHB related liver disease is still unknown.[42-44] Previous studies indicated that approximately one-third of patients with CHB suffer from NAFLD, although the reported prevalence varied widely.[42-44] In patients with CHB, the association between *PNPLA3*, liver histology and long-term clinical outcome has not been studied so far. Therefore, it is currently unknown whether there may be a clinical advantage in testing this candidate SNP for diagnostic or prognostic purposes for individual CHB patients.

Next to host genetics, recent studies have investigated the association and performance of risk scores for cirrhosis, HCC and mortality among patients with a CHB or HCV infection.[45-56] These risk scores consists of readily available clinical parameters, such as ALT, AST, thrombocytes, HBeAg status, HBV DNA levels and the gender and age of patients. None of these studies however have assessed the added value of non-invasive markers combined with liver histologic characteristics in detail. Moreover, it is unknown whether the scores developed in HCV infected patients may be useful for CHB patients.

TREATMENT GOALS AND OPTIONS

The goal of antiviral therapy is to improve the quality of life and survival by preventing the progression of liver disease, initially by means of immunologic control of the hepatitis B virus (HBV), and ultimately through complete eradication.[13] The indications for treatment are based on the combination of serum HBV DNA and ALT levels, and the severity of liver disease. Patients should be considered for treatment when having HBV DNA levels >2,000 IU/mL and serum ALT levels above the upper limit of normal (ULN), with moderate to severe active necroinflammation and/or at least moderate fibrosis by liver biopsy.[12, 13] The treatment of CHB has considerably been improved over the past decades with the emergence of pegylated interferon (PEG-IFN), an immune modulator with antiviral capacity, and the potent nucleos(t)ide analogues (NA), which inhibit HBV DNA polymerase and suppress HBV DNA replication. NA is given orally once-daily, and PEG-IFN is given as a once-weekly subcutaneous injection. The duration of treatment with NA is indefinite, while the standard duration of a PEG-IFN course is 48 weeks.[12, 57]

With the current first-line treatment regimens complete eradication of HBV is only rarely achieved, as cccDNA persists in hepatocytes, often even after the loss of serum HBsAg.[28] Moreover, serum HBsAg loss during antiviral therapy occurs in only in a minority of patients. Other surrogate endpoints are therefore adopted to evaluate treatment efficacy.[12, 13] These endpoints are associated with an improvement in clinical outcome and occur more frequently, and differ according to HBeAg status (positive or negative) and treatment regimen (IFN or NA).[12] The different therapeutic endpoints are categorized in biochemical, serological, virological and histological response. Biochemical response is defined as a normalization of serum ALT levels. Serological response for HBeAg applies to HBeAg-positive patients who have HBeAg loss and appearance of anti-HBe (HBeAg seroconversion), and serological response for HBsAg applies to all CHB patients and is defined as HBsAg loss with the appearance of anti-HBs (HBsAg seroconversion). The definition of virological response depends on the type of antiviral therapy. For PEG-IFN, this is usually an HBV DNA level <2,000 IU/mL (i.e., off-treatment immune control), while this is <20 IU/mL for patients on NA (i.e., maintained suppression). The efficacy of PEG-IFN therapy is evaluated at the end of treatment, and 6 and 12 months during off-treatment follow-up. For HBeAg-positive patients the aim of treatment is HBeAg seroconversion with virological response, and for HBeAg-negative patients a sustained virological response.[12, 13]

TREATMENT STRATEGIES AND CURRENT UNMET NEEDS

Present recommended first-line NA's with a high-barrier to viral resistance are entecavir (ETV) and tenofovir (TDF). Other less potent NA's, which are associated with viral resistance, are adefovir (ADV), telbivudine (LdT) and lamivudine (LAM). Treatment with potent NA maintains viral suppression in over 90% during continuous therapy, improves liver histology, reduces the risk of liver failure and HCC development.[58-62] NA's have gained popularity as these are easy to use and are generally well-tolerated. However, as HBsAg loss is seldom achieved and virological and serological response are not sustained off-treatment in most cases, the duration of NA therapy is indefinite, and possibly lifelong.[28, 29] Moreover, long-term administration is associated with moderate adherence, may select for drug-resistant mutants, and has a considerable impact on health care costs.[63, 64] Furthermore, the long-term safety profile is unknown. This urges physicians to find the best strategy to achieve a sustained off-treatment response. In contrast, response to PEG-IFN is frequently sustained after a finite treatment course due to its immune modulating capacity.[65] After one year of treatment, a subset of patients achieve HBeAg loss (approximately 30%), which leads to high rates of HBsAg loss during long-term follow-up in those with an initial favorable response. This results in a reduced incidence of HCC and a prolonged survival.[65-67] However, a limitation of PEG-IFN therapy is the occurrence of considerable side-effects. Most frequent side-effects observed during PEG-IFN therapy are flu-like symptoms, fatigue, myalgia, headache, bone-marrow suppression, depression, alopecia and other skin reactions such as rash, eczema and pruritus.[57, 68, 69]

As NA therapy does not seem to induce off-treatment immune control of HBV,[70, 71] HBsAg loss or seroconversion appears the best endpoint.[62, 72] As these endpoints are seldom achieved with NA therapy, new treatment strategies facilitating the discontinuation of NA are required.[73] Previous studies have shown that HBV influences the innate and adaptive immune response, primarily through HBeAg, which may aid in viral persistence.[74] ETV therapy has been shown to partially restore this adaptive immunity, while IFN prevents the formation of HBV proteins and depletes the intrahepatic cccDNA pool. This leads to more HBsAg loss when compared to ETV.[75-78] Combining these potent first-line treatment regimens may therefore lead to higher response rates. Previous randomized trials showed a beneficial effect in terms of on-treatment HBsAg decline and HBeAg response for a *de novo* combination of LAM or TDF with PEG-IFN. However, the achieved response rates were not sustained off-treatment, questioning the role of NA consolidation therapy after combining PEG-IFN and NA.[68, 69, 79-81] It remains elusive whether other treatment strategies such as PEG-IFN add-on or sequential NA – PEG-IFN

therapy may lead to higher response rates, and may result in a larger proportion of patients able to discontinue NA therapy.

HOST GENETICS AND VIRAL MARKERS TO SELECT PATIENTS WITH A HIGH PROBABLITY OF RESPONSE TO PEG-IFN: WHAT IS CURRENT PRACTICE?

Viral markers and response to PEG-IFN

As only a small subset of patients respond favourably to PEG-IFN therapy, it is of importance to only select those patients with a high chance to achieve a response. Response to PEG-IFN therapy is associated with age, gender, HBV genotype and baseline levels of HBV DNA and serum ALT, and possibly with failure of previous IFN therapy.[82, 83] Response has also been associated with intrahepatic HBV DNA and cccDNA concentrations,[84] however, this can only be assessed invasively with liver biopsy, limiting the clinical use of these markers. Recently, several independent studies have shown that the change of serum HBsAg levels during PEG-IFN therapy mimics the change of both intrahepatic cccDNA and intrahepatic HBsAg. This suggests that a decline in serum HBsAg levels is associated with the induction of an effective anti-HBV immune response.[26, 77, 85, 86] Also, baseline HBsAg levels are associated with response to PEG-IFN.[87] It has been shown that based on on-treatment HBsAg levels patients with a very low probability of response can be identified at week 12 of PEG-IFN therapy. Stopping rules based on HBV DNA and HBsAg levels have therefore been developed for both HBeAg-positive and HBeAg-negative patients.[88, 89] Nevertheless, the current stopping rules have a suboptimal performance for HBeAg-negative patients and therefore other predictors of response, especially before treatment is initiated, are demanded. One possible viral candidate-marker is the hepatitis B core-related antigen (HBcrAg), which is a combined measure of three proteins coded by the precore/core region of the cccDNA: HBcAg, HBeAg, and a 22-kDa precore protein (p22cr). It was recently demonstrated that HBcrAg correlates with intrahepatic cccDNA in CHB patients.[90] Moreover, HBcrAg could be detected in patients with loss of HBsAg or in patients with undetectable serum HBV DNA levels.[91, 92] A decline in serum HBcrAg levels could therefore reflect a decline in intrahepatic cccDNA and may as a consequence predict response to treatment. HBcrAg dynamics have been described in natural history and during NA therapy,[93, 94] but it is unknown how HBcrAg levels are influenced by PEG-IFN therapy.

Host genetics and response to PEG-IFN

Recently, several genome wide association studies (GWAS) identified genetic polymorphisms of the *IL28B* gene that were shown to be associated with treatment response to IFN and ribavirin in patients with chronic hepatitis C.[95-98] The same polymorphisms were also associated with natural clearance of hepatitis C virus.[95] In CHB patients, this association also seemed apparent for PEG-IFN treatment response in both HBeAg-positive and HBeAg-negative patients.[99, 100] Therefore this candidate SNP may play a role in the response to PEG-IFN in CHB, although more recent reports on the association of *IL28B* and response to PEG-IFN have been contradictory.[99, 101] This has limited the use of *IL28B* polymorphisms in clinical practice. Whether the candidate *HLA-DP* polymorphisms that are associated with the natural clearance of HBV are also associated with PEG-IFN response remains to be determined.

SCOPE AND AIMS OF THIS THESIS

The aims of the current thesis were 1) to assess the risk of hepatitis activity and disease progression during long-term follow-up for individual CHB patients, by using host genetic and viral markers; 2) to investigate a PEG-IFN add-on approach to highly potent NA for HBeAg-positive CHB patients, in order to optimize the treatment strategy; and 3) to assess the association between viral and host genetic markers and PEG-IFN response to improve the pre-treatment selection of CHB patients with the highest probability of response.

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PART I

INDIVIDUALIZED MANAGEMENT OF CHRONIC HEPATITIS B PATIENTS

CHAPTER 2

HBsAg levels to define HBeAg negative chronic hepatitis B phases during long-term follow-up using repeated measurements

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Submitted

ABSTRACT

Background & aims

HBsAg levels in combination with HBV DNA may help to identify inactive carriers (IC). We assessed the performance of repeated HBsAg measurements through long-term follow-up.

Methods

We enrolled 292 HBeAg-negative non-cirrhotic patients with a normal ALT and HBV DNA <20,000 IU/mL at 8 tertiary centers. IC (HBV DNA <2,000 IU/mL and normal ALT) or HBV activity (HBV DNA fluctuation >2,000 IU/mL ± abnormal ALT) was assessed after each follow-up year. Patients were followed for a median of 8 years (range 4 – 9). Dynamic regression analysis was used to study changes in HBsAg levels and HBV phase and to update the HBV activity risk.

Results

After 1 year, 189 (65%) patients were IC and 103 (35%) had HBV activity. The probability to remain IC any given following year was 97% for HBsAg levels <100, versus 85% and 76% for 100 – 1,000 or >1,000 IU/mL respectively ($p<0.001$). IC during 2 consecutive years was predictive of IC in the following year, still 15% of patients with HBsAg >100 IU/mL showed HBV activity. An HBsAg <100 IU/mL combined with HBV DNA <2,000 IU/mL could predict maintained IC with a specificity of 98% and positive predictive value of 97% across all HBV genotypes. Patients with HBV activity and an HBV DNA <5,000 IU/mL & HBsAg decline of ≥0.5 log IU/mL had a high probability to become IC.

Conclusion

HBsAg levels <100 IU/mL are highly specific for the IC phase and should be used to define HBV phases in HBeAg negative HBV infection.

INTRODUCTION

The natural history of chronic hepatitis B (CHB) is generally regarded as a five phase disease continuum, with an immune tolerance and immune activity phase in hepatitis B e antigen (HBeAg) positive patients, and an inactive or active phase in HBeAg-negative patients.[1-3] The last phase of CHB infection would be the loss of serum hepatitis B surface antigen (HBsAg), which is a rare event. Patients in the inactive carrier (IC) phase have a favourable long-term outcome and a minimal risk of developing hepatocellular carcinoma (HCC), and a higher probability of achieving serum HBsAg loss over time.[4, 5] However, these patients may also progress to have chronic HBeAg-negative hepatitis, which is associated with further deterioration to liver cirrhosis and subsequent risk of decompensation and HCC development.[1-4] It is therefore of importance to accurately distinguish those patients who are regarded IC and still at risk of developing hepatitis.

To date, there is no definite consensus on the definition of the different phenotypes of CHB. According to American and European guidelines patients are IC if HBV DNA is below 2,000 IU/mL with persistently normal serum ALT, while patients with an HBV DNA below 20,000 IU/mL are regarded as IC according to Asian-Pacific guidelines. The management of patients with an HBV DNA above 2,000 IU/mL but below 20,000 IU/mL is a grey area, as these patients may both progress to either active hepatitis or IC.[1-3] Nonetheless, the REVEAL HBV study showed that compared to patients with low HBV DNA levels, patients with HBV DNA >2,000 IU/mL have a ≥3-fold higher risk of HCC development, which may indicate that these patients should be regarded at risk of disease progression.[4, 6]

In HBeAg-negative patients, the HBsAg production and secretion seems to be separated from the production of virions, as HBsAg is produced in excess of the complete virion particles and circulates mainly as defective subviral fragments. Hence serum HBsAg levels are thought to better reflect the transcriptionally active HBV in the liver than serum HBV DNA levels in these patients, and may thus better reflect the HBV phase and the level of immune control.[7-10] Moreover, several studies attributed HBsAg levels to disease progression and development of HCC in patients with low viral loads.[11-13] It was therefore attempted to use the combination of both HBsAg and HBV DNA levels to predict the true IC phase. It was shown that a single-point measurement of HBsAg combined with HBV DNA could be used as a marker to identify IC patients one year later with high specificity and sensitivity in HBV genotype D patients, as well as patients with a high risk of reactivation.[14, 15] However, HBsAg levels at a single-point were shown to have a poor diagnostic performance over 13 years of follow-up in a large Taiwanese cohort.[12] Because HBeAg-negative CHB is associated with a remittent pattern of

HBV fluctuations followed by long-term remissions, and thus may be a recurrent event, it seems that the risk assessment should be updated over time.[16] Nevertheless, studies on long-term risk of progression to active disease in relation to repeated HBsAg level measurements have not yet been performed.

The aim of the current study was therefore to investigate whether we could further improve the existing rules by using repeated HBsAg level measurements for the prediction of IC through long-term follow-up, using a dynamic model with an annual update of the risk prediction.

PATIENTS AND METHODS

Patient selection. The medical charts of all consecutive non-cirrhotic CHB patients who visited at the outpatient clinic of 8 tertiary care centers during the period from 1990 to 2011 were reviewed. Treatment-naïve HBeAg-negative CHB (HBsAg positive for more than 6 months) with an HBV DNA ≤20,000 IU/mL and normal serum alanine aminotransferase (ALT) (below the upper limit of normal [ULN]) at presentation, and with available repeated HBsAg measurements, were initially eligible. Patients were excluded in case of liver cirrhosis, a history of a co-infection with hepatitis C, hepatitis delta, human immunodeficiency virus or in case of evidence of HCC, hepatic decompensation (jaundice, variceal bleeding, encephalopathy or ascites) and in case of a history of liver transplantation. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice and was approved by each ethical review board of the participating centers.

Data acquisition and follow-up assessment. Data on demographics (gender, age, race, ethnicity, height, weight, route of HBV transmission) and clinical history was obtained from the (electronic) chart by the treating physician. At every visit, data on chemistry (ALT, aspartate aminotransferase (AST), gamma-glutyltransferase (y-GT), bilirubin, albumin), hematology (platelet count, prothrombin time) and virology (serum quantitative HBsAg, anti-HBs, HBV DNA load, HBV genotype) were obtained from the clinical laboratory of each participating centre. The follow-up time was calculated from the date of inclusion until the last visit.

Laboratory measurements. Serum HBsAg was quantified using the Architect platform (Abbott Laboratories, Abbott Park, IL, USA) or the Elecsys HBsAg (Roche Diagnostics, Indianapolis, IN, USA). There is an excellent correlation between the HBsAg measurements for these two assays.[17] Antibodies to HBsAg (anti-HBs), hepatitis B core antigen (anti-HBc), hepatitis C, hepatitis D and human immunodeficiency virus were detected by commercially available

immunoassays. Serum HBV DNA levels were quantified by COBAS Amplicor Monitor 2.0 HBV assay (Roche Diagnostic Systems Inc, Mannheim, Germany) with a lower limit of detection of 200 copies/mL and linearity range from 200 to 20,000 copies/mL (conversion factor, 5.6 copies = 1 IU) until 2005 and thereafter by COBAS TaqMan assay, sensitivity 12 IU/mL, dynamic range 6.0 – 1.10 × 10^8 IU/mL. Gender, centre and time-dependent ULN values were used for the analysis of serum ALT.

Definitions and endpoints. At inclusion, all patients had an HBV DNA <20,000 IU/mL, a normal serum ALT (≤ULN) and no liver cirrhosis (HBV remission at single measurement). After each follow-up year patients were defined according to their biochemical and virological profile as follows: "Inactive carriers (IC)": HBV DNA ≤2,000 IU/mL and a persistently normal ALT observed at each visit within that follow-up year, according to clinical practice guidelines.[1, 2] Patients were regarded potentially IC in case of an HBV DNA <2,000 IU/mL with an occasional ALT 1 – 2 x ULN. "Intermediate": an HBV DNA fluctuation >2,000 IU/mL – 20,000 IU/mL observed while having a persistently normal ALT within that follow-up year. "Active carriers (AC)": patients with an HBV DNA >20,000 IU/mL, or at least a one-time fluctuation of HBV DNA >2,000 IU/mL – 20,000 IU/mL with an abnormal ALT (>ULN), or an ALT >2 x ULN while having an HBV DNA <2,000 IU/mL.[1, 2] In both dynamic as well as survival analysis, the event of transition from IC to intermediate or the AC state is referred to as "HBV activity", as this corresponds to HBV DNA levels >2,000 IU/mL and/or abnormal ALT levels, with a ≥ 3 fold increased probability of clinical disease progression.[1, 2, 4, 6, 12, 14] Other endpoints studied included hepatic decompensation, HCC, liver transplantation and death. The diagnosis of HCC was based on histopathology and when not available, on 2 imaging modalities (magnetic resonance imaging, computed tomography or contrast enhanced ultrasound).

Statistical analysis. Survival analysis was used to study the cumulative probability of HBV activity for IC patients over time. For this analysis the HBsAg level at inclusion was used and categorized in HBsAg levels <100, 100 – 1,000, and >1,000 IU/mL.[11-15] For the association analysis of multiple-point HBsAg measurements, a dynamic model was constructed, in which every 2 or 3 consecutive follow-up years were regarded as a random block in the natural history, to rule out potential time-dependent confounding (moving timeframe, supplementary figure 1). All information from multiple blocks were analyzed simultaneously for each patient. This approach allowed for changes in HBsAg, HBV DNA and thus the HBV phase over time. Both the maximum HBsAg level as well as the maximum combined HBV DNA and HBsAg level in each follow-up year was used to determine the probability of IC or HBV activity in the next year. Thus, a "visit-year" in dynamic analysis is an umbrella term for multiple visits per patient

within that follow-up year (the most aberrant combined measurement). Furthermore, the following sensitivity analyses were carried out: 1) defining IC patients after 3 years of inactivity according to the APASL guidelines,[3] 2) excluding patients with possible advanced liver disease, 3) excluding patients with a persistently low HBV DNA <2,000 IU/mL with an occasional ALT elevation <2 x ULN, and 4) excluding each participating centre one by one to rule out centre-specific effects (Supplementary file 1). SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.3 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

Role of the funding source. The study was organized and sponsored by the Foundation for Liver research, Rotterdam, the Netherlands. An unrestricted grant was provided by F. Hoffmann-La Roche Ltd., Basel, Switzerland. The funding sources did not have any influence on study design, data collection, analysis and interpretation of the data, writing of the report nor the decision to submit for publication.

RESULTS

Patient characteristics and follow-up. Of 529 HBeAg-negative patients, 110 (21%) were AC at baseline, 121 (23%) had no follow-up data and 6 (1%) had cirrhosis and were excluded from the analysis. Hence, 292 treatment-naïve CHB patients with repeated HBsAg measurements were included in the study. After 1 year of follow-up, 189 (65%) were defined as inactive carriers, 85 (29%) patients were intermediate and 18 (6%) were AC (thus in total 103 patients with HBV activity). The patient characteristics are shown in table 1. HBsAg levels differed across HBV genotype A, B, C and D with a mean level of 3.6, 2.4, 2.8 and 2.8 log IU/mL, respectively ($p<0.001$), and were in weak correlation with HBV DNA levels ($r=0.35$, $p<0.001$). In total, there were 2095 visits, with a median number of 11 visits (interquartile range [IQR] 5 – 15) per patient within a median of 7.8 years (IQR 4.1 – 9.2) of follow-up. The median number of visits per patient in the first year of follow-up was 3 (IQR 2 – 3), and was 2 (IQR 1 – 2) every follow-up year up to 8 years thereafter.

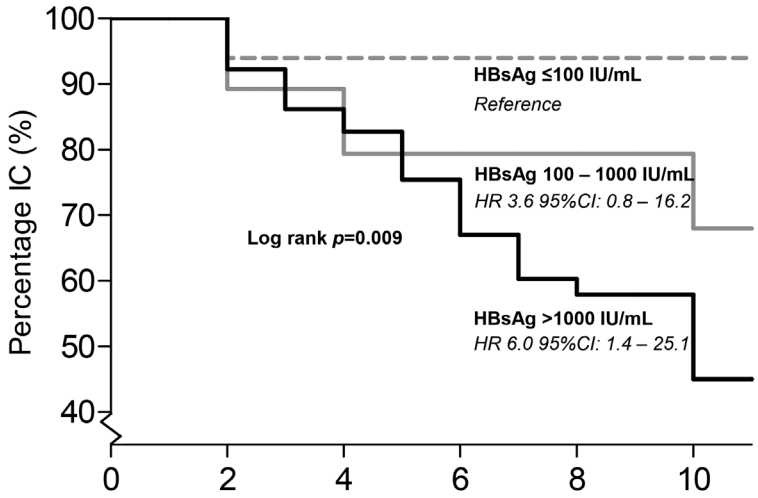
Cumulative probability of HBV activity during follow-up. After the first year of follow-up, 35 and 11 of the initial 189 IC patients became intermediate or AC, respectively. Comparing the risk of progressing to AC for IC versus intermediate patients showed a significant difference: the respective 5 and 10 year cumulative probability for IC patients was 3.6% and 9.8%, which was 23.5% and 35.4% for intermediate patients (Hazard ratio [HR] 5.0, 95%CI:

2.4 – 10.3, p<0.001). For IC patients, the 5 and 10 year cumulative probability of HBV activity (intermediate or AC) was 19.9% and 42.5%, respectively. This probability differed according to HBsAg categories at inclusion: the 5 and 10 year cumulative probability for IC patients with a baseline HBsAg level ≤100 IU/mL was 6.1%, vs. 20.9% and 32.0% for patients with HBsAg levels of 100 – 1,000 IU/mL, vs. 24.6% and 55.0% for patients with an HBsAg level >1,000 IU/mL, respectively (log-rank p=0.009, figure 1). A similar pattern was observed for IC and intermediate patients for progression to AC (log-rank p=0.008).

Table 1 Characteristics of the cohort at inclusion (definition after 1 year of follow-up)

Characteristics	Total cohort (n=292)	Inactive carrier (n=189)	HBV activity (n=103)	*p*-value
Demography				
Age, years*	43.2 (13.3)	44.2 (13.3)	41.3 (13.1)	0.08
Male, n (%)	162 (55)	108 (57)	54 (52)	0.44
Ethnicity, n(%)				0.72
Asian	83 (29)	46 (24)	37 (36)	
Caucasian	157 (54)	110 (59)	47 (46)	
African/black	17 (6)	13 (7)	4 (4)	
Other/unknown	35 (12)	20 (10)	15 (15)	
HBV genotype, n(%)[α]				0.206
A	47 (16)	30 (16)	17 (17)	
B	42 (14)	23 (12)	19 (18)	
C	32 (11)	17 (9)	15 (15)	
D	109 (37)	73 (38)	36 (35)	
Other	13 (5)	11 (6)	2 (2)	
Laboratory measurements*				
Serum ALT (x ULN)	0.6 (0.2)	0.6 (0.2)	0.6 (0.2)	0.58
Log HBV DNA (IU/mL)	2.6 (0.9)	2.2 (0.7)	3.4 (0.5)	<0.001
Log HBsAg (IU/mL)	2.9 (1.1)	2.8 (1.1)	3.2 (1.0)	0.001
HBsAg categories, n (%)				0.001
≤100 IU/mL	46 (16)	36 (19)	10 (10)	
100 – 1,000 IU/mL	97 (33)	71 (38)	26 (25)	
>1,000 IU/mL	149 (51)	82 (43)	67 (65)	
Follow-up measurements				NA***
Total visits, n	2095	1190	905	
Follow-up (years)**	7.8 (4.1-9.2)	7.1 (3.9-9.1)	8.4 (4.6-9.2)	
Visits per patient**	11 (5-15)	9 (5-14)	12 (7-17)	
Months between visits**	8.0 (6.1-13.6)	8.5 (6.3-15.1)	7.0 (5.9-10.9)	

* At baseline, mean (standard deviation)
[α] HBV genotype information present in 83%
** Median (interquartile range)
*** Not applicable, survival analysis



# Patients at risk							# Events
≤100 IU/mL	35	33	23	14	9	3	**2**
100 – 1000 IU/mL	70	65	36	19	12	7	**12**
>1000 IU/mL	82	77	50	36	25	18	**32**

Figure 1 Cumulative probability to remain IC during follow-up given HBsAg at inclusion

* *HBsAg 100 – 1,000 IU/mL versus >1,000 IU/mL: HR 0.61, 95%CI: 0.3 – 1.2, p=0.144*

HBV phase switching during follow-up. By dynamic analysis using repeated HBV DNA and ALT measurements, the probability to remain IC in the following year was 86% when a patient was IC during a random follow-up year. Moreover, when a patient was IC during 2 consecutive years the probability to remain IC in the next year increased to 92%. Fifty-two percent of the patients who were first IC and had HBV activity in the subsequent year returned to the IC phase again in the year thereafter. Vice versa, for patients with HBV activity at first and who were IC during the following year there was a 38% chance of reverting back to HBV activity in the subsequent year.

Factors associated with HBV phase switching. Using repeated HBsAg, HBV DNA and ALT levels, the probability to remain IC in any following year significantly differed according to different HBsAg level categories one year prior: for levels <100, 100 – 1,000 or >1,000 IU/mL, this probability was 97%, 85% and 74%, respectively (p<0.001). Furthermore, when a patient was IC during any 2 consecutive years, the probability to remain IC was 99% in the third year when combined with an HBsAg level <100 IU/mL one year prior, and was 94% and 76% for HBsAg levels of 100 – 1,000 or >1,000 IU/mL, respectively (p<0.001, figure 2).

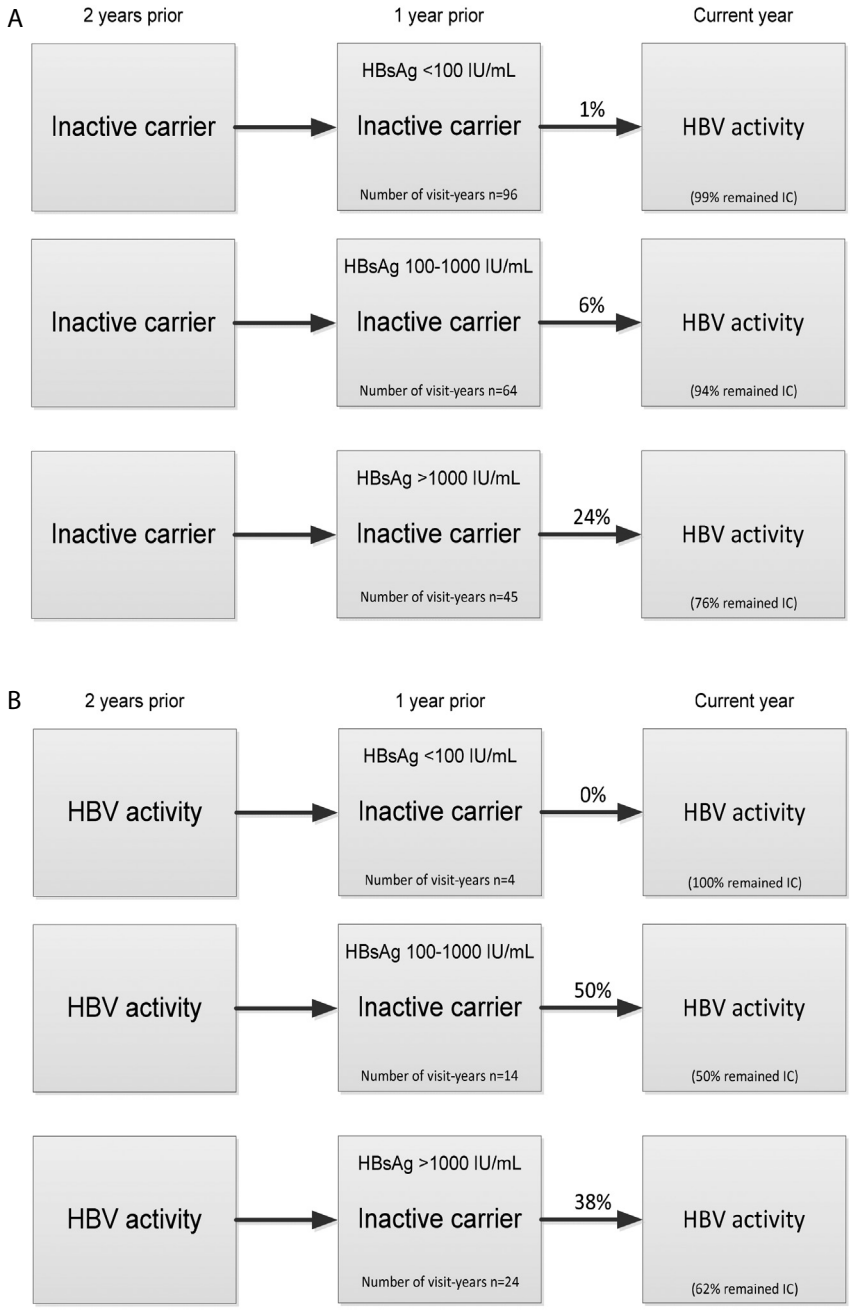


Figure 2 Probability of switching given IC (A) or HBV activity (B) and the combination of HBsAg levels in the prior years

Total number of visit-years in this analysis is n=234 (A) and n=131 (B). A "visit-year" is a maximum HBV phase and HBsAg level within a follow-up year and thus an umbrella term for multiple visits within a follow-up year

The results for patients who were IC during 3 consecutive years were comparable and can be found in the supplementary file. By multivariable dynamic regression analysis, HBV phenotype switching was associated with male gender (OR 2.0, 95%CI: 0.9 – 4.4, p=0.082), younger age (OR 0.68 per 10 years older, 95%CI: 0.5 – 0.9, p=0.014), higher HBsAg (p=0.005) and HBV DNA levels in the previous year (p<0.001), and with HBV genotype (p=0.002): versus HBV genotype A, patients with genotype B (OR 4.8, 95%CI: 1.5 – 15.3) or genotype C (OR 2.6, 95%CI: 0.8 – 8.5) were more likely to switch, and patients with genotype D had a probability comparable to patients with HBV genotype A (OR 0.91, 95%CI: 0.4 – 2.3). The FIB-4 score during follow-up was not associated with switching (p=0.806), and there was no interaction between HBsAg levels, HBV genotype or the FIB-4 score on the probability to switch (all p>0.5). Stratified by HBV genotype in dynamic analysis, the observed HBV activity rates in the following year for patients who were IC during any given follow-up year with HBsAg levels of <100, 100 – 1,000, and >1,000 was as follows: for HBV genotype A: 0%, 8%, 29%; for genotype B: 5%, 42%, 60%; for genotype C: 11%, 30%, 20%; and for genotype D: 2%, 10% and 23%, respectively (all p<0.001).

Diagnostic performance of HBsAg measurements by dynamic analysis. We investigated the performance of repeated measurements of an HBV DNA ≤2,000 IU/mL and HBsAg ≤1,000 IU/mL during a random follow-up year for the probability to remain IC 1 year later. (Table 2) Patients complied with these criteria in 46% (286/622 visit-years). We observed a specificity and sensitivity of 88% and 62% for maintained IC during the following year, with a positive predictive value (PPV) of 92% and a negative predictive value (NPV) of 53%. The specificity and PPV increased to 98% and 97% respectively when an HBsAg level ≤100 IU/mL in combination with an HBV DNA ≤2,000 IU/mL was used, of which 24% (151/622 visit-years) of the cohort complied with. Supplementary tables 1 – 5 show the performance of different prediction rules stratified by HBV genotype, and excluding the intermediate group.

Dynamic prediction of transition from HBV activity to a sustained inactive carrier phase. HBsAg levels showed to decline over time, with a mean annual decline of -0.15 log IU/mL (standard error [SE] 0.02). Patients who were IC throughout the complete follow-up had a more prominent mean HBsAg decline than did patients who experienced HBV activity (-0.23 versus -0.09 log IU/mL, respectively, p=0.004). Figure 3 shows the HBsAg level decline according to true IC versus patients with HBV activity.

Table 2 HBsAg and HBV DNA levels (IU/mL) one year prior and the dynamic predictive performance for the inactive carrier state (combined HBV DNA <2,000 IU/mL and normal ALT for one year) one year later

Prediction rule#	Inactive carrier versus HBV activity					
	HBsAg decline ≥0.50 log	HBsAg ≤1,000	HBsAg ≤100	HBsAg ≤1,000 & HBV DNA ≤2,000	HBsAg ≤100 & HBV DNA ≤2,000	HBsAg ≤100 & HBV DNA ≤200
Sensitivity	43%	66%	37%	62%	35%	26%
Specificity	89%	59%	93%	88%	98%	99%
PPV	90%	78%	92%	92%	97%	99%
NPV	41%	43%	40%	53%	42%	39%
Diagnostic accuracy	57%	64%	54%	71%	55%	50%
Percentage of visit-years*	33%	58%	27%	46%	24%	17%

Measured one year prior, maximum levels within that follow-up year. Values are presented in IU/mL
* Percentage of visit-years at which patients complied with these criteria through long-term follow-up

For patients with an HBV DNA >2,000 IU/mL at any given visit-year, we sought to predict the probability of becoming IC in the following year. By multivariable regression, factors associated with becoming IC in the following year were an HBsAg decline of ≥0.5 log IU/mL in the previous year (OR 6.2, 95%CI: 1.9 – 20.6, p=0.003) and lower HBV DNA levels (OR 0.21, 95%CI: 0.1 – 0.5, p<0.001). Figure 4 shows the predicted probability as a function of HBV DNA levels and HBsAg decline in the prior year. Using a rule of an HBV DNA ≤5,000 IU/mL and an HBsAg decline of ≥0.5 log IU/mL in the previous year we could identify patients becoming IC in the next year with a specificity of 99%, sensitivity of 16%, a PPV of 83% (10/12 visit-years) and a NPV of 72% (142/196 visit-years).

Probability of HBsAg loss during follow-up. During follow-up, 43 patients lost serum HBsAg. The 5 and 10 year cumulative probability of HBsAg loss was highest for patients with an HBsAg <100 IU/mL at inclusion: 39.1% and 63.3%, respectively, and decreased with HBsAg levels 100 – 1,000 IU/mL (7.6%, and 43.1%) and >1,000 IU/mL (0% and 3.1%, p<0.001, figure 5). By Cox regression analysis, only baseline HBsAg levels (HR 0.34, 95%CI: 0.3 – 0.4, p<0.001) were independently associated with the time to HBsAg loss.

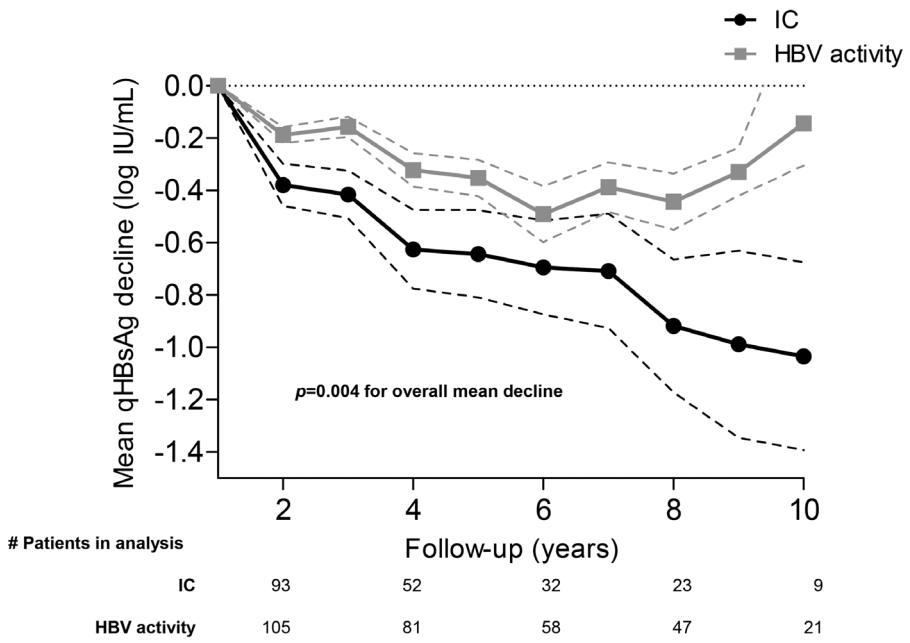


Figure 3 Mean HBsAg level decline from inclusion in inactive carriers versus patients with HBV activity

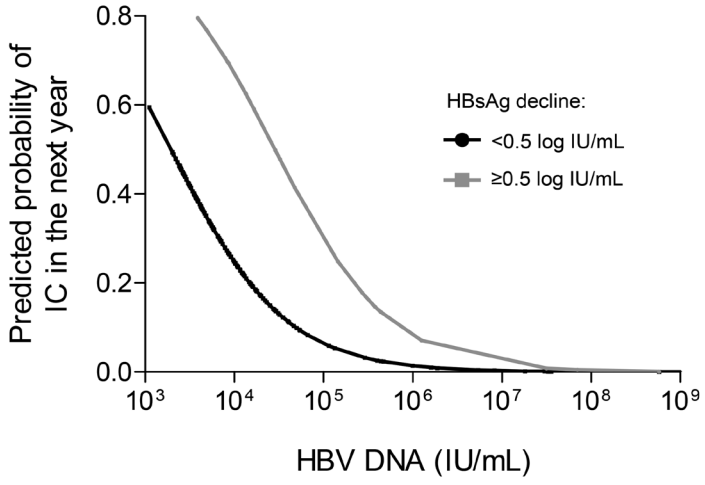
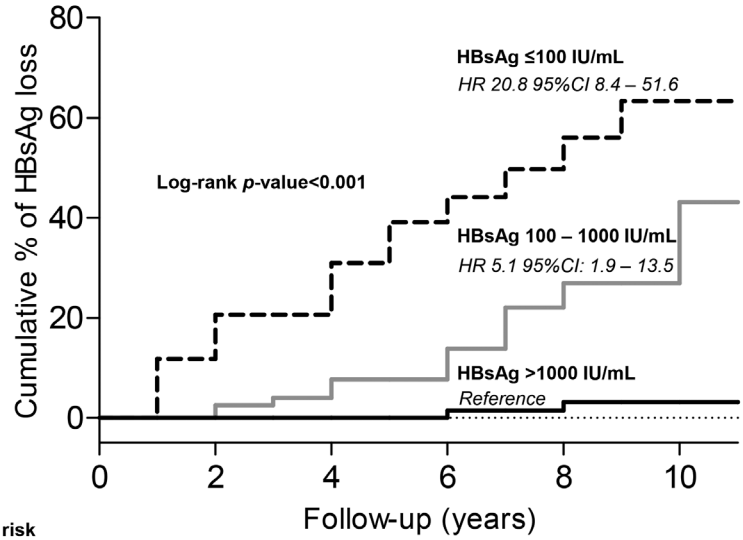


Figure 4 Predicted probability of becoming IC the next year given HBsAg decline and HBV DNA levels



# Patients at risk							# Events*
≤100 IU/mL	34	30	26	12	8	6	**18**
100 – 1000 IU/mL	89	82	52	30	16	9	**13**
>1000 IU/mL	137	130	97	72	57	34	**6**

Figure 5 Cumulative probability of HBsAg loss given baseline HBsAg levels

Six patients with HBsAg ≤100 IU/mL lost HBsAg during the first follow-up year, and 26 patients did not have follow-up beyond the first year and were not included in this analysis

Clinical events. In total, 7 patients developed a clinical event. Five patients had HBV activity during follow-up, and 2 were considered IC throughout. Of the 5 patients with HBV activity, 1 had an HBsAg level <100, 1 an HBsAg level >100 and 3 had HBsAg levels >1,000 IU/mL. Of these 5 patients, 3 had an episode of hepatic decompensation (after 6 and 10 years of follow-up, while 1 patient had an hepatic flare with ALT >4 x ULN and HBV DNA levels $>10^6$ IU/mL after 3 years of follow-up), 1 patient developed an HCC and 1 patient died of a non-liver related cause. Of the 2 IC patients, 1 died of a non-liver related cause after 7 years of follow-up, and 1 developed an HCC and died after 8 years of follow-up. The latter patient was infected with HBV genotype C, had low levels of HBsAg throughout follow-up (<100 IU/mL), and did not have evidence of advanced fibrosis. Of the patients with an event, 3 were infected with HBV genotype B, 3 with genotype C and 1 with genotype A. In total, 3 patients started antiviral therapy during follow-up.

DISCUSSION

In this collaborative real-life cohort study we have investigated for the first time the performance of repeated HBsAg and HBV DNA measurements for the prediction of the IC phase using a dynamic model, updating the risk of progressing or remaining IC each follow-up year. We have shown that HBsAg levels <100 IU/mL in patients with HBV DNA <2,000 IU/mL could identify inactive carriers through long-term follow-up with a specificity of 98% and a positive predictive value of 97%. Patients with HBsAg levels <100 IU/mL had a high probability of HBsAg loss over time. In contrast, patients with HBsAg levels >100 IU/mL were still at risk of HBV activity, of whom approximately 40% switched back and forth during follow-up. Furthermore, the probability of becoming IC in the following year was highest for those patients with both an HBV DNA ≤5,000 IU/mL and an HBsAg level decline of ≥0.5 log IU/mL in the year prior.

It is generally well-established that patients with HBV DNA levels >2,000 IU/mL are at risk of disease progression and HCC development [4, 6], although clinical practice guidelines still leave open the possibility that inactive carriers may have HBV DNA levels <20,000 IU/mL.[2, 3] In a large Taiwanese study it has recently been shown that patients with a low to intermediate HBV DNA load and higher HBsAg levels at inclusion are at risk of hepatitis activity and HCC development.[11-13] In patients infected with HBV genotype D, an HBsAg level <1,000 IU/mL in combination with HBV DNA levels <2,000 IU/mL at the start of follow-up was highly predictive of the IC state during further follow-up, and HBsAg levels >1,000 were associated with the risk of reactivation in patients infected with HBV genotype A – E.[14, 15] The prediction rule combining HBV DNA <2,000 and HBsAg <1,000 IU/mL however had a poor diagnostic performance over a decade of follow-up in the Taiwanese study (sensitivity of 54% and specificity of 56%). A possible explanation for this observation may be that HBV DNA and HBsAg levels fluctuate over time, making a single-point measurement at inclusion less optimal for a risk analysis in the long-term, also because of the possibility of including HBV activity patients observed during a remission phase.[12, 16, 18-20] Another reason may be differences in HBV genotype and the criteria for the frequency and duration of monitoring of the HBV carriers.

In the current study we observed a very low risk of HBV activity for patients with an HBsAg level of <100 IU/mL, and these levels were highly specific for the inactive carrier state. Importantly, patients with HBsAg levels >100 IU/mL were at risk of HBV activity regardless of how many years they were considered IC. Even when patients were defined IC after 3 years of follow-up according to the APASL guidelines [3], HBsAg levels remained significantly predictive of the IC phase after 3 years with no HBV activity in patients with HBsAg <100 IU/mL, versus 12% and 17% for patients with HBsAg 100 – 1,000 and above 1,000 IU/mL respectively.(Supplementary

file) Given these lines of evidence, it seems reasonable to define IC patients across all HBV genotypes as those with both an HBV DNA ≤2,000 IU/mL and HBsAg <100 IU/mL (table 3).

Table 3 Proposed new criteria for natural history in HBeAg-negative patients

Phenotype	HBsAg* (IU/mL)	and/or	HBV DNA* (IU/mL)	and/or	Serum ALT*
Inactive carrier	≤100	*and*	≤2,000	*and*	Normal
HBV remission	100 – 1,000	*and*	≤2,000	*and*	Normal
Active carrier	>1,000	*or*	>2,000	-	-

* Maximum levels within a follow-up year

Patients with an HBV DNA ≤2,000 and a persistently normal ALT during a follow-up year, but HBsAg levels 100 – 1,000 IU/mL should be defined as "HBV remission patients" and should be kept under close surveillance for at least one more year to confirm IC status.

The management of HBeAg-negative CHB patients who have HBV DNA levels <20,000 IU/mL remains a matter of debate. These patients have a high probability of developing HBV DNA levels >20,000 IU/mL during follow-up such as indicated in the current study, and given the increased risk of HCC, may thus be regarded as active carriers.[4-6] We have shown that inactive carriers had a more prominent HBsAg decline, and we hypothesized that patients with HBV activity who will become IC in the next years would be having a more prominent annual HBsAg decline. Indeed, those patients with an HBV DNA level between 2,000 and 5,000 IU/mL and with an HBsAg level decline of ≥0.5 log IU/mL in the prior year had a high probability of progression to a sustained IC phase one year later. Patients who did not fulfil these criteria were less likely to become IC. This is clinically important as it may be worthwhile to monitor these patients instead of offering lifelong antiviral therapy.[21-23]

An essential difference between the current study and the previous report is that we have now used a dynamic analysis, including HBV genotypes other than D as well.[14] The results of the dynamic analysis were largely in line with the previous report, as we observed only 6% of patients with HBsAg levels between 100 – 1,000 IU/mL who developed HBV activity after consecutive years of follow-up, which was 3% of all patients with an HBsAg <1,000 IU/mL. When aiming for the largest diagnostic accuracy, the repeated combination of an HBV DNA <2,000 IU/mL with HBsAg <1,000 IU/mL was consistently the best performing, with an accuracy of 71% versus 55% compared to the repeated combined measurement of HBV DNA <2,000 IU/mL and HBsAg levels <100 IU/mL. Even so, it may be important to utilize a rule with a high specificity and PPV to classify IC patients, and this could best be achieved with a combination of an HBV DNA <2,000 IU/mL and HBsAg <100 IU/mL. Using the prediction

rule of an HBsAg <1,000 IU/mL we observed lower sensitivities than previously described for HBV genotype D patients.[14, 15] An explanation for this observation could be that HBV genotypes behave differently, as HBsAg levels are known to differ across HBV genotype.[18-20, 24, 25] Indeed, we observed a higher probability of phenotype switching for HBV genotype B and C patients, and in a stratified analysis a higher sensitivity for HBV genotype D patients was obtained. (Supplementary table 4) Yet, a separate analysis to identify the best HBV genotype-specific HBsAg cut-off was not possible since the number of patients was too low in a stratified analysis. Hence, future studies should further elucidate the role of HBV genotype for the different HBsAg cut-offs in IC prediction. Nonetheless, when using an HBsAg level <100 IU/mL there seems to be a very low probability that patients would be wrongly classified as IC irrespective of HBV genotype.

This study was conducted at 8 tertiary centres globally, which may have resulted in heterogeneity of this real-life cohort. To control for this, we have excluded every participating centre one by one, and have not detected a centre-specific effect. Also, different centres may apply various criteria for the diagnosis of cirrhosis. Patients with cirrhosis at baseline were not included in the current study. This diagnosis was based on multiple modalities and according to different pathologists and radiologists, which could have led to a less accurate diagnosis and possible influence on the results of the study. We have further assessed this by excluding patients with potentially advanced liver disease on basis of less stringent criteria in a sub-analysis (supplementary file). This analysis did not provide other results than observed in the main analysis. In line with this observation, there was also no significant association between repeated measures of the FIB-4 score during follow-up and HBV phenotype switching. This may further strengthen our findings as the mode of diagnosis of cirrhosis probably resembles the daily clinical practice more closely. One patient who was considered IC with low levels of HBsAg and without evidence of advanced fibrosis developed an HCC after 8 years of follow-up. This underlines that next to the clinical phase defined using HBsAg and HBV DNA levels, other risk factors for HCC should always be considered in the management of CHB.

Concluding, HBeAg-negative CHB patients with HBsAg levels <100 IU/mL and HBV DNA <2,000 IU/mL can be considered inactive with a high chance of HBsAg loss. CHB patients with an HBV DNA level >2,000 IU/mL and below <5,000 IU/mL with an annual HBsAg level decline of ≥0.5 log IU/mL have a high probability to become IC and may therefore not require antiviral therapy. HBsAg levels seem to better reflect the natural history and a revision of the definitions including HBsAg levels may ease the management of HBeAg negative CHB.

SUPPLEMENTARY: SENSITIVITY ANALYSES

1) The APASL guidelines for the management of CHB state that patients are IC in case of a persistently normal ALT and HBV DNA <20,000 IU/mL during 3 consecutive years. In this sub-analysis we assess a modified APASL rule: results for patients who have persistently normal ALT and HBV DNA <2,000 IU/mL during 3 consecutive years, in relation to HBsAg levels.

2) Excluding patients with possible advanced liver disease (i.e. advanced fibrosis, possible cirrhosis) diagnosed either by liver biopsy or one of the following: abdominal ultrasound, liver stiffness through Fibroscan (>11 kPa), a Bonacini cirrhosis discriminant score >7 (which includes platelets, ALT/AST ratio and international normalized ratio), a FIB-4 score ≥3.25, and/or stigmata of chronic liver disease by physical examination). [26, 27]

3) Excluding patients with an occasional elevation of ALT>1 – <2x ULN while having a low HBV DNA <2,000 IU/mL during follow-up.

4) Assessment of the results excluding every participating centre one by one.

1) **Modified APASL rule: maintained HBV DNA <2,000 IU/mL and persistenty normal ALT during 3 consecutive years.**

   By survival analysis, it was shown that the hazard of HBV activity differed across HBsAg categories for patients who were IC for 3 consecutive years. Using baseline HBsAg levels, none (0/19) of these patients with HBsAg levels <100 IU/mL showed HBV activity, versus 2/31 (cumulative probability 8.5% in year 5) and 9/24 (cumulative probability of 17.6% in year 5) patients with HBsAg levels of 100 – 1000 IU/mL and >1000 IU/mL, respectively (log-rank p=0.016). By dynamic regression analysis, the results remained comparable to the main analysis: it was observed that of patients who were IC during three random consecutive years, 93% remained IC in the fourth year. This probability also showed to differ across HBsAg level categories of <100, 100 – 1000 and >1000 IU/mL: 100%, 88% and 83% of the patients maintained IC through follow-up.

2) **Exclusion of patients with possible advanced liver disease.**

   We excluded an additional 41 patients with possible advanced liver disease, 25 of which belonged to the 189 IC patients and 16 to the 103 HBV activity patients. By time-dependent analysis, the probability of HBV activity for the 164 IC patients remained comparable to the main analysis and showed to differ across HBsAg categories at inclusion: the 5 and 10 year cumulative probability was and remained 7%, was 19.4% and 39.6% and was 20.0% and 53.2% for HBsAg levels <100, 100 – 1000 and > 1000 IU/mL (p=0.044). By dynamic analysis, similar results were obtained. When a patient was IC during 2 consecutive years with HBsAg levels <100, 100 – 1000 and >1000, the probability to remain IC in the third year was 99%, 94% and 76% (p<0.001).

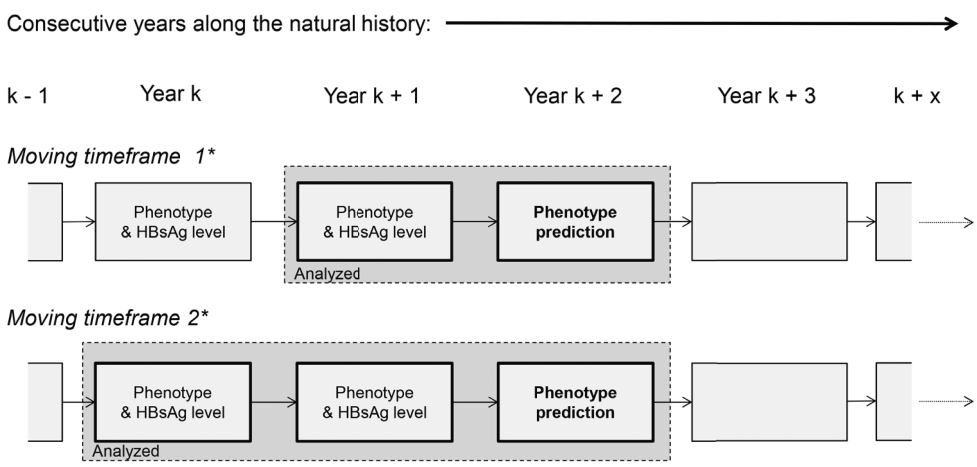
3) **Exclusion of patients who were potential inactive carriers during follow-up.**

   When we excluded 28 patients who were potentially inactive during follow-up (i.e., a patient with low HBV DNA levels and an ALT between 1 and 2 times the upper limit of normal), similar results were obtained in both survival as well as in dynamic analysis. The cumulative 5 and 10 year probability was and remained 7%, was 15.9% and 38.3%, and was 24.8% and 54.6% for the respective HBsAg categories at inclusion (p=0.024). Also the rates of HBV switching remained similar, with 99% of patients who were IC during 2 consecutive years with HBsAg <100 remaining in the IC phase, versus 94% and 76% for the other categories (p<0.001).

4) **Exclusion of every participating centre one by one.**

   When we excluded every of the 8 participating centers one by one, the results of the main analysis remained comparable, indicating no center effect on the results.

SUPPLEMENTARY FIGURES



**Moving timeframe 1 includes information of 2 consecutive years, moving timeframe 2 includes information of 3 consecutive years. All information from multiple blocks (using timeframe 1 or 2) were analyzed simultaneously for each patient*
Pt: patient. Phenotype: Inactive carrier, indeterminate or active carrier. Patients were only IC when they were observed to be in this state for the complete follow-up year. The maximum HBsAg level and HBV phase in each follow-up year was used

Figure 1 Schematic example of dynamic regression analysis using a moving timeframe

SUPPLEMENTARY TABLES

Suppl. table 1a Diagnostic performance of different prediction rules for HBV genotype A

Prediction rule#	Inactive carrier versus HBV activity		
	HBsAg ≤1000 & HBV DNA ≤2000	HBsAg ≤100 & HBV DNA ≤2000	HBsAg ≤100 & HBV DNA ≤200
Sensitivity	41%	19%	10%
Specificity	97%	99%	99%
PPV	96%	99%	99%
NPV	45%	39%	36%
Diagnostic accuracy	60%	46%	40%
Percentage of visit-years*	28%	12%	7%

Suppl. table 1b Diagnostic performance of different prediction rules for HBV genotype B

Prediction rule#	Inactive carrier versus HBV activity		
	HBsAg ≤1000 & HBV DNA ≤2000	HBsAg ≤100 & HBV DNA ≤2000	HBsAg ≤100 & HBV DNA ≤200
Sensitivity	69%	54%	41%
Specificity	80%	93%	99%
PPV	82%	91%	99%
NPV	67%	61%	57%
Diagnostic accuracy	74%	71%	52%
Percentage of visit-years*	48%	33%	23%

Suppl. table 1c Diagnostic performance of different prediction rules for HBV genotype C

Prediction rule#	Inactive carrier versus HBV activity		
	HBsAg ≤1000 & HBV DNA ≤2000	HBsAg ≤100 & HBV DNA ≤2000	HBsAg ≤100 & HBV DNA ≤200
Sensitivity	54%	40%	26%
Specificity	79%	92%	96%
PPV	82%	90%	92%
NPV	49%	46%	42%
Diagnostic accuracy	63%	58%	51%
Percentage of visit-years*	42%	28%	18%

Suppl. table 1d Diagnostic performance of different prediction rules for HBV genotype D

Prediction rule#	Inactive carrier versus HBV activity		
	HBsAg ≤1000 & HBV DNA ≤2000	HBsAg ≤100 & HBV DNA ≤2000	HBsAg ≤100 & HBV DNA ≤200
Sensitivity	70%	36%	24%
Specificity	90%	99%	99%
PPV	94%	98%	99%
NPV	59%	43%	39%
Diagnostic accuracy	76%	56%	49%
Percentage of visit-years*	50%	24%	16%

Measured one year prior, maximum levels within that follow-up year. Values are presented in IU/mL.
* Number of visit-years at which patients complied with these criteria through long-term follow-up
PPV, positive predictive value; NPV, negative predictive value

Suppl. table 2 Diagnostic performance of different prediction rules for all HBV genotypes excluding the intermediate group

Prediction rule#	Inactive carrier versus active carrier		
	HBsAg ≤1000 & HBV DNA ≤2000	HBsAg ≤100 & HBV DNA ≤2000	HBsAg ≤100 & HBV DNA ≤200
Sensitivity	63%	35%	26%
Specificity	95%	98%	98%
PPV	99%	99%	99%
NPV	27%	18%	16%
Diagnostic accuracy	67%	43%	35%
Percentage of visit-years*	55%	31%	23%

Measured one year prior, maximum levels within that follow-up year. Values are presented in IU/mL.
* Number of visit-years at which patients complied with these criteria through long-term follow-up
PPV, positive predictive value; NPV, negative predictive value

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CHAPTER 3

The impact of *PNPLA3* (*rs738409*C>G) polymorphisms on liver histology and long-term clinical outcome in chronic hepatitis B patients

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ABSTRACT

Background & aims

We aimed to assess the association between the patatin-like phospholipase domain-containing-3 (PNPLA3) I148M polymorphism, liver histology and long-term outcome in chronic hepatitis B (CHB) patients.

Methods

We enrolled 531 consecutive treatment-naïve CHB patients diagnosed from 1985 to 2012 with an available liver biopsy for reassessment, and sample for genetic testing. Data on all-cause mortality and hepatocellular carcinoma (HCC) at long-term follow-up were obtained from national database registries.

Results

The prevalence of steatohepatitis increased with PNPLA3 CC (14%), CG (20%) and GG (43%) ($p<0.001$). The association was altered by both gender ($p=0.010$) and overweight ($p=0.015$): the effect of PNPLA3 on steatohepatitis was most pronounced among non-overweight females (adjusted OR 13.4, 95%CI: 3.7-51.6, $p<0.001$), and non-overweight males (adjusted OR 2.4, 95%CI: 1.4-4.3, $p=0.002$). Furthermore, PNPLA3 GG genotype was associated with iron depositions (OR 2.8, 95%CI: 1.2-6.4, $p=0.014$) and lobular inflammation (OR 2.2, 95%CI: 1.1-4.5, $p=0.032$), but not with advanced fibrosis (OR 1.1, 95%CI: 0.7-1.8, $p=0.566$). The median follow-up was 10.1 years (interquartile range 5.6 – 15.8), during which 13 patients developed HCC and 28 died. Steatohepatitis was associated with all-cause mortality (Hazard ratio [HR] 3.1, 95%CI: 1.3-7.3, $p=0.006$) and HCC (HR 2.8, 95%CI: 0.9-9.2, $p=0.078$), but no significant association was observed for PNPLA3.

Conclusions

In this cohort of biopsied CHB patients, PNPLA3 was independently associated with steatosis, steatohepatitis, lobular inflammation and iron depositions, but not with advanced fibrosis, HCC development or all-cause mortality. The effect of PNPLA3 on steatohepatitis was particularly pronounced among female patients without severe overweight.

INTRODUCTION

Chronic hepatitis B (CHB) infection affects approximately 350 million people worldwide.[1] When patients are infected with the hepatitis B virus (HBV) at birth or in early childhood, and to a lesser extent in adulthood, an ineffective immune response directed at the virus can result in chronic liver inflammation. Subsequently, this can lead to liver cirrhosis, hepatocellular carcinoma (HCC), liver failure and death.[1, 2] Globally, 30% of cirrhosis and 53% of HCC is attributable to CHB.[2]

Over the past decades obesity and its associated morbidities has reached epidemic proportions, particularly in developed countries. Nowadays, there are approximately 500 million obese men and women, a number which has almost doubled since 1980.[3] Non-alcoholic fatty liver disease (NAFLD) is one of the many expressions of corpulence and affects up to 30% of adults.[4] Steatosis could progress to steatohepatitis, a condition which subsequently leads to cirrhosis in 10-29% of individuals within 10 years. Moreover, 4-27% of patients with steatohepatitis associated cirrhosis develop HCC.[4] Steatosis is a risk factor for disease progression and HCC development in patients chronically infected with the hepatitis C virus (HCV) as well.[5, 6] However, its impact on the progression of CHB related liver disease is still unknown.[7-9] Previous studies indicated that approximately one-third of patients with CHB suffer from NAFLD, although the reported prevalence varied widely.[7-9]

NAFLD is a complex disorder, and a cumulative result of many biological interactions in different pathways.[10, 11] Only recently it was shown that a common variation in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene predisposes for NAFLD.[12] This susceptibility for NAFLD in patients with the unfavourable variation was also observed in patients with alcoholic liver disease or HCV infection. Moreover, the 148M variation was associated with the progression of liver disease, and with the development of HCC,[13-17] and possibly with the presence of iron depositions.[18] Recently, it was found that *PNPLA3* was also associated with hepatic steatosis in CHB patients.[19] However, whether this unfavourable variation is also associated with advanced fibrosis and the long-term clinical outcome in CHB patients, remains to be elucidated.

Therefore the aim of the current study was to investigate the association between *PNPLA3* I148M and the severity of liver histologic changes in relation to the long-term outcome of a large multi-ethnic population of treatment naïve CHB patients.

PATIENTS AND METHODS

Patient selection. The electronic medical charts of all consecutively biopsied CHB patients during the period from 1985 to 2012 at the Erasmus University Medical Center in Rotterdam, the Netherlands, were reviewed. Treatment naïve CHB (hepatitis B surface antigen [HBsAg] positive for more than 6 months) patients with an available liver biopsy for structured histological assessment and an available sample for genetic testing, were eligible for inclusion. Patients were excluded in case of a current or past co-infection with HCV, hepatitis D, hepatitis E or human immunodeficiency virus, presence of auto-immune liver disease, primary biliary cirrhosis, Wilson's disease, hemochromatosis or any other co-existing primary liver disease, antiviral treatment for more than 1 month or treatment with immune suppressive medication for more than 6 months prior to or at the time of biopsy.
This study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice. Due to the retrospective nature of this study, written informed consent was not obtained from each patient. The ethical review board of the Erasmus Medical Center, Rotterdam, The Netherlands approved of the study as it was considered a low-risk study using anonymized patient data.

Data acquisition. At first biopsy, data on demographics (sex, age, race, ethnicity, height, weight, route of HBV transmission) and clinical data (history, diagnosis of diabetes mellitus, daily alcohol intake, history of alcohol abuse, smoking) were obtained by a single investigator (WB) as stated in the electronic chart by the treating physician. Alcohol use was defined as ≥1 units of alcohol/day, binge drinking inclusive. Alcohol abuse was defined as an history or current use of ≥5 units/day, corresponding to 40-50 grams alcohol per day.[20] The body-mass index (BMI) was calculated using height and weight, and severe overweight was defined as a BMI of ≥27.5 kg/m^2.[3] Data on chemistry (alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutyltransferase (y-GT), bilirubin, albumin), hematology (platelet count, prothrombin time) and virology (serum qualitative HBsAg, anti-HBs, HBeAg, anti-HBe, HBV DNA load, HBV genotype) at the time of biopsy were obtained from the clinical laboratory and the department of Virology at the Erasmus Medical Center. At long-term follow-up, data on all-cause mortality was obtained from the municipal record database and development of HCC was obtained from the national HCC registry database.

Endpoints. The following outcomes were studied: presence of steatosis and steatohepatitis, advanced fibrosis, hepatic inflammation, iron depositions and time to all-cause mortality and

HCC at long-term follow-up. The diagnosis of HCC was based on histopathology and when not available, on 2 imaging modalities (magnetic resonance imaging, computed tomography or contrast enhanced ultrasound).

Histological evaluation. All liver biopsies were percutaneously obtained and re-scored in a uniform structured manner by a single experienced hepato-pathologist (FK) who was blinded to the patient characteristics. The length of biopsy and the number of portal fields were determined for quality assessment. Biopsies were scored according to the Ishak fibrosis score (0-6), the hepatic activity index (HAI 0-18, which is a cumulative score of lobular (0-4), portal (0-4) and interface (0-4) inflammation, as well as confluent necrosis (0-6)),[21] the Brunt score for steatosis (Brunt 0: 0-5%, Brunt 1: 5-33%, Brunt 2: 33-66% and Brunt 3: >66% of steatosis droplets present) and steatohepatitis, defined as the combined presence of macrovesicular or microvesicular steatosis, lobular inflammation, lipogranulomas and ballooning degeneration.[22] Significant lobular inflammation was defined as a lobular score ≥2 out of 4.[23] Furthermore, the presence of iron depositions was assessed (none, mild, moderate). Patients were classified as having any degree of steatosis (Brunt ≥1) or severe steatosis (Brunt ≥2). Advanced fibrosis was defined as an Ishak score of ≥4, corresponding to portal to portal bridging and probable or definite cirrhosis.[21] Iron depositions were regarded as a binary outcome, either present or absent. To minimize the chance of histological misclassification due to sample size, liver biopsies with a length of less than 10 mm and with less than 10 portal fields were excluded from the analysis.[24, 25]

Laboratory tests. The gender and time-dependent upper-limit of normal (ULN) values were used for the analysis of serum ALT and AST. The HBV DNA level was expressed in units/millilitre (IU/ml) and, when required, calculated using the conversion of 1.0 picogram/ml = 5.15×10^4 IU/ml or 1.0 copies/ml = 0.1818 IU/ml. HBV genotype was determined using the INNO-LiPA HBV Genotyping assay (Innogenetics, Ghent, Belgium) in case data on HBV genotype was missing.

Host genotyping. Competitive allele-specific PCR assays (KASP™, LGC genomics, Huddleston, United Kingdom) were employed for the detection of the reference SNP *PNPLA3 rs738409*.[12] Serum samples stored at -20^0 or -80^0 Celsius were used for DNA extraction and genotyping procedures, which were carried out centrally at LGC genomics. Purified genomic DNA of ≥5 nanogram was used for genotyping. Genotypes were assigned using all of the data from the study simultaneously. The genotype sequence was derived from NCBI.

Follow-up assessment. The follow-up time was calculated from the date of biopsy until the date of diagnosis of HCC, the date of (all-cause) mortality, the date of the last outpatient visit, or the last date of the evaluation of national registry databases which was May 14th 2014. Patients were censored at the time of emigration.

Statistical analyses. 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. Continuous variables were expressed as mean ± standard deviation (SD) or median (interquartile range (IQR)). Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. *PNPLA3 rs738409* was subject to Hardy-Weinberg equilibrium tests for the total cohort as well as for subgroups.[26] The association analysis between *PNPLA3* and liver histology was performed using an additive genetic model (linear OR per addition of an unfavourable G-allele).[27] For the association analysis between *PNPLA3*, iron depositions and liver inflammation a recessive model (GG versus non-GG) was considered as well. Kaplan-Meier and Log-rank analyses were used to compare survival curves. Cox proportional hazard models were used to estimate the association between *PNPLA3* polymorphisms, liver histology and all-cause mortality or HCC at long-term follow-up. To address potential population stratification, gender-specific effects and gene-environmental interactions, logistic regression analysis was performed taking into account possible interactions between *PNPLA3*, race, age, gender, BMI, diabetes mellitus, alcohol (ab) use, chemistry and virology parameters. All statistical tests were two-sided and evaluated at the 0.05 level of significance.

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RESULTS

Patient characteristics. From 1985 to 2012, a liver biopsy was performed in 880 consecutive HBV infected patients. Of 880 patients, 163 did not meet the eligibility criteria (75 patients with co-infection, 53 antiviral therapy before biopsy, 20 immune suppressive medication, 12 passed infection before biopsy and 3 acute HBV) and 127 were excluded because of a missing chart (n=6), liver biopsy slide (n=68) or sample for genetic testing (n=53); 33 (6%) of the 590 remaining patients showed to have an inadequate liver biopsy sample and 26 (5%) were not successfully genotyped. In total, the study cohort thus consists of 531 patients, of which the characteristics are shown in table 1. In total, 101 (19%) patients were severely overweight, 181 (34%) used ≥ 1 unit of alcohol/day and 27 (5%) had diabetes mellitus. The distribution of *PNPLA3* genotypes CC, CG and GG was 56%, 36% and 8%, respectively. *PNPLA3* was in Hardy-Weinberg equilibrium for the total cohort and when stratified by ethnicity, gender or severe overweight. Long-term follow-up data was available for 491 (92%) patients, and 40 (8%) patients emigrated. The median follow-up was 10.1 years (interquartile range [IQR] 5.6 – 15.8), which did not differ across *PNPLA3* polymorphisms (table 1). Three-hundred and thirty-six (64%) patients received antiviral therapy (nucleos(t)ide analogues or (peg)interferon) after biopsy.

Association between *PNLPA3* and hepatic steatosis. A total of 169 (32%) patients had steatosis, which was severe in 58 (34%). The presence and severity of steatosis increased per additional *PNPLA3* G-allele: for CC, CG and GG steatosis was present in 26%, 37% and 53% (p<0.001), while severe steatosis was present in 8%, 12% and 28% (p=0.001), respectively. Most important factors associated with steatosis were older age, male gender, BMI, alcohol use and diabetes mellitus. (Supplementary table 1) The prevalence of steatosis did not differ across race (p=0.190). By multivariable analysis, we observed a statistically significant interaction between *PNPLA3* and gender on the presence of steatosis (p=0.009): the magnitude of the association between *PNPLA3* and steatosis differed significantly for females (OR 8.6, 95%CI: 2.7 – 27.2, p<0.001, table 2) compared to males (OR 1.7, 95%CI: 1.1 – 2.6, p=0.015) adjusted for age, race, severe overweight, diabetes mellitus and alcohol use. There was no significant interaction between race and *PNPLA3* genotypes (table 2). Figure 1 shows the prevalence of steatosis for females and males according to the *PNPLA3* polymorphisms.

Table 1 Characteristics of the study cohort

Characteristics	*PNPLA3* polymorphisms (n=531)			
	CC (n=296)	CG (n=195)	GG (n=40)	*p*-value[µ]
Demography				
Age at biopsy, years (SD)	35.4 (12.9)	33.4 (12.1)	34.7 (12.1)	0.259
Male, n(%)	200 (67.6)	128 (65.6)	26 (65.0)	0.883
Body mass index kg/m^2 (SD)[α]	24.7 (4.0)	24.7 (4.2)	25.1 (4.4)	0.860
Severe overweight, n(%)[α]	57 (22.4)	39 (23.6)	5 (15.2)	0.566
Diabetes, n(%)	17 (5.8)	8 (4.1)	2 (5.0)	0.717
Alcohol intake, n(%)[β]	108 (36.5)	63 (66.3)	10 (25.0)	0.282
History of alcohol abuse, n(%)[β]	17 (6.6)	5 (3.0)	2 (5.0)	0.267
AVT after biopsy, n(%)¥	181 (61)	130 (67)	25 (63)	0.417
Race, n(%)*				<0.001
Caucasian	146 (49.3)	90 (46.2)	12 (30.0)	
Asian	73 (24.7)	73 (37.4)	23 (57.5)	
African/negroid	72 (24.3)	24 (12.3)	4 (10.0)	
Other	5 (1.7)	8 (4.1)	1 (2.5)	
Virology				
HBeAg-positive, n(%)	145 (49.0)	105 (53.8)	22 (55.0)	0.509
log HBV DNA, IU/ml (SD)	5.8 (2.7)	5.8 (2.8)	5.9 (2.6)	0.974
HBV genotype A/B/C/D/E (%)[δ]	33/11/14/32/9	17/16/20/40/5	8/11/50/29/0	<0.001
Chemistry/hematology (IQR)				
ALT U/L, xULN	1.5 (1.0-2.7)	1.7 (1.1-2.9)	1.6 (1.0-2.9)	0.310
AST U/L, xULN	1.1 (0.8-1.6)	1.1 (0.8-1.6)	1.1 (0.8-1.7)	0.972
Thrombocytes x10^9/L	203 (170-241)	214 (179-252)	208 (174-244)	0.259
Albumin, g/L	45.0 (42.0-47.0)	45.0 (42.0-47.0)	45.6 (44.0-47.5)	0.419
Bilirubin, µmol/L	10.0 (8.0-15.0)	11.0 (8.0-14.0)	9.5 (7.3-15.8)	0.771
γ-GT, U/L	28.0 (18.0-46.0)	26.0 (18.0-39.5)	23.0 (14.0-40.8)	0.377
Prothrombin time, sec	12.0 (10.9-13.2)	12.0 (11.4-13.0)	12.3 (11.1-13.4)	0.598
Liver histology				
Biopsy length, mm (SD)	19.6 (6.5)	20.5 (7.1)	19.1 (6.8)	0.266
Portal fields, n (SD)	19.5 (8.4)	20.7 (8.8)	17.8 (6.8)	0.094
Ishak fibrosis, (IQR)	2.0 (1.0-3.0)	2.0 (1.0-3.0)	2.0 (1.0-3.0)	0.127
Iron depositions, n (%)[π]	75 (27.7)	45 (25.6)	16 (42.1)	0.118
Hepatic Activity index (IQR)	4.0 (2.0-6.0)	4.0 (2.0-5.0)	3.5 (2.3-5.0)	0.247
Advanced fibrosis, n(%)	64 (21.6)	34 (17.4)	6 (15.0)	0.391
Cirrhosis, n (%)	35 (11.8)	17 (8.7)	4 (10.0)	0.546

Table 1 Continued

Characteristics	*PNPLA3* polymorphisms (n=531)			
	CC (n=296)	CG (n=195)	GG (n=40)	*p*-value[μ]
Steatosis, n(%)*				<0.001
<5%	220 (74.3)	123 (63.1)	19 (47.5)	
5-33%	52 (17.6)	49 (25.1)	10 (25.0)	
33-66%	19 (6.4)	19 (9.7)	7 (17.5)	
>66%	5 (1.7)	4 (2.1)	4 (10.0)	
Severe steatosis, n (%)	24 (8.1)	23 (11.8)	11 (27.5)	0.001
Steatohepatitis, n(%)	42 (14.2)	38 (19.5)	17 (42.5)	<0.001
Follow-up, years (min-max)	10.1 (0.1-26.7)	10.1 (0.1-27.3)	10.0 (0-24.4)	0.842

[μ] p-value represents comparisons across polymorphisms of PNPLA3 (one-way analysis of variance for normally distributed variables and Kruskal Wallis tests for skewed variables)
[α] Available in 453 (85%) patients
[β] Available in 460 (87%) patients
[*] Chi-square test
[δ] Available in 478 (90%) patients, Chi-square test
[π] No cases of hemochromatosis
[¥] AVT, antiviral therapy (interferon-based or nucleos(t)ide analogues)

Association between *PNPLA3* and steatohepatitis. Ninety-seven (18%) patients had steatohepatitis. The percentage of patients with steatohepatitis increased with *PNPLA3* CC (14%), CG (20%) and GG (43%) (p<0.001). The same factors associated with steatosis were also associated with steatohepatitis. (Supplementary table 1) The association between *PNPLA3* and steatohepatitis was significantly modified by both gender and the presence of severe overweight (interactions p=0.010 and p=0.015, respectively): the OR per additional G-allele of *PNPLA3* among non-overweight females was 13.4 (95%CI: 3.7 – 51.6, p<0.001) and for males 2.4 (95%CI: 1.4 – 4.3, p=0.002), while the effect per G-allele was decreased in severely overweight females (OR 4.0, 95%CI: 1.0 – 16.2, p=0.053) and absent in severely overweight males (OR 0.7, 95%CI: 0.3 – 1.7, p=0.433) adjusted for age, race, diabetes mellitus and alcohol use. (Table 2) There was no significant interaction between race and *PNPLA3* genotypes.

Association between *PNPLA3* and hepatic iron depositions. One-hundred thirty-six (26%) patients had presence of hepatic iron depositions, which were more frequently found in *PNPLA3* GG (42%) versus CC and CG (27%) (p=0.044). Factors associated with iron depositions included older age, male gender, BMI, alcohol use, a higher HAI score and the presence of steatohepatitis or steatosis. (Supplementary table 2) *PNPLA3* was significantly associated with hepatic iron depositions (adjusted OR GG versus non-GG 2.8, 95%CI: 1.2 – 6.4, p=0.014), independent of age (OR per 10 years 1.4, 95%CI:1.1 – 1.6, p=0.002), male gender (OR 4.9, 95%CI: 2.5 – 9.7, p<0.001), alcohol use (OR 1.8, 95%CI: 1.1 – 2.8, p=0.023), the presence

of steatohepatitis (OR 1.6, 95%CI: 0.9 – 2.8, p=0.107) and the HAI score (OR 1.1, 95%CI: 1.0 – 1.3, p=0.028).

Table 2 Multivariable analysis for the presence of steatosis or steatohepatitis

Variable	Steatosis (n=169)		Steatohepatitis (n=97)	
	OR (95%CI)	***p*-value**	**OR (95%CI)**	***p*-value**
Age at biopsy (per 10 years)	1.5 (1.2-1.9)	<0.001	1.6 (1.2-2.0)	0.001
Severe overweight[a]	5.6 (3.1-10.2)	<0.001	-	-
Alcohol use	1.0 (0.6-1.8)	0.916	0.9 (0.5-1.6)	0.637
Diabetes mellitus	1.5 (0.6-4.1)	0.387	1.2 (0.4-3.4)	0.783
Race*		0.451		0.633
– Caucasian	Reference		Reference	
– Asian	0.9 (0.5-1.6)		1.4 (0.7-2.8)	
– African	0.6 (0.3-1.2)		0.9 (0.4-2.3)	
PNPLA3 genotype per G-allele[¥] within:				
– Females	8.6 (2.7-27.2)	<0.001	-	-
– Males	1.7 (1.1-2.6)	0.015	-	-
– Non-overweight females	-	-	13.4 (3.7-51.6)	<0.001
– Non-overweight males	-	-	2.4 (1.4-4.3)	0.002
– Severe overweight females	-	-	4.0 (1.0-16.2)	0.053
– Severe overweight males	-	-	0.7 (0.3-1.7)	0.433

[a] No significant interaction between severe overweight and PNPLA3 on steatosis (p=0.346)

* No significant interaction between race and PNPLA3 genotypes (p=0.951 for steatosis and p=0.618 for steatohepatitis)

[¥] Additive (linear) model of PNPLA3: with every addition of an unfavorable G-allele, the probability for the outcome increases with the presented odds. The additive OR of PNPLA3 significantly differs for gender (interaction term for steatosis p=0.009 and for steatohepatitis p=0.010) and severe overweight (interaction term for steatohepatitis p=0.015); Hence, the OR per G-allele within each subgroup is presented, which is a display of these interaction term(s)

Association between *PNPLA3*, advanced fibrosis and hepatic inflammation. By univariable analysis, *PNPLA3* was not associated with advanced fibrosis (OR 0.8, 95%CI: 0.5 – 1.1, p=0.175), found in 104 (20%) patients. Factors associated with advanced fibrosis are found in supplementary table 3. In multivariable analysis, advanced fibrosis was associated with male gender (OR 2.9, 95%CI: 1.4 – 6.0, p=0.004) alcohol abuse (OR 3.3, 95%CI: 1.1 – 10.0, p=0.032), AST/ALT ratio (OR 5.5, 95%CI: 1.8 – 16.5, p=0.003), a higher HAI score (OR 1.8, 95%CI: 1.5 – 2.1, p<0.001), a higher HBV DNA load among HBeAg-negative patients (OR 1.2, 95%CI: 1.0 – 1.6, p=0.088) and older age (OR per 10 years 1.2, 95%CI: 0.97 – 1.5, p=0.094), but not with *PNPLA3* (OR 1.1, 95%CI: 0.7 – 1.8, p=0.566). Finally, *PNPLA3* GG was associated with significant lobular inflammation, which was found in 55% versus 34% of patients with GG versus CC and CG, respectively (p=0.008). By multivariable analysis,

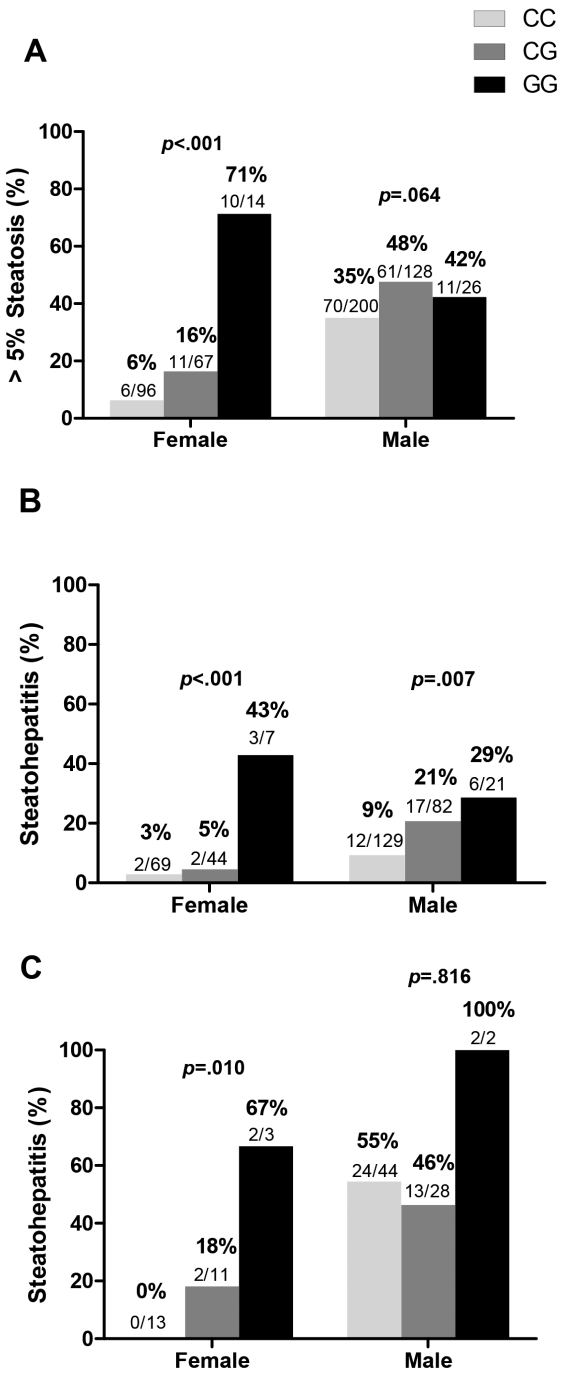


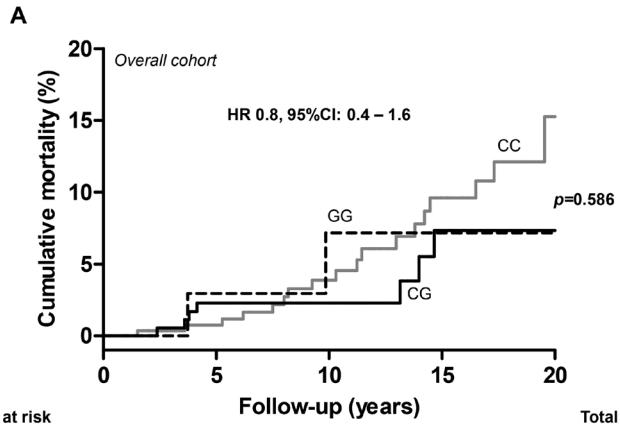
Figure 1 The percentage of patients with steatosis (A), steatohepatitis in patients without severe overweight (B), and steatohepatitis in patients with severe overweight (C), according to gender and *PNPLA3* genotypes

PNPLA3 GG was associated with significant lobular inflammation (OR 2.2, 95%CI: 1.1 – 4.5, p=0.032) independent of the presence of advanced fibrosis, steatohepatitis, and serum ALT or AST. *PNPLA3* was not associated with the (cumulative) HAI score (β=-0.2, 95%CI: -0.5 – 0.1, p=0.149).

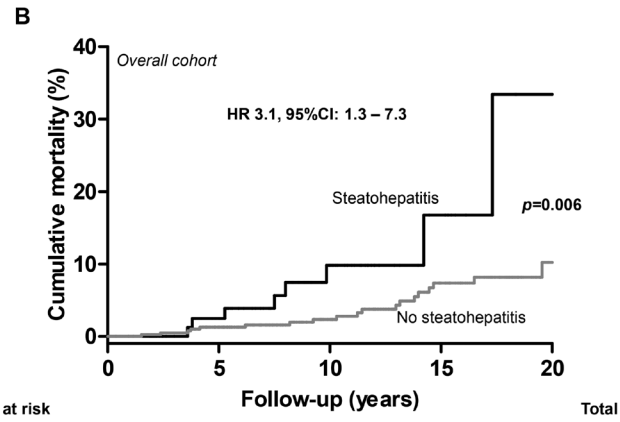
Association between *PNPLA3* and all-cause mortality. During long-term follow-up, 28 patients died, of which 8 due to a liver-related cause, 8 of other causes and 12 unknown. Sixteen of these patients received antiviral therapy after liver biopsy. By Kaplan-Meier analysis, the time to all-cause mortality was not associated with polymorphisms of *PNPLA3* (p=0.586, figure 2). In contrast, both steatosis (Hazard ratio [HR] 2.1, 95%CI: 1.0 – 4.4, p=0.051) and steatohepatitis (HR 3.1, 95%CI: 1.3 – 7.3, p=0.006, figure 2) were associated with all-cause mortality, also in those patients who received antiviral therapy after their liver biopsy (HR 2.4, 95%CI: 0.9 – 6.6, p=0.071 for steatosis and HR 5.2, 95%CI: 1.7 – 15.9, p=0.001 for steatohepatitis, supplementary figure 2). Other factors associated with all-cause mortality are shown in supplementary table 4. When separately including significant variables at biopsy within a multivariable model, steatohepatitis remained a risk factor for all-cause mortality (table 3). Due to the limited number of events we could only include one baseline variable besides steatohepatitis within the models.

Table 3 Multivariable Cox models including steatohepatitis and different baseline variables for all-cause mortality and HCC

	All-cause mortality (n=28 events)		**HCC (n=13 events)**	
	HR (95%CI) of Steatohepatitis	***p*-value**	**HR (95%CI) of Steatohepatitis**	***p*-value**
Model 1				
Steatohepatitis + age	1.9 (0.8-4.5)	0.151	1.6 (0.5-5.4)	0.436
Model 2				
Steatohepatitis + cirrhosis	3.4 (1.4-7.9)	0.005	3.1 (0.9-10.4)	0.066
Model 3				
Steatohepatitis + gender	2.5 (1.1-5.7)	0.038	2.3 (0.7-7.5)	0.183
Model 4				
Steatohepatitis + alcoholabuse	2.6 (1.0-6.5)	0.048	2.2 (0.5-8.7)	0.269
Model 5				
Steatohepatitis + diabetes	1.9 (0.7-4.7)	0.184	1.9 (0.6-6.7)	0.298
Model 6				
Steatohepatitis + HAI	3.6 (1.5-8.7)	0.004	3.2 (0.9-10.8)	0.061

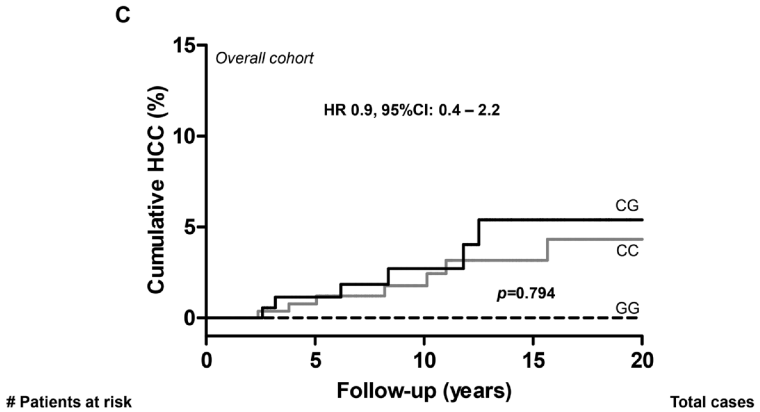


# Patients at risk						Total cases
PNPLA3 CC	295	235	150	93	27	18
CG	195	162	102	50	16	8
GG	39	32	21	9	4	2

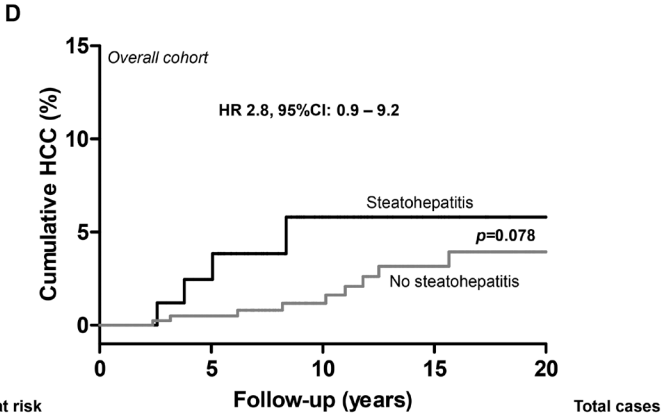


# Patients at risk						Total cases
No steatohepatitis	433	354	236	139	44	20
Steatohepatitis	97	74	37	12	2	8

Figure 2 Kaplan-Meier survival curves for the cumulative probability of all-cause mortality (A, B) and HCC (C, D) according to polymorphisms of *PNPLA3* or the presence of steatohepatitis



# Patients at risk						Total cases
PNPLA3 CC	292	231	150	92	26	7
CG	194	162	101	50	16	6
GG	38	31	20	8	3	0



# Patients at risk						Total cases
No steatohepatitis	433	355	235	138	43	9
Steatohepatitis	97	73	36	12	2	4

Figure 2 (continued) Kaplan-Meier survival curves for the cumulative probability of all-cause mortality (A, B) and HCC (C, D) according to polymorphisms of *PNPLA3* or the presence of steatohepatitis

Association between *PNPLA3* and HCC development. Thirteen patients developed HCC, of which 11 received antiviral therapy after liver biopsy, and of which 8 died. By Kaplan-Meier analysis, the time to HCC was not associated with *PNPLA3* polymorphisms (p=0.794, figure 2). Moreover, both steatosis (HR 2.2, 95%CI: 0.7 – 6.5, p=0.153) and steatohepatitis (HR 2.8, 95%CI: 0.9 – 9.2, p=0.078, figure 2) showed a non-significant association with the time to

HCC development both in the overall cohort, and a significant association in patients who received antiviral therapy after liver biopsy (HR 3.7, 95%CI: 1.1 – 12.2, p=0.023 for steatosis and HR 5.4, 95%CI: 1.5 – 19.6, p=0.004 for steatohepatitis, supplementary figure 2). Factors in univariable cox regression associated with HCC development are found in supplementary table 4. Again we separately included significant variables at biopsy within the multivariable model due to the limited number of events, and observed that steatohepatitis remained a risk factor for HCC development (table 3).

DISCUSSION

In this large-scale, single-centre study, we have shown that a common variant of *PNPLA3* is associated with the presence and severity of steatosis, steatohepatitis, lobular inflammation and iron depositions in CHB patients. Importantly, the effect of *PNPLA3* on steatosis and steatohepatitis was significantly modified by gender and the presence of severe overweight independent of ethnicity, viral factors or metabolic risk factors. Furthermore, we have shown that in the current cohort *PNPLA3* was not associated with all-cause mortality or HCC development, and we confirmed that *PNPLA3* was not associated with advanced fibrosis in CHB patients.[19]

Romeo et al. were the first to describe the association between *PNPLA3* and liver steatosis in the general population.[12] *PNPLA3* showed to influence the severity of liver steatosis independently of metabolic disorders, such as diabetes mellitus,[27] and showed to aggravate liver disease in HCV-infected patients,[15, 16] in patients with alcoholic liver disease[13] and was associated with the development of HCC.[17] *PNPLA3* was also associated with steatosis, but not with advanced fibrosis, in CHB patients.[19] Recent evidence from a knockin mice study showed that the isoleucine (I) to methionine (M) variation at position 148 of *PNPLA3 rs738409* directly results in the accumulation of hepatic steatosis, probably through a loss rather than a gain of catalytic function of *PNPLA3*.[11]

Previous studies indicated that the effect of *PNPLA3* on steatosis may be modified by environmental factors. In HCV-infected patients, *PNPLA3* led to hepatic fat accumulation in patients with moderate, but not in those with excessive, alcohol consumption.[28] In contrast, *PNPLA3* was only associated with severe steatosis in CHB patients with excessive alcohol consumption and/or severe overweight. However, the latter finding was probably biased by a low number of cases in the group without co-factors.[19] Our study advances these reports, in that we have shown that the additive effect of *PNPLA3* on steatohepatitis depended on gender as

well as on the presence of severe overweight, independent of other (behavioural) risk factors. We observed that the additional effect per *PNPLA3* G-allele on steatohepatitis development was significantly more distinct in patients without severe overweight compared to severe overweight patients. Among severe overweight male patients, the prevalence of steatohepatitis was high both for the favourable CC and unfavourable GG genotypes. A possible explanation for this finding could be that the predisposing factors for and the effects of severe overweight prevail over the effect of the favourable CC genotype. This finding however needs further confirmation, as there was only a limited number of severe overweight patients with the unfavourable GG genotype, restricting the analysis of the additive effect of *PNPLA3* in this subgroup.

In line with a recent meta-analysis which showed that the effect of *PNPLA3* on steatosis decreased with the proportion of male patients studied,[27] we found that females had a significantly higher probability of steatosis or steatohepatitis in the presence of an unfavourable *PNPLA3* G-allele compared to male patients. Counterintuitively, female gender was also associated with a reduced probability of steatosis and steatohepatitis. This underlines the strong effect of the unfavourable variation of *PNPLA3* in females. The main hypothesis for this finding is that the effect of *PNPLA3* may be amplified by an interaction with female sex hormones.[29]

In accordance with findings of a genome-wide association study in NAFLD, we have shown for the first time that the GG variation of *PNPLA3* was independently associated with the presence of hepatic iron depositions in CHB patients.[18] The accumulation of hepatic iron has previously been associated with disease progression and HCC development.[30, 31] In line with these findings, we also found an association between hepatic iron and the presence of advanced fibrosis, and a non-significant higher risk for mortality and HCC.

We observed that steatosis and steatohepatitis was a risk factor for all-cause mortality and the development of HCC in CHB patients, confirming observations in other liver disease.[5, 6, 17] Despite the fact that we found a significant association between *PNPLA3*, steatohepatitis and iron depositions, we could not detect a significant association between *PNPLA3* and advanced fibrosis at baseline, and *PNPLA3* and the development of HCC and mortality at long-term follow-up. There are several explanations for these observations. The current cohort was relatively young of age at biopsy (mean 35 years) and at long-term follow-up (mean 45 years), while the effect of *PNPLA3* through histologic changes on further progression of fibrosis, HCC development and ultimately mortality may become more distinct over time. Furthermore, the number of HCC and mortality events in the current cohort was low, limiting the power for the analysis of the effect of *PNPLA3* on these outcomes. With regard to advanced fibrosis we could only perform a cross-sectional analysis. Our findings confirmed the results from a previous CHB study, which may indicate that HBV related factors such as HBeAg status and HBV DNA

load may be more important risk factors for the progression of liver fibrosis in CHB patients.[19] Nevertheless, the additive effect of an unfavourable *PNPLA3* variation on fibrosis progression during long-term follow-up remains to be elucidated. Moreover, as we found an association between steatosis, steatohepatitis and clinical outcome, *PNPLA3* may still be a significant risk factor in the long-term.

An important asset of the current study is that all liver biopsies were uniformly assessed by one highly experienced, academic hepato-pathologist. This excludes problems with inter-observer variation, improves the consistency and accuracy of the histological assessments and minimizes potential problems related to sample size.[24] Furthermore, it minimizes the chance of misdiagnosis of steatohepatitis, which could be a potential problem due to viral hepatitis. Moreover, this cohort of CHB patients is one of the largest histologically studied thus far, was well balanced in terms of age and HBeAg status, and showed to be representative for the HBV-infected population in daily practice as all major viral genotypes and ethnicities were included. Limitations of the current study are mainly related to the retrospective collection of data on insulin resistance and the use of alcohol. Nonetheless, the prevalence of diabetes and alcohol consumption in our cohort was similar to the prevalence in CHB patients reported in previous studies.[9, 19] Furthermore, previous publications also indicate that the association between *PNPLA3* and liver steatosis was independent of these factors,[27] and our data were in line with these findings.

In conclusion, *PNPLA3* was independently associated with hepatic steatosis, steatohepatitis, lobular inflammation and hepatic iron depositions, but not with advanced fibrosis, HCC development or mortality in the current cohort of biopsied CHB patients. The effect of *PNPLA3* on steatohepatitis was particularly pronounced among female patients without severe overweight.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY TABLES

Supplementary table 1 Univariable analysis for steatosis (>5%) and steatohepatitis

Variable	Steatosis (>5%)		Steatohepatitis	
	OR (95%CI)	p-value	OR (95%CI)	p-value
Age at biopsy (per 10 years)	1.1 (1.0-1.1)	<0.001	1.1 (1.0-1.1)	<0.001
Female gender	0.3 (0.2-0.4)	<0.001	0.3 (0.2-0.5)	<0.001
Race		0.190		0.677
– Caucasian	Reference		Reference	
– Asian	0.7 (0.5-1.1)		1.2 (0.7-1.9)	
– African	0.6 (0.4-1.0)		1.0 (0.6-1.8)	
BMI	1.3 (1.2-1.3)	<0.001	1.3 (1.2-1.3)	<0.001
BMI ≥ 27.5kg/m^2	5.5 (3.5-8.8)	<0.001	5.8 (3.5-9.6)	<0.001
Alcohol use	1.8 (1.2-2.7)	0.003	1.6 (1.0-2.5)	0.043
Alcohol abuse	1.4 (0.6-3.2)	0.412	2.1 (0.9-5.0)	0.098
Diabetes mellitus	3.2 (1.5-6.9)	0.003	2.9 (1.3-6.4)	0.007
Serum ALT (per 2x ULN)	0.9 (0.8-1.1)	0.322	0.8 (0.7-1.0)	0.051
Serum AST (per 2x ULN)	0.9 (0.7-1.1)	0.251	0.7 (0.5-1.1)	0.101
Thrombocytes (per 10x10^9/L)	1.0 (1.0-1.1)	0.367	1.0 (1.0-1.1)	0.042
Iron depositions	2.3 (1.5-3.4)	<0.001	2.7 (1.7-4.3)	<0.001
Hepatic Activity Index	1.0 (0.9-1.1)	0.666	1.0 (0.9-1.1)	0.457
– Lobular inflammation	1.5 (1.2-2.0)	0.002	1.9 (1.4-2.5)	<0.001
– Portal inflammation	0.9 (0.7-1.2)	0.479	0.9 (0.7-1.2)	0.678
– Interface inflammation	1.0 (0.8-1.1)	0.624	1.0 (0.8-1.2)	0.827
HBeAg-negative	2.2 (1.5-3.1)	<0.001	2.3 (1.5-3.6)	<0.001
HBV DNA load among:	0.9 (0.8-1.0)	0.001	0.9 (0.8-0.9)	<0.001
– HBeAg(+)	1.0 (0.9-1.2)	0.996	0.9 (0.8-1.1)	0.398
– HBeAg(-)	1.0 (0.9-1.1)	0.841	0.9 (0.8-1.1)	0.373
HBV genotype		0.786		0.617
– A	Reference		Reference	
– B	0.9 (0.4-2.1)		0.7 (0.2-1.8)	
– C	1.1 (0.6-2.3)		1.1 (0.5-2.5)	
– D	1.1 (0.6-2.1)		1.1 (0.5-2.2)	
– E	0.8 (0.3-2.0)		1.0 (0.4-3.0)	
PNPLA3 genotype per G-allele[¥]:	1.8 (1.3-2.3)	<0.001	1.9 (1.4-2.6)	<0.001
– Female	5.6 (2.7-11.4)	<0.001	[x]	[x]
– Male	1.4 (1.0-1.9)	0.064	[x]	[x]
– Female severe overweight	[x]	[x]	4.7 (1.5-15.4)	0.010
– Female non-overweight	[x]	[x]	8.6 (2.9-25.8)	<0.001
– Male severe overweight	[x]	[x]	1.1 (0.5-2.3)	0.816
– Male non-overweight	[x]	[x]	2.0 (1.2-3.3)	0.007

[¥] Additive (linear) model of PNPLA3: with every addition of an unfavorable G-allele, the probability for the outcome increases with the displayed odds. [x] Not applicable for this endpoint

Supplementary table 2 Univariable analysis for iron depositions

Variable	Iron	
	OR (95%CI)	p-value
Age at biopsy (per 10 years)	1.0 (1.0-1.1)	<0.001
Female gender	0.2 (0.1-0.3)	<0.001
Race		0.209
– Caucasian	Reference	
– Asian	1.5 (0.9-2.3)	
– African	1.7 (1.0-2.8)	
BMI	1.1 (1.0-1.1)	0.042
BMI ≥ 27.5kg/m^2	1.5 (0.9-2.4)	0.116
Alcohol use	2.7 (1.8-4.2)	<0.001
Alcohol abuse	2.3 (0.9-5.6)	0.065
Diabetes mellitus	1.4 (0.6-3.4)	0.467
Serum ALT (per 2x ULN)	1.0 (0.9-1.2)	0.376
Serum AST (per 2x ULN)	1.1 (0.9-1.4)	0.308
Thrombocytes (per 10x10^9/L)	0.9 (0.9-1.0)	<0.001
Hepatic Activity Index	1.2 (1.1-1.3)	<0.001
– Lobular inflammation	1.5 (1.1-2.0)	0.006
– Portal inflammation	1.5 (1.2-2.0)	0.001
– Interface inflammation	1.2 (1.0-1.4)	0.048
Steatosis	2.2 (1.5-3.4)	<0.001
Severe steatosis	2.6 (1.4-4.6)	0.001
Steatohepatitis	2.6 (1.6-4.2)	<0.001
HBeAg-negative	2.4 (1.6-3.7)	<0.001
HBV DNA load among:		
– HBeAg(+)	1.5 (1.1-2.1)	0.007
– HBeAg(-)	1.2 (1.0-1.3)	0.028
HBV genotype		0.456
– A	Reference	
– B	1.1 (0.5-2.2)	
– C	1.2 (0.7-2.3)	
– D	0.7 (0.4-1.2)	
– E	1.4 (0.6-3.4)	
PNPLA3 genotype per G-allele[¥]:	1.2 (0.9-1.6)	0.316
PNPLA3 GG versus non-GG	2.0 (1.0-3.9)	0.044

[¥] Additive (linear) model of PNPLA3: with every addition of an unfavorable G-allele, the probability for the outcome increases with the displayed odds

Supplementary table 3 Univariable analysis for advanced fibrosis

Variable	Advanced fibrosis	
	OR (95%CI)	p-value
Age at biopsy (per 10 years)	1.0 (1.0-1.1)	<0.001
Female gender	0.4 (0.2-0.6)	<0.001
Race		0.995
– Caucasian	Reference	
– Asian	0.9 (0.6-1.5)	
– African	1.3 (0.8-2.2)	
BMI	1.0 (1.0-1.1)	0.494
BMI ≥ 27.5kg/m^2	1.5 (0.9-2.5)	0.112
Alcohol use	1.1 (0.7-1.8)	0.559
Alcohol abuse	5.3 (2.3-12.0)	<0.001
Diabetes mellitus	3.0 (1.4-6.4)	0.005
Serum ALT (per 2x ULN)	1.1 (1.0-1.3)	0.014
Serum AST (per 2x ULN)	1.6 (1.2-2.0)	<0.001
Thrombocytes (per 10x10^9/L)	0.8 (0.8-0.9)	<0.001
Iron depositions	2.6 (1.7-4.1)	<0.001
Hepatic Activity Index	1.7 (1.5-1.9)	<0.001
– Lobular inflammation	2.0 (1.4-2.7)	<0.001
– Portal inflammation	4.7 (3.4-6.5)	<0.001
– Interface inflammation	3.3 (2.5-4.2)	<0.001
Steatosis	1.2 (0.8-1.9)	0.406
Severe steatosis	0.9 (0.5-1.8)	0.795
Steatohepatitis	1.4 (0.9-2.4)	0.167
HBeAg-negative	1.1 (0.7-1.6)	0.704
HBV DNA load among:	1.1 (1.0-1.2)	0.022
– HBeAg(+)	1.0 (0.8-1.1)	0.667
– HBeAg(-)	1.5 (1.2-1.7)	<0.001
HBV genotype		0.089
– A	Reference	
– B	0.8 (0.4-1.6)	
– C	0.8 (0.4-1.5)	
– D	0.5 (0.3-0.9)	
– E	0.6 (0.2-1.7)	
PNPLA3 genotype per G-allele[¥]:	0.8 (0.5-1.1)	0.175

[¥] Additive (linear) model of PNPLA3: with every addition of an unfavorable G-allele, the probability for the outcome increases with the displayed odds

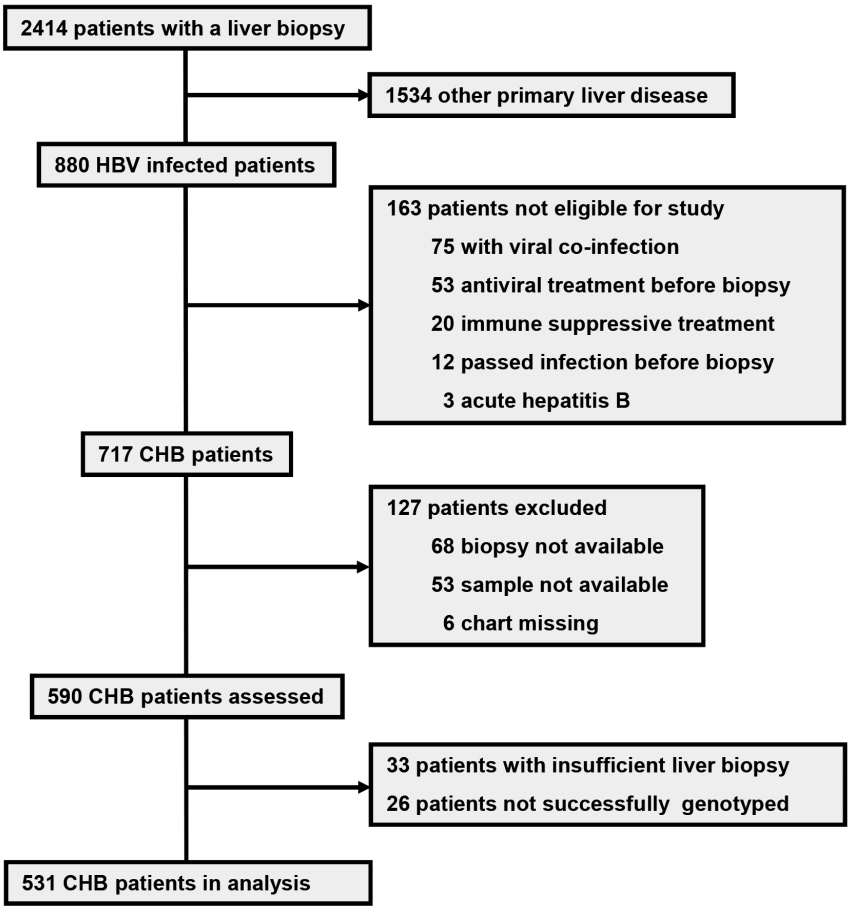
Supplementary table 4 Univariable Cox regression analysis for HCC and all-cause mortality

Variable	HCC		All-cause mortality	
	HR (95%CI)	p-value	HR (95%CI)	p-value
Age at biopsy (per 10 years)	2.4 (1.6-3.7)	<0.001	2.2 (1.7-2.9)	<0.001
Female gender	0.1 (0.02-1.2)	0.073	0.1 (0.03-0.6)	0.008
Race		0.739		0.911
– Caucasian	Reference		Reference	
– Asian	0.6 (0.2-2.2)		0.7 (0.3-1.8)	
– African	[x]		0.9 (0.3-2.8)	
BMI	1.1 (0.9-1.2)	0.349	1.1 (1.0-1.2)	0.147
BMI ≥ 27.5kg/m^2	1.9 (0.6-6.2)	0.285	1.8 (0.7-4.4)	0.191
Alcohol use	1.2 (0.3-4.3)	0.768	1.4 (0.6-3.2)	0.420
Alcohol abuse	5.4 (1.1-25.4)	0.035	8.0 (3.1-20.5)	<0.001
Diabetes mellitus	6.8 (2.1-22.1)	0.001	5.6 (2.5-13.0)	<0.001
Serum ALT (per 2x ULN)	0.9 (0.6-1.4)	0.569	1.0 (0.8-1.2)	0.672
Serum AST (per 2x ULN)	1.1 (0.7-1.8)	0.694	1.2 (0.9-1.6)	0.303
Thrombocytes (per 10x10^9/L)	0.8 (0.7-0.8)	<0.001	0.9 (0.8-0.96)	0.001
Iron depositions	1.6 (0.4-6.2)	0.507	2.2 (0.9-5.1)	0.078
Hepatic Activity Index	1.2 (1.0-1.4)	0.036	1.2 (1.0-1.3)	0.010
– Lobular inflammation	1.1 (0.5-2.4)	0.734	1.3 (0.8-2.1)	0.377
– Portal inflammation	2.0 (1.0-3.9)	0.039	1.5 (0.97-2.4)	0.071
– Interface inflammation	1.3 (1.1-1.6)	0.008	1.3 (1.1-1.5)	0.009
Steatosis	2.2 (0.7-6.5)	0.153	2.1 (1.0-4.4)	0.051
Steatohepatitis	2.8 (0.9-9.2)	0.078	3.1 (1.3-7.3)	0.006
Advanced fibrosis	14.5 (4.0-52.7)	<0.001	4.3 (2.1-9.1)	<0.001
Cirrhosis	12.5 (4.1-38.3)	<0.001	5.3 (2.5-11.3)	<0.001
HBeAg-negative	1.5 (0.5-4.6)	0.436	1.6 (0.8-3.4)	0.205
HBV DNA load among:				
– HBeAg(+)	0.9 (0.6-1.2)	0.401	1.0 (0.8-1.2)	0.885
– HBeAg(-)	1.7 (1.1-2.7)	0.014	1.1 (0.8-1.6)	0.550
HBV genotype		0.597		0.234
– A	Reference		Reference	
– B	[x]		[x]	
– C	0.3 (0.03-2.5)		1.1 (0.3-4.5)	
– D	0.5 (0.1-1.9)		1.2 (0.4-3.8)	
– E	[x]		1.5 (0.2-13.2)	
PNPLA3 per G-allele[¥]:	0.8 (0.4-2.2)	0.785	0.8 (0.4-1.6)	0.587
PNPLA3 GG versus non-GG	1.1 (0.4-3.3)	0.855	0.8 (0.3-1.6)	0.478

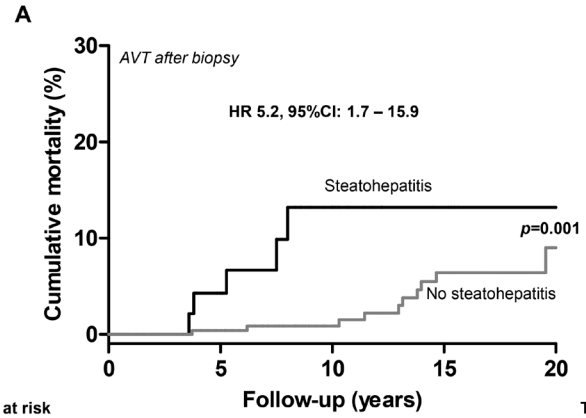
[¥] Additive (linear) model of PNPLA3: with every addition of an unfavorable G-allele, the probability for the outcome increases with the displayed odds

[x] Not applicable for this endpoint

SUPPLEMENTARY FIGURES



Supplementary Figure 1 Study workflow



# Patients at risk						Total cases
No steatohepatitis	282	237	166	95	35	11
Steatohepatitis	54	42	19	6	2	5

# Patients at risk						Total cases
No steatohepatitis	282	237	165	94	34	7
Steatohepatitis	54	42	18	6	2	4

Supplementary Figure 2 Kaplan-Meier survival curves for the cumulative probability of all-cause mortality (A) and HCC (B) according to the presence of steatohepatitis in the subcohort of patients treated after liver biopsy

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CHAPTER 4

Prediction of long-term clinical outcome in a diverse chronic hepatitis B population: role of the PAGE-B score

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Submitted

ABSTRACT

Background & Aims

An abundance of non-invasive scores have been associated with fibrosis and hepatocellular carcinoma (HCC) development. We aimed to compare the prognostic ability of these scores in relation to liver histology in chronic hepatitis B (CHB) patients.

Methods

Liver biopsies from treatment-naïve CHB patients at one tertiary care centre were scored by a single hepato-pathologist. Laboratory values at liver biopsy were used to calculate the PAGE-B, REACH-B, GAG-HCC, CU-HCC, and FIB-4 scores. Any clinical event was defined as HCC development, liver failure, transplantation and mortality. HCC and mortality data was obtained from national database registries.

Results

Of 557 patients, 40 developed a clinical event within a median follow-up of 10.1 (IQR 5.7-15.9) years. The PAGE-B score predicted any clinical event (C-statistic 0.87, 95%CI: 0.81-0.92), HCC development (C-statistic 0.89) and reduced transplant-free survival (C-statistic 0.84) with good accuracy, also when stratified by ethnicity, AVT after biopsy or advanced fibrosis. The C-statistics (95%CI) of the REACH-B, GAG-HCC, CU-HCC, and FIB-4 scores for any event were 0.70 (0.59-0.81), 0.82 (0.75-0.89), 0.73 (0.63-0.84), and 0.79 (0.69-0.89), respectively. The PAGE-B event risk assessment improved modestly when combined with the Ishak fibrosis stage (C-statistic 0.88, 95%CI: 0.83-0.93), with a total net risk reclassification improvement of 0.1 and 0.09 at year 5 and 10.

Conclusions

The PAGE-B score showed the best performance to assess the likelihood to develop a clinical event among a diverse CHB population over 15 years of follow-up. Additional liver histologic characteristics did not appear to provide with a clinically significant improvement.

INTRODUCTION

Approximately 350 million patients worldwide have chronic hepatitis B (CHB). Long-term CHB can lead to liver cirrhosis, decompensation, hepatocellular carcinoma (HCC) development and death. Nearly 30% of cirrhosis and 53% of all HCC is attributable to CHB, and about 650.000 patients die to the complications of CHB each year.[1, 2] Disease progression may be halted by antiviral therapy, and therefore it is important to assess the risk of deterioration for individual patients to be able to provide a timely intervention for those who benefit most.[3-5] Since decades liver biopsy has been the gold standard to assess the severity of liver disease and the patients' related prognosis. However, this procedure is associated with potentially severe complications, sampling error, as well as inter- and intra-observer variation.[6, 7] Non-invasive objective surrogate scores for the long-term prognosis are therefore warranted.

Recently the FIB-4 and APRI score have been developed to estimate the fibrosis severity with moderate to good accuracy, and the FIB-4 score has additionally been associated with survival and HCC development during 5 years of follow-up.[8-12] Furthermore, the PAGE-B score has been developed to estimate the probability of HCC development in Caucasian CHB patients treated with entecavir or tenofovir, as an alternative to the REACH-B, CU-HCC and GAG-HCC scores which were only associated with HCC development in Asian patients.[13-18] It is unknown how the prognostic accuracy of these non-invasive serum scores compare to that of liver histology with respect to the long-term outcome in CHB patients, especially with regard to event-free and transplant-free survival. Also, the prognostic benefit in performing a liver biopsy in addition to these non-invasive scores has, to our knowledge, not been assessed in detail.

The aims of the current study therefore were 1) to assess the prognostic performance of simple non-invasive serum scores, and 2) to assess whether liver histologic characteristics could improve this performance in CHB patients.

PATIENTS AND METHODS

Patient selection. The patient population and selection has been described previously.[19] In short, mono-infected treatment-naïve CHB (HBsAg positive for >6 months) patients consecutively biopsied in the period of 1985 – 2012 were retrospectively identified in a tertiary care centre in Rotterdam, the Netherlands. Patients were excluded in case of a history of antiviral therapy for the duration of > 1 month prior to or at the time of biopsy, a current or past co-infection with hepatitis C, D, E or human immunodeficiency virus, presence of auto-immune liver disease,

primary biliary cirrhosis, Wilson's disease, hemochromatosis or any other co-existing primary liver disease, or treatment with immune suppressive medication for more than 6 months prior to or at the time of biopsy. The study was conducted in accordance with the guidelines of the Declaration of Helsinki and the principles of Good Clinical Practice and was approved by the ethical review board of the Erasmus Medical Center, Rotterdam, The Netherlands.

Data acquisition. Data on all-cause mortality was obtained from the municipal record database, and the development of HCC was obtained from the national HCC registry database. These databases were reviewed on May 14, 2014. The event of liver transplantation or decompensation was obtained from the (electronic) medical chart. Data on demographics (sex, age, race, ethnicity, height, weight, route of HBV transmission, presumed date of infection) and clinical data (history, diagnosis of diabetes mellitus, daily alcohol intake, history of alcohol abuse, smoking) were obtained by a single investigator (WB) from the chart in a standardized way. Alcohol use was defined as ≥1 units of alcohol/day and alcohol abuse was defined as an history or current use of ≥5 units/day, corresponding to 40-50 grams alcohol per day.[20] Data on chemistry (alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutyltransferase (γ-GT), bilirubin, albumin), hematology (platelet count, prothrombin time) and virology (HBsAg, anti-HBs, HBeAg, anti-HBe, HBV DNA load, HBV genotype) at the time of biopsy were obtained from the clinical laboratory and the department of Virology at the Erasmus Medical Center.

Liver histology. All liver biopsies were obtained percutaneous. These biopsies were re-scored by a single experienced hepato-pathologist (FK) who was blinded to the patient characteristics and outcome. Biopsies were scored in a uniform manner according to the Ishak fibrosis score, the hepatic activity index (HAI) [21], the Brunt score for steatosis, and NASH, defined as the combined presence of macrovesicular or microvesicular steatosis, lobular inflammation, lipogranulomas and ballooning degeneration.[22] Advanced fibrosis was defined as an Ishak score of ≥4, corresponding to portal to portal bridging and probable or definite cirrhosis. To minimize the chance of histological misclassification due to sample size, liver biopsies with a length of less than 10 mm and with less than 10 portal fields were excluded from the analysis.[23, 24]

Laboratory measurements. The gender and time-dependent upper-limit of normal (ULN) values were used for the analysis of serum ALT and AST. The HBV DNA level was expressed in units/millilitre (IU/ml) and, when required, calculated using the conversion of 1.0 picogram/ml = 5.15×10^4 IU/ml or 1.0 copies/ml = 0.1818 IU/ml. HBV genotype was

determined using the INNO-LiPA Genotype assay (Innogenetics, Ghent, Belgium) in case data on HBV genotype was missing.

Outcome measures. The occurrence of liver failure (defined as an episode of jaundice, ascites, hepatic encephalopathy or gastro-duodenal bleeding due to varices), HCC development, liver transplantation and all-cause mortality was studied. We assessed the occurrence of these events as a composite endpoint (any clinical event) as well as separately. In case of multiple events in an individual patient, only the first event was considered for the composite endpoint. The cause of death was determined by the treating physician. Death caused by liver failure or HCC was considered liver-related. The diagnosis of HCC was based on histopathology and when not available, on 2 imaging modalities (magnetic resonance imaging, computed tomography or contrast enhanced ultrasound).[25]

Statistical analysis. Baseline was defined as the date of first liver biopsy. At baseline, the association between the non-invasive serum scores and advanced fibrosis or cirrhosis was assessed using logistic regression analyses and the area under the receiver-operating characteristic curve (AUROC). The association between (non-)invasive scores at baseline and clinical outcome at long-term follow-up was subsequently estimated using the Cox proportional hazards method. Deceased patients were censored at the time of death for the non-mortality outcomes. Patients who experienced liver failure were not censored in the analysis for HCC, and vice versa. If there was no clinical event, patients were censored at the last follow-up visit. The C-statistic was calculated for the (non-)invasive scores to assess the predictive ability for any clinical outcome. The risk of any clinical event was estimated for each individual patient using the baseline survival from the Cox regression model using the non-invasive score(s) with the highest overall C-statistic. After addition of liver biopsy characteristics to the Cox regression model, we re-assessed the updated C-statistic and the updated estimation of this event risk. Subsequently the net reclassification change was calculated, in order to obtain the change in the estimated risk and thus the clinical added value of a liver biopsy when combined with the best performing non-invasive score.[26, 27] Scores for cirrhosis and outcome were constructed as previously described and their respective cut-offs were utilized in the analysis when appropriate.[9, 10, 13-15, 18, 28] (Supplementary table 1) Skewed variables were log-transformed prior to the analyses. SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.3 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All statistical tests were two-sided and evaluated at the 0.05 level of significance.

Table 1 Patient characteristics.

Characteristics	All patients (n=557)
Demography	
Age at biopsy, years (SD)	34.7 (12.5)
Male, n(%)	371 (67)
Duration of infection, years (SD)	31.3 (12.9)
Body mass index kg/m^2 (SD)*	24.8 (4.1)
Diabetes, n(%)	29 (5)
History of alcohol abuse, n(%)$^{\beta}$	25 (5)
AVT after biopsy, n (%):	348 (63)
First course / Last course:	
– NA only	184 (53)
– (Peg)IFN only	68 (19)
– (Peg)IFN / NA	84 (24)
– NA / (Peg)IFN	7 (2)
– Other	5 (2)
Ethnicity, n(%)	
Caucasian	261 (47)
Asian	175 (31)
African/negroid	104 (19)
Other	17 (3)
Virology	
HBeAg-positive, n (%)	280 (50)
log HBV DNA, IU/ml (SD)	5.7 (2.8)
HBV Genotype A/B/C/D/E**	126/64/98/171/31
Chemistry/hematology	
ALT, x ULN (SD)	2.6 (3.6)
AST, x ULN (SD)	1.6 (1.9)
Trombocytes, (IQR)	206 (172 – 243)
Histology, n (%)	
Biopsy length, mm (SD)	19.9 (6.8)
Portal fields, n (SD)	19.9 (9.1)
Median Ishak fibrosis, (IQR)	1.0 (1.0 – 2.0)
Advanced fibrosis, n(%)	113 (20)
Hepatic Activity index (SD)	4.2 (2.3)
Steatosis (>5%), n(%)	179 (32)
Steatohepatitis, n(%)	103 (19)
Follow-up, years from biopsy (IQR)	10.1 (5.7 – 15.9)

** Available in 469 (85%) patients, $^{\beta}$ Available in 477 (86%) patients;*
*** Available in 498 (90%) patients,*
AVT, antiviral therapy; NA, nucleos(t)ide analogue; (Peg)IFN, (pegylated) interferon

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RESULTS

Patient characteristics. Of 880 biopsied CHB patients, 163 did not meet eligibility criteria, 127 were excluded because of a missing chart or liver biopsy and 33 were excluded because of an inadequate liver biopsy sample. In total 557 patients were thus included, of which 371 (67%) were male. The mean age at biopsy was 34.7 years. Patient characteristics are presented in table 1. Patients were Caucasian in 47% (n=261), Asian in 31% (n=175) and African in 19% (n=104). HBV genotype A, B, C, D, E and other/mixed was present in 126 (23%), 64 (12%), 98 (18%), 171 (31%), 31 (6%) and 8 (1%) of patients, respectively. At baseline, 113 (20%) patients had advanced fibrosis and 63 (11%) cirrhosis. By univariable and multivariable analysis, the non-invasive scores all showed a significant association with advanced fibrosis (supplementary table 2). The ability to detect advanced fibrosis or cirrhosis for the non-invasive serum scores is presented in table 2.

Table 2 Area Under the Receiver-Operating Characteristic Curve for the non-invasive biomarkers for prediction of advanced fibrosis or cirrhosis at baseline

Serum score* ***AUROC (95%CI)***	**Advanced fibrosis (n=113)**	**Cirrhosis (n=63)**
PAGE-B	0.74 (0.69-0.80)	0.80 (0.74-0.85)
REACH-B	0.67 (0.62-0.73)	0.69 (0.61-0.77)
FIB-4	0.80 (0.75-0.85)	0.84 (0.79-0.89)
Log APRI	0.79 (0.74-0.83)	0.80 (0.74-0.85)

* *The GAG-HCC and CU-HCC risk scores were not tested as these already include the factor "cirrhosis" and are thus by default related to the endpoints*

AUROC, area under the receiver-operating characteristic curve

Events during follow-up. Survival and HCC status was available for 515 (92.6%) patients, 41 (7.2%) patients emigrated and were censored at the last follow-up visit; follow-up data of 1 patient (0.2%) could not be retrieved. The mean duration of follow-up after liver biopsy was 10.1 years (interquartile range 5.7 – 15.9, maximum 27.3 years). After liver biopsy, 348 (63%) patients received antiviral therapy (interferon-based or nucleos(t)ide analogue therapy). Fifty-one patients lost HBsAg (median time from biopsy 3.1 years, IQR 1.2 – 8.2, maximum 19.2 years): 15 (8%) spontaneous, 24 (36%) after (pegylated) interferon ((Peg)IFN) treatment, 10 (5%) to nucleos(t)ide analogues (NA) and 2 (4%) after sequential NA/PegIFN (p<0.001). During long-term follow-up, 40 patients experienced a clinical event: 10 patients developed liver failure, 15 patients were diagnosed with HCC, 7 patients underwent liver transplantation, and 31 patients died. Ten patients died of a liver-related cause (7 of whom due to HCC, and 3 as a result of liver failure), 8 died of liver-unrelated causes (of which 1 patient had an HCC) and for 13 patients the cause of death was unknown. The overall 5, 10 and 20-year event-free survival was 97.6%, 94.0% and 86.8%, respectively.

Factors associated with long-term clinical outcome. The (non-)invasive scores were all significantly associated with the development of a clinical event, HCC and a reduced transplant-free survival.(Supplementary table 3) In addition to the Ishak score (Hazard ratio [HR] 1.74, 95%CI: 1.5 – 2.1, p<0.001), liver biopsy characteristics significantly associated with the development of any clinical event were the HAI score (HR 1.17, 95%CI: 1.1 – 1.3, p=0.005), and presence of steatosis (HR 2.38, 95%CI: 1.3 – 1.4, p=0.006) or NASH (HR 2.56, 95%CI: 1.3 – 5.2, p=0.009). Antiviral therapy after liver biopsy (HR 1.10, 95%CI: 0.6 – 2.1, p=0.788) nor the type of therapy was associated with the development of a clinical event. Also, there was no significant interaction between (the type of) antiviral therapy and HBsAg loss on the occurrence of any clinical event.

Non-invasive scores versus liver biopsy for the prediction of clinical outcome. The C-statistic for the PAGE-B score for the prediction of any clinical event was 0.87 (95%CI: 0.81 – 0.92, table 4), and was 0.84 (95%CI: 0.78 – 0.91) for reduced transplant-free survival and 0.89 (95%CI: 0.80 – 0.98) for HCC development. The other non-invasive prognostic measures showed a lower C-statistic for all respective outcomes (table 3). When the Ishak stage was combined with the PAGE-B, the prediction for clinical outcome improved (C-statistic 0.88, 95%CI: 0.83 – 0.93). For PAGE-B scores <4, 4 – 7 and >7, the observed cumulative probability of any clinical event was 0.9%, 2.5% and 13.6% at year 5, 0.9%, 7.2% and 35.2% at year 10 and 4.0%, 11.3% and 50.3% at year 15, respectively (log-rank p<0.001, figure 1). The estimated

event risk and HCC risk for individual patients using the PAGE-B score is shown in figure 2. For patients with a PAGE-B score <4, 4 – 7 and >7 the estimated 5-year event risk was <2%, 2 – 5% and ≥5%; the 10-year event risk <5%, 5 – 12% and ≥12%; and the 15-year event risk <6%, 6 – 20% and ≥20%, respectively. By multivariable analysis, the only factors independently associated with clinical outcome were the PAGE-B score and the Ishak fibrosis stage.(Table 4)

Table 3 C-statistic for the prediction of clinical outcome by prognostic biomarkers

Serum score ***C-statistic (95%CI)***	**Any event (n=40)**	**Transplantation or mortality (n=31)**	**HCC development (n=15)**
Non-invasive			
PAGE-B	**0.87 (0.81-0.92)**	**0.84 (0.78-0.91)**	**0.89 (0.80-0.98)**
REACH-B	0.70 (0.59-0.81)	0.66 (0.53-0.80)	0.83 (0.75-0.92)
FIB-4	0.79 (0.69-0.89)	0.76 (0.65-0.88)	0.86 (0.75-0.98)
Log APRI	0.69 (0.58-0.80)	0.65 (0.53-0.78)	0.81 (0.70-0.91)
Invasive			
PAGE-B + Ishak	**0.88 (0.83-0.93)**	**0.86 (0.80-0.92)**	**0.93 (0.86-0.99)**
GAG-HCC$^{\beta}$	0.82 (0.75-0.89)	0.78 (0.70-0.87)	0.91 (0.86-0.96)
CU-HCC$^{\beta}$	0.73 (0.63-0.84)	0.69 (0.55-0.82)	0.84 (0.73-0.95)
Ishak fibrosis	0.78 (0.70-0.86)	0.75 (0.66-0.84)	0.87 (0.81-0.94)

$^{\beta}$ *The GAG-HCC and CU-HCC scores already include the diagnosis of cirrhosis, which in the current study is solely based on liver biopsy, and are therefore "invasive markers"*

In ***bold*** *the non-invasive or invasive marker with the highest C-statistic*

Table 4 Multivariable Cox regression model for any clinical event

Variables	***Full model***		***Final model***	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
PAGE-B	1.33 (1.2-1.5)	<0.001	1.31 (1.2-1.4)	<0.001
HBsAg loss*	0.38 (0.1-1.6)	0.179	-	
Diabetes mellitus	1.12 (0.4-2.8)	0.806	-	
Alcohol abuse	2.29 (0.9-6.1)	0.095	-	
Ishak stage	1.46 (1.1-2.0)	0.010	1.42 (1.2-1.7)	0.001
Hepatic activity index	0.90 (0.7-1.1)	0.383	-	
NASH	1.70 (0.7-3.9)	0.207	-	

* *Entered as a time-dependent covariate; no significant interaction with antiviral therapy after biopsy (not shown in table)*

NASH, non-alcoholic steatohepatitis

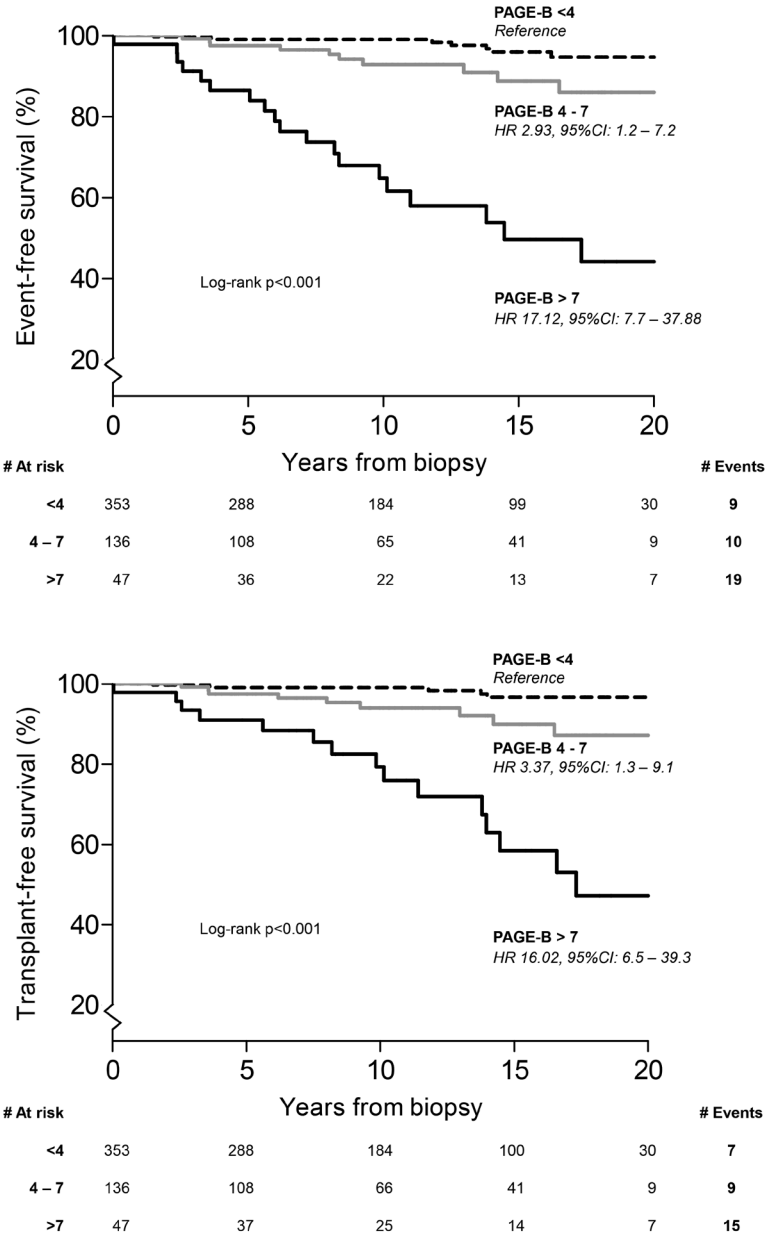

Figure 1 Kaplan meier curves for event-free survival (A), transplant-free survival (B) and HCC-free follow-up (C) according to PAGE-B risk score <4, 4 – 7 and >7 points.

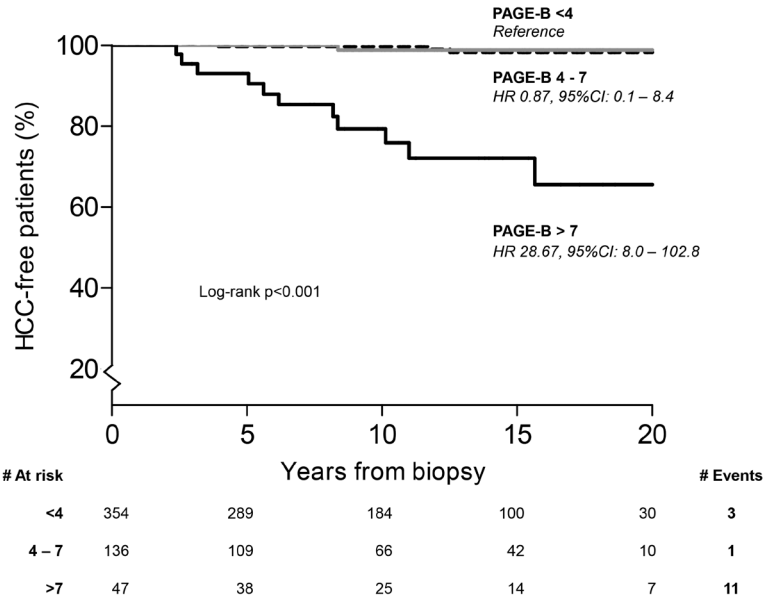

Figure 1 (continued) Kaplan meier curves for event-free survival (A), transplant-free survival (B) and HCC-free follow-up (C) according to PAGE-B risk score <4, 4 – 7 and >7 points.

Additional prognostic value of the Ishak stage combined with PAGE-B. The individual risk for patients was also estimated for the combination of the PAGE-B with the Ishak fibrosis stage using the hazard function derived from the final multivariable Cox regression model. (Table 4) This updated risk was compared to the original estimated risk (PAGE-B only) versus the actual observed events and non-events at the respective time intervals to obtain the net reclassification improvement. With the addition of the Ishak fibrosis stage, 1/12 (8.3%) of events were correctly reclassified into the intermediate – high risk group (PAGE-B score >4, corresponding to a 5-year event risk >2%), and 10/429 (2.3%) were correctly reclassified into the low-risk group at year 5 (total net reclassification improvement [NRI]=0.106). At year 10, 2/24 (8.3%) of events was incorrectly reclassified into the low-risk group (10-year event risk <5%) and 46/268 (17.2%) was correctly reclassified into the low-risk group (total NRI=0.088). Within the first 10 years of follow-up, the PAGE-B score alone correctly classified all patients who developed HCC into the intermediate – high HCC risk group (PAGE-B >4 corresponding to an HCC risk >0.3% at year 5 [4/4 cases] and >0.9% at year 10 [10/10 cases]).

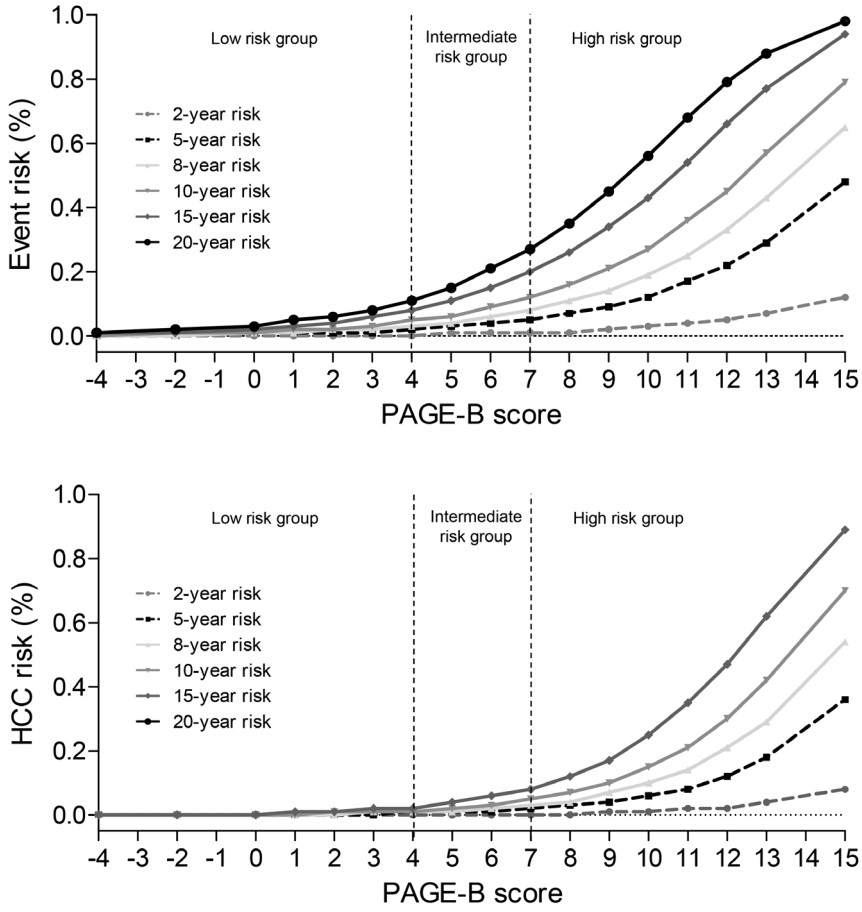

Figure 2 2-year to 20-year risk of any clinical event (A) or HCC (B) as a function of the PAGE-B score.

Performance of non-invasive scores for clinical outcome in subgroups. For the prediction of any clinical event, the PAGE-B had the highest C-statistic compared to the other non-invasive scores stratified by antiviral therapy, advanced fibrosis and ethnicity. Moreover, the C-statistics obtained with the PAGE-B for the prediction of any event or HCC development in Asian patients who received antiviral therapy after liver biopsy were higher than for the REACH-B. This was 0.89 (95%CI: 0.80 – 0.97) and 0.80 (95%CI: 0.70 – 0.91) for the PAGE-B, versus 0.85 (95%CI: 0.72 – 0.97) and 0.69 (95%CI: 0.61 – 0.78) for the REACH-B, respectively.

DISCUSSION

In the current study, we have compared different prognostic scores for their ability to predict the clinical outcome over more than 15 years of follow-up in CHB patients of diverse ethnic origin and infected with all major HBV genotypes. We found that the PAGE-B score was the overall best performing score to predict any clinical outcome. The PAGE-B score also showed a superior prognostic performance within different subgroups compared to all other scores. After addition of the Ishak fibrosis stage, the risk assessment obtained by the PAGE-B modestly improved. The clinical significance of this improvement seemed limited as there was only a minor improvement observed in the risk reclassification.

Nowadays there is an abundance of (non-)invasive scores to assess liver fibrosis and long-term outcome. This may lead to uncertainty which score best to use in clinical practice. It is of great importance to select those patients in need of antiviral therapy and HCC surveillance by assessing the risk of clinical disease progression. Comparing the different scores, we have shown for the first time that the PAGE-B score had the best overall discriminative ability to predict the risk of any clinical event, reduced transplant-free survival and HCC development over more than 15 years of follow-up and across different ethnicities. We observed a better ability for the PAGE-B score to assess the HCC risk among treated Asian patients compared to the REACH-B. Importantly, the validated PAGE-B score was previously constructed in a selected group of Caucasian CHB patients under highly potent NA therapy only.[17, 18] The findings of the current study therefore further underline the robustness of the score, also for other outcomes among untreated CHB patients of different origin.

For decades, the gold standard to assess the severity of liver disease has been liver biopsy.[7] To our knowledge, no study has assessed the additional prognostic value of liver biopsy next to non-invasive scores in detail. In the current study we have shown that the PAGE-B score both had a good relation with the severity of liver fibrosis at baseline and with the clinical outcome during long-term follow-up. With the combination of the PAGE-B score and the Ishak stage, the long-term risk prediction improved only minimally (from a C-statistic of 0.87 to 0.88). This observation was further quantified by analysing the net reclassification improvement.[26, 27] By performing a liver biopsy in 557 patients, a correct reclassification was achieved for 1/12 patients at year 5. However, 2/24 patients who developed an event at year 10 were incorrectly reclassified into the low 10-year risk group. It could be debated whether the slight improvement in the detection of events at year 5 justifies a liver biopsy with its accompanying risk for potentially severe complications and other limitations, while the PAGE-B score is an objective, readily available score. In patients with a PAGE-B score above 4, a liver biopsy may still be considered.

Table 5 C-statistic for the prediction of any clinical event within different subgroups

Biomarker ***C-statistic (95%CI)***	**Within ever AVT after biopsy (n=348)**	**Within never AVT after biopsy (n=209)**	**Within Advanced fibrosis (n=113)**	**Within no advanced fibrosis (n=444)**	**Caucasian patients (n=261)**	**Asian patients (n=175)**	**African patients (n=104)**
Non-invasive							
PAGE-B	**0.86 (0.80-0.92)**	**0.87 (0.77-0.99)**	**0.84 (0.76-0.92)**	**0.80 (0.72-0.88)**	**0.89 (0.84-0.94)**	**0.91 (0.84-0.97)**	**0.76 (0.56-0.96)**
REACH-B	0.73 (0.62-0.85)	0.66 (0.46-0.86)	0.65 (0.49-0.82)	0.65 (0.46-0.84)	0.71 (0.57-0.85)	0.87 (0.77-0.98)	0.49 (0.23-0.76)
FIB-4	0.79 (0.69-0.88)	0.77 (0.53-0.99)	0.82 (0.70-0.93)	0.65 (0.47-0.83)	0.83 (0.73-0.92)	0.81 (0.67-0.96)	0.61 (0.24-0.99)
Log APRI	0.67 (0.56-0.78)	0.68 (0.42-0.98)	0.68 (0.55-0.82)	0.50 (0.31-0.68)	0.72 (0.62-0.83)	0.77 (0.62-0.91)	0.48 (0.10-0.86)
Invasive							
PAGE-B + Ishak	**0.88 (0.82-0.94)**	**0.89 (0.80-0.98)**	**0.85 (0.79-0.92)**	**0.80 (0.72-0.89)**	**0.89 (0.84-0.95)**	0.95 (0.90-0.99)	**0.78 (0.56-0.90)**
GAG-HCC[β]	0.83 (0.76-0.90)	0.82 (0.70-0.94)	0.71 (0.58-0.83)	0.74 (0.62-0.86)	0.79 (0.70-0.88)	**0.97 (0.94-0.99)**	0.68 (0.46-0.91)
CU-HCC[β]	0.78 (0.68-0.88)	0.67 (0.46-0.88)	0.77 (0.64-0.90)	0.52 (0.36-0.68)	0.68 (0.54-0.82)	0.95 (0.90-0.99)	0.61 (0.28-0.94)
Ishak fibrosis	0.77 (0.68-0.87)	0.80 (0.67-0.93)	0.72 (0.61-0.83)	0.61 (0.49-0.72)	0.73 (0.63-0.84)	0.92 (0.85-0.99)	0.75 (0.52-0.98)

[β] *The GAG-HCC and CU-HCC scores already include the diagnosis of cirrhosis, which in the current study is solely based on liver biopsy, and are therefore "invasive markers"*

AVT, antiviral therapy

*In **bold** the non-invasive or invasive marker with the highest C-statistic*

It could however be questioned whether this would be clinically relevant as these patients already carry a higher risk of any event and as such already require more intensive follow-up, especially when the HCC risk exceeds 0.2%/year.[7, 25]

A strength of the current study is that all liver biopsies were scored by a single experienced hepato-pathologist, which excludes inter-observer, and also minimizes intra-observer variation through experience and repetition.[23] Moreover, the long-term follow-up data was both obtained from the municipal record database as well as from the national HCC registry database. As a result, data on HCC development and survival was complete for 93% of the cohort. A limitation may be the absence of transient elastography data. Nevertheless, we aimed to investigate simple, readily available serum scores for the prediction of clinical outcome. It would be important to assess the added value of transient elastography to the PAGE-B score in future research. Moreover, previous studies have shown that quantitative HBsAg levels may be associated with fibrosis and could be useful to determine the phase of infection and the risk of HCC in patients with low viral loads.[29-32] In the current study, we did not have HBsAg levels available.

In conclusion, we have shown that the PAGE-B score was the best performing non-invasive score to predict the clinical outcome of CHB patients of different origin. The Ishak stage did not clinically improve the risk prediction of the PAGE-B score. When further validated, this score could additionally be used to assess the need for antiviral therapy and HCC surveillance.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY TABLES

Supplementary table 1 Biomarkers and their components

Serum score	Components	Cut-offs	Ref
PAGE-B	Platelets, gender, age	<4, 4-7 >7	17, 18
REACH-B	Gender, age, ALT, HBeAg status, HBV DNA load (copies/mL)	<8, >8	14
FIB-4	Platelets, age, AST, ALT	>3.25	9
Log APRI	Platelets, AST	>1.4	10
GAG-HCC	Gender, age, HBV DNA load (copies/mL), cirrhosis (US+)	<101, >101	15
CU-HCC	Age, albumin, bilirubin, HBV DNA load (copies/mL), cirrhosis (US+)	<5, >5	13

US+, ultrasound+: ultrasound and other factors indicating cirrhosis. In the current study, the diagnosis of cirrhosis was solely based on liver biopsy

Supplementary table 2 Univariable and multivariable regression analysis for advanced fibrosis

Advanced fibrosis at baseline	Univariable		Multi-variable Full model		Multi-variable Final model	
	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p
Host and viral factors						
Male gender	2.78 (1.7-4.7)	<0.001	1.95 (0.9-4.2)	0.090	-	
Age at biopsy (per 10 years increase)	1.57 (1.3-1.9)	<0.001	1.43 (1.1-1.9)	0.008	-	
BMI	1.02 (1.0-1.1)	0.494	-			
Diabetes mellitus	2.98 (1.4-6.4)	0.005	0.78 (0.2-3.8)	0.762	-	
Alcohol abuse	2.09 (0.9-5.0)	0.098	4.32 (1.3-14.8)	0.020	3.52 (1.3-9.8)	0.016
Ethnicity*		0.325		0.912		0.624
– Caucasian	Reference		Reference		Reference	
– Asian	0.91 (0.6-1.5)		1.29 (0.6-2.8)		1.37 (0.7-2.6)	
– African	1.31 (0.8-2.2)		1.23 (0.5-2.9)		1.20 (0.6-2.5)	
HBV genotype A vs non-A	1.51 (0.9-2.5)	0.094	0.84 (0.4-1.9)	0.673	-	
HBeAg positive	1.1 (0.7-1.6)	0.704	-		-	
Higher HBV DNA load among:	1.10 (1.0-1.2)	0.022	-			
– HBeAg positive	1.0 (0.8-1.1)	0.667	-		-	
– HBeAg negative	1.5 (1.2-1.7)	<0.001	1.54 (1.2-2.0)	0.002	1.56 (1.3-1.9)	<0.001
Platelet count (per 10 units increase)	0.82 (0.8-0.9)	<0.001	0.85 (0.8-0.9)	<0.001	-	
AST (2 x ULN)	1.56 (1.2-2.0)	<0.001	2.02 (1.0-4.1)	0.055	-	
ALT (2 x ULN)	1.13 (1.0-1.3)	0.015	0.78 (0.5-1.1)	0.198	-	
Non-invasive score						
FIB-4	2.71 (2.1-3.6)	<0.001	**		-	
APRI	1.80 (1.5-2.2)	<0.001	**		-	
PAGE-B	1.26 (1.2-1.3)	<0.001	**		1.26 (1.2-1.4)	<0.001
REACH-B	1.30 (1.2-1.4)	<0.001	**		-	

*** These scores were tested separately and its components were omitted from the full model. The OR for the separate scores adjusted for the mentioned variables were as follows: FIB-4 (age, platelets, AST, ALT omitted from model): OR 3.01, 95%CI 2.0 – 4.5, p<0.001 ; APRI (AST and platelets omitted): OR 2.77, 95%CI 1.7 – 4.5, p<0.001; PAGE-B (age, gender, platelets omitted): OR 1.27, 95%CI: 1.2 – 1.4, p<0.001; REACH-B (Age, gender, ALT, HBeAg and HBV DNA omitted): OR 1.27, 95%CI: 1.1 - 1.5, p=0.002; HCV mortality score (age, platelets, ast/alt ratio and gender omitted): OR 2.34, 95%CI: 1.8 – 3.1, p<0.001.*

The GAG-HCC and CU-HCC risk scores were not tested as these already include the factor "cirrhosis" and are thus by default related to the endpoint

Supplementary table 3 Factors in univariable Cox regression analysis associated with clinical outcome

Variable	Any clinical event (n=40)		Mortality or transplantation (n=33)		HCC development (n=15)	
	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
Host and viral factors						
Male gender	4.76 (1.7-13.4)	0.003	3.80 (1.3-10.8)	0.012	7.36 (1.0-56.0)	0.054
Age at biopsy (per 10 years increase)	2.29 (1.8-2.9)	<0.001	2.28 (1.8-3.0)	<0.001	2.42 (1.6-3.6)	<0.001
BMI	1.12 (1.0-1.2)	0.002	1.10 (1.0-1.2)	0.020	1.05 (0.9-1.2)	0.456
Diabetes mellitus	5.64 (2.7-11.6)	<0.001	6.02 (2.8-13.0)	<0.001	5.68 (1.8-17.8)	0.003
Alcohol abuse	4.40 (1.8-10.7)	0.001	5.69 (2.3-14.1)	<0.001	4.52 (1.0-21.0)	0.054
Ethnicity		0.339		0.621		0.905
– Caucasian	Reference		Reference		Reference	
– Asian	0.49 (0.2-1.1)		0.54 (0.2-1.4)		0.64 (0.1-5.0)	0.675
– African	0.66 (0.3-1.7)		0.87 (0.3-2.3)		0.47 (0.1-4.2)	0.496
HBV genotype		0.215		0.163		0.724
– A	Reference		Reference		Reference	
– B	0.18 (0.02-1.4)		0.29 (0.03-2.3)		-	
– C	0.60 (0.2-1.9)		0.72 (0.2-2.8)		0.60 (0.1-3.1)	
– D	0.71 (0.3-1.7)		0.88 (0.3-2.5)		0.48 (0.1-2.0)	
– E	0.69 (0.1-5.4)		1.16 (0.1-9.5)		-	
HBeAg positive*	0.65 (0.3-1.2)	0.173	0.58 (0.3-1.2)	0.120	0.71 (0.3-2.0)	0.505
HBV DNA load*	0.96 (0.9-1.1)	0.444	0.93 (0.8-1.0)	0.230	1.12 (0.9-1.4)	0.274
Platelet count (per 10 units increase)	0.85 (0.8-0.9)	<0.001	0.87 (0.8-0.9)	<0.001	0.78 (0.7-0.8)	<0.001
AST (2 x ULN)	2.35 (0.6-9.4)	0.228	1.20 (0.9-1.6)	0.224	1.87 (0.2-15.8)	0.566
ALT (2 x ULN)	0.71 (0.2-2.1)	0.527	0.95 (0.8-1.2)	0.634	0.49 (0.1-4.4)	0.529
Invasive characteristics						
Ishak stage	1.74 (1.5-2.1)	<0.001	1.66 (1.4-2.0)	<0.001	2.32 (1.6-3.3)	<0.001
Advanced fibrosis	5.39 (2.9-10.1)	<0.001	4.56 (2.3-9.0)	<0.001		
Hepatic activity index	1.17 (1.1-1.3)	0.005	1.18 (1.0-1.3)	0.007	1.20 (1.0-1.4)	0.032
Steatosis	2.38 (1.3-4.4)	0.006	2.23 (1.1-4.4)	0.023	1.83 (0.7-4.8)	0.221
NASH	2.56 (1.3-5.2)	0.009	2.67 (1.2-5.9)	0.014	1.94 (0.6-6.0)	0.251
Iron depositions	1.77 (0.9-3.7)	0.121	1.90 (0.9-4.3)	0.118	2.03 (0.6-7.0)	0.262
Non-invasive scores						
FIB-4	1.60 (1.4-1.8)	<0.001	1.55 (1.4-1.7)	<0.001	1.53 (1.4-1.7)	<0.001
Log APRI	3.98 (1.9-8.5)	<0.001	3.49 (1.5-8.0)	0.003	8.98 (3.1-25.8)	<0.001
PAGE-B	1.37 (1.3-1.5)	<0.001	1.34 (1.2-1.5)	<0.001	1.50 (1.3-1.7)	<0.001
REACH-B	1.43 (1.2-1.7)	<0.001	1.37 (1.1-1.6)	<0.001	1.82 (1.4-2.4)	<0.001
GAG-HCC	1.05 (1.04-1.1)	<0.001	1.04 (1.03-1.1)	<0.001	1.07 (1.05-1.1)	<0.001
CU-HCC	1.09 (1.06-1.1)	<0.001	1.08 (1.05-1.1)	<0.001	1.09 (1.05-1.1)	<0.001

* *Entered as a time-dependent covariate*

** *No interaction between HBeAg status and viral load for "any event" or "mortality or transplantation". HCC development was associated with a higher HBV DNA load among HBeAg negative patients (HR 1.77, 95%CI 1.2 – 2.6, p=0.005)*

AVT, antiviral therapy

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CHAPTER 5

Validation of a mortality risk score to assess the long-term prognosis of chronic hepatitis B patients

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Submitted

To the Editor:

Recently, our group reported on a mortality and clinical disease progression risk score in hepatitis C virus (HCV) infected patients with advanced but compensated hepatic fibrosis.[1] These scores were based on readily available objective clinical parameters, and showed a good prognostic ability to assess the likelihood of disease progression during long-term follow-up. Importantly, the scores were validated in an independent cohort of patients with cirrhosis.[1] Shortly after, Giannini et al. described that the mortality risk score could adequately assess the short-term prognosis among HCV-infected patients with decompensated liver disease as well.[2] It is currently unknown whether this risk score can be used in patients with advanced liver disease of other etiology. Each year approximately 0.6 million patients die of complications of a chronic hepatitis B (CHB) infection, such as hepatocellular carcinoma (HCC) development and liver decompensation. It is important to assess the risk of clinical disease progression for individual CHB patients to provide a timely therapeutic intervention and intensify follow-up. Previous risk scores in CHB have shown variable performance among different patient groups.[3] We therefore aimed to assess whether the mortality risk score could be used as a universal score to assess the clinical event risk in CHB patients.

We studied 557 consecutive treatment-naïve mono-infected CHB patients who underwent a liver biopsy.[4] The liver biopsies were re-scored by a single hepato-pathologist. The risk score [Rm=(6 x age [years]) – (platelet count x 10^9/L) + (258.8 x $\log_{10}$ (AST/ALT) + (64.5 for males)] was applied on laboratory values obtained at the time of biopsy. For visualization purposes, low, medium and high-risk groups were formed on basis of a mortality score <87.1, ≥87.1 and ≤221.2 and >221.2 as previously described.[1] Data on HCC and mortality were obtained from national database registries, and data on decompensation and liver transplantation from the chart. The clinical endpoints combined were defined as any event. Reduced transplant-free survival was defined as the event of mortality or liver transplantation.

Of 557 patients, 371 (67%) were male, mean age was 34.7 years (SD 12.5), and 261 (47%), 175 (31%), and 104 (19%) were Caucasian, Asian and African, respectively. Mean ALT and AST levels (times upper limit of normal) were 2.6 (SD 3.6) and 1.6 (SD 1.9), respectively, and median platelet count was 206 x 10^9/L (interquartile range [IQR] 172 – 243). The median Ishak fibrosis score was 1.0 (IQR 1.0 – 2.0) and 113 (20%) had advanced fibrosis (F3/F4). After liver biopsy, 348 (63%) patients received antiviral therapy (AVT). The follow-up was complete for 93% (n=515) of the cohort; 7% (n=41) of patients emigrated and were censored at the last follow-up visit. During a median follow-up of 10.1 years (IQR 5.7 – 15.9), 40 patients developed a clinical event; 10 developed liver decompensation, 15 an HCC, and 31 died or

underwent liver transplantation. The event-free survival for the low, medium and high-risk groups were 0.99 (0.98 – 1.0), 0.99 (0.73 – 1.0), and 0.82 (0.70 – 0.95) at year 5, and 0.99 (0.97 – 1.0), 0.95 (0.90 – 1.0) and 0.52 (0.33 – 0.71) at year 10, respectively.(Figure 1) The C-statistic for any clinical event was 0.82 (95%CI: 0.73 – 0.91) for the complete cohort and 0.84 (95%CI: 0.74 – 0.94) for the subgroup of patients with advanced hepatic fibrosis. The C-statistics were comparable for patients who received AVT (0.83, 95%CI: 0.73 – 0.92) and for those who never received AVT (0.81, 95%CI: 0.61 – 0.99). The mortality score also showed a good performance among patients from different ethnicity (C-statistic 0.86, 0.82 and 0.71 for Caucasian, Asian and African patients, respectively). The C-statistics for transplant-free survival or HCC development were 0.79 (95%CI: 0.68 – 0.90) and 0.87 (95%CI: 0.75 – 0.99), respectively.

In conclusion, we showed that the mortality risk score could adequately assess the prognosis of CHB patients. If further corroborated, this score may be used to assess the need for antiviral therapy and more stringent follow-up, particularly for CHB patients with advanced hepatic fibrosis. Future studies should further elucidate the role of this mortality score in advanced liver disease of non-viral etiology.

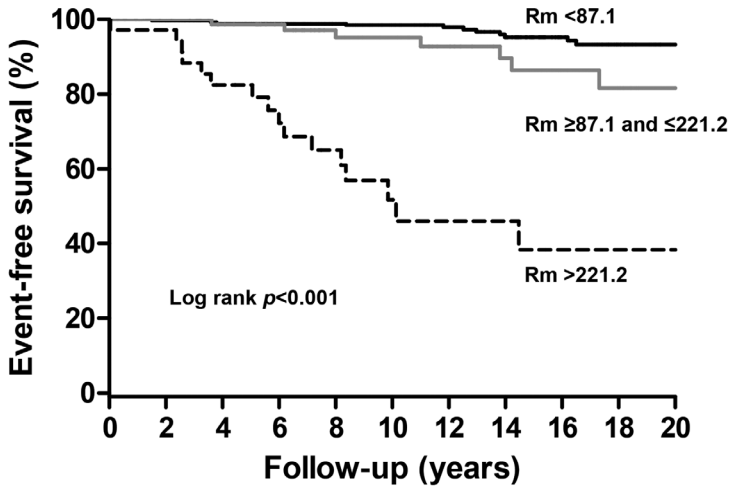

Figure 1 Event-free survival according to the mortality risk score categories.

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PART II

OPTIMIZATION OF TREATMENT STRATEGIES FOR CHRONIC HEPATITIS B PATIENTS

CHAPTER 6

Adding peginterferon to entecavir for HBeAg-positive chronic hepatitis B: A multicentre randomized trial (ARES study)

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ABSTRACT

Entecavir (ETV) is a potent inhibitor of hepatitis B viral replication, but long-term therapy may be required. We investigated whether adding-on peginterferon (PEG-IFN) to ETV therapy enhances serologic response rates. In this global investigator-initiated, open-label, multicentre randomized trial, HBeAg-positive chronic hepatitis B (CHB) patients with compensated liver disease started on ETV monotherapy (0.5mg/day) and were randomized in a 1:1 ratio to either PEG-IFN add-on therapy (180µg/week) from week 24 to 48 (n=85), or to continue ETV monotherapy (n=90). Response was defined as HBeAg loss with HBV DNA <200 IU/mL at week 48. Responders discontinued ETV at week 72. All patients were followed until week 96. Response was achieved in 16/85 (19%) patients allocated to the add-on arm versus 9/90 (10%) in the monotherapy arm (p=0.095). Adjusted for HBV DNA levels prior to randomized therapy, PEG-IFN add-on was significantly associated with response (OR 4.8, 95%CI: 1.6 – 14.0, p=0.004). Eleven (13%) of add-on treated patients achieved disease remission after ETV cessation, versus 2/90 (2%) of patients treated with monotherapy (p=0.007), which was 79% (11/14) versus 25% (2/8) of those who discontinued ETV (p=0.014). At week 96, 22 (26%) patients assigned add-on versus 12 (13%) assigned monotherapy achieved HBeAg seroconversion (p=0.036). PEG-IFN add-on led to significantly more decline in HBsAg, HBeAg and HBV DNA (all p<0.001). Combination therapy was well-tolerated. ***Conclusion:*** Although the primary endpoint was not reached, 24 weeks of PEG-IFN add-on therapy led to a higher proportion of HBeAg response compared to ETV monotherapy. Add-on therapy resulted in more viral decline and appeared to prevent relapse after stopping ETV. Hence PEG-IFN add-on therapy may facilitate the discontinuation of nucleos(t)ide analogues. Clinicaltrials.gov number: NCT00877760.

INTRODUCTION

In chronic hepatitis B (CHB) infected patients, the goal of antiviral therapy is to stop the progression of liver disease, initially by means of immunologic control over the hepatitis B virus (HBV), and ultimately through eradication of the virus.[1] Serum hepatitis B surface antigen (HBsAg) loss is considered the closest to eradication and is associated with disease remission and an improved prognosis.[2] However, complete eradication of HBV is only rarely achieved, as covalently closed circular DNA (cccDNA) often persists in hepatocytes, even after the loss of HBsAg.[3]

The treatment of CHB has considerably been improved over the past decade with the emergence of pegylated interferon (PEG-IFN), an immune modulator with antiviral capacity, and the more potent nucleos(t)ide analogues (NA). Entecavir (ETV) profoundly suppresses HBV DNA levels through inhibition of viral DNA polymerase. Both PEG-IFN and ETV are recommended as first-line monotherapies for CHB.

Treatment with ETV maintains viral suppression in more than 90% during continuous therapy, improves liver histology, reduces the risk of liver failure and probably of HCC development.[4-7] ETV has gained popularity as it is easy to use and is generally well-tolerated. However, long-term administration is associated with moderate adherence, may select for drug-resistant mutants, and has a considerable impact on health care costs.8, 9 NA therapy does not seem to induce off-treatment immune control of HBV, as hepatitis B e antigen (HBeAg) loss is not sustained in a large proportion of patients after treatment has been stopped.10, 11 Hence, HBsAg loss or even seroconversion appears the best endpoint for NA treatment.7, 12 Nevertheless, these endpoints are seldom achieved with ETV therapy and new treatment strategies facilitating the discontinuation of ETV are required.[13]

Response to PEG-IFN is frequently sustained after a finite treatment course due to its immune modulating capacity.[14] After one year of treatment, HBeAg loss is achieved in approximately 30% of patients, leading to high rates of HBsAg loss during long-term follow-up in those with an initial favorable response, which results in a reduced incidence of HCC and a prolonged survival.[14-16] However, the downside of PEG-IFN therapy are the considerable side-effects.

Previous studies have shown HBV to influence the innate and adaptive immune response, primarily through HBeAg, which leads to viral persistence.[17] ETV therapy has been shown to partially restore the adaptive immunity, while IFN prevents the formation of HBV proteins and depletes the intrahepatic cccDNA pool, which leads to more HBsAg loss when compared to ETV.[18-21] By utilizing the concept of immune restoration through ETV therapy, PEG-IFN add-on may enhance serologic response rates. This strategy could result in a larger proportion of

patients who are able to successfully discontinue ETV therapy and achieve a durable response without the need for further antiviral therapy.
Therefore, in this randomized controlled trial we investigated the efficacy and safety of adding-on PEG-IFN for 24 weeks during ETV therapy.

PATIENTS AND METHODS

Trial design

This global investigator-initiated, open-label, multicentre, randomized controlled trial (the ARES study, ClinicalTrials.gov registration number NCT00877760) was conducted at 14 centres in 5 countries in Europe and Asia. The recruitment of patients started in May 2009, the follow-up ended June 2013, and the database closed October 2013. All patients started ETV monotherapy (Baraclude, Bristol Myers Squibb, New York, United States, 0.5 mg once daily) at week 0 and were randomized at week 24 in a 1:1 ratio to either receive PEG-IFN α-2a add-on therapy (Pegasys, F. Hoffmann-La Roche Ltd., Basel, Switzerland, 180 μg once weekly) from week 24 to week 48, or to continue ETV monotherapy. For both arms, responders at week 48 received ETV consolidation therapy for 24 weeks until week 72 and were then followed for another 24 weeks off-therapy, while non-responders continued ETV monotherapy for 48 weeks until week 96. (Figure 1)
Randomization was done centrally and stratified by study centre. We used a computer-generated randomization sequence, prepared by the trial statistician (BH). Randomization was done sequentially and communicated to the sites by e-mail based on the randomization sequence. Treatment was assigned in random blocks of 2-6. The study was performed in accordance with the declaration of Helsinki and the Good Clinical Practice guidelines. The study was approved by the ethics committee of each participating centre. All subjects gave written informed consent prior to screening. All centres were monitored by an independent company. All authors had access to the study data and have reviewed and approved the final manuscript.

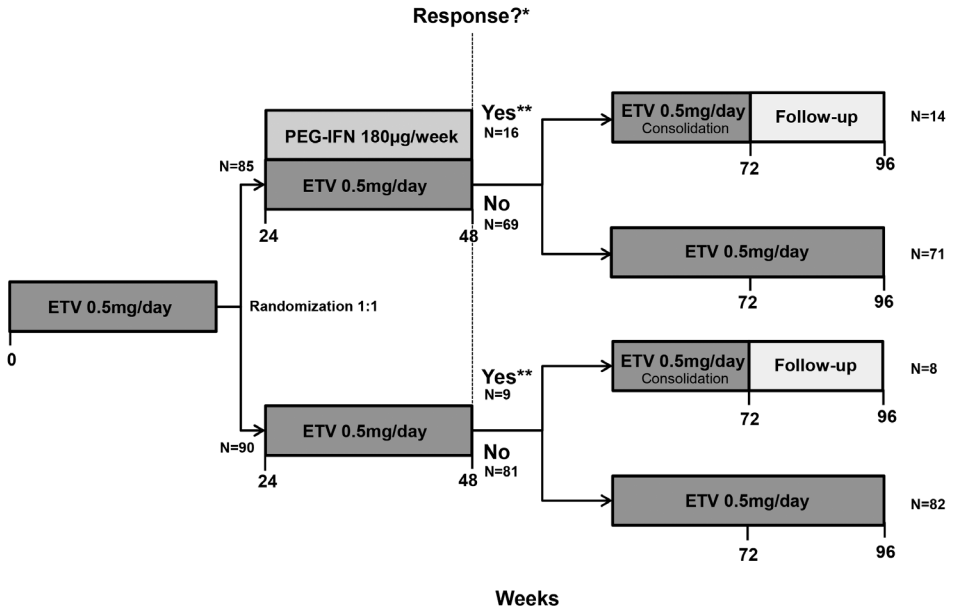


Figure 1 Trial design

Study population

Adult patients with documented CHB (serum HBsAg positive >6 months) were eligible if they were HBeAg-positive and anti-HBe negative at screening and had a serum alanine aminotransferase (ALT) level of more than 1.3 times the upper limit of normal (ULN) on two occasions within 60 days prior to and during screening, and had a liver biopsy available performed within the preceding 2 years. Exclusion criteria included antiviral therapy for HBV or immune suppressive medication within the preceding 6 months; any prior lamivudine or telbivudine experience for more than six months; any investigational treatment within 30 days prior to screening; pregnancy, lactation; evidence of a co-infection with hepatitis C or human immunodeficiency virus or any other acquired or inherited liver disease; a serum ALT of more than 10 times the upper limit of normal (ULN); pre-existent neutropenia (neutrophils ≤1,500 mm^3), thrombocytopenia (platelets ≤90,000/mm^3), alpha fetoprotein level of >50 ng/mL, uncontrolled thyroid disease; substance abuse in the past 2 years (such as alcohol (≥80 g/day), intravenous drugs or inhaled drugs); a history of decompensated cirrhosis defined as jaundice in the presence of cirrhosis, ascites, bleeding gastric or oesophageal varices or encephalopathy; any medical condition requiring or likely to require chronic systemic administration of

corticosteroids during the course of the study; or any other contra-indication for PEG-IFN therapy.

Efficacy analysis

The efficacy analysis included all patients who fulfilled the inclusion criteria, were HBeAg-positive at week 0, were randomized and received at least one dose of the assigned medication (modified intention-to-treat (MITT) population). The predefined primary endpoint was the combined presence of HBeAg loss with an HBV DNA level <200 IU/mL at week 48. Secondary endpoints included serologic response, viral dynamics over time, and ALT normalization. A subgroup analysis for the off-treatment response at week 96 was performed for patients who stopped ETV treatment at week 72: off-treatment response at week 96 was defined as HBeAg loss with HBV DNA <200 IU/mL (sustained primary response) and combined HBeAg loss with an HBV DNA <2000 IU/mL and ALT normalization (disease remission).

Safety analysis

The safety analysis included all patients who were randomized and received at least one dose of study medication. Safety measures included adverse events, vital signs and chemistry and hematology data, analyzed according to the World Health Organization recommendations for toxicity grading, adapted for chronic liver disease. The causality of adverse events was determined by the local investigator.

Measurements

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. Routine biochemical and hematological tests were performed locally. Serum ALT levels were standardized by calculating the value times the 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 Cobas TaqMan 48 polymerase chain reaction assay (Roche Diagnostics, Basel, Switzerland, lower limit of quantification 20 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). HBV genotype analysis was performed using the INNO-LiPA HBV genotype assay (Innogenetics, Ghent, Belgium). Liver biopsies were assessed at one European (Erasmus Medical Center, Rotterdam, The Netherlands) and one Asian centre (Zong Shan Hospital,

Fudan university, Shanghai) by two experienced pathologists (FK and YJ, respectively), according to the Ishak fibrosis stage (0-6) and hepatic inflammation index (HAI, grade 0-18).

Statistical analysis

This trial was designed as a superiority study. The power analysis was based on the primary endpoint at week 48. The assumed response rates were 20% for the ETV monotherapy group versus 40% for the PEG-IFN add-on group. The calculated number of patients per treatment arm needed to detect a statistical significant difference at the α-level of 0.05 with a power of 80%, when anticipating a 10% drop-out rate, was 90 patients per treatment-arm. Hence, 180 patients were needed for this study. Patients were classified as non-responders in case of missing HBeAg status or HBV DNA levels at week 48. A statistical analysis plan was defined before closure of the database. This pre-specified statistical analysis plan included adjustment of covariates possibly of influence on the primary outcome using logistic regression analysis techniques, and followed the European Medicines Agency guidelines for the adjustment of baseline variables.[22] The most relevant factors included HBV genotype and HBV DNA, HBeAg, HBsAg and serum ALT levels at the start of randomized therapy. Skewed laboratory values were log-transformed prior to analyses. The primary endpoint was assessed using Chi-square and regression analysis. Patient characteristics were compared using Students t-test or its non-parametric equivalent when appropriate. SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.3 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All analyses were performed two-sided at the 0.05 level of significance.

Role of the funding source

The study was organized and sponsored by the Foundation for Liver research, Rotterdam, the Netherlands. Financial support was provided by Bristol Myers Squibb (BMS, New York, United States), Roche International (F. Hoffmann-La Roche Ltd., Basel, Switzerland), and the Virgo consortium, funded by the Dutch government project number FES0908, and by the Netherlands Genomics Initiative (NGI) project number 050-060-452. The funding sources did not have any influence on study design, data collection, analysis and interpretation of the data, writing of the report nor the decision to submit for publication.

RESULTS

Study cohort. Of the 211 patients screened, 185 met the eligibility criteria and started ETV monotherapy. Figure 2 shows the patient disposition. One-hundred and eighty-two patients were randomized at week 12 to either receive PEG-IFN add-on therapy at week 24 (n=89) or to continue ETV monotherapy (n=93). In total, 175 patients were included in the final modified intention to treat (MITT) analysis: one patient was withdrawn from the study at the week 0 visit because of a pre-existent hepatic flare, two patients dropped-out before week 12 and thus were not randomized, one patient who was assigned to PEG-IFN add-on therapy withdrew his consent before receiving any dose at week 24 and six patients showed to be HBeAg-negative at week 0. Among the treatment arms, the frequency of exclusion of patients from MITT-analysis was equal (p=0.656). The characteristics for patients in the MITT-analysis were comparable. However, at the start of the randomized treatment phase (week 24) HBV DNA levels were higher in the add-on arm as compared to the monotherapy arm (Table 1). Only four patients in the add-on and two patients in the monotherapy arm did not complete treatment or follow-up.

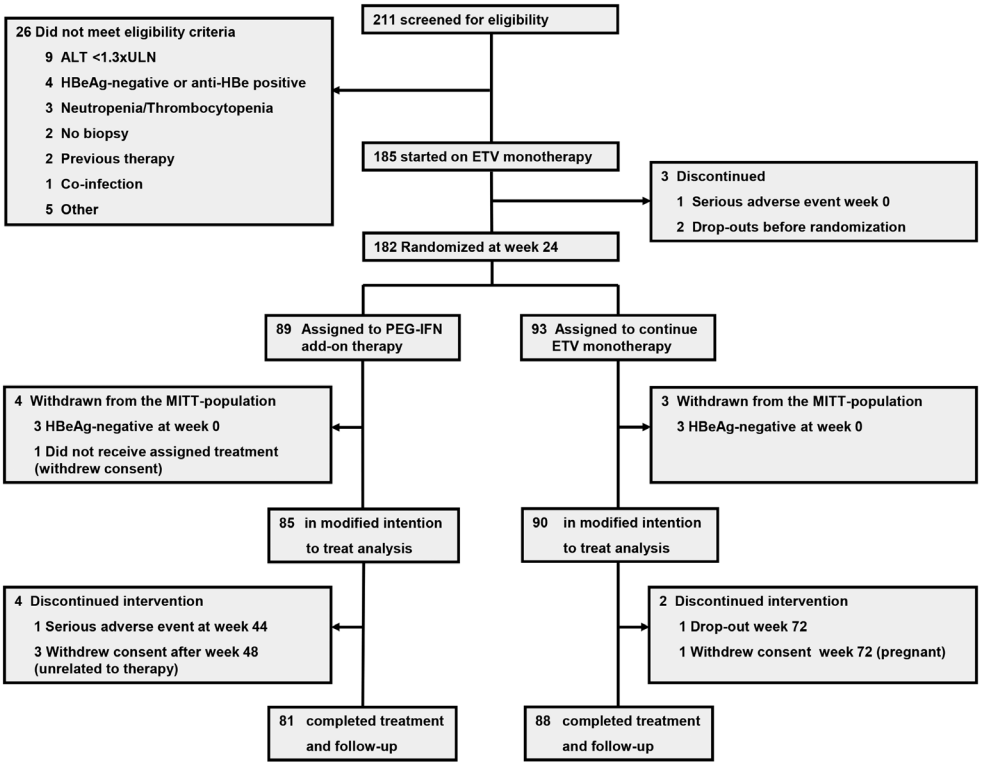


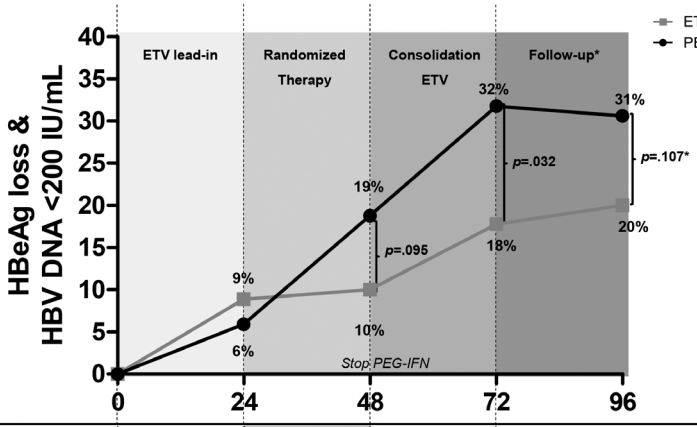
Figure 2 Patient disposition

Table 1 Characteristics of the MITT population

Characteristics	ETV monotherapy (n=90)	PEG-IFN add-on (n=85)
Demography		
Age, years	31 (9)	32 (10)
Male, n (%)	62 (69)	63 (74)
Body-mass index (kg/m^2)	23.5 (3.3)	23.2 (3.3)
Mode of transmission, n (%)		
– Vertical	10 (11)	11 (13)
– Sexual	2 (2)	1 (1)
– Parental/blood	10 (11)	12 (14)
– Unknown/other	68 (76)	61 (72)
Race, n (%)		
Caucasian	35 (39%)	30 (35%)
Asian	54 (60%)	53 (63%)
Other	1 (1%)	2 (2%)
Previous antiviral therapy, n (%)		
(PEG-)interferon$^{\pi}$	15 (17%)	6 (7%)
Nucleos(t)ide analogue*	3 (4%)	8 (9%)
Lamivudine (<6 months)	1 (1%)	4 (4%)
HBV Genotype: A/B/C/D (%)	10/14/46/30	5/23/39/33
Week 0 laboratory results μ		
serum ALT**	2.7 (2.1)	3.1 (3.3)
HBV DNA$^{\alpha}$	7.8 (1.1)	7.8 (1.3)
– HBV DNA ≥10^8 IU/mL	41 (46%)	47 (55%)
Quantitative HBsAg$^{\alpha}$	4.1 (0.8)	4.2 (0.8)
Quantitative HBeAg$^{\alpha}$	2.3 (1.0)	2.3 (1.0)
Week 24 laboratory results		
serum ALT*	0.8 (0.6)	0.8 (0.4)
HBV DNA$^{\alpha,\beta}$	2.3 (1.4)	2.8 (1.5)
Quantitative HBsAg$^{\alpha}$	3.7 (0.8)	3.7 (0.7)
Quantitative HBeAg$^{\alpha}$	0.9 (1.2)	1.1 (1.1)
Histology		
Ishak fibrosis	2.4 (1.3)	2.0 (1.3)
Hepatic Activity Index	4.8 (2.3)	4.5 (2.1)
Cirrhosis	5 (6%)	3 (4%)

$^{\pi}$ proportion of patients with previous interferon use differs between the arms (p=0.049)
$^{\mu}$ Patients were randomized at week 24 and thus not yet allocated at week 0
* Patients were excluded in case of previous telbivudine or lamivudine use for >6 months
** Multiples of upper limit of the normal range (Mean (SD))
$^{\alpha}$ logarithmic scale, IU/mL (Mean (SD))
$^{\beta}$ week 24 HBV DNA levels differed between the two arms (p=0.021)

Response at week 48. In total, 16/85 (19%) patients assigned PEG-IFN add-on versus 9/90 (10%) patients assigned ETV monotherapy reached the primary endpoint of HBeAg loss with an HBV DNA level below 200 IU/mL (p=0.095, figure 3). For HBV genotype A/B/C/D, response was achieved in 50%/25%/18%/11% patients assigned add-on versus 22%/15%/7%/7% patients assigned monotherapy, respectively. By univariable analysis, week 24 factors associated with a higher chance of response were lower HBV DNA (Odds-ratio (OR) 0.3, 95% confidence interval [CI]: 0.2 – 0.5, p<0.001), lower HBsAg (OR 0.4, 95%CI: 0.2 – 0.7, p=0.002), lower HBeAg (OR 0.2, 95%CI: 0.1 – 0.4, p<0.001), and serum ALT levels (OR 0.1, 95%CI: 0.02 – 0.5, p=0.004), while HBV genotype D was associated with a reduced probability of response (OR 0.2, 95%CI: 0.05 – 1.0, p=0.050). Age (p=0.675), sex (p=0.586), race (p=0.980), previous (PEG-)IFN, NA or lamivudine treatment (all p>0.5), or the presence of precore/basal core promotor mutations (p=0.302) were not associated with response. (supplementary table 1) Adjusted for the difference in HBV DNA at week 24, PEG-IFN add-on therapy was significantly and independently associated with the primary outcome (OR 4.8, 95%CI: 1.6 – 14.0, p=0.004, supplementary table 1). Table 2 shows the secondary outcome measures.



HBeAg loss & HBV DNA <200 IU/mL		Week 0 – 24	Week 24 – 48	Week 48 – 72	Week 72 – 96*		
		ETV lead-in	Randomized	Consolidation	All patients	Continued ETV	Discontinued ETV
PEG-IFN add-on	Response at begin interval	0	5/85	16/85	27/85	13/85	14/85 stopped ETV*
	Extra response within interval	5/85	11/85	11/85	5/85	5/85	--
	Relapse within interval	0	0	0	6/85	1/85	5/85
	Interval total	**5/85 (6%)**	**16/85 (19%)**	**27/85 (32%)**	**26/85 (31%)**	**17/85 (20%)**	**9/85 (11%)**
ETV mono	Response at begin interval	0	8/90	9/90	16/90	8/90	8/90 stopped ETV*
	Extra response within interval	8/90	1/90	7/90	8/90	8/90	--
	Relapse within interval	0	0	0	6/90	0	6/90
	Interval total	**8/90 (9%)**	**9/90 (10%)**	**16/90 (18%)**	**18/90 (20%)**	**16/90 (18%)**	**2/90 (2%)**
p-value: add-on vs. mono		0.448	0.095	0.032	0.107	0.707	0.023

**Overall results at week 96 includes both patients who continued or discontinued ETV at week 72; 14/16 and 8/9 responders assigned add-on and monotherapy, respectively stopped ETV at week 72 according to protocol. The 3 responders who continued ETV after week 72 sustained the primary response and are included in the on-treatment group at week 96.*

Figure 3 Evolution of HBeAg loss & HBV DNA <200 IU/mL throughout the study.
The primary outcome was assessed at week 48 (grey box in table)

Response at week 72. From week 48 to 72, 11/85 (13%) and 7/90 (8%) patients achieved a response for add-on and monotherapy. Hence, a total of 27/85 (32%) assigned add-on versus 16/90 (18%) assigned monotherapy achieved HBeAg loss with HBV DNA <200 IU/mL (p=0.032, figure 3).

Response at week 96. Overall, 26/85 (31%) patients assigned add-on versus 18/90 (20%) assigned monotherapy achieved HBeAg loss with an HBV DNA <200 IU/mL (p=0.107, figure 3). Add-on therapy showed to be independently associated with response after adjustment for the difference in HBV DNA at week 24 (OR 3.1, 95%CI: 1.3 – 6.9, p=0.007).

On-treatment response. Among patients who continued ETV, 24% (17/71) versus 20% (16/82) for add-on versus monotherapy achieved HBeAg loss with HBV DNA <200 IU/mL (p=0.506), which was 20% (17/85) versus 18% (16/90) of the total cohort (p=0.707, figure 3).

Off-treatment response. Three patients continued ETV throughout the study while they showed to have had achieved the primary response after central HBeAg and HBV DNA tests. As a result, ETV was discontinued in 14/16 and 8/9 responders assigned add-on and monotherapy, respectively. After ETV discontinuation, 64% (9/14) versus 25% (2/8) assigned add-on versus monotherapy sustained the primary response (p=0.076), which was 11% (9/85) versus 2% (2/90) of the total cohort (p=0.023, figure 3). For add-on versus monotherapy, disease remission (HBeAg negative, HBV DNA <2000 IU/mL and ALT normalization) was achieved in 11/14 (79%) versus 2/8 (25%) patients (p=0.014, figure 4), which was 13% (11/85) versus 2% (2/90) of the total cohort (p=0.007), respectively. Table 3 shows the secondary outcomes for patients who (dis)continued ETV.

Table 2 Secondary outcome measures at week 48 – 96

Response	Week 48 (End of PEG-IFN)			Week 72 (Consolidation)			Week 96** (Follow-up)		
	ETV mono (n=90)	PEG-IFN Add-on (n=85)	*p*	ETV mono (n=90)	PEG-IFN Add-on (n=85)	*p*	ETV mono (n=90)	PEG-IFN Add-on (n=85)	*p*
Serologic response									
HBeAg loss	9 (10%)	16 (19%)	0.095	16 (18%)	27 (32%)	0.032	20 (22%)	29 (34%)	0.080
HBeAg seroconversion	8 (9%)	14 (17%)	0.131	10 (11%)	22 (26%)	0.012	12 (13%)	22 (26%)	0.036
HBsAg seroconversion	0	1 (1%)	0.302	0	0	1	0	1 (1%)	0.302
HBsAg <1000 IU/mL	16 (18%)	24 (28%)	0.100	16 (18%)	21 (25%)	0.262	13 (14%)	22 (26%)	0.059
Virologic response									
HBV DNA <200 IU/mL	64 (71%)	71 (84%)	0.051	67 (75%)	64 (77%)	0.779	65 (72%)	65 (77%)	0.520
HBV DNA <20 IU/mL*	45 (50%)	52 (61%)	0.137	51 (57%)	47 (57%)	0.929	49 (54%)	48 (57%)	0.788
– *Week 0 HBV DNA ≥108 IU/mL*	12 (29%)	20 (43%)	0.196	17 (42%)	17 (38%)	0.727	16 (39%)	21 (45%)	0.592
– *Week 0 HBV DNA <108 IU/mL*	33 (67%)	32 (84%)	0.073	34 (71%)	30 (79%)	0.392	33 (67%)	27 (71%)	0.711
Biochemical response									
ALT normalization	70 (78%)	42 (49%)	<0.001	75 (83%)	73 (86%)	0.641	76 (84%)	75 (88%)	0.466

* The HBV DNA response differed according to the week 0 HBV DNA level. Percentages displayed are calculated using the number of patients with week 0 HBV DNA levels ≥10^8 IU/mL (n=41 and n=47 for ETV monotherapy versus add-on) or HBV DNA levels <10^8 IU/mL (n=49 and n=38 for ETV monotherapy versus add-on)

** Results shown are for the complete cohort regardless of (dis)continuation of ETV at week 72

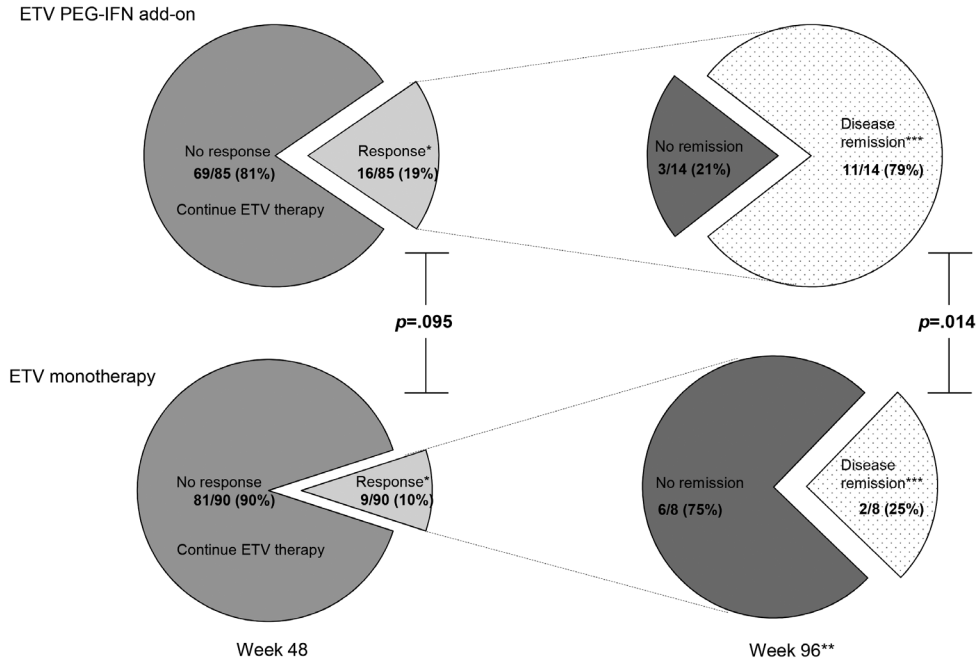
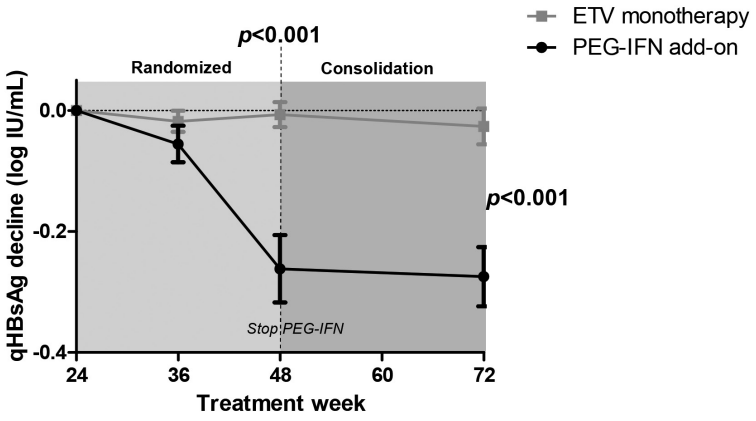
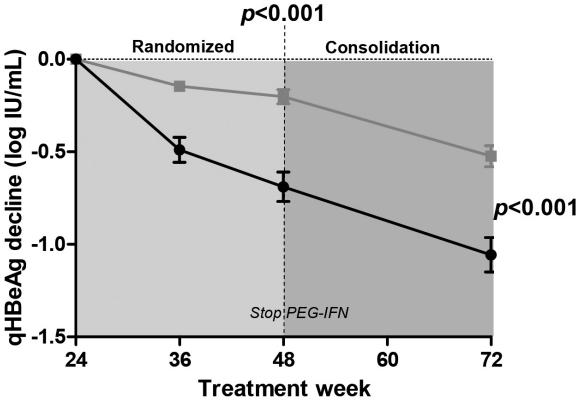


Figure 4 Disease remission (HBeAg negative, HBV DNA <2000 IU/mL and ALT normalization) through week 96 after discontinuation of ETV

Viral dynamics. During randomized therapy from week 24 to 48, patients in the add-on arm had more decline in HBsAg (-0.3 (SD 0.5) versus -0.01 (SD 0.2) log IU/mL, p<0.001), HBeAg (-0.7 (SD 0.7) versus -0.2 (SD 0.4) log IU/mL, p<0.001) and HBV DNA levels (-1.5 (SD 1.1) versus -0.7 (SD 0.6) log IU/mL, p<0.001, figure 5). From week 48 to 72, there was an incline observed in HBV DNA levels after stopping PEG-IFN add-on (mean change 0.2 (SD 1.0) versus -0.2 (SD 0.5), p=0.001 for add-on versus monotherapy), while there was no difference between the treatment arms in HBeAg or HBsAg decline during this period (both p>0.5). At week 72, mean viral levels differed for add-on versus monotherapy for HBsAg (3.4 versus 3.7 log IU/mL, p=0.052), and for HBeAg (0.01 versus 0.4 log IU/mL, p=0.026), but not for HBV DNA (1.5 versus 1.5 log IU/mL, p=0.831). Week 96 viral levels differed for add-on versus monotherapy for HBsAg (3.4 versus 3.7 log IU/mL, p=0.028), but not for HBeAg (-0.05 versus 0.25 log IU/mL, p=0.070) or HBV DNA (1.5 versus 1.5 log IU/mL, p=0.866).

Safety and tolerability. Combined PEG-IFN and ETV therapy was generally well-tolerated. No adverse events were deemed related to the combination of PEG-IFN and ETV therapy. Adverse events were most frequently observed in the PEG-IFN add-on arm during randomized therapy, while after week 48 frequencies of reported adverse events were comparable among the two arms (p=0.253). The most common side-effects in the add-on arm were those known to occur with PEG-IFN therapy (table 3). From week 24 to 96, there were three (3%) severe adverse events observed in the PEG-IFN add-on arm, versus two (2%) in the ETV monotherapy arm (p=0.615). Two patients in the add-on arm experienced a grade 4 neutropenia, which resolved in both patients subsequent to a dose reduction according to protocol. One patient discontinued PEG-IFN because of coronary heart disease at week 44 which was deemed unrelated to PEG-IFN therapy. In the monotherapy arm, one patient was diagnosed with an inguinal hernia and one patient with a jejunal perforation due to tuberculosis. In total, 2/5 (40%) of the severe adverse events were considered related to therapy. One (7%) patient assigned add-on experienced an ALT flare (7 x ULN) after stopping ETV, while no patients assigned ETV monotherapy experienced an ALT flare after cessation of ETV (p=0.439).





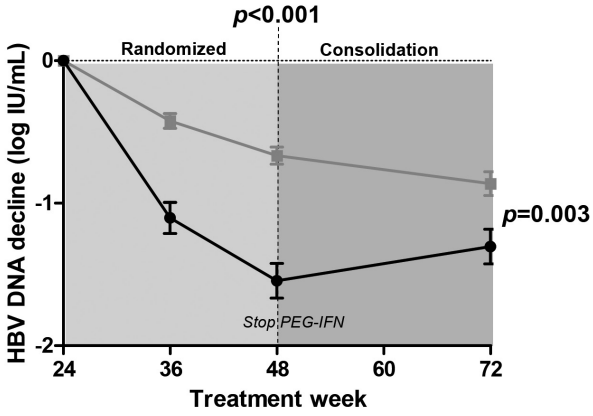


Figure 5 Decline in HBsAg (A), HBeAg (B) and HBV DNA (C) from week 24 – 72 according to therapy allocation. The p-values reflect the difference in decline from week 24 – 48, and the overall decline from week 24 – 72

Table 3 Secondary outcome measures at week 96 according to (dis)continuation of ETV at week 72

Response	Week 96 (Continued ETV**)			Week 96 (Discontinued ETV**)		
	ETV mono (n=82)	PEG-IFN Add-on (n=71)	*p*	ETV mono (n=8)	PEG-IFN Add-on (n=14)	*p*
Serologic response						
HBeAg loss	16 (20%)	17 (24%)	0.506	4 (50%)	12 (86%)	0.070
HBeAg seroconversion	8 (10%)	11 (16%)	0.283	4 (50%)	11 (79%)	0.166
HBsAg seroconversion	0	1 (1%)	0.281	0	0	1
HBsAg <1000 IU/mL	12 (15%)	17 (24%)	0.143	1 (13%)	5 (36%)	0.240
Virologic response						
HBV DNA <200 IU/mL	63 (77%)	56 (79%)	0.762	2 (25%)	9 (64%)	0.076
HBV DNA <20 IU/mL*	48 (59%)	44 (62%)	0.665	1 (13%)	4 (29%)	0.387
– *Week 0 HBV DNA ≥108 IU/mL*	16 (41%)	20 (48%)	0.551	0	1 (20%)	0.495
– *Week 0 HBV DNA <108 IU/mL*	32 (74%)	24 (83%)	0.404	1 (17%)	3 (33%)	0.475
Biochemical response						
ALT normalization	69 (84%)	62 (87%)	0.576	7 (88%)	13 (93%)	0.674

* The HBV DNA levels differed according to the week 0 HBV DNA level. Percentages displayed are calculated using the number of patients with week 0 HBV DNA levels $\geq 10^8$ IU/mL (denominator in continued ETV subgroup n=39 and n=42 for ETV monotherapy versus add-on, in the discontinued subgroup n=2 versus n=5 for ETV versus add-on) or HBV DNA levels $<10^8$ IU/mL (denominator in continued subgroup n=43 and n=29 for ETV monotherapy versus add-on, in the discontinued subgroup n=6 versus n=9 for ETV versus add-on, respectively)

** 8/9 responders in the ETV monotherapy and 14/16 responders in the PEG-IFN add-on arm discontinued ETV according to protocol

DISCUSSION

In this international study, we have shown that adding-on PEG-IFN to ETV results in more on-treatment viral decline and more off-treatment response after discontinuation of ETV. Although the primary endpoint was not reached, 24 weeks of PEG-IFN add-on therapy led to a higher proportion of HBeAg response as compared to ETV monotherapy. Combining PEG-IFN and ETV was safe and no (severe) adverse events were deemed related to combination therapy.

Current recommended treatment regimens for HBeAg-positive CHB patients include long-term treatment with a potent NA, such as ETV, or finite treatment with PEG-IFN.[1] ETV monotherapy has an excellent safety profile and maintains high rates of HBV suppression, while PEG-IFN treatment leads to a more sustained serologic response in a subset of patients through induction of immune control.[4, 14-16] However, these regimens are unsatisfactory as long-term ETV does not induce immune control, may select for drug-resistant mutants and is associated with significant problems in adherence, while PEG-IFN is poorly tolerated and only leads to a favorable response in a subgroup of patients.[8-10, 14, 16, 23, 24]

We found that patients treated with PEG-IFN add-on achieved a numerically higher rate of serologic response when compared to ETV monotherapy. At week 48, the primary response was achieved in 19% of patients assigned to the add-on arm versus 10% in the monotherapy arm. This difference however did not reach statistical significance, as the assumed response rates were not met and there was an imbalance between the treatment arms in terms of HBV DNA levels at the start of the randomized treatment phase. Lower HBV DNA levels at randomization were also significantly associated with response. Pre-specified analysis allowed to study the effect of treatment after adjustment for factors associated with response, and add-on therapy was significantly associated with the primary outcome after adjustment for HBV DNA levels.

Previous studies reported on the efficacy of PEG-IFN combined with lamivudine (LAM), an inferior nucleoside analogue with a low barrier to resistance. It was shown that this de novo combination led to more virologic suppression during treatment, but not to higher virologic or serologic response rates during off-treatment and long-term follow-up as compared to PEG-IFN monotherapy.[23-25] Nonetheless, the new generation NAs with high barriers to resistance have replaced LAM as first-line treatment, resolving the resistance issues associated with LAM.[9] As a consequence, the combination of PEG-IFN with LAM has not been implemented in clinical practice. Furthermore, it was recently shown that switching patients from ETV to PEG-IFN led to higher HBeAg seroconversion rates. However, a major caveat of the study was that only patients with low HBeAg levels (<100 PEIU/mL) were included, and no off-treatment results

were reported.[26] The results of this switch study were therefore difficult to interpret and may not be applicable to the daily clinical practice.

Table 4 Safety profile

Variable	ETV mono-therapy (n=93)	PEG-IFN add-on (n=89)	*p*
Adverse events:			
Serious adverse event	2 (2%)	5 (6%)	0.270
week 24 – 48	1 (1%)	3 (3%)	0.291
week 48 – 96	1 (1%)	0	0.327
One or more adverse event	28 (30%)	85 (96%)	<0.001
week 24 – 48	9 (10%)	84 (94%)	<0.001
week 48 – 96	12 (13%)	17 (19%)	0.253
Most common adverse events:			
Flu-like syndrome*	1 (1%)	46 (52%)	<0.001
Headache	5 (5%)	10 (11%)	0.151
Fatigue	3 (3%)	16 (18%)	0.001
Myalgia	0	15 (17%)	<0.001
Abdominal pain	6 (6%)	6 (7%)	0.937
Arthralgia	0	5 (6%)	0.020
Nausea**	2 (2%)	4 (4%)	0.376
Leukopenia (<2.0x10^9/L)	0	9 (10%)	0.002
Neutropenia (<1.0x10^9/L)	0	26 (29%)	<0.001
Thrombocytopenia (<75x10^9/L)	3 (3%)	20 (23%)	<0.001
Skin reaction***	1 (1%)	9 (10%)	0.007
Alopecia	0	10 (11%)	0.001
Hyperthyroidism	0	3 (3%)	0.074
Hypothyroidism	0	2 (2%)	0.146

* *including only pyrexia*
** *including vomiting*
*** *rash, eczema, pruritus*

The strategy of adding-on PEG-IFN for six months appeared to result in more off-treatment response, which was achieved in a higher proportion of patients assigned add-on. Moreover, 79% of patients assigned add-on who stopped all treatment achieved disease remission at week 96, versus only 25% of patients assigned monotherapy. This observation suggests that PEG-IFN add-on may prevent relapse after the cessation of ETV. However, it should be acknowledged that the subgroup analysis was hampered by the limited number of patients who achieved a response and the relatively short duration of off-treatment follow-up.

PEG-IFN add-on showed to induce more decline in HBV DNA, HBeAg and HBsAg levels than did ETV monotherapy, and led to a higher proportion of patients achieving a HBsAg level <1000 IU/mL, which is a level associated with long-term disease remission.[27] However, the HBeAg response rate with 24 weeks PEG-IFN add-on was disappointing low and comparable to that achieved with 24 weeks of PEG-IFN monotherapy. This may have been related to the HBV genotype distribution in our study as we observed that patients were predominantly infected with HBV genotype C or D.[28] As a result, we were able to stop only a small subgroup of patients, which consequently led to a low rate of patients who could achieve off-treatment disease remission. Taken together, these observations raise the question if extension of combined treatment to 48 weeks may lead to more serologic response.[29] Additionally, as lower levels of HBV DNA at the start of add-on therapy were associated with response, it may be more beneficial to add-on PEG-IFN once viral suppression has been achieved.

Considering safety, PEG-IFN add-on was generally well-tolerated without observing unexpected adverse events. Indeed, adverse events were increased when compared to ETV monotherapy, which should be taken into account when considering PEG-IFN add-on therapy.

A limitation of the current study is that we could not evaluate the additive benefit of ETV to PEG-IFN, since we did not include a PEG-IFN monotherapy arm. Nevertheless, this trial was designed to assess whether PEG-IFN add-on could lead to more serologic response than ETV alone and thus may allow for cessation of ETV therapy. This is an important question given the extensive use of NA. Six months after the cessation of ETV we observed that 75% of ETV monotherapy treated patients did not sustain response. Therefore, one could argue that the control group was possibly disadvantaged as a six month consolidation period may not have been long enough to achieve an off-treatment response. In contrast, the control group was possibly advantaged as 8/9 (89%) responders to ETV monotherapy achieved the primary response at week 24 and thus received 48 weeks of consolidation therapy, versus only 5/16 (31%) patients in the PEG-IFN add-on arm. Nevertheless, it is unknown whether consolidation duration indeed impacts on the durability of response as the results have been contradictory, probably as most studies on consolidation therapy duration are retrospective and heterogeneous in nature, and possibly confounded by indication.[10, 11, 30-32] Yet in spite of the potential advantages for the monotherapy group, we still observed higher response rates for the PEG-IFN add-on arm. Therefore the strategy of adding-on PEG-IFN to ETV warrants further investigation.

Concluding, in this randomized controlled trial we have shown that 24 weeks of PEG-IFN add-on was safe and resulted in more viral decline, and may lead to more HBeAg loss when compared to ETV monotherapy. PEG-IFN add-on therapy appeared to prevent relapse after stopping ETV and may therefore facilitate the discontinuation of nucleos(t)ide analogues.

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Supplementary table 1 Univariable and multivariable analysis for the primary response at week 48 (response n=27)

Week 48: HBeAg loss & HBV DNA <200 IU/mL	Univariable		Multivariable (Full model week 24)		Multivariable (Final Model week 24)	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
PEG-IFN add-on	2.1 (0.9-5.1)	0.095	5.1 (1.6-16.6)	0.006	4.5 (1.6-14.0)	0.004
HBV genotype*		0.153		0.225		
– A	4.4 (1.0-19.8)		2.1 (0.3-14.1)			
– B	2.7 (0.8-9.3)		0.6 (0.1-2.9)			
– C	1.4 (0.4-4.4)		0.3 (0.1-1.2)			
– D	Reference		Reference			
Precore/basal core promotor	3.0 (0.4-23.6)	0.302				
Gender	0.8 (0.3-2.0)	0.586				
Asian ethnicity	0.9 (0.4-2.1)	0.752				
Age	1.0 (1.0-1.1)	0.675				
Body-mass Index	1.0 (0.9-1.2)	0.786				
Week 0 ALT (x ULN)	1.0 (0.9-1.2)	0.705				
Week 0 HBsAg (log IU/mL)	0.6 (0.4-0.9)	0.012				
Week 0 HBV DNA (log IU/mL)	0.7 (0.5-1.0)	0.061				
Week 0 HBeAg (log IU/mL)	0.7 (0.5-1.0)	0.095				
Week 24 HBV DNA <200 IU/mL	11.3 (3.2-39.5)	<0.001				
Week 24 HBV DNA <20 IU/mL	9.2 (3.7-23.2)	<0.001				
Week 24 HBV DNA (log IU/mL)*	0.3 (0.2-0.5)	<0.001	0.2 (0.1-0.5)	<0.001	0.2 (0.1-0.4)	<0.001
Week 24 ALT (x ULN)	0.1 (0.02-0.5)	0.004	0.2 (0.03-2.1)	0.169		
Week 24 HBsAg (log IU/mL)	0.4 (0.2-0.7)	0.002	**	**		
Week 24 HBeAg (log IU/mL)	0.2 (0.1-0.4)	<0.001	**	**		
Any previous antiviral therapy	0.9 (0.2-3.2)	0.855				
Previous (PEG)-IFN	1.0 (0.3-3.7)	1.0				
Previous Lamivudine	1.5 (0.2-14.2)	0.713				
Previous Nucleos(t)ide analogue	0.6 (0.1-4.8)	0.615				

* HBV DNA at week 24 (randomization) differed significantly among the two arms (p=0.021) and is collinear with HBsAg (r=0.5, p<0.001) and HBeAg (r=0.7, p<0.001) at week 24. Therefore only HBV DNA at week 24 is added to the final model

** When HBV DNA levels were replaced with HBsAg levels or HBeAg levels at week 24, the OR for PEG-IFN add-on therapy in this model was as follows: adjusted for HBsAg: OR 3.3, 95%CI: 1.2 – 9.6, p=0.026; adjusted for HBeAg: OR 5.5, 95%CI: 1.6 – 19.8, p=0.008

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CHAPTER 7

Peginterferon add-on results in more HBsAg decline compared to monotherapy in HBeAg positive chronic hepatitis B patients

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ABSTRACT

Background

It is unknown whether peginterferon (PEG-IFN) add-on to entecavir (ETV) leads to more HBsAg decline compared to PEG-IFN monotherapy or combination therapy, and whether ETV therapy may prevent HBsAg increase after PEG-IFN cessation.

Methods

We performed a post-hoc analysis of 396 HBeAg-positive patients treated for 72 weeks with ETV + 24 weeks PEG-IFN add-on from week 24 – 48 (add-on, n=85), 72 weeks with ETV monotherapy (n=90), 52 weeks with PEG-IFN monotherapy (n=111) and 52 weeks PEG-IFN + lamivudine (combination, n=110) within 2 randomized trials. HBsAg decline was assessed at end-of PEG-IFN (EOP) and 6 months after PEG-IFN (EOF) discontinuation. Differences in baseline characteristics were accounted for using inversed probability of treatment weights.

Results

At EOP, a HBsAg reduction of ≥1$\log_{10}$ IU/mL was more frequently achieved for patients in the add-on or combination therapy arms (both 36%), compared to PEG-IFN mono (20%) or ETV (8%) (add-on versus PEG-IFN mono p=0.050). At EOF, the HBsAg reduction ≥1$\log_{10}$ IU/mL was only sustained in patients treated with ETV consolidation (add-on versus combination and PEG-IFN mono: 40% versus 23% and 18%, p=0.029 and p=0.003 respectively). For add-on, combination, PEG-IFN mono and ETV the mean HBsAg level change at EOF was -0.84, -0.81, -0.68, and -0.33 $\log_{10}$ IU/mL, respectively (p>0.05 for PEG-IFN arms). HBeAg loss at EOF was 36%, 31%, 33% and 20%, respectively (p>0.05).

Conclusions

PEG-IFN add-on for 24 weeks results in more on-treatment HBsAg decline than does 52 weeks of PEG-IFN monotherapy. ETV therapy may maintain the HBsAg reduction achieved with PEG-IFN.

INTRODUCTION

Chronic hepatitis B (CHB) infection remains a global burden affecting approximately 350 million people worldwide despite screening and vaccination programs. Both peginterferon (PEG-IFN) and nucleos(t)ide analogues (NA) are effective first-line therapies which have proven to halt the progression and even reverse the extent of liver disease and as a result improve patients' prognosis in the long-term.[1-8] Nevertheless, there are significant disadvantages of both first-line therapies which urges physicians to find the best strategy to achieve the highest rate of sustained off-treatment immune control.

Hepatitis B surface antigen (HBsAg) is the hallmark of CHB infection, and loss of serum HBsAg is considered the closest to eradication of hepatitis B virus (HBV) infection. It is however well-recognized that a mini chromosome – covalently closed circular DNA (cccDNA), which is the template for HBV replication – may still persist in hepatocytes after HBsAg loss.[9-11] Nevertheless, HBsAg levels are thought to better reflect the amount of intrahepatic cccDNA than any other serum HBV marker, and are currently used to monitor on-treatment and off-treatment response to PEG-IFN.[12-16]

PEG-IFN and NA have different modes of action. The immune modulator PEG-IFN has the ability to deplete the intrahepatic cccDNA pool, as reflected by a decline in HBsAg levels, and to induce off-treatment immune control without the need for further antiviral therapy.[11, 13-16] NA on the other hand inhibits viral replication, but does not seem to have a direct effect on cccDNA and generally does not result in off-treatment immune control. Nonetheless, NA is thought to partially restore immune function.[17, 18] It is therefore hypothesized that combining PEG-IFN with lead-in therapy of NA may lead to more (off-treatment) response.

Previous randomized trials showed a beneficial effect in terms of on-treatment HBsAg decline and HBeAg response for a combination of lamivudine (LAM) or tenofovir (TDF) with PEG-IFN. However, the achieved response rates were not sustained off-treatment, questioning the role of NA consolidation therapy after combining PEG-IFN and NA.[19-23] We have recently shown that a 24 week PEG-IFN add-on strategy results in more decline in HBsAg, HBeAg and HBV DNA compared to ETV monotherapy.[24] Moreover, it was shown that PEG-IFN add-on could possibly prevent relapse after ETV discontinuation. Nevertheless, we could neither investigate the added value of combined therapy with ETV, nor the added value of ETV lead-in therapy compared to PEG-IFN monotherapy.

Therefore, the aims of the current study were 1) to compare the achieved HBsAg declines achieved with PEG-IFN add-on versus PEG-IFN monotherapy versus ETV monotherapy or de

novo combination therapy, and 2) to describe the role of NA therapy after PEG-IFN cessation in HBeAg-positive CHB patients treated within 2 multicenter randomized controlled trials.[20, 24]

PATIENTS AND METHODS

Patients. We performed a post-hoc analysis of two international randomized controlled trials in which 396 HBeAg-positive patients were treated with ETV monotherapy ± 24 weeks PEG-IFN add-on from week 24 – 48 (n=175, ARES study [24]) or with PEG-IFN ± LAM for 52 weeks (n=221, HBV 9901 study [20]). Figure 1 shows the study design and number of patients per treatment arm for both trials. Patients in the ARES study were started on ETV monotherapy at week 0, and were randomized towards PEG-IFN add-on or ETV monotherapy continuation at week 24. In this trial patients were treated for another 24 weeks with ETV after PEG-IFN withdrawal.

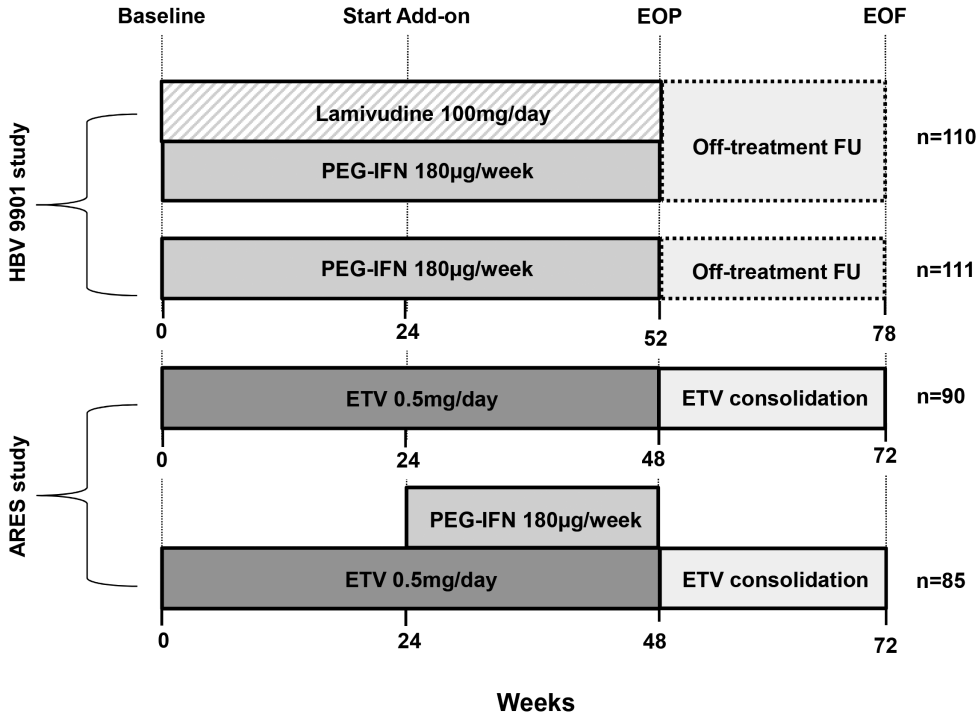


Figure 1 Study design

EOP, end of PEG-IFN treatment; EOF, end of follow-up; FU, follow-up

The inclusion and exclusion criteria for the original trials were similar and are described elsewhere.[20, 24] In short, adult patients with documented CHB (serum HBsAg positive >6 months) and compensated liver disease were eligible if they were HBeAg-positive and anti-HBe negative at screening and had an elevated serum alanine aminotransferase (ALT) level. Patients were excluded in case of any contra-indication for PEG-IFN therapy. For the current study the availability of HBsAg level measurements was required. The studies were 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. The primary focus was to compare the HBsAg declines achieved with each treatment strategy, assessed both on-treatment, at end of PEG-IFN treatment (EOP, week 48 and 52 for ARES and HBV 9901 respectively) as well as at end of follow-up (EOF), 6 months post PEG-IFN treatment (weeks 72 and 78). Since the achievement of low levels of HBsAg confer a high probability of immune control and HBsAg loss over time, we both investigated the proportion of patients with a HBsAg decline of ≥1 $\log_{10}$ IU/mL from baseline as well as mean HBsAg declines.[13-15, 25, 26] Moreover, the frequency of HBeAg loss was described both at EOP and EOF.

Measurements. 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. Routine biochemical and hematological tests were performed locally. Serum ALT levels were standardized by calculating the value times the 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 Cobas TaqMan 48 polymerase chain reaction assay (Roche Diagnostics, Basel, Switzerland, lower limit of quantification 20 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). HBV genotype analysis was performed using the INNO-LiPA HBV genotype assay (Innogenetics, Ghent, Belgium).

Statistical analysis. The data of the current study origins from two randomized controlled trials. Therefore, to be able to compare the 4 treatment arms the inversed probability of treatment weights approach was used to reduce significant differences in baseline characteristics.[27] This method allows an optimal use of the total data without loss of subjects such as with the propensity

score method. Firstly, the probability of being included in the randomized trial (ARES versus HBV 9901) was estimated using the following baseline factors in a logistic regression analysis: age, gender, ethnicity, HBV genotype, serum HBsAg, HBV DNA, HBeAg and ALT levels. These factors were chosen as these are extensively described in previous studies to be associated with PEG-IFN treatment response.[13, 14, 20, 28] Secondly, patients were weighted by this inverse probability, which were stabilized prior to the analyses to exclude bias from extreme weights. Weighted least squares regression analysis was finally used to analyse HBsAg declines and HBeAg loss according to therapy allocation and adjusted for baseline HBsAg levels, both for the overall cohort as well as stratified by HBV genotype. Due to the limited number of patients stratified by HBV genotype, declines were only observational and statistical testing was not performed for this stratified analysis. Skewed laboratory values were log-transformed prior to analyses. SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.3 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. All analyses were performed two-sided at the 0.05 level of significance.

Role of the funding source. This investigator initiated study was organized and sponsored by the Foundation for Liver research, Rotterdam, the Netherlands. Financial support for the original trials was provided by Schering-Plough (now Merck Sharpe & Dohme)[20], Hoffmann-La Roche (Basel, Switzerland), Bristol Myers Squibb (BMS, New York, United States), and the Virgo consortium, funded by the Dutch government project number FES0908, and by the Netherlands Genomics Initiative (NGI) project number 050-060-452.[24] The funding sources did not have any influence on study design, data collection, analysis and interpretation of the data, writing of the report nor the decision to submit for publication.

RESULTS

Study cohort. In total, 396 patients were included of which 90 were treated with ETV monotherapy for the duration of 72 weeks, 85 were treated with ETV + 24 weeks PEG-IFN add-on, 110 patients received PEG-IFN + LAM for 52 weeks and 111 patients received PEG-IFN monotherapy for 52 weeks. (Figure 1) The baseline patient characteristics were comparable after adjustment with inversed probability of treatment weights. (Table 1)

Table 1 Baseline Patient Characteristics

Characteristics	ETV mono (n=90)	PEG-IFN add-on (n=85)	PEG-IFN + LAM (N=110)	PEG-IFN mono (n=111)	Un-adjusted *p*-value	IPTW adjusted *p*-value
Demography						
Age, years (SD)	31 (9)	32 (10)	33 (12)	34 (13)	0.198	0.762
Male, n (%)	62 (69)	63 (74)	83 (76)	90 (81)	0.257	0.685
Race, n (%)					<0.001	0.424
Caucasian	35 (39)	30 (35)	80 (73)	80 (72)		
Asian	54 (60)	53 (63)	19 (17)	25 (23)		
Other	1 (1)	2 (2)	11 (10)	6 (5)		
HBV Genotype A/B/C/D (%)	10/14/46/30	5/23/39/33	31/9/13/42	36/9/16/37	<0.001	0.454
Laboratory results						
ALT *	2.7 (2.1)	3.1 (3.3)	4.2 (3.0)	4.3 (3.0)	<0.001	0.910
HBV DNA**	7.8 (1.1)	7.8 (1.3)	8.4 (1.0)	8.4 (0.8)	<0.001	0.869
HBsAg**	4.1 (0.8)	4.2 (0.8)	4.4 (0.7)	4.4 (0.6)	0.011	0.724
HBeAg**	2.3 (1.0)	2.3 (1.0)	2.4 (0.7)	2.5 (0.7)	0.345	0.559
Liver Histology						
Ishak fibrosis (SD)	2.3 (1.3)	2.0 (1.3)	2.6 (1.5)	2.4 (1.6)	0.082	0.337
Cirrhosis, n (%)	5 (6)	3 (4)	10 (9)	9 (8)	0.470	0.423
Hepatic activity index (SD)	4.8 (2.3)	4.5 (2.1)	5.1 (2.1)	5.3 (2.2)	0.083	0.204

* times the upper limit of normal (standard deviation)
** $\log_{10}$ IU/mL (standard deviation)
IPTW, inversed probability of treatment weights

Changes in HBsAg during treatment. At EOP, a larger proportion of patients treated in the add-on and combination therapy arms showed a ≥ 1 $\log_{10}$ reduction in HBsAg levels compared to the monotherapy arms: for both add-on and combination treated patients this was 36%, versus 20% and 8% for PEG-IFN and ETV monotherapy, respectively (add-on versus PEG-IFN monotherapy p=0.050, combination versus PEG-IFN monotherapy p=0.042, figure 2). A steep decline in estimated mean HBsAg levels was observed both after initiation of PEG-IFN monotherapy (mean change from baseline -0.30 $\log_{10}$ IU/mL, figure 3A) as well as after initiation of ETV monotherapy up till week 12 of treatment (mean change -0.35 $\log_{10}$ IU/mL, p=0.656 for difference). From week 12 – 24, the HBsAg decline decelerated for patients treated with ETV monotherapy whereas PEG-IFN monotherapy treated patients showed a continuous steep decline (-0.42 versus -0.59 IU/mL, p=0.161). From week 24 – 48, PEG-IFN add-on to ETV led to a pronounced HBsAg decline, while patients who continued ETV monotherapy did not show a further decline in HBsAg. The mean HBsAg decline at EOP was comparable

between PEG-IFN add-on therapy and PEG-IFN monotherapy (mean change -0.80 versus -0.77 $\log_{10}$ IU/mL, respectively, p=0.874), while patients treated with ETV alone showed less mean decline (-0.31 $\log_{10}$ IU/mL, p=0.002 versus add-on and p=0.006 versus PEG-IFN monotherapy). The most prominent on-treatment HBsAg decline was achieved with PEG-IFN + LAM combination therapy (-1.27 $\log_{10}$ IU/mL at EOP, p=0.045 versus add-on).

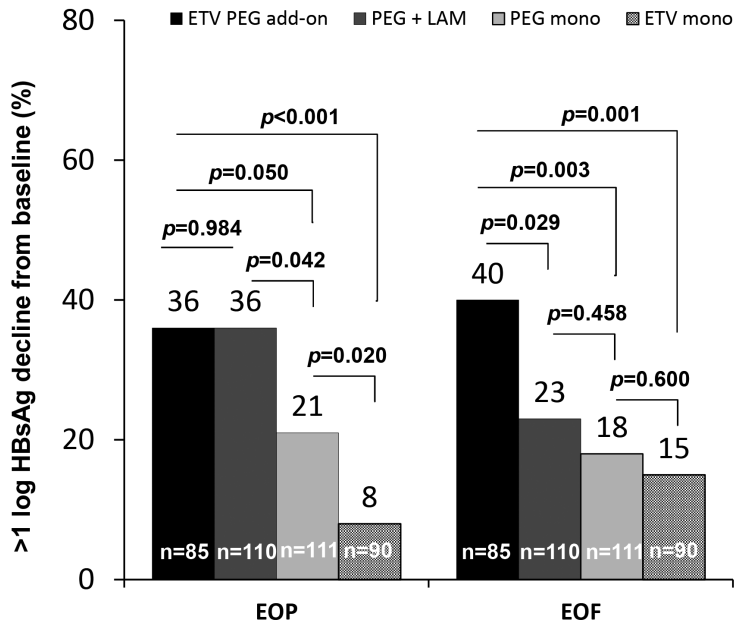
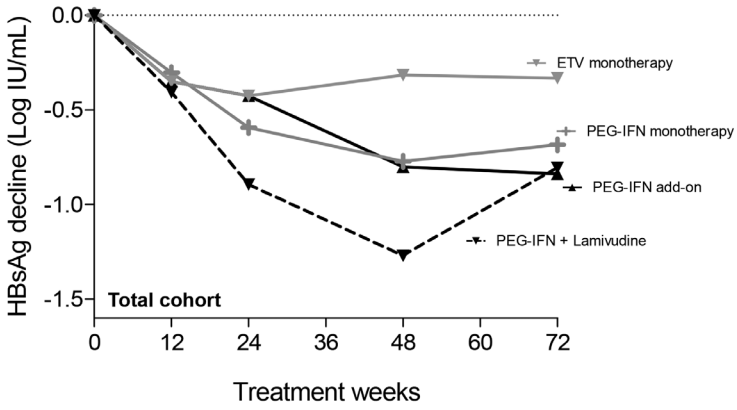


Figure 2 Proportion of patients with a HBsAg reduction of ≥1 $\log_{10}$ from baseline at EOP and EOF estimated using inversed probability of treatment weights

Changes in HBsAg during off-treatment follow-up and ETV consolidation therapy. At EOF, 40% of add-on treated patients achieved ≥1 $\log_{10}$ decline, versus 23%, 18% and 15% of patients in the combination, PEG-IFN monotherapy and ETV monotherapy arms respectively (p=0.029 and p=0.003 for add-on versus combination and PEG-IFN monotherapy, respectively, figure 2). The percentage of patients who achieved a HBsAg decline of ≥ 1 $\log_{10}$ IU/mL at EOP but subsequently relapsed to < 1 $\log_{10}$ IU/mL decline at EOF was 4%, 46%, 38% and 0% for add-on, combination, monotherapy and ETV, respectively (p<0.001). The discontinuation of PEG-IFN + LAM and PEG-IFN monotherapy resulted in an increase in mean HBsAg levels (EOF HBsAg level change -0.81 and -0.68 $\log_{10}$ IU/mL from baseline, respectively). In contrast, add-on treated patients receiving ETV therapy experienced a stabilization of the HBsAg level decline after PEG-IFN withdrawal (mean HBsAg change -0.84 $\log_{10}$ IU/mL, p>0.05 for PEG-

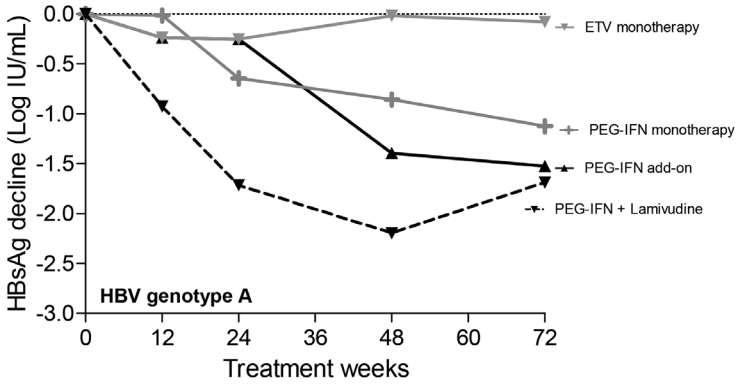
IFN add-on versus combination or PEG-IFN monotherapy, figure 3A). At EOF, no patients in the ETV monotherapy or add-on arms had achieved HBsAg loss, versus 5% and 6% of patients in the combination and monotherapy arm, respectively (p=0.027).

HBsAg decline according to HBV genotype. We observed the extent of HBsAg decline to differ across HBV genotype, with greatest declines seen for HBV genotype A and B. For all HBV genotypes, the most pronounced declines were observed for combination or add-on therapy. For HBV genotype C PEG-IFN add-on led to more decline than de novo combination therapy. (Figure 3B-E) Patients with HBV genotype D only had modest HBsAg declines regardless of therapy strategy.

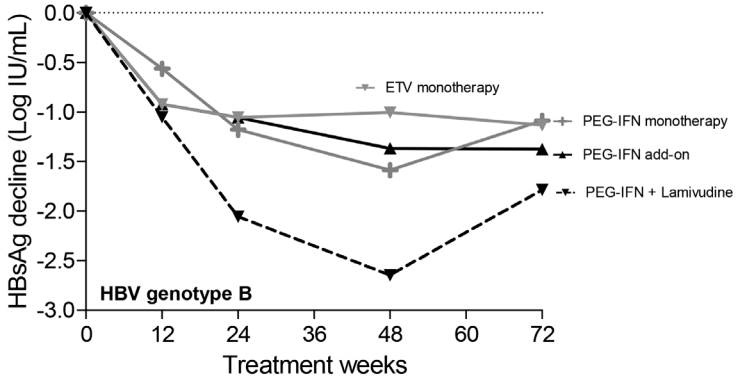


Decline log_{10} HBsAg (IU/mL)	PEG-IFN add-on (N=85)	PEG-IFN + LAM (N=110)	PEG-IFN mono (N=111)	ETV mono (N=90)
Week 12	-0.35	-0.41	-0.30	-0.35
Week 24	-0.42	-0.89	-0.59	-0.42
Week 48	-0.80	-1.27	-0.77	-0.32
Week 72	-0.83	-0.81	-0.68	-0.33

Figure 3 Mean HBsAg decline according to therapy regimen estimated using inversed probability of treatment weights and baseline HBsAg level, for the total cohort (A), and stratified by HBV genotype (B-E)

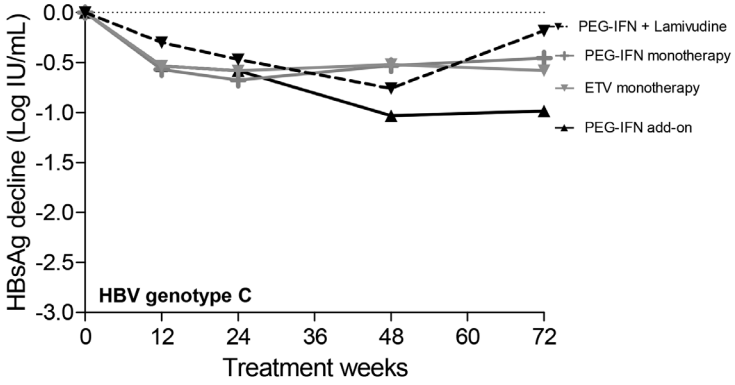


Decline log_{10} HBsAg (IU/mL)	PEG-IFN add-on (N=4)	PEG-IFN + LAM (N=34)	PEG-IFN mono (N=40)	ETV mono (N=9)
Week 12	-0.24	-0.93	-0.01	-0.24
Week 24	-0.25	-1.72	-0.64	-0.25
Week 48	-1.40	-2.20	-0.86	-0.02
Week 72	-1.52	-1.69	-1.12	-0.08

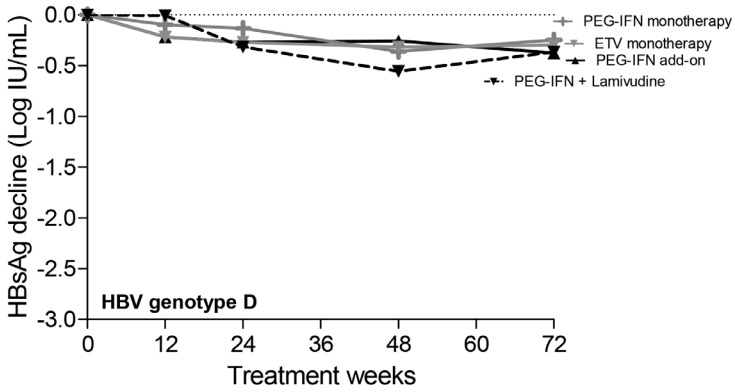


Decline log_{10} HBsAg (IU/mL)	PEG-IFN add-on (N=20)	PEG-IFN + LAM (N=10)	PEG-IFN mono (N=10)	ETV mono (N=13)
Week 12	-0.92	-1.05	-0.56	-0.92
Week 24	-1.05	-2.06	-1.18	-1.05
Week 48	-1.37	-2.65	-1.59	-1.01
Week 72	-1.37	-1.79	-1.09	-1.13

Figure 3 (continued) Mean HBsAg decline according to therapy regimen estimated using inversed probability of treatment weights and baseline HBsAg level, for the total cohort (A), and stratified by HBV genotype (B-E)



Decline log_{10} HBsAg (IU/mL)	PEG-IFN add-on (N=33)	PEG-IFN + LAM (N=14)	PEG-IFN mono (N=18)	ETV mono (N=41)
Week 12	-0.53	-0.30	-0.57	-0.53
Week 24	-0.58	-0.47	-0.67	-0.58
Week 48	-1.03	-0.76	-0.53	-0.52
Week 72	-0.98	-0.18	-0.46	-0.58



Decline log_{10} HBsAg (IU/mL)	PEG-IFN add-on (N=28)	PEG-IFN + LAM (N=46)	PEG-IFN mono (N=41)	ETV mono (N=27)
Week 12	-0.22	-0.01	-0.10	-0.22
Week 24	-0.27	-0.32	-0.13	-0.27
Week 48	-0.26	-0.56	-0.36	-0.32
Week 72	-0.38	-0.37	-0.25	-0.30

Figure 3 (continued) Mean HBsAg decline according to therapy regimen estimated using inversed probability of treatment weights and baseline HBsAg level, for the total cohort (A), and stratified by HBV genotype (B-E)

HBeAg response. At EOP, an estimated 22%, 40%, 27% and 11% of patients achieved HBeAg loss for PEG-IFN add-on, combination, monotherapy and ETV monotherapy, respectively. (Figure 4) The estimated proportion of patients achieving HBeAg loss at EOF was 36%, 31%, 33% and 20% for add-on, combination, PEG-IFN monotherapy and ETV monotherapy, respectively (p>0.05 across the treatment arms).

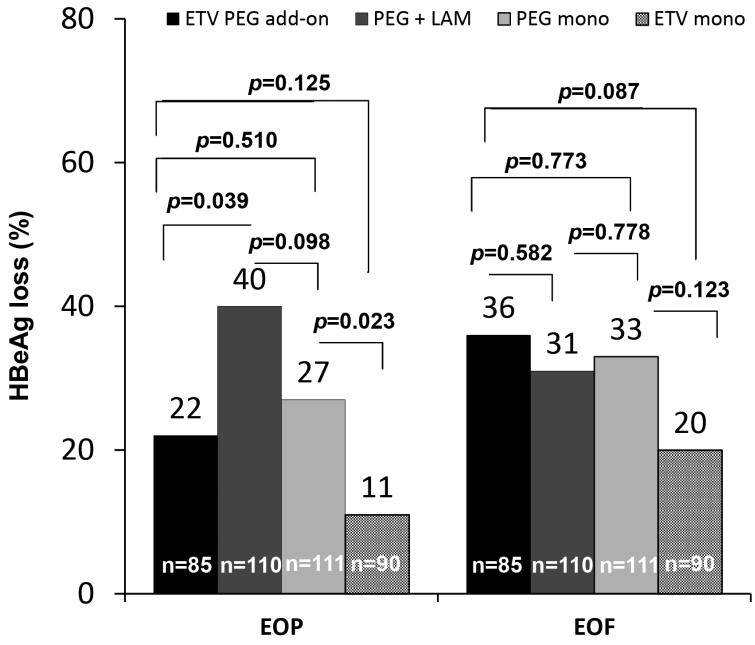


Figure 4 Proportion of patients with HBeAg loss according to therapy, estimated using inversed probability of treatment weights

DISCUSSION

In the current post-hoc analysis of 2 international randomized controlled trials, we have compared the HBsAg level declines achieved with a PEG-IFN add-on strategy versus PEG-IFN combination or monotherapy. We found that with the combination of PEG-IFN and NA more pronounced HBsAg declines were achieved, which was only maintained in patients who received ETV therapy after PEG-IFN withdrawal. Moreover, both after one year of treatment and after 6 months post PEG-IFN therapy, the proportion of patients achieving at least a 1 $\log_{10}$ reduction of HBsAg was significantly higher for 24 weeks of PEG-IFN add-on compared to

52 weeks of PEG-IFN monotherapy. Furthermore, equal HBeAg loss rates were achieved at 6 months post PEG-IFN treatment.

Currently, the best approach for the combination of PEG-IFN and NA in CHB is still unknown. We have previously investigated a 24 week PEG-IFN add-on strategy, which showed pronounced declines of HBsAg, HBeAg and HBV DNA levels both during and post PEG-IFN treatment.[24] Previous randomized trials have not indicated a beneficial off-treatment effect of a de novo PEG-IFN and LAM combination strategy, and also a NA add-on or a short NA pre-treatment switch strategy were unsuccessful.[19, 20, 22, 29, 30] More recently, a large trial investigating a de novo TDF with PEG-IFN combination strategy in 740 HBeAg-positive and HBeAg-negative patients indicated a beneficial effect for the combination strategy in terms of HBsAg decline and on-treatment HBsAg loss when compared to PEG-IFN monotherapy, but again the achieved response was not sustained off-treatment.[23] These findings therefore have raised the question whether there could be a role for NA consolidation therapy after PEG-IFN cessation.

In the current study, we have shown that there may indeed be a role for NA therapy in terms of prevention of HBsAg level increase after PEG-IFN withdrawal. We found that the combination of NA with PEG-IFN led to more HBsAg reduction compared to PEG-IFN monotherapy, also when PEG-IFN therapy duration was shortened by 24 weeks. The achieved HBsAg response was only sustained in those patients who received consolidation therapy after PEG-IFN withdrawal. We observed a prominent HBsAg decline in the first 12 weeks comparable for both the ETV and PEG-IFN monotherapy arms. This observation in the ETV monotherapy treated patients confirms findings from a recent study to ETV ± TDF in HBeAg-positive and negative patients, in which a prominent HBsAg reduction in the first 12 weeks was observed as well.[31] A possible explanation for this phenomenon should probably be sought in the pre-treatment immune activity against infected hepatocytes. The rapid HBsAg level reduction could be explained by a further improvement of immune function, a concept which is exploited with the PEG-IFN add-on strategy.[17, 18] Interestingly, as a result of this prominent decline in the first 12 weeks with ETV alone, at least equal mean HBsAg declines were observed with only 24 weeks of PEG-IFN add-on therapy compared to 52 weeks of PEG-IFN monotherapy. Moreover, an even larger proportion of patients treated with 24 weeks of PEG-IFN add-on achieved more than 1 $\log_{10}$ HBsAg decline, which was a comparable proportion achieved with 52 weeks of combination therapy.

There are several important questions left unanswered, which at the moment may limit the use of a PEG-IFN add-on strategy in clinical practice. First, it is unknown for what duration patients should receive ETV before initiating PEG-IFN, and whether HBV DNA levels should be undetectable or very low but detectable. Second, it is unknown what the optimal duration

of PEG-IFN add-on therapy is. Given our results, prolonging PEG-IFN add-on therapy with another 24 weeks may lead to even more HBsAg decline compared to combination therapy. Furthermore, we could not investigate the duration and effect of consolidation therapy on the sustainability of an off-treatment response. In the add-on arm, only 14 patients – 88% of add-on treated patients with HBeAg loss at week 48 – discontinued ETV after 24 weeks of consolidation therapy at week 72.[24] Of these, 3 patients achieved a HBsAg decline >1$\log_{10}$ IU/mL at week 48, which was sustained in 2 out of 3 patients at week 96. The low number of patients thus limit the interpretation of the effect of consolidation therapy on off-treatment response. Nevertheless, previous studies have shown that NA therapy may be discontinued in patients with low HBsAg levels or a rapid reduction of ≥1 $\log_{10}$.[26, 32]

Our study in almost 400 HBeAg-positive CHB patients infected with all major HBV genotypes is the first to indicate that 24 weeks of PEG-IFN add-on may result in more HBsAg reduction compared to 52 weeks of PEG-IFN monotherapy. It therefore provides with important directions for future randomized controlled trials or prospective cohort studies. Even so, a limitation of the current study may be the post-hoc exploratory analyses using the inversed probability of treatment weights approach. Indeed, at first there was an imbalance between the treatment arms in terms of HBV genotype distribution and other baseline factors such as HBsAg, HBV DNA and ALT levels. However, the current approach enabled us to adjust for these differences and compare different treatment strategies with a valid statistical method, mimicking a randomized controlled trial altogether.

In conclusion, the combination of a NA with PEG-IFN either as de novo for one year or as add-on for 24 weeks results in more on-treatment HBsAg decline than does one year of PEG-IFN alone. Continuation of ETV after PEG-IFN discontinuation may prevent HBsAg level increase. Future randomized trials should evaluate the role of NA consolidation therapy in PEG-IFN treated patients.

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PART III

OPTIMIZATION OF THE PRE-TREATMENT SELECTION OF CHRONIC HEPATITIS B PATIENTS

CHAPTER 8

Polymorphisms of *HLA-DP* are associated with response to peginterferon in Caucasian patients with chronic hepatitis B

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ABSTRACT

Background

Polymorphisms of the *HLA-DP* gene are associated with the natural clearance of the hepatitis B virus in Asian patients. We aimed to investigate the association of *HLA-DP* polymorphisms with response to peginterferon (PEG-IFN) in Caucasian chronic hepatitis B (CHB) patients.

Methods

We studied 262 Caucasian CHB patients infected with HBV genotype A or D, treated with PEG-IFN for one year in two randomized controlled trials (HBV 99-01 and PARC study). Response was defined as an HBV DNA <2000 IU/mL at 6 months post-treatment. Variations at *HLA-DPA1* and *HLA-DPB1* were genotyped.

Results

Of the 262 patients, 58% was HBeAg-positive and HBV genotype A and D was observed in 32% and 68%, respectively. At six months post-treatment, 57 (22%) patients had achieved an HBV DNA <2000 IU/mL. *HLA-DPB1* was independently associated with virologic response (adjusted odds ratio (OR) 1.8, 95% confidence interval [CI]:1.1 – 3.0, p=0.025), and with an undetectable HBV DNA (adjusted OR 2.4 95% CI: 1.2 – 4.7, p=0.015) when adjusted for HBeAg status and other known response modifiers. In HBeAg-positive patients, combined HBeAg seroconversion with HBV DNA <2000 IU/mL was increasingly observed with each addition of an *HLA-DPB1* G-allele (adjusted OR 2.7, 95%CI: 1.2 – 5.9, p=0.012). Furthermore, *HLA-DPA1* and *HLA-DPB1* haplotype block GG showed comparable results for virologic and combined response.

Conclusion

In this large cohort of Caucasian CHB patients infected with HBV genotypes A or D, polymorphisms of *HLA-DP* are independently associated with both virologic and serologic response to PEG-IFN therapy at six months post-treatment.

INTRODUCTION

Peginterferon (PEG-IFN) is a first line treatment option for patients with chronic hepatitis B (CHB) and is the only agent that can reasonably be expected to induce a sustained off-treatment response after a relative short treatment course.[1-3] However, a favourable response is only achieved in a subset of patients and comes at the price of considerable side-effects.[4] Therefore, it is of great importance to identify pre-treatment factors that are associated with response to PEG-IFN, in order to select patients with high probabilities of response before initiating treatment.

Over the past years, comprehensive research has led to identification of pre-treatment host and viral factors associated with response.[2, 5-8] The identification of these pre-treatment factors have introduced personalized medicine into clinical practice for CHB patients, but are generally only applicable to hepatitis B e antigen (HBeAg) positive patients while there are no strong pre-treatment predictors for virologic response in HBeAg-negative patients.[9] Moreover, the combination of host and viral factors used for the pre-treatment prediction of response have shown suboptimal discrimination at the individual level, suggesting that there are other response-modifying factors left undiscovered.

There is increasing evidence that host genetics play an important role in the natural history of hepatitis B virus (HBV) related liver disease and in response to treatment.[10] Single-nucleotide polymorphisms (SNPs) near interleukin-*28B* (*IL28B*) were first discovered to be associated with natural clearance and treatment response in hepatitis C (HCV)[11] and were recently associated with response to PEG-IFN in HBeAg-positive CHB.[12] Additionally, for both HBeAg-positive[12] as well as HBeAg-negative patients,[13] *IL28B* was associated with HBsAg clearance after long-term follow-up. Following these initial promising publications conflicting results have been reported,[14, 15] probably limiting the clinical applicability of *IL28B* in CHB patients treated with PEG-IFN.[16] Hence, other genetic variations, particularly when associated with the host immune response and immunologic control of HBV, may play a more explicit role in the response to PEG-IFN.

Recent genome-wide association studies (GWAS) in Asian CHB patients showed that SNPs in the human leukocyte antigen (HLA) region, *HLA-DPA1* and *HLA-DPB1*, are associated with protection against chronicity and thus immune control of HBV,[17, 18] findings that were confirmed in subsequent studies.[19, 20] Moreover, a study in HBeAg-positive Asian CHB patients indicated a role of *HLA-DPA1* in HBeAg seroconversion after PEG-IFN therapy.[15] However, whether these beneficial *HLA-DP* variations are associated with response to PEG-IFN in Caucasian patients has never been studied.

Therefore, our aim is to investigate the relationship of *HLA-DP* polymorphisms with response to PEG-IFN in Caucasian CHB patients.

PATIENTS AND METHODS

Patients. CHB patients who were treated within two international randomized controlled trials with PEG-IFN alfa (2a, Pegasys, F. Hoffmann-La Roche Ltd., Basel, Switzerland or 2b, PegIntron, Merck Sharpe & Dohme, Whitehouse Station, NJ, USA) for one year, either alone or in combination with ribavirin (HBeAg-negative patients, Copegus, F. Hoffmann-La Roche Ltd., Basel, Switzerland) or lamivudine (HBeAg-positive patients, Zeffix, GlaxoSmithKline, Greenford, UK), were eligible for the current study. Inclusion criteria for the initial trials are described elsewhere. [2, 21] In short, all patients were required to be hepatitis B surface antigen (HBsAg) positive for more than 6 months, and to have two episodes of a raised serum alanine aminotransferase (ALT). HBeAg-negative patients were required to have an HBV DNA concentration >100,000 copies/mL (18,181 IU/mL). For the current study, only Caucasian patients infected with HBV genotype A or D were included to avoid potential confounding by population stratification.[22] The follow-up period of patients was 24 weeks (6 months) post-treatment according to the clinical trial protocols.
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. The primary endpoint assessed at six months post-treatment was virologic response, defined as an HBV DNA below 2000 IU/mL according to clinical practice guidelines.[9, 23] Since relapse rates are not uncommon for HBeAg-negative patients,[3] HBV DNA below 80 IU/mL, HBsAg levels below 1000 IU/mL and HBsAg loss at six months post-treatment were also considered, as these endpoints are associated with sustained disease remission and an improved clinical outcome over time.[1, 3, 24] For HBeAg-positive patients, the combined presence of HBeAg seroconversion with HBV DNA <2000IU/mL (referred to as combined response) was evaluated as well.[9]

Laboratory measurements. Serum HBV DNA, HBeAg and HBsAg were quantified in samples taken at baseline, during the treatment period and at 6 months post-treatment. HBV DNA was measured using an in-house developed TaqMan polymerase chain reaction (PCR)

assay (lower limit of quantification 80 IU/mL) and HBsAg was measured using the Abbott ARCHITECT HBsAg assay (Abbott laboratories; range 0.05 – 250 IU/mL). The presence of precore (PC, nucleotide position G1896) and/or basal core promotor (BCP, nucleotide positions A1762 and G1764) was assessed using the INNO-LiPA HBV PreCore assay (Innogenetics, Ghent, Belgium).

Host genotyping. Competitive allele-specific PCR assays (KASP™, LGC genomics, Huddleston, United Kingdom) were employed for the detection of the reference SNPs *HLA-DPA1* (at *rs3077*) and *HLA-DPB1* (at *rs9277535*).[17] Serum samples stored at -20^0 or -80^0 Celsius were used for DNA extraction and genotyping procedures which were carried out centrally at LGC genomics. Purified genomic DNA of ≥5 nanogram was used for genotyping. Genotypes were assigned using all of the data from the study simultaneously. Genotype sequences were derived from NCBI.

Statistical analysis. SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) and the SAS 9.3 program (SAS Institute Inc., Cary, NC, USA) were used to perform statistical analyses. Associations between variables were tested using Student's t-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. The genetic association analysis consisted of an additive model (representing the effect on the outcome with each addition of an allele) as well as a haplotype block analysis (constructed by combining *HLA-DPA1* and *HLA-DPB1*). Both SNPs were subject to Hardy-Weinberg equilibrium tests.[25] Binary logistic regression modelling was used to identify independent factors associated with the outcome measures at six months post-treatment. Since combination treatment with lamivudine [2] or ribavirin [21] did not influence response rates at six months post-treatment, data from the monotherapy and combination arms were pooled for the current analysis. Treatment allocation was controlled for whenever applicable. All statistical tests were two-sided and were evaluated at the 0.05 level of significance.

Role of the funding source. Financial support for this study was provided by Merck Sharpe & Dohme (MSD), The Netherlands, the Foundation for Liver and Gastrointestinal Research (SLO) in Rotterdam, The Netherlands and by the Virgo consortium, funded by the Dutch government project number FES0908, and by the Netherlands Genomics Initiative (NGI) project number 050-060-452. Financial support for the original trials was provided by Schering-Plough (now Merck Sharpe & Dohme)[2] and Hoffmann-La Roche.[21] The funding sources did not have any influence on study design, data collection, analysis and interpretation of the data, writing of the report nor the decision to submit for publication.

RESULTS

Patient characteristics. Of the initial 399 patients treated with PEG-IFN, 307 patients were Caucasian infected with viral genotype A or D. Of these, 262 patients had a sample available for host DNA isolation and were included in the analysis of this study. The characteristics of the enrolled patients are shown in table 1. Two-hundred and one patients were male (78%) and 153 were HBeAg-positive (58%). Viral genotypes A and D were found in 84 (32%) and 178 (68%) patients. Patients were treated with either PEG-IFN monotherapy (n=133), PEG-IFN + lamivudine (n=74)[2] or PEG-IFN + ribavirin (n=55).[21]

Distribution of *HLA-DP* polymorphisms. *HLA-DP* polymorphisms were in a weak linkage disequilibrium (D'=0.55, R^2=0.30). The distributions of *HLA-DPA1* AA/GA/GG and *HLA-DPB1* AA/GA/GG were 63%/26%/6% (95% successfully genotyped) and 62%/26%/6% (94% successfully genotyped), respectively. Host genotype distributions did not statistically differ between HBeAg-positive and HBeAg-negative patients (table 1).

***HLA-DP* polymorphisms and virologic response.** At six months post-treatment, a total of 57 patients (22%) achieved an HBV DNA below 2000 IU/mL, of which 32 (56%) were HBeAg-positive and 25 (44%) were HBeAg-negative (p=0.696). Patients with *HLA-DPB1* genotype GG achieved this response in 50% (8/16) versus 30% (20/67) and 17% (28/162) in patients with genotypes GA and AA respectively (p=0.001). Both when stratified by HBeAg status as well as by HBV genotype the response rates increased per addition of an *HLA-DPB1* G-allele (figure 1). In univariate analysis both *HLA-DPB1* (additive OR 2.1, 95% CI: 1.3 – 3.4, p=0.001) and *HLA-DPA1* (additive OR 1.6, 95% CI: 1.0 – 2.5, p=0.050) were associated with an HBV DNA below 2000 IU/mL at six months post-treatment. Furthermore, older age (OR 1.0, 95%CI: 1.0 – 1.1, p=0.003) and the absence of PC/BCP mutants (OR 2.6, 95%CI: 1.4 – 4.8, p=0.004) were associated with virologic response while gender, baseline serum ALT, previous IFN therapy and baseline HBV DNA and HBsAg levels did not show a significant association.

By multivariate analysis, *HLA-DPB1* was significantly associated with virologic response (additive OR 1.8, 95%CI: 1.1 – 3.0, p=0.025, figure 2 & table 2), independent of HBeAg status, HBV genotype, the presence of PC and/or BCP mutant virus and baseline HBV DNA or HBsAg levels. The probability of response was highest for HBV genotype A infected HBeAg-positive patients, and response rates increased per addition of a G-allele independent of HBeAg status and HBV genotype (figure 2). Gender, age, and baseline serum ALT levels did not add to the multivariate model. There were no interactions between *HLA-DPB1* and the other factors

investigated in this model (table 2). The effect of *HLA-DPA1* showed a similar direction (adjusted OR 1.5, 95% CI: 0.9 – 2.4, p=0.138). Combined analysis of both *HLA-DPB1* and *HLA-DPA1* showed that haplotype block GG was significantly associated with virologic response (OR 2.6, 95%CI: 1.3 – 5.4, p=0.010).

Table 1 Characteristics of the study cohort

Characteristics	**HBeAg(+) (n=153)**	**HBeAg(-) (n=109)**	***p*-value***
Demography			
Mean (SD) age, years	34.3 (13.6)	42.1 (10.5)	<0.001
Male, n (%)	122 (80)	79 (73)	0.180
Previous IFN therapy, n (%)	35 (23)	17 (16)	0.137
Therapy allocation, n (%)			
PEG-IFN Monotherapy	79 (52)	54 (50)	0.740
PEG-IFN + Lamivudine	74 (48)	NA	NA
PEG-IFN + Ribavirin	NA	55 (50)	NA
Laboratory results, mean (SD)			
ALT (U/L)**	4.3 (3.2)	3.3 (2.6)	0.004
HBV DNA, log IU/mL	8.6 (0.8)	6.8 (1.2)	<0.001
HBsAg, log IU/mL	4.5 (0.5)	3.8 (0.6)	<0.001
HBV Genotype, n (%)			<0.001
A	71 (46)	13 (12)	
D	82 (54)	96 (88)	
INNO-LiPA result, n (%)			<0.001
Wildtype	55 (36)	4 (4)	
Precore	37 (24)	20 (18)	
Basal core promoter	32 (21)	8 (7)	
Precore and basal core	23 (15)	77 (71)	
Minor allele frequencies (%)			
HLA-DPA1 (G)	20	19	0.729
HLA-DPB1 (G)	20	17	0.182
Haplotype block, n (%)[a]			0.306
AA	70 (53)	67 (63)	
AG	18 (14)	8 (7)	
GA	14 (10)	8 (7)	
GG	31 (23)	23 (23)	
Liver histology			
Mean (SD) Fibrosis (Ishak)	2.5 (1.5)	2.3 (1.4)	0.246
Mean (SD) Hepatic Activity Index	5.1 (2.2)	5.2 (2.2)	0.804
Ishak stage 5 and 6, n (%)	13 (8)	3 (3)	0.019

* Comparison of HBeAg-positive versus HBeAg-negative patients
** Multiples of upper limit of the normal range
[a] Haplotype block consisting of HLA-DPA1 and HLA-DPB1, respectively

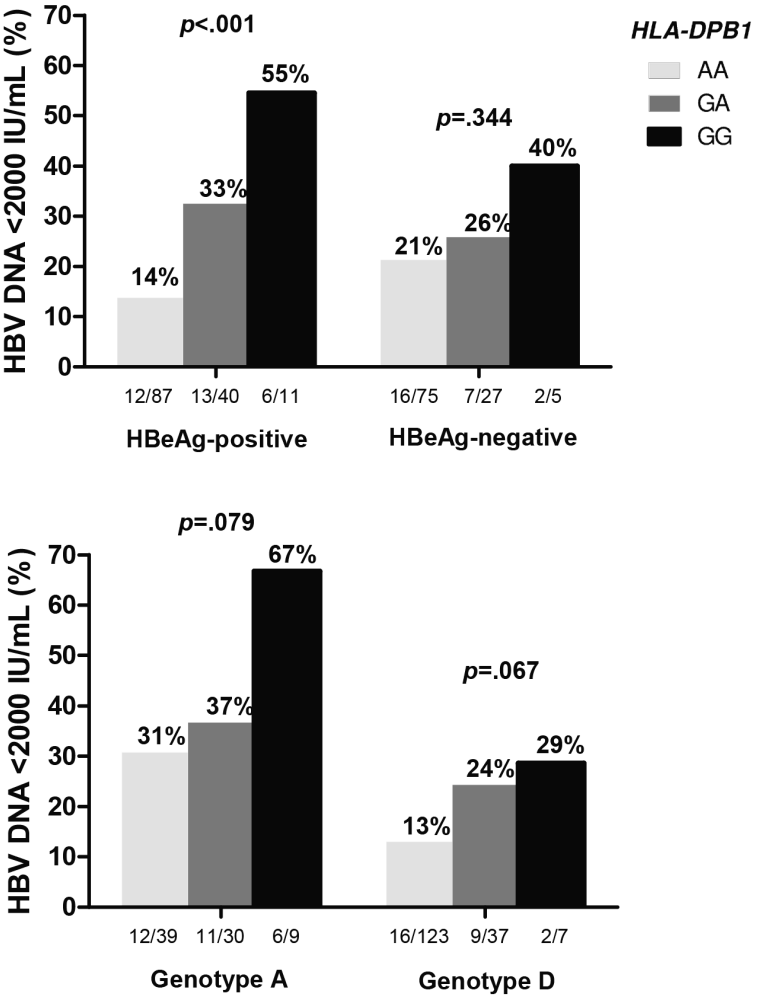

Figure 1 Observed rates of HBV DNA <2000 IU/mL at 6 months post-treatment stratified by HBeAg status (A) and HBV genotype (B) according to polymorphisms of *HLA-DPB1*

When using a lower threshold for virologic response, comparable results were observed. Twenty-two (8%) patients achieved an HBV DNA below 80 IU/mL. Forty percent of the GG-allele carriers achieved this response, compared to 8% and 6% for patients with GA and AA, respectively (p<0.001). Adjusted for the aforementioned factors, the additive OR for *HLA-DPB1* for achieving an undetectable HBV DNA was 2.4 (95% CI: 1.2 – 4.7, p=0.015).

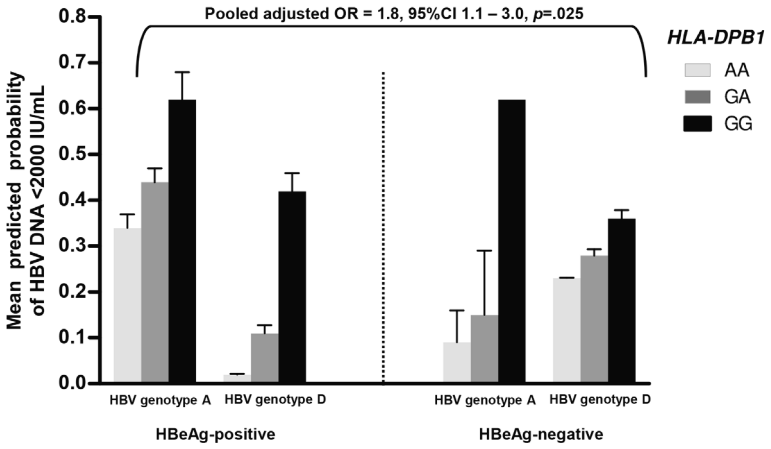

Figure 2 Mean predicted probability of a HBV DNA <2000 IU/mL at six months post-treatment according to polymorphisms of *HLA-DPB1*, by HBeAg-status and HBV genotype *(adjusted for baseline HBV DNA load, HBsAg levels and the presence of PC/BCP mutants by logistic regression)*. Per additional G-allele the overall odds for an HBV DNA <2000 IU/mL increases with 1.8

***HLA-DP* polymorphisms and combined HBeAg seroconversion with low HBV DNA levels.** Of the 153 HBeAg-positive patients included in the analysis, 25 (16%) achieved a combined HBeAg seroconversion with HBV DNA levels <2000 IU/mL at six months post-treatment. Forty-six (30%) patients achieved HBeAg seroconversion. Combined response rates increased significantly with *HLA-DPB1* AA (9%), GA (28%) and GG (46%) (p<0.001, figure 3).

Table 2 Multivariate analysis for an HBV DNA <2000 IU/mL at 6 months post-treatment.

Variable	OR (95% CI)[α]	*p*-value
HLA-DPB1 [β]	1.8 (1.1-3.0)	0.025
Baseline HBV DNA (log IU/mL)	1.0 (0.7-1.5)	0.812
Baseline HBsAg (log IU/mL)	0.6 (0.3-1.2)	0.116
Absence of PC/BCP mutations	1.4 (0.5-3.8)	0.494
HBeAg-positive versus negative*		
– among HBV genotype A	8.7 (2.4-31.2)	0.001
– among HBV genotype D	0.1 (0.1-0.4)	0.001

[α] Stepwise regression analysis (forward likelihood ratio) approach of variables with a p-value <0.2 in univariate analysis and those considered of importance for the endpoint

[β] Additive model (OR per addition of an allele)

* Interaction between HBeAg status and HBV genotype (p=0.006)

By multivariate regression analysis, the occurence of a combined response was significantly associated with *HLA-DPB1* polymorphisms (additive OR 2.7, 95%CI: 1.2 – 5.9, p=0.012), HBV genotype A (OR 7.1, 95%CI: 1.6 – 32.1, p=0.011), and previous IFN therapy (OR 0.2, 95%CI: 0.1 – 0.9, p=0.035), while absence of PC/BCP mutations (OR 1.7, 95%CI: 0.5 – 5.8, p=0.370), baseline HBsAg (OR 0.7, 95%CI: 0.2 – 2.8, p=0.663) and HBV DNA levels (OR 0.8, 95%CI: 0.3 – 1.7, p=0.530) were not associated with combined response. Gender, age and serum ALT levels did not add to the multivariate model.
The effect of *HLA-DPA1* on combined response showed a similar direction: in multivariate analysis adjusted for the same variables, *HLA-DPA1* showed a trend towards an association with combined response (additive OR 2.1, 95%CI: 0.9 – 4.8, p=0.086), and was significantly associated with HBeAg seroconversion (adjusted OR 2.0, 95%CI: 1.0 – 3.9, p=0.045). In addition, combining *HLA-DPB1* and *HLA-DPA1* showed that patients carrying haplotype block GG had a significantly higher probability of achieving HBeAg seroconversion with an HBV DNA <2000 IU/mL (OR 6.8, 95%CI: 1.9 – 24.1, p=0.003) compared to patients carrying haplotype block AA.

***HLA-DP* polymorphisms and HBsAg loss.** At six months post-treatment, a total of 52 (20%) patients achieved an HBsAg level <1000 IU/mL. Figure 4 shows the HBsAg response rates. The rate of HBsAg levels below 1000 IU/mL increased with both the addition of a *HLA-DPB1* G-allele (OR 1.5, 95%CI: 0.9 – 2.4, p=0.086) and a *HLA-DPA1* G-allele (OR 1.5, 95%CI: 0.9 – 2.5, p=0.077). Furthermore, a total of 16 patients (6%) lost HBsAg. There was an increasing rate of HBsAg loss across the polymorphisms of *HLA-DPB1* (figure 4), with an unadjusted additive OR of 1.7 (95% CI: 0.8 – 3.6, p=0.122).

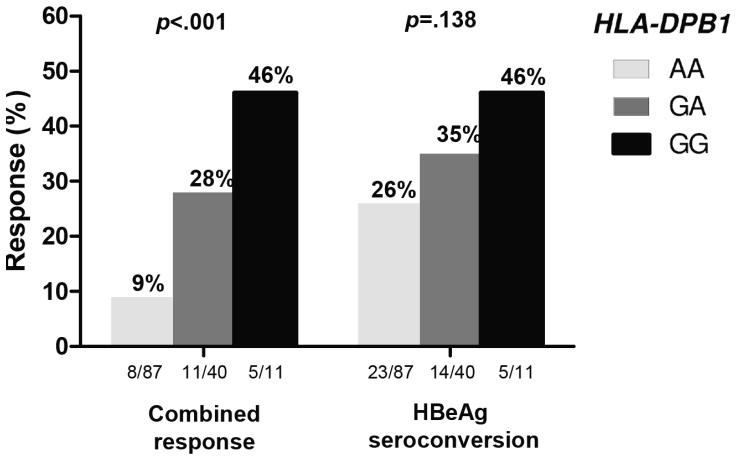

Figure 3 Observed rates of combined response and HBeAg seroconversion at six months post-treatment, according to *HLA-DPB1* polymorphisms for HBeAg-positive patients

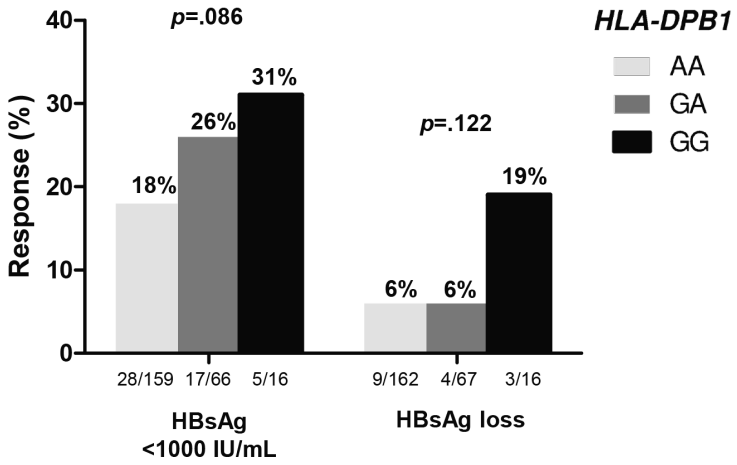

Figure 4 Observed rates of HBsAg levels <1000 IU/mL and HBsAg loss at six months post-treatment, according to *HLA-DPB1* polymorphisms

DISCUSSION

As PEG-IFN is generally poorly tolerated and only leads to a successful response in a subgroup of patients, it is essential to select patients who have a high probability of achieving a response before initiating therapy. Previously described predictors of response are suboptimal for pre-treatment stratification at the individual level,[5, 6] while there is increasing evidence that host genetic variations may modify the response to PEG-IFN treatment.[10, 12, 13, 16]

In the current study we have shown for the first time that polymorphisms of the *HLA-DP* gene are associated with both virologic and serologic response to PEG-IFN six months post-treatment in Caucasian CHB patients. There was a predominant association of *HLA-DPB1* with virologic and combined response. The probability of response increased with the addition of each G-allele, irrespective of HBeAg status or HBV genotype.

Recent studies showed that polymorphisms of *HLA-DP* are protective against disease progression and thus associated with immune control of CHB.[17-20] Additionally, it was found that *HLA-DPA1* was associated with higher HBeAg seroconversion rates in Asian patients treated with PEG-IFN.[15] However, robust pre-treatment predictors for virologic response to PEG-IFN are currently lacking for HBeAg-negative patients,[6, 9, 13] while to date, the role of genetic predictors for serologic or virologic response in HBeAg-positive patients has been unclear. [14, 15]

In the current study, investigating only Caucasian CHB patients, we have shown that *HLA-DPB1* is associated with virologic response to PEG-IFN in both HBeAg-positive and HBeAg-negative patients. These results provide new insight into the role of host immunity in the response of PEG-IFN therapy and potentially of other immune modulating treatment which are currently under investigation. [26, 27] Patients with the favourable *HLA-DP* genotypes more frequently achieved low levels of HBV DNA and HBeAg seroconversion after PEG-IFN therapy. These endpoints are highly durable and of clinical significance given the prolonged survival and the reduced risk of HCC development.[7, 28, 29]

HLA-DPA1 and *HLA-DPB1* are members of the major histocompatibility complex (MHC) class II molecules, which are expressed on B-lymphocytes, dendritic cells and macrophages. These molecules present antigens to specific $CD4^+$ helper T-lymphocytes, which may lead to antiviral immune responses and production of antibodies.[30] Genetic variations in the *HLA-DP* gene may lead to a stronger interaction of antigen-presenting cells and T-cells and thus to a more effective antiviral immune response. These features may work synergistically with PEG-IFN, as this agent induces a profound immune response and has the potential to inhibit viral replication. The findings of the current study could help guide functional follow-up studies to elucidate the role of these polymorphisms in the biological pathway associated with response to PEG-IFN.

The results of our study were in line with a previous report on the association between *HLA-DP* and response to PEG-IFN in Asian HBeAg-positive patients, as we confirmed the association of the *HLA-DPA1* G-allele with HBeAg seroconversion.[15] However, the results differed from studies related to the natural clearance of HBV.[17-20] There are several explanations for this observation. First, it should be appreciated that the genotype distribution of the *HLA-DP* genes are different for Caucasian patients as compared to Asian patients; in Caucasian patients, the G-allele is the minor allele for both *HLA-DPA1* and *HLA-DPB1*, whereas it is the major allele found in Asian patients.[31] Second, the distribution of viral genotypes – known for the association with antiviral therapy response – vary with ethnicity, where HBV genotypes A and D predominate in Caucasian patients and HBV genotype B and C are most prevalent in Asian patients.[22] Nonetheless, we speculate that Asian or Caucasian CHB patients carrying the presumed 'high clearance' A-allele, who previously have failed to clear the virus, represent a subset of patients that are less susceptible to spontaneous or PEG-IFN mediated clearance of HBV.[15]

In the current cohort, *HLA-DP* was significantly associated with low levels of HBV DNA after PEG-IFN treatment, independent of HBeAg status or HBV genotype. However, it is important to underline that both HBeAg status and HBV genotype are important response

modifiers. HBeAg-positive HBV genotype A infected patients had the highest probability of achieving a virologic response, regardless of *HLA-DP* status. The findings of our study suggest that polymorphisms of *HLA-DP* may play a key role in the response to PEG-IFN and therefore the *HLA-DP* status may be of valuable addition to these known virologic determinants.

A strength of our study encompasses the analysis of a well-defined, large cohort of only Caucasian patients, which limits the chance of confounding due to population stratification.[22] A potential caveat of the current study could be the combined analysis of HBeAg-positive and HBeAg-negative patients. However, we have shown that pooling of the two cohorts was possible, as there were no interactions between *HLA-DP* polymorphisms and other response modifiers, while the interaction between HBV genotype and HBeAg status did not impact on the effect of *HLA-DPB1* on virologic response. This actually demonstrates a homogeneous effect of this polymorphism across the different phases of infection, and highlights the importance of HBV genotype in the probability of response to PEG-IFN. HBeAg-negative patients who achieve an HBV DNA load below 2000 IU/mL are still at risk for virologic relapse, especially in the first year after treatment.[3] Nevertheless, we have shown that *HLA-DP* was also associated with an undetectable viral load and showed a trend for HBsAg levels <1000 IU/mL and HBsAg loss at six months post-treatment. These strict endpoints are associated with a sustained response and improved long-term clinical outcome,[1, 3, 24] which supports the clinical importance of the *HLA-DP* polymorphisms.

In conclusion, in this large cohort of Caucasian HBeAg-positive and HBeAg-negative CHB patients infected with viral genotypes A or D, we have shown that polymorphisms of *HLA-DP* have an independent association with virologic and serologic response six months after PEG-IFN therapy.

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CHAPTER 9

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

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ABSTRACT

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). 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). 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. In summary, 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.[1, 2] PEG-IFN treatment induces a reduction of intrahepatic cccDNA [3], 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.[4] 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.[5] 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.[5-7] HBcrAg dynamics have been described in natural history and during NA therapy[8-10], 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).[11] 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. 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.[11] 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.[12] We specifically chose week 72 as the time-point for assessment of on-treatment response, because 22 patients stopped ETV after week 72.[11] 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.

Serum HBcrAg measurements. HBcrAg was measured using the Lumipulse® *G* HBcrAg assay (Fujirebio Europe, Belgium) in serum samples stored at -20^0 Celsius. A pre-heat treatment in presence of a provided detergent solution was applied for extracting denatured precore/core proteins and for inactivating 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). 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 1.0kU/mL and the measurement range is from 1.0 to 10,000 kU/mL.[13] In the present study units were expressed as U/mL.

Laboratory measurements. Serum samples were taken at baseline and during treatment. 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 promotor (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 1). 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 1). 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 2). By Bland-Altman analysis, standardized HBcrAg and qHBeAg measurements showed close agreement at baseline (Supplementary figure 3).

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	7 (8%)	9 (12%)
Precore	13 (16%)	21 (27%)
Basal core promotor	16 (19%)	7 (9%)
Precore & Basal core promotor	48 (57%)	40 (52%)
Histology		
Ishak fibrosis	2.4 (1.3)	2.0 (1.3)
Hepatic Activity Index	4.8 (2.3)	4.5 (2.1)
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 (-2.62 log U/mL vs. -1.74 U/mL, p=0.02).

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. 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 (-3.16 log U/mL vs. -1.76 U/mL, p=0.01).

One patient lost HBsAg at week 48, and still had detectable HBcrAg (215,000 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 (2,000 U/mL).

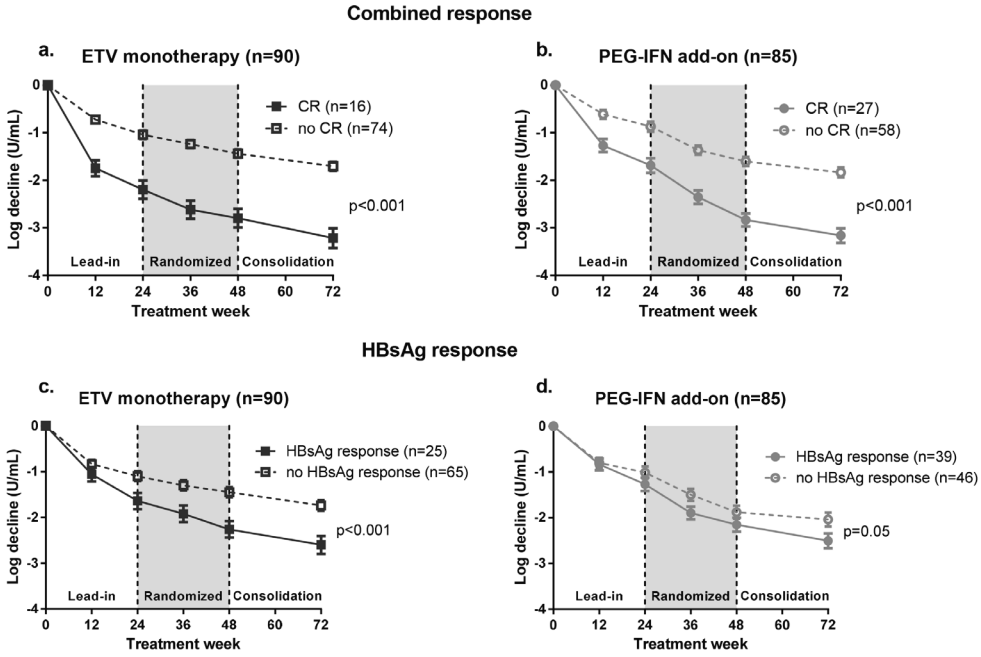

Figure 1a HBcrAg decline according to week 72 response

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).

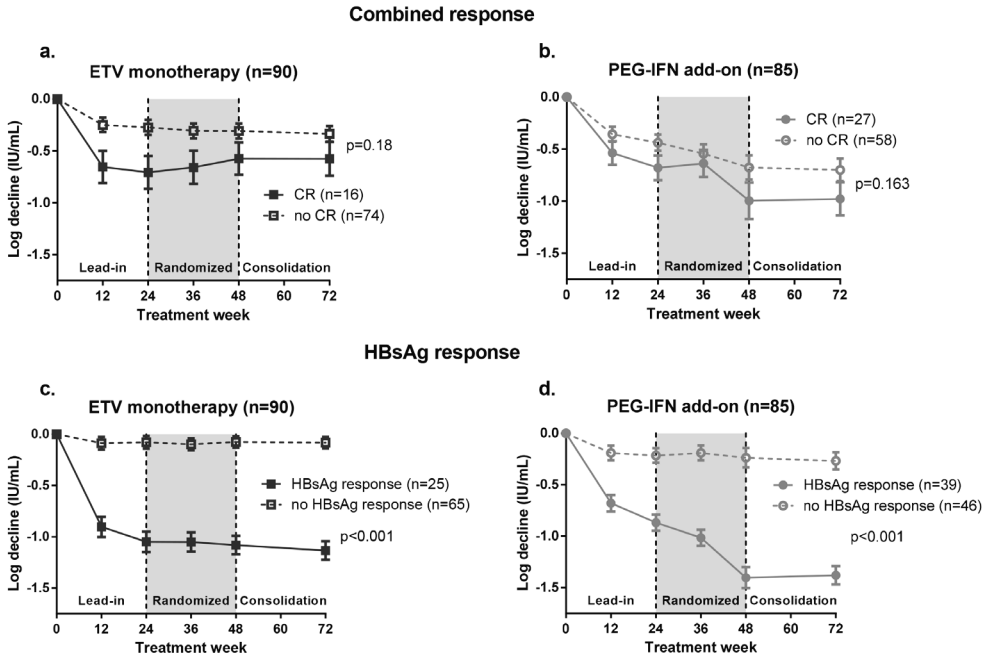

Figure 1b qHBsAg decline according to week 72 response

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.

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 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).

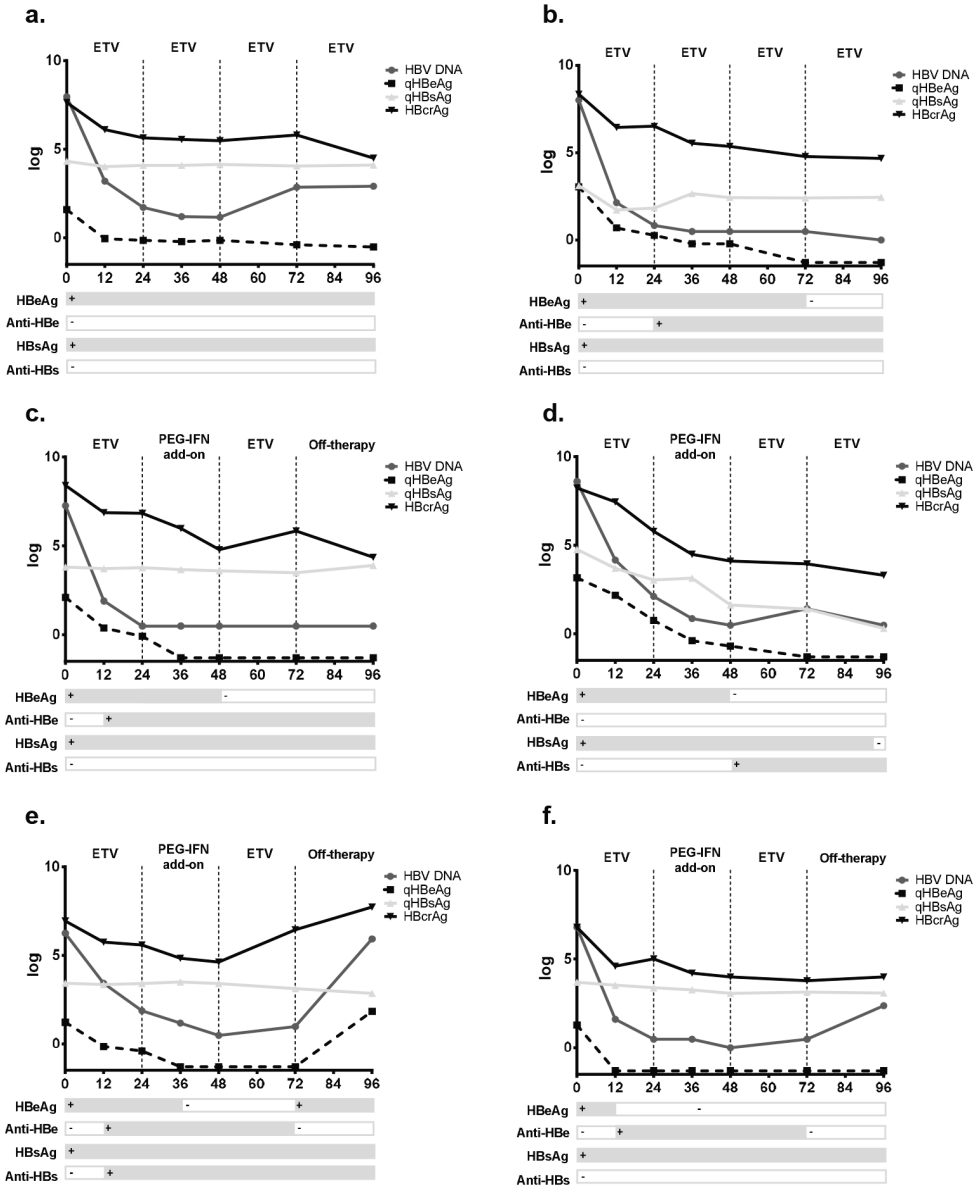

Figure 2 Dynamics of HBcrAg, qHBeAg, qHBsAg and HBV DNA in 6 example patients.

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 HBeAg loss & HBV DNA <200 IU/mL	Univariable OR (CI-95%)	p-value	Multivariable (Full model week 24) OR (CI-95%)	p-value	Multivariable (Final model week 24) 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						
– Wildtype	Reference					
– Gender	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						
– 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).*

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).

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, even after HBeAg loss. HBcrAg dynamics strongly resembled those of qHBeAg. HBcrAg levels at randomization were associated with response to both ETV monotherapy and PEG-IFN add-on therapy, but were not superior to qHBsAg, qHBeAg or HBV DNA levels in response prediction. 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.[4] In addition, cccDNA can still be detected after loss of HBsAg, acting as a substrate for viral reactivation during immunosuppressive states.[14] There is a need for serum makers that correlate with cccDNA and HBcrAg has been shown to do that, as well as correlate with cccDNA in both HBeAg-positive and HBeAg-negative patients.[5, 6]

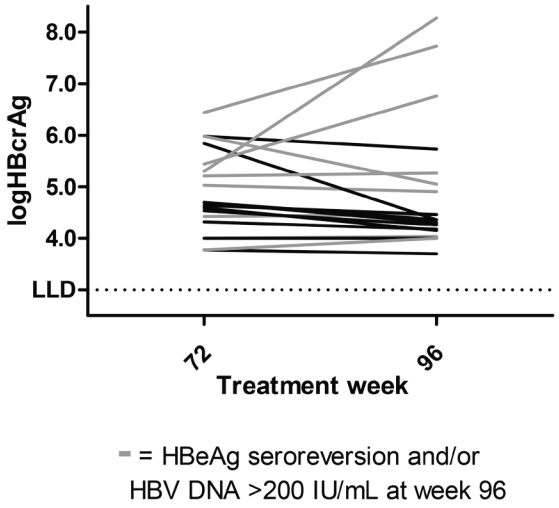

Figure 3 Individual HBcrAg dynamics in patients who stopped ETV at week 72.

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).

Mean HBcrAg levels at baseline in our study population were in the same range as the mean levels reported for HBeAg-positive patients in natural history studies.[8, 9] Previously, it has been described that the correlation of HBcrAg to serum HBV DNA and HBsAg is strongest for HBeAg-positive CHB.[6, 15, 16] 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.[15, 16] 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.[6, 7] In addition to possibilities for monitoring treatment response and relapse in CHB, it is therefore interesting to explore HBcrAg as a marker for reinfection after liver transplantation, or for reactivation during immunosuppression.[7]

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.[11, 17] The performance of HBcrAg in response prediction was comparable to that of qHBsAg, qHBeAg and HBV DNA. The close agreement of the results of HBcrAg and qHBeAg measurements in HBeAg-positive CHB illustrates that HBeAg comprises a considerable fraction of HBcrAg.[13] For HBeAg-positive disease, we did not find HBcrAg

measurements to be superior to HBsAg 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 highlights the importance of investigating HBcrAg measurements in HBeAg-negative disease. The predictive ability of HBcrAg levels for relapse after NA cessation has previously been described by a Japanese group.[18] Due to the limited number of patients that stopped treatment in our study, we could not confirm this specific role of HBcrAg.

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 in monitoring treatment response in HBeAg-positive patients, 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.

ACKNOWLEDGEMENTS

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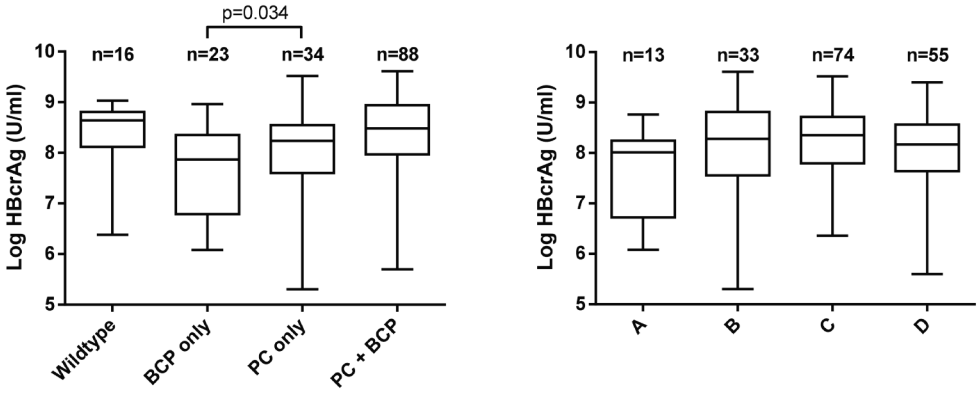
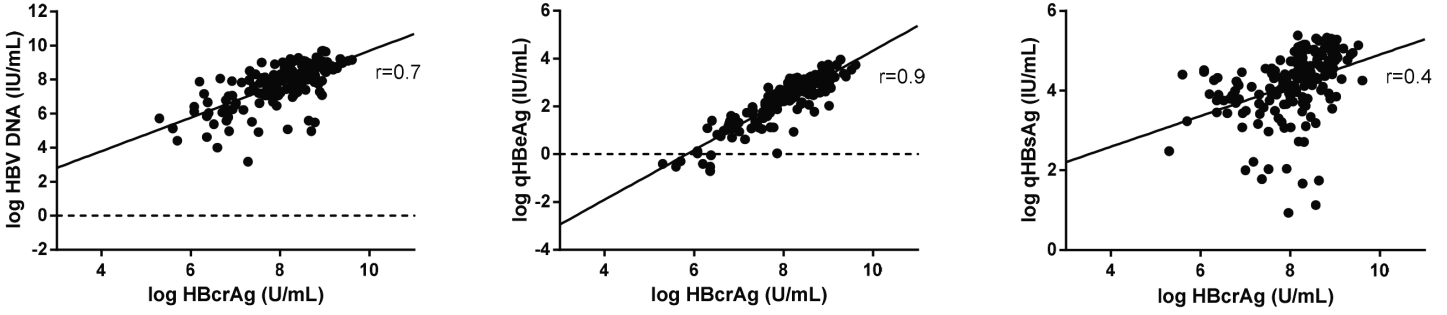
SUPPLEMENTARY FIGURES

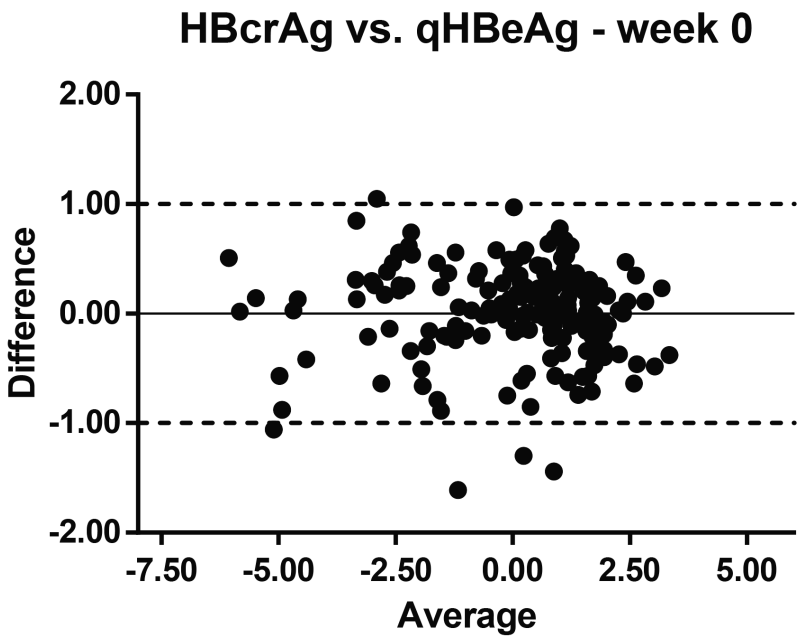


Supplementary figure 1 Baseline HBcrAg according to precore/core mutations and genotype.

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 2 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 3 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.

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CHAPTER 10

Studies of *IL28B* Genotype and Response to Peginterferon in Chronic Hepatitis B Should Be Stratified by HBV Genotype

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Hepatology 2013;57:1283

To the Editor:

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

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

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

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PART IV

SUMMARY & APPENDICES

CHAPTER 11

Summary and discussion

BACKGROUND

Patients with chronic hepatitis B (CHB) infection are at high risk of developing longstanding hepatic inflammation due to an ineffective immune response directed at the non-cytopathic hepatitis B virus (HBV). When infected in early childhood over 90% of patients become chronically infected. Annually, approximately 650.000 patients worldwide die of complications of CHB infection such as liver decompensation or hepatocellular carcinoma (HCC) development. Some CHB patients do not develop liver inflammation and seem to have achieved immunological control of the virus. These so called inactive carriers have an improved prognosis comparable to the general population, and may be monitored less frequently. Still hepatitis reactivation as a result of immune escape can occur. In this case, patients are at high risk of developing liver cirrhosis, decompensation and HCC, and therefore require antiviral therapy. It is important to identify those patients at risk of hepatitis reactivation, and to assess the risk of deterioration for individual patients to be able to provide a timely intervention for those who benefit most.[1-3] Both pegylated interferon (PEG-IFN) and nucleos(t)ide analogues (NA's) have shown to achieve an improved prognosis in those patients with a good response. For NA's, this is achieved by maintained HBV DNA suppression. A large proportion of patients however develop hepatitis relapse after cessation of NA, and therefore such a course is indefinite and may even be lifelong. In contrast, PEG-IFN therapy leads to more serological response (Hepatitis B e antigen [HBeAg] loss and Hepatitis B surface antigen [HBsAg] loss), however only a subset of patients achieve this favourable outcome at the cost of significant side-effects. Therefore optimizing the treatment strategies, and selecting patients with a high probability of response to a finite course of PEG-IFN may help to make this treatment modality more acceptable to patients, physicians and health-care policy makers.

OPTIMIZATION OF THE MONITORING OF CHRONIC HEPATITIS B PATIENTS

Viral markers in the natural history of CHB

For decades the gold standard to assess a patients' risk of liver disease progression has been liver biopsy. However, this diagnostic and prognostic modality is associated with several major limitations, which urges physicians to look for other ways to assess a patients' individual prognosis.

It has been shown that patients with HBV DNA levels above 2,000 IU/mL carry a higher risk of HCC development.[4, 5] Also quantitative HBsAg levels have been associated with hepatitis activity, disease progression and HCC development in patients with a low HBV DNA load.[6-12] It has been shown that a single-point measurement of HBsAg combined with HBV DNA could be used as a marker to identify patients at low risk of disease progression (i.e. inactive carriers) one year later with a high specificity and sensitivity in HBV genotype D patients.[6] However, this prediction rule showed a variable performance in other studies.[11, 13] One of the reasons for these discrepant observations may be a remittent pattern of HBV fluctuations followed by long-term remissions in patients with HBeAg-negative CHB. A single measurement may therefore be not enough to predict the risk of reactivation, and the HBsAg and HBV DNA levels should probably be assessed annually.[14] In **chapter 2**, we have studied the performance of repeated HBsAg and HBV DNA measurements for the prediction of the inactive carrier phase using a dynamic model, updating the risk of progressing to active hepatitis or remaining inactive carrier. We have shown that HBsAg levels <100 IU/mL in patients with HBV DNA <2,000 IU/mL could identify inactive carriers over 8 years of follow-up with a high specificity and positive predictive value across all HBV genotypes. Patients with HBsAg levels <100 IU/mL also had a high probability of HBsAg loss over time. These patients therefore can be considered inactive carriers and may be monitored less frequently. In contrast, patients with HBsAg levels >100 IU/mL were still at risk of HBV activity. Of these patients approximately 40% switched back and forth between HBV phases during follow-up. These patients may therefore require close monitoring. Furthermore, the probability of becoming inactive carrier in the following year was highest for those patients with both an HBV DNA ≤5,000 IU/mL and an HBsAg level decline of ≥0.5 log IU/mL in the year prior. This is clinically important as it may be worthwhile to monitor these patients instead of offering lifelong antiviral therapy.[15-17]

Host genetics in the natural history of CHB

Recently improved techniques have enabled researchers to assess common variations in the human genome, known as single-nucleotide polymorphisms (SNPs). Many genome-wide association (GWA) and candidate SNP studies have been performed among patients with alcoholic liver disease, fatty liver disease and viral hepatitis. Common SNPs were associated with fatty liver disease, fibrosis progression, cirrhosis, HCC development, and also with the natural or therapy-induced clearance of HBV.[18-23] However, studies on the impact of these SNPs on liver-related clinical outcomes in CHB patients have not yet been performed. In **chapter 3**, we have assessed the association between a common variation in the *PNPLA3* gene and hepatic steatosis and non-alcoholic steatohepatitis (NASH) in 531 CHB patients who all underwent

a liver biopsy. In this study we showed that *PNPLA3* was associated with the presence and severity of hepatic steatosis and steatohepatitis, iron depositions and significant lobular inflammation, but not with advanced fibrosis, all-cause mortality or HCC development. The association between *PNPLA3* and the presence of steatosis was particularly pronounced among female patients, and the effect of the unfavorable *PNPLA3* 148M variation on the development of steatohepatitis was most marked in female patients without severe overweight. Although we observed an association between steatohepatitis and an increased risk for all-cause mortality and HCC development, we could not indicate a significant role for the I148M polymorphism of *PNPLA3* in the current study. However, as polymorphisms in the *PNPLA3* gene showed a significant association with detrimental histological features, this genetic marker may have implications for CHB patients in the long-term. Future studies should therefore further assess the role of different candidate SNPs, such as *PNPLA3*, and long-term outcome of CHB-related liver disease.

Recent studies have investigated the meaning of risk scores for cirrhosis, HCC and mortality among patients with CHB or HCV infection.[24-35] None of these studies however have assessed the added value of non-invasive markers combined with liver histologic characteristics in detail. In **chapter 4**, we have therefore investigated the prognostic performance of different serum risk scores and the added value of liver histology in 557 patients during a follow-up of more than 15 years. In this study, we have shown that the PAGE-B score was the overall best performing marker to assess the event and HCC risk in patients with CHB. Moreover, we have shown that liver histology did not provide with a clinically meaningful update of the risk classification obtained by the PAGE-B score alone. Therefore, when this PAGE-B score is further validated, a subjective liver biopsy for prognostic purposes may become obsolete, and a simple objective serum score may help to stratify patients at high risk for clinical disease progression, and thus in need of antiviral therapy and HCC surveillance.

Our group recently developed a validated mortality risk score for the long-term risk of mortality and clinical disease progression for patients with advanced hepatic fibrosis due to a chronic hepatitis C infection.[35] This score could be easily obtained by using objective clinical parameters. Shortly after this report, Giannini et al. reported on the prognostic performance of this marker for the short-term prognosis of hepatitis C infected patients with decompensated liver cirrhosis.[36] In **chapter 5**, we assessed whether this new mortality risk score could also be utilized in patients with a CHB infection. Indeed, this risk score could adequately assess the prognosis of CHB patients, especially of those with advanced hepatic fibrosis. Future studies should further confirm the role of both the PAGE-B and 'HCV mortality risk' scores for both CHB patients as well as for those patients with advanced liver disease of other etiology.

OPTIMIZATION OF THE TREATMENT STRATEGY FOR CHB PATIENTS

Over the past decades, the treatment of CHB has considerably improved with the emergence of pegylated interferon (PEG-IFN) and more potent nucleos(t)ide analogues (NA's) with a high barrier to viral resistance. Both treatment modalities are recommended as first-line therapy for CHB patients.[37-39] PEG-IFN is given as a finite course of 48 weeks, during which approximately 20 – 30% of patients achieve a favorable response. In those patients with a good response the long-term prognosis is improved.[40-42] Nonetheless, PEG-IFN therapy is associated with significant side-effects. NA therapy on the other hand maintains viral suppression in over 90% of patients, and leads to reversal of cirrhosis, a reduced risk for HCC and an improved prognosis.[3, 43-45] However, NA therapy may be indefinite as most patients develop a relapse of active hepatitis after NA cessation.[46, 47] Therefore HBsAg loss seems to be the desired endpoint for NA therapy, however, this is only achieved in a small proportion of patients.[45, 48] PEG-IFN leads to more HBsAg loss and HBsAg decline on-therapy, leads to a reduction of the cccDNA pool and prevents formation of viral proteins. NA therapy may improve the T-cell function and may therefore enhance the immune response.[49-52] Combining these 2 first-line treatment modalities after NA lead-in therapy may therefore lead to higher serological response rates and may enable patients to safely stop NA therapy. In **chapter 6**, we investigated a PEG-IFN add-on strategy for 24 weeks after 6 months of NA lead-in therapy. In this study we have shown that PEG-IFN add-on leads to more viral response reflected in more HBsAg, HBeAg and HBV DNA decline. Furthermore, patients who achieved a favorable response to PEG-IFN add-on more frequently sustained this response after ETV treatment cessation when compared to those patients with a good response to ETV monotherapy, of whom the majority relapsed. Nevertheless, in this study we were not able to compare the PEG-IFN add-on strategy to a PEG-IFN monotherapy arm to study the effect of pre-treatment immune restoration with NA therapy before addition of PEG-IFN. In **chapter 7**, we have therefore compared the results of this study with that of a previous randomized trial in which a *de novo* combination of PEG-IFN and LAM was compared to PEG-IFN monotherapy.[53] We have shown that with only 24 weeks of PEG-IFN add-on therapy equal HBsAg declines could be achieved compared to 52 weeks of PEG-IFN monotherapy. Moreover, when patients were treated with NA after PEG-IFN cessation, there was no HBsAg level rebound observed, suggesting that NA therapy may maintain the HBsAg decline achieved with PEG-IFN, and could possibly be used as consolidation therapy. Future trials should further elucidate the role of the duration of NA lead-in therapy and the timing of PEG-IFN add-on, and the role of NA consolidation therapy after PEG-IFN withdrawal, to further improve serological response rates.

OPTIMIZATION OF PRE-TREATMENT SELECTION OF CHB PATIENTS

As only a minority of patients achieve a favorable response to PEG-IFN at the cost of significant side-effects, it is of great importance to only select those patients with the highest chance of response to PEG-IFN treatment. Previous studies have indicated factors such as HBV genotype, a low baseline HBV DNA and higher serum ALT, older age, female gender and previous PEG-IFN therapy (for Caucasian patients infected with HBV genotype A or D a detrimental effect, and for Asian patients infected with HBV genotype B or C a beneficial effect) as factors associated with a good response to IFN.[42, 53-56] Nonetheless, combining these factors still fall short to accurately predict the chance of response to PEG-IFN at the individual level. Little is known about host genetics in relation to PEG-IFN response. These genetic factors may further help in pre-treatment stratification. Recently our group published a report on *IL28B* polymorphisms and response to PEG-IFN, and showed that the major alleles were associated with response and the long-term sustainability thereof.[57] Polymorphisms of *IL28B* were first found to be associated with the natural clearance and therapy response in HCV patients.[58] Following publications in CHB patients were however contradictory and therefore *IL28B* polymorphisms do not seem to have a major impact on response.[59-61] Other polymorphisms identified by a genome wide association study (GWAS) in CHB patients could possibly have more potential. Recently several Asian GWA studies indicated an association between polymorphisms in the *HLA-DPA1* and *HLA-DPB1* genes and the natural clearance of HBV infection.[62-65] In **chapter 8**, we therefore investigated the association between these SNPs and response to PEG-IFN in 262 Caucasian CHB patients infected with HBV genotype A or D, and found that the minor alleles of these genes indeed were associated with a better response to PEG-IFN. These results were apparent also when stratified by HBV genotype and HBeAg status at baseline. The findings of this study thus suggests that these polymorphisms may play a key role in response to PEG-IFN, next to known viral and host response modifiers. Still, the response alleles had a low prevalence (~20%) in Caucasian patients. These *HLA-DP* SNPs may therefore not be ready for clinical use. As PEG-IFN response occurs in approximately 30% of patients, other SNPs discovered by performing a GWAS in CHB patients may have additional value.

In **chapter 9**, we have investigated the role of hepatitis B core-related antigen (HBcrAg), a new virologic marker which is a combined measure of hepatitis B core antigen (HBcAg), HBeAg and p22cr.[66] In 175 HBeAg-positive patients, HBcrAg showed a strong association with response to both PEG-IFN add-on as well as ETV monotherapy. Nevertheless, serum HBcrAg levels showed a close resemblance to HBeAg levels. For response prediction, HBV DNA, HBeAg or HBsAg levels could be interchanged with HBcrAg levels, but serum HBcrAg was not superior

to HBsAg levels. Therefore, the role of HBcrAg levels in PEG-IFN response prediction for HBeAg-positive patients seems limited. However, in HBeAg-negative patients, markers of response are scarce.[55] Though HBsAg levels at baseline may have an impact on response, currently only on-treatment HBsAg levels can be used to assess the chances of response to PEG-IFN.[67-70] Therefore, HBcrAg levels may have additional clinical value in the subgroup of HBeAg-negative patients. Future studies should further focus on response prediction for this growing group of CHB patients.

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CHAPTER 12

Samenvatting en discussie

ACHTERGROND

Patiënten met een chronische hepatitis B (CHB) infectie hebben een hoog risico op langdurige leverontsteking, welke veroorzaakt wordt door een ineffectieve immuunrespons gericht tegen het niet-cytopathische hepatitis B-virus (HBV). Ongeveer 90% van de patiënten die besmet raakt in de eerste levensjaren, raakt ook chronisch geïnfecteerd. Jaarlijks sterven er ongeveer 650.000 patiënten wereldwijd aan de complicaties van een CHB infectie zoals door lever decompensatie of door de ontwikkeling van leverkanker (HCC). Sommige CHB patiënten ontwikkelen geen verdere ontsteking en lijken immunologische controle van het virus te hebben bereikt. Deze zogenaamde inactieve dragers hebben een verbeterde prognose vergelijkbaar met de algemene populatie, en kunnen minder frequent worden gecontroleerd. Door mutaties in het HBV genoom kunnen deze patiënten echter toch hepatitis reactivering ontwikkelen. Deze patiënten lopen een hoog risico op de ontwikkeling van levercirrose, decompensatie en HCC, en vereisen daarom antivirale therapie. Daarom is het belangrijk om het risico van hepatitis reactivering en het risico van verdere leverbeschadiging in te kunnen schatten voor individuele patiënten om zodoende tijdig te kunnen interveniëren met antivirale therapie.[1-3] Zowel met gepegyleerd interferon (PEG-IFN) als met nucleos(t)ide analogen (NA) kan een verbeterde prognose bij patiënten worden bereikt indien een goede antivirale respons wordt behaald. Voor NA wordt dit bereikt door suppressie van het HBV DNA middels remming van het HBV DNA polymerase. Een groot deel van de patiënten ontwikkelt echter hepatitis reactivatie na beëindiging van de NA, en daarom wordt deze vorm van behandeling momenteel voor onbepaalde tijd en mogelijk zelfs levenslang gegeven. Daarentegen leidt PEG-IFN therapie tot meer serologische respons (Hepatitis B e antigen [HBeAg] verlies en hepatitis B oppervlakte-antigeen [HBsAg] verlies), maar slechts een derde van de patiënten behalen dit gunstige resultaat ten koste van significante bijwerkingen. Daarom is het belangrijk om de behandelstrategie te optimaliseren en patiënten te selecteren met een hoge waarschijnlijkheid op response na een jaar PEG-IFN. Dit zou zodoende kunnen helpen om deze behandelingsvorm meer aanvaardbaar te maken voor patiënten, artsen en beleidsmakers in de gezondheidszorg.

HET OPTIMALISEREN VAN DE MONITORING VAN PATIËNTEN MET CHB

Virale markers en het natuurlijke beloop van een CHB infectie

Al decennia lang is het leverbiopt de gouden standaard om het risico op leverziekte progressie te beoordelen voor een patiënt met CHB. Deze modaliteit is echter geassocieerd met een aantal

belangrijke beperkingen: het potentieel van ernstige complicaties, inter- en intra-observer variabiliteit en sampling error. Het is daarom dat onderzoekers op zoek zijn naar andere manieren om niet-invasief de mate van leverfibrose, ontsteking en de daarmee de verband houdende prognose te beoordelen. Patiënten met een hoog risico op progressie van leverziekte hebben dan mogelijk een intensievere follow-up en eventueel antivirale therapie nodig.

Het is aangetoond dat in vergelijking met patiënten met lage HBV DNA waarden in het bloed, patiënten met HBV DNA waarden boven 2000 IU/mL een hoger risico hebben op het ontwikkelen van HCC.[4, 5] Quantitatieve HBsAg niveaus blijken sterk te variëren tijdens de verschillende stadia van chronische HBV-infectie en lijken te correleren met de staat van immuun controle.[6-9] Recent onderzoek toonde aan dat de hoogte van deze quantitatieve HBsAg waarden de kans op ziekteprogressie en ontwikkeling van HCC konden voorspellen bij patiënten met lagere HBV DNA waarden.[10-12] Daarom werd getracht HBsAg gehalten te gebruiken om de waarschijnlijkheid van reactivering voor inactieve dragers te voorspellen. Er werd aangetoond dat een één-puntmeting van HBsAg in combinatie met de hoogte van het HBV DNA kon worden gebruikt als een marker om reactivatie één jaar later te voorspellen met een hoge specificiteit en sensitiviteit onder HBV genotype D geïnfecteerde patiënten.[13] Deze voorspellingsregel bleek echter een wisselende diagnostische prestatie te hebben in opeenvolgende studies.[11, 14] Een van de redenen voor deze afwijkende observaties is dat HBeAg-negatieve CHB geassocieerd is met een remitterend patroon van HBV schommelingen gevolgd door langdurige remissies. Eén meting kan daarom mogelijk niet voldoende zijn om het risico van reactivering voorspellen, en de HBsAg en HBV DNA niveaus moet waarschijnlijk jaarlijks worden beoordeeld.[15] In **hoofdstuk 2** hebben we daarom de prestatie van herhaalde HBsAg en HBV DNA metingen onderzocht voor het voorspellen van inactief dragerschap met behulp van een dynamisch model. Hierbij hebben we aangetoond dat we inactieve dragers konden identificeren door middel van het gebruik van de combinatie van een HBsAg-waarde <100 IU/mL en HBV DNA waarde <2000 IU/mL met een hoge positief voorspellende waarde en specificiteit. Patiënten met HBsAg levels <100 IU/mL hadden ook een grotere kans op HBsAg verlies tijdens de follow-up. Deze patiënten kunnen daarom worden beschouwd als inactief dragers en kunnen mogelijk minder frequent worden gecontroleerd. Patiënten met HBsAg niveaus >100 IU/ml daarentegen behielden nog steeds een risico op HBV activiteit, waarvan ongeveer 40% een fluctuerend patroon van hepatitis liet zien tijdens follow-up. Deze patiënten vereisen daarom een intensievere follow-up. De kans om inactief drager te worden in het volgende jaar was het grootst voor die patiënten met zowel HBV DNA waarden ≤ 5,000 IU/mL en een HBsAg niveau daling van ≥0.5 log IU/ml in het jaar voorafgaand. Dit is klinisch van belang omdat het de moeite waard is om deze patiënten te

controleren in plaats van behandelen met antivirale middelen die mogelijk levenslang gegeven moeten worden.[16-18]

Gastheer genetica en het natuurlijke beloop van een CHB infectie

Recent verbeterde technieken hebben het mogelijk gemaakt om eenvoudige en veelvoorkomende variaties in het humane genoom, bekend als single nucleotide polymorphisms (SNPs), te beoordelen. Zodoende is het gemakkelijker geworden om de impact van de gastheer genetica op vele ingewikkelde menselijke kenmerken en ziektes te bestuderen. Veel van dergelijke studies hebben verbanden tussen SNPs en leverziekte aangetoond, zoals een predispositie voor alcoholische leverziekte, niet-alcoholische vette leverziekte (NAFLD) en progressie van leverfibrose.[19-24] Er zijn echter geen studies uitgevoerd om het effect te evalueren van deze SNPs op leverschade in CHB patiënten.

In de afgelopen decennia hebben obesitas en de geassocieerde morbiditeit epidemische proporties bereikt, met name in ontwikkelde landen. Tegenwoordig zijn er ongeveer 500 miljoen zwaarlijvige mannen en vrouwen, een aantal dat bijna is verdubbeld sinds 1980.[25] NAFLD is één van de vele uitingen van corpulentie en treft tot 30% van de volwassenen.[26] Steatohepatitis is een uitingsvorm van NAFLD, een aandoening die vervolgens leidt tot lever cirrose in 10-29% van individuen binnen 10 jaar. Bovendien ontwikkelt 4-27% van de patiënten met cirrose door steatohepatitis HCC.[26] Steatose is een risicofactor voor de progressie van de ziekte en de ontwikkeling van HCC bij patiënten die chronisch geïnfecteerd zijn met het hepatitis C virus (HCV),[27, 28] echter, de impact ervan op de progressie van CHB verwante leverziekte is onduidelijk.[29-31] Eerdere studies hebben aangetoond dat ongeveer 1/3 van de patiënten met CHB lijden aan NAFLD, hoewel de prevalentie zeer sterk varieerde in deze studies.[29-31] Een veelvoorkomende variatie in het patatin-achtige fosfolipase-domein bevattende 3 (*PNPLA3*) gen is recentelijk geassocieerd met een verhoogde vatbaarheid voor het ontwikkelen NAFLD.[32] Deze gevoeligheid voor NAFLD onder patiënten met de ongunstige 148M variatie werd eveneens waargenomen bij patiënten met alcoholische leverziekte of een HCV-infectie. Bovendien werd de 148M variatie geassocieerd met de progressie van leverziekte, en de ontwikkeling van HCC.[19-23]

In **hoofdstuk 3** hebben we het verband tussen deze variatie in het *PNPLA3* gen en hepatische steatose en steatohepatitis geëvalueerd in 531 CHB patiënten die allen een leverbiopt hebben ondergaan. In deze studie hebben we de beschreven associatie van *PNPLA3* met NAFLD en steatohepatitis verder bevestigd. Ook was er een associatie met ijzer deposities en significante lobulaire ontsteking van de lever, maar niet met gevorderde fibrose, HCC ontwikkeling of mortaliteit. De associatie tussen *PNPLA3* en de aanwezigheid van steatose was bijzonder uitgesproken bij vrouwelijke patiënten en het effect van de ongunstige *PNPLA3* 148M variatie op

de ontwikkeling van steatohepatitis was het meest uitgesproken bij vrouwelijke patiënten zonder ernstig overgewicht. Hoewel we een associatie tussen steatohepatitis en een verhoogd risico op mortaliteit en HCC ontwikkeling observeerden, konden wij geen associatie tussen het 148M polymorfisme van *PNPLA3* en deze klinische uitkomsten aantonen. Aangezien polymorfismen in het *PNPLA3* gen wél een significante associatie met nadelige lever histologische kenmerken toonde, zou deze genetische marker toch gevolgen kunnen hebben voor CHB patiënten op de lange termijn. Daarom zou de rol van verschillende SNPs, zoals *PNPLA3*, en de impact op de lange-termijn uitkomsten voor patiënten met CHB-gerelateerde leverziekte verder onderzocht moeten worden in toekomstige studies.

Recente studies hebben de betekenis van risicoscores voor cirrose, HCC en sterfte onderzocht bij patiënten met een CHB of HCV infectie.[33-44] Geen van deze studies onderzocht echter de toegevoegde waarde van niet-invasieve markers gecombineerd met lever histologie in detail. In **hoofdstuk 4** hebben we daarom de associatie tussen verschillende serum risico scores en de toegevoegde waarde van de leverhistologie in 557 patiënten geëvalueerd tijdens een follow-up van meer dan 15 jaar. In deze studie hebben we aangetoond dat de PAGE-B score de best presterende serum marker was om het HCC en sterfte risico bij patiënten met CHB in te schatten. Bovendien toonden we aan dat de lever histologie geen klinisch relevante toevoeging toonde in het beoordelen van het risico op klinische uitkomsten op de lange termijn. Als deze PAGE-B score verder kan worden bevestigd, zou een leverbiopsie voor prognostische doeleinden mogelijk vermeden kunnen worden in de toekomst, en kan een eenvoudige serum marker al helpen om patiënten met een hoog risico op klinische uitkomsten te selecteren voor een intensievere follow-up en mogelijk voor antivirale therapie.

Onze groep ontwikkelde recent een gevalideerde risicoscore voor de lange-termijn kans op sterfte en klinische ziekteprogressie voor patiënten met gevorderde leverfibrose op basis van een chronische hepatitis C infectie.[44] Deze score kan gemakkelijk worden verkregen door het gebruik van objectieve klinische parameters. Kort na deze publicatie rapporteerden Giannini et al. over de prestaties van deze prognostische marker voor de korte-termijn prognose van hepatitis C geïnfecteerde patiënten met gedecompenseerde lever cirrose.[45] **In hoofdstuk 5** hebben we onderzocht of deze nieuwe risicoscore ook kan worden gebruikt bij patiënten met een CHB infectie. Deze risicoscore liet inderdaad zien dat het de prognose van CHB patiënten op lange-termijn adequaat kon inschatten, in het bijzonder voor degenen met geavanceerde leverfibrose. Toekomstig onderzoek moet de rol van zowel de PAGE-B als de 'HCV risicoscore' verder bevestigen in zowel CHB patiënten en patiënten met gevorderde leverziekte van andere etiologie.

HET OPTIMALISEREN VAN DE BEHANDELSTRATEGIE IN PATIËNTEN MET CHB

In de afgelopen decennia is de behandeling van CHB aanzienlijk verbeterd met de opkomst van gepegyleerde interferon (PEG-IFN) en krachtigere NA's met een hoge barrière voor virale resistentie. Beide behandelingen worden aanbevolen als eerste-lijn therapie voor patiënten met CHB.[46-48] PEG-IFN wordt gegeven voor de duur van 48 weken, waarin ongeveer 20 – 30% van de patiënten een gunstige respons behalen. Bij die patiënten met een initieel goede respons is er op lange termijn een verbetering van de prognose meetbaar.[49-51] PEG-IFN therapie gaat echter wel gepaard met significante bijwerkingen. NA behandeling wordt daarentegen vaak goed verdragen en het virus wordt hierbij effectief onderdrukt in meer dan 90% van de patiënten, wat leidt tot omkering van cirrose, een verminderd risico op HCC en een verbeterde prognose.[3, 52-54] Het nadeel van NA therapie is echter dat het voor een onbepaalde tijd gegeven dient te worden omdat de meeste patiënten weer actieve hepatitis ontwikkelen na het stoppen van NA.[55, 56] Daarom lijkt HBsAg verlies het gewenste eindpunt voor NA therapie, maar dit wordt slechts in een klein deel van de patiënten bereikt.[54, 57] PEG-IFN leidt tot meer HBsAg verlies en daling, leidt tot een vermindering van de cccDNA pool en voorkomt de vorming van virale eiwitten. NA therapie kan de T-cel functie verbeteren en kan daarmee het immuunsysteem en daarmee de respons op antivirale therapie versterken.[58-61] De combinatie van deze 2 eerstelijns behandelopties kan daarom leiden tot meer serologische respons en kunnen patiënten in staat stellen om veilig te stoppen met NA therapie zonder het ontwikkelen van een terugval van hepatitis activititeit. In **hoofdstuk 6** onderzochten we daarom een PEG-IFN additie (add-on) strategie voor 24 weken na 6 maanden NA lead-in therapie. In deze gerandomiseerde studie hebben we aangetoond dat PEG-IFN add-on leidt tot meer virale respons in vergelijking met continue NA therapie. Patiënten die een goede respons behaalden op PEG-IFN lieten over het algemeen een aanhoudende reactie zien, in tegenstelling tot patiënten met een goede respons op NA monotherapie, waarvan de meerderheid een recidief hepatitis ontwikkelde na het stoppen van behandeling. In deze studie waren we echter niet in staat de PEG-IFN add-on strategie te vergelijken met een PEG-IFN monotherapie arm om het effect van de voorbehandeling op het herstel van de immuun respons met NA behandeling te bestuderen. In **hoofdstuk 7** hebben we daarom de resultaten van deze studie met die van een eerdere gerandomiseerde studie vergeleken waarbij een *de novo* combinatie van PEG-IFN en LAM vergeleken werd met PEG-IFN monotherapie.[62] We hebben aangetoond dat met slechts 24 weken PEG-IFN add-on therapie een gelijke HBsAg daling kan worden bereikt in vergelijking met 52 weken van de PEG-IFN monotherapie. Wanneer patiënten na PEG-IFN beëindiging bovendien werden

doorbehandeld met NA, werd er geen HBsAg level stijging waargenomen, wat suggereert dat NA therapie de HBsAg daling verkregen met PEG-IFN kan behouden en eventueel kan worden ingezet als consolidatietherapie. Toekomstige studies moeten verder ophelderen wat de rol van de duur van een NA voorbehandeling, PEG-IFN add-on en de duur en het effect van NA consolidatie therapie is.

HET OPTIMALISEREN VAN DE PRE-THERAPEUTISCHE SELECTIE VAN CHB PATIËNTEN

Aangezien slechts een minderheid van patiënten een gunstige response behalen op PEG-IFN ten koste van significante bijwerkingen, is het van groot belang om alleen die patiënten te selecteren met de grootste kans op een goede respons. Eerdere studies hebben aangetoond dat factoren zoals HBV genotype, een lage uitgangswaarde HBV DNA en hogere serum ALT, hogere leeftijd, vrouwelijk geslacht en eerdere PEG-IFN therapie (voor blanke patiënten die geïnfecteerd zijn met HBV genotype A of D een nadelig effect, en voor de Aziatische patiënten besmet met HBV genotype B of C een gunstig effect) geassocieerd zijn met respons op PEG-IFN.[51, 62-65] Een combinatie van deze factoren is echter nog steeds niet genoeg om de kans op respons op PEG-IFN op individueel niveau te voorspellen.

Er is weinig bekend over de gastheer genetica in relatie tot de reactie op PEG-IFN. Genetische factoren zouden verder kunnen helpen bij de pre-therapeutische selectie van patiënten. Onlangs publiceerde onze groep een rapport over *IL28B* polymorfismen en de respons op PEG-IFN, en toonde aan dat de meest voorkomende allelen van deze variatie in dit gen werden geassocieerd met de duurzame respons op PEG-IFN op zowel korte als op lange termijn.[66] Polymorfismen nabij het *IL28B* gen werden voor het eerst geassocieerd met de natuurlijke klaring en therapie respons bij HCV geïnfecteerde patiënten.[67] Latere publicaties in patiënten met CHB waren vervolgens tegenstrijdig. Daarom lijken *IL28B* polymorfismen niet een grote impact op therapie respons te hebben.[68-70] Andere polymorfismen geïdentificeerd door een genoom-wijde associatie studie (GWAS) in CHB patiënten zouden mogelijk meer potentie kunnen hebben. Onlangs zijn er verscheidene GWA studies gepubliceerd in Aziatische patiënten welke een rol voor polymorfismen in de *HLA-DPA1* en *HLA-DPB1* genen in relatie tot de natuurlijke klaring van HBV infectie aantoonden.[71-74] **In hoofdstuk 8** hebben we daarom de rol van deze variaties in deze HLA genen en de reactie op PEG-IFN in 262 Kaukasische patiënten onderzocht. We vonden dat de minder prevalente allelen van deze genen inderdaad geassocieerd waren met een betere respons op PEG-IFN. Voor deze gunstige variaties in deze kandidaat SNPs werd echter

een lage prevalentie waargenomen. Daar PEG-IFN-respons bij ongeveer 30% van de patiënten voorkomt is het waarschijnlijk dat er andere SNPs zijn die mogelijk een grotere invloed op de respons hebben.

In **hoofdstuk 9** hebben we de rol van het hepatitis-B-core-gerelateerde antigeen (HBcrAg) onderzocht. HBcrAg is een nieuwe virologische marker welke een gecombineerde meting is van het hepatitis B core-antigeen (HBcAg), HBeAg en p22cr.[75] We hebben deze marker onderzocht in 175 HBeAg-positieve patiënten die werden behandeld met ETV of PEG-IFN add-on. In deze studie liet HBcrAg een sterke associatie zien met respons op zowel PEG-IFN add-on evenals op ETV monotherapie. Serum HBcrAg niveaus toonde echter een nauwe gelijkenis met HBeAg niveaus. Voor het voorspellen van een goede respons op PEG-IFN konden HBV DNA, HBeAg of HBsAg-niveaus worden afgewisseld met HBcrAg niveaus, maar HBcrAg was niet superieur aan HBsAg. De rol van HBcrAg levels in response predictie voor HBeAg-positieve patienten lijkt daarom gelimiteerd. In HBeAg-negatieve patiënten zijn respons markers echter schaars.[64] Mogelijk is er een associatie tussen HBsAg niveaus voor de start van de behandeling en PEG-IFN respons. Op dit moment worden echter alleen HBsAg waarden gebruikt om de respons tijdens de therapie te beoordelen.[76-79] Toekomstig onderzoek moet zich daarom verder richten op serum HBcrAg niveaus en de associatie met PEG-IFN response voor de steeds groeiende groep van HBeAg-negatieve patiënten.

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CHAPTER 13

Appendices

DANKWOORD

Eindelijk mag ik dan de hand leggen aan het dankwoord. Dit is wellicht het belangrijkste gedeelte van mijn proefschrift, omdat alle mensen die mij hebben gesteund, van groot belang zijn geweest in de totstandkoming ervan.

Beste Harry (Prof.dr. H.L.A. Janssen), dank dat ik deel uit heb mogen maken van jouw succesvolle hepatitis B team. Helaas ben je binnen een jaar naar Toronto vertrokken, waarna het contact voornamelijk via Skype is verlopen. Je hebt voor mij veel mogelijkheden gecreëerd en je hebt me alle ruimte gegeven om mezelf te kunnen ontplooien. In combinatie met jouw kritische blik heeft me dit de onderzoeker gemaakt, die ik nu ben. Daar ben ik je zeer dankbaar voor. De 6 maanden in Toronto zijn fantastisch geweest, ik kom daar graag nog eens terug!

Lieve Bettina (Dr. B.E. Hansen), ik had me geen leukere copromotor kunnen wensen. Na Harry's vertrek heb jij de begeleiding van de hepatitis B groep op je genomen. Het is bewonderenswaardig hoe je deze nieuwe rol hebt weten te vervullen. Jouw veelzijdigheid is ongekend, van het uitvoeren van zeer complexe statistiek tot aan gezellig stappen op congressen. Ik hoop dat we nog veel kunnen blijven samenwerken.

Een goede link tussen de kliniek en het laboratorium is van groot belang voor het doen van wetenschappelijk onderzoek. Beste Andre (Dr. A. Boonstra), jij bent voor mij die link. Het werken met jou is heel inspirerend. Je hebt hart voor je vak en je neemt alle tijd voor het brainstormen over translationeel en basaal onderzoek. Je hebt mij geleerd de positieve kant te zien van tegenslagen, op momenten dat menigeen het bijltje erbij neer zou gooien. Bedankt voor al je hulp de afgelopen jaren! Ik hoop dat we in de toekomst samen nog veel meer studies kunnen uitvoeren.

Prof.dr. F.J.W. ten Kate, beste Fiebo, toen ik kwam met het idee om leverbiopten te gaan scoren voor een genetische studie, heeft u geen moment getwijfeld om mij te helpen. Met veel plezier hebben we heel wat (vroege) uurtjes samen achter de microscoop doorgebracht. Ik voel me bevoorrecht zoveel van u te hebben mogen leren. Bedankt voor alles, want zonder uw onvoorwaardelijke inzet had dit boekje er nu niet gelegen.

Prof.dr. H.J. Metselaar, prof.dr A.M. Hoepelman en Prof.dr. C.A.B. Boucher, bedankt voor het plaatsnemen in de kleine commissie en het beoordelen van dit proefschrift. Dr. R.A. de Man, beste Rob, bedankt voor het in mij gestelde vertrouwen en de bereidheid mij op te leiden tot Maag-, Darm-, en Leverarts. Beste Dr. van den Dorpel, ik kijk er erg naar uit om weer terug te keren naar het Maasstad ziekenhuis.

Beste Rob (Dr. R.J. de Knegt), nadat Harry was vertrokken heb je plaatsgenomen tijdens onze HBV meetings, en begeleidde je me op de hepatitis B poli. Je hebt een groot relativeringsvermogen en buitengewone interesse in allerhande maatschappelijke zaken, zowel binnen als buiten het ziekenhuis. Dit heeft voor mij bijgedragen aan het werkplezier.

Dear David Wong, dear Jordan Feld, I am grateful for your hospitality during the 6 months that I spend in Toronto. It was a great experience. Dear Matt, Mayur, Geraldine, Seham, Orlando, Colina, Catalina, Doinita, Danie, Lucie and everyone else of the Hepatology unit in the Western Hospital, thank you all for the wonderful collaboration.

Zonder een goede ondersteuning omtrent alle regelgeving, contracten en uitvoering van studies zou onze afdeling veel minder succesvol zijn. Dames van het CRB, jullie vormen samen de "backbone" van de research afdeling. Elke, Judith, Irene en Edith, dank voor jullie hulp en inzet. Elke, jij hebt er voor gezorgd, dat de ARES studie van begin tot eind goed is verlopen en je hebt me waanzinnig geholpen en gesteund bij het uitvoeren van de GIANT-B studie. We hebben heel veel lol gehad en we hebben ons vaak mogen "verbazen" (niet ergeren!) over de gang van zaken en manier van werken van bepaalde centra. Fijn dat ik bij jou en Judith altijd even kon binnenvallen om weer een uurtje deze verbazing te delen. Ook onze trialnurses, Heleen, Melek, Lucille, Esther, dank voor de fijne samenwerking de afgelopen jaren op de trialpoli. Ik denk dat het hebben van gespecialiseerde verpleegkundigen een luxe is en dat er zonder jullie weinig terecht zou komen van veel klinische studies. Alle poli-dames en -heer, *en* in het bijzonder Wilma, wil ik bedanken voor het ondersteunen van de hepatitis B poli de afgelopen jaren.

Marion en Margriet, ons geweldige secretaresse-duo. Bedankt voor alle gezelligheid en voor alle hulp de afgelopen jaren. De roddeluurtjes met jullie zijn fantastisch! Wat zou het leuk zijn als ik ook de komende tijd in het Maasstad op de hoogte zou kunnen blijven…

Tijdens het uitvoeren van mijn studies heb ik heel wat uurtjes gespendeerd in een zeer kleine en koude (-20°C) vriesruimte. Collega's van de afdeling Viroscience, Sandra en Mark, bedankt voor jullie hulp bij het zoeken en rapen van de vele serum samples in deze barre omstandigheden van Rotterdam tot in Wijk bij Duurstede! Annemiek (Dr. A.A. van der Eijk) en Suzan (Dr. S.D. Pas), bedankt voor de prettige samenwerking en voor het uitvoeren van alle virologische testen. Ook op het MDL lab zijn er aardig wat samples uit de -20 of -80 vriezer getoverd. Hanneke (Dr. A.J. van Vuuren), Anthonie en Gertine, dank voor jullie hulp bij de logistiek, het uitzoeken van de samples, de DNA extractie voor de GIANT-B studie en het meten van de HB core-related antigen levels in Gent.

Verder heb ik allerlei hoogte- en dieptepunten beleefd met een grote groep mensen op de beruchte "dakpoli". Michelle, Daphne, Edith, Jeoffrey, Jorie, Vivian, Edmee, Lisanne H, Susan,

Renate, Femme, Ludi, Wim, Lisanne P, Mitchel, Jihan, Maren, Atija, Alison, Shannon, Eline, Anniek, Esmee, Floor, Ingrid, Joany, Loes, Louisa, Marjolein, Priscilla, Rik, Rosalie, Sophie, Vincent, Wesley, Elmer, Jihan, de tijd op het dak was geweldig. Wintersporten, schaatsen, naar (Vrijmi)borrels, BDDLs, feestjes, RotJong games, you name it. Ik kijk terug op een fantastische tijd! Een aantal van jullie wil ik in het bijzonder bedanken. Roeland, in mijn eerste maandjes als onderzoeker gingen we direct op roadtrip van San Francisco naar Las Vegas. Geen enkel congres heeft deze trip kunnen overtreffen. Daarnaast hebben we er al heel wat fietstochtjes op zitten en hoop ik dat er nog vele zullen volgen. Pauline, tevens mijn vaste vrijmibo-buddy. Jij bent altijd in voor een gezellige borrel. Jammer dat je naar 020 bent vertrokken, ik hoop dat je snel terugkomt! Succes met je verdere opleiding. Heng, onze trip naar China was geweldig. Vooral het nachtleven van Shanghai was fantastisch en gelukkig iets beter dan de nachtelijke, gigantische "knoflook oesters" in Guangzhou. Bedankt voor al je tolkactiviteiten op de HBV poli tot in China! Margo, dank voor de gezellige tijd op het dak! Ik wens je veel succes met de "*lange termijn studies*" PAS en ARES, en het verder afronden van je promotie.

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Ad, toen ik startte was jij degene die me heeft opgevangen en mij op weg heeft geholpen. Je hebt geen idee hoe belangrijk dat voor me is geweest. Ik heb veel geleerd van je kritische blik én het accepteren van commentaar, je revisies zijn echt onverbeterlijk. De tijd op kamer CA 409 heb ik als een geweldig leuke periode ervaren. Uiteindelijk is er (naast mijn proefschrift) nog heel veel meer van gekomen. Onze vriendschap is mij heel waardevol.

Terug uit Toronto ben ik al snel naar CA 425 verhuisd; een van mijn betere beslissingen tijdens mijn promotie. Lieve Els, ik had me geen gezelligere kamergenoot kunnen wensen. We hebben een superleuke klik, en we hebben in korte tijd een hele goede vriendschap opgebouwd. Dank je voor je betrokkenheid, de gezelligheid, de véle (goede en minder goede) bakjes koffie en bovenal onze discussies over onderzoek en alles daarbuiten.

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onze levens steeds maar drukker worden, hoop ik dat we elkaar nog veel kunnen blijven zien. Jullie zijn heel belangrijk voor me. Lieve Vaughn, Gaby, Paula, Mirelle, Telma, Victoria, Robert, Karen en alle andere studiegenootjes, "*roomies around the world*" en hockeyteam-genoten van HCR H4, bedankt voor alle gezelligheid.

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12. **Brouwer WP**, van der Meer AJ, Boonstra A, Plompen EPC, Pas SD, Knegt RJ, de Man R, ten Kate FJW, Janssen HLA, Hansen BE. The PAGE-B score accurately predicts clinical outcome over 15 years of follow-up in a diverse cohort of chronic hepatitis B patients. Submitted.
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14. **Brouwer WP**, Sonneveld MJ, Xie Q, Guo S, Zhang NP, Zeuzem S, Tabak F, Zhang Q, Simon K, Akarca US, Streinu-Cercel A, Hansen BE, Janssen HLA. Peginterferon add-on results in more HBsAg decline compared to monotherapy in HBeAg-positive chronic hepatitis B patients. Submitted.
15. van Campenhout MJH, **Brouwer WP**, van Oord GW, Xie Q, Zhang Q, Zhang N, Guo S, Tabak F, Streinu-Cercel A, Wang JY, Pas SD, Sonneveld MJ, de Knegt RJ, Boonstra A, Hansen BE, Janssen HLA. Hepatitis B core-related antigen levels are associated with response to entecavir and peginterferon add-on therapy in HBeAg-positive chronic hepatitis B patients. Submitted.
16. Maan R, van der Meer A, **Brouwer WP**, Plompen EPC, Sonneveld MJ, Roomer R, van der Eijk AA, Groothuismink ZMA, Hansen BE, Veldt BJ, Janssen HLA, Boonstra A, de Knegt RJ. ITPA polymorphisms are associated with hematological side effects during pegylated interferon and ribavirin therapy for chronic hepatitis C infection. Submitted.
17. **Brouwer WP**, Van der Meer AJ, Janssen HLA. Validation of a mortality risk score to assess the long-term prognosis of chronic hepatitis B patients. Submitted.
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CURRICULUM VITAE

Willem Pieter Brouwer werd geboren op 18 januari 1987 te Schiedam. In 2005 behaalde hij het atheneum diploma aan de scholengemeenschap Spieringshoek te Schiedam. In hetzelfde jaar startte hij met de opleiding Geneeskunde aan de Erasmus Universiteit te Rotterdam. Het artsexamen werd behaald in 2011. Tijdens de opleiding Geneeskunde heeft hij 2 maanden stage gelopen op de afdeling Hepatologie van het Queen Elizabeth Hospital te Birmingham, Engeland, onder begeleiding van Dr. David Mutimer. Tevens werkte hij tijdens de opleiding Geneeskunde als studententeamleider op de afdeling Vaat- en Transplantiechirurgie van het Erasmus MC. In januari 2012 startte hij met zijn promotieonderzoek aan de afdeling Maag-, Darm-, en Leverziekten onder supervisie van Prof.dr. Harry Janssen en Dr. Bettina Hansen. Tijdens dit promotietraject heeft hij een half jaar gewerkt in het Toronto Western Hospital te Toronto, Canada, als research-fellow onder begeleiding van Prof.dr. Harry Janssen. Sinds september 2015 is hij in opleiding tot Maag-, Darm-, en Leverarts in het Erasmus MC (opleider Dr. R. de Man), en is thans in vooropleiding in het Maasstad Ziekenhuis Rotterdam (opleider Dr. R. van den Dorpel). Hij woont samen met Lieke Hol en hun zoon Victor in Rotterdam.

PHD PORTFOLIO

SUMMARY OF PHD TRAINING AND TEACHING

Name PhD student: Willem Pieter Brouwer
PhD period: 2012-2015
Erasmus MC Department: Gastroenterology and Hepatology
Promotor: Prof. Dr. H.L.A. Janssen
Copromotor: Dr. B.E. Hansen

1. PHD TRAINING

Courses in methodology and biostatistics	**Year**	**Workload**
Biostatistical methods I: Basic principles, NIHES, Rotterdam	2012	104 hours
Regression analysis, NIHES, Rotterdam	2013	30 hours
Survival analysis, NIHES, Rotterdam	2014	30 hours
SNP course VIII, Molecular Medicine Postgraduate school	2012	40 hours
Workshop Browsing Genes and Genomes with UCSC, Molecular Medicine Postgraduate school	2012	40 hours
Research integrity		
Basiscursus Regelgeving en Organisatie van Klinisch onderzoek, Erasmus MC, Rotterdam	2011	30 hours
UHN Good Clinical Practice – Principles. Toronto Western Hospital, Toronto, Canada	2013	24 hours
Presentations and workshops		
Association between Interferon Gamma Inducible Protein 10 and Liver inflammation in chronic hepatitis B – Najaarsvergadering NVH, Zeist	2012	12 hours
Significant fibrosis in liver biopsies from HBeAg-positive chronic hepatitis B patients with a low serum ALT – Najaarsvergadering NVH, Zeist	2012	12 hours

Polymorphisms near the IL28B gene are not associated with response to peginterferon in HBeAg-negative chronic hepatitis B patients – oral poster presentation, EASL ILC 2013, Amsterdam, Nederland	2013	36 hours
Hepatitis B surface antigen (HBsAg) levels differ across HBV genotype and phenotype: Results from the Adult Cohort Study of the NIDDK-sponsored Hepatitis B Research Network – Hepatitis B&E clinical session, EASL ILC 2014, Londenpeginterferon alfa-2b.	2014	12 hours
Adding peginterferon to entecavir increases response rates in HBeAg-positive chronic hepatitis B patients: Week 96 results of a global multicenter randomized trial (ARES) – General session 1 and Opening, EASL ILC 2014, Londen	2014	36 hours
Combination therapy for chronic hepatitis B. Erasmus Liver day, Rotterdam, the Netherlands	2014	20 hours
HBsAg levels to define HBeAg-negative chronic hepatitis B phases during long-term follow-up using repeated measurements – Early morning workshop EASL ILC 2015, Vienna, Austria	2015	36 hours
2nd Post-EASL symposium: Update on HBV, HEV and HDV	2015	20 hours

Poster presentations

Significant fibrosis in liver biopsies from HBeAg-positive chronic hepatitis B patients with a low serum ALT – AASLD 2012, Boston, Verenigde Staten	2012	32 hours
Association between Interferon Gamma Inducible Protein 10 and Liver inflammation in chronic hepatitis B – AASLD 2012, Boston, Verenigde Staten	2012	32 hours
Polymorphisms near the IL28B gene are not associated with response to peginterferon in HBeAg-negative chronic hepatitis B patients –EASL ILC 2013, Amsterdam, Nederland	2013	32 hours
Fibrosis progression in chronic hepatitis B is biphasic and influenced by multiple single-nucleotide polymorphisms – AASLD 2013, Washington, Verenigde Staten	2013	32 hours
Single-nucleotide polymorphism PNPLA3 I148M is associated with steatosis and steatohepatitis in patients with chronic hepatitis B – AASLD 2013, Washington, Verenigde Staten	2013	32 hours

Polymorphisms of HLA-DPB1 are associated with virologic response to peginterferon in Caucasian patients with chronic hepatitis B – AASLD 2013, Washington, Verenigde Staten	2013	32 hours
Hepatitis B core related antigen levels are associated with response to ETV and PEG-IFN treatment in HBeAg-positive chronic hepatitis B patients – AASLD 2014, Boston, Verenigde Staten	2014	32 hours
Hepatitis B core related antigen levels differ during the natural history of chronic hepatitis B infection – AASLD 2014, Boston, Verenigde Staten	2014	32 hours
Consolidation therapy with entecavir can prevet post-treatment HBsAg rebound in HBeAg-positive chronic hepatitis B patients treated with peginterferon alpha – EASL ILC 2015, Vienna, Austria	2015	32 hours
HBsAg levels to define HBeAg-negative chronic hepatitis B phases during long-term follow-up using repeated measurements – EASL ILC 2015, Vienna, Austria	2015	32 hours

Awards

Young investigator bursary for best abstract (European Association for the study of the Liver)	2014
Young investigator bursary for best abstract (European Association for the study of the Liver)	2015

(Inter)national conferences

The Liver Meeting 2011, 62nd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). San Francisco, CA, United States of America.	2011	28 hours
47th Annual Meeting of the European Association for the Study of the Liver (EASL). Barcelona, Spain.	2012	28 hours
Annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands.	2012	12 hours
The Liver Meeting 2012, 63rd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). Boston, MA, United States of America.	2012	28 hours
48th Annual Meeting of the European Association for the Study of the Liver (EASL). Amsterdam, Nederland.	2013	28 hours

The Liver Meeting 2013, 64th Annual Meeting of the American Association for the Study of Liver Diseases (AASLD). Washington, DC, United States of America.	2013	28 hours
49th Annual Meeting of the European Association for the Study of the Liver (EASL). London, United Kingdom.	2014	28 hours
Annual meeting of the Netherlands Association of Hepatology, Veldhoven, the Netherlands.	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
49th Annual Meeting of the European Association for the Study of the Liver (EASL). Vienna, Austria.	2014	28 hours

Attended seminars and workshops

9th Post-AASLD symposium. Rotterdam, the Netherlands	2011	2 hours
4e Lagerhuisdebat Hepatitis B en C. Utrecht, the Netherlands	2012	2 hours
10th Post-AASLD symposium. Rotterdam, the Netherlands	2012	2 hours
6e Lagerhuisdebat Hepatitis B en C. Utrecht, the Netherlands	2014	2 hours
Post-EASL symposium, Amsterdam, the Netherlands	2014	2 hours
12th Post-AASLD symposium. Rotterdam, the Netherlands	2014	2 hours
2nd Post-EASL symposium, Amsterdam, the Netherlands	2015	2 hours
Review activities for scientific journals		
Clinical Infectious Diseases, Digestive Disease and Science, Gastroenterology, Hepatology, Journal of Clinical Virology, Journal of Hepatology, the Lancet, Liver international, Nature scientific reports, scientific abstracts for the DDW, EASL and AASLD international meetings.		60 hours

2. TEACHING

Lecturing	**Year**	**Workload**
Master Infection and Immunity 2014: Treatment of viral hepatitis, immune-modulation or antiviral action?	2014	12 hours
Master Infection and Immunity 2015: Treatment of viral hepatitis, immune-modulation or antiviral action?	2015	12 hours

ABBREVIATIONS

AC	Active carrier
ALT	Alanine aminotranferase
AUC	Area under the receiver-operating curve
anti-HBe	Antibody against HBeAg
Anti-HBs	Antibody against HBsAg
BCP	Basal core promoter
cccDNA	Covalently closed circular DNA
CHB	Chronic hepatitis B
CI	Confidence interval
ETV	Entecavir
GWAS	Genome-wide association study
HAI	Hepatic Activity index
HBcAg	Hepatitis B core antigen
HBcrAg	Hepatitis B core-related antigen
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B surface antigen
HBxAg	Hepatitis B X antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HBV DNA	Hepatitis B virus DNA
HCC	Hepatocellullar carcinoma
HIV	Human immunodeficiency virus
HLA-DPA1	Human Leukocyte antigen-DP alpha 1
HLA-DPB1	Human Leukocyte antigen-DP beta 1
HR	Hazard ratio
IC	Inactive carrier
IL28B	Interleukin 28-B
IPTW	Inversed probability of treatment weight
ITT	Intention to treat
LAM	Lamivudine
MITT	Modified intention to treat

NA	Nucleo(s)tide analogues
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NPV	Negative predictive value
PC	Precore
PCR	Polymerase chain reaction
PEG-IFN	Pegylated interferon
PNPLA3	Patatin-like phospholipase domain-containing 3
PPV	Positive predictive value
SE	Standard error
SNP	Single-nucleotide polymorphism
TDF	Tenofovir disoproxil fumarate
ULN	Upper limit of normal
WT	Wildtype