New treatment strategies for chronic hepatitis B: Viral, genetic and immunological factors predicting treatment outcome

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NEW TREATMENT STRATEGIES FOR CHRONIC HEPATITIS B
Viral, Genetic and Immunological Factors Predicting Treatment Outcome

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New Treatment Strategies for chronic hepatitis B
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Viral, Genetic and Immunological Factors Predicting Treatment Outcome

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General Introduction
BACKGROUND HEPATITIS B

Chronic hepatitis B (CHB) is a major health problem; approximately 30% of the world’s population encountered HBV infection, resulting in 240 million chronically infected persons worldwide. The virus can be transmitted via blood or body fluids. In endemic regions such as Asia and Africa (HBsAg prevalence >8%), transmission is predominantly perinatal (from mother to child) or horizontal (from exposure to infected blood) during the first 5 years of life. In low prevalence regions (prevalence <2%) transmission is mainly caused by sexual contact. HBsAg prevalence in The Netherlands is estimated between 0.3-0.5%, but a higher prevalence is found among immigrants.

When the virus is acquired during adolescence or adulthood, a phase of acute hepatitis leads to resolution of the infection in almost all (90-95%) of the cases. In contrast, when the infection is acquired perinatally, 90% of newborns become chronically infected. CHB patients have a lifetime risk of 15-40% to develop cirrhosis, liver failure or hepatocellular carcinoma.

NATURAL HISTORY

During chronic hepatitis B four consecutive phases of infection are distinguished:

(1) The immune-tolerance phase is characterized by high levels of circulating hepatitis B virus (HBV DNA), high hepatitis B e and s antigen (HBeAg, HBsAg), but normal alanine-aminotransferase (ALT) levels. This phase of ‘tolerance’ can last for decades.

(2) During the consecutive immune-active phase HBV DNA levels are lower, but liver disease is markedly more severe and ALT levels increase, with the risk of progression to liver cirrhosis.

(3) Transition from the immune-active phase to the inactive low-replicative phase is marked by clearance of HBeAg from the serum and development of HBeAg-specific antibodies. In this phase, serum HBV DNA is low, ALT levels normalize, and necro-inflammatory liver disease improves.

(4) A proportion of inactive patients (estimated 20-30%) enter the reactivation phase and experience recurrent high-level HBV replication and marked necro-inflammatory liver disease.

Among all chronically infected individuals worldwide, the majority has HBeAg negative disease. Approximately 70% of the HBeAg negative carriers is estimated to have inactive disease with low HBV-DNA levels and normal ALT levels.

IMMUNE RESPONSE DURING HBV INFECTION

HBV is a non-cytopathic virus and the incubation phase of the disease, representing the active viral replication phase before development of an effective host immune response, is protracted compared to other viral infections. The onset of symptoms occurs when a CD8- and CD4-mediated cytolytic immune response against the infected hepatocytes develops.
However, maximal reduction of HBV DNA takes place before the main cytolytic loss of hepatocytes by non-cytolytic mechanisms. These mechanisms could be mediated by cytokines, such as IFN-γ, produced by NK cells, CD8 and CD4 T cells that have been shown to reside in the liver. Why certain people resolve infection - either spontaneously or in a treatment setting - whereas others don’t, is a question of ongoing research. Several clues have been found in failure of innate and adaptive HBV immune responses.

**Deviation of innate immune response during HBV infection**

Natural killer (NK) cells are important in the first line of defense against viruses. NK cells exert their action as a result of the balance between inhibitory and activating killer cell immunoglobulin-like cell surface receptors (KIR) combined with the cytokine milieu. NK cells are enriched in the liver composing up to 30% of the intrahepatic mononuclear cell population. NK cells can exert direct antiviral actions or modulate T cell responses. They can control HBV infection in a non-cytolytic manner shortly after viral contraction by the production of cytokines such as IFN-γ. Also, during active HBV infection, they could play a role in direct lysis of virus-infected hepatocytes. However, transiently impaired NK cell function was demonstrated as viral load increased and coincided with increasing IL-10 levels during peak viremia.

Importantly, during chronic HBV infection NK cells could delete HBV-specific CD8 T cells. In this way, NK cells diminish ongoing liver damage but disarm adaptive immune function necessary for viral control.

**Deviation of adaptive immune response during HBV infections**

Initiation of an efficient adaptive immune response against viruses starts with the detection of pathogens by pattern recognition receptors (PRRs) such as toll like receptors (TLRs). Upon TLR ligand recognition, Kupffer cells and liver sinusoidal endothelial cells (LSECs) produce interleukin-6 (IL-6) and type I interferon (IFN-1: IFN-α, IFN-β). IL-6 activates innate effector molecules, whereas type I IFN has antiviral effects, activates natural killer (NK) cells and improves antigen presentation.

As HBV resides in the hepatocytes, a strong adaptive immune response appropriately primed by the innate immune system is required for viral clearance. In the liver, immune responses can cause severe tissue damage. Liver damage is prevented by the induction of immune tolerance through presentation of antigens by non-professional antigen presenting cells (APCs) e.g. immature dendritic cells (DCs), non-paranchymal liver cells or hepatocytes. When cytotoxic T cells are primed in the liver by these non-professional APC’s, they are deviated to a suppressive or regulatory phenotype with partial activation that leads to apoptosis of the T cell. However, effective immune priming, resulting in an efficient cytotoxic T cell response, can be induced in the liver during inflammation. During inflammation, DC maturation and type I IFN production promote differentiation of T cells to full activation. HBV has the ability to avoid the development of an effective immune response. First, HBV avoids initial sensing by PRR-TLR mechanisms and does not activate the innate immune system. Furthermore, HBV inhibits the plasmacytic DC
function and is a weak inducer of type I IFN and IFN stimulating genes. In this way HBV can induce a situation of immune tolerance, which will have a positive effect on liver damage, but hampers viral clearance. In addition repetitive T cell receptor stimulation by high HBV antigen levels is believed to play a role in inducing T cell exhaustion. Lack of signaling from CD4 helper cells and unfavorable soluble factors such as inhibitory cytokines (IL-10) or inhibitory molecules as arginase released by apoptotic hepatocytes could further aggravate CD8 T cell exhaustion.

Features of exhaustion are reflected in the phenotypic and functional profiles of HBV-specific CD8 T cells as they upregulate several inhibitory molecules such as PD-1 and are shown to have impaired function. Furthermore, the upregulation of death receptors on HBV-specific cells has been shown to increase their susceptibility to apoptosis.

Still, it remains a question of debate whether the impairment of T cell responses are a cause or consequence of persistent HBV infection.

TREATMENT FOR CHRONIC HEPATITIS B

In the last decade therapeutic options for chronic hepatitis B have dramatically improved, which resulted in more patients achieving a state of inactive disease. Unfortunately treatment is not yet optimal, and a cure (i.e. loss of HBsAg) is still far away.

Two types of therapies are currently available for the treatment of chronic hepatitis B:

1) Nucleos(t)ide analogues, which are antiviral agents inhibiting the activity of reverse transcriptase, a viral DNA polymerase. Several nucleos(t)ide analogues are licensed for the treatment of HBV infection in The Netherlands; lamivudine, entecavir, telbivudine, which are nucleoside analogues and adefovir dipivoxil (ADF) and tenofovir disoproxil fumarate (TDF), which are a nucleotide analogues. The mode of action of nucleotide and nucleoside analogues is the same: they inhibit the HBV reverse transcriptase.

2) Conventional interferon and pegylated interferon. Pegylated interferon (Peginterferon alpha2a (Peg-IFN)) has a dual mode of action, with both antiviral and immunomodulatory effects and has been recommended as one of the first-line therapies for both HBeAg-positive and HBeAg-negative chronic hepatitis B patients according to the EASL guidelines 2012. Peg-IFN can be administered once weekly due to attachment of interferon alfa-2a to 40 KD branched-chain polyethylene glycol (Peg). These types of therapies have suboptimal efficacy. Peg-IFN is administered once weekly subcutaneously and is associated with dose-limiting adverse events. Nucleos(t)ide analogues are very well tolerated but require lifelong administration.

Development of drug resistance of HBV during nucleoside analogue treatment, except for tenofovir, has been widely described.

For example, the long-term use of lamivudine promotes viral resistance at an estimated rate of 14 to 32% per year. The 5 year resistance for lamivudine was 70%, for adefovir 29%, for entecavir
1.2%, for telbuvidine unknown but 17% after 2 years. In contrast, resistance remains 0% for tenofovir after up till now 7 years of use. Emergence of resistant mutants or discontinuation of antiviral therapy has been associated with acute exacerbations of liver disease with viral rebound and ALT elevation.

Differences in expert opinion exist about the appropriate HBV DNA threshold beyond which treatment is indicated. European guidelines (EASL) advise treatment of CHB patients with \( >2 \times 10^3 \) IU/mL and elevated alanine aminotransferase (ALT) levels independent of HBeAg status, whereas American guidelines (AASLD) advise treatment of all CHB patients with HBV DNA \( >2 \times 10^4 \) IU/mL and elevated ALT levels > 2x upper limit of normal (ULN) or a liver biopsy with a significant degree of necroinflammation. In addition treatment is indicated in HBeAg negative patients with HBV DNA \( >2 \times 10^3 \) IU/mL and a significant degree of necroinflammation. Dutch guidelines advise treatment of all CHB patients with HBV-DNA \( >2 \times 10^4 \) IU/mL with persistent or intermittent elevation of ALT above ULN and with moderate/severe hepatitis on biopsy using a standardized scoring system (e.g. at least grade A2 or stage F2 by METAVIR scoring), irrespective of HBeAg status.

In HBeAg-positive patients, HBeAg loss has long been selected as the primary goal of treatment. This is because studies on the natural history of HBV infection indicate that, in the majority of patients with HBeAg-positive chronic hepatitis B, HBeAg loss (either spontaneous or induced by antiviral therapy) is closely correlated with suppression of HBV DNA replication, ALT normalization and disease remission.

Ideally treatment is directed at complete elimination of the virus. However eradication of HBV infection is rendered difficult because stable, long enduring covalently closed circular DNA (cccDNA) and HBV DNA becomes integrated in the hepatocyte nuclei.

As the course for chronic hepatitis B into developing cirrhosis/HCC is slow, the major goals of therapy are the long-term prevention of progression to irreversible liver damage. Relatively short-term follow up studies use different surrogate outcomes, which have been proven to be durable and predictive for further progression of the disease. As HBV DNA levels are associated with the progression to irreversible liver damage, the goal of treatment at present has been effective suppression of HBV replication. More recently however, increasing attention has been paid to the loss of HBsAg and durable formation of anti-HBs antibodies as the primary goal for treatment. Serum HBsAg is a marker of HBV infection, and antibodies against HBsAg (anti-HBs) may signify recovery.

When in current strategies loss of HBsAg and anti-HBs conversion have been achieved, it is considered as the closest outcome to ‘functional’ cure of chronic hepatitis B infection and indicates complete immunological control. HBsAg loss is the most reliable efficacy parameter for both HBeAg-positive and HBeAg-negative chronic hepatitis B. Especially in patients who clear HBsAg before they have developed cirrhosis, the prognosis is excellent compared to patients who remain HBsAg positive. Treatment with nucleos (t)ide analogues for one year does not increase the loss of HBsAg for both HBeAg positive and HBeAg negative patients and remains
approximately 1%. One year treatment with Peg-IFN results in HBsAg loss of approximately 3% in the HBeAg positive patients and 4% in the HBeAg negative patients.\(^5\)

HBsAg clearance rates 24 weeks post treatment between 3-10% were observed after a 48 weeks course of Peg-IFN and lamivudine therapy. Variation was probably related to the heterogeneity of genotypes among the different studies. HBsAg clearance rates were comparable between Peg-IFN mono- and Peg-IFN/lamivudine combination therapy\(^{55–58}\). In a large recently completed randomised controlled study, HBsAg clearance rates of 9% were reported 24 weeks after Peg-IFN and TDF combination therapy for 48 weeks, which were higher than HBsAg clearance rates after Peg-IFN or tenofovir monotherapy (both 2.8%)\(^{39}\).

**AIM OF THE DISSERTATION**

One of the major issues in chronic hepatitis B virus (HBV) infection is the question why some people become chronically infected whereas other people clear the virus and form protective immunity against the virus. Once chronic infection is established, spontaneous viral clearance rate is low (<1% a year) and currently approved antiviral treatment rarely cures infection.

The aim of this thesis is to investigate efficacy of new therapies for chronic hepatitis B patients, specifically the possibility to achieve functional cure marked by hepatitis B surface antigen (HBsAg) clearance. Baseline and on treatment viral and host factors were assessed to find predictive markers for therapy outcome.

Next to this, the aim is to advance our understanding of HBV specific immunity, including factors that shift the balance from immune tolerance to immune clearance. Insight in these mechanisms could reveal predictive markers for treatment response and assist the development of novel immunotherapy strategies to improve treatment outcome.

**OUTLINE OF THIS THESIS**

In **part 1** of this thesis the findings of two prospective clinical trials in chronic hepatitis B patients with high and low viral load are described that formed the framework of our investigations. In these trials markers of response are discussed.

**Peg-IFN and adefovir in patients with high viral load**

Ninety-two HBeAg positive (n=44) and HBeAg negative (n=44) patients with active chronic hepatitis B (alanine aminotransferase (ALT)<10xULN and HBV DNA>17,000 IU/mL) were part of a clinical trial in which they received peg-IFN and adefovir for 48 weeks and were subsequently followed up for a period of 144-196 weeks. Results are discussed in chapter 2. Furthermore this study formed the basis for the genetic host factors that were analysed to find predictors of response to therapy. In chapter 4 and 5 we analysed host genome variations and its association with therapy outcome. In chapter 6 we analysed tissue specific host factors by transcriptome analysis (mRNA) in the liver.
General Introduction. Chapter 1

Peg-IFN and nucleotide analogues in patients with low viral load

Hundred-and-thirty-four HBeAg negative patients with chronic hepatitis B patients and a low viral load (LVL) \((\text{ALT}<2\times\text{ULN and HBV DNA}<20,000)\) were randomized in a 1:1:1 ratio to receive 48 weeks of treatment with peg-IFN and tenofovir, peg-IFN and adefovir or no treatment. Results are discussed in chapter 3.

In part 2 of this thesis immunological aspects were investigated in chronic hepatitis B patients with and without anti-viral treatment. Specifically we investigated the HBV-specific CD8 T cell profile in chronic hepatitis B patients with active disease (high viral load) that cleared HBsAg after therapy with immune modulators (peg-IFN alfa2a) and nucleotide analogues (adefovir) (chapter 7). Next we investigated whether chronic infection in the liver in general caused by hepatitis B or C virus was associated with a change in the total T cell population (chapter 8). In chapter 9 we investigated the B cell compartment by quantifying the production of anti-HBs and the formation of immune complexes between anti-HBs and HBsAg. In chapter 10 we analysed the association between NK cell markers and treatment outcome.

In part 3 radiologic aspects for detection of liver fibrosis were analysed (chapter 11).

In supplementary part 4 clinical aspects of chronic viral hepatitis caused by the hepatitis E virus are described (chapter 12).
REFERENCES


General Introduction. Chapter 1


PART I

Clinical Outcome in Patients with Chronic hepatitis B with High and Low Viral Load Treated with Peg-Interferon Based Therapy
Baseline hepatitis B surface antigen (HBsAg) as predictor of sustained HBsAg loss in chronic hepatitis B patients treated with pegylated interferon-α2a and adefovir.

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ABSTRACT

Background
In this study we aimed to identify baseline predictors of response in chronic hepatitis B patients treated with a combination of pegylated interferon α-2a (peg-IFN) and adefovir.

Methods
We treated 92 chronic hepatitis B patients (44 HBeAg-positive and 48 HBeAg-negative) with HBV-DNA >100,000 copies/mL (>17,182 IU/mL) with peg-IFN and adefovir for 48 weeks, and followed them up for 2 years. Baseline markers for HBeAg loss, combined response (HBeAg negativity, HBV-DNA levels ≤ 2,000 IU/mL and ALT normalization), and HBsAg loss were evaluated.

Results
Two years after treatment, rates of HBeAg loss and HBsAg loss in HBeAg-positive patients were 18/44 (41%) and 5/44 (11%), respectively. In HBeAg-negative patients, rates of combined response and HBsAg loss were 12/48 (25%) and 8/48 (17%), respectively. HBeAg-negative patients with HBsAg loss had lower baseline HBsAg levels than those without HBsAg loss (mean HBsAg 2.35 versus 3.55 log_{10} IU/mL, p <0.001). They also had lower HBV-DNA levels and were more often (peg-) interferon experienced. Baseline HBsAg was the only independent predictor of HBsAg loss (OR 0.02, p = 0.01).

Conclusions
With combination therapy of peg-IFN and adefovir for 48 weeks, a high rate of HBsAg loss was observed in both HBeAg-positive (11%) and HBeAg-negative (17%) patients two years after treatment ended. In HBeAg-negative patients, a low baseline HBsAg level was a strong predictor for HBsAg loss.
Baseline HBsAg as predictor of sustained HBsAg loss  Chapter 2

ABBREVIATIONS
ALT, alanine aminotransferase; anti-HBe, antibody to hepatitis B e antigen; anti-HBs, antibody to hepatitis B surface antigen; BCP, basal core promoter; CR, combined response; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NUCs, nucleos(t)ide analogues; PC, pre-core; Peg-IFN, pegylated interferon alfa 2a; ULN, upper limit of normal range; week 48, end of treatment; week 72, short term follow-up; week 144, long term follow-up.
INTRODUCTION

Worldwide, approximately 400 million people have chronic hepatitis B virus (HBV) infection. Such chronic infection increases the risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC). About 25% of people who acquire chronic HBV infection early in life will develop cirrhosis or HCC during their lifetime.\(^{(1)}\)

Treatment of chronic hepatitis B has improved over the decades, and can be divided into direct antiviral or immunomodulatory therapy. The nucleos(t)ide analogues (NUCs) lamivudine, adefovir, entecavir, telbivudine and tenofovir, currently registered for such treatment, inhibit HBV-DNA synthesis and viral replication. They are well tolerated and have few side effects. But they require prolonged, probably lifelong, use because reactivation is common if treatment is stopped.\(^{(2-4)}\)

Pegylated interferon alfa 2a (peg-IFN) is mainly immunomodulatory, although it also has limited direct anti-viral effects. Unfortunately, treatment with peg-IFN entails significant side-effects. Therefore, treatment is usually limited to one year. More importantly, most patients do not benefit from peg-IFN treatment.\(^{(2;3;5)}\)

Given the different target sites of peg-IFN and NUCs, a potential way to improve therapeutic efficacy is by combining them. Large randomized trials investigated whether combination therapy might have an additive effect and thereby achieve more effective sustained viral suppression. However, such studies showed no beneficial effect of combining peg-IFN with lamivudine or ribavirin compared with peg-IFN alone in both HBeAg-positive and -negative patients.\(^{(2;3;5-7)}\) The combination of peg-IFN with other more potent NUCs remains of interest, but has not so far been reported in prospective studies.

Considering the low efficacy and significant side effects of peg-IFN based therapy, there is a need to establish predictors of response (or non-response) to allow selection of patients likely to benefit from treatment. Several pre-treatment markers have been found to predict combined response, defined as sustained viral suppression (HBV-DNA < 2,000 IU/mL) with alanine aminotransferase (ALT) normalization, in both HBeAg-positive and -negative patients treated with peg-IFN. In HBeAg-positive patients, HBV genotype, lower baseline HBV-DNA level (≤ 2 x 10^8 IU/mL), high ALT level, female sex, older age and absence of previous interferon therapy were predictors of response.\(^{(8)}\) In HBeAg-negative patients, besides high ALT and low HBV-DNA levels, younger age, female sex and a lower Ishak fibrosis score were found to be baseline markers of combined response.\(^{(9;10)}\) For HBsAg seroconversion in HBeAg-positive patients, genotype A and B, and HBeAg seroconversion before week 32 of treatment were favorable markers.\(^{(11-13)}\)

In a prospective study we treated 92 chronic HBV patients (44 HBeAg-positive and 48 HBeAg-negative) with a combination of peg-IFN and adefovir for 48 weeks. In this report, we present the results of up to 2 years of treatment-free follow-up and investigate baseline markers that predict the outcome of treatment.
Study design
This investigator-initiated, prospective and open label study was carried out in two hospitals in the Netherlands; the Academic Medical Centre (AMC) in Amsterdam and the Erasmus University Medical Centre (EMC) in Rotterdam.

All patients received a combination of peginterferon alfa 2a (Pegasys®; Hoffman La Roche, Basel, Switzerland) 180 µg subcutaneously once a week, and adefovir dipivoxil (Hepsera®; Gilead Sciences, Foster City CA) 10 mg daily for 48 weeks. We offered patients a liver biopsy before treatment. After 48 weeks, we discontinued treatment and began a follow-up period of up to five years. Patients attended the outpatient clinic every 4 – 6 weeks for routine examination and laboratory tests up to week 72, and every 24 weeks afterwards (for schedule of assessments see the additional information).

Patients
Patients with chronic HBV infection (HBeAg-positive and -negative) aged 18 years or older were enrolled after assessment of eligibility. To be eligible, patients must have had documented HBsAg positivity for longer than 6 months. Other inclusion criteria were HBV-DNA >100,000 copies/mL (17,182 IU/mL), normal or elevated alanine aminotransferase (ALT) levels, but ≤ 10 times upper limit of normal (ULN), or histological signs of chronic active hepatitis. Exclusion criteria were concurrent infection with hepatitis C virus, hepatitis Delta virus, or HIV; decompensated liver disease, HCC or a history of bleeding from esophageal varices; direct antiviral or immune modulatory therapy within previous 6 months; women with ongoing pregnancy or breast feeding; a history of auto-immune related disease, significant cardiac disease or renal impairment; evidence of chronic liver disease other than HBV; neutrophil count < 1,500 cells/mm$^3$, platelet count < 90,000 cells/mm$^3$ or total serum bilirubin > twice ULN; evidence of current hard drug(s) and/or alcohol abuse.

The study complied with the Declaration of Helsinki and with the principles of Good Clinical Practice and was approved by the ethical committees of the corresponding sites (controlled-trials.com; ISRCTN 77073364). All patients gave written informed consent.

Laboratory assays
Biochemical and virological analyses
Local laboratories carried out biochemical and hematological analyses in accordance with good laboratory practice. ALT levels were expressed relative to the upper limit of normal range (x ULN). ALT reference values were 45 U/L for males, and 34 U/L for females. Plasma HBV-DNA was extracted by COBAS® Ampliprep (F. Hoffmann-La Roche Ltd, Diagnostics Division, Basel, Switzerland) according to the manufacturer's instructions. Quantitation of plasma HBV-DNA levels was done by the Roche COBAS® TaqMan 48® assay (F. Hoffmann-La Roche Ltd, Diagnostics
Division, Basel, Switzerland), with a dynamic range between 20 and 1.70x10^8 IU/mL. HBV genotype was determined using the INNO-LiPA assay (Innogenetics, Gent, Belgium) or by sequencing a part of the polymerase gene with dideoxynucleotide technology. Presence of precore (PC) or basal core promoter (BCP) mutations was determined by sequencing the PC and BCP regions. Qualitative detection of serum hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), hepatitis B e antigen (HBeAg) and antibody to hepatitis B e antigen (anti-HBe) was performed by enzyme immunoassay (AxSYM; Abbott Laboratories, Abbott Park, IL, USA), and expressed as sample to cut-off ratio (S/CO) with a lower limit of detection of HBsAg 0.05 IU/mL, HBeAg 1.0 S/CO and anti-HBe 1.0 S/CO. Serum HBsAg quantitation was performed by the Abbott Architect (Abbott Diagnostics, Abbott Park, IL, USA), with a dynamic range between 0.05 and 250 IU/mL. If HBsAg levels were above 250 IU/mL, we diluted ten-fold with Abbott Manual Diluent (Abbott Diagnostics, Abbott Park, IL, USA) until a quantitative value was achieved.

**Histological analyses**

For histological assessment of liver biopsies we used the modified Ishak scoring system, based on a zero to 6 score for fibrosis (fibrosis score) in which score 5 and 6 represent marked bridging (incomplete cirrhosis) and cirrhosis, respectively.

**Response definitions**

Response was determined after 48 weeks of treatment (end of treatment; week 48), after 24 weeks of treatment-free follow-up (short-term follow-up; week 72) and after 96 weeks of treatment-free follow-up (long-term follow up; week 144).

We defined responses to comply with most recent AASLD and EASL guidelines. HBeAg loss was defined as undetectable HBeAg. HBeAg seroconversion was defined as HBeAg loss with formation of anti-HBe. HBsAg loss was defined as undetectable HBsAg (Abbott AxSYM: HBsAg < 0.05 IU/mL). HBsAg seroconversion was defined as HBsAg loss with formation of anti-HBs (anti-HBs > 10 IU/mL).

Combined response (CR) was defined as combination of virological (HBeAg negativity and HBV-DNA levels ≤ 2,000 IU/mL) and biochemical response (persistent normal ALT levels) in both HBeAg-positive and -negative patients. Patients were considered non-responder when not meeting one or both criteria for combined response, or when re-treatment with NUCs had been initiated.

**Statistical analysis**

Statistical comparisons were tested with IBM SPSS Statistics, version 19.0.0.1 (SPSS Inc., Chicago, IL, USA). Analyses were based on the intention-to-treat (ITT) model, in which patients who prematurely discontinued treatment were scored as non-responders. Differences by normally distributed variables (age, log HBsAg and log HBV-DNA) were tested using the Student’s t test, whereas differences in variables with skewed distribution (ALT) were tested using the Mann-Whitney U test. For comparison of categorical variables, the Chi-square test (Χ^2) or Fisher’s exact
test was used. The associations between variables as potential predictors of combined response or HBsAg loss as dependent variables were examined by logistic regression analysis. We used multivariable logistic regression analysis, including all variables with a $p$-value below 0.05, to determine independent predictive factors for response. All $p$-values are two sided and values below 0.05 were considered statistically significant.

RESULTS

Patient characteristics

Ninety-six patients signed informed consent (additional information). Four patients did not meet inclusion criteria at (re-)screening, all four because of a viral load <17,182 IU/mL. Ultimately, 92 patients (32 from AMC and 10 from EMC) received at least one dose of combination therapy (ITT). Six patients prematurely discontinued treatment due to adverse events and were considered as non-responders in all analyses. One patient stopped because of general side effects at week 18 (fatigue and flu like symptoms), one became pregnant while on therapy at week 10, one had a myocardial infarction at week 10, one developed autoimmune hepatitis at week 25, one had severe neutropenia despite dose reduction from week 2, and one developed Child-Pugh B cirrhosis at week 4. Common side-effects included fatigue, myalgia, other flu-like symptoms, pruritus, and insomnia (additional information). This side-effect pattern was similar to seen before in interferon treatment, and no new side-effects that could be attributable to combination therapy were reported. In sum, 91 of 92 patients (99%) completed at least 2 years of treatment-free follow-up. One patient with combined response at week 72 was lost to follow-up after virological relapse at week 96, and was regarded as a non-responder at week 144.

Table 1 shows baseline characteristics of HBeAg-positive and -negative patients. HBeAg-positive patients were younger, were more frequently Caucasian or Asian, had higher ALT, higher baseline HBV-DNA and HBsAg levels, and a lower prevalence of PC and BCP mutations. Although patients with genotype A were more frequently HBeAg positive, and genotype D and E more frequently HBeAg negative, this difference was not significant in our cohort. Data on the association of IL28B polymorphisms and treatment outcome in these patients has been published earlier by A. de Niet et al. in 2012.(18)

HBeAg-positive patients

Response

Table 2 shows an overview of clinical outcomes at week 48, week 72, and week 144 in HBeAg-positive patients. Thirteen of 44 patients (30%) lost HBeAg at week 48, which increased to 18 (41%) at week 144. The majority also had HBeAg seroconversion: 11 of 44 patients (25%) at week 48 and 15 of 44 (34%) at week 144. In none of the patients, reversion to HBeAg positivity was documented throughout follow-up. Of 18 patients with HBeAg loss, 12 had a combined response at week 144. We considered the other 6 patients who lost HBeAg as non-responders.
Table 1. Baseline characteristics of all HBeAg-positive and -negative patients included in intention to treat (ITT) analysis.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HBeAg-positive (N=44)</th>
<th>HBeAg-negative (N=48)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years) (SD, range)</td>
<td>35.8 (9.5, 19-54)</td>
<td>43.1 (9.7, 24-69)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>9 (20.5)</td>
<td>15 (31.3)</td>
<td>0.24</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Caucasian (%)</td>
<td>16 (36)</td>
<td>12 (25)</td>
<td></td>
</tr>
<tr>
<td>Asian (%)</td>
<td>20 (46)</td>
<td>14 (29)</td>
<td></td>
</tr>
<tr>
<td>African (%)</td>
<td>8 (18)</td>
<td>22 (46)</td>
<td></td>
</tr>
<tr>
<td>Median ALT (xULN) (range)</td>
<td>2.3 (0.6-27.9)</td>
<td>1.6 (0.5-9.2)</td>
<td>0.04</td>
</tr>
<tr>
<td>Interferon treatment naïve (%)</td>
<td>35 (79.5)</td>
<td>33 (68.8)</td>
<td>0.24</td>
</tr>
<tr>
<td>NUC treatment naïve (%)</td>
<td>41 (93)</td>
<td>44 (92)</td>
<td>0.78</td>
</tr>
</tbody>
</table>

**Viral characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HBeAg-positive (log_{10} IU/ml) (SD, range)</th>
<th>HBeAg-negative (log_{10} IU/ml) (SD, range)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean HBV-DNA (log_{10} IU/ml) (SD, range)</td>
<td>8.1 (1.2, 4.8-10.4)</td>
<td>5.5 (1.1, 3.6-7.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean HBSAg (log_{10} IU/ml) (SD, range)</td>
<td>4.3 (0.7, 1.9-5.3)</td>
<td>3.3 (0.7, 1.6-4.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HBV genotype</td>
<td></td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td>A (%)</td>
<td>18 (42)</td>
<td>11 (23)</td>
<td></td>
</tr>
<tr>
<td>B (%)</td>
<td>8 (18)</td>
<td>7 (15)</td>
<td></td>
</tr>
<tr>
<td>C (%)</td>
<td>7 (16)</td>
<td>5 (10)</td>
<td></td>
</tr>
<tr>
<td>D (%)</td>
<td>9 (21)</td>
<td>18 (38)</td>
<td></td>
</tr>
<tr>
<td>E (%)</td>
<td>2 (5)</td>
<td>7 (15)</td>
<td></td>
</tr>
<tr>
<td>Pre-core mutation</td>
<td>6 (14)</td>
<td>36 (78)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Basal core promoter mutation</td>
<td>16 (38)</td>
<td>29 (62)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Baseline liver biopsy**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Baseline liver biopsy</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median fibrosis score (range)</td>
<td>1 (0-6)</td>
<td>1 (0-6)</td>
</tr>
<tr>
<td>Cirrhosis (%)</td>
<td>2 (7)</td>
<td>9 (23)</td>
</tr>
</tbody>
</table>

(a) p-value for Student’s t test, Mann-Whitney U test, Fisher’s exact or Chi-square test
(b) 70 patients had baseline liver biopsy material available for evaluation
Baseline HBsAg as predictor of sustained HBsAg loss  Chapter 2

This included 3 with increased HBV-DNA levels after HBeAg loss, and 3 with HBeAg loss after the initiation of re-treatment with NUCs. In addition, 2 patients lost HBeAg after premature discontinuation of study treatment (drop outs) and counted as non-responders. Twenty of 32 non-responders (63%) were re-treated with NUCs before week 144.

At end of week 144, 5 of 44 patients (11%) had lost HBsAg, four of them with HBsAg seroconversion before week 48. The other remained HBsAg-negative, without detectable anti-HBs. One patient with HBeAg loss and HBsAg loss reverted to an HBsAg positive state at week 68.

Baseline predictors for combined response at week 144 in HBeAg-positive patients
The additional information shows baseline characteristics in relation to combined response. We found no significant differences between the baseline characteristics of patients who had combined response at week 144 and those with non-response. Patients with combined response tended to have higher ALT levels (median 3.6 vs 1.9 x ULN, p = 0.17).

Baseline predictors for HBsAg loss at week 144 in HBeAg-positive patients
Table 3 shows the baseline characteristics in relation to HBsAg loss at week 144. An overview of all evaluated characteristics is shown in the additional information. The 5 patients who lost HBsAg at week 144 tended to be older than those with HBsAg persistence (mean 42 vs 35 years, p = 0.12) and had a relatively high proportion of HBV genotype A (80 vs 36 %, p = 0.06). Liver biopsies were available of 4 patients with HBsAg loss, which showed higher Ishak fibrosis scores than those with HBsAg persistence (median 2.5 vs 1.0, p = 0.03).

HBeAg-negative patients
Response
Table 2 shows an overview of the clinical outcomes at week 48, week 72, and week 144 in HBeAg-negative patients. In HBeAg-negative patients, 37 of 48 (77%) attained combined response at week 48, decreasing to 17 (35%) and 12 (25%) during treatment-free follow-up (week 72 and week 144 respectively). Re-treatment with NUCs was initiated in 15/31 (48%) patients with non-response at week 72, and in 27/36 (75%) patients with non-response at week 144. Five non-responder patients at week 144 did not meet the treatment criterion of HBV-DNA >20,000 IU/mL. One patient developed HCC during follow-up, and was re-treated with NUC therapy. The percentage of patients with HBsAg loss increased during 2 years of follow-up. At week 48, 3 (6%) had achieved HBsAg loss, increasing to 8 (17%) at week 144. All but one patient with HBsAg loss had developed HBsAg seroconversion at week 144. Of note, the one patient without HBsAg seroconversion at week 144 did develop anti-HBs at week 177.

Baseline predictors for combined response at week 144 in HBeAg-negative patients
The additional information shows all baseline characteristics in relation to combined response in HBeAg-negative patients. HBV-DNA and HBsAg levels were lower in patients achieving a combined response at week 144, as compared to non-responders (mean HBV-DNA 4.85 versus...
5.76 log_{10}IU/mL, p = 0.01, and mean HBsAg 2.87 versus 3.50 log_{10}IU/mL, p = 0.04). Higher levels of HBV-DNA correlated with higher levels of HBsAg (Pearson’s correlation coefficient 0.334, p = 0.02).

Using multivariable logistic regression only the HBsAg level was an independent predictor of combined response at week 144 (OR 0.30 per 1log_{10}IU/mL HBsAg decline [95% CI 0.09-0.93], p=0.04).

**Baseline predictors for HBsAg loss at week 144 in HBeAg-negative patients**

Table 3 shows baseline characteristics in relation to HBsAg loss in HBeAg-negative patients. An overview of all evaluated characteristics is shown in the additional information. Both HBV-DNA and HBsAg levels were significantly lower in HBeAg-negative patients who had lost HBsAg at week 144 (mean HBV-DNA 4.76 versus 5.69 log_{10}IU/mL, p = 0.03, and mean HBsAg 2.35 versus 3.55 log_{10}IU/mL, p < 0.001). Moreover, fewer patients with HBsAg loss were naïve to interferon treatment than those with HBsAg persistence; 2/8 (25%) versus 31/40 (78%), p = 0.02. In multivariable analysis, HBsAg level was the only independent predictor of HBsAg loss at week 144 (OR 0.02 per 1log_{10}IU/mL increase [95% CI, 0.00-0.39] p = 0.01).

Figure 1 shows the distribution of baseline HBsAg levels in all HBeAg-negative patients of different genotypes. Six of 8 (75%) patients with HBsAg loss had a baseline HBsAg level below 2.60 log_{10}IU/ml (400 IU/mL), corresponding to a positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 95%.
Table 2. Response rates of HBeAg-positive patients and HBeAg-negative patients at 48 weeks of treatment (end of treatment; week 48), 24 weeks of treatment-free follow-up (short-term follow-up; week 72) and 96 weeks of treatment-free follow-up (long term follow-up; week 144). NUC; nucleos(t)ide analogue.

<table>
<thead>
<tr>
<th></th>
<th>HBeAg-positive (n = 44)</th>
<th>HBeAg-negative (n = 48)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wk 48</td>
<td>wk 72</td>
</tr>
<tr>
<td>n</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td><strong>Biochemical response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT normalization*</td>
<td>33 (75)</td>
<td>22 (50)</td>
</tr>
<tr>
<td><strong>Virological response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV-DNA &lt; 2,000 IU/mL</td>
<td>29 (66)</td>
<td>14 (32)</td>
</tr>
<tr>
<td>Non-response</td>
<td>12 (27)</td>
<td>27 (61)</td>
</tr>
<tr>
<td>NUC re-treatment</td>
<td>-</td>
<td>3 (7)</td>
</tr>
<tr>
<td>Drop out</td>
<td>3 (7)</td>
<td>3 (7)</td>
</tr>
<tr>
<td>Lost to follow up</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Serological response</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBeAg loss</td>
<td>13 (30)</td>
<td>14 (32)</td>
</tr>
<tr>
<td>HBeAg loss after NUC re-treatment</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>HBeAg seroconversion</td>
<td>11 (25)</td>
<td>12 (27)</td>
</tr>
<tr>
<td>HBeAg seroconversion after NUC re-treatment</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>HBsAg loss</td>
<td>4 (9)</td>
<td>4 (9)</td>
</tr>
<tr>
<td>HBsAg seroconversion</td>
<td>4 (9)</td>
<td>4 (9)</td>
</tr>
<tr>
<td>HBsAg loss after NUC re-treatment</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td><strong>Combined response</strong></td>
<td>29 (66)</td>
<td>14 (32)</td>
</tr>
</tbody>
</table>

(a) ALT reference values: male 45 U/L, female 34 U/L
(b) Combined response was defined as HBV-DNA < 2,000 IU/mL and ALT normalization
### Table 3. Baseline characteristics of HBeAg-positive and -negative patients, who lost HBsAg at long term follow-up (week 144) compared with those with HBsAg persistence.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HBsAg loss at long-term follow-up (week 144)</th>
<th>HBsAg persistence</th>
<th>p²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBsAg loss; n (%)(SD)</td>
<td>HBsAg persistence</td>
<td></td>
</tr>
<tr>
<td>HBeAg-positive; n (%)</td>
<td>5 (11)</td>
<td>39 (52)</td>
<td></td>
</tr>
<tr>
<td>Mean age (years) (SD)¹</td>
<td>42.0 (6.9)</td>
<td>35.0 (9.6)</td>
<td>0.12</td>
</tr>
<tr>
<td>IFN treatment naïve (%)^³</td>
<td>4 (80)</td>
<td>31 (80)</td>
<td>0.98</td>
</tr>
<tr>
<td>Median ALT (xULN) (iqr)^²</td>
<td>5.3 (1.8-6.7)</td>
<td>2.2 (1.1-4.8)</td>
<td>0.23</td>
</tr>
<tr>
<td>Mean HBV-DNA (log_{10} IU/ml) (SD)^¹</td>
<td>8.09 (1.1)</td>
<td>8.04 (1.2)</td>
<td>0.94</td>
</tr>
<tr>
<td>Mean HBsAg (log_{10} IU/ml) (SD)^¹</td>
<td>4.16 (1.0)</td>
<td>4.33 (0.7)</td>
<td>0.64</td>
</tr>
<tr>
<td>HBV genotype A (%)^³</td>
<td>4 (80)</td>
<td>14 (36)</td>
<td>0.06</td>
</tr>
<tr>
<td>Baseline liver biopsy⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median fibrosis score (range)^²</td>
<td>4 (80)</td>
<td>27 (69)</td>
<td>0.62</td>
</tr>
<tr>
<td>HBeAg-negative; n (%)</td>
<td>8 (17)</td>
<td>40 (83)</td>
<td></td>
</tr>
<tr>
<td>Mean age (years) (SD)¹</td>
<td>46.9 (12.9)</td>
<td>42.3 (9.0)</td>
<td>0.22</td>
</tr>
<tr>
<td>IFN treatment naïve (%)^³</td>
<td>2 (25)</td>
<td>31 (78)</td>
<td>0.02</td>
</tr>
<tr>
<td>Median ALT (xULN) (iqr)^²</td>
<td>1.1 (0.8-2.4)</td>
<td>1.8 (1.1-2.9)</td>
<td>0.18</td>
</tr>
<tr>
<td>Mean HBV-DNA (log_{10} IU/ml) (SD)^¹</td>
<td>4.76 (0.9)</td>
<td>5.69 (1.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Mean HBsAg (log_{10} IU/ml) (SD)^¹</td>
<td>2.35 (0.6)</td>
<td>3.55 (0.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HBV genotype A (%)^³</td>
<td>3 (38)</td>
<td>8 (20)</td>
<td>0.28</td>
</tr>
<tr>
<td>Baseline liver biopsy⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median fibrosis score (range)^²</td>
<td>7 (88)</td>
<td>32 (80)</td>
<td>0.62</td>
</tr>
<tr>
<td>Multivariable logistic regression (adjusted)</td>
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<tr>
<td>HBeAg-negative patients^c</td>
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<td></td>
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<tr>
<td>HBV-DNA (log_{10} IU/ml)</td>
<td>0.40</td>
<td>0.09-1.73</td>
<td>0.21</td>
</tr>
<tr>
<td>HBsAg (log_{10} IU/ml)</td>
<td>0.02</td>
<td>0.00-0.39</td>
<td>0.01</td>
</tr>
<tr>
<td>IFN treatment naïve</td>
<td>0.05</td>
<td>0.02-3.86</td>
<td>0.32</td>
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</tbody>
</table>

(a) p-value for Student’s t test¹, Mann-Whitney U test², Fisher’s exact or Chi-square test³
(b) 70 patients had baseline liver biopsy material available for evaluation
(c) multivariable analysis was performed in HBeAg-negative patients only (n=48)
Figure 1: Baseline serum HBsAg levels in HBeAg-negative patients according to viral genotype. Patients with HBsAg loss at long term follow up (week 144) are marked in green. Error bars represent the mean and SEM.

HBsAg kinetics – on treatment and during follow-up

HBeAg-positive patients

Figure 2A shows the course of serum HBsAg during treatment and long term follow-up in patients with combined response and those who did not respond. Patients with combined response at week 144 had significantly lower HBsAg levels than patients with non-response at all timepoints, except at baseline. Non-responder patients receiving NUC therapy were censored in this analysis.

At end of week 72, 7 HBeAg-positive patients had a serum HBsAg level below 20 IU/mL, including 5 with HBsAg loss at week 144. In contrast, none of the 35 patients with serum HBsAg level of >20 IU/mL at week 72 had lost HBsAg at week 144. This corresponds to a PPV of 71% and an NPV of 100% for HBsAg loss at week 144.

Individual figures of HBsAg kinetics in all HBeAg-positive patients (n = 5) with HBsAg loss at week 144 are shown in the additional information.

HBeAg-negative patients

Figure 2B shows the course of serum HBsAg during treatment and long term follow-up in patients with combined response and non-response. Those with combined response at week 144 had significantly lower HBsAg levels than non-responding patients at all timepoints, including baseline. Non-responder patients receiving NUC therapy were censored in this analysis.

At end of week 72, 9 HBeAg-negative patients had a serum HBsAg level below 20 IU/mL, including 8 with HBsAg loss at week 144. Of note, the one patient who had not lost HBsAg at week 144 had lost it after 174 weeks with no anti-HBs formation up to 296 weeks. In contrast, none of the 36 patients with serum HBsAg level of >20 IU/mL at week 72 had lost HBsAg at week 144. Using a cut-off of 20 IU/mL at week 72, we observed a PPV of 89% and an NPV of 100% for predicting HBsAg loss at week 144.

Individual figures of HBsAg kinetics in all HBeAg-negative patients (n = 8) with HBsAg loss at week 144 are shown in the additional information.
Part I

**Figures 2A and 2B:** Mean serum HBsAg levels in HBeAg-positive (2A) and –negative (2B) patients with combined response and in those with non-response. The lines represent mean serum HBsAg levels in patients with non-response (black lines), and patients with combined response (CR) at week 144 (gray line). CR was defined as combination of HBV DNA < 2,000 IU/mL and ALT normalization. Patients with non-response were further subdivided into patients receiving retreatment with nucleotide analogues (black striped line) or not (black line). $P$-values of the difference in HBsAg level between patients with CR and untreated non-responders are represented as * ($p < 0.05$), ** ($p < 0.005$) and *** ($p < 0.0005$), respectively. Error bars represent the SEM. Total number of available patient samples at each time point is given in the table below.

<table>
<thead>
<tr>
<th>Non Response (NR)</th>
<th>Untreated</th>
<th>NUC re-treated</th>
<th>Combined response</th>
</tr>
</thead>
<tbody>
<tr>
<td>start</td>
<td>32</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>24</td>
<td>30</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>48</td>
<td>30</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>72</td>
<td>26</td>
<td>-</td>
<td>11</td>
</tr>
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<td>96</td>
<td>12</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>144</td>
<td>10</td>
<td>16</td>
<td>11</td>
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<table>
<thead>
<tr>
<th>peg-IFN and ADV</th>
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<th>33</th>
<th>33</th>
<th>21</th>
<th>13</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUC re-treated</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>18</td>
<td>25</td>
<td></td>
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<td>12</td>
<td>11</td>
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DISCUSSION

Here we report the results of a prospective study of predictive markers for response in HBeAg-positive and -negative chronic hepatitis B patients treated with peg-IFN and adefovir. In our study, we found a high rate of HBsAg loss in both HBeAg-positive (11%) and HBeAg-negative (17%) patients at two years of treatment-free follow-up (week 144). The main outcome of the study was that HBeAg-negative patients with a low baseline HBsAg level were more likely to achieve HBsAg loss.

In HBeAg-negative patients in particular, the extent of HBsAg loss (17%) and HBsAg seroconversion (15%) at two year follow-up was high compared with those treated with peg-IFN for 48 weeks as monotherapy or combined with lamivudine. For example Marcellin et al. (19) reported 4% and 5% HBsAg loss respectively in 116 peg-IFN and 114 peg-IFN + lamivudine treated HBeAg-negative patients after a follow-up of 2 years. Although we can make no direct comparison because of the absence of a monotherapy arm in our study, the high rate of HBsAg loss suggests an additive therapeutic effect of adefovir. Next to antiviral activity, adefovir enhances innate immune functions in mice (20;21), indicating that the immune modulatory effect of adefovir might be synergistic when combined with peg-IFN. Three earlier studies have involved combination treatment of peg-IFN and adefovir. Two studies found a relatively high rate of HBsAg loss in HBeAg-positive patients, but were either small (22), or used a different treatment regimen.(23) In contrast to our study, a relatively small randomized trial in HBeAg-negative patients (n=30), mainly infected with viral genotype D, did not show an increase in HBsAg loss using peg-IFN and adefovir combination treatment.(10)

We observed that HBsAg level at baseline was significantly lower in HBeAg-negative patients with HBsAg loss compared with those with HBsAg persistence (p < 0.001). To our knowledge, this is the first prospective study indicating that HBsAg at baseline is an independent predictor of HBsAg loss in HBeAg-negative patients. Only one retrospective study associated lower baseline HBsAg in a subset of HBeAg-negative patients treated with IFN-alpha-2b with HBsAg loss over time.(24) Interestingly, all HBeAg-negative patients in our study with a baseline HBsAg level below 400 IU/mL (n=6) had lost HBsAg at week 144. Although most HBeAg negative patients had HBsAg levels above this value, demographic studies on quantitative HBsAg showed that 2-13% of HBeAg negative patients with high viral load had HBsAg levels below 400 IU/mL, and thus may be good candidates for peg-IFN based combination therapy.(25-27)

Due to a lack of studies and the usually low rate of HBsAg loss, there is a paucity of clear mechanistic insight into HBsAg loss in peg-IFN based treatment. The decline in serum HBsAg seems to be associated with eradication of cccDNA through clearance of infected hepatocytes by cytotoxic T cells.(22;28) There is evidence that HBsAg impairs antigen presenting cells by repetitive toll like receptor triggering.(29;30) Lower antigenic loads of HBsAg may therefore be associated with a less refractory innate immune system, thus lowering the threshold for response to exogenous interferon. Interestingly, a higher proportion of HBeAg-negative patients with
HBsAg loss was IFN-experienced, compared with patients not losing HBsAg. We also showed that both responders and non-responders had a sustained decline in HBsAg levels after therapy. This may suggest that lower baseline HBsAg levels in some patients could be explained by an earlier IFN-related decrease in HBsAg. Based on these findings, HBeAg-negative patients who relapse after peg-IFN treatment but retain low levels of serum HBsAg may be good candidates for re-treatment with peg-IFN based therapy.

In contrast to our findings in HBeAg-negative patients, neither baseline HBsAg nor HBV-DNA predicted HBeAg or HBsAg loss in HBeAg-positive patients. HBeAg-positive patients with HBsAg loss did have higher fibrosis scores in liver biopsy specimen (p = 0.03), although only 4 biopsies of patients with HBsAg loss were available for analysis.

Both HBeAg-positive and -negative patients with combined response showed a significant decline in serum HBsAg level during treatment, which was sustained during long term follow-up. The definition of combined response (HBV-DNA < 2000 IU/mL with normal ALT at end of follow-up), is accepted by recent international guidelines and relates to epidemiological studies showing that progression of cirrhosis and HCC significantly diminishes when HBV-DNA is below 2,000 IU/mL.(31-33) However, it is a relatively weak endpoint since after longer follow-up many patients relapse. Those with HBeAg-negative disease in particular may show increased HBV-DNA and elevated ALT necessitating re-treatment, most often with nucleos(t)ide analogues.(19) For example in our study, 5/13 (38%) HBeAg-negative patients with combined response at week 72, but without HBsAg loss, relapsed during long term follow-up. Conversely, a large proportion of HBeAg-negative patients who did achieve a combined response at week 144 (n=19) experienced HBsAg loss (9/19, 47%) and subsequent HBsAg seroconversion (8/19, 42%), which is regarded as the closest outcome to clinical cure in HBV treatment.(17) In addition, we found that HBeAg-negative patients with HBsAg levels <20 IU/mL at 24 weeks of treatment-free follow-up, had high predictive values for HBsAg loss at long term follow-up (PPV of 89% and NPV of 100%). Overall, these findings suggest that HBsAg loss with HBsAg seroconversion may be an important objective for future intervention studies to focus on.

This is a non-randomized study on a limited number of patients. Therefore we have to be careful with our conclusions. We cannot exclude that differences in study population characteristics may account in part for the high rate of HBsAg loss found this study. In our study for example, a relatively high percentage of patients with genotype A was present, known to easier clear HBsAg in peg-IFN based therapy. However, in HBeAg negative patients, in whom higher rates of HBsAg loss occurred specifically, we observed HBsAg loss across all major genotypes. Nevertheless, our results have to be validated in a comparable cohort of patients treated with peg-IFN based combination therapy. Partly for this reason, large randomized studies, comparing peg-IFN and tenofovir or entecavir in combination versus monotherapies have been initiated. Next to therapeutic and viral factors, other yet undefined host and viral factors may play a role in inducing HBsAg loss in HBeAg-positive and -negative patients. In this regard, it would be of interest to study additional viral, host genetic and immunological markers that could be associated with HBsAg loss.
In conclusion, in HBeAg-negative patients with active disease and treated with peg-IFN and adefovir combination therapy, a low baseline HBsAg is an excellent predictor for sustained HBsAg loss. Thus, selection of patients by baseline HBsAg levels may substantially increase the rate of HBsAg loss and avoid unnecessary IFN-related adverse events in patients with a poor chance of responding to peg-IFN and nucleos(t)ide analogue combination therapy.

ACKNOWLEDGEMENTS

We thank Dr Martin Wagtmans (Flevo Hospital, Almere) for recruiting patients for this trial. We thank Dr Martin de Vries (Roche, The Netherlands) and Michiel Plugge (Gilead Sciences, The Netherlands) for financial support of this study in the form of unrestricted grants.

GRANT SUPPORT

This study was sponsored by Roche the Netherlands and Gilead in the form of an unrestricted grant.
ADDITIONAL INFORMATION:

Supplementary figure 1: Overview of patients included in the study. In sum, 44 HBeAg-positive and 48 HBeAg-negative patients received at least one dose of study drug and formed the intention-to-treat (ITT) population in which drop-outs are regarded as non-responders.
Baseline HBsAg as predictor of sustained HBsAg loss  Chapter 2

Supplementary table 1: Schedule of Assessments; Treatment and Short term follow-up.

<table>
<thead>
<tr>
<th>Assessment/ Procedure</th>
<th>Screening (weeks)</th>
<th>Study treatment Period Peg-IFN and ADF (weeks)</th>
<th>Short term Follow-up (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study weeks</td>
<td>-4 to 0</td>
<td>8 day 3 1 2 4 6 8 12 18 24 30 36 42 48 50 52 56 60 64 68 72</td>
<td></td>
</tr>
<tr>
<td>Weeks from end-of-treatment</td>
<td></td>
<td></td>
<td>2 4 8 12 16 20 24</td>
</tr>
<tr>
<td>Informed consent, medical history</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physical examination</td>
<td>X X X X X X X X X X X X X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver biopsy (preceding 12 months)</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Urine or serum pregnancy test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chest X-ray, selected patients</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultrasound, CT or MRI, selected patients</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrocardiogram, selected patients</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ophthalmologic examination</td>
<td>X</td>
<td>Repeat in patients with pre-existing findings or those developing symptoms</td>
<td></td>
</tr>
<tr>
<td>Anti-HAV IgM, anti-HIV, anti-HCV, HCV-RNA, anti-HDV</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B: baseline
a. Or upon and following discontinuation – the follow-up visits being at 4, 12 and 24 weeks after the end of therapy
b. For females of child bearing potential only, a pregnancy test will be performed within 24 hours prior to first dose. A pregnancy test must be done at any time after a secondary amenorrhea of more than 1 week occurs.
c. Only for patients with pre-existing pulmonary disease [Not necessary if (1) a chest X-ray available to the investigator has been obtained within the past 12 months and (2) the patient's pulmonary disease has been clinically stable]
d. Patients with cirrhosis or marked fibrosis on liver biopsy or raised AFP need to have a liver imaging study during the screening period to rule out hepatic neoplasia.(see inclusion criterion 6)
e. For anyone with pre-existing cardiac disease.
f. All patients should have a baseline eye examination. Any patient complaining of decrease or loss of vision must have a prompt and complete eye examination. Patients with pre-existing ophthalmologic disorders (eg, diabetic or hypertensive retinopathy) should receive periodic ophthalmologic exams during Pegasis therapy. Pegasis treatment should be discontinued in patients who develop new or worsening ophthalmologic disorders.
### Supplementary table: Schedule of Assessments; Treatment and Short term follow-up (continued)

<table>
<thead>
<tr>
<th>Assessment / Procedure</th>
<th>Screening (weeks)</th>
<th>Study treatment Period Peg-INF and ADF (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study weeks</td>
<td>-4 to 0</td>
<td></td>
</tr>
<tr>
<td>Weeks from end-of-treatment INF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete hematology with differential WBC, and platelets.</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Chemistry 1ᵃᵇ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemistry 2ᵍ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemistry 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinalysis, dipstick.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceruloplasmin, alfa-1-antitrypsin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alfa-fetoprotein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMA, ANA, ASMA, thyroid peroxidase antibodies,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>selected patientsʰ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBeAg, anti-HBe (frozen for central processing)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>HBsAg, anti-HBc (frozen for central processing)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV-DNA (frozen for central processing)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Alpha-2 macro globulin, Haptoglobin, Apolipoprotein A1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma bank, viral sequencing</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>PBMC’s (frozen for central processing, genetic markers)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Compliance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concomitant medication</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Adverse events</td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

Chemistry 1: ASAT, ALAT, Chemistry 2: sodium, chloride, potassium, bilirubin, creatinine, urea, prothrombin time, alkaline phosphatase, calcium, phosphorus, total protein, albumin, uric acid, TSH, free T₄, and glucose.

Chemistry 3: cholesterol, triglycerides, sober glucose.

(a) Or upon and following discontinuation – the follow-up visits being at 4, 12 and 24 weeks after the end of therapy

(g) If there are clinically significant laboratory abnormalities, repeat no less frequently than every 2 weeks or as clinically indicated, with appropriate toxicity management, until they return to normal or baseline values. Urinalysis to be performed via dipstick, with subsequent microscopic evaluation if positive for hemoglobin at the discretion of the investigator.

(h) Only for certain patients at risk, don’t repeat if data from previous assessments are available

(i) Roche TaqMan®; when HBV DNA is more than the maximal detection limit, ten or hundred time dilution is proceeded.

(k) Two elevated (> ULN < 10 x ULN) ALAT determinations should be established at least 14 days apart during the six months before the first dose, with at least one determination during the screening period (see inclusion criterion 5).
Supplementary table: Schedule of Assessments; Long term follow-up.

<table>
<thead>
<tr>
<th>Assessment / Procedure</th>
<th>Long-term follow-up (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Years after end of treatment</td>
<td>1.0</td>
</tr>
<tr>
<td>Complete hematology with, hemoglobin, differential WBC, and platelets</td>
<td>X</td>
</tr>
<tr>
<td>ALAT, ASAT, Alfa-fetoprotein</td>
<td>X</td>
</tr>
<tr>
<td>HBeAg, anti-HBe (frozen for central processing)</td>
<td>X</td>
</tr>
<tr>
<td>HBsAg, anti-HBs (frozen for central processing)</td>
<td>X</td>
</tr>
<tr>
<td>Serum bank (frozen for central processing)</td>
<td>X</td>
</tr>
<tr>
<td>HBV-DNA</td>
<td>X</td>
</tr>
<tr>
<td>Plasma bank (viral sequencing,)</td>
<td>X</td>
</tr>
<tr>
<td>PBMC’s (frozen for central processing)</td>
<td>X</td>
</tr>
</tbody>
</table>

Baseline HBsAg as predictor of sustained HBsAg loss Chapter 2
### Supplementary table 2: Incidence of common Adverse Events, Dose Modifications, and Treatment Discontinuation.*

<table>
<thead>
<tr>
<th>Adverse Event</th>
<th>Number of patients (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td>73 (91)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>55 (69)</td>
</tr>
<tr>
<td>Fever (after first injection)</td>
<td>46 (58)</td>
</tr>
<tr>
<td>Headache</td>
<td>43 (54)</td>
</tr>
<tr>
<td>Flu-like symptoms</td>
<td>41 (51)</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>34 (43)</td>
</tr>
<tr>
<td>Arthralgia</td>
<td>33 (41)</td>
</tr>
<tr>
<td>Dizziness</td>
<td>32 (40)</td>
</tr>
<tr>
<td>Cough</td>
<td>24 (30)</td>
</tr>
<tr>
<td>Palpitations</td>
<td>15 (19)</td>
</tr>
<tr>
<td><strong>Digestive tract</strong></td>
<td></td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>35 (44)</td>
</tr>
<tr>
<td>Nausea</td>
<td>28 (35)</td>
</tr>
<tr>
<td>Flatulence</td>
<td>27 (34)</td>
</tr>
<tr>
<td>Sore throat</td>
<td>20 (25)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>18 (23)</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>17 (21)</td>
</tr>
<tr>
<td>Constipation</td>
<td>17 (21)</td>
</tr>
<tr>
<td>Loss of &gt;10% bodyweight</td>
<td>13 (16)</td>
</tr>
<tr>
<td><strong>Dermatological</strong></td>
<td></td>
</tr>
<tr>
<td>Pruritus</td>
<td>33 (41)</td>
</tr>
<tr>
<td>Injection-site reaction</td>
<td>26 (33)</td>
</tr>
<tr>
<td>Hair loss</td>
<td>21 (26)</td>
</tr>
<tr>
<td><strong>Psychiatric</strong></td>
<td></td>
</tr>
<tr>
<td>Insomnia</td>
<td>33 (41)</td>
</tr>
<tr>
<td>Depression, including mood changes</td>
<td>31 (39)</td>
</tr>
<tr>
<td>Irritability</td>
<td>29 (36)</td>
</tr>
<tr>
<td><strong>Haematological</strong></td>
<td></td>
</tr>
<tr>
<td>Anemia (&lt;7.5 mmol/L)</td>
<td>23 (29)</td>
</tr>
<tr>
<td>Neutropenia (&lt;0.75 x 10⁹/L)</td>
<td>19 (24)</td>
</tr>
<tr>
<td>Thrombocytopenia (&lt;75 x 10⁹/L)</td>
<td>14 (18)</td>
</tr>
<tr>
<td><strong>Dose modification</strong></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25 (31)</td>
</tr>
<tr>
<td>Adverse event</td>
<td>15 (19)</td>
</tr>
<tr>
<td>Laboratory abnormality</td>
<td>8 (10)</td>
</tr>
<tr>
<td>Weight adjusted modification</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Discontinuation</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6 (7)</td>
</tr>
</tbody>
</table>

*Values are based on all randomized patients who received at least one dose of study medication and of whom at least one safety assessment after baseline was available (n=80).
Supplementary table 3: Baseline characteristics of HBeAg-positive patients who had combined response at long term follow-up (week 144) compared with those with non-response. Combined response was defined as HBeAg negativity, HBV-DNA < 2,000 IU/mL and ALT normalization.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Combined response</th>
<th>Non-response</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td>12 (27)</td>
<td>32 (73)</td>
<td></td>
</tr>
<tr>
<td>Mean age (years) (SD, range)</td>
<td>38.8 (9.4, 19-49)</td>
<td>34.7 (9.5, 19-54)</td>
<td>0.21</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>4 (33)</td>
<td>5 (16)</td>
<td>0.20</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>Caucasian (%)</td>
<td>6 (50)</td>
<td>10 (31)</td>
<td></td>
</tr>
<tr>
<td>Asian (%)</td>
<td>4 (33)</td>
<td>16 (50)</td>
<td></td>
</tr>
<tr>
<td>African (%)</td>
<td>2 (17)</td>
<td>6 (19)</td>
<td></td>
</tr>
<tr>
<td>Median ALT (xULN) (range)</td>
<td>3.6 (1.1-7.3)</td>
<td>1.9 (0.6-27.9)</td>
<td>0.17</td>
</tr>
<tr>
<td>Interferon treatment naïve (%)</td>
<td>9 (75)</td>
<td>25 (78)</td>
<td>0.83</td>
</tr>
<tr>
<td>NUC treatment naïve (%)</td>
<td>12 (100)</td>
<td>29 (91)</td>
<td>0.27</td>
</tr>
</tbody>
</table>

**Viral characteristics**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Combined response</th>
<th>Non-response</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean HBV-DNA (log_{10} IU/ml) (SD, range)</td>
<td>8.0 (1.1, 5.5-9.0)</td>
<td>8.0 (1.3, 4.8-10.5)</td>
<td>1.00</td>
</tr>
<tr>
<td>Mean HBsAg (log_{10} IU/ml) (SD, range)</td>
<td>4.3 (0.6, 2.9-5.0)</td>
<td>4.3 (0.8, 1.9-5.3)</td>
<td>0.80</td>
</tr>
<tr>
<td>HBV genotype</td>
<td></td>
<td></td>
<td>0.53</td>
</tr>
<tr>
<td>A (%)</td>
<td>7 (58)</td>
<td>11 (34)</td>
<td></td>
</tr>
<tr>
<td>B (%)</td>
<td>2 (17)</td>
<td>6 (19)</td>
<td></td>
</tr>
<tr>
<td>C (%)</td>
<td>2 (17)</td>
<td>5 (16)</td>
<td></td>
</tr>
<tr>
<td>D (%)</td>
<td>1 (8)</td>
<td>8 (25)</td>
<td></td>
</tr>
<tr>
<td>E (%)</td>
<td>0 (-)</td>
<td>2 (6)</td>
<td></td>
</tr>
<tr>
<td>Pre-core mutation</td>
<td>1 (8)</td>
<td>5 (16)</td>
<td>0.51</td>
</tr>
<tr>
<td>Basal core promoter mutation</td>
<td>3 (25)</td>
<td>13 (43)</td>
<td>0.27</td>
</tr>
<tr>
<td><strong>Baseline liver biopsy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median fibrosis score (range)</td>
<td>1.5 (1-6)</td>
<td>1 (0-4)</td>
<td>0.25</td>
</tr>
<tr>
<td>Cirrhosis (%)</td>
<td>2 (20)</td>
<td>0 (-)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

(a) p-value for Student’s t test¹, Mann-Whitney U test², Fisher’s exact or Chi-square test³
(b) 31 patients had baseline liver biopsy material available for evaluation
Supplementary table 4: Baseline characteristics of HBeAg-positive patients who lost HBsAg at long term follow-up (week 144) compared with those with HBsAg persistence.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HBsAg loss</th>
<th>HBsAg persistence</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td>5 (11)</td>
<td>39 (89)</td>
<td></td>
</tr>
<tr>
<td>Mean age (years) (SD, range)</td>
<td>42.0 (6.9, 31-49)</td>
<td>35.0 (9.6, 19-55)</td>
<td>0.12</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>1 (20)</td>
<td>8 (21)</td>
<td>0.98</td>
</tr>
<tr>
<td>Ethnicity¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian (%)</td>
<td>6 (50)</td>
<td>12 (31)</td>
<td>0.09</td>
</tr>
<tr>
<td>Asian (%)</td>
<td>4 (33)</td>
<td>19 (49)</td>
<td></td>
</tr>
<tr>
<td>African (%)</td>
<td>2 (17)</td>
<td>8 (21)</td>
<td></td>
</tr>
<tr>
<td>Median ALT (xULN) (range)²</td>
<td>5.3 (1.8-7.3)</td>
<td>2.2 (0.6-27.9)</td>
<td>0.23</td>
</tr>
<tr>
<td>Interferon treatment naïve (%)³</td>
<td>4 (80)</td>
<td>30 (77)</td>
<td>0.88</td>
</tr>
<tr>
<td>NUC treatment naïve (%)³</td>
<td>5 (100)</td>
<td>36 (92)</td>
<td>0.52</td>
</tr>
<tr>
<td>Viral characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean HBV-DNA (log₁₀ IU/ml) (SD, range)¹</td>
<td>8.1 (1.1, 6.9-9.0)</td>
<td>8.0 (1.2, 4.8-10.4)</td>
<td>0.94</td>
</tr>
<tr>
<td>Mean HBsAg (log₁₀ IU/ml) (SD, range)¹</td>
<td>4.2 (1.0, 2.9-5.0)</td>
<td>4.3 (0.7, 1.9-5.3)</td>
<td>0.64</td>
</tr>
<tr>
<td>HBV genotype¹</td>
<td></td>
<td></td>
<td>0.33</td>
</tr>
<tr>
<td>A (%)</td>
<td>4 (80)</td>
<td>14 (36)</td>
<td></td>
</tr>
<tr>
<td>B (%)</td>
<td>0 (-)</td>
<td>8 (21)</td>
<td></td>
</tr>
<tr>
<td>C (%)</td>
<td>1 (20)</td>
<td>6 (15)</td>
<td></td>
</tr>
<tr>
<td>D (%)</td>
<td>0 (-)</td>
<td>9 (23)</td>
<td></td>
</tr>
<tr>
<td>E (%)</td>
<td>0 (-)</td>
<td>2 (5)</td>
<td></td>
</tr>
<tr>
<td>Pre-core mutation¹</td>
<td>0 (-)</td>
<td>6 (16)</td>
<td>0.34</td>
</tr>
<tr>
<td>Basal core promoter mutation¹</td>
<td>2 (40)</td>
<td>14 (38)</td>
<td>0.93</td>
</tr>
<tr>
<td>Baseline liver biopsy¹</td>
<td>4 (80)</td>
<td>27 (69)</td>
<td>0.62</td>
</tr>
<tr>
<td>Median fibrosis score (range)²</td>
<td>2.5 (2-6)</td>
<td>1 (0-5)</td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>Cirrhosis (%)²</td>
<td>1 (25)</td>
<td>1 (4)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

(a) p-value for Student’s t test¹, Mann-Whitney U test², Fisher’s exact or Chi-square test³
(b) 31 HBeAg-positive patients had baseline liver biopsy material available for evaluation
### Supplementary table 5: Baseline characteristics of HBeAg-negative patients who had combined response at long term follow-up (week 144) compared with those with non-response. Combined response was defined as HBV-DNA < 2,000 IU/mL and ALT normalization.

<table>
<thead>
<tr>
<th>HBeAg negative (n=48)</th>
<th>Combined response</th>
<th>Non-response</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td>12 (25)</td>
<td>36 (75)</td>
<td></td>
</tr>
<tr>
<td>Mean age (years) (SD, range)</td>
<td>44.4 (12.0, 24-69)</td>
<td>42.6 (9.0, 26-62)</td>
<td>0.57</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>2 (17)</td>
<td>13 (36)</td>
<td>0.21</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian (%)</td>
<td>1 (8)</td>
<td>11 (31)</td>
<td>0.18</td>
</tr>
<tr>
<td>Asian (%)</td>
<td>3 (25)</td>
<td>11 (31)</td>
<td></td>
</tr>
<tr>
<td>African (%)</td>
<td>8 (67)</td>
<td>14 (39)</td>
<td></td>
</tr>
<tr>
<td>Median ALT (xULN) (range)</td>
<td>1.3 (1.1-7.3)</td>
<td>1.6 (0.6-27.9)</td>
<td>0.52</td>
</tr>
<tr>
<td>Interferon treatment naive (%)</td>
<td>7 (58)</td>
<td>27 (75)</td>
<td>0.27</td>
</tr>
<tr>
<td>NUC treatment naive (%)</td>
<td>10 (83)</td>
<td>34 (94)</td>
<td>0.23</td>
</tr>
<tr>
<td>Viral characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean HBV-DNA (log_{10} IU/ml) (SD, range)</td>
<td>4.8 (0.8, 3.6-6.4)</td>
<td>5.7 (1.1, 3.8-7.7)</td>
<td>0.01</td>
</tr>
<tr>
<td>Mean HBsAg (log_{10} IU/ml) (SD, range)</td>
<td>2.9 (0.9, 1.6-4.3)</td>
<td>3.5 (0.5, 2.6-4.5)</td>
<td>0.04</td>
</tr>
<tr>
<td>HBV genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (%)</td>
<td>3 (25)</td>
<td>8 (22)</td>
<td></td>
</tr>
<tr>
<td>B (%)</td>
<td>1 (8)</td>
<td>6 (17)</td>
<td></td>
</tr>
<tr>
<td>C (%)</td>
<td>2 (17)</td>
<td>3 (8)</td>
<td></td>
</tr>
<tr>
<td>D (%)</td>
<td>3 (25)</td>
<td>15 (42)</td>
<td></td>
</tr>
<tr>
<td>E (%)</td>
<td>3 (25)</td>
<td>4 (11)</td>
<td></td>
</tr>
<tr>
<td>Pre-core mutation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal core promoter mutation</td>
<td>7 (64)</td>
<td>29 (83)</td>
<td>0.18</td>
</tr>
<tr>
<td>Baseline liver biopsy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median fibrosis score (range)</td>
<td>1.0 (0-6)</td>
<td>1.5 (0-6)</td>
<td>0.66</td>
</tr>
<tr>
<td>Cirrhosis (%)</td>
<td>2 (20)</td>
<td>7 (23)</td>
<td>0.83</td>
</tr>
</tbody>
</table>

**Multivariable logistic regression (adjusted)**

<table>
<thead>
<tr>
<th>HBeAg-negative patients</th>
<th>OR</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV-DNA (log_{10} IU/ml)</td>
<td>0.46</td>
<td>0.20-1.05</td>
<td>0.07</td>
</tr>
<tr>
<td>HBsAg (log_{10} IU/ml)</td>
<td>0.30</td>
<td>0.09-0.93</td>
<td>0.04</td>
</tr>
</tbody>
</table>

(a) p-value for Student’s t test, Mann-Whitney U test, Fisher’s exact or Chi-square test
(b) 39 HBeAg-negative patients had baseline liver biopsy material available for evaluation
Supplementary table 6: Baseline characteristics of HBeAg-negative patients who lost HBsAg at long term follow-up (week 144) compared with those with HBsAg persistence.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HBsAg loss</th>
<th>HBsAg persistence</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (%)</td>
<td>8 (17)</td>
<td>40 (83)</td>
<td></td>
</tr>
<tr>
<td>Mean age (years) (SD, range)†</td>
<td>46.9 (12.9, 24-69)</td>
<td>42.3 (9.0, 26-62)</td>
<td>0.22</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>1 (13)</td>
<td>14 (35)</td>
<td>0.21</td>
</tr>
<tr>
<td>Ethnicity†</td>
<td></td>
<td></td>
<td>0.54</td>
</tr>
<tr>
<td>Caucasian (%)</td>
<td>1 (13)</td>
<td>11 (28)</td>
<td></td>
</tr>
<tr>
<td>Asian (%)</td>
<td>2 (25)</td>
<td>12 (30)</td>
<td></td>
</tr>
<tr>
<td>African (%)</td>
<td>5 (63)</td>
<td>17 (43)</td>
<td></td>
</tr>
<tr>
<td>Median ALT (xULN) (range)‡</td>
<td>1.1 (0.6-9.2)</td>
<td>1.8 (0.5-6.6)</td>
<td>0.18</td>
</tr>
<tr>
<td>Interferon treatment naïve (%)†</td>
<td>3 (38)</td>
<td>31 (78)</td>
<td>0.02</td>
</tr>
<tr>
<td>NUC treatment naïve (%)†</td>
<td>6 (75)</td>
<td>38 (95)</td>
<td>0.06</td>
</tr>
</tbody>
</table>

**Viral characteristics**

| Mean HBV-DNA (log10 IU/ml) (SD, range)† | 4.8 (0.9, 3.6-6.4) | 5.7 (1.1, 3.8-7.7) | 0.03|
| Mean HBsAg (log10 IU/ml) (SD, range)† | 2.4 (0.6, 1.6-3.2) | 3.5 (0.5, 2.6-4.5) | <0.001|

**HBV genotype†**

<table>
<thead>
<tr>
<th>A (%)</th>
<th>B (%)</th>
<th>C (%)</th>
<th>D (%)</th>
<th>E (%)</th>
<th>Pre-core mutation†</th>
<th>Basal core promoter mutation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 (38)</td>
<td>1 (13)</td>
<td>1 (13)</td>
<td>2 (25)</td>
<td>1 (13)</td>
<td>4 (50)</td>
<td>4 (50)</td>
</tr>
</tbody>
</table>

**Baseline liver biopsy**

| Median fibrosis score (range)‡    | 2 (0-6) | 1 (0-6) | 0.65 |
| Cirrhosis (%)†                   | 2 (29)  | 7 (21)  | 0.67 |

(a) p-value for Student’s t test†, Mann-Whitney U test‡, Fisher’s exact or Chi-square test†
(b) 39 HBeAg-negative patients had baseline liver biopsy material available for evaluation
Supplementary figure 7: Individual figures of 5 HBeAg-positive patients with HBsAg loss at week 144
HBV DNA (grey area), HBsAg (black line) and ALT (red line)
Part I

Supplementary figure B: Figures of 8 HBeAg-negative patients with HBsAg loss at week 144

HBV DNA (grey area), HBsAg (black line) and ALT (red line)
Baseline HBsAg as predictor of sustained HBsAg loss  Chapter 2

REFERENCE LIST


A Randomized Prospective Open-label Trial Comparing Peginterferon Plus Adefovir or Tenofovir Combination Therapy Versus No Treatment in HBeAg-Negative Chronic Hepatitis B Patients with a Low Viral Load.


1 Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, 2 Gastroenterology and Hepatology, Sint Lucas Andreas Hospital, Amsterdam, 3 Internal Medicine, Medical Center Zuiderzee, Lelystad, 4 Gastroenterology and Hepatology, VU Medical Center, Amsterdam, 5 Medical Microbiology, Academic Medical Center, Amsterdam, 6 Blood-borne Infections, Sanquin, Amsterdam, 7 Virus Diagnostic Services, Sanquin, Amsterdam 8 Pathology, Academic Medical Center, Amsterdam, Netherlands.

* Shared first authors
** Shared second authors

Submitted for publication
ABBREVIATIONS

ALT, alanine aminotransferase; cccDNA, CHB, chronic hepatitis B; cccDNA, covalently closed circular DNA; CR, combined response; HBeAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; iqr, interquartile range; LVL, low viral load; NUCs, nucleot(s)ide analogues; peg-IFN, pegylated interferon alfa 2a; ULN, upper limit of normal; Week 72, 24 weeks of treatment free follow-up.
ABSTRACT

Background and aims
Antiviral treatment is currently not recommended for chronic hepatitis B (CHB) patients with a low viral load (LVL). However, they are still at risk to develop cirrhosis or hepatocellular carcinoma. Here, we assessed HBsAg loss and decline during peginterferon-alfa (peg-IFN) and nucleotide analogue combination therapy in CHB patients with LVL.

Methods
134 patients (HBeAg-negative, HBV-DNA <20,000 IU/mL) were randomized 1:1:1 to receive peg-IFN plus adefovir (arm I; n=46), peg-IFN plus tenofovir (arm II; n=45) or no treatment (arm III; n=43) for 48 weeks, followed by 24 weeks of treatment-free follow-up (Week 72).

Results
At Week 72, 4 patients receiving either of the combination therapies but none of the untreated patients had achieved HBsAg loss (4.4% vs 0%, p=0.31). HBsAg levels had declined significantly in all study arms at Week 72: -0.53 (p<0.001), -0.59 (p<0.001), and -0.15 (p<0.001) mean log_{10} IU/mL reduction for arms I, II, and III, respectively. HBsAg declined more strongly in treatment arms I (p=0.004) and II (p=0.004) than in the control arm III.

An HBsAg decline of >1.0 log_{10} IU/mL at end-of-treatment was observed in 17 treated patients (21%), but in none of the untreated patients (p<0.001); independent predictors were a higher on-treatment ALT level (p=0.003), and a lower Week 12 HBsAg level (p=0.002).

Conclusion
In CHB patients with LVL, combination treatment can result in HBsAg loss and a stronger HBsAg decline compared to controls, which may indicate a further increase in the rate of HBsAg loss if the follow up is extended.
Part I

INTRODUCTION

Despite the availability of a safe and effective vaccine for more than three decades, infection with hepatitis B virus (HBV) is still a major public health problem, with approximately 350 million chronically infected individuals worldwide [1].

The goal of antiviral treatment in patients with chronic hepatitis B (CHB) is to prevent progression to cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC) by reducing viral replication [2, 3]. The decision to start treatment is obvious in patients with a high viral load (HVL), progressive liver inflammation and fibrosis. In contrast, for patients with a low viral load (LVL) there is currently no indication for treatment, since the progression of liver disease tends to be slower [2, 4]. There is, however, no solid evidence that HBV infection is harmless in these patients, who still carry a significant risk for the development of cirrhosis and HCC [5-8]. It is difficult to reliably identify the so-called 'carriers of inactive HBV infection with persistently normal alanine aminotransferase (ALT) levels and HBV DNA <2,000 IU/mL by single laboratory measurements [9, 10]. Importantly, 20-30% of HBeAg-negative patients with LVL will develop reactivation, with progression to an immune active phase with persistently or transiently elevated ALT and high serum HBV DNA levels [9, 11-14].

The most favorable outcome in the treatment of CHB patients is the clearance of hepatitis B surface antigen (HBsAg) with a durable formation of anti-HBs antibodies, indicating complete immunological control [15-17]. Unfortunately, with a finite course of pegylated interferon-α (peg-IFN) or long-term viral suppression with nucleos(t)ide analogues (NUCs), this functional cure is rarely achieved [18].

Previous attempts to improve response rates with Peg-IFN and NUC combination therapy have been disappointing when lamivudine was used [19-21]. Nevertheless, combining Peg-IFN with more potent NUCs remained of interest because of the dual effect on both the innate and adaptive immune responses [22]. In our previous study, in which active CHB patients with HVL were treated with peg-IFN and adefovir, a relatively high rate of HBsAg loss (17%) was observed in HBeAg-negative patients 2 years after therapy [23]. Moreover, lower HBsAg levels at baseline were associated with HBsAg loss. The majority (70-80%) of all CHB patients worldwide have LVL. In contrast to CHB patients with HVL, they exhibit low HBsAg levels [24]. Hypothetically, these patients may be more susceptible to HBV treatment as they have an antiviral immune response that is already capable of keeping HBV DNA and HBsAg at low levels, possibly related to the finding that T-cell inhibition is less severe in the presence of low HBV levels [25]. Furthermore, the innate immune activation induced by Peg-IFN is known to improve with lower HBV DNA levels [26]. Next to its antiviral effect, adefovir has been shown to enhance innate immune functions in mice, suggesting a possible synergistic when combined with Peg-IFN [27]. More recently, adefovir has been largely replaced by tenofovir, which has a similar mechanism of action, but is more potent against HBV [28]. Indeed, a large randomized trial with active CHB patients, showed a higher rate of HBsAg loss in patients treated with peg-IFN and tenofovir than in those receiving monotherapy [29].
These observations led to the hypothesis that combination treatment with Peg-IFN and adefovir, and possibly tenofovir, leads to improved rates of HBsAg loss in CHB patients with LVL. However, no randomized controlled studies on the efficacy of peg-IFN or NUCs in LVL patients have been performed. In this prospective, randomized controlled study, we compared combination therapies of peg-IFN plus adefovir or tenofovir versus no treatment in HBeAg-negative CHB patients with LVL. The aim of this study was to investigate the rate of HBsAg loss and decline, and to study markers of response.

METHODS

Patients
CHB patients, aged 18-70 years, with HBV-DNA < 20,000 IU/mL were enrolled after assessment of eligibility. Major inclusion criteria were documented HBsAg positivity, HBeAg negativity, and anti-HBe positivity for more than 6 months. Exclusion criteria were concurrent infection with hepatitis C virus, hepatitis delta virus, or HIV; decompensated liver disease, HCC or a history of bleeding from esophageal varices; pregnancy or breast feeding; and ALT levels > 5 xULN. Patients were either treatment naive, or had received (peg-)interferon or nucleos(t)ide analogues more than 3 or 6 months before inclusion, respectively. The full eligibility criteria are provided in Supplementary information S1.

The study complied with the Declaration of Helsinki and the principles of Good Clinical Practice and was approved by a legally instituted ethical committee (ClinicalTrials.gov; NCT00973219). All patients gave written informed consent.

Study design
This investigator-initiated, prospective, open-label, randomized controlled trial was performed at the Academic Medical Center (AMC), Amsterdam, The Netherlands.

Patients were randomized 1:1:1 to receive pegylated interferon alfa-2a (peg-IFN (Pegasys®; Hoffmann La Roche, Basel, Switzerland)180 µg/week, subcutaneously) in combination with adefovir dipivoxil (Hepsera®; Gilead Sciences, Foster City, CA, USA, 10 mg once daily) in arm I, peg-IFN plus tenofovir disoproxil fumarate (Viread®; Gilead Sciences, Foster City, CA, USA, 245 mg once daily) in arm II, or no treatment in arm III. Randomization was stratified according to HBV genotype A, non-A (B-G), or indeterminable genotype. After 48 weeks, treatment was discontinued and all patients were followed till Week 72.

Laboratory assays

Biochemical and virological analyses
Routine examinations and laboratory tests were performed at regular intervals (Supplementary information S1). Plasma HBV-DNA level was determined by the COBAS TaqMan assay (F. Hoffmann-La Roche Ltd, Basel, Switzerland). Serum HBsAg level was quantified by the Architect
(Abbott Diagnostics, Abbott Park, IL, USA) [7]. Qualitative detection of serum HBsAg, antibody to HBsAg (anti-HBs), HBeAg and antibody to HBeAg (anti-HBe) was performed by an enzyme immunoassay (AxSYM; Abbott Laboratories, Abbott Park, IL, USA). ALT levels were expressed as absolute values (U/L) or relative to the ULN range. ALT reference values were 45 U/L for males and 34 U/L for females.

**Histological analyses**

For histological assessment of liver biopsies the modified Ishak scoring system was applied, based on a zero to 18 score for necroinflammation and a zero to 6 score for fibrosis [30]. Immunohistochemical detection of HBsAg by monoclonal anti-HBs antibodies (Neomarkers, Fremont, CA, USA) was expressed as the percentage of all hepatocytes.

**Response definitions**

The primary end point was HBsAg loss at Week 72. HBsAg loss was defined as undetectable serum HBsAg by AxSYM (<0.05 IU/mL). HBsAg seroconversion was defined as HBsAg loss with the formation of anti-HBs (anti-HBs >10 IU/mL).

Additional end points included HBsAg loss at Week 48 and >0.5 or >1 log₁₀ IU/mL HBsAg decline at Week 48 and 72. Patients were considered to be non-responder when not meeting the criteria for HBsAg loss or HBsAg decline.

**Statistical analysis**

The sample size calculation was based on the primary endpoint. The assumed response rates were 20% for patients treated in each combination arm versus 1% for patients in the control group (receiving no treatment). A group sample of 44 patients in the control group was needed to achieve a power of 81% to detect a statistically significant difference with either of the treatment arms at the α-level of 0.05 (two-sided Fisher’s exact test). Assuming a 10% drop-out rate, 150 patients were needed for this study. For the primary analysis on the proportion of patients with HBsAg loss, a modified intention-to-treat (ITT) model was applied, including all patients who received at least one dose of study medication (treatment arms) or performed at least one study visit (control arm). Patients who prematurely discontinued treatment were scored as non-responders. In the secondary analysis on HBsAg decline, only patients who completed 48 weeks of treatment and 24 weeks of treatment-free follow-up were included (per-protocol (PP) model).

Baseline and on-treatment variables were compared between study arms using Student’s t, Mann-Whitney U, Chi-square or Fisher’s exact test. The associations between variables as potential predictors of HBsAg loss or HBsAg decline were examined by multivariable logistic regression analysis. Statistical comparisons were performed using IBM SPSS Statistics, version 21 (IBM Corp., Chicago, IL, USA). All p-values are two sided and values below 0.05 were considered statistically significant.
RESULTS

1. Patients
In total, 167 patients were screened for participation in the study, of whom 151 patients were randomized. Between September 2009 and November 2014, 134 patients received intervention or no-treatment. The 17 patients who withdrew consent after randomization were equally distributed among the randomization arms (Figure 1). Details on patient characteristics are shown in Table 1. Patients were predominantly male (58%) and in their fifth decade (mean 43.0 ± 11.1 years). Ethnical background was mixed, as well as the corresponding HBV genotypes. All patients were HBeAg-negative, with a relatively low viral load (mean HBV-DNA 2.74 log$_{10}$ IU/mL, normal ALT levels (median 27 U/L, iqr 21-37) and minimal liver fibrosis (mean Fibroscan value 5.4 ± 1.9 kPa).

Figure 1: CONSORT flow diagram.

2. Safety
Of the 134 patients who received study interventions or no-treatment (intention-to-treat analysis; ITT), 12 patients prematurely discontinued: 11 in the treatment arms and 1 in the control arm (Figure 1). Reasons for treatment discontinuation in the peg-IFN + adefovir arm were alcohol-related pancreatitis (n=1; at 6 weeks), dizziness (n=1; at 8 weeks), hair loss (n=1; at 24 weeks), back pain (n=1; at 38 weeks), and laboratory abnormalities with concomitant alcohol abuse (n=1; at 42 weeks). Treatment in the peg-IFN + tenofovir arm was discontinued because of general interferon-related side effects (n=2; at 1 and 8 weeks), nausea and vomiting (n=1; at
Table 1: Baseline characteristics. Variables are shown for all randomized patients (n=151) according to treatment arm.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Treatment arms</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arm I; Peg-IFN + adefovir</td>
<td>Arm II; Peg-IFN + tenofovir</td>
<td>Arm III; No Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>21 (40)</td>
<td>25 (49)</td>
<td>18 (38)</td>
<td>16 (40)</td>
<td>.479 c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age, years (SD)</td>
<td>44.0 (11.8)</td>
<td>42.8 (11.5)</td>
<td>42.0 (9.9)</td>
<td>64.4 (9.9)</td>
<td>.684 a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian, n (%)</td>
<td>11 (24)</td>
<td>14 (31)</td>
<td>17 (40)</td>
<td>24 (39)</td>
<td>.585 c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian, n (%)</td>
<td>7 (15)</td>
<td>11 (24)</td>
<td>6 (16)</td>
<td>7 (11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African, n (%)</td>
<td>20 (43)</td>
<td>11 (24)</td>
<td>14 (33)</td>
<td>24 (38)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South American, n (%)</td>
<td>8 (17)</td>
<td>9 (20)</td>
<td>5 (12)</td>
<td>8 (12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN naïve, n (%)</td>
<td>43 (93)</td>
<td>42 (93)</td>
<td>43 (100)</td>
<td>93 (100)</td>
<td>.227 c</td>
<td></td>
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<tr>
<td><strong>Laboratory</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median ALT, U/L (iqr)</td>
<td>27 (21-40)</td>
<td>26 (20-30)</td>
<td>30 (22-43)</td>
<td>27 (20-30)</td>
<td>.189 b</td>
<td></td>
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</tr>
<tr>
<td>HBV Genotype</td>
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<td></td>
<td></td>
<td>.893 c</td>
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<td>Indeterminable, n (%)</td>
<td>12 (23)</td>
<td>13 (25)</td>
<td>9 (19)</td>
<td>17 (29)</td>
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<tr>
<td>A, n (%)</td>
<td>11 (21)</td>
<td>10 (20)</td>
<td>8 (17)</td>
<td>10 (17)</td>
<td></td>
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<tr>
<td>B, n (%)</td>
<td>5 (10)</td>
<td>3 (6)</td>
<td>2 (4)</td>
<td>2 (4)</td>
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<tr>
<td>C, n (%)</td>
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<td>2 (4)</td>
<td>3 (6)</td>
<td>2 (4)</td>
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<tr>
<td>D, n (%)</td>
<td>11 (21)</td>
<td>15 (29)</td>
<td>14 (29)</td>
<td>15 (29)</td>
<td></td>
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<tr>
<td>E, n (%)</td>
<td>10 (19)</td>
<td>8 (16)</td>
<td>11 (23)</td>
<td>9 (16)</td>
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<tr>
<td>F, n (%)</td>
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<td>G, n (%)</td>
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<td>0 (0)</td>
<td>1 (2)</td>
<td>0 (0)</td>
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<tr>
<td>Mean HBV-DNA, log_{10} IU/mL (SD)</td>
<td>2.65 (1.23)</td>
<td>2.79 (1.03)</td>
<td>2.79 (1.04)</td>
<td>2.79 (1.04)</td>
<td>.796 a</td>
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<td>Mean HBsAg, log_{10} IU/mL (SD)</td>
<td>3.21 (0.98)</td>
<td>3.31 (0.76)</td>
<td>3.07 (0.87)</td>
<td>3.07 (0.87)</td>
<td>.431 a</td>
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<tr>
<td>Fibroscans performed, n (%)</td>
<td>41 (79)</td>
<td>36 (71)</td>
<td>40 (83)</td>
<td>36 (71)</td>
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<tr>
<td>Mean value, kPa (SD)</td>
<td>5.0 (1.8)</td>
<td>5.4 (1.8)</td>
<td>5.8 (2.0)</td>
<td>5.8 (2.0)</td>
<td>.169 a</td>
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<tr>
<td><strong>Liver biopsy</strong></td>
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<tr>
<td>Liver biopsies performed, n (%)</td>
<td>40 (77)</td>
<td>43 (84)</td>
<td>21 (44)</td>
<td>84 (44)</td>
<td>.003 a</td>
<td></td>
<td></td>
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<tr>
<td>Mean biopsy length, mm (SD)</td>
<td>17 (7)</td>
<td>21 (9)</td>
<td>14 (6)</td>
<td>21 (9)</td>
<td>.003 a</td>
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<td>Mean portal fields, n (SD)</td>
<td>11 (5)</td>
<td>13 (6)</td>
<td>10 (6)</td>
<td>13 (6)</td>
<td>.078 a</td>
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<tr>
<td>Median inflammatory score, (iqr)</td>
<td>2 (2-3)</td>
<td>2 (1-3)</td>
<td>2 (2-2)</td>
<td>2 (2-2)</td>
<td>.871 b</td>
<td></td>
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<tr>
<td>Median Ishak fibrosis score, (iqr)</td>
<td>1 (1-1)</td>
<td>1 (1-1)</td>
<td>1 (1-1)</td>
<td>1 (1-1)</td>
<td>.627 b</td>
<td></td>
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<tr>
<td>Median steatosis, grade (iqr)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>.942 b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median % HBsAg staining, (iqr)</td>
<td>10 (1-35)</td>
<td>25 (5-45)</td>
<td>10 (5-25)</td>
<td>25 (5-45)</td>
<td>.232 b</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Differences in baseline variables between randomization arms were assessed by Oneway ANOVA (a), Kruskal-Wallis test (b), or Pearson’s Chi-squared test (c). SD, standard deviation; iqr, interquartile range.
Combination therapy in CHB patients with low viral load  Chapter 3

12 weeks), depression (n=2; at 12 and 23 weeks), and hair loss (n=1; at 24 weeks). One patient in the no treatment arm was lost to follow-up after 6 weeks.

The most common adverse events and all serious adverse events are summarized in Supplementary Table S2. During treatment 48/91 (53%) of patients in the intervention arms had ALT levels $>2 \times$ ULN, compared to 3/43 (7%) of the controls ($p<0.001$). During follow-up, the rate of ALT levels $>2 \times$ ULN was comparable in the two treated groups: 7/91 (8%) vs 4/39 (9%), respectively ($p=0.751$).

3. Efficacy

3.1 HBsAg loss

HBsAg response rates at Week 48 and Week 72 are shown in Table 2. At Week 48, 4 patients receiving combination therapy but none of the untreated patients had achieved HBsAg loss (ITT 4.4% vs 0%, $p=0.31$ and per-protocol 5.3% vs 0%, $p=0.30$). Patients with HBsAg loss were in arm I (n=1) or arm II (n=3), and had HBV genotype A (n=1), B (n=1), or indeterminable (n=2). Three of 4 patients had anti-HBs $>10$ IU/L. During the follow-up, the patient without anti-HBs sero-conversion (treatment arm II, indeterminable genotype) sero-reverted to HBsAg positivity to levels around the detection limit at week 72. One patient who was HBsAg positive at week 48 (arm I, indeterminable HBV genotype) became HBsAg negative at week 72, without detectable anti-HBs.

Figures of virological parameters in individual patients who achieved HBsAg loss during treatment and/or follow-up (n=5) are shown in Figure 2.

Table 2: HBsAg response rates. Variables are shown for all patients who completed study interventions (per-protocol population; n=122) according to treatment arm. Arms represent Peg-IFN plus adefovir (Arm I; n=41), Peg-IFN plus tenofovir (Arm II, n=39), and untreated patients (Arm III, n=42), respectively.

<table>
<thead>
<tr>
<th>HBsAg response</th>
<th>Arm I</th>
<th>Arm II</th>
<th>Arm I + II (Treated)</th>
<th>Arm III (Untreated)</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>End-of-treatment (Week 48)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg negative, n (%)</td>
<td>1 (2)</td>
<td>3 (8)</td>
<td>4 (5)</td>
<td>0 (0)</td>
<td>$0.143^b$</td>
</tr>
<tr>
<td>HBsAg &lt; 10 IU/mL, n (%)</td>
<td>4 (10)</td>
<td>6 (15)</td>
<td>10 (12)</td>
<td>1 (2)</td>
<td>$0.069^b$</td>
</tr>
<tr>
<td>Mean HBsAg decline, Log$_{10}$ IU/mL (SD)</td>
<td>-.61 (.92)</td>
<td>-.61 (.96)</td>
<td>-.61 (.94)</td>
<td>-.06 (.18)</td>
<td>$0.000^a$</td>
</tr>
<tr>
<td>$&gt;1$ Log$_{10}$ IU/mL decline, n (%)</td>
<td>9 (22)</td>
<td>8 (21)</td>
<td>17 (21)</td>
<td>0 (0)</td>
<td>$0.001^b$</td>
</tr>
<tr>
<td>$&gt;0.5$ Log$_{10}$ IU/mL decline, n (%)</td>
<td>15 (37)</td>
<td>10 (26)</td>
<td>25 (31)</td>
<td>0 (0)</td>
<td>$0.000^b$</td>
</tr>
<tr>
<td>End-of-follow-up (Week 72)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg negative, n (%)</td>
<td>2 (5)</td>
<td>2 (5)</td>
<td>4 (5)</td>
<td>0 (0)</td>
<td>$0.143^b$</td>
</tr>
<tr>
<td>HBsAg &lt; 10 IU/mL, n (%)</td>
<td>5 (12)</td>
<td>5 (13)</td>
<td>10 (12)</td>
<td>2 (5)</td>
<td>$0.185^b$</td>
</tr>
<tr>
<td>Mean HBsAg decline, Log$_{10}$ IU/mL (SD)</td>
<td>-.53 (.77)</td>
<td>-.59 (.85)</td>
<td>-.56 (.81)</td>
<td>-.16 (.22)</td>
<td>$0.000^a$</td>
</tr>
<tr>
<td>$&gt;1$ Log$_{10}$ IU/mL decline, n (%)</td>
<td>5 (12)</td>
<td>6 (15)</td>
<td>11 (14)</td>
<td>0 (0)</td>
<td>$0.012^b$</td>
</tr>
<tr>
<td>$&gt;0.5$ Log$_{10}$ IU/mL decline, n (%)</td>
<td>13 (32)</td>
<td>14 (36)</td>
<td>27 (34)</td>
<td>3 (7)</td>
<td>$0.001^b$</td>
</tr>
</tbody>
</table>

Differences between treated (arm I + II) and untreated patients (arm III) were assessed by Welch’s T-test (a), or Pearson Chi-square test (b).
3.2 HBsAg decline at end-of-treatment (week 48)
In a per-protocol analysis, mean HBsAg level had declined significantly in all study arms at week 48; mean -0.61 (p<0.001), -0.62 (p<0.001), and -0.06 (p=0.042) log$_{10}$ IU/mL reduction for arms I, II, and III, respectively (Table 2). No difference in HBsAg decline was observed between the two treatment arms. However, HBsAg declined more strongly in the treatment arms I (p<0.001) and II (p=0.002) than in the control arm III (Figure 3AB). An HBsAg decline of >1.0 log$_{10}$ IU/mL was observed in 17 treated patients (21%), but in none of the untreated patients (p<0.001).

3.3 HBsAg decline at end-of-follow-up (week 72)
During follow-up, HBsAg levels remained significantly lower than pretreatment levels in all arms; -0.53 (p<0.001), -0.59 (p<0.001), and -0.15 (p<0.001) mean log$_{10}$ IU/mL reduction at week 72 for arms I, II, and III, respectively (Table 2 and Figure 3AB). Despite the slight increase in mean HBsAg
levels during follow-up of treated patients, the decline in HBsAg remained significantly larger in the treatment arms I ($p=0.004$) and II ($p=0.004$) compared to that in the control arm.

The increase in HBsAg level during the treatment-free follow-up (HBsAg rebound) was particularly pronounced in patients with an HBsAg decline of $>1.0 \log_{10} \text{IU/mL}$ at week 48, who remained HBsAg-positive ($n=13$) (Figure 3C). Of these, 12/13 had an HBsAg rebound during the treatment-free follow-up (mean $+0.84 \log_{10} \text{IU/mL}$ increase at week 72 compared to week 48).

The decline in HBV-DNA level and subsequent HBV-DNA rebound did not differ between patients with or without $>1.0 \log_{10}$ HBsAg decline (Figure 3D).

**Figure 3:** HBsAg and HBV-DNA decline according to treatment arm. Mean $\log_{10}$ IU/mL decline in HBsAg (A) and HBV-DNA (B) compared to baseline. Data is shown for the per-protocol population; no therapy ($n=42$), Peg-IFN plus adefovir ($n=41$), and Peg-IFN plus tenofovir ($n=39$). Mean $\log_{10}$ IU/mL decline in HBsAg (C) and HBV-DNA (D) compared to baseline in patients with a strong on-treatment HBsAg decline. Data is shown for the per-protocol population; no therapy ($n=42$), treated patients with $<1 \log_{10}$ IU/mL HBsAg decline at Week 48 ($n=63$), and treated patients with $>1 \log_{10}$ IU/mL HBsAg decline at Week 48 ($n=17$).
4. HBsAg response prediction

Baseline and early on-treatment variables were compared between treated patients with >1 log reduction in HBsAg level at Week 48 and those with <1 log reduction (Supplementary table S3). Because the decline in HBsAg between treatment arms I and II were comparable, these were combined for further analysis. Significant predictors of HBsAg decline in treated patients in univariable analysis were male sex ($p=0.041$), higher maximum on-treatment ALT level ($p=0.003$), and lower Week 12 HBsAg level ($p=0.002$). Both on-treatment ALT increase, as well as HBsAg level at Week 12 were independent predictors of HBsAg decline at Week 48, in different multivariable logistic regression models (Table 3).

Table 3: Multivariable analysis of on-treatment HBsAg decline. Baseline and early on-treatment variables significantly associated with >1 log HBsAg reduction at Week 48 in univariable logistic regression analysis were evaluated in 2 multivariable models. B, regression coefficient; SE, standard error; p, p-value.

<table>
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<th>Univariable Analysis</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 2</th>
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<tr>
<td></td>
<td>B</td>
<td>SE</td>
<td>p</td>
<td>B</td>
</tr>
<tr>
<td>Female sex</td>
<td>-1.27</td>
<td>.63</td>
<td>.041</td>
<td>-1.92</td>
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<td>.02</td>
<td>.397</td>
<td>-</td>
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<td>Maximum ALT (log$_{10}$ U/L)</td>
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<td>1.12</td>
<td>.003</td>
<td>-</td>
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<td>.006</td>
<td>.46</td>
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<td>HBV genotype A</td>
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<td>.683</td>
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<tr>
<td>Baseline HBV-DNA (log$_{10}$ U/L)</td>
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<td>.24</td>
<td>.341</td>
<td>-</td>
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<td>Week 12 HBV-DNA (log$_{10}$ U/L)</td>
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<td>.52</td>
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<td>.28</td>
<td>.160</td>
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<td>Week 12 HBsAg (log$_{10}$ U/L)</td>
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<td>.27</td>
<td>.002</td>
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<tr>
<td>Week 12 HBsAg decline</td>
<td>-3.80</td>
<td>1.12</td>
<td>.001</td>
<td>-4.21</td>
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</tbody>
</table>

DISCUSSION

There is a clinical need for finding therapeutic strategies to clear HBsAg, currently considered as the ultimate endpoint in the treatment of CHB patients [16, 31]. This is the first study in which it is shown that HBsAg clearance and strong HBsAg decline can be achieved upon peg-interferon/NUC combination therapy in CHB patients with LVL, currently not eligible for treatment.

Worldwide more than 200 million CHB patients with LVL are not selected for treatment since current treatment strategies are directed at viral suppression and not at HBsAg loss. However, studies in HBeAg-negative CHB patients with HVL with peg-IFN mono- or combination therapy showed that a low baseline HBsAg level and its reduction during treatment increase the chance of HBsAg loss [23, 32]. These findings support a beneficial effect of treating patients with LVL and low HBsAg levels [24]. The observation that HBV infection in patients with LVL is not completely harmless and may still progress to active CHB, cirrhosis or HCC, further supports the suggestion that these patients may benefit from treatment-induced HBsAg loss and complete viral control.
We showed that 48 Weeks of peg-IFN and nucleotide analogue combination therapy resulted in a $>1 \log$ HBsAg decline and even HBsAg loss in 21% and 4% of patients with LVL at week 72, respectively. In contrast to those, no strong HBsAg decline ($>1 \log_{10}$ IU/mL) was observed in the untreated control group.

Although treatment was generally well tolerated and no unexpected severe adverse events occurred, peg-IFN based treatment strategies are costly and associated with a range of side effects. It may therefore be too early to state that combination therapy needs to be commenced in all LVL patients. First, longer follow-up needs to reveal whether the strong HBsAg decline observed in our study may lead to higher rates of HBsAg loss. Next, several findings from this study could be used to further optimize treatment strategies.

We observed that in most patients with $>1 \log_{10}$ IU/mL HBsAg decline (who were still HBsAg positive at Week 48) HBsAg levels increased during the treatment-free follow-up period (HBsAg rebound). This may suggest the need for a period of consolidation therapy to ensure that HBsAg decline is sustained. Indeed, consolidation therapy was effective in preventing post-treatment HBsAg rebound in peg-IFN treated CHB patients with an initial high viral load. Continuing entecavir for an additional 24 weeks in HBeAg-positive patients after 48 weeks of Peg-IFN and entecavir combination therapy resulted in a weaker HBsAg rebound than in patients treated with Peg-IFN alone [33, 34]. A different approach may be increasing the peg-IFN therapy duration to 96 weeks, which has been shown to result in higher rates of HBsAg decline $<10$ IU/ml, compared to 48 weeks of peg-IFN [35].

Patients in our study in general had normal pre-treatment ALT levels with limited liver fibrosis or inflammation. Nevertheless, patients with a strong HBsAg decline showed a marked rise in ALT, which was more pronounced than in patients with a limited HBsAg decline. This could indicate that immune responses necessary to clear infected hepatocytes can indeed be enhanced by therapy in this patient group. The finding of HBsAg loss in patients with LVL may have future implications for immunological research. HBV-specific T cells are more abundantly present in the peripheral blood of patients with LVL than with HVL, and could therefore be more easily studied [36-38]. Understanding the immunological mechanisms of protection in these patients may help to develop novel immune-modulating strategies to restore effective antiviral responses in CHB patients. In addition, these immunological markers, as well as genetic or on-treatment virological markers, may be used to select those LVL patients who are most likely to benefit from combination therapy by achieving a durable off-treatment response.

In conclusion, this is the first study which showed that strong HBsAg decline and HBsAg clearance can be achieved upon peg-interferon based combination therapy in CHB patients with LVL. Although it remains to be investigated whether such strong HBsAg decline and loss further improves outcome in terms of cirrhosis or HCC-free survival in these patients, our finding may lead to extension of therapy indications for CHB patients with LVL.
ACKNOWLEDGEMENTS

We would like to express great appreciation to Martine Peters, Jeltje Helder, and Anja van Ek for patient follow-up and sampling. We thank Marjan Sinnige for handling patient material and performing additional immunological analyses. Valuable language suggestions have been provided by prof. Engelfriet.
SUPPLEMENTARY INFORMATION S1: ADDITIONAL INFORMATION FROM STUDY PROTOCOL

STUDY DESIGN
This is a three arm open-label prospective randomized controlled trial. (n=150) The trial is designed to compare the efficacy of treatment with Peg-IFN and ADF or Peg-IFN and TDF versus no treatment for clearance of the HBsAg in chronic hepatitis B patients with low viral load.

Patients will be enrolled into the study after assessment of eligibility. Group 1 will consist of patients treated with Peg-IFN 180µg once weekly and ADF 10mg once daily (standard doses), group 2 will consist of patients treated with Peg-IFN 180µg once weekly and TDF 300mg (equivalent to 245 mg tenofovir disoproxil) once daily (standard doses) and group 3 will consist of untreated patients as negative controls.

The trial consists of a screening period of approximately 4 weeks, a 48 week treatment period, a 24 week follow-up period and a long term follow up period of 5 year. A schematic overview of the study design is presented in table 1 and 2 (see appendix).

150 patients will be randomized in a 50:50:50 ratio to one of the treatment groups. Randomization will be stratified to genotype A to optimize balance between treatment groups. All patients will receive medication for the period of 48 weeks. For enrolment into the study a liver biopsy at time of enrolment is compulsory for patients selected in the treatment groups. For enrolment of patients that are selected in the non-treatment group a liver biopsy is optional but not compulsory for participation. In patients selected in the treatment group a second liver biopsy at week 48 is recommended for evaluation of therapeutic responses and to guide any further possible therapy. Due to the burden of undergoing a liver biopsy, the second liver biopsy is not compulsory to take part into the study. Patients that are selected in the non treatment group will not undergo a biopsy at week 48. Patients will be clinically monitored intensively according to the assessment schedule (table 1). In the long term follow up period patients will be clinically monitored each year.

Amendments that were made to the protocol:
Due to the burden of undergoing a liver biopsy, the requirements for undergoing a liver biopsy were altered during inclusion of the last 20 patients. For patients selected in the treatment groups a liver biopsy before the start of treatment was not advisable but not compulsory for participation in the study.

STUDY POPULATION
Population (base)
Patients will be enrolled into the study after assessment of eligibility. Target population will consist of adult patients (≥ 18 years) with chronic hepatitis B (HBsAg positivity ≥ 6 months), HBeAg negativity, HBV DNA < 20,000 IU/ml and ALT < 5* upper limit of normal.
Part I

Inclusion criteria
1. Male and female patients ≥ 18 and ≤ 70 years of age
2. Positive HBsAg for more than 6 months.
3. Negative for HBeAg for more than 6 months.
4. HBV DNA < 20,000 IU/ml
5. Patients with chronic hepatitis B who are either naive to antiviral treatment, or have received either interferon (IFN) or nucleoside/nucleotide analogues in the past but are still positive for HBsAg.
6. Serum ALT ≤ 5 * ULN as determined by two values taken ≥ 14 days apart during the six months before the first dose of study drug with at least one of the determinations obtained during the screening period.
7. Negative urine or serum pregnancy test (for women of childbearing potential) documented within the 24-hour period prior to the first dose of test drug.

Exclusion criteria
1. Patients co-infected with HCV, HIV or who have decompensated liver disease, hepatocellular carcinoma, significant cardiac disease, significant renal disease, seizure disorders or severe retinopathy.
2. Patients who have received nucleos(t)ide analogues for their chronic hepatitis B within 6 weeks before enrolment or have received Peg-IFN within 3 months before enrolment.
3. Patients must not have received any other systemic anti-viral, anti-neoplastic or immunomodulatory treatment (including supraphysiologic doses of steroids or radiation) ≤ 3 months prior to the first dose of study drug or the expectation that such treatment will be needed at any time during the study.
4. Positive test at screening for anti-HAV IgM, anti-HIV, HCV RNA. (Patients that have cleared the hepatitis C virus can be included in the study)
5. Patients who are expected to need systemic antiviral therapy other than that provided by the study at any time during their participation in the study are also excluded. Exception: patients who have had a limited (≤ 7 day) course of acyclovir for herpetic lesions more than 1 month prior to the first administration of test drug are not excluded.
6. Evidence of decompensated liver disease (Child pugh B-C)
7. Serum total bilirubin > twice the upper limit of normal at screening
8. History or other evidence of bleeding from esophageal varices or other conditions consistent with decompensated liver disease.
9. History or other evidence of a medical condition associated with chronic liver disease.
other than HBV (e.g., hemochromatosis, autoimmune hepatitis, metabolic liver diseases including Wilson’s disease and alfa1-antitrypsin deficiency, alcoholic liver disease, toxin exposures, thalassemia).

10. Women with ongoing pregnancy or who are breast feeding.

11. Neutrophil count <1500 cells/mm$^3$ or platelet count <80,000 cells/mm$^3$ at screening.

12. Hemoglobin < 7.1 mmol/L (<11.5 g/dL) for females and < 7.8 mmol/L (<12.5 g/dL) for men at screening.

13. Serum creatinine level >1.5 times the upper limit of normal at screening.

14. Unstable ongoing severe psychiatric disease, especially depression (stable patients can be included).

15. History of immunologically mediated disease (e.g., inflammatory bowel disease, idiopathic thrombocytopenic purpura, lupus erythematosus, autoimmune hemolytic anemia, scleroderma, severe psoriasis, rheumatoid arthritis).

16. History or other evidence of chronic pulmonary and cardiac disease associated with functional limitation. Severe cardiac disease (e.g., NYHA Functional Class III or IV, myocardial infarction within 6 months, ventricular tachyarrhythmias requiring ongoing treatment, unstable angina or other significant cardiovascular diseases).

17. History of a severe seizure disorder or current anticonvulsant use and clinically unstable disease.

18. Evidence of an active or suspected cancer or a history of malignancy where the risk of recurrence is ≥20% within 5 years. Patients with a lesion suspicious of hepatic malignancy on a screening imaging study will only be eligible if the likelihood of carcinoma is ≤10% following an appropriate evaluation.

19. Major organ transplantation. (patients with skin, cornea or bone transplantation are allowed to be included into the study)

20. Thyroid disease with thyroid function poorly controlled on prescribed medications. Patients with elevated thyroid stimulating hormone or T4 concentrations, with elevation of antibodies to thyroid peroxidase and any clinical manifestations of thyroid disease that are not stable on prescribed medication are excluded. Stable patients can be included.

21. History or other evidence of severe retinopathy (e.g. CMV retinitis, macula degeneration) or clinically relevant ophthalmological disorder due to diabetes mellitus or hypertension.

22. Inability or unwillingness to provide informed consent or abide by the requirements of the study.
Part I

23. History or other evidence of severe illness or any other conditions which would make the patient, in the opinion of the investigator, unsuitable for the study.

24. Patients with a value of alfa-fetoprotein >100 ng/mL are excluded, unless stability (less than 10% increase) has been documented over at least the previous 3 months.

25. Evidence of current hard drug(s) (i.e. cannabis products are allowed) and/or alcohol abuse (20g/day for women and 30g/day for men).

26. Patients included in another trial or having been given investigational drugs within 12 weeks prior to screening.
## Schedule of Assessments

**Table 1: Schedule of Assessments**

<table>
<thead>
<tr>
<th>Assessment/Procedure</th>
<th>Screening (weeks)</th>
<th>Study treatment Period Peg-INF and ADF, Peg-IFN and tenofovir or no treatment (weeks)</th>
<th>Follow-up (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study weeks</td>
<td>-4 to 0</td>
<td>8 Day 3 1 2 4 6 8 12 18 24 30 36 42 48 2 4 8 12 16 20 24</td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Informed consent,</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>medical history</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Physical examination</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Liver biopsy (preceding 12 months)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Urine or serum</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>pregnancy test a</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Chest X-ray, selected patients b</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Ultrasound of the liver</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Fibroscan</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CT or MRI, selected patients c</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Electrocardiogram, selected patients d</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Anti-HAV IgM, anti-HIV, anti-HCV, HCV-RNA, anti-HDV</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Ophthalmologic exam-</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>ination e (on indication)</td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>
### Part I

**Assessment/Procedure** | Long term Follow-up (years)
---|---
**Years** | 1 | 2 | 3 | 4 | 5
**Physical examination** | X | X | X | X | X
**Complete hematology with differential WBC, and platelets a (on indication)** | X | X | X | X | X
**Chemistry 1 b** | X | X | X | X | X
**Chemistry 2 b (on indication)**
**Chemistry 3 (on indication)**
**Fibroscan c** | X | X | X | X | X
**HBeAg, anti-HBe (frozen for central processing)** | X | X | X | X | X
**HBV-DNA (frozen for central processing)**
**HBsAg, anti-HBs (frozen for central processing)**
**Ultrasound, CT or MRI (on indication) c** | X | X | X | X | X
**PBMC's (frozen for central processing)** | X | X | X | X | X
**Paxgene™ RNA (k)** | X | X | X | X | X
**Plasmatube (proteomics profiling) l** | X | X | X | X | X
**Quality of life m** | X | X | X | X | X
Combination therapy in CHB patients with low viral load  Chapter 3

B: baseline
a. For females of child bearing potential only, a pregnancy test will be performed within 24 hours prior to first dose. A pregnancy test must be done at any time a secondary amenorrhea of more than 1 week occurs.
b. Only for patients with pre-existing pulmonary disease (Not necessary if (1) a chest X-ray available to the investigator has been obtained within the past 12 months and (2) the patient’s pulmonary disease has been clinically stable)
c. Patients with cirrhosis or marked fibrosis on liver biopsy or raised AFP need to have a liver imaging study during the screening period to rule out hepatic neoplasia.
d. For anyone with pre-existing cardiac disease.
e. Eye examination will be done on indication. Any patient complaining of decrease or loss of vision must have a prompt and complete eye examination. Patients with pre-existing ophthalmologic disorders (eg, diabetic or hypertensive retinopathy) should receive periodic ophthalmologic exams during Peg-IFN therapy. Peg-IFN treatment should be discontinued in patients who develop new or worsening ophthalmologic disorders.
f. Non-invasive measurement of the liver to detect fibrosis and cirrhosis
g. Only for patients with decreased kidney function. If there are clinically significant laboratory abnormalities, repeat no less frequently than every 2 weeks or as clinically indicated, with appropriate toxicity management, until they return to normal or baseline values. Urinalysis to be performed via dipstick, with subsequent microscopic evaluation if positive for hemoglobin at the discretion of the investigator.
h. Only for certain patients at risk, don’t repeat if data from previous assessments are available
i. Roche TaqMan®; when HBV DNA is more than the maximal detection limit, ten or hundred time dilution is proceeded.
j. Paxgene TM DNA sample (tube) will be collected. The DNA samples will be used exclusively for exploratory DNA research (genotyping of hostomics) to evaluate drug disposition genes (e.g. metabolic enzymes and drug transporters), to explore possible underlying genetic variants involved in therapy response in HBV chronically infected. Samples will only be processed in a number of subjects. No other testing will be performed on these samples.
k. Paxgene TM RNA sample (tube) will be collected to assess the expression of RNA in peripheral blood using whole genome microarray technology. These samples are collected as part of an effort to better understand the effects of medication. Samples will only be processed in a number of subjects. No other testing will be performed on these samples.
l. Plasma tube will be collected for proteomics profiling. These samples are collected as part of a study to better understand the effects of medication on liver activity. Samples will only be processed in a number of subjects. No other testing will be performed on these samples. Possible protein candidates include: SR-B1(CD36), CD81, certain Claudins, STAT-1 and 2, ApoE and more.
m. During the study a quality of life index according to SF36 standardized questionnaires will be administered
### Table 2: (cont.) Schedule of Assessments

<table>
<thead>
<tr>
<th>Assessment / Procedure</th>
<th>Screening (weeks)</th>
<th>Study treatment Period (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B day 3</td>
</tr>
<tr>
<td>Complete screening</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Chemistry 1 a</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Chemistry 2 a</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Chemistry 3 -</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Urinalysis, dipstick</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Ceruloplasmin, alfa-1-antitrypsin</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Alfa-fetoprotein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMA, ANA, ASMA, thyroid peroxidase antibodies, selected patients b</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>HBeAg, anti-HBe</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>HBsAg, anti-HBs</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>HBV-DNA (frozen for central processing)</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Alpha-2 macro globulin, Haptoglobin, Apolipoprotein A1</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Viral sequencing</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>PBMC’s (frozen for central processing)</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>PAXgene DNA (j) (human genomics)</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>PAXgene RNA (k) (human genomics)</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Plasma tube (proteomics profiling)</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Adverse events</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Quality of life</td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

Chemistry 1: ASAT, ALAT.
Chemistry 2: sodium, chloride, potassium, bilirubin, creatinine, urea, prothrombin time, APTT, antitrombine III, alkaline phosphatase, calcium, phosphorus, total protein, albumin, uric acid, TSH, free T4 and glucose.
Chemistry 3: cholesterol, triglycerides, sober glucose
### Combination therapy in CHB patients with low viral load

#### Chapter 3

#### Assessment / Procedure

<table>
<thead>
<tr>
<th>Study weeks</th>
<th>50&lt;sup&gt;a&lt;/sup&gt;</th>
<th>52&lt;sup&gt;a&lt;/sup&gt;</th>
<th>56&lt;sup&gt;a&lt;/sup&gt;</th>
<th>60&lt;sup&gt;a&lt;/sup&gt;</th>
<th>64</th>
<th>68</th>
<th>72&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete hematology with differential WBC, and platelets&lt;sup&gt;g&lt;/sup&gt;</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemistry 1&lt;sup&gt;g&lt;/sup&gt;</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Chemistry 2&lt;sup&gt;g&lt;/sup&gt;</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Chemistry 3&lt;sup&gt;g&lt;/sup&gt;</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Urinalysis, dipstick (on indication)&lt;sup&gt;g&lt;/sup&gt;</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Ceruloplasmin, alfa-1-antitrypsin</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Alfa-fetoprotein</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>AMA, ANA, ASMA, thyroid peroxidase antibodies, selected patients&lt;sup&gt;b&lt;/sup&gt;</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>HBeAg, anti-HBe (frozen for central processing)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>HBsAg, anti-HBs (frozen for central processing)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>HBV-DNA (frozen for central processing)&lt;sup&gt;l&lt;/sup&gt;</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Alpha-2 macro globulin, Haptoglobin, Apolipoprotein A1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Viral sequencing</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>PBMC’s (frozen for central processing)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>PAXgene&lt;sup&gt;TM&lt;/sup&gt; DNA (j) (human genomics)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>PAXgene&lt;sup&gt;TM&lt;/sup&gt; RNA&lt;sup&gt;TM&lt;/sup&gt; (human genomics)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Plasma tube (proteomics profiling)&lt;sup&gt;l&lt;/sup&gt;</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Adverse events</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Quality of life&lt;sup&gt;m&lt;/sup&gt;</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

---

<sup>a</sup> The follow-up schedule is based on the patient's viral load response.

<sup>b</sup> Laboratory tests are conducted on a subset of patients as indicated.

<sup>c</sup> Tests are performed at specific weeks as determined by the study protocol.

<sup>d</sup> Additional testing is performed at the discretion of the treating physician.

<sup>e</sup> Human genomics refer to advanced genetic testing techniques.

<sup>f</sup> Proteomics profiling involves the analysis of protein expression patterns.

<sup>g</sup> Differential WBC refers to the examination of white blood cell count and differential.

<sup>h</sup> AMA, ANA, ASMA are autoimmune markers.

<sup>i</sup> HBeAg, anti-HBe are markers related to hepatitis B e antigen.

<sup>j</sup> HBsAg, anti-HBs are markers related to hepatitis B surface antigen.

<sup>k</sup> HBV-DNA tests are used to detect hepatitis B viral DNA.

---

501524-L-bw-deNiet
## Part I

### SCREENING ASSESSMENTS

**Table 3.: Screening Assessments**

<table>
<thead>
<tr>
<th>Medical History And Physical Examination</th>
<th>Includes body weight, vital signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye examination</td>
<td>Eye examination by an ophthalmologist at screening will be done on indication. Patients with preexisting ophthalmologic disorders (e.g., diabetic or hypertensive retinopathy) should receive periodic ophthalmologic exams during Peg-IFN therapy. Peg-IFN treatment should be discontinued in patients who develop new or worsening ophthalmologic disorders.</td>
</tr>
<tr>
<td>Clinical Chemistry</td>
<td>ASAT, ALAT, bilirubin, creatinine, urea, sodium, chloride, potassium, calcium, phosphorus, prothrombin time, alkaline phosphatase, total protein, albumin, BUN, uric acid, cholesterol, triglycerides, glucose</td>
</tr>
<tr>
<td>Hematology</td>
<td>Leukocyte count, differential WBC, red blood count, platelets</td>
</tr>
<tr>
<td>Immunology and Special Chemistry</td>
<td>HBeAg, anti-HBe, HBsAg, anti-HBs, anti-HIV, anti-HCV, anti-HDV, Alfa-fetoprotein, ceruloplasmin, alfa1-antitrypsin, ferritin</td>
</tr>
<tr>
<td></td>
<td>Only for patients at risk without previous data being available: AMA, ANA, ASMA, thyroid peroxidase antibodies.</td>
</tr>
<tr>
<td>Virology</td>
<td>Quantitative HBV-DNA measurement</td>
</tr>
<tr>
<td>Thyroid Function Tests</td>
<td>TSH, free T&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>Urinalysis</td>
<td>Dipstick with subsequent microscopic evaluation if positive for hemoglobin</td>
</tr>
<tr>
<td>Chest X-Ray</td>
<td>Will be done during screening (Not necessary if (1) a chest X-ray available to the investigator has been obtained within the past 12 months and (2) the patient’s pulmonary disease has remained clinically stable.)</td>
</tr>
<tr>
<td>Electrocardiogram</td>
<td>Only for anyone with a history of pre-existing cardiac disease.</td>
</tr>
<tr>
<td>Liver Biopsy (a)</td>
<td>Performed at start of the study and repeated after 48 weeks.</td>
</tr>
<tr>
<td>Fibroscan (b)</td>
<td>Performed at start of the study and repeated after 48 weeks.</td>
</tr>
<tr>
<td>Liver Imaging (ultrasound)</td>
<td>Performed at screening or start of the study, repeated after 48 weeks and on indication.</td>
</tr>
<tr>
<td>Liver Imaging (CT or MRI)</td>
<td>Only for patients suspected to have hepatic neoplasia.</td>
</tr>
<tr>
<td>HCG Pregnancy Test</td>
<td>For women of childbearing potential a negative urine (or serum) HCG test needs to be documented within 24 hours prior to the first dose.</td>
</tr>
<tr>
<td>(a)</td>
<td>When a participant has objections against a second liver biopsy at week 48, he or she can refuse it. The participant will than not be excluded from the study.</td>
</tr>
<tr>
<td>(b)</td>
<td>A fibroscan measures the liver stiffness, e</td>
</tr>
</tbody>
</table>

(a) When a participant has objections against a second liver biopsy at week 48, he or she can refuse it. The participant will than not be excluded from the study.

(b) A fibroscan measures the liver stiffness, e.
**Supplementary Table S2: Overview of (serious) adverse events ITT population.**

| Serious adverse events* | Peg-IFN+ADV  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 46</td>
<td>n = 45</td>
<td>n = 43</td>
</tr>
<tr>
<td>General</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>16</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>Headache</td>
<td>18</td>
<td>19</td>
<td>2</td>
</tr>
<tr>
<td>Fatigue</td>
<td>23</td>
<td>28</td>
<td>2</td>
</tr>
<tr>
<td>Myalgia</td>
<td>15</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Other flu-like symptoms</td>
<td>9</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Dizziness</td>
<td>8</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Back pain</td>
<td>2</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Cough</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Change in menstrual pattern</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Digestive tract</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>14</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Change in stool consistency</td>
<td>10</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Nausea</td>
<td>17</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>7</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Dysgeusia</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Gingivitis</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Dermatological</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin rash</td>
<td>7</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Pruritus</td>
<td>9</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Alopecia</td>
<td>8</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Dry mucous membranes</td>
<td>3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Dry skin</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Psychiatric</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depression, including mood changes</td>
<td>10</td>
<td>12</td>
<td>1</td>
</tr>
<tr>
<td>Concentration problems</td>
<td>2</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Insomnia</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Laboratory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anemia (&lt; 6.0 mmol/L)</td>
<td>4</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Neutropenia (&lt;0.75x10^9 cells/L)</td>
<td>15</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Thrombocytopenia (&lt;75x10^9 cells/L)</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thyroid abnormalities</td>
<td>2</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>On-treatment ALT elevation**</td>
<td>24</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>Off-treatment ALT elevation**</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

* Serious adverse events. Arm I: hospitalization for alcohol-related pancreatitis (week 6), pregnancy, which was electively aborted (week 9). Arm II: hospitalization after a suicide attempt during a severe depression (week 23), hospitalizations for abdominal pain (week 2) and an elective laminectomy (week 40). Arm III: hospitalizations for septic arthritis (week 72), endocarditis (week 5), and hyperthyroidism (week 20).

** ALT elevation of more than two times the upper limit of normal (45 U/L for males, 34 U/L for females).
### Supplementary Table S3: Predictors of on-treatment HBsAg decline. Baseline and early on-treatment variables were compared between treated patients with >1 log reduction in HBsAg level (n=17) and those with <1 log reduction (n=63). Variables are shown for all treated patients who completed study interventions (per-protocol population; n=122).

<table>
<thead>
<tr>
<th>Demographics</th>
<th>HBsAg response at Week 48 (end-of-treatment)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt; 1 log decline</td>
<td>&lt; 1 log decline</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>4 (24)</td>
<td>33 (52)</td>
</tr>
<tr>
<td>Mean age, years (SD)</td>
<td>40.6 (3.0)</td>
<td>44.9 (1.5)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian, n (%)</td>
<td>4 (24)</td>
<td>16 (25)</td>
</tr>
<tr>
<td>Asian, n (%)</td>
<td>6 (29)</td>
<td>22 (19)</td>
</tr>
<tr>
<td>African, n (%)</td>
<td>5 (43)</td>
<td>12 (35)</td>
</tr>
<tr>
<td>South American, n (%)</td>
<td>2 (12)</td>
<td>13 (21)</td>
</tr>
<tr>
<td>IFN naïve, n (%)</td>
<td>15 (88)</td>
<td>59 (94)</td>
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<tr>
<td>Laboratory</td>
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<td></td>
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<tr>
<td>Median baseline ALT, U/L (iqr)</td>
<td>25 (20-32)</td>
<td>24 (19-37)</td>
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<tr>
<td>Median zenith ALT, U/L (iqr)</td>
<td>106 (86-204)</td>
<td>72 (45-103)</td>
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<tr>
<td>Mean baseline ALT, log10 U/L (SD)</td>
<td>1.42 (.12)</td>
<td>1.44 (.24)</td>
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<tr>
<td>Mean zenith ALT, log10 U/L (SD)</td>
<td>2.11 (.27)</td>
<td>1.87 (.26)</td>
</tr>
<tr>
<td>HBV genotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indeterminable, n (%)</td>
<td>5 (29)</td>
<td>14 (22)</td>
</tr>
<tr>
<td>A, n (%)</td>
<td>3 (18)</td>
<td>14 (22)</td>
</tr>
<tr>
<td>B, n (%)</td>
<td>4 (24)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>C, n (%)</td>
<td>0 (0)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>D, n (%)</td>
<td>3 (18)</td>
<td>14 (22)</td>
</tr>
<tr>
<td>E, n (%)</td>
<td>2 (12)</td>
<td>15 (24)</td>
</tr>
<tr>
<td>F, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>G, n (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mean baseline HBV-DNA, (SD)</td>
<td>2.51 (.26)</td>
<td>2.81 (.14)</td>
</tr>
<tr>
<td>Mean HBV DNA week 12, (SD)</td>
<td>.84 (.22)</td>
<td>1.03 (.09)</td>
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<tr>
<td>Mean baseline HBsAg, (SD)</td>
<td>2.96 (.20)</td>
<td>3.31 (.11)</td>
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<tr>
<td>Mean HBsAg week 12, (SD)</td>
<td>2.13 (.34)</td>
<td>3.23 (.12)</td>
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<tr>
<td>Fibroscan</td>
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<tr>
<td>Mean value, kPa (SD)</td>
<td>5.0 (1.8)</td>
<td>5.1 (.2)</td>
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<tr>
<td>Liver biopsy</td>
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<tr>
<td>Median inflammatory score, (iqr)</td>
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<td>2 (2-2)</td>
</tr>
<tr>
<td>Median Ishak fibrosis score, (iqr)</td>
<td>1 (1-1)</td>
<td>1 (1-1)</td>
</tr>
<tr>
<td>Median % HBsAg staining, (iqr)</td>
<td>15 (5-40)</td>
<td>15 (2-35)</td>
</tr>
</tbody>
</table>

*Differences between variables were assessed by Student’s t (a), Mann-Whitney U (b), or Pearson’s Chi-squared test (c).*
REFERENCES


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Combination therapy in CHB patients with low viral load  Chapter 3
Genetic variation in IL28B and treatment outcome in HBeAg-positive and -negative chronic hepatitis B patients treated with Peginterferon alfa-2a and adefovir

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5. KIT Biomedical Research, Amsterdam, The Netherlands.
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ABSTRACT

In a cohort of 95 chronic hepatitis B patients, who were treated with peg-interferon and adefovir for one year, and who had 15% HBsAg loss (overall), no association was found between IL28B polymorphisms and HBeAg seroconversion or HBsAg clearance. These findings suggest that any association with outcome, if present, is less than that seen in chronic hepatitis C. Additional studies are needed to enlarge sample size and to refine our understanding of IL28B biology in the context of chronic hepatitis B response to immunomodulatory and direct antiviral therapy.
BACKGROUND

Chronic hepatitis B virus (HBV) infection affects approximately 450 million people worldwide.¹ ² Current therapeutic options for chronic hepatitis B include pegylated interferon-alfa (peg-IFN) and the nucleot(s)ide analogues lamivudine, telbivudine, adefovir, entecavir and tenofovir. In HBeAg-positive patients treated with peg-IFN for one year, HBeAg seroconversion was achieved in 25%-30%.³,⁴ Predictive factors for HBeAg seroconversion include genotype A, lower baseline HBV DNA levels (≤10⁷ IU/mL), lower baseline HBeAg levels, higher baseline alanine aminotransferase (ALT) level, high inflammation activity on liver biopsy, and absence of previous interferon therapy.³,⁴ In HBeAg-negative patients with HBV DNA > 2.10³-10⁴ IU/mL treated with peg-IFN, virological response (VR) was achieved in approximately 30%.⁷ Favourable baseline markers for VR were higher ALT, lower HBV DNA levels, younger age and female sex.⁸ The limited treatment efficacy and the potential serous side effects of peg-IFN highlight the importance of identification of more determinants of response. A major breakthrough in the investigation of host factors predictive of response to interferon therapy of chronic hepatitis C has been the discovery that single nucleotide polymorphisms (SNP) in the IL28B gene were associated with treatment response to peg-IFN and Ribavirin.⁹ Specifically, the single nucleotide polymorphism (SNP) rs12979860, situated 3kb upstream of the IL28B gene, was strongly associated with spontaneous and treatment induced viral clearance in patients with chronic hepatitis C.⁵,¹⁰ In these studies, significantly more genotype 1 patients with genotype CC of the polymorphism achieved sustained virological response (SVR) than did those with a T allele. Other SNPs associated with SVR in chronic hepatitis C were rs12980275 and rs8099917 which are located within a 17-kb region around the IL28B gene. Both rs12980275 and rs8099917 were found to be in linkage disequilibrium with rs12979860 meaning that their effects can be largely explained by rs12979860. After accounting for the latter, rs8099917 was the second most significant SNPs associated with treatment outcome.⁹

Recently the relation between the IL28B polymorphism and spontaneous clearance of acute hepatitis B was assessed in 610 patients. In this study no significant difference was seen in the rs12979860 C allele frequency between subjects with HBV persistence and those with HBsAg clearance.¹¹ As opposed to the former study a recent report showed that an association could be present between rs12979860 and treatment outcome (HBeAg and HBsAg seroconversion) in chronic hepatitis B patients.¹² In light of conflicting reports, it remains unclear whether IL28B polymorphisms are associated with treatment outcome in chronic hepatitis B. Therefore we investigated the association between three most significant IL28B polymorphisms rs12979860, rs12980275 and rs8099917 and treatment outcome in a cohort of chronic hepatitis B patients, treated with a combination of peg-IFN and adefovir for one year who achieved a high proportion of HBsAg clearance.
Part I

METHODS

Patients
Ninety five chronic hepatitis B patients (46 HBeAg positive and 49 HBeAg negative) were part of a clinical trial in which they were treated with a combination of peginterferon-alfa-2a 180µg subcutaneously once a week, and adefovir-dipivoxil 10 mg daily for 48 weeks. Subsequently they entered a 24 week follow-up period. Inclusion criteria were HBV DNA > 20,000 IU/mL (100,000 copies/mL), alanine aminotransferase (ALT) ≤ 10x upper limit of normal (ULN), and histological signs of chronic active hepatitis in the liver biopsy. This investigator-initiated open labelled prospective study was approved by the local ethics committee, was carried out in two centres in The Netherlands and was in accordance with the principles of the Declaration of Helsinki, ISRCTN 77073364.

Virological and IL28B analyses
Plasma HBV DNA was extracted by the COBAS® Ampliprep (F. Hoffman-La Roche Ltd, Diagnostics Division, Basel, Switzerland) according to manufacturing instructions.

Quantification of plasma HBV-DNA was performed using the Roche COBAS® TaqMan48® (F. Hoffman-La Roche Ltd, Diagnostics Division, Basel, Switzerland) assay with a dynamic range between 20 and 1.70x10⁸ IU/mL. Serum HBsAg quantification was performed by the Abbott Architect (Abbott diagnostics, Abbott park, IL, USA) with a dynamic range between 0.05 and 250 IU/mL. If HBsAg levels were above 250 IU/mL a ten-fold dilution with Abbott Manual diluent was performed until a quantitative value was achieved. For human genotyping we focused on the polymorphism rs12979860, rs8099917, rs12980275 near the IL28 gene. Human samples were genotyped according to the Illumina-I-Infinium(r)-HD Assay Super, manual protocol using the HumanOmni1 Quad (v1.0) chips (Illumina inc., San Diego, California, USA). To study the relation between IL28B and ethnic background, patients were classified as African, Asian or Caucasian according to their country of origin.

Response definitions
HBeAg seroconversion was defined as the disappearance of HBeAg with formation of anti-HBe antibodies. Virological response (VR) was defined as HBV DNA levels ≤ 2,000 IU/mL at end of follow-up in combination with persistent normal ALT levels (24 months after stopping therapy). HBsAg clearance or seroconversion was defined as loss of HBsAg (HBsAg <0.05 IU/mL) with the appearance of anti-HBs antibodies (anti-HBs ≥ 10 IU/L).

Statistical analysis
Statistical comparisons were performed using the Statistical Program for Social Sciences (SPSS 16.02 for Windows, SPSS, Chicago, IL). Analyses were based on the intention-to-treat model. Continuous variables were tested for normality using the Kolmogorov-Smirnov test. Differences by normally distributed variables were tested using the Student t test, whereas differences by variables with skewed distribution were tested using the Mann-Whitney U test. For comparison of
categorical variables, chi-squared test ($X^2$) or Fisher’s exact test was used. The associations between IL28B polymorphisms as potential predictors of HBeAg seroconversion, VR or HBsAg seroconversion as dependent variables were examined by logistic regression analysis and log rank test.

**RESULTS**

**Patients and response**

HBeAg seroconversion was achieved in 14/46 (30%) patients at end of follow up. In HBeAg negative patients, 23/49 (47%) attained VR at end of follow up. At end of follow-up, 5/46 (11%) HBeAg positive and 9/49 (18%) HBeAg negative patients had achieved HBsAg seroconversion (Table 1).

**Table 1: Treatment outcome according to rs12979860 genotype**

<table>
<thead>
<tr>
<th>rs12979860</th>
<th>CC</th>
<th>CT/TT</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBeAg+ n=46</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBeAg seroconversion</td>
<td>7/31</td>
<td>23%</td>
<td>7/15</td>
</tr>
<tr>
<td>HBsAg loss</td>
<td>3/31</td>
<td>10%</td>
<td>2/15</td>
</tr>
<tr>
<td>HBeAg- n=49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBV DNA&lt; 2.000IU/mL(SVR)</td>
<td>13/25</td>
<td>52%</td>
<td>10/24</td>
</tr>
<tr>
<td>HBsAg loss</td>
<td>5/25</td>
<td>20%</td>
<td>4/24</td>
</tr>
</tbody>
</table>

**IL28B polymorphisms and ethnic background**

The rs12979860 polymorphism was associated to ethnic background. Presence of the major genotype CC was high in Asians and Caucasians and low in Africans. Frequency of the minor genotype TT was highest in African patients, whereas no TT frequency was observed in the Asian patients. (Figure1). Similar results were observed for the major genotypes of rs12980275 and rs8099917.

**Figure 1: rs12979860 genotype distributions according to ethnic background**

![Graph showing genotype distributions according to ethnic background](image-url)
Part I

IL28B polymorphism and baseline HBsAg and HBV DNA

Baseline HBV DNA and HBsAg levels were associated with the IL28B polymorphism in HBeAg negative but not in HBeAg positive patients. In HBeAg negative patients baseline HBsAg (p=0.033) and HBV DNA (p=0.024) were significantly lower in patients with CC genotype (Figure 2). In HBeAg positive, but not in HBeAg negative patients, baseline HBV DNA and HBsAg levels were associated with viral genotype.

Figure 2A: Baseline HBsAg levels in patients with CC genotype compared to patients with CT/TT genotype.

Figure 2B: Baseline HBV DNA levels in patients with CC genotype compared to patients with CT/TT genotype.
IL28B polymorphisms and treatment outcome
Neither HBeAg positive nor negative patients showed an association between the rs12979860 polymorphism and HBsAg seroconversion. Likewise, neither HBeAg positive nor negative patients demonstrated an association between polymorphism in rs12979860 and HBeAg seroconversion or VR (Table 1). Similar results were found for the other IL28B SNPs (rs12980275 and rs8099917).
DISCUSSION

Polymorphisms near the IL28B gene are strongly associated with spontaneous and treatment induced viral clearance in chronic hepatitis C patients. However, the role of IL28B in the prediction of treatment outcome in chronic hepatitis B patients is currently debated.

In our study, treatment of chronic hepatitis B patients with a combination of peg-IFN and adefovir provoked a high HBsAg seroconversion rate of approximately 15%. This provided the opportunity to assess whether the IL28B polymorphisms are associated with treatment induced clearance of HBsAg in chronic hepatitis B. We found no clear association between the IL28B polymorphisms and HBsAg seroconversion. These results are in line with a previous report in which 226 acute hepatitis B patients with HBV persistence were compared with 384 patients with spontaneous HBV recovery and no association was found between rs12979860 C allele frequency and spontaneous clearance in this patient group. Similar results were found in another study in China in which SNPs rs12979860, rs12980275 and rs8099917 were not associated with persistence of HBV in patients with acute hepatitis B.

In our study no correlation was found between the ‘favourable’ IL28B genotypes and HBeAg seroconversion. Data on the relation of IL28B and treatment outcome in chronic hepatitis B patients is limited. So far the role of IL28B in treatment outcome of chronic hepatitis B has been studied by one other group. In this study an association was observed between both rs12979860 and rs12980275 and HBeAg seroconversion. In this retrospective and heterogeneous study of 205 HBeAg positive patients treated with different interferon based treatment strategies (peg interferon with or without lamivudine therapy), the CC and AA genotype of rs12979860 and rs12980275 respectively were associated with HBeAg seroconversion across all viral genotypes (aOR 2.68, p=0.04 for CC and 3.08, p=0.016 for AA). Additionally, a positive correlation was found between the major genotype (AA) of rs12980275 and HBsAg clearance. These distinct results raise questions concerning the putative role of IL28B polymorphism as response marker for treatment outcome in chronic hepatitis B.

While the precise mechanism of IL28B in chronic hepatitis C is not well understood, it has been shown that IL28B activates signal transducers and activators of transcription 1 (STAT1) through downstream signalling from heterodimeric class II cytokine receptors that consists of IL-10 receptor B, (IL-10RB) and IL-28 receptor a (IL-28Ra), and shares signalling pathways with interferon alpha, resulting in up regulation of interferon stimulating genes (ISGs). One study suggested that a pre-activated ISG system is associated with unfavourable outcomes in clearing HCV infection after treatment. Patients with the minor genotype (TT) of rs12979860 or the minor genotype (GG) of IL28B SNP rs8099917, a SNP located in the same haplotype block as rs12979860, have been shown to have more ISGs than patients with the major genotype (CC or TT). This could indicate that IL28B polymorphisms determine treatment outcome by regulating ISG’s in the liver and thereby alter antiviral signalling pathways that are involved in innate immunity.
After infection, HBV and HCV may develop mechanisms to interfere with the IFN signalling pathway. In chimpanzee models it has been shown that, whereas HCV (RNA) directly stimulates ISGs in the liver after acute infection, HBV (DNA) does not activate ISGs and acts as a 'stealth virus', escaping parts of the innate immune system.\textsuperscript{18, 19} Interestingly, a recent study in immunodeficient mice showed that HBV can antagonize the induction of IFN-α signalling by inhibiting STAT-1 and thus interfere with the transcription IFN-stimulating genes.\textsuperscript{20} Since IFN-α and IFN-λ (The biological substrate of the IL28B gene) activate transcription of a similar set of IFN-stimulating genes it seems likely that IFN-λ (IL28B) acts by similar mechanisms as IFN-α. Although we cannot extrapolate these findings to humans, presumably the antiviral pathways and the activated interferon stimulated genes, through which the host exerts its action against HBV, differ profoundly from HCV and these findings indicate that the IL28B pathway has a more dominant response to HCV than to HBV.

This is supported by the difference in cure attainable with IFN mediated immune modulation in HCV as compared to HBV. While up to 50% of patients with genotype 1 HCV may achieve cure on a 48 week pegylated interferon and Ribavirin based regimen, the clearance rate of chronic hepatitis B (i.e. loss of HBsAg and conversion to anti-HBs) on (peg) IFN is much lower, in the order of 3% at one year.

We question whether the IL28B pathway is involved in triggering immune modulation leading to HBeAg seroconversion. HBeAg seroconversion is a critical prerequisite to the transition from the active immune clearance phase to the inactive carrier phase of HBV infection, still the exact mechanism remains unknown\textsuperscript{21}. Response rates for HBeAg seroconversion vary from 30-40% on a 48 week IFN based regimen indicating that the IFN system has a more dominant role in HBeAg seroconversion than in HBsAg clearance. Furthermore HBeAg has a role in developing chronicity and may function in tolerization of T cells.\textsuperscript{22} HBeAg seroconversion is typically preceded by hepatic flares characterized by increases in ALT levels, which are presumed to signify an enhanced endogenous cellular T lymphocyte-mediated response against HBV.\textsuperscript{21}

IL28B may activate alternate antiviral pathways involved in the adaptive immune response. This is supported by a study in which IL28B, when used as a vaccine adjuvant, significantly decreased splenic regulatory T cells, increased splenic and peripheral blood CD8+ T cells, and led to increased T cell function.\textsuperscript{23} The role of IL28B and specific ISG's in HBeAg seroconversion is presently under investigation.

In contrast to HCV, where the favorable genotype is associated with a high viral load, we found that HBeAg negative patients with genotype CC of rs12979860 had a significantly lower viral load than patients with the CT or TT genotype. Similar results were observed in a recent study in which the minor genotypes (CT/TT) were more frequently present in HBV infected patients with a high viral load.\textsuperscript{13} These findings may reflect differences in the underlying biology and immune response of HBeAg positive versus HBeAg negative disease, and is consistent with differential outcomes of (peg) IFN therapy for HBeAg positive and HBeAg negative patients.
In conclusion, in contrast to previous studies in HCV, in which the rs12979860 SNP was shown to be a strong predictor for spontaneous and treatment induced viral clearance, we found no relation between rs12979860 and a favourable treatment outcome (HBsAg clearance, HBeAg seroconversion and VR). Although limited patient numbers and the heterogeneity of our study population may limit the ability to draw definite conclusions, in our study still 14 out of 46 HBeAg positive patients and 14 out of all 95 patients underwent HBeAg or HBsAg seroconversion respectively and we suggest that if a relation between IL28B and treatment outcome did exist, this should at least have been shown as a trend. We posit that there are biologically plausible reasons to infer that an association of IL28B and clearance of chronic hepatitis B is less than that seen in chronic hepatitis C. Prospective studies with sufficient power to enable analysis of IL28B with respect to treatment outcome and viral load dynamics are lacking. In contrast to HCV studies with large numbers of genotype 1 infected individuals, HBV infected patients comprise a heterogeneous population with large variation in host and viral factors. Additional studies are needed to enlarge sample size in both HBeAg positive and negative disease, which may require separate investigations to refine our understanding of IL28B biology in the context of chronic hepatitis B response to immunomodulatory and direct antiviral therapy.

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Genetic variation in IL28B and treatment outcome in CHB patients

Chapter 4

REFERENCE LIST


Part I


Genetic variation in IL28B and treatment outcome in CHB patients Chapter 4
HBsAg loss in patients treated with peginterferon alfa-2a and adefovir is associated with SLC16A9 gene variation and lower plasma carnitine levels.

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• Both authors contributed equally to this work.

ABBREVIATIONS

ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; CD8+, cluster of differentiation 8 positive; CHB, chronic hepatitis B; CR, combined response; FACS, fluorescent activated cell sorter; GWAS, genome-wide association study; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HLA-DP, major histocompatibility complex, class II, DP; HWE, Hardy Weinberg Equilibrium; IL28B, interleukin 28B; NUCs, nucleos(t)ide analogues; PBMC, peripheral blood mononuclear cells; Peg-IFN, pegylated interferon alfa-2a; SLC16A9, solute carrier family 16 member 9; SNP, single nucleotide polymorphism; ULN, upper limit of normal range; week 96, one year of treatment free follow-up.

Supplementary information can be found online.
ABSTRACT

Background & Aims
Achievement of HBsAg loss remains the hallmark of chronic hepatitis B treatment. In order to identify host factors contributing to treatment-induced HBsAg loss, we performed a genome-wide screen of single nucleotide polymorphisms (SNPs) and studied its immunological consequence.

Methods
Chronic hepatitis B patients (40 HBeAg-positive and 44 HBeAg-negative) treated with peginterferon alfa-2a and adefovir were genotyped for 999,091 SNPs, which were associated with HBsAg loss at week 96 (n=9). Plasma carnitine levels were measured by tandem-mass spectrometry, and the effect of carnitine on the proliferative capacity of hepatitis B virus (HBV)-specific and non-specific CD8 T-cells was studied in vitro.

Results
One polymorphism, rs12356193 located in the SLC16A9 gene, was genome-wide significantly associated with HBsAg loss at week 96 (p=1.84x10^{-8}). The previously reported association of rs12356193 with lower carnitine levels was confirmed in our cohort, and baseline carnitine levels were lower in patients with HBsAg loss compared to patients with HBsAg persistence (p=0.02). Furthermore, we demonstrated that carnitine suppressed HBV-specific CD8 T-cell proliferation.

Conclusions
In chronic hepatitis B patients treated with peginterferon and adefovir, we identified strong associations of SLC16A9 gene variation and carnitine levels with HBsAg loss. Our results further suggest that a lower baseline plasma carnitine level increases the proliferative capacity of CD8 T-cells, making patients more susceptible to the immunological effect of this treatment. These novel findings may provide new insight into factors involved in treatment-induced HBsAg loss, and play a role in the prediction of treatment outcome.
INTRODUCTION

Chronic hepatitis B (CHB) is a major health problem, affecting more than 240 million people worldwide [1]. Persons with prolonged hepatitis B virus (HBV) infection are at increased risk of developing liver fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC) and death [2]. The disease spectrum of CHB infection is diverse, and current guidelines recommend treatment of CHB patients with high viral load and active liver inflammation. There are two approved treatment options: a finite course of pegylated interferon-alfa (Peg-IFN) or long-term administration of nucleos(t)ide analogues (NUCs) [3, 4]. Although NUCs potently suppress viral replication and prevent disease progression in most patients, they must be administered long-term (potentially for life) as they do not affect the viral reservoir – the covalently closed circular DNA (cccDNA) of HBV in hepatocytes. In contrast, patients on Peg-IFN appear to have a higher chance to achieve clearance of HBsAg compared with patients on NUCs. HBsAg loss is considered the closest outcome to cure, and is associated with improved survival [5]. Nevertheless, while HBsAg loss occurs more frequently on Peg-IFN than on NUC based therapy, this clinical outcome is rarely achieved with rates of 3-7% in large clinical trials with or without lamivudine [6-8]. In addition, clinical use of Peg-IFN is limited by significant side-effects.

It is therefore of utmost importance to be able to select those patients who are most likely to benefit from Peg-IFN monotherapy or combination with NUCs. Previously, higher rates of HBsAg loss were observed in HBeAg-positive patients with HBV genotype A and B [9], and in some studies, including the cohort studied here, lower baseline HBsAg levels were associated with HBsAg loss in HBeAg-negative patients [10, 11].

Unfortunately, these mainly virological markers account for only a small proportion of the variance in response to treatment. However, host genetic factors may also play a role in response of CHB to Peg-IFN. Genome-wide association studies (GWAS) have proven useful as a method for identifying genetic variations associated with diseases or treatment outcome. For example in hepatitis C, where genetic variation near the interleukin 28B (IL28B) gene is widely accepted as a strong predictor of viral clearance after treatment with Peg-IFN and ribavirin [12]. In contrast, studies on the effect of the IL28B genotype in hepatitis B treatment have yielded conflicting findings [13-16]. Although still under debate, the influence of the IL28B polymorphism is definitely not as pronounced in CHB as it is for chronic hepatitis C. Several other candidate gene studies have reported possible associations with IFN treatment response, but none had a level of significance that would lead to general acceptance of the finding. GWAS in East-Asian populations did find that genetic variants in the HLA-DP locus were strongly associated with persistent HBV infection [17]. However, the question of IFN response in patients with established chronic HBV infection was not addressed.

In this study we aimed to identify novel human genetic contributions to treatment-induced loss of serum HBsAg. We performed a genome-wide screen of single nucleotide polymorphisms (SNPs) in patients treated with Peg-IFN and adefovir. We identified a SNP (rs12356193), which
is associated with HBsAg loss with genome-wide significance ($p=1.84 \times 10^{-08}$) and confirmed its association with plasma carnitine levels [18]. In concordance with this finding, we observed a strong association between lower carnitine levels in plasma and HBsAg loss. In addition, we demonstrated that presence of carnitine limited the proliferation of HBV-specific T-cells in vitro.

**PATIENTS AND METHODS**

**Subjects**
This study was performed in 92 CHB patients who participated in an investigator-initiated study, carried out at the Academic Medical Center (AMC) in Amsterdam and the Erasmus University Medical Center (EMC) in Rotterdam, The Netherlands. The inclusion and exclusion criteria had been described elsewhere [11]. In summary, patients had documented HBsAg positivity for longer than 6 months, were either HBeAg-positive or -negative, and had HBV-DNA levels above 17,182 IU/mL (100,000 copies/mL). All patients were treated with peginterferon alfa-2a 180 µg subcutaneously once a week, and adeovir dipivoxil 10 mg daily. After 48 weeks, treatment was discontinued and a treatment-free follow-up period started. The study was conducted according to the guidelines of the Declaration of Helsinki, with the principles of Good Clinical Practice and was approved by local ethics committees (controlled-trials.com; ISRCTN 77073364). All patients gave written informed consent.

Inclusion criteria for the present study were completion of the 48 weeks of treatment and 48 weeks of follow-up (week 96), and availability of peripheral blood samples for genotyping. Of the 92 patients treated in the initial study, 84 fulfilled these criteria (supplementary information).

**Laboratory assays**
Routine biochemical and virological analyses were performed at the AMC in accordance with good laboratory practice. We determined quantitative HBsAg levels by the Architect (Abbott, IL, USA), and used the AxSYM (Abbott, IL, USA) as a qualitative test to define presence or absence of HBsAg. Both tests use a lower limit of detection of <0.05 IU/mL. Quantitation of plasma HBV-DNA levels was done by the Roche COBAS® TaqMan 48® assay (F. Hoffmann-La Roche Ltd, Basel, Switzerland), with a dynamic range between 20 and $1.70 \times 10^8$ IU/mL. Concentrations of plasma carnitine and carnitine derivatives were assessed retrospectively in plasma stored at -80 degrees Celsius, using a Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, UK) with 50 microliters input, as described earlier [19].

**DNA extraction and genotyping**
Total DNA was extracted from peripheral blood mononuclear cells (PBMC) of patients by the Magna Pure LC (Roche Applied Science, Mannheim, Germany) and the DNA isolation kit I (Roche Diagnostics) at the AMC, according to the manufacturer’s recommendations. DNA samples were genotyped according to the IlluminaI-INFINUM(r)-HD Assay Super manual protocol, using the HumanOmn1-Quad BeadChip (v1.0, Illumina inc., San Diego, California, USA) at Hoffman-La Roche (Nutley, NJ, USA).
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In total 999,091 SNPs were genotyped in DNA samples from 84 patients and further analyzed in the GenABEL package in R statistical software (v2.15.2). In total 743,673 (74.4%) SNPs and 84 (100%) patients passed all quality control criteria, and were used for further analysis (supplementary information).

Association analysis
Response was determined after 48 weeks of treatment free follow-up (week 96). Primary and secondary outcomes for both HBeAg-positive and HBeAg-negative patients were HBsAg loss (undetectable HBsAg levels by AxSYM; <0.05 IU/mL), and combined response (HBeAg negativity, HBV-DNA levels ≤ 2,000 IU/mL, normalization of alanine aminotransferase (ALT) levels), respectively.

The genome-wide scan was performed with the ‘egscore’-function in GenABEL, which corrects for population stratification by principal components analysis (PCA) as implemented in the Eigenstrat algorithm [20]. In our analysis, the first two principal components were used, which reflected population ancestry well (supplementary information).

Statistical significance was assessed with the 1-degree-of-freedom Cochran-Armitage trend test. Genomic control was applied to correct for any residual genomic inflation ($p_c$-values). Secondly, the same association analysis was performed using a model with sex, HBV genotype A, and HBV-DNA level as covariates. After Bonferroni correction for multiple testing, associations with a $p_c$-value of $<6.7 \times 10^{-8}$ were considered genome-wide significant. Odds ratios and confidence intervals were calculated using the effect allele as a reference. A linkage disequilibrium plot of rs12356193 was created using SNAP (BROAD Institute) based on phased genotype data from the International HapMap Project.

Other statistical comparisons were performed using IBM SPSS Statistics (v19.0.0.1).

HBV-specific and non-specific T-cell proliferation in presence of carnitine
A detailed description of this assay is provided in the supplementary information. In short, PBMC from 7 CHB patients were isolated. To study the effect of carnitine on HBV-specific T-cell proliferation, 0.5-1.0x10^6 cells were cultured for 10 days with HBVcore18-27 peptide in the presence of 0, 10 or 20 mM carnitine. After 10 days, cells were stained with APC-labeled-HBVcore tetrameric complexes. In addition, 0.5-1.0x10^6 CFSE labelled cells were cultured for five days with or without anti-CD3/anti-CD28 in the presence of 0, 10 or 20 mM carnitine. All cells were analyzed in a fluorescence-activated cell sorter (FACS) Canto (BD Biosciences, San Jose, USA) with FlowJo-software MacV9.7.2 (TreeStar, Ashland, USA).
RESULTS

Patient characteristics
In total, 84 patients were included in the study, and patient characteristics are summarized in table 1. In total, 9 (11%) patients achieved HBsAg loss at week 96, of which 6 (7%) had also developed anti-HBs. Of the other 3 patients, one had disappearance of anti-HBs after week 48, and two developed anti-HBs after week 96. Combined response at week 96 was attained by 25 patients (30%). The presence of HBV genotype A versus non-A was the only significantly different baseline characteristic when comparing patients with HBsAg loss and patients without HBsAg loss (p=0.02).

Table 1: Baseline characteristics of all patients included in the association analysis.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Main study population (n = 84)</th>
<th>HBsAg loss (n = 9)</th>
<th>HBsAg persistence (n = 75)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean age, years (SD)</td>
<td>39.5 (10.3)</td>
<td>44.9 (11.8)</td>
<td>38.9 (10.0)</td>
<td>0.10</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>21 (25)</td>
<td>1</td>
<td>20</td>
<td>0.44</td>
</tr>
<tr>
<td>Caucasian, n (%)</td>
<td>27 (32)</td>
<td>5 (56)</td>
<td>22 (29)</td>
<td>0.18</td>
</tr>
<tr>
<td>African, n (%)</td>
<td>26 (31)</td>
<td>3 (33)</td>
<td>23 (31)</td>
<td></td>
</tr>
<tr>
<td>Asian, n (%)</td>
<td>31 (37)</td>
<td>1 (11)</td>
<td>30 (40)</td>
<td></td>
</tr>
<tr>
<td>IFN naive, n (%)</td>
<td>62 (73)</td>
<td>5 (56)</td>
<td>57 (76)</td>
<td>0.19</td>
</tr>
<tr>
<td>Laboratory characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median ALT, xULN (iqr)</td>
<td>1.8 (1.1-3.2)</td>
<td>1.5 (0.8-4.0)</td>
<td>2.0 (1.1-3.2)</td>
<td>0.31</td>
</tr>
<tr>
<td>HBeAg positive, n (%)</td>
<td>40 (48)</td>
<td>4</td>
<td>36</td>
<td>1.00</td>
</tr>
<tr>
<td>Mean HBV-DNA, log_{10} IU/ml (SD)</td>
<td>6.67 (1.73)</td>
<td>6.18 (2.22)</td>
<td>6.73 (1.67)</td>
<td>0.37</td>
</tr>
<tr>
<td>Mean HBsAg, log_{10} IU/ml (SD)</td>
<td>3.78 (0.87)</td>
<td>3.30 (1.30)</td>
<td>3.84 (0.79)</td>
<td>0.25</td>
</tr>
<tr>
<td>HBV genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A, n (%)</td>
<td>26 (31)</td>
<td>6 (67)</td>
<td>20 (27)</td>
<td>0.02</td>
</tr>
<tr>
<td>B, n (%)</td>
<td>14 (17)</td>
<td>0 (0)</td>
<td>14 (19)</td>
<td>0.35</td>
</tr>
<tr>
<td>C, n (%)</td>
<td>12 (14)</td>
<td>1 (11)</td>
<td>11 (15)</td>
<td>0.00</td>
</tr>
<tr>
<td>D, n (%)</td>
<td>23 (27)</td>
<td>1 (11)</td>
<td>22 (29)</td>
<td>0.43</td>
</tr>
<tr>
<td>E, n (%)</td>
<td>9 (11)</td>
<td>1 (11)</td>
<td>8 (11)</td>
<td>1.00</td>
</tr>
<tr>
<td>Liver biopsy characteristics, n (%)</td>
<td>68 (81)</td>
<td>8 (89)</td>
<td>60 (80)</td>
<td></td>
</tr>
<tr>
<td>Inflammatory score (iqr)</td>
<td>5 (3-8.8)</td>
<td>6 (3.5-9.8)</td>
<td>5 (3-7.8)</td>
<td>0.35</td>
</tr>
<tr>
<td>Ishak fibrosis score (iqr)</td>
<td>1 (1-3)</td>
<td>2 (1.3-4.5)</td>
<td>1 (1-3)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

P-values are shown for Student’s t test¹, Mann-Whitney U test², Fisher’s exact or Chi-square test³.
SNP rs12356193 is genome-wide significantly associated with HBsAg loss

One SNP, rs12356193, was significantly associated with HBsAg loss ($p = 1.84 \times 10^{-8}$) (figure 1). The top 10 SNPs are summarized in table 2, and all SNPs with $p < 1.0 \times 10^{-4}$ are listed in the supplementary information. One other SNP, rs7094971 located upstream of rs12356193 in the SLC16A9 gene was in high linkage disequilibrium with rs12356193 and highly associated with HBsAg loss as well ($p = 1.27 \times 10^{-8}$). No other SNPs genotyped are known to yield significant linkage to rs12356193 (figure 1). After correcting for the calculated genomic inflation factor $\lambda$ (1.09), SNP rs12356193 still reached genome-wide significance ($p$-value in table 2). Incorporating sex, with either HBeAg status and HBV genotype A ($p = 5.93 \times 10^{-5}$), baseline HBV-DNA ($p = 1.88 \times 10^{-4}$), or baseline HBsAg ($p = 4.05 \times 10^{-4}$) as covariates into the association analysis, resulted in similar $p$-values for rs12356193.

No SNP associated with combined response at week 96 reached genome-wide significance (supplementary information).

**Figure 1:** Manhattan plot of association analysis for HBsAg loss.

P-values of the Cochran-Armitage trend test after genomic control are shown as $-\log_{10}(P$-value). The threshold for genome-wide significance ($p < 6.7 \times 10^{-8}$) is depicted as a red striped line. The outlier circled by red corresponds to the significantly associated SNP rs12356193. A detailed plot of chromosome 10 is depicted in the lower figure, showing R-squared values of Linkage Disequilibrium (LD) for SNP rs12356193 with other SNPs genotyped (HapMap 1000 genomes database; based on reference populations of European ancestry).
HBsAg loss is associated with SLC16A9 gene variation  Chapter 5

Table 2: Top 10 single nucleotide polymorphisms (SNPs) associated with HBsAg loss at week 96.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chr.</th>
<th>Nearest gene</th>
<th>Allele</th>
<th>Allele freq. (2)</th>
<th>HBsAg loss</th>
<th>No HBsAg loss</th>
<th>Odds ratio</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs12356193</td>
<td>10</td>
<td>SLC16A9</td>
<td>A/G</td>
<td>0.56</td>
<td>0.06</td>
<td>19.6 (6.2-61.7)</td>
<td>1.84x10^6</td>
<td></td>
</tr>
<tr>
<td>rs11574</td>
<td>1</td>
<td>ID3</td>
<td>G/A</td>
<td>0.33</td>
<td>0.03</td>
<td>18.3 (4.5-73.7)</td>
<td>4.37x10^7</td>
<td></td>
</tr>
<tr>
<td>rs9926783</td>
<td>16</td>
<td>SHISA9</td>
<td>A/G</td>
<td>0.39</td>
<td>0.05</td>
<td>13.0 (3.9-43.8)</td>
<td>7.46x10^7</td>
<td></td>
</tr>
<tr>
<td>rs525413</td>
<td>6</td>
<td>SNORA18</td>
<td>A/G</td>
<td>0.50</td>
<td>0.07</td>
<td>12.6 (4.2-38.3)</td>
<td>9.51x10^7</td>
<td></td>
</tr>
<tr>
<td>rs16962380</td>
<td>16</td>
<td>SHISA9</td>
<td>G/A</td>
<td>0.50</td>
<td>0.06</td>
<td>15.7 (5.0-49.2)</td>
<td>1.09x10^6</td>
<td></td>
</tr>
<tr>
<td>rs7094971</td>
<td>10</td>
<td>SLC16A9</td>
<td>A/G</td>
<td>0.50</td>
<td>0.07</td>
<td>14.0 (4.5-43.1)</td>
<td>1.27x10^6</td>
<td></td>
</tr>
<tr>
<td>rs17241543</td>
<td>16</td>
<td>GNPATP</td>
<td>G/A</td>
<td>0.67</td>
<td>0.17</td>
<td>9.5 (3.3-27.7)</td>
<td>1.44x10^6</td>
<td></td>
</tr>
<tr>
<td>rs12214948</td>
<td>6</td>
<td>EYS</td>
<td>G/A</td>
<td>0.61</td>
<td>0.09</td>
<td>16.6 (5.5-50.0)</td>
<td>1.53x10^6</td>
<td></td>
</tr>
<tr>
<td>SNP6-66700749</td>
<td>4</td>
<td>SNAPC2</td>
<td>A/C</td>
<td>0.39</td>
<td>0.03</td>
<td>23.2 (5.9-91.7)</td>
<td>1.88x10^6</td>
<td></td>
</tr>
</tbody>
</table>

1 Allele frequency of effect allele (Allele 2).
2 Unadjusted odds ratio and 95% confidence interval of effect allele (Allele 2).
3 p-value of Cochrane-Armitage's trend test (1 d.f.) corrected for inflation factor λ (1.09).
4 rs16962380, rs12214948, SNP6-66700749, and rs9654 violate Hardy-Weinberg equilibrium in the main study population (p < 0.05)

Ethnic distribution in rs12356193 genotypes and HBsAg loss

The prevalence of the rs12356193 G allele was higher in Caucasians (0.22) than in Africans (0.12) and Asians (0.02), which was comparable to those published by HapMap. The rate of HBsAg loss in the three major ethnic groups within our study population appeared to be associated with frequency of the G allele for rs12356193 (supplementary information).

Plasma carnitine levels are associated with SNP rs12356193 and HBsAg loss

SNP rs12356193 is located on chromosome 10 in an intronic region of the solute carrier family 16, member 9 (SLC16A9) gene, and was reported to be strongly associated with plasma carnitine levels in other GWAS studies [18]. This association was confirmed in our study cohort: mean baseline plasma carnitine levels were significantly different in patients with the GG, AG and AA genotype (p=0.02, figure 2A). In addition, carnitine levels were lower in patients with HBsAg loss than in patients with HBsAg persistence (p=0.02, figure 2A). This effect was independent of other predictors, such as viral genotype A and HBsAg at baseline and week 12 (table 3). Receiver-operator characteristic analysis showed a comparable predictive value for baseline carnitine and HBsAg at week 12 (supplementary information). Furthermore, there was an additive effect of carnitine to rs12356193 genotype as patients with the favorable AG genotype and HBsAg loss had significantly lower plasma carnitine levels than patients with the AG genotype and HBsAg persistence (p=0.05). A similar association of lower baseline carnitine levels was found...
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in patients with combined response compared to non-responders (33.9 vs 37.8 µmol/L, respectively, \( p=0.03 \)). Values for all carnitine derivatives are shown in the supplementary information.

**Table 3**: Multivariable analysis on the effect of plasma carnitine and other predictors in relation to HBsAg loss at week 96.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds ratio (95% CI)</th>
<th>p-value</th>
<th>Odds ratio (95% CI)</th>
<th>p-value</th>
<th>Odds ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline carnitine</td>
<td>0.87 (0.77-0.98)</td>
<td>0.03</td>
<td>0.88 (0.78-0.98)</td>
<td>0.02</td>
<td>0.86 (0.75-0.99)</td>
<td>0.03</td>
</tr>
<tr>
<td>HBeAg positive</td>
<td>0.52 (0.10-2.65)</td>
<td>0.43</td>
<td>2.59 (0.44-15.2)</td>
<td>0.29</td>
<td>0.22 (0.03-1.75)</td>
<td>0.15</td>
</tr>
<tr>
<td>HBV genotype non-A</td>
<td>0.13 (0.02-0.71)</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Baseline HBsAg</td>
<td>-</td>
<td>-</td>
<td>0.36 (0.13-0.97)</td>
<td>0.04</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Week 12 HBsAg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.19 (0.06-0.57)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

P-values are shown for binary logistic regression including maximal 3 predictors.

**Association of SNP rs12356193 genotype and plasma carnitine levels with HBsAg loss after longer follow-up duration**

During longer (treatment-free) follow-up (week 144), 4 additional patients (1 HBeAg-positive; 3 HBeAg-negative) became HBsAg undetectable. For this timepoint SNP rs12356193 remained strongly associated with HBsAg loss, although not genome-wide significant \((p_c=1.14\times10^{-5})\). In addition, the associations of baseline carnitine levels with HBsAg loss at week 96 or week 144 were similar (supplementary information).

**Factors influencing plasma carnitine levels**

Various factors are known to influence the level of plasma carnitine, e.g. dietary intake, sex, age, and disease state [21, 22]. In our cohort, carnitine and acetylcarnitine level varied mostly with sex and ethnicity, but not with age, creatinine, ALT, HBV-DNA or HBsAg level (supplementary information).

Most patients with HBsAg loss were non-Asian males (8/9). When analyzed in a non-Asian male subpopulation, mean plasma carnitine levels were significantly lower in patients with HBsAg loss than those with HBsAg persistence (28.4 vs 38.3 µmol/L, respectively, \( p<0.001 \)).

In addition, carnitine levels after 42 weeks of treatment were compared between 8 non-Asian males with HBsAg loss (rs12356193 genotypes AG/GG) and 7 sex- and ethnicity matched patients without HBsAg loss (rs12356193 genotype AA). Carnitine levels increased in both groups compared to baseline (figure 2B), but remained lower in patients with HBsAg loss compared to those without HBsAg loss (35.1 vs 44.6 µmol/L, respectively, \( p=0.01 \)). A similar pattern was observed for acetyl- and propionylcarnitine levels (supplementary information).
HBsAg loss is associated with SLC16A9 gene variation.

Figure 2: Association of carnitine with rs12356193 genotype and HBsAg loss.

(A) Baseline plasma carnitine levels according to rs12356193 genotype and HBsAg loss. (B) Baseline and week 42 (on-treatment) carnitine levels in 8 patients with HBsAg loss (genotype AG/GG) and 7 patients without HBsAg loss (genotype AA). Patients with HBsAg loss at week 96 are marked by open symbols. The lines represent mean and SEM. Reference values for carnitine are represented as dotted lines.
Carnitine limits proliferation of HBV-specific and non-specific CD8 T-cells

Previously, in murine studies, carnitine was shown to inhibit T-cell proliferation and functionality after non-specific stimulation in vitro [23, 24]. We explored the influence of carnitine on T-cell responses in PBMCs of 7 CHB patients. The proportion of HBV tetramer-positive CD8 T-cells in PBMCs after 10 days of HBV-peptide stimulation was significantly lower in the presence of 10 or 20 mM carnitine compared to untreated cells (figure 3A). This effect was present in all CHB patients analyzed (figure 3B), which indicates that carnitine suppresses HBV-specific CD8 T-cell responses. Furthermore, we observed less proliferation of total CD8 T-cells during 5 days of stimulation with anti-CD3/anti-CD28 in the presence of 20 mM carnitine compared to untreated cells (supplementary information). Figure 3C shows representative FACS plots of T-cell responses in one CHB patient. There was no evidence for a toxic effect of carnitine on lymphocytes with the used concentrations (figure 3D).

Figure 3: Effect of carnitine on HBV-specific and non-specific CD8 T-cells.
(A) Percentage of HBV tetramer-positive CD8 T-cells among all CD8 T-cells in PBMC from 7 CHB patients after 10 days of peptide stimulation in the presence of 0, 10, or 20 mM carnitine. Bars represent the mean values for 10 and 20 mM carnitine relative to 0 mM carnitine. (B) Individual plots of HBV tetramer-positive CD8 T-cells for 0 and 20 mM carnitine. Each type of symbol represents one CHB patient. P-values represent Wilcoxon signed-rank tests (* p<0.05).
(C) Representative FACS plot of one CHB patient. Numbers represent the percentage HBV tetramer-positive CD8 T-cells (upper row), and the percentage CFSE-low CD8 T-cells among all CD8 T-cells (lower row).
(D) Flow cytometric analysis of Live/Dead and Annexin-V staining in samples from fig. 3A.
HBsAg loss is associated with SLC16A9 gene variation.
DISCUSSION

In this study we identified a SNP (rs12356193) in the SLC16A9 gene to be strongly associated with HBsAg loss in patients treated with Peg-IFN and adefovir. The functional relevance of this SNP was investigated, revealing lower carnitine levels in plasma of patients with the favorable rs12356193 genotype. Patients with HBsAg loss had lower carnitine levels, an effect which was independent of other confirmed predictors such as HBV genotype A. In addition, an inhibitory effect of carnitine was observed on the proliferative capacity of HBV-specific T-cells.

Although several attempts have been made to find associations between candidate genes and IFN response in CHB therapy, no genome-wide screens have been published regarding this question to date. Although GWAS have the advantage of providing an unbiased screen of multiple genetic variations, it can be difficult to generate relevant biological hypotheses explaining the associations. Strikingly, a large GWAS on blood metabolite concentrations in healthy subjects identified a strong association of rs12356193 with plasma carnitine levels ($p=4.0 \times 10^{-26}$) [18]. Another GWAS on metabolic traits confirmed this association, and provided evidence that SLC16A9 acts as a unidirectional carnitine efflux transporter [25]. Since SLC16A9 is predominantly expressed in kidney tissue it is possibly involved in the regulation of plasma carnitine levels by reabsorption of carnitine at the renal proximal epithelial cells. We are the first to confirm the association of rs12356193 with baseline plasma levels of carnitine in CHB patients, and observed significantly lower plasma levels of carnitine in patients with treatment-induced HBsAg loss.

Carnitine is an endogenous compound mainly known for its essential function in fatty acid metabolism and cellular energy production [26]. Carnitine is present in all body tissues, and maintained by diet, synthesis in the liver, and tubular reabsorption [27]. The liver is, together with the kidney, a central organ for carnitine metabolism and distribution. From the liver, carnitine can be distributed to other organs in the form of free carnitine or as a carnitine derivative. Therefore, it is not surprising that carnitine metabolism was found to be altered in liver disease. However, findings regarding plasma carnitine levels in patients with chronic hepatitis B or C have been somewhat contradictory, as different studies reported lower [28-30], unchanged [31], or even higher [32] plasma carnitine levels compared to controls. Interestingly, two of these studies additionally reported lower carnitine levels in patients with a beneficial outcome to (Peg-)IFN based therapy [28, 30].

With the data presented here a new model can be proposed in which having a higher plasma carnitine level results in a less effective T-cell response, which makes these patients less susceptible to the immune modulatory effect of Peg-IFN.

These findings may add to the knowledge on the complex virus-host interplay involved in CHB
HBsAg loss is associated with SLC16A9 gene variation  Chapter 5

infection and Peg-IFN treatment. For example, Interferon has been shown to have additional non-cytolytic antiviral effects, such as promotion of core particle decay and epigenetic cccDNA modifications [33]. Furthermore, Peg-IFN and NUCs have been shown to have differential effects on the recovery of innate and adaptive immune responses [34]. In this regard, part of the high rate of HBsAg loss found in our study cohort could be induced by the combination of Peg-IFN with adefovir, either by the direct anti-viral effect of adefovir or by an indirect effect caused by immune recovery with NUC therapy in general. While treatment with adefovir is described to lower plasma carnitine levels [35], IFN therapy was shown to increase plasma carnitine in HCV patients [30]. We also observed an increase of carnitine levels during treatment. However, plasma carnitine levels remained lower in patients with HBsAg loss, suggesting that having lower carnitine levels throughout therapy is beneficial.

Support for this new model is provided in several lines of evidence suggesting that carnitine displays immune suppressive properties [23, 24]. Fortin et al. showed that carnitine treatment significantly inhibited both antigen presenting cells and CD4+ T-cell function in carnitine-supplemented and deficient cell culture systems [23]. Furthermore, they demonstrated that carnitine deficiency resulted in T-cell hyper activation in mice, which was reversed by carnitine supplementation. Next to this non-specific immune suppressive effect, we are the first to present data on the specific inhibitory effect of carnitine on human PBMC and HBV-specific CD8+ T-cell proliferation.

Importantly, a direct relation between this inhibitory effect on HBV-specific CD8+ T-cells and subsequent Peg-IFN induced HBsAg loss remains speculative. However, the role of carnitine in relation to other immunosuppressive mechanisms favoring viral persistence in CHB infection, such as the expression of inhibitory receptors on the surface of HBV-specific CD8+ T-cells, the action of suppressive cytokines on NK cells and T-cells, or the action of regulatory T-cells [34], has to be elucidated in further studies.

Despite the relatively high rate of HBsAg loss found in our study, our cohort was small with respect to recently published genetic studies. An important tool to correct for possible confounding due to population stratification which we applied is the EIGENSTRAT algorithm incorporated in GenABEL [20]. To further decrease the risk of false positive findings, Bonferroni adjustment and genomic control was applied to all p-values. The Bonferroni adjustment is known to be a conservative approach because it does not account for dependency between single marker tests. However, with the inconsistent results of previous genetic association studies, we feel that this conservative approach is appropriate. Furthermore, the association of SNP rs12356193 and carnitine levels with HBsAg loss appeared independent of other known predictors, such as HBV genotype A and baseline HBsAg, since introduction of these variables as covariates resulted in similar p-values.

An interesting observation was the low allele frequency of SNP rs12356193 and high carnitine levels in people from Asian origin. This may make our findings of less practical use in the Asian
population. On the other hand, our data suggests that rs12356193 genotype and higher carnitine levels could at least partly explain the low rate of HBsAg loss in the Asian patients in our cohort.

In addition to the effect of the rs12356193 genotype and carnitine levels, other yet undefined host genetic factors may play a role in achieving HBsAg loss in HBeAg-positive and HBeAg-negative patients. In our study, no other SNP reached the threshold of genome-wide significance, which might be attributed to the limited power of our study due to limited patient numbers.

Ideally, our results have to be confirmed in independent populations. Unfortunately, HBsAg loss in the treatment of CHB remains a rare event, and Peg-IFN in combination with adefovir or tenofovir has not been commonly used. Although a large randomized study comparing Peg-IFN and tenofovir in combination versus monotherapies has been initiated (clinicaltrials.gov: NCT01277601), long-term off therapy results as presented in our study are not expected for several years. For this reason, further exploration of a possible biological consequence of genetic associations, as presented here, provides a more practical option.

In summary, the finding of both genetic and functional consequences of SLC16A9 gene variation on carnitine levels and HBsAg loss, in addition to the immune-suppressive potential of carnitine, suggests a possible role for these factors in Peg-IFN and adefovir induced viral clearance. First, these associations open novel avenues to broaden our understanding of the host immune response to IFN in CHB. Second, identifying those at greatest chance of HBsAg loss through SNP genotyping or carnitine measurement may assist in making the decision of whether to start with Peg-IFN based combination treatment, or to prefer NUC therapy only. Moreover, we could speculate that creating a therapeutic condition with low plasma carnitine could improve the immune response in Peg-IFN treated patients. Further research is needed to fully elucidate the role of the SLC16A9 gene and carnitine in hepatitis B infection.

ACKNOWLEDGEMENTS

We thank Nico Abeling, Frédéric Vaz, and Ronald Wanders for the interpretation of carnitine measurements. We acknowledge Vincent Rijckborst and Harry Janssen for the inclusion of study participants.
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REFERENCES


Part I


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An intrahepatic transcriptional signature of enhanced immune activity predicts response to peginterferon in chronic hepatitis B.

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ABSTRACT

Background
Differences in intrahepatic gene expression patterns may be associated with therapy response in peginterferon-treated chronic hepatitis B (CHB) patients.

Methods
We employed gene expression profiling in baseline liver biopsies of 40 CHB patients (19 HBeAg-positive; 21 HBeAg-negative) treated with peginterferon and adefovir for 48 weeks, and compared expression patterns of combined responders (HBeAg loss, HBV-DNA <2,000 IU/mL, alanine aminotransferase normalization after one year of treatment-free follow-up) with non-responders. Genes identified by transcriptome analysis in 15 biopsies were confirmed in 25 additional biopsies by RT-qPCR.

Results
Transcriptome analysis demonstrated significant differences in expression of 41 genes between responders and non-responders. In responders, pathway analysis showed specific upregulation of genes related to the immune response, including chemotaxis and antigen processing and presentation. Genes upregulated in responders exhibited strongest similarity with a set of genes induced in livers of chimpanzees with acute Hepatitis B infection. Differential expression was confirmed for 8 selected genes. A 2-gene subset (HLA-DPB1, SERPIN-E1) was found to predict response most accurately. Incorporation of these genes in a multivariable model with HBeAg status, HBV genotype, and baseline HBsAg level correctly classified 90% of all patients, in which HLA-DPB1 and SERPIN-E1 were independent predictors of response.

Conclusion
We identified an intrahepatic transcriptional signature associated with enhanced immune activation which predicts therapy response. These novel associations could lead to better understanding of responsiveness to peginterferon in CHB patients, and may assist in selecting possible responders to interferon-based treatment.

Key points
In this unbiased screen of variations in gene expression, we identified a novel intrahepatic signature strongly associated with enhanced immune activation which predicts response to peginterferon treatment.

Genes upregulated in responders exhibited strong similarities to genes induced in livers of chimpanzees with acute hepatitis B infection.

Differential expression of selected genes was confirmed in an independent cohort by RT-qPCR, and a 2-gene subset (HLA-DPB1, SERPIN-E1) predicted response most accurately.

Incorporation of these genes in a multivariable analysis correctly classified 90% of patients from both cohorts.
ABBREVIATIONS

ACTB, beta actin; ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; CD4, cluster of differentiation 4; CD8, cluster of differentiation 8; CR, combined response; FDR, false discovery rate; FNAB, fine-needle aspiration biopsy; GSEA, gene-set enrichment analysis; GUSB, beta glucuronidase; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HLA-DPB1, major histocompatibility complex, class II, DP beta 1; ISGs, interferon-stimulated genes; LOOCV, leave-one-out cross-validation; MHC, major histocompatibility complex; mRNA, messenger RNA; NUCs, nucleotide analogues; PBMCs, peripheral blood mononuclear cells; Peg-IFN, pegylated interferon alfa 2a; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SERPIN-E1, serpin peptidase inhibitor, clade E, member 1; ULN, upper limit of normal; Week 96, one year of treatment free follow-up; Week 144, two years of treatment free follow-up; WV, weighted voting algorithm; YF17D, Yellow Fever 17D vaccination.
INTRODUCTION

Prolonged hepatitis B virus (HBV) infection increases the risk of morbidity and mortality due to liver disease, including liver cirrhosis and hepatocellular carcinoma [1]. Chronic hepatitis B (CHB) patients with a high viral load (HBV-DNA > 2,000 IU/mL) and active liver inflammation can currently be treated with either nucleos(t)ide analogues (NUCs) or pegylated-interferon alfa-2a (Peg-IFN) [2, 3].

While NUCs potently reduce HBV-DNA and its associated complications [4], discontinuation of NUCs usually results in a recurrence of disease activity since the HBV covalently closed circular DNA (cccDNA) within hepatocytes remains transcriptionally active. In contrast, treatment with Peg-IFN results in higher rates of sustained off-treatment viral suppression [3], but is associated with significant side-effects. Furthermore, loss of serum HBsAg, as the outcome closest to a cure, is also rarely achieved by Peg-IFN treatment [3].

Attempts to achieve improved outcomes with a Peg-IFN and NUC combination therapy have been disappointing when lamivudine was used [5-7]. Nevertheless, the use of Peg-IFN with more potent NUCs remains of interest because of their proven differential effects on the innate and adaptive immune responses [8].

However, in the majority of patients treated with a Peg-IFN based therapy the outcome is not satisfactory. It is therefore crucial to identify those patients who will benefit from this treatment before the start of therapy, thereby avoiding unnecessary use of Peg-IFN in other patients. Previous studies suggested several potential viral and host characteristics that were associated with response to treatment in both HBeAg-positive [9, 10] and HBeAg-negative [11] patients.

However, only part of the variation in response to Peg-IFN treatment can be explained by these characteristics. Treatment response might also be determined by other factors measurable at the actual site of infection: the liver. Here, we present histological characteristics and gene expression signatures associated with therapy outcome in pre-treatment liver biopsies of CHB patients treated with Peg-IFN and adefovir. These novel associations could lead to better understanding of responsiveness to IFN in CHB patients, and possibly to the selection of those patients most likely to benefit from peg-IFN based treatment.

PATIENTS AND METHODS

Subjects

This study was performed in 40 CHB patients from a cohort of 92 patients who participated in an investigator-initiated study, which has been described in detail previously [12]. In summary, patients were documented to be HBsAg-positive for longer than 6 months, were either HBeAg-positive or -negative, and had HBV-DNA levels above 17,182 IU/mL (100,000 copies/mL). Patients received a combination of peginterferon alfa-2a 180 µg subcutaneously once a week, and adefovir dipivoxil 10 mg daily for 48 weeks. The study was conducted according to the
guidelines of the Declaration of Helsinki, with the principles of Good Clinical Practice and was approved by local ethics committees (controlled-trials.com; ISRCTN 77073364). All patients gave written informed consent. Inclusion criteria for the present study were completion of 48 weeks of treatment, 2 years of treatment-free follow-up, and the availability of frozen liver biopsy material for gene expression analysis. Forty patients fulfilled the inclusion criteria for the present study (consort chart in supplementary figure S1).

For the transcriptome analysis, 15 patients were selected based on their serological response: HBeAg-positive patients with HBeAg loss (n=2), HBeAg-positive patients with both HBeAg and HBsAg loss (n=3), HBeAg-positive non-responders (n=3), HBeAg-negative patients with HBsAg loss (n=4), and HBeAg-negative non-responders (n=3). All serological responders had a combined response throughout follow-up (responders; n=9) and were compared with non-responders (n=6). Biopsies from the remaining 25 patients were used for confirmation of selected genes (second cohort).

**Therapy response definitions**
Response was determined after 1 year (week 96) and 2 years (week 144) of treatment-free follow-up. Primary clinical outcome was a combined response (CR) at year 1, defined as the combination of a virological (HBeAg negativity, and HBV-DNA levels ≤ 2,000 IU/mL) and a biochemical response (persistent normal alanine aminotransferase (ALT) levels) in both HBeAg-positive and -negative patients, according to EASL guidelines [3]. HBsAg loss at year 2 (HBsAg<0.05 IU/mL) was assessed as a secondary clinical outcome.

**Histological analysis**
For histological assessment we used the modified Ishak scoring system, based on a zero to 18 score for necroinflammation (inflammatory score) and a zero to 6 score for fibrosis (fibrosis score) [13]. Immunohistochemical detection of HBsAg and HBeAg by monoclonal anti-HBs antibodies (Neomarkers, Fremont, CA, USA) and polyclonal anti-HBc antibodies (Dako, Glostrup, Denmark) was expressed as the percentage of all hepatocytes.

**RNA extraction**
After obtaining the liver biopsy, the tissue was directly processed at the department of pathology where approximately 3 to 5 millimeter was OCT-embedded and stored in liquid nitrogen for further studies. Detection of cccDNA was performed as described previously [14]. Total RNA for transcriptome analysis was extracted with QIAzol reagent (Qiagen, CA, USA), and purified on columns (Nucleospin kit; Macherey-Nagel, Oensingen, Switzerland) according to manufacturer’s instructions. Alternatively, RNA was extracted with the AllPrep isolation kit (Qiagen, CA, USA). Concentration (ng/µL) and purity of RNA was assessed using the Nanodrop-1000 spectrophotometer (Thermo Scientific, USA).
Part I

Transcriptome analysis
RNA samples for transcriptome analysis were used as a template for reverse transcription. The resulting cDNA was fragmented, labelled and hybridized overnight to Human Gene ST 1.0 arrays (Affymetrix, CA, USA).

Scanned images of the arrays were normalized and background-corrected by the RMA algorithm implemented in GenePattern [15]. Only fully annotated genes were considered for further analysis ($n=21,462$). Genes differentially expressed in responders and non-responders were determined using filtering on minimal average expression, fold change (1.5 fold) and p-values from 2-sided t-tests (0 permutations) in GenePattern. Data reported here have been deposited in the NCBI's Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo, accession no. GSE54747).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)
Three micrograms of RNA were reverse transcribed using M-MLV reverse transcriptase and random hexamer primers. Relative quantification of gene expression was determined with the LightCycler 480 Real-Time PCR System (Roche Applied Science) using the SYBR Green PCR Master Mix and gene specific primers (supplementary table S2). PCR product specificity was assessed by melting curve analysis. mRNA expression levels were normalized to the arithmetic mean of 2 housekeeping genes ($ACTB$ and $GUSB$) using the comparative Ct method, and log$_{10}$ transformed for analysis.

Statistical analysis
Baseline- and liver biopsy characteristics were compared among response groups using 2-sided Student’s $t$ tests, Mann-Whitney $U$ tests or Chi-square test where appropriate. All statistical comparisons were performed using IBM SPSS Statistics, v19.0.0.1 (SPSS Inc., IL, USA).

The Database for Annotation, Visualization and Integrated Discovery (v6.7, http://david.abcc.ncifcrf.gov) was used to assess gene ontology terms enrichment in all genes upregulated more than 1.5 fold in responders or non-responders, with the remaining genes on the array as a background. Data on interferon-stimulated genes (ISGs) were retrieved from the Interferome database (v2.01, http://interferome.its.monash.edu.au/).

Gene-set enrichment analysis (GSEA) was used in the default settings [16]. Briefly, GSEA tests whether genes from pre-defined gene-sets were randomly distributed or showed specific upregulation in responders or non-responders. Gene-sets were retrieved from curated- ($n=3655$) and immunological gene-set collections ($n=1910$). The resulting enrichment scores and false discovery rates were generated by 1000 gene-set permutations. In addition, the leading-edge analysis tool was used to cluster gene-sets representing similar biological signals.

Differentially expressed genes were quantified by RT-qPCR in 39 patients, and the association with combined response was examined by 1-sided Student’s $t$ tests. For response class prediction in all patients with RT-qPCR data ($n=39$), the weighted voting (WV) algorithm with leave-one-out
cross-validation (LOOCV) was used. In LOOCV, one patient from the set is excluded, and the rest of the patients are used to build the classifier. Then, the classifier is used to predict the response class of the patient that was left out. After iteratively training and testing all patient combinations, the results are combined to determine the classifier. Genes included in this classifier were subsequently evaluated in a multivariable logistic regression analysis.

RESULTS

1. Patient characteristics

1.1 Response rates

Baseline characteristics of the 40 patients included in this study are summarized in table 1. At year 1, 13 (33%) patients had a combined response (CR), and 12 (30%) patients at year 2. The number of patients with HBsAg loss was 7 (18%) at year 1, which increased to 8 (20%) at year 2, of whom all but one had developed anti-HBs. This particular patient developed anti-HBs at week 177. HBeAg seroconversion occurred in all HBeAg-positive patients with a CR at year 1 (n=6).

1.2 Baseline characteristics associated with treatment response

HBeAg-positive patients with a CR were predominantly infected with HBV genotype A (5/6, 83%). Patients with a CR had higher inflammatory scores in baseline liver biopsies than non-responders (median 9 vs 3, p=0.03, supplementary table S3). In addition, a trend of higher fibrosis scores was observed in patients with a CR compared to non-responders (median 2 vs 1, p=0.07). The inflammatory score was positively correlated with both fibrosis score and ALT (r=0.70, p<0.001 and r=0.58, p=0.01 respectively).

HBeAg-negative patients with a CR or HBsAg loss had significantly lower plasma HBsAg levels at baseline (2.58 vs 3.46 log_{10} IU/mL, p=0.02, and 2.10 vs 3.50 log_{10} IU/mL, p<0.001, respectively, supplementary table S4). In addition, a lower proportion of HBsAg-positive hepatocytes was associated with HBsAg loss. Patients with a percentage of HBsAg-positive hepatocytes between 0-20% had higher rates of HBsAg loss (3/5, 60%) than patients with 21-60% (2/10, 20%) or 61-100% (0/6, 0%) HBsAg-positive hepatocytes (p=0.02). The proportion of HBsAg-positive hepatocytes showed a moderate correlation with plasma HBsAg level (r=0.44, p=0.05).

2. Gene expression signature associated with treatment response

2.1 Transcriptome analysis

Baseline liver gene expression values of patients with CR (responders; n=9) and non-response (n=6) were compared for 21,462 genes. We identified 182 genes whose mean expression differed >1.5-fold, of which 53 were upregulated in non-responders and 129 in responders (supplementary table S5). In total, 41 genes were differentially expressed with a p<0.05. Unsupervised hierarchical clustering using these 41 genes clustered all non-responders and all but one responder in separate groups (figure 1A). From these genes, 22/41 (54%) were ISGs of which 19 were upregulated in responders, and 3 in non-responders.
Part I

2.2 Functional annotation analysis
Pathway analysis, based on gene ontology terms enrichment, was performed for all genes whose expression differed on average more than 1.5-fold between responders and non-responders (figure 1B, and supplementary table S5). A significant number of genes upregulated in responders were related to the immune response (25/129 genes). The most significantly enriched biological processes included "antigen processing and presentation" (n=9 genes), and more specifically "presentation via MHC class II" (n=6; HLA-DMA, HLA-DOA, HLA-DPA1, HLA-DPB1, HLA-DQB1, HLA-DRB5). Other significantly enriched terms were related to chemotaxis (n=9 genes) and chemokine activity (n=5; CCL18, CCL4L1, CXCL9, CXCL10, CXCL11).

For non-responders the only significant enrichment was found for gene localization to the plasma membrane (p=0.03).

2.3 Gene-set enrichment analysis (GSEA)
Gene-sets for GSEA were retrieved from curated- and immunological gene signature databases. The algorithm tests for each set of genes whether they are enriched in expression in the responder group relative to the non-responder group, or vice versa.

GSEA predominantly showed enrichment of gene-sets in responder patients (supplementary GSEA file). In the curated gene signatures, the strongest enrichment was found for a set of genes induced in the liver of chimpanzees during clearance of acute HBV infection [17]. Mean expression values of all 92 genes included in this set were higher in responders than in non-responders (figure 1C). More specifically, 13 genes from this set were also present among the 129 genes upregulated >1.5 fold in responders, of which 6 were related to antigen processing and presentation (HLA-DQB1, HLA-DPA1, HLA-DPB1, HLA-DMA, HLA-C, HLA-F) and 4 to chemotaxis (CXCL9, CXCL10, CXCL11, C3AR1).

In the immunological gene signatures, a large number of gene-sets were found to be significantly enriched in responders (596/1804 gene-sets). Despite the large variety of experimental conditions these gene-sets originate from, the 15 most significantly enriched gene-sets in responders could be clustered into 2 main functionally related groups by the leading-edge analysis (supplementary figure S6). One cluster comprised gene-sets related to CD8 effector T-cells and activated CD4 memory T-cells. The other cluster included sets of genes upregulated in PBMCs of individuals in response to Yellow Fever (YF17D) vaccination, and genes upregulated after in vitro stimulation of microglia with IFN-gamma.
Intrahepatic transcriptional signature predicts response to peg-IFN

Chapter 6

Table 1: Baseline characteristics of patients included in the present study.

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Identification cohort (n=15)</th>
<th>Second cohort (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (SD)a</td>
<td>39.1 (12.4)</td>
<td>40.1 (9.9)</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>2 (13)</td>
<td>9 (36)</td>
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<tr>
<td>Ethnicity</td>
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<tr>
<td>Caucasian, n (%)</td>
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<td>9 (36)</td>
</tr>
<tr>
<td>African, n (%)</td>
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<tr>
<td>Asian, n (%)</td>
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</tr>
<tr>
<td>IFN naive, n (%)</td>
<td>8 (53)</td>
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<tr>
<td>Laboratory</td>
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</tr>
<tr>
<td>ALT, xULN (iqr)b</td>
<td>1.5 (0.8-2.9)</td>
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<tr>
<td>HBeAg positive, n (%)</td>
<td>8 (53)</td>
<td>11 (40)</td>
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<td>HBV-DNA, log10 IU/ml (SD)a</td>
<td>6.37 (2.17)</td>
<td>6.89 (1.58)</td>
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<tr>
<td>HBsAg, log10 IU/ml (SD)a</td>
<td>3.59 (1.08)</td>
<td>3.93 (0.92)</td>
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<tr>
<td>A, n (%)</td>
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<td>9 (36)</td>
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<td>B, n (%)</td>
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<td>7 (28)</td>
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<td>C, n (%)</td>
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<td>D, n (%)</td>
<td>5 (33)</td>
<td>6 (24)</td>
</tr>
<tr>
<td>E, n (%)</td>
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<td>1 (4)</td>
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<td>Liver Biopsy</td>
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<tr>
<td>Time to start of therapy, weeks (iqr)b</td>
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<td>2.4 (0.9-8.0)</td>
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<tr>
<td>Length, mm (iqr)b</td>
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<td>18 (12-21)</td>
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<td>Portal fields, n (iqr)b</td>
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<td>16 (10-20)</td>
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<td>Inflammatory score (iqr)b</td>
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<td>Steatosis grade, n (%)</td>
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<tr>
<td>&lt;5%</td>
<td>10 (67)</td>
<td>17 (71)</td>
</tr>
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<td>5-33%</td>
<td>4 (27)</td>
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<td>33-66%</td>
<td>1 (7)</td>
<td>3 (13)</td>
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<td>HBsAg positive cells, % (iqr)b</td>
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<td>50 (10-80)</td>
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<td>ALT closest to biopsy, xULN (iqr)b</td>
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<tr>
<td>Treatment Response</td>
<td></td>
<td></td>
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<tr>
<td>Combined response at week 96, n (%)</td>
<td>9 (60)</td>
<td>4 (16)</td>
</tr>
<tr>
<td>HBsAg loss at week 144, n (%)</td>
<td>6 (40)</td>
<td>2 (8)</td>
</tr>
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</table>

* Mean value, † Median value.
Figure 1: Transcriptome and pathway analysis.

(A) Hierarchical clustering of patients and genes differentially expressed (p<0.05, n=41) between responders and non-responders. Responders (CR) are shown in dark gray and non-responders (NR) in light gray. The heatmap shows scaled expression values with highest values in red and lowest in blue. Interferon-stimulated genes are marked with asterisks.

(B) Functional annotation analysis of genes significantly upregulated in responders (>1.5-fold) compared to non-responders. The number of corresponding genes are given for each term. P-values represent Bonferroni-corrected term enrichment: * p<0.05, ** p<0.01, *** p<0.001.

(C) Graphical view of the enrichment score for the gene-set most significantly enriched in responders; genes induced in the liver of chimpanzees during clearance of acute HBV infection. The bottom plot shows the correlation with response for each gene from the transcriptome analysis, which are ranked to the left if correlated to response and to the right if correlated with non-response. The middle plot shows where the members of the gene-set appear in the ranked list of genes.

3. Validation of genes associated with response

3.1 RT-qPCR validation of selected genes

From the most significant genes identified in the transcriptome analysis, we selected 17 genes for replication by RT-qPCR in the second cohort (n=25). Genes were selected based on lowest p-value (n=6) and/or proven immune response related function (n=11). In this cohort, expression levels of 8 genes were significantly different in responders and non-responders (figure 2A, and supplementary table S7).
3.3 Association of gene expression with other variables

The expression level of 6 of the identified genes were positively correlated with inflammatory scores and ALT levels. No significant correlations were observed between gene expression values and other baseline or virological characteristics (table 2). When analyzing the association with combined response at year 1 in HBeAg-positive and –negative patients separately, expression levels of \textit{HCP5}, \textit{IL17RB}, and \textit{SERPIN-E1} were associated with response in HBeAg-positive patients ($p=0.045$, $p=0.013$, $p=0.002$, respectively), whereas \textit{HCP5}, \textit{HLA-DPB1}, and \textit{CPVL} expression was associated with response in HBeAg-negative patients ($p=0.036$, $p=0.017$, $p=0.035$, respectively). Next to predicting combined response at year 1, higher expression levels of \textit{IL17RB} and \textit{CPVL}, or lower levels of \textit{SERPIN-E1} were associated with HBsAg loss at year 2 ($p=0.013$, $p=0.015$, $p=0.020$, respectively).

Table 2: Correlation of gene expression values and other baseline characteristics.

Upper limit of normal for ALT values are 45 U/L for males and 34 U/L for females. One patient from the identification cohort did not have RNA available for RT-qPCR.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CXCL11</th>
<th>HCP5</th>
<th>HLA-DPB1</th>
<th>HLA-DMA</th>
<th>IL17RB</th>
<th>SCIMP</th>
<th>CPVL</th>
<th>SERPIN-E1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT at biopsy, xULN $a$</td>
<td>0.64*</td>
<td>0.67*</td>
<td>0.54*</td>
<td>0.63*</td>
<td>0.42*</td>
<td>0.51*</td>
<td>0.19</td>
<td>-0.38*</td>
</tr>
<tr>
<td>Inflammatory score $b$</td>
<td>0.44*</td>
<td>0.42*</td>
<td>0.38*</td>
<td>0.61*</td>
<td>0.35*</td>
<td>0.27</td>
<td>0.19</td>
<td>-0.35*</td>
</tr>
<tr>
<td>Fibrosis score $b$</td>
<td>-0.09</td>
<td>0.05</td>
<td>0.03</td>
<td>0.02</td>
<td>-0.08</td>
<td>0.14</td>
<td>0.00</td>
<td>-0.19</td>
</tr>
<tr>
<td>HBsAg pos. cells, % $b$</td>
<td>0.01</td>
<td>-0.12</td>
<td>-0.09</td>
<td>-0.06</td>
<td>-0.10</td>
<td>0.06</td>
<td>-0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>cccDNA, copies/cell $b$</td>
<td>0.23</td>
<td>0.25</td>
<td>0.31</td>
<td>0.38*</td>
<td>0.12</td>
<td>0.05</td>
<td>0.09</td>
<td>-0.07</td>
</tr>
<tr>
<td>HBsAg, log10 IU/ml $a$</td>
<td>-0.12</td>
<td>-0.14</td>
<td>-0.01</td>
<td>-0.06</td>
<td>-0.16</td>
<td>-0.22</td>
<td>-0.06</td>
<td>0.07</td>
</tr>
<tr>
<td>HBV-DNA, log10 IU/ml $a$</td>
<td>0.13</td>
<td>-0.03</td>
<td>0.14</td>
<td>0.17</td>
<td>-0.11</td>
<td>-0.19</td>
<td>0.04</td>
<td>-0.08</td>
</tr>
</tbody>
</table>

$^a$ Pearson’s-, and $^b$ Spearman’s correlation coefficients. Significant ($p<0.05$) correlations are marked with asterisks.

3.3 Response class prediction by weighted voting

The 8 genes whose expression level were found to be significantly associated with a combined response at year 1 in both cohorts were evaluated for classification accuracy by weighted voting. First, the weighted voting algorithm used RT-qPCR measurements from all patients (n=39) to select a given number of marker genes by LOOCV. With this algorithm a combination of 2 genes (\textit{HLA-DPB1} and \textit{SERPIN-E1}) was found to predict response most accurately (supplementary table S8). This set of marker genes was then used to classify patients into responder
and non-responder categories. In total, 11/14 (79%) of patients in the identification cohort and 19/25 (76%) in the second cohort were correctly classified, resulting in an overall response classification accuracy of 30/39 (77%).

3.4 Multivariable analysis
To assess the independent predictive effect of the most important virological parameters and the most predictive gene-set in our cohort, different models were compared in a multivariable logistic regression analysis (table 3). First, a model including HBeAg status, HBV genotype A vs non-A, and baseline HBsAg, showed a significantly improved fit to the data compared to a model comprising only a constant ($p=0.041$). Introduction of HLA-DPB1 and SERPIN-E1 expression as independent variables into the model further improved its fit significantly ($p=0.006$). Both HLA-DPB1 and SERPIN-E1 were independent predictors of response in this model, which showed an overall classification accuracy of 90% (figure 2B).

Figure 2: RT-qPCR validation of selected genes and supervised response classification.

(A) Genes differentially expressed in the second cohort by RT-qPCR. For all genes, 4 patients with combined response at year 1 (CR) were compared with 21 non-responders (NR). The lines in boxes represent median values, and the whiskers 10th and 90th percentiles. P-values represent one-sided t-tests.

(B) Response classification of patients from both cohorts ($n=39$) using model 3 from the multivariable logistic regression analysis, including HLA-DPB1 and SERPIN-E1 expression. The cut-off for predicted probability of response was set at 0.5, and predictions in favor of combined responders (CR) and non-responders (NR) are presented as up- or downward facing bars, respectively. The actual responses of patients are indicated in dark- (CR), or light gray (NR). PPV, positive predictive value; NPV, negative predictive value.
Intrahepatic transcriptional signature predicts response to peg-IFN Chapter 6

Table 3: Multivariable analysis.

Multivariable logistic regression on the effect of independent variables on combined response at year 1 in three models. B, logistic regression coefficient; SE, standard error; df, degrees of freedom.

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Model 1</th>
<th></th>
<th></th>
<th>Model 2</th>
<th></th>
<th></th>
<th>Model 3</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>SE</td>
<td>p-value</td>
<td>B</td>
<td>SE</td>
<td>p-value</td>
<td>B</td>
<td>SE</td>
<td>p-value</td>
</tr>
<tr>
<td>Constant</td>
<td>3.84</td>
<td>1.93</td>
<td>0.047*</td>
<td>2.58</td>
<td>2.16</td>
<td>0.233</td>
<td>3.76</td>
<td>2.39</td>
<td>0.116</td>
</tr>
<tr>
<td>HBeAg positive</td>
<td>1.60</td>
<td>1.18</td>
<td>0.175</td>
<td>1.09</td>
<td>1.23</td>
<td>0.377</td>
<td>1.77</td>
<td>1.36</td>
<td>0.192</td>
</tr>
<tr>
<td>HBV genotype non-A</td>
<td>-1.00</td>
<td>0.79</td>
<td>0.202</td>
<td>-0.97</td>
<td>0.81</td>
<td>0.232</td>
<td>0.83</td>
<td>1.14</td>
<td>0.465</td>
</tr>
<tr>
<td>Baseline HBsAg</td>
<td>-1.28</td>
<td>0.59</td>
<td>0.032*</td>
<td>-1.11</td>
<td>0.61</td>
<td>0.066</td>
<td>-1.63</td>
<td>0.78</td>
<td>0.038*</td>
</tr>
<tr>
<td>Inflammatory score</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.15</td>
<td>0.13</td>
<td>0.230</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HLA-DPB1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.14</td>
<td>2.07</td>
<td>0.046*</td>
</tr>
<tr>
<td>SERPIN-E1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-2.43</td>
<td>1.19</td>
<td>0.042*</td>
</tr>
</tbody>
</table>

Model summary

| -2 Log likelihood         | 41.38   | 39.29    | 31.04    |
| Chi-square (df)           | 8.27    | (3) 0.041* | 1.49a (1) 0.223 | 10.33b (2) 0.006* |
| Classification accuracy   | 69.2%   | 76.3%    | 89.7%    |

a, b Improvement for model 2 and 3 was calculated based on the change in -2 Log likelihood compared to a model comprising the variables of model 1 only

DISCUSSION

In this study we evaluated intrahepatic characteristics associated with IFN response in CHB patients. Using an unbiased screen of variations in gene expression across the genome, we identified a novel intrahepatic signature strongly associated with enhanced immune activation which predicts therapy response.

CHB patients, and more specifically HBeAg-positive patients, with a favorable response showed more disease activity in the liver before the start of treatment. This is consistent with previous data showing increased inflammatory activity in liver biopsies of HBeAg-positive IFN responders [18]. Similarly, baseline ALT levels have been reported to be higher in HBeAg-positive [9] and –negative [11] patients who responded to peg-IFN therapy. Nevertheless, inflammatory scores correlated only moderately with serum ALT in HBeAg-positive patients, and even less in HBeAg-negative patients.
Because available serologic tests only marginally reflect the immunological response to HBV in the liver, identifying novel intrahepatic markers may improve the prediction of IFN response. For this, we performed a genome-wide screen of gene transcripts and studied their association with treatment outcome.

In patients with a combined response we observed a marked upregulation of genes related to the immune response, and more specifically to antigen processing and presentation or chemotaxis. In addition, there was strong enrichment of a gene-set previously described to be induced in the liver of chimpanzees during the clearance phase of an acute HBV infection [17]. In this set, a large number of genes that enhance antigen processing and presentation were present, and induction of these genes was associated with the appearance of virus-specific T-cells. In contrast to the vigorous T-cell responses seen in acute HBV infection, there is abundant evidence suggesting that these responses are functionally impaired during chronic HBV infection [19]. These findings suggest that those CHB patients who display an intrahepatic transcriptional profile at baseline with more similarities to the clearance signature seen in acute HBV infection, are more likely to respond to IFN treatment. In contrast, a clear downregulation of these genes has been shown in CHB patients [20], suggesting that a more suppressed immune response favoring viral persistence is present in non-responders.

It remains speculative what the additive effect of adefovir is in our cohort. Efficient HBV suppression with NUCs has been shown to restore HBV-specific T-cell responses in some patients [21]. However, rates of combined response appear similar to previous studies with Peg-IFN monotherapy.

Ultimately, one could use this immunological gene signature to predict treatment outcome in CHB patients who are evaluated for receiving treatment. In an independent cohort we were able to validate differential expression of 8 genes. Inclusion of the most predictive gene-set (HLA-DPB1, SERPIN-E1) in a model with other important virological parameters improved the classification accuracy to 90% in both cohorts.

Interestingly, genome-wide association studies previously associated variations in the HLA-DPB1 gene with chronicity in HBV infection [22]. The same variations have also been shown to influence IFN response in CHB patients [23]. Furthermore, the favorable gene variants result in significantly higher expression of HLA-DPB mRNA in the liver [24], thereby providing a possible link between the beneficial effect of these genetic variations and intrahepatic HLA-DPB1 expression. In addition to the relative up-regulation of these immune response related genes in responders, the baseline expression of SERPIN-E1 was higher in non-responders. SERPIN-E1, which is an inhibitor of fibrinolysis, belongs to the group of ISGs. In chronic hepatitis C patients a broad upregulation of ISGs was observed in pre-treatment liver biopsies of future non-responders, resulting in a refractory immune response to exogenous IFNs [25]. A previous transcriptome analysis on IFN response in CHB also showed enhanced ISG expression in non-responders at baseline [26]. In contrast, we did not observe such a pattern in HBV non-responders. A possible
explanation for this discrepancy is provided by previous studies, which showed important differences for the role of ISG induction in HCV- and HBV infection. For example, liver biopsy studies in chimpanzees with acute HBV infection showed no induction of ISGs in the early phase, while in HCV many ISGs were induced [17]. Furthermore, the association between IL28B genotype and ISG expression in the liver appears to specifically depend on infection with HCV [27].

An important limitation of the use of intrahepatic markers in day-to-day clinical practice are the risks involved in taking core-needle biopsies, especially since non-invasive diagnostic tools for fibrosis staging have become widely available. Hence, serum markers would obviously provide the easiest method for response prediction. However, plasma levels of IL25 (ligand of IL17RB), CXCL11, and SERPIN-E1 did not predict response, and did not correlate with liver disease activity or ALT level (data not shown). This suggests that these plasma proteins do not reflect the specific immunological signature in the liver associated with response.

Alternatively to serum markers, cytological fine-needle aspiration biopsy (FNAB) of the liver could represent a non-traumatic way to study the intrahepatic compartment more specifically. Ideally, a technique such as FNAB could lower the threshold for studying liver gene expression in larger patient numbers.

In summary, we identified an intrahepatic transcriptional signature of IFN treatment response in CHB patients. This signature showed strong similarities with the pattern seen during the HBV clearance phase, suggesting that a more activated adaptive immune response at baseline makes these patients more susceptible to the immunomodulatory effect of IFN therapy. These findings aid to our understanding of the host immune response in relation to IFN treatment in CHB patients. Furthermore, pre-treatment determination of a specific gene expression signature may assist in identifying CHB patients with the greatest chance of achieving a response to IFN-based treatment.

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REFERENCES


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Chapter 6


PART II

Immunologic Aspects of Chronic Hepatitis B Virus Infection
Restoration of T cell function in chronic hepatitis B patients upon treatment with interferon based combination therapy.

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Part II

LIST OF ABBREVIATIONS: (in the order of appearance)

HBV: hepatitis B virus
HBsAg: hepatitis B surface antigen
CHB: chronic hepatitis B
HBeAg: Hepatitis B e antigen
peg-IFN: peginterferon alpha 2a
NUC: nucleotide analogues
HLA: human leucocyte antigen
ALT: alanine aminotransferase
LVL: low viral load
HVL: high viral load
CR: combined response
NR: non-response
CMV: cytomegalo virus
PBA: phosphate buffer/albumin
PBS: phosphate buffered saline
FACS: Fluorescence-Activated Cell Sorting
PMA: phorbol 12-myristate 13-acetate
LFU: long term follow up

Keywords (3-10): Hepatitis B, combination therapy, HBV-specific T cells, adaptive immunity, HBsAg loss.

Conflict of interest: none.

Financial support: This work was funded by Roche.

Author's contributions: AN, FS, MJS, EBM designed the study. AN, FS, MJS performed the experiments and analyses. AN, FS wrote the manuscript. NAK, HWR, RAWL, EMML supervised the study and critically reviewed the manuscript. AN, LJ and RBT were involved in the clinical supervision of the patients and critically reviewed the manuscript.
ABSTRACT

Background & Aims: Chronic hepatitis B virus (HBV) infection is characterized by functional impairment of HBV-specific T cells. Understanding the mechanisms behind T cell dysfunction and restoration is important for the development of optimal treatment strategies.

Methods: In this study we have first analysed the phenotype and function of HBV-specific T cells in patients with low viral load (HBV DNA <20,000 IU/mL) and spontaneous control over the virus. Subsequently, we assessed HBV-specific T cells in patients with high viral load (HBV DNA >17,182 IU/mL) treated with peginterferon/adefovir combination therapy who had various treatment outcomes.

Results: HBV-specific T cells could be detected directly ex vivo in 7/22 patients with low viral load. These showed an early differentiated memory phenotype with reduced ability to produce IL-2 and cytotoxic molecules such as granzyme B and perforin, but with strong proliferative potential.

In a cohort of 28 chronic hepatitis B patients with high viral load treated with peg-interferon and adefovir, HBV-specific T cells could not be detected direct ex vivo. However, HBV-specific T cells could be selectively expanded in vitro in patients with therapy induced HBsAg clearance (HBsAg loss n=7), but not in patients without HBsAg clearance (n=21). Further analysis of HBV-specific T cell function with peptide pools showed broad and efficient antiviral responses after therapy.

Conclusions: Our results show that peg-interferon based combination therapy can induce HBV-specific T cell restoration. These findings may help to develop novel therapeutic strategies to reconstitute antiviral functions and enhance viral clearance.
INTRODUCTION

Patients who are chronically infected with hepatitis B (CHB) are at increased risk of liver related morbidity and mortality. Treatment of these patients is aimed at prevention of progression to advanced liver disease [1-3]. Ideally, hepatitis B surface antigen (HBsAg) loss and development of anti-HBs antibodies is achieved upon treatment. This outcome is considered the closest to cure and is associated with complete and durable control of the infection.

Available data on acute, self-limiting HBV infection show strong and multispecific HBV-specific T cell responses [4, 5]. In patients with low viral load CHB, HBV core-specific T cells can be detected directly ex vivo[5-7]. However, not much is known about the exact phenotype and function of these cells. In patients with high viral load and active disease, low HBV-specific immune responses are found. HBV is thought to have evolved several mechanisms to avoid the development of an effective immune response [4, 8-10]. Repetitive T cell receptor stimulation by persistently high hepatitis B antigen (HBsAg and HBeAg) levels is believed to play a role in T cell exhaustion [11].

Treatment options for patients with chronic active hepatitis B consist of peginterferon alpha 2a (peg-IFN) and nucleotide analogues (NUC). The in vitro proliferative capacity of HBV-specific T cells can be restored upon successful treatment with NUCs, which inhibit viral replication [12-14]. Recent data from CHB patients treated with peg-IFN did not show restoration of HBV-specific T cells upon successful treatment, as these cells remained at low frequencies during and after treatment [15, 16].

In this study we first performed an extensive analysis of the phenotype and function of HBV-specific CD8+ T cells in patients with CHB with a low viral load. These patients represent a unique CHB subpopulation because they spontaneously control the virus without need for treatment and in general have minimal or no liver damage. Next, we longitudinally investigated HBV-specific CD8+ T cells in patients with active chronic HBV infection and a high viral load, who were treated with combination therapy of peg-IFN and adefovir. HBV-specific CD8+ T cell responses were compared in patients with HBsAg loss upon therapy versus patients who did not respond to therapy. Our results reveal that combination therapy can induce reconstitution of HBV-specific T cells in patients with HBsAg loss. These data show that HBV-specific T cell activation and differentiation can be restored in CHB patients treated with peg-IFN and adefovir and foster development of combination therapy strategies to enhance viral clearance.

PATIENTS AND METHODS

In order to achieve optimal reactivity with HBV-tetramers and genotype-specific peptides, a selection of CHB patients was made based on viral genotype (genotype A, D or E) and HLA-type (HLA-A2 positivity).

Twenty-two to out of the 80 non-treated HBeAg negative patients with a low viral load (LVL) (ALT<2x upper limit of normal (ULN) and HBV DNA<20,000 IU/mL) that visited our outpatient clinic, were selected for analysis in the present study (table 1 and supplementary figure 1).
Patients with high viral load (HVL) and active CHB (44 HBeAg positive and 48 HBeAg negative, HBSAg positivity > 6 months, normal or increased alanine aminotransferase (ALT) < 10x ULN and HBV DNA > 17,182 IU/mL (> 100,000 copies/mL) were part of a clinical trial in which they received peg-IFN alpha 2a (Pegasys®; Hoffman La Roche, Basel, Switzerland) 180 mg subcutaneously once a week and adefovir dipivoxil (Hepsera®; Gilead Sciences, Foster City, CA, USA) 10 mg daily for 48 weeks and were subsequently followed up for a period of 144-196 weeks [17], (table 1 and supplementary figure 1).

Table 1: Baseline characteristics of subjects with chronic hepatitis B and healthy controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HVL</th>
<th>HVL</th>
<th>HVL</th>
<th>LVL</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response</td>
<td>peg-IFN + ADV</td>
<td>peg-IFN + ADV</td>
<td>peg-IFN + ADV</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>HBsAg loss</td>
<td>7 (100)</td>
<td>4 (57)</td>
<td>12 (86)</td>
<td>13 (59)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Combined response (CR)</td>
<td>7 (100)</td>
<td>4 (57)</td>
<td>12 (86)</td>
<td>13 (59)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Non-response (NR)</td>
<td>7 (100)</td>
<td>4 (57)</td>
<td>12 (86)</td>
<td>13 (59)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Number of subjects</td>
<td>7</td>
<td>7</td>
<td>14</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>Gender, male (%)</td>
<td>7 (100)</td>
<td>4 (57)</td>
<td>12 (86)</td>
<td>13 (59)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Age, median (range)</td>
<td>47 (38-49)</td>
<td>42 (25-62)</td>
<td>42 (27-53)</td>
<td>46 (20-65)</td>
<td>32 (29-56)</td>
</tr>
<tr>
<td>ALT U/L, median (range)</td>
<td>80 (26-275)</td>
<td>108 (26-199)</td>
<td>98 (22-1256)</td>
<td>26 (19-152)</td>
<td>n.a.</td>
</tr>
<tr>
<td>HBV DNA, log10IU/mL, mean ±sd</td>
<td>6.45 ±2.30</td>
<td>5.64 ±2.14</td>
<td>7.10 ±1.72</td>
<td>2.52 ±1.15</td>
<td>n.a.</td>
</tr>
<tr>
<td>HBeAg status, pos (%)</td>
<td>3 (42)</td>
<td>2 (28)</td>
<td>7 (50)</td>
<td>0 (0)</td>
<td>n.a.</td>
</tr>
<tr>
<td>Viral genotype</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>4</td>
<td>2</td>
<td>8</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2</td>
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<tr>
<td>non measurable</td>
<td></td>
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</tr>
</tbody>
</table>

Response definitions of the clinical study, defined at week 72 (6 months after treatment) and follow up (week 144-196), were as follows (with patient numbers included in the present study):

HBsAg loss (n=7): persistently undetectable HBsAg combined with undetectable HBV DNA or HBV DNA <20 IU/mL. For HBeAg positive patients this also included HBeAg seroconversion (HBeAg loss with development of anti-HBe antibodies). Two patients had very low HBsAg levels at week 72 and lost HBsAg at week 84 and 144, and were therefore included in this analysis. All patients developed anti-HBs antibodies.

Combined response (CR)(n=7): HBV DNA <17,182 IU/mL combined with persistently normal ALT values (< ULN) but with still detectable HBsAg. For HBeAg positive patients this also included HBeAg seroconversion.
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Non response (NR) (n=14): HBV DNA >17,182 IU/mL in 2 consecutive measurements at least 3 months apart after cessation of treatment. These patients were all retreated with NUCs.

All patients were HIV seronegative and were not co-infected with hepatitis C or hepatitis delta virus.

For comparison, 8 HLA-A2 or HLA-B7 positive healthy controls were included. These were all seropositive for cytomegalovirus (CMV), which enabled us to compare characteristics of HBV-specific T cells (chronic infection with detectable viral load) with CMV-specific T cells (latent infection with undetectable viral load).

Viral Assessments

Quantification of plasma HBV DNA, HBsAg and HBV genotyping was assessed as described previously (15). CMV serostatus was determined by anti-CMV IgG in serum using the AxSYM microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL) according to the manufacturer's instructions.

Peripheral Blood Mononuclear Cells (PBMCs)

Heparinized peripheral blood samples were obtained at baseline (all patients), and for chronic hepatitis B patients with HVL subsequently during treatment (week 4, 12 and 48), after treatment (week 52 and week 72) and at long term follow up (range 144-196 weeks). PBMCs were isolated using standard density gradient centrifugation and subsequently cryopreserved until the day of analysis.

Immunofluorescent staining and flow cytometry

PBMCs were washed in PBA (PBS containing 0.01% (w/v) NaN3, 0.5% (w/v) bovine serum albumin and 2 mM EDTA). Thawed PBMCs (1.0 x 10^6 cells) were incubated for 30 min in the dark at 4°C with different combinations of fluorescent label-conjugated mouse antibodies. For phenotypic analysis, the following mAbs were used: CD45RA FITC, CD38 PE, CD161 PE, HLA-DR PerCP-Cy5.5, CD8 PerCP-Cy5.5, CD45RA PE-Cy7, CCR7 PE-Cy7 (BD Biosciences, San Jose, USA), CX3CR1 PE (MBL International, Naka-ku Nagoya, Japan), CXCR3 PE (R&D Systems, Minneapolis, USA), CD3 PE-Alexa610, CD27 APC-Alexa750 (Invitrogen, Camarillo, USA), CD127 PerCP-Cy5.5, CD8 Alexa700, PD-1 PE (eBioscience, San Diego, USA). For intracellular staining, cells were fixed after surface staining with FACS Lysing Solution (BD) and subsequently permeabilized (FACS Permeabilizing Solution 2 (BD)). Cells were incubated for 30 min in the dark at 4°C with one or more of the following antibodies: perforin FITC, Ki-67 FITC (BD Biosciences), granzyme B PE (Sanquin, Amsterdam, The Netherlands), granzyme K FITC (ImmunoTools, Germany) or T-bet PerCP-Cy5.5 (eBioscience, San Diego, USA). Measurements were done using BD FACS Canto or LSR Fortessa flow cytometer (BD Biosciences, Europe) and FACS Diva Software. Analysis was done using FlowJo MacV8.6.3.

Antigen-specific T cells

For detection of antigen-specific CD8+ T cells, PBMCs were stained directly ex vivo or after 10 days of culture with HBV peptides (HBV core\textsubscript{18-27} (sequence, FLPSDFFPSV) or HBV envelope\textsubscript{335-343}...
(sequence; WLSLLVPFV) and addition of 50 U/mL IL-2 at day 3 and day 6. PBMCs were incubated with tetrameric complexes for 30 min in the dark at 4°C and subsequently phenotyped as described above. The following APC conjugated HLA-peptide tetrameric complexes were used: HLA-A2 tetramer loaded with HBV core\textsubscript{18-27}, HLA-A2 tetramer loaded with HBV envelope\textsubscript{335-343}, HLA-A2 tetramer loaded with CMV pp65-derived NLVPMVATV peptide; HLA-B7 tetramer loaded with CMV pp65-derived TPRVTGGGAM peptide (Sanquin, Amsterdam, the Netherlands). HLA class I genotyping was carried out by PCR for HLA-A2 and HLA-B7.

**Functional assays**

For LVL patients functional assays, PBMCs were stimulated directly ex vivo for 4 hours with phorbol 12-myristate 13-acetate (PMA) /ionomycine in the presence of Brefeldin A. For HVL patients, PBMC of 7 responders (HVL) and 7 matched non-responders (HVL) were stimulated with a panel of 315 15-mer peptides (HBV genotype A), overlapping by 10 residues, pooled in 5 mixtures, and restimulated for 6 hours at day 10 in the presence of Brefeldin A, CD107a-PE and monensin. The production of cytokines was evaluated by intracellular staining with IFN-γ BV421, MIP-1β Pe-Cy7, TNF-α AF700, IL-2 APC IFNγ FITC and IL-2 PE monoclonal antibodies (BD Biosciences, San Jose CA) after staining with surface markers – and in the case of LVL patients after staining with tetramer complexes - as described above. Measurements and analyses were done as described above.

**Statistical analysis**

The two-tailed Mann-Whitney U test was used for analysis of differences between groups. The Wilcoxon Signed rank test was used for longitudinal analysis in individual patients. P values < 0.05 were considered statistically significant.

**RESULTS**

**Phenotype of HBV-specific T cells in low viral load patients**

HBV-core-specific CD8+ T cells were detected directly ex vivo in 7 out of 22 LVL patients (median 0.052% of CD8+ T cells, range 0.02-0.12) and were selected for direct phenotyping and functional analyses. The phenotype of these HBV-specific CD8+ T cells was compared to the total CD8+ population and to CMV-specific CD8+ T cells of healthy controls (n=8). In this way the differentiation status, level of exhaustion, homing capabilities, activation status and cytotoxic potential of HBV- and CMV-specific T cells were analysed. (figure 1, supplementary figure 2).

In order to distinguish between different CD8+ T cells subsets along the human T cell differentiation pathway, a panel of cell surface markers was used [18]. Analysing expression of CD27 and CD45RA allows discrimination of naïve cells (CD27+CD45RA+) from memory cells (CD27+CD45RA-) and resting effector-type cells which have lost CD27 but re-expressed CD45RA. HBV-specific T cells predominantly showed a memory-like phenotype (CD27+CD45RA-) as described before [19] and had high IL-7 receptor alpha (CD127) and low CCR7 expression (median 83.3 and 14.3%
respectively) consistent with an early differentiated phenotype [19-21]. Although no significant differences were found in the percentage of effector or memory T cells between HBV- and CMV-specific CD8+ T cells, which is in line with our earlier data [22], CMV-specific T cells were predominantly CD27 low and CD45RA high, and had significantly lower expression of CD127 and CCR7 (p=0.0025 and p=0.03 respectively, figure 1b,c).

**Figure 1**: Phenotypic analysis of ex vivo HBV-core-specific and total CD8+ T cells in LVL patients compared to ex vivo CMV-specific and total CD8+ T cells in healthy controls (HC), a) Amount of detected virus-specific cells and b) expression of differentiation markers with representative FACS plots of both HBV- and CMV specific T cells. c) Proportion of memory, effector, CD127 and CCR7 positive cells. d) Amount of chemokine receptors and activation markers, amount of transcription factor T-bet and CD161, amount of exhaustion and cytotoxic markers.

The chemokine receptor CXCR3, associated with T cell recruitment to the liver, was significantly more expressed on HBV-specific cells of CHB patients compared to CMV-specific T cells of healthy controls. In contrast, CMV-specific cells showed high expression of CX3CR1 (fractalkine receptor), a chemokine receptor involved in T cell recruitment to activated endothelium [23] (figure 1d).
HBV-specific T cells generally exhibited low levels of the activation markers HLA-DR and CD38, but were more activated than CMV-specific cells as expression of the cell-cycle marker Ki67 was significantly higher in HBV-specific cells (p=0.0047) (figure 1d).

The T-box transcription factor T-bet, which is seen as a regulator of cell mediated immunity capable of controlling effector molecule gene expression of CD8+ T cells [24], was low in HBV-specific cells but high in CMV-specific cells (p=0.0003). CD161, a marker associated with CXCR6 up regulation, which has been shown to be highly expressed on hepatitis C virus-specific CD8+ T cells [25], was significantly higher on HBV-specific cells compared to CMV-specific CD8+ T cells (figure 1d).

Except for CXCR3 which was higher expressed on total CD8+ T cells of HBV-infected individuals, no other phenotypic differences were found between total CD8+ T cells of CHB patients or healthy controls (figure 1d). This is in line with our former study in which we demonstrated higher expression of CXCR3 on total CD8+ T cells of HBV- and HCV-infected patients [22].

During chronic viral infection, T cells can become exhausted due to continuous antigen exposure and express the inhibitory receptor PD-1 and other inhibitory molecules. As we analysed LVL patients with spontaneous control of the virus who have low or negative HBV DNA level, it was not possible to correlate the expression of exhaustion markers with viral load. Although on average 53.2% of HBV-specific T cells expressed PD-1, this was not significantly higher than on CMV-specific T cells and could also indicate T cell activation instead of exhaustion [26] (figure 1d).

**Cytotoxic potential of HBV-specific T cells in LVL patients**

The phenotypic data showed that HBV-specific T cells in LVL patients have an early differentiated memory phenotype. We next investigated the intracellular presence of granzyme B and perforin, reflecting cytotoxic potential. HBV-specific T cells contained very little granzyme B or perforin, much less than in CMV-specific T cells which are known to have a strong cytotoxic potential (p=0.0003)[18, 27] (figure 1d). In contrast, about 50% of HBV-specific T cells contained granzyme K, which is indeed mostly expressed by memory-type T cells [28].

**Function of HBV-specific T cells in LVL patients**

In order to assess cytokine production, HBV-specific T cells were stimulated with PMA/ionomycine ex vivo. This induced IFNγ production in 58.7% of HBV-specific T cells whereas on average only 22.1% produced IL-2 (figure 2a).

A potent proliferative potential is another hallmark of memory T cells. We therefore analysed the proliferation capacity of HBV-specific T cells using an in vitro stimulation model with HBV-specific peptides. HBV-specific T cells against core peptide could be detected in 19 out of 22 LVL patients after in vitro expansion (median 1.56% of CD8+ T cells, range 0.06-14.9% of CD8+ T cells) and showed a median fold increase of 21.9 (range 0.0-321.6) (figure 2b,c,d). When LVL patients were divided into two groups consisting of patients with a HBV DNA load <2,000 IU/mL and patients with a HBV DNA load > 2,000, no differences were observed (supplementary figure 3). These
data show that indeed, HBV-specific memory T cells found in peripheral blood of LVL patients are very well capable of clonal expansion.

**Figure 2.** Direct ex vivo detection and in vitro stimulation as well as in vitro proliferation of HBV-specific core CD8+ T cells LVL patients with spontaneous viral control. a) overview of cytokine production of HBV core-specific CD8+ T cells after direct ex vivo stimulation with PMA and ionomycin in LVL patients. b) Overview of amount of HBV-core-specific CD8+ T cells of all LVL CHB patients, direct ex vivo and after 10-days of in vitro proliferation (Wilcoxon Signed rank test). c) fold change after 10-days in vitro proliferation of HBV core CD8+ specific T cells. d) Two representative examples of detection of HBV core-specific CD8+ T cells direct ex vivo and after 10 days of proliferation.

Proliferative potential of HBV specific cells in HVL patients is related to treatment outcome
Next, we aimed to characterize HBV-specific T cells in chronic hepatitis B patients with a high viral load and active disease (HVL). As these cells could not be detected ex vivo, we again used the in vitro expansion assay. To investigate whether viral control after combination therapy was associated with the restoration of HBV-specific T cells, we compared the proliferation of HBV core peptide- and HBV envelope peptide-specific T cells between 3 groups of patients with HVL at 3 different time points (week 0, 72 and long term follow-up, LFU). These included HVL patients with HBsAg loss (n=7), HVL patients with combined response (CR, n=7) and HVL patients with non-response who were retreated with NUCs after week 72 (NR, n=14). In the latter patient group, all NUC treatment resulted in long term viral suppression with undetectable HBV DNA.

HVL patients with CR and NR did not show HBV-specific proliferation during follow-up (figure 3a-c). In HVL patients with HBsAg loss however, a strong HBV-specific response at week 72 and at LFU
was observed (wk72: range 0.01-0.98%, LFU: range 0.01-5.8 % of CD8+ T cells). During (weeks 4, 12 and 24) and at end of therapy (week 48) however, very low amounts of HBV-specific T cells could be detected in these patients after in vitro expansion (figure 3a, supplementary figure 4). Remarkably, when NR patients were retreated with long term NUC therapy, HBV-specific T cells did become detectable after expansion in 3 out of 12 patients during LFU (range 0.2-2.2 % of CD8+ T cells) (figure 3b,c). For all three groups of HVL patients, similar observations were made for HBV-specific T cells responses against the envelope epitope as for the core epitope (figure 3b,c). Compared to the HVL patients, LVL patients had higher frequencies of HBV core-peptide -specific T cells after in vitro expansion, however in none of the LVL patients a specific T cells response against the envelope peptide was observed (figure 3b,c).

Figure 3: Longitudinal analysis of HBV-specific CD8+ T cells responses after 10 days of culture with HBV-specific peptide, measured with tetrameric complexes in patients with HBsAg loss, non-responders to trial therapy retreated with nucleot(s)ide retreatment from week 72 onwards (NR), combined responders with sustained viral response but positive HBsAg (CR), low viral load patients with spontaneous viral control (LVL). a) Representative example of a patient with HBsAg loss (at week 36) versus a non-responder patient without HBsAg loss, samples measured during treatment (week 0, 4, 48) and short-term follow up (week 72). HBV core(b) and envelope-specific (c) CD8+ T cell responses at baseline (week 0), short term follow up (week 72) and long term follow up (week 144-196). Numbers of measured samples are indicated. Significant differences are indicated, Wilcoxon signed rank test (longitudinal analysis in individual patients) and Mann Whitney U test (comparing groups). LVL patients had significantly higher core responses (p-value <0.05, not shown in graph), and no measurable envelope responses.
Part II

Broad cytokine responses of HBV specific cells in responders to combination therapy.
The breadth of the HBV-specific T cell response was measured upon expansion and re-stimulation using peptide pools in 7 patients with HBsAg loss and 7 matched non-responders during LFU. Analyses of cytokine production as well as degranulation by CD8+ T cells revealed reactivity against all parts of the HBV proteome and, for patients with HBsAg loss upon combination therapy, particular reactivity against envelope peptides (figure 4a). Non-responders who were on long term NUC treatment also showed cytokine responses in reaction to stimulation with peptide pools (figure 4b), in accordance with the epitope specific data (figure 3b,c) and earlier data [14]. Similarly, broad CD4+ T cell responses were observed in patients with HBsAg loss upon combination therapy, and to a lesser extent in patients with non-response who were retreated with NUCs (supplementary figure 5).

**Figure 4:** Cytokine production by peptide-pool expanded HBV-specific T cells. The mean percentage of cytokine producing CD8+ T cells (>0.01% above background) after 10 days in vitro expansion and re-stimulation with overlapping peptide pools. Cytokine responses in 7 patients with HBsAg loss upon combination therapy (a) and 7 non-responders, retreated with NUCs (b). c) representative FACS plots showing CD107a expression and cytokine production of CD8+ T cells.

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Proliferative potential of HBV specific cells is related to antigen levels
The proliferative potential of HBV core-specific CD8+ T cells was then related to HBV-DNA and HBsAg levels during therapy and LFU for the treated HVL patients and the untreated LVL patients (figure 5). The improved proliferative potential of HBV-specific T cells in patients with HBsAg loss
coincided with the decline and clearance of HBsAg and HBV DNA (figure 5a). A slightly improved proliferative potential of HBV-specific T cells was also observed during viral load suppression in NR retreated with NUCs, whereas in these patients HBsAg levels remained unchanged (figure 5c). Furthermore, our results show that even though LVL patients with spontaneous partial control and HVL patients developing a CR after therapy have similar HBsAg levels, the proliferative potential of HBV-specific T cells was significantly higher in LVL patients compared to patients who needed therapy to achieve CR (fig 5b,d).

**Figure 5:** Proliferative potential of HBV specific cells after 10-days of culture in relation to antigen levels.

a-d) HBV core-specific CD8+ T cell responses (right Y-axis) after 10 days of culture (as in figure 3b) in relation to antigen levels: viral HBV DNA load (IU/mL) and HBsAg antigen levels (IU/mL) (left Y-axis) for patients with HBsAg loss (a), CR (b), NR (c) and LVL (d) respectively. Note the adjusted right Y-axes scales per patient group for core tetramer responses.
DISCUSSION

Chronic hepatitis B virus infection is characterized by functional impairment of HBV-specific T cells which fail to successfully eradicate the virus from the body. It is not known what kind of anti-viral T cell restoration is required (upon therapy) to achieve complete viral control and HBsAg loss. In patients with low viral load (LVL), HBV-specific T cells can be detected directly ex vivo (24). As these patients are able to control viral replication without the need for treatment, we were interested in the phenotype of these HBV-specific T cells. This might give insight into a distinct HBV-specific T cell-profile that is associated with viral control. We therefore first analysed the phenotype and function of HBV-specific cells circulating in the blood of CHB patients with LVL relative to CMV-specific T cells of healthy controls. CHB patients with LVL predominantly displayed a HBV-specific T cell response directed against the core protein. These T cells had an early differentiated phenotype and a low ability to produce IL-2. Furthermore, low levels of cytotoxic proteins such as granzyme B and perforin were observed. During exhaustion, loss of effector function is believed to occur in a hierarchical manner. Functions such as IL-2 production, high proliferative capacity and ex vivo killing are lost first [9], whereas loss of homeostatic proliferation and physical deletion take place at the final stages. This could indicate that HBV-specific T cells in patients with LVL have preserved part of their function and are not terminally exhausted.

Next, we analysed longitudinal HBV-specific T cell dynamics in relation to HBsAg antigen load in patients with high viral load (HVL) and active disease who were treated with combination therapy of peginterferon and adefovir. Previous data has shown that a broad and strong HBV-specific T cell response can be detected after in vitro proliferation when HBsAg is cleared upon NUC therapy. Functional recovery however, was not observed for these HBV-specific T cells [14].

In a small group of patients with HBsAg loss upon peginterferon monotherapy, proliferated HBV-specific cells were able to kill HBV-peptide specific target cells [29]. However, peg-interferon monotherapy does not seem to be able to revert proliferative capacity of HBV-specific T cells [15, 16]. Moreover, no downregulation of exhaustion markers such as PD-1 and CTLA-4 has been observed on HBV-specific T cells after peginterferon treatment [16]. In the current study we have observed a significant increase in HBV-specific T cell proliferation in patients that cleared HBsAg upon combination treatment. While in LVL patients only HBV-specific CD8 T cell responses against the core peptide were observed, in HVL patients with HBsAg loss upon combination treatment, HBV-specific T cells specific for the core as well as envelope protein could be induced. Upon re-stimulation after in vitro expansion with HBV-peptide pools, broad production of cytokines was observed in response to different parts of the HBV-proteome. This suggests a possible causal relationship between the action of HBV-specific T cells and HBsAg clearance in these patients. In patients with NR to therapy, no HBV-specific T cells could be detected at any time before retreatment with NUCs, and hardly any HBV-specific T cells were detectable in patients with combined response, even though viral HBV DNA load and HBsAg levels in these patients were similar to LVL patients. The extent of anti-viral T cell restoration that
is required to achieve complete viral control and HBsAg clearance upon therapy in patients with HVL is currently unknown.

Our results show that HBsAg clearance after combination therapy is associated with a broad and functional HBV-specific T cell response in the circulatory compartment. This observation was made in HBeAg positive and –negative patients with HBsAg loss. As we were only able to analyse a limited set of patients, we were not able to objectify any possible differences between these two entities. Furthermore, the exact phenotype of HBV-specific T cells in HVL patients with active disease could not be studied as these were only detected after in vitro expansion. It is well possible that these cells express different phenotypic markers reflecting a more severe exhausted state and diminished proliferative potential than HBV-specific T cells in LVL patients. Furthermore, our inability to detect specific T cells before therapy even after in vitro expansion, could be the result of allocation of HBV-specific T cells to the active site of inflammation, i.e. the liver. Detection of HBV-specific T cells could derive from relocation to the blood after cessation of inflammation and antigen decline. However, no increased frequency of HBV-specific T cells was observed previously in the liver during active infection [10]. Still, whether the increase in HBV-specific T cell proliferation is a direct consequence of therapy on HBV-specific cells or an indirect result of therapy-induced antigen decline remains to be further investigated.

Our data may indicate that HBV-specific T cells in patients with HVL are present at frequencies below the threshold of detection but retain the flexibility to restore their antigen responsiveness. Indeed earlier advances in improving T cell function have been made by blockade of PD-1, indicating that these cells are not terminally differentiated and have the plasticity to become fully functional early differentiated memory cells [30, 31]. Furthermore, other lymphocyte subsets have proven to play a role in HBsAg clearance upon therapy. Regulatory T cells and natural killer cells, that make up a considerable part of the lymphocytes in the liver, have not been analysed in this study. However, the increased proliferation observed in patients with HBsAg loss could be related to an alteration in NK cell function, as these cells have been identified as regulators of HBV-specific T cells in the liver [31].

To summarize, our data indicate that in patients with LVL, relatively narrow HBV-core-specific T cells with a resting effector-memory phenotype and low ability to produce IL-2, but strong proliferative potential, seem to be sufficient to retain the virus at low levels. In patients with HBsAg loss upon treatment with peginterferon and adefovir, as well as in some patients on long term nucleos(t)ide therapy, there is a partial recovery of HBV-specific T cells. In these patients a broader repertoire of HBV-specific T cell restoration was observed. The extent to which HBV-specific T cell restoration is needed in order to achieve HBsAg loss, as well as the interaction with other players of the immune system remains to be elucidated. Future therapeutic strategies may be based on the modulation of protective T cell responses, and reconstitute their antiviral function enabling cure of CHB virus infection.
SUPPLEMENTARY MATERIAL

Supplementary figure 1: Patient breakdown. (A) Active CHB patients (HBV DNA>17,182 IU/mL, ALT <10X upper limit of normal (ULN)) were treated for 48 weeks with 180 ug peg-interferon alfa-2a weekly and 10 mg adefovir once daily and followed up in this analysis for a period of 96-144 weeks (HVL patients). (B) Untreated LVL HBeAg negative CHB patients with low viral load and spontaneous viral control (HBV DNA <20.000 IU/mL, ALT <2x ULN) were used as control (LVL patients). (C) Healthy controls were used as HBV-negative controls. Selection of samples was based upon viral genotype (a,d,e) and HLA status (HLA-A2 positivity) of the patients and on CMV seropositivity and HLA status (HLA-A2 of HLA-B7 positivity for healthy controls).
Supplementary figure 2: Phenotypic analyses of HBV-core-specific CD8+ T cells in CHB patients with LVL and CMV-specific CD8+ T cells in HC. Representative FACS staining of expression of phenotypic markers on HBV- and CMV-specific T cells. HBV, hepatitis B virus; LVL, low viral load; CMV, cytomegalovirus; HC, healthy control.
Supplementary figure 3: In vitro proliferation of HBV-specific core CD8+ T cells LVL patients divided in two groups. HBV DNA > 2,000 IU/mL and/or HBsAg > 1,000 IU/mL and HBV DNA < 2,000 IU/mL + HBsAg < 1,000 IU/mL. Mann Whitney U test.

Supplementary figure 4: Longitudinal analysis of HBV-specific CD8+ T cells responses after 10 days of culture with HBV-specific peptide, measured with tetrameric complexes in patients with HBsAg loss after combination therapy. Samples measured: start (week 0), during treatment (week 0, 4, 12, 24), end of treatment (week 48), short (week 72) and long-term follow up (LFU).
Supplementary figure 5: Cytokine production by peptide-pool expanded HBV-specific T cells. The mean percentage of cytokine producing CD4+ T cells (>0.01% above background) after 10 days in vitro expansion and re-stimulation with overlapping peptide pools. Cytokine responses in 7 patients with HBsAg loss upon combination therapy (A) and 7 non-responders, retreated with NUCs (B).
REFERENCES


Upregulation of CXCR3 expression on CD8+ T cells due to the pervasive influence of chronic hepatitis B and C virus infection.
ABSTRACT:
Chronic systemic ‘latent’ viral infections such as Cytomegalovirus infection (CMV) are known to leave a fingerprint in the total T-cell population. We investigated whether chronic infections with a ‘persistent’ viremia, such as chronic hepatitis B and C (CHB, CHC), characterized by local organ-specific inflammation, also impact the total peripheral T-cell population or other virus specific T-cells that do not target hepatitis viruses.

No phenotypic or functional differences were found between CD8+ T-cells or CMV- or Epstein-Barr virus specific T-cells in viral hepatitis and healthy controls (HC). However, expression of chemokine-receptor CXCR3 was significantly higher on total peripheral CD8+ T-cells of CHB or CHC patients compared to HC (p<0.005) which may reflect the pervasive influence of a persistent viral infection, even when restricted to the liver. In CHB higher CXCR3 expression was associated with positive HBeAg-status and correlated with the percentage of HBsAg expressing hepatocytes found in liver biopsies, both pointing to a relation between CXCR3 expression and disease activity. In fact chemokine-receptors such as CXCR3 are important for T-cell recruitment to the liver and chemokine-ligands specific for CXCR3 are upregulated in chronic hepatitis. Modulating chemokine(receptor) expression could be a potential target for future therapy to optimize the anti-viral immunologic environment in the liver.

Supplementary figures can be found online
INTRODUCTION

Infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most common causes of liver disease. Worldwide more than 500 million people are chronically infected [1,2] and annually more than 1 million people die of infection mediated liver cirrhosis and hepatocellular carcinoma. T cells directed against viruses that are cleared from the host (such as influenza) differ from virus-specific T cells responsive to latent or chronic infections. This indicates that the differentiation phenotypes mostly seem to be determined by the chronicity and level of viral load during infection together with the inflammatory milieu. Latent infection with Cytomegalovirus (CMV) has been shown to leave a phenotypical fingerprint even in the total T cell population, identified by an increased percentage of highly differentiated effector-memory type T cells. This is most prominent in immunosuppressed and older individuals as CMV-specific T cell responses continue to increase throughout life and can come to dominate the entire T cell repertoire in the elderly, perhaps impairing responses to other antigens [3]. Contrary to systemic latent virus infections such as CMV with undetectable viral load in healthy individuals, HBV and HCV together with HIV are characterized by continuous presence of high viral loads and are the three clinically most important persistent viral infections. It is not clear whether viruses like HBV or HCV, which cause a predominantly localized, organ-specific chronic infection can also impact the total peripheral T-cell population.

Accumulating evidence shows that CD8+ T cell phenotypes also largely depend on the signals they receive from the tissue environment. Recent data in humans show that CMV-specific T cells in peripheral blood differ in phenotype and function from CMV-specific cells in lymph nodes [4] and T cells directed against influenza diverge between lung and blood [5,6].

As T cells continuously recirculate through organs, lymph nodes and peripheral blood, it can well be envisaged that a local infection will be also reflected in the total peripheral T cell population in affected individuals. Indeed, one study showed that the effect of chronic HCV infection on T cell phenotype extends beyond the T cells that target this virus. CMV-specific CD8+ T cells were shown to have a more immature phenotype in HCV-infected individuals compared with healthy controls [7].

When studying T cell phenotypes, analysis of chemokine receptor expression is especially interesting since this provides information on the migration properties of the cells in response to chemokines produced in inflamed sites. Several reports have described the upregulation of chemokines during chronic viral hepatitis [8-12]. The way in which CD8 T cells are polarized with regard to their chemokine receptor profile depends on the tissue cytokine profile. Under influence of type 1 related inflammatory cytokines IFN-γ and IL-2, hepatocytes and liver endothelial sinusoid cells produce CXCL9, 10, and 11, which can ligate to their receptor CXCR3. CXCR3 expression is described to be restricted to activated T cells, natural killer cells and a small proportion of B cells and appears to mark a subset of lymphocytes with a capacity for migration to inflammatory sites [13]. During chronic hepatitis C infection it has been shown that expression of chemokine receptors, such as CXCR3, has been associated with hepatic infiltrates [9].
In the present study we investigated whether indeed chronic HCV or HBV infection, which is predominantly located in the liver, can alter the total peripheral CD8+ T-cell population in a similar way as has been described for CMV. We compared the phenotypic and functional characteristics of the total CD8+ T-cell population of CMV-seropositive individuals with and without chronic hepatitis B or C. Furthermore, to determine the effect of eventual clearance (HCV) or suppression (HBV) of the virus upon therapy, we compared T cells from responders and non-responders before and after therapy.

**MATERIALS AND METHODS**

**Subjects**
Peripheral blood mononuclear cells (PBMC) were collected from patients with chronic hepatitis B, chronic hepatitis C and healthy controls. All subjects were HIV seronegative. Patients co-infected with HBV, HCV or hepatitis D virus were excluded from this study.

Chronic hepatitis B patients participated in a clinical study designed to assess markers of response during treatment with peg-IFN and adefovir (Supp.fig.1a). The treatment regime consisted of weekly Peg-IFN-alfa-2a 180ug and 10mg adefovir daily for 48 weeks, followed by a 24-week treatment-free follow-up. Sustained virological response (SVR) was defined as HBV DNA levels ≤2.0 x 10^3 IU/mL and normalization of ALT for HBeAg negative patients. For HBeAg positive patients SVR was defined as HBeAg seroconversion and ALT normalization.

Chronic hepatitis C patients participated in a clinical study designed to assess the efficacy of high-dose interferon–alfa induction therapy (Supp.fig.1b). HCV-infected patients were treated with triple therapy consisting of amantadine hydrochloride (200 mg/day) and ribavirin (1000 or 1200 mg/day, based on body weight) for a total of 24 or 48 weeks, combined with IFN-alfa-2b induction during the first 6 weeks and thereafter combined with weekly pegylated IFN-alfa-2b (1.5 ug/kg). The scheme for IFN induction was as follows: weeks 1 and 2, 18 MU/day in 3 divided doses; weeks 3 and 4, 9 MU/day in 3 divided doses; and weeks 5 and 6, 6 MU/day in 2 divided doses. Treatment was stopped in all patients who were HCV RNA positive at week 24. SVR was defined as HCV RNA negative 24 weeks after cessation of treatment. Non-response was defined as detectable HCV RNA at all time-points during treatment and at end of treatment.

Healthy CMV-seropositive volunteers without viral hepatitis were used as healthy controls. All subjects gave written informed consent prior to inclusion in the study and the study was approved by the Ethical Review Board (ERB) of the Academic Medical Center Amsterdam.

**Peripheral Blood Mononuclear Cells (PBMCs)**
Heparinized peripheral blood samples were obtained at baseline, during (week 4 and 12) and after antiviral treatment (week 52 or week 72). PBMC were isolated using standard density gradient centrifugation and subsequently cryopreserved until the day of analysis.
Viral Assessments
Quantification of plasma HBV DNA was assessed using the Roche COBAS TaqMan 48 assay (F. Hoffmann-La Roche Ltd, Diagnostics Division, Basel, Switzerland), with a dynamic range between 20 and 1.70x10^8 IU/mL. HBV genotypes were determined using the INNO-LiPA assay (Innogenetics, Gent, Belgium) or by sequencing with didioxy technology with SQL Lims system software.

HCV RNA was assessed using a quantitative bDNA assay (Versant HCV assay, version 3.0; linear dynamic range between 615 and 7.7 x 10^6 IU/mL), qualitative PCR (COBAS Amplicor HCV test, version 2.0; lower limit of detection (LLD), 50 IU/mL), and qualitative transcription-mediated amplification (TMA; Versant HCV qualitative assay; LLD of 5 IU/mL). The Truegene assay was used to determine the HCV genotype.

CMV serostatus was determined by anti-CMV IgG in serum using the AxSYM microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL) according to the manufacturer’s instructions. The EBV serostatus was investigated by determination of IgG to EBV viral capsid antigen (Biotest, Dreieich, Germany).

Immunofluorescent staining and flowcytometry
PBMC were washed in PBS containing 0.01% (w/v) NaN3, 0.5% (w/v) bovine serum albumin and 2 mM EDTA (PBA). Thawed PBMCs (1.0 x 10^6 cells) were incubated for 30 min in the dark at 4°C with different combinations of fluorescent label-conjugated mouse antibodies. For analysis of expression of surface markers, the following mAbs were used: CD45RA FITC, CD38 PE, CD161 PE, CD4 PerCP-Cy5.5, HLA-DR PerCP-Cy5.5, CD8PerCP-Cy5.5, CD45RA PE-Cy7, CCR7 PE-Cy7 (BD Biosciences, San Jose, USA), CX3CR1 PE (MBL International, Naka-ku Nagoya, Japan), CXCR3 PE (R&D Systems, Minneapolis, USA), CD3 PE-Alexa610, CD27 APC-Alexa750 (Invitrogen, Camarillo, USA), CD127 PerCP-Cy5.5, CD8 Alexa700, PD-1 PE, IL18 receptor alpha FITC (eBioscience, San Diego, USA). Cells were labelled according to manufacturer’s instructions and washed and analyzed in PBA and analyzed using BD FACS Canto flow cytometer and FACS Diva Software. Analysis was done using FlowJo MacV8.6.3.

Analysis was done on all or a subset of patients (table 1) based on HLA subtype and availability of sample material.

In total 1 million cells were measured for each staining.

Antigen-specific T cells
For detection of antigen-specific CD8^+ T cells, PBMC were incubated with tetrameric complexes for 30 min in the dark at 4°C and subsequently with other fluorescent antibodies as described above. HLA-peptide tetramer complexes were obtained from Sanquin Reagents (Amsterdam, The Netherlands). For CMV, we used 8 different tetramers loaded with pp65- and IE-derived peptides: for EBV, we used 6 different tetramer loaded with BMLF1-, EBNA3A-, and BZLF1-derived peptides. Ex vivo HCV-specific CD8 T cell responses were analyzed using two APC-labeled tetramers. HLA-A2 tetramers contained the HCV NS3 CINGVCWTV peptide. CMV- and EBV-specific T cell responses were measured at baseline in chronic hepatitis B patients (n=8/8), chronic hepatitis C patients (n=8/7) and healthy controls (n=8/4) respectively.
Part II

Five HLA-A*02 chronic hepatitis C patients were analyzed to detect changes in HCV-specific T cell responses between responders and non-responders after antiviral therapy at week 52. HLA class I genotyping for HLA A2 was carried out by PCR.

Intracellular staining
For intracellular perforin, granzyme B and Ki-67 staining, the following technique was used: cells were fixed after surface staining with FACS Lysing Solution (BD). After permeabilization (FACS Permeabilizing Solution 2 (BD) cells were incubated for 30 min in the dark at 4°C with one or more of the following antibodies: perforin FITC, Ki-67 FITC (BD Biosciences), granzyme B PE (Sanquin, Amsterdam, The Netherlands), granzyme K FITC (Immunotools, Germany) or T-bet PerCP-Cy5.5 (eBioscience, San Diego, USA). After incubation cells were washed and analyzed in PBS containing 0.01% (w/v) NaN₃ and 0.5% (w/v) bovine serum albumin (PBA) and analyzed using BD FACSCanto flow cytometer and FACSDiva Software. Analysis was done using FlowJo MacV8.6.3.

Statistical analysis
The two-tailed Mann-Whitney U test was used for analysis of differences between groups. The spearman test for non-parametric correlation was used. P values < 0.05 were considered statistically significant.

RESULTS

No changes in peripheral CD8⁺ T cell differentiation phenotype and function in viral hepatitis
To examine whether chronic liver infection can have a pervasive influence on the immune system we analyzed peripheral blood T cells for differentiation, migratory and activation properties and compared CD8⁺ T cells of chronic hepatitis patients with healthy controls. An overview of the study population is shown in table 1.

The percentage of CD3⁺ T cells within the PBMCs did not differ between patients infected with HBV or HCV and healthy controls (data not shown). Using a combination of CD27 and CD45RA expression, a phenotypic analysis was performed to discriminate between naive (CD45RA⁺CD27⁺), memory (CD45RA⁻CD27⁺) and effector (CD45RA⁻CD27⁻) CD8⁺ T cells [14]. The distribution of effector, memory and naive T cells did not significantly differ between HBV or HCV infected individuals and healthy controls (Fig.1a,b). In addition, no significant differences were seen in expression of CD28, CCR7, CD127, activation markers CD38,HLADR, Ki67 and mucosal associated invariant T cell (MAIT) markers CD161 and IL18Ra (data not shown). Functional characteristics of CD8⁺ T cells were examined by analysis of the cytotoxic molecules granzyme B and perforine, which represent the cell-mediated killing ability. In line with the equal percentages of effector/memory T cells, no differences were seen in the expression of these cytotoxic molecules between HBV and HCV infected patients and healthy controls (Fig.1c) nor in the expression of T-bet, the transcription factor associated with differentiation to effector cells (data not shown). In addition no upregulation was seen in PD-1 expression in either percentage positive cells or mean fluorescence intensity (MFI) between HBV or HCV infected patients and healthy controls (Fig.1d).
CXCR3 expression on T cells in CHB and CHC patients  Chapter 8

Table 1: Baseline characteristics of hepatitis B-, hepatitis C-infected patients and healthy controls. (Fisher exact test for sex, one sided ANOVA with Kruskal-Wallis test for age, n.s.= not significant)

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<th>HCV+</th>
<th>Healthy controls</th>
<th>p value</th>
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<td>log viraemia, IU/mL, median (range)</td>
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<td>log HBsAg serum,median (range) IU/mL</td>
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<td>HBsAg (%) median (range)</td>
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N.D.= not determined, *SVR: sustained viral response 24 weeks after therapy for HBV: HBeAg seroconversion HBV DNA<2,000 IU/mL, for HCV: HCV RNA negative. NR: non response after therapy **According to ISHAK ***: HBsAg: % of HBsAg positive hepatocytes in liverbiopsy, n.s.= not significant

Ethnicity of patients:
HBV: Caucasian n=12 (40%), Central African n=11 (37%), North African n=1 (3%), Central Asian n=7 (23%)
HCV: Caucasian n=12 (55%), North African (mostly Egypt) (45%)
Healthy controls n=10 Caucasian (100%)

It is largely unclear why some patients with chronic hepatitis infection respond to therapy whereas others do not. CD8+ T cells are crucial in clearing virus-infected cells. Therefore we studied T cell phenotype and function before and after therapy and compared responders to non-responders. In this study phenotypic CD8+ T cells markers associated with differentiation and activation properties were not altered after a favourable treatment response to therapy in HBV (sustained virologic response:HBV DNA<2,000 IU/mL +/- HBeAg seroconversion, or HBsAg seroconversion) or HCV (viral clearance) (data not shown).

Pervasive influence of chronic hepatitis infection on other virus specific cells
An earlier study showed that in patients with chronic HCV, CMV-specific CD8+ T cells lost markers associated with effector differentiation; they had increased expression of CCR7 and reduced expression of Fas and perforin[7].

Table 1: Baseline characteristics of hepatitis B-, hepatitis C-infected patients and healthy controls. (Fisher exact test for sex, one sided ANOVA with Kruskal-Wallis test for age, n.s.= not significant)
Part II

Another study found that the exhaustion marker PD-1 was not only markedly upregulated on HCV-specific cells in peripheral blood and liver, but also on CMVpp65-specific cytotoxic lymphocytes in chronically infected patients compared to normal controls. These findings suggest a global effect of HCV infection on the phenotype of all T cells [15].

We analyzed CMV- and EBV-specific CD8+ T cells from HBV- and HCV-infected patients and healthy controls. No differences were seen in frequencies of CD8+ T cells directed against CMV or EBV (Supplement fig.2a). As to the differentiation status, no differences were seen in CMV- or EBV-specific cells between the groups (Supplement fig.2b). Figure 2b shows similar percentages of effector-phenotype (CD27-CD45RA+), CCR7+ cells and perforin or granzyme B-containing cells within CMV- and EBV-specific CD8-T cells.

Also no changes were seen in PD-1 expression on CMV specific cells between HBV- and HCV-infected patients and healthy controls (Supplement fig.2c). Therefore, our data do not show a more exhausted or less mature phenotype of CMV- or EBV-specific CD8+ T cells in chronic hepatitis patients compared to healthy controls. If anything, CMV-specific T cells in HBV have an increased effector-phenotype based upon CD27 and CD45RA expression (Supplement fig. 2b) but this was not reflected in i.e. perforin expression.

Upregulation of inflammatory CXCR3 chemokine receptor expression in viral hepatitis

Chronic HBV and HCV infections are characterized by T cell mediated hepatic inflammation, eventually leading to hepatic fibrogenesis. As the recruitment of immune cells into the infected liver is orchestrated by chemokines, we investigated the presence of chemokine receptors on peripheral blood T cells in patients with HBV or HCV compared to healthy individuals. As T-helper (Th) 1 inflammatory cells, characterized by interferon (IFN)-gamma and interleukin (IL)-2 secretion, have been shown to predominate in the liver during chronic HCV infection,[11] we focused on Th1 associated chemokine receptors, in particular CXCR3. As shown in Figure 2, expression of chemokine receptor CXCR3 was significantly higher on CD8+ T cells of patient chronically infected with HBV (p<0.005) compared to healthy controls. The higher expression of CXCR3 was observed on the total CD8+ T cell population and across all T cell subsets (effector, memory and naïve T cells) and expression remained high after therapy (week 52 or 72) irrespective of the outcome. Remarkably, in HCV infected patients a significant difference was observed in CXCR3 expression between baseline and end of therapy (all subsets p<0.01) which was not correlated to treatment outcome as both responders and non-responders to therapy showed an increase in CXCR3 expression. CXCR3 was significantly higher after therapy (p<0.05) compared to healthy controls. Before therapy CXCR3 expression did only differ between naïve T cells of HCV patients and healthy controls.

Next we analyzed CXCR3 expression on HCV specific cells (Fig.2c,d) on available samples after end of therapy. HCV specific cells were CD45RA CD27+ indicating that they have a memory-like phenotype. CXCR3 expression on HCV-specific cells was similar compared to total memory CD8+ T cells in HCV patients but significantly higher than CXCR3 expression on total memory cells of
Figure 1: Distributions of T cell subsets and functional profile.

Fig.1. (a) In order to discriminate between differentiation of CD8+ T cells a phenotypic analysis was performed by using a combination of CD27 and CD45RA expression: naïve T cells: CD45RA+CD27+, memory T cells: CD45RA-CD27+, effector T cells: CD45RA+CD27-. (a) representative FACS plots. (b) distributions of effector, memory and naïve T cells in hepatitis B (HBV) patients, hepatitis C (HCV) patients (before therapy) and healthy controls (HC) (two tailed-Mann whitney U). (c,d) perforine, granzyme B (c) and PD-1 (d) expression on total CD8 T cells in HBV, HCV (before therapy) and HC.
Part II

healthy controls (Fig. 2d). Detection of HBV specific cells directly ex vivo by HBV tetramers was not possible due to low frequencies. To investigate whether CXCR3 upregulation was also detectable on virus specific T cells directed to viruses not affecting the liver, CXCR3 expression on CMV and EBV specific T cells was analyzed. No differences were found in CXCR3 expression on CMV and EBV specific cells between viral hepatitis patients and healthy controls (Supplement fig. 2d).

Since CXCR3 expression was increased on the total CD8+ T cell population (including naive) and is therefore not likely to be induced only by TCR signalling, we addressed the question what signals could lead to upregulation of CXCR3. *In vitro* experiments showed that indeed TCR stimulation (by antiCD3 + antiCD28) increased CXCR3 expression on T cells, whereas culture in the presence of several inflammatory cytokines (IFNy, IL-2, IL-12, IL-15, IL-18, IP-10) did not, pointing to an *in vivo* mechanism that cannot be mimicked *in vitro* (data not shown).

We also analyzed expression of CX3CR1, a chemokine involved in endothelial inflammation, but no differences were found between HBV, HCV patients and healthy controls (data not shown).

**CXCR3 expression on CD8+ T cells correlates with disease activity in chronic HBV infection.** In HBV, we investigated whether CXCR3 expression on CD8+ T cells was associated with disease activity according to HBeAg status. HBeAg, the product of transcription of the HBcore open reading frame is involved in formation of the viral capsid and has been shown to have immunomodulatory properties. A high level of serum HBeAg is a marker of active viral replication and is associated with high viral loads in the serum [16]. In our study ALT levels were significantly higher in HBeAg positive patients compared to HBeAg negative patients (p<0.05, data not shown). CXCR3 expression on all T cell subsets was increased in HBeAg positive patients compared to HBeAg negative patients (Fig. 3a). Effector CD8+ T cells have the highest capacity for direct cytotoxicity and are likely involved in maintaining a situation of chronic inflammation. Therefore we analyzed whether CXCR3 expression on effector T cells correlated with liver pathology and viral load. Neither in HBV nor HCV infection a correlation was observed between CXCR3 expression on effector T cells and HAI or ISHAK fibrosis score in liverbiopsies, serum alanine aminotransferase (ALT) levels or viral load in plasma (Fig. 3b) (HAI scores were only available for HBV). However, in HBV infection the percentage of hepatocytes which stained positive for HBsAg, which is a measure for the amount of infected hepatocytes in the liverbiopsy, was associated with CXCR3 expression on effector T cells in peripheral blood (Fig. 3c,d).
Figure 2: Chemokine receptor CXCR3 expression on CD8+ T cells of patients chronically infected with HCV and HBV and healthy controls. (a) Representative flow cytometric analysis of CXCR3 and CD8 staining within CD3+ cells in HBV, HCV patients and HC. (b) CXCR3 expression on total CD8+ T cells and CD8+ T cell subsets (effector, memory, naive cells, based on CD27 and CD45RA expression as shown in Fig 2) of patients chronically infected with HBV and HCV (before and after treatment) and HC. Ns: P>0.05 (non-significant), *P<0.05, **P<0.005, ***P<0.001 (Two tailed Mann Whitney u). (c) Differentiation status and CXCR3 expression on HCV specific CD8+ cells. (d) CXCR3 expression on HCV specific CD8+ T cells and on total CD8+ T cells with similar differentiation status (memory cells) of HCV patients and healthy controls.
Figure 3: CXCR3+ expression on CD8+ T cells in relation to disease activity in HBV infected patients (before therapy). (a) CXCR3 expression on total CD8+ T cells in HBeAg+ and HBeAg- patients and healthy controls. (b) CXCR3+ expression on effector CD8+ T cells and liver fibrosis grade in both HBV and HCV (Spearman test, non parametric correlation). (c) Representative immunohistochemistry analysis of HBsAg-staining (black) of hepatocytes in a liver biopsy of a chronic hepatitis B patient taken at start of therapy. (d) CXCR3+ expression on effector CD8+ T cells and the amount of HBsAg+ hepatocytes (p-value for correlation). HBsAg+ cells in the liver biopsy were counted by two independent pathologists.
DISCUSSION

In the present study we investigated whether chronic HCV and HBV infection, both causing a localized organ-specific infection with persistent high viral loads, could have a pervasive influence on the total peripheral CD8⁺ T-cell population and on other virus specific T cells. We showed that CD8⁺ T cells in patients with chronic viral hepatitis are not different from healthy controls when focusing on differentiation phenotype. In contrast to an earlier report in which a reduced expression of perforin on total CD8 T cells and a reduced effector phenotype of CMV specific cells was shown in HCV patients [7], in our study both in the total CD8⁺ T cell population or in CMV- and EBV-specific T cells of patients with chronic HCV or HBV no reduction in cytoxicity or other clear changes in maturation were observed. These characteristics also did not change after antiviral therapy and were independent of the treatment outcome. It should be taken into account that dissimilarities with the earlier study may be influenced by differences in study population characteristics such as numbers of healthy controls and variations in viral genotype.

However, we did find a pervasive influence of the chronic liver infections with respect to expression of the chemokine receptor CXCR3. CXCR3 expression was increased on peripheral blood CD8⁺ T cells chronic HBV and HCV patients with a localized liver inflammation compared to healthy individuals infected with a latent CMV, infection (Fig.3). The CXCR3 upregulation was also found on HCV-specific T cells. CMV and EBV virus-specific cells that do not specifically target the liver did not seem to be affected by the upregulation of CXCR3, this may indicate that CMV and EBV specific T cells that do not specifically home to the chronic inflammatory milieu in the liver, avoid trigger signals that lead to upregulation of CXCR3. However these results may be difficult to interpret as numbers of patients with detectable CMV and EBV cells were low. Also the finding that CXCR3 upregulation was most prominent on naive cells while these cells do not enter the liver, supports the hypothesis of a systemic inflammatory component that is responsible for this chemokine receptor induction.

Interestingly, the inflammatory effect seems not to be restricted to T cells as also the total B cell population in patients with HCV and HBV infection has been shown to have increased activation markers and CXCR3 expression[17].

Thus far it is unclear which factors in the inflammatory environment directly regulate CXCR3 expression. Perhaps observations of HBV and HCV infection in extrahepatic tissues [18,19], suggesting that also other cell types are accessible for both HBV and HCV replication in humans, could play a role in systemic changes observed in both HBV and HCV infection.

The upregulation of CXCR3 in HBV and HCV infected patients was most prominent on naive CD8 T cells. It has been shown in mice that naive phenotype bystander CD8 T cells could indeed be sensitized by acute viral infections via low affinity MHC-TCR interactions in combination with type I interferon such that they temporarily upregulate cytotoxic molecules prior to cognate antigen stimulation[20]. Perhaps this phenomenon plays a role in the ‘bystander’ CXCR3 upregulation on naive CD8 T cells.
Our *in vitro* experiments showed upregulation of CXCR3 upon TCR-mediated activation of the T cells but could not confirm CXCR3 upregulation upon cytokine stimulation. However, the *in vivo* environment is difficult to simulate and upregulation of CXCR3 may only occur in the context of a “cytokine storm” with an accumulation of inflammatory cytokines. Next to inflammatory cytokines also alterations in chemokine ligands, direct effects of interferon and induction of chemokines by viral proteins could be associated with CXCR3 expression. CXCR3-associated chemokines, CXCL9, CXCL10 (IP10) and CXCL11 have been shown to be overexpressed in hepatic parenchyma of patients with viral or non-viral chronic liver disease [10,21]. These observations suggest a specific role for CXCR3 and its ligands in the recruitment of T cells into these otherwise restricted sites. Recent studies in mice have revealed that CXCR3 may play a crucial role in directing T cell migration to the proximity of inflammatory cytokines or antigen. These studies suggest that in this way CXCR3 expression can influence cell fate decision that controls effector versus memory commitment early after T cell activation [22,23]. It is unclear what determines the feedback loop between the presence of chemokine ligands and expression of their chemokine receptor; both increased and decreased chemokine levels have been associated with CXCR3 upregulation [9,24].

In HCV patients we found that CXCR3 expression was significantly increased upon therapy. Indeed it has been shown that HCV proteins such as NS5a and core could directly induce synthesis of CXCR3 ligands CXCL10 and CXCL9 in the liver [25] and thereby interfere with CXCR3 expression. In a small study the stronger reduction of viral load observed in HCV patients with a favourable treatment response was also associated with an increase in CXCR3 expression at week 24 of therapy [9]. In our study we could not confirm the relation between the increase in CXCR3 expression and therapy outcome, however this may be explained by the later time point of analysis at 24 weeks after therapy withdrawal. As upregulation of CXCR3 on CD8+ T cells is not seen during treatment in HBV-infected patients who also receive exogenous interferon, upregulation due to a direct effect of type I interferon seems less likely. Furthermore these findings could indicate that pathways responsible for CXCL10 levels, CXCR3 expression and consecutive T cell recruitment from the liver are differentially regulated in HBV and HCV.

It is of interest to measure CXCL10 levels at baseline and during HBV therapy and investigate their predictive value in treatment outcome.

In HCV patients levels of CXCR3 ligands have been associated with liver damage. However the correlation with liver inflammation appeared to be stronger than the correlation between CXCR3 ligands and liver fibrosis [8-11]. Also CXCR3 expression has been associated with liver inflammation but a clear relation between CXCR3 expression and liver fibrosis has not been observed [8]. In our study no correlation between CXCR3 expression and fibrosis grade in viral hepatitis patients was present. This indicates that other factors such as the duration of liver inflammation but also genetic and non-genetic factors are necessary to develop fibrosis. Although in our study no association was found between CXCR3 expression and inflammation score in liver biopsies, the higher CXCR3 expression observed in HBeAg positive chronic hepatitis B patients...
could reflect the higher disease activity according to ALT levels seen in these patients compared to patients without detectable serum HBeAg. The finding that CXCR3 expression on T cells correlated with the amount of HBV antigen in the liver (Fig.5) is certainly of interest. It is however plausible that increased antigen expression is accompanied by elevated inflammation, making it difficult to distinguish one from the other.

Summarizing, we have shown in a well defined cohort of patients that chronic HCV infection, just like chronic HBV infection, does have a pervasive influence on the peripheral blood CD8\(^+\) T cell pool. Not so much by changes in the maturation or differentiation of the T cells, but by increased expression of the chemokine receptor CXCR3 which is involved in migration to the inflamed liver. The continuous antigen load and inflammation may alter the functional status of both HBV/HCV-specific and non-specific T cells in order to redirect immune cells to the site of infection.

Further research is necessary in order to investigate whether modulating chemokine expression could be a potential target for future therapy optimizing the anti-viral immunologic environment in the liver.

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REFERENCES


Experimental HBsAg/anti-HBs complex assay for prediction of HBeAg loss in chronic hepatitis B patients treated with peg-interferon and adefovir.

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ABSTRACT

Background and aim
We studied whether HBsAg/anti-HBs immune-complex levels in chronic hepatitis B (CHB) patients receiving anti-viral therapy could be used as a response marker at baseline (BL), or early during treatment to predict treatment outcome.

Methods
An experimental array-based assay (IMPACT - Immunological Multi-Parameter Chip Technology, Roche Diagnostics) served to determine HBsAg, anti-HBs and complex levels. We tested a panel of serum samples of 40 HBeAg-positive and 44 HBeAg-negative patients who received pegylated-interferon and adefovir for 48 weeks.

Results
HBsAg loss occurred in 4 of 40 HBeAg-positive and 4 of 44 HBeAg-negative patients. Fourteen of 40 HBeAg positive patients lost HBeAg and 12 of them formed anti-HBe. At BL complexes were present in 83 (99%) patients, whereas free anti-HBs levels were detectable in 5 patients. Complex levels at BL and WK 12 were higher in HBeAg-positive patients with HBeAg loss, compared to patients who retained HBeAg (p=0.002 and p=0.005 respectively). ROC analysis for HBeAg loss in HBeAg positive patients at BL and WK 12 showed AUC 0.79 (p=0.002) and AUC 0.82 (p=0.003) for complex levels. We found no correlation in either HBeAg-positive or -negative patients between complex levels and HBsAg loss.

Discussion
We demonstrated for the first time that before and during treatment HBsAg/anti-HBs immune-complex levels can predict HBeAg loss in HBeAg-positive CHB patients treated with peg-interferon and adefovir. Complexes were present in almost all patients at BL and were higher in patients that lost HBeAg. In conclusion, determining HBsAg/anti-HBs immune-complex levels before and early during treatment could select CHB patients with an optimal chance to achieve HBeAg loss.

Supplementary figures can be found online
INTRODUCTION

Persistent hepatitis B virus infection affects over 350 million individuals worldwide and causes more than 1 million deaths from liver failure or hepatocellular carcinoma (1). The primary aim in the treatment of chronic hepatitis B (CHB) patients with pegylated interferon (peg-IFN) and/or nucleos(t)ide analogues, is to achieve a sustained HBV-DNA suppression and thereby reduce liver pathology. When we consider using peg-IFN treatment, we want to select patients with the best chance of response by evaluating various viral and host factors, such as HBV DNA levels and aspartate aminotransferase (ALT) at baseline (BL) and viral genotype (2-4). However the positive and negative predictive value (PPV, NPV) of these markers is limited and we need better markers to select patients with the best chance of response. In both HBeAg-positive and -negative patients treated with peg-IFN, a decline in HBsAg levels seems to be associated with sustained viral response (SVR: HBV DNA<2,000 IU/mL, normalisation of ALT) (5-9). Although the HBV guidelines recently included HBsAg, the glycosylated envelope protein of the mature HBV virion, as a marker to indicate treatment response, HBsAg levels and their decline during peg-IFN treatment appear to differ across HBV genotypes (10) and it remains unclear whether HBsAg levels have practical advantages in predicting response before treatment (11, 12).

Generally immunological control seems to depend on the function of virus specific T-cells (13-15). Most adult patients (95%) resolve acute infection and lose serological HBsAg. These patients show broad and strong HBV specific T-cell responses, whereas the HBV specific T-cell repertoire is narrowly focused and barely detectable in patients with chronic active infection, mostly acquired during childhood or birth. We do not clearly know the role of the humoral immune response in controlling the virus. Several findings highlight the crucial role of anti-HBs antibodies in control of HBV infection. B-cells contribute to viral elimination by production of neutralizing antibodies (anti-HBs). Anti-HBs is necessary for sustained immune control as B-cell depletion after rituximab therapy in cancer patients sometimes leads to reactivation of the virus(16-18).

Simultaneous presence of HBsAg and anti-HBs antibodies has been reported in 10-32 % of chronic HBV infections (19-21) Only limited studies are available on the interaction between anti-HBs and HBsAg, employing precipitation of immune complexes and subsequent detection of HBsAg by the aid of labelled anti-HBs (22, 23). No clear data are available on the role of B-cells and quantitative anti-HBs antibody responses in the presence of antigen excess in relation to treatment outcome. In this study we analysed the quantitative dynamics of anti-HBs, HBsAg and anti-HBs/HBsAg immune complex formation in relation to treatment outcome by an experimental immune complex assay. We demonstrated that anti-HBs is present in virtually all CHB patients, and we showed that the level of these immune complexes before and early during peg-IFN-based treatment can predict HBeAg loss.
MATERIAL AND METHODS

Patients
Ninety-two CHB patients were part of a clinical trial in which they received peg-IFN alpha2a and adefovir for 48 weeks. We quantitatively tested patient samples under code for HBsAg/anti-HBs complexes, HBsAg and anti-HBs (fig 1.) to assess correlation between the test results and treatment outcome. The original clinical study included an intent–to-treat population, but for the complex test analysis we changed to a per protocol analysis. Of the 92 patients enrolled into the clinical study 6 patients prematurely discontinued treatment and dropped out of the study. Two (one HBeAg positive and one HBeAg negative) patients who did not lose HBeAg or HBsAg did not have samples collected for the complex test and were left out of the analysis. Consequently, we analysed immune complex in a total of 84 patients: 40 HBeAg positive and 44 HBeAg negative patients. Baseline characteristics are shown in table 1 and 2.

Table 1: Baseline Characteristics HBeAg positive patients. P values for significant differences are: *p<0.05, **p<0.005. ALT, alanine aminotransferase; HBeAg, hepatitis B e Antigen; HBsAg, Hepatitis B surface Antigen; ULN, Upper limit of normal

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<td>female sex (%)</td>
<td>5 (19)</td>
<td>4 (29)</td>
<td>0.69</td>
</tr>
<tr>
<td>Median ALT(xULN) (IQR)</td>
<td>1.9 (1.0-3.7)</td>
<td>3.6 (1.8-6.3)</td>
<td>0.08</td>
</tr>
<tr>
<td>Mean HBV DNA (log10 IU/mL) (SD)</td>
<td>8.2 (1.3)</td>
<td>7.9 (1.1)</td>
<td>0.55</td>
</tr>
<tr>
<td>mean HBsAg (log10 IU/mL) (SD)</td>
<td>4.42 (0.75)</td>
<td>4.22 (0.70)</td>
<td>0.42</td>
</tr>
<tr>
<td>mean complex (log10 IU/mL) (SD)</td>
<td>2.54 (0.95)</td>
<td>3.51 (0.66)</td>
<td>0.0016 **</td>
</tr>
<tr>
<td>HBV genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A(%)</td>
<td>9 (35)</td>
<td>8 (57)</td>
<td>0.19</td>
</tr>
<tr>
<td>B(%)</td>
<td>5 (19)</td>
<td>2 (14)</td>
<td>1.0</td>
</tr>
<tr>
<td>C(%)</td>
<td>4 (15)</td>
<td>3 (21)</td>
<td>0.68</td>
</tr>
<tr>
<td>D(%)</td>
<td>6 (23)</td>
<td>1 (7)</td>
<td>0.39</td>
</tr>
<tr>
<td>E(%)</td>
<td>2 (8)</td>
<td>0 (0)</td>
<td>0.53</td>
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</table>
**Table 2:** Baseline Characteristics HBeAg negative patients. P values for significant differences are: *p<0.05, **p<0.005. ALT, alanine aminotransferase; HBeAg, hepatitis B e Antigen; HBsAg, Hepatitis B surface Antigen; ULN, Upper limit of normal

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HBeAg negative patients (n=44)</th>
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<tbody>
<tr>
<td></td>
<td>no HBsAg loss</td>
<td>HBsAg loss</td>
<td>p=value</td>
<td></td>
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<tr>
<td>N (%)</td>
<td>40 (91.0)</td>
<td>4 (9.0)</td>
<td></td>
<td></td>
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<tr>
<td>mean age (years) (SD)</td>
<td>43 (9.1)</td>
<td>45 (18.8)</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>female sex (%)</td>
<td>13 (33)</td>
<td>1 (25)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Median ALT (xULN) (IQR)</td>
<td>1.9 (1.1-2.9)</td>
<td>1.0 (0.8-1.4)</td>
<td>0.069</td>
<td></td>
</tr>
<tr>
<td>Mean HBV DNA(log10 IU/mL) (SD)</td>
<td>5.6 (1.1)</td>
<td>4.5 (0.8)</td>
<td>0.05 *</td>
<td></td>
</tr>
<tr>
<td>mean HBsAg(log10 IU/mL) (SD)</td>
<td>3.46 (0.6)</td>
<td>2.45 (0.7)</td>
<td>0.003 **</td>
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</tr>
<tr>
<td>mean complex (log10 IU/mL) (SD)</td>
<td>2.23 (0.60)</td>
<td>2.32 (0.77)</td>
<td>0.78</td>
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</tr>
<tr>
<td>HBV genotype</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A(%)</td>
<td>7 (18)</td>
<td>1 (25)</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>B(%)</td>
<td>7 (18)</td>
<td>0 (0)</td>
<td>1.0</td>
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<tr>
<td>C(%)</td>
<td>4 (10)</td>
<td>1 (25)</td>
<td>0.39</td>
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<tr>
<td>D(%)</td>
<td>16 (40)</td>
<td>1 (25)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>E(%)</td>
<td>6 (15)</td>
<td>1 (25)</td>
<td>0.51</td>
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</table>

**Assays**

Assays to determine HBsAg, anti-HBs, HBeAg, anti-HBe and HBV-DNA were described previously for this study cohort (26). With a new technique developed by Roche, quantitative levels of HBsAg/anti-HBs immune complexes in serum or plasma were determined by an experimental assay (IMPACT - Immunological Multi-Parameter Chip Technology, Roche Diagnostics), based on the following principle (fig 1). HBsAg capture antibodies, as used in the Elecsys HBsAg assay (Roche Diagnostics, Mannheim, Germany) were coated on a solid phase. These antibodies detect various isoforms and mutants of HBsAg (24). Detection of the immune complexes was done using a mouse IgM antibody against oligomeric human IgG, as used in Elecsys antibody detection assays (Roche Diagnostics, Mannheim, Germany). An immune complex standard was constituted by mixing a pool of high titer anti-HBs positive plasma with a pool of high titer HBsAg positive plasma. The corresponding signal in the complex assay was defined as 500 AU/mL. From the mixture a 2-fold serial dilution in HBsAg and anti-HBs negative plasma was prepared as calibrators. Linearity of the assay was studied in 3 CHB individuals, applying 2-fold serial dilutions, see fig 2a. A high-titer HBsAg/anti-HBs complex sample was diluted with negative material in 10% steps (e.g. 90+10, 80+20, 70+30 etc.) and the expected recovery was compared to the measured recovery (fig 2b). This experiment was repeated with 3 patient
samples, showing similar results within a range of +/- 20% (data not shown). Specificity of the assay was studied using 50 HBsAg and anti-HBs negative human control samples; 48/50 showed negative complex test results; 2 samples showed borderline reactivity. For this study, all patient samples were tested in duplicate and mean values were reported. In general the duplicate signals showed a covariance variability of 2-3%.

HBsAg and anti-HBs levels were quantified using the IMPACT Immunological Multi-Parameter Chip Technology assay (Roche Diagnostics, Mannheim, Germany). Plasma HBV DNA was extracted by the COBAS® Ampliprep and quantified using the Roche COBAS® TaqMan48® assay (Hoffman-La Roche Ltd, Diagnostics Division, Basel, Switzerland), with a dynamic range between 20 and 1.70x10^8 IU/mL.

Figure 1: Principle of the experimental assay for detection of HBsAg/anti-HBs complexes. A plate coated with biotinylated anti-HBs binds the immune complex. The immune complex is detected by a monoclonal mouse IgM antibody. (Dig: digoxine, HBsAg: Hepatitis B surface antigen).
Figure 2: Linearity data of the IMPACT complex assay. a) Results of 3 patient samples, diluted in 2-fold steps. We started each sample in a suitable dilution to yield results within the measuring range in order to be able to determine dilution linearity. Linearity is shown for the 3 samples in the different dilutions. b) Linearity experiment according to CLSI (Clinical and Laboratory Standards Institute) standards. A high-titer sample was diluted with a negative sample in 10% steps, e.g. 90+10, 80+20, 70+30 etc. The expected recovery is compared to the measured recovery.

Statistical analysis
We used the Statistical Program for Social Sciences (SPSS 16.02 for Windows, SPSS, Chicago, IL) and Graphpad Prism 5 (2007, GraphPad Software Inc., San Diego CA). Analyses were based on the per-protocol model. The Kolmogorov-Smirnov test served to check for normality in continuous variables. The Student t test assessed differences between normally distributed variables, while the Mann-Whitney U test was used for differences between variables with skewed distribution. The chi-squared test ($\chi^2$) or Fisher’s exact test was applied for comparison of categorical variables. Using the ROC curve analysis, we examined the associations between complex levels and HBsAg as potential predictors of HBeAg loss or HBsAg loss as dependent variables. Logistic regression analysis was used to examine the associations between complex levels as potential predictors of HBeAg loss or HBsAg loss as dependent variables.
RESULTS

Clinical outcome after peg-interferon and adefovir treatment

Eighty-four patients were tested for presence of HBsAg/anti-HBs complexes. At end of follow-up (week 72) 14 of the 40 HBeAg positive patients lost HBeAg (35%) of whom 4 also lost HBsAg (10%), all with subsequent anti-HBs seroconversion. Twelve out of 14 patients with HBeAg loss, also developed anti-HBe antibodies. Of two patients that lost HBeAg without detectable anti-HBe one patient lost HBsAg and had formed anti-HBs and one lost HBeAg durably without detectable anti-HBe. Thirteen out of 14 patients that lost HBeAg also developed combined response at week 72 (normalisation of ALT levels, HBV DNA< 2.000 IU/mL, HBeAg negativity). Four of 44 HBeAg negative patients (9%) lost HBsAg and subsequently seroconverted to anti-HBs. (supplementary fig. 1).

Results of complex test

At baseline in 83 out of 84 patients (99%) HBsAg/anti-HBs immune complexes were detected, whereas free anti-HBs was positive in 5 out of 84 (6%) of the patients.

Relation of HBsAg/anti-HBs complex levels and treatment outcome

HBeAg positive patients

In HBeAg positive patients complex levels at BL and WK 12 were higher in patients who lost HBeAg compared to patients without HBeAg loss at week 72 (p=0.0016 and p=0.0047 respectively). ROC analysis at BL and WK 12 showed AUC 0.79 (p=0.002) and AUC 0.82 (p=0.003) for complex levels and AUC 0.55 (not significant) and AUC 0.62 (not significant) for HBsAg levels (figure 3.a, supplementary figure 2.a). For BL complex levels < 468 AU/mL NPV and PPV for HBeAg loss were 0.93 and 0.5 respectively (sensitivity 93%, specificity 50%). At WK 12, complex levels <397 AU/mL showed a NPV and PPV of 0.95 and 0.53 for HBeAg loss (sensitivity 90%, specificity 69%).

HBeAg negative patients

Takkenberg et al (11, 23) reported that lower baseline HBsAg levels (using the Abbott Architect assay) were found in HBeAg negative patients with HBsAg loss. We similarly observed this in the present study in which quantititation of HBsAg levels was performed by the IMPACT assay (p<0.05). Although complex levels at baseline were higher in patients with HBsAg loss than in those without HBsAg loss, this difference did not reach statistical significance. ROC analysis at BL and WK 12 showed AUC 0.59 (non significant) and AUC 0.52 (non significant) for complex levels and AUC 0.83 (p<0.05) and AUC 0.83 (p=0.06, non significant) for HBsAg levels (figure 3.b, supplementary figure 2.b).
**Figure 3:** a) HBsAg and complex levels at baseline and week 12 in HBeAg positive patients with or without HBeAg loss. P values of statistical tests (unpaired T-test on log values) and mean levels are indicated. b) HBsAg and complex levels at baseline and week 12 in HBeAg negative patients with or without HBsAg loss. P values of statistical tests (unpaired T-test on log values) are indicated. Patients that reached HBeAg or HBsAg loss before week 12 were left out of the week 12 analysis.

**Quantitative HBeAg levels in HBeAg positive patients**
Mean quantitative HBeAg levels at baseline and week 12 of patients with and without HBeAg loss were not significantly different (supplementary figure 3).

**Correlation between levels of HBsAg/anti-HBs complex, HBsAg and HBV DNA**
At baseline, we found a weak correlation between immune complex and HBV DNA levels ($r=0.33, p=0.0026$). However we found no significant correlation between complex and HBsAg.
Correlation between HBV genotype and levels of HBsAg/anti-HBs complex and HBsAg

In both HBeAg-positive and -negative patients baseline immune complex levels were not significantly different between individual viral genotypes (A-E) but there was more variation in HBsAg levels (supplementary fig.5a,b). More detailed analysis of HBeAg-positive patients, showed immune complex levels higher in those infected with genotype A than with other genotypes (p=0.02) (supplementary figure 5.c,d). In HBeAg positive patients we analysed the individual HBV genotypes for their effect on response to treatment. Although we found no association between HBsAg levels and HBeAg loss, immune complex levels were higher in genotype A infected patients who had lost HBeAg compared to those who had not (supplementary figure 5.d). The difference in complex levels between patients with or without HBeAg loss was not as clear in the non-genotype A infected patients and did not reach statistical significance. The differences in response rates for HBeAg loss in genotype A and non-genotype A, 8/17 (47%) and 6/23 (26%) respectively, did not reach statistical significance, (Fisher’s exact test (p=0.20)), suggesting that the response rates for loss of HBeAg do not account for the finding that genotype A patients have higher immune complex levels. Also in patients who lost HBeAg, we found no differences in baseline complex levels between genotype A patients who had lost HBeAg and non-genotype A patients who also lost HBeAg (supplementary figure 5.d). In addition, when we analysed immune complex levels and HBV genotype in a multivariate model as potential predictors for HBeAg loss, only immune complex levels were significantly associated with treatment outcome (p<0.05).

Patterns of decline of immune complexes

In HBeAg-positive patients no difference in the decline in complex levels occurred at week 12 between patients who lost HBeAg (and HBsAg) and those who did not lose HBeAg (figure 4.a,b). In patients who did not lose HBeAg, immune complex levels declined during therapy but relapsed after its cessation. There was no difference in HBsAg decline at week 12 between patients who did or did not lose HBeAg. (figure 4.c,d).

HBeAg-negative patients showed no difference in decline of complex or HBsAg levels between patients who did or did not lose HBsAg (figure 4.c,d).

We analyzed immune complex levels in detail in patients achieving HBeAg or HBsAg loss during therapy. Generally, reduction in immune complex levels followed the decline of HBsAg (supplementary figure 6, 7.a,b) and became negative within a month after HBsAg loss. The increase of (free) serum anti-HBs coincided with the decline of complex levels.
Figure 4: Mean complex levels and decline during treatment of all HBeAg positive and negative patients. 

a) Complex levels in HBeAg positive patients (mean levels and unpaired T-test. 

b) Decline in complex levels in HBeAg positive and negative patients. 

c) HBsAg levels in HBeAg positive patients (mean levels and unpaired T-test. 

d) Decline in HBsAg levels in HBeAg positive and negative patients.
Part II

Discussion

In this study we demonstrated that high levels of HBsAg/anti-HBs immune complexes before- and during-treatment are associated with HBeAg loss following IFN-based therapy, whereas no association was found between baseline and week 12 HBsAg or HBeAg levels and HBeAg loss. Approximately 70% of HBeAg-positive CHB patients with active disease (HBV DNA >2,000 IU/mL, ALAT >ULN, liver inflammation > grade 2) do not respond to a 48 weeks course of Peg-IFN, and subsequently require long-term treatment with nucleotide therapy to maintain viral suppression indicating the need for better predictors of response pre-treatment. Until present, only weak baseline markers such as lower baseline HBV DNA level (<2x10^8 IU/mL), high ALT level, female sex, older age and absence of previous peg-interferon therapy have been described to predict treatment response to interferon base therapy in HBeAg positive patients (3). Other on-treatment markers, such as HBV DNA and HBsAg kinetics, were only predictive for response depending on the treatment the patients received; In our clinical study these earlier described on treatment markers were not found to be predictive for response (23). We found that low immune complex levels at baseline and week 12 have a high negative predictive value (NPV) for HBeAg loss, and such patients have little chance of responding to IFN-based treatment. Hence, for HBeAg positive patients the complex test may add value to the presently used pre- and on-treatment markers and may enable cost saving by not commencing treatment in patients unlikely to respond.

The strongest association between complex levels and loss of HBeAg was found for HBV genotype A, which is known to have the highest rate of response to IFN treatment. Complex levels were higher in genotype A as compared to other genotypes. It seems unlikely that the observed genotype specific differences were caused by the IMPACT test, as the antibodies for capture and detection of complexes have been shown to bind equally well to all genotypes. Because in our study the rate of HBeAg loss in genotype A patients was slightly higher, further investigation in larger clinical trials is needed to determine whether indeed higher complex levels in genotype A patients are related to the higher response rates to peg-interferon observed for this ‘more favourable’ genotype.

Loss of HBeAg with subsequent anti-HBe seroconversion and HBsAg loss with anti-HBs seroconversion are associated with a durable control of the virus. However when we analyzed HBeAg-positive and -negative patients separately, we found no association between complex levels and HBsAg loss. Neither did we find a correlation between baseline or week 12 complex levels and combined response at week 72 in HBeAg negative patients (data not shown). The small sample size could account for the lack of association between complex levels and HBsAg response in this patient category, as only 4 of the HBeAg positive and 4 of the HBeAg negative patients lost HBsAg. In HBeAg positive patients, complex levels at baseline and week 12 did not differ significantly between patients that developed HBeAg loss alone and those with both HBeAg and HBsAg loss. In HBeAg negative patients who lost HBsAg, HBsAg levels at baseline and week 12 were significantly lower compared to patients without HBsAg loss. This indicates that the
dynamics of immune complex levels are not straightforward and that complexes behave differently in response to treatment between HBeAg-positive and -negative patients.

We found that almost all (99%) patients had immune complexes before the start of treatment, while free anti-HBs was detected at baseline in 6% (5/84) of the patients. Traditionally, HBsAg loss and the occurrence of anti-HBs antibodies is seen as the closest thing to cure of the disease, reflecting the state in which the patient has developed immune control over the virus. Although HBsAg and anti-HBs often co-exist during the natural course of chronic HBV infection, with a prevalence of 10-32% (19-21), it was long assumed that a sustained anti-HBs response only occurs after the loss of HBsAg. Interestingly, during superinfection with hepatitis delta virus (HDV), the inhibitory effect exerted by HDV on the replication of HBV occasionally lead to a picture of temporary HBsAg negative hepatitis, during which anti-HBs transiently becomes detectable (25), indicating the 'hidden' presence of anti-HBs in chronic HBV infection.

Our study confirms that in chronic HBV infection, production and presence of anti-HBs antibodies is the rule, not the exception. The finding of immune complexes during chronic HBV infection, shows that antibodies against HBsAg always are present, but in complexed form. When serum HBsAg levels decline due to the reduction of active cccDNA transcription, fewer complexes are formed and more free anti-HBs antibodies become available. We found that complex levels at baseline were higher in patients that lost HBeAg during or after IFN-based therapy. Since, in HBeAg positive patients, HBsAg levels at baseline did not differ between patients who did or did not lose HBeAg to IFN based therapy, the higher immune complex levels can be explained by higher anti-HBs production at baseline, which can only be detected in complexed form. It is an interesting question whether higher levels of antibody production against HBsAg are necessary for viral eradication or merely reflect a better (humoral) immune response against the virus during therapy. In this setting 'B-cell help' by presentation of antigens or production of cytokines may enhance T cell immunity (26) required for HBeAg loss. Importantly, in our study we saw no difference in quantitative HBeAg levels at baseline between patients that did or did not lose HBeAg. This supports our hypothesis that indeed immunological pressure rather than a viral factor determines the loss of HBeAg.

The finding of HBsAg/anti-HBs complexes in nearly all chronic hepatitis B patients, and the higher complex levels in patients who lose HBeAg after treatment, changes our idea about the humoral immune response in chronic HBV infection. Detection of immune complexes could give more insight to the extent of changes in total (free and complexed) anti-HBs levels during treatment. It is of interest to know to what extent this new marker could also enhance on-treatment prediction in HBeAg positive patients, and whether it adds value to viral markers such as HBsAg and HBV DNA decline during treatment. Further research is necessary to describe the changes of total anti-HBs during treatment and during untreated hepatitis B, to gain more insights in the interplay between humoral and cellular immune response that is responsible for HBeAg loss and viral clearance.
In conclusion we found that higher HBsAg/anti-HBs complex levels in HBeAg positive patients are associated with HBeAg loss. If additional studies confirm our findings, then measuring HBsAg/anti-HBs immune complex levels before and early during immune modulatory treatment could select CHB patients with an optimal chance to achieve loss of HBeAg while avoiding unnecessary IFN-related adverse events in patients with low complex levels and a low chance of HBeAg response.
HBsAg/anti-HBs complex assay for prediction of HBeAg loss  Chapter 9

REFERENCE LIST


NK Cell Characteristics in Chronic Hepatitis B Patients are Associated with HBsAg Loss after Combination Treatment with Peg-interferon Alpha-2a and Adefovir.
ABSTRACT

Background
It is currently unknown what the role of NK cells is in the process of HBsAg loss and whether their phenotype is related to treatment outcome in chronic hepatitis B patients.

Methods
Chronic hepatitis B (CHB) patients (HBV-DNA>17,000 IU/ml) were treated with peg-interferon alpha-2a and adefovir for 48 weeks. NK cell phenotype and function was analysed in 7 responders (HBsAg loss at week 72; 3 HBeAg+/ 4 HBeAg-), 7 matched non-responders and 7 healthy controls (HC). Subsequently, 34 baseline samples of HBeAg+ CHB patients were analysed.

Results
During treatment, the percentage as well as absolute numbers of CD56\textsuperscript{bright} NK cells increased significantly, whereas CD56\textsuperscript{dim} NK cells decreased. At baseline, responders had a significantly lower expression of chemokine receptor CX\textsubscript{CR1} on CD56\textsuperscript{bright} and inhibitory receptor NKG2A on CD56\textsuperscript{dim} NK cells compared to non-responders. In addition, responders had higher CD56\textsuperscript{bright} TRAIL expression and IFN\gamma production at end of treatment. These baseline differences were not found in HBeAg positive patients who had HBeAg seroconversion without HBsAg clearance.

Conclusions
Combination therapy significantly influences NK cell phenotype and function. Differences between CHB patients with HBsAg loss and non-responders suggests that NK cells play a role in the clearance of HBsAg upon interferon-based combination therapy.
INTRODUCTION

Hepatitis B virus (HBV) infection, with approximately 240 million chronically infected people worldwide, is a global health problem(1). Patients with chronic hepatitis B (CHB) are at increased risk of developing serious hepatic complications such as cirrhosis and hepatocellular carcinoma(2). Current treatment options for CHB include pegylated interferon alpha-2a (IFNα) or nucleos(t)ide analogue (NUC) treatment. While NUCs can potently block viral replication, IFNα exerts its action via direct antiviral activity as well as immunomodulatory effects.

The immunomodulatory effects of IFNα have been investigated in innate and adaptive effector cells. IFNα therapy has been shown to cause expansion and activation of CD56^{bright} NK cells(3). T cells on the other hand, remain present at low frequency in the peripheral blood, due to bone-marrow depressive and anti-proliferative effects of IFNα(4,5). However, in patients with a long-term response to IFNα therapy, a restoration of HBV-specific CD8^{+} T cells has been shown (6) though no reversion of the exhausted phenotype were shown early during treatment (3,7,8). Conversely, the effect of NUC therapy on innate immune cell functionality is controversial (9,10), whereas HBV-specific CD8^{+} T cell responses are restored in patients in whom HBsAg is cleared upon NUC treatment(11). Together these data imply that a combination of IFNα and a NUC is a promising treatment option for CHB patients, optimally restoring both the innate and adaptive functions of the immune system(12).

Early studies of combination regimens in which a combination of IFNα with lamivudine was used have not shown superior responses to those obtained by IFNα monotherapy(13). Treatment of IFNα combined with a newer generation NUC however, could give improved antiviral responses as suggested in several studies(14–18). In our cohort of CHB patients treated with a combination of IFNα and adefovir, we indeed observed a relatively high rate of HBsAg loss in both HBeAg positive as well as HBeAg negative patients(14). We have previously shown that the function of HBV-specific CD8^{+} T cells is restored in responders to combination therapy(19). It is unknown however, what the effect of this treatment regimen is on the innate immunity. Furthermore, the role of NK cells in the clearance of HBsAg has not yet been identified. In the present study we have characterized changes in the NK cell compartment during IFNα/NUC combination therapy in CHB patients and related these to treatment outcome.
MATERIALS AND METHODS

Patient samples
In a previously described investigator-initiated study (controlled-trials.com; ISRCTN 77073364) (14) CHB patients with a high viral load (HBV DNA>17,000 IU/ml) received combination therapy of pegylated interferon alpha-a2 and adefovir for 48 weeks. All patients gave informed consent and the study was approved by the Ethical Review Board (ERB) of the Academic Medical Center Amsterdam. Samples were obtained at baseline, during treatment (day 3, week 1, week 24, week 48) and during follow-up (week 72). PBMCs were isolated using standard density gradient centrifugation and cryopreserved for later analysis.

From the cohort, 14 CHB patients were selected (supplementary figure 1A). This group consisted of 7 responders with HBsAg loss at week 72, defined according to EASL guidelines(20) as having persistently undetectable HBsAg with negative HBV DNA with or without development of anti-HBs antibodies (for HBeAg positive patients this also included HBeAg seroconversion) and 7 matched (based on genotype, ethnicity and HBeAg status at baseline) non-responders with no HBsAg loss and no HBeAg loss (for HBeAg positive patients)(table 1). All 7 non-responders had to be retreated with NUC within 2 years after end of treatment (viral kinetics are depicted in supplementary figure 1B). For comparison, 7 healthy controls (blood donors) were analysed.

Secondly, baseline PBMCs of a cohort of 34 HBeAg positive patients, of which 9 patients had HBeAg seroconversion without HBsAg loss at week 72 were analysed. All patients were HIV negative and not co-infected with hepatitis C or D virus.

HBV genotype determination, quantification of plasma HBV DNA, serum HBsAg and detection of anti-HBs, HBeAg and anti-HBe were performed as described before(14).

Flowcytometry
PBMCs were stained in PBS containing 0.01% (w/v) NaN3, 0.5% (w/v) bovine serum albumin and 2 mM EDTA (PBA) with different combinations of fluorescent label-conjugated mouse monoclonal antibodies (mAbs) (supplementary material and methods). For intracellular staining, cells were fixed after surface staining, permeabilized and subsequently stained with fluorescent label-conjugated mAbs (supplementary material and methods). All measurements were done on an LSR Fortessa or FACS Canto flow cytometer (BD Biosciences) and analysed by FlowJo MacV9.7.5 software. When positive and negative expression could be identified for a specific marker, gates were used to indicate cell frequency. For markers that are gradually expressed, gMFI (geometric mean fluorescent intensity) was used to define expression level.
Table 1: Baseline characteristics of patients with chronic hepatitis B treated with combination therapy of peg-interferon α with adefovir. A persistently undetectable HBsAg combined with negative HBV DNA or HBV DNA<20 IU/mL, b HBsAg loss with subsequent development of anti-HBs antibodies. M, male; F, female; HBeAg, hepatitis B e antigen; neg, negative; pos, positive; HBV DNA, hepatitis B virus DNA; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; IFN, interferon based treatment; T72, 72 weeks after start of treatment.

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<th>HBsAg log10 IU/mL</th>
<th>ALT U/L</th>
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NK cell functional assays

For functional measurements, PBMCs were co-cultured with K562 cells for 3 hours (effector:target ratio 5:1) in the presence of monensin (BD Biosciences, San Jose, USA), brefeldin (Invitrogen, Camarillo, USA) and CD107a FITC (Affymetrix eBioscience, San Diego, USA) after overnight incubation with either medium or rhIL-2. After surface staining, cells were fixed, permeabilised and stained with IFNγ PE and TNFα PE-Cy7 (Affymetrix eBioscience, San Diego, USA). To assess target cell apoptosis, cells were stained with DiOC₆. Measurements and analyses were performed as described above.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software. For paired data, the Wilcoxon signed rank test was used, for non-paired data, the Mann Whitney U test was used. For analysis of correlation, the Spearman correlation coefficient was calculated. 2-Sided p values <0.05 were considered statistically significant.
RESULTS

Changes in the NK cell compartment in CHB patients receiving combination therapy.

NK cell characteristics were analysed in 14 CHB patients (table 1) who were treated with combination therapy, and compared to 7 healthy controls (HC). The gating strategy that was used excluded double, non-viable, CD3+, CD14+ and CD19+ cells. From these, the CD56+ and/or CD16+ cells were considered NK cells. NK cells were divided into the classical subsets CD56<sup>bright</sup> (CD16<sup>−/−dim</sup>) and CD56<sup>dim</sup> (CD16+) NK cells (figure 1C, supplementary figure 2A). At baseline (BL), CHB patients had a significantly increased proportion of NK cells compared to HC, which is consistent with a previous report(21). The CD56<sup>bright</sup> and CD56<sup>dim</sup> subset distribution in patients at BL was similar to HC (figure 1B). While the percentage of NK cells within the lymphocyte compartment in patients increased at end of treatment (EoT) (13.3 at BL to 19.0% at EoT, p<0.05, figure 1A), the absolute number of NK cells was unchanged (253 to 231 cells/µl, supplementary figure 2B). The percentages of NK cell subsets however, changed considerably towards end of treatment (figure 1B,C) as well as their absolute numbers (supplementary figure 2B). This resulted in an increase in CD56<sup>bright</sup> NK cells (11.3 to 44.4% at EoT, p<0.0001) and a decrease in CD56<sup>dim</sup> NK cells (74.2 to 39.9% EoT, p<0.0001). Longitudinal analysis of NK cell subsets showed no changes when measured at early time points (day 3, week 1), suggesting a gradual change during combination therapy, with normalization during follow-up at week 72 (supplementary figure 2C).

Figure 1: Combination treatment with peg-interferon α and adefovir changes the natural killer (NK) cell compartment. NK cells were measured in 7 HC and 14 CHB patients at BL and EoT. Frequency of total (A) and CD56<sup>bright</sup> and CD56<sup>dim</sup> (B) NK cells. (C) Representative FACS staining of CD14-CD19-CD3- cells with gating for total NK cells (CD56 and/or CD16 positive cells,CD56bright(CD16−/−dim) and CD56dim(CD16+) NK cells. Frequencies in graph represent percentage of CD56<sup>bright</sup>/ total NK cells and CD56<sup>dim</sup>/ total NK cells. Statistical analyses unpaired data: Mann Whitney U test, paired data: Wilcoxon test: *p<0.05; **p<0.01 ***p<0.001; ns non-significant. HC, healthy control; CHB, chronic hepatitis B; BL, baseline; EoT, end of treatment.
NK cell proliferation and chemokine receptors.

To investigate the possible cause of the changes in NK cell subset distribution, the expression of various markers, including proliferation marker Ki67 and several chemokine receptors on NK cells were analysed. The percentage of Ki67$^+$ CD56$^{\text{bright}}$ as well as CD56$^{\text{dim}}$ NK cells in patients was significantly higher at EoT compared to BL (figure 2A). As the distribution of these subsets drastically changed, absolute numbers were also taken into account. In parallel, the absolute number of Ki67$^+$ CD56$^{\text{bright}}$ NK cells increased (3 at BL to 16 cells/µl at EoT, $p<0.001$, data not shown) and even though the CD56$^{\text{dim}}$ subset decreased in absolute number, the number of Ki67$^+$ CD56$^{\text{dim}}$ cells increased during therapy (14 and 28 cells/µl, at BL and EoT respectively, $p<0.01$, data not shown), illustrating an overall increased proliferation tendency in both subsets.

Figure 2: Proliferation and expression of chemokine receptors is altered in NK cell subsets after treatment with combination therapy. Frequencies of Ki67 positive cells (A), gMFI levels of CX3CR1 (B), gMFI levels of CXCR3 (C) and frequencies of CXCR6 (D) in CD56$^{\text{dim}}$ and CD56$^{\text{bright}}$ NK cells in 7 HC and 14 CHB patients at BL and EoT. Representative dot plots (E) showing an overlay of CD56$^{\text{dim}}$ and CD56$^{\text{bright}}$ (with percentage) NK cells. Statistical analyses unpaired data: Mann Whitney U test, paired data: Wilcoxon test: *$p<0.05$; **$p<0.01$; ***$p<0.001$; ns non-significant. HC, healthy control; CHB, chronic hepatitis B; BL baseline; EoT, end of treatment; gMFI geometric mean fluorescence intensity.

CX3CR1, also known as the fractalkine receptor, which is associated with the migration of cells to sites of inflammation, is generally expressed at a higher level on CD56$^{\text{dim}}$ cells than CD56$^{\text{bright}}$ NK cells. The expression of CX3CR1 on CD56$^{\text{dim}}$ NK cells in CHB patients at BL was higher compared to HC. In the CD56$^{\text{bright}}$ subset CX3CR1 expression was similar in HC and CHB patients at BL, but increased during combination therapy (figure 2B). The expression of CXCR3, which is associated with homing to sites of inflammation including the liver, was higher in both subsets
in CHB patients at BL as compared to HC. During therapy however, CXCR3 expression decreased in the CD56<sup>bright</sup> NK cell subset (figure 2C). CXCR6, also associated with homing to the liver, was higher expressed on CD56<sup>bright</sup> NK cells of patients with CHB at BL compared to HC, but significantly decreased during combination therapy (27.5 to 4.6%; p < 0.001, figure 2D). A similar decrease in CXCR6 expression during therapy was seen in CD56<sup>dim</sup> NK cells (figure 2D).

**Increased natural cytotoxicity receptors and activation status.**

The effector function of NK cells is regulated by the sum of activating and inhibitory signals from the environment combined with the surrounding cytokine milieu. Inhibitory receptors include the heterodimer NKG2A/CD94, whereas CD16, NKG2C/CD94, CD161 and the natural cytotoxicity receptors (NCR) Nkp30, Nkp44, Nkp46 are activating receptors. No differences were found in the expression of NKG2A, CD16, NKG2C, CD94, CD161, Nkp44 and Nkp46 on the different NK cell subsets in HC compared to CHB patients at BL. However, the expression of Nkp30 on CD56<sup>dim</sup> NK cells was decreased in patients at BL compared to HC (data not shown). During therapy, the expression of the inhibitory receptor NKG2A significantly increased on both CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells. The expression of CD94, that can form a heterodimer with NKG2A, followed a similar pattern. The expression of activating receptors CD16 (Fc-γ receptor III) on CD56<sup>dim</sup> NK cells and NKG2C on the CD56<sup>bright</sup> subset decreased, whereas the expression of the NCRs Nkp30 and Nkp46 increased significantly in both subsets during therapy (supplementary figure 3). CD161 expression, which has been implicated in triggering NK-cell mediated cytotoxicity, decreased in both NK cell subsets during therapy.

When the activation status (HLA-DR, CD38) of NK cells was assessed in CHB patients at BL and in HC, no differences were found (data not shown). During therapy, the activation status of both CD56<sup>dim</sup> and CD56<sup>bright</sup> cells significantly increased, illustrated by an increase in HLA-DR and CD38 expression (figure 3A-C). The strongest increase was seen in gMFI of CD38 which increased by a factor 1.5 for CD56<sup>bright</sup> and 2.6 for CD56<sup>dim</sup> NK cells (figure 3A, p < 0.001).

**Figure 3:** Activation status in NK cells increases during combination therapy in CHB patients. CD38 (A) and HLA-DR (B) expression in CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells. (C) Representative staining for CD38 and HLA-DR in CD56<sup>bright</sup> and CD56<sup>dim</sup> NK cells at BL and EoT. Statistical analyses Wilcoxon test: *p < 0.05; **p < 0.01; ***p < 0.001. CHB, chronic hepatitis B; BL baseline; EoT, end of treatment; gMFI geometric mean fluorescence intensity.
NK cell effector potential.

CD56<sup>bright</sup> NK cells of CHB patients at BL showed higher expression of both perforin and granzyme B compared to HC (supplementary figure 4D,E). During therapy, the expression of perforin further increased in this subset, while the level of granzyme B remained the same. In CD56<sup>dim</sup> NK cells the expression of both of these cytotoxic proteins increased in expression during therapy (supplementary figure 4A,B). TRAIL expression on CD56<sup>bright</sup> NK cells was significantly higher in patients compared to HC, and increased even further during therapy (gMFI 284 at BL to 385 at EoT, \( p<0.01 \), supplementary figure 4C). On CD56<sup>dim</sup> NK cells, TRAIL expression also increased significantly during therapy (supplementary figure 4F).

**Figure 4:** Combination therapy results in a decrease in functional activity of NK cells. Longitudinal analysis of interferon-γ positive NK cells upon co-culture with K562 cells (A). K562 apoptosis as measured by DiOC6 negative cells (n=11-14 per timepoint) in two E:T ratios (B). Functionality of activated NK cells at BL and EoT (C) with representative FACS plots at baseline (gates identify positive cells out of total NK cells) (D). Statistical analyses Wilcoxon test: \(* p<0.05\); ** \( p<0.01 \); CHB, Chronic hepatitis B; BL baseline; d3, day 3 of treatment; T24, at week 24 of treatment; EoT, end of treatment; E:T, effector: target ratio; polyfunct, polyfunctionality.

NK cell functionality upon target cell recognition.

Next, we evaluated the functionality of NK cells by measuring cytokine production (IFNγ and TNFα) and degranulation (CD107a) in response to target cells, as well as the killing capacity of NK cells, measured by apoptosis of these K562 cells in response to co-culture with PBMCs. IFNγ and TNFα production as well as CD107a expression significantly decreased during therapy (figure 4A,
only IFNγ is shown). Apoptosis induced in K562 cells at on-treatment time points (day 3, week 24 and week 48) significantly decreased compared to baseline, but normalized during follow-up (figure 4B). When activated overnight with IL-2, which in vitro has a similar effect as activation with IL-15,(24) NK cell function increased compared to unstimulated NK cells, however, still a decrease was seen when comparing baseline with end of treatment (figure 4C,D). The percentage of NK cells capable of all three functions (CD107a, IFNγ and TNFα positive) also decreased (figure 4C).

Differential expression of TRAIL, CX3CR1, NKG2A and IFNγ in patients with HBsAg loss, HBeAg seroconversion and non-responders.

Subsequently, we analysed whether changes in NK cell phenotype and function differed in CHB patients with regard to therapy response. Therefore, the group was divided in responders (n=7, HBsAg loss with or without anti-HBs formation at week 72) and matched non-responders (n=7) (table 1). There were no differences in either total NK cell numbers or NK cell subsets in responders and non-responders (data not shown). At BL we observed a significantly lower expression of CX3CR1 expression on CD56bright NK cells in responders than in non-responders, however no significant differences were found at EoT (figure 5A). NKG2A expression on CD56dim cells was also significantly lower in responders (37.3%) than in non-responders (50.9%) at BL ($p<0.05$) (figure 5B). Furthermore, in responders to therapy the expression of TRAIL on CD56bright NK cells was significantly higher at EoT (figure 5C). Even though there was no significant difference in IFNγ production at baseline, at EoT IFNγ production of activated NK cells was twice as high in responders than in non-responders (14% compared to 7.5% respectively, $p=0.026$ figure 5D). A similar trend was seen for TNFα production and CD107a expression (data not shown).

We next investigated whether these markers were also differentially expressed in patients who developed a partial response to combination therapy, represented by HBeAg seroconversion without HBsAg loss (supplementary table 1). In this second group of patients however, no significant differences were observed in responders who had HBeAg seroconversion at week 72 and in patients who did not have HBeAg seroconversion (supplementary figure 5A-F). Subsequent analyses using combined response (HBV DNA<2,000 with ALT normalization) at week 72 and HBeAg seroconversion at week 144 as endpoint, did not change the outcome (data not shown).

Association between CD56bright NK cells and ALT.

In order to investigate whether NK cells could have a role in liver injury, we studied the relation between NK cell phenotype and ALT levels at BL in the group of HBeAg positive patients. The percentage of CD56bright NK cells at BL was positively correlated with ALT ($p=0.0355$, figure 6A). Furthermore, TRAIL and CD38 expression on CD56bright NK cells were also positively correlated with ALT at BL in HBeAg positive patients ($p=0.005$, figure 6B and $p=0.015$, figure 6C respectively). No correlation between NK cell markers and viral parameters (HBsAg, HBV DNA) were found.
Figure 5: Differential expression of CX3CR1, NKG2A, TRAIL and IFNγ between CHB patients with HBsAg loss at week 72 compared to non-responders. BL and EoT expression in responders and non-responders to therapy of CX3CR1 (gMFI) on CD56bright cells (A), NKG2A (percentage) on CD56dim NK cells (B), TRAIL (gMFI) on CD56bright cells (C) and IFNγ (percentage) of total NK cells (D) compared to HC. Horizontal bars indicate mean with SEM. Statistical analyses Mann Whitney U test: *p<0.05; **p<0.01. BL baseline; EoT, end of treatment; gMFI geometric mean fluorescence intensity.

Figure 6: Baseline CD56bright NK cells associated with ALT in HBeAg positive patients. The CD56bright NK cell percentage correlated positively with ALT (A), gMFI of TRAIL on CD56bright NK cells correlated positively with ALT (B), gMFI of CD38 on CD56bright NK cells correlated positively with ALT (C). Spearman correlation coefficient r and p values indicated in graph. ALT; alanine aminotransferase.
**DISCUSSION**

Immune modulatory effects have been reported in patients with CHB treated with nucleos(t)ide analogues as well as in those treated with IFNα. (3, 7, 8, 10, 11) We have previously reported a restoration in the function of HBV-specific CD8+ T cells upon successful treatment with a combination of IFNα and adefovir (19). However, the effect of combination treatment on the innate immune compartment is currently unknown, and there is little understanding of its role in HBsAg clearance. In this study, NK cell characteristics of CHB patients treated with combination therapy of IFNα and adefovir were extensively analysed. In our previously described cohort, high levels of HBsAg clearance were achieved with combination therapy, which resulted in unique patient material (14).

As shown previously for IFNα monotherapy, treatment with IFNα and adefovir combination induced a shift from primarily CD56dim to more immature CD56bright NK cells in our cohort (3). CD56bright NK cells are known as the precursors of CD56dim NK cells and gradually differentiate via CD56dim NKG2a+ KIR- CD57- to terminally differentiated CD56dim NKG2A KIR+ CD57+. (25) We observed an increase in NKG2A expression on both NK cell subsets in our cohort (supplementary figure 3), supporting a shift in the overall NK cell compartment towards a more immature phenotype upon combination therapy. Furthermore, a decreased expression of CD16 on CD56dim NK cells as well as increased CD94, Nkp30 and Nkp46 on this subset also suggests more immature CD56dim NK cells (25–27). These results are in line with a shift towards more immature NK cells after INFα treatment for other conditions such as hepatitis C and hepatitis delta infection, but also myeloproliferative neoplasms (28–30).

This skewing towards an immature NK phenotype in the periphery could have different etiologies, for example (I) an increased egress of progenitors from the bone marrow, (II) increased proliferation of immature cells, (III) altered migration of NK cell subtypes or (IV) a loss of mature NK cells. When analysing Ki67 expression in NK cells, we found an increase in proliferation of CD56bright NK cells, as shown previously (3). However, in increase of Ki67 expression was also observed in CD56dim NK cells, whilst these cells decreased in absolute numbers in the periphery. Immature CD56dim NKG2A+ KIR- CD57- NK cells express more Ki67 than terminally differentiated CD56dim NKG2A KIR+ CD57+ NK cells. Thus, a shift within the CD56dim NK cell compartment towards immaturity towards end of treatment with interferon-based therapy could possibly explain the relatively increased expression of Ki67 in this subset. (25) Furthermore, the observed changes in chemokine receptor expression, suggest that migration to other organs might contribute to the increased CD56bright NK cells and loss of proliferating CD56dim NK cells. However, we were unable to study this assumption as measurements of NK cells were performed only in the peripheral blood and not in other compartments such as the lymph nodes and liver. Lastly, the susceptibility to apoptosis could be altered within specific NK cell subsets, such as CD56dim NK cells, upon IFNα monotherapy (3).

NK cells carry the IFNα receptor and can therefore be directly activated by type I interferons. Indeed, an increase in the activation status was seen in both CD56bright and -dim NK cell subsets during therapy as it has been shown previously only for CD56bright NK cells after IFNα monotherapy (3).
The functional activity of NK cells has been reported to be increased as well as decreased upon IFNα monotherapy(3,29). In our cohort, combination therapy was associated with a functional impairment of NK cells. We did however find higher IFNγ production in response to target cells in responders compared to non-responders at EoT (figure 5D), suggesting target cell-induced cytotoxicity and cytokine release by NK cells may play an important role in the viral clearance induced by IFNα/afenovir combination therapy.

Another difference between responders and non-responders was found with regard to TRAIL expression (figure 5C). Not only did TRAIL expression increase during treatment with combination therapy as reported in IFNα monotherapy(3,31), at end of treatment expression was found to be higher in responders compared to non-responders. TRAIL on NK cells has contrasting functional abilities. Whereas TRAIL on NK cells can induce apoptosis in virally infected hepatocytes and fibrosis-inducing stellate cells, it has also been suggested that NK cells can kill HBV-specific CD8+ T cells via the TRAIL pathway, potentially impairing viral clearance(32,33).

Higher expression of TRAIL on CD56bright in responders to therapy at end of treatment suggests a beneficial role of this ligand in which HBV-infected hepatocytes and stellate cells are eliminated by NK cells. Furthermore, lower CD56bright CX3CR1 expression in responders may indicate that migratory abilities of these cells to sites of inflammation are crucial for their action. Lower expression of the inhibitory receptor NKG2A on CD56dim NK cells at baseline, as found in responders in this study, has previously been related to long-term virologic response in HCV patients treated with IFNα and ribavirin(34) and could render these cells more susceptible to activation. Furthermore, blocking of NKG2A has previously been shown to augment NK cell function in CHB(21). Future studies will have to be conducted to assess whether these NK cell characteristics could become predictive markers of response early during IFNα based therapy.

In a second group, which consisted of HBeAg positive CHB patients who either had HBeAg seroconversion or not, no relation between NK cell phenotype and response was found. This underlines the fact that HBeAg seroconversion is driven by a different immunological mechanism than HBsAg clearance. A relation between ALT at BL and CD56bright NK cells, TRAIL expression and CD38 expression of these cells was found in HBeAg positive patients (figure 6). This suggests that NK cells do play a role in hepatocyte lysis, either directly or indirectly, similar to the relationship between CD56bright TRAIL expression and ALT that has been shown previously(9,33).

Combination therapy with IFNα/ NUC therapy for patients with chronic hepatitis B is a successful treatment option which can give rise to relatively high proportion of HBsAg clearance. Here, we show that combination therapy induces radical changes in the NK cell compartment, increasing their activation. Furthermore, significant differences were found in NK cell phenotype in patients with HBsAg loss and patients who had no response to therapy. A correlation between the NK cell phenotype and ALT suggests a role for NK cells in liver damage. While the exact role of NK cells with respect to viral clearance remains to be determined, better understanding of the actions of this immunological subset may help in generating better treatment options for CHB patients in the future.
ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

Supplementary figure 1: (A) Schematic overview of selected patients. Definitions of response as mentioned in methods section. *Note that due to restriction by availability of samples only 7 out of initial 8 responders at week 72 were used in cohort I. **In cohort II the HBeAg positive patients from cohort I were excluded (n=6) (B) Viral and clinical parameters of responders and non-responders of cohort I.

Supplementary figure 2: Absolute numbers of NK cell subsets at BL and EoT. Gating strategy (A) total NK cells, CD56bright and CD56dim NK cells (B) and longitudinal analysis of CD56bright and CD56dim NK cells subsets in patients with CHB (n=13-14 per timepoint) (C). *p<0.05; **p<0.01***p<0.001; ns non-significant. FSC, forward scatter; SSC, side scatter; H, hight; W, width; CHB, chronic hepatitis B patients; BL baseline; EoT, end of treatment.
Supplementary figure 3: Combination therapy in CHB patients results in a change in expression of activating and inhibiting markers. Representative staining for CD16, NKG2A, CD94, NKG2C, NKp30, NKp46, CD161 (A), frequencies or gMFI levels of different NK cells markers on CD56bright (B) and CD56dim (C) NK cells. Graphs show 14 patients with CHB at BL and EoT. Statistical analyses Wilcoxon test:

*p<0.05; **p<0.01; ***p<0.001. HC, healthy control; CHB, chronic hepatitis B; BL baseline; EoT, end of treatment; gMFI geometric mean fluorescence intensity. (Internal negative = CD14+/CD19+ cells for CD16, NKG2A, CD94, NKG2C, NKp30, CD161 and CD3+ cells for NKp46)
Supplementary figure 4: Perforin, granzyme B and TRAIL expression increase during therapy. Expression of perforin (A), granzyme B (B) and TRAIL (C) in CD56bright and CD56dim NK cells. (D) Representative staining for perforin, granzyme B and TRAIL in the total NK cell gate at BL and EoT. Statistical analyses: unpaired data: Mann Whitney U test, paired data: Wilcoxon test: *p<0.05; **p<0.01; ***p<0.001. CHB chronic hepatitis B; BL baseline; EoT, end of treatment; gMFI geometric mean fluorescence intensity.

Supplementary figure 5: HBeAg positive patients who achieve HBeAg seroconversion without HBsAg clearance at T=72 after therapy do not have different baseline NK cell markers or functional activity than non-responders. Horizontal bars indicate mean with SEM. ns, non significant. BL baseline; EoT, end of treatment; gMFI geometric mean fluorescence intensity.
REFERENCES


PART III

Novel Non-Invasive Methods for Monitoring of Liver Fibrosis in Chronic Hepatitis B
Non-invasive evaluation of liver fibrosis: a comparison of ultrasound-based Transient Elastography and MR elastography in patients with viral hepatitis B and C.


Accepted for publication in European Radiology 2014 Mar;24(3):638-48
ABSTRACT

Objective
To compare diagnostic accuracy of TE and MRE and establish cut-off levels and diagnostic strategies for both techniques, enabling selection of patients for liver biopsy.

Methods
103 patients with chronic hepatitis B or C and liver biopsy were prospectively included. Areas under curves (AUROC) were compared for TE and MRE for METAVIR fibrosis grade ≥F2 and ≥F3. We defined cut-offs for selection of patients with F0–F1 (sensitivity >95%) and for significant fibrosis F2–F4 (specificity >95%).

Results
Following exclusions, 85 patients were analysed (65 CHB, 19 CHC, 1 co-infected). Fibrosis stages were F0 (n=3); F1 (n=53); F2 (n=15); F3 (n=8) and F4 (n=6). TE and MRE accuracy were comparable (AUROC_{TE}≥F2: 0.914 [95%CI: 0.857–0.972] vs. AUROC_{MRE}≥F2: 0.909 [0.840–0.977], P=0.89; AUROC_{TE}≥F3: 0.895 [0.816–0.974] vs. AUROC_{MRE}≥F3: 0.928 [0.874–0.982], P=0.42). Cut-off values of <5.2 and ≥8.9 kPa (TE) and <1.66 and ≥2.18 kPa (MRE) diagnosed 64% and 66% of patients correctly as F0–F1 or F2–F4. Conditional strategy in inconclusive test results increased diagnostic yield to 80%.

Conclusion
TE and MRE have comparable accuracy for detecting significant fibrosis, which was reliably detected or excluded in two-thirds of patients. A conditional strategy further increased diagnostic yield to 80%.
INTRODUCTION

Ultrasound-based Transient Elastography (TE) and MR Elastography (MRE) can detect liver fibrosis non-invasively by measuring viscoelastic properties of the liver [1–4]. Both techniques have shown excellent accuracy in detecting cirrhosis, and in excluding the presence of significant liver fibrosis [5–14]. In fact, a reduction in the number of liver biopsies performed is already seen in those centres that have TE available [15]. However, studies directly comparing TE with MRE are scarce; to our knowledge only one study compared the two techniques in a patient cohort [11].

Important clinical events in chronic hepatitis B (CHB) and chronic hepatitis C (CHC) are the development of significant fibrosis (METAVIR stage F2 or higher) and of cirrhosis (stage F4). From stage F2, the risk of progression to cirrhosis and subsequent development of hepatocellular carcinoma increases, and antiviral treatment needs to be considered to slow down or reverse progressive liver fibrosis or cirrhosis [16–18]. Therefore, non-invasive selection of those patients with stage F2 fibrosis and higher, and those without (i.e. with stage F0 and F1 fibrosis) would be useful. The primary aim of this study was to compare TE and MRE with liver biopsy as the reference standard. The secondary aim of this study was to define lower and upper cut-off values for both TE and MRE, based on diagnostic values (≥95% sensitivity for the lower cut-off and ≥95% specificity for the higher cut-off). With these cut-off values patients who do or do not need a liver biopsy before starting anti-viral therapy can be selected by employing either a single technique strategy or a conditional technique strategy.

METHODS

Patients and study procedures

This was a single centre study, with patients initially being recruited from four different hospitals in The Netherlands: Academic Medical Center, Amsterdam; Free University Medical Center, Amsterdam; Onze Lieve Vrouwe Gasthuis, Amsterdam and Erasmus University Medical Center, Rotterdam. The institutional review boards of all four participating centres approved of this study and all participants signed informed consent. Patients of 18 years and over with CHB or CHC were prospectively and consecutively included from gastroenterology and hepatology departments of the four participating centres. Patients were eligible if they had a liver biopsy taken. Biopsies were taken either for clinical reasons, or as part of a clinical trial recruiting patients with low viral load CHB (NCT00973219). Exclusion criteria were pregnancy or other contra-indications for MRI and initiation of antiviral therapy before all study procedures were finalised. After inclusion, study procedures (TE and MRE examination) were carried out at the Academic Medical Center, Amsterdam.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined at the time of liver biopsy and expressed relative to the upper limit of normal (xULN).
Liver biopsy
Percutaneous liver biopsies were performed using a 14- or 16-gauge needle. Tissue was fixed in formalin and paraffin embedded. Two expert liver pathologists evaluated all biopsies for fibrosis, necro-inflammatory activity, steatosis and iron, independently (J.V. and V.T.). Any discrepancies were resolved by consensus. Both were blinded to clinical details, except for type of liver disease. In consensus by the participating pathologists (J.V. and V.T.) and a third, independent expert liver pathologist, biopsies with less than 7 portal tracts were excluded from analysis. The METAVIR scoring system was used for fibrosis assessment (F0: no fibrosis; F1 portal fibrosis without septa; F2 few septa; F3 numerous septa; F4: cirrhosis) [19]. A METAVIR fibrosis score of F2 or higher was referred to as significant fibrosis. The modified hepatic activity index (m-HAI) was used to assess the degree of inflammation, based on a zero to 18 score as follows: no inflammation (score 0), minimal (score 1-3), mild (score 4-8), moderate (score 9-12) and severe (score 13-18) [20]. Steatosis was scored from zero to three based on the percentage fat-containing hepatocytes: grade 0 (<5%, none), grade 1 (5–33%, mild), grade 2 (33–66%, moderate) or grade 3 (>66%, severe) [21]. Presence of iron was scored from 0 to 4 [22].

Ultrasound-based transient elastography
TE (FibroScan®, Echosens, France) measures the speed by which a shear wave propagates through tissue using ultrasound. A vibrating device, positioned on the ultrasound transducer, generates shear waves. The speed of the shear wave correlates with tissue stiffness. TE measures liver stiffness in a cylinder of 1 x 4 cm [4]. TE was performed by one of two examiners (A.E.B. and A.d.N.). Examiners were not blinded with respect to clinical data. A minimum of 10 TE measurements was performed. Median liver stiffness measurements (LSM), success rates (SR) and interquartile ranges (IQR) were recorded. The following reliability criteria for TE were applied [23]:

- **Very reliable**: IQR/LSM ≤ 0.10;
- **Reliable**: IQR/LSM between 0.10 and 0.30, or IQR/LSM > 0.30 with LSM < 7.1 kPa;
- **Poorly reliable**: IQR/LSM > 0.30 with LSM ≥ 7.1 kPa.

MR based elastography
For the acquisition of MR elastograms of the liver, mechanical waves of 50 Hz were applied to the liver by a portable transducer, which was placed against the right side of the chest. MRE was performed on a 3.0-Tesla MR system (Intera, Philips Healthcare, The Netherlands). Transverse slices were obtained through the mid-level (feet–head direction) of the liver using a motion-sensitive 2D spin-echo based echo-planar imaging (SE-EPI) sequence with the following acquisition parameters: repetition time 560 ms, echo time 40 ms, flip angle 90°, number of slices 7, slice thickness 4 mm; field of view 320x320 mm and matrix size 80x80. MRE acquisition time was 78 s, divided over six breath holds on expiration. Elastograms of the liver were reconstructed using dedicated post-processing software (provided by R. Sinkus, Clichy, France) [24]. As a minimum of two neighbouring voxels is needed for reliable calculation of elasticity, only the middle three of
the seven acquired slices are considered reliable. These three middle slices were merged into a single, 12 mm thick slice for further analysis. Next, the wave images were examined for adequate wave amplitude (Figure 1). Adequate wave amplitude was defined by: the amplitude of the propagating exceeding at least three times the noise value (with noise being measured in the left chest compartment where the mechanical waves do not propagate). A physician experienced in MRE (A.E.B.) manually drew a single region of interest (ROI) in those areas of the liver with adequate wave amplitude, avoiding liver margins, gall bladder and major central blood vessels. The post-processing software takes into account wave interference and reflections, areas of which therefore do not have to be excluded from the ROI. MRE acquisitions were anonymised to ensure the physician’s blinding to the patient’s clinical information while drawing the ROIs. The elasticity value (kPa) measured inside this region of interest was used for analysis.

It should be noted that the examination results of both TE and MRE are expressed in kPa. However, in TE the one-dimensional stiffness measurement is related to the Young’s modulus, whereas elasticity with MRE is derived from the three-dimensional shear elasticity modulus [11]. Therefore, examination results of both techniques cannot be directly compared.

Figure 1: Region of interest (ROI) placement in magnetic resonance elastography (MRE). (A) ROI placement on a magnitude image, avoiding liver margins and large vessels. (B) Wave image showing that waves penetrate well throughout the entire ROI. (C) Elastogram of the liver. Voxels are colour-coded from 0 to 2.5 kPa. The mean elasticity value within this selected ROI was 1.79 kPa (61-year-old woman with chronic hepatitis C (CHC) and F1 fibrosis on liver biopsy).

Statistical analysis
Baseline characteristics of F0–F1 and F2–F4 groups were compared with unpaired T-tests, Mann-Whitney U tests or Chi Square tests, as appropriate. Correlation of MRE with TE was calculated using Spearman’s rho ($r_s$). For the comparison of the diagnostic values, the areas under the receiver operating characteristic curves (AUROC) of MRE and TE (with liver biopsy as the reference standard) were calculated for dichotomised groups: F0–F1 vs. F2–F4 and F0–F2 vs. F3–F4. AUROCs were compared using paired analysis [25].

Based on AUROC analysis results we defined for the F0–F1 vs. F2–F4 group:

a) The cut-off value by which patients without significant liver fibrosis (F0–F1) are reliably selected, defined as sensitivity ≥ 95% (lower cut-off);
Part III

b) The cut-off value by which patients with significant liver fibrosis (F2–F4) are reliably selected, defined as specificity ≥ 95% (higher cut-off).

The accompanying sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and positive/negative likelihood ratios (LR) for each cut-off, with 95% confidence intervals (95% CI), were calculated. Sensitivity and specificity were compared using McNemar's test for paired data; PPV and NPV were compared by calculating the z-ratios of proportions.

The possible confounding effect on TE and MRE of fibrosis stage (F0-F1 vs. F2-F4), age (continuous), sex (male vs. female), BMI (continuous), disease aetiology (CHB vs. CHC), inflammation (m-HAI score 0-3 vs. ≥4), steatosis (grade 0-1 vs. grade 2-3), presence of any iron (grade 0 vs. grade 1-3), ALT and AST levels (continuous) and the number of portal tracts on liver biopsy (continuous) was analysed with univariate and subsequently multivariate linear regression analysis.

A P value of < 0.05 was considered statistically significant. All analyses were performed with IBM SPSS statistics 19 (SPSS, Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Patients and study procedures
Between November 2009 and March 2012, 103 patients were included (n=76 from Academic Medical Center, Amsterdam; n=12 from Free University Medical Center, Amsterdam; n=6 from Onze Lieve Vrouwe Gasthuis, Amsterdam and n=9 from Erasmus Medical Center, Rotterdam, The Netherlands). Patients with unreliable or unsuccessful MRE, TE or liver biopsy results were excluded from analysis. In total, 18 patients were excluded for reasons related to the quality of the biopsy, MRE and TE (as summarised in the flowchart in Figure 2). A direct comparison of MRE and FS with a reliable reference standard was therefore conducted in 85 patients. Twelve patients had been included in a previous study on the reproducibility of MRE [26].

Baseline characteristics are summarised in Table 1. The median interval between MRE and TE was 0 days (IQR 0–19 days, range 167 days). Median intervals between MRE and biopsy and between TE and biopsy were 18 days (IQR 6–31 days, range 178 days) and 25 days (IQR 10–35 days, range 167 days), respectively.

The TE results were “very reliable” for 25 patients and “reliable” for 59 patients (n=52 with IQR/LSM between 0.1–0.3; n=7 with IQR/LSM > 0.3 and LSM < 7.1 kPa). TE result of one patient had “poor reliability”.

Liver biopsy
After initial independent scoring, the pathologists disagreed on the METAVIR score of 13 biopsy samples. Disagreement was one stage for 12 biopsies and two stages for 1 biopsy. These biopsies were consequently scored in consensus. Overall, fibrosis was scored as METAVIR F0 (n=3), F1 (n=53), F2 (n=15), F3 (n=8) and F4 (n=6). Four patients had co-existent steatohepatitis (2 CHB, 2 CHC). Distribution of disease aetiology per fibrosis stage is outlined in Figure 2.
Transient Elastography

Of all 103 patients who signed informed consent, 3 patients were excluded from analysis because of technical problems with the TE machine. These patients had all started antiviral therapy by the time the machine was available again. Attempts to perform TE were unsuccessful in 5 patients. The success rate of TE was therefore 95% (95 out of 100). Of the 85 patients included, TE results were “very reliable” for 25 patients; “reliable” for 59 patients (n=52 with IQR/LSM between 0.1–0.3; n=7 with IQR/LSM > 0.3 and LSM < 7.1 kPa). The TE result of one patient had “poor reliability”. This was a male patient with CHB, LSM of 8.3 kPa, IQR/LSM of 0.34 and success rate of 63%. Liver biopsy showed F1 fibrosis. MRE elasticity value was 1.57 kPa.

Figure 2: Study flow chart. There was overlap in the reasons for exclusion: the 24 cases with a reason for exclusion represented 18 patients. METAVIR scores represent consensus scores obtained by two experienced pathologists.

MR elastography

Of all consenting patients, 7 were excluded from analysis for reasons related to MRE, giving a success rate of MRE of 93% (93 out of 102). Of the 85 cases analysed, selected ROIs in the liver comprised an average 679 voxels (SD 187 voxels), which corresponds to a liver parenchymal volume of 43.4 cm³ (SD 11.9 cm³).

Comparison of TE with MRE

The TE results correlated with those for MRE \( r_s = 0.71, P<0.01 \). AUROC values when comparing F0–F1 with F2–F4 were 0.914 (95% CI: 0.857, 0.972) for TE and 0.909 (95% CI: 0.840, 0.977) for MRE. These values were not significantly different (\( P = 0.89 \)).
### Table 1: Baseline characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>All participants (n=85)</th>
<th>F0–F1 (n=56)</th>
<th>F2–F4 (n=29)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>45 (12) a</td>
<td>43 (12) a</td>
<td>48 (12) a</td>
<td>0.63</td>
</tr>
<tr>
<td><strong>Male gender (n)</strong></td>
<td>55 (65%)</td>
<td>34 (61%)</td>
<td>21 (72%)</td>
<td>0.29</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>25.5 (3.9) a</td>
<td>25.2 (3.3) a</td>
<td>26.0 (4.9) a</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Aetiology (n)</strong></td>
<td>65 CHB; 19 CHC; 1 CHB/CHC</td>
<td>50 CHB; 6 CHC</td>
<td>15 CHB; 13 CHC; 1 CHB/CHC</td>
<td></td>
</tr>
<tr>
<td><strong>ALT (xULN) a</strong></td>
<td>1.04 (0.66,1.89) (n=82)</td>
<td>0.88 (0.56,1.36) (n=54)</td>
<td>1.69 (1.05,3.33) (n=28)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>AST (xULN) c</strong></td>
<td>0.88 (0.65,1.46) (n=82)</td>
<td>0.73 (0.62,1.01) (n=54)</td>
<td>1.50 (0.99,2.72) (n=28)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Biopsy length (mm)</strong></td>
<td>18 (7) a</td>
<td>16 (7) a</td>
<td>22 (7) a</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Portal tracts (n)</strong></td>
<td>15 (6) a</td>
<td>13 (5) a</td>
<td>19 (6) a</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>m-HAI (score 0–18)</strong></td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none (n)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.98</td>
</tr>
<tr>
<td>minimal (n)</td>
<td>38 (44%)</td>
<td>32 (57%)</td>
<td>6 (21%)</td>
<td></td>
</tr>
<tr>
<td>mild (n)</td>
<td>37 (44%)</td>
<td>24 (43%)</td>
<td>13 (45%)</td>
<td></td>
</tr>
<tr>
<td>moderate (n)</td>
<td>9 (11%)</td>
<td>-</td>
<td>9 (31%)</td>
<td></td>
</tr>
<tr>
<td>severe (n)</td>
<td>1 (1%)</td>
<td>-</td>
<td>1 (3%)</td>
<td></td>
</tr>
<tr>
<td><strong>Iron (grade 0–4)</strong></td>
<td>(n=79)</td>
<td>(n=53)</td>
<td>(n=26)</td>
<td>0.98</td>
</tr>
<tr>
<td>Grade 0 (n)</td>
<td>66 (84%)</td>
<td>44 (83%)</td>
<td>22 (85%)</td>
<td></td>
</tr>
<tr>
<td>Grade 1 (n)</td>
<td>10 (13%)</td>
<td>7 (13%)</td>
<td>3 (12%)</td>
<td></td>
</tr>
<tr>
<td>Grade 2 (n)</td>
<td>3 (4%)</td>
<td>2 (4%)</td>
<td>1 (4%)</td>
<td></td>
</tr>
<tr>
<td><strong>Steatosis (grade 0–3)</strong></td>
<td>0.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 0 (n)</td>
<td>42 (49%)</td>
<td>31 (55%)</td>
<td>11 (38%)</td>
<td></td>
</tr>
<tr>
<td>Grade 1 (n)</td>
<td>30 (35%)</td>
<td>19 (34%)</td>
<td>11 (38%)</td>
<td></td>
</tr>
<tr>
<td>Grade 2 (n)</td>
<td>11 (13%)</td>
<td>6 (11%)</td>
<td>5 (17%)</td>
<td></td>
</tr>
<tr>
<td>Grade 3 (n)</td>
<td>2 (2%)</td>
<td>-</td>
<td>2 (7%)</td>
<td></td>
</tr>
<tr>
<td><strong>MRE elasticity (kPa)</strong></td>
<td>1.73 (1.50,2.16)</td>
<td>1.58 (1.48,1.79)</td>
<td>2.29 (1.92,3.07)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>TE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stiffness (kPa)</td>
<td>5.3 (4.5,7.9)</td>
<td>4.7 (4.3,5.9)</td>
<td>8.3 (6.9,14.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IQR (IQR/SM)</td>
<td>0.14 (0.09,0.22)</td>
<td>0.15 (0.09,0.22)</td>
<td>0.13 (0.08,0.20)</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>Success rate (%)</strong></td>
<td>100 (83,100)</td>
<td>100 (83,100)</td>
<td>100 (80,100)</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Data are presented as median (25th, 75th percentile) or as mean (SD) a

a ALT reference values: 45 U/L (men), 35 U/L (women) ; ULN: upper limit of normal

b AST reference value: 40 U/L
AUROC values for F0–F2 vs. F3–F4 were also not significantly different: 0.895 (0.816, 0.974) for TE and 0.928 (0.874, 0.982) for MRE (P= 0.42). ROC curves are shown in Figure 3. The small number of patients with F0 (n=3) and F4 (n=6) precluded AUROC analyses for F0 vs. F1–F4 and for F0–F3 vs. F4, respectively.

Figure 3: Area under the receiver operating characteristic curve (AUROC) curves for MRE (blue) and transient elastography (TE; purple) with liver biopsy as the reference standard. Data between brackets are 95% confidence intervals. There were no significant differences in the area under the curves between MRE and TE in this population.

Selection of patients with and without significant fibrosis (F2–F4 vs. F0–F1)

The distribution of TE and MRE results per histological fibrosis stage are illustrated in Figure 4. When comparing non-significant fibrosis (F0–F1) with significant fibrosis (F2–F4), the cut-off value to rule out non-significant fibrosis – defined by a specificity of at least 95% – was ≥ 8.9 kPa for TE and ≥ 2.18 kPa for MRE. Specificity of these cut-off values was 96% for both techniques. The accompanying PPV and positive LR (LR+) were high: 88% and 90% (P=0.81) and 14.0 and 17.4 for TE and MRE, respectively. This high specificity and PPV, however, come at a cost: the accompanying sensitivity and NPV were 48% and 62% (P=0.22), and 78% and 83% (P=0.48) for TE and MRE, respectively. The negative LR (LR–) were 0.54 and 0.39 for TE and MRE, respectively (Table 2).

When selecting patients with F0–F1 fibrosis, as defined by a sensitivity of a least 95%, the lower cut-offs were < 5.2 kPa for TE and < 1.66 kPa for MRE. For these cut-offs, sensitivity and NPV were 97% for TE and MRE. LR– was 0.05 (TE) and 0.06 (MRE). In return, the accompanying specificity and PPV dropped to 66% and 63% (P= 0.80) and 60% and 57% (P=0.81) for TE and MRE, respectively. LR+ was 2.85 (TE) and 2.57 (MRE).
Figure 4: Distribution of TE and MRE results per fibrosis stage. Horizontal dotted lines represent cut-off values. Upper cut-off line: selection of patients with F2–F4 fibrosis; lower cut-off line: selection of patients with F0–F1 fibrosis; shaded grey area: inconclusive test result. Per fibrosis stage, medians and interquartile ranges are indicated.

Table 2: Cut-offs and accompanying diagnostic accuracy of TE and MRE.

<table>
<thead>
<tr>
<th>Cut-offs</th>
<th>Sensitivity (%)(%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>LR+</th>
<th>LR-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selecting patients with F2–F4 fibrosis (higher cut-off)</td>
<td>TE ≥8.9 kPa</td>
<td>48 (30,67)</td>
<td>88 (60,98)</td>
<td>78 (66,87)</td>
<td>14.0</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>MRE ≥2.18 kPa</td>
<td>62 (42,79)</td>
<td>90 (67,98)</td>
<td>83 (71,91)</td>
<td>17.4</td>
<td>0.39</td>
</tr>
<tr>
<td>Selecting patients with F0–F1 fibrosis (lower cut-off)</td>
<td>TE &lt;5.2 kPa</td>
<td>97 (80,100)</td>
<td>60 (44,73)</td>
<td>97 (85,100)</td>
<td>2.85</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>MRE &lt;1.66 kPa</td>
<td>97 (80,100)</td>
<td>57 (42,71)</td>
<td>97 (84,100)</td>
<td>2.57</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Data between brackets are 95% confidence intervals; numbers in italic represent raw data.

Defining strategies

Single technique strategy:
For MRE, results were comparable: 56 of the 85 patients (66%) were diagnosed correctly when applying the higher and lower cut-offs: 36 as F0–F1 (including 1 false negative) and 20 as F2–F4 (including 2 false positives). In total, the results of 29 out of 85 (34%) patients were inconclusive and still required a liver biopsy.

For both TE and MRE, the false negative result concerned the same patient: a 47-year-old male CHC patient with a histological confirmation of F2 fibrosis, but with MRE and TE results suggestive of F0–F1: 1.45 kPa and 4.9 kPa, respectively. Mild steatosis and iron accumulation
Figure 5: depicts a flow-chart with the diagnostic yield of the proposed cut-off values of TE and MRE. When applying both the higher and lower TE cut-offs in our population, 54 of the 85 patients (64%) were correctly diagnosed: 38 patients as F0–F1 (including 1 false negative) and 16 patients as F2–F4 (including 2 false positives). TE results of 31 of 85 (36%) were inconclusive (in between the two cut-offs: ‘grey’ zone) – and would still require a liver biopsy.

(both grade 1) were confirmed histologically. MRE and TE were performed on the same day, 26 days after the biopsy. The four false positives for the higher cut-off concerned 4 CHB patients with fibrosis stage F1 on biopsy. The two MRE false positives (2.20 and 2.27 kPa) both had inconclusive TE values of 5.3 and 6.4 kPa, respectively. The two TE false positives (9.0 and 9.4 kPa) had intermediate MRE results of 2.17 and 1.82 kPa, respectively. ALT levels were not explanatory with values of 0.46, 4.23, 1.62 and 1.04 xULN, respectively. MRE and TE measurements were performed on the same day and immediately following one another in all four cases; the maximum time interval between biopsy and MRE/TE was 33 days.

Conditional strategy
The bottom part of Figure 5 shows the additional yield of a conditional strategy. If all 31 patients with an inconclusive TE result had been offered an MRE, 14 additional correct diagnoses (45%) would have been obtained: 7 F0–F1 and 7 F2–F4. The additional value of a conditional TE in the case of an inconclusive MRE (although this is a less likely clinical scenario) is similar: 12 out of 29 (41%) patients were diagnosed with TE (9 as F0–F1; 3 as F2–F4). Examination results of 17 of the 85 patients (20%) remained inconclusive by using a conditional strategy; these patients would have benefited from a liver biopsy to determine the presence of significant fibrosis. The characteristics of these 17 inconclusive patients are outlined in Table 3. The patient details show no discrepancies from the total group characteristics (Table 1) that could explain the inconclusive test results.
Table 3: Characteristics of 17 inconclusives after the conditional strategy.

<table>
<thead>
<tr>
<th>#</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Aetiology</th>
<th>Metavir</th>
<th>MRE (kPa)</th>
<th>TE (kPa)</th>
<th>TE IQR/LSM</th>
<th>TE Reliability</th>
<th>Biopsy #</th>
<th>portal tracts</th>
<th>m-HAI score</th>
<th>Iron grade</th>
<th>Steatosis grade</th>
<th>ALT (ULN)</th>
<th>AST (ULN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>56</td>
<td>CHB</td>
<td>0</td>
<td>1.73</td>
<td>6.4</td>
<td>0.20</td>
<td>Reliable</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0.74</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>54</td>
<td>CHB</td>
<td>1</td>
<td>1.66</td>
<td>6.8</td>
<td>0.57</td>
<td>Reliable</td>
<td>19</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0.74</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>61</td>
<td>CHC</td>
<td>1</td>
<td>1.79</td>
<td>5.4</td>
<td>0.09</td>
<td>Very rel.</td>
<td>23</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>0.76</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>48</td>
<td>CHB</td>
<td>1</td>
<td>2.12</td>
<td>6.6</td>
<td>0.09</td>
<td>Very rel.</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td></td>
<td>1.02</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>42</td>
<td>CHB</td>
<td>1</td>
<td>2.15</td>
<td>5.3</td>
<td>0.06</td>
<td>Very rel.</td>
<td>18</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>3.31</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>29</td>
<td>CHB</td>
<td>1</td>
<td>1.96</td>
<td>6.2</td>
<td>0.24</td>
<td>Reliable</td>
<td>14</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>2.49</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>32</td>
<td>CHB</td>
<td>1</td>
<td>2.07</td>
<td>6.1</td>
<td>0.08</td>
<td>Very rel.</td>
<td>23</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>5.24</td>
<td>1.75</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>46</td>
<td>CHB</td>
<td>1</td>
<td>1.97</td>
<td>6.2</td>
<td>0.11</td>
<td>Reliable</td>
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<td>7</td>
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<td>M</td>
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<td>CHB</td>
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<td>6.8</td>
<td>0.35</td>
<td>Reliable</td>
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<td>CHB</td>
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<td>Reliable</td>
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<td>CHC</td>
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<td>7.4</td>
<td>0.07</td>
<td>Very rel.</td>
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<td>Reliable</td>
<td>9</td>
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<td>1.05</td>
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<td>51</td>
<td>CHC</td>
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<td>8.3</td>
<td>0.27</td>
<td>Reliable</td>
<td>16</td>
<td>6</td>
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<td>1</td>
<td>1.82</td>
<td>1.05</td>
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<td>CHB</td>
<td>2</td>
<td>1.66</td>
<td>5.9</td>
<td>0.09</td>
<td>Very rel.</td>
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<td>0</td>
<td>4.40</td>
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<td>7.7</td>
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<td>Reliable</td>
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<td>7</td>
<td>-</td>
<td></td>
<td>0.74</td>
<td>3.53</td>
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</table>

*aMissing data.*
Analysis of possible confounders

Table 4 displays the influence of several baseline characteristics on TE and MRE results. Fibrosis stage had a statistically significant influence on both MRE and TE results ($p < 0.001$). Age had a significant influence on TE results ($p = 0.04$), but not on MRE results ($p = 0.08$). All other parameters had no significant influence on TE and MRE results in this dataset.

Table 4: Multivariate linear regression analysis of possible confounders.

<table>
<thead>
<tr>
<th></th>
<th>TE</th>
<th>P</th>
<th>MRE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate*</td>
<td>P</td>
<td>Multivariate*</td>
<td>P</td>
</tr>
<tr>
<td>Fibrosis stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(F0-F1 vs. F2-F4)</td>
<td>5.81</td>
<td>&lt;0.01</td>
<td>5.44</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(4.25, 7.38)</td>
<td></td>
<td>(3.47, 7.41)</td>
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</tr>
<tr>
<td>Age (y)</td>
<td>0.11</td>
<td>&lt;0.01</td>
<td>0.07</td>
<td>0.04</td>
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<tr>
<td></td>
<td>(0.04, 0.19)</td>
<td></td>
<td>(0.00, 0.14)</td>
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</tr>
<tr>
<td>Sex (m vs. f)</td>
<td>-1.44</td>
<td>0.15</td>
<td>-0.23</td>
<td>0.17</td>
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<tr>
<td></td>
<td>(-3.41, 0.53)</td>
<td></td>
<td>(-0.57, 0.10)</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>-0.04</td>
<td>0.74</td>
<td>-0.03</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>(-0.29, 0.21)</td>
<td></td>
<td>(-0.07, 0.01)</td>
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<tr>
<td>Aetiology (CHB vs. CHC)</td>
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<td>0.01</td>
<td>-0.28</td>
<td>0.79</td>
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<tr>
<td></td>
<td>(0.69, 5.13)</td>
<td></td>
<td>(0.27, 1.80)</td>
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<tr>
<td>Inflammation (m-HAI 0-3 vs. ≥4)</td>
<td>2.69</td>
<td>&lt;0.01</td>
<td>1.07</td>
<td>0.25</td>
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<tr>
<td></td>
<td>(0.86, 4.52)</td>
<td></td>
<td>(-0.76, 2.89)</td>
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<tr>
<td>Steatosis (grade 0-1 vs. 2-3)</td>
<td>1.37</td>
<td>0.30</td>
<td>0.09</td>
<td>0.69</td>
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<tr>
<td></td>
<td>(-1.26, 4.01)</td>
<td></td>
<td>(-0.36, 0.54)</td>
<td></td>
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<tr>
<td>Iron (grade 0 vs. 1-4)</td>
<td>0.87</td>
<td>0.53</td>
<td>-0.06</td>
<td>0.81</td>
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<tr>
<td></td>
<td>(-1.86, 3.59)</td>
<td></td>
<td>(-0.52, 0.41)</td>
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<tr>
<td>ALT (xULN)</td>
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<td>&lt;0.01</td>
<td>-1.18</td>
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<td></td>
<td>(0.42, 1.60)</td>
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<td>(-1.34, 0.98)</td>
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<tr>
<td>AST (xULN)</td>
<td>0.05</td>
<td>&lt;0.01</td>
<td>0.03</td>
<td>0.23</td>
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<td></td>
<td>(0.03, 0.07)</td>
<td></td>
<td>(-0.02, 0.08)</td>
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<tr>
<td>Portal tracts (n)</td>
<td>0.20</td>
<td>0.01</td>
<td>-0.16</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>(0.04, 0.36)</td>
<td></td>
<td>(-0.32, 0.00)</td>
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</table>

* Univariate and multivariate analyses are expressed as unstandardized B coefficients with 95% confidence intervals.

DISCUSSION

The diagnostic accuracies of TE and MRE for detecting METAVIR fibrosis stage F2 and higher and for detecting stage F3 and higher in patients with CHB and CHC did not differ significantly in this study. Based on the proposed higher and lower cut-off values, approximately two in every three patients who participated in this study were diagnosed using either technique as F0–F1 fibrosis or F2–F4 fibrosis, obviating the need for a liver biopsy. When employing a conditional strategy, by means of an additional MRE examination in the case of an inconclusive TE examination and vice versa, four in every five patients may not have needed a liver biopsy.
In the literature, various cut-off values have been defined for TE in different patient populations. In meta-analyses, covering mainly CHB and CHC, the pooled optimal cut-off value for detecting F2–F4 fibrosis ranged from 7.3 to 7.9 kPa [7, 8, 27, 28]. These cut-offs, however, optimised the combination of sensitivity and specificity whereas we aimed for 95% sensitivity for the lower cut-off and 95% specificity for the higher cut-off, irrespective of the corresponding specificities and sensitivities. Viganò et al employed a strategy similar to ours by defining higher and lower cut-offs for selecting patients with and without significant fibrosis using TE in a cohort of 125 CHB patients [5]. In addition, the defined cut-off values were internally validated in an additional 92 CHB patients. Their higher cut-off was slightly higher than ours at 9.4 kPa. Accuracy results were identical to ours: specificity of 96% and LR+ of 14. The lower cut-off was ≤ 6.2 kPa with sensitivity of 94% and LR- of 0.1 (our results: sensitivity 97% and LR- 0.05). No noticeable differences between the two populations with respect to AST or ALT levels, age or gender were present. One can however argue that our lower cut-off may have been higher had we been able to include more patients with significant fibrosis, especially with stage F3 or F4. On the other hand, a large French multicentre TE study (FIBROSTIC study) favoured a more conservative lower cut-off: at 5.2 kPa, 90% sensitivity and LR- of 0.29 were reported for the prediction of significant fibrosis in 1307 patients (913 CHC, 284 CHB, 110 HIV) [6]. Of note is that no difference was found with respect to the diagnostic accuracy of CHC and CHB subgroups at this cut-off level. Verveer et al [29] studied the diagnostic accuracy of TE in 241 patients (125 CHB, 116 CHC), all with large liver biopsies (≥25 mm). A sensitivity level of 90% was achieved for detecting METAVIR ≥F2 at a cut-off of 6.0 kPa for CHB and 5.0 kPa for CHC.

The absolute elasticity values obtained with MRE in this study cannot be compared directly with those obtained in other studies. First of all, the elasticity parameter as calculated with our MRE post-processing method, is not the same as the “stiffness” values that are measured with other available MRE techniques [24, 26]. Secondly, MRE is under on-going development and no clear consensus with respect to the acquisition parameters, the mechanical wave frequency, or the reconstruction algorithm exists [30]. The only other study that compared TE and MRE directly in a large patient population is the study by Huwart and colleagues. They compared TE and MRE in 96 patients and found a diagnostic accuracy of MRE that was significantly higher than that of TE [11]. The AUROCs that we found for TE ≥ F2 and TE ≥ F3 were 0.914 and 0.895, compared with 0.837 and 0.905 in Huwart’s paper. For MRE, the respective AUROCs for MRE were 0.909 and 0.928 in our population vs. 0.994 and 0.985 in Huwart’s. Differences in the patient population and distribution of fibrosis stages may explain these different results.

Our multivariate analysis showed, besides a significant association between fibrosis stage and both TE and MRE results, a significant association between age and TE results. Age is a known cofactor of fibrosis progression [31]. Other known confounders of TE include for instance inflammation, ALT and AST levels and steatosis [32–36]. In the present dataset, these variables were not significantly associated with either TE or MRE results. However, this study was not powered to investigate the confounding effects of these parameters, for which larger patient samples would be needed.
The fact that all patients underwent both TE and MRE is an advantage of this study. Also, the double read of all liver biopsies by two experienced pathologists independently is a strong point. Limitations of this study should also be mentioned. Firstly, this study could have been stronger had we been able to use both a training set and a validation set for the defined cut-off values. However, as relatively few patients with viral hepatitis B and C received a liver biopsy in the participating centres, this could not be incorporated into this study. Our cut-off values should therefore be validated on existing datasets or in a new prospective study. Secondly, the distribution of liver fibrosis stages is not well balanced in this study. Most patients had F1 fibrosis. This precluded accuracy analysis for F0 vs. F1–F4 and for F0–F3 vs. F4, and may have caused the lower cut-offs to be somewhat conservative, as pointed out before. We did not correct for this spectrum bias, since our primary aim was to compare the diagnostic accuracy of TE with MRE. Spectrum bias affects the AUROC values when dichotomizing results, but does not affect the comparison between the two techniques. Thirdly, 32 biopsies of all 103 included patients did initially not meet the AASLD criteria of at least 11 portal tracts and 2–3 cm in length [37]. We therefore chose a less strict criterion of at least 7 portal tracts. Biopsies with less than 7 portal tracts were excluded. Fourthly, although we aimed to perform all study procedures within a time frame of 60 days, this was not always possible for logistic reasons. In 9 patients this 60-day limit was exceeded. However, a sub-analysis without these patients did not change the cut-off values or the diagnostic yield, nor were the accuracy results significantly affected (data not shown). Fifth, of the 85 analysed biopsies, 22 had 7–10 portal tracts. Of these, only 2 cases (both F1) were misclassified when applying the proposed cut-off values (false positives): 1 with MRE and 1 with TE. These concerned: a 56 year old female patient, CHB, TE 6.4 kPa, MRE 2.3 kPa, ALT 4.2 xULN, AST 3.2 xULN, m-HAI 2, no steatosis, interval MRE - TE 0 days, interval TE/MRE and biopsy 33 days; the other a 19 year old male patient, CHB, TE 9.4 kPa, MRE 1.82 kPa, ALT 1.04 xULN, AST 0.73 xULN, m-HAI 2, no steatosis, interval MRE – TE 0 days, interval TE/MRE and biopsy 20 days. Explanation for the false positivity of the former could be the elevated transaminases, of the latter possible underestimation of fibrosis stage. Sixth, we did not assess interobserver variability or reproducibility of elastography findings, as previous studies have shown excellent reproducibility and interobserver agreement [26, 38, 39]. TE readers were not blinded with respect to clinical data. TE reading cannot be done without interaction with the patient. It is however not likely that knowing the clinical data of the patient would affect the TE examination result since the technique does not allow manipulation of the examination result. Finally, we asked most – but not all – of our patients to fast before the examination. Studies have shown that a meal shortly before the examination influences elasticity results [40, 41]. We acknowledge that this limitation may have influenced our results. Our defined cut-off values need to be validated. For TE, this could be done on existing large population study data or on prospective studies. The high diagnostic accuracy of MRE for fibrosis staging shows promise for the future. Because of its short acquisition time, MRE could easily be added to a comprehensive liver MRI examination. At present, MRE is already being used in clinical practice in some centres.
Part III

In conclusion, this study shows that TE and MRE have comparable accuracy in detecting liver fibrosis. TE and MRE could reliably detect or exclude significant liver fibrosis in two-thirds of patients in this study; a conditional strategy of performing MRE in the case of an inconclusive TE examination further increased the diagnostic yield to 80% correct diagnoses. Furthermore, TE and possibly also MRE, have the advantage that they are non-invasive techniques that can be used in the follow-up of patients on prolonged anti-viral therapy. This is particularly relevant because antiviral therapy can reverse fibrosis, and potentially even reverse cirrhosis [42].
REFERENCES


PART IV

Supplementary chapter
Chronic hepatitis E after solid organ transplantation: Case report and review of the literature

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Departments of 1 Gastroenterology & Hepatology, 2 Virology and 3 Nephrology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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ABSTRACT:

Large outbreaks of acute hepatitis E, caused by hepatitis E virus (HEV) genotypes 1 and 2, are known from developing countries with suboptimal sanitation infrastructure. An increasing incidence of HEV infections is being reported in industrialized countries, caused mainly by HEV genotypes 3 and 4, which are often found among pigs. Recent evidence suggests that in immunocompromised patients about 50% of the cases of acute hepatitis E evolve to chronic hepatitis with rapid progression to cirrhosis. Thus, HEV should be considered as a cause of chronic hepatitis in immunocompromised patients, such as solid organ transplant recipients. Because an antibody response to HEV may be absent in these patients, an HEV RNA test should be carried out when serum liver tests are elevated over months. Ribavirin has been shown in small case series to represent a promising treatment option for chronic HEV infection. To increase the awareness for HEV infection in immunocompromised patients, a representative case report of a HEV-infected renal transplant recipient with chronic hepatitis E, successfully treated with ribavirine, is presented. Studies are required to determine the optimal duration of ribavirin therapy and to assess outcome for solid organ transplant recipients with chronic HEV infection.
INTRODUCTION

Hepatitis E virus (HEV) is a global pathogen that can cause epidemic, endemic, sporadic and zoonotic cases of acute hepatitis. Large outbreaks of hepatitis E are seen in developing countries with suboptimal sanitation infrastructure. However, an increasing incidence of HEV infection is found in industrialized countries of the Western hemisphere. In the Netherlands, hepatitis E virus infection is considered as one of the three most important emerging infectious diseases: whereas less than 0.5% of the population had contact with HEV 15 years ago, a recent survey showed that more than 15% of the population has meanwhile developed HEV antibodies as a sign of former exposure to HEV (see below). In the Western world, the course of endemic HEV infection is generally self-limiting and asymptomatic in immunocompetent individuals. In contrast, immunocompromised patients are at risk to develop chronic HEV infection with progressive liver disease as reported in various case series since 2008. Patients with haematological disorders 1-3, HIV infection 4, 5 and after solid organ transplantation under immunosuppressive therapy 6-9 appear at particular risk to develop chronic hepatitis E that rapidly evolves to cirrhosis. Potentially effective therapeutic interventions by use of ribavirine in chronic hepatitis E have only recently been documented. To further raise the awareness of the community in the Western hemisphere for the risk of chronic hepatitis due to HEV infection in immunocompromised patients, but also to demonstrate the most recent development of promising therapeutic approaches, we report a case of a renal transplant patient with Bechterew disease, who developed chronic hepatitis E, but was cured with ribavirin monotherapy.

Case report: A 51-year old bank manager was known with a medical history of arterial hypertension, hypertension-related renal insufficiency, and Bechterew disease. In July 2010 he received a living related kidney transplant. After transplantation, serum liver tests increased temporarily, but returned to normal levels within two weeks. His further postoperative course was uneventful. Before transplantation, serologic tests for hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), and varicella zoster virus were all negative. The immunosuppressive regimen after kidney transplantation consisted of prednisolone 10 mg bid, mycophenolate mofetil 1 g bid and tacrolimus 4 mg bid. For his Bechterew disease, he received etanercept 25mg twice weekly. One year after kidney transplantation alanine aminotransferase (ALT [normal < 45 U/L]: 132 U/L), aspartate aminotransferase (AST [ < 40]: 65 U/L), and gamma-glutamyltransferase (yGT [ < 60]: 139 U/L) became elevated (fig. 1). Protrombine time and total bilirubine remained within the normal range. Ultrasound of the liver did not show any abnormalities. Serologic and molecular testing, respectively, for HAV, HBV, HCV, and CMV remained negative. Unexpectedly, serologic tests for hepatitis E were positive (HEV IgM positive, HEV IgG weakly positive). Retrospective testing of former specimens revealed positive HEV RNA (genotype 3) in serum one year after transplantation onwards, coinciding with the rising serum liver tests. The patient (95 kg, 198 cm) was treated with ribavirin at a dose of 800mg bid for three months. Maintenance immunosuppressive drug treatment was diminished (prednisolone 5 mg bid, mycophenolate mofetil 1500mg bid and...
Part IV

tacrolimus 500mg bid) and etanercept was temporarily discontinued, but had to be restarted because of severe symptoms of Bechterew disease. At the start of ribavirin therapy, creatinine clearance was >60ml/min, haemoglobin was 7.2 mmol/l, and HEV RNA in serum was strongly positive (fig. 1). No deterioration of kidney function or hemoglobin count was observed during treatment. Two months after the start of ribavirin therapy all serum liver tests had returned to normal and HEV RNA was negative. Three months after the end of ribavirin treatment serum liver tests remained normal and serum HEV RNA remained negative. The patient is doing well.

Figure 1

A 51-year old patient with arterial hypertension-associated renal insufficiency and Bechterew disease developed chronic hepatitis E one year after living-related kidney transplantation and was treated with ribavirin monotherapy for three months. His immunosuppressive medication, HEV RNA, and serum ALT (ALAT, normal < 40 U/L) are documented between 2009 and early 2012. Immunosuppressive drug therapy with prednisolone, mycophenolate mofetil and tacrolimus was diminished after the diagnosis of HEV.
BACKGROUND

Biology and Epidemiology

HEV is a hepevirus, consisting of a capsid containing a positive stranded RNA genome, and it is not enveloped. Phylogenetic studies have unravelled four genotypes. HEV genotype 1 and 2 infections are restricted to humans whereas genotypes 3 and 4 appear to be zoonotic, infecting various animal species such as pigs, wild boar and deer as well as humans.

Epidemiological studies revealed that genotypic distribution of HEV is area specific. Genotypes 1 and 2 are prevalent in the highly disease-endemic regions, which cover the tropical and subtropical parts of Asia, Africa and Central America. HEV genotype 1 and 2 infections are most commonly transmitted through fecally contaminated water via the oral route. Materno-fetal and transfusion related transmissions have only occasionally been reported. Various endemic outbreaks of hepatitis E genotype 1 and 2 have been documented. Young adults are most often infected. Pregnant women are at particular risk: they have a higher disease attack rate and are much more likely to develop fulminant liver failure with fatal outcome in up to 22% (all genotype 1 infections). The pathophysiologic mechanisms leading to fulminant liver failure after HEV infection in pregnant women are not understood, but have been attributed to pregnancy-related immunological or hormonal alterations.

In regions with lower HEV infection rates, such as Western Europe, the United States, and developed countries of Asia and the Pacific region, hepatitis E was long thought to be related to travel to endemic regions. This view has recently changed since an increasing number of sporadic, autochthonous HEV infections has been reported particularly in Western Europe. Viral strains isolated from HEV-infected patients without a history of travel to high risk areas were classified as genotype 3 and 4. This strongly suggests a zoonotic origin. First, human HEV isolates genotype 3 and 4 appear to be the same strains that also frequently infect pigs. Secondly, interspecies transmission from human to pig and from swine to nonhuman primates has been demonstrated. Thirdly, molecular typing in Japan demonstrated a link between cases of hepatitis E after eating raw or undercooked pig meat and HEV strains isolated from domestic swine, wild boar and sika deer.

In contrast to predominant infection of young adults in the endemic areas in Asia and Africa, in Western Europe and the U.S. mostly elderly subjects and people with coexisting illness are affected, while fulminant disease in pregnant women is not reported. It is assumed that HEV genotype 3 is less virulent than genotype 1 and 2 strains, which may explain the predilection of elderly and immunocompromised persons for HEV genotype 3 infection. In addition, this may explain why epidemic outbreaks with genotype 3 were not observed so far in the Western world. Subclinical infection with HEV genotype 3 may explain the relatively high seroprevalence of anti-HEV antibodies in these areas. The geographical distribution of genotype 4 is limited to Asian countries and has not been associated with disease in immunocompromised patients so far.
Seroprevalence in Europe

A study in 1995 among Dutch blood donors reported a seroprevalence of 0.4%\(^23\). Seroprevalence in the South-West of France (according to testing of blood donors) has meanwhile dramatically increased from 16.6 to 52.5% suggesting that the disease is highly endemic in this region\(^24\). In the UK, HEV RNA was detected in 6 of 880 pools of blood donations (with 48 donations per pool), indicating a considerable rate of silent ongoing HEV infections among British blood donors\(^25\). The incidence of de novo HEV infections after kidney transplantation was calculated as 3.2 cases/100 person-years in a recent analysis\(^22\). Accordingly, seroprevalence studies had previously reported anti-HEV IgG antibodies in 6-16% of renal transplant recipients\(^26\).

Clinical Manifestations

Clinical features of HEV infection differ between the hyperendemic and the low prevalence areas. Incubation time is approximately 30 days. In hyperendemic areas the most commonly observed clinical presentation is a typical acute icteric hepatitis resembling hepatitis A and other forms of acute viral hepatitis. A pre-icteric and an icteric phase of the illness can be distinguished. The initial pre-icteric phase is characterized by fever, anorexia, distaste for food, vomiting, abdominal pain and diarrhea. These symptoms last for about 3 days. Onset of the icteric phase is marked by disappearance of the prodromal symptoms. It is usually self limiting and improves within 10-24 days. Hepatomegaly and splenomegaly are common during the icteric phase\(^11,27\). Less frequently, liver injury is mild and can be asymptomatic or accompanied by only non-specific symptoms. Acute hepatic decompensation may be particularly seen in persons with pre-existing liver disease or with genotype 1 infections during pregnancy.

In low prevalence areas, patients with symptomatic hepatitis E genotype 3 typically are older, predominantly male with higher frequency of underlying liver disease, alcohol abuse, or an immunocompromised state. The clinical presentation is often non-specific, the spectrum ranges from asymptomatic elevation of serum transaminases to a severe icteric hepatitis. In clinical case series, jaundice was the most common symptom (68-86%) followed by asthenia, fever, joint and muscle pains and abdominal discomfort\(^11,27\). Extrahepatic clinical manifestations such as pancreatitis, haematological manifestations, autoimmune and neurological disorders have infrequently been reported\(^11\).

Chronic hepatitis E

Chronic hepatitis E, genotype 3, in low-endemic regions has first been described in immunocompromised patients in 2008. Various case series reported chronic hepatitis E in patients who developed elevation of serum transaminases after kidney-, liver- and heart-transplantation\(^9,20,28-30\). Chronic HEV infection was also reported in patients with haematological malignancies, human immunodeficiency virus infection or those receiving anticancer chemotherapy\(^11,5,31\). Symptoms in these patients are mild or unnoticed and elevation of serum transaminases may be the only sign during routine follow-up. Rapid progression of chronic hepatitis E to cirrhosis is observed in these patients. Furthermore, extrahepatic manifestations such as HEV induced glomerulonephritis, that recovered after HEV clearance, have been observed after organ transplantation\(^32\).
All recently described cases of chronic hepatitis E in low prevalence areas were caused by HEV genotype 3. The way of transmission of HEV genotype 3 has been a matter of intense discussion since. Apart from zoonotic infection, other modes of transmission have been suggested in immunocompromised organ transplant recipients. Transmission of the virus from the donor organ has been reported in a liver transplant recipient who subsequently developed cirrhosis and hepatic decompensation. Transmission via blood transfusions was unravelled in a number of hemodialysis patients. Although reactivation of the virus has not been observed after solid organ transplantation, as is seen in other chronic viral infections such as hepatitis B during immunosuppression, reactivation of HEV has been discussed in one patient with allogenic stem cell transplantation.

Diagnosis
The diagnosis of HEV infection is based on detection of HEV IgG and IgM antibodies in blood and of HEV RNA in blood and stool. Both HEV IgM and IgG ELISA based assays are available, but have not been standardized so far. Sensitivity and specificity may vary considerably between different assays and even between batches of a given assay. HEV IgM antibodies are detectable as soon as symptoms occur. IgG antibodies may reach a sensitivity of 72% to 98% and a specificity of 78% to 96% to diagnose HEV infection in immunocompetent patients, but are less accurate in immunocompromised individuals. HEV IgG may persist for years.

Molecular detection of HEV RNA can be achieved by PCR from serum, bile and stool. Nucleic acid testing as a diagnostic marker of HEV infection has limitations in immunocompetent patients, because the period of viral shedding is limited to only 1-2 weeks around the time of ALT elevation and jaundice. Still, HEV PCR is a crucial tool in immunocompromized patients. In these patients, the diagnosis can be easily missed as they often remain seronegative, whereas HEV RNA remains detectable during chronic infection. Furthermore, diagnostic testing for hepatitis E in transplant patients may be delayed as increased levels of serum liver tests (ALT, AST) are frequently seen as a consequence of drug toxicity or are related to other hepatotropic viral infections.

Histological findings in liver biopsies may vary from portal hepatitis with dense lymphocytic infiltrates, piecemeal necrosis and fibrosis to cases with severe fibrosis or cirrhosis and are not specific for hepatitis E.

Treatment of chronic hepatitis E
Acute hepatitis E is self-limited and usually does not need any treatment. In contrast, up to 60% of immunocompromized patients develop chronic hepatitis after HEV infection (HEV RNA persistently positive, >6 months in serum or stool) without treatment. Decreasing the number and dose of immunosuppressive drugs can be the first approach to control chronic hepatitis E in (renal) transplant recipients. Most recently, advances have been made by the use of peg-interferon and ribavirin in chronic hepatitis E.
Peg-interferon therapy of chronic hepatitis E in transplant patients

Three case series have reported successful results with 3-12 months of peg-interferon therapy in 6 liver transplant recipients and in one patient on hemodialysis after kidney transplantation. Sustained viral response (SVR), defined as negative serum HEV RNA 6 months after cessation of therapy, was reported in 5 of the 7 patients. In the first report, 12 months of peg-interferon therapy resulted in sustained response in 2 of 3 liver transplant recipients chronically infected with hepatitis E. Similar results were obtained with a 3-month course of peg-interferon therapy in 2 of 3 patients with HEV infection that also underwent liver transplantation. In the third report a 3 months of peg-interferon therapy also led to SVR in a patient with kidney transplant failure and hemodialysis who did not spontaneously clear HEV after withdrawal of immunosuppressive therapy.

Of note, as the use of peg-interferon therapy could enhance allogenic immunity, it has to be given with precautions in transplant patients and should be avoided in kidney transplant patients because of a high risk of acute organ rejection in these patients.

Ribavirin therapy of chronic hepatitis E in transplant recipients

Three case series have reported promising results with three months of ribavirin therapy in patients with kidney, pancreas and heart transplantation. Sustained viral response was reported in 78% of the reported cases. In the largest series, ribavirin therapy in kidney transplant recipients led to a SVR 6 months after cessation of therapy in 4 out of 6 patients. The second report documented SVR in two patients with chronic hepatitis E after kidney and pancreas transplant, respectively, following 3 months of ribavirin therapy. A third case report showed SVR after ribavirin therapy in a patient with chronic hepatitis E after heart transplantation.

The major adverse effect of ribavirin is haemolytic anemia that usually occurs within the first 2 weeks of treatment and may be reversible after dose reduction. Close monitoring of hemoglobin levels is therefore advised. Nevertheless, the otherwise good tolerability of ribavirine monotherapy makes this medication an attractive option for treatment of chronic hepatitis E after solid organ transplantation. The case reported by us above confirms this conclusion.

Therapy in patients with HIV or hematologic malignancies

SVR after a combined regimen of peg-interferon and ribavirin for chronic HEV infection was reported in a case of co-infection with HIV-1 and HEV. Successful treatment of chronic hepatitis E by 3-months ribavirin treatment was reported for three cases with hematologic malignancies. Although the afore mentioned results are promising, only small case series are available and larger trials with longer follow up are necessary.

Preventive strategies

In endemic areas the main measures to prevent hepatitis E are better public education regarding hygiene and provision of safe drinking water. In areas of zoonotic transmission proper cooking of pig and deer meat is advised. However, it is questionable whether direct contact
with HEV-positive pork meat explains the current rise in infections with endemic hepatitis E in the Western world. Other food products may also be involved, for example fruit and vegetables may be contaminated via the use of HEV-positive water 48.

Two HEV recombinant protein vaccines have been successfully developed against proteins from the highly conserved open reading frame 2 of the virus 49, 50. The vaccines showed 96 and 100% efficacy, respectively, but they are not yet commercially available.

Conclusion
Hepatitis E virus (HEV) (human genotype 1 and 2) is a global pathogen that is responsible for large outbreaks of hepatitis E in developing countries. HEV (genotype 3) has been found to cause occasional autochthonous infections also in low endemic, developed areas in Europe or the United States. In immunocompromized patients, HEV can cause chronic hepatitis with rapid progression to cirrhosis. Because the anti-HEV antibody response can be absent or delayed in immunocompromized patients, an HEV RNA test should be carried out when serum liver tests are elevated. Recent advances have been made with peg-IFN and ribavirin treatment for chronic hepatitis E. Considering side effects of peg-IFN and its potential to induce allogenic immunity, ribavirin treatment should be the first choice for chronic hepatitis E in transplant patients. Adequate follow-up is necessary to assess the long-term outcome for immunocompromized patients with chronic HEV infection. Hepatitis E vaccines, currently under investigation, may be beneficial for patients infected with HEV genotype 1 or 2 in the near future.

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REFERENCE LIST


Summary and Conclusion
The major goals of therapy for chronic hepatitis B patients are the prevention of progression to irreversible liver damage, ideally by sustained HBsAg loss and formation of anti-HBs antibodies (functional cure). In the light of the suboptimal response to therapies currently available, e.g. the dose limiting adverse reactions of Peg-IFN treatment and the development of drug resistance for nucleos(t)ide analogues, it is of great practical value to develop new treatment strategies and establish predictors of response (or non-response) before or early in the course of anti-viral treatment to guide therapeutic interventions in chronic hepatitis B.

1. Combination therapy with Peg-IFN and nucleos(t)ide analogues for CHB patients with high and low viral load

Peg-IFN and nucleos(t)ide analogues are regarded as first line therapies for CHB. Whereas peg-IFN has both antiviral and immunomodulatory effects, nucleos(t)ide analogues act by direct inhibition of viral replication. HBsAg loss is uncommon with either treatment strategy. Previous attempts to improve response rates and rates of HBsAg loss by combining Peg-IFN and nucleos(t)ide analogue therapy have been disappointing when lamivudine was used. Nevertheless, combining Peg-IFN with more potent nucleos(t)ide analogues remained of interest because of the dual effect on both the innate and adaptive immune responses.

In chapter 2 we showed the results of 48 weeks of combination treatment of peg-IFN and adefovir in patients with a high viral load (HBV DNA ≥ 17,000 IU/mL). A comparably high rate of HBsAg loss in both HBeAg-positive (11%) and HBeAg-negative (17%) patients at 2 year follow-up was found compared to patients treated with peg-IFN for 48 weeks as monotherapy or combined with lamivudine in earlier studies. Although we can not make a direct comparison because of the absence of a monotherapy arm in our study, the higher rate of HBsAg loss suggests an additive therapeutic effect of adefovir.

Furthermore, a recent publication showed that combination therapy with Peg-IFN and tenofovir in HBeAg positive and negative patients resulted in a higher rate of HBsAg loss (9.1%) compared to either monotherapy (2.8% and 2.8%).

As patients may experience considerable side effect and treatment has limited efficacy, it is important to avoid unnecessary treatment in unresponsive patients. Although several pre- and on-treatment markers, such as HBV DNA, HBeAg status, HBV genotype are currently used to guide treatment, more predictors of response need to be identified to improve individual probability of response. Recently, increasing attention has been paid to serum HBsAg levels. Serum HBsAg reflects the transcriptional activity of cccDNA and is secreted from the hepatocyte during viral replication. Several studies showed that quantitative on-treatment HBsAg levels can be used as a biomarker for prognosis and treatment response. In our study we found that HBeAg-negative patients with HBsAg loss had lower baseline HBsAg levels than those without HBsAg loss, which supports the importance of HBsAg levels as pre-treatment viral marker of response.

A major breakthrough in the investigation of host factors predictive of response to interferon therapy for chronic hepatitis C has been the discovery that single nucleotide polymorphisms...
(SNPs) in the IL28B gene were associated with treatment response to peg-IFN and ribavirin\textsuperscript{16}. Therefore it was of interest to investigate the association between IL28B polymorphisms and treatment outcome in chronic hepatitis B patients. In our study we did not find a significant relation between the three most significant IL28B polymorphisms and HBeAg or HBsAg loss (chapter 4). Although the exact molecular mechanisms are yet unclear, IL28B is assumed to play a role in the regulation of interferon stimulating genes and the development of the innate anti-viral immune response. Likely anti-viral pathways and the activated interferon-stimulated genes, through which the host exerts its action against HBV, differ profoundly from those against HCV. Our findings suggest that the IL28B pathway is more relevant for elimination of HCV than HBV.

This is also supported by the difference in cure attainable with IFN-mediated immune modulation in HCV as compared with HBV infection. While up to 50% of patients with HCV genotype 1 may achieve cure on a 48-week peg-IFN- and ribavirin-based regimen, the clearance rate in chronic hepatitis B (i.e., loss of HBsAg and conversion to anti-HBs) on (peg) IFN is much lower, in the order of 3% at 1 year\textsuperscript{1,11}. In our genome-wide association study (chapter 5), one polymorphism, rs12356193 located in the \textit{SLC16A9} gene, was strongly associated with HBsAg loss at week 96 ($p = 1.84 \times 10^{-8}$). The previously reported association of rs12356193 with lower carnitine levels was confirmed in our cohort, and baseline carnitine levels were lower in patients with HBsAg loss compared to patients with HBsAg persistence. Here, we demonstrated that carnitine suppressed HBV-specific CD8 T cell proliferation, possibly an explanation for the striking association of rs12356193 in the SLC16A9 gene with HBsAg loss (chapter 5).

In addition to variations in host DNA associated with therapy outcome, we analysed mRNA isolated from baseline liver biopsies from chronic hepatitis B patients that participated in the clinical trial. We demonstrated an intrahepatic transcriptional signature, which was associated with enhanced immune activation and predicted therapy response (chapter 6). In responders (with HBeAg or HBsAg loss), pathway analysis showed specific upregulation of genes related to the immune response, including chemotaxis and antigen-processing and -presentation.

The decision to start treatment is obvious in patients with high viral load (HVL) and progressive liver inflammation and fibrosis. However patients with low HBV-DNA viral load (LVL) (<20,000 IU/mL) currently do not have an indication for treatment according to international guidelines, since these patients tend to have slower progression of liver disease. However HBV infection can be harmful in these patients\textsuperscript{1,2}. Since considerable rates of HBsAg loss were observed in our peg-IFN- adefovir trial, we aimed to investigate the effectiveness of peg-IFN combination therapy in achieving HBsAg loss in patients with LVL (chapter 3). As recent studies had shown that tenofovir monotherapy is a more potent viral suppressor than adefovir both combination regimens with peg-IFN- adefovir and peg-IFN- tenofovir were used in this study\textsuperscript{12}. This is the first intervention study done in CHB patients with low viral load and we show that strong HBsAg decline and HBsAg clearance can be achieved upon peg-interferon based combination therapy.
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in these patients. Although it remains to be investigated whether long lasting decline and loss of HBsAg further improves outcome in terms of cirrhosis or HCC free survival in patients with LVL, our finding may lead to extension of therapy indications for patients with chronic hepatitis B.

2. Immunologic Aspects of Chronic Hepatitis B Virus Infection

We investigated HBV-specific immunity in different CHB patient groups. Insight in these mechanisms could reveal predictive markers for treatment response and assist the development of novel immunotherapeutic strategies to reconstitute antiviral T cell function and improve treatment outcome. In the first study (chapter 7) phenotypic and functional properties (cytokine production, proliferative and cytotoxic potential) of HBV-specific T cells were investigated. In patients with LVL, relatively narrow HBV specific T cells responses, only directed towards the HBV core antigen, were identified. These HBV specific T cells showed a resting effector-memory phenotype and low ability to produce IL-2, but strong proliferative potential, and are probably able to retain the virus at low levels.

We found that HBV-specific T cells in patients with HVL are present at frequencies below the threshold of detection but retain the flexibility to restore their antigen responsiveness upon stimulation. In patients with HBsAg loss upon treatment with peg-interferon and adefovir, as well as in some patients on long term nucleos(t)ide therapy, there is a partial recovery of HBV-specific T cells. In these patients HBV-specific T cell restoration with a broader repertoire of antigen responsiveness, against HBV core and HBV envelope, was observed.

In the subsequent study (chapter 8) we found that chronic hepatitis B and C virus infection both leave a general signature in the total non-viral specific CD8^+ T cell population. We found an increase in CXCR3 expression, a chemotactic receptor of which the ligand is present in the liver, thereby enabling recruitment of T cells to the liver. This indicates the pervasive influence of localized chronic virus infections on the peripheral immune compartment. Next to CD8^+ T cell responses, also B cell responses and the generation of anti-HBs/HBsAg immune complexes was associated with therapy response. Immune complexes were significantly higher in HBeAg positive patients with HBeAg loss (chapter 9). As described earlier, HBV infection has the capability to influence the innate (non-specific) part of the immune system. We found that combination therapy significantly altered natural killer (NK) cell phenotype and function. Differences between patients with HBsAg clearance and non-responders suggest that NK cells play a critical role in the clearance of HBsAg during interferon-based combination therapy (chapter 10).


In chapter 11 we found that non-invasive monitoring of the liver with fibroscan and magnetic resonance elastography, as compared with liver biopsy as golden standard, was accurate in predicting the degree of liver fibrosis and disease progression. Thus, future monitoring of patients with CHB could rely on non-invasive methods without the need of serial liver biopsies and the associated risks.
CONCLUSION

In this thesis we investigated the efficacy of new therapies for chronic hepatitis B patients. The studies described in this thesis allow additional insight into antiviral mechanisms and the impact of genetic and immunological host factors associated with favorable or unfavorable treatment outcome. The novel genetic associations with treatment response described in this thesis; baseline HBsAg levels, SLC16A9 gene variations, intrahepatic transcriptional signature, restoration of HBV specific T cells, CXCR3 expression on CD8+ T cells, HBsAg/anti-HBs complex levels at baseline and NK cell characteristics, could improve our understanding of the variable responsiveness to therapy in CHB patients. Our findings may contribute to enable the selection of potential responders to treatment and may help to avoid unnecessary adverse events in putative non-responders.

The extent to which HBV-specific T cell restoration is required to achieve HBsAg loss, as well as the interaction with other players of the immune system, such as NK cells, remains to be further elucidated in subsequent studies.

There is an enormous clinical need to further improve treatment strategies for chronic hepatitis B patients in order to achieve HBsAg clearance. In addition to the currently approved therapies, new compounds that interact with the viral life cycle may be promising for the future. Direct antivirals (such RNA interferences and HBsAg reducing agents) and host-acting antivirals (such as viral entry inhibitors and immune enhancers such as Toll like receptor 7 agonists) are currently under clinical development.
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Nederlandse Samenvatting en Conclusie
Het belangrijkste doel van de behandeling van chronische hepatitis B (CHB) is het voorkomen van progressie van irreversible leverschade, idealiter door verlies van HBsAg en het ontwikkelen van anti-HBs antistoffen (functionele genezing) 1,2. Omdat de huidig beschikbare therapie nog niet optimaal is, peg-interferon (peg-IFN) bijwerkingen heeft en er resistentie ontwikkeling is voor nucleot(s)ide analogen, is het van groot belang om nieuwe behandelstrategieën te ontwikkelen en ‘respons-markers’ te vinden voor of tijdens therapie om de behandeling te kunnen sturen en patiënten te kunnen selecteren die baat hebben bij deze behandeling.

1. Peg-IFN en nucleos(t)ide analogen combinatie therapie voor CHB patiënten met een hoge of lage virale load

Bij de behandeling van CHB zijn zowel Peg-IFN en nucleos(t)ide analogen de middelen van eerste keus. De werking van peg-IFN berust op zowel antivirale als immunomodulerende eigenschappen terwijl nucleos(t)ide analogen voornamelijk zorgen voor directe remming van virale replicatie1,2. Bij beide behandelingen is de kans om HBsAg te verliezen laag. Eerdere pogingen om met combinatietherapie van Peg-IFN en lamivudine meer HBsAg verlies te bereiken vielen tegen 3–5. Echter, gezien het tweeledige effect op zowel de aangeboren (of niet specifieke) als de verworven (of specifieke afweer), blijft het combineren van Peg-IFN met sterker werkende nucleos(t)ide analogen potentieel interessant.

In hoofdstuk 2 laten we de resultaten zien van 48 weken combinatie therapie van peg-IFN en adefovir bij patiënten met een hoge virale load (HBV DNA ≥ 17,000 IU/mL). Vergeleken met eerdere studies met mono- of combinatietherapie zagen we een hoog percentage HBsAg verlies na 2 jaar follow up in zowel HBeAg-positieve (11%) als HBeAg-negatieve (17%). Alhoewel we de resultaten niet direct kunnen vergelijken door het ontbreken van een peg-IFN monotherapie arm in onze studie, suggereert de hoge mate van HBsAg verlies een aanvullend therapeutisch effect van adefovir. Daarnaast liet een recent gepubliceerde studie zien dat combinatietherapie met peg-IFN en tenofovir in HBeAg-positieve en -negatieve patiënten resulteerde in een hoger percentage HBsAg verlies (9.1%) vergeleken met beide monotherapie armen (2.8% and 2.8%). Omdat patiënten aanzienlijke bijwerkingen kunnen krijgen en de effectiviteit van de behandeling matig is, is het belangrijk om onnodige behandelingen te vermijden bij patiënten die niet zullen reageren. Alhoewel er momenteel verscheidene ‘respons markers’, zoals HBV DNA, HBeAg status en HBV genotype, voor of tijdens therapie gebruikt worden, is er behoefte aan betere voorspellende ‘respons markers’ om de individuele behandelkans te verbeteren. De laatste tijd is er toenemende interesse voor serum HBsAg-waarden. Serum HBsAg reflecteert de transcriptionele activiteit van het cccDNA en wordt uitgescheiden door de levercel tijdens virale replicatie. Meerdere studies hebben laten zien dat de kwantitatieve bepaling van HBsAg-waarden tijdens behandeling gebruikt kan worden als een biomarker voor prognose en therapie respons 6-8. In onze studie vonden we dat HBeAg-negatieve patiënten met HBsAg verlies een significant lagere ‘baseline’ HBsAg-waarde hadden dan patiënten zonder HBsAg verlies.
Dit ondersteunt de relevantie van quantitatieve HBsAg-waarden als virale ‘respons marker’ voorafgaand aan therapie. Een belangrijke doorbraak in het onderzoek naar voorspellende gastheer (host) factoren bij de respons op therapie bij chronische hepatitis C patiënten, was de ontdekking dat single nucleotide polymorphismen (SNPs) naast het IL28B-gen sterk geassocieerd bleken met respons op de behandeling met peg-IFN en ribavirine\textsuperscript{16}. Het is de vraag of deze associatie tussen IL28B polymorphismen en de uitkomst van therapie ook bestaat in CHB patiënten. In onze studie vonden we geen significant verschil tussen de drie belangrijkste IL28B polymorphismen en HBeAg of HBsAg verlies (hoofdstuk 4). Alhoewel precieze mechanismen onduidelijk zijn, wordt verondersteld dat het IL28B gen een rol speelt in de regulatie van interferon-gestimuleerde genen en de tootstandkoming van de niet specifieke anti-virale immuunspons. Waarschijnlijk zijn de anti-virale ‘pathways’ en de geactiveerde interferon-gestimuleerde genen, door middel waarvan de gastheer zijn afweer tegen het hepatitis B virus uitoefent, zeer verschillend ten opzichte van het hepatitis C virus (HCV). Onze bevindingen geven aan dat de IL28B ‘pathway’ een veel minder prominente rol speelt bij klaring van HBV dan bij HCV, hetgeen verder ondersteund wordt door het verschil in genezing dat bereikt kan worden met interferon gemedierte immuunmodulatie bij CHB vergelijkt met de resultaten van CHC patiënten. Terwijl zo'n 50% van de patiënten met een chronische hepatitis C (CHC) genotype 1 infectie kunnen genezen na 48-weken behandeling met peg-IFN en ribavirine, wordt bij CHB slechts 3% genezing (d.w.z. verlies van HBsAg en seroconversie naar anti-HBs na peg-IFN) bereikt na 1 jaar peg-IFN behandeling\textsuperscript{1,11}.

In onze studie bleek het polymorphisme, rs12356193, gelokaliseerd in het SLC16A9 gen significant geassocieerd met het verlies van HBsAg op week 96 ($p = 1.84 \times 10^{-8}$). De eerder beschreven associatie tussen rs12356193 en lagere carnitine-waarden werd bevestigd in ons cohort en baseline carnitine-waarden waren significant lager in patienten met HBsAg verlies vergeleken met patienten die HBsAg positief bleven. Verder lieten we zien dat carnitine de proliferatie van HBV-speifieke CD8$^+$ T-cellen onderdrukt (hoofdstuk 5).

Naast gastheer DNA, analyseerden we mRNA uit ‘baseline’ lever-biopten van CHB patiënten die deelnamen aan de klinische studie. We vonden dat een bepaald intrahepatisch transcriptioneel profiel, geassocieerd met toegenomen immuun activatie, de respons op therapie kon voorspellen (hoofdstuk 6). In responders (met HBeAg of HBsAg verlies) zagen we door middel van ‘pathway’ analyse dat specifieke genen gerelateerd aan de immuunrespons, waaronder chemotaxis en antigeenverwerking en -presentatie, waren opgereguleerd.

De beslissing om behandeling te starten is duidelijk in patiënten met een hoge HBV DNA virale load (HVL) en progressieve leverinflammatie en -fibrose. Daarentegen hebben patiënten met een lage HBV DNA virale load (LVL) (<20,000 IU/mL) volgens de internationale richtlijnen momenteel geen behandelindicatie, omdat zij een langzamere progressie hebben van de leverziekte. Echter, de HBV-infectie in deze patiënten kan wel degelijk leverschade veroorzaken\textsuperscript{1,2}. Gezien het hoge percentage HBsAg verlies bij patiënten met HVL in onze peg-IFN-adefovir trial, wilden wij in een volgende studie nagaan of hetzelfde effect bereikt kon worden bij

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patiënten met LVL (hoofdstuk 3). Omdat recente studies lieten zien dat tenofovir monotherapie een krachtigere virale onderdrukker is dan adefovir, onderzochten we zowel peg-IFN-adeovir als peg-IFN-tenofovir combinatietherapie in onze studie en vergeleken de resultaten met een onbehandelde controle groep\(^1\). Dit is de eerste interventie studie gedaan in CHB-patiënten met een LVL en we konden vaststellen dat met deze combinatie therapie er een sterke daling van HBsAg (21%) en HBsAg verlies (4,4%) op trad. Alhoewel nog onderzocht moet worden of langdurige HBsAg daling en verlies van HBsAg de lange termijn uitkomst qua levercirrose of hepatocellulair-carcinoom-vrije overleving kan verbeteren, kunnen onze bevindingen mogelijk leiden tot uitbreiding van de behandeldindicatie voor CHB-patiënten.

2. Immunologische Aspecten van een Chronische Hepatitis B Virus Infectie

In deel 2 onderzochten we de HBV-specifieke afweer in verschillende stadia van CHB. Door inzicht in afweermechanismen kunnen nieuwe immuuntherapiën ontwikkeld worden om de antivirale T-cel functie te herstellen en behandelfoutkomst te verbeteren. In de eerste studie (hoofdstuk 7) bestudeerden we fenotypische en functionele eigenschappen (cytokine productie en de potentie tot proliferatie en cytotoxische respons) van HBV-specifieke T-cellen. In patiënten met LVL vonden we relatief beperkte HBV specifieke T-cel responsen, slechts gericht tegen het HBV core antigen. Deze HBV specifieke T-cellen toonden een rustend effector-memory fenotype, een laag potentieel tot IL-2 productie, maar een sterk potentieel tot proliferatie. Deze cellen waren in staat om het virus te onderdrukken en de virale load laag te houden.

We vonden dat HBV-specifieke T-cellen in patiënten met HVL aanwezig waren in lage frequentie onder het detectie niveau, maar dat deze cellen de mogelijkheid behielden om hun respons op antigenen te herstellen na stimulatie met dit antigenen. In zowel patiënten met HBsAg verlies na behandeling met peg-IFN en adefovir, als in sommige patiënten die langdurig nucleos(t)ide therapie kregen, was er een gedeeltelijk herstel van HBV-specifieke T-cellen. In deze patiënten zagen we een herstel van HBV-specifieke T-cellen met een breder repertoire van de respons tegen antigenen, gericht tegen zowel tegen HBV core als HBV envelop antigenen.

In de volgende studie (hoofdstuk 8) vonden we dat chronische hepatitis B en C virus infecties beide een effect hadden op de niet-HBV-specifieke circulerende CD8\(^+\) T-cel populatie. We vonden een toename in de baseline expressie van CXCR3, een chemotactische receptor waarvan het ligand aanwezig is in de lever, en daarbij rekrutering van T-cellen naar de lever mogelijk maakt. Dit geeft het pervasieve (doordringende) effect aan van een in de lever gelokaliseerd virus op het periferie immuuncompartiment.

Naast de CD8\(^+\) T-cel respons, vonden we ook dat de B-cel respons en de formatie van anti-HBs/ HBsAg immuuncomplexen geassocieerd waren met therapie uitkomst. Immuuncomplexen op ‘baseline’ waren significante hoger in HBeAg-positieve patiënten met HBeAg verlies (hoofdstuk 9).

Zoals we eerder beschreven, kan het HBV-virus de aangeboren (niet-specifieke) afweer beïnvloeden. Uit ons onderzoek bleek dat natural killer (NK)-cellen een rol spelen in het verlies van HBsAg na combinatietherapie (hoofdstuk 10). We vonden dat combinatie therapie het
phenotype en de functie van NK-cellen kon veranderen en zagen verschillen tussen patiënten met HBsAg verlies en non-responders.

In hoofdstuk 11 vonden we dat vergeleken met het leverbiopt als gouden standaard zowel het onderzoek met de fibroscan als de ‘magnetic resonance elastography’ (MRI) van de lever nauwkeurig de mate van leverfibrose konden voorspellen. Mogelijk zou daarom in de toekomst een leverbiopt daarom achterwege gelaten kunnen worden.

CONCLUSIE
In deze dissertatie onderzochten we de effectiviteit van nieuwe therapiën voor chronische hepatitis B patiënten. Het bestuderen van genetische en immunologische gastheer factoren heeft meer inzicht gegeven in de antivirale mechanismen die een rol spelen bij de respons op therapie. De nieuw ontdekte associaties die we beschreven in deze disseratie: ‘baseline’ HBsAg-waarden, SLC16A9 gen variaties, intrahepatische transcriptionele factoren, herstel van de functie van HBV specifieke T-cellen, CXCR3-expressie op CD8+ T-cellen, HBsAg/anti-HBs immuuncomplex waarden op ‘baseline’ en NK-cel karakteristieken verbeteren de mogelijkheden om potentiële responders te selecteren en onnodige bijwerkingen te vermijden in non-responders.

De mate waarin het herstel van HBV-specifieke T-cellen van belang is voor het bereiken van HBsAg verlies, alsmede de interactie met andere factoren van het immuunsysteem, zoals NK-cellen dient verder onderzocht te worden in volgende studies.

Er is een duidelijke klinische noodzaak om de behandeling van chronische hepatitis B patiënten te verbeteren om meer HBsAg verlies en daarmee een functionele genezing te bereiken. Naast de huidige ‘standard-of-care’ therapieën zijn er veelbelovende middelen voor de toekomst die ingrijpen op virale levenscyclus van het HBV. Directe antivirale middelen (zoals RNA interferases en HBsAg remmende middelen) en de op de gastheer gerichte antivirale middelen (zoals virale entry inhibitors en immuun versterkers zoals Toll like receptor 7 agonisten) zijn momenteel in ontwikkeling.
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Part IV

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Annikki de Niet was born on 18th of July 1982 in Assen. She went to high school at the Dr. Nassau College in Assen from which she graduated in 2000. Thereafter she started studying medicine at the University of Groningen and studied piano for which she did preliminary training at the Converservatory of Groningen for 1 year. During her medicine studies she did her research internship about immunology in Melbourne, Australia. After her medicine studies she worked at the intensive care department in the University Medical Center of Groningen and at the department of internal medicine in the Deventer Hospital. In 2009 she started her PhD at the department of gastroenterology and hepatology and the department of experimental immunology at the Academic Medical Center (AMC) under supervision of dr. H.W. Reesink, dr. E.M.M. van Leeuwen, prof. dr. H.L. Zaaijer and prof. dr. U.H.W. Beuers. In 2013 she started her ophthalmology residency at the AMC in Amsterdam under supervision of prof. dr. M.P. Mourits.
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HBsAg loss in patients treated with peginterferon alfa-2a and adefovir is associated with SLC16A9 gene variation and lower plasma carnitine levels.

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Genetic variation in IL28B and treatment outcome in HBeAg-positive and -negative chronic hepatitis B patients treated with Peg interferon alfa-2a and adefovir.

SUBMITTED FOR PUBLICATION

A Randomized Prospective Open-label Trial Comparing Peginterferon Plus Adefovir or Tenofovir Combination Therapy Versus No Treatment in HBeAg-Negative Chronic Hepatitis B Patients with a Low Viral Load.
*participated equally to the study
** participated equally to the study

Human liver CD8+ T cells have a tissue-resident phenotype which is altered in the presence of chronic viral infection
*participated equally to the study
PhD Portfolio Summary
Part IV

General courses
Basic course: The AMC world of Science 2009 ECTS 0.7
Practical Biostatistics 2010 ECTS 1.1
Excel course 2010 ECTS 1.1

Specific courses
Postgraduate course, advanced immunology, Sanquin, Amsterdam The Netherlands 2010 ECTS 2.5
EASL basic school: Hepatitis B and C: Molecular Virology and Antiviral Targets, Lausanne Switzerland 2010 ECTS 0.7

Seminars, workshops and masterclasses.
Weekly department seminars Gastroenterology 2009-2013 ECTS 1.5
Weekly department seminars immunology ECTS 1.5
Tweede lagerhuisdebat Hepatitis B en C. Amsterdam, the Netherlands 2009 ECTS 0.1

Poster presentations
European Association for the Study of the Liver (EASL)
Augmented hepatitis B specific T cell response after clearance of chronic hepatitis B, poster prize 2011 ECTS 0.5
Upregulation of CXCR3+ expression on total CD8+ T cells due to the pervasive influence of chronic hepatitis B and C infection 2012 ECTS 0.5
Gene set enrichment analysis in baseline liver biopsies of chronic hepatitis B patients treated with peginterferon and adefovir, (second author) 2014 ECTS 0.2
A randomized prospective open-label trial comparing peginterferon + adefovir and peginterferon + tenofovir versus no treatment in HBeAg negative chronic hepatitis B patients with low viral load: interim analysis of week 48 results, late breaker poster 2015 ECTS 0.5

American Association for the study of liver diseases (AASLD)
T cell immune responses in chronic hepatitis B patient treated with peg-interferon and adefovir 2011 ECTS 0.5
Transcriptome analysis of chronic hepatitis B patients and its relation to the treatment outcome 2011 ECTS 0.5
new HBsAg/anti-HBs immune complex assay for prediction of treatment outcome in chronic hepatitis B patients 2011 ECTS 0.5
Polymorphism at rs12979860 and rs12980275 near the IL28B and treatment outcome in chronic hepatitis B patients treated with peg-interferon and adefovir 2011 ECTS 0.5
### PhD Portfolio Summary

#### Chapter 13

**A Genome Wide Association Study of Chronic Hepatitis B Patients Identifies a Variation in the SLC16A9 Gene Associated with HBsAg Loss after Treatment with Peginterferon and Adefovir, (second author)**

2012 | ECTS 0.2

**HBsAg/anti-HBs immune complex levels predict HBeAg seroconversion in patients treated with peginterferon and adefovir**

2012 | ECTS 0.5

**Characteristics of the adaptive immune responses in clearing or controlling chronic hepatitis B**

2012 | ECTS 0.5

**T cell characteristics during acute hepatitis B infection, AASLD**

2013 | ECTS 0.5

**A Randomized Prospective Open-label Trial Comparing Peginterferon Plus Adefovir or Tenofovir Combination Therapy Versus No Treatment in HBeAg-Negative Chronic Hepatitis B Patients with a Low Viral Load**

2015 | ECTS 0.5

### Keystone meeting

**Augmented hepatitis B specific T cell response after clearance of chronic hepatitis B**

2011 | ECTS 0.5

### Nederlands Vereniging voor Gastroenterologie (NVGE)

**Augmented hepatitis B specific T cell response after clearance of chronic hepatitis B**

2011 | ECTS 0.5

**A new HBsAg/anti-HBs immune complex assay predicts HBeAg loss in chronic hepatitis B patients**

2013 | ECTS 0.5

### Nederlandse Vereniging voor Immunologie (NVVI)

**Augmented hepatitis B specific T cell response after clearance of chronic hepatitis B**

2010 | ECTS 0.5

### Oral presentations

**scientific symposium London: New developments in viral load monitoring - implications for patient management. HBV viral load dynamics in the management of chronic hepatitis B**

2011 | ECTS 0.7

**Several presentations at regional hepatology meetings in AMC-VUMC (conference summaries and research results)**

2009-2013 | ECTS 0.5

### (Inter)national conferences

**Annual Meeting of the European Association for the Study of the Liver (EASL) Europe**

2009-2013 | ECTS 3.0

**Annual Meeting of the American Association for the Study of Liver diseases (AASLD). Boston and San Francisco**

2009-2012 | ECTS 2.4

**Keynote symposium viral immunology, Calgary, Al, Canada**

2011 | ECTS 0.6

**Nederlands Vereniging voor Gastroenterologie (NVGE)**

2009-2010 | ECTS 0.6

**Nederlandse Vereniging voor Immunologie (NVVI)**

2009-2012 | ECTS 1.2
Part IV

Teaching
Hepatitis B. Medical students participating in a 4-week Gastroenterology and Hepatology training program. Amsterdam the Netherlands 2010 ECTS 0.1
Hepatitis B. Internal Medicine residents in training. 2011 ECTS 0.1
Hepatitis B. Pathology residents in training. 2011 ECTS 0.1
Part IV