Natural course and effects of treatment in chronic hepatitis B

Cohort study in non-Asian women in Amsterdam

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Een cohortstudie in niet-Aziatische vrouwen in Amsterdam

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

General Introduction

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Hepatitis B viral load and risk of HBV-related liver disease: from East to West?

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Introduction

Of the different viral causes of hepatitis, hepatitis B virus (HBV) has the highest rate of chronically infected patients worldwide and poses a global health problem[1]. Approximately one third of the global population has ever been infected with HBV with an estimated 240 million people currently being chronically infected[1]. Chronic HBV has a variable course in disease activity with a risk of clinical complications like liver cirrhosis and hepatocellular carcinoma. As clinical symptoms present in a late stage of the disease, identification of risk factors is important for early detection and therefore improvement of prognosis. In 2006, two REVEAL-HBV studies from Taiwan have shown a positive correlation between viral load at any point in time and the development of cirrhosis and hepatocellular carcinoma[2,3]. This natural course of chronic HBV can be modulated by antiviral therapy improving the long-term outcome of the infection[4-6]. A recent multicenter cohort study demonstrated that treatment with TDF reversed liver fibrosis progression, regardless of HBeAg status[5]. Therefore, it is necessary to gain insight in the natural course of HBV to trace patients at high risk for development of HBV-related liver complications.

Epidemiology of hepatitis B

The HBV prevalence varies between different parts of the world, from the highest prevalence (>8%) in Southeast Asia and sub-Saharan Africa to the lowest prevalence (≤1%) in North America and Northwest Europe[7]. Annually, the virus is responsible for the death of approximately 620.000 to 786.000 patients due to HBV-related complications[8, 9]. The Netherlands has a stable low seroprevalence of HBV (HBsAg positive prevalence 0.2%)[10]. Since HBV is a notifiable disease, both incidence and prevalence data are available. In 2013, there were 1.108 registries of persons with

chronic hepatitis B[11]. In the Dutch chronic HBV population, approximately 6.500 patients are at risk dying within the next 10 years due to HBV-related complications[12]. It is estimated that currently 60.431 patients in the Netherlands are chronically infected with HBV[13], with immigrants from intermediate and high endemic countries contributing greatly to this prevalence[14]. A recent study showed that between 58% and 72% of all chronic HBV carriers in the Netherlands were first-generation immigrants, with the majority originating from Turkey, Surinam or Morocco[13]. The prevalence of HBV varies across these three countries with a low prevalence in Morocco (1.81%), an intermediate prevalence in Surinam (5%) and a high prevalence in Turkey (around 10 percent)[15-17]. A relative under exposed ethnic group in the Netherlands who originate from a high HBV prevalence country (around 9% HBsAg positive prevalence[18]) are Chinese migrants. Recently, several screening projects in Chinese migrants in the Netherlands showed that HBsAg positivity was detected in up to 8.4% of the included peoples[12, 19].

Complications of chronic hepatitis B virus infections

The hallmark of chronic HBV infections is the continuous hepatic inflammation during the course of the disease. Histological studies have shown that during chronic hepatitis B the liver has a varying degree of portal inflammation with lymphocytic infiltration leading to interface hepatitis[20]. Subsequently, persistent inflammation can lead to hepatic cell necrosis ultimately resulting in loss of liver parenchyma and substitution into fibrous tissue[20, 21]. Moreover, progression of liver fibrosis increases the risk of HBV-related liver disease, i.e. cirrhosis, hepatocellular carcinoma (HCC) and end-stage liver disease[22, 23]. Since liver inflammation and fibrosis progression may occur without clinical symptoms, patients often only seek medical help in the

more advanced stages of the disease. For instance, a study in 141 asymptomatic HBeAg negative patients, histologically evaluated whether there were signs of liver inflammation and fibrosis and found that 26% of the included patients had significant necroinflammation and that in 17% there was severe fibrosis in the liver biopsy (≥ F3 fibrosis)[24]. Therefore, it is mandatory to monitor chronic HBV patients for the development of fibrosis or HCC. In clinical practice, liver inflammation can be suspected by biochemical changes in liver specific parameters, such as elevated alanine aminotransferase (ALT) levels. Liver biopsy with liver cell necrosis and inflammation can confirm this clinical suspicion[25, 26]. Once cirrhosis has developed, there is a 5-year cumulative risk to develop HCC between 10% in West Europe and 17% in East Asia[27]. However, HBV can also cause HCC in the absence of cirrhosis due to integration of HBV DNA in the host genome leading to chromosomal instability[28, 29] or the formation of oncogenic protein HBx which can modulate several signalling pathways which promote carcinogenesis[30]. Therefore, HCC screening criteria have been proposed for early detection of HCC [31].

Until recently, risk factors associated with development of HBV-related liver disease were age, male gender, alcohol consumption, smoking, carrier state of hepatitis B virus e-antigen (HBeAg), the presence of core or precore mutations, coinfection with hepatitis C, hepatitis D or HIV, exposure to aflatoxin and diabetes mellitus[32-41]. More recently, several studies in South-East Asian adults chronic HBV patients have demonstrated that viral load at any point in time also positively correlates with progression to cirrhosis and development of HCC[2, 3, 42, 43]. Following these large Taiwanese studies, most international guidelines have changes their indications for initiating anti-HBV therapy[36, 44].

Differences between Asian and non-Asian patients in chronic hepatitis B

While the majority of risk factors for HBV-related liver complications are extracted from studies performed in HBV patients of Asian descent, it is unclear whether these can be extrapolated to other populations. In the course of chronic hepatitis B, at least three factors, which could influence the natural history, differ between Asian and non-Asian patients: 1) age of acquisition of HBV infection, 2) HBV genotype and 3) rate of HBeAg seroconversion. In the South-East Asian region, the predominant mode of HBV transmission is either vertically from mother-to-child at birth or by horizontal transfer in early childhood resulting in a 90% risk of becoming chronically infected[45]. Contrary, in other areas of the world, with low HBV prevalence like Northwest Europe, HBV transmission is mainly acquired through sexual or blood-blood contact at older age.

Furthermore, the HBV genotype (defined as HBV-DNA discongruity of 8% of more in genomic HBV sequencing), differs between Asian and non-Asian HBV patients. HBV genotypes dominant in Asian patients are genotype B and C, while in Europe genotype A and D are more prevalent[46, 47]. Several studies have shown that genotype C infection is associated with a less favorable clinical outcome compared to genotype B[48-53]. For example, Yang et al. found that genotype C, when compared to genotype B, was associated with a higher rate of HCC development[54]. Also, response to treatment with pegylated-interferon-alfa (PEG-IFN-α) varies between genotypes with more favourable results in patients with genotype A and B compared to genotype C and D[55-57].

Finally, Asian patients differ from non-Asians in the rate of HBeAg seroconversion might influence progression to HBV-related liver disease[58-62]. Comparison of studies in HBsAg positive Greece women aged between 20 and 30 to same aged Chinese

women showed a higher prevalence in the latter group of patients (6% versus 42%)[59, 61]. Though not described in the Greek women, in the Asian patients perinatal transmission was the major transmission route. Another longitudinal cohort study in Asian HBeAg positive patients showed an annual progression rate to cirrhosis of 5%[61], while after HBe seroconverion the yearly incidence of cirrhosis considerably dropped to 0.5% to 0.9%[60, 63].

These observations indicate that Asian chronic HBV patients may have an increased risk of liver complications due to a higher prevalence and longer duration in HBeAg positivity compared to the European population.

Immunology in hepatitis B

In the late nineties, studies demonstrated that the cellular immune response, in particular the HBV-specific T-cell, play an important role in the development of chronic HBV[64]. Patients with acute HBV who were able to clear the virus had a vigorous and broad HBV-specific T-cell response[64, 65]. Contrary, those who remained infected and developed chronic HBV had a weak and limited HBV-specific T-cell response[64, 65]. Over the years, several hypotheses have been developed to explain why the T-cell function is impaired in chronic HBV patients. Apart from theories describing ways to blunt the T-cell response, such as the presence of dysfunctional antigen presenting cells (APC) and the ability of the virus to escape from the immune system (viral escape mutations)[66, 67], two theories have gained interest as a possible explanation for the dysfunctional HBV-specific T-cell response in adult chronic HBV patients. First, there is the concept of T-cell tolerance, in which T-cells were not able to withstand the high viral burden of HBV[68]. The consequence of this high viral burden could be a loss of T-cell functionality due to an up-regulated expression of

inhibitory receptors like programmed death 1 (PD-1) and T-cell immunoglobulin domain and mucin domain 3 (Tim-3) resulting in T-cell exhaustion[68, 69]. Second, persistent HBV infection could also be caused by the predominance of regulatory T-cells (Tregs), characterized by among others the expression of CD25 and Foxp3[70]. Tregs are a specific T-cell population with strong anti-inflammatory properties which are able to downregulate the HBV-specific T-cell response leading to HBV persistence[70]. These Tregs are elevated in peripheral blood as well as in the liver[70]. NUCs are able to inhibit HBV DNA replication which reduce the antigen stimuli and restore the T-cell response by reducing the frequency of Tregs and improvement of pro-inflammatory T-cell function.

Treatment of patients with chronic hepatitis B

Current guidelines recommend initiating anti-HBV therapy based on a combination of three parameters: 1) HBV DNA level, 2) alanine aminotransferase (ALT) and 3) assessment of the severity of liver disease. Patients with a HBV DNA level > 2000 IU/mL, an ALT higher than the upper limit of normal and moderate necro-inflammation and/or fibrosis should be considered for treatment[36, 44]. The purpose of antiviral therapy is to achieve HBsAg seroconversion (HBsAg negative and formation of anti-HBs). Secondary endpoints for therapy are achieving HBe seroconversion (loss of HBeAg and formation of anti-HBe), achieving sustained viral response (SVR, undetectable HBV DNA <20 IU/mL during therapy), biochemical response (normalization of liver enzymes) and regression of fibrosis[36, 44]. These are all associated with an improvement in liver histology[5]. Currently, there are two groups of drugs approved for the treatment of chronic HBV infection; PEG-IFN-α and the nucleos(t)ide analogs (NUCs; lamivudine, adefovir, telbuvudine, entecavir and

tenofovir). The advantage of PEG-IFN-α is that it is a temporary treatment for up to 48 weeks with a possible prolonged immunological control (i.e. HBe seroconversion) in 30% of patients[56]. However, PEG-IFN-α therapy is associated with frequent side effects and is most successful only in a selected group of HBeAg positive patients with genotype A, high ALT values and relatively low HBV DNA. The other option of NUCs, with tenofovir and entecavir being the preferred options, can be administered orally with few side effects[36, 44]. However, NUC therapy often requires life-long therapy and lower HBs seroconversion rates compared to PEG-IFN-α achieves. PEG-IFN-α has both direct antiviral effects and immune-modulating effects, while NUCs exclusively inhibit the replication of HBV DNA. Although current guidelines recommend monitoring HBV DNA and ALT levels during therapy to evaluate the effectiveness, a relative new virological parameter, quantitative HBsAg measurement, may be promising to help evaluate response during treatment. Quantitative HBsAg is produced during viral replication and is a good reflection of the amount of covalent circular closed DNA (cccDNA) in the liver in HBeAg positive patients[71]. This is important since cccDNA is thought to be responsible for persistent viral replication in the liver[72, 73]. Several studies have shown that quantitative HBsAg may be a reliable parameter to monitor treatment response during PEG-IFN-α treatment[74-79]. Asian HBeAg positive patients treated with PEG-IFN-α with a quantitative HBsAg level <300 IU/mL after 24 weeks of treatment and a decrease of ≥1 log 10 IU/ml at 24 weeks compared to baseline quantitative HBsAg level had a good chance to achieve sustained virological (SVR)[78]. In Dutch patients a value of quantitative HBsAg level >20,000 IU/mL at week 24 or no decrease in quantitative HBsAg level at week 24 during treatment were predictive for treatment failure with a negative predictive value of 99-100%[76, 77]. In European HBeAg negative patients a

decrease of ≥0.5 log quantitative HBsAg level at week 12 and a quantitative HBsAg level <10 UI/mL at the end of therapy were associated with SVR and HBsAg clearance[74]. In chronic HBV-infected patients treated with NUCs, only in those being HBeAg positive, an association between quantitative HBsAg and treatment success was demonstrated[75, 79]. In a large international trial in HBeAg positive patients treated with TDF, with or without 48 week preceding treatment with ADV, a decrease of more than 2log10 IU/mL in quantitative HBsAg during TDF treatment at week 24 was associated with a 8% chance of HBsAg loss after 3 years therapy[75]. Quantitative HBsAg is less reliable in HBeAg negative patients due to less prominent decline in values during treatment[75]. A possible explanation for this observation is that in HBeAg negative patients the intrahepatic amount of cccDNA is lower than in HBeAg positive patients[80].

Aim and outline of this thesis

This thesis outlines the natural course of chronic hepatitis B in non-Asian women in Amsterdam. Data were collected from a cohort of treatment-naïve, multi-ethnic chronic HBV positive women in Amsterdam, who had participated in a vaccination program between 1990 and 2004 and of whom serum samples were stored at the Public Health Service Amsterdam[81]. The rationale of this thesis was based on two large Asian studies[2, 3] published in 2006 that found a strong positive association between single HBV DNA measurement and the occurrence of liver cirrhosis and hepatocellular carcinoma. Since the disease course of HBV is related to several factors, like HBV genotype and the transmission route of HBV infection, the main topic of this thesis concerned whether same association could be found in non-Asian chronic HBV patients.

The aim of this thesis was (1) to study the predictive value of HBV viral parameters and fibrosis-related serological tests to identify patients with liver cirrhosis; (2) to identify serological determinants related to HBsAg loss and (3) focus on immunological effects and renal complications of antiviral therapy in chronic HBV patients.

After a short introduction on hepatitis B (**chapter 1**), the second and third chapters focus on the occurrence of cirrhosis in non-Asian chronic HBV patients. First, **Chapter 2** described the predictive value of historic and recent HBV DNA levels and quantitative HBsAg levels in relation to the occurrence of liver cirrhosis in non-Asian chronic HBV patients. In **Chapter 3** the diagnostic accuracy of the serum ELF-test, was compared to liver stiffness measurement for accuracy to detect severe fibrosis and cirrhosis in chronic HBV patients.

Next, this thesis focuses on factors associated with spontaneous HBsAg loss in relation to reversal of liver fibrosis. **Chapter 4** describes whether serum hyaluronic acid, a component of the ELF-test and an extracellular matrix substance is lower in HBsAg negative patients compared to HBsAg positive patients.

In **Chapter 5** health related quality of life was assessed in chronic HBV patients focusing on the influence of ethnic origin.

In the following chapters first an overview of the literature summarizing the effects of antiviral therapy on HBV-specific T-cell responses in chronic HBV patients is given (**chapter 6**). Subsequently, in **Chapter 7,** one of the known adverse events of

tenofovir which is used in HBV therapy, Fanconi syndrome, is described. Based on this case report, an overview the literature on tenofovir-induced proximal tubular dysfunction is provided.

Finally, in **Chapter 8,** the main findings of this thesis are summarized and discussed, and future perspectives are provided.

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Chapter 2

Historic and current Hepatitis B viral DNA and quantitative HBsAg level are not associated with cirrhosis in non-Asian women with chronic hepatitis B

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Abstract

Background: Some studies done in Asian patients show that serum levels of HBV DNA predict development of cirrhosis. However, it is unclear whether this also applies for non-Asian patients. This study investigates historic and current HBV DNA and quantitative HBsAg levels as predictors for cirrhosis in non-Asian women with chronic HBV.

Methods: A retrospective cohort study of non-Asian women infected as neonates with chronic HBV. HBV DNA and quantitative HBsAg level were measured in stored historic serum samples (period 1990-2004) during pregnancy and current serum samples (period 2011-2012) among other variables for association with liver cirrhosis by liver stiffness measurement (LSM).

Results: 119 asymptomatic, treatment-naïve non-Asian women were included with a median (IQR) time of 17 (13-20) years between historic and current sample. Median historic log HBV DNA and quantitative log HBsAg level were 2.5 (1.9–3.4) IU/mL and 4.2 (3.6–4.5) IU/mL. LSM diagnosed 14 patients (12%) with F3-F4 fibrosis, i.e. stiffness >8.1 kPa. No association with cirrhosis was found with historic HBV DNA (RR 0.34 (0.05–2.44)) nor with quantitative HBsAg level (HBsAg levels >1000 IU/mL, RR 0.35 (0.11–1.11)). Multivariable analysis, included current variables only, alcohol consumption OR 6.4 (1.3–30.1), AST >0.5xULN OR 15.4 (1.9–122.6) and prothrombin time OR 12.0 (1.2–120.4), but neither HBV DNA nor quantitative HBsAg level, were independent predictors for the presence of cirrhosis.

Conclusion: Neither historic nor current HBV DNA or quantitative HBsAg level are associated with development of HBV-related cirrhosis in non-Asian women.

Introduction

Liver cirrhosis with risk of developing hepatocellular carcinoma (HCC) is an increasing clinical problem in patients infected with hepatitis B virus (HBV)[1]. In recent years, HBV DNA has been identified as an important predictor for the development of HBV-related liver disease[2-9]. Studies from the REVEAL-HBV group showed a clear association between the serum HBV DNA and the development of both cirrhosis and HCC in Taiwanese HBV-infected patients[4, 5]. This resulted in a more important role for HBV DNA measurement in international hepatitis B guidelines[10-12].

Asian people may differ in viral and host factors from those originating from other regions of the world[13]. For instance, HBV genotypes B and C predominate in Asian patients compared to genotypes A and D in Northern Europe[14] and Asian patients have different cardiovascular risk factors[15].

Given these viral and host differences, the role of HBV DNA in the progression of liver fibrosis may also differ between Asian patients and other populations. Therefore, we investigated amongst other variables the association between HBV DNA and the development of liver cirrhosis in non-Asian patients with chronic HBV.

Methods and materials

Patients and study design

HBV is a notifiable disease in the Netherlands. Blood samples of women in Amsterdam, who were diagnosed with chronic HBV during pregnancy (as being HBsAg positive) between 1990 and 2004, were stored at the Public Health Service Amsterdam (historic sample). Criteria for chronic HBV infection were either a persistent HBsAg positivity during 6 months after diagnosis, an already known chronic HBV patient or a history during source and contact tracing without factors

associated with a recent infection. Between September 2011 and May 2012 these women, assumed to be infected as neonates, were invited for a short history, physical examination focusing on symptoms of chronic liver disease, liver stiffness measurement (LSM) and additional blood tests (current sample).

Women with HIV or hepatitis C coinfection, younger than 18 years, with parents or ancestors of Asian descent, receiving HBV therapy or those who could not give informed consent, were excluded from this study. Hepatitis D (HDV) was only tested in patients with moderate to severe fibrosis (F2 or more).

All patients provided written informed consent and the study was conducted in accordance with the declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice with the approval by the local medical ethical committee of the Academic Medical Center Amsterdam. Clinical trials number NCT01462981.

Liver stiffness measurement

LSM was performed with a Fibroscan® (model F402 Echosens, France) by an experienced researcher (S.H.) according to standard operating procedures as described before[16]. A valid LSM was defined as at least 10 valid measurements, a success rate of at least 60% and an interquartile range (IQR) less than 33% of the median stiffness. The extent of fibrosis was staged according to the METAVIR classification for HBV[17], with F0/F1 <7.2 kPa, F2 between 7.2 and 8.0 kPa, F3 between 8.1 and 11.0 kPa and F4 greater than 11.0 kPa[18,19].

Laboratory tests

Biochemical parameters for assessment of liver function (total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamine transpeptidase (GGT), alkaline phosphatase (ALP) and prothrombine time) together with renal (blood urea nitrogen, creatinine) and hematogical parameters (hemoglobin, leucocytes, thrombocytes) and alphafetoprotein (AFP) were determined by local standard laboratory procedures. All these parameters were only measured in the current blood sample. The cut-off values for women of ALT and AST were 30 IU/ml and 35 IU/ml respectively.

HBV DNA and quantitative HBsAg level were determined in both the historic sample and the current sample. HBV DNA was determined with the COBAS Taqman (Roche, Meylan, France) with a lower detection limit of 20 IU/ml. Qualitative and quantitative HBsAg, anti-HCV, anti-HIV were performed with the ADVIA Centaur XP assay (Siemens, Erlangen, Germany). The quantitative HBsAg level had a detectable limit of 1 IU/mL. The HBeAg was performed with the AxSYM immunochemical automated analyzer (Abbott, Illinois, USA) and the anti-HDV was performed with a qualitative ELISA, the ETI-AB-Deltak-2 (DiaSorin S.p.A., Turin, Italy). HBV genotype was determined by the COBAS Ampliprep/COBAS Taqman HBV test 2.0 (Roche, Meylan, France). The genotype could only be determined in patients with a HBV DNA of 1000 IU/ml or higher.

Statistical analysis

Sample-size calculation was based on a 5.5% prevalence rate of liver cirrhosis in the low viral DNA group (HBV DNA $<10^4$ IU/mL) and 23.4% in high viral DNA group (HBV DNA $>10^4$ IU/mL) as reported by the REVEAL-HBV group[5]. The calculated sample size was 172 patients, with the assumption of a difference in prevalence rate

of cirrhosis of 10% between the low viral DNA group (HBV DNA level <20.000 IU/mL) and high viral DNA group (HBV DNA level >20.000 IU/mL), a 80% power at a 5% significance level and a 3-to-1 allocation to the low viral DNA group. With an estimated refusal rate of 50%, a total of at least 344 patients had to be contacted.

To analyze the association between HBV DNA and cirrhosis, HBV DNA were classified according to the four viral DNA groups defined by the EASL guideline[12]. These four HBV DNA groups were: undetectable (HBV DNA: <20 IU/mL); low (HBV DNA: 20-2.000 IU/mL); intermediate (HBV DNA: 2.000-20.000 IU/mL) and high viral DNA (HBV DNA: >20.000 IU/mL). The quantitative HBsAg level was also divided in two groups (below and above 1000 IU/mL) in accordance to the EASL guideline[12].

Continuous variables were summarized as a median with interquartile range (IQR) and categorical variables as frequencies with percentage. The HBV DNA and quantitative HBsAg level were expressed logarithmically. Differences between two groups were calculated with the Mann-Whitney U test for continuous variables and the Chi square test for categorical variables. The historic data were analyzed with univariable and multivariable Poisson regression models with time adjustment for variable follow-up. Outcomes were reported as relative risks with 95% confidence intervals (CI). The data of the study visit were analyzed with univariable and multivariable logistic regression model. Outcomes were reported as odds risks with 95% CI. A p-value <0.05 was considered significant. The statistical analysis was performed with SPSS v17 (version 17.0; SPSS Inc., Chicago, IL, USA) and Stata 11 (StataCorp LP, College Station, TX, USA).

Results

Patient characteristics

Of 1113 women with stored blood samples, 380 were eligible for this study, of which a total of 174 patients consented to participate (figure 1). After exclusion of 55 patients, mainly because of inconclusive LSM results, data of 119 women were included for final analysis. The patient characteristics are given in Table 1. Of the known HBV genotypes, D was the most prevalent (46%). The percentages of HBeAg positive women in the historic and current samples were 6% and 0% respectively. Only 2 women had increased ALT levels, 54 and 78 U/L. The median follow-up time between pregnancy screening and the study visit was 17 (13–20) years.

We explored whether patients with an inconclusive LSM (47 patients) were different from the main study population (119 patients) leading to a bias in study results. There was no difference with regard to age at pregnancy screening, and in historic and current HBV DNA and quantitative HBsAg levels (data not shown). However, the excluded patients had a higher body mass index (BMI) (32 (28–37) kg/m2 versus 29 (26–32) kg/m2, $p<0.001$) and a slightly longer time between pregnancy screening and the recent visit to the outpatient clinic (19 (16–21) years versus 17 (13–20) years, $p= 0.03$).

We also studied the AST to platelet ratio (APRI) score for differences between the excluded and study patients but no difference was found and no F3-F4 fibrosis patients were identified in the excluded ones (data not shown).

Association between HBV DNA and cirrhosis

Historic sample

To explore the association between historic viral DNA and the diagnosis of severe fibrosis or cirrhosis (F3-F4 fibrosis) at the study visit, we did an analysis-adjusted difference in follow-up time. Patients were stratified into 4 categories based on the

viral DNA at time of HBV diagnosis (Table 2). More patients with undetectable or low HBV DNA (less than 2000 IU/mL) than with intermediate or high viral DNA (more than 2000 IU/mL) were diagnosed with F3-F4 fibrosis (11 patients versus 3 patients; p=0.62).

At time of recruitment into this study, it appeared that 4 had deceased. These patients had to be excluded due to ethical country constraints. Hypothesizing that F3-F4 fibrosis with complications led to their death, they were assigned to the high viral DNA group and the number of cases in the high viral DNA group with F3-F4 fibrosis increased from 1 (6%) to 5 (25%). However, this did not affect the main finding of the study being that HBV DNA was associated with fibrosis progression (cases of F3-F4 fibrosis in the undetectable and low viral DNA versus intermediate and high viral DNA would be 11 patients versus 7 patients; p=0.21).

Contrary to HBV DNA, more patients with HBsAg levels above than below 1000 IU/mL in the historic sample were diagnosed with severe fibrosis or cirrhosis compared to those with quantitative HBsAg levels below 1000 IU/mL (10 patients versus 4 patients; p=0.09). In both univariable and multivariable analysis, there was no significant association between F3-F4 fibrosis, viral DNA and quantative HBsAg level (table 2).

Current sample

Next, we explored the association between the current HBV DNA and quantitative HBsAg level and the diagnosis of severe fibrosis or cirrhosis. At current evaluation, one patient (0.8%) with F2 fibrosis in the intermediate viral DNA group had a hepatitis D coinfection. Compared with an undetectable HBV DNA in the current sample, HBV DNA was not a predictor for severe fibrosis or cirrhosis, odds ratio (OR) low viral

DNA group 1.9 (0.5–8.0) and OR intermediate viral DNA group 1.8 (0.4–9.0) respectively (Table 3). Similarly, quantitative HBsAg level of >1000 IU/mL, was not associated with F3-F4 fibrosis (OR 1.4 (0.5–4.3)). In multivariable analysis alcohol consumption, AST level more than 0.5 above upper limit of normal (ULN) and a prolonged prothrombin time were independent predictors for F3-F4 fibrosis (alcohol consumption OR 6.4 (1.3–30.1), AST >0.5 ULN OR 15.4 (1.9–122.6) and prothrombin time of >13 sec OR 12.0 (1.2–120.4))(Table 3).

Discussion

In contrast to the Asian studies that have shown a relationship between HBV DNA and the development of severe liver fibrosis or cirrhosis (F3-F4), the main finding of our study are that such an association was not found in treatment-naïve non-Asian women with chronic HBV infection. Moreover, after a median of 17 years since pregnancy screening, only a small number of these asymptomatic HBV-infected non-Asian women developed severe fibrosis or cirrhosis. Finally, this study showed that only alcohol consumption, AST level and increased prothrombin time were predictors for the presence of severe fibrosis and cirrhosis (F3-F4). Interestingly, we found that the number of patients with severe fibrosis to cirrhosis was higher in the group of HBV DNA less than 2,000 IU/mL compared to the group with a HBV DNA more than 2,000 IU/mL (11 versus 3 patients). One explanation for this finding was the difference in alcohol consumption between both groups more likely in those patients with a HBV DNA less than 2,000 IU/mL. Alternatively, factors such as difference in hepatic steatosis among the viral DNA groups, might also contribute to this finding. Hepatitis D coinfection did not attribute to this difference

since only one patient with a viral DNA more than 2,000 IU/mL (and F2 fibrosis) had a hepatitis D coinfection.

Our study findings are in contrast with data from other studies. The REVEAL-study showed an increasing risk for development of cirrhosis with increasing baseline HBV DNA[5]. However, their study population only consisted of Asian patients with an HBV genotype distribution assumed being B and C. Our data are also in contrast to the study results of Zacharakis et al. who prospectively showed in native Greek patients with predominantly genotype D that HBV DNA >2,000 IU/mL served as an independent risk factor for liver disease progression[20].

An interesting observation from our study is that patients with low ALT levels can also be at risk for the presence of fibrosis which was also observed by others[21]. A prospective study in India, with a cut-off value for ALT similar to the one in our study, showed a comparable percentage of 14% for moderate to severe fibrosis in HBeAg negative patients with normal ALT[21]. However, the Indian study showed a significant relationship between HBV DNA levels and histological fibrosis grade that was not found in our study. A possible explanation might be differences in other risk factors such as alcohol consumption and smoking as these factors were poorly described in the Indian study. Since several studies (mainly in patients with genotype A, C and D), like our study, already showed that patients are capable of developing severe fibrosis even with normal ALT levels, it should be considered to amend the currently used values for ALT and AST in which HBeAg negative chronic HBV-infected patients should be referred for monitoring of disease activity[21, 22].

Our study was distinctive since a multi-ethnic group of non-Asian women, infected as neonates, was included. Furthermore, the time between the pregnancy screening and the study visit for evaluation of liver fibrosis was almost two decades and the

time between assumed infection and evaluation of liver fibrosis four to five decades. Finally, next to historic HBV DNA, also quantitative HBsAg level in a historic sample was evaluated as a possible predictor for development of cirrhosis.

This study has some limitations. First, similar to the study of the REVEAL-HBV group, no liver biopsies were performed. However, several studies in chronic HBV patients have already shown that LSM can reliably detect the presence of severe fibrosis and cirrhosis[23, 24]. Second, due to the method of recruitment, using an opt-in procedure, a possible selection bias cannot be ruled out. Restrictions to attain information about the cause of death in deceased patients might have caused a selection bias. However, assuming that all these women had died due to cirrhosis with a high viral DNA, this did not have any impact on our results. Finally, though the beforehand calculated number of study patients was reached, due to unsuccessful LSM due to obesity, a number of women had to excluded resulting in a smaller sample size than planned.

In summary, we conclude that in non-Asian women with chronic HBV infection neither historic nor current HBV DNA viral DNA and quantitative HBsAg level are not associated with the development of HBV-related cirrhosis. A larger study in non-Asian women should be performed to confirm these findings.

Table 1. Patient characteristics

Patients included *(n)*	119
Clinical parameters	
Age at pregnancy screening *years*	28 (22–33)
Follow-up time *years**	17 (13-20)
Diabetes *n(%)*	7 (6)
BMI *kg/m2*	29 (26–32)
Smoking *n(%)**	18 (15)
Alcohol consumption *n(%)**	13 (11)
Illicit drug abuse *n(%)**	1 (1)
Country of origin *n(%)*	
Turkey	34 (29)
Ghana	32 (27)
Surinam	22(19)
Morocco	21 (18)
Others	10 (8)
Genotype *n(%)*	
A	11 (9)
B	1 (1)
D	21 (18)
E	14 (12)
Indeterminate	72 (61)
Current virological parameters	
HBeAg positive *n(%)*	7 (6)
Log HBV DNA DNA *IU/mL*[#]	2.5 (1.9–3.4)
log quantitative HBsAg level *IU/mL*[#]	4.2 (3.6–4.5)
Current virological parameters	
HBeAg positive *n(%)*	0 (0)
Log HBV DNA DNA *IU/mL*[#]	2.4 (1.3–3.5)
log quantitative HBsAg level *IU/mL*[#]	3.0 (0.0–4.0)
Current biological parameters	
ALT *U/L*	18 (15–24)
AST *U/L*	17 (13–23)
Thrombocytes 10^6*/L*	245 (197–281)
Prothrombin time *sec*	13.1 (12.8–13.5)
Alphafetoprotein *µg/L*	2.7 (1.9–3.7)

*Definitions: Follow-up time was defined as time between pregnancy screening and study visit. Smoking was defined as the inhalation of 1 or more tobacco products per day. Alcohol was defined as more than 1 alcohol containing unit per week. Illicit drug abuse was defined as consumption of mood-altering substances. #Expressed logarithmically. n= number, kg/m2= kilogram per square meters, IU/mL= international units per millilitre, U/L= units per litre, 10^6/L= million per liter, sec= seconds, a. Reference values: ALT <35 U/L, AST <30 U/L.

Table 2. Relative risks of severe fibrosis or cirrhosis by historic parameters

Characteristics of	Total	F3-F4	Univariable analysis	Multivariable analysis[b]
HBV DNA [#]				
Undetectable	22	4 (18,2)	1 (reference)	1 (reference)
Low	65	7 (10.8)	0.5 (0.2–1.6)	0.6 (0.2–1.8)
Intermediate	16	2 (12.5)	0.7 (0.1–3.2)	0.9 (0.2–4.4)
High	16	1 (6.2)	0.3 (0.0–2.4)	0.3 (0.1–2.4)
Quantitative HBsAg level,				
> 1000 IU/mL	101	10 (9.9)	1 (reference)	1 (reference)
< 1000 IU/mL	18	4 (22.2)	0.4 (0.2–1.1)	0.4 (0.1–1.1)
Country of origin				
Morocco	21	1 (4.8)	1 (reference)	1 (reference)
Turkey	34	3 (8.8)	1.6 (0.2–14.5)	1.6 (0.2–12.9)
Ghana	32	4 (12.5)	2.5 (0.3–21.1)	3.1 (0.5–20.1)
Surinam	22	4 (18.2)	3.3 (0.4–26.8)	3.4 (0.5–24.4)
Other	10	2 (20.0)	4.3 (0.4–42.9)	3.6 (0.4–36.3)

a. Relative risks are adjusted for follow-up period. # Following HBV DNA categories: Undetectable viral DNA below 20 IU/mL; low viral DNA was between 20 and 2,000 IU/mL; intermediate viral DNA between 2,000 and 20,000 IU/mL and high viral DNA more than 20,000 IU/mL. n/a= not applicable, IU/mL= international units per millilitre, 95% CI= 95 percent confidence interval. b. All variables tested at univariable level were included in a direct multivariable analysis.

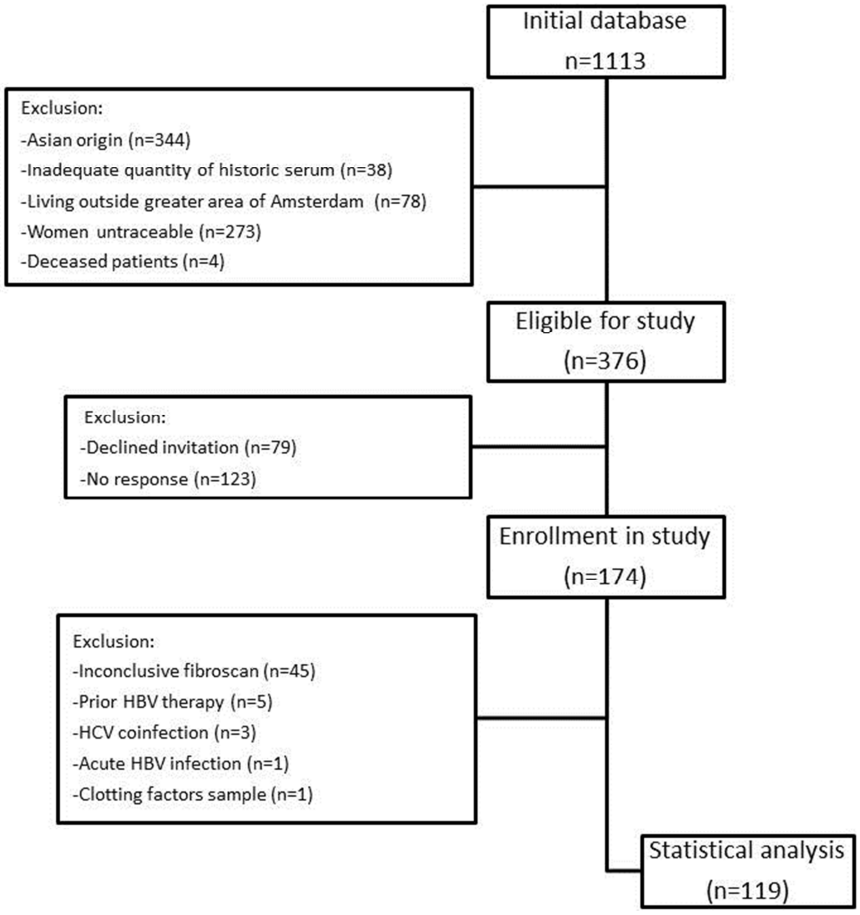
Table 3. Prevalence of severe fibrosis to cirrhosis by characteristics

		Total	F3-F4 cases (%)	Univariable analysis	Multivariable analysis
Age at enrolment					
	≤ 45 years	66	8 (12.1)	1 (reference)	
	> 45 years	53	6 (11.3)	0.9 (0.3–2.9)	
Country of origin					
	Turkey	34	3 (8.8)	1 (reference)	
	Ghana	32	4 (12.5)	1.5 (0.3–7.2)	
	Surinam	22	4 (18.2)	2.3 (0.5–11.4)	
	Morocco	21	1 (4.8)	0.5 (0.1–5.3)	
	Other	10	2 (20.0)	2.6 (0.4–18.1)	
Smoking					
	No	101	11 (10.8)	1 (reference)	
	Yes	18	3 (16.7)	1.4 (0.4–5.6)	
Alcohol consumption					
	No	84	7 (8.3)	1 (reference)	1 (reference)
	Yes	35	7 (20.0)	2.8 (0.9–8.5)	6.4 (1.3–30.1)*
Body mass index					
	≤ 25 kg/m2	22	3 (13.6)	1 (reference)	
	> 25 kg/m2	97	11 (11.3)	0.8 (0.2-3.2)	
Diabetes					
	No	112	12 (10.7)	1 (reference)	
	Yes	7	2 (28.6)	3.3 (0.6–19.1)	
ALT					
	≤ 0.5 x ULN	63	7 (11.1)	1 (reference)	
	> 0.5 x ULN	56	7 (12.5)	1.1 (0.4–3.5)	
AST					
	≤ 0.5 x ULN	112	11 (9.8)	1 (reference)	1 (reference)
	> 0.5 x ULN	7	3 (42.9)	6.9 (1.4–34.8)*	15.4 (1.9–122.6)*
Trombocytes*					
	< 150 x 10^9/L	3	0 (0)	1 (reference)	
	> 150 x 10^9/L	111	13 (11.7)	n/a	
	Indeterminate	5	1 (20.0)	n/a	

Prothrombin time*					
	< 13 sec	41	1 (2.4)	1 (reference)	1 (reference)
	> 13 sec	76	13 (17.1)	8.3 (1.0–65.6)*	12.0 (1.2–120.4)*
	Indeterminate	2	0 (0)	n/a	
Alpha fetoprotein*					
	< 9 µg/L	114	14 (12.3)	1 (reference)	
	> 9 µg/L	5	0 (0)	n/a	
HBV DNA group#					
	Undetectable	36	3 (8.3)	1 (reference)	
	Low	47	7 (14.9)	1.9 (0.5–8.0)	
	Intermediate	28	4 (14.3)	1.8 (0.4–9.0)	
	High	8	0 (0.0)	n/a	
Quantitative HBsAg					
	≤ 1000 IU/mL	59	6 (10.2)	1 (reference)	
	> 1000 IU/mL	59	8 (13.6)	1.4 (0.5–4.3)	
	Indeterminate	1	0 (0.0)	n/a	
Genotype					
	A	11	3 (27.3)	1 (reference)	
	B	1	0 (0.0)	n/a	
	C	0	0 (0.0)	n/a	
	D	21	0 (0.0)	n/a	
	E	14	2 (14.3)	0.4 (0.1–3.3)	
	Indeterminate	72	9 (12.5)	n/a	

*Cut-off based on reference value for parameter. #Following HBV DNA categories: Undetectable viral DNA below 20 IU/mL; low viral DNA was between 20 and 2,000 IU/mL; intermediate viral DNA between 2,000 and 20,000 IU/mL and high viral DNA more than 20,000 IU/mL. OR= odds ratio, 95% CI= 95 percent confidence interval n/a= not applicable, ULN= upper limit of normal. *p ≤ 0.05.

Figure 1 Flowchart summarizing the process of patient selection



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Chapter 3

ELF-test less accurately identifies liver cirrhosis diagnosed by liver stiffness measurement in non-Asian women with chronic hepatitis B

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Abstract

Background: The enhanced liver fibrosis test (ELF-test) has been validated for several hepatic diseases. However, its performance in chronic hepatitis B virus (CHB) infected patients is uncertain. This study investigates the diagnostic value of the ELF-test for cirrhosis identified by liver stiffness measurement (LSM) in non-Asian women with CHB.

Methods: Women of non-Asian origin with perinatally acquired CHB infection, detected during pregnancy in the period 1990-2003, returned to our center between September 2011 and May 2012 for LSM and blood sampling to perform an ELF-test and to calculate, APRI and FIB-4 scores. Fibrosis stages were classified by the METAVIR system.

Results: A total of 119 women were included in this study with a median age of 43 years, all ALT levels being <2x ULN and all being HBeAg negative. The overall median LSM (IQR) stiffness and ELF-test were 5.5 kPa (4.0–6.8) and 8.4 (7.8–9.2) respectively. LSM and ELF-test classified 14 (12%) and 19 (16%) patients with severe fibrosis to cirrhosis (>= F3, i.e. liver stiffness >8.1 kPa), however in only 4 (3%) patients there was an agreement between LSM and ELF-test. With LSM as reference, the area under receiver operating characteristic curve (AUROC) for detection of >= F3 fibrosis were for ELF 0.65 (95%CI 0.51–0.80; p=0.06), APRI 0.66 (0.50–0.82; p=0.07) and FIB-4 0.66 (0.49–0.82; p=0.07).

Conclusion: The ELF-test less accurately discriminates severe fibrosis or cirrhosis when compared to LSM in our cohort of non-Asian women with CHB.

Introduction

Annually, approximately 600,000 patients with chronic active hepatitis B virus (CHB) infection die worldwide due to complications of advanced liver disease[1]. Since development of liver cirrhosis is an asymptomatic process, timely detection of progression to cirrhosis is essential to improve prognosis of CHB patients.

The enhanced liver fibrosis (ELF) test is a minimal-invasive blood test composed of three components, hyaluronic acid (HA), tissue inhibitor of matrix metalloproteinases-1 (TIMP-1), and aminoterminal propeptide of procollagen type 3 (P3NP) and is able to predict the occurrence of liver fibrosis. It has been shown promising in several liver diseases such as chronic hepatitis C and non-alcoholic fatty liver disease[2, 3]. For example, in a study of 347 patients with chronic hepatitis C, the ELF-test reliably detected severe fibrosis (>F2 by METAVIR-score, AUROC of 0.85 (95% CI 0.81-0.89)), sparing the need for liver biopsy in 81% of the patients[2]. Furthermore, the ELF-test was capable to predict the outcome of in a variety of liver diseases, with a score above 12.5 giving a hazard ratio of 75.7 (17.6 to 325.4) for liver related morbidity and mortality[4].

However, the diagnostic value of the ELF-test in patients with CHB is less well established[5-8]. In a study performed in Asian CHB-infected patients (n= 170), the ELF-test was compared to liver stiffness measurement (LSM) and fibrotest with liver biopsy as reference test[6]. Although the ELF-test was comparable to LSM for the detection of F2 fibrosis (AUROC 0.901 versus 0.937;), its accuracy decreased when identifying F3 or higher fibrosis (AUROC 0.860 versus 0.956;). Importantly, the studies describing the ELF-test in CHB were performed in predominant male patient populations. However, gender has recently been shown to influence in the accuracy of the ELF-test[9].

This study aims to investigate the diagnostic value of the ELF-test for liver cirrhosis identified by LSM in a multi-ethnic population of non-Asian women with CHB.

Methods

Patient selection

Non-Asian women, who were diagnosed with CHB during pregnancy screening between 1990 and 2004 were contacted between September 2011 and May 2012 by letter to participate in this study. Women who were willing to participate were invited for a single study visit at the Public Health Service Amsterdam for a short history, physical examination focusing on symptoms of chronic liver disease, LSM and a blood sample (for biochemical and virological tests, the APRI score, FIB4 score and the ELF-test). Patients were eligible if they were of non-Asian descent, older than 18 years, CHB treatment-naive and capable of giving informed consent. Co-infections with HIV and/or hepatitis C were exclusion criteria. All patients provided written informed consent and the study protocol was approved by the Ethical Committee of the Academic Medical Center Amsterdam in accordance with the declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. Clinical trials number NCT01462981.

Laboratory tests

Biochemical parameters, i.e. total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) gamma glutamine transpeptidase (GGT), alkaline phosphatase (ALP), blood urea nitrogen, creatinine and hematogical parameters i.e. hemoglobin, leucocytes, thrombocytes and prothrombine time, were determined by local standard laboratory procedures. The upper limit of normal (ULN) for ALT and

AST were 30 IU/ml and 35 IU/ml respectively. Hepatitis B DNA load was determined with the COBAS Taqman (Roche, Meylan France) with a lower detection limit of 20 IU/ml. HBeAg, Anti-HCV and anti-HIV status were measured with the ADVIA Centaur XP assay (Siemens, Erlangen Germany).

Hepatic fibrosis tests

To calculate the ELF-test, the P3NP, HA and TIMP-1 were measured on serum by using the ADVIA Centaur XP (Siemens, Germany). The ELF-test was calculated by the following algorithm provided by the manufacturer: ELF-test= 2.278+0.851 ln(Concentration(Conc)HA)+0.751 ln(ConcP3NP)+0.394 ln(ConcTIMP1). An ELF-test up to 7.7 indicates no to mild fibrosis, a score between 7.8 and 9.8 moderate fibrosis and a score above 9.8 severe fibrosis or cirrhosis as indicated by the manufacturer.

Two other markers for fibrosis were calculated, the AST to platelet ratio index (APRI: [AST (/ULN)/PLT (10^9/L)] × 100) and the fibrosis 4 score (FIB-4: age ([yr] x AST [U/L]) / ((PLT [10(9)/L]) x (ALT [U/L])(1/2))[10, 11]. Used cut-off values for the APRI score were: ≤0.5 = absence of fibrosis; between 0.5 and 1.5 = presence of fibrosis and ≥1.5 = severe fibrosis. Used cut-off values for the FIB-4 score were: ≤1.45= absence of fibrosis; between 1.45 and 3.25= presence of fibrosis and ≥ 3.25= severe fibrosis.

Liver stiffness measurement

LSM was performed with a Fibroscan® (model F402; Echosens, Paris, France) by an experienced researcher (S.H.) according to standard procedures[12] on the same day as the study visit. A successful LSM was defined as at least 10 valid measurements, a success rate of at least 60% and an interquartile range (IQR) less than 33% of the

median stiffness. The extent of fibrosis was staged according to the METAVIR classification[13], with cut-off values published specific for CHB-infected patients; F0/F1 <7.2 kPa, F2 between 7.2 and 8.0 kPa, F3 between 8.1 and 11.0 kPa and F4 greater than 11.0 kPa[14].

Statistical analysis

Quantitative variables were expressed as median (range), categorial variables as absolute numbers (percentage). Correlation between ELF and LSM was calculated with the Spearman rank correlation coefficient. The discriminatory values of the ELF-test, APRI score and FIB-4 test in predicting severe fibrosis and cirrhosis (F3-F4), compared to LSM, were assessed by the receiver operator characteristic (ROC) curve. The areas-under-the-ROC-curves (AUROC) as well as 95%-CI of AUROC were calculated. The various tests were compared using descriptive statistics using the Chi squared test. A p-value <0.05 was considered significant. The statistical analysis was performed with SPSS v17 (version 17.0; SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics

A total of 174 women participated in this study, of which 119 patients were included in the final analysis (Figure 1). The baseline characteristics of the patients are given in Table 1. The median age was 43 years and the BMI had a median of 29 Kg/m^2. All patients were HBeAg negative, treatment-naïve and except for 2 women with normal ALT values. The median (interquartile range, IQR) follow-up time since pregnancy screening related CHB diagnosis was 17 (13–20) years. The major ethnic groups were: Turkey (34%), Ghana (32%), Surinam (22%) and Morocco (21%). LSM was

used as reference test. Of the 55 excluded patients, 47 patients had an inconclusive LSM, mainly due to a higher body mass index (BMI) than the included patients (32 (28–37) kg/m2 versus 29 (26–32) kg/m2, p<0.001).

ELF-test versus LSM

First, the diagnostic value of the ELF-test to detect severe fibrosis or cirrhosis was explored using LSM as reference. The median (IQR) ELF-test per LSM fibrosis stage was: 8.3 (7.8–9.0) in F0/F1; 9.0 (7.9–10.1) in F2; 9.0 (8.6–9.5) in F3 and 8.3 (8.0–11.4) in F4 (figure 2a). When looking at severe fibrosis and cirrhosis, LSM and ELF-test detected F3 and F4 stages in 14 (12%) and 19 (16%) patients respectively. However, in only 4 (3%) patients with >=F3 fibrosis there was an agreement between LSM and ELF-test. There was no correlation between the ELF-test and LSM (r= 0.13, p= 0.10).

Overall, for the whole study group, there was an agreement in fibrosis stage between LSM and the ELF-test in 60 patients (50%) that was most evident in the lesser fibrosis stages F0/F1 (45%). In 4 (3%) patients with severe fibrosis and cirrhosis, diagnosed by LSM, the ELF-test classified them as no to mild fibrosis. Similarly, in 11 (9%) patients classified by LSM as F0/F1 fibrosis, the ELF-test diagnosed them as severe fibrosis (table 2). The receiver operator curve (ROC) of the ELF-test, compared to LSM was low (r=0.65 (95%CI 0.51–0.80), p= 0.06)(Figure 2b). With the cut-off value provided by the manufacturer for detection of F3-F4 fibrosis, i.e. ELF-test above 9.8, the sensitivity, specificity, positive predictive value and negative predictive values were 21%, 24%, 22% and 23% respectively. There was no difference in performance of the ELF-test compared to LSM between different ethnic groups (data not shown).

Thus, the ELF-test had only a moderate diagnostic value in detection of severe fibrosis to cirrhosis in relation to LSM.

Comparison of ELF-test to APRI and FIB-4 score

Next, the ELF-test was compared with the APRI and FIB-4 score to determine its accuracy against other minimal-invasive tests for severe fibrosis or cirrhosis detection (figure 3a). While the ELF classified severe fibrosis in 16% of the patients, the APRI score and FIB-4 score classified less patients with F3-F4 fibrosis, 1 (0.8%) and 2 (1.7%) patients respectively (data not shown). The difference in detection of F3-F4 fibrosis between ELF-test, APRI score and FIB-4 score were comparable (figures 2a and 3a). In the 14 patients who were identified by LSM as severe fibrosis or cirrhosis (F3-F4), the ELF-test, APRI score or FIB-4 score identified them as severe fibrosis in only 3, 0 and 2 patients. In only one patient with F3-F4 fibrosis, there was an agreement between LSM, ELF-test and FIB-4 score. All three tests (ELF, APRI, FIB-4) were equally less accurate in identifying F3-F4 fibrosis identified by LSM with an AUROC for ELF, APRI and FIB-4 of 0.65 (95%CI 0.51–0.80; p= 0.06), 0.66 (0.50–0.82; p= 0.07) and 0.66 (0.49–0.82; p= 0.07)(Figure 3b). Combining ELF-test with APRI score or FIB-4 score did not result in an improvement to identify F3-F4 fibrosis (data not shown).

Discussion

Minimal-invasive tests for assessment of liver fibrosis, like the ELF-test, are emerging alternatives to liver biopsy, which have reduced the need for histological evaluation of the liver for fibrosis staging. This study showed that the ELF-test less accurately identifies women with severe fibrosis or cirrhosis whom have been identified with this

disease by LSM. Furthermore, the ELF-test had a similar moderate discriminatory value for severe fibrosis or cirrhosis compared to the APRI and FIB-4 scores.

Our study is in line with others in which the ELF score was shown to be less accurate in diagnosing patients with severe fibrosis or cirrhosis[6,7]. In an Italian cohort study, with liver biopsy as reference, LSM performed better than the ELF score in the detection of F3-F4 fibrosis. However, the AUROC values for the ELF-test reported in the Italian study (0.80 for severe fibrosis and 0.83 for cirrhosis) were higher than those found in our study (0.65)[7]. A possible explanation for this finding might be due to differences in study populations since in our study the majority of patients had normal ALT values and low CHB-DNA indicating low liver inflammation. Furthermore, the hyaluronic acid (HA) levels in our study were lower than reported by others[8]. Several studies, in both patients with CHB and in patients with other liver diseases, have described that an increase in (hepatic) inflammation is positively associated with an increased hepatic fibrogenesis with elevated fibrotic markers such as HA and PN3P, which are major components of the ELF-test[15-18]. Therefore, we hypothesize that in CHB patients with signs of low disease activity, as is the case in our study, the ELF-test may less accurately diagnose patients with cirrhosis when compared to other fibrosis tests like LSM.

Generation of extracellular matrix components like HA, might also serve as explanation for the observed differences in performance of the ELF-test in different populations. For example, in hepatitis C infected patients, the performance of the ELF-test is more accurate compared to CHB. This might reflect the fact that in chronic hepatitis C fibrosis progression occurs at a higher level resulting in higher levels of fibrotic markers, such as hyaluronic acid, from which the ELF-test is composed.

In our study, LSM was used as reference test instead of the liver biopsy. Although liver biopsy has long been the gold standard for assessment of fibrosis, LSM was shown to be a good alternative for detection of severe fibrosis and cirrhosis in several liver diseases, including CHB[14, 19-21]. For instance, in a meta-analysis of 50 studies, with the majority conducted in hepatitis C patients, LSM showed excellent distinctive property to detect patients with cirrhosis compared to patients without cirrhosis (AUROC 0.94 (0.93–0.95))[19]. For patients with CHB, others have already showed that LSM had a good diagnostic accuracy to detect F3-F4 fibrosis compared to liver biopsy with an AUROC for severe fibrosis of 0.95 (0.91-0.98) and cirrhosis of 0.98 (0.96-0.99)[14, 22]. Based on these data, we believe that LSM is reliable as a reference test to examine the accuracy of the ELF-test.

Different CHB-specific cut-off values for LSM did not influence the discriminatory value of the ELF-test. In this study we used the CHB-specific LSM cut-off values defined by Marcellin et al. which have been adopted in most studies examining LSM in CHB patients[19]. Lower cut-off values, as suggested by Kim et al. with 6.0 kPa, 7.5kPa and 10.1 for F2, F3 and F4 fibrosis, increased the cases with F3-F4 fibrosis from 14 (12%) to 21 (18%) patients in our cohort[23]. However, the outcomes of the study remained similar (AUROC ELF, APRI and FIB-4 of 0.66, 0.67 and 0.69).

In the years after the introduction of LSM, optimizing the cut-off values for every different liver disease and establishing its role in tracking fibrosis progression over time had to be defined. A similar path might lay ahead for the ELF-test which is relatively new in CHB and its place in the diagnostic assessment for fibrosis will further have to be established. In our study, we used the cut-off values for the ELF-test that were provided by the manufacturer. However, in time it might be shown that cut-off values might vary between different liver diseases and even within CHB

studies as given in table 3 which is similar to LSM[24]. Therefore, the optimal cut-off value of the ELF-test in CHB has to be established. Moreover, in this study the ELF-test was measured one time only to assess fibrosis stage. It is not clear whether changes in ELF-test over time (similar to LSM) might give a better diagnostic accuracy for detection of cirrhosis or might be a better marker for progression of fibrosis. Therefore, although the ELF-test at this stage may not be useful in patients with CHB, it might be of potential value in the future after extensive further clinical evaluation.

In conclusion, the ELF-test less accurately discriminates severe fibrosis or cirrhosis when compared to LSM in non-Asian women with CHB.

Table 1. Patient characteristics

Age [years]	43 (39–49)
Follow-up [years]	17 (13-20)
BMI *kg/m2*	29 (26–32)
Smoking *n (%)*	18 (15)
Alcohol consumption *n (%)*	13 (11)
Ethnicity	
- Turkey	34 (29)
- Ghana	32 (27)
- Surinam	22(18)
- Morocco	21 (18)
- Others	10 (8)
ALT (U/L)	18 (15–24)
HBeAg negative (%)	119 (100)
HBV DNA (IU/mL)	267 (20–2,810)
ELF-test	8.4 (7.8–9.2)
- HA (ng/mL)	23.8 (11.5–41.3)
- PN3P (ng/mL)	7.0 (5.5–9.1)
- TIMP1 (ng/mL)	210.6 (180.0–250.9)
LSM (kPa)	5.5 (4.0–6.8)
- F0/F1	94 (79)
- F2	11 (9)
- F3	10 (8)
- F4	4 (3)

Expressed as median and interquartile range (IQR) or number and percentage. BMI= body mass index; ALT= alanine aminotransferase; HBeAg= hepatitis B e antigen; CHB DNA= hepatitis B viral load; HBsAg= hepatitis B surface antigen; ELF= enhanced liver fibrosis; HA= hyaluronic acid; P3NP= propeptide of procollagen type

3; TIMP-1= tissue inhibitor of matrix metalloproteinases-1= LSM, liver stiffness measurement; U/L= units per litre; IU/mL= international units per millilitre; ng/mL= nanogram per millilitre; kPa= kilopascal.

Table 2. Distribution of patients according to fibrosis stages

		Liver stiffness measurement			Total
		F0/F1	F2	F3-F4	
ELF-test	No/mild fibrosis	53 (45)	4 (3)	4 (3)	61 (51)
	Moderate fibrosis	30 (25)	3 (3)	6 (5)	39 (33)
	Severe fibrosis	11 (9)	4 (3)	4 (3)	19 (16)
Total		94 (79)	11 (9)	14 (12)	119 (100)

Light gray boxes represent concordance between ELF-test and LSM, dark gray boxes represent discordance between ELF-test and LSM with difference of one fibrosis stage, black boxes represent discordance between ELF and LSM with difference of two fibrosis stages. Parameters are expressed in numbers of patients with percentages between brackets ().

Table 3. Comparison of studies investigating the ELF-test in patients with chronic CHB

Author	**Country**	**Sample size (n)**	**% Female patients**	**% HBeAg positive patients**	**Fibrosis scoring system**	**Reference test**	**ELF-test cut-offs (≥F3) and AUC**	**LSM cut-offs (≥F3) and AUC**
Wong et al. *(5)*	China	323	15	26	METAVIR	Liver biopsy	≥F3: >9.8 0.69 (0.63-0.75)	≥F3: >6.0 0.83 (0.76–0.91)
Kim et al. *(6)*	Taiwan	170	40	?	METAVIR	Liver biopsy	≥F3: 10.1 0.86 (0.81-0.92)	≥F3: 10.1 0.96 (0.93–0.98)
Gumusay et al. *(8)*	Turkey	58	46	19	Ishak	Liver biopsy	≥F3: >5.2 0.83 (? - ?)	-
Trembling et al. *(7)*	Italy	182	29	71	METAVIR	Liver biopsy	≥F3: >8.75 0.80 (0.73–0.87)	≥F3: >6.9 0.90 (0.85–0.95)
Harkisoen et al.	**The Netherlands**	**164**	**100**	**0**	**METAVIR**	**LSM**	**>F3: >9.8 0.65 (0.51 – 0.80)**	**≥F3: >8.1 -**

Bolded text refers to data from this article. HBeAg= hepatitis B e-antigen; ELF= enhanced liver fibrosis; LSM= liver stiffness measurement; kPa= kiloPascal.

Figure 1. Flow chart describing the patient selection.

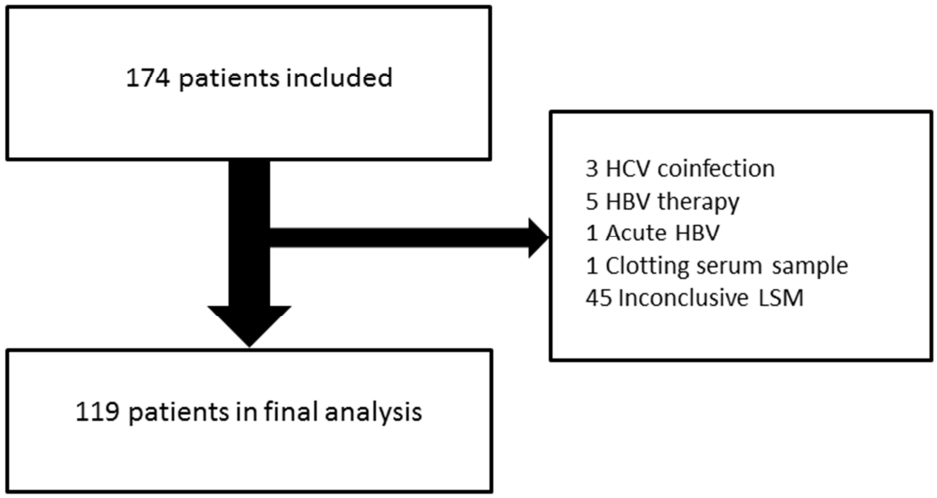
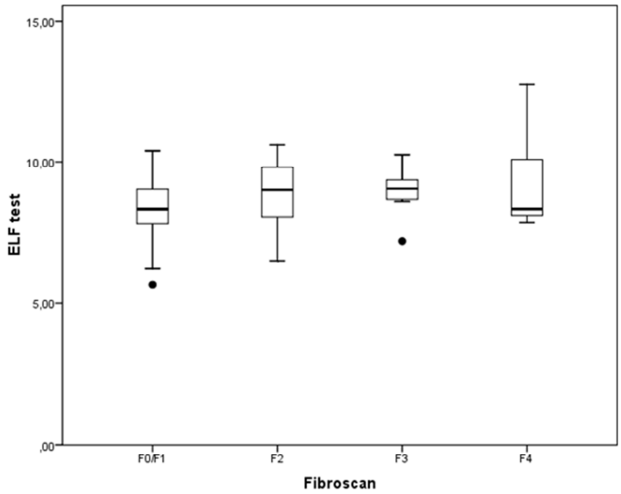
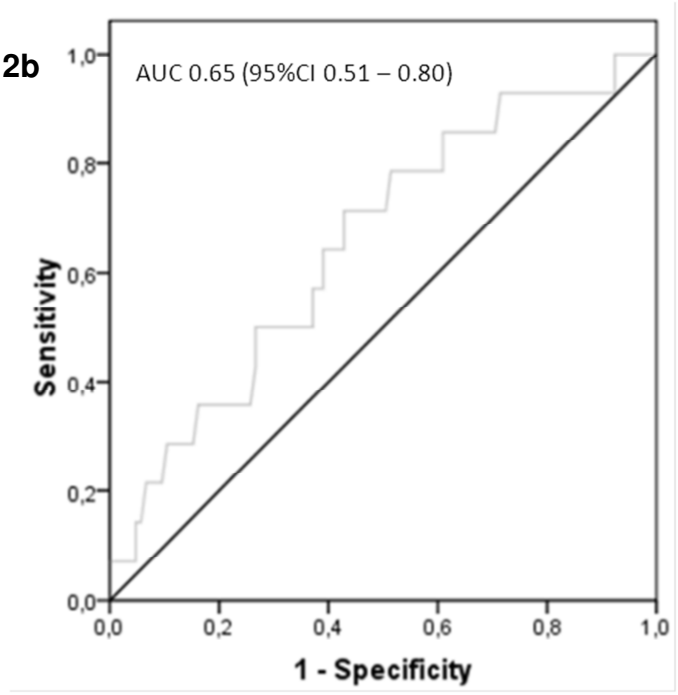


Figure 2. Performance of the ELF-test compared to LSM

2a



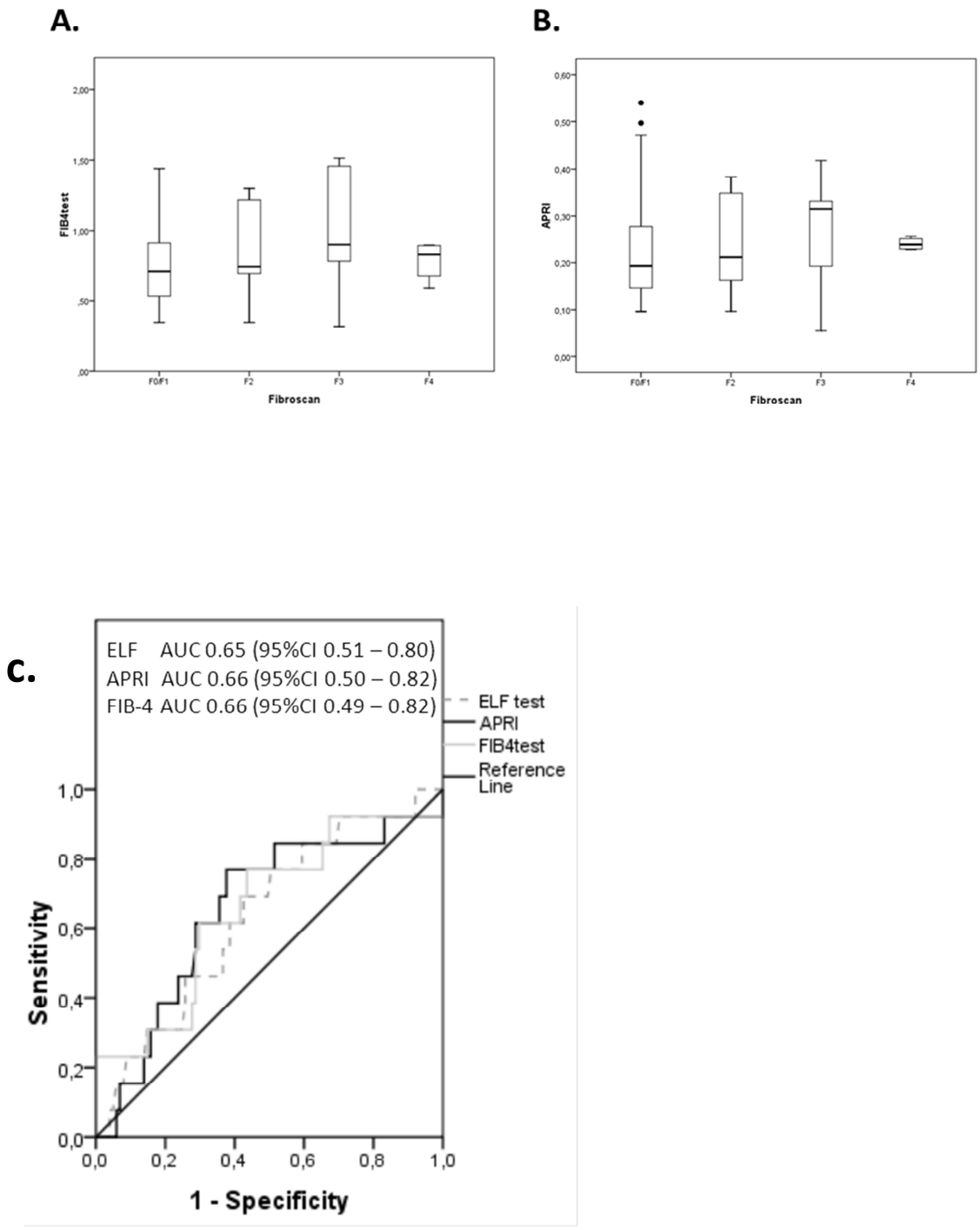
2b



a. Boxplot of ELF-test according to LSM fibrosis stage. Horizontal lines within boxes and boxes represent median and interquartile ranges (IQR). ELF-test expressed as median (IQR) per fibrosis stage. Cut-off values LSM: F0/F1 <7.2 kPa; F2 7.2–8.0 kPa; F3 8.1–11.0 kPa; F4> 11.0 kPa. (Cut-off values adapted from reference *14*). **b.** Receiver operating characteristic (ROC) curve for ELF-test in relation to LSM in

detection of severe fibrosis and cirrhosis (F3-F4). AUC = area under curve, 95%CI = 95 per cent confidence interval.

Figure 3. Performance of the APRI and FIB-4 score compared to LSM



Boxplot of FIB4-score (**a**) and APRI-score (**b**) according to LSM fibrosis stage. Horizontal lines within boxes and boxes represent median and interquartile ranges (IQR). Cut-off values LSM: F0/F1 <7.2 kPa; F2 7.2–8.0 kPa; F3 8.1–11.0 kPa; F4 >11.0 kPa. (Cut-off values adapted from reference *14*). **c.** Receiver operating

characteristic (ROC) curves for ELF-test, APRI score and FIB4 score in relation to LSM in detection of severe fibrosis and cirrhosis (F3-F4). AUC= area under curve, 95%CI= 95 per cent confidence interval.

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Chapter 4

Low serum hyaluronic acid levels associated with spontaneous HBsAg clearance

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Submitted

Abstract

Background: The pathophysiological underlying mechanism of spontaneous HBsAg clearance in hepatitis B virus (HBV) infected patients is largely unknown. However, serum Hyaluronic Acid (sHA) plays a role in liver fibrosis progression and reversely could serve as a potential biomarker for HBsAg clearance. This study investigates whether low sHA is associated with HBsAg loss in non-Asian HBV patients.

Methods: Non-Asian women living in Amsterdam with known chronic HBV infection between 1990-2003 were invited for a single follow-up visit at the Municipal Health Service Amsterdam between September 2011 to May 2012. Serum hyaluronic acid and liver stiffness measurement together with clinical evaluation, biochemical and virologic blood tests were performed.

Results: Of the 160 women, HBsAg loss occurred in 38 (23%) patients between diagnosis and follow-up. sHA levels was lower in HBsAg negative patients compared to HBsAg positive patients (14.5 (9.4–27.2) ng/mL versus 25.0 (12.3–42.5) ng/mL, p <0.01). A similar distinction in sHA between low and high HBV DNA was noted. sHA had a significant discriminatory ability to differentiate between HBsAg positive and HBsAg negative patients, AUC 0.65 (95% CI 0.55–0.75), p<0.01. In multivariable analysis only sHA level was associated with HBsAg loss (OR 0.4 (0.2–0.9)). Finally, F3-F4 fibrosis (cut-off >8.1 kPa) was diagnosed in 3% in HBsAg negative patients compared to 10% in HBsAg positive patients (p=0.15).

Conclusion: Serum HA levels are lower in patients who experience spontaneous HBsAg loss compared to HBsAg positive patients.

Introduction

Liver inflammation is the hallmark of hepatotropic viruses like Hepatitis B virus (HBV). During this chronic hepatic inflammation, synthesis and turnover of the extracellular matrix (ECM) is modulated by several cytokines with serum hyaluronic acid (sHA) being one of its major ECM components[1, 2]. sHA levels have already been shown to be higher in patients with chronic hepatitis C or active autoimmune hepatitis than those of healthy individuals without signs of hepatitis[3-5]. In addition, in chronic HBeAg negative HBV patients, high serum sHA positively correlates with fibrosis progression and level of inflammation[6]. Based on these observations, sHA level might be a reflection of the intensity of the immune response in chronic liver diseases.

Annually, approximately 2% of all chronic hepatitis B virus (HBV) infected patients experience HBV surface antigen (HBsAg) loss without antiviral therapy (i.e. spontaneous HBsAg clearance)[7]. Although the exact pathophysiological mechanism of spontaneous HBsAg loss in patients with chronic HBV has not been fully understood, there is some evidence that a T-cell mediated immune response may be essential in this process[8]. Rehermann et al. demonstrated that chronic HBV patients who cleared HBsAg, spontaneously or after interferon-alfa treatment, had a strong HBV multi-specific cytotoxic T-lymphocyte response that was similar to those clearing HBsAg after an acute infection[9]. Several histological studies have shown that after spontaneous HBsAg loss, liver inflammation declined over time[10, 11]. However, it is unknown whether sHA levels also decline after spontaneous HBsAg loss and if sHA could serve as a potential biomarker for HBsAg clearance. Therefore, the aim of this study is to investigate whether a low sHA level is associated with HBsAg loss in patients with chronic HBV.

Methods

Patient selection

The study population has already been described previously[12]. Briefly, non-Asian women in the greater Amsterdam area, who were registered as chronic HBV patients between 1990 and 2004, were invited for a single study visit between September 2011 and May 2012 at the Municipal Health Service of Amsterdam. History taking and physical examination, along with a liver stiffness measurement (LSM) and blood tests to determine the hyaluronic acid level and biochemical and virological tests were performed. For this study 14 patients were excluded from the 174 included patients (3 with HCV co-infection, 5 with previous HBV treatment, 1 with acute HBV infection and 5 due to indeterminate sHA), resulting in a study population of 160 participants.

Spontaneous HBsAg loss was defined as having a positive HBsAg antigen in the historic sample with subsequent HBsAg negativity during the follow-up visit without previously being subjected to antiviral therapy. The study was approved by the local medical ethical committee (Clinical trials number NCT01462981).

Laboratory tests

Hyaluronic acid levels were measured in serum at the follow-up visit as part of the ELF-test by the ADVIA Centaur XP (Siemens, Erlangen, Germany). This assay has a lower detection limit of 1.6 ng/ml, a linear range from 1.6–1,000 ng/ml; higher concentrations are expressed as >1000 ng/ml. Linearity, sensitivity and precision were evaluated according to CLSI guidelines. HBV DNA was determined with the COBAS Ampliprep/COBAS Taqman assay, v.2.0 (Roche Molecular Diagnostics, California, USA) with a lower detection limit of 20 IU/ml. Qualitative Anti-HCV, anti-

HIV and qualitative HBsAg were also performed with the ADVIA Centaur XP assay. Lastly, liver-related biochemical parameters were determined according to local standard laboratory procedures. HBV DNA levels were divided in more or less than 2,000 IU/mL that was derived from current international guidelines[13, 14].

Liver stiffness measurement

An experienced researcher (S.H.) performed the LSM with a Fibroscan® (model F402 Echosens, France) according to standard operating procedures supplied by the manufacturer and as described by others[15]. The METAVIR classification was used to determine the fibrosis stage, categorizing no to mild fibrosis (F0/F2) with a score less or equal to 8.1 kPa and severe fibrosis to cirrhosis (F3/F4) with a score above 8.1 kPa based on a cut-off value from previous studies in chronic HBV patients[16, 17].

Statistical analysis

Continuous variables were expressed as a median with interquartile range (IQR) and categorical variables as frequencies with percentage. Differences between HBsAg positive and HBsAg negative groups were calculated with the Mann-Whitney U test (continuous variables) or Chi square test (categorical variables). Factors associated with HBsAg loss were analyzed with univariable and multivariable logistic regression. Factors with a p-value <0.10 in univariable analysis were included in the multivariable logistic regression. Correlation between ALT levels and sHA levels were calculated with the Spearman rho correlation. A receiver-operator characteristic (ROC) curve was constructed to assess the value of sHA in discriminating HBsAg positive patient from HBsAg negative patients and to determine an optimal cut-off value for the sHA. A ROC curve with an area under curve (AUC) less than 0.60 and a p-value >0.05

was considered unreliable for ROC curve. Outcomes were reported as odds ratio (OR) with 95% confidence intervals (CI) and a p-value <0.05 was considered significant. The statistical analysis was performed with SPSS v17 (version 17.0; SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics are given in table 1. At the time of the follow-up study visit, HBsAg loss was documented in 38 (23%) patients resulting in 122 HBsAg positive patients, all being HBeAg negative. All HBsAg negative patients had an undetectable HBV DNA level (i.e. <20 IU/ml). Of the 117 patients with a liver stiffness result, occurrence of F3-F4 fibrosis was lower in the HBsAg negative patients compared to HBsAg positive patients (1 in 38 HBsAg negative patients (3%) versus 12 in 122 HBsAg positive patient (10%), p=0.15).

Hyaluronic acid level and HBV parameters

To explore whether sHA levels were different between HBsAg positive and HBsAg negative patients, sHA levels were compared in both groups. The median (IQR) sHA level of all included patients was 21.9 (11.7–41.2) ng/mL. HBsAg negative patients had a significant lower sHA level compared to HBsAg positive patients (14.5 (9.4–27.2) ng/mL versus 25.0 (12.3–42.5) ng/mL, p <0.01) (figure 1). In addition, when patients were categorized into low HBV DNA level (HBV DNA less than or equal to 2000 IU/mL) and high HBV DNA level (HBV DNA >2,000 IU/mL), there was a trend towards a lower sHA levels in the low HBV DNA patients compared to the patients with high HBV DNA (20.6 (11.2–40.4) ng/mL versus 26.3 (14.7–44.4) ng/mL, p= 0.07). sHA levels were higher in patients with F3-F4 fibrosis 37.4 (18.1–50.0) ng/mL

compared to patients with F0-F2 fibrosis 22.4 (11.2–40.0) ng/mL, though this was not statistically significant (p=0.25).

Overall, there was a positive correlation between sHA levels and ALT level in the whole study population (r= 0.20, p= 0.01) (data not shown). However, when patients were divided by HBsAg status, this correlation was lost (HBsAg positive group r=0.16, p=0.08 and HBsAg negative group, r=0.11, p=0.49). The sHA had a significant diagnostic discriminatory power to detect HBsAg negative patients, AUC 0.65 (95% CI 0.55–0.75), p<0.01. A cut-off value of 16.9 ng/mL was selected to have the optimum in both sensitivity and specificity. Subsequently, patients were divided in two groups, either low or high sHA. There were 68 (43%) patients with a low sHA level compared to 92 (57%) patients with a high sHA level. Of the 38 HBsAg negative patients, 25 (66%) had a low sHA level (i.e. below 16.9 ng/mL) and 13 (34%) a high sHA level.

Factors associated with HBsAg loss

Next, we performed an univariable and multivariable analysis, to identify factors that were associated with HBsAg loss. The results are shown in table 2. In univariable analysis, which included age, BMI, ethnic origin, smoking, alcohol consumption, ALT level, AST level, sHA level, HBV DNA and fibrosis stage as factors, alcohol consumption OR 0.3 (0.1–0.9) and a high sHA level OR 0.3 (0.1–0.6) were associated with increased chance to loose HBsAg. BMI, smoking, and ALT level had a close, but statistical not significance association with HBsAg loss (BMI OR 0.5 (0.2–1.1), smoking OR 0.3 (0.1–1.0) and ALT OR 0.5 (0.2–1.0)). When all significant or borderline significant factors from univariable analysis were included in a

multivariable analysis, only those patients with a high sHA level had a lower chance to loose HBsAg (OR 0.4 (0.2–0.8)).

Discussion

Spontaneous HBsAg loss in chronic HBV-infected patients is a phenomenon that is still not well understood. This study clearly shows that chronically HBV infected patients who lost HBsAg had lower sHA levels compared to patients who remained positive.

Though not reported in the literature previously, there are several arguments to explain this finding. First, sHA is a ligand of CD44 that is expressed in numerous cells, including liver sinus endothelial cells, neutrophils and regulatory T-cells (Tregs)[18, 19]. Data from experimental studies in other diseases have shown that there is an interplay between sHA and the immune system during chronic inflammation. On the one hand, studies in lung fibroblasts have shown that cytokines such as TNF-a activate the production of sHA[20]. Subsequent sHA-CD44 binding then promotes T-cell adhesion and migration to the endothelium to engage in an inflammatory process. On the other hand, there is also evidence that sHA stimulates the anti-inflammatory pathway of the immune system[21]. In an *in vitro* study, sHA binding to CD44 was correlated with a high suppressive activity of Tregs[21].

Second, in chronic HBV-infected patients experiencing spontaneous HBsAg loss, early studies have shown that the inflammatory process in the liver declined after HBsAg clearance[10, 11]. Although the interplay between sHA, inflammation and HBsAg loss has to be further elucidated, we hypothesize that HBsAg loss dampens the immune system which in turn suppresses the synthesis of sHA leading to low sHA levels.

Several studies have investigated the value of sHA (or sHA as component of the enhanced liver fibrosis (ELF) test) in patients with chronic HBV[6, 22-29]. However, these studies differ in several aspects from our study. First, until now studies in HBV patients have only focused on the role of sHA in the identification of liver fibrosis. Second, the study population of previous studies differed from our study, since previous studies either included only HBsAg positive patients or did not separate patients based on their HBsAg status (when HBsAg status of the included patients was not mentioned). Third, the cut-off value of the sHA concentration in our study is much lower than the cut-off values of other studies which varied between 52 and 300 ng/mL[23, 25]. Since we have shown that lower sHA values than previously reported are necessary to differentiate between patients with and without spontaneous HBsAg loss, this association could have gone unnoticed by others.

In this study, although not statistically significant, there were less HBsAg negative patients with severe fibrosis and cirrhosis (F3-F4) compared to HBsAg positive patients. This is in line with other studies. In a Spanish study on 612 Caucasian chronic HBV-infected patients who were followed for 15 years, those who remained HBsAg positive had significantly more cirrhosis than patients who had experienced HBsAg loss of whom 28% received antiviral therapy[30]. Another study with Taiwanese chronic HBV patients who were followed up to 179 months after spontaneous HBsAg loss, only 1.6% of the patients developed cirrhosis after HBsAg loss[31]. Thus, our study further supports the finding that spontaneous HBsAg loss is associated with minimal fibrosis progression.

Since this was a cross-sectional study, we could not explore whether the sHA levels could be of predictive value to identify future candidates for spontaneous HBsAg loss. A prospective longitudinal study with multiple time points in which sHA levels

would be determined and in which the moment of HBsAg loss is documented is needed to clarify this question. This would also allow for exploration of additional variables, which could be fitted into an algorithm, to identify chronic HBV patients who will experience spontaneous HBsAg loss in an early stage to avoid unnecessary antiviral therapy. The advantage of sHA is that it is easily accessible and available, relatively inexpensive and a small amount of serum is required for the assay.

In conclusion, serum HA levels are significantly lower in chronic HBeAg negative HBV patients who experience spontaneous HBsAg loss in comparison to those still being HBsAg positive. Further validation studies are needed to determine the predictive value of sHA with potential other variables in the natural history of chronic HBV.

Table 1. Characteristics of the study population by HBsAg status

	HBsAg positive patients (n=122)	HBsAg negative patients (n=38)	p-value
Age, *years median (IQR)*	45 (41–49)	44 (39–48)	0.57
Follow-up, *years median (IQR)*	18 (13–20)	18 (14–20)	0.85
BMI, *kg/m^2 median (IQR)*	30 (27–33)	29 (25–34)	0.85
Alcohol, *n (%)*[a]	39 (31)	5 (13)	0.02
Smoking, *n (%)*[b]	28 (23)	3 (8)	0.03
Origin, *n (%)*			0.04
Turkey	33 (27)	19 (50)	
Ghana	31 (25)	3 (8)	
Surinam	25 (21)	5 (13)	
Morocco	24 (20)	7 (18)	
Other	9 (7)	4 (11)	
ALT, *U/L median (IQR)*	18 (15–26)	16 (12–21)	0.11
AST, *U/L median (IQR)*	17 (13–23)	15 (12–19)	0.16
Trombocytes, *10^9/L median (IQR)*	249 (201–287)	245 (218–284)	0.94
Protrombin time, *seconds median (IQR)*	13.1 (12.8–13.5)	13.2 (12.6–13.5)	0.82
Log HBV DNA*, IU/mL median (IQR)*	2.7 (1.9–3.5)	n/a	n/a
F3-F4 fibrosis, *n (%)*[c]	12 (10)	1 (3)	0.15

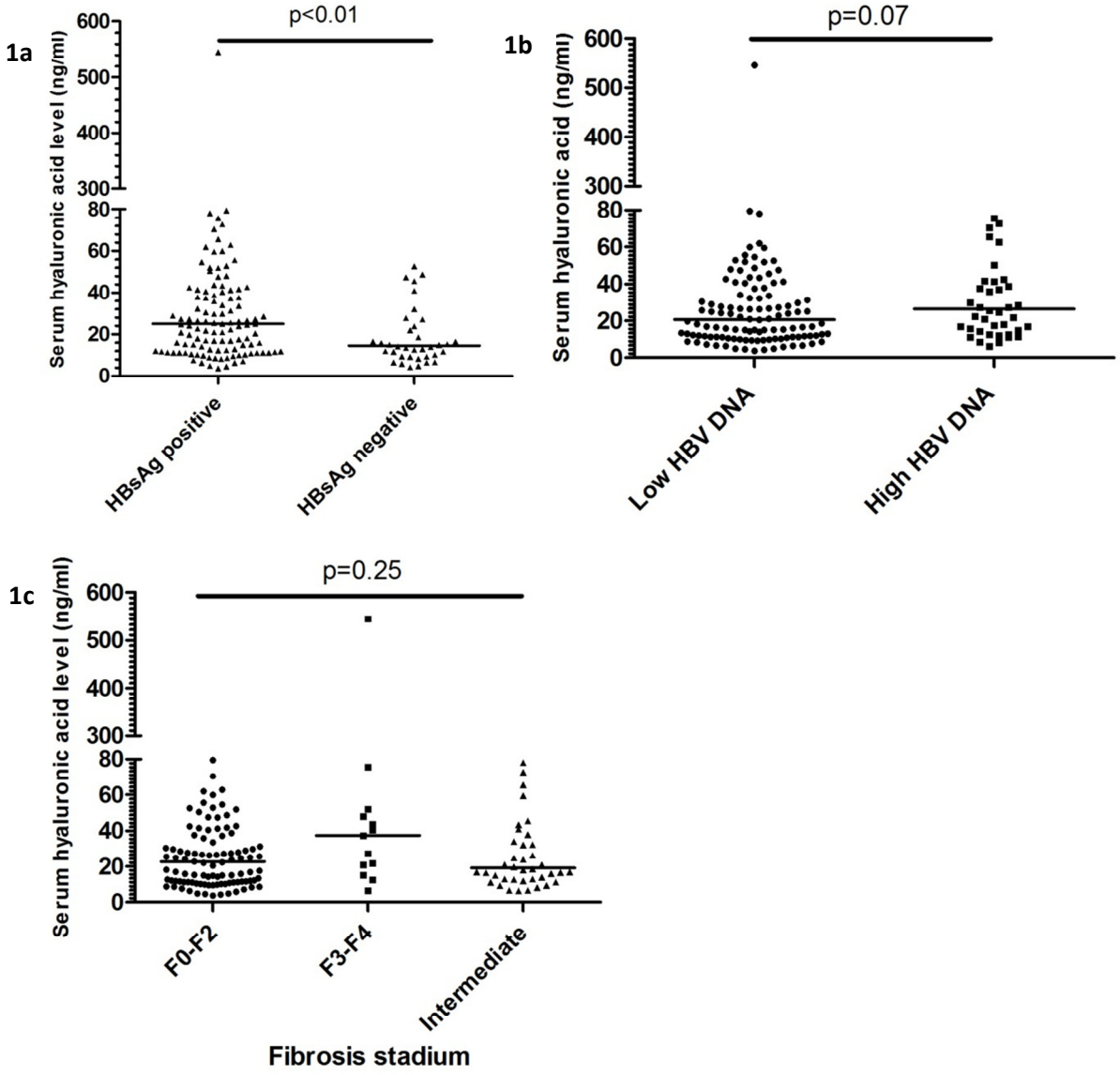
BMI= body mass index, ALT= alanine aminotransferase, AST= aspartate aminotransferase, IQR= interquartile range, kg/m^2= kilograms per square meter, U/L= units per liter, IU/mL= international units per milliliter, n/a= not applicable. a= Alcohol consumption defined as more than 1 glass per week, b= Smoking defined as more than one cigarette per week, c= determined by liver stiffness measurement

Table 2. Analysis of factors associated with spontaneous HBsAg loss

	Total (n)	HBsAg negative cases (%)	Univariable analysis OR (95% CI)	p-value	Multivariable analysis OR (95% CI)	p-value
Age						
- ≤ 45 years	85	22 (26)	1 (reference)	0.50	1 (reference)	
- > 45 years	75	16 (21)	0.8 (0.4–1.6)			
BMI						
- ≤ 25 kg/m^2	24	9 (38)	1 (reference)	0.09	1 (reference)	0.10
- > 25 kg/m^2	136	29 (21)	0.5 (0.2–1.1)		0.4 (0.2–1.2)	
Ethnic origin						
- Turkey	52	19 (37)	1 (reference)	0.18	1 (reference)	
- Ghana	34	3 (9)	1.3 (0.4–4.9)			
- Surinam	30	5 (17)	0.2 (0.1–1.2)			
- Morocco	31	7 (23)	0.5 (0.1–2.1)			
Smoking[#]						
- No	129	35 (27)	1 (reference)	0.05	1 (reference)	0.06
- Yes	31	3 (10)	0.3 (0.1–1.0)		0.3 (0.1–1.1)	
Alcohol consumption[*]						
- No	116	33 (28)	1 (reference)	0.03	1 (reference)	0.17
- Yes	44	5 (11)	0.3 (0.1–0.9)		0.5 (0.2–1.4)	
ALT						
- ≤ 0.5 x ULN	88	26 (30)	1 (reference)	0.06	1 (reference)	0.26
- > 0.5 x ULN	72	12 (17)	0.5 (0.2–1.0)		0.6 (0.3–1.4)	
AST						
- ≤ 0.5 x ULN	150	38 (25)	1 (reference)		1 (reference)	
- > 0.5 x ULN	10	0 (0)	n/a			
HA level						
- Low	68	25 (37)	1 (reference)	<0.01	1 (reference)	0.01
- High	92	13 (14)	0.3 (0.1–0.6)		0.4 (0.2–0.8)	
HBV DNA level						
- Low/undetectable	118	38 (32)	1 (reference)		1 (reference)	
- High	42	0 (0)	n/a			
Fibrosis stage						
- F3-F4	13	1 (8)	1 (reference)	0.17	1 (reference)	
- F0-F2	105	23 (22)	5.8 (0.7–49.1)			
- Indeterminate	42	14 (33)	3.4 (0.4–27.6)			

BMI= body mass index, ALT= alanine aminotransferase, AST= aspartate aminotransferase, kg/m^2= kilograms per square meter, ULN= upper limit of normal, OR= odd ratio, 95% CI= 95 per cent confidence interval, n/a= not applicable. a= Alcohol consumption defined as more than 1 glass per week, b= smoking defined as more than one cigarette per week

Figure 1. Distribution of serum hyaluronic acid according to HBsAg status (a), HBV DNA level (b) and fibrosis stage (c)



ng/ml= nanogram per milliliter, LSM= liver stiffness measurement.

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Chapter 5

Descent affects health-related quality of life in therapy-naïve non-Asian women with chronic hepatitis B

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Abstract

***Background**:* Patients with asymptomatic chronic hepatitis B (HBV) have a lower health related quality-of-life (HRQoL) than healthy controls. It unclear whether ethnicity, which is an important factor in assessing HRQoL in several chronic disease, is of influence in chronic HBV patients. This study aims to investigate the role of ethnicity on HRQoL in women with chronic HBV.

***Methods**:* Non-Asian women with chronic HBV mono-infection, diagnosed during pregnancy screening in the 80-90ties, were invited at the Public Health Service of Amsterdam between September 2011 and May 2012 for a single visit. HRQoL was assessed with an amended questionnaire extracted from the Short Form 36 (SF36). Norm-based scores were used for the analysis of the questionnaire outcomes. Quality of life was divided in summaries of a psychical component (PCS) and a mental component (MCS).

***Results**:* A total of 171 non-Asian chronic HBV patients were included in this study. Patients were divided in five group based on their origin: Turkey (n=55), Ghana (n=37), Surinam (n=34), Morocco (n=31) and other origins (n=14). A low psychical HRQoL (PCS<50) was observed in 47%; 64% Turkish patients, 24% Ghanaian patients, 44% Surinamese patients, 48% Moroccan patients and 50% patients of other origins (p<0.01). A low mental HRQoL (MCS<50) was observed in 49% patients, 67% Turkish patients, 27% Ghanaian patients, 47% Surinamese patients, 42% Moroccan patients and 50% patients from other origins (p<0.01). The PCS scores per ethnic group were: Turkish patients 45±8, Ghanaian patients 53±7, Surinam patients 49±9, and Moroccan patients 48±7 (p<0.01). The MCS scores per ethnic group were: Turkish patients 42±14, Ghanaian patients 53±11, Surinamese patients 47±14, Moroccan patients 47±16 (p<0.01). Overall, age OR 0.94 (0.88–0.99)

and comorbidity 3.41 (1.19–9.80) were additional predictors for PCS and comorbidity OR 3.26 (1.12–9.52) and psychiatric disease OR 6.69 (2.75–16.27) were additional predictors for MCS.

***Conclusion**:* Ethnic origin is an important determinant when assessing HRQoL in chronic HBV patients.

Introduction

According to the World Health Organization (WHO) approximately 240 million people worldwide are chronically infected with hepatitis B virus (HBV)[1]. Chronic HBV patients can remain asymptomatic for decades until liver related complications may develop. Despite the lack of symptoms, chronic HBV infection may still have an impact on the psychical and mental condition of patients. For instance, several studies have shown that liver fibrogenesis develops and progresses in HBV carriers during their asymptomatic course[2-4].

Chronic hepatitis B can also influence the health-related quality of life (HRQoL). Several observational studies, mainly in chronic HBV patients of Asian descent, have shown that HRQoL is lower in patients with chronic hepatitis B compared to healthy controls[5-7]. For example, Zhuang et al. investigated the HRQoL in 460 Asian chronic HBV patients and 460 Asian healthy controls. They found that both psychical and mental components of HRQoL were lower in HBV patients compared to the healthy controls[7]. Even between chronic HBV patients, cirrhosis can negatively impacting HRQoL. Wong et al. found that HBV patients with progressive liver complications such as cirrhosis and hepatocellular carcinoma had a lower HRQoL compared to HBV patients without cirrhosis[8]. However, in both studies the influence of HBV parameters in the patients, such as HBeAg status and HBV DNA level, were not described. Moreover, previously we have described that the natural course of HBV infection differs between Asian patients and non-Asian patients[2]. Ethnicity is a determinant which may be of influence when assessing HRQoL and therefore interpretation of Asian studies should be done with caution. In other chronic diseases, ethnic differences have already shown to be of influence on HRQoL[9, 10]. For example, Han et al. showed that HRQoL was lower in African Americans

compared to Caucasian Americans who were hospitalized with an COPD exacerbation[9]*).* In another study in patients over 60 years of ages with diabetes mellitus, Caucasian patients had a lower psychical HRQoL compared to Filipino, Asian, Hispanic and Black patients[10]. However, in patients with chronic HBV, especially in patients of non-Asian origin, the impact of ethnicity on HRQoL is less clear. Therefore, the aim of this study is to investigate the influence of ethnic origin on HRQoL in non-Asian women with chronic HBV.

Methods

Patient selection

The patient selection and inclusion has been described previously[2]. In brief, non-Asian women who had been diagnosed with HBV during pregnancy screening (HBsAg positive) in the 80-90ties were invited between September 2011 and May 2012, with an opt-in procedure, for a single visit at the Public Health Service Amsterdam. During this visit, history taking, physical exam, collection of one blood sample and a liver stiffness measurement were performed to assess the HBV activity and the rate of liver fibrosis. During this visit these women were also asked to fill out a quality of life questionnaire.

Virological parameters, i.e. HBsAg, HBeAg, HBV DNA level, quantitative HBsAg level, anti-HCV and ant-HIV were determined with the COBAS Taqman (Roche, Meylan, France) and the ADVIA Centaur XP assay (Siemens, Erlangen, Germany). Liver stiffness measurement was performed as described by others[11] with a Fibroscan® (model F402 Echosens, France). Fibrosis was staged according to the METAVIR classification for HBV[12] and cut-off values were derived from previous studies in HBV patients[13].

Patients were excluded when they were HIV and/or hepatitis C coinfected, had parents or ancestors of Asian descent or those who could not give informed consent.. All patients provided written informed consent. The study was conducted in accordance with the declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice with the approval by the local medical ethical committee of the Academic Medical Center Amsterdam (clinical trials number NCT01462981).

HRQoL questionnaire

Health-related quality of life was measured by a modified version of the short form 36 (SF-36)[14], a 36-item questionnaire. Modifications included 1) the time scales since the SF-36 form uses a relatively short period to assess the HRQoL and chronic HBV patients have a long asymptomatic period and 2) the physical questions which were focused more on liver related symptoms.

The questionnaire quantifies HRQoL in eight subscales, consisting of 4 psychical and 4 mental components. The physical subscales are: physical functioning (PF), bodily pain (BP) role limitations due to physical problems (RP) and general perception of health (GH). The mental subscales are: role limitations due to emotional problems (RE), vitality (VT), social functioning (SF) and mental health (MH). From these subscales a physical component summary (PCS) and mental component summary (MCS) is generated. Algorithms and scores from the questions were coded and transformed according to previous studies describing the Dutch female populations norms[15]. Scores from the subscales, PCS and MCS varied between 0 to 100. Standardized norm-based scores of the Dutch female population (mean (SD)= 50 (±10))[15] were used to assess HRQoL. A norm-based score (NBS) of 50

demonstrates HRQOL equivalent to the Dutch female population; scores below or above this threshold demonstrate lower or respectively higher rated HRQOL. A difference of 0.2–0.5 SD corresponding to 2.5–5.0 points were be considered clinically important[16] .

Statistical analysis

The baseline characteristics were summarized as continuous variables expressed as medians with interquartile range (IQR) and categorical variables expressed as frequencies with percentage. The scores of the different psychical and mental subscales as well as the PCS and MCS were expressed as mean and standard deviation. Differences in different variables between ethnic groups were calculated with the Chi square test. Differences in subscales, PCS and MCS between different ethnic groups were calculated with one-way ANOVA with the LSD post hoc analysis. Univariable, bivariable and multivariable logistic regression was used to calculate predictors of HRQoL overall. Outcomes were reported as odds risks with 95% CI. A p-value <0.05 was considered significant. The statistical analysis was performed with SPSS v17 (version 17.0; SPSS Inc., Chicago, IL, USA) and Stata 11 (StataCorp LP, College Station, TX, USA).

Results

Patient characteristics

A total of 171 patients were included in this study with patient selection shown in figure 1. Patients were divided in 5 major groups based on ethnic origin: 32% Turkish, 22% Ghanaian, 20% Surinamese, 18% Moroccan and 8% other origin. The baseline characteristics of these groups are given in table 1. Turkish chronic HBV

patients had more psychiatric comorbidity than other origins (47% Turks versus 5% Ghanaians, 32% Surinamese, 23% Moroccans and 29% other ethnic origins, p<0.01). HBV DNA level and fibrosis stage did not differ between the groups (p= 0.25 and p= 0.64), but Surinamese and Moroccan patients had higher quantitative HBsAg levels than patients in other groups (3.6 (1.9-4.2) IU/mL and 3.7 (0.0-4.2) IU/mL versus 1.9 (0.0-3.0); 2.9 (0.0–4.0) and 3.3 (0.0-3.9), p=0.004). One (2%) Turkish patient was HBeAg positive and 5 patients were previously treated with antiviral therapy, 2 (4%) Turkish patients, 2 (6%) Surinamese patients and 1 (7%) patient from another origin.

Ethnic differences in psychical HRQoL

Overall, when all patients were considered as one group, 81 patients (47%) had a low psychical HRQoL (PCS<50). The scores of the physical and mental subscales and the PCS and MCS between the different ethnic groups are given in table 2 and figure 2 shows how the PCS, MCS, and subscales of different ethnic groups relate to the norm-based scores. A PCS less than 50 was found in 35 (64%) Turkish patients, 9 (24%) Ghanaian patients, 15 (44%) Surinamese patients, 15 (48%) Moroccan patients and 7 (50%) patients from other origins. When Turkish patients were compared to Ghanaian patients, both the PCS score and frequencies of patients with a psychical HRQoL (PCS<50) were significant lower in the Turkish patients compared to Ghanaian patients (PCS score 45±8 versus 53±7, p<0.01; frequency 64% versus 24% patients, p<0.01). Furthermore, although not statistically significant, there Turkish patients had a lower PCS compared to the Surinamese and Moroccan patients, respectively 45±8 versus 49±9, p=0.07 and 45±8 versus 48±7, p=0.06. Contrary, when the Ghanaian patients were compared to the Surinamese and

Moroccan patients, Ghanaian patients had a higher PCS than those of Surinamese and Moroccan patients, respectively 53±7 versus 49±9, patients (p=0.08) and 53±7 versus 48±7, p=0.04. Finally, Surinamese and Moroccan patients had a similar PCS (49±9 and 48±7, p=0.73).

Ethnic differences in mental HRQoL

Similar to the psychical HRQoL, when all patients were considered as one group, 83 patients (49%) had a low mental HRQoL (MCS<50). A MCS less than 50 was found in 37 (67%) Turkish patients, 10 (27%) Ghanaian patients, 16 (47%) Surinamese patients, 13 (42%) Moroccan patients and 7 (50%) patients from other origins. Comparable with the PCS, Turkish patients had a significantly lower MCS and frequency of patients with a low mental HRQoL (MCS<50) compared to Ghanaian patients (MCS 42±14 versus 53±11, p=<0.01, frequency 67% versus 27% patients, p<0.01). Furthermore, Turkish patients had a lower MCS compared to Surinamese and Moroccan patients which was not statistical significant, respectively 42±14 versus 47±14, p=0.17 and 42±14 versus 47±16, p=0.16. Opposed to the Turkish patients, Ghanaian patients had a non-significant higher MCS than Surinamese and Moroccan patients (53±11 versus 47±14, p=0.44 and 53±11 versus 47±16, p=0.59). Finally, Surinamese and Moroccan patients had a comparable MCS (47±14 versus 47±16, p=0.94).

Predictors of physical and mental HRQoL overall

Next, all patients were combined to explore for additional factors which could influence HRQoL. In univariable analysis, age odds ratio (OR (95%CI)) 0.95 (0.91–0.99), time since diagnosis OR 2.60 (1.39–4.82), comorbidity OR 5.08 (1.97–13.11),

including psychiatric disease OR 3.35 (1.67–6.72), ethnicity OR 5.44 (2.15–13.81) and quantitative HBsAg level OR 2.03 (1.09–3.76) significantly influenced PCS. Similar, univariable analysis identified comorbidity OR 6.91 (2.51–18.98), psychiatric disease OR 10.24 (4.40–23.84), ethnicity OR 5.55 (2.22–13.91) and quantitative HBsAg level OR 2.44 (1.31–4.55) to influence MCS. After adjusting for ethnic origin, age OR 0.95 (0.91–0.99), time since diagnosis OR 2.13 (1.11–4.10) and comorbidity OR 4.07 (1.50–11.01), including psychiatric disease OR 2.50 (1.20–5.23) remained significant factors for PCS. For MCS, comorbidity OR 5.31 (1.86–15.16) and psychiatric disease OR 8.23 (3.44–19.67) remained significant factors for HRQoL. Multivariable logistic regression identified age OR 0.94 (0.88–0.99) and comorbidity 3.41 (1.19–9.80) as predictors for PCS and comorbidity OR 3.26 (1.12–9.52) and psychiatric disease OR 6.69 (2.75–16.27) as predictors for MCS.

Discussion

This study demonstrated that ethnicity could affect HRQoL in patients with chronic HBV. Furthermore, this study showed that age and comorbidity were significant determinants of HRQoL in women with chronic HBV.

Apart from the Ghanaian patients, the groups had a low HRQoL compared to a representative Dutch population of healthy females which were used for the norm-based scores[15]. This is in line with other studies. In a Turkish study, 30 patients with chronic HBV were compared with 30 HBV carriers and 30 healthy controls (criteria for chronic HBV and HBV carriers were not described)[17]. Healthy controls had significant higher scores in all eight subscales of HRQoL compared to patients with chronic HBV and HBV carriers. Patients with chronic HBV patients had comparable HRQoL with HBV carriers, except for the role emotional subscale which was significantly higher in

HBV carriers. Also, as previously mentioned, studies in Asian patients have shown that chronic HBV patients have lower HRQoL compared to healthy controls[7, 18, 19]. Of these studies, Zhuang et al. have the largest sample size in which they demonstrated that HRQoL was negatively influenced in all domains in Chinese patients with chronic HBV compared to the healthy Chinese population[7]. In a more heterogenous group, Levy et al. also showed that chronic HBV infected patients had a lower HRQoL compared to uninfected patients[20]. Of note, the virological characteristics of the studied populations in these studied were poorly described, making it difficult to interpret whether HBV infection itself or social cultural and other non-disease related factors may be related to the negative influence on HRQoL

In the group of Turkish chronic HBV patients there was a higher frequency of patients with a low HRQoL as well as a lower mean PCS and MCS score compared to the other ethnic groups. A possible explanation for these findings may be that HBV infection may have a great negative impact within the Turkish community. Van der Veen et al. previously identified that shame, stigma and an association with sexual behavior negatively influenced the intention to screen for HBV in Turkish people in the Netherlands[21]. However, it is unknown whether chronic HBV has the same impact within the communities of the other studied ethnic origins.

In this study, we have shown that ethnic differences have to be taken into account when assessing HRQoL in patients with chronic HBV. In clinical practice, ethnic origin with their underlying social and cultural factors have to be explored for an optimal guidance of patients. Social and cultural effects could influence the expectation from patients towards their health care providers. For example, in one study in a American Indian population, one barrier to access health care was that these patients, compared to white patients, experienced significantly more that their

doctor did not understand their culture, did not respect their religious beliefs and they felt discriminated by their doctor[22]. This may negatively impact the doctor-patient relationship, their health care perception and their HRQoL.

This study has several limitations. The healthy controls, which were used for the norm based scores, were not investigated during this study. However, the healthy controls are a representative and multi-ethnic group in the same area as our study population and also already used in several studies as reference[15]. Furthermore, due to the patient selection procedure, a possible selection bias cannot be ruled out. However, except the Ghanaian patients, all patients had a low HRQoL, we assume that this would not influence the main findings of this study. Finally, since the study had a cross-sectional design, the impact of disease progression between different ethnic groups could not be studied.

In conclusion, ethnic origin is an important determinant when assessing HRQoL in chronic HBV patients.

Table 1. Baseline characteristics between different ethnic groups

	Turkey n=55	**Ghana** n=37	**Surinam** n=34	**Morocco** n=31	**Other** n=14	**P-value**
Age *(years, mean (IQR))*	43 (40–47)	44 (41–50)	48 (46–53)	42 (38–48)	44 (39–51)	<0.01
Time since diagnosis *(years, mean (IQR))*	19 (16–21)	15 (12–20)	19 (14–21)	17 (11–19)	16 (13–19)	0.04
Comorbidity *(n (%))*						
Yes	51 (93)	23 (62)	30 (88)	22 (71)	13 (93)	0.01
No	4 (7)	14 (38)	4 (12)	9 (29)	1 (7)	
Type of comorbidity *(n (%))*						
Psychiatric	26 (47)	2 (5)	11 (32)	7 (23)	4 (29)	0.01
Cardiovascular	4 (7)	1 (3)	6 (18)	4 (13)	1 (7)	0.24
Diabetes	5 (9)	3 (8)	3 (9)	4 (13)	0 (0)	0.73
Malignancy	1 (2)	0 (0)	1 (3)	0 (0)	0 (0)	0.72
Smoking *(n (%))*	16 (29)	3 (8)	12 (35)	2 (7)	1 (7)	<0.01
Alcohol consumption *(n (%))*	7 (13)	17 (46)	20 (59)	0 (0)	5 (36)	<0.01
Prior HBV treatment *(n (%))*	2 (4)	0 (0)	2 (6)	0 (0)	1 (7)	0.40
HBeAg positive *(n (%))*	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0.71
Log HBV DNA *(IU/mL)*	2.5 (1.3–3.3)	2.3 (1.3–3.2)	2.6 (1.3–3.4)	2.3 (1.3–3.7)	1.3 (1.3–2.6)	0.25
Log quantitative HBsAG level *(IU/mL)*	1.9 (0.0–3.0)	2.9 (0.0–4.0)	3.6 (1.9–4.2)	3.7 (0.0–4.2)	3.3 (0.0–3.9)	<0.01
F3-F4 fibrose *(n (%))*	3 (6)	4 (11)	4 (12)	1 (3)	3 (21)	0.64

Continuous variables are expressed in median and interquartile range (IQR) or frequency (n) and percentage (%). HBV= hepatitis b virus, HBeAg= hepatitis e-antigen, Log HBV DNA= logarithmically expressed hepatitis b DNA level, HBsAg= hepatitis B surface antigen.

Table 2. Differences between ethnic groups in subscales and components of the HRQoL questionnaire

	Turkey	**Ghana**	**Surinam**	**Morocco**	**Other origin**	**p-value**
Psychical functioning (PF)	48 ± 11	53 ± 7	48 ± 12	49 ± 13	50 ± 8	0.02
Role psychical (RP)	42 ± 12	52 ± 9	48 ± 11	48 ± 11	46 ± 12	<0.01
Bodily pain (BP)	49 ± 9	52 ± 5	50 ± 11	50 ± 6	50 ± 7	0.29
General Health (GH)	42 ± 12	53 ± 10	47 ± 11	45 ± 10	47 ± 12	<0.01
Vitality (VT)	40 ± 15	55 ± 10	47 ± 11	47 ± 15	48 ± 14	<0.01
Social functioning (SF)	45 ± 14	52 ± 10	48 ± 12	48 ± 15	52 ± 10	0.09
Role emotional (RE)	44 ± 13	51 ± 10	47 ± 13	47 ± 12	47 ± 12	0.11
Mental health (MH)	43 ± 13	55 ± 11	47 ± 14	48 ± 16	51 ± 12	<0.01
Main scores						
Psychical component summary (PCS)	45 ± 8	53 ± 7	49 ± 9	48 ± 7	48 ± 7	<0.01
Mental component summary (MCS)	42 ± 14	53 ± 11	47 ± 14	47 ± 16	50 ± 13	<0.01

PF= psychical functioning, RP= role physical, BP= bodily pain, GH= general health, VT= vitality, SF= social functioning, RE= role emotional, MH= mental health, PCS= psychical component summary, MCS= mental component summary.

Figure 1. Patient selection and inclusion

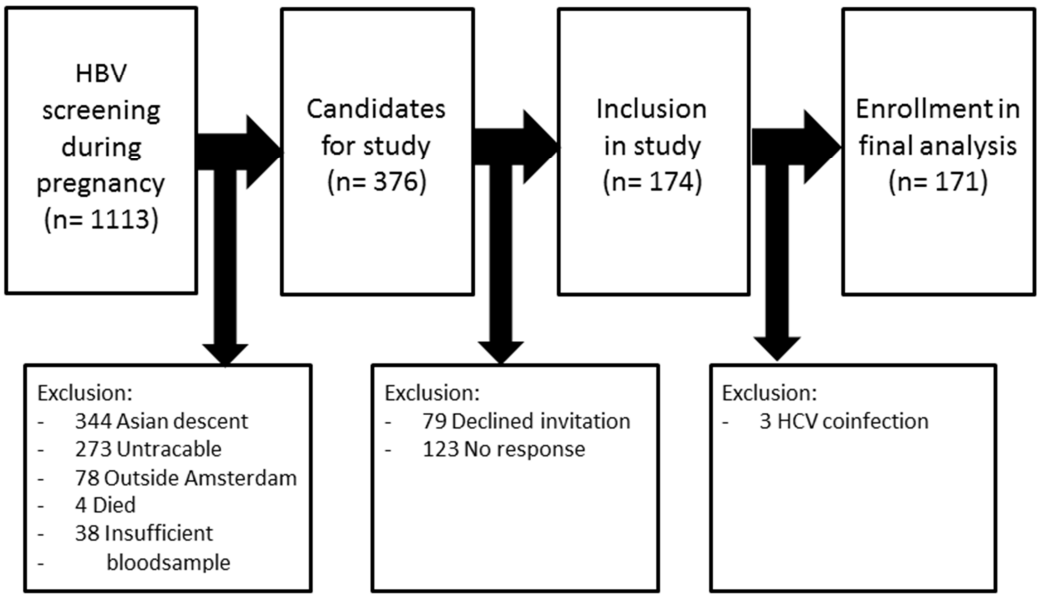
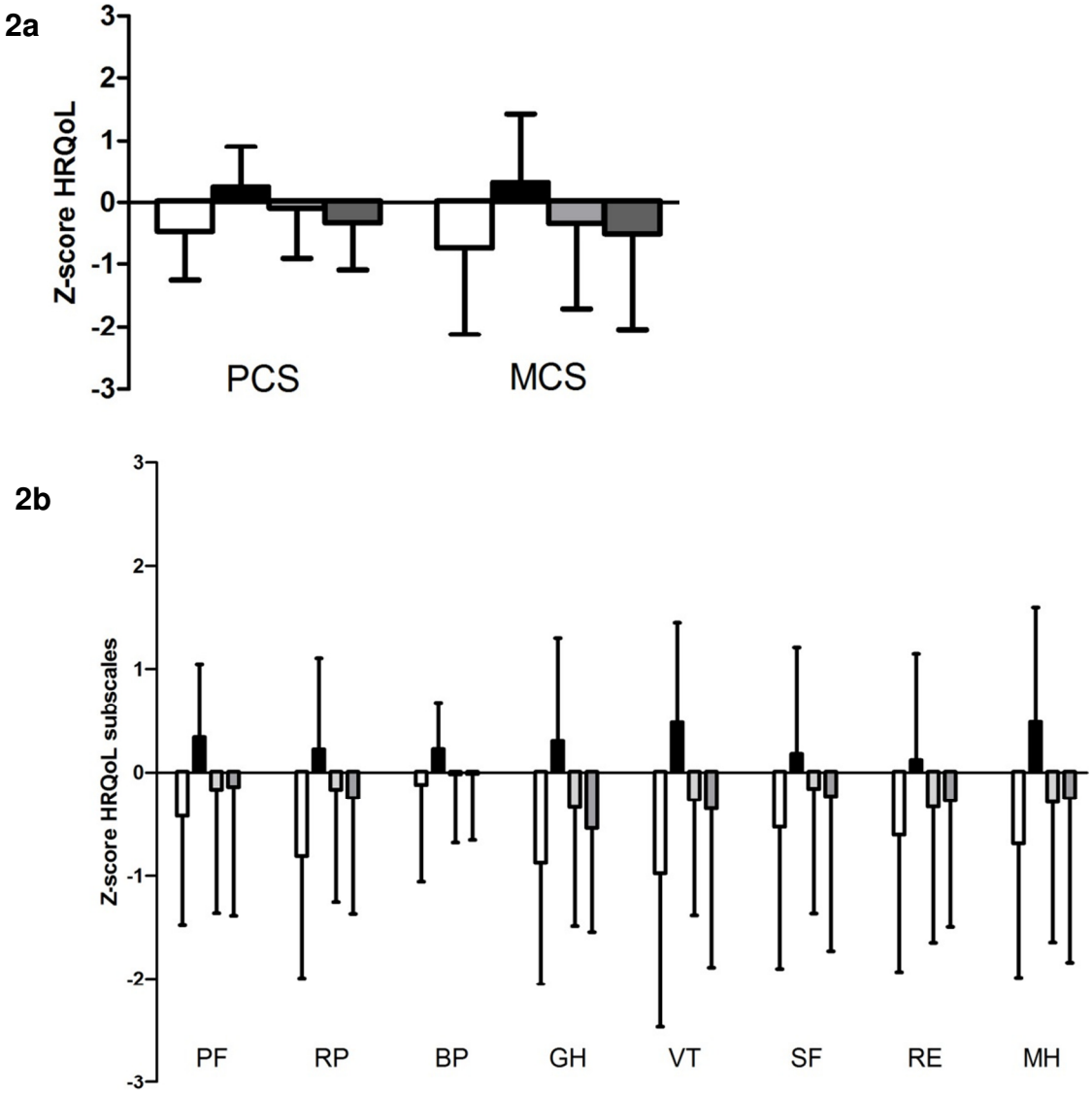


Figure 2. Differences between ethnic groups in overall psychical and mental HRQoL (a) and HRQoL subscales (b).



The White histograms represent the Turkish patients, the black histograms the Ghanaian patients, the light gray histograms the Surinamese patients and the dark gray histograms the Moroccan patients. PCS= psychical component summary; MCS= Mental component summary. PF= psychical functioning, RP= role physical, BP= bodily pain, GH= general health, VT= vitality, SF= social functioning, RE= role emotional, MH= mental health.

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Chapter 6

The influences of antiviral therapy on the T-cell function in adult patients with chronic hepatitis B

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Abstract

T-cells play an important role in the clearance of acute infection and control of hepatitis B virus (HBV) infection during the chronic phase. Chronic HBV is characterized by a weak and limited T-cell response. Several hypotheses, such as presence of regulatory T-cells (Tregs) or occurrence of T-cell exhaustion have been proposed to explain these observations. The two registered classes of anti-HBV drugs; (pegylated-)interferon-alfa (PEG-IFN-α) and nucleos(t)ide analogues (NUCs) have, next to their antiviral effect, also an immunomodulatory effect. Although NUCs have no direct immunomodulatory effects, they may indirectly positively affect the T-cell response through their viral suppressive action. In this review, effects of both (PEG-)IFN-alfa and NUC therapy will be discussed with regard to the cellular immune response against HBV.

Introduction

Approximately 2 billion people worldwide have been exposed to hepatitis B virus (HBV)[1]. After infection with HBV, in adults 10% become chronically infected[2]. These patients are at increased risk of developing liver complications like cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC)[2]. Although it is not fully understood which factors contribute to the development of chronic HBV in these patients, it appears that the adaptive immunity, especially the cellular immune response (i.e. HBV-specific T-cells), is critical in determining the outcome of this process. For example, patients with acute HBV exhibit a vigorous and broad HBV-specific T-cell response[3, 4] that correlates with spontaneous clearance of the virus. This is in contrast to chronic HBV, which is characterized by a weak and limited T-cell response[3, 4]. Besides the T-cell response, other aspects of the immune system, such as the activation of NK cells in the liver and the innate immune system, are also involved in the HBV pathogenesis. However, this is already reviewed by others[5] and outside the topic of this review.

The underlying mechanisms for these observations are unclear but different explanations have been proposed. Aside from theories describing indirect ways to blunt the T-cell response, which include the presence of dysfunctional antigen presenting cells (APC) and the ability of the virus to escape from the immune system (viral escape mutations), several explanations have been proposed on how T-cells become functionally impaired[6, 7]. In adult chronic HBV patients, two presumptions have gained interest as a possible explanation for the weak HBV-specific T-cell response. On the one hand, the concept of T-cell tolerance, which includes HBV-specific T-cell exhaustion and/or anergy, could occur when T-cells are unable to withstand the viral burden[8]. This was nicely illustrated in both human and animal

studies by Jung and colleagues and Milich and colleagues and also have been reviewed by others[8-11]. The high viral burden results in a loss of T-cell functionality due to an increase in expression of several inhibitory receptors like programmed death 1 (PD-1) and T-cell immunoglobulin domain and mucin domain 3 (Tim-3)[8, 12]. On the other hand, there could be predominance of regulatory T-cells (Tregs), characterized by the expression of CD25 and Foxp3, which are able to downregulate the HBV-specific T-cell response leading to HBV persistence[13].

The purpose of antiviral therapy, consisting of (pegylated-) interferon alfa (PEG-IFN-α) or nucleos(t)ide analoques (NUC) therapy, is to achieve immunological control by the host through of HBsAg and/or HBeAg clearance. The focus of this review is to describe the effects of current approved antiviral therapy on the cellular immune response in patients with chronic HBV.

Interferon-alfa therapy

The immunomodulatory effects of interferons (IFN) have already been extensively described by others[14, 15]. In hepatitis C for example, it was nicely described that (PEG-)IFN-α could activate interferon stimulating genes (ISGs) resulting in the production of cytokines/ chemokines leading to the differentiation of cytotoxic T-cells, i.e. T-cells which were able to kill infected and/or damaged cells[16]. Although similar mechanisms of IFN might also steer T-cells in HBV patients, these data in humans are scarce. Studies in patients with chronic HBV treated with IFN-α or PEG-IFN-α have shown that downregulation of STAT1 expression inhibits IFN-α signaling[17-19].

Numerous studies have evaluated the role of HBV-specific T-cells in chronic HBV-infected patients treated with (PEG-)IFN-α therapy[20-23]. Although (PEG-)IFN-α therapy may result in a reasonable HBe seroconversion in 32% of treated patients,

the majority of patients on (PEG-)IFN-α therapy experience treatment failure (defined as not experiencing HBeAg seroconversion)[24]. Failure of (PEG-)IFN-α therapy to achieve virological response, although not fully understood, might be the result of multiple mechanisms, such as modulation of the T-cell responses.

Upon treatment initiation with (PEG)-IFN-α in a prospective study with 23 HBeAg positive Chinese patients, a shift toward a pro-inflammatory CD4+ Thelper 1 cytokine profile was demonstrated in peripheral blood. During treatment, CD4+ Thelper 1 cell-related cytokines such as IL-12, TNF-alfa and IFN-y were increasingly produced[21]. This was found for both HBeAg positive and negative patients, who had been treated with (PEG-)IFN-α for up to 48 weeks[20]. At the same time, anti-inflammatory CD4+ Thelper 2 cell-related cytokines, like IL-4, IL-6 and IL-10 were decreased[20]. Thus, one of the mechanisms by which (PEG-)IFN-α could improve the immune response is by skewing the T-cell response towards the CD4+ Thelper 1 cell type.

Also, treatment with (PEG-)IFN-α changes the composition of specific T-cell populations in peripheral blood. During treatment, patients who responded to (PEG-)IFN-α therapy by reaching sustained clearance of HBeAg and undetectable plasma HBV DNA within 1 year of initiating treatment, had a significant rise in their CD4/CD8 ratio due to a decrease in peripheral CD8 numbers[22]. A favorable viral and immunological response was associated with an increase in frequency of new HBe- and HBcAg specific CD4+ T-cells and a decrease of regulatory T-cells (Tregs) characterized by CD4+CD25+Foxp3-positivity[25-29]. However, it was unclear whether this increase in HBV specific T-cells also led to an increase in virological response leading to viral control. In HBeAg negative patients with genotype D, a less favorable group for (PEG-)IFN-α treatment, 24 weeks of (PEG-)IFN-α therapy did not improve CD4+ and CD8+ T-cell responses (no improvement of IFN-y production after

longitudinal ex vivo analysis) despite a small increase in HBV specific T-cells[30]. Data on T-cell function from other patient cohorts with more favorable host and viral characteristics is currently lacking.

In contrast to findings in peripheral blood, data from studies examining the intrahepatic T-cells frequencies have shown less convincing results with respect to the effects of (PEG-)IFN-α[31-33]. Although the intrahepatic CD4+ T-cells numbers did not change during (PEG-)IFN-α therapy, the numbers of CD8+ T-cells varied between studies during (PEG-)IFN-α therapy[31-33]. For example, Tang et al. demonstrated an increase in intrahepatic CD8+ T-cells after initiation of (PEG-)IFN-α, in the liver of HBeAg positive patients[32]. Other studies found a decrease of intrahepatic CD8+ T-cells at the end of (PEG-)IFN-α therapy (up to 52 weeks), which was associated with an increase in plasma IL-10 level[31, 33]. The latter finding could be explained by a possible modulatory effect of (PEG-)IFN-α therapy on antigen presenting cells (APCs) which resulted in inhibitory effects on lymphocytes with the purpose to reduce tissue damage[33].

Although several hypothesis exist on the failure of (PEG-)IFN-α therapy, to date studies have only described data on two mechanisms which might be associated with failure of (PEG-)IFN-α therapy being increased regulatory T-cells (Tregs) and core mutations in the HBV virus. Sprengers et al. suggested that Tregs might be related to an unsuccessful outcome of (PEG-)IFN-α therapy. Their study in 14 HBeAg positive patients found that treatment failure after one year of therapy was related to an elevated level of Tregs compared to low numbers in responder patients[34]. The underlying mechanism how Tregs increase during (PEG-)IFN-α treatment remains to be resolved, however their cytokine production with anti-inflammatory properties probably result in a diminished T-cell response.

Finally, although there is limited evidence available, mutations within the core region of the HBV virus could lead to viral escape mutations[35]. This subsequently could impair T-cell recognition and loss of T-cell responses resulting in unsuccessful treatment with (PEG-)IFN-α[35]. In the study by Naoumov et al., the precore/core region of responders and non-responders on (PEG-)IFN-α therapy was analysed. Response was defined as achieving anti-HBe positive, loss of HBV DNA and normalization of serum aminotransferase levels. All non-responders had substitutions in amino acids 21-27 of the core protein, while none of the responders had these substitutions[35]. Another previously published study suggesting impaired T-cell recognition because of HBV core protein mutations illustrated that substitutions in this region were associated with a 10-20 fold reduced binding affinity of CD8+ T-cells[36].

All together, (PEG-)IFN-α therapy on the one hand enhances the pro-inflammatory components of the T-cell response and on the other hand dampens the anti-inflammatory elements resulting in a better immunological control of HBV. Furthermore, Tregs and viral escape mutations are linked to failure of (PEG-)IFN-α therapy.

Nucleoside and nucleotide analogues

Next to (PEG-)IFN-α therapy, the nucleoside and nucleotide analogues (NUC) are the other class of drugs recommended for the treatment of hepatitis B. At present, five NUCs have been approved for the treatment of patients with chronic hepatitis B being lamivudine (LAM), adefovir (ADV), entecavir (ETV), tenofovir (TDF) and telbivudine (LdT)[37]. Unlike (PEG-)IFN-alfa, NUCs do not have direct immunomodulatory capacities to alter the T-cell response. Their mechanism of action is by interfering with the viral DNA polymerase and act as chain terminators resulting

in inhibition of viral replication as already reviewed by others[38]. In general, inhibition of HBV replication will lead to decreased antigen stimulation of specific T-cells which results in improved T-cell function including T-cell proliferation and cytokine production, as will have an effect on T-cell population distribution[39, 40]. Also, a lower viral load requires less T-cells for an adequate viral control[39, 40].

Treatment with NUCs has an effect on the proliferation of HBV-specific T-cells[39-42]. In an early Italian study, it was already shown that LAM treatment was associated with an enhancement of both HBV-specific T-cell proliferation and function[39, 40]. This finding was already observed within 2 weeks after initiation of LAM therapy, with frequencies that were similar to those of patients with acute self-limiting infection[39]. In more recent studies, these observations of enhanced T-cell proliferation and function were also confirmed with ETV and LdT[41, 42]. However, the moment at which T-cell proliferation occurred, differed between the NUCs. Although all NUCs achieved a rapid decline in viral burden after initiation of therapy, T-cell proliferation started around 12 weeks with ETV and LdT, which was much later compared to LAM[39, 41, 42]. Nevertheless, this difference in moment of T-cell proliferation does not influence clinical outcomes within the first years of treatment, i.e. HBeAg or HBsAg seroconversion rates are similar for LAM when compared to LdT and ETV[43, 44].

Another indirect consequence of NUC therapy on the T-cell response is a change in T-cell populations, as given in Table 1. The most obvious changes were observed in the CD4+ T-cell counts and the Tregs counts. Besides TDF, which is not well studied, all NUCs increased the number of CD4+ T-cells[40, 41, 45-50]. In contrast, the number of Tregs, characterized by CD25 and FoxP3 expression, were low in patients on the majority of NUCs[51-56]. With regard to the numbers of 2 other major T-cell populations, the CD8+ T-cells and the CD4+ Thelper 17 (Th17+) cells, the overall effect of NUCs

is less evident. Th17 cells are a subset of pro-inflammatory CD4+ T-cells that activate neutrophils by producing interleukine-17 (IL-17) and are associated with viral clearance as well as liver cell injury in HBV infection as already reviewed by others[57]. For example, while LdT increased CD8+ T-cell numbers, they decreased during LAM, ADV and ETV therapy[41, 47, 58]. Also, the number of Th17+ T-cells, has been described to be variable during NUC therapy[51, 53, 59]. It is unclear whether the presence of Th17+ T-cells could be beneficial in controlling the virus or that they ultimately could harm the liver due to increased inflammation. Thus, NUC therapy indirectly changes the T-cell populations mainly with suppression of Tregs and stimulation of CD4+ T-cells leading to a shift in immunological balance towards a pro-inflammatory T-cell response. The overall effect of both NUC and (PEG-)IFN on the T-cell response is shown in Figure 1.

Finally, NUC therapy also increases the T-cell functionality by indirectly altering the production of cytokines. After up to 1 year of treatment, ADV, ETV and LdT increased pro-inflammatory cytokines, such as IL-2, TNF-α, IFN-y on the one hand and decreased anti-inflammatory cytokines like IL-10 and inhibitory molecules on the T-cell membrane like programmed death 1 (PD-1)[48, 54, 56].

Thus, NUC therapy indirectly affects the T-cell response in different manners with a net effect in skewing of the immune balance towards the proinflammatory pathway. However, despite these effects, which are comparable to the immunological effects of (PEG-)IFN-α, the rate of viral clearance (HBeAg and HBsAg seroconversion) is lower than with (PEG-)IFN-α suggestion that other factors might also play a role in immunological control of HBV.

Combining (PEG-)IFN-α therapy with NUCs

While current therapy strategies in hepatitis B are mainly focused on single agent therapy, one may consider that the combination of NUCs with their antiviral qualities together with the immunomodulatory activities of (PEG-)IFN-α might be more effective in inhibiting viral replication and restoring the T-cell response leading to increased immunological control. To date, only a few studies, with small sample sizes, using the combination of (PEG-)IFN-α and LAM and one study with (PEG-)IFN-α and TDF have addressed the effect of dual treatment on the T-cell function in chronic HBV patients.

As expected, all studies showed a rapid decline of HBV DNA in both HBeAg positive as well as HBeAg negative patients within the first weeks of combination therapy with LAM and (PEG-)IFN-α[60-62]. The rates of patients achieving an undetectable HBV in these studies ranged from 42% to 100%[60, 61]. During combination therapy, a HBcAg-specific proliferative T-cell response (especially of CD4+ T-cells) was detected[60, 62]. However, when LAM was discontinued, there was an increase of HBV DNA in the majority of patients[60]. Also, the observed HBcAg-specific T-cell response was temporary and disappeared at the end of combination therapy[60]. Furthermore, when LAM and (PEG-)IFN-α combination therapy was compared to (PEG-)IFN-α monotherapy in HBeAg positive patients, there were no overall significant differences between the two therapy regimens with respect to viral clearance/ control, although in one study a subgroup analysis suggested an increase in HBeAg seroconversion in patients on combination therapy[61, 63].

Thus, in these small studies with short follow-up periods that are mainly conducted in HBeAg positive patients, despite a rapid improvement in viral suppression, combination therapy does not seem to work synergistically in the restoration of T-cell

function. Even when T-cell responses are observed, it is highly questionable whether they additionally contribute to viral clearance and viral control.

Conclusion & future perspective

Chronic hepatitis B in adults is characterized by T-cell dysfunction resulting in viral persistence. Studies describing the influence of antiviral therapy on the T-cell indicate that antiviral therapy might positively affect the T-cell response. On the one hand they might achieve this by reducing inhibitory stimuli, like blocking HBV DNA replication and altering the numbers of Tregs. On the other hand they might improve pro-inflammatory impulses that indirectly affect the T-cell function, like improving the T-cell in producing pro-inflammatory cytokines.

In the future, it would be interesting to explore the effect of combination therapy with (PEG-)IFN-α therapy plus tenofovir or entecavir on the T-cell response. While in other virus infections, combination therapy has proven to be more effective than monotherapy, in HBV, this strategy to date does not seem more effective in improving the T-cell function compared to monotherapy. The optimal strategy and duration of NUC and (PEG-)IFN-α combination therapy on clinical outcomes has to be determined. Several studies examining this issue are still ongoing. It will be interesting to await immunological data of these studies.

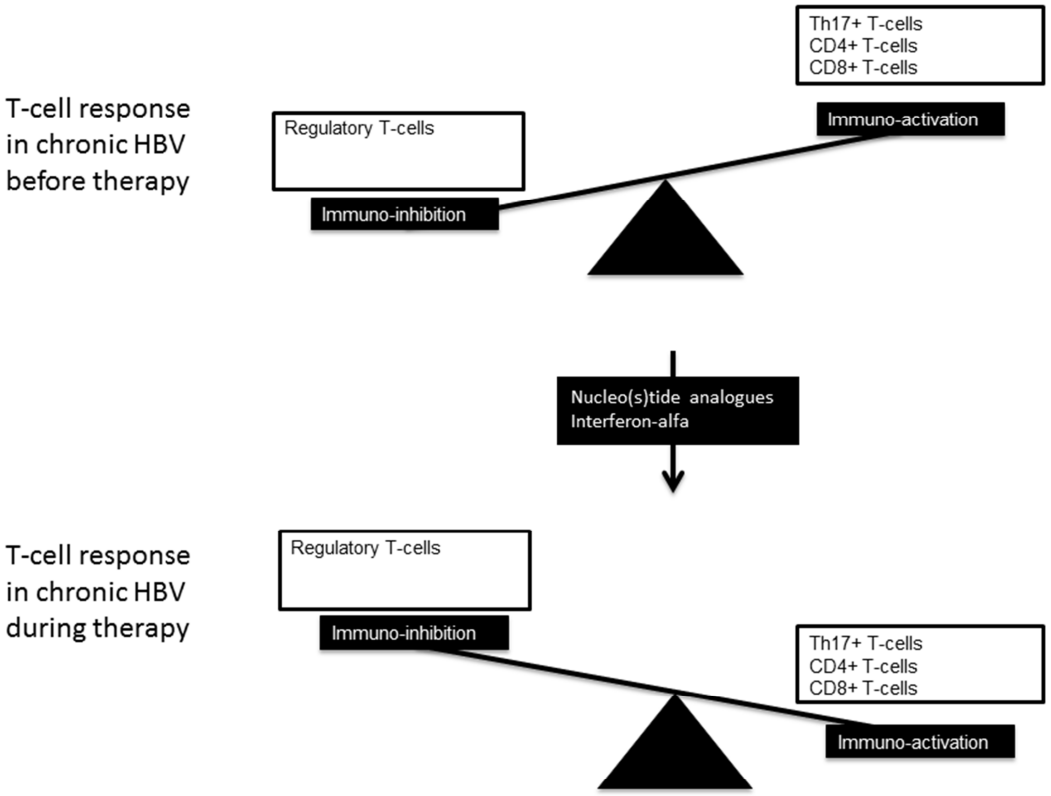
To gain more insight into the mechanisms how antiviral therapy and HBeAg status affects the T-cells on the long term further studies with serial measures and a prolonged follow-up period are needed. Also, the relationship between the restoration of the T-cell function and the ultimate goal of antiviral therapy, i.e. anti-HBs seroconversion, remains to be unraveled.

Table 1. Proliferation of different T-cell subsets during HBV therapy

	T-cell populations			
	CD4+	CD4+Treg *(CD25+Foxp3+)*	CD4+Th17	CD8+
Antiviral therapy (NUC)				
LAM *Ref: 39, 40, 53*	↑	↓		↓
LdT *Ref: 47, 59*	↑	↓	↓	↑
ADV *Ref: 45, 53, 54, 55, 58*	↑	↓	↑	↓
ETV *Ref: 41, 51, 53, 54*	↑	↓	↑	↓
TDF *Ref: 56*		↓		↑
Immunomodulatory therapy (IFN)				
(PEG-)IFN-alfa *Ref: 20, 23, 27*	↑	↓		↑ or ↓

Drugs divided in antiviral therapy and immunomodulatory drugs. ↑ represents increase in frequency of T-cell population, ↓ represents decrease in frequency of T-cell population. NUC= nucleos(t)ide analogue, LAM= lamivudine, LdT= telbivudine, ADV= adefovir, ETV= entecavir, TDF= tenofovir, IFN= interferon, (PEG-)IFN-alfa= (pegylated-)interferon-alfa.

Figure 1. T-cell response before and during therapy



Balance between immuno-inhibition and immuno-activation T-cell response in chronic hepatitis B before antiviral therapy and change in balance during antiviral therapy. Th17+ T-cells = T helper 17 cells, CD4+ T-cells= cluster of differentiation 4 positive T helper cells, CD8+ T-cells= cluster of differentiation 8 positive T helper cells.

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Chapter 7

Renal proximal tubular dysfunction due to tenofovir in a patient with chronic hepatitis B mono-infection: case report and review of the literature

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Abstract

Tenofovir (TDF) is increasingly used as first line therapy in chronic hepatitis B virus (HBV) infection. While TDF use in HIV patients can be complicated by development of renal proximal tubular acidosis (rPTA), this is seldom reported in HBV mono-infected patients. We describe a case of TDF associated rPTA in a patient with HBV monoinfection along with an overview of the literature.

Introduction

Currently two groups of drugs, the interferon-alfa's and the nucleos(t)ide analogues (NUCs), have been registered for treatment of patients with chronic hepatitis B virus infection (HBV). Within the group of NUCs, tenofovir (TDF) and entecavir (ETV) are recommended as first line treatment by international guidelines, because of an effective viral suppression and good tolerability[1, 2]. NUC therapy improves the long-term course and outcome of chronic HBV mono-infection. For instance, in a large multicenter cohort study, treatment with TDF has shown to reverse fibrosis and cirrhosis in chronic HBV mono-infected patients, regardless of HBeAg status[3]. The advantage of TDF over ETV is that so far no viral resistance has been documented in chronic HBV mono-infected patients treated with TDF.

Next to its antiviral properties against HBV, TDF is also active against HIV, wherein it is an important component of combination antiviral therapy (cART) in the treatment of HIV patients. Previous studies in HIV patients have clearly shown that TDF therapy can induce renal proximal tubular acidosis (rPTA)[4, 5]. However, this complication has rarely been reported in HBV mono-infected patients during TDF therapy[6-9]. We here report a case of a chronic HBV patient who developed rPTA during TDF treatment and summarize previous data.

Case

A 55-year-old Caucasian male with HBeAg (HIV negative) negative chronic HBV (initial HBV DNA level $2.0x10^5$ IU/mL, determined by an in house realtime PCR) was diagnosed and referred to our hospital in 1999. His past medical history was unremarkable. Previously, he had been treated with interferon-alfa for sixteen weeks in 1999 resulting in primary non-response. From 2001 onwards he received

lamivudine (100mg once a day) mono-therapy which was stopped in 2004 because of non-response with persistent elevated transaminases and no decline in HBV DNA level ($1.9x10^7$ IU/mL before start and $8.2x10^7$ IU/mL at the end). Although development of lamivudine resistance was suspected, this was not genotypically tested. In 2004 lamivudine was switched to adefovir (10 mg once a day) leading to normal transaminases and a partial virological response (HBV DNA declined to $9.5x10^3$ IU/mL two years after adefovir was started). In 2007, lamivudine was again prescribed in addition to adefovir based on studies suggesting that "add on" of adefovir to lamivudine was associated with better maintenance of virological suppression and less adefovir resistance in HBV patients with lamivudine resistance than replacement of lamivudine by adefovir[10, 11]. Subsequently, complete virological response was achieved (HBV DNA <200 IU/mL) and in 2008 he experienced HBe-seroconversion (HBeAg and anti-HBe determined by Abbott AxSYM assays (Abbott Diagnostics, Abbott Park, IL, USA)). A liver stiffness measurement (Fibroscan, Echosens, Paris, France) at that time showed no significant fibrosis (6.0 kiloPascal, F0-F1 fibrosis). Since the patient retained an undetectable HBV DNA after HBe-seroconversion for more than one year, lamivudine and adefovir were discontinued in accordance with international guidelines[1]. During adefovir treatment, he had developed a slight but stable renal impairment with a creatinine between 100 and 123 µmol/L (between 1.13 mg/dL and 1.39 mg/dL, reference value 64–104 µmol/L or 0.72–1.18 mg/dL) which was 83 µmol/L (0.94 mg/dL) before initiation of adefovir. Unfortunately, in subsequent months, after cessation of the antiviral drugs, both HBV DNA and ALT levels increased to $1.1x10^4$ IU/mL and 87 U/L (reference value 0–45 U/L) respectively. Therefore, it was deemed necessary to resume antiviral therapy. Since the patient had been treated twice with lamivudine and resistance to

lamivudine was a concern, TDF was considered the treatment of choice. Before initiation of TDF, his creatinine level was 115 µmol/L (1.30 mg/dL) with an estimated glomerular filtration rate (eGFR) above 60 mL/min/1.73m^2 (reference value more than 90 mL/min/1.73m^2). During routine follow-up after 3 months, HBV DNA level became undetectable (HBV DNA limit of detection 200 IU/mL) again and ALT normalized. However, he developed a persistent hypophosphatemia (initial serum phosphate level before TDF 0.87 mmol/L (2.69 mg/dL) which declined to 0.55 mmol/L (1.70 mg/dL) during treatment (reference value 0.80–1.50 mmol/L or 2.48–4.64 mg/dL)) with a further deterioration of kidney function (creatinine level 123 umol/L (1.39 mg/dL) and an eGFR of 57 mL/min/1.73m^2) as shown in figure 1. Additional plasma biochemical evaluation revealed a hypouricemia (0.18 mmol/L, reference value 0.30–0.50 mmol/L), normal bicarbonate (26.2 mmol/L, reference value 23.0–29.0 mmol/L) and normal glucose level (5.0 mmol/L, reference value 3.6–5.6 mmol/L). Urine-analysis showed proteinuria (0.51g/L, reference value 0.01–0.14 g/L) and uric acid of 3.0 mmol/L without glucosuria. A 24-hours collection of urine revealed a phosphate level of 39.5 mmol/24h (reference value 12.9–42.0 mmol/24h), a beta-2-microglobelinemia of 11.7 mg/24h (reference value 0.2 mg/24h), a creatinine of 12.0 mmol/24h (reference value 9.0–18.0 mmol/24h) and negative glucose screening. The patient had normal serum calcium and vitamin D levels, and had never been exposed to heavy metals such as cadmium and copper. An additional dual-energy x-ray absorptiometry (DXA scan) showed osteoporosis of the lumber spine (T-score -3.3 and Z-score -2.9, reference value T-score ≥ -1.0 and Z-scores ≥ -2.0) and osteopenia of the hip (T-score -2.0 and Z-score -1.6). No renal biopsy was performed. Based on these findings, and after exclusion of other causes for renal insufficiency such as multiple myeloma, he was diagnosed with rPTA due to TDF. Entecavir therapy was

considered unattractive because of earlier presumed lamivudine resistance. Since there was a sustained virological response and patient responded well to sodium phosphate treatment, TDF was continued under intensive monitoring of phosphate and creatinine levels. Currently, his electrolytes and phosphate levels are normal, his creatinine remains slightly elevated but stabile (between 108 and 117 µmol/L, 1.22 and 1.32 mg/dL) and he still has an undetectable viral load with normal transaminases.

Discussion

TDF is becoming more prominent in the treatment of HBV mono-infected patients. Its indication in HBV mono-infected patients is further extending, illustrated by the latest international guideline where TDF is now also recommended during pregnancy rather than lamivudine which for long was the only treatment advised in this special population[2]. Although it has been well documented in HIV patients that TDF can cause rPTA, data on HBV mono-infected patients with TDF are very limited. So far, including our case, the association between TDF-related rPTA and HBV mono-infection has been reported in eight patients[6-9]. A summary of patient characteristics of all reported patients in the literature (including ours) is given in Table 1.

In one of the reported HBV mono-infected patients in which renal biopsy was performed during TDF treatment there were profuse large mitochondria in the proximal tubules cells resembling those seen in HIV-infected patients with TDF induced rPTA[7]. Histologically, TDF related changes in the tubular cells can be reflected by widespread morphological abnormalities in the mitochondria of the proximal tubules which are also enlarged[12-15]. Previously, it has already been demonstrated in HIV patients that rPTA is a consequence of accumulation of TDF in

the mitochondria of renal proximal tubular cells[12]. In HIV patients, two theories have been proposed why TDF could accumulate in the proximal tubular cell. First, since HIV patients are usually treated with multiple antiretroviral drugs, there is a distinct possibility that drugs could interact at the level of the nephron which could cause toxicity. For example, didanosine, a nucleotide reverse transcriptase inhibitor, shares the same organic anion transporter with TDF at the basolateral side of the proximal tubules cell and can interact with TDF to potentiate renal toxicity[16, 17]. Also ritonavir, a protease inhibitor, can cause TDF accumulation in the proximal tubule by blocking the multidrug resistance-associated protein 2 (MRP2) on the apical side of the proximal renal tubules cell and thereby inhibiting TDF secretion[18]. Other protease inhibitors, such as lopinavir and atazanavir, have also been associated with TDF-related tubular dysfunction[19, 20]. Second, in HIV patients there is some evidence that genetic variations in a gene of a proximal tubule transporter might be associated with the development of rPTA. Polymorphisms in this ABCC2 gene, which encodes the multidrug resistance transporter 2 (MRP2) protein at the apical proximal tubular cell has been associated with development of rPTA during TDF treatment[21]. Until now, one HBV mono-infected patient has been reported who exhibited a CC polymorphism in the ABCC2 gene[8]. It remains to be seen whether similar to HIV patients, this genetic susceptibility is also a relevant factor for rPTA development in HBV mono-infected patients. Moreover, HBV mono-infected patients differ from HIV patients in that they generally are not exposed to multiple (nephrotoxic) drugs with exception of those who had earlier treatment with adefovir, as in our case.

In HIV patients, age, low body weight, pre-existing impaired renal function and nephrotoxic medication are established risk factors for development of rPTA[22]. In HBV mono-infected patients reported until now several observations could be made

which may be potential risk factors for development of rPTA. Of all eight reported HBV cases, seven were older than 50 years with the majority being Caucasian males. Also, five patients were pretreated with adefovir and two patients with inhibitors of the renin-angiotensin system blocking therapy which are known to alter the renal function. However, except for our patient, none of the patients had a pre-existing impaired renal function.

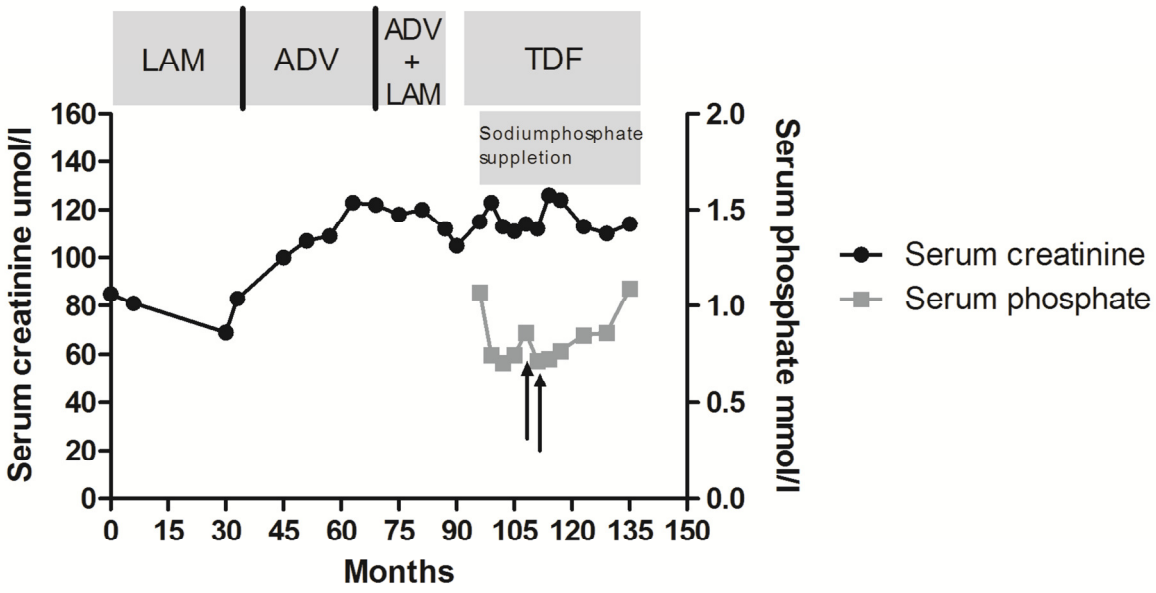
Our patient was diagnosed with osteoporosis under TDF therapy. This was also observed in other HBV mono-infected patients who were treated with TDF[8]. Several reports in HIV patients with TDF therapy have shown that TDF can cause a hypophosphatemic osteomalacia[23, 24]. This was also observed in a HBV mono-infected patients during treatment with adefovir[25]. However, it unclear whether TDF related hypophosphatemia is associated with osteoporosis. In a study with 146 Asian-American HBV mono-infected patients who were treated with either TDF or ETV for 18 months, those treated with TDF turned out to have a higher prevalence of hypophosphatemia without an accompanying higher prevalence of osteopenia or osteoporosis compared to ETV[26]. It remains to be seen whether hypophosphatemic HBV mono-infected patients on long term maintenance TDF could be at increased risk for osteoporosis.

In conclusion, rPTA should be considered in patients with chronic HBV mono-infection during TDF treatment when hypophosphatemia develops. Especially, older patients who have been pre-treated with adefovir or other potentially nephrotoxic medication, those for years exposed to TDF and those with pre-existing nephropathy are at increased risk of rPTA development and should be closely monitored.

Table 1. Comparison between reported cases of rPTA in HBV mono-infected patients

	Case	Age *(years)*, sex	Ethnic origin	HBeAg	Initial HBV-DNA (IU/mL)	Fibrosis Stage (METAVIR)	Previous HBV therapy	Comedication	Years on TDF	Before TDF		During TDF		Intervention	Outcome
										Serum Creatinine (umol/l)	Serum phosphate (mmol/l)	Serum Creatinine (umol/l)	Serum phosphate (mmol/l)		
Gara et al. 2012	1	62, M	NM	NM	NM	NM	None	NM	3.9	NM	NM	NM	NM	Switch to ETV	Favorable
	2	66, M	NM	NM	NM	NM	ADV	NM	3.7	NM	NM	NM	NM	Switch to ETV	Favorable
Gracey et al. 2013	3	39, M	Asian	Negative	1.1x10E5	F1	ADV	Telmisartan	7.0	95	1.0	150	0.6	Switch to ETV	Favorable
	4	54, M	Caucasian	Negative	6.4x10E6	F2	None	None	2.0	94	±0.9[b]	135	±0.7[b]	Switch to ETV	Favorable
Vigano et al. 2014	5	58, M	Caucasian	Negative	<12	NM	ADV	None	2.5	79	0.84[c]	114	0.69[c]	Switch to ETV	Favorable
	6	62, M	Caucasian	Negative	1.22x10E5	NM	None	Beta-blocker, ACE-inhibitor	3.8	80	0.84[c]	296	0.55[c]	Switch to ETV	Favorable
Samarkos et al. 2014	7	87, M	NM	NM	NM	F4	ADV+LAM	NM	NM	NM	NM	NM	NM	Stop TDF	Unfavorable
Harkisoen et al. 2014	8	55, M	Caucasian	Negative	1.9x10E7[a]	F0-F1	IFN-a/LAM/ADV +LAM	None	0.4	115	0.87	123	0.51	Continu TDF, sodium phosphate suppletion	Favorable, Stable renal function

Figure 1. Time course of serum creatinine (black line with round dots) and serum phosphate (gray line with gray squares) before and during TDF treatment.



Black arrows represent adjustment in sodiumphosphate dose. ADV= adefovir, LAM= lamivudine, TDF= tenofovir

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Chapter 8

General discussion and future perspectives

Introduction

Since the discovery of the hepatitis B virus (HBV) almost five decades ago, there has been a growing interest in its pathogenesis and natural history. Although progress has been made, many issues remain unsolved. For instance, it is unknown how the virus is able to bypass the immune system upon infection in a subset of patients (i.e. evading spontaneous clearance) and silently invades the liver where it starts replicating. Also, it is still not fully understood why HBV can cause fibrosis in some patients and not in others.

Physicians have for long tried to discover a suitable treatment for patients with chronic HBV, but progress was made in 1976 when Greenberg et al. showed that intravenous interferon-alfa was able to produce a rapid fall in all Dane-particles in three chronic HBV patients[1]. After the official approval of interferon-alfa in 1992, and later the nucleos(t)ide analogues (NUCs), physicians have been able to alter the disease course of chronic HBV patients thereby improving their prognosis[2].

Although the pathogenesis and clinical course of chronic HBV is increasingly being unraveled, there are still many aspects that need to be further clarified. In 2009 the idea of this thesis emerged when shortly before two studies of the REVEAL-HBV group had been published showing a clear positive association between HBV DNA level at any given time and the occurrence of HBV-related liver disease[3, 4]. Although these results were evident leading to changes in international treatment guidelines, questions arose whether these findings were applicable to chronic HBV populations other than those of Asian descent. For it was already known that several aspects during the course of chronic HBV, such as the transmission route and genotype, differed between Asian chronic HBV patients and non-Asian populations. Although between 2009 and now many articles have been published in the field of hepatitis B,

the main topic of this thesis still remains unanswered. Therefore, the primary aim of this thesis focuses on the natural course of non-Asian chronic HBV patients evaluating factors associated with cirrhosis and HBsAg loss.

HBV DNA and liver cirrhosis in chronic hepatitis B

During the course of chronic hepatitis B several immunological phases can be identified as given in figure 1. One of the main problems during chronic HBV which has not been clarified is what triggers the immunotolerant phase to transform into the immune reactive phase in which the immune system tries to achieve HBe seroconversion and control the virus. A possibility might be that this process is initiated by HBV itself. Although HBV is believed to be non-cytopathic, evidence suggests that caspase-induced apoptosis is involved in this process of transition.

HBV can induce apoptosis of hepatocytes, in part mediated by HBx (with several pleiotropic actions and also involved in HCC development) which is able to activate the caspase pathway through activation of the TNF-α related apoptosis-inducing ligand (TRAIL) signaling pathway[5-9]. Following interaction with the TRAIL receptor, cytoplasmatic different executory caspases (caspase 3, 6 and 7) become activated resulting in hepatocyte cell death[10-13].

Although the exact trigger for this apoptotic mechanism initiating transition from immune tolerant to immune reactive phase is still unknown, it is becoming more clear that hepatic apoptosis forms a crucial inflammatory impulse[14]. At the same time, T-cells try to control HBV by clearance of HBeAg and activation of B-cells to form anti-HBe antibodies[15, 16]. This process, which constantly occurs at a variable intensity, activates hepatic stellate cells to produce fibrotic tissue[17]. The progressive

replacement of damaged liver cells by fibrous tissue, which occurs at a subclinical level, leads to liver fibrosis and ultimately cirrhosis.

It is unclear why some patient with chronic HBV develop fibrosis and cirrhosis while others do not. In our study, 12% of the women had developed cirrhosis (**chapter 2**) and in the REVEAL-HBV study 10% of the included patients developed cirrhosis over a period of many years[4]. The findings from our study, illustrate that differences in lifestyle could be in part be responsible, since there were more patients who consumed alcohol in the group with F3-F4 liver fibrosis compared to the group F0-F2 fibrosis. Also, the intensity and duration of the immune response with subsequent liver damage might play a role in fibrosis progression. A prolonged period of the immune reactive phase or frequent hepatic flares in HBeAg negative patients could be responsible for the fact that some patients have more liver fibrosis than other patients.

As discussed in **chapter 2** of this thesis, there are now several studies demonstrating HBV DNA at any given time is a risk factor for the development of liver complications, while our study opposes this finding[3, 4, 18-20]. This contradiction emphasizes that the natural course of chronic HBV can differ between different ethnic groups and apart from HBV DNA other factors are also of influence in the prediction of liver cirrhosis. First, this difference is explained by the global genotypic distribution between different ethnic groups. The majority of Asian patients who have been included in the REVEAL-HBV studies were infected with HBV genotype B or C[3]. Both genotypes are associated with HBV-related liver complications, with genotype C having a five times more risk of developing HCC compared to other genotypes[20]. This more fibrogenic potential of genotype C is explained by the association of a prolonged immune reactive phase with higher HBV DNA levels and ALT levels, delayed HBeAg

seroconversion and active disease in the HBeAg negative phase (compared to genotype B)[21, 22]. Therefore, in Asian HBV patients a combination of genotype and HBV DNA could cause this HBV-related liver disease, while in patients with other genotypes, HBV DNA does not relate with development of cirrhosis. Finally, another factor which has become more clear in the past few years and which was shown to protect patients from progression to liver cirrhosis is liver steatosis as seen in patients with the metabolic syndrome. In the REVEAL-HBV study population, a low HBV DNA was associated with HBeAg positive men who were extremely obese or had central obesity and HBeAg negative men and women who had hypertriglyceridemia[23]. The patients from our cohort could largely be classified as overweight according to the current World Health Organization (WHO) definition based on body mass index[24]. They also had relatively low HBV DNA levels compared to the Asian HBV patients. Although other factors related to metabolic syndrome were not analyzed in our studies, this may have been of importance in the association between HBV DNA level and liver cirrhosis in our cohort. Our observations are supported by other studies confirming the inversely association between steatosis hepatis or triglyceride level and HBV infection[23, 25].

The relationship between chronic HBV and obesity is largely unknown, but androgen levels have been implicated since high androgen levels have been positively associated with viral transcription and high HBV DNA levels[26]. While not fully understood, this may on the one hand be driven by HBx that has been shown to induce the expression of androgen receptor in liver cells in Asian chronic HBV patients with HCC[27]. In vitro and mouse models have proposed that HBx may enhance the androgen receptor activation by interfering in two cytosolic kinases activity[28]. On the other hand the variations in the length of the CAG repeats on exon

1 of the androgen receptor gene, might be another route in which androgen levels and the viral activity during chronic HBV may be related[26]. Therefore, obese patients low androgen levels and short CAG repeats could lead to lower HBV DNA levels, which in turn leads to lower disease progression.

Another hypothesis that might explain the low HBV DNA levels in obese patients is through the inhibition of transcription factors which are able to activate both the glucose metabolism and HBV transcription. A major metabolic regulator and a coactivator of key gluconeogenic genes is peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α). PGC-1α is able to coactivate HBV transcription and increase HBV replication in vitro experiments[29-31]. This could explain why patients with a low BMI may have higher HBV DNA levels.

To unravel the interaction between liver steatosis and liver fibrosis progression is important for two reasons. First, the percentage of people worldwide suffering from obesity is high and still increasing. Second, metabolic syndrome (i.e. abdominal obesity) has clinical implications for the assessment of liver fibrosis in chronic HBV patients. In the past few years, several non-invasive methods have been developed to assess liver fibrosis and cirrhosis in patients with chronic liver diseases. One of these methods is liver stiffness measurement (LSM). With this method, liver fibrosis (and if present, the severity of fibrosis) can be estimated with a specialized transcutaneous ultrasound transducer. Several studies in different chronic liver diseases have shown that LSM is accurate to distinguish between no and mild fibrosis (F0/F1 versus F2) and patients with severe fibrosis and cirrhosis (F3/F4 fibrosis)[32]. Previous studies have shown that LSM is less reliable and often leads to failure of measurements in obese patients[33, 34]. For example, in our cohort we have also seen that in 26% of HBV patients, LSM could not produce valid results due to a

high BMI. This has led to a search for serum markers to detect fibrosis such as the AST to Platelet Ratio Index (APRI), fibrosis-4 (FIB-4) score or the ELF-test.

Identification of liver cirrhosis in patients with chronic hepatitis B

For long, liver biopsy has been the gold standard for evaluation of liver fibrosis in chronic HBV patients as well as patients with other chronic liver diseases. However, liver biopsy has its disadvantages since it is patient unfriendly, has a risk of complications such as bleeding and infection[35, 36] and there is the possibility of sampling error or an inconclusive outcome if the liver specimen does not fulfill the required criteria for evaluation (i.e. a specimen with a length of at least 1.5 cm with at least six portal tracts[37]). These shortcomings have led to a quest for alternative methods to evaluate the fibrosis stage. As a result, several tests have been developed with algorithms, in which various blood tests were incorporated as biomarkers, to predict whether liver fibrosis and/or liver cirrhosis was present in patients with chronic liver diseases. The two most popular tests, the APRI score and FIB-4 score, are only moderately able to detect significant fibrosis in chronic HBV patients which was illustrated in a recent meta-analysis (APRI sensitivity 70% and specificity 60% to detect significant fibrosis; FIB-4 sensitivity 65% and specificity 74% to detect significant fibrosis)[38]. An alternative score to predict liver fibrosis is the enhanced liver fibrosis test (ELF-test). This test, developed in 2004 by Rosenberg et al., based on an algorithm of three serum markers from constituents of extracellular matrix and enzymes involved in fibrosis and fibrolysis, had already earned its value to identify severe fibrosis in patients with chronic hepatitis C and patients with non-alcoholic fatty liver disease (NAFLD)[39-42]. One study reported that 81% of the biopsies could be avoided by using the ELF-test[40]. However, in chronic hepatitis B

patients, there are little data regarding the accuracy of the ELF-test[43]. Therefore, in **chapter 3**, the diagnostic value of the ELF-test has been evaluated in non-Asian chronic HBV patients. The results of this study showed that compared to LSM, the accuracy of the ELF-test to identify patients with severe fibrosis in non-Asian patients was poor. The strength of the ELF-test lies in the interplay between the high turnover of the extracellular matrix and the disease activity of the liver disease[41]. If there is an increased rate of hepatic cell necrosis with inflammation, this results in a higher production of extracellular matrix components[44]. However, chronic HBV is associated with a lower rate of fibrosis development compared to chronic hepatitis C[45]. This might explain why the ELF-test is less accurate in chronic HBV patients compared to other chronic liver diseases.

Another possibility why the ELF-test may not always reflect the fibrosis stage in chronic HBV patients is the variable course of chronic HBV. In patients with an active hepatitis, i.e. elevated ALT levels and high HBV DNA level, the ELF-test seems to have a higher predictive value. This was demonstrated in two studies in Italian and Asian HBeAg positive patients in which the Area under the Cure (AUC) of the ELF-test was 0.86 to detect F4 fibrosis if ALT levels were elevated[43, 46]. In our cohort, all patients were HBeAg negative with a normal ALT level and low HBV DNA levels which reflect a more stable and low inflammatory state. In this condition, the ELF-test poorly discriminates between severe fibrosis/cirrhosis versus milder forms of liver fibrosis. Another way to illustrate the close relationship between disease activity during chronic HBV and the extracellular matrix is by looking at HBsAg loss. In **chapter 4** we showed that hyaluronic acid levels, a major component of the ELF-test, decrease after HBsAg loss when compared to patients who remain HBsAg positive. This may be the result of a diminished inflammatory response after HBsAg loss. This

assumption is supported by liver biopsies of patients who experienced HBsAg loss showing that there was a significant improvement of necroinflammation[47, 48].

Altogether, the variable course of disease activity and inflammation of chronic HBV may affect the reliability of certain serum markers to detect fibrosis and/or cirrhosis in non-Asian chronic HBV patients.

Influence of antiviral therapy on T-cell immunity during chronic hepatitis B

The immune system plays an important role in the control and elimination of HBV. Early studies have already shown that a strong and multi-specific T-cell response against different HBV antigens is able to clear HBV during an acute infection[49, 50]. These findings have also been observed in patients with chronic HBV who spontaneously clear the infection after certain years of chronic infection[51]. Therefore, it is essential to understand how antiviral therapy may affect the T-cell response which is summarized in **Chapter 6**. During antiviral therapy there is a shift from a immune controlling anti-inflammatory T-cell response in which regulatory CD4+CD25+ T-cells (Tregs) dominate the immune response towards a pro-inflammatory T-cell response in which pro-inflammatory CD4+ and CD8+ T-cells dominate the immune response[52-56]. This is directly induced by (pegylated-)interferon (PEG-IFN) which, similar to hepatitis C[57], could activate IFN stimulating genes resulting in production of pro-inflammatory cytokines and chemokines and leading to increased frequencies of proinflammatory CD4+ and CD8+ T-cells[52-56]. Furthermore NUCs can indirectly change the T-cell response by interfering with the viral replication, by blocking the HBV DNA polymerase, which reduces the viral burden, improves pro-inflammatory CD4+ T-cell responses and decreases the frequencies of anti-inflammatory regulatory T-cells (Tregs)[58-61]. However, despite the promising

effect of antiviral therapy on the T-cell function, current antiviral therapy only achieves a low success rate. The success rate to achieve HBe seroconversion during PEG-IFN-α2, entecavir or tenofovir mono-therapy is 32%, 21% and 21% respectively[62-64]. The success rates to achieve HBs seroconversion for both PEG-IFN and NUCs are even lower (under 10%), regardless of HBeAg status[63, 65, 66].

Although it is not fully understood why antiviral therapy exhibit such a low efficacy, several possible explanations may be given. One explanation for this low success rate may rely on the ability of the virus to mutate and reduce its susceptibility to antiviral therapy. Naoumov et al. have demonstrated that patients who did not respond to PEG-IFN-α2, had a higher rate of mutations in the precore/core region of the virus[67]. Since HBeAg is an immunogenic antigen[16] and the precore/core region is responsible for HBeAg production, mutations in this area could provide the virus an opportunity to avoid the improved T-cell response which is accomplished by PEG-IFN. In all NUCs (except tenofovir in which it is not yet documented), mutations in the virus at certain nucleotide segments of the DNA where the NUC exhibits its action, it can reduce the efficacy of the NUC. For instance, viral resistance rates are high during lamivudine therapy, where mutations within the virus can occur in up to 70% of the patients after 5 years treatment[68]. This probably impairs the function of pro-inflammatory T-cells again and shifts the balance back to the predominant anti-inflammatory immune system.

Another possible explanation for the low efficacy of antiviral therapy is that antiviral therapy does not sufficiently improves the T-cell function, since it cannot optimally overcome processes like T-cell exhaustion, to reach a strong response which is needed to overcome the anti-inflammatory immune response and control and eliminate the virus. Data from non-responders to PEG-IFN-α treatment showed a

progressive rise of Tregs during treatment or a rebound of Tregs after discontinuation of therapy compared to patients who responded to therapy[56, 69]. Therefore, it could be speculated that in some patients treatment success of PEG-IFN-α therapy cannot be reached due to the inability to correct the strong inhibition of the immune response.

In the past, PEG-IFN-α2 has been combined with NUCs (mainly combination with lamivudine) to examine whether combination therapy could improve T-cell function and achieve higher rates of HBe seroconversion[70-73]. In theory, combining these agents may have a benefit over monotherapy, since both the inhibition of viral replication by NUCs and improvement of T-cell function by IFN could synergistically improve the T-cell at a level that it could be able to achieve a sustained viral control. During combination therapy an undetectable HBV DNA could be reached between 42 and 100% (in one study defined as HBV DNA < 400 Eq/ml, in the other study defined as < 3pg/ml) with an increase in HBV-specific T-cell response[70, 71]. HBe seroconversion in these studies were 33% and 32% during combination therapy, but after discontinuation of treatment, in one study all patients and in the other study half of the patients who experienced HBe seroconversion became HBeAg positive again (i.e. HBe seroreversion)[70, 71]. Moreover, after discontinuation of lamivudine, the HBV DNA rebounded and the T-cell response declined to levels before start of therapy. Moreover, the overall treatment outcome was not better than PEG-IFN-α2 monotherapy or lamivudine monotherapy[70]. Therefore, the results of combination therapy up to recently were disappointing.

Interestingly, recent publications have again sparked the interest in combination therapy, albeit now with the newer NUC entecavir combined with PEG-IFN-α. Enomoto et al. treated 24 Japanese HBeAg positive patients (95% male and 95% genotype C) sequentially with entecavir alone for 36-52 weeks, followed by entecavir

plus PEG-IFN-α for 4 weeks, and lastly by PEG-IFN-α alone for 20 weeks[74]. With this regime, 29% achieved a sustained HBe seroconversion defined as loss of HBeAg and formation of anti-HBe. Two randomized clinical trials combined PEG-IFN-α with entecavir and compared it to entecavir monotherapy[75, 76]. A Chinese randomized controlled trial in 218 patients examined PEG-IFN-α for 48 weeks, either as monotherapy (group A), or with 24 weeks of entecavir added at week 13 (group B), or pretreatment with a 24-week course of entecavir, starting PEG-IFN-α at week 21 (group C)[76]. The HBe seroconversion rates were comparable between all groups (group A 31%, group B 25% and group C 26%, p= 0.07). A second randomized controlled trial in a multi-ethnic chronic HBV study population, patients started on entecavir monotherapy and were randomized in a 1:1 ratio to either PEG-IFN-α add-on therapy from week 24 to 48, or to continue entecavir monotherapy[75]. Add-on therapy had a higher but non-significant HBe seroconversion rate compared to entecavir monotherapy (31% versus 20%, p=0.11).

Two possible reasons may explain why the combination of entecavir with PEG-IFN-α was not superior to entecavir monotherapy. First, genotype could be a major determinant of treatment success in the combination group. Brouwer et al. showed that compared to genotype D, there was a trend that patients with genotype A had a higher rate of treatment success, while patients with genotype B and C had lower of success rates (genotype A odds ratio (95% confidence interval) 2.1 (0.3-14.1) versus genotype B OR 0.6 (0.1-2.9) and genotype C 0.3 (0.1- 1.2), p=0.23)[75]. However, these differences were not statistically significant. Second, the HBV DNA level before adding PEG-IFN-α could also be of importance for treatment success, because patients with lower HBV DNA levels before the start of PEG-IFN had a higher change of treatment success[75].

Since all three studies, with different treatment strategies did not improve overall HBe seroconversion rate, alternative approaches have to be considered. Similar to HIV and hepatitis C, triple therapy seems a reasonable next approach for chronic HBV patients. Two approaches with triple therapy could be interesting. One approach could be to treat patients with triple therapy, such as entecavir plus tenofovir (which exhibit their actions on different reverse transcriptase domains of HBV polymerase[77]) and PEG-IFN-α and after 48 weeks of therapy discontinue PEG-IFN-α. Another approach, with the theoretical knowledge of the T-cell response mentioned above, could be to start with two NUCs with the rational to reach an optimal suppression of the viral replication and once an undetectable HBV DNA has been achieved, the T-cell response could be stimulated by adding a 48 week course of PEG-IFN-α. This is currently investigated in the Dutch PADD-study of which the results are expected in 2016. Alternatively, initiating therapeutic vaccination upon HBV-DNA undetectability, after NUC pretreatment, seems a viable strategy to investigate in the future.

Next to alternative treatment regiments, it would be interesting to further investigate how the combination of PEG-IFN with the newer NUCs may affect T-cell response and alter the frequencies and function of T-cell subsets. Since newer NUCs are virologically more potent than older NUCs in inhibition of viral replication, the T-cell restoration may be more pronounced during therapy. Also, nowadays with the introduction of immunotherapy, such as monoclonal antibodies, which can alter the T-cell function in different ways, it might be a promising field to examine whether the combination of NUCs and/or PEG-IFN with these newer drugs can produce higher (more favourable) success rates.

Timing of antiviral therapy in chronic HBV patients

Currently, antiviral therapy is indicated in patients with an active hepatitis B disease defined as elevated ALT (more than 3x ULN) high HBV DNA levels and signs of liver fibrosis in histological or non-invasive tests)[78, 79]. However, with increasing knowledge in the behavior of the virus, it is questionable whether this combination of criteria is still suitable for the management of patients with chronic HBV. Current guidelines are focusing on the reduction of liver injury (and progressive fibrosis) from active hepatic inflammation and clearance of HBsAg. Some authors believe that HBs seroconversion might be a big step towards the 'cure' of chronic HBV[2]. Therefore, persistent HBsAg seroclearance is now generally accepted as a novel and more desirable end point of therapy than real seroconversion. The newest European guideline even states that in HBeAg negative patients who have attained HBs seroconversion for at least 12 months during NUC therapy may be candidates for stopping NUC treatment. However, it is questionable whether the clearance of HBsAg will also cure patients from HBV complications. On the one hand, there is evidence that HBV DNA can still be found in the liver after HBsAg loss and patients can still be at risk for HBV reactivation in times of severe immunosuppression[80, 81]. On the other hand, HBV DNA can be incorporated into host DNA making patients still at risk to develop HCC after HBsAg seroconversion[82, 83].

Recently, the treatment of immune-tolerant chronic HBV patients has become a hot topic since several studies implicated that antiviral therapy may reduce the risk of HCC development[84-87]. Opponents of early therapy in immune-tolerant patients have two major concerns. First, early treatment in the immune tolerant phase might reduce chances of HBeAg seroconversion based on one retrospective study in Asian HBV patients[88]. In this study, within the group of entecavir treated HBeAg positive patients with a high HBV DNA, patients with a baseline ALT level more than 2 times upper

limit of normal (ULN) had more HBe seroconversion compared to patients with a baseline ALT level below 2x ULN (26% versus 8%, p-value not reported)[88]. Another concern from opponents of early treatment is that the immunotolerant patients have no or insignificant liver fibrosis and therefore prolonged treatment would be unnecessary with only risk for development of viral resistance and adverse events[89] . However, since in Asian patients there is a strong positive relationship between HBV DNA level and HCC independent of HBeAg status[3], an important long-term outcome of antiviral therapy is the prevention of liver complications. Studies have shown that entecavir or tenofovir treatment is associated with reduction in risk of HCC[84-86] and therefore it should be advocated to treat certain chronic HBV patients in the immune tolerance phase who have a higher risk of developing HCC, such as Asian patients with genotype C.

However, there are currently two major limitations of NUC therapy that impede the applicability in immune tolerant patients such as the low success rate and the infinite duration of therapy to control HBV infection. Additionally, NUC discontinuation can trigger HBV reactivation which is observed in 9% of the patients who have achieved therapy induced HBe seroconversion[90]. Given these shortcomings, first efforts must be made to increase the success rate of therapy before early treatment outweighs the shortcomings in immune tolerant patients to let them benefit from antiviral therapy. Also, an ideal moment when to start antiviral therapy in immunotolerant patients' needs further exploration. The quantitative HBsAg level, as a new virological parameter, might be of help in this setting. Previously, quantitative HBsAg level, in combination with HBV DNA, could distinguish which patients in the carrier phase were at risk for HBV reactivation[91]. With the expanding diagnostic and clinical value of quantitative HBsAg, it could be possible that similar algorithms with quantitative

HBsAg in combination with other parameters, such as HBV DNA and genotype, may provide information about the timing of initiation of antiviral therapy in eligible immune tolerant patients.

Monitoring of viral activity in chronic HBV patients

In **chapter 2** both the HBV DNA level and the quantitative HBsAg level were examined in relation to occurrence of liver cirrhosis in non-Asian patients. For long HBV DNA has been the only virological parameter to monitor the viral activity of HBV. However, with the arrival of the quantitative HBsAg level, as diagnostic assay to monitor the natural course and treatment response in chronic HBV patients, it is time to rethink whether HBV DNA is still useful for clinical practice. In the natural course of HBV, together with ALT levels and HBeAg status, HBV DNA was able to distinguish the different immunological phases of the infection with the highest levels during the immunotolerant phase and the lowest levels during the HBsAg carrier phase. Similar to HBV DNA, quantitative HBsAg also shows a dynamic course during the different immunological phases with higher levels (between 4 and 5 $\log_{10}$IU/ml) during the immunotolerance and immunoclearance phase (HBeAg positive) phase and lower levels (between 2 and 3 log(10)IU/ml) during the inactive carrier phase[92-96]. In HBeAg negative patients with genotype A to E, quantitative HBsAg level in combination with HBV DNA is able to discriminate between true inactive carriers (HBsAg level < 1000 IU/mL and HBV DNA < 200 IU/mL) and those at risk for reactivation (HBsAg level > 1000 IU/mL and HBV DNA level > 200 IU/mL)[91]. Since levels of HBV DNA are more pronounced between different phases of chronic HBV compared to quantitative HBsAg, HBV DNA is more preferable than HBsAg as virological marker in this setting. However, combinations of HBV DNA and quantitative HBsAg may be

valuable to identify patients in the transition from one phase to another. Similar to the distinction between true HBsAg carriers and patients at risk for HBV reactivation, it is worth rethinking how this combination of HBV DNA and quantitative HBsAg could distinguish the timing when immune tolerant patients will go into the immune reactive phase.

During the treatment of HBV patients, quantitative HBsAg seems a promising diagnostic tool. Sonneveld et al. have elegantly demonstrated in HBeAg positive patients how genotype and quantitative HBsAg level at week 12 and week 24 after initiation of PEG-IFN-α2 aids the decision to continue or stop PEG-IFN-α2 therapy[97]. For instance, if patients with genotype A had a quantative HBsAg level <1500 IU/mL at week 24 after PEG-IFN-α2 therapy, they had a 86% chance of achieving response (defined as HBeAg loss and HBV DNA level < 2000 IU/mL), while patients with genotype D with a quantitative HBsAg level <1500 IU/mL at week 24 had 0% chance of treatment response[97]. Moreover, they showed that patients who had a quantitative HBsAg level > 20,000 IU/mL at week 24, independent of genotype, had a minimal chance of achieving HBsAg loss during PEG-IFN-α2 treatment[97]. For NUC therapy, this has to be further explored, but it seems likely that a similar quantitative HBsAg guided therapy will be used to direct future therapy. Furthermore, in the future quantitative HBsAg could further help to define more accurate starting points, in combination with HBV DNA and ALT level, when to initiate antiviral therapy in selected patients who may have higher chances to achieve treatment success compared to current criteria to start therapy. Also, contrary to starting points of therapy, another possible use of quantitative HBsAg might be to identify patients who will not benefit from neither NUCs nor PEG-IFN-α, such as patients eligible for

spontaneous HBsAg clearance, and treatment should be withheld to prevent unnecessary harm of therapy.

With the results of our thesis in mind, quantitative HBsAg level alone does not seem to be predictive for the occurrence of cirrhosis in non-Asian chronic HBV patients. However, since clinical algorithms are more and more being used in the management of chronic HBV patients, combination of HBsAg level with other HBV- or host-specific factors in specific algorithms, may be used in future in this setting.

Thus, although quantitative HBsAg is a promising tool in the monitoring of treatment in chronic HBV patients, it will not replace the clinical value of HBV DNA within the coming years. A combination of both quantitative HBsAg and HBV DNA will be able to help the physician in clinical decision making and further improve the management of chronic HBV patients.

Future perspectives

Nowadays there is a trend towards a tailor-made treatment in medicine. For instance, in clinical oncology, several methods, such as gene-expression profiling tests, are being developed to provide detailed information about tumor characteristics to adjust the chemotherapeutic regimen for achievement an optimal treatment responses possibly reduced chance of toxicity[98]. With the results of this thesis in mind, together with the growing insight of the behavior of HBV, it is more and more becoming evident that such a tailor-made treatment would also be appropriate (and inevitable) in chronic HBV patients. Although such a treatment has already been used in chronic HBV to select candidates for PEG-IFN-α treatment, in the future this will also be used in the selection of patients eligible for NUC treatment or combination therapy. Within this individualized approach, patient characteristics, such as body mass index and

waist circumference as well as quantitative HBsAg and HBV genotype will have a more pronounced place. In the near future, quantitative HBsAg will establish its place in the monitoring of PEG-IFN-α treatment and in a later stage also for the monitoring during NUC therapy in both HBeAg positive and HBeAg negative patients. Quantitative HBsAg will have a prominent place in the starting, monitoring and stopping of PEG-IFN-α and possible stop of NUC therapy.

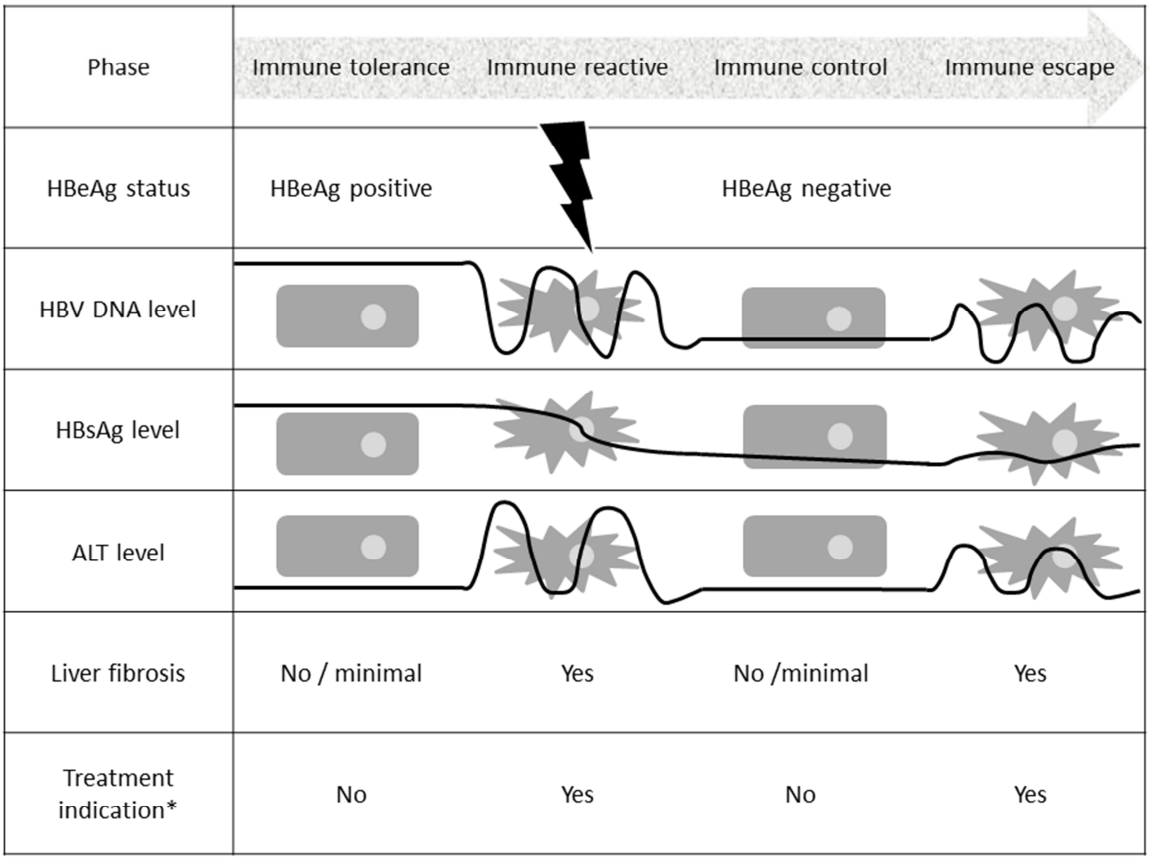
Furthermore, experiences in other fields, such as HIV and HCV have taught us that a multidrug regime improves the efficacy of treatment and results in better treatment outcomes. In chronic HBV, up to now international guidelines have mainly focused on single drug therapy regimens since the clinical outcomes were comparable with combination therapy with two drugs[78, 79]. In the future, multiple drugs regimes consisting of three or more drugs may be necessary to achieve more desirable treatment outcome. Moreover, results of newer agents in the pipeline, such as ARC-520 which is a liver-targeted RNA interfering therapy, designed to control the viral activity of HBV by hampering its replication and HBV-related protein production through interfering with the produced viral RNA (although it has no effect on cccDNA) and is now being tested in a phase II study in combination with entecavir, are eagerly awaited and their role in triple drug regimens will have to be evaluated.

Finally, next to challenges regarding treatment in the future, the quest for an ideal test or combination of tests to predict liver fibrosis or HBsAg loss continues. Based on what is already known from the literature and the findings in **chapter 4**, serum hyaluronic acid may be a potential biomarker which might be of diagnostic value on the one hand to identify chronic HBV patients at risk for liver fibrosis and on the other hand patients eligible for spontaneous HBsAg loss. Also, combination of biomarkers with imaging techniques such as liver stiffness measurement or magnetic resonance

elastrography may further reduce the indication for liver biopsy in clinical decision making.

While a few giant steps have been made up to now in the understanding of the natural course of chronic HBV and identification of patients at risk for liver complications, there is still a road ahead to further optimize the management of chronic HBV patients. However, with the high efficacy of vaccination regimes to prevent HBV infection combined with the ongoing development of better treatment options for chronic infected patients, in the future there is hope that HBV may be eradicated within a century from the moment of its discovery.

Figure 1. Schematic presentation of the different immunological phases during the natural course of chronic hepatitis b.



Schematic cells drawn in different phases represent liver cells. HBeAg = hepatitis B e-antigen, HBV DNA= hepatitis B virus Deoxyribonucleic acid, HBsAg= hepatitis B surface antigen, ALT= alanine aminotransferase. *= treatment indication according to current AASLD guideline[77]

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Chapter 9

Summary

Introduction

Hepatitis B (HBV) is a virus that is transmitted via blood-blood contact, and leads to inflammation of the liver. From its discovery in 1965 up to now, it has had a major impact worldwide. Approximately one-third of the world's population has been infected with HBV. Of these people, it is estimated that 240 million are chronically infected of which about 60,000 patients are located in the Netherlands.

The inflammation of the liver causes liver damage and loss of hepatocytes which is replaced by fibrotic tissue. If the inflammation persists, this subsequently leads to hepatic fibrosis and ultimately liver cirrhosis resulting in severely impaired liver function. Moreover, chronic HBV patients have an increased risk of developing hepatocellular carcinoma (HCC). These conditions negatively influence the prognosis of chronic HBV patients. About 15 to 25% of adult chronic HBV patients who are perinatally infected with hepatitis B will eventually die as a consequence of the diseases.

The transition from liver inflammation to liver fibrosis and cirrhosis in chronic HBV patients is slow and takes several decades. Also, it is a process that generally remains asymptomatic. This explains why patients often consult a doctor in an (advanced) stage of liver cirrhosis. Due to the liver complications of HBV, it is important that patients who are at risk of developing liver cirrhosis and HCC are early identified.

In Asian chronic HBV patients, viral load (the amount of hepatitis B virus per millilitre of blood) at a random moment in time was predictive for the development of liver cirrhosis and HCC. However, it is not known whether the hepatitis B viral load in non-Asian chronic HBV patients also is predictive for liver cirrhosis development. This has been the basis for the studies described in this thesis.

In **chapter 2 and 3** studied, within a cohort of non-Asian women with chronic hepatitis B, whether there was a correlation between viral load and the occurrence of liver cirrhosis and the diagnostic value of the ELF-test to detect liver cirrhosis. Next, **chapter 4** describes the serum hyaluronic acid level between patients who remain persistent HBsAg positive compared to those who have cleared the virus (who achieved HBs seroconversion). **Chapter 5** explores the impact of ethnicity on the quality of life of chronic HBV patients. Finally, while **chapter 6 and 7** provide a summary of the literature on the effects of anti-viral therapy on HBV-specific T-cells in chronic HBV patients and an overview of the occurrence of a resorption disorder in the kidney (Fanconi syndrome) due to tenofovir, one of the most widely used drugs in the treatment of chronic HBV patients.

Liver cirrhosis and hepatitis B

Since liver cirrhosis has such a negative influence on the course of chronic HBV patients, it is important to identify risk factors for development of liver cirrhosis, especially factors that can be modified by antiviral therapy against HBV. While in Asian chronic HBV patients there is a positive association between hepatitis B viral load and the development of liver cirrhosis, it is unclear whether this also applies to chronic HBV patients of non-Asian origin. The natural history of HBV infection differs in Asian patients compared to non-Asian patients in some aspects. First, Asian patients are mainly infected with hepatitis B during birth or in early childhood, while in other parts of the world, for example in North West Europe, infection occurs at later age when people are sexually active. There are also differences in HBV genotype between Asians and non-Asians. Differences in genotypes occur if the DNA of two hepatitis B viruses are distinctive for at least 8%. Asian patients have genotype B and

C, whereas in other parts of the world other genotypes are more prevalent, like for example, A and D in Europe. Finally, HBe seroconversion occurred in Asian patients slower and at a later age than in non-Asian patients. HBe seroconversion means that the immune system has an immune response against one of the proteins of the hepatitis B virus, the HBeAg protein, with the formation of antibodies (anti-HBe). HBe seroconversion results in a decrease of viral replication. These differences in the natural course of HBV, might explain the results in **chapter 2** that hepatitis B viral load was not predictive for the occurrence of liver cirrhosis in non-Asian women. Furthermore, after a long follow-up only a small number of patients developed cirrhosis. A possible explanation could be that the included women had little HBV reactivation after HBe seroconversion. Finally, a relatively newer determination, the quantitative measurement HBsAg, which can reflect the amount of intracellular HBV DNA in hepatocytes, was also not predictive for the development of cirrhosis of the liver.

Besides the detection of patients who have an increased risk of developing liver cirrhosis, it is also important to improve the detection of liver fibrosis and cirrhosis in HBV patients. To date, a liver biopsy is the gold standard in this determination. However, obtaining a liver biopsy is an invasive procedure which is patient unfriendly and not without risks of complications. Therefore, it is important to look for alternatives for assessment of the fibrosis stage. One alternative to a liver biopsy is measurement of the scar tissue in the liver by a fibroscan. This technique has shown to reliably discriminate between people with and without liver fibrosis and among people between mild and severe fibrosis (and cirrhosis). Another alternative to determine the rate of liver fibrosis is through serum markers. The ELF-test (enhanced liver fibrosis test) is a blood test in which three substances in the serum, the

hyaluronic acid, the procollagen III amino-terminal peptide (PIIINP), and the tissue inhibitor metalloproteinase 1 (TIMP-1) could determine if someone has liver fibrosis with the severity of fibrosis. **Chapter 3** evaluates the diagnostic value of the ELF test in chronic HBV patients. Our research showed that the ELF test, in relation to Fibroscan, poorly predicted the occurrence of cirrhosis. The low disease activity in our study population may be a possible explanation for the descripancy between our findings an other publications showing a good association between ELF-test and liver fibrosis.

Spontaneous HBsAg clearance

Next to the identification of liver cirrhosis, it is also important to investigate whether HBV patients are able to clear the virus by themselves. Clearance of HBV is considered to be achieved if HBs seroconversion occurs, which is characterized by the disappearance of HBsAg and formation of antibodies (anti-HBs). Individuals who achieve HBs seroconversion have a favourable prognosis with a reduced risk of liver cirrhosis (if not yet occurred) and HCC. Annually, this is achieved in about two percent of all chronic HBV patients worldwide. To date, it is difficult to predict which patients will experience HBs seroconversion. In **chapter 4** it is investigated whether there is an association between serum hyaluronic acid and HBsAg loss. Patients who are HBsAg negative appear to have lower serum hyaluronic acid levels than patients who remain HBsAg positive. Serum hyaluronic acid is found to be the only factor that is associated with loss of HBsAg in the study population. Based on these results serum hyaluronic seems an interesting test for the future which could provide more insight into the natural history of HBV patients.

Quality of life and hepatitis B

Chronic illnesses have an important influence on the quality of life. Ethnicity could also influence quality of life. In some chronic diseases, such as asthma, it has already been described that ethnicity can influence the quality of life in these patients. Therefore, it is interesting to examine the role of ethnicity in HBV patients. This has led to the study described in **chapter 5**. In this study, the difference in quality of life was explored between Turkish, Ghanaian, Surinamese and Moroccan chronic hepatitis B patients since these were the major ethnic groups in the COBRA study. Turkish HBV patients were found to have a significantly lower quality of life in comparison with the other ethnic groups, while in Ghanaian HBV patients quality of life was significantly higher compared to the other ethnicities. With these results in mind, it could be concluded that ethnicity was a factor that negatively influences quality of life in HBV patients. This may result in more medical care consumption in ethnic groups with a low quality of life compared to others.

Effects of antiviral therapy

Currently, there are two groups of antiviral drugs for the treatment of HBV patients, (gepegyleerd-)interferon-alpha (PEG-IFN-α) and nucleos(t)ide analogues (NUC). Antiviral therapy can reduce liver inflammation and inhibit the formation of liver fibrosis. Moreover, in the past years it is demonstrated that antiviral therapy could reduce fibrosis in HBV patients who have liver fibrosis or cirrhosis. The immune system, and particular the HBV-specific T-cell, seems to play an important role in the disease course of HBV patients. **Chapter 6** gives an overview of the literature how antiviral therapy affects T-cells. PEG-IFN-α enhances the HBV-specific T-cell response by changing the composition of different T-cell populations, with an

increase in the number of pro-inflammatory HBV-specific CD4 + T-cells during treatment, while the anti-inflammatory T-cells, such as the regulatory T-cells (Tregs), decline during treatment. During NUC therapy, the HBV-specific T-cell response is indirectly influenced by inhibiting the viral burden. The T-cell, which is impaired by the high viral load, then has the ability to recover and achieve better viral control.

Besides the positive effects of antiviral therapy, there are also disadvantages to antiviral therapy such as bone marrow toxicity during PEG-IFN-α therapy a the lifelong duration of NUC therapy in most patients. Tenofovir is a NUC which is often used as the first choice in the treatment of HBV patients. It has both antiviral effects against HBV and human immunodeficiency virus (HIV). In HIV patients, it is already known that tenofovir may cause damage in the kidney by causing proximal tubular dysfunction (Fanconi syndrome). In HBV patients, this side effect has rarely been reported. In **chapter 7**, the reported literature on Fanconi syndrome in HBV patients is summarized. Here it is concluded that if a low level of phosphate develops in the blood during tenofovir treatment, Fanconi syndrome should be suspected, especially in patients over 50 years, those with pre-existing renal insufficiency or those receiving ACE-inhibitors or adevofir (another antiviral drug against HBV).

Conclusions of this thesis

1. In non-Asian patients, both hepatitis B viral load and quantitative HBsAg are not associated with the development of liver cirrhosis
2. The ELF test poorly identifies liver cirrhosis in HBV patients
3. Serum hyaluronic acid levels are lower at HBsAg negative patients compared to HBsAg positive patients and also is able to predict HBsAg clearance
4. Ethnicity is a factor which negatively affects the quality of life in HBV patients

Chapter 9

Nederlandse samenvatting

Introductie

Hepatitis B virus (HBV) is een virusinfectie die via bloed-bloed contact wordt overgedragen en zich kenmerkt door een ontsteking van de lever. Vanaf het moment waarop het virus in 1965 werd ontdekt, heeft het wereldwijd een grote impact gehad. Naar schatting hebben één op de drie mensen wereldwijd ooit een infectie met HBV doorgemaakt. Hiervan heeft ongeveer 240 miljoen een chronische infectie waarvan in Nederland ongeveer 60.000 patiënten met chronische HBV besmet zijn.

Door de ontsteking van de lever treedt er leverschade op en gaan levercellen kapot. Als reactie op de schade wordt er littekenweefsel gevormd in de lever. Als de ontsteking persisteert, gaat er steeds meer leverweefsel verloren en vindt er steeds meer verlittekening van de lever (leverfibrose) plaats. Als dit proces aanhoudt, zoals bij chronische HBV patiënten, kan er op den duur levercirrose optreden waardoor de werking van de lever ernstig wordt aangetast. Naast de verhoogde kans op levercirrose, hebben chronische HBV patiënten ook een verhoogd risico op het ontwikkelen van een vorm van leverkanker, hepatocellulair carcinoom (HCC) genaamd. Door de verhoogde kans op het krijgen van deze aandoeningen hebben patiënten met chronische HBV een slechtere prognose. Ongeveer 15 tot 25% van de volwassen chronische HBV patiënten die tijdens hun jeugd zijn geïnfecteerd met hepatitis B zullen uiteindelijk overlijden ten gevolge van leverfalen of leverkanker.

De overgang van leverontsteking naar leverfibrose en levercirrose bij chronische HBV patiënten is een langzaam proces wat enkele decennia kan duren. Bovendien is het een proces dat veelal geen symptomen geeft. Dit verklaart waarom patiënten zich vaak pas presenteren bij een arts in een (vergevorderd) stadium van levercirrose. Het is daarom van belang om patiënten met chronische HBV vroeg op te sporen om zo het risico op het ontwikkelen van levercirrose en HCC te verkleinen.

Bij Aziatische chronische HBV patiënten is aangetoond dat de hepatitis B virale lading (hoeveelheid hepatitis B virus per milliliter bloed) op een willekeurig moment in de tijd voorspellend is voor het ontwikkelen van levercirrose en HCC. Het is echter niet bekend of de hepatitis B virale lading bij niet-Aziatische chronische HBV patiënten ook voorspellend is voor levercirrose. Dit is de basis geweest voor de onderzoeken die worden beschreven in dit proefschrift.

In de **hoofdstukken 2 en 3** wordt bij een groep van niet-Aziatische vrouwen met hepatitis B enerzijds het verband tussen virale lading en het voorkomen van levercirrose bestudeerd en anderzijds de diagnostische waarde voor het opsporen van levercirrose via de ELF-test. Daarna wordt in **hoofdstuk 4** bestudeerd of het serum hyaluronzuur verschilt tussen patiënten die nog een chronische infectie hebben ten opzichte van personen die het virus hebben geklaard (ookwel HBs seroconversie genoemd). Vervolgens wordt in **hoofdstuk 5** de invloed van etniciteit op de kwaliteit van leven van chronische HBV patiënten beschreven. Ten slotte geven **hoofdstuk 6 en 7** een overzicht van de literatuur over de effecten van antivirale therapie op de T-cel van chronische HBV patiënten en een overzicht van het voorkomen van een opnamestoornis in de nier (Fanconi syndroom) ten gevolge van tenofovir, een van de meest gebruikte medicijnen bij de behandeling van chronische HBV patiënten.

Levercirrose en hepatitis B

Omdat levercirrose het leven van chronische HBV patiënten nadelig kan beïnvloeden, is het belangrijk om risicofactoren te identificeren die kunnen leiden tot levercirrose, vooral factoren die kunnen worden beïnvloed door anti-HBV

behandeling. Hoewel bij Aziatische chronische HBV patiënten een positieve associatie is gevonden tussen de hepatitis B virale lading en ontwikkeling van levercirrose, is het de vraag of dit ook voor chronische HBV patiënten van niet-Aziatische oorsprong geldt. Bij Aziatische patiënten verschilt het natuurlijk beloop van een HBV infectie met die van niet-Aziatische patiënten op een aantal punten. Aziatische patiënten worden hoofdzakelijk geïnfecteerd met hepatitis B tijdens de zwangerschap en geboorte of in de vroege kinderjaren, terwijl in andere delen van de wereld, bijvoorbeeld in Noordwest Europa, besmetting met name optreedt tussen 20 en 30 jaar als mensen seksueel actief zijn. Ook zijn er verschillen in HBV genotype tussen Aziaten en niet-Aziaten. Verschillen in genotypen ontstaan als het DNA van twee hepatitis B virussen voor tenminste 8% van elkaar verschillen. Bij Aziaten komen met name genotype B en C voor terwijl in delen van de wereld andere genotypen voorkomen, in Europa bijvoorbeeld meer genotype A en D. Tenslotte, treedt HBe seroconversie bij Aziatische patiënten langzamer en later op dan bij niet-Aziatische patiënten. HBe seroconversie betekent dat het afweersysteem een immuunreactie tegen één van de eiwitten van het hepatitis B virus, het HBeAg eiwit, heeft opgewekt en hierbij zijn er antistoffen tegen dat eiwit (anti-HBe) gevormd. HBe seroconversie zorgt voor een afname in de vermenigvuldiging van het virus (virusreplicatie). Deze verschillen in natuurlijk beloop verklaren mogelijk het in **hoofdstuk 2** gevonden resultaat chronische HBV dat de hepatitis B virale lading niet voorspellend is voor het optreden van levercirrose in niet-Aziatische vrouwen. Opvallend in onze onderzoekspopulatie was dat er na bijna twee decennia sinds de diagnose hepatitis B er maar een klein aantal patiënten zijn die levercirrose hebben ontwikkeld. Een mogelijke verklaring hiervoor is dat er in de geïncludeerde patiënten weinig HBV reaktivaties hebben plaatsgevonden. Ook een relatief nieuwere bepaling,

de kwantitatieve HBsAg meting, welke een maat voor het hoeveelheid HBV DNA in de levercellen zou kunnen weergeven, bleek niet voorspellend te zijn voor het ontwikkelen van levercirrose.

Naast het opsporen van patiënten die een verhoogd risico hebben op het ontwikkelen van levercirrose, is het ook belangrijk om de diagnostiek naar leverfibrose en levercirrose bij HBV patiënten te verbeteren. Tot op heden is een leverbiopt de gouden standaard voor het vaststellen van leverfibrose en/of levercirrose bij chronische HBV patiënten. Echter, het verkrijgen van een leverbiopt is een belastend onderzoek welke niet patiëntvriendelijk is en niet zonder risico op complicaties is. Daarom is het belangrijk om te zoeken naar alternatieven om informatie over het fibrose stadium te krijgen. Eén alternatief voor een leverbiopt is het echografisch meten van het littekenweefsel in de lever door middel van een fibroscan. Met deze techniek is het bij mensen met chronische leverziekten gebleken dat er goed onderscheid kan worden gemaakt tussen mensen met en zonder leverfibrose en tussen mensen met milde of ernstige fibrose (en levercirrose). Een ander alternatief is het bepalen van de mate van leverfibrose door middel van bloedtesten. De ELF-test (enhanced liver fibrosis test) is een bloedtest waarbij met behulp van drie stoffen in het serum, het hyaluronzuur, de procollageen III aminoterminaal peptide (PIIINP) en de weefselremmer metalloproteinase 1 (TIMP-1) kan worden vastgesteld of iemand leverfibrose heeft en hoe ernstig de fibrose is. In **hoofdstuk 3** is de diagnostische waarde van de ELF-test onderzocht bij chronische HBV patiënten. Uit het onderzoek bleek dat de ELF-test slecht voorspelde of er sprake was van levercirrose (de fibroscan was hierbij het referentieonderzoek). Een mogelijke verklaring hiervoor zou kunnen zijn dat bij de onderzochte HBV patiënten weinig ziekteactiviteit was.

Spontane HBsAg klaring

Behalve het opsporen van levercirrose is het ook belangrijk om te onderzoeken of HBV patiënten in staat zijn om het virus uit zichzelf te klaren. Klaring van HBV wordt gezien als het bereiken van HBs seroconversie. Hierbij verdwijnt het HBsAg eiwit van het virus en worden antistoffen (anti-HBs) gevormd. Personen die HBs seroconversie bereiken hebben een gunstige prognose waarbij het risico op levercirrose (indien nog niet opgetreden) en HCC sterk is verlaagd. Jaarlijks wordt dit bij ongeveer twee procent wereldwijd bereikt in de groep van chronische HBV patiënten. Tot op heden is het moeilijk te voorspellen welke patiënten HBs seroconversie zullen bereiken. In **hoofdstuk 4** wordt onderzocht of er een associatie is tussen het serum hyaluronzuur en HBsAg verlies. Patiënten die HBsAg negatief zijn blijken lagere serum hyaluronzuurwaarden te hebben dan patiënten die HBsAg positief blijven. Het serum hyaluronzuur blijkt de enige factor te zijn die is geassocieerd met HBsAg verlies in de studiepopulatie. Met deze resultaten lijkt serum hyaluronzuur een interessante test voor de toekomst die meer inzicht zou kunnen geven in het natuurlijk beloop van HBV patiënten.

Kwaliteit van leven en hepatitis B

Een chronische ziekte heeft een grote invloed op de kwaliteit van leven. Ook etniciteit zou hier een rol bij kunnen spelen. Bij sommige chronische ziekten, zoals astma, is eerder gebleken dat etniciteit een rol speelt bij de kwaliteit van leven van deze patiënten. Daarom is het interessant om ook bij HBV patiënten te onderzoeken wat de rol van etniciteit is op de kwaliteit van leven. Dit heeft geleid tot het onderzoek welke is beschreven in **hoofdstuk 5**. In onze studiepopulatie werd met name gekeken naar verschillen tussen Turkse, Ghanese, Surinaamse en Marokkaanse

chronisch hepatitis B patiënten omdat zij de belangrijkste etniciteiten vormden van de COBRA-studie. Turkse HBV patiënten bleken een significant lagere kwaliteit van leven te hebben in vergelijking met de andere bevolkingsgroepen, terwijl van Ghanese HBV patiënten de kwaliteit van leven significant hoger was ten opzichte van de andere etniciteiten. Hieruit werd geconcludeerd dat etniciteit wel een factor was die de kwaliteit van leven bij HBV patiënten nadelig zou kunnen beïnvloeden. Dit zou er toe kunnen leiden dat HBV patiënten van bepaalde etnische origines, welke meer geassocieerd zijn met een lagere kwaliteit van leven, vaker medische zorg nodig zouden kunnen hebben dan andere groepen.

Effecten van antivirale therapie

Momenteel bestaan er twee groepen antivirale medicijnen voor de behandeling van HBV patiënten, (gepegyleerd-) interferon-alfa (PEG-IFN-α) en nucleos(t)ide analogen (NUC). Antivirale therapie kan ervoor zorgen dat de ontsteking van de lever vermindert en de vorming van leverfibrose wordt geremd. Daarnaast is er de laatste jaren gebleken dat bij HBV patiënten die al leverfibrose of levercirrose hebben, antivirale therapie kan zorgen dat de verlittekening van de lever afneemt. De afweer, en specifiek de HBV-specifieke T-cel, speelt een belangrijke rol in het ziektebeloop van HBV patiënten. in **hoofdstuk 6** wordt een overzicht gegeven van de bestaande literatuur over de manier waarop antivirale therapie de T-cel beïnvloedt. PEG-IFN-α versterkt de HBV-specifieke T-cel respons door de samenstelling van verschillende T-cel populaties te veranderen. Hierbij neemt het aantal pro-inflammatoire HBV-specifieke CD4+ T-cellen tijdens de behandeling toe terwijl de anti-inflammatoire T-cellen, zoals de regulatoire T-cellen (Tregs), in aantal afnemen. Tijdens NUC therapie wordt de HBV-specifieke T-celrespons indirect beïnvloed doordat de

hoeveelheid virus door de therapie wordt geremd. Hierdoor krijgt de HBV-specifieke T-cel, die vanwege de grote virale belasting niet meer goed functioneert, de mogelijkheid om te herstellen en betere controle te bewerkstelligen.

Naast de positieve effecten zijn er ook nadelen van antivirale therapie op zoals beenmergtoxische bijwerkingen bij PEG-IFN-α en het vaak levenslang duur in het gebruik van NUCs. Tenofovir is een NUC die vaak als eerste keus wordt gebruikt bij de behandeling van HBV patiënten. Het heeft zowel antivirale effecten tegen HBV als tegen het humaan immuundeficiëntie virus (HIV). Bij HIV patiënten is het al bekend dat tenofovir in de nieren schade kan veroorzaken waarbij er proximale tubulaire dysfunctie (Fanconi syndroom) kan optreden. Bij HBV patiënten is deze bijwerking zelden gerapporteerd. In **hoofdstuk 7** wordt er aan de hand van een patiëntencasus een overzicht gegeven van de literatuur met betrekking tot deze bijwerking bij HBV patiënten. Hierin wordt geconcludeerd dat als er tijdens tenofovirgebruik een laag fosfaatgehalte in het bloed optreedt, dit een voorbode kan zijn op het ontwikkelen van Fanconi syndroom, Dit treedt met name op bij patiënten boven de 50 jaar, patiënten met pre-existente nierfunctiestoornissen of patiënten die ACE-remmers of adefovir (andere antivirale middel tegen HBV) hebben gebruikt.

Conclusies van dit proefschrift

1. Bij niet-Aziatische HBV patiënten is zowel de hepatitis B virale lading als de kwantitatieve HBsAg niet geassocieerd met het ontwikkelen van levercirrose.
2. Bij HBV patiënten is de ELF-test minder betrouwbaar in het opsporen van levercirrose in vergelijking met de fibroscan

3. Serum hyaluronzuur waarden zijn lager bij HBsAg negatieve patiënten ten opzichte van HBsAg positieve patiënten en voorspeld het optreden van spontane klaring van het virus
4. Etniciteit is een factor die negatief van invloed is op de kwaliteit van leven bij HBV patiënten

Chapter 9

List of publications

List of publications

Harkisoen S, Arends JE, Hoepelman IM, van Erpecum KJ. Renal proximal tubular dysfunction due to tenofovir in a patient with chronic hepatitis B monoinfection. *Accepted for publication in Clin Res Hepatol Gastroenterol*

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Chapter 9

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Aum bhadra kalyai namo nityam, Sarasvatyai namo namah,
veda vedanga vedanta vidya sthanebhya eva cha.

Chapter 9

Curriculum vitae

Curriculum vitae

Soeradj Harkisoen werd op 18 Januari 1978 geboren te Amsterdam. Na het behalen van zijn VWO diploma in 1996 aan het Amstellyceum in Amsterdam ging hij geneeskunde studeren aan de Universiteit van Antwerpen (eerder RUCA) waar hij zijn 1^{e} kandidatuur behaalde. In 1998 verhuisde hij terug naar Amsterdam waar hij geneeskunde studeerde aan het Academisch Medisch Centrum (AMC). Tijdens zijn studie verrichte hij een jaar onderzoek naar de veneuze diameter en capillaire densiteit in het tandvlees en onder de tong met OPS (orthogonal polarization spectral) imaging op de afdelingen Tandheelkunde en Experimentele Anesthesiologie in het AMC onder begeleiding van dr. J.A.H. Lindeboom en prof. dr. C. Ince. In 2005 behaalde hij zijn artsendiploma. Hierna werkte hij als arts-assistent geneeskunde niet in opleiding op de afdeling Interne Geneeskunde in het Antoni van Leeuwenhoek ziekenhuis te Amsterdam en vervolgens in het Sint Antonius ziekenhuis te Nieuwegein. In 2006 begon hij aan zijn opleiding tot internist in het Sint Antonius ziekenhuis te Nieuwegein onder leiding van dr. D.H. Biesma en later dr. A.B.M. Geers. In 2009 vervolgde hij zijn opleiding in het Universitair Medisch Centrum Utrecht (UMCU) onder leiding van prof. dr. E. van der Wall, later prof. dr. D.H. Biesma en vervolgens prof. dr. M.M.E. Schneider. In 2011 onderbrak hij de opleiding voor promotieonderzoek op het gebied van hepatitis B onder leiding van dr. J.E. Arends en prof. dr. I.M. Hoepelman. Vanaf 2013 combineerde hij zijn promotieonderzoek met het aandachtsgebied infectieziekten in het UMCU onder leiding van prof. dr. I.M. Hoepelman. In oktober 2014 heeft hij zijn opleiding tot internist-infectioloog succesvol afgerond.