MicroRNA’s in chronic hepatitis B and C virus infection
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CHAPTER 9

HBSAG LOSS AFTER PEGINTERFERON AND NUCLEOTIDE COMBINATION TREATMENT IN CHRONIC HEPATITIS B PATIENTS: 5 YEARS OF FOLLOW-UP

ABSTRACT

Background and aims
Combining peginterferon alfa-2a (pegIFN) with a nucleotide analogue can result in higher rates of HBsAg loss than either therapy given alone. Here we investigated the durability of the response to combination therapy in chronic hepatitis B (CHB) patients after 5 years of follow-up.

Methods
In the initial study, 92 CHB patients (44 HBeAg positive, 48 HBeAg negative) with HBV DNA > 100,000 c/mL (~20,000 IU/mL) and active hepatitis were treated 48 weeks with pegIFN 180 mcg/week and 10 mg adefovir dipivoxil daily. For the long term follow-up study, patients were followed for 5 years after the end of treatment. At year 5, 70 (32 HBeAg positive, 38 HBeAg negative) patients were still included.

Results
At year 5, 19% (6/32) of HBeAg positive patients and 16% (6/38) of HBeAg negative patients had HBsAg loss, and no HBsAg sero-reversion was observed. The 5-year cumulative Kaplan-Meier estimate for HBsAg loss was 17.2% for HBeAg positive patients and 19.3% for HBeAg negative patients. 14/16 patients who lost HBsAg at any time point during follow-up developed anti-HBs antibodies (>10 IU/L). At year 5, in total 63% (20/32) of HBeAg positive and 71% (27/38) of HBeAg negative patients were retreated with nucleos(t)ide analogues during follow-up. The cumulative Kaplan-Meier estimate for retreatment was 60% of patients at year 5.

Conclusion
At year 5 of follow-up, 18% of CHB patients treated with pegIFN/ nucleotide analogue combination therapy had durable HBsAg loss and 88% of these had developed anti-HBs antibodies.
INTRODUCTION

Hepatitis B virus (HBV) infection is a major public health issue. Worldwide over 240 million patients are chronically infected with HBV.\(^1\) Patients who have chronic hepatitis B (CHB) virus infection are at increased risk of developing hepatic complications such as cirrhosis and hepatocellular carcinoma.\(^2,3\) Current treatment options for patients with CHB consist of pegylated interferon alfa (pegIFN) or nucleos(t)ide analogue (NUC) therapy. PegIFN is thought to directly interfere with HBV and boost the immune system and is usually given as a yearlong course. NUCs have a direct antiviral mode of action and generally have to be given lifelong. As it is particularly difficult to eradicate the covalently closed circular HBV DNA (cccDNA) from the infected hepatocyte, the outcome closest to cure is currently clearance of HBV DNA with HBsAg loss, referred to as ‘functional cure’. This outcome is rare, therefore treatment is mainly focused on reducing the viral load and liver inflammation. Achieving these goals is referred to as a ‘combined response’, in which the HBV DNA viral load is under 2,000 IU/mL and ALT levels are normal.\(^4\)

Experimental data suggests a combination of pegIFN and a NUC may have supplementary effects on outcome.\(^5\) To improve treatment outcome, several trials have been conducted with combination regimens of pegIFN combined with a NUC.\(^6\) Recently, a multicenter randomized controlled trial was performed which compared pegIFN or NUC monotherapy with combination therapy in HBeAg positive or –negative CHB patients. At six months follow-up after the end of treatment 9% of patients had HBsAg loss in the pegIFN + tenofovir combination arm, as compared to 2.8% in the pegIFN mono-treatment arm.\(^7\) In the past, we have completed a study in which HBeAg positive and negative CHB patients were treated with a combination of pegIFN and adefovir. In this study, similar rates of HBsAg loss (9%) were observed at 6 months after the end of treatment.\(^8\)

In our study, we observed increasing rates of HBsAg loss (13/92, 14%) at two years of follow-up after end of treatment. However, whether this proportion can further increase after more years of follow-up is unknown. Furthermore, the durability of response to combination therapy is unknown. We therefore assessed the outcome of pegIFN and NUC combination treatment after five years of follow-up.

PATIENTS AND METHODS

Patients

Initial study: Ninety-two CHB patients were included in the initial study in the Academic Medical Center (AMC) in Amsterdam and the Erasmus University Medical Center (EMC) in Rotterdam (controlled-trials.com; ISRCTN 77073364).\(^8\) Patients were treated for 48 weeks with pegylated interferon alfa-2a once a week (Pegasys® 180 mcg subcutaneously; Hoffman La Roche, Basel, Switzerland) and adefovir dipivoxil daily (Hepsera® 10 mg orally;
Gilead Sciences, Foster City, CA, USA). Patients that could be included were aged 18 years or older, had chronic HBV infection (documented HBsAg positivity for longer than six months, HBeAg-positive or -negative), had a viral load of HBV DNA >100,000 copies/ml (~20,000 IU/ml) and had normal or increased alanine aminotransferase (ALT) levels (≤10x upper limit of normal (ULN)) or histological signs of chronic active hepatitis.

Follow-up (FU) study: Patients attended the outpatient clinic once a year up to five years after end of treatment for laboratory testing and routine examination. Screening for hepatocellular carcinoma (HCC) was performed routinely by abdominal ultrasound. Both parts of the study were conducted according to the guidelines of the Declaration of Helsinki and with the principles of Good Clinical Practice and were approved by the local ethics committee. All patients gave written informed consent. All authors had access to the study data and reviewed and approved the final manuscript.

Outcome
The primary outcome of the long term follow-up (LTFU) study was sustainability of the functional cure i.e. HBsAg loss, with or without anti-HBs antibody formation (defined as anti-HBs >10 IU/L). The secondary outcomes were defined as the need for re-treatment and the sustainability of ‘combined response’ (CR) defined as sustained viral suppression (HBV DNA <2,000 IU/ml) with ALT normalization. In HBeAg positive patients this included HBeAg loss.

Laboratory testing
Local laboratories carried out biochemical and hematological analyses in accordance with good laboratory practice. ALT levels were expressed relative to the ULN range (45 U/l for males and 34 U/l for females). Plasma HBV DNA was extracted by COBAS® Ampliprep (F. Hoffmann-LaRoche Ltd, Diagnostics Division, Basel, Switzerland) according to the manufacturer’s instructions. Quantitation of plasma HBV DNA levels was done by the Roche COBAS® TaqMan 48® assay (F. Hoffmann-LaRoche Ltd, Diagnostics Division, Basel, Switzerland), with a dynamic range between 20 and 1.70×10⁸ IU/ml.

Qualitative detection of serum hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), hepatitis B e antigen (HBeAg) and antibody to hepatitis B e antigen (anti-HBe) was performed by enzyme immunoassay (AxSYM; Abbott Laboratories, Abbott Park, IL, USA), and expressed as sample to cutoff ratio (S/CO) with a lower limit of detection for HBsAg of 0.05 IU/ml. Quantitative detection of serum HBsAg was performed using the Architect quantitative HBsAg assay (Abbott Diagnostics, Abbott Park, IL, USA) with a lower limit of detection of 0.05 IU/ml.
HBSAG LOSS AFTER COMBINATION TREATMENT

Statistical analyses
Statistical analyses were performed in SPSS (IBM SPSS Statistics for Windows, Version 23.0 Armonk, NY: IBM Corp.). Analyses were either based on the per protocol study population, which included all patients who were still included in the study at a specific time-point, or the initial study population which was analysed with Kaplan Meier estimates. Patients who became eligible for retreatment were regarded as non-responders in the LTFU study. Patients with missing data (no show for the study visit) were classified as non-responders (qualitative data) or excluded from the analysis (quantitative data). For Kaplan-Meier analyses, patients who were lost to follow up as well as patients who had not reached the endpoint at year five were censored. For the identification of factors influencing HBsAg loss univariable Cox regression analysis was used. The hazard ratio (HR) and 95% confidence interval (CI) are given for factors that were associated with HBsAg loss. Statistical significance was based on a p-value below 0.05.

RESULTS
Patients
In the initial pegIFN and adefovir combination treatment study, 92 CHB patients were included (baseline characteristics are depicted in Table 1). At two years after the end of treatment, 85 patients (92%) were still participating. Five years after end of treatment, 70 patients (76%) were still in follow-up. A per-year overview of the patients included in the LTFU study is given in Supplementary Figure 1. At year five, 22 patients were excluded from the study of which 18 patients were lost to follow-up (including 6 drop-outs during treatment). Of these, three had HBsAg loss with the formation of anti-HBs antibodies during last visit, four had a combined response during last visit, three were retreated with NUC due to relapse of HBV DNA with ALT elevation, and one had a HBV DNA relapse without an indication for retreatment. In addition, due to participation in another experimental treatment study, two patients were excluded from the LTFU study who at the time of exclusion had a combined response. Furthermore, two patients had died of which one had HBsAg loss with anti-HBs formation, and one was previously retreated due to relapse.

HBsAg loss
From year two to year five of follow-up, three additional patients lost HBsAg, of which two had development of anti-HBs antibodies. These patients all had a combined response before becoming HBsAg negative. One of these patients (HBeAg negative at baseline) lost HBsAg at year three after end of treatment, one at year four, and one at year five after end of treatment (the latter were both HBeAg positive at baseline). None of these patients received any additional antiviral treatment after the cessation of the study medication. No HBsAg sero-reversions were observed in this study.
In a per protocol analysis at year five, 18.8% (6/32) of HBeAg positive patients had HBsAg loss (of which five had developed anti-HBs), and 15.8% (6/38) of HBeAg negative patients had HBsAg loss (of which five had developed anti-HBs). A year to year overview of patients with HBsAg loss is depicted in Table 2.

At year five the cumulative Kaplan-Meier estimate for HBsAg loss was 17.2% for HBeAg positive patients and 19.3% for HBeAg negative patients (Figure 1). In total 16 patients lost HBsAg of which 12 patients reached end of follow-up (year five); the other four patients had died (n=2), or were lost to follow up (n=2). 14/16 patients (87.5%) who lost HBsAg developed anti-HBs antibodies at some point during follow-up. Patients with HBsAg loss were infected with HBV genotype A (n=9), B (n=1), C (n=2), D (n=2), and E (n=2).

The baseline factors associated with HBsAg loss are depicted in Table 3. In patients who were HBeAg positive at baseline, factors associated with HBsAg loss were age and HBV genotype A. In patients who were HBeAg negative at baseline, HBsAg loss was significantly associated with HBV DNA, and HBsAg levels at baseline.

**Quantitative HBsAg**

During LTFU, plasma levels of HBsAg decreased in patients who were not retreated. Patients who were already HBsAg negative at year two of follow-up were excluded from
Table 2. Year to year overview of patients and outcome during follow-up in a per protocol analysis.

<table>
<thead>
<tr>
<th></th>
<th>HBeAg positive</th>
<th>HBeAg negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Year 2  n (%)</td>
<td>Year 3  n (%)</td>
</tr>
<tr>
<td>Patients n=</td>
<td>41 (12)</td>
<td>38 (11)</td>
</tr>
<tr>
<td>HBsAg loss</td>
<td>5 (8)</td>
<td>4 (11)</td>
</tr>
<tr>
<td>Anti-HBs*</td>
<td>4 (80)</td>
<td>3 (75)</td>
</tr>
<tr>
<td>HBeAg loss**</td>
<td>18 (44)</td>
<td>14 (37)</td>
</tr>
<tr>
<td>HBeAg conversion</td>
<td>15 (37)</td>
<td>10 (26)</td>
</tr>
<tr>
<td>Retreated</td>
<td>20 (49)</td>
<td>20 (53)</td>
</tr>
<tr>
<td>HBsAg loss after NUC</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*anti-HBs as a percentage of patients with HBsAg loss. **n=1 HBeAg sero-reversion observed.

Figure 1. Kaplan-Meier estimate of HBsAg loss in HBeAg positive and HBeAg negative patients (intention-to-treat population).

this analysis. Likewise, in patients who were retreated with NUCs, HBsAg levels declined over time (Supplementary figure 2), but in this group HBsAg loss was not observed.

LTFU outcome – combined response

At year two of follow-up after cessation of therapy, in total 11 of 85 patients (12.9%) had a combined response with HBV DNA < 2,000 IU/ml and ALT normalization, including HBeAg loss for HBeAg positive patients. This excluded patients with HBsAg loss and patients that met the criteria for combined response after having been retreated with NUC. Of these 11 patients, two had a combined response at year five, while one patient
relapsed (HBV DNA > 2,000 IU/mL). The other eight patients with a combined response at year two either lost HBsAg (n=3), were included in another treatment study (n=2), or were lost to follow-up (n=3). Between years two and five of follow-up, three additional patients (two HBeAg negative, one HBeAg positive at baseline) met the criteria for combined response without being retreated.

In a per protocol analysis, at the end of LTFU 5/70 (7%) of patients had a durable combined response five years after having received combination therapy (two HBeAg positive, three HBeAg negative) (Table 2).

**LTFU outcome – retreatment**

Between year two and year five of follow-up, six additional patients became eligible for retreatment due to an increase in HBV DNA and ALT level. These patients all had a relapse of HBV DNA without the indication for retreatment at year two of follow-up. Only one of these patients had a combined response at some time point after treatment (six months after the end of treatment) but HBV DNA levels relapsed after that, meeting treatment criteria at year three of follow-up. At two years follow-up after stopping treatment, 45/85 (52.9%) patients who were participating in the trial had been retreated with a NUC. At year five, 62.5% (20/32) of HBeAg positive and 71.1% (27/38) of HBeAg negative patients were retreated during follow-up (Table 2).

Of the 86 patients who completed 48 weeks of treatment, 51 patients were retreated. The cumulative Kaplan-Meier estimate for retreatment was 60% (54.7% for HBeAg positive and 64.9% for HBeAg negative patients, p=0.20) at year five (Figure 2). Half of the patients had been retreated at year two of follow-up after the cessation of treatment (95% CI 0.6-3.4 years). No HBsAg loss was observed in any of the retreated patients (Table 2).

**LTFU clinical outcome**

During follow-up two patients died, of which one patient had a myocardial infarction, and one patient died due to a drug overdose. One patient, who had cirrhosis at baseline,
developed HCC during follow-up, shortly after the end of treatment. No other patients developed HCC between year two and year five. Furthermore, no hepatic decompensation was observed in 14 patients with cirrhosis (including two dropouts) during follow-up in the study. At year five, nine patients with cirrhosis were still included in the study.

**DISCUSSION**

In this study we showed that the high rate of HBsAg loss that was observed 2 years after the cessation of treatment in CHB patients was sustained until year 5 of follow-up. From year two to five of follow-up, only three additional patients lost HBsAg resulting in 17 and 19% HBsAg loss after five years in HBeAg positive and -negative patients respectively. Durable HBsAg loss is the closest to a cure and associated with improved survival and a lower incidence of HCC.9–11

In the past, several controlled studies have analysed the effect of pegIFN and NUC combination treatment for patients with CHB.6,7,12,13 Long term follow-up data on the efficacy of such regimens however, is scarce. In a recent study, rates of HBsAg loss were 9.1% at six months after the end of treatment in patients treated with pegIFN and tenofovir, compared to 2.8% in the pegIFN monotherapy arm.7 This short term follow-up outcome reflects the rate of HBsAg loss (9%) that also was observed in our study 6 months after the cessation of treatment. This could suggest that, in contrast to when used as monotherapy, adefovir may not be inferior to tenofovir when used in combination with pegIFN.14 In another study in HBeAg negative patients, HBsAg loss was observed in 8.7% of patients three years after the end of treatment with pegIFN and lamivudine therapy, as compared to 0% in lamivudine-only treated patients.13 A study in HBeAg positive patients
reported 15% HBsAg loss in patients treated with pegIFN (alfa-2b) and lamivudine combination treatment after a mean of three years follow-up, compared to 8% in patients treated with pegIFN monotherapy, however this difference was not significant. In the absence of a monotherapy arm in our study, comparison of data to these results is difficult, even so, the rates of HBsAg loss in the combination treatment arms appear to be comparable with the results of other combination therapy studies.

In our study, 11/85 (13%) of patients had a combined response at year 2 after the cessation of therapy (excluding patients with HBsAg loss). This outcome was sustainable as only one of these patients had HBV DNA relapse (>2,000 IU/mL). The remaining patients with a previous combined response had a durable combined response during LTFU, had HBsAg loss or were lost to follow-up.

One of the limitations of our study was that after five years of follow-up, only 76% of patients were still included, introducing the risk of a selection bias. However, the participation rate was still relatively high, as compared to other studies reporting 59-65% CHB patients still participating three years after end of treatment. To compensate for the cases lost to follow-up, we analyzed the data not only in a per protocol analysis but also calculated Kaplan Meier estimates in which all patients are taken into account, and patients lost to follow-up are censored.

In the past, many studies have assessed predictive markers of response to therapy. Due to a low number of patients, the study was underpowered for multivariable analyses of factors associated with HBsAg loss. Univariable analyses resulted in a high level of significance for low baseline HBsAg level as a predictor for HBsAg loss in HBeAg negative patients. Measuring quantitative HBsAg levels has previously been suggested to be of help in making clinical decisions. Others found that during therapy, an early drop in HBsAg levels during pegIFN treatment was associated with a sustained therapy response in HBeAg positive and negative patients. Here, baseline HBsAg levels were significantly correlated with HBsAg loss in HBeAg negative patients, confirming the data from our initial study. During LTFU, HBsAg levels showed a stable decline over time.

Other factors associated with HBsAg loss were HBV genotype A in HBeAg positive patients, as previously shown, while in HBeAg negative patients, this was not associated with response.

None of the patients who became eligible for retreatment with NUC therapy during LTFU (n=6) had a combined response at two years after end of treatment. The cumulative probability for retreatment was 60% after five years. Retreatment occurred mostly in the first two years after the end of treatment, which is consistent with previous data on
interferon based treatment in which HBV DNA relapse was usually seen within the first year after treatment.\textsuperscript{13}

No HBsAg loss was observed in patients who were retreated with NUC therapy. Similarly, when comparing pegIFN and lamivudine combination therapy with lamivudine monotherapy in HBeAg negative patients, no HBsAg loss was observed in the lamivudine monotherapy arm.\textsuperscript{13}

In conclusion, five years after the end of treatment with pegIFN and NUC combination treatment, a durable functional cure was achieved in approximately 18\% of CHB patients. Even though high levels of HBsAg loss were observed in this study, the majority of patients had to be retreated with NUC therapy. There is an urgent need for improved treatment of CHB patients, in order to achieve a durable functional cure. Hopefully the recent advances in development of new drugs targeting the HBV replication cycle will lead to more effective treatment options for CHB patients to achieve a durable functional cure.
REFERENCES


CHAPTER 9

FUNDING
This study was funded by Roche.

SUPPLEMENTARY MATERIALS

Supplementary Figure 1. Year to year overview of included patients. Abbreviations: CR; combined response (HBV DNA < 2,000 IU/mL and normal ALT), PR; partial response (HBV DNA > 2,000 IU/mL, no retreatment indication).
Supplementary Figure 2. Quantitative HBsAg levels. (A) Slight drop in HBsAg levels between previous measurements and measurements for LTFU study. This is probably due to 4 year interval between measurements, i.e. new architect batch and longer storage time for samples. (B) Only samples measured in the same batch (year 3 year 4 and year 5) were analysed for the LTFU study. Left side: plasma HBsAg levels of patients who were not retreated during LTFU and not HBsAg negative at 2 years after cessation of therapy. Right side: plasma HBsAg levels of patients who were retreated at year 2 of follow-up.