Clinical studies on hepatitis B, C, and E virus infection
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Citation for published version (APA):

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CHAPTER 2

Intrahepatic IP-10 mRNA and Plasma IP-10 Levels as Response Marker for HBeAg-positive Chronic Hepatitis B Patients Treated with Peginterferon and Adefovir


Antiviral Research 2016; 131: 148–155
ABSTRACT

Introduction
Interferon-y–inducible protein-10 (IP-10), also called CXCL10, is produced by different types of cells such as monocytes, neutrophils and hepatocytes. IP-10 functions as an inflammatory cytokine, which after binding to its receptor CXCR3, expressed on T-lymphocytes, leads to immune activation. We aimed to establish if IP-10 expression in liver tissue and in plasma of chronic hepatitis B (CHB) patients correlated with each other and further to investigate if IP-10 levels before and during therapy with peginterferon and adefovir could predict treatment outcome in CHB patients.

Patients and methods
A total of 86 CHB patients (41 HBeAg-positive and 45 HBeAg-negative) received combination therapy of peginterferon and adefovir for 48 weeks. Combined Response (CR) (HBeAg-negativity, HBV-DNA ≤ 2,000 IU/mL, ALT normalization) and non-response (NR) were assessed at Week 72. Plasma IP-10 levels were measured at baseline and during treatment at Day 3 (D3) and Week 1 (W1). Pre-treatment liver biopsies from 40 of 86 patients were obtained and stored in liquid nitrogen for the analysis of intrahepatic IP-10 mRNA expression.

Results
CR was achieved in 14/41 HBeAg-positive and 17/45 HBeAg-negative patients. Mean baseline plasma IP-10 levels were significantly higher in HBeAg-positive patients with CR than NR (3.20 vs 3.00 log pg/mL p = 0.03); but not in HBeAg-negative patients. Baseline IP-10 levels correlated with ALT-levels in HBeAg-positive and -negative patients (both p < 0.001), and with a decline of HBsAg-levels of ≥ 0.5 log IU/mL at Week 12 in HBeAg-positive patients (p = 0.001). Plasma IP-10 levels were associated with intrahepatic IP-10 mRNA expression, however, more strongly in HBeAg-positive (R=0.79, p<0.001) than in HBeAg-negative patients (R=0.53, p = 0.011). IP-10 levels only correlated with HAI-scores in HBeAg-positive patients (R = 0.40 p = 0.025). Mean plasma IP-10 levels of both HBeAg-positive and -negative patients increased significantly at D3 compared to baseline (+0.30 log pg/mL p = 0.003), to then decline subsequently at W1 to a level still significantly higher than baseline (+0.14 log pg/mL p < 0.001). The increase of IP-10 was significantly higher in HBeAg-positive patients with NR than in those with CR (+0.35 versus +0.11 log pg/mL p = 0.003).

Conclusions
Baseline plasma IP-10 levels and IP-10 mRNA expression in the liver are correlated with each other, suggesting that plasma IP-10 reflects intrahepatic immune activation. Higher IP-10 levels at baseline seem to be associated with CR in HBeAg-positive patients treated with peginterferon and adefovir, but not in HBeAg-negative patients.
INTRODUCTION

Worldwide, there are over 350 million people chronically infected with the hepatitis B virus (HBV), with the highest prevalence in South-East Asia and Africa.\textsuperscript{1} Every year HBV infection is responsible for over 780,000 deaths.\textsuperscript{2} In acute infection an adequate immune response and resolution of the virus with lifelong protective immunity is seen in approximately 95% of adult people. Perinatal transmission of HBV from mother to neonate has a higher chance to result in chronic infection.\textsuperscript{3} When the host immune response is inadequate and the infection persists, patients become chronically infected and are at risk to develop liver cirrhosis and hepatocellular carcinoma.\textsuperscript{4} The mechanism of developing chronic hepatitis B virus (HBV) infection, rather than clearing acute infection, is not fully understood. It has been associated with impairment of the innate and adaptive immune responses. In most individuals with acute HBV infection that spontaneously resolve, strong and broad virus specific T-cell responses directed against the HBV-infected hepatocytes can be detected. However, these responses are weak and narrowly focused in patients who develop chronic HBV infection, resulting in low levels of antiviral cytokines and attenuated cytotoxic T-lymphocyte (CTL) activity.\textsuperscript{5–12} During chronic HBV infection inflammatory activity and HBV load may fluctuate in some patients whereas in others inflammation is absent and HBV load remains low.

Many cytokines and chemokines have been identified to be involved in immune reactions in response to HBV infection. Interferon-\(\gamma\)-inducible protein-10 kDa (IP-10/ CXCL10) is a non-EXR-CXC chemokine\textsuperscript{13, 14} and is a chemo-attractant for T-lymphocytes, monocytes, and NK-cells. This cytokine and especially its relation to viral clearance and response to antiviral therapy have extensively been described in acute and chronic hepatitis C virus (HCV) infection.\textsuperscript{15–24} Next to this, it was shown in chronic HCV patients that intrahepatic IP-10 mRNA expression and plasma IP-10 levels are correlated with each other.\textsuperscript{18, 23, 24} In chronic HBV patients, serum IP-10 seems to be higher than in healthy controls.\textsuperscript{25, 26} Serum IP-10 is positively correlated with HBV-DNA, ALT levels and progressive disease in chronic HBV infection.\textsuperscript{8, 27–31} Next to this, high pre-treatment IP-10 levels have been associated with HBeAg-loss during or after treatment with peginterferon\textsuperscript{32} or nucleo(s)tide analogues (NAs).\textsuperscript{33} Combination therapy with peginterferon and adefovir\textsuperscript{34} or tenofovir\textsuperscript{35} in active CHB patients showed higher response rates than monotherapy with either medicament alone (peginterferon or NAs).

It is not entirely clear whether IP-10 levels at baseline or changes in IP-10 levels during treatment predict response to treatment in active CHB patients, treated with a combination of peginterferon and a nucleo(s)tide analogue (NA). Aside from this, a correlation between intrahepatic IP-10 mRNA expression and plasma IP-10 levels in chronic HBV infection has not been investigated. Our aim was to establish if IP-10 levels at baseline and early during therapy can predict treatment outcome, and to establish if IP-10 mRNA expression in liver tissue is correlated with plasma IP-10 levels in CHB patients.
PATIENTS AND METHODS

Patients, Treatment regimen and Sample collection
From 2005 – 2008, an open label prospective study was performed in which 86 CHB patients with active CHB (HBV-DNA ≥ 20,000 IU/mL and ALT above upper limit of normal (45 U/L for males and 34 U/L for females) or histological signs of chronic active hepatitis) were included (41 HBeAg-positive and 45 HBeAg-negative patients). Inclusion of patients was done according to the Dutch national guidelines on the indication for treatment of chronic HBV-infection at the time. The results of this study were reported in 2013 (34). All patients were treated for 48 weeks with peginterferon-alpha-2a 180 ug subcutaneously once per week (Roche, Switzerland) and adeovir diperoxil 10 mg daily (Gilead Sciences, USA). Plasma samples were obtained and stored at -80° C at baseline (BL), Day 3 (D3), Week 1 (W1), Week 18 (W18) during treatment, end of treatment (EOT) and at Week 24 after cessation of treatment (Week 72).

Plasma measurements
HBV-DNA was measured quantitatively at BL and at Week 72 using the Roche COBAS Taqman 48® assay (dynamic range 20–1.70 *10^8 IU/ml, F. Hoffmann-La Roche Ltd, Diagnostics Division, Switzerland). HBsAg-levels were measured quantitatively at BL, Week 12 (W12) and Week 72 (W72) using the Abbott Architect (dynamic range 0.05–250 IU/mL, Abbott Diagnostics, USA). HBV genotype was assessed at BL using the INNO-LiPa-assay (Innogenetics, Gent, Belgium) or by sequencing a part of the polymerase gene with dideoxynucleotide technology. ALT, anti-HBs, HBeAg, anti-HBe were measured at BL by our local laboratory in accordance to good laboratory practice. Plasma IP-10 levels at baseline were measured using a Luminex polystyrene bead-based assay (Bio-Plex Pro, BioRad, Hercules CA, USA). Then, we performed longitudinal measurement of plasma IP-10 at BL, D3 and W1, using a solid base sandwich enzyme linked immunosorbent assay (sensitivity 4.46 pg/ml, assay range 7.8–500 pg/ml; Quantikine human CXCL10/IP-10 immunoassay, R&D Systems). Accordance of the test results between the two tests and time points was seen in 82 of 86 patients, so for the longitudinal analysis only these patients were included. Of these 82 patients, further longitudinal measurement of plasma IP-10 at W18 and at EOT was performed in 22 patients, of which 12 were HBeAg-positive and 10 HBeAg-negative.

Liver biopsy specimens and Measurements
Pre-treatment liver biopsy specimens were obtained in 69 of 86 patients. Of these specimens, 40 were stored in liquid nitrogen, enabling the analysis of intrahepatic IP-10 mRNA expression using an in-house developed reverse transcription quantitative polymerase chain reaction (RT-qPCR). First, three micrograms of RNA were reverse transcribed using M-MLV reverse transcriptase and random hexamer primers. Relative quantification of gene expression was determined with the LightCycler 480 Real-Time PCR System (Roche Applied Science, Rotkreuz, Switzerland) using the SYBR Green PCR Master Mix and for IP-10 gene specific primers. PCR product specificity was assessed by melting curve analysis. Thereafter, mRNA expression levels were normalized to the arithmetic mean of two housekeeping genes (ACTB and GUSB) using the comparative Ct method, and log_{10} transformed for analysis. All liver biopsy
specimens were assessed by an experienced pathologist on activity score using the modified Ishak histopathologic activity index (HAI)\textsuperscript{37, 38} and on fibrosis stage using the Ishak score.\textsuperscript{38}

**Assessment of treatment outcome**
Assessment of response to therapy was based upon HBsAg-loss, HBeAg-loss and HBV-DNA decline (≤2,000 IU/mL at W72). Responses were defined in accordance to the most recent AASLD and EASL guidelines.\textsuperscript{39, 40} HBeAg-loss was defined as undetectable HBeAg at W72, and HBeAg seroconversion was defined as HBeAg-loss with formation of anti-HBe at W72. HBsAg-loss was defined as undetectable HBsAg at W72, and HBsAg seroconversion as HBsAg-loss with formation of anti-HBs at W72. Combined response (CR) was defined as a combination of virological (HBeAg-negative and HBV-DNA ≤2,000 IU/mL) and biochemical response (persistent normal ALT values) in all patients. Patients were considered non-responder (NR) if they did not meet the definition for one or both criteria for CR, when there was a virological and/or biochemical relapse, or when re-treatment with a NA was started after end of therapy. Relapse was defined as HBV-DNA >2,000 IU/mL (virological relapse) and/or ALT above upper limit of normal (biochemical relapse) after stopping therapy.

**Statistical analysis**
Levels of plasma IP-10, HBsAg and HBV-DNA were logarithmically transformed to achieve a normal distribution. Graphic representation was performed using Graphpad Prism version 5 and 6 for Windows\textsuperscript{®} (GraphPad Software Inc., San Diego, California, USA). Statistical comparisons were performed using IBM\textsuperscript{®} SPSS Statistics, v20.0.0.1 and v22 (SPSS Inc., Chicago, Illinois, USA). Accordance between measurements was assessed using the Bland-Altman test. Differences between groups were examined using the Student’s t-test and the Mann-Whitney U test where appropriate and correlations of parameters were analysed using Spearman’s rank correlation with 95\% confidence interval (CI 95\%). P-values <0.05 were considered statistically significant. Means were expressed plus/minus standard error of the mean (SEM) or standard deviation (SD) where appropriate.

**RESULTS**

**Baseline characteristics and Treatment outcome**
Baseline characteristics of the 86 patients included in the study are shown in Table 1. A total of 8 patients had HBsAg-loss (4 HBeAg-positive and 4 HBeAg-negative patients) and 11 of 41 HBeAg-positive patients had HBeAg-loss. CR was achieved in 14/41 HBeAg-positive and 17/45 HBeAg-negative patients. Mean log IP-10 plasma level at baseline was 3.03 log pg/mL (+/- 0.03 log pg/mL). Mean log IP-10 plasma levels at baseline were slightly higher in HBeAg-positive patients compared to HBeAg-negative patients (3.07 +/- 0.03 versus 2.99 +/- 0.04 log pg/mL), but this was not statistically significant. Mean intensity of IP-10 mRNA expression in liver tissue was 0.156 log (+/- standard deviation (SD) 1.05 log). There was no difference in baseline IP-10 levels and IP-10 mRNA expression in liver between HBeAg-positive and HBeAg-negative patients.
Table 1. Baseline characteristics of patients treated with peginterferon and adefovir for 48 weeks, according to HBeAg-status.

<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>HBeAg-positive</th>
<th>HBeAg-negative</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>41</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Sex (male / female) (%)</td>
<td>32 (78) / 9 (22)</td>
<td>31 (69) / 14 (31)</td>
<td>NS</td>
</tr>
<tr>
<td>Age (mean, years) (SD)</td>
<td>35.2 (9.3)</td>
<td>43.1 (9.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interferon-naive (N) (%)</td>
<td>32 (78)</td>
<td>32 (71)</td>
<td>NS</td>
</tr>
<tr>
<td>HBV-DNA (mean log IU/mL) (SD)</td>
<td>8.1 (1.2)</td>
<td>5.5 (1.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HBV Genotype (N) (%)</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>A</td>
<td>17 (42)</td>
<td>9 (20)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>8 (20)</td>
<td>7 (16)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>7 (17)</td>
<td>5 (11)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>7 (17)</td>
<td>17 (38)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2 (5)</td>
<td>7 (16)</td>
<td></td>
</tr>
<tr>
<td>HBsAg (mean log IU/mL) (SD)</td>
<td>4.31 (0.75)</td>
<td>3.33 (0.69)</td>
<td>NS</td>
</tr>
<tr>
<td>ALT (xULN)* (SD)</td>
<td>4.2 (5.6)</td>
<td>2.2 (1.7)</td>
<td>0.04</td>
</tr>
<tr>
<td>HBsAg-loss (N) (%)</td>
<td>4 (10)</td>
<td>4 (9)</td>
<td>NS</td>
</tr>
<tr>
<td>HBeAg-loss (N) (%)</td>
<td>11 (27)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined Response (N) (%)</td>
<td>14 (34)</td>
<td>17 (38)</td>
<td>NS</td>
</tr>
<tr>
<td>IP-10 plasma (mean log pg/mL) (SD)</td>
<td>3.07 (0.27)</td>
<td>2.99 (0.29)</td>
<td>NS</td>
</tr>
<tr>
<td>Liver biopsy (N) (%)</td>
<td>18 (44)</td>
<td>22 (49)</td>
<td>NS</td>
</tr>
<tr>
<td>HAI-score (median) (range)</td>
<td>5 (1-13)</td>
<td>5 (1-13)</td>
<td>NS</td>
</tr>
<tr>
<td>Ishak fibrosis score (median) (range)</td>
<td>1 (0-6)</td>
<td>1 (0–6)</td>
<td>NS</td>
</tr>
<tr>
<td>Cirrhosis (N) (%)</td>
<td>2 (7)</td>
<td>9 (23)</td>
<td>0.06</td>
</tr>
<tr>
<td>IP-10 mRNA expression (mean log copies vs 2 housekeeping genes) (SD)</td>
<td>0.23 (0.56)</td>
<td>0.09 (0.45)</td>
<td>NS</td>
</tr>
</tbody>
</table>

* ULN: upper limit of normal, > 45 U/L for men and > 34 U/L for women

Baseline plasma IP-10 levels in HBeAg-positive and HBeAg-negative patients

Baseline IP-10 plasma levels were slightly higher in both HBeAg-positive (3.10 +/- 0.12 log pg/mL) and -negative (3.07 +/- 0.05 log pg/mL) patients with HBsAg-loss at W72, although not statistically different. In HBeAg-positive patients, plasma IP-10 levels at baseline were significantly higher in patients with CR versus NR (3.20 +/- 0.06 log pg/mL versus 3.00 +/- 0.05 log pg/mL) (p=0.03). This difference was not observed in HBeAg-negative patients (3.00 +/- 0.07 log pg/mL versus 3.00 +/- 0.06 log pg/mL), which is shown in Figure 1.

In HBeAg-positive patients, baseline plasma IP-10 levels were higher in patients with a decline in HBsAg of ≥0.5 log IU/mL at W12 compared to those without this decline (3.28
Part I | Chapter 2: Intrahepatic IP-10 mRNA and Plasma IP-10 in Chronic Hepatitis B Patients

Figures

IP-10 (log pg/mL +/- SEM)

HBeAg
n=14 n=27 n=17 n=28

Week12HBsAg

Week72HBsAg

Figure 1. Baseline IP-10 plasma levels in HBeAg-positive and -negative patients according to response to therapy. HBeAg-positive patients with CR have higher IP-10 levels at baseline as compared to patients with NR.

Figure 2. High baseline IP-10 is associated with a more pronounced decline in HBsAg at Week 12 and Week 72 in HBeAg-positive patients.

+/- 0.06 log pg/mL versus 2.98 +/- 0.05 log pg/mL) (p = 0.001). The same was observed in HBeAg-positive patients with a decline in HBsAg of ≥ 0.5 log IU/mL at W72 compared to those without this decline (3.19 +/- 0.07 log pg/mL versus 3.01 +/- 0.05 log pg/mL (p = 0.049)). These differences were not observed in HBeAg-negative patients. This is shown in Figure 2.

Baseline IP-10 plasma levels and decline in HBsAg of ≥ 0.5 log IU/mL at W12 were combined in HBeAg-positive patients to predict combined response. If baseline IP-10 levels were higher than the mean (3.07 log pg/mL) and there was a decline in HBsAg of ≥ 0.5 log IU/mL at W12, the chance to achieve CR was 63%. In patients with baseline IP-10 plasma levels higher than the mean (3.07 log pg/mL) without a decline in HBsAg of ≥ 0.5 log IU/mL at W12, the chance to achieve CR was 33%. In patients with baseline IP-10 plasma levels below the mean without a decline in HBsAg, CR was only achieved in 19%. This is shown in Table 2.

Correlation of baseline IP-10 plasma levels with different markers of response

Baseline plasma IP-10 levels were correlated with baseline ALT in both HBeAg-positive and -negative patients (both p < 0.001). In HBeAg-negative patients baseline IP-10 levels were also correlated with baseline HBV-DNA (p = 0.01), but this was not the case in HBeAg-positive patients. In HBeAg-positive, but not in HBeAg-negative patients, a correlation was seen between baseline plasma IP-10 levels and HAI-score (p = 0.025). There was no correlation seen in HBeAg-positive or -negative patients between plasma IP-10 levels at baseline and HBsAg-levels or Ishak-score (liver fibrosis) in liver biopsy specimens (Table 3).
**Table 2.** Combination of baseline IP-10 plasma levels ($\geq$ or $<$ mean 3.07 log pg/mL) and decline in HBsAg-levels $\geq$ 0.5 log IU/mL at W12

<table>
<thead>
<tr>
<th>IP-10 BL $\geq$ 3.07 log</th>
<th>HBsAg W12 $\geq$ 0.5 log ↓</th>
<th>Combined Response</th>
<th>Total</th>
<th>Spearman's Correlation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>7</td>
<td>4</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>4</td>
<td>8</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>3</td>
<td>13</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>+</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>14</td>
<td>26</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.** Correlation of IP-10 levels with different baseline parameters (ALT, HAI-score, HBV DNA, HBsAg, Ishak Fibrosis score).

<table>
<thead>
<tr>
<th>IP-10 plasma baseline Baseline</th>
<th>HBeAg-positive</th>
<th>HBeAg-negative</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spearman's Rho</td>
<td>p-value</td>
<td>Spearman's Rho</td>
<td>p-value</td>
<td>Spearman's Rho</td>
</tr>
<tr>
<td>ALT</td>
<td>0.65</td>
<td>&lt;0.001</td>
<td>0.53</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>HAI-score</td>
<td>0.40</td>
<td>0.025</td>
<td>0.07</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>HBV DNA</td>
<td>0.08</td>
<td>NS</td>
<td>0.38</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>HBsAg</td>
<td>0.03</td>
<td>NS</td>
<td>-0.13</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Ishak Fibrosis score</td>
<td>0.14</td>
<td>NS</td>
<td>-0.05</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.** Correlation of intrahepatic IP-10 mRNA expression with plasma IP-10 levels at baseline: stronger correlation in HBeAg-positive than in HBeAg-negative CHB patients

**IP-10 mRNA expression in liver tissue at baseline**

In 18 of 41 HBeAg-positive patients and in 22 of 45 HBeAg-negative patients IP-10 mRNA expression in liver tissue at baseline was determined and correlation with IP-10 plasma levels at baseline was assessed. Mean expression of IP-10 mRNA in liver
was 0.156 log copies vs 2 housekeeping genes (+/- SD 1.05 log copies) in general. In HBeAg-positive patients mean expression was 2.30 log copies vs 2 housekeeping genes (+/- SD 0.56 log copies), and in HBeAg-negative patients mean mRNA expression was 1.01 log copies vs 2 housekeeping genes (+/- SD 0.45 log copies). There was a correlation between IP-10 mRNA expression in liver and plasma IP-10 levels (R=0.55, p=0.002) in the total group, as well as in both HBeAg-positive and HBeAg-negative patients separately (Figure 3). This correlation, however, was stronger in HBeAg-positive patients (R = 0.79, 95% CI 0.51–0.92, p < 0.001) than in HBeAg-negative patients (R = 0.53, 95% CI 0.12–0.78, p = 0.01).

**Correlation of intrahepatic IP-10 mRNA expression with different markers of response**

Intrahepatic IP-10 mRNA expression was correlated with baseline ALT levels and HAI score in both HBeAg-positive and -negative patients. In HBeAg-negative patients intrahepatic IP-10 mRNA expression was also correlated with baseline HBV-DNA level, but this was not the case in HBeAg-positive patients. There was no correlation between intrahepatic IP-10 mRNA expression and HBsAg-levels or Ishak-score (liver fibrosis), both in HBeAg-positive and HBeAg-negative patients (Table 4).

**Plasma IP-10 levels during treatment**

At D3, mean IP-10 plasma levels increased two-fold (0.3 log pg/mL) compared to baseline (from 1.95 log pg/mL to 2.15 log pg/mL). At W1 IP-10 levels decreased 0.2 log pg/mL compared to D3, to a level still 0.14 log pg/mL higher than at baseline (2.09 log pg/mL, p<0.001). These levels were not significantly different between HBeAg-positive and HBeAg-negative patients at D3 (2.21 versus 2.22 log pg/mL) or W1 (both 2.08 pg/mL). At W18 and at EOT IP-10 plasma levels declined further to a level comparable to the baseline value. No significant differences in plasma IP-10 levels in HBeAg-positive and HBeAg-negative patients were observed (Figure 4).

In HBeAg-positive patients, the increase from baseline to D3 was significantly greater in patients with NR versus patients with CR (0.35 log pg/mL versus 0.08 log pg/mL, p=0.003) (Figure 5).

**Table 4. Correlation of intrahepatic IP-10 mRNA expression with different baseline parameters (ALT, HAI-score, HBV DNA, HBsAg, Ishak Fibrosis score).**

<table>
<thead>
<tr>
<th>IP-10 plasma mRNA expression</th>
<th>HBeAg-positive</th>
<th>HBeAg-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Spearman’s Rho</td>
<td>p-value</td>
</tr>
<tr>
<td>ALT</td>
<td>0.48</td>
<td>0.046</td>
</tr>
<tr>
<td>HAI-score</td>
<td>0.47</td>
<td>0.051</td>
</tr>
<tr>
<td>HBV DNA</td>
<td>-0.27</td>
<td>NS</td>
</tr>
<tr>
<td>HBsAg</td>
<td>-0.35</td>
<td>NS</td>
</tr>
<tr>
<td>Ishak Fibrosis score</td>
<td>0.19</td>
<td>NS</td>
</tr>
</tbody>
</table>

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DISCUSSION

In this study we found that in HBeAg-positive patients, but not in HBeAg-negative patients, baseline IP-10 plasma levels were higher in patients with combined response (HBeAg-loss, ALT-normalization and HBV-DNA < 2,000 IU/mL) compared to those with non-response. These findings are in concordance with earlier studies. We saw no significant relation between IP-10 levels in plasma and HBsAg-loss. Earlier studies showed contradicting results. Some studies demonstrated higher baseline IP-10 levels in patients with HBsAg-loss, whereas others did not, which may be explained by the small proportion of patients who achieved HBsAg-loss. We did observe, however, higher baseline IP-10 levels in HBeAg-positive patients who had a decline of HBsAg-level of more than 0.5 log IU/mL at Week 12. This is in agreement with earlier studies showing that high baseline IP-10 plasma levels are associated with HBsAg-decline in both HBeAg-positive and -negative patients treated with interferon-based therapy or with NA. Our results show that baseline IP-10 level in plasma was valuable in predicting response to (finite) peginterferon-based therapy in HBeAg-positive CHB patients, especially when used in conjunction with decline of HBsAg level at Week 12.

Figure 4. Plasma IP-10 levels during treatment in A: all patients and B: HBeAg-positive and -negative patients.

Figure 5. Plasma IP-10 levels during treatment: a higher increase from BL to D3 was observed in HBeAg-positive CHB patients with NR versus CR.
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during treatment. Combining these two markers may help to identify patients with high and low chance of achieving a combined response. To translate this into clinical practice, early cessation (after Week 12) of peginterferon-based treatment may be considered in those patients with a low likelihood of achieving combined response, being those with low baseline IP-10 levels (< 3 log pg/mL) and no decline in HBsAg at Week 12.

As the combination of peginterferon and adefovir is not a widely used treatment modality, we believe that our findings may be applicable to finite peginterferon-based treatment options such as the recently described combination of peginterferon and tenofovir or peginterferon monotherapy.

We observed a clear correlation between IP-10 in plasma and IP-10 mRNA expression in the liver in CHB patients, which was earlier described in CHB and in chronic hepatitis C (CHC) patients. This suggests that plasma IP-10 is a reflection of intrahepatic immune activity in both in CHB and CHC patients. The fact that IP-10 mRNA expression in the liver was also associated with ALT-levels supports this hypothesis. Given the function of IP-10 being a chemo-attractant for inflammatory cells, high intrahepatic IP-10 expression is essential for migration of leukocytes into the liver in response to HBV infection. Another inflammatory cytokine, CXCL-9, related to IP-10 was also important for the immune response against HBV and may predict treatment outcome. High baseline expression of the chemokine CXCL9, which shares its receptor (CXCL3) with IP-10, was associated with response to interferon-based therapy in both HBeAg-positive and -negative CHB patients. Furthermore, the ligand programmed death-1 (PD-1), expressed on activated CD8+ and CD4+ T-cells, has been associated with T-cell exhaustion. A relation between PD-1, CXCL9 and expression of IP-10 (intrahepatic and peripheral) is mechanistically explained by the fact that IP-10 and CXCL9 are encoded by an interferon-stimulated gene (ISG) which is induced by type I interferon and activates T-lymphocytes.

The relation between IP-10 (in plasma and liver) and ALT level or HAI score we observed in HBeAg-positive patients as well as between IP-10 (in plasma and liver) and HBV-DNA in HBeAg-negative patients was shown in earlier studies. This supports our suggestion that IP-10 is a reflection of intrahepatic immune activation. In addition, pre-existent immune activation is important for virological response in HBeAg-positive patients, whereas in HBeAg-negative patients, HBV-DNA level is a more important response marker.

Our study is the first study to describe IP-10 kinetics early after start of interferon-based treatment, showing a clear increase in plasma IP-10 levels at D3 after start of treatment. Subsequently, at W1 IP-10 levels declined to a level still higher than baseline, followed by a level comparable to baseline at W18 and at EOT. This is in agreement with other studies, which showed a decline in plasma IP-10 levels during IFN-based therapy at W12 and W24 compared to baseline. The two-to threefold increase in IP-10 plasma levels shortly after start of therapy in our study was also described in patients with CHC. In CHC patients a dose-dependent two- to nine fold rise in plasma IP-10 levels was seen at day 1-3 after start of interferon-based therapy. The fact that in all patients in our study at D3 a significant rise in plasma IP-10 was observed indicates that early after start of interferon-based treatment, the immune activation of responders and non-responders was similar. In CHC patients treated with high-dose interferon a similar increase of IP-10 level was observed. This suggests that the increase in IP-10
levels early after start of (peg)interferon-based treatment is the result of a non-specific stimulation of the Interferon type I immune response, causing the induction of multiple ISG’s, which is not specific for an immune response provoking response to therapy.

Our study population consists of HBeAg-positive and HBeAg-negative patients, and as a consequence the numbers of patients in the different groups remain relatively small. As a result, we might have missed possible relationships between serum IP-10 or intrahepatic IP-10 mRNA and treatment response parameters in HBeAg-negative patients. However, the fact that we did find a statistically significant relation between IP-10 and treatment response in HBeAg-positive patients of comparable size, suggests that there is a difference between HBeAg-positive and HBeAg-negative CHB patients. This is also in line with earlier study results of serum IP-10 levels in HBeAg-positive and negative CHB patients.32

In conclusion, we found that plasma IP-10 levels and IP-10 mRNA expression in the liver at baseline were correlated with each other, especially in HBeAg-positive patients. Higher IP-10 levels in plasma seem to be associated with combined response in HBeAg-positive, but not in HBeAg-negative CHB patients treated with peginterferon-based therapy. Our findings underline the importance of pre-treatment immune activation in HBeAg-positive CHB patients as a predictor of response to antiviral immune modulating therapy, of which plasma IP-10 levels and intrahepatic IP-10 mRNA expression are a reflection.

ACKNOWLEDGEMENTS AND DISCLOSURES

Acknowledgements
We thank R. Engwerda for his critical review of our manuscript.

Disclosures
This study was financially funded by Gilead Sciences with a non-restricted grant. The sponsor has had no involvement in the design of the study, the collection, analysis and interpretation of data, in writing the report, and in submitting the data for publication.
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Clinical Studies on Hepatitis B, C, and E Virus Infection


