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HBsAg plasma level kinetics: a new role for an old marker as a therapy response predictor in vertically infected children on combination therapy

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1 - study concept and design
2 - acquisition of data
3 - analysis and interpretation of data, statistical analysis, manuscript drafting
4 - medical care
5 – obtaining funding, study supervision

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ABSTRACT

We aimed to investigate the ability of HBsAg plasma level kinetics to predict therapy response by studying 23 children with infancy-acquired chronic hepatitis B (CHB) during combination sequential therapy with lead-in lamivudine (LAM) and add-on interferon-α (IFN-α) [5 responders (R=anti-HBs seroconversion) and 18 non-responders (NR)] and to assess their relationship with pre-treatment intrahepatic HBV-DNA and cccDNA and HBsAg and HBcAg liver expression.

Methods: Plasma HBsAg levels were measured in samples before (treatment week 0=TW0), during (TW9, TW28, TW52) and after (follow-up week=FUW24) therapy by Abbott ARCHITECT® assay [log_{10}IU/ml]. Baseline liver HBV-DNA and cccDNA were quantified by real-time TaqMan PCR [log_{10}copies/ng genomic DNA]. HBsAg and HBcAg liver expression was evaluated by immunostaining of formalin-fixed, paraffin-embedded specimens [number of positive cells/1000 hepatocytes]. All results are presented as medians.

Results: Plasma: at baseline, on-treatment and during follow-up, HBsAg levels were lower in R than NR (TW0: 4.36 vs. 4.75;TW28: 2.44 vs. 4.35;TW52: 0 vs. 4.08 and FUW24: 0.17 vs. 4.35, all p<0.05). Liver: baseline HBV-DNA (3.82 vs. 4.71, p=0.16) and cccDNA (1.98 vs. 2.26, p=0.18) tended to be lower in R than NR, HBsAg expression was lower in R than NR (0.5 vs. 4.7, p=0.03) and HBcAg expression was similar between R and NR. There were positive correlations between plasma HBsAg levels and liver HBV-DNA (r=0.44,p=0.04), cccDNA (r=0.41,p=0.04) and HBsAg liver expression (r=0.38,p=0.05).

Conclusions: Lower baseline HBsAg plasma levels, lower HBsAg expression in liver and on-treatment decline of plasma HBsAg levels heralds HBsAg clearance and response to treatment in tolerant children with CHB.
INTRODUCTION

Approximately 400 million people worldwide are chronically infected with hepatitis B virus (HBV) resulting in over 1 million related deaths per year [1-2]. HBsAg clearance is the ultimate goal of antiviral therapy for HBV infection [3]. In plasma HBsAg circulates in variable forms, namely as a part of mature virions enveloping viral DNA (42nm size Dane particles) or as non-viral (subviral) particles, i.e. 20 nm diameter filaments and 20-22 nm size spherical defective particles corresponding to empty viral envelopes [4-7]. HBsAg plasma levels and HBsAg kinetics during antiviral therapy with pegylated interferon or/and nucleos(t)ide analogues has become a clinical tool for monitoring on-therapy response in adults, as recommended by NICE guidelines [8-13, 31]. No data are available on children.

Acquiring HBV infection perinatally leads to HBV persistence, characterised by a prolonged immunotolerant phase of disease during childhood [1-2,13-14]. Data from two cross-sectional studies in adults demonstrated HBsAg plasma levels to be higher in the immunotolerant phase than in the immunoactive phase of HBeAg positive (HBeAg+) chronic hepatitis B (CHB) infection, suggesting that high HBsAg levels are involved in promoting immune tolerance [9-15]. While a few studies have evaluated HBsAg decline during combination therapy with nucleos(t)ide analogues and pegylated IFN-α commenced simultaneously in adult HBeAg+ CHB [23-30], there are no data on HBsAg kinetics during sequential lead-in therapy with nucleos(t)ide analogue followed by add-on IFN-α and its impact on predicting therapy responses in immunotolerant children.
HBV-DNA replication and HBsAg secretion pathways are distant processes within hepatocytes. It is suggested that HBsAg plasma levels reflect transcriptional activity of covalently closed circular DNA (cccDNA) activity rather than total liver cccDNA content [6-8]. Translation of HBsAg depends on transcription of the appropriate mRNAs from cccDNA [32]. cccDNA acts as a transcriptional template of HBV within the nucleus and represents the intrahepatic reservoir for HBV; cccDNA concentrations reflect different stages of CHB infection [11,16, 33-37]. Correlations between HBsAg plasma levels, liver relaxed circular (RC) HBV-DNA and cccDNA have been demonstrated in HBeAg+ CHB patients [11,16]. Additionally, a relationship between cccDNA and hepatitis B core antigen (HBcAg) has been reported [38], but no data are available on the association between HBsAg and HBcAg expression in the liver, intrahepatic cccDNA and HBsAg plasma levels in immunotolerant perinatally infected children [38].

Therapy response has also been shown to be influenced by HBV genotypes and presence/absence of changes within the basal core promoter (BCP) and pre-core region (stop codon 1896), changes in BCP and/or pre-core regions being linked with poorer response in adult patients with active disease [39].

We have investigated longitudinally plasma HBsAg kinetics and their relationship with plasma HBV-DNA and with pre-treatment levels of liver RC HBV-DNA and cccDNA and expression of liver HBsAg and HBcAg in tolerant children during antiviral therapy with lead-in lamivudine and add-on interferon-α.
The importance of HBsAg kinetics and changes in BCP and pre-core regions on predicting therapy response were also evaluated.

**PATIENTS**

Twenty-three children with perinatally acquired CHB, treated with combination antiviral therapy, were investigated (Table 1). They were all HBeAg+, HBV-DNA positive, and all but 2 had persistently normal transaminases levels. Their pre-treatment liver biopsies showed minimal/mild inflammation and no fibrosis. Patients were treated for 52 weeks in total; initially lamivudine lead-in (3 mg/kg/day) was administered once daily on its own for 8 weeks and for a further 44 weeks in combination with add-on interferon-α (IFN-α) 2b (5MU/m² subcutaneously), given daily for the first 5 doses [treatment week 9 (TW9)] and then thrice weekly for the remaining 44 weeks [40]. The informed written consent to participate in the study was obtained from patients’ legal guardians and the study was conducted following the principles of the World Medical Association Declaration of Helsinki.

Clinical and laboratory monitoring is summarized in Figure 1. Sustained virological response (SVR) was defined as decline of HBV-DNA below detection limits of quantitative real-time polymerase chain reaction (PCR) assay (20 IU/ml) and anti-HBe seroconversion lasting for >6 months after antiviral therapy discontinuation; complete response was defined as normal aminotransferase levels, HBeAg seroconversion, decrease of HBV-DNA below 20 IU/ml, loss of HBsAg, anti-HBs seroconversion persisting during the 5-year follow-up period [40]. At treatment-end (TW52), 5 patients were anti-HBe positive, 4 of whom were also HBsAg negative/anti-HBs positive (all genotype B). During follow-up [follow-up
week 52 (FUW52)], the fifth patient (genotype A) seroconverted to anti-HBs. These five children are defined as 'responders'. Two further patients (both genotype D) seroconverted to anti-HBe 36 and 48 months after treatment-end, but their HBV-DNA remained above 2000 IU/ml. Two children with mildly abnormal baseline transaminases levels did not respond to treatment. No lamivudine-related mutations were detected during therapy and follow-up.

Controls included 7 untreated HBeAg+ children with perinatally acquired CHB (Table 1) with minimal/mild inflammation and no fibrosis on liver biopsy, and assessed regularly every 6 months during a 2-year follow up period.

**MATERIALS AND METHODS**

**Materials**

Plasma samples were obtained at baseline, during and after antiviral therapy at different time-points (Figure 1). Liver biopsy was performed in all patients prior to starting antiviral therapy.

**Methods**

**HBV serological markers**

Hepatitis B envelope antigen (HBeAg), antibodies against HBeAg (anti-HBe), hepatitis B surface antigen (HBsAg) and anti-HBsAg antibody (anti-HBs) were evaluated using commercially available enzyme immunoassays (MEIA, Abbott, USA).

**HBV-DNA viral load quantitation**

Plasma concentrations of HBV-DNA were determined by a commercially available quantitative real-time polymerase chain reaction (PCR) assay, the
COBAS AmpliPrep-COBAS Taqman HBV test (CAP-CTM; Roche Molecular Systems Inc., Branchburg, NJ, USA) with a lower detection limit of 20 IU/ml. The results are presented as log_{10} IU/ml.

**HBsAg quantitation assay**

Plasma HBsAg levels at different therapy time-points were measured using the Abbott ARCHITECT® HBsAg chemiluminescent microparticle immunoassay (CMIA) (Abbott Diagnostics, Abbott Park, IL, USA). The test has a dynamic range of 0.05-250 IU/ml. Samples were initially diluted 1:500 according to the manufacturer’s instructions. Samples with HBsAg levels less than 25 IU/ml were retested neat. The results are presented as log_{10} IU/ml [4-5].

**HBV genotypes, YMDD and HBV pre-core region mutation testing**

HBV genotypes and HBV polymerase YMDD mutation were determined by direct sequencing of PCR-amplified DNA fragments from the pre-S1/pre-S2 and S gene and overlapping polymerase gene regions [41] and compared with known sequences. To identify mutations within the BCP region and 1896 stop codon, the purified and sequenced PCR products were compared with known sequences at codons 1762, 1764 and 1896.

**HBV-DNA isolation from liver**

HBV-DNA in liver was isolated from ~1-2mm of tissue as previously described [42].

**Real-time PCR for HBV-DNA quantitation in liver**

HBV-DNA viral load in liver (RC HBV-DNA) was quantified by real-time TaqMan™ PCR [41]. The concentration of liver genomic DNA was determined by Quantifiler™ Human DNA quantification kit [Applied Biosystems, Foster City, CA,
Liver DNA was amplified with 2 primers and the HBV probe labelled with fluorophore 6-carboxyfluorescein (FAM) and non-fluorescent quencher (AB) using ABI PRISM 7000 SDS Software (AB) as described previously [38]. HBV concentration was determined using a 12-point standard curve generated with plasmid pHBV991 (10^{10}-1 copies/ml), and results were expressed as log_{10} number of HBV-DNA copies per ng of liver genomic DNA. Intra-assay coefficient of variation (CV) for triplicate experiments was 0.25-1.04%; the inter-assay CV, determined on eight samples in 6 different runs, 0.32–0.98%.

**Real-time PCR for cccDNA Quantitation**

Liver HBV cccDNA was quantified by real-time TaqMan™ PCR after treatment of liver DNA with 25U ATP-dependent DNAse (PlasmidSafe™, Epicentre, Madison, WI, USA) [38]. The digestion product containing HBV cccDNA was amplified in the presence of 2 primers and HBV probe labelled with FAM and tetramethylrhodamine (TAMRA) quencher as described previously [38]. cccDNA concentration was determined using a 12-point standard curve generated with plasmid pHBV991 (10^{7}-10^{-3} copies/ml). Concentration of liver genomic DNA was measured by Quantifiler™ Human DNA. Results are expressed as log_{10} number of cccDNA copies/ng of liver genomic DNA (detection limit 5x10^{-1} copies/ng), intra- and inter-experimental assay CV ranges being 1.34–2.21% and 0.62–1.93%.

**Immunohistochemical detection of HBcAg and HBsAg expression in the liver**

HBcAg and HBsAg expression within the liver was evaluated by immunostaining deparaffinised and re-hydrated sections of formalin-fixed, paraffin-embedded liver specimens, using rabbit anti-HBc and monoclonal anti-HBs as primary antibodies and EnVision System anti-rabbit horseradish peroxidase as
revealing reagent for HBcAg and EnVision anti-mouse kit for HBsAg (all Dako, Ely, UK). Number of positive cells expressing nuclear and/or cytoplasmic HBcAg and HBsAg per 1000 hepatocytes was scored semi quantitatively at 250x magnification [43].

**Statistical Analysis**

Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, Illinois, USA). Continuous variables are presented as median and ranges and were compared using the Mann-Whitney test. Categorical variables were assessed using the Chi-squared or Fisher exact tests. Multivariate analysis was performed using linear regression analysis for continuous variables and binary logistic regression for categorical variables. Bivariate correlations between continuous variables were evaluated by Spearman’s rank test. P-values less than 0.05 were considered significant. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated using Bayes’ theorem.

**RESULTS**

**Plasma HBV-DNA**

At baseline, HBV-DNA plasma levels (Figure 2A&B) were similar in responders and non-responders (median 8.21, range 7.03-8.68 vs. 8.13, range 7.13-8.9 log_{10} IU/ml). In responders HBV-DNA plasma levels declined significantly from TW9, persisting low throughout treatment, and remained below 2.5 log_{10} IU/ml at FUW24 (1.33, range 1.1-2.3 log_{10} IU/ml) and below the limit of detection at FUW52 (Figure 2A). In non-responders levels of HBV-DNA were lower than baseline only at TW28 and TW52, returning to levels similar to
baseline at FUW24 and FUW52 (Figure 2B). A difference in HBV-DNA viral load between responders and non-responders became apparent from TW9 and persisted at TW28, TW52, FUW24 and FUW52 (Figure 2C). In 7 untreated patients HBV-DNA viral load did not change during the follow up period of 2 years and was similar to the levels in treated patients at baseline (median 8.32, range 7.23–8.96 log_{10}IU/ml) (Figure 2D).

### Plasma HBsAg levels

At baseline, HBsAg levels were lower in responders than non-responders (Figure 3A&B) (median 4.36, range 3.64-4.64 vs. 4.75, range 3.74-5.10 log_{10}IU/ml, p=0.02) and the proportion of patients with HBsAg < 20 000 IU/ml =4.3 log_{10}IU/ml was higher in responders vs. non-responders (60% vs. 22%, p=0.05). In responders HBsAg levels decreased significantly compared to baseline at TW28, remaining low throughout treatment and at FUW24 (0.17, range 0-1.1 log_{10}IU/ml, p<0.01) (Figure 3A). In non-responders HBsAg levels declined only slightly during treatment and returned to baseline values at FUW24 (4.53, range 2.33-5.1 log_{10}IU/ml) (Figure 3B). At end of therapy, HBsAg levels were undetectable in 4 out of 5 responders in contrast to none of the non-responders. Only 2 of 18 non-responders had HBsAg levels below 2-log_{10}IU/ml by the end of therapy (TW52). In 2 non-responders with HBsAg levels below 2.0-log_{10}IU/ml at TW52, HBsAg levels increased after stopping treatment (1.6 vs. 4.41 and 1.67 vs. 4.22 log_{10}IU/ml respectively).

When compared to baseline, HBsAg plasma levels were similar at TW9 in responders and non-responders, but from TW28 HBsAg plasma levels declined markedly in responders and only slightly in non-responders (median difference –
1.84 vs. –0.36, p=0.01) (Figure 3C). HBsAg levels persisted below 0.5-\log_{10}\text{IU/ml} in responders at TW52 and FUW24 (Figure 3A&C).

In the seven untreated control patients baseline HBsAg levels were similar to those of treated patients and did not change throughout the follow up period (median 4.5, range 3.89–5.1 \log_{10}\text{IU/ml}) (Figure 3D).

**HBsAg plasma level kinetics**

According to changes in HBsAg plasma level kinetics between baseline and TW28 (i.e. after 20 weeks of IFN treatment), four different patterns of HBsAg plasma decline were seen: HBsAg decline >2-\log_{10}\text{IU/ml} (3 patients, all responders), HBsAg decrease <2-\log_{10}\text{IU/ml}, but >1-\log_{10}\text{IU/ml} (5 patients; 2 responders and 3 non-responders), HBsAg reduction <1-\log_{10}\text{IU/ml} >0.5-\log_{10}\text{IU/ml} (4 non-responders) and HBsAg decline <0.5-\log_{10}\text{IU/ml} (11 non-responders). Among patients with a decline >1.0-\log_{10}\text{IU/ml}, 2 responders had baseline levels < 4.3-\log_{10}\text{IU/ml}, while levels were > 4.3-\log_{10}\text{IU/ml} in the 3 non-responders. Only 4 non-responders, all with an HBsAg decrease <1.0-\log_{10}\text{IU/ml} during treatment, had baseline HBsAg plasma levels < 4.3-\log_{10}\text{IU/ml} (Figure 4). All 3 patients with a decline > 2-\log_{10}\text{IU/ml} responded to therapy (PPV 100% and NPV 90%), while, 15 patients with a decline <1-\log_{10}\text{IU/ml} were non-responders (sensitivity 100%, specificity 83%, PPV 63% and NPV 100%). Two responders with reduction between 1-2-\log_{10}\text{IU/ml} by TW28 had lower baseline levels (median 4.08) in comparison to 3 non-responders with the same HBsAg kinetics (median 4.67) (p=0.03).

**Correlation between HBV-DNA and HBsAg levels in plasma**
In treated patients there was no correlation between HBV-DNA and HBsAg plasma levels at baseline (r=0.35, p=0.1) in contrast to a strong positive correlation during therapy and follow-up (TW9: r=0.46, p=0.03; TW28: r=0.6, p=0.007; TW52: r=0.64, p=0.002 and FUW24: r=0.58, p=0.008) (Figures 5A-E). No bivariate correlations were noted in the untreated population at any time-point.

Hepatitis B genotype and changes in pre-core region (BCP and stop codon 1896 mutations)

HBsAg loss occurred in 4 out of 14 genotype B and 1 of 2 genotype A patients. No other genotypes were associated with HBsAg loss. Two genotype D patients had anti-HBe seroconversion more than 2-years after completing therapy. In responders, no changes within BCP (A1762T/G1764A) or stop codon 1896 (G1896A) were present, while mutations were observed in 14 out of 18 non-responders. Baseline changes within both BCP and stop codon 1896 were detected in 2 non-responders with genotype D who seroconverted to anti-HBeAg more than 2-years after stopping therapy. The results of genotype and pre-core region mutations in relation to response are summarised in Table 2.

Relaxed circular HBV-DNA viral load in liver

Median RC HBV-DNA concentration in pre-treatment liver biopsies was 4.41 log_{10} copies/ng of liver genomic DNA (lg DNA) (range 1.71–5.14) in treated patients and tended to be lower in responders than non-responders (3.82, 1.71–4.18 vs. 4.71, 2.78-5.14 log_{10} copies/ng of lg DNA, p=0.16). The median concentration of RC HBV-DNA in untreated patients (4.22, 2.2-5.2 log_{10} copies/ng of lg DNA, p=0.45) did not differ from the treated cohort.
cccDNA concentration in liver

The tissue concentration of cccDNA was similar in treated and untreated patients (2.08, 0.57-3.56 vs. 1.88, 0.76-3.49 log\text{10} copies/ng of Ig DNA) and tended to be lower in responders than non-responders (1.98, 0.57-3.56 vs. 2.26, 1.32-2.37 log\text{10} copies/ng of Ig DNA, p=0.18). Levels of liver RC HBV-DNA and liver cccDNA were positively correlated (r=0.67, p=0.04).

Expression of HBcAg and HBsAg in liver

HBcAg expression was observed predominantly within the nucleus of hepatocytes and was similar between responders and non-responders (8.1, 1.1-42.5 vs. 10.5, 0.5-83.6 positive cells/1000 hepatocytes, p=0.24). When assessed semi-quantitatively, HBcAg nuclear and cytoplasmatic expression was similar in all treated patients, whether responders (median nuclear staining 45% and median cytoplasmic staining 32.5%) or not (40% and 30%), and in untreated HBV+ controls (45% and 30%). In contrast, HBsAg was predominantly expressed in the cytoplasm focally associated with membranous staining [2.9 (0-56.9) positive cells/1000 hepatocytes] and was significantly lower in responders than non-responders [0.5, (0-16) vs. 4.7 (0-56.9) positive cells/1000 hepatocytes, p=0.03]. There were positive bivariate correlations between plasma HBsAg levels and liver cccDNA (r=0.44, p=0.04), HBcAg liver expression and cccDNA (r=0.41, p=0.04) and between pre-treatment plasma HBsAg levels and HBsAg liver expression (r=0.38, p=0.05). Testing was not performed in untreated patients.

Multivariate analysis
HBV-DNA load at TW52 ($p=0.003$), FUW24 ($p=0.001$) and FUW52 ($p=0.001$) and HBsAg plasma levels at TW28 ($p=0.001$), TW52 ($p=0.001$) and FUW24 ($p=0.001$) were significantly lower in responders than non-responders.

**DISCUSSION**

Loss of HBsAg with anti-HBs seroconversion is the ultimate goal of antiviral therapy for chronic hepatitis B infection [3]. The present longitudinal study firstly demonstrates the role of HBsAg plasma pre-treatment levels and their on-treatment kinetics in predicting therapy response; secondly, it illustrates the relationship between pre-treatment liver cccDNA, relaxed circular HBV-DNA and liver expression of HBsAg; and thirdly, it highlights the importance of changes in BCP and 1896 codon of the pre-core region in relation to therapy response (HBsAg loss) in children with immunotolerant perinatally acquired CHB infection treated with lead-in lamivudine followed by add-on interferon [45].

Of the 23 vertically infected patients participating in a pilot treatment study, 5 (22%) achieved seroconversion to anti-HBs either during treatment or within 1 year post therapy, reflecting transition from an immune tolerance state to viral control [40]. Patients achieving viral control were characterized by lower pre-treatment HBsAg plasma levels, lower HBsAg expression in the liver, sharper decline of HBV-DNA within the first 9 weeks of therapy during the lamivudine lead-in phase, followed by a steep decrease in HBsAg plasma levels ($>1-\log_{10}\text{IU/ml}$) by treatment week 28, during the IFN-α add-on phase. Although concentrations of intrahepatic relaxed circular HBV-DNA and cccDNA tended to be lower in responders, HBCAg expression in the liver was similar between
responders and non-responders. The therapy response was associated with lower cytoplasmic expression of HBsAg in the liver. Plasma HBsAg levels correlated with intrahepatic RC HBV-DNA, cccDNA and HBsAg expression within the liver reflecting transcriptional activity of intrahepatic viral DNAs.

Though on-treatment viral kinetics are currently used to monitor treatment response and to decide treatment duration in adults with CHB [16-31], data are not available in children. In particular there is no information on HBsAg kinetics in paediatric immunotolerant patients during antiviral therapy. In our cohort, the combination of pre-treatment HBsAg plasma levels <4.3-\log_{10}IU/ml and the HBsAg kinetics profile, i.e. a decline >1-\log_{10}IU/ml, during the first 6 months of therapy after adding INF-\alpha, was highly predictive of response. Lower HBsAg plasma levels at baseline also predict therapy response in adult immunoactive patients on therapy with pegylated interferon (Peg-IFN) alone or in combination with lamivudine (LAM) commenced simultaneously [18-21-22].

In a Dutch study investigating the impact of HBsAg plasma levels and their on-treatment kinetics during pegylated-interferon ± lamivudine therapy, HBsAg <1500 IU/ml (3.17-\log_{10}IU/ml) at TW24 predicted HBeAg seroconversion and HBV-DNA <2000 IU/ml 6 months after stopping therapy (PPV 54% and NPV 76%) [22]. Applying these criteria to our paediatric cohort, at TW28 (20 weeks after adding IFN-\alpha) all five patients who lost HBsAg after therapy had HBsAg levels <1000 IU/ml, while none of fifteen patients with an HBsAg decline <1-\log_{10}IU/ml achieved response (NPV 100%).

In the Dutch study, patients treated with a combination of lamivudine and Peg-IFN-\alpha 2b achieved a higher and earlier anti-HBe seroconversion rate during
treatment and a higher rate of HBsAg loss at follow up than patients treated with Peg-IFN-α 2b alone [44]. In agreement with these observations, all our patients who lost HBsAg had HBeAg seroconversion between the first 12-20 weeks of therapy, while two patients who achieved HBeAg seroconversion during the post-treatment follow-up never lost HBsAg. Therefore, early anti-HBe seroconversion heralds treatment-induced complete viral control.

In our cohort, responders tended to have lower intrahepatic concentrations of RC HBV-DNA and cccDNA and a lower expression of HBsAg within the liver. Baseline HBsAg plasma levels correlated positively with cccDNA, liver RC HBV-DNA and liver HBsAg expression akin to what observed in adult HBeAg+ patients, in whom pre-treatment HBsAg plasma levels correlate with liver cccDNA and liver HBV-DNA, suggesting that plasma HBsAg levels may act as a surrogate marker of cccDNA and intrahepatic relaxed circular HBV-DNA [11,16].

In our patients, the switch between immune tolerance to immune control and HBsAg clearance was linked to a significant reduction of HBV-DNA replication initially, followed by a sharp decline in HBsAg production as a likely consequence of the immune enhancing effect of IFN-α. In the same cohort of patients, we have previously shown that combined treatment elicits virus-specific immune responses [38]. Thus, HBsAg decline and its kinetics are likely to reflect strong and multispecific adaptive immune responses fostering the progression from immunotolerance to complete viral control [38]. The predominance of genotype B in the responders might contribute to the lower pre-treatment HBsAg levels, as there was no difference in HBV-specific reactivity at baseline between responders and non-responders [38].
In immunoactive adult patients concentrations of cccDNA and intrahepatic RC HBV-DNA have been variably reported to predict HBsAg plasma levels and response to treatment [16, 35, 37]. In our cohort of immunotolerant children - a yet untested patient population - there was only a tendency for cccDNA and relaxed circular DNA tissue levels to be lower in responders than non-responders, the difference from adult studies probably reflecting the small number of our patients, their immunotolerant status and different HBV genotypes.

Similar to reports in adults [45], the children who lost HBsAg in our cohort had genotypes A or B. In agreement with the report by Sonneveld et al [39], all our patients who lost HBsAg had wild type BCP and 1896 codon in the pre-core region, while mutations were present in 14 of the 18 non-responders.

Although the present study is not focused on therapeutic approaches in children with infancy-acquired infection who are tolerant to HBV, it is important to highlight the lack of data in this group of patients. In general, the goal of antiviral HBV therapy in children, as in adults, is to improve the long-term survival and quality of life by reducing the risk of liver disease progression, preventing the development of cirrhosis and hepatocellular carcinoma (HCC) [3, 46]. Long-term high HBV-DNA viral load, as seen during the immunotolerant phase of the disease, is an independent risk factor for evolution to cirrhosis and HCC [47]. In addition, childbearing female patients in the immunotolerant stage of HBV infection have a high risk of transmitting the virus to the next generation. All these factors should be taken into account when designing treatment in paediatric patients. There is a limited number of anti-HBV drugs licensed for children, in particular in those younger than <12 years. Most studies in
paediatrics use monotherapy and include children with immunoactive disease. There is little information on the treatment of immunotolerant children [46]. However, on the basis of the encouraging results in terms of HBsAg loss of two pilot studies using lamivudine/standard interferon combination therapy [40, 51], currently two large clinical trials of sequential combination therapy with a nucleoside analogue (lamivudine or entecavir) and pegylated interferon are ongoing [48-49].

Recently, several pilot adult studies have also focused on a sequential combination therapy using nucleos(t)ide analogues lead-in followed by addition of interferon [50-53]. This approach is based on the immunopathology knowledge that harnessing the immune response is important to achieve a ‘functional cure’ of HBV infection (HBsAg loss). On the one hand, long-term anti-viral therapy with a nucleos(t)ide analogue improves HBV-specific the cytotoxic T lymphocyte reactivity, which is a critical component of the anti-HBV response [43,54], on the other hand interferon based therapy stimulates innate immune system natural killer cell activity, which increases in patients during therapy and persists during the post-treatment follow-up period [55]. The concerted action of innate and adaptive immune responses is needed to achieve HBV replication control, including that of nuclear cccDNA, and attain HBsAg loss/anti-HBs seroconversion. An additional effect of pro-inflammatory cytokines on HBV replication, namely of IFN-α on the epigenic regulation of the nuclear cccDNA minichromosome (critical component of HBV persistence) has been demonstrated in-vitro and in animal models [56-57].
High HBV-DNA levels are linked to high concentrations of viral antigens, leading to immune system exhaustion and immune tolerance in the long-term [58]. Reduction of the HBV-DNA viral load with consequent decrease of HBV antigens enhances the immune system recognition [15]. In our cohort, although HBV-DNA declined after 9 weeks of therapy with lamivudine, there were no or minimal changes in HBsAg levels between baseline and TW9. Adding interferon at TW9 contributed to the HBsAg decline and to the restoration of the immune responses in all patients, but more significantly in the responders, indicating a concerted effect of the innate and adaptive immune responses [38]. Whether serological immune markers like chemokine and cytokine levels including CXCL10, in conjunction with HBV-DNA viral load, HBsAg and HBeAg plasma levels and HBsAg subtype (viral vs subviral particles ratio) would be helpful in determining the best timing for adding interferon to nucleos(t)ide analogues needs to be investigated.

In conclusion, the present longitudinal study demonstrates that assessment of pre-treatment HBsAg plasma levels and monitoring of their on-treatment kinetics during combination therapy, in particular during the early phase of add-on interferon treatment, are useful tools for predicting therapy response and for tailoring treatment in immunotolerant children, as recently advocated for adult patients [30-31]. However, a degree of caution should be applied when universally implementing these therapy response predictors owing to the genotypic variability and variable immunological stages of CHB [24]. Larger combination treatment trials of tolerant patients, using sequential therapy with more powerful new generation nucleot(s)ide analogues lead-in, to efficiently suppress HBV-DNA and
prevent emergence of mutations, followed by pegylated-interferon add-on, are warranted to validate the use of these markers in predicting treatment response.

Table 1

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<td><strong>Gender</strong></td>
<td>8/15</td>
<td>3/4</td>
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<td>2</td>
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<tr>
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<td>4</td>
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<tr>
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<tr>
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<tr>
<td><strong>Genotype:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>AST [IU/L]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>29</td>
<td>32</td>
</tr>
<tr>
<td>Range</td>
<td>18-90</td>
<td>22-68</td>
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<tr>
<td><strong>HBV-DNA [log_{10}IU/ml]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>8.17</td>
<td>8.32</td>
</tr>
<tr>
<td>Range</td>
<td>7.03-8.90</td>
<td>7.23-8.96</td>
</tr>
</tbody>
</table>
AST: aspartate aminotransferase (normal value <50IU/L); 
HBV-DNA: hepatitis B virus DNA

Table 2
Table 2: HBV genotypes and pre-core region mutations

<table>
<thead>
<tr>
<th>Genotype:</th>
<th>Treated (N=23)</th>
<th>Untreated (N=7)</th>
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<tbody>
<tr>
<td></td>
<td>Responders (N=5)</td>
<td>Non-responders (N=18)</td>
</tr>
<tr>
<td>* A</td>
<td>1 patient (WT)</td>
<td>1 patient (A1762T/G1764A)</td>
</tr>
<tr>
<td>* B</td>
<td>4 patients (WT)</td>
<td>7 patients (A1762T/G1764A)</td>
</tr>
<tr>
<td>* C</td>
<td>0 patients</td>
<td>2 patients (A1762T/G1764A)</td>
</tr>
<tr>
<td>* D</td>
<td>0 patients</td>
<td>2 patients (G1896A)</td>
</tr>
<tr>
<td>* E</td>
<td>0 patients</td>
<td>1 patient (A1762T/G1764A)</td>
</tr>
</tbody>
</table>

WT: wild type
A1762T/G1764A: basal core promoter (BCP) mutations
G1896A: stop codon 1896 mutation
REFERENCES:


21. Ma H, Yang RF, Wei L. Quantitative serum HBsAg and HBeAg are strong predictors of sustained HBeAg seroconversion to pegylated interferon alfa-2b in HBeAg-positive patients. J Gastroenterol Hepatol. 2010; 25(9): 1498-506.


49. Roche. Lamivudine/pegylated interferon in immune tolerant children with chronic hepatitis B virus infection. Single center clinical trial at the Paediatric Liver Centre, King’s College Hospital, London, United Kingdom. Study identifier: NV2536.


