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Article type : Original Scientific Paper

First decision date: 09-Sep-2017

HBeAg levels at week 24 predict response to 8 years of tenofovir in HBeAg-positive chronic hepatitis B patients

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Key Words: HBV; quantitative HBeAg; nucleos(t)ide analogue therapy; HBeAg seroconversion; antiviral therapy

Word count

Title: 18 words

Abstract: 241 words

Main text: 3532 words

Number of figures: 3

Number of tables: 3

Financial Disclosure:

Gilead Sciences funded and conducted the original study, including the collection and storage of serum samples. For this study, Gilead provided funds for the DNA sequencing and HBeAg quantification. They were not involved in the analysis or interpretation of data, or the decision to submit for publication.

Acknowledgements:

We thank the patients who participated in the parent study and their families.

Authorship Statement

Associate Professor Peter Revill is the guarantor of this work. All authors reviewed and approved the final version of the manuscript. DW was involved study design, data collection, statistical analysis, HBeAg quantification, and manuscript preparation and revision. ML, LY, KJ, JB, and GR were responsible for sequencing. HM performed HBeAg quantification. AT and PR were involved in study conception, design, and manuscript preparation. AG, KK, MS, PM, MB, HJ, EG, and SL all reviewed the manuscript.

ABSTRACT

Background

HBeAg seroconversion is a treatment endpoint for HBeAg-positive CHB, and a necessary precursor to HBsAg loss. Biomarkers that predict serological outcomes would be useful.

Aim

The aim of this study was to evaluate the utility of measuring HBeAg levels for predicting HBeAg SC and HBsAg loss under long-term tenofovir (TDF) therapy.

Methods

266 patients were enrolled into a phase III study of TDF vs. adefovir (ADV) for 48 weeks in HBeAg-positive patients, followed by open-label TDF up to 384 weeks. Serum HBeAg levels were measured for subjects with samples available at both baseline and week 24 of treatment (n=200). Analysis compared subjects who achieved HBeAg seroconversion by week 384 vs. no HBeAg seroconversion.

Results

HBeAg seroconversion rate was 52% by week 384. Time to HBeAg seroconversion was 80 weeks [IQR:36-162]. HBeAg decline at week 24 was associated with HBeAg seroconversion (1.63 vs 0.90 $\log_{10}$ PEIU/ml, p = 0.002). The optimal threshold for identifying HBeAg seroconversion was HBeAg decline ≥2.2 $\log_{10}$ PEIU/mL at week 24, with HBeAg seroconversion achieved by 76% of patients, compared to 44% if HBeAg decline <2.2 $\log_{10}$ (p<0.0001). HBeAg decline ≥2.2 $\log_{10}$ PEIU/mL at week 24 was associated with HBsAg loss in genotype A or D patients (38% vs 15%, p=0.03). Precore/basal core promotor variants were associated with lower baseline HBeAg levels, but not HBeAg seroconversion.

Conclusion

Decline in HBeAg levels by week 24 was associated with HBeAg seroconversion and HBsAg loss in HBeAg-positive chronic hepatitis B patients treated with long-term TDF.

Study GS-US-174-103 (NCT00116805).

INTRODUCTION

Chronic hepatitis B infection is a complex disease affecting approximately 257 million people worldwide [1]. The natural history of infection evolves through multiple phases, currently defined by clinical parameters including the patient's hepatitis B e antigen (HBeAg) status, the presence of hepatic inflammation (alanine aminotransferase (ALT) levels), as well as HBV DNA levels [2-4]. Therapeutic strategies focus on achieving sustained virological control. Most patients are treated with nucleos(t)ide analogues and although these are potent antiviral agents, therapy is usually long-term.

HBeAg seroconversion, defined by the loss of HBeAg, and the appearance of anti-HBe antibodies, is usually associated with suppression of HBV DNA to <2000 IU/ml, and reduction in risk of progression to advanced liver disease or hepatocellular carcinoma [5-7]. HBeAg seroconversion is also considered a necessary precursor to HBsAg loss [8, 9], and has been recognised as a treatment end-point by international guidelines [4, 10, 11]. Despite this, little is known about the molecular events that precede HBeAg seroconversion, and it is not possible to accurately predict which patients are likely to achieve this outcome whilst on nucleos(t)ide analogue therapy. This is important, as patients who do not serologically respond are likely to require lifelong treatment to maintain viral suppression. Fried and colleagues published a detailed analysis of on-treatment quantitative HBeAg levels in the setting of 48 weeks of pegylated interferon therapy, and showed that the magnitude of HBeAg decline could predict for HBeAg seroconversion (and non-response) [6]. Whether HBeAg levels predict for HBeAg seroconversion during nucleos(t)ide analogue therapy is unknown.

Study GS-US-174-0103 was a randomised, double-blind, phase 3 study of 266 HBeAg-positive chronic hepatitis B patients with immune clearance disease that compared the antiviral efficacy of tenofovir disoproxil fumarate (TDF) vs. adefovir dipivoxil (ADV) monotherapy for an initial 48 weeks, followed by open label TDF for a further 336 weeks [12, 13]. Primary clinical trial endpoints included plasma HBV DNA <400 copies/ml and histological improvement. HBeAg and/or HBsAg loss and seroconversion were secondary endpoints. The clinical outcomes at weeks 48 through 384 have been previously published [12-16].

The aim of this analysis was to evaluate the association between on-treatment quantitative HBeAg levels and serological outcomes among subjects enrolled in study GS-US-174-0103.

METHODS

Patients

A total of 266 HBeAg-positive patients with chronic HBV infection were enrolled into GS-US-174-0103 [12]. The current analysis included only those patients infected with the four major HBV genotypes A to D (n=249); patients infected with other genotypes were excluded from the analysis due to small subject numbers, which would have confounded the analysis (n=17). Of these, 235 had serum available at week 0, and 214 had serum available at week 24. These two cohorts only partially overlapped, leaving 200 subjects that had sufficient serum for inclusion. Clinical data including patient demographics, biochemistry, histologic activity scores, and HBV DNA levels at baseline were provided from the clinical database of the GS-US-174-0103 study. Serological status (HBeAg, anti-HBe, HBsAg and anti-HBs) at week 384 was provided. The primary outcome for the current analysis was HBeAg seroconversion (HBeAg undetectable, anti-HBe detectable) by week 384 or last visit.

Viral load and serological characterisation

HBeAg levels were measured using the Roche Elecsys HBeAg assay (Roche Diagnostics, Mannheim, Germany). The upper limit of quantification was 6,000 PEIU/ml and the lower limit was 0.3 PEIU/ml. Serial dilution of serum was performed to obtain an exact level if the dynamic range of the test was exceeded. HBV viral load and HBsAg quantification were determined previously using the Roche COBAS TaqMan (Roche Diagnostics, Mannheim, Germany) and Abbott Architect (Abbott Laboratories, Illinois, USA) platforms, respectively [12]. The lower limit of detection for HBV DNA was 29 IU/ml, and for HBsAg was 0.05 IU/ml.

PCR Amplification and Direct Sequencing

To interrogate the association between presence of BCP (A1762T/G1764A) and/or PC (G1896A) mutations, and HBeAg levels and treatment response, the PC/core region was amplified from baseline, and on-treatment samples to week 48, by PCR. Oligonucleotides were synthesized by Geneworks, Adelaide, Australia and first round primers were PC5 (5' TCG CAT GGA GAC CAC CGT GA3') and 1437 (5' CAT GCT GTA GCT CTT GTT CC3'), second round primers were PC5 and 1094 (5' CGA AAT AAG AAG ATG ACA TGG3'). Samples were amplified in a 50 µl reaction containing 4µl extracted DNA template, 1X Qiagen amplification buffer containing 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.2µM each primer and 2 units of HotStar Taq Polymerase (Qiagen). Second round amplification was carried out using 2µl of first round product in a 50µl reaction as for the first round. Cycling parameters for first round consisted of an initial denaturation/Taq activation step at 95°C for 5 min, followed by a further 50 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 50 s). A final cycle of extension (72°C for 10 min) was used for completion of the products. Cycling parameters for second round consisted of an initial denaturation/Taq activation

step at 95°C for 5 min, followed by 25 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 40 s). A final cycle of extension (72°C for 10 min) was used for completion of the products. These primers amplified the negative regulatory element (NRE), core upstream regulatory sequence, BCP regulatory regions, and the complete PC/core coding region. The region amplified in the first-round PCR was from nts 1624 to 2836 inclusive, and the second-round nested PCR amplified nts 1624 to 2493. Numbering commenced from the HBV EcoR1 start site [17]. The amplicon was purified using PCR purification columns from MO BIO Laboratories Inc. (Carlsbad, CA, USA) as per the manufacturer's protocol. PCR products were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit Version 3.1 (Applied Biosystems, Foster City, CA, USA) using the PCR primers. Gel electrophoresis of the sequencing reactions was carried out by MicroMon (Department of Microbiology, Monash University, Clayton, Australia). Cut-off for variant detection is approximately 20-25%.

Next Generation Sequencing

The frequency of BCP and PC mutations in a subset of this cohort was also determined by next generation sequencing using the Illumina MiSeq protocol, the results of which have been previously published [18, 19].

Definition of HBeAg Seroconversion

HBeAg seroconversion was defined as the development of anti-HBe and loss of HBeAg, confirmed on two samples taken at least 6 months apart. If participants did not continue follow up for the full 384 weeks of the study, the last available serologic outcome was taken as final. Regardless of the development of HBeAg seroconversion, all patients had ongoing treatment and follow up, as only HBsAg loss was an endpoint allowing treatment discontinuation in the parent study.

Statistical analysis

Statistical analyses were performed using Stata 14.2 (StataCorp, College Station, TX). Parametric data is reported as mean ± standard deviation (sd). Non-parametric data is reported as median (interquartile range; IQR). Categorical data is reported as number (percentage). Exploratory, bivariate analyses of outcome variables were conducted using parametric or non-parametric tests as appropriate for continuous data, and chi-square or Fisher's exact test for categorical data. Regression analyses with stepwise elimination were performed using all variables. Where multivariate regression was used, all baseline variables were included in the model, with stepwise elimination of non-significant factors to create the final model. Cox proportional hazards modelling

was used to determine independent factors associated with on-treatment response. Bonferroni corrections for repeated measures testing were applied. A two-tailed p-value of 0.05 was considered statistically significant.

RESULTS

Cohort characteristics.

The characteristics of the overall cohort have been described in detail previously [12]. Baseline and on-treatment samples from 200 patients were evaluated in this analysis; characteristics of those included did not differ significantly from those excluded (Supplementary Table 1). Of these, 104 (52%) patients achieved HBeAg seroconversion by week 384 (Supplementary Figures 1 & 2). A total of 178 (89%) had at least two years of follow up, with 147 (74%) subjects completing the study (Supplementary Table 2). The median time to HBeAg seroconversion was 80 weeks [IQR: 36-162]. Baseline predictors of HBeAg seroconversion were older age, HBV genotype A, and higher grade necro-inflammatory activity on liver histology (Table 1). There was no association with other baseline variables including ALT, HBV DNA level or HBsAg level.

Baseline HBeAg levels varied by genotype.

Median HBeAg level was 1,369 PEIU/ml [IQR: 300-3,340] (3.14 $\log_{10}$ PEIU/ml [IQR: 2.48-3.52]) across the cohort (Figure 1A). Only one sample required dilution to fall within the dynamic range of the HBeAg assay. The remainder returned results within range as tested on undiluted serum. There were no statistically significant differences in baseline virologic parameters between those who achieved HBeAg seroconversion vs. not (Table 1). Specifically, median HBeAg levels in those achieving seroconversion were 1,690 PEIU/ml [IQR 187-3,441] vs. 1,175 PEIU/ml [IQR 547-3,001] in non-seroconversion participants (p=0.95).

HBeAg levels at baseline varied by HBV genotype (Table 2, Figure 1B). When compared to genotype A subjects, genotype C subjects had significantly lower baseline HBeAg levels (2,595 PEIU/ml [IQR: 335-3,960] vs. 843 PEIU/ml [IQR: 201-2,920], p=0.02). However, on-treatment median values at week 24 were not significantly influenced by genotype (Table 2), although there was a trend towards genotype A subjects achieving a larger reduction in HBeAg levels by week 24 (reduction of 1,969 PEIU/ml [IQR: 273-2,955] vs. 927 PEIU/ml [IQR: 205-2,149], p=0.07).

Baseline HBeAg levels varied by the presence of PC / BCP variants.

Population sequencing of the BCP/PC region was successful for 190 of 200 baseline samples, with ten failures due to insertions-deletions within the BCP sequence. Next generation sequencing (NGS) data was available for 147 of the subjects at baseline. There was no significant difference in the

serological profile of these samples compared to the complete dataset (data not shown). The frequency of BCP and PC variants at baseline by population sequencing is presented in Table 1. The presence of BCP variants at baseline was associated with lower baseline HBeAg level, except for genotype B infection, although there were only four subjects in this group (Table 2, Figure 1C) [18]. NGS data demonstrated that BCP variants, when detected, were present as the dominant quasispecies. There was a negative correlation between baseline HBeAg levels and the proportion of the quasispecies with a BCP variant detected by NGS. An increasing proportion of BCP variants resulted in lower HBeAg levels (Figure 1D). BCP variants were also associated with lower HBeAg levels at week 24. In contrast, HBeAg levels were not significantly lower in subjects with a detectable PC mutation. These variants were uncommon, and when present were a minor quasispecies in this cohort with HBeAg-positive disease. PC variants were detectable at a median proportion of only 15% [IQR: 2-43] vs. BCP variants 85% [IQR: 47-96].

Using bivariate analysis, baseline HBeAg levels were associated with the presence of both BCP variants (Pearson's r = -0.41, p<0.0001), genotype (p=0.006), HBV DNA (Pearson's r=0.47, p<0.0001) and HBsAg levels (Pearson's r=0.33, p<0.0001). BCP variants were independently associated with baseline HBeAg levels after adjustment for HBV DNA and HBsAg level (p=0.003, data not shown).

HBeAg level decline reflects potency of viral suppression.

In contrast to baseline HBeAg levels, the magnitude of HBeAg decline by week 24 of treatment did not vary by genotype (p=0.53) or the presence of BCP/PC variants (p=0.84). There was a statistically greater reduction in week 24 HBeAg levels in those subjects randomised to initial treatment with TDF vs. ADV (mean reduction 1.50 [sd: 1.14] $\log_{10}$ PEIU/ml vs 1.01 [sd: 0.91] $\log_{10}$ PEIU/ml, p=0.002), in keeping with the greater potency of TDF. After controlling for the concomitant decline in HBV DNA by week 24, the association with initial TDF treatment was rendered non-significant (p=0.23). This indicates that the timing of the decline in HBeAg levels reflects the degree of viral suppression, and is not related to a specific drug effect.

A greater decline in HBeAg level at week 24 predicted HBeAg seroconversion.

Overall, HBeAg levels declined by a mean of 1.31 (sd 1.09) $\log_{10}$ PEIU/ml by week 24. The magnitude of this decline was significantly greater in seroconversion vs. non-seroconversion patients (1.63 $\log_{10}$ PEIU/ml vs 0.90 $\log_{10}$ PEIU/ml respectively, p=0.002) (Figure 2B). Subjects attaining seroconversion had significantly lower HBeAg levels at week 24 with mean values of 1.23 $\log_{10}$ PEIU/ml [sd 1.33] vs.

1.85 $\log_{10}$ PEIU/ml [sd 1.10] in non-seroconversion participants (p=0.0004) (Figure 2A). There was no significant difference in the magnitude of HBV DNA decline by week 24 between the two groups.

To ascertain the optimal cut-off for HBeAg decline by week 24, the Youden index was calculated for all degrees of HBeAg decline at week 24 of treatment, showing that the association was strongest when HBeAg decline was ≥2.2 $\log_{10}$ HBeAg PEIU/ml (Table 3, Figure 3A).

The association between a decline in HBeAg to an absolute level of ≤10 PEIU/ml at week 24 and HBeAg seroconversion was also investigated. This cut off was chosen as it intersected the lower tertiles of HBeAg values at week 24, and coincided with previously published data [6]. Subjects achieving this HBeAg level had a significantly higher likelihood of seroconversion (Table 3, Figure 3B). Detailed information on the rates of HBeAg seroconversion according to both thresholds is presented in Supplementary Table 3. Additionally, amongst HBeAg seroconverters, those achieving the ≥2.2 $\log_{10}$ HBeAg PEIU/ml threshold did so after a median of 36 weeks [IQR: 24-80], whereas those who did not meet the threshold did so after a median of 108 weeks [IQR: 48-204], p=0.0001.

Multivariate modelling identified several factors independently associated with on-treatment HBeAg seroconversion.

Using Cox proportional hazards modelling, increased likelihood of HBeAg seroconversion was independently associated with age (HR 1.02, 95% CI: 1.01-1.04, p=0.004), genotype A infection (HR 2.11, 95% CI: 1.38-3.24, p=0.001), and HBeAg decline of ≥2.2 $\log_{10}$ PEIU/ml by week 24 (HR 2.66 95% CI: 1.77-3.99, p<0.0001). We have previously shown that higher baseline HBsAg titres were associated with increased likelihood of HBsAg loss, although there was no association with HBeAg seroconversion [18]. Although we identified a statistical association with HBsAg decline at week 24 and eventual HBeAg seroconversion in a univariate analysis (HBsAg decline of ≥1 $\log_{10}$ IU/ml – HR 1.81, 95% CI: 1.09-3.01, p=0.02), this association was no longer significant after adjustment for HBeAg decline. The presence of BCP or PC mutations by population sequencing at baseline did not affect the likelihood of HBeAg seroconversion, despite the observation that BCP variants were associated with a reduction in baseline HBeAg levels. HBeAg level ≤10 PEIU/ml was independently associated with HBeAg seroconversion in a second model that excluded HBeAg decline of ≥2.2 $\log_{10}$ PEIU/ml by week 24. There was a statistical interaction in the model between an HBeAg decline of ≥2.2 $\log_{10}$ HBeAg PEIU/ml by week 24 and reduction of HBeAg to ≤10 PEIU/ml at week 24. If both

thresholds were reached, the association with HBeAg was significantly stronger (HR 3.37 95% CI: 2.15 – 5.27, p<0.0001).

Decline in on-treatment HBeAg level at week 24 was associated with HBsAg loss in subjects infected with genotype A or D.

We have previously shown that the presence of a PC/BCP variant population at baseline has a high negative predictive value for HBsAg loss [18]. The additional predictive value of on-treatment HBeAg decline was explored. We focused on the 112 genotype A or D-infected subjects, as only one study subject with genotype B or C infection achieved HBsAg loss, and therefore additional predictive rules for this group are unnecessary. There was a significant association between a week 24 HBeAg decline of ≥2.2 $\log_{10}$ PEIU/ml and HBsAg loss, occurring in 38% (10/26) meeting this threshold, vs 15% (13/86) who did not (p=0.03). The positive predictive value was 38%, but the negative predictive value (ie, if this threshold was not met) was 85%. Using the absolute HBeAg level of ≤10 PEIU/ml at week 24 returned similar results (PPV 31%; NPV 85%), but this did not reach statistical significance. Following multivariate logistic regression analysis, factors associated with HBsAg loss were genotype A infection (OR 3.89, p=0.01) and a HBeAg decline of ≥2.2 $\log_{10}$ PEIU/ml at week 24 (OR 4.19, p=0.02). The presence of a BCP mutation at baseline detected by population sequencing was a significant negative predictor for HBsAg loss in the multivariate model (OR 0.17, p=0.005).

DISCUSSION

This study is a detailed evaluation of the use of HBeAg level monitoring to predict for HBeAg seroconversion in the context of long-term nucleos(t)ide analogue therapy for chronic HBV in a large patient cohort. The decline in on-treatment HBeAg level from baseline to week 24 was shown to strongly predict for HBeAg seroconversion. HBeAg decline ≥2.2 $\log_{10}$ PEIU/mL was identified as an important threshold for predicting HBeAg seroconversion. In addition, achieving a reduction in HBeAg to below 10 PEIU/ml was also associated with HBeAg seroconversion, and provided additional predictive value to the on-treatment decline in the model. Decline in HBeAg level was a stronger predictor of HBeAg seroconversion than HBsAg decline (HBsAg decline was not associated with HBeAg seroconversion after adjustment for HBeAg level). Furthermore, in subjects infected with a so-called 'Western' HBV genotype (ie, genotype A or D), HBeAg decline also independently identified subjects who later achieved HBsAg loss.

The results do have clinical relevance. Monitoring of HBeAg levels will inform likelihood of HBeAg seroconversion and will be of interest to patients and clinicians, using a paradigm where HBeAg seroconversion is a treatment endpoint, particularly relevant to resource-limited regions. An

important question for future studies will be whether the rate of HBeAg decline predicts for durable off-treatment HBV DNA suppression or the need/duration of consolidation therapy. Although treatment withdrawal was not directly studied in this cohort, there is extensive literature supporting this as a reasonable strategy with a period of >12 months consolidation therapy, an approach which has cost benefits [4, 10, 11, 20]. Whilst the 2.2 $\log_{10}$ PEIU/ml decline threshold identified in this study has strong statistical significance, it will require prospective validation in future study cohorts.

Using population-based Sanger sequencing, we showed that BCP variants were common, being detected in 41% of the study cohort. As previously observed, the detection of BCP variants was associated with lower HBeAg levels [18]. Conversely, the PC variant was uncommon in this cohort (19%), and when present existed as a minor quasispecies. The association of the BCP variant with significantly reduced baseline HBeAg level was again demonstrated, with the suggestion of a gradient effect (ie, a higher proportion of BCP variants was significantly associated with lower HBeAg level) in the subset of study subjects with NGS data available. Despite lower HBeAg levels, BCP variants at baseline did not predict for on-treatment HBeAg seroconversion, either in the entire cohort, nor after adjustment for BCP / PC variants.

The association between HBeAg kinetics and serological end-points is potentially relevant to clinical trial design for novel candidates aiming for HBV cure [21]. Assays to quantify HBeAg longitudinally should be included in biomarker discovery panels for clinical development studies, as identifying patients that are unlikely to respond serologically on nucleos(t)ide analogue therapy may enable early intervention with alternative treatments to promote seroconversion. Finally, HBeAg status has been associated with HCC risk [22]. The association between HBeAg decline and treatment response suggests a plausible role for HBeAg level as a marker for natural history - HBeAg levels may refine existing risk models. It will be important for future studies to evaluate the association between HBeAg level and HCC risk in the setting of nucleos(t)ide analogue therapy. As the global HBV cure effort moves forward, biomarkers that identify subsets of patients who may more rapidly benefit from novel therapeutic interventions will be helpful. Measuring HBeAg levels at baseline and on treatment is a simple assay that may guide such interventions. As data characterizing the anti-HBV immune response was not available for the current study, we also suggest that future studies should investigate the correlation between HBeAg level, anti-HBV immunity and clinical outcomes.

The observations made are subject to several limitations. The first is that off-treatment samples from the parent study were not available to determine durability of serologic response. Secondly, is

the reduced granularity of results by grouping genotypes A and D vs. genotypes B and C. Whilst we acknowledge that there are inherent differences between all genotypes, reporting of results by grouping subjects in this way is common in the literature, and insufficient subject numbers precluded a more detailed analysis. Thirdly, there was no available immunological data for this cohort which would allow derivation of mechanisms underlying the observations made in this study. These limitations need to be addressed in future studies.

The findings provide further support for the incorporation of quantitative assays for HBeAg in patient management [23-29]. Although widely available outside of the United States, assays that measure HBeAg have yet to be submitted to the FDA for approval. The data demonstrate clinical relevance to nucleos(t)ide analogue therapy, and identify HBeAg as an important biomarker for clinical trials moving forwards.

In conclusion, this study demonstrates the potential to use quantitative HBeAg levels as an on-treatment predictor of HBeAg seroconversion and HBsAg loss. The magnitude of the decline in HBeAg from baseline was strongly associated with this current treatment endpoint. Quantitative HBeAg levels should be included as a routine component of clinical trial laboratory analysis, particularly to identify patients who are unlikely to reach current treatment endpoints and the therapeutic targeting of HBeAg also warrants investigation.

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470 surface antigen loss in patients with HBeAg-positive chronic hepatitis B treated with tenofovir
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472

473 **Table 1:** Baseline clinical characteristics of patients analysed from study GS-US-174-0103, stratified by HBeAg
474 seroconversion (n=200).

	HBeAg Seroconversion		
	No	Yes	
	n=96	n=104	p-value
Age, median (IQR)	28 (23 - 39)	36 (26 - 47)	0.001
Male, n (%)	68 (71)	71 (68)	0.65
Ethnicity, n (%)			
White	48 (50)	55 (53)	0.67
Black	0 (0)	7 (7)	0.01
Asian	45 (47)	36 (35)	0.08
Other	3 (3)	6 (5)	0.47
Body Mass Index, median (IQR)	23.5 (20.6 – 26.4)	24.0 (21.4 – 27.7)	0.21
Estimated Years of Infection, median (IQR)	4.6 (1.5 - 15.7)	3.4 (0.9 – 12.7)	0.26
ADV for First 48 Weeks, n (%)	32 (45)	39 (55)	0.41
Knodell Histologic Activity Index*, median (IQR)	10 (8-11)	11 (10-13)	0.007
ALT (IU/L), median (IQR)	112 (80 - 160)	109 (85 - 190)	0.79
HBV DNA (log_{10} IU/ml), mean (sd)	8.06 (1.01)	7.83 (1.04)	0.12
Baseline HBsAg (IU/ml), median (IQR)	42,463 (16,120 – 77,165)	31,855 (7,325 – 76,608)	0.18
Baseline HBeAg (PEIU/ml), median (IQR)	1,175 (547 – 3,001)	1,690 (187 – 3,441)	0.95
Genotype, n (%)			
A	9 (20.9)	34 (79.1)	0.0001
B	18 (66.7)	9 (33.3)	0.08
C	28 (46.7)	32 (53.3)	0.61
D	41 (58.6)	29 (41.4)	0.15
Baseline PC/BCP Variants by population sequencing (n=190), n (%)	n=90	n=100	(vs. WT)
Wildtype	38 (42)	45 (45)	-
PC G1896A	16 (18)	9 (9)	0.16
BCP A1762T + G1764A	32 (36)	37 (37)	0.86
PC + BCP Double Variant	4 (4)	9 (9)	0.55

475 * The Knodell Histologic Activity Index is a validated composite score incorporating inflammatory activity and fibrosis
476 scores

Table 2: HBeAg levels (PEIU/ml) by genotype and baseline PC/BCP variants, at weeks 0 and 24. Values are represented as median (IQR). PC/BCP variants were detected by population sequencing. P-values calculated comparing all groups in the row, following correction for multiple comparisons. Subject numbers are the same for each cell at baseline and week 24.

	Genotype				
	A *n=43*	B *n=27*	C *n=60*	D *n=70*	*p*-value
Baseline					
Wildtype, *n=83*	3,446 (2,043-4,277) *n=27*	2,619 (2,128-3,608) *n=9*	2,729 (888-3,662) *n=13*	2,083 (1,056-3,732) *n=34*	0.25
PC Variant, *n=25*	- *n=0*	2,823 (1,256-3,436) *n=13*	3,051 (2,995-3,696) *n=3*	219 (42-1,672) *n=9*	0.02
BCP Variant, *n=69*	229 (125-884) *n=12*	3,231 (2,240-3,670) *n=4*	626 (103-1,320) *n=34*	336 (122-876) *n=19*	0.03
PC-BCP Double Variant, *n=13*	124 (124-124) *n=1*	1 (1-1) *n=1*	681 (233-1,426) *n=8*	30 (19-160) *n=3*	0.10
Untypeable, *n=10*	735 (335-3,464) *n=3*	- *n=0*	126 (104-148) *n=2*	3,591 (3,364-3,756) *n=5*	0.05
Overall, *n=200*	2,595 (335-3,960)	2,823 (1,256-3,507)	843 (201-2,920)	1,029 (233-2,723)	0.006
Week 24					
Wildtype, *n=83*	604 (6-1,254)	26 (4-389)	269 (9-1,169)	269 (13-775)	0.49
PC Variant, *n=25*	-	144 (12-288)	1,283 (2-2,537)	10 (1-27)	0.07
BCP Variant, *n=69*	3 (1-5)	4 (1-66)	7 (1-107)	28 (2-79)	0.38
PC-BCP Double Variant, *n=13*	1 (1-1)	1 (1-1)	16 (2-247)	3 (0-12)	0.39
Untypeable, *n=10*	24 (22-704)	-	11 (6-16)	61 (50-62)	0.29
Overall, *n=200*	26 (2-720)	86 (2-288)	13 (2-329)	58 (3-557)	0.66

Table 3: HBeAg seroconversion by defined cut-off values after 24 weeks of treatment.

	Cut-off	HBeAg seroconversion	p-value
HBeAg level	≤10 PEIU/ml >10 PEIU/ml	57/79 (72%) 47/121 (39%)	<0.0001
HBeAg decline	≥2.2 $\log_{10}$ PEIU/ml <2.2 $\log_{10}$ PEIU/ml	38/50 (76%) 66/150 (44%)	<0.0001

482

483

484 **FIGURE LEGENDS**

485 **Fig. 1. Baseline HBeAg levels in the study cohort.** Distribution of HBeAg levels at baseline A) across
486 the whole study cohort (n=200); B) stratified by genotype; C) stratified by the presence of precore
487 (PC) and/or basal core promotor (BCP) variants identified by population sequencing; and D)
488 scatterplot of HBeAg level vs BCP variant frequency as determined by next generation sequencing.

489

490 **Fig. 2. A significant difference in HBeAg levels is shown after 24 weeks of treatment in those who**
491 **achieved HBeAg seroconversion.** A) Mean HBeAg levels were similar at baseline, with significant
492 differences developing on treatment. B) Reduction in HBeAg levels from baseline showed a larger
493 relative reduction in HBeAg seroconversion subjects. Error bars indicate standard error of the mean.

494

495 **Fig. 3. Week 24 HBeAg kinetics are associated with HBeAg seroconversion.** Kaplan-Meier curves
496 show that A) a threshold of 2.2 $\log_{10}$ PEIU/ml reduction in HBeAg at week 24 was significantly
497 associated with HBeAg seroconversion; and B) an absolute reduction in HBeAg to ≤10 PEIU/ml at
498 week 24 was also strongly associated with HBeAg seroconversion. At risk tables show the number of
499 HBeAg-positive subjects remaining in each group at each time point.

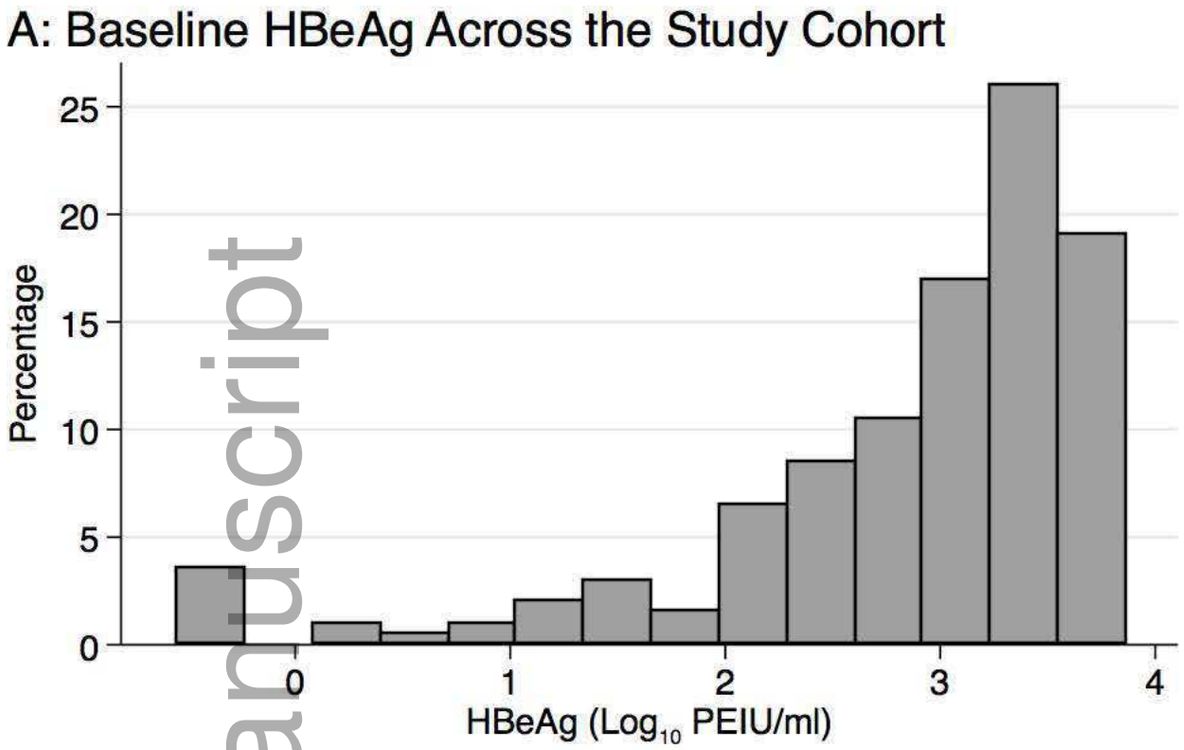
500

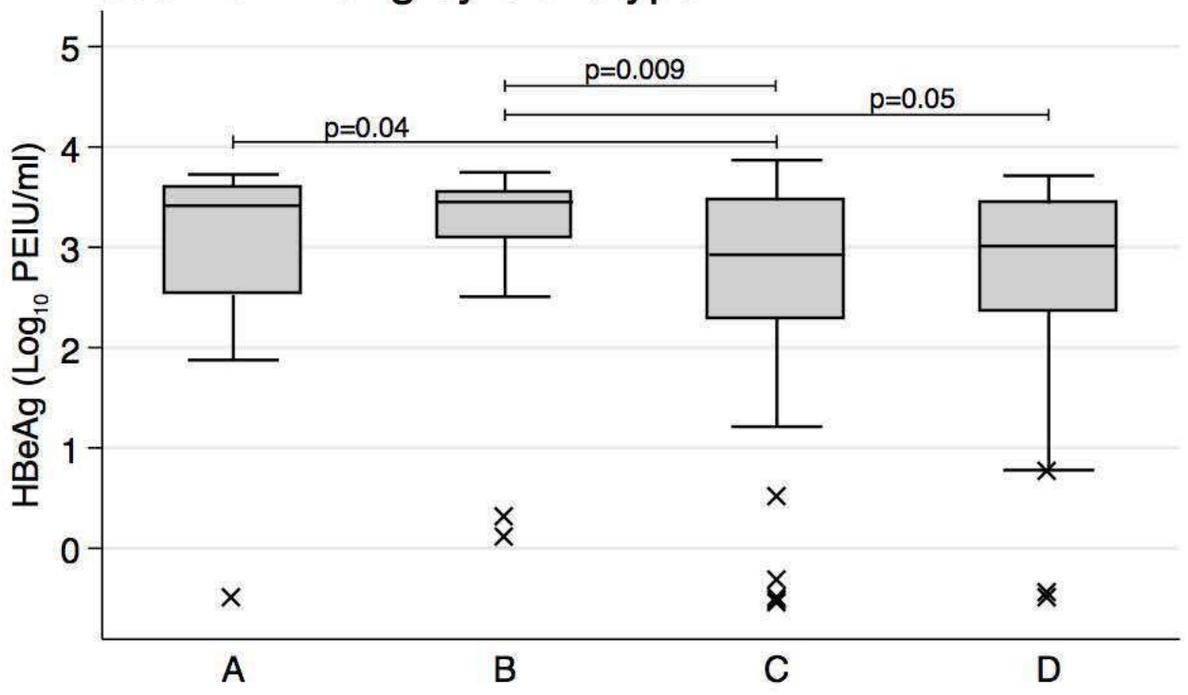
501 **Statement of Interests**

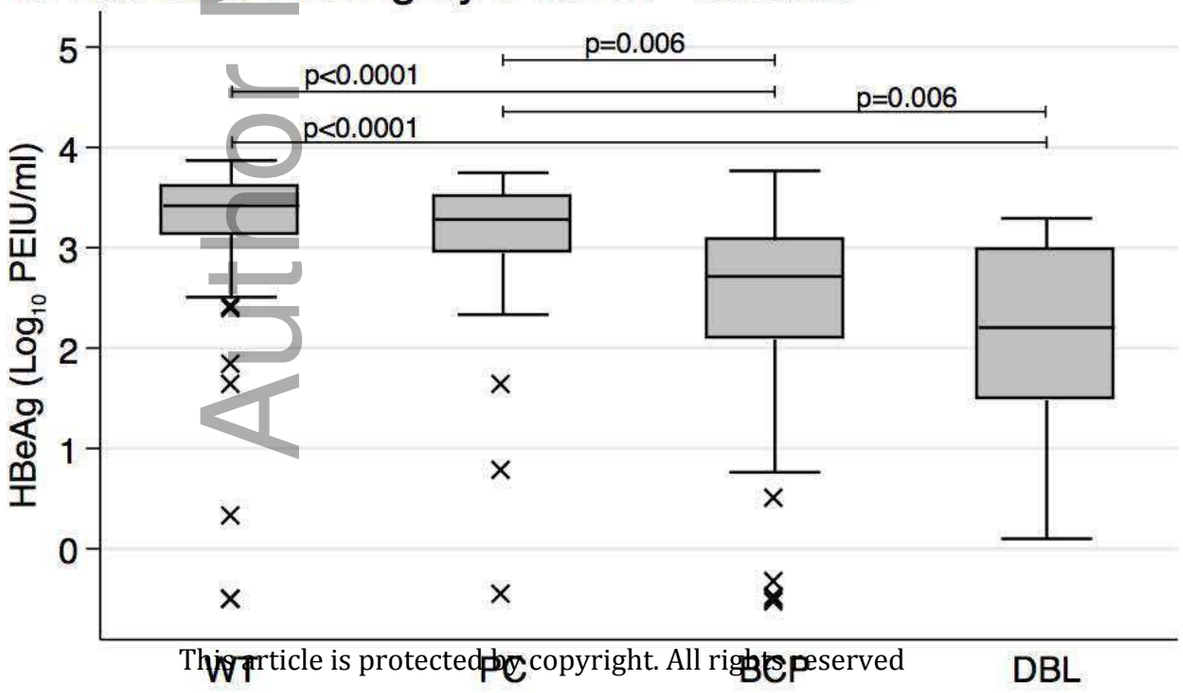
502 AG, KK, and GMS are employees of Gilead Sciences Inc., and own stocks and shares in Gilead
503 Sciences Inc. PM has served as a consultant for Abbot, Boehringer Ingelheim, Bristol-Myers Squibb,
504 Gilead, Janssen/Tibotec, Merck, Novartis, Pfizer, Roche, and Vertex, and has received honoraria from
505 Bristol-Myers Squibb, Gilead, Janssen/Tibotec, Merck, Novartis, and Roche. MB has received
506 honoraria and research grants from Brisol-Myers Squibb, and Gilead. HLJ has received consulting
507 fees and research support from Roche, Merck, Gilead, Bristol-Myers Squibb, Novartis, Santaris,
508 Medtronic, Anadys, Innogenetics, and Kirin. EG has served as a consultant for Achillion, Gilead,

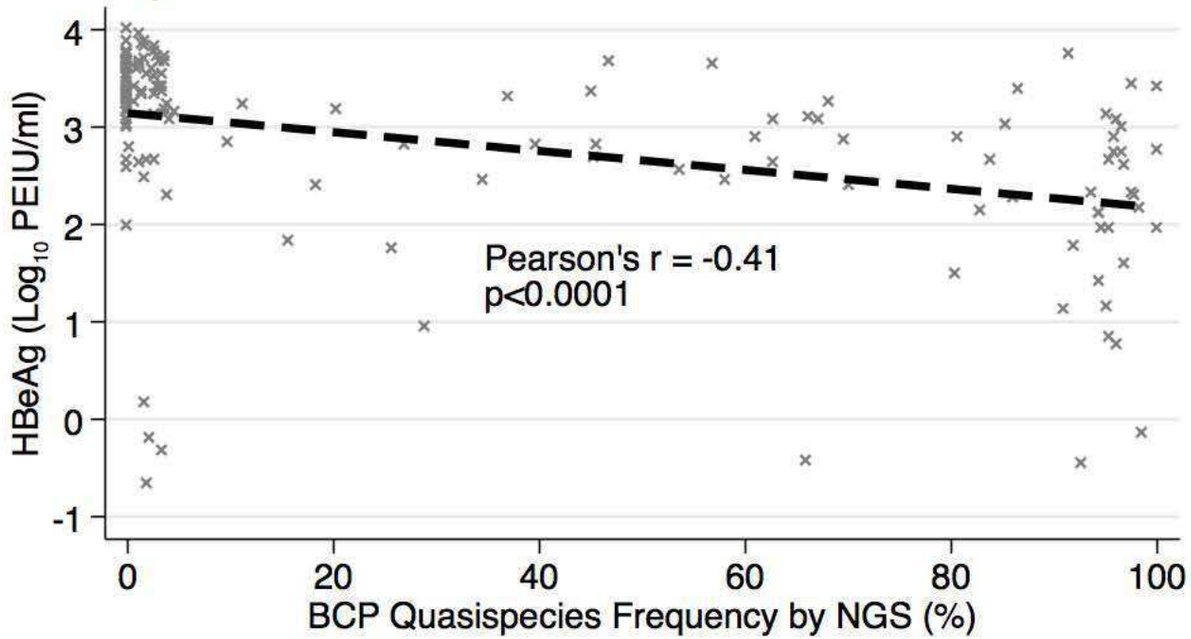
Idenix, Janssen, Merck, Novartis, Novira, and Roche. SL has served as a consultant for Gilead. The remaining authors have no financial disclosures.

This study was funded in part by Gilead.

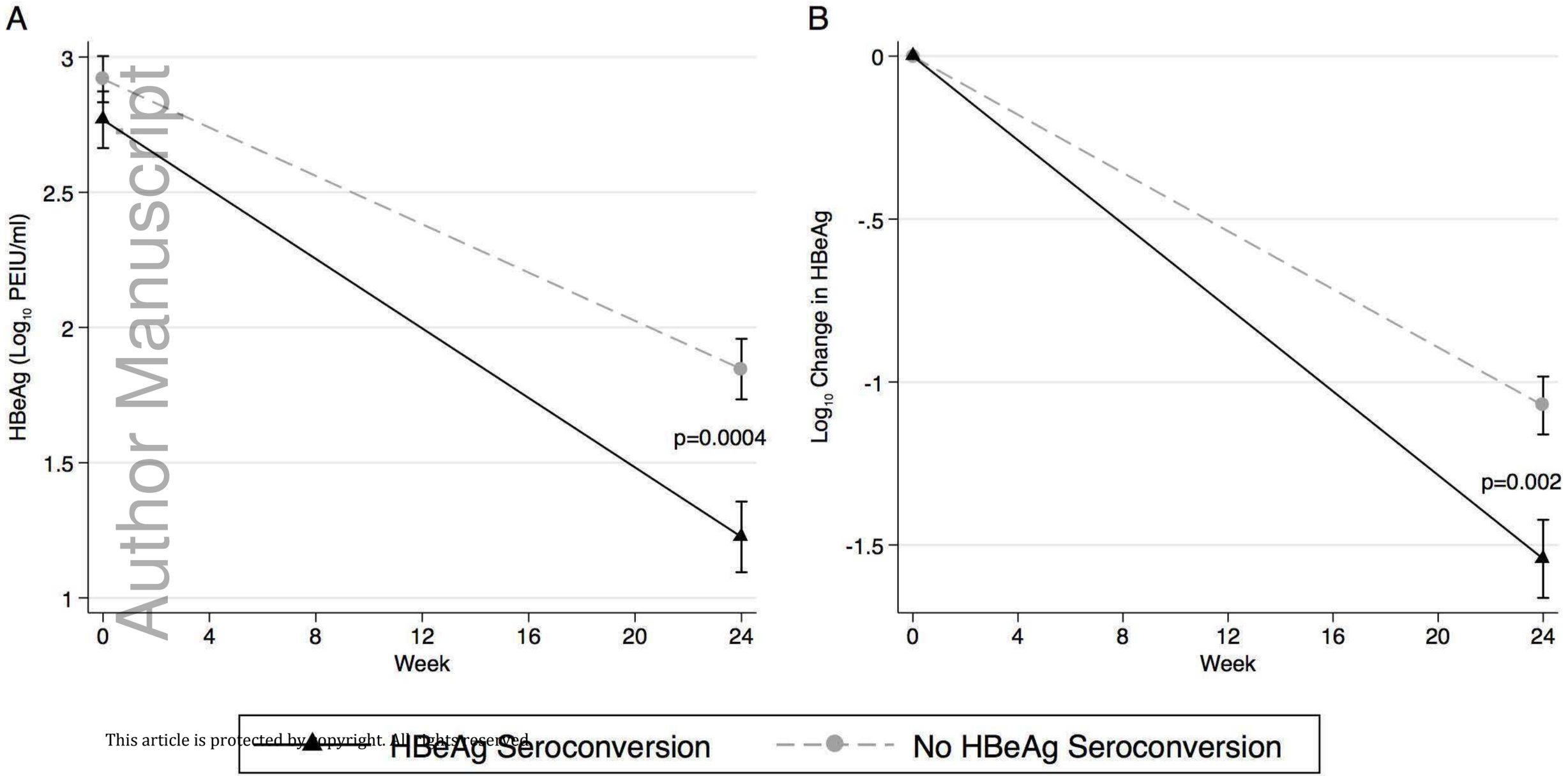








Decline in HBeAg Levels is Associated with HBeAg Seroconversion



On-Treatment Changes in HBeAg at Week 24 are Associated with HBeAg Seroconversion