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Comparison of Novel Biomarkers with Conventional HBV Markers among Untreated Adults with Chronic Hepatitis B in North America

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Abbreviations: CHB, Chronic hepatitis B, HBV, hepatitis B virus, HBsAg, hepatitis B surface antigen, HBeAg, hepatitis B e antigen, ALT, alanine aminotransferase, HBcrAg, hepatitis B core related antigen, cccDNA, covalently closed circular DNA, HBcAg, hepatitis B core antigen, HBRN, Hepatitis B Research Network, e+, HBeAg positive, IT(e+) immune tolerant HBeAg positive, IA(e+), immune active HBeAg positive, IND(e+), indeterminant HBeAg positive, e-, HBeAg negative, IA(e-), immune active HBeAg negative, IC(e-), inactive carriers HBeAg negative, IND(e-)DNA-L indeterminant HBV DNA low HBeAg negative, IND(e-)DNA-H , indeterminant HBV DNA high HBeAg negative, Rec(s-), recovered HBsAg loss, qHBeAg, quantitative HBeAg, qHBsAg, quantitative HBsAg, APRI, AST to platelet ratio index, FIB-4, Fibrosis 4 marker.

Abstract

The clinical utility of two novel biomarkers, hepatitis B virus (HBV) RNA and hepatitis B core-related antigen (HBcrAg), as compared to conventional markers of HBV replication and disease activity is unclear. Untreated participants in the North American Hepatitis B Research Network Adult Cohort Study were categorized by chronic hepatitis B (CHB) phases based on HBsAg and HBeAg status, and HBV DNA and ALT levels. HBV RNA and HBcrAg were measured (Abbott HBV pgRNA Research Assay and Fujirebio Lumipulse Immunoassay, respectively) and cross-sectional associations with conventional CHB markers were tested. Among 1409 participants across all CHB phases, median HBV DNA was 3.8 $\log_{10}$ IU/mL and ALT was 34 U/L. HBV RNA was quantifiable in 99% of HBeAg+ and 58% of HBeAg- participants; HBcrAg was quantifiable in 20% of HBeAg+ (above linear range in the other 80%) and 51% of HBeAg- participants. Both markers differed across CHB phases (p<.001), with higher levels in the HBeAg+ and HBeAg- immune active phases. HBV RNA and HBcrAg correlated moderately-strongly with HBV DNA in both HBeAg+ and HBeAg- phases (HBV RNA: e+ ρ=.84; e- ρ=.78; HBcrAg: e+ ρ=.66; e- ρ=.56; p for all <.001), but with HBsAg levels among HBeAg+ phases only (HBV RNA: e+ ρ=.71; p<.001; e- ρ=.18; p=.56; HBcrAg: e+ ρ=.51; p<.001; e- ρ=.27; p<.001). Associations of higher HBV RNA and HBcrAg levels with higher ALT, APRI and FIB-4 levels were consistent in HBeAg- but not HBeAg+ phases. ***Conclusion:*** Despite clear relationships between HBV RNA

and HBcrAg levels and CHB phases, these markers have limited additional value in differentiating CHB phases because of their strong association with HBV DNA and to a lesser extent with clinical disease indicators.

Introduction

Chronic hepatitis B (CHB) is a dynamic infection with different phases reflecting the complex immune interaction between the hepatitis B virus (HBV) and the host. Accurately identifying the phase of CHB is important for providing advice on prognosis, monitoring disease activity, and determining need for treatment.(1) Typically, a combination of serological (hepatitis B surface antigen [HBsAg], hepatitis B e antigen [HBeAg]), virological (HBV virus DNA) and biochemical testing (alanine aminotransferase [ALT]) are used to obtain these goals.(1) However, these tests are not sufficient in discriminating the phases of CHB because many patients fall into grey zones or indeterminant phases.(2)

Two novel HBV serum biomarkers, HBV RNA and hepatitis B core related antigen (HBcrAg),(3) provide an opportunity to better characterize CHB, beyond currently available HBV assays.(4-7) Understanding how these biomarkers compare to other serological and virological markers, as well as their utility in quantifying transcriptionally active covalently closed circular DNA (cccDNA) might yield valuable clinical insights.(8-10) These markers might also be used to characterize the natural history of the chronic phases of infection, to assess risk of disease reactivation after withdrawal of nucleos(t)ide analogs (NAs), and to understand mechanisms of action of new antiviral agents in development for achieving functional cure.

It is well known that HBV RNA can be detected in the serum of patients with CHB. However, there is controversy as to the source of this RNA. A recent *in-vitro* study suggested that HBV RNA in serum represents partially reverse transcribed encapsidated pgRNA in virus-like particles.(11) Since the pgRNA is transcribed directly from the cccDNA, levels of HBV RNA can potentially serve as a surrogate marker for transcriptionally active cccDNA.(12) HBcrAg is a composite biomarker incorporating several viral antigens expressed from the pre-Core/Core gene: the hepatitis B core antigen (HBcAg), HBeAg, and p22 core-related antigen.(13) HBcrAg can be detected as a defective particle without a HBV genome, in virions containing pregenomic RNA, circulating virus and HBeAg. Serum HBcrAg has also been shown to correlate with cccDNA particularly in HBeAg positive patients and may reflect the amount of cccDNA in hepatocytes.(14)

Although several recent studies have reported on the clinical utility of these two novel virological biomarkers in differentiating phases of CHB, they have been limited by small sample size, omission of some phases of CHB and inclusion of subjects with predominantly Asian or

European genotypes.(10, 15-19) We took advantage of the Hepatitis B Research Network (HBRN) Cohort Study (20) to perform a cross-sectional analysis of levels of HBV RNA and HBcrAg across the entire spectrum of CHB phases including genotypes A-D, in a large North American sample of adults with active, as well as recovered CHB, not receiving treatment. We evaluated the associations of these biomarkers with conventional biomarkers of HBV replication and disease activity among those with active CHB.

Methods

HBRN

The HBRN is a research network of 28 clinical sites throughout the U.S. and Canada, funded by the National Institutes of Health, initiated to study the natural history of CHB and to conduct clinical trials in both children and adults. The Adult Cohort study (NCT01263587) enrolled HBsAg positive subjects ≥18 years, between 2012 and 2017, who were not currently on antiviral therapy.(20) Participants underwent evaluation at entry, at weeks 12, 24, and every 24 weeks thereafter. Follow-up ended in January 2020. The HBRN study protocols were approved by the institutional review boards, (Research Ethics Board in the case of the Toronto site), of each participating institution and each participant provided written, informed consent. Details of the study protocol were previously described.(20)

Participant Selection

Adult Cohort participants were selected for this report if they had serum available for HBV RNA and HBcrAg testing at a time point in which they tested HBsAg negative, or a timepoint within the first 48 weeks of study entry at which required test results necessary to categorize their phase of CHB were available. Participants' first qualifying time point was selected. Participants without the required laboratory data or stored serum were excluded, as were participants with acute HBV, history of hepatocellular carcinoma, or co-infection with human immunodeficiency virus, hepatitis C virus or hepatitis delta virus.

CHB Phase Definitions

Phase of disease was determined based on results of HBeAg status and HBV DNA level obtained from the same visit and an ALT level within 12 weeks of that study visit using pre-defined criteria developed by the HBRN.(2) HBeAg positive (e+) participants were categorized as immune tolerant (IT(e+)) if HBV DNA $\geq 10^5$ IU/mL and ALT was normal, immune active (IA(e+)) if HBV DNA $\geq 10^5$ IU/mL and ALT was elevated, or indeterminant (IND(e+)) if HBV DNA $<10^5$ IU/mL, regardless of ALT level. HBeAg negative (e-) participants were categorized as immune active (IA(e-)) if HBV DNA $>10^4$ IU/mL and ALT level was elevated, inactive carriers (IC(e-)), if HBV DNA $\leq 10^4$ IU/ml and ALT was normal, indeterminant HBV DNA low, (IND(e-)DNA-L) if HBV DNA $\leq 10^4$ IU/ml and ALT was elevated, and indeterminant HBV DNA high (IND(e-)DNA-H) if HBV DNA $>10^4$ IU/mL and ALT was normal. Participants with HBsAg loss during follow-up were categorized as recovered (Rec(s-)).

Conventional Assays

Assessments during the study included detailed medical history, physical examination, health surveys, and routine blood tests including HBV DNA level, and HBV serologies. Antibodies against HIV, HCV, and HDV were tested at enrollment. Quantitative HBeAg (qHBeAg) was tested every 24 weeks for those who were HBeAg positive at enrollment and quantitative HBsAg (qHBsAg) every 48 weeks. Local HBV serology testing was performed using commercially available ELISA assays. Standardized gender-specific cutoff values were chosen to define the upper limit of normal (ULN) for ALT: 30 U/L for men and 20 U/L for women, and categories were defined as ≤1.0, >1.0-2.0, >2.0 xULN. AST to platelet ratio index (APRI) and Fibrosis 4 marker (FIB-4) were calculated as previously described and standard thresholds were applied (APRI: ≤0.5, 0.5-2.0, >2.0)(21) and (FIB-4: <1.45, 1.45-3.25, >3.25).(22)

HBV DNA, qHBeAg and qHBsAg testing were performed at a HBRN-funded virology laboratory (University of Washington, Seattle, WA) using research blood samples stored at -70C.(20) HBV DNA levels were determined using a real-time PCR assay (COBAS Ampliprep/COBAS TaqMan HBV Test, v2.0; Roche Molecular Diagnostics, Branchburg, NJ), and qHBeAg and qHBsAg were determined using Roche Diagnostics' Elecsys platform for research purposes (i.e., Elecsys HBeAg II Quant and Elecsys HBsAg II Quant assay; Roche Molecular Systems, Inc). The lower limit of quantification and detection for HBV DNA were 20 IU/mL and 10 IU/mL, respectively; values below these thresholds were randomly imputed using uniform distributions (10-<20 IU/mL and 0-<10 IU/mL, respectively). The lowest quantifiable/detectable value for qHBsAg was

0.05 IU/mL and for qHBeAg was 0.30 IU/mL; evaluation of qHBeAg and qHBsAg was limited to participants with detectable values. HBV DNA, qHBsAg and qHBeAg are reported on the log_{10} scale. HBV genotype was determined based on mass spectrometry, at the Molecular Epidemiology and Bioinformatics Laboratory in the Division of Viral Hepatitis at the Centers for Disease Control and Prevention.(23)

Novel Assays

HBV RNA was isolated from plasma and amplified as described by Butler et al.(24), using the m2000 system (Abbott Molecular; Department of Infectious Diseases, Abbott Diagnostics, Abbott Park, USA) and results are presented as log_{10} U/mL. Levels below quantification (<1.65 log_{10} U/mL), were randomly imputed using a uniform distribution (0.01-<1.65 log_{10} U/mL). Non-detected HBV RNA levels were set to 0 log_{10} U/mL. HBcrAg serum concentrations were measured using a chemiluminescence enzyme immunoassay (Lumipulse G® HBcrAg assay by Fujirebio Europe, Gent, Belgium). The assay has a linear measurement range of 3.0 log_{10} to 6.8 log_{10} U/ml, with 3 log_{10} U/ml being the detection limit. Dilution was not performed for samples with concentration >6.8 log_{10} U/ml. HBcrAg levels were categorized as <3, 3-<4, 4-<5, 5-<6, 6-<6.8, ≥6.8 log_{10} U/ml.

Statistical Analysis

Demographic and clinical characteristics of the participants are summarized overall and by HBV phase, as median and interquartile range (IQR; 25th-75th percentiles) for continuous variables, and frequencies (percentage) for categorical variables. All analyses were stratified by HBeAg status and limited to HBsAg positive participants. Characteristics were compared across CHB phases using the Kruskal-Wallis, Chi-Square, or Fisher's Exact, as appropriate. Box plots and stacked bar charts were used to visualize the distributions of HBV RNA (continuous) and HBcrAg (ordinal), respectively, by phases and by genotypes, and the Kruskal-Wallis and Fisher's Exact tests were used for comparisons.

Scatter plots and box plots were used to visualize the distribution of HBV DNA, qHBeAg and qHBsAg by HBV RNA and by HBcrAg, respectively. Associations were tested with the Spearman's Rank correlation (ρ). Box plots and stacked bar charts were used to visualize the distributions of HBV RNA, HBcrAg, HBV DNA and qHBsAg, respectively, by ALT, APRI and FIB-4 categories, and a series of multinomial logistic regression models were used to test the

odds of higher ALT, APRI or FIB-4 categories, versus the lowest category, by HBV RNA, HBcrAg, HBV DNA and qHBsAg, respectively. Modeling was repeated, adjusting for age and body mass index (BMI), with the exception that the FIB-4 models, which were adjusted for BMI only since age is part of the FIB-4 score. Finally, box plots were used to visualize the distribution of HBV RNA by HBcrAg categories and the association was tested with the Spearman's Rank correlation (ρ). For box plots, each box represents the first (lower end) to third (upper end) quartiles (IQR); the horizontal line in each box represents the median. The vertical line at either end of the box extends to the most extreme values or is cut off at 1.5 times the IQR. Analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC, USA).

Results

Characteristics of the cohort

Among 2018 adult participants, 1409 (373 HBeAg positive, 978 HBeAg negative and 58 who lost HBsAg in follow-up) met inclusion criteria, 609 having been excluded (167 for acute HBV infection or coinfection with HIV, HCV or HDV and 442 for lack of a research serum sample available at the qualifying time points, **Figure S1**). Per exclusion criteria no participants were currently taking antiviral therapy. However, 14% had previously received antiviral therapy; the median (IQR) time between last use of antiviral therapy and assessment was 4.1 (1.3-7.9) years. Median age of participants was 41 years, 49% were female, 76% Asian, 10% White, 11% Black and 3% other/mixed race. Genotype distribution was A-16%, B-40%, C-34%, D-7% and other-3%. Median HBV DNA was 3.8 $\log_{10}$ IU/mL and median ALT was 34 U/L. CHB phase allocation is shown in **Figure S2,** with the highest percentage (29%) in IND(e-)DNA-L phase, roughly 20% in each of IA(e+), IA(e-), and IC(e-) phases, and 4% in Rec(s-) phase. Demographic and clinical characteristics of the participants, overall and by phase, are reported in **Table 1**. Among HBeAg positive phases, there were significant differences in distributions of age, sex, ALT categories, platelets, APRI categories, and all viral makers (HBV DNA, qHBeAg, qHBsAg, HBV RNA, HBcrAg), but not in race, treatment history, genotype or FIB-4 categories. Among HBeAg negative phases, there were significant differences in the distributions of all examined factors except treatment history.

HBV RNA and HBcrAg levels and phases of CHB

HBV RNA was quantifiable in 99% of HBeAg positive, 58% of HBeAg negative, and 4% of HBsAg negative participants. HBcrAg was present within the quantifiable range of the assay in 20% of HBeAg positive (detectable but above limit of quantification in the other 80%), 51% of HBeAg negative and 12% of HBsAg negative participants. HBV RNA and categories of HBcrAg levels were strongly correlated independent of HBeAg status (**Figure S3;** e+: ρ=.65; p<.001; e-: ρ =.61; p<.001), and both differed across HBeAg positive as well as HBeAg negative phases **Figures 1a and 1b**. In general, HBV RNA and HBcrAg levels were higher among HBeAg positive than HBeAg negative participants, and lowest in HBsAg negative participants. However, levels were similar in IND(e+) and IA(e-). Among HBeAg positive participants, median HBV RNA levels were similar among those in IT(e+) and IA(e+) phases, 7.1 and 7.3 $\log_{10}$ U/mL, respectively, and markedly lower among those in IND(e+) phase (3.1 $\log_{10}$ U/mL). Among the HBeAg negative participants, HBV RNA levels were highest among those in IA(e-) phase (3.6

log_{10} U/mL), followed by those in IND(e-)DNA-H phase (2.9 log_{10}U/mL), and lowest among participants in IC(e-) and IND(e-)DNA-L phases, 1.3 and 1.4 log_{10} U/mL, respectively. Median HBV DNA/HBV RNA ratios were approximately 1 among HBeAg positive participants, >1 among HBeAg negative participants with highest ratios among IC(e-) and IND(e-)DNA-L phases, 1.9 and 1.7, respectively, and <1 among HBsAg negative participants, **Table 1** and **Figure S4**. Median HBV DNA/HBV RNA ratios were similar across genotypes A-D irrespective of HBeAg status, **Figure S5.**

Among the HBeAg positive participants, the majority of IT(e+) (82%) and IA(e+) (87%) participants had HBcrAg values above the upper limit of quantification (≥6.8 log_{10} U/mL) compared to only 4% of IND(e+) participants (**Table 1**). Among the HBeAg negative participants, the majority of IA(e-) (92%) participants had values above the lower limit of quantification with most having values 3-<6 log_{10} U/mL, and only 7% with values above the upper limit of quantification. By contrast, the majority of those in IC(e-) (70%) and IND(e-)DNA-L (60%) phases had values below the lower limit of quantification and most of the remainder had values between 3-<5 log_{10} U/mL (**Table 1**). IND(e-)DNA-H participants had intermediate HBcrAg levels between that of IA (e-) and IC(e-)/IND(e-)DNA-L participants.

Associations between HBV RNA and HBcrAg levels and quantitative HBV DNA, HBeAg and HBsAg levels

HBV RNA levels correlated strongly with HBV DNA levels independent of HBeAg status (**Figure 2A;** HBeAg positive ρ=.84; p<.001; HBeAg negative ρ =.78; p<.001), and with qHBeAg (**Figure S6A**; ρ=.55, p<.0001), and qHBsAg (**Figure 3A**; HBeAg positive ρ=.71; p<.001) among HBeAg positive phases. Correlation between HBV RNA and qHBsAg was significant but weak among HBeAg negative phases (**Figure 3A**; ρ=.18; p<.0001).

Similarly, HBcrAg levels correlated strongly with HBV DNA levels independent of HBeAg status (**Figure 2B**; HBeAg positive ρ=.66; p<.001; HBeAg negative ρ=.56; p<.001), and with qHBeAg (**Figure S6B**; ρ=.67, p<.0001), and moderately with qHBsAg among HBeAg positive phases (**Figure 3B**; HBeAg positive ρ=.51; p<.001). Correlation between HBcrAg and qHBsAg was significant but weak among HBeAg negative phases (**Figure 2B**; ρ=.27; p<.001).

Associations between HBV RNA and HBcrAg levels and genotype

To assess whether the observed associations with virological markers (HBV DNA, qHBeAg and qHBsAg) were influenced by genotype, we explored the HBV RNA and HBcrAg distributions by genotype. Among HBeAg positive participants, there were no significant differences in either HBV RNA (**Figure S7**; p=0.48) or HBcrAg (**Figure S8A**; p=0.43) levels by genotype. However, among HBeAg negative participants, there were significant differences in both HBV RNA and HBcrAg levels by genotype (**Figures S7** and **S8B;** p<.0001, p<.01, respectively); with higher median HBV RNA levels (log_{10} U/L) in genotype B (2.3) and lower levels in genotype D (1.5) compared to genotypes A, C, and E (all 1.9). HBcrAg levels also appeared highest in genotype B (e.g., 29.4% HBcrAg ≥4 log_{10} U/L), followed by C (24.7%), E (20.0%), A (15.9%), and D (14,5%). As a sensitivity analysis, among HBeAg negative participants, multivariable linear and ordinal logistic regression models evaluated whether genotype was related to HBV RNA and HBcrAg levels, respectively, independent of HBV DNA. Genotype was not independently related to HBV RNA (p=.28) but was independently related to HBcrAg (p<.01), with higher adjusted values in genotypes B and C versus A and D. HBV genotypes did not influence the association between HBV RNA and HBcrAg levels and other virological markers (data not shown).

Associations between HBV RNA, HBcrAg, HBV DNA and HBsAg levels and liver disease markers

Among HBeAg positive participants, there was a weak positive association between HBV RNA levels and ALT categories (p<0.01), while associations with APRI (p=.25) and FIB-4 (p=.56) categories were not significant (**Figure 4**). HBcrAg levels were not associated with ALT (p=.08), APRI (p=0.67) or FIB-4 (p=0.27) (**Figures S9A, C, E**), nor were HBV DNA levels (ALT p=.06; APRI p=0.71; FIB-4 p=0.59). Finally, there was not a significant association between qHBsAg level (log10 U/mL) and ALT (p=.25), while associations with APRI (p<.001) and FIB-4 (p<.001) were in the opposite direction with higher HBsAg level associated with lower odds of higher FIB-4 categories (**Figures S11**). In general, adjusting for age and BMI strengthened associations (**Table S1**); e.g., HBV RNA was associated with higher APRI (p=.045), and HBcrAg and HBV DNA were associated with higher ALT (p=.01 and p<.01, respectively). However, qHBsAg was no longer inversely associated with APRI (p=.10).

Among HBeAg negative participants, there were significant associations between higher HBV RNA levels (**Figure 4**) and HBcrAg levels (**Figure S9 B, D, F**), respectively, with higher ALT, APRI and FIB-4 categories, respectively (p for all<.0001). Associations with HBV DNA level

(log10 U/mL) mimicked those with HBV RNA (**Figures S10**; p for all <.001). In contrast, the association between qHBsAg level (log10 U/mL) and ALT (p<.001) was weaker, not quite significant with APRI (p=.08) and in the opposite direction with FIB-4 (p=.01), with higher HBsAg level associated with lower odds of a higher FIB-4 category (**Figures S11**). Adjusting these models for age and BMI had negligible impact on associations (**Table S2**). However, the positive association between qHBsAg and APRI categories was strengthened with adjustment for age (p=.01).

Discussion

In this study of 1409 North American participants with CHB, we examined whether the novel HBV biomarkers, HBV RNA and HBcrAg, which are claimed to be better surrogates of hepatic cccDNA transcriptional activity, (12, 14), can further discriminate CHB phase compared to conventional viral markers (HBeAg, HBV DNA, and quantitative HBsAg). We observed that while both HBV RNA and HBcrAg levels were significantly correlated with HBV DNA levels, they had little to no correlation with ALT levels in HBeAg positive participants. Although HBV RNA and HBcrAg levels were correlated with ALT levels in HBeAg negative participants they did not provide substantial discriminating capability to separate IND(e-)DNA-L, from the IC(e-) group as they essentially mirrored HBV DNA levels in this cross-sectional analysis. We postulate that many of these HBeAg negative indeterminant participants with low HBV DNA, yet elevated ALT may have concomitant fatty liver disease, as reflected by higher body mass index and higher prevalence of diabetes, but this will require longitudinal follow-up to confirm. However, this large study that includes all phases of chronic HBV infection and major HBV genotypes, provides valuable insights on our understanding of CHB.

First, similar to prior studies, we confirmed that there are strong associations between HBV RNA and HBV DNA levels among both HBeAg positive and HBeAg negative participants.(15-17, 25) Regardless of phase of CHB, HBV RNA levels mirrored those of HBV DNA albeit 1-2 logs lower, with the highest HBV RNA values seen in IT(e+) and IA(e+), intermediate in IA(e-), IND(e+) and IND(e-)DNA-Hi and lowest in IC(e-) and IND(e-)DNA-L. The ratio of HBV DNA/HBV RNA was ~1 among HBeAg positive, >1 among HBeAg negative and <1 among HBsAg negative participants. The reason for apparent increase in HBV DNA level over HBV RNA level among HBeAg negative participants is uncertain. It is possible that this represents integrated

HBV DNA detected by the PCR assay. In HBeAg positive participants, the contribution of HBV DNA from integrated HBV DNA would likely be minimal but may increase as cccDNA levels decline following HBeAg loss. Alternatively, after HBeAg loss, viral transcription may be more repressed compared to replication.(26) In contrast to the relationship with HBV DNA, the correlation between HBV RNA and qHBsAg levels was modest and limited to HBeAg positive CHB patients. The lack of correlation between HBsAg levels and HBV RNA in HBeAg negative patients supports the emerging concept that the source of circulating HBsAg in many HBeAg negative patients is integrated HBV DNA, not cccDNA.(27) The inclusion of indeterminant phases is a unique aspect of this study and shows that HBV RNA values add little beyond HBeAg and HBV DNA in determining assigned CHB phase.

Second, we found a small but significant difference in HBV RNA levels across genotypes A-E among HBeAg negative, but not HBeAg positive patients, with higher levels in HBV genotype B and lower levels for genotype D, compared to genotypes A, C and E. This corroborates a previous study from Europe where patients with HBV genotype B had the highest HBV RNA levels compared to HBV genotype D, although these differences were no longer significant after adjustment for HBeAg status.(28) Sequence differences between genotypes that affect the secondary structure of the pgRNA (epsilon) that binds the HBV polymerase has been suggested as an explanation for the differential detection of HBV RNA by HBV genotype.(25) However, differences in HBV RNA by genotype were no longer significant after adjustment for HBV DNA, indicating the associations with genotype and HBV RNA may have been driven by differing HBV DNA levels (e.g., higher HBV DNA in genotype B). Conversely, genotype, was associated with HBcrAg level in unadjusted and adjusted analysis (i.e., independent of HBV DNA). This finding is contrary to most other studies,(9, 14, 29) though previous data are generally more limited. The reasons for the difference in results between HBV RNA and HBcrAg are not clear but may be related to the strong correlation between HBV DNA and HBV RNA versus between HBV DNA and HBcrAg.

Third, we found that both HBV RNA and HBcrAg levels were consistently positively associated with liver disease markers (ALT, APRI and FIB-4) among HBeAg negative, but not HBeAg positive participants, although some associations among HBeAg positive participants were stronger after adjustment for age and BMI. The exact reasons for these differences are unclear. As potential biomarkers of cccDNA transcription, they reflect and mirror the high viral replication

rates among HBeAg positive participants but do not provide information on the host immune response, and therefore cannot discriminate between those with normal and elevated ALT levels. In contrast, among HBeAg negative patients where viral replication is more closely linked with disease activity, HBV RNA and HBcrAg levels might provide additional evidence of virally mediated liver disease. In this regard, it would be of interest to determine whether there are associations between the HBV transcriptional biomarkers and immunological correlates of disease activity, such as pro-inflammatory cytokines, in those with and without elevated ALT levels. Similarly, other studies have found that qHBsAg levels, which are a more indirect marker of cccDNA activity, provide additional prognostic information in HBeAg negative patients with low HBV DNA but not in those with high HBV DNA levels.(30-32) Additionally, the ability of HBV RNA and HBcrAg to differentiate HBV phases among HBeAg negative subjects is hampered by their limited sensitivity, 1.65 log U/ml for HBV RNA and 3.0 log U/ml for HBcrAg. Thus, in HBeAg positive patients with high HBV DNA levels, HBV RNA and HBcrAg levels provide more direct evidence of HBV replication. By contrast, in HBeAg negative patients, other markers of cccDNA activity, HBV RNA, HBcrAg and qHBsAg, may provide additional prognostic information on HBV-mediated liver disease.

Although the novel biomarkers HBV RNA and HBcrAg did not contribute additional information compared to conventional markers in classifying phases of CHB, they provide support for hypotheses that the IND(e+) group was probably on the way to spontaneous HBeAg clearance given lower HBV RNA and HBcrAg levels, while the IND(e-)DNA-L group likely represent inactive carriers with concomitant fatty liver disease accounting for the elevated ALT levels, as they not only had low HBV DNA, but also low HBV RNA, low HBcrAg levels and similar HBV DNA/HBV RNA ratios to IC(e-). The two biomarkers also provided assurance that the HBsAg negative participants likely had low cccDNA activity because only 3% had quantifiable HBV RNA and only 12% had HBcrAg above the lower limit of quantification.

There were several limitations to this study. First, as a cross-sectional analysis, we were unable to assess the role of the two markers to predict phase transitions. It is possible that changes in HBV RNA and HBcrAg levels may predate or predict phase transitions and provide better indications when antiviral treatment should be initiated or deferred. Future evaluation of longitudinal samples in our cohort will address these issues. Second, we were unable to examine the role of HBV RNA and HBcrAg in monitoring response during antiviral treatment or

in predicting relapse after treatment withdrawal in this study as all participants were required to be off treatment at study enrollment. Indeed, monitoring therapeutic responses appears to represent the major utility of HBV RNA and HBcrAg testing.(3, 12) Presently, these biomarkers should continue to be used as research tools until more studies are performed to confirm their clinical utility.

In summary, HBV RNA and HBcrAg, at a single timepoint, offer limited advantages over currently approved assays in characterizing phase of chronic HBV infection but may have a role in assessing efficacy of novel antiviral agents in development. Characterization of CHB phases may be relevant in the rapidly evolving arena of HBV therapeutics, where potentially aligning subtypes of patients based on these novel markers with specific therapeutic approaches may be envisioned. The detailed virological and clinical characterization of CHB phases provided by this representative North American cohort study provides a solid foundation for such future studies.

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Figure Legends

Figure 1. HBV RNA and HBcrAg categories by chronic hepatitis B (CHB) phase

A. HBV RNA by CHB phase
B. HBcrAg categories by CHB phase

A. HBeAg positive participants depicted by red boxes and HBeAg negative participants by blue boxes. In box-whisker plots, upper and lower ends of the boxes = upper and lower quartlies, horizontal line = median, and upper and lower whiskers = highest and lowest observations. IT(e+) = immunetolerant, IA(e+) = HBeAg positive immuneactive, IND(e+) = indeterminant HBeAg positive, IA(e-) = HBeAg negative immuneactive, IC(e-) = inactive carrier, (IND(e-)DNA-L) = indeterminant HBeAg negative HBV DNA low, IND(e-)DNA-H = indeterminant HBeAg negative HBV DNA high, and Rec(s-) = Recovered HBsAg negative. n= numbers of participants in each CHB phase. B. Green bars = HBcrAg below lower limit of detection, brown bars = HBcrAg 3-<4 U/L, blue bars = HBcrAg 4-<6.8 U/L and red bars = HBcrAg≥6.8 U/L.

Figure 2. HBV DNA (log10 IU/mL) by HBV RNA (log10 U/mL) and HBcrAg (log10 U/mL) categories, respectively

A. HBV DNA by RNA
B. HBV DNA by HBcrAg categories

A. HBeAg positive participants represented by red triangles and HBeAg negative participants represented by blue circles. B. HBeAg positive participants depicted by red boxes and HBeAg negative participants by blue boxes. In box-whisker plots, upper and lower ends of the boxes = upper and lower quartlies, horizontal line = median, and upper and lower whiskers = highest and lowest observations.

Figure 3. HBsAg (log10 IU/mL) by HBV RNA (log10 U/mL) and HBcrAg (log10 U/mL), respectively

A. HBsAg by HBV RNA
B. HBsAg by HBcrAg categories

A. HBeAg positive participants represented by red triangles and HBeAg negative participants represented by blue circles. B. HBeAg positive participants depicted by red boxes and HBeAg negative participants by blue boxes. In box-whisker plots, upper and lower ends of the boxes = upper and lower quartlies, horizontal line = median, and upper and lower whiskers = highest and lowest observations. *The lowest value of -3.0 HBsAg (log10 IU/mL) corresponds to 0.001 HBsAg (IU/mL).

Figure 4. HBV RNA (log10 U/mL) by ALT (ULN), APRI and FIB-4 categories

A. HBV RNA by ALT
B. HBV RNA by APRI
C. HBV RNA by FIB-4

A, B and C, HBeAg positive particpants represented by red boxes and HBeAg negative participants by blue boxes. In box-whisker plots, upper and lower ends of the boxes = upper and lower quartlies, horizontal line = median, and upper and lower whiskers = highest and lowest observations.

Table 1. Demographics and clinical characteristics among adults with CHB, overall and by phase

		HBeAg+ phases				HBeAg- phases					
	Total	IT(e+)	IA(e+)	IND(e+)		IA(e-)	IC(e-)	IND(e-)DNA-L	IND(e-)DNA-H		Rec(s-)
	n=1409	n=62	n=284	n=27	P	n=260	n=274	n=401	n=43	P	n=58
Age, years					0.03					0.051	
Median (25th-75th%-ile)	41 (33: 51)	30 (25: 36)	35 (26: 44)	32 (27: 38)		45 (38: 53)	43 (34: 52)	42 (34: 52)	43 (35: 57)		50 (44: 59)
Female, n (%)	696 (49.4%)	42 (67.7%)	136 (47.9%)	19 (70.4%)	0.003	98 (37.7%)	141 (51.5%)	220 (54.9%)	22 (51.2%)	<.001	18 (31.0%)
Race, n (%)	n=1408				0.20		n=273			<.001	
Asian	1065 (75.6%)	59 (95.2%)	235 (82.7%)	22 (81.5%)		214 (82.3%)	199 (72.9%)	263 (65.6%)	39 (90.7%)		34 (58.6%)
Black	161 (11.4%)	1 (1.6%)	18 (6.3%)	1 (3.7%)		25 (9.6%)	43 (15.8%)	59 (14.7%)	2 (4.7%)		12 (20.7%)
White	143 (10.2%)	0 (0.0%)	21 (7.4%)	3 (11.1%)		19 (7.3%)	23 (8.4%)	65 (16.2%)	2 (4.7%)		10 (17.2%)
Other/Mixed	39 (2.8%)	2 (3.2%)	10 (3.5%)	1 (3.7%)		2 (0.8%)	8 (2.9%)	14 (3.5%)	0 (0.0%)		2 (3.4%)
Treatment history, n (%)	202 (14.3%)	7 (11.3%)	52 (18.3%)	4 (14.8%)	0.39	28 (10.8%)	35 (12.8%)	57 (14.2%)	7 (16.3%)	0.55	12 (20.7%)
Genotype, n (%)	n=1319	n=61	n=283	n=26	0.14	n=257	n=244	n=361		<.001	n=44
A	213 (16.1%)	1 (1.6%)	37 (13.1%)	4 (15.4%)		28 (10.9%)	50 (20.5%)	76 (21.1%)	3 (7.0%)		14 (31.8%)
B	526 (39.9%)	20 (32.8%)	96 (33.9%)	9 (34.6%)		135 (52.5%)	97 (39.8%)	124 (34.3%)	29 (67.4%)		16 (36.4%)
C	453 (34.3%)	39 (63.9%)	133 (47.0%)	12 (46.2%)		67 (26.1%)	72 (29.5%)	113 (31.3%)	7 (16.3%)		10 (22.7%)
D	87 (6.6%)	0 (0.0%)	13 (4.6%)	1 (3.8%)		17 (6.6%)	20 (8.2%)	29 (8.0%)	3 (7.0%)		4 (9.1%)
E	35 (2.7%)	1 (1.6%)	4 (1.4%)	0 (0.0%)		8 (3.1%)	5 (2.0%)	16 (4.4%)	1 (2.3%)		0 (0.0%)
Other	5 (0.4%)	0 (0.0%)	0 (0.0%)	0 (0.0%)		2 (0.8%)	0 (0.0%)	3 (0.8%)	0 (0.0%)		0 (0.0%)
ALT (U/mL)	n=1409	n=62	n=284	n=27	<.001	n=260	n=274	n=401	n=43	<.001	n=58
Median (25th-75th%-ile)	34 (22: 53)	18 (15: 23)	58 (37: 105.5)	30 (21: 42)		53 (38: 81)	19 (15: 23)	36 (28: 46)	20 (17: 24)		22 (14: 29)
ALT (x ULN), n (%)	n=1409	n=62	n=284	n=27	<.001	n=260	n=274	n=401	n=43	<.001	n=58
≤1	431 (30.6%)	62 (100.0%)	0 (0.0%)	8 (29.6%)		0 (0.0%)	274 (100.0%)	0 (0.0%)	43 (100.0%)		44 (75.9%)
>1-2	596 (42.3%)	0 (0.0%)	121 (42.6%)	13 (48.1%)		130 (50.0%)	0 (0.0%)	319 (79.6%)	0 (0.0%)		13 (22.4%)
>2	382 (27.1%)	0 (0.0%)	163 (57.4%)	6 (22.2%)		130 (50.0%)	0 (0.0%)	82 (20.4%)	0 (0.0%)		1 (1.7%)
Platelets ($x10^3/mm^3$)	n=1212	n=57	n=255	n=24	0.04	n=221	n=231	n=344	n=34	<.001	n=46
Median (25th-75th%-ile)	218 (180: 257)	231 (199: 268)	213 (175: 248)	218.5 (173: 252.5)		205 (169: 246)	223 (185: 259)	227 (186: 267)	229.5 (187: 268)		219.5 (176: 254)
APRI, n (%)	n=1208	n=57	n=254	n=24	<.001	n=221	n=231	n=341	n=34	<.001	n=46
≤0.5	889 (73.6%)	56 (98.2%)	122 (48.0%)	17 (70.8%)		114 (51.6%)	224 (97.0%)	278 (81.5%)	34 (100.0%)		44 (95.7%)
>0.5-1.5	260 (21.5%)	1 (1.8%)	103 (40.6%)	7 (29.2%)		81 (36.7%)	5 (2.2%)	61 (17.9%)	0 (0.0%)		2 (4.3%)
>1.5	59 (4.9%)	0 (0.0%)	29 (11.4%)	0 (0.0%)		26 (11.8%)	2 (0.9%)	2 (0.6%)	0 (0.0%)		0 (0.0%)

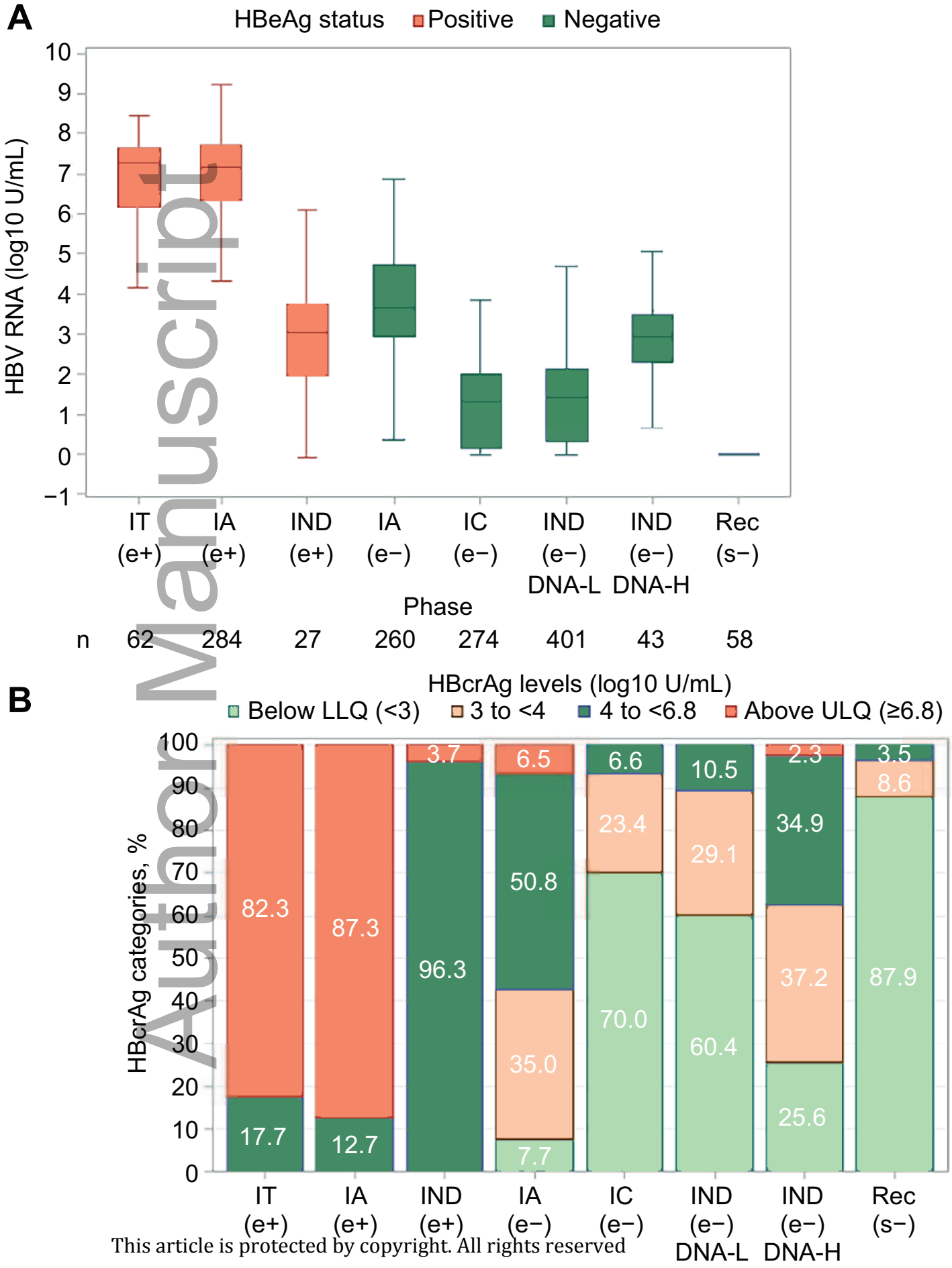
FIB-4, n (%)	n=1208	n=57	n=254	n=24	0.01	n=221	n=231	n=341	n=34	<.001	n=46
<1.45	945 (78.2%)	52 (91.2%)	191 (75.2%)	23 (95.8%)		143 (64.7%)	189 (81.8%)	284 (83.3%)	28 (82.4%)		35 (76.1%)
1.45-3.25	227 (18.8%)	5 (8.8%)	48 (18.9%)	1 (4.2%)		66 (29.9%)	39 (16.9%)	51 (15.0%)	6 (17.6%)		11 (23.9%)
>3.25	36 (3.0%)	0 (0.0%)	15 (5.9%)	0 (0.0%)		12 (5.4%)	3 (1.3%)	6 (1.8%)	0 (0.0%)		0 (0.0%)
qHBeAg (log10 IU/mL) among HBeAg+	n=372	n=62	n=283	n=27	<.001	NA	NA	NA	NA		NA
Median (25th-75th%-ile)	3.1 (1.6: 3.3)	3.3 (2.8: 3.4)	3.1 (1.9: 3.3)	0.1 (-0.2: 0.4)							
qHBsAg (log10 IU/mL) among HBsAg+	n=1255	n=61	n=263	n=26	<.001	n=248	n=252	n=367	n=38	0.002	NA
Median (25th-75th%-ile)	3.5 (2.8: 4.2)	4.6 (4.3: 4.9)	4.5 (3.7: 4.9)	3.6 (3.0: 3.9)		3.3 (2.9: 3.7)	3.0 (2.1: 3.6)	3.2 (2.5: 3.9)	3.1 (2.6: 3.7)		
HBV DNA (log10 IU/mL)					<.001					<.001	
Median (25th-75th%-ile)	3.8 (2.6: 6.2)	8.3 (7.9: 8.5)	8.1 (7.1: 8.4)	4.2 (3.1: 4.7)		5.1 (4.6: 5.9)	2.7 (2.1: 3.3)	2.8 (2.1: 3.3)	4.5 (4.3: 4.9)		0.9 (0.5: 1.2)
HBV RNA quantifiable, n (%)	938 (66.6)	62 (100.0)	284 (100.0)	23 (85.2)	<.001	254 (97.7)	103 (37.6)	170 (42.4)	40 (93.0)	<.001	2 (3.5)
HBV RNA (log10 U/mL) [T]					<.001					<.001	
Median (25th-75th%-ile)	2.4 (1.1: 5.1)	7.3 (6.2: 7.6)	7.1 (6.3: 7.7)	3.1 (2.0: 3.7)		3.6 (3.0: 4.7)	1.3 (0.2: 1.9)	1.4 (0.3: 2.1)	2.9 (2.3: 3.4)		0.0 (0.0: 0.0)
HBV DNA/RNA ratio	n=1245			n=25 [+]			n=221 [+]	n=338 [+]			n=12 [c]
Median (25th-75th%-ile)	1.3 (1.1 : 1.9)	1.1 (1.1 : 1.2)	1.1 (1.1 : 1.2)	1.2 (1.1 : 1.5)	<.001	1.4 (1.2 : 1.6)	1.9 (1.4 : 3.0)	1.7 (1.3 : 3.0)	1.6 (1.3 : 2.0)	<.001	0.8 (0.3 : 1.2)
HBcrAg quantifiable, n (%)	575 (40.8)	11 (17.7)	36 (12.7)	26 (96.3)	<.001	223 (85.8)	82 (29.9)	159 (39.7)	31 (72.1)	<.001	7 (12.1)
HBcrAg (log10 U/mL), n (%)					<.001					<.001	
Below LLQ (<3)	516 (36.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)		20 (7.7%)	192 (70.1%)	242 (60.3%)	11 (25.6%)		51 (87.9%)
3-<4	293 (20.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)		91 (35.0%)	64 (23.4%)	117 (29.2%)	16 (37.2%)		5 (8.6%)
4-<5	142 (10.1%)	0 (0.0%)	1 (0.4%)	4 (14.8%)		70 (26.9%)	16 (5.8%)	40 (10.0%)	9 (20.9%)		2 (3.4%)
5-<6	86 (6.1%)	4 (6.5%)	6 (2.1%)	20 (74.1%)		47 (18.1%)	2 (0.7%)	2 (0.5%)	5 (11.6%)		0 (0.0%)
6-<6.8	54 (3.8%)	7 (11.3%)	29 (10.2%)	2 (7.4%)		15 (5.8%)	0 (0.0%)	0 (0.0%)	1 (2.3%)		0 (0.0%)
Above ULQ (≥6.8)	318 (22.6%)	51 (82.3%)	248 (87.3%)	1 (3.7%)		17 (6.5%)	0 (0.0%)	0 (0.0%)	1 (2.3%)		0 (0.0%)

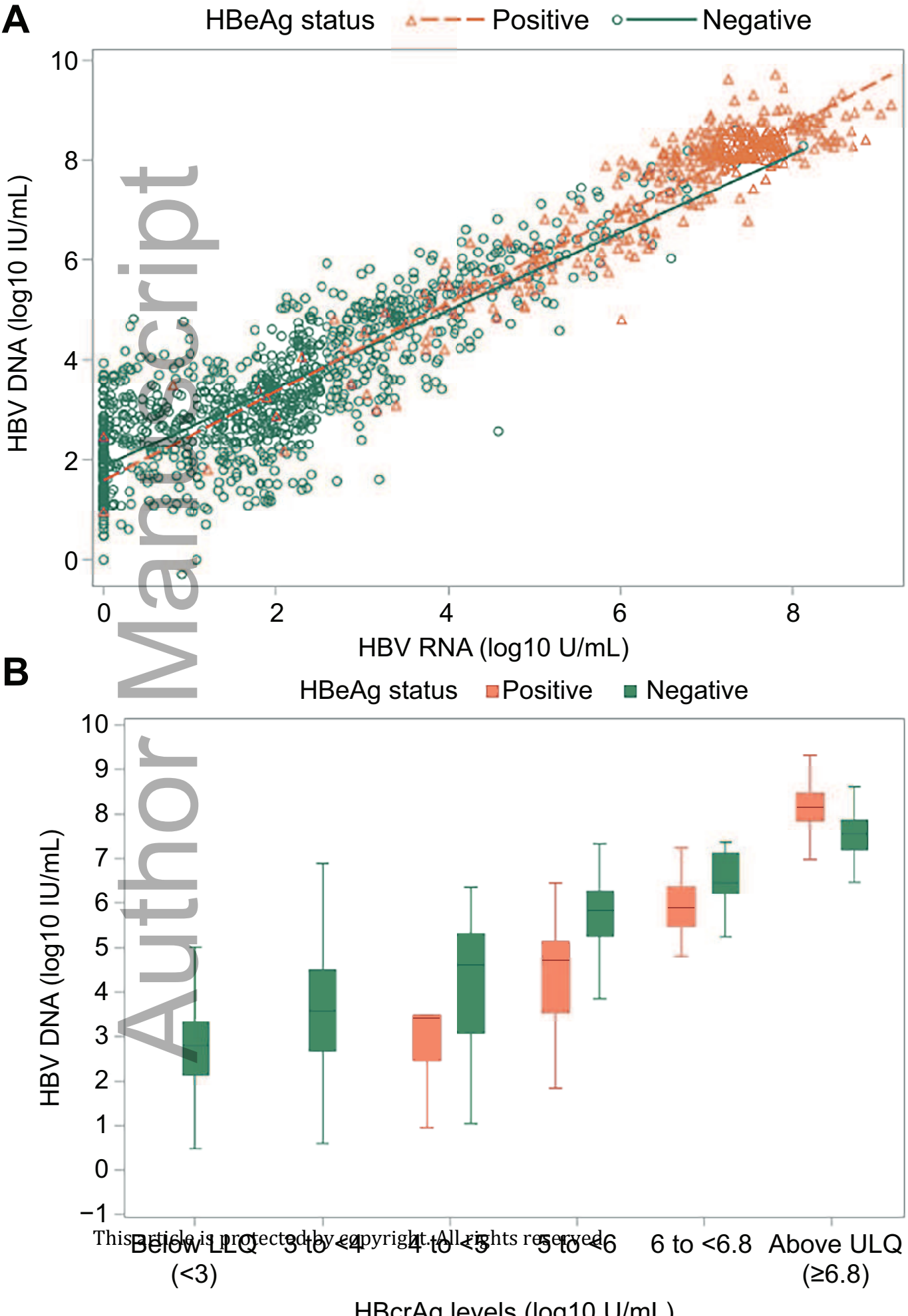
Abbreviations: ALT, Alanine Aminotransferase; APRI, AST to Platelet Ratio Index; CHB, Chronic Hepatitis B; DNA, Deoxyribonucleic Acid; FIB-4, Fibrosis-4; HBcrAg, Hepatitis B Core-Related Antigen; HBeAg, Hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, Hepatitis B Virus; IH, Immunohistochemistry; RNA, Ribonucleic Acid; ULN, Upper Limit of Normal.

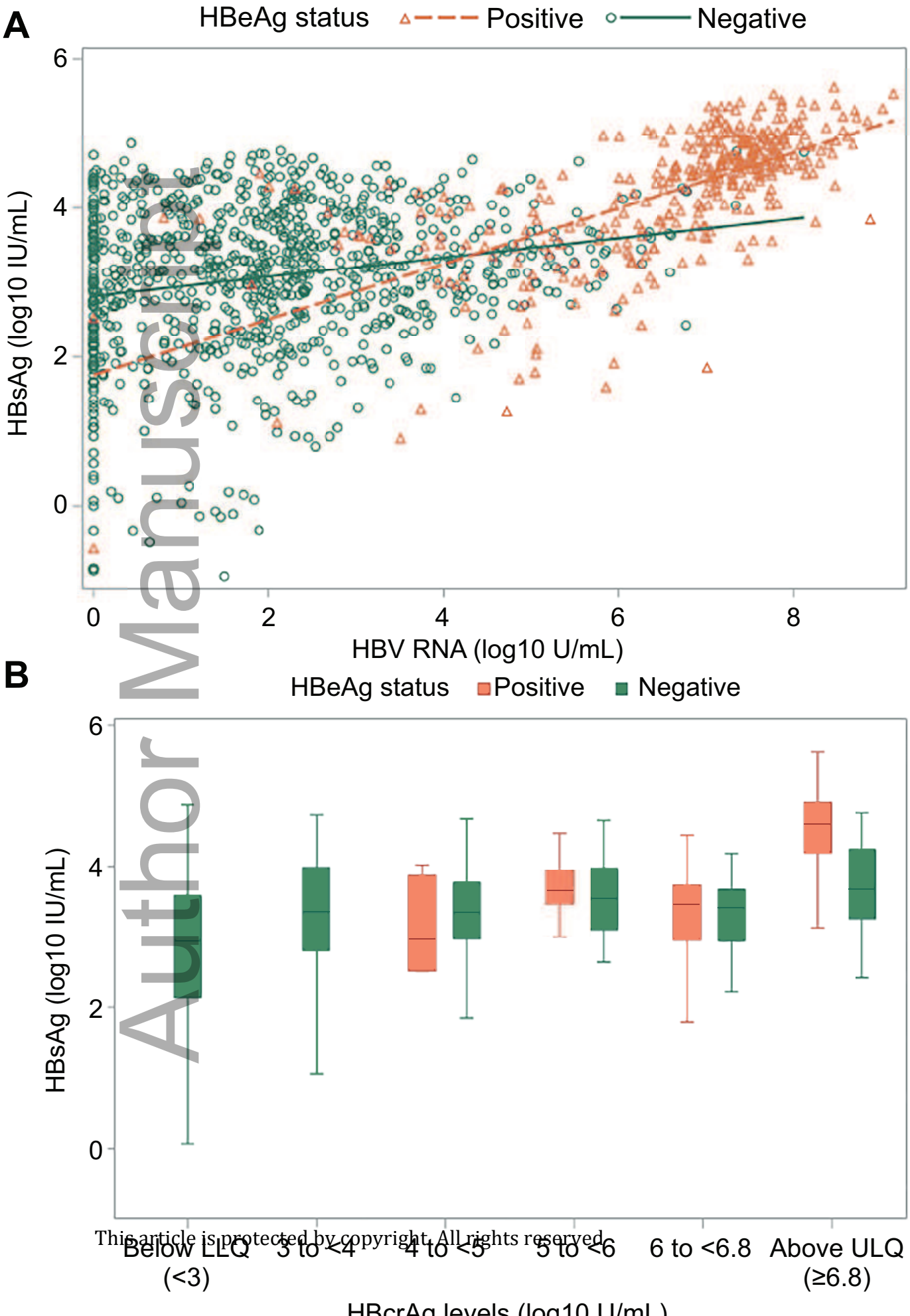
* Imputed values used when below level of quantification (<20 IU/mL) and detection (<10 IU/mL): uniform imputation ranged from 10.0 to 19.9 and 0.0 to 9.9, respectively.

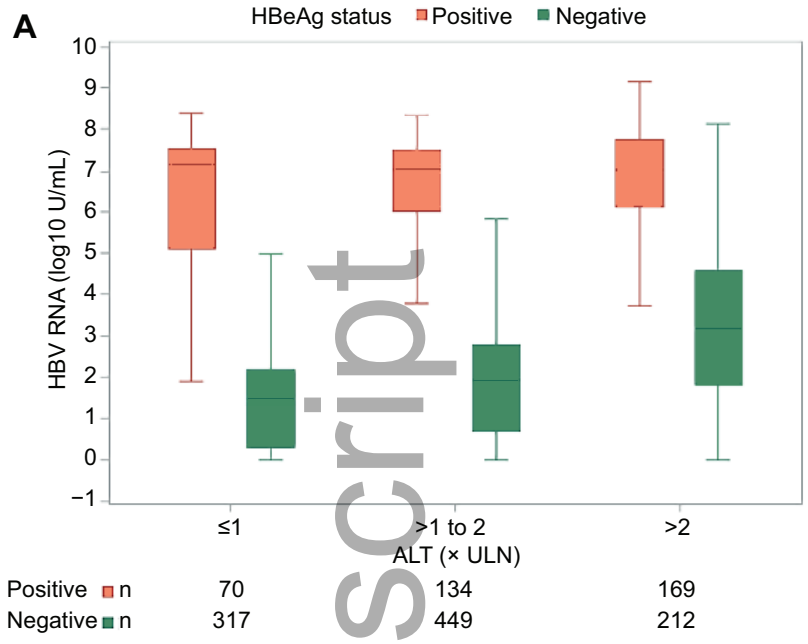
† Imputed values used when below level of quantification (<1.65 log10 U/mL): uniform imputation ranged from .01 and 1.64. Non-detected HBV RNA levels were set to 0.

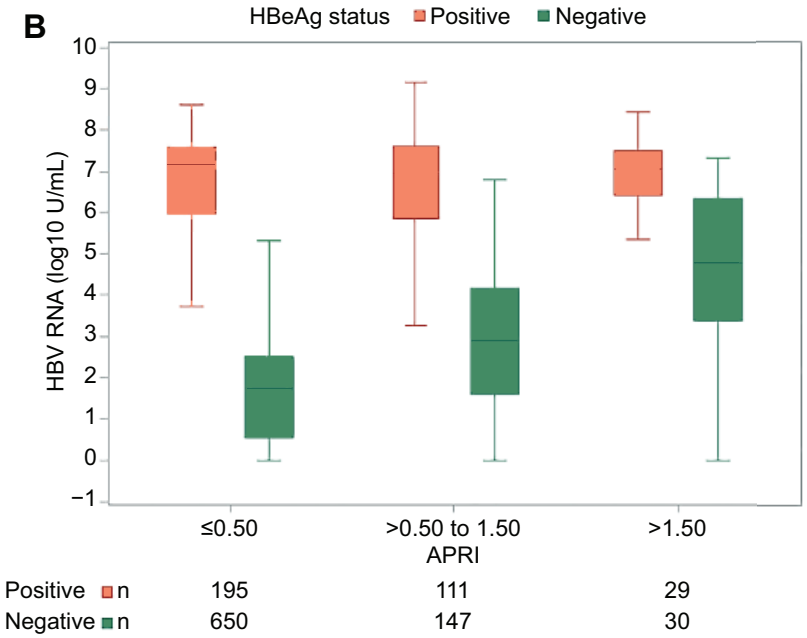
‡ The ratio is missing in participants with undetectable HBV RNA.

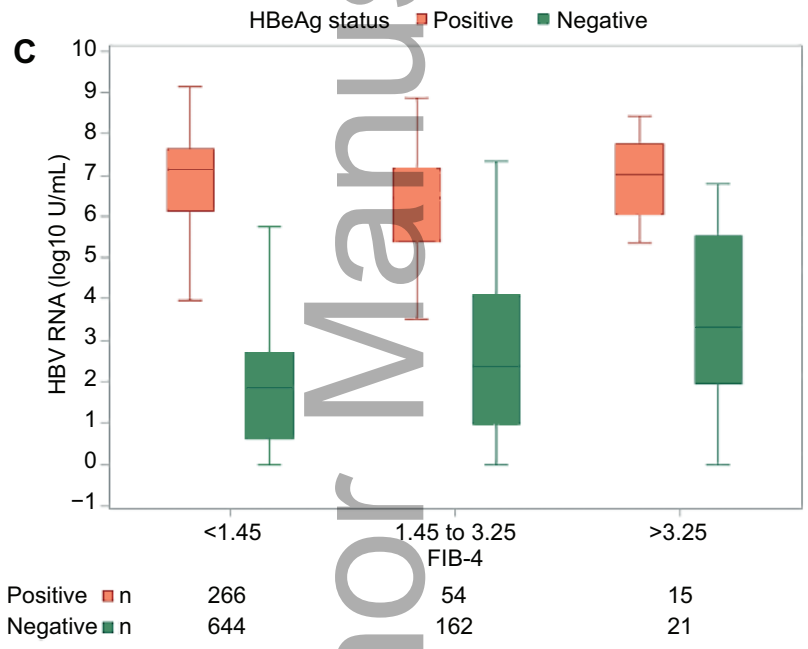












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