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Precore and basal core promoter hepatitis B virus (HBV) variants are present from a young age and differ across HBV genotypes

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Abstract

Background: HBV precore (PC) and dual basal core promoter (BCP) mutations halt and downregulate HBeAg production respectively. PC mutation is rarely associated with HBV genotype A. We sought to examine the association of these variants with HBV genotypes, age and HBeAg status in a racially diverse population in North America.

Methods: Prospective study included 1036 (808 adults, 228 children) participants in the Hepatitis B Research Network. PC and BCP variants were determined by Sanger sequencing and dominant HBV species (>50%) were reported.

Results: Median age was 36.3 years (range 2-80), 44.6% HBeAg(+), 74.2% Asians, 13.3% black and 9.7% white. The dominant PC variant was present in 29.4% participants including 20 with sub-genotype A1 or A2. 17/20 participants with genotype A and PC had a compensatory C1858T mutation. In the HBeAg(+) cohort, the prevalence of PC and/or BCP variants increased from 14.4% in the first 2 decades to 51% after 40 years of age. Among those aged 2-18, 52% and 83% with dominant PC and BCP variants were HBeAg(+) compared to 3.8% and 29% in the >40 years age group. HBeAg clearance rates were significantly higher for those with dominant PC or BCP variants, 24.4 and 15.0 per 100 person-years compared to 6.0 in WT HBV ($p < 0.0001$).

Conclusions: PC variants can be present in HBV genotype A and are usually associated with C1858T that preserves the pregenome encapsidation sequence. Selection of PC and BCP variants occurred at a young age with increasing prevalence across age groups. HBeAg(+) participants with dominant PC and BCP variants progressed to the HBeAg(-) phase of chronic HBV infection significantly faster. This finding has potential clinical and therapeutic implications.

Keywords:

chronic HBV infection, HBeAg, genotypes, Precore mutation, Basal Core Promoter mutations

Introduction:

Hepatitis B virus (HBV) infection is a significant public health problem worldwide and a major cause of chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC). According to the World Health Organization (WHO) Global Hepatitis Report in 2017, an estimated 257 million people world-wide have chronic HBV infection with the highest prevalence in Asia and Africa.

At least 9 genotypes of HBV, designated A-I, have been described that vary by >8% in nucleotide sequence and differ greatly in geographic distribution.[1, 2] Genotype A is the predominant cause of hepatitis B in Northern Europe and was formerly the most frequent genotype found in the U.S. Genotype D is most commonly found in Mediterranean areas of Europe, Northern Africa, India and the Middle East. Genotypes B and C are found most frequently in East Asia, with genotype C being predominant in Northern and genotype B in Southern Asia. Genotype E is found predominantly in Africa, where genotype A is also prevalent, but designated A1 as it differs slightly (4%) but distinctly from the European strains, which are designated A2.[3, 4] In the U.S., HBV genotype usually matches the country of origin (B and C among Asian Americans; E among African Americans).

HBV genotypes also vary in clinical and virologic features, risk of HCC and response to therapy.[5-9] A prominent difference among genotypes is its association with a variant in the pre-core region

where a G [Guanine] is replaced by an A [Adenine] at nucleotide (nt) position 1896 (Codon 28), the "classical" precore (PC) stop codon variant: G1896A. (Figure 3B) This mutation abrogates the production of hepatitis B e antigen (HBeAg).[10] The PC G1896A variant is most frequently associated with genotypes B and D followed by genotype C. HBV genotype A is believed to be incompatible with the PC G1896**A** variant because it harbors 1858**C** (Cytosine), which is paired to nt1896 in the stem-loop structure of epsilon (ε) at the pregenome encapsidation sequence. A change from G to A at nt1896 would cause instability of the stem-loop structure. [11] In contrast, genotype non-A usually harbors 1858**T.** The G1896**A** mutation, in this case, actually strengthens the stability of the HBV pregenome encapsidation sequence. (Figure 3B)

The most common mutations in the basal core promoter (BCP) region (A1762T [Thymine], G1764A) can down regulate but not abolish HBeAg production. Contrary to the PC G1896A variant, the BCP variant can be found across all genotypes. [12]

We determined the prevalence of the PC G1896A and BCP variants across the HBV genotypes among participants enrolled in the Hepatitis B Research Network (HBRN) Cohort Study; and examined the association of these variants with virological parameters and age in this large racially diverse cohort of children and adults with chronic HBV infection living in North America.

Patients and Methods:

HBRN is a cooperative network of 21 adult and 7 pediatric clinical centers in the U.S. and in Toronto, Canada, a Data Coordinating Center, a virology testing laboratory, an immunology core and a serum and tissue repository. [13] The HBRN Cohort Study enrolled persons with hepatitis B surface antigen (HBsAg) in serum who were without evidence of hepatic decompensation, HCC, organ transplant, human immunodeficiency virus (HIV) infection, and were not receiving antiviral treatment at enrollment, as detailed in our previous publications. [13, 14] All protocols were approved by the HBRN Steering Committee, the Institutional Review Board or Research Ethics Board at each participating site and a central Data Safety and Monitoring Board selected by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) which was responsible for funding the Network. All participants provided written informed consent/assent prior to participation.

As of July 2018, a total of 2112 (1795 adults, 317 children) participants with chronic hepatitis B enrolled in the study had BCP and PC testing. Participants were excluded from this analysis if

they had acute hepatitis B, or were co-infected with HIV, hepatitis C virus (HCV) or hepatitis D virus (HDV). We also excluded participants who were targeted for other HBRN studies (e.g., immunology and pregnancy projects) in order to keep "randomness" of the sample analyzed so that the prevalence of BCP/PC and other variables represent an unbiased estimate of those in the HBRN study. A total of 322 participants met the exclusion criteria. The resulting 1790 participants were potentially eligible for this study. The final cohort was further reduced to 1036 (808 adults, 228 children) for the following reasons: 699 in whom BCP/PC sequence could not be determined due to technical problems including sample condition and/or low HBV DNA as well as assay sensitivity, and 55 who received antiviral therapy ≤48 weeks prior to the BCP/PC sample collection. [Supplementary Figure] Participant information at the initial evaluation including sex, age at the time blood sample was collected for BCP/PC testing, race, continent of birth, and presumed mode of HBV transmission were recorded.

Laboratory tests

Routine laboratory testing: Complete blood counts and chemistry panels including serum aminotransferase levels were tested at local sites and the results recorded in a central database maintained by the HBRN Data Coordinating Center. Additional serum samples were shipped to a central repository and laboratories for virological testing. HBV DNA levels were determined using a real-time PCR assay (COBAS Ampliprep/COBAS TaqMan HBV Test, v2.0; Roche Molecular Diagnostics, Branchburg, NJ) with a lower limit of detection of 20 IU/mL. HBeAg status was determined by a quantitative HBeAg (qHBeAg) test (Roche Diagnostics). If the result was below limit of quantification, qualitative enzyme immunoassays for HBeAg and hepatitis B e antibody (anti-HBe) at local sites were used to determine the status of HBeAg and anti-HBe. HBeAg (+) participants had repeat HBeAg and anti-HBe testing every 6 months to determine the time of HBeAg clearance.

Quantitative HBsAg (qHBsAg) and qHBeAg: Both tests were made available by Roche Diagnostics for research purposes using their Elecsys platform and performed at the HBRN central virology laboratory at the University of Washington. qHBsAg assay had a measuring range of 0.05-130,000 IU/mL. Results for qHBsAg are presented as $\log_{10}$ IU/mL. qHBeAg testing was modified as previously described. [15, 16] Initial testing was done by utilizing the Paul Ehrlich Institute HBeAg standard material (PEI Units/mL), and then converted to the International WHO HBeAg quantitative standard (1 PEI Unit X 1.1 = IU/mL). Utilizing standardized sample dilutions, the assay had a measuring range of 0.3- 149,500 IU/mL. Samples with results lower than 0.3 IU/mL

are reported as “below the limit of detection” (BLD) and participants with a qHBeAg result BLD were considered to be HBeAg (-).

HBV genotyping: Testing was performed by an automated mass spectrometry test using a 420 bp fragment of the S gene, including the a-determinant. Subtyping was done based on Sanger sequence of a larger fragment spanning nt311 to nt1026 of the HBV genome, followed by phylogenetic clustering to reference sequences.[17] The sensitivity of the genotyping method is 50 genome equivalents per reaction.

Basal Core Promoter (BCP) and Pre-core (PC) variants: The BCP fragment was amplified by first round PCR with primers 1521sense GGGGCGCACCTCTCTTTACG and 2324anti-sense CTGGAGGAGTGCGAATCCACAC at 55°C annealing temperature for 35 cycles, followed by 30 cycles of a nested PCR reaction with primers 1572sense GGACCGTGTGCACTTCGCTTCA and 2169 anti-sense GGATCTTCC AAATTAITICC CAC CCA GG at 50°C annealing temperature. The sensitivity of this approach is estimated to be 500 genome equivalents per reaction. The presence of BCP mutations, A1762T and/or G1764A, and the PC mutations G1896A, C1858T was determined by the majority call of the Sanger chromatograms of both strands of a fragment spanning nt1572-2187 of the HBV genome. BCP and PC results were presented using validated scoring system based on dominant viral species (>50%) of the Sanger chromatogram at nt 1762, 1764, and 1896. Both HBV genotyping and BCP and PC variant testing were performed at the Molecular Epidemiology and Bioinformatics Laboratory in the Division of Viral Hepatitis at the Centers for Disease Control and Prevention (CDC).

Stem-loop structure prediction and energy calculation: Only the stem-loop region of the core promoter sequences was selected for RNA structure calculations. Sequences that had large gaps in the stem-loop region and/or had more than one IUPAC code assignment in any position of the stem-loop in the majority call sequence were removed from the analysis in order to avoid ambiguities in the structure predictions, leaving the number of available fragments at n=605. Structure prediction and stem-loop energy (delta G) was calculated by CLC Genomics Workbench v 11.0 Secondary RNA structure Tool. Stem-loops were rendered by the RNAfold webserver (The Vienna RNA web services). [18]

Statistical Analyses

Demographic, clinical, and virological characteristics were summarized using frequencies and percentages for categorical variables. As appropriate, mean values with standard deviation, or median values with 25th and 75th percentiles were used to summarize continuous variables. Since the distribution of clinical variables such as HBV DNA, qHBsAg, and qHBeAg levels were skewed, they were summarized using medians and 25th and 75th percentiles. We used Wilcoxon's test to compare continuous characteristics and Chi-square test to compare categorical characteristics between wild types and variants. Fisher exact tests were used to compare the distribution of wild types (WT) and variants of interests across each age group and genotype, and to compare HBeAg positivity between wild types and variants of interests within each age group. Wilcoxon's tests were used to compare quantitative HBeAg between WT and variants of interests within each age group among HBeAg positive group. P-values from these tests were adjusted for multiple comparisons using Holm's step-down procedure. Statistical software package SAS version 9.4 (SAS Institute, Cary NC, USA) was used to perform statistical analyses.

Results:

The demographic, clinical and virologic features of the 1036 participants included in this study are shown in Table 1. The majority (74.2%) of the participants was Asians, followed by blacks (13.3%) and whites (9.7%). Roughly half (48.9%) of the cohort was male. In this cohort, 461 (44.6%) were HBeAg (+) and 228 (22.0%) were 18 years or younger.

Of the samples from these 1036 participants, 478 (46.3%) had dominant WT sequences in both BCP and PC regions, 250 (24.2%) had dominant BCP variant only, 190 (18.4%) dominant PC variant only, and 114 (11.0%) dominant BCP and PC variants.

Among the HBeAg (+) participants, 67.7% had dominant WT HBV, 24.9% had dominant BCP only, 5.4% had dominant PC only, and 2% had dominant BCP+PC variants. In contrast, among HBeAg (-) participants, dominant wild type, BCP only, PC only and BCP+PC variants were present in 28.9%, 23.6%, 29%, and 18.5%, respectively. (Figure 1)

Participants born in Asia (n=680) were infected predominantly with genotypes B (47.5%) or C (40.1%). Genotype A was identified in 5.4% (A1 4.1%, A2 1.2%, other A 0.1%) of Asians. The majority of African-born persons (n=106) had genotype A (54.7%; A1 51.9%, A2 2.8%) followed by genotype E (31.1%). Participants born in North America (n=205) had more diverse genotypes,

the majority having genotype A (39%: A2 34.1% and A1 4.9%) followed by C (28.8%), B (22.4%), D (7.3%) and E (0.5%).

Distribution of PC and BCP variants across the HBV genotypes

Predominant classical PC G1896A variant was identified in 304 (29.3%) participants. Other PC variants including mixtures of G1896A, G1896C, G1896T without a majority call were identified in only 13 (1.3%) participants. Therefore, PC variant will hereafter refer to G1896A variant unless specified otherwise.

For HBeAg (+) participants, dominant PC variant was infrequent and was mostly associated with genotype B (11.6%) followed by genotype C (4.5%) and genotype D (3%). (Figure 2A) Among the HBeAg (-) participants, PC variant as the dominant viral species, either alone or in combination with BCP, was most frequently associated with genotype B (64%) and genotype D (63.5%), followed by genotype C (43.9%). (Figure 2B) There were only 10 HBeAg (+) and 26 HBeAg (-) participants with genotype E in this study; 5 (50%) of the HBeAg(+) participants had dominant PC and 21 (81%) of the HBeAg (-) ones had dominant PC variant either alone or in combination with BCP.

The dominant PC variant was detected in 16.4% HBeAg (-) and 2.3% HBeAg (+) participants with genotype A infection. PC variants had similar prevalence in sub-genotypes A1 and A2: 11 of 92 (12%) and 9 of 84 (11%) participants, respectively. (Figure 3A) All 20 participants with the dominant PC variant and genotype A were adults and all but one were HBeAg (-). Among the 11 participants with sub-genotype A1 and dominant PC variant, 4 (36.4%) were born in Africa and 7 (63.6%) were born in Asia. In contrast, 8 of 9 (89%) participants with sub-genotype A2 and the dominant PC variant were born in North America and only 1 (11%) was born in Asia. The nucleotide at 1858 was T and not C in 18 participants with sub-genotype A1 and in 17 with sub-genotype A2 infection. The PC G1896A variant was the dominant species in 17 (49%) of these 35 with C1858**T** allowing base-pairing with **A**denine at nt1896. Two participants with sub-genotype A1, but none of those with sub-genotype A2, and 1858C were noted to have the G1896A variant.

The stem-loop region contains the HBV pregenome encapsidation sequence (ε) that is critical for HBV replication. The stem-loop energy (kcal/mol) indicates the thermodynamic stability of the structure of epsilon (ε); with lower numbers indicating stronger bonds. The stem-loop energy

levels across wild type HBV genotypes B to E were similar and ranged between -31.6 and -32.8 kcal/mol (data not shown). Wild type HBV genotype A had lower stem-loop energy at -30.6 kcal/mol compared to other genotypes (P<0.0001). The stem-loop structures and energy stabilities of HBV genotype A G1896A PC variant in association with T or C at the nt1858 position were examined. (Figure 3B). Compared to WT sequence with G1896 and C1858, the bond in the stem-loop is significantly weakened (-25.1 kcal/mol) when G1896A is present in association with C1858. The bond is restored with similar energy level (-30.5 kcal/mol) when G1986A is paired with C1858T.

The double BCP TA variant was the most dominant species (82.4%) among the BCP variants in all the genotypes. The single A1762T or G1764A mutation was identified in 57 (6.3%), and other variants in BCP region without majority call in only 17 (1.9%) participants. Therefore, BCP variant will hereafter refer to double BCP TA variant unless specified otherwise. The distribution of BCP variants across the HBV genotypes for HBeAg (-) and HBeAg (+) participants is shown in Figure 2A and 2B. The double BCP TA variant, alone or in combination with PC, was most frequently associated with genotype C followed by genotype D and A, for both HBeAg (+) and HBeAg (-) participants.

Distribution of PC and BCP variants for HBeAg (+) and (-) participants across the age groups

For the HBeAg (+) participants, the prevalence of dominant WT HBV progressively decreased with age from 79.9% in those age 2-18 years to 44.2% in those age >40 years. (Figure 4A) Dominant PC and BCP variants, either alone or in combination, were already present in 7.2% and 14.4%, respectively in participants between 2 and 18 years, and increased to 10.2% and 50.7% among those >40 years. Among the HBeAg (-) participants, dominant PC and BCP variants, either alone or in combination, were present in 42.8% and 25% of participants between age 2-18 years respectively, and increased to 49.8 and 46.7% among those >40 years. (Figure 4B)

Comparison of HBeAg status and qHBeAg levels in participants with WT, PC and BCP variants across the age groups

Since the emergence of PC and BCP variants is associated with abolished or reduced production of HBeAg, the relationship between HBeAg status and HBV variants across the age groups was examined. [Figure 5A] Among participants with dominant WT HBV, prevalence of HBeAg was >90% in those between 2-18 years of age, decreasing steadily with age; however, 28.9% of those

older than 40 years of age remained HBeAg positive. The prevalence of HBeAg across the age groups for those with dominant BCP variants was similar to those with wild type HBV ($p>0.05$). As expected, the prevalence of HBeAg was lower among participants with dominant PC variant compared to those with WT HBV in each age group ($p< 0.001$). However, 12 of 23 (52%) participants aged 2-18 years of age were HBeAg (+) decreasing to 15% by the 4th decade and only 4% in those older than 40 years. Participants with both dominant PC and BCP variants had similar prevalence of HBeAg across the age groups as those with dominant PC variant only.

Of the 461 HBeAg (+) participants included in this study, 410 (89%) had follow-up data on HBeAg status. The rates of HBeAg clearance among these HBeAg (+) participants, with dominant WT HBV (n=272), BCP (n=109) and dominant PC with or without BCP variants (n=29) were further examined. After a median follow up of 3.2 years, 22% participants with WT HBV achieved HBeAg clearance compared to 62% of those with dominant PC and 45% of those with dominant BCP variants. The rates of HBeAg clearance per 100 person-years were significantly higher for participants with dominant PC or BCP variants, 24.4 (95%CI: 15.4, 38.8) and 15.0 (95%CI: 11.4, 19.9), respectively compared to those with WT HBV, 6.0 (95%CI: 4.7, 7.7) ($p <0.0001$). [Supplementary table] This finding was true for both adults and children. Specifically among the 167 HBeAg(+) children age 2-18, the incidence rates of HBeAg loss per 100 person-year were significantly higher for those with dominant PC variants, 25.1 (95% CI 12.6, 50.2; $P<0.0001$) and those with BCP variants, 9.9 (95% CI 4.4, 22.0; $P=0.03$) compared to those with WT HBV, 3.4 (95% CI 2.1, 5.5).

Among HBeAg (+) participants, the median qHBeAg levels for those with predominant WT HBV were consistently >3 $\log_{10}$ IU/ml across the age groups. (Figure 6B) The qHBeAg levels were more variable for those with dominant BCP or PC variants but tended to be lower compared to WT HBV especially for those >40 years of age.

Comparison of HBV DNA and qHBsAg levels in participants with WT HBV, BCP or PC variants across the age groups

HBV DNA levels were generally higher in HBeAg (+) than in HBeAg (-) participants. (Figure 6) Among the HBeAg (+) participants, median HBV DNA levels were >8.2 log IU/ml for those with WT HBV across the different age groups. Those with dominant PC and BCP variants also had relatively high HBV DNA levels, >6 log IU/ml. (Figure 6A) HBeAg (-) participants with WT HBV had low median HBV DNA levels (2.6 - 3.4 $\log_{10}$ IU/ml) consistent with an inactive phase of

hepatitis B across the age groups; these levels tended to be lower than those with dominant PC (3.4 - 4 log_{10} IU/ml) or BCP (3 – 4.9 log_{10} IU/ml) variants. (Figure 6B)

The qHBsAg levels were higher in HBeAg (+) than HBeAg (-) participants. [Data not shown] There were no differences in qHBsAg levels regardless of the presence or absence of dominant BCP or PC variants in both HBeAg (+) and HBeAg (-) groups.

Discussion:

We examined the prevalence and patterns of HBV PC G1896A and BCP A1762T and G1764A variants among the racially diverse pediatric and adult participants enrolled in the HBRN Cohort Study. Our study is unique in its size (>1,000 participants), wide age range (2 to 80 years), and robust representation of the four most common HBV genotypes (A-D). The majority (67%) of our participants was born in Asia with a substantial proportion born in North America (19%) and Africa (10%), allowing us to examine HBV PC and BCP variants in persons who acquired HBV infection from different continents. One of our major findings was the presence of PC G1896**A** variant among participants with HBV genotype A and a T but not a C at nt1858. In addition, we observed that PC and BCP variants were detected at a young age in both HBeAg (+) and HBeAg (-) participants. We also noted that the prevalence of HBeAg remained high in young persons with dominant PC or BCP variants, with a more rapid decline with age in those with dominant PC than those with dominant BCP variants.

In this study which included 22% participants younger than 18 years, we found that PC G1896A and BCP variants were present at a very young age, being detected as dominant species in 7.2% and 14.4% of the HBeAg (+) participants age 2-18 years. Of note, while these variants block or decrease HBeAg production, some participants with these variants as dominant HBV sequences remained HBeAg (+) suggesting that these variants can be selected and become dominant even prior to HBeAg seroconversion. In fact, more than 50% of our participants younger than 18 years who had dominant PC or BCP variants were HBeAg (+). In contrast, a recent study on African children living in Australia reported 25 of 30 (83.3%) children infected with BCP or PC variants were HBeAg (-). [19] This could be explained by the difference in genotypes; all the African children with HBeAg (-) hepatitis in the Australian study had HBV genotype D or E; while 38 of 57 (67%) children with BCP or PC variants in our study had HBV genotype B or C.

Findings of mixed viral populations of WT and PC and/or BCP variants in HBeAg (+) patients had been previously reported and this observation was more common in patients who cleared HBeAg during short-term follow-up[20]. In our study, we observed that the prevalence of HBeAg among participants with dominant PC variants markedly declined between subjects age 2-18 and those age >18-40 years while the decline was much smaller in those with dominant BCP variants attesting to the difference in impact of these two variants on HBeAg production. The rate of HBeAg clearance was highest for participants with dominant PC variants, followed by those with dominant BCP variants and lowest for those with dominant WT HBV. As expected, we found that quantitative HBeAg levels were lower in participants with PC or BCP variants compared to those with WT HBV. This difference was more striking in those above the age of 40.

Among the HBeAg (+) participants, those with WT HBV had higher HBV DNA levels compared to those with dominant PC or BCP variants. In contrast, HBeAg (-) participants with dominant PC or BCP variants tended to have higher serum HBV DNA levels than those with WT HBV across age groups. These observations might suggest that patients with PC and BCP variants likely evolve to immune active hepatitis B, whereas those with wild type HBV are more likely to remain in the inactive phase after HBeAg clearance. Similar findings were observed in an earlier study conducted in 17 sites in the U.S. but that study included adults only.[21] It has been postulated that selection of PC and BCP variants may facilitate escape of immune control. Prospective studies from Taiwan reported the annual rates of active hepatitis B after spontaneous HBeAg seroconversion ranged from 1.5% to 3.3%. [22, 23] Chu et al reported 37 patients with reactivation of hepatitis B (HBV DNA ≥10^4 copies/mL) after HBeAg seroconversion. Of these, 21 (56.8%) had solely PC variant, 4 (10.8%) had solely BCP variant, 10 (27.0%) had both variants, and only 2 (5%) had wild type HBV. [23] Follow-up of our participants will be important to determine whether HBeAg (+) participants with dominant PC or BCP variants are more likely to evolve to active hepatitis B after HBeAg clearance.

A striking finding in our study is the detection of dominant PC G1896**A** variant in 13% and 10% of participants with sub-genotypes A1 and A2. The maintenance of the stem-loop structure is critical for the function of epsilon (Ɛ) which plays a pivotal role in pregenome encapsidation and HBV replication. [11] Nucleotides 1858 and 1896 are located opposite each other at the base of the stem-loop structure. In genotype A, the nt1858 is usually a C; a G to A change at nt1896 would weaken the pairing between these two nucleotides and the Ɛ signal structure. PC G1896**A** variant is thus rarely detected in patients with genotype A infection. We found that 17 of 20 participants

with genotype A and dominant G1896A variant actually had C1858**T** that allow base-pairing with A at nt1896 in the G1896**A** variant.

There is a paucity of reports on the prevalence of PC mutations among patients with genotype A infection. The publications to date have focused on Africans with predominantly sub-genotype A1. In a study in South Africa[24], variations of the PC sequence in HBV sub-genotype A1 were examined in 25 patients (20 with HIV coinfection) with HBeAg negative chronic hepatitis B. Six (24%) had start codon mutations: A1814C/T and G1816T but none had a G1896**A** stop codon mutation and all the samples had 1858**C**. Similar start codon mutations at nt1814 were described in another study in Africa on patients with HBV genotype A. [25] In another report focused on HIV and HBV co-infected individuals prior to antiretroviral therapy in South Africa, 44 sequences derived from HBeAg negative samples were analyzed for mutations that are known to down-regulate or abolish HBeAg production. [26] All had sub-genotype A1 and PC G1896**A** variant was identified in 5 of the 39 samples that were successfully sequenced. Similar to our findings, in 4 cases the G1896**A** variant occurred together with C1858**T**.

We found that PC G1896**A** variant was detected not only in sub-genotype A1 but also in sub-genotype A2 which is usually found in Europe and North America. In fact, the prevalence of PC G1896**A** variant was almost identical in sub-genotypes A1 and A2, 12% and 11%, respectively. Furthermore, while 4 of 11 participants with sub-genotype A1 and G1896**A** variant were born in Africa and the remaining 7 in Asia, 8 of 9 participants with sub-genotype A2 and G1896**A** variant were born in North America while the remaining one was born in Asia. Surprisingly, 2 of 11 participants (one born in Asia and one in Africa) with sub-genotype A1 and dominant PC G1896**A** variant had C but not T at nt 1858. The predicted stem-loop energy would indicate a very unstable structure. There could be other compensatory mutations in these two participants that we have not identified. Unfortunately, we do not have sequences of follow-up samples from these two participants to determine whether changes at nt 1858 or 1896 occurred over time.

The prevalence of PC 1896A variants in this study (29.3%) is comparable to a previous study conducted on 694 patients in 17 centers in the United States (27%).[21] As has been reported by others, we found that prevalence of PC 1896A variants is related to HBV genotype with the highest prevalence in genotypes B and D. Similarly, the prevalence of BCP variants in this study (35.1%) is similar to the earlier study in the United States cited above (44%), with a higher prevalence in genotypes C and A.

The strengths of our study were the large cohort of racially diverse participants with chronic HBV infection with ages spanning from 2 to 80 years and the follow up data allowing us to compare the rates of HBeAg clearance between WT and PC and BCP HBV variants. However, there are several limitations. Since HBRN only enrolled participants who were not receiving antiviral therapy, their liver disease tended to be less active and less advanced. Only samples with detectable HBV DNA could be applied to characterize the various HBV species that could skew the distribution of virological parameters of the cohort. Our study was also limited by the lack of sequencing data of serial samples to determine the temporal relationship of the changes in the PC and BCP regions and HBV DNA levels during the course of infection.

This study is novel for a number of reasons. Firstly, we reported the presence of precore G1896A variant in 11.4% of participants with genotype A, with similar prevalence in sub-genotypes A1 and A2. In most but not all cases, the G1896A variant was present in association with a compensatory C1858T mutation preserving base-pairing in the stem-loop structure of the pregenome encapsidation sequence. This new finding has potential clinical significance; the selection of PC (G1896A) mutation may be important for genotype A HBV that harbor C1858T to persist by escaping immune surveillance.

Secondly, the study is unique because we were able to evaluate the pediatric and adult populations in a continuum. We found that PC and BCP variants were present and could be dominant during the first two decades of life with increasing prevalence over time. While most adults with PC variant were HBeAg (-), majority of children between 2 and 18 years old with dominant PC variant were HBeAg (+). Our study demonstrates that the selection of HBV variants, especially PC, results in more rapid HBeAg clearance compared to dominant WT HBV. These novel findings have clinical and therapeutic implications in understanding variations in rates of spontaneous HBeAg clearance and potentially response to antiviral therapy especially for children and young adults. Longer-term observations will be critical to determine if HBeAg (+) participants with PC and BCP variants are more likely to evolve to an HBeAg (-) immune active state than those with WT HBV after HBeAg clearance.

Supplemental Material

Supplemental Figure. Participant Selection in the BCP/PC study from the HBRN cohort study

Supplemental Table. HBeAg clearance rates for dominant WT HBV, PC and BCP variants in longitudinal study

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Table 1. Demographic, clinical and virologic features of the participants

Variable	*Total n=1036*	*Wild type n=478 (46.3%)*	*PC G1896A only n=190 (18.4%)*	*BCP only n=250 (24.2%)*	*BCP & PC G1896A n=114 (11.0%)*
Sex	**n=1036**	**n=478**	**n=190**	**n=250**	**n=114**
Male	507 (48.9%)	208 (41.1%)	104 (20.6%)	130 (25.7%)	64 (12.6%)
Female	529 (51.1%)	270 (51.3%)	86 (16.3%)	120 (22.8%)	50 (9.5%)
Race	**n=1031**	**n=475**	**n=188**	**n=250**	**n=114**
White	100 (9.7%)	49 (49.0%)	13 (13.0%)	23 (23.0%)	15 (15.0%)
Black	137 (13.3%)	64 (47.1%)	24 (17.6%)	34 (25.0%)	14 (10.3%)
Asian	765 (74.2%)	353 (46.3%)	143 (18.8%)	185 (24.3%)	81 (10.6%)
Other/Mixed	29 (2.8%)	9 (31.0%)	8 (27.6%)	8 (27.6%)	4 (13.8%)
Age (years)	**n=1036**	**n=478**	**n=190**	**n=250**	**n=114**
0-18	228 (22.0%)	171 (75.0%)	21 (9.2%)	31 (13.6%)	5 (2.2%)
>18-40	377 (36.4%)	185 (49.1%)	66 (17.5%)	95 (25.2%)	31 (8.2%)
>40	431 (41.6%)	122 (28.6%)	103 (24.1%)	124 (29.0%)	78 (18.3%)
HBeAg status	**n=1033**	**n=476**	**n=190**	**n=249**	**n=114**
Negative	572 (55.4%)	164 (28.9%)	165 (29.0%)	134 (23.6%)	105 (18.5%)
Positive	461 (44.6%)	312 (67.7%)	25 (5.4%)	115 (24.9%)	9 (2.0%)
Genotype	**n=1028**	**n=475**	**n=189**	**n=247**	**n=113**
A	185 (18.0%)	106 (57.6%)	14 (7.6%)	54 (29.3%)	10 (5.4%)
B	374 (36.4%)	180 (48.3%)	112 (30.0%)	45 (12.1%)	36 (9.7%)

Variable	*Total* *n=1036*	*Wild type* *n=478 (46.3%)*	*PC G1896A only* *n=190 (18.4%)*	*BCP only* *n=250 (24.2%)*	*BCP & PC G1896A* *n=114 (11.0%)*
C	339 (33.0%)	142 (41.9%)	28 (8.3%)	127 (37.5%)	42 (12.4%)
D	87 (8.5%)	35 (41.2%)	17 (20.0%)	16 (18.8%)	17 (20.0%)
E	36 (3.5%)	10 (27.8%)	16 (44.4%)	3 (8.3%)	7 (19.4%)
Other (F, G, and multiple)	7 (0.7%)	2 (28.6%)	2 (28.6%)	2 (28.6%)	1 (14.3%)
HBV DNA (log_{10} IU/mL)	**n=1036**	**n=478**	**n=190**	**n=250**	**n=114**
<4	386 (37.3%)	134 (35.0%)	108 (28.2%)	79 (20.6%)	62 (16.2%)
4 -<6	207 (20.0%)	42 (20.4%)	55 (26.7%)	71 (34.5%)	38 (18.4%)
6 -<8	146 (14.1%)	67 (45.9%)	14 (9.6%)	54 (37.0%)	11 (7.5%)
>=8	297 (28.7%)	235 (79.1%)	13 (4.4%)	46 (15.5%)	3 (1.0%)

Figure Legends:

Figure 1. Precore and basal core promotor variants by HBeAg status

Figure 2. Prevalence of precore and basal core promotor variants across HBV genotypes and HBeAg status.

2A. HBeAg(+) participants, **2B.** HBeAg(-) participants

Figure 3.

3A. Prevalence of PC G1896A Variant in HBV Genotype A

3Bi. HBV pregenome encapsidation sequence

3Bii. Stem loop configurations of HBV genotype A with and without substitution at nt1986 (Precore) and nt1858 positions

The stem-loop energy (kcal/mol) indicates the thermodynamic stability of the HBV pregenome encapsidation sequence. Lower energy numbers indicate stronger bonds between base pairs.

Figure 4. Precore and basal core promotor variants by age and HBeAg status

4A. HBeAg(+) participats, **4B.** HBeAg(-) participants

Figure 5. Distribution of WT, BCP, PC, combination of BCP and PC variants across age groups

Wild type (WT) indicates dominant WT at both BCP and PC regions.
Overall p-value <0.001 for the prevalence of BCP or PC variants across age groups.

Figure 5. Distribution of quantitative HBeAg by age among HBeAg(+) participants with WT, BCP, PC, combination of BCP and PC variants

Wild type (WT) indicates dominant WT at both BCP and PC regions.

Figure 6. Distribution of HBV DNA by HBV variants, age and HBeAg status

6A. HBeAg(+) participats, **6B.** HBeAg(-) participants

Reference:

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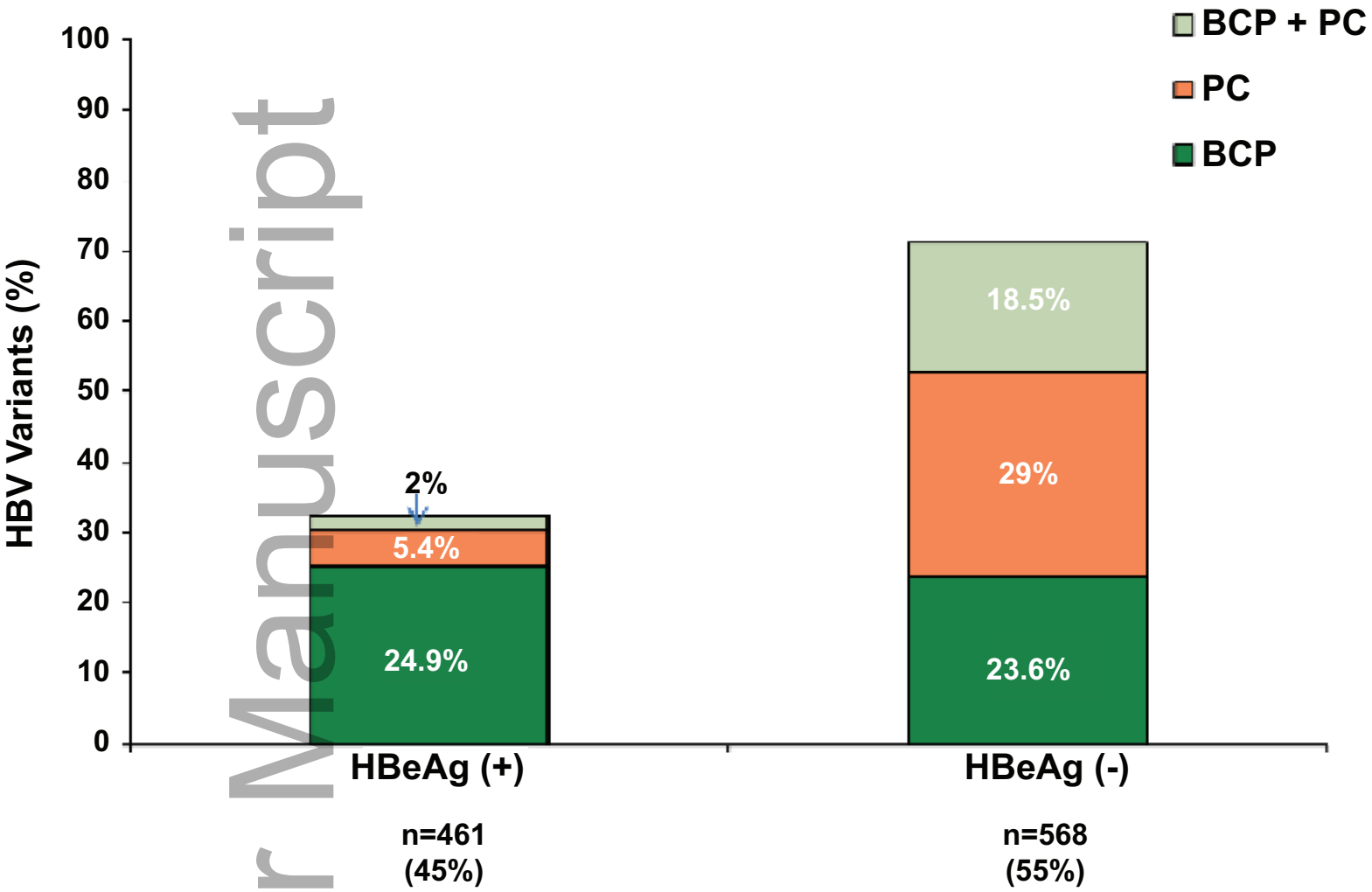
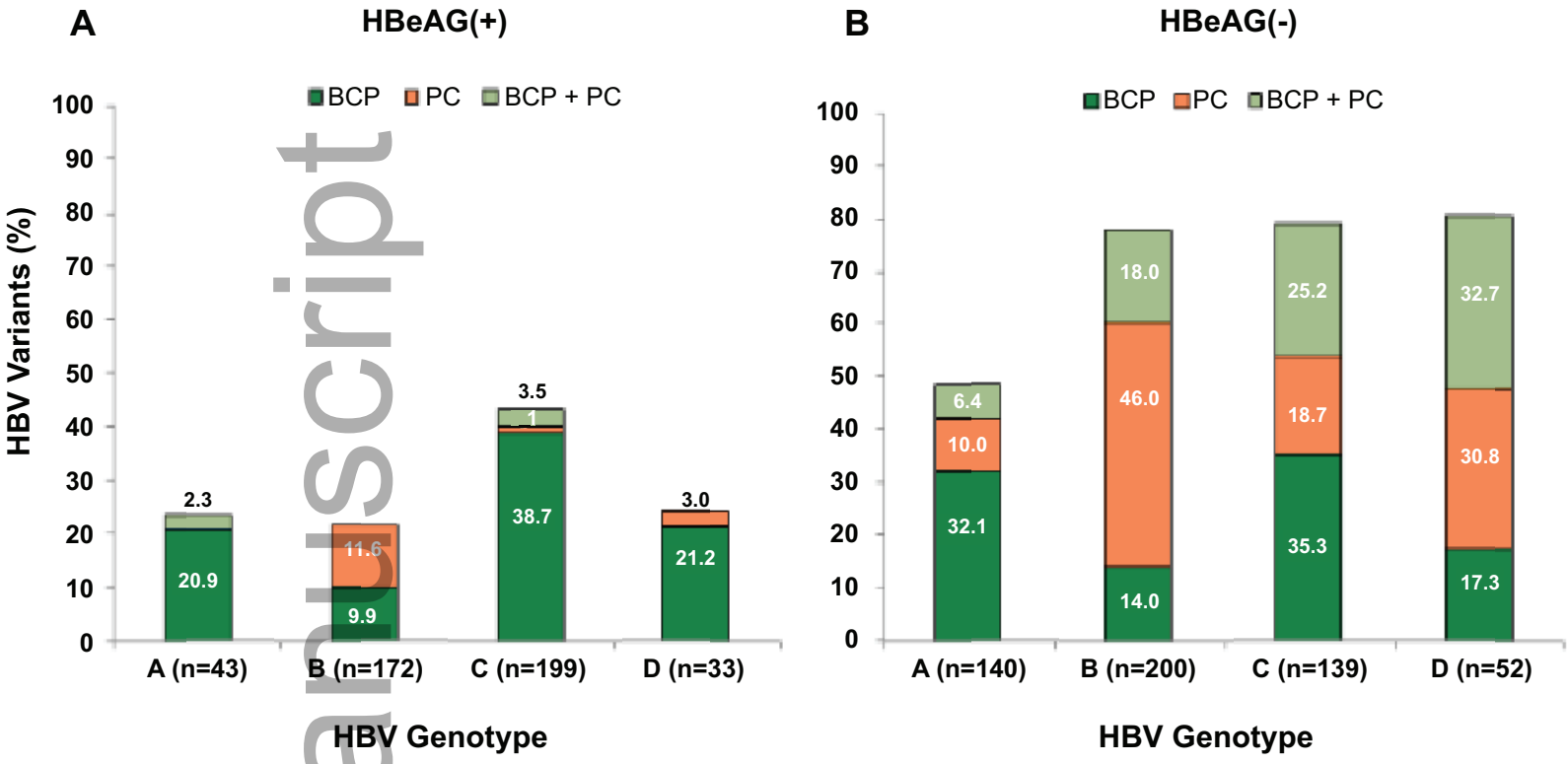
Figure 1.



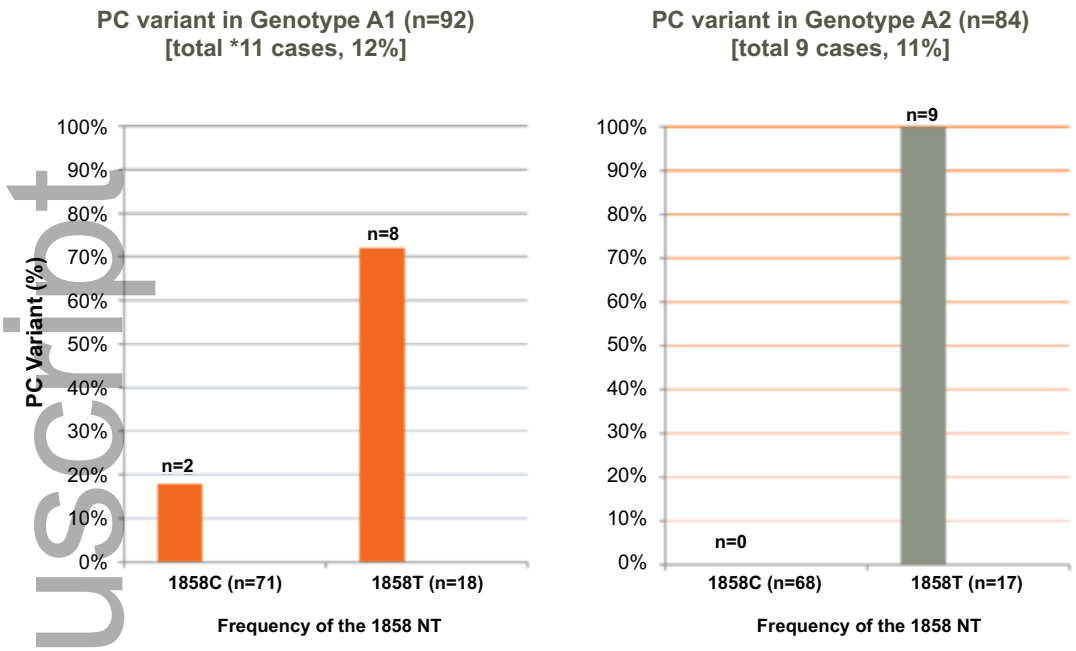
hep_31506_f1.eps

Figure 2.



hep_31506_f2.eps

Figure 3A.



*Sequence at nt1858 cannot be determined in 1 subject with PC variant

Figure 3Bi.

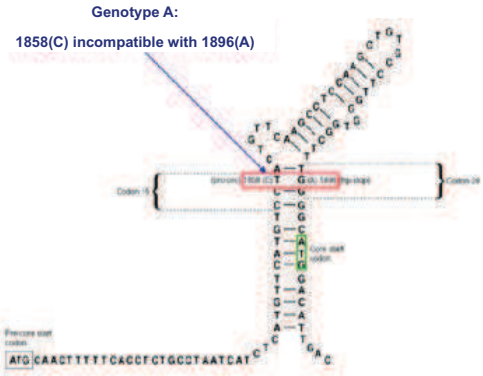
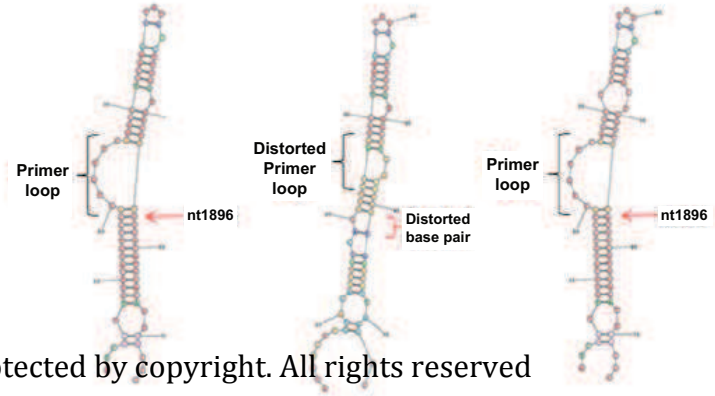
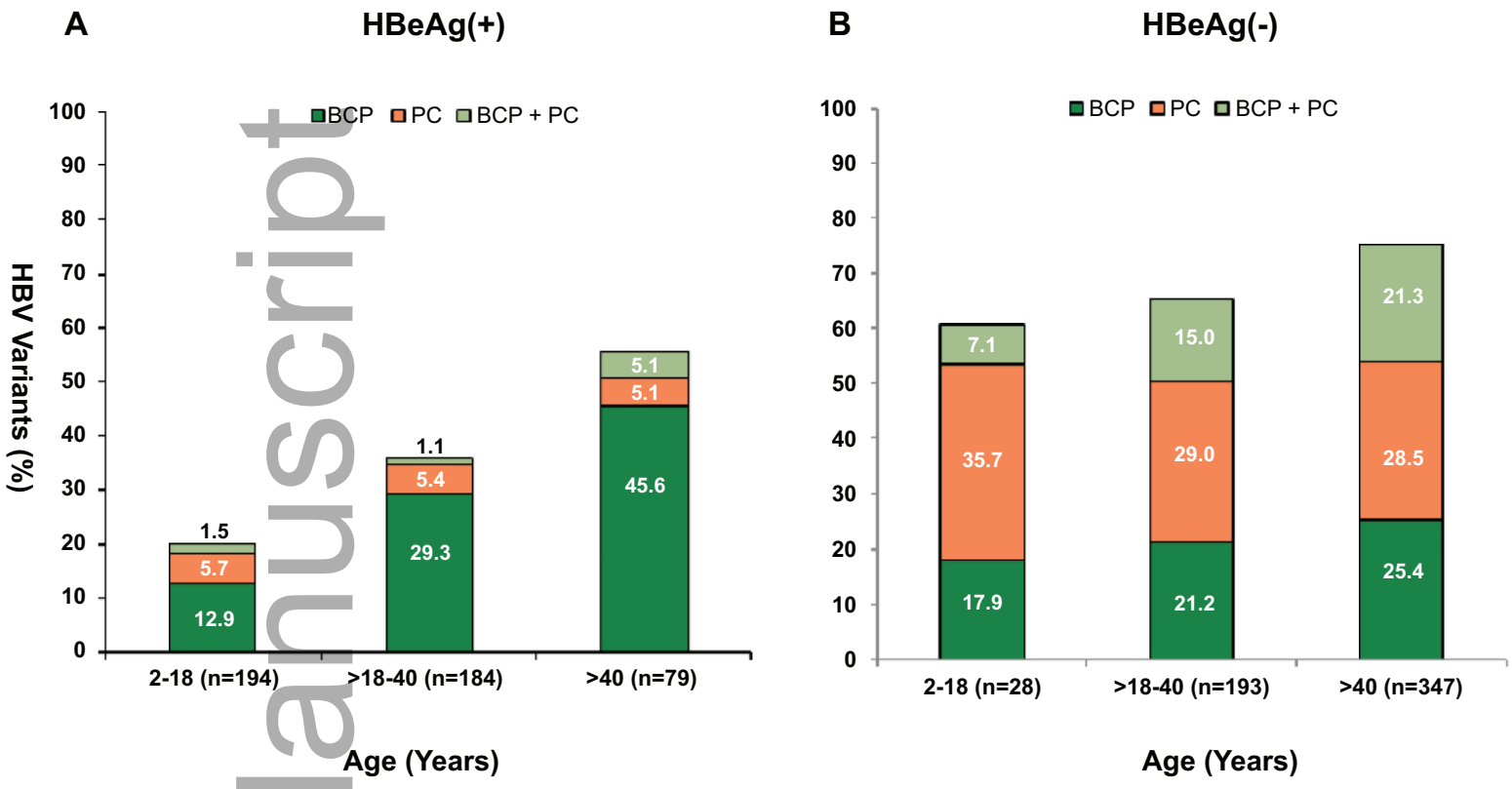


Figure 3Bii.



Wild Type	Precore Variant	Precore Variant with
C1858	C1858	nt1858 mutation
G1896	G1896A	C1858T

Figure 4.



hep_31506_f4.eps

Figure 5A.

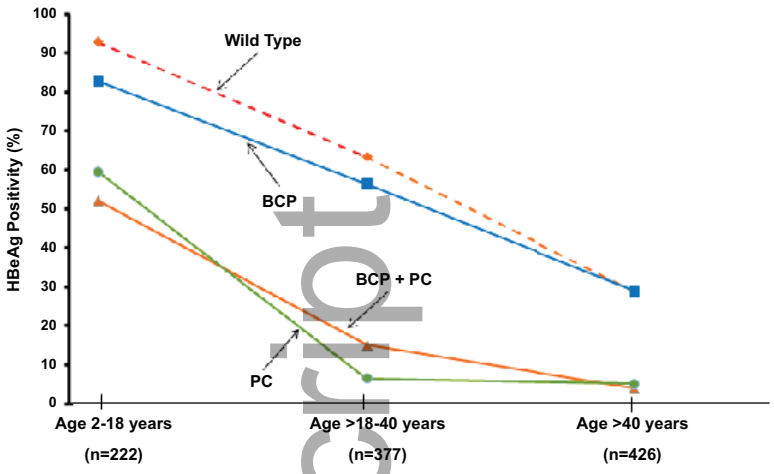
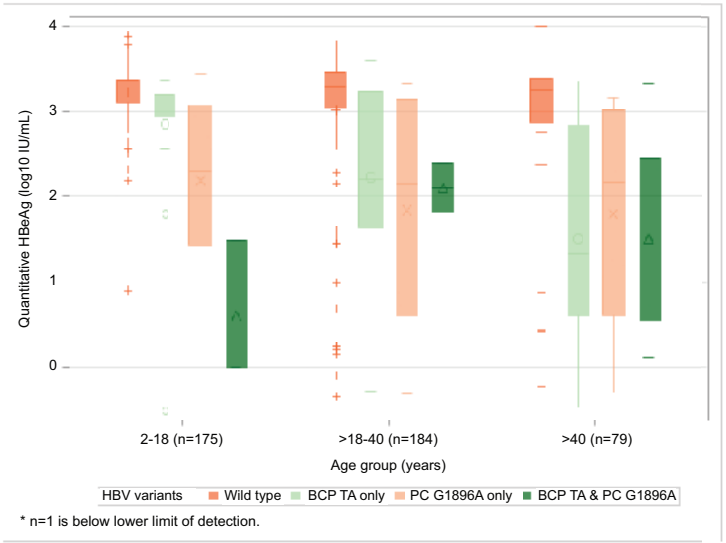


Figure 5B.



hep_31506_f5.eps