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9 **Original Article**

10 **TITLE:** HBV variants are common in the "immune-tolerant" phase of chronic hepatitis B

11 **Running Title:** HBV variants in immune-tolerant phase

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27 **Conflict of Interest:**

28 • Peter Revill has received research funding from Gilead Sciences.
29 • Susanna Tan is an employees of Gilead Sciences
30 • Anuj Gaggar, Kathryn Kitrino and Mani Subramanian are employees and stockholders of Gilead Sciences
31 • Ed Gane is a member of the Scientific Advisory Board and Speakers Bureau for Gilead Sciences and on
32 Scientific Advisory Board for Janssen, VIR, Dicerna and Roche.
33 • Henry Chan is an advisor and speaker for Gilead Science
34 • Alexander Thompson is on the advisory board for Gilead Sciences, research grant from Gilead Sciences,
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36 • All remaining co-authors do not have no conflict of interest

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39 **Ethics:**

40 All subjects in the study have signed an informed consent form prior to screening and in accordance with
41 local regulatory and ethics committee requirements. Experimental protocol in these trials was approved by
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43 0101 was NCT0050750.

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ABSTRACT

Nucleos(t)ide analouges (NUC) treatment prevents progression of liver fibrosis in subjects with chronic hepatitis B (CHB). However, risk for hepatocellular carcinoma (HCC) persists despite viral suppression. Specific HBV variants have been associated with adverse outcomes, including HCC, however the frequency of these variants during the seemingly benign immunotolerant (IT) phase is unknown. Next generation sequencing and detailed virological characterization on a cohort of treatment-naïve IT subjects was performed to determine the frequency of clinically relevant viral variants. Samples from 97 subjects (genotype B/C 55%/45%, median HBV-DNA 8.5 log10 IU/mL, median HBsAg 4.8 log10IU/mL, median HBeAg 3.6 log10 PEIU/mL) were analysed. Despite subjects being in the IT phase, clinically relevant HBV variants were common at baseline, particularly in the basal core promoter (BCP, overlaps the hepatitis B X (HBx) gene), precore, and PreS regions. BCP/HBx variants were independently associated with lower baseline HBeAg, HBsAg and HBV-DNA titres. Precore variants were independently associated with higher baseline ALT. Increased viral diversity was associated with increased age and lower HBV DNA, HBsAg and HBeAg levels. Low level (<5%) drug resistance associated amino acid substitutions in the HBV reverse transcriptase were detected in 9 (9%) subjects at pre-treatment but were not associated with reduced antiviral activity. Future studies should evaluate whether detection of HBV variant during IT CHB is predictive of progression to immune clearance and poor prognosis, and whether early initiation of antiviral therapy during IT CHB to prevent the selection of HBV variants is clinically beneficial.

KEYWORDS: hepatitis B virus; chronic hepatitis B; immune tolerance; HBV variants; viral diversity

INTRODUCTION

Persistence of hepatitis B virus (HBV) infection is most common in individuals who are exposed as neonates or young children.(1)The earliestphaseof chronic hepatitis B (CHB)infection, termed the immunotolerant (IT) or the “HBeAg-positive chronic HBV infection” phase of disease, in these subjects is characterized by the presence ofserum HBeAg, very high levels of serum HBV DNA and persistently normal serum ALT levels.The IT phase has traditionally been thought to represent a benign phase of disease asnecroinflammation is mild and fibrosis progression is unusual.(2)

While current guidelines do not recommend initiation of antiviral therapy for IT subjects(3-5),there is increasing scientific rationale to consider treating these subjects.Sustained viral suppression from a young age may be associated with reducedhepatocellular carcinoma(HCC) risk.(6)IT subjects have recently been reported to have ahigh level of HBV DNA integration and clonal hepatocyteexpansionsimilar to subjects in

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74 the HBeAg positive immune clearance phase of CHB infection, suggesting that hepatocarcinogenesis may
75 alreadybe underway in this early phase of the infection.(7)Recent data also suggest that HBV specific T cell
76 function is intact in childhoodrather than being in a state of immune tolerance, consistent with an early
77 initiation of an anti-HBV immune response.(8)Few studies evaluating for viral variants using next-generation
78 sequencing (NGS) technology have been performed in CHB subjects, particularly in IT subjects. This is
79 important because the selection of HBV variants, including HBeAg-defective variants, such as those with
80 mutations in the basal core promoter (BCP, A1762T/G1764A) which overlaps the gene of the hepatitis B x
81 protein(HBx) and/orin the precore (PC, G1896A) of the viral genome may contribute to disease progression
82 including HCC.(9, 10)The frequency of HBV variants withthese mutations or other mutations known to be
83 associated with adverse disease outcomes, referred to as clinically relevant HBV variants,in the IT phase of
84 chronic HBV infection is unknown. Identification of these variants and early initiation of antiviral therapy to
85 prevent their selection may contribute to a reduction in progressive liver disease and HCC risk.

86 StudyGS-US-203-0101 wasa randomized, double blind study that evaluated the efficacy of antiviral therapy
87 inITsubjects.(11)HBeAg positive, treatment-naïve individuals with high HBV DNA and normal ALTreceived
88 either oral tenofovir disoproxil fumarate (TDF, 300 mg) and placebo (n=64) or a combination of TDF (300 mg)
89 and emtricitabine (FTC, 200 mg, n=62) for 192 weeks.The primary end point of study was to determine the
90 proportion of subjects who achieved viral suppression to <69 IU/mL at week 192. Study showed the
91 treatment was safe and efficacious, and TDF/FTCprovided better viral suppression than TDF alone(11), with
92 55% and 76% of subjects who received TDF and TDF/FTC respectively achieved HBV DNA levels of <69 IU/mL
93 at end of treatment.Among subjects with on-going low level viraemia, no confirmed TDF resistance
94 wasidentified by Sanger sequencing.However, the rate of HBeAg loss was low (5%) and no subjects achieved
95 HBsAg seroclearance, which wasconsiderably lower compared to observations in IC subjects.(12)No HCC was
96 observed, although the study was not designed to evaluate HCC as an outcome.

97 To datethere have been few detailed studies profiling HBV virology among ITsubjects. The aim of the
98 currentstudy was to perform a detailed virological investigation of baseline and on-treatment samples
99 fromsubjects enrolled inStudy GS-US-203-0101. We have previously shown that the detection by NGS of HBV
100 variants with BCP(A1762T/G1764A) and/orPC (G1896A)mutations at baseline was associated with reduced
101 likelihood of HBsAg loss, even when present in serum at levels as low as 1% of the viral quasispecies
102 pool(14). We hypothesized that these and other clinically relevant HBV variants that are associated with
103 disease progression and HCC risk may begin to emerge during the IT phase.Whole HBV genome
104 sequencingbyNGS was performed to identify the presence and frequency of HBV variants with these genetic
105 changes, which were tested for association with baseline and on-treatment levels of serum HBeAg, HBsAg
106 and HBV DNA.

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107 **MATERIALS AND METHODS**

108 **Subjects**

109 The inclusion criteria for GS-US-203-101 were: age 18-69yrs., serum HBV DNA level >1.7 x 10^7 IU/mL, serum
110 ALT ≤upper limit of normal (ULN) (men:43 U/L, women:34 U/L), positive serum HBeAg and HBsAg, and
111 creatinine clearance ≥70 mL/min. Subjects with decompensated liver disease, history of HCC, and
112 co-infection with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus were excluded.(11)A
113 total of 126 subjects received treatment in GS-US-203-0101, 64 received TDF and 62 received TDF/FTC. This
114 study was restricted to subjects infected with HBV genotypes B and C (n = 114, 90% of the cohort).

115 **Viral load and serological characterisation**

116 HBV viral load and HBsAg quantification were determined previously(11)using the Roche COBAS TaqMan
117 (Roche Diagnostics, Mannheim, Germany) and Abbott Architect (Abbott Laboratories, Abbott Park, IL)
118 assays, respectively.The lower limit of quantification for HBV DNA was 29 IU/mLand HBsAg was 0.05
119 IU/mL.HBeAg levels were determined using the Roche Elecsys HBeAg assay (Roche Diagnostics, Basel,
120 Switzerland) at the Victorian Infectious Diseases Reference Laboratory (VIDRL) as previously
121 described.(13)The upper limit of quantification was>6000 PEIU/mLand the lower limit was 0.3 PEIU/mL.(13)

122 **Next Generationsequencing (NGS)**

123 Whole HBV genome amplification was performed with HBV DNA extracted from 200 µl serum using the
124 QIAamp DNA Minikit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. A genome
125 length PCR product and an overlapping PCR product covering the primer binding region were generated
126 usingthe Hi-Fidelity FastStart DNA polymerase (Roche Diagnostics, Basel, Switzerland). PCR products
127 generated for Sanger sequencing were pooled in a 1:1 molar ratio. Library preparation was performed using
128 the Nextera XT kit (Illumina, San Diego, CA, USA) and NGS was donewiththe MiSeq platform (Illumina)
129 according to manufacturer's protocolat Micromon Genomics (Melbourne, Australia).Analysis of NGS data
130 was performed using the in-house HBV-QuasiMiner software package, as previously described(14) with
131 minor modifications. Briefly, the package was composed of a number of modules:raw data processing,
132 quality assurance testing, HBV genotype determination, significant single/multiple nucleotide
133 variant(SNV/MNV) calling, and auto-deduction of nucleotide to amino acid changes of the respective HBV
134 genes.The threshold for detection of SNV/MNVusing this approach was established at 1%, compared to
135 Sanger sequencing (limit of detection >20%).NGS was successfully performed on 97 baseline and 27 on-
136 treatment (last sample with sufficient viral load for amplification) samples. Sanger sequencing was
137 performed for validation purposes and mutation analysis across the whole genome was carried out against
138 genotype specific consensus sequences as described previously.(15-17)

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139 **Clinically relevant HBV variants**

140 Each HBV infected subjectharboursa uniquecollection of HBV variants some of which may be associated with
141 clinically important outcomes.The clinically relevant HBV variants analysed in this study included those with
142 BCP mutation/deletionatA1762T and G1764A (BCP/HBx variant), PC mutation at G1896Awhich created a
143 stop codon at position 28 of the HBe proteinsequence (PC variant), PreS1/2 amino acid substitution or
144 deletion at M1 (PreS variant), S amino acid substitutions within the 'a'-determinant region of the surface
145 protein sequence (S variant), S amino acid substitution at G145R (vaccine-escape variant), drugresistance
146 associated amino acid substitutions (RAS) in the RT of HBV polymerase (RAS variants), and the NRE mutation
147 G1613A (NRE variant).

148 **Viral Diversity Analysis**

149 HBV variants in individual subjects occur at different relative frequencies, and their diversity level can be
150 assessed byhaplotype reconstruction (refer to Supplementary Material and Methods section for detail).
151 Eachreconstructed haplotype was equated to the genome sequence of a specific HBV variant in this study.
152 Briefly, the NGS short reads were processed using Trimmomatic v0-2.36(18), mapped to a reference genome
153 using SMALT v0.7.4(Wellcome Sanger Institute, Cambridge UK), and the HBV genome haplotypes were
154 reconstructed using CliqueSNV v1.4.8(19). All haplotypes with a minimum abundance of 1% were used for
155 analysis.

156 **Phylogenetic Analysis**

157 A maximum likelihood (ML) tree was generated in MEGA7(20)to show the phylogenetic relationships
158 between all the haplotypes determined from baseline samples with a minimum abundance of 1%.

159 **Statistical Analysis**

160 Statistical analysis between clinical factors and single nucleotide or amino acid variants (SNV or SAV)
161 detected in baseline and on-treatment samples of subjects were performed with R Statistical Software
162 (v2.3.1; R Foundation for Statistical Computing, Vienna, Austria).For bivariate analysis, categorical data were
163 evaluated using the Fisher's exact test, and continuous data using thet-test for parametric data and
164 Wilcoxon Rank Sum test (or Kruskal-Wallis if more than 2 groups were analysed) for non-parametric data.
165 Normality of continuous data was assessed using the Shapiro-Wilk normality test. Multiplelogistic regression
166 analysis was performed to determine independent factors associated with treatment response using the R
167 package MuMIn.(21)All reported P values were 2-sided.

168 Statistical tests on the reconstructed haplotypes were performed using GraphPad Prism version 7.0e
169 (GraphPad Software, La Jolla California USA, www.graphpad.com) (refer to Supplementary for detail). The t-

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170 test was used to evaluate parametric data and Mann Whitney Test for non-parametric data. Normality of
171 continuous data was tested with both D'Agostino & Pearson and Shapiro-Wilk normality tests. Correlations
172 between clinical factors and haplotype frequency was assessed by measuring the Pearson correlation
173 coefficient for parametric data and Spearman correlation coefficient for nonparametric data.

174 Results

175 i) Subject characteristics

176 Baseline samples from 53 HBV genotype B and 44 HBV genotype C subjects from the GS-US-203-101 study
177 were successfully analysed using NGS(97/114 (85%) subjects, Table1). Samples of the remaining 17subjects
178 could not be amplified or had failed NGS quality control. Thesubjects at baselinewere predominantly young
179 (median age 31 [IQR 26-40] yrs.), Asian (96%), had high HBV DNA (median viral load 8.4[IQR
180 8.2-8.6]$\log_{10}$IU/mL) and normal ALT (median 26 [IQR 21-31] U/L), consistent with baseline characteristics
181 described in the overall GS-US-203-101 cohort.(11)

182 ii) Clinically relevant HBV variants were commonat baseline

183 Whole HBV genome NGS data was generated from baseline samples of 97 subjects, and analysis revealed
184 presence of clinically revelvantHBV variants was frequent despite the subjects were in the IT phase of CHB
185 (Table 2), with 87% of subjects had at least one of the described HBV variants that are associated with
186 adverse disease outcomes. BCP/HBx (n=19, 20%) and NRE (n=72, 74%) variants weremore common in
187 subjects infected with HBV genotype C than those with genotype B(BCP/HBx 41% vs. 2%, NRE 100% vs. 53%;
188 p<0.001). Other HBV variants detected include those with genetic variations in the PC, PreS and S regions of
189 the viral genome. Subjects harbouring HBV variants with BCP/HBx, PC or PreS1/2 M1 immune evasion
190 associated changes or deletions were older (35yrs vs. 30yrs, p=0.042) and had higher ALT levels (27 U/L vs.
191 23 U/L, p=0.006). RAS variants(22) were identified at low frequency (<5%) in 9 subjects.

192 iii) BCP/PC variants were associated with HBeAg, HBsAg, HBV DNA and ALT levels at baseline

193 The associations betweenthe BCP/HBx andPC variantsandserumHBsAg, HbeAg, HBV DNA and ALT levelsof
194 subjects at baselinewere assessed. The frequency of BCP/HBx variants when present at>1% was negatively
195 correlated withHBsAg (r=-0.503; p=0.028),HbeAg (r=-0.651; p=0.003), and HBV DNA levels (r=-0.446;
196 p=0.056).Furthermore, subjects withBCP/HBx variantspresent at>20% of the quasispecies pool (n=9,
197 detectable by Sanger sequencing) had significantly lower HbeAg (p=0.033), HBsAg (p=0.001) and HBV DNA
198 (p=0.016) levelsthan those with only wildtype (WT) variants (n=75) (Figure 1). Notably, subjects with PC
199 variants even at low levels (>1%, n=11)had significantly higher ALT levels than those infected with WT

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200 variants (n=43)(p=0.015).However, since all IT subjects by definition have low ALT levels, the biological
201 relevance of these small yet statically significant differences is unclear.

202 **iv) An increase in viral diversitymay indicatetransition towards immune clearance phase**

203 The median number of haplotypes determined from the IT cohort at baseline was 3 [IQR: 2 to 5].
204 Therelationship between viral diversity level in subjects and clinical factorsat baseline were
205 assessed.Haplotype reconstructionshowed that the level of viral diversity, expressed as number of
206 haplotypes per sample, correlated with baseline serum ALT level (r=0.314 and p=0.002)(Figure 2).In addition,
207 the level of viral diversity correlated with age of subjects (r = 0.367 and p <0.001), negatively correlated
208 albeit weakly with HBV DNA (r = -0.264 and p = 0.012), HbeAg (r = -0.254 and p <0.001) and HBsAg (r = -0.418
209 and p <0.001) levels at baseline. Viral diversity level also differed significantly by HBV genotype (p
210 <0.001,Figure3), being higher in subjects infected with HBV genotype C (median = 4; IQR 2 to 6) than HBV
211 genotype B(median = 2; IQR 2 to 3). No subjects were infected with mixed HBV genotypes by phylogenetic
212 analysis, and allhaplotypes clustered correctly in the maximum likelihood tree by the expected HBV
213 genotype (data not shown). Interestingly, the majority of the BCP/HBx variant haplotypes were determined
214 fromHBVgenotype Csubjects (data not shown).

215 **v) Baseline HBV variants were not associated with treatment response**

216 Finally, we assessed whether baseline HBV variants can inform response to antiviral therapy in IT subjects.Of
217 77 subjects with a complete set of serologicialdata available at week 192,66% achieved viral suppression of
218 serum HBV DNA to <29 IU/mL(59% among TDF treated and 76% among FTC/TDF treated), 15% had >1
219 log_{10}IU/mL decline in serum HBsAg, and 29% had >1 log_{10}PEIU/mL decline in serum HbeAgat end of
220 treatment.Female subjects in this cohort were more to likely to achieve viral suppression to <29 IU/mL at
221 week 192 than male subjects (Table 3), consistent with the parent study.(11)

222 Baseline clinically relevant HBV variantswere not associated with reductions in HBV viral load or HBsAg, but
223 presence of the BCP/HBx variant (p=0.006), and higher viral diversity (haplotype count, p=0.005) at baseline
224 was independently associated with >1 log_{10} decline in serum HbeAg at week 192 by univariate analysis
225 (Table 3). The dynamics of HBsAg and HbeAg change were also explored, with both markers exhibiting a
226 biphasic decline over 192 weeks of treatment (Supplementary Figure 1).

227 **vi) Persistent viraemia was not associated with selection of drug resistance associated substitutions**

228 RASvariants were detectedat low frequency in 9(9%)subjects at baseline (Tables 2 and4).The lamivudine
229 (LMV) resistance associated rtM204Isubstitution(22)was detected in 5 of the 9subjects,the LMV and adefovir
230 (ADV) resistance associated rtA181S/Tsubstitution(22) was detectedin 3subjects, and a subject had viral
231 variants with both rtM204I and rtA181T substitutions. All RAS were detected at levels below 5% of the HBV

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232 quasipecies pool, well below the 20% Sanger sequencingdetection threshold, and were not associated with
233 viral suppressionin response to TDF treatment.

234 The quadruple mutation (rtS106C, rtH126Y, rtD134E and rtL269I, collectively referred to as the CYEI
235 mutation) that may be associated with reduced sensitivity to TDF(23), recently identified in two patients(23),
236 was not detected in the samples of any of the subjects.

237 No subjects with RAS variants at baseline experienced virological breakthrough nor HBeAg lossby week 192.
238 Moreover, on-treatment samples from 4 subjects were successfully analysed by NGS, and the RAS variants
239 were no longer detected (Table 4).

240 **DISCUSSION**

241 This is the first detailed study of HBV sequence variability in the setting of IT CHB.The standard clinical
242 definition of ITCHB includes HbeAg positivity, high HBV DNA, normal ALT and minimal hepatic
243 necroinflammation(1), with current clinical guidelines not recommending treatment for this phase.(3-5)This
244 study has identified prevalent clinically revlevant HBV variants among a cohort of subjects at baseline in the
245 IT phase of CHB. Increase HBV diversity was associated with clinical factorssuggestive of transition to IC
246 phase disease, despite persistently normal ALT. HBV variants previously associated with disease progression
247 (cirrhosisand HCC) were common using NGS and were detected in 87% of the cohort.

248 Of note, the BCP/HBx mutations/deletions located at nucleotide positions 1762 and 1764of the HBV
249 genome(BCP/HBx variant) was detected at baseline by NGS in 20% of the ITsubjects.Presence of the
250 BCP/HBxvariant was associated with lower baseline levels of HbeAg, HBsAg and HBV DNA. This association
251 was independent of gender and HBV genotype. Similarly, mutations or amino acid subustitutions in the PC,
252 core Pre-S and NRE regions of HBV genomes were detected in IT subjects.Analysis of viral diversity by
253 haplotype reconstruction of NGS data confirmed that increased diversity was associated with clinical
254 features suggestive of progression towards the immune clearance phase of CHB, including lower HBV DNA
255 levels, HBsAg and HbeAg levels. Sequence diversity also varied by HBV genotype, with higher levels of
256 sequence diversity in HBV genotype C than genotype B in IT subjects. The data suggest thatthe viral
257 population is diverse during the IT phase of CHB, which we speculate reflects host-virus interaction but this
258 requires further experimental confirmation. Future studies should evaluate the association between
259 selection of HBV variants during the IT phase and markers of anti-HBV immunity.

260 The presence of clinically relevant HBV variants has been associated with clinical outcomes.
261 BCP/HBxvariantshave been associated with disease progression, cirrhosis,and HCC.(10, 24)Variants with
262 mutations/deletions in the PC and Pre-S regions of viral genomes have also been associated with increased
263 HCC risk(10, 25).Long-term viral suppression with NUC therapy reduces HCC risk when treating subjects with

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264 cirrhosis (26). Given the excellent long-term efficacy, resistance and safety profiles, the European
265 guidelinessuggest that treatment be considered in IT subjects if they are older than 30 years, regardless of
266 the severity of liver histological lesions(27)). Detection of HBV variants associated with HCC risk in IT subjects
267 with a mean age of 32 yrs in our study supports this approach. Prospective studies are required to evaluate
268 whether commencing immunotolerant subjects on NUC therapy before the selection of HBV variants will be
269 associated with a risk reduction for progression to HCC.

270 Another important clinical question is whether commencing treatment in IT subjects with very high viral
271 loads would be associated with an increase in the risk of antiviral resistance. Despite subjects in this study
272 having very high baseline HBV DNA levels, variants with resistance to tenofovir were not identified, although
273 very low levels of variants with resistance to other NUC's were detected.

274 In summary, our data shows that clinically relevant HBV variants associated with increased risk of liver
275 morbidity and mortality were detected in IT subjects. We surmise that individuals who may be transitioning
276 to IC phase have an increase in viral haplotype diversity, and suggest that the classification of
277 "immunotolerant CHB" may need to be revisited to consider the role of detectable viral evolution within the
278 clinical definition. Further studies of subjects who transition from IT to IC phases are required, as are studies
279 to evaluate the clinical benefit of early antiviral therapy to prevent the selection of HBV variants and reduce
280 the risk of HCC.

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357 **Table 1:**Comparison between baseline characteristicsof subjects who had samples successfully assessed by
358 NGS and those of the original study. (11)

		NGS Cohort (N = 97)		Original Study (N = 114)		p-values
Age (yrs), median [IQR]		33	[26-42]	34	[26-40]	0.535
Male, n (%)		45	(46)	54	(47)	0.891
Asian, n (%)		92	(95)	109	(96)	1
TDF monotherapy, n (%)		53	(55)	56	(49)	0.490
ALT (U/m), median [IQR]		25	[20-31]	26	[21-31]	0.679
HBV DNA (log_{10} IU/mL), median [IQR]		8.5	[8.2-8.6]	8.4	[8.2-8.6]	0.962
HBsAg (log_{10} IU/mL), median [IQR]		4.8	[4.6-5.0]	4.7	[4.6-5.0]	0.571
HbeAg (log_{10} PE IU/mL), median [IQR]		3.6	[3.4-3.7]	3.5	[3.4-3.7]	0.872
HBV Genotype, n (%)	B	53	(55)	64	(56)	0.890
	C	44	(45)	50	(44)	0.899

359

360 **Table 2:** BaselineHBV variants with specific mutations or amino acid substitutions in the viral genome that
361 have previously been associated with clinical phenotype, present at >1% frequency, that were detected by
362 NGS from 97 subjects.

Mutations/Amino Acid Subsitutions (†)	Associated phenotype	N (%) (‡)	Median frequency [IQR] when detected by NGS
PreS1 M1	Loss of large HBsAg, associated with infectivity(28), disease progression(29) and decreased HBsAg(30)	33 (34%) (B=17; C=16)	2% [1-9] (B: 1% [1-2]) (C: 11% [4-25])
PreS2 M1	Loss of medium HBsAg, associated with viral secretion(28), disease progression(31) and decreased HBsAg(30)	10 (11%) (B=2; C=8)	4% [3-20] (B: 22% [12-32]) (C: 4% [3-13])
PreS deletions (excluding those that involved M1)	Truncated large/medium HBsAg associated with decreased HBsAg(30) and immune escape(32)	5 (5%) (C=5)	20% [6-27] -
HBsAg 'a' determinant (codons 120-150)	Major antigenic region of the protein(33) and variants can lead to vaccine, immune, and diagnostic escape	43 (61%) (B=26; C=17)	2% [1-3] (B: 1% [1-2]) (C:2% [1-3])
HBsAg G145G/R	Known vaccine escape variant(34)	10 (10%) (C=10)	12% [4-20] -

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RT L80, A169, V173, L180, A181*, T184, S202, M204*, N236*, M250	Known drug resistance and compensatory variants(22)	9 (9%) § (B=4; C=5)	1% [1-2] (B: 1% [1-2]) (C:2% [1-2])
NRE G1613A	Associated with an increased risk of HCC in genotype C and alongside the BCP mutation(35), increase viral expression(35) and decreased HBeAg secretion(35)	72 (74%) (B=28; C=44)	4% [1-19] (B: 4% [1-25]) (C:4% [2-17])
BCP (mutations/deletions involving sites A1762 and G1764)	Associated with lower HBeAg expression, higher viral loads, disease progression(33). Suggestive of upcoming HBeAg seroconversion(36).	19 (20%) (B=1; C=18)	28% [4-73] (B: 53%]) (C:25% [4-80])
Precore (G1896G/A)	Associated with loss of HBeAg(33). Suggestive of upcoming HBeAg seroconversion(36).	13 (15%) (B=7; C=6)	9% [3-20] (B: 13% [3-29]) (C: 9% [7-18])

363 †RT = reverse transcriptase, NRE = negative regulatory region, BCP = basal core promoter

364 ‡ Number (percentage) of subjects with the mutation/amino acid substitution

365 §10 HBVRT amino acid substitutions are associated with drug resistance(37) and were screened for in this study, but only viral
366 variants with at least one of the 3 primary resistance substitutions flagged with asterisk are considered a drug resistant variant. The
367 others are secondary resistance associated substitutions. The RAS profiles of the 9 subjects are shown in Table 4.

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368 **Table 3:** Association ofviral variantswith clinically relevant nucleotide/amino acid changes detected by NGS at baseline and associated clinical markers with
369 antiviral therapy response at 192 weeks

Baseline characteristics	Viral suppression (†)			HBsAg >1 log_{10} decline			HBeAg >1 log_{10} decline		
	R (N = 54)‡	**NR(N = 27)‡**	**p-value§**	**R(N = 12)‡**	**NR (N = 67)‡**	**p-value§**	**R (N = 24)‡**	**NR (N = 57)‡**	**p-value§**
TDF monotherapy, n (% of N)	26 (48)	19 (70)	0.068	6 (50)	37 (55)	0.763	15 (63)	30 (53)	0.470
Age, median [IQR]	34 [26-45]	32 [27-39]	0.627	30 [23-44]	33 [27-42]	0.461	36 [26-46]	32 [27-41]	0.612
Female Gender, n (% of N)	32 (59)	9 (33)	***0.035***	4 (33)	36 (54)	0.224	11 (46)	30 (53)	0.632
ALT,median [IQR]	24 [21-30]	28 [23-35]	0.218	28 [24-35]	24 [20-31]	0.127	28 [24-35]	24 [20-30]	***0.006***
HBV DNA, median [IQR]	8.4 [8.2-8.6]	8.5 [8.3-8.8]	0.331	8.6 [8.4-8.6]	8.4 [8.2-8.6]	0.541	8.4 [8.0-8.6]	8.5 [8.3-8.7]	0.102
HBsAg,median [IQR]	4.8 [4.5-5.0]	4.9 [4.8-5.1]	0.070	4.8 [4.6-5.1]	4.8 [4.6-5.0]	0.778	4.5 [4.3-4.8]	4.9 [4.7-5.0]	***0.005***
HBeAg,median [IQR]	3.6 [3.3-3.7]	3.6 [3.4-3.6]	0.670	3.5 [3.3-3.6]	3.6 [3.4-3.7]	0.244	3.5 [3.3-3.7]	3.6 [3.4-3.7]	0.757
Genotypes C, n (% of N)	31 (57)	12 (44)	0.346	3 (25)	39 (58)	0.057	13 (54)	30 (53)	1.000
BCP/HBx (A1762T, G1764A),n (% of N)	14 (26)	3 (11)	0.155	1 (8)	15 (22)	0.442	10 (42)	7 (12)	***0.006***
Precore (G1896A),n (% of N)	7 (13)	4 (15)	1.000	2 (17)	9 (13)	0.671	4 (17)	7 (12)	0.724
PreS1/2 (M1, deletion),n (% of N) ¶	19 (35)	10 (37)	1.000	5 (42)	22 (33)	0.742	9 (38)	20 (35)	1.000
HBsAg ('a' determinant), n (% of N)	19 (35)	14 (52)	0.161	4 (33)	27 (40)	0.756	13 (54)	20 (35)	0.140
HBsAg (G145R), n (% of N)	7 (13)	2 (7)	0.710	1 (1)	8 (12)	0.674	6 (25)	3 (5)	***0.017***
Drug-resistant changes,n (% of N)	12 (22)	8 (30)	0.586	4 (33)	15 (22)	0.469	5 (21)	15 (26)	0.779
NRE (G1613A),n (% of N)	41 (76)	24 (89)	0.240	7 (58)	56 (84)	0.060	18 (75)	47 (82)	0.543
Haplotype Count,median [IQR]	3 [2-5]	2 [1-4]	0.089	3 [2-4]	3 [2-5]	0.989	4 [2-8]	2 [2-4]	***0.005***

370 †Number of subjects who achieved viral suppression of serum HBV DNA to <29 IU/mL

371 ‡ R = responders; NR = non-responders

372 § p-values in bold and italic font are statistically significant

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373 ¶ PreS: include the 3amino acid changes/deletionscategories listed in Table 2

374 **Table 4:** Summary of drug-resistance associated amino acid substitutions (RAS)identified in the reverse transcriptase domain of HBV polymerase

375 and overlapping HBsAg,at baseline in 9/97subjects by NGS.

Subject† (Genotype)	Treatment	RAS Profile at baseline (%)	Overlapping HBsAg changes at baseline	HBeAg atBaseline (log_{10} PEIU/mL)	VL at Baseline (log_{10} IU/mL)	VL at week 192 (IU/mL)	VL first week <29 IU/ml	ALT at Baseline (U/L)	HBeAg >1 log_{10} decline at week 192	HBsAg >1 log_{10} decline at week 192
1 (C)	TDF	A181T (1.1) + M204I (1.9) + M250I (1.1)	W172*+W196*	3.60	8.36	<29	96	31	N	N
2 (C)	TDF	M204I (1.3) + M250I (1.2)	W196*	3.25	7.65	<29	48	21	Y	Y
3 (B)	TDF	A181T (1.4) + M250I (3.6)	W172*	3.66	8.71	44	160	15	N	N
4 (B)	TDF + FTC	M204I (1.5) + M250I (3.6)	W196L	3.20	8.61	<29	96	24	N	N
5 (C)	TDF	A181S (1.7)	W172C	3.35	9.02	90	72	29	N	N
6 (B)	TDF	M204I (1.6) + M250I (2.7)	W196L	3.26	8.45	early EOT	-	29	N	N
7 (B)	TDF	M204I (1.2) + M250I (1.6)	W196L	3.29	8.52	32	160	17	N	N
8 (C)	TDF + FTC	A181S/T (3.6/2.7)	W172C/*	1.33	7.87	<29	48	26	N	N
9 (C)	TDF	M204I (2.0)	W196*	1.97	7.42	<29	48	23	Y	N

376 † Second samples of Subjects 3 (week 24), 5 (week 24), 6 (week 24), and 7 (week 8) were successfully analysed by NGS, and no RAS variants were detected.

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378 **FIGURE LEGENDS**

379 **Figure 1:** Baseline levels of HBsAg, HBeAg, HBV DNA and ALT determined from subjects infected with
380 immune/drug susceptible viral variants (WT), and those with BCP/HBx (BCP), PC and BCP+PC variants present
381 at different frequencies (>20%, >10% and >1%). Subject groups with significantly different serological
382 biomarker levels are marked with a bar above the boxplots.

383 **Figure 2:**Correlation relationships between viraldiversity level (number of haplotypes), serological markers,
384 and age of subjectsat baseline. Number of haplotypes versus: (a) ALT level, (b) HBV DNA level, (c) HBsAg
385 level, (d) HBeAg level, and (e) subject age.

386 **Figure 3:** Distribution of haplotype frequency determined from subjects with HBV genotype B and C
387 infections.

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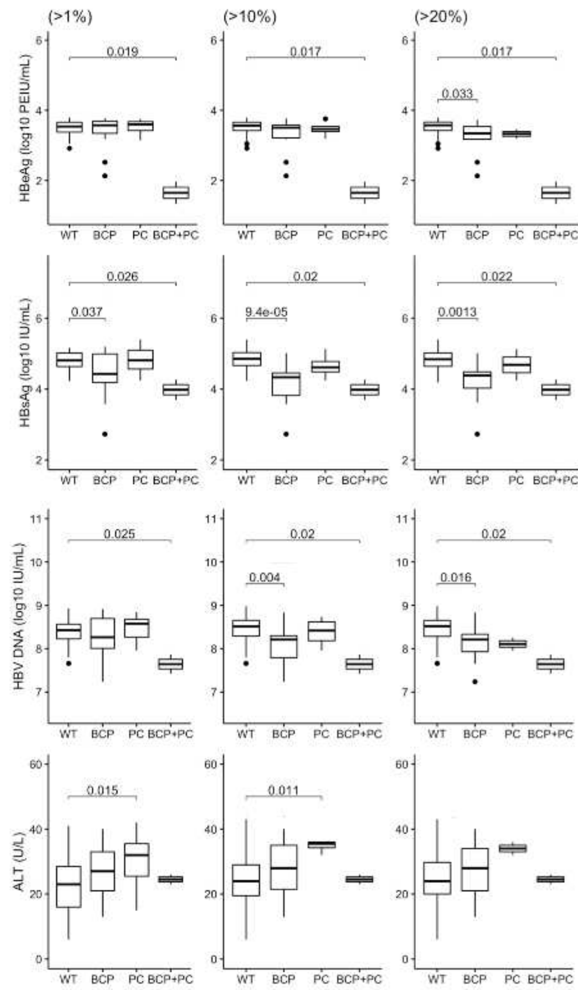

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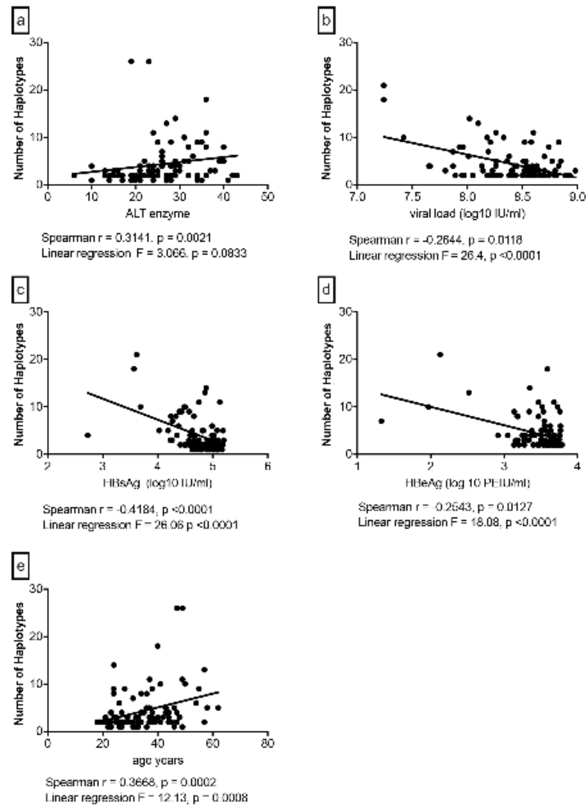

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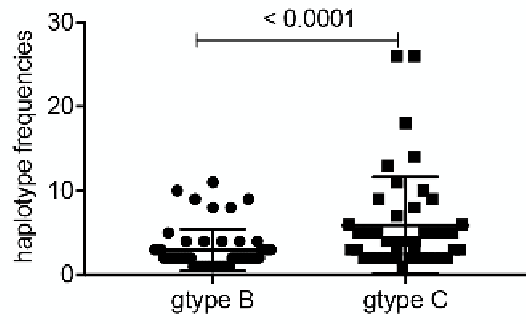

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