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1 **Evolution and phenotypic characterization of HBV quasispecies in compliant patients**
2 **experiencing unexplained entecavir treatment failure**

3

4 **Running title: Entecavir, viral resistance & whole genome**

5

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19

20 **ABSTRACT**

21 Entecavir treatment failure can be observed in compliant patients despite an absence of
22 detectable resistance mutations by Pol/RT Sanger sequencing. We hypothesized that these
23 unexplained treatment failures could rely on other mechanisms of viral resistance, especially
24 on mutations selected outside of the Pol/RT domain. Partial virological response to entecavir
25 was observed in three patients treated with immunosuppressive drugs, without selection of
26 Pol/RT resistance mutations. Mutations selected in the whole HBV genome during entecavir
27 treatment and potentially associated with resistance were searched for using deep sequencing
28 and characterized using a phenotypic resistance assay. Mutations Q206K (pre-core/core),
29 Q120K (pre-S1/pre-S2, T-cell epitope) and A300E (spacer domain) were selected during
30 entecavir treatment in patient #1 but were not associated with an increased level of resistance
31 to entecavir or an increase in HBV replication capacity. Core promoter mutations T1753G,
32 A1762T and G1764A were present before treatment in patient #1. HBs Ag immune escape
33 mutations were present before treatment in patients #2 (sK122R, sT126I, sP127S and sG145R)
34 and #3 (sM133I). These results provided a unique insight into the evolution of HBV
35 quasispecies during entecavir treatment, addressing the whole HBV genome and not only the
36 Pol/RT domain. Our results suggest that mutations selected outside of the Pol/RT domain could
37 contribute to entecavir treatment failure, not by an increased level of resistance, but by an
38 immune escape mechanism, in synergy with immunosuppressive drugs.

39

40 INTRODUCTION

41 Chronic hepatitis B virus infection affects 292 million people worldwide and is associated with
42 an increase in the risk of cirrhosis and hepatocellular carcinoma (HCC) (Razavi-Shearer et al.,
43 2018). Nucleoside or nucleotide analogs are used as a first-line treatment to prevent these
44 complications (Li et al., 2020; Papatheodoridis et al., 2020; Xu et al., 2015). Two of these
45 molecules, entecavir and tenofovir (disoproxil fumarate or alafenamide) have been associated
46 with higher rates of viral response and a higher drug genetic barrier than previous antiviral
47 drugs (lamivudine, adefovir). Entecavir resistance mutations in the HBV Pol/RT domain are
48 rarely selected (< 1.2%, after five years of treatment) (Tenney et al., 2009) except in patients
49 with a history of lamivudine treatment (Hayashi et al., 2015; L. Liu et al., 2019; Y. Liu et al.,
50 2019; Rose et al., 2018). These mutations have been associated with primary non-response and
51 viral breakthrough on entecavir treatment, but not with a partial virological response (PVR) to
52 entecavir (Lampertico et al., 2017). This last form of treatment failure is defined as a decrease
53 in HBV DNA levels of more than 1 log₁₀ IU/mL, with HBV DNA detectable after at least 12
54 months of treatment in compliant patients (Lampertico et al., 2017). It occurs in 10-33% of
55 patients treated with entecavir (Chang et al., 2006; Lai et al., 2006) and has been associated
56 with HBe Ag positivity, and with low levels of ALAT and a high HBV DNA viral load at
57 baseline (Chen et al., 2014; Lee et al., 2015; Li et al., 2015; Zoutendijk et al., 2011).

58

59 The management of patients experiencing entecavir treatment failure is based on the
60 identification of known resistance mutations by Pol/RT Sanger sequencing (Lampertico et al.,
61 2017; Lok et al., 2007) and, if relevant, the characterization of new resistance mutations in
62 phenotypic assays (Zoulim and Locarnini, 2013). However, this conventional strategy has
63 several limitations. First, it does not take into account polymorphisms selected outside of the

64 Pol/RT domain and associated with different clinical phenotypes of chronic hepatitis B (Huang
65 et al., 2013; Peng et al., 2017; Podlaha et al., 2019). Second, unlike deep sequencing, it is
66 unsuitable for the sensitive detection of minor variants before (Chevaliez et al., 2019) or after
67 treatment failure (Lee et al., 2018; Lowe et al., 2016).

68 This conventional approach fails to detect any Pol/RT resistance mutations in some patients
69 experiencing treatment failure on entecavir. We hypothesized that a more thorough exploration,
70 based on deep sequencing of the whole HBV genome before and after treatment failure, would
71 provide a more comprehensive overview of the viral factors associated with treatment failure.

72

73 We applied this strategy to compliant patients experiencing entecavir treatment failure without
74 the selection of mutations of the HBV Pol/RT domain. Relevant mutations selected during
75 entecavir treatment were characterized in a phenotypic resistance assay, based on whole HBV
76 genome sequences from clinical isolates.

77

78 **MATERIALS AND METHODS**

79 **Patients and samples**

80 Plasma samples were collected between 2011 and 2018, at Tours University Hospital, for the
81 diagnosis or follow-up of HBV infection (Marlet et al., 2020). We included patients
82 experiencing entecavir treatment failure with no detectable Pol/RT resistance mutations
83 (Lampertico et al., 2017). HBV infection and liver disease were followed, and therapeutic drug
84 monitoring was performed, as previously described (Marlet et al., 2020). This study was
85 approved by the Tours University ethics committee (no. 2019 055).

86

87 **Genotypic drug resistance testing**

88 Genotypic drug resistance testing was performed by amplifying the Pol/RT domain (aa rt1-
89 rt344) and subjecting it to Sanger sequencing , as previously described (Villeneuve et al., 2003).
90 We searched for resistance mutations as previously described (Marlet et al., 2020) and
91 interpreted them in accordance with the latest EASL guidelines (Lampertico et al., 2017).

92 **Deep sequencing of the whole HBV genome**

93 Two overlapping regions of the HBV genome were amplified by PCR with primer P1
94 (CCGGAAAGCTTGAGCTCTTCTTTTTCACCTCTGCCTAATCA) plus primer 786R
95 (GGACTIONAAGATGTTGTACAGACTTGGCC), and with primer P2
96 (CCGGAAAGCTTGAGCTCTTCAAAAAGTTGCATGGTGCTGG) plus primer BB
97 (CTGGATGTGTCTGCGGCG), described elsewhere (Günther et al., 1995; Marlet et al.,
98 2020). These PCRs were performed with 300 nM of each primer, 2.5 µL of DNA extract and
99 12.5 µL of Platinum™ PCR SuperFi PCR Master Mix 2X (Thermo Fisher Scientific,
100 Massachusetts, USA). Cycling reactions were performed on a T3000 (Biometra) with the
101 following cycle parameters: 2 min at 94°C, followed by 40 cycles of 40 s at 94°C, 90 s at 60°C,
102 90 s at 68°C, with an increment of 12 s after each cycle and a final extension step of 10 min at
103 68°C. We fused these two overlapping regions of the HBV genome together by PCR, using 1
104 µL of each diluted PCR product (equimolar) obtained in the first step and 12.5 µL of Platinum™
105 SuperFi PCR Master Mix 2X. Fusion PCR was performed on the T3000 with the following
106 cycle parameters: 30 s at 98°C, followed by 10 cycles of 10 s at 98°C, 45 s at 72°C and 2 min
107 at 72°C. The whole genome of HBV was amplified by PCR with 300 nM primers P1 and P2,
108 and 25 µL of fusion PCR product. Cycling reactions were performed on a T3000 Biometra with
109 the following cycle parameters: 2 min at 94°C, followed by 40 cycles of 40 s at 94°C, 90 s at

110 60°C, 180 s at 68°C, with an increment of 12 s after each cycle and a final extension step of 10
111 min at 68°C.

112 Next-generation sequencing was performed on the Miseq platform (Illumina, San Diego, USA),
113 with a previously described workflow (Guinoiseau et al., 2017). Briefly, the sequencing library
114 was prepared with the Nextera XT DNA sample preparation kit (Illumina, San Diego, USA),
115 in accordance with the manufacturer's protocol. Paired-end sequencing reads of 150 base pairs
116 were obtained with the Miseq (Illumina, San Diego, USA) platform. Illumina sequencer output
117 files matching of up to 151-base pair sequencing reads were processed with the Galaxy platform
118 after filtering and checking read quality (FastQC algorithm). Trinity software was used for *de*
119 *novo* assembly. The constructed sequences were checked with the Blast option from the
120 National Center for Biotechnology Information (NCBI). Reads were mapped with the Bowtie2
121 tool onto the best sequence given by the *de novo* assembly sequences. Nucleotide analysis was
122 performed position-by-position using the mpileup program. Positions with a sequencing depth
123 of over 100X were retained for further analysis. Only variants representing 10% or more of the
124 HBV quasispecies were considered for analysis. For each patient, consensus sequences of the
125 whole HBV genome were compared between samples collected before treatment and at
126 treatment failure. We searched for known HBs Ag escape mutations (Colagrossi et al., 2018;
127 Coppola, 2015; Lazarevic, 2014; Weber, 2005), core promoter and pre-core mutations (Parekh
128 et al., 2003) in these sequences, after alignment with other sequences from the same genotype
129 and reference sequences: HBV/E (AY738145), HBV/D (AF121240) and HBV/I (AB231908)
130 extracted from HBV database (HBVdb) (Hayer et al., 2013). HBV strain X02763 from HBVdb
131 (Hayer et al., 2013), with the usual numbering, was used as the reference sequence for the
132 annotation of mutations. Mutations were identified on the basis of their position in the pre-S1,

133 HBe, HBx and polymerase proteins or their position in the HBs Ag (s) or reverse transcriptase
134 domain (rt), if relevant. Core promoter mutations (CPM) were annotated with their nucleic acid
135 position in the X02763 HBV genome.

136 **Phenotypic drug resistance testing**

137 We had 1.1 units of the HBV genome (pCI_HBV1.1x), corresponding to a consensus sequence
138 identified by NGS on isolates collected before treatment, synthesized *in vitro* (by GenScript
139 Biotech, the Netherlands) and inserted between MluI and SallI restriction sites of a pCI vector
140 (Promega, Toulon, France), downstream from a CMV promoter. This construct allowed HBV
141 replication after transfection in HuH7 cells. Relevant mutations selected during entecavir
142 treatment were introduced into these clones by site-directed mutagenesis (GenScript Biotech).

143 Phenotypic resistance assays were performed as previously described (Marlet et al., 2020).
144 Briefly, Huh7 cells were transfected with pCI_HBV1.1x. Entecavir monohydrate (Merck,
145 Missouri, USA) was added to the cells at six different final concentrations, ranging from 2.5 to
146 50 000 nM. After four days of culture, intracapsid viral DNA was quantified by duplex real-
147 time PCR. We determined 50% inhibitory concentrations (IC₅₀) by least squares regression in
148 GraphPad Prism v5.0 (GraphPad Software, California, USA). The fold-change in resistance
149 corresponded to the IC₅₀ of entecavir for a given isolate, relative to the IC₅₀ of entecavir for the
150 wild-type HBV genotype D *ayw3* strain (pCI_HBVpg1820), which was tested in parallel.
151 Entecavir IC₅₀ and the fold-change in resistance were determined in three independent
152 experiments and compared in Mann-Whitney tests. Replication capacity relative to the same
153 wild-type strain as before was determined by quantifying HBV DNA levels in six different
154 wells after four days of culture without antiviral drugs. Results are represented as the means
155 and standard errors.

156

157 **Data availability**

158 All sequences were deposited in the GenBank database (Accession numbers: [SAMN16263252-](#)
159 [SAMN16263257](#) for MiSeq data and MW082636-MW082641 for consensus sequences).

160 **RESULTS**

161 **Patients**

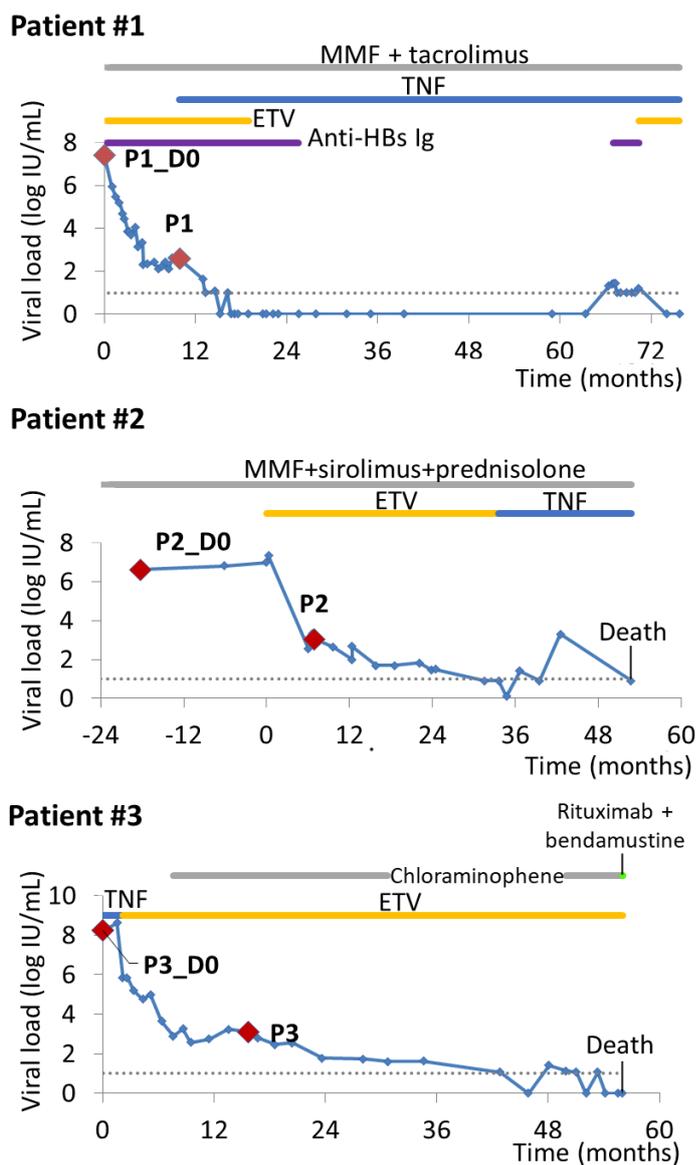
162 During the study period, HBV genotypic drug resistance testing was performed on 110 clinical
163 isolates. We identified seven isolates associated with entecavir treatment failure but with no
164 detectable Pol/RT resistance mutations. Four isolates were not included because of poor patient
165 compliance with treatment ($n=3$) or because viral DNA load was too low for amplification by
166 PCR ($n=1$, 1.3 log IU/mL). The three remaining isolates (P1, P2 and P3) were associated with
167 a partial virological response (PVR) to entecavir in immunocompromised patients (Figure 1).

168 Patient #1 underwent liver transplantation in the context of decompensated alcohol-related
169 cirrhosis. He was negative for HBs Ag, HBs Ab and HBc Ab before transplantation, but he
170 received a graft from a donor with positive HBc Ab. He received one dose of anti-HBs
171 immunoglobulins (IVHEBEX, 10 000 IU, LFB Biomédicaments) during the anhepatic phase,
172 to prevent HBV infection from the graft. Despite this prophylaxis, he experienced HBV
173 infection leading to acute hepatitis one year later, whilst on mycophenolate mofetil (MMF) and
174 tacrolimus treatment.

175 Patient #2 underwent kidney transplantation and was treated with MMF, sirolimus and
176 prednisolone. She was anti-HBc Ab-positive and HBs Ag-negative before transplantation. She

177 was diagnosed with asymptomatic HBV infection six years later (she had tested negative for
178 HBs Ag the year before).

179 Patient #3 had a history of resolved HBV infection (HBc Ab-positive, HBs Ag-negative) before
180 he was diagnosed with chronic lymphocytic leukemia (CLL). He was treated with one dose of
181 rituximab, and with cyclophosphamide and fludarabine for two years before remission of CLL
182 was achieved and treatment stopped. HBV reactivation was diagnosed six years later, in the
183 context of a relapse of CLL with cytotoxicity. Rituximab is known to be associated with a high
184 risk of HBV reactivation and could have triggered HBV reactivation in this patient (Loomba
185 and Liang, 2017). Interestingly, this patient remained negative for HBc Ab throughout follow-
186 up, probably due to the strong B-cell depletion induced by rituximab (Feeney et al., 2013).
187 Patients #1, #2 and #3 reported good treatment compliance, which was confirmed by
188 therapeutic drug monitoring (entecavir plasma concentrations = 0.45, 0.78 and 0.45 ng/mL,
189 respectively). No relevant drug interactions between HBV treatment and concomitant
190 medication were detected. Retrospective genotypic drug resistance testing identified the same
191 Pol/RT sequences in pretreatment (P1_D0, P2_D0 and P3_D0) and post-treatment (P1, P2 and
192 P3) isolates (GenBank MW082636-MW082641). These findings suggest that the PVR to
193 entecavir in these three patients was not associated with the selection of resistance mutations in
194 the Pol/RT domain, or with poor patient compliance.



195

196 **Figure 1: Biological and clinical follow-up of patients experiencing a partial virological**
 197 **response to entecavir**

198 HBV viral loads are represented from the diagnosis of HBV infection (month 0) to the end of
 199 follow-up. The clinical isolates included in this study are identified with red diamonds and
 200 characters in bold typeface. ETV, entecavir; TNF, tenofovir disoproxil fumarate; MMF,
 201 mycophenolate mofetil. HBV viral loads were quantified with the Abbott HBV real-time
 202 assay. Its lower limit of detection (10 IU/mL) is represented with a dashed line. Patients #2
 203 and #3 died from graft rejection and acute complications of hemopathy, respectively.

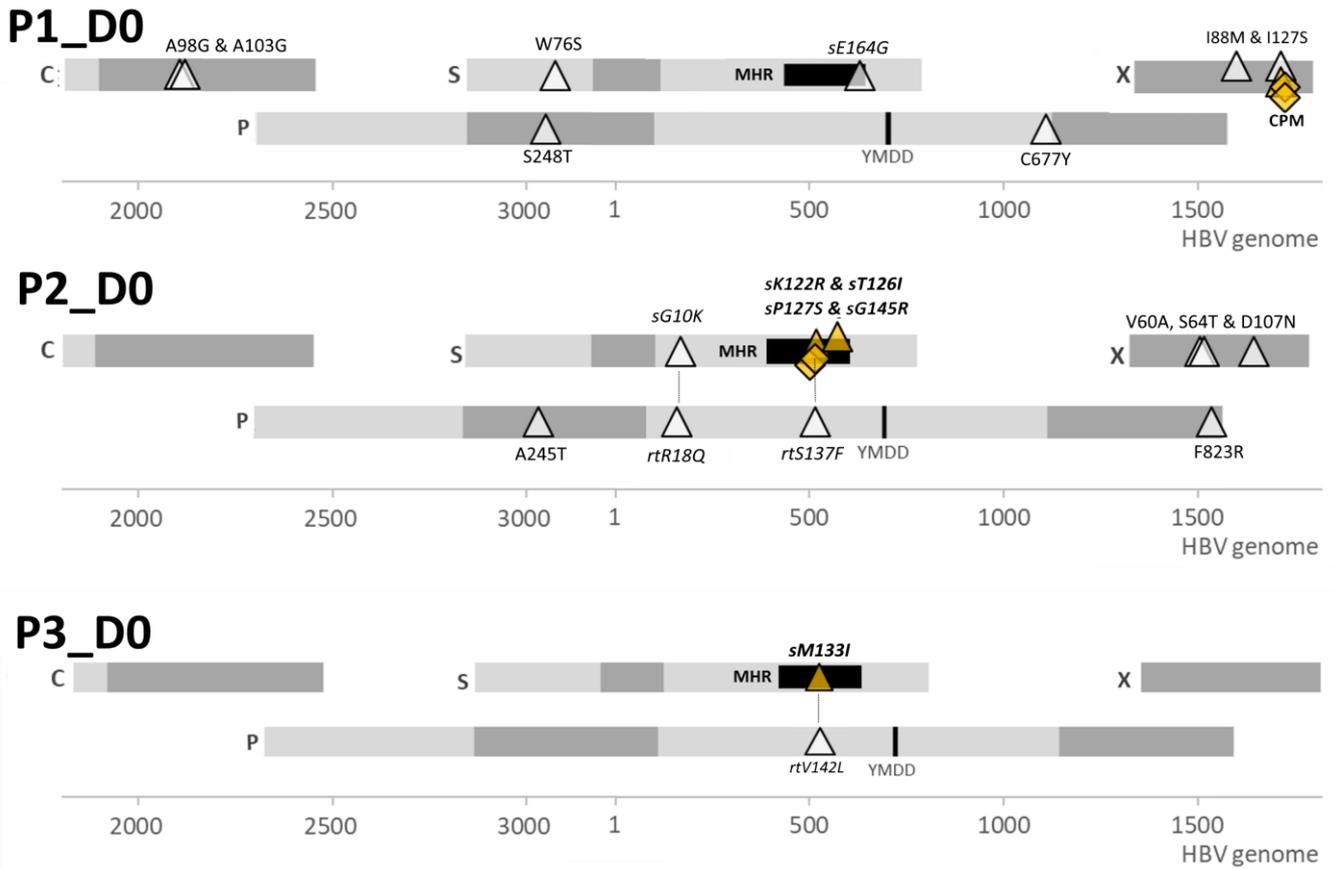
204 Table 1: Clinical and virological profiles of patients before entecavir treatment

Patient	Sex	Age (years)	Immunosuppression		HBV genotype	Serotype	Metavir score	ALAT (IU/L)	HBV DNA ^a (log IU/mL)	HBs Ag ^b	HBs Ab ^b	HBe Ag ^b
			Context	Delay before HBV infection (years)								
#1	M	41	Liver transplant	1	E	<i>ayw4</i>	A2F2	400	7.4	3760	0	924
#2	F	75	Kidney transplant	6	I ^c	<i>ayw^c</i>	A0F2	26	6.6	2051	5	2094
#3	M	73	Chronic lymphocytic leukemia	17	D	<i>ayw3</i>	F2	57	8.3	320	0	1622

205 ^a HBV real-time assay (Abbott); ^b Architect HBs Ag qualitative, HBs Ab and HBe Ag assays (Abbott); ^c This rare genotype originates from
206 a recombination between genotypes A, C and G, and circulates in Asian countries, including Laos, the patient's home country (Hannoun et
207 al., 2000; Huy et al., 2008). The subtype of this isolate remained undetermined, because aa s127S was not included in the current
208 classification (Kay and Zoulim, 2007; Purdy et al., 2007).

209 **Mutations detected before entecavir treatment**

210 Clinically relevant mutations (HBs immune escape or core promoter mutations, CPM) were detected
211 in the consensus sequence of the three HBV isolates collected before entecavir treatment (Figure 2).
212 Isolate P1_D0 harbored core promoter mutations (CPM) T1753G, A1762T and G1764A (Figure 2).
213 The last two of these mutations are known to be associated with poor HBe Ag secretion and an
214 increased HBV replication capacity, with no impact on susceptibility to entecavir (Leng et al., 2015;
215 Parekh et al., 2003). Interestingly, this unique combination of three CPMs has been described once
216 before, in a patient experiencing HBV reactivation following liver transplantation (Kim et al., 2003).
217 Mutation T1753G has not been previously characterized, but mutation T1753C increased the level of
218 replication associated with mutations A1762T and G1764A (Parekh et al., 2003).
219 Isolates P2_D0 and P3_D0 harbored HBs Ag immune escape mutations, which are frequently
220 detected in this context of HBV reactivation (Salpini et al., 2015). Isolate P2_D0 harbored a unique
221 set of mutations in the “a” determinant of the HBs Ag: sK122R, sT126I, sP127S and sG145R. The
222 sK122R, sT126I and sG145R mutations have been associated with immune escape and the restoration
223 of viral fitness in lamivudine-resistant isolates (Amini-Bavil-Olyaei et al., 2010; Bock et al., 2002).
224 The sP127S mutation has been observed only once before, in an isolate from Laos (Olinger et al.,
225 n.d.) but another mutation at this position (sP127L) has been described as an immune escape mutation
226 (Colagrossi et al., 2018). This suggests that the sP127S mutation may also be an immune escape
227 mutation. Isolate P3_D0 harbored the sM133I mutation of the “a” determinant of the HBs Ag MHR
228 region. This mutation has been associated with HBV reactivation (Pei et al., 2014), mother-to-child
229 HBV transmission despite vaccination (Latthaphasavang et al., 2019) and major antigenic changes in
230 the HBs Ag (Ie et al., 2010).



231

232 **Figure 2: Mutations detected before entecavir treatment**

233 Mutations of the consensus sequence detected in HBV isolates collected before entecavir treatment.

234 Clinically relevant mutations (HBs Ag escape mutations and core promoter mutations CPM

235 T1753G, A1762T and G1764A) are represented with orange symbols and labeled in bold typeface,

236 whereas mutations of unknown significance are shown in white. Mutations rarely (<3%) found in

237 other isolates of the same genotype are represented with a triangle, whereas more frequent

238 mutations are represented with a diamond. Mutations associated with a change in two ORFs are

239 indicated with a dashed line between the two ORFs. Shades of gray correspond to the pre-C, Core,

240 pre-S1, pre-S2, S and X genes (upper row) and the terminal protein, spacer, polymerase/reverse

241 transcriptase and RNase domains of the polymerase gene (lower row). MHR, major hydrophilic

242 region. HBV strain X02763, with the usual numbering, was used as the reference sequence for

243 annotation.

244 **Mutations selected during entecavir treatment**

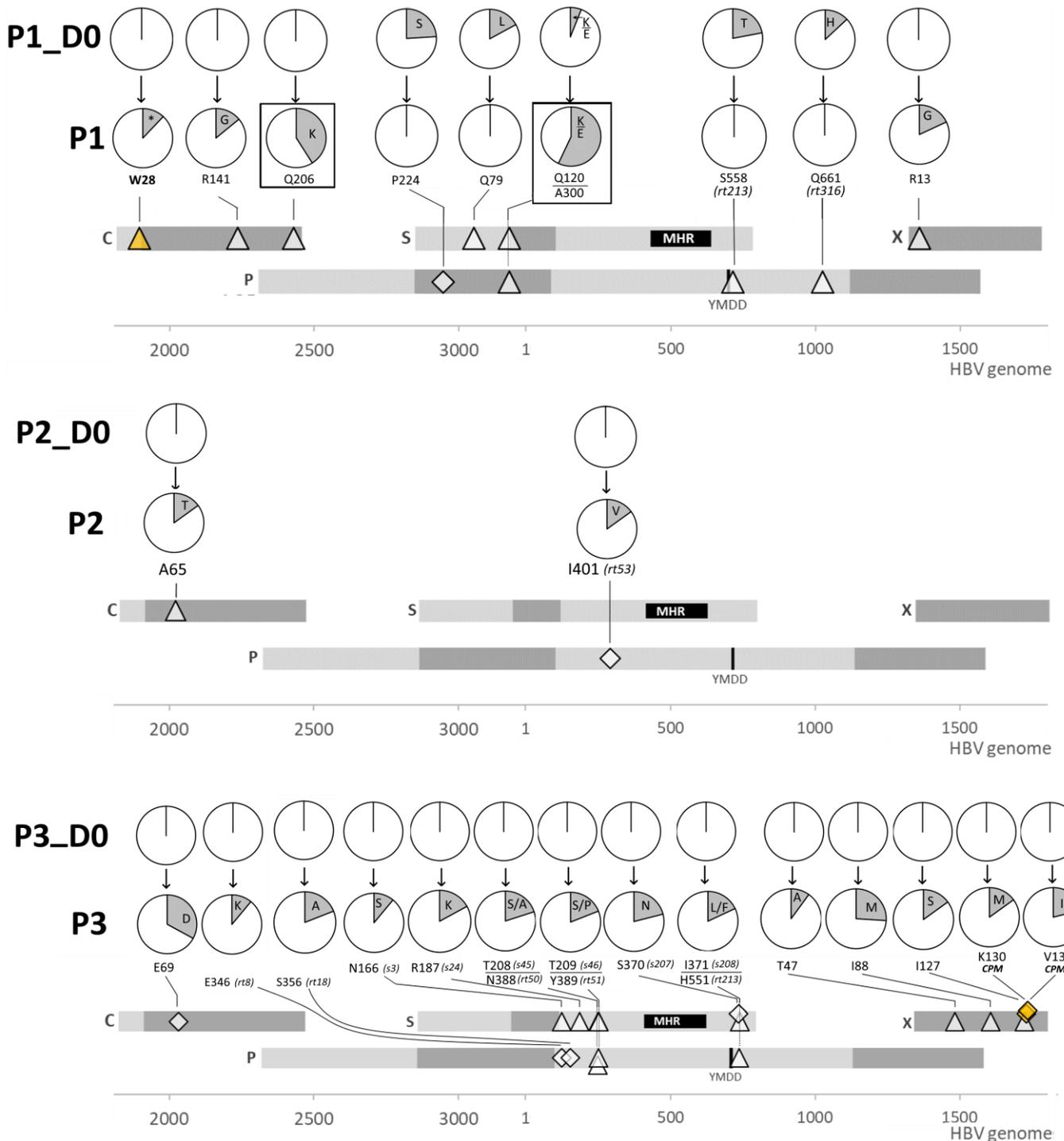
245 We analyzed the evolution of HBV quasispecies by studying the whole HBV genome by NGS
246 and comparing isolates collected before (P1_D0, P2_D0 and P3_D0) and after (P1, P2 and P3)
247 entecavir treatment failure. Twenty-five non-synonymous mutations had been selected in
248 isolates P1 ($n=9$), P2 ($n=2$) and P3 ($n=14$) (Figure 3). Two of these mutations, both in isolate
249 P1, were considered to be major variants. Their proportion in the HBV quasispecies increased
250 from 0-6 % before treatment to 41-57 % during treatment (Figure 3). The first mutation resulted
251 in simultaneous changes in the spacer domain of the polymerase (A300E) and in the pre-S1
252 protein (Q120K) (Figure 3). The latter mutation was localized at the N-terminus of pre-S2
253 (Q2K) in a T-cell epitope (Barnaba et al., 1989; Pajot et al., 2006). This mutation has been
254 observed in 1.2% of HBV/E isolates (Abdou Chekaraou et al., 2010; Candotti et al., 2016;
255 Mulders et al., 2004). The second mutation, in the pre-core (Q206K) and core (Q177K)
256 proteins, was located at the C-terminal end of the core protein (Figure 3), in a highly conserved
257 protamine-like DNA-binding domain crucial for HBV pgRNA encapsidation and DNA
258 replication (Jung et al., 2012). Interestingly, this is the first description of a mutation at this
259 position. These two mutations were not known to be associated with resistance to entecavir.

260 Phenotypic assays were performed on recombinant viruses harboring one or both of these
261 mutations, to determine whether they had any impact on the level of resistance to entecavir.

262 The other 23 mutations were selected during entecavir treatment, but only as minor variants
263 (frequency <40% at treatment failure), suggesting that they were probably not required for a
264 PVR to entecavir. Eight of these mutations were located in the sequence corresponding to the
265 Pol/RT domain, all as minor variants (<25%). None was detected with Sanger sequencing,
266 coherent with their distribution as minor variants. Three mutations were of clinical interest. The

267 first was pre-core mutation W28*, selected in patient #1. The other two were basal core
268 promoter mutations A1762T and G1764A, selected in patient #3 (Figure 3). These pre-
269 core/basal core promoter mutations are known to be associated with increases in viral
270 replication and decreases in HBe Ag expression (Parekh et al., 2003). However, as minor
271 variants, they were considered unlikely to account for entecavir treatment failure and were not
272 further characterized.

273



274

275 **Figure 3: HBV mutations selected or repressed during entecavir treatment**

276 Mutations selected or repressed during entecavir treatment are indicated with pie charts
 277 representing their proportion in the HBV quasispecies, together with their position in the HBV
 278 genome. Clinically relevant mutations are represented with orange symbols and in bold
 279 typeface: core promoter mutations (CPM) A1762T and G1764A and pre-core mutation W28*.
 280 Mutations rarely (<3%) found in other isolates of the same genotype are represented with a
 281 triangle, whereas more frequent mutations are represented with a diamond. Mutations

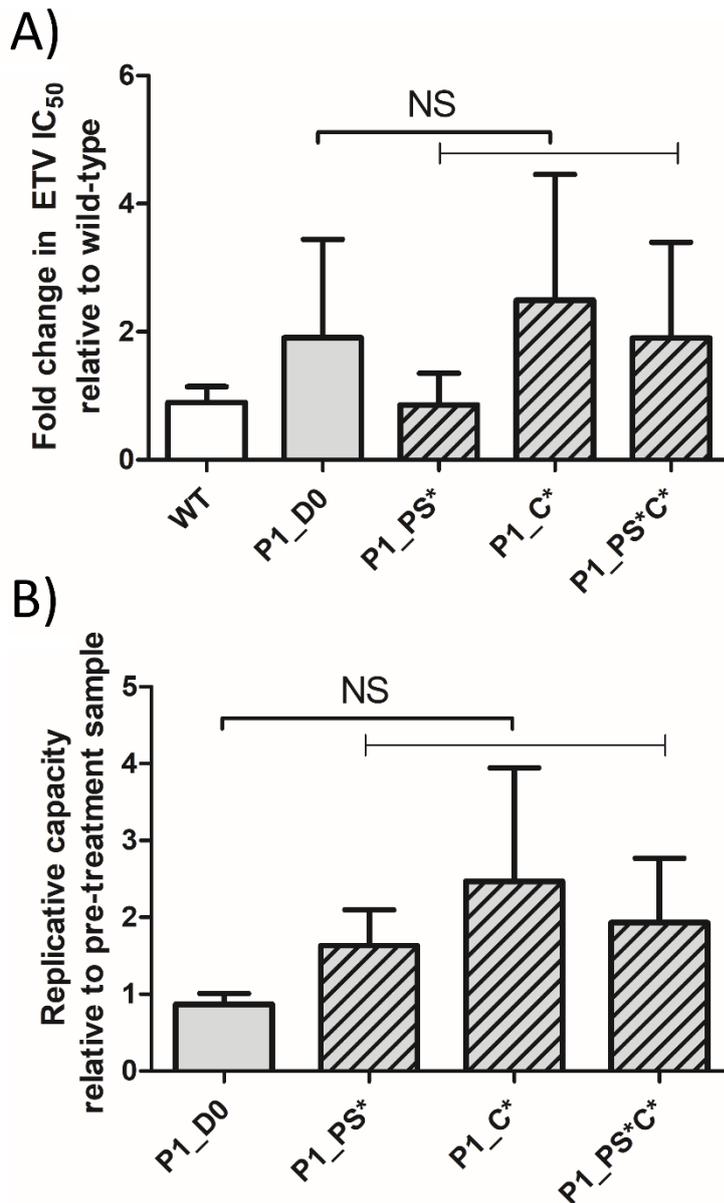
282 associated with a change in two ORFs are indicated with a dashed line between the two ORFs.
283 Shades of gray correspond to the pre-C, Core, pre-S1, pre-S2, S and X genes (upper row) and
284 the terminal protein, spacer, polymerase/reverse transcriptase and RNase domains of the
285 polymerase gene (lower row). MHR, major hydrophilic region. HBV strain X02763, with the
286 usual numbering, was used as the reference sequence for annotation.

287

288 **Phenotypic characterization of mutations selected during entecavir treatment**

289 Recombinant viruses harboring the whole genome of isolate P1_D0, with or without the two
290 relevant mutations selected during entecavir treatment in patient #1, were obtained by *in vitro*
291 synthesis and site-directed mutagenesis, followed by transfection of the Huh7 cell line. The
292 level of resistance to entecavir and the replication capacity of these recombinant viruses are
293 shown in Figure 4. Variants harboring one or both of these mutations were as susceptible to
294 entecavir and had a similar replicative capacity to the pretreatment isolate P1_D0 (Figure 4).
295 This suggests that these mutations, alone or in combination, played no direct role in entecavir
296 treatment failure.

297



298

299 **Figure 4: Phenotypic resistance testing of HBV isolates selected during entecavir**
 300 **treatment in patient #1.**

301 Major variant before treatment (P1_D0, plain bar) and variants detected at treatment failure
 302 (hatched bars) harboring the A300E mutation in the polymerase and the Q120K mutation in
 303 the pre-S1/S2 proteins (P1_PS*), the Q206K mutation in the Core protein (P1_C*) or both
 304 (P1_PS*C*). NS, non-significant ($p > 0.05$). Six drug concentrations, ranging from 2.5 to 50
 305 000 nM, were tested in triplicate, in three independent experiments.

306

307 **DISCUSSION**

308 In a series of 110 patients tested for HBV drug resistance, we identified three patients
309 experiencing entecavir treatment failure despite having good treatment compliance and no
310 detectable Pol/RT resistance mutations. These three patients experienced a PVR to entecavir in
311 a context of immunosuppression.

312 Previous studies demonstrated that PVR to entecavir does not require selection of resistance
313 mutations in the Pol/RT domain but is associated with HBe Ag positivity, low levels of ALAT
314 and a high HBV DNA viral load at baseline (Chen et al., 2014; Lee et al., 2015; Li et al., 2015;
315 Zoutendijk et al., 2011). Despite these findings, most cases of PVR to entecavir remain
316 unexplained. We hypothesized that mutations selected outside of the Pol/RT domain could
317 contribute to PVR to entecavir. Mutations Q206K (pre-core/core), Q120K (pre-S1/pre-S2, in a
318 T-cell epitope) and A300E (spacer domain) were selected during entecavir treatment in patient
319 #1 but were not associated with an increased level of resistance to entecavir or an increase in
320 HBV replication capacity. No mutations were selected in the whole HBV genome of patients
321 #2 and #3. This study provided a unique insight into the evolution of HBV quasispecies during
322 entecavir treatment, addressing the whole HBV genome and not only the Pol/RT domain. We
323 demonstrated that PVR to entecavir does not require selection of any resistance mutation in the
324 whole HBV genome. Our results demonstrate that mutations can be selected outside of the
325 Pol/RT domain during entecavir treatment. These mutations could contribute to entecavir
326 treatment failure by other mechanisms than an increased level of resistance.

327

328 Mutations of unknown significance can be selected in the whole HBV genome during entecavir
329 treatment. In this study, mutation Q120K in the pre-S1/pre-S2 protein was selected during
330 entecavir treatment in patient #1. This mutation was localized in a T-cell epitope (pre-S2 1-15)

331 (Barnaba et al., 1989; Pajot et al., 2006) and in a domain required for viral secretion (pre-S2 1-
332 5) (Le Seyec et al., 1998). Mutations and deletions at this position tend to be selected in
333 asymptomatic carriers and are associated with reduced secretion of HBs Ag *in vitro* and *in vivo*
334 (Fan et al., 2001; Taghiabadi et al., 2019; Wang et al., 2018). This mutation could have been
335 associated with viral escape from cellular immune responses and could have contributed to the
336 partial virological response observed in patient #1. In contrast, in patients #2 and #3, no
337 mutations were selected in the whole HBV genome during treatment, suggesting that PVR to
338 entecavir did not rely on viral escape or resistance. HBs Ag immune escape mutations were
339 selected before treatment in these two patients, as previously observed in the context of HBV
340 reactivation (Corti et al., 2018; Rehermann and Nascimbeni, 2005).

341 It is unclear whether treatment with immunosuppressive drugs, such as rituximab, is associated
342 with PVR to entecavir. These drugs have been associated with an increased risk of HBV
343 reactivation (Loomba and Liang, 2017) and delayed response to entecavir treatment in a
344 minority of patients (Brost et al., 2010; Yap et al., 2014). In addition, low ALAT levels at
345 baseline have been associated with an increase in the risk of PVR to entecavir. It has been
346 hypothesized by others that these low ALAT levels were the result of an impaired host immune
347 response, which could increase the risk of PVR to entecavir (Lee et al., 2015; Li et al., 2015).
348 Our findings support this hypothesis and suggest that mutations selected outside of the Pol/RT
349 domain could contribute to entecavir treatment failure, not by an increased level of resistance,
350 but by an immune escape mechanism, in synergy with immunosuppressive drugs.

351 We developed an original approach to study entecavir treatment failure, based on whole HBV
352 genome analysis, combining deep sequencing and a phenotypic resistance assay. This made it
353 possible to perform a comprehensive evaluation of mutations selected before or during
354 entecavir treatment, potentially associated with treatment failure. The main limitation of this

355 study is the small sample size, which made it impossible to draw reliable conclusions on the
356 association between immune escape mutations and PVR to entecavir.

357 This study argues for a systematic approach to entecavir treatment failure. In addition to Pol/RT
358 sequencing, therapeutic drug monitoring seems to be useful for detecting poor compliance with
359 treatment or underdosing in some patients. Screening for HBs immune escape mutations may
360 be relevant in immunocompromised patients experiencing HBV reactivation, to confirm the
361 association between these mutations and the PVR to entecavir and to guide treatment adaptation
362 more effectively. By contrast, deep sequencing does not seem more relevant than Sanger
363 sequencing for detecting resistance mutations at treatment failure, as they can be detected as
364 major variants in this context (Lee et al., 2018; Lowe et al., 2016). However, in the near future,
365 HBV treatment strategies will target other steps of the viral cycle and/or aim to modulate the
366 host immune response (Cornberg et al., 2020; Fanning et al., 2019). In this context, deep
367 sequencing and phenotypic characterization of whole HBV genomes from sequential clinical
368 isolates, as performed in this study, will probably be essential, for the identification of viral
369 factors associated with the response to these new antiviral treatments.

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