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Running title: *Precore* G1896A mutations in SSA

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ABSTRACT

The nucleotide substitution G1896A on the *precore* (pc) region has been implicated in virological and serological responses during treatment in hepatitis B virus (HBV)-infected patients. Whether this mutation affects the therapeutic course of HIV-HBV co-infected patients, especially from Western Africa, is unknown. In this prospective cohort study, 86 antiretroviral (ARV)-naïve HIV-HBV co-infected patients from Côte d'Ivoire, initiating ARV-treatment containing lamivudine (*n*=53) or tenofovir (*n*=33), had available baseline pc sequences. Association of the pcG1896A mutation with time-to-undetectable HBV-DNA, hepatitis B "e" antigen (HBeAg)-seroclearance (in HBeAg-positive patients), and hepatitis B surface antigen (HBsAg)-seroclearance was evaluated using Cox proportional hazards regression. At ARV-initiation, median HBV-DNA was 6.04 $\log_{10}$ copies/mL (IQR=3.70-7.93) with 97.7% harboring HBV genotype E. Baseline pcG1896A mutation was identified in 51 (59.3%) patients, who were more commonly HBeAg-negative (*p*<0.001) and had basal core promotor A1762T/ G1764A mutations (*p*<0.001). Patients were followed for a median 36 months (IQR=24-36). Cumulative proportion of undetectable HBV-DNA was significantly higher in patients with baseline mutation (pcG1896A=86.6% versus no pcG1896A=66.9%, *p*=0.04), but not after adjusting for baseline HBV-DNA levels and anti-HBV agent (*p*=0.2). No difference in cumulative proportion of HBeAg-seroclearance was observed between mutation groups (pcG1896A=57.1% versus no pcG1896A=54.3%, *p*=0.7). Significantly higher cumulative proportion of HBsAg-seroclearance was observed in patients without this mutation (pcG1896A=0% versus no pcG1896A=36.9%, *p*<0.001), even after adjusting for baseline HBsAg-quantification and anti-HBV agent (*p*<0.001). In conclusion, lacking the pcG1896A mutation before ARV-initiation appeared to increase HBsAg-seroclearance rates during treatment. The therapeutic implications of this mutation need further exploration in this setting.

Keywords: antiviral treatment; basal core promotor; genetic variability; immunosuppression; precore.

INTRODUCTION

In sub-Saharan Africa (SSA), it is estimated that approximately 10% of HIV-infected patients have chronic hepatitis B virus (HBV) infection (1). HIV-HBV co-infection is known to increase the risk of severe liver disease and accelerate progression to liver-related death compared to either infection individually (2,3). Circulating HBV is a strong contributor of the increased risk in both liver-related and overall mortality (4,5), hence the importance of reducing serum HBV DNA viral loads through effective antiviral therapy (6,7).

There are, however, certain virological factors of the HBV genome that might impact response during therapy with an anti-HBV nucleoside/nucleotide analogue (NA). For example, patients harboring the *precore* (pc) G1896A mutation are less likely to achieve lower HBV DNA viral loads during treatment with lamivudine (LAM) (8). Pc mutant variants have been shown *in vitro* to increase replication yields of LAM-resistant strains, which are normally deficient in replicative capacity (9), and have been clinically associated with faster development of LAM-resistance (10). Importantly, the presence of pc mutations, even when existing as a minority quasi-species, hampers hepatitis B surface antigen (HBsAg) loss during treatment with the potent NA tenofovir (TDF) (11). The pcG1896A mutation would then appear to impose serious consequences on the therapeutic course of NA-treated patients.

Within the continent, data on the prevalence of pc mutations in both HBV mono-infected and HIV-HBV co-infected patients are certainly available (12–16). However, their implications with respect to virological and serological response during anti-HBV NA therapy remain to be elucidated. In the study herein, we used unique data from a cohort of antiretroviral treatment (ART)-naïve HIV-HBV co-infected patients from Côte d'Ivoire initiating treatment with an anti-HBV containing regimen. We first evaluated the distribution of mutations observed on the basal

core promoter (BCP) and pc regions before treatment initiation, while examining risk-factors of harboring the pcG1896A mutation. We then aimed to determine the effect of harboring this mutation on virological and serological response during LAM- or TDF-containing ART.

MATERIALS AND METHODS

Study design and visits

The VarBVA study (17) is an observational cohort including patients from two prospective, randomized, open-label, multi-center trials in Abidjan, Côte d'Ivoire: Trivacan ANRS 1269 (NCT00158405), aimed at evaluating the benefits and risks of structured treatment interruption; and Temprano ANRS 12136 (NCT00495651), aimed at evaluating the benefits and risks of starting ART earlier than current World Health Organization (WHO) recommendations. Study randomization and follow-up procedures have been detailed elsewhere (18,19).

Inclusion criteria were as follows – both studies: age ≥18 years, HIV-1 or mixed HIV-1/2 infection, and ART-naïve (with the exception of short-course treatment for the prevention of mother-to-child HIV transmission); *Trivacan*: CD4 cell count between 150-350/mm^3 or CD4 percentage between 12.5%-20.0%; *Temprano*: CD4 cell count <800/mm^3 and no concurrent criteria for ART-initiation according to most recent WHO guidelines. Non-inclusion criteria are summarized in the Supplementary methods. All participants gave written informed consent and approval of the study protocols were obtained by the Ministry of Health of Côte d'Ivoire and the French National Agency for Research on AIDS and Viral Hepatitis (ANRS, Paris, France).

For this study, we included patients testing HBsAg-positive at study inclusion (Mini Vidas® assay; Biomerieux, Marcy l'Etoile, France), confirmed by the HBsAg Qual II Architect assay (Abbott Laboratories, Rungis, France), and who started ART containing LAM and/or TDF at inclusion or any time during participation in the trial (Supplementary Methods). We defined the "baseline" visit at ART-initiation and "follow-up" visits at each yearly visit thereafter until the date of last follow-up (with available frozen sample), study termination, permanent treatment discontinuation, or treatment switch. In the Temprano study, if the last follow-up visit was not on the yearly interval, the closest 6-month visit was used instead.

HBV-related parameters

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were quantified at baseline. From frozen samples stored at -80°C, HBV-DNA viral loads were quantified at baseline and every follow-up visit using an in-house PCR-based assay (QuantiFast SYBR® Green PCR kit, Qiagen, Courtaboeuf, France; Light Cycler 480, Roche, Boulogne-Billancourt, France) with a detection limit of 12 copies/mL (17). Qualitative HBsAg was detected using the HBsAg Qual II test (detection limit=0.03 IU/mL, Architect, Abbott Laboratories, Rungis, France) at baseline and during follow-up. Qualitative HBeAg and anti-HBe antibodies (anti-HBeAb) were detected using the Elecsys assay (Roche Diagnostics, Meylan, France) for the same visits. *HBeAg-seroclearance* was defined as HBeAg-loss from the previous visit in HBeAg-positive patients. *HBsAg-seroclearance* was defined as loss of HBsAg from the previous visit for all patients.

HBeAg quantification (qHBeAg) and HBsAg quantification (qHBsAg) were quantified for HBeAg and HBsAg positive samples, respectively, using the Elecsys assay with the Modular E170 analyzer (Roche Diagnostics, Meylan, France). qHBeAg levels were provided as a semi-

quantitative result and were interpreted as the ratio of sample relative light units to a cut-off value. These units were converted to Paul Ehrlich Institute units (PEI U/mL) from a previously established protocol (20).

Detection of HBV mutations on viral genome sequences

Genotypic analysis was performed at baseline for all patients with an HBV DNA viral load >1000 copies/mL (17). Nucleotide (nt) sequences from the BCP and pc regions were examined by direct sequencing after nested-PCR amplification of the *preC/C* gene (nt 1743-2362). Individual sequences were aligned using the ClustalW full multiple alignment program with 1000 bootstraps in BioEdit (v7.0.5.3, Carlsbad, CA) and were compared to a consensus sequence from 454 genotype E and 500 genotype A *preC/C* nucleotide sequences retrieved from the HBVdb (21).

Amino acid sequences of the *pol* and *S*-genes were also examined after PCR amplification of the reverse transcriptase (rt) and surface antigen (s) encoding regions (between rt107-rt385 and s99-s226, respectively). Mutant variants were detected using similar methods as described above (17).

Phylogenetic analysis

HBV genotypes were determined via phylogenetic analysis conducted on aligned sequences of the pc region (nt 1814-2452), which were compared to several referent sequences of HBV genotypes A-H (GenBank accession numbers listed in Supplementary Table 1). The neighbor-joining method was employed with 1000 bootstrap replications using a Kimura-2 parameter substitution model that included transitions and transversions (d). A discrete gamma model with

mean equal to one was also used to incorporate rate variation among sites. The resulting phylogenetic tree was constructed using MEGA6 software (22) and is provided in Supplementary Figure 1.

Statistical analysis

The distribution of specific mutations of the *preC/C* gene (13) at baseline was described. We chose to focus further analysis on the pcG1896A mutation due to its strong association with virological and serological responses (23). Determinants of harboring this mutation at inclusion were examined using logistic regression. Demographic, treatment, and clinical characteristics related to HIV and HBV-infection with a $p \leq 0.1$ in univariable analysis were retained and used to create a predictive, multivariable model. A backwards-stepwise selection process was then performed, removing any co-variable greater than this *p*-value threshold.

We then assessed the effect of harboring the pcG1896A mutation at inclusion on virological and serological parameters during treatment. The cumulative probability of time until undetectable HBV, time until HBeAg-seroclearance (for patients with positive HBeAg at baseline), and time until HBsAg-seroclearance was calculated using Kaplain-Meier curves. Survivor functions were stratified on mutation groups and compared using the log-rank test. Cox proportional hazards regression was also used to compare rates of these events, both unadjusted and adjusted for baseline quantified parameter (HBV DNA, qHBeAg or qHBsAg) and anti-HBV treatment (LAM versus TDF).

Additionally, qHBsAg levels were summarized for each study visit. Changes in antigen levels from baseline were modeled using a mixed-effect linear regression with a random-intercept to account for between-patient variability. The model included time and mutation group as

independent variables along with the interaction between the two, which was tested in order to determine differences in overall on-treatment antigen decline between mutation groups.

All analyses were performed using STATA (v12.1, College Station, TX, USA). All statistical tests were two-sided and a *p*-value of <0.05 was considered significant.

RESULTS

Description of the study population

Among the 259 HBsAg-positive patients enrolled in both trials, 173 were not included in analysis for the following reasons: did not have confirmed HBsAg-positive serology (*n*=31) or had suspected acute HBV-infection (*n*=1), never initiated ART (*n*=30), had only one follow-up visit (*n*=16), discontinued ART <6 months due to pregnancy (*n*=3) or adverse event (*n*=2), had missing data on HBV viral loads (*n*=8) or missing data on BCP or pc genetic variability (*n*=82). In total, 86 patients were included in analysis. Demographic, HIV-related and HBV-related characteristics at baseline are summarized in Table 1. These characteristics are compared between source studies in Supplementary Table 2, demonstrating a significantly lower CD4+ T-cell count (*p*<0.001) and higher proportion with WHO clinical stage III/IV (*p*=0.007) among participants enrolled in the Trivacan versus Temprano study.

Basal core promoter/*precore* mutant variants at baseline

Table 2 describes specific BCP and pc mutations identified at baseline. The most common mutations were A1850T and C1858T nt substitutions on the pc region. Roughly two-thirds of

patients (59.3%) harbored the G1896A or mixed G1896G/A pc mutation and almost one-quarter of patients harbored the A1762T (21.2%) or G1764A (24.7%) BCP mutations. Almost all patients with a BCP mutation at these nt positions also had the pcG1896A mutation (*n*=19/21, 90.5%).

As shown in Table 3, the pcG1896A mutation at baseline was associated with HBeAg-negative status ($p<0.001$), anti-HBe antibody positive status ($p<0.001$), lower HBV DNA levels ($p=0.001$), and presence of BCP mutations ($p=0.004$). Of note, median ALT/AST levels were not significantly different in patients with versus without the pcG1896A mutation (32/39 IU/mL versus 25/40 IU/mL, respectively, $p=0.10/0.6$). Only two patients harbored a genotype other than E (genotype A) in whom no pcG1896A mutation was detected. In multivariable analysis, HBeAg-negative status ($p<0.001$) and presence of BCP mutations ($p<0.001$) were significantly and independently associated with baseline presence of pcG1896A mutations (Table 3), while considering the strong collinearity between associated variables.

Baseline pcG1896A mutation is not associated with virological response and antiviral resistance during treatment

Patients were followed for a median 36 (IQR=24-36) months. Of the 53 patients undergoing LAM-containing ART, 24 (45.3%) had per protocol treatment interruptions after a median 12 (IQR=11-37) months of follow-up. Median cumulative duration of treatment was 26 (IQR=19-34) months for all those undergoing LAM. All 33 TDF-treated patients underwent continuous ART for a median 24 (IQR=17-30) months.

Individual and median HBV DNA viral loads during ART containing an anti-HBV agent are provided in Figure 1A, while stratified on baseline pcG1896A status. During follow-up,

undetectable HBV DNA was achieved in 66 patients after a median 12 (IQR=11-23) months of follow-up (cumulative proportion=78.9%). Time to undetectable HBV DNA was significantly faster for patients with versus without baseline pcG1896A (cumulative proportion= 86.6% versus 66.9%, respectively, log rank test *p*=0.04) (Figure 1B). However, this association was no longer significant after adjusting for baseline HBV DNA levels and anti-HBV agent (*p*=0.2). At the end of follow-up, 31 (36.1%) patients had detectable HBV DNA, with no significant difference between mutation groups (with pcG1896A=31.4% versus without pcG1896A=42.9%, *p*=0.3). Thirteen (41.9%) of these patients had an HBV DNA viral load >10,000 copies/mL, only one of whom developed the LAM-resistance rtV173L+rtL180M+rtM204V mutation during treatment (without the pcG1896A mutation at baseline).

Lack of baseline pcG1896A mutation is associated with HBsAg, but not HBeAg, quantification and serological response during treatment

In the 35 HBeAg-positive patients at baseline, median qHBeAg levels decreased from 160.4 PEI U/mL (IQR=24.9-355.4) at treatment initiation to <0.05 PEI U/mL (IQR=<0.05-115.7) at the end of follow-up. At baseline, qHBeAg levels were lower in patients with versus without the pcG1896A mutation (24.9 PEI U/mL, *n*=7 versus 164.6 PEI U/mL, *n*=27, respectively), yet this association was not significant (*p*=0.13). No significant differences between mutation groups were observed in change of qHBeAg levels from baseline (*p*=0.9), owing to the low antigen levels overall during anti-HBV treatment (Figure 2A). HBeAg-seroclearance occurred in 18 patients after a median 12 months (IQR=11-23) of follow-up (cumulative proportion=55.3%), with no significant difference in time to HBeAg-seroclearance between patients with versus without the pcG1896A mutation (cumulative proportion=57.1% versus 54.3%, respectively, *p*=0.7) (Figure 2B).

Overall, median qHBsAg levels decreased from 4.00 $\log_{10}$ IU/mL (IQR=3.36-4.36) at treatment initiation to 3.79 $\log_{10}$ IU/mL (IQR=3.10-4.15) at the end of follow-up. qHBsAg levels at baseline were significantly lower in patients with versus without the pcG1896A mutation (3.81 versus 4.24 $\log_{10}$ IU/mL, respectively, p=0.02). Over time, change in qHBsAg levels from baseline was significantly faster in patients without the pcG1896A mutation (p<0.001) with substantial between-patient variability (Figure 2C). HBsAg-seroclearance occurred in 12 patients after a median 12 months (IQR=12-21) of follow-up (cumulative proportion=14.5%). Baseline and 12-month characteristics of patients are compared between those with versus without HBsAg-seroclearance in Table 4. Ten and two of these patients were HBeAg-positive and HBeAg-negative, respectively, with HBeAg-positive patients more likely to lose HBsAg (log-rank test p=0.001). As shown in Figure 2D, only patients without the pcG1896A mutation exhibited HBsAg-seroclearance (p<0.001). This significant association held when adjusting for baseline qHBsAg levels and anti-HBV treatment regimen (p<0.001).

Low incidence of pcG1896A mutation during treatment

Of the 35 patients without the pcG1896A mutation at baseline, two patients developed incident pcG1896A mutations during treatment (IR=2.5/100 person-years). The first patient had high HBV DNA levels at treatment initiation (7.57 $\log_{10}$ copies/mL). The patient was undergoing LAM-containing ART with CD4+ T-cell guided therapeutic interruptions. The G1896G/A mutation was detected at the month-24 visit with an HBV DNA viral load at 228,000 copies/mL, which gradually declined to 9260 copies/mL at the last study visit. The patient remained HBeAg-positive and HBsAg-positive during follow-up. The second patient also had high HBV DNA at treatment initiation (7.93 $\log_{10}$ copies/mL). After initiating continuous LAM-containing ART, HBV DNA viral loads steadily decreased to 1074 copies/mL at month-24, when the G1896G/A mutation was detected, and finally to 810 copies/mL at the last study visit. The patient lost both

HBeAg and HBsAg during follow-up, while no apparent mutations on the “a” determinant of the *S*-gene emerged. No LAM-resistance mutations were observed for either patient.

DISCUSSION

The pcG1896A mutation is frequently observed around the time of HBeAg-seroconversion, allowing abrogated HBeAg-production with persistent HBV DNA replication and possibly immune escape from HBeAg-directed immune responses (23). Since most patients in Western Africa are HBeAg-negative and infected with HBV genotypes prone to the G1896A nt substitution (24,25), it is assumed that a substantial proportion with detectable HBV DNA replication would be infected with this mutation.

At treatment initiation, we did indeed identify pcG1896A mutant variants in almost two-thirds of co-infected patients harboring genotype E, reflecting other studies in this region (26–28). The presence of this mutation was associated with widely-recognized determinants of more inactive forms of HBV infection, such as low HBV DNA replication and HBeAg-negative status. Since the G to A substitution at position pc1896 replaces tryptophan for a stop codon during translation of the HBeAg and thereby limits its production (23), it is unsurprising that this mutation was not frequently observed in HBeAg-positive individuals. BCP mutations at either nt 1762 or 1764 were almost always present with the pcG1896A mutation in our study and are also associated with reduced HBeAg production and HBeAg-negative status (29). Meanwhile, CD4+ T-cell concentrations failed to show an association with baseline pc mutation and would suggest that HIV-induced immunosuppression might not play an essential role on the genetic variability of the pc region (30).

We further our understanding on the therapeutic role of the pcG1896A mutation in genotype E infection, while clearly demonstrating that patients with this mutation at treatment initiation will likely have difficulty in clearing HBsAg during NA-based therapy. Reduced HBsAg-seroclearance rates have been described with this mutation, as well as with increased genetic diversity of the pc encoding region, during TDF-treatment in HBV mono-infected patients (11). The importance of this mutation was further evidenced when deep-sequencing was applied, in which pcG1896A mutant strains detected as even minority variants (>1% of the HBV quasispecies pool) were able to produce greater sensitivity in predicting HBsAg-seroclearance (11). Interestingly, these results originated from a study population with HBV genotypes A-D, high transaminase levels, and male and HBeAg-positive predominance. The fact that our study comprised mostly HBeAg-negative women with genotype E would suggest the robustness of this association across different HBV-infected populations.

The pathophysiological explanations of this result remain largely unknown. HBsAg-seroclearance is mainly viewed as the consequence of complex and appropriate immune responses during the course of infection (31). NA-based therapy does not have substantial immunomodulatory effects compared to other treatment options, such as pegylated-interferon (32). Regardless, higher rates of HBsAg-seroclearance are often observed during HBeAg-positive phases of HBV when patients are undergoing treatment with any antiviral agent (33). When replacing HBeAg-positive status with the lack of pcG1896A mutation, we were able to obtain a marginally higher sensitivity associated with loss of HBsAg in our study. Two HBeAg-negative individuals had HBsAg-seroclearance, both of whom did not harbor the pcG1896A mutation. From our data, it is difficult to determine if these individuals recently had HBeAg-seroclearance prior to study inclusion or if the pcG1896A was present as a minority quasi-species. As increased pc genetic diversity during HBeAg-seroconversion spans over several years (34), perhaps the presence of pc mutations might be a more accurate reflection of later

stages of HBV-infection when HBsAg-seroclearance is most difficult to clear. Whether this means that earlier treatment prior to pc mutant emergence should be considered remains to be determined.

Patients harboring the pcG1896A mutation at baseline did not have increased rates of HBeAg-seroclearance, which was somewhat unexpected (8). One reason could be the few patients with HBeAg-positive serology harboring this mutation at baseline, thereby reducing the power to establish any difference. On the other hand, qHBeAg production did seem to persist for some patients with the pc mutation. It could be speculated that the quasi-species make-up of pcG1896A mutant variants remained at consistent levels during follow-up. Substantial evolutionary changes on the pc encoding region over time are needed in order for HBeAg-seroconversion to occur, at least during the natural history of infection (34), which might not have been the case in our study. This might also explain why there was no significant association with this mutation and qHBeAg at treatment initiation and during treatment, despite the well-established impact that the pcG1896A mutation has on HBeAg-production in clinical settings (35,36). This hypothesis could be further clarified by examining the specific viral subpopulations during treatment using next-generation sequencing technologies.

We did not observe any association with the pcG1896A mutation at baseline and virological response or LAM-resistance, which is in contrast to other studies evaluating the use of NA-based agents in HBV mono-infected patients (8,10). Several noteworthy features of our cohort could explain this finding. Persistent viremia observed in these patients was mostly due to insufficient follow-up, immunocompromised status, or for some, LAM-interruption (17). TDF is also highly effective in suppressing HBV DNA among co-infected patients, even when pcG1896A mutations are present (37). These factors would have eclipsed any purported role of pcG1896A mutations on HBV suppression. In addition, HBV DNA viral loads and transaminase

levels were for the most part low at treatment initiation. These conditions might have been ideal to abate the emergence of LAM-resistance (38) and have appeared to be associated with low LAM-resistance rates in other treated, co-infected populations from SSA (39). Considering that only one patient in this study had LAM-resistance, there was an insufficient number of events to appropriately address this question.

As previously shown in treated co-infected patients (40), the pcG1896A mutation emerged infrequently during follow-up. It is debatable, however, whether this pc mutation was truly incident. Appearance of the G1896A nt substitution did not occur in concert with ALT increases, which is a common event in HBeAg-seroconversion when genetic alterations on the pc region are readily observed (34,41). Furthermore, mixed wildtype and pcG1896A was observed in the two patients identified with incident mutation. These patients potentially had minority variants at treatment initiation and displayed fluctuations in mutant populations detected with population sequencing during therapy. This is an important consideration given that one of the patients with incident pcG1896G/A mutation lost HBsAg. Had this mutant variant been detected by deep-sequencing, it would have reduced the sensitivity in predicting HBsAg-seroclearance. Nevertheless, this patient had a unique progression of HBV infection with continuing HBV DNA replication despite negative HBsAg serology.

Other limitations of our study need to be addressed. First, we excluded patients with severely elevated transaminases or clinical signs of severe liver disease at study inclusion. This criterion probably resulted in the lack of liver-related clinical events observed during follow-up and thus the relationship between BCP or pc mutations and liver-related morbidity was unable to be assessed. Conversely, individuals with occult HBV infection, which is rather prevalent in SSA (16), were not included in our study. Although the low levels of HBV DNA often observed in these patients make it difficult to conduct sequencing, the few reports of occult infection in SSA

do not support high prevalence of the pcA1896G mutations during this infection “phase” (42). Second, almost all patients undergoing LAM were taking part in the Trivacan study, with most interrupting treatment during follow-up. ART-interruptions could have affected time to undetectable HBV DNA (17) and some caution should be given when interpreting the association between pc mutations and virological response. Third, almost all patients with the pcG1896A mutation also harbored mutations on the BCP. Although previous research has shown differential clinical outcomes for patients with various BCP and pc mutation combinations (43), this stratification was unfeasible in our analysis. Fourth, HBsAg was tested only once during study enrollment. Chronic HBV infection (i.e. minimum 6 months of HBsAg-positive serology) was not firmly established at study inclusion and those with HBsAg-seroclearance could have had acute HBV infection. Nevertheless, the vast majority of HBV-infected patients from SSA acquire HBV horizontally during childhood or adolescence (44), hence this scenario would be considered fairly unlikely.

Another consideration worth mentioning is the higher rate of HBeAg- and HBsAg-seroclearance from what would be expected among treated HBV mono-infected patients (33). Other studies in European and Asian co-infected cohorts have observed this phenomenon, which has been linked to accelerated ART-induced immunorestauration under severe immunosuppression (45,46). Individuals with versus without HBsAg-seroclearance did have somewhat lower CD4 T-cell count at baseline, yet the lack of difference in on-treatment CD4 increases would suggest that immunocompromised status might not explain most of the seroclearance events observed in our study.

In conclusion, the lack of pcG1896A mutation at baseline, as determined by population sequencing, was strongly linked to HBsAg-seroclearance in this group of NA-treated co-infected patients. However, rates of HBeAg-seroclearance and virological response were no different

between patients with or without this pc mutation. In light of these findings, there could be a strong rationale to initiate NA-based therapy in HBV-infected patients from this setting before the pcG1896A mutation emerges. Nevertheless, future research would be needed to investigate the genetic diversity of the pc and BCP encoding regions of these strains and confirm its therapeutic and clinical utility.

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TABLES

Table 1. Description of the study population at treatment initiation

	N=86
Demographic characteristics	
Gender, male/female (% males)	27/59 (31.4)
Age, years†	36 (31-40)
BMI, kg/m²†	21.3 (19.2-23.4)
Current smoker‡ [N=85]	9 (10.6)
HIV characteristics	
WHO clinical stage III/VI‡	30 (34.9)
HIV-RNA >300 copies/mL‡ [N=85]	84 (98.8)
HIV-RNA $\log_{10}$ copies/mL†§	5.07 (4.53-5.70)
CD4+ cell count, /mm³†	281 (195-364)
CD4+ cell count†	15.0 (10.9-20.9)
CD4+ count >350 cells/mm³‡	23 (26.7)
Initial antiretroviral regimen‡	
LAM-based	53 (61.6)
TDF-based	33 (38.4)
HBV characteristics	
HBeAg-positive‡	35 (40.7)
Anti-HBe antibody positive‡	53 (61.6)
HBV DNA $\log_{10}$ copies/mL†	6.04 (3.70-7.93)
HBV genotype‡	
A	2 (2.3)
E	84 (97.7)
ALT, IU/L† [N=76]	29 (21-48)
AST, IU/L† [N=52]	40 (29-60)
ALT or AST >40 IU/L‡	36 (41.9)

†Median (IQR). ‡Number (%).

§Only among patients with detectable HIV viremia.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HBeAg, hepatitis B “e” antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; LAM, lamivudine; TDF, tenofovir; WHO, World Health Organization.

Table 2. Distribution of specific basal core promotor (BCP) and precore (PC) mutations

Mutation	*n* (%)
BCP mutation	
A1762T or mixed A/T [N=85]	18 (21.2)
G1764A or mixed A/G or A/T [N=85]	21 (24.7)
HBeAg start-codon PC mutation	
A1814C	2 (2.3)
HBeAg PC stop-codon apparition/pregenomic RNA encapsidation signal mutation	
C1817T	0 (0)
A1850T	84 (97.7)
C1857T	0 (0)
C1858T	84 (97.7)
G1896A or mixed A/G	51 (59.3)
Characteristic PC mutations	
G1862C	2 (2.3)
G1862A	1 (1.2)
Frequent PC mutations	
G1899A or mixed A/G	24 (27.9)

Abbreviations: BCP, basal core promoter; HBeAg, hepatitis B “e” antigen; PC, precore.

Table 3. Determinants of *precore* G1896A mutation at baseline

	Univariable		Multivariable[†]	
	OR (95%CI)	*p*	OR (95%CI)	*p*
Age per year	1.03 (0.97-1.09)	0.4		
Male versus female	1.00 (0.40-2.53)	0.9		
BMI >25 kg/m²	2.63 (0.51-13.46)	0.2		
WHO Stage III or IV	0.69 (0.28-1.68)	0.4		
HIV RNA per $\log_{10}$ copies/mL	1.03 (0.63-1.68)	0.9		
CD4 cell count				
>500 mm^3	0.67 (0.13-3.51)	0.6		
>350 mm^3	1.41 (0.52-3.79)	0.5		
>250 mm^3	2.12 (0.88-5.12)	0.10		
HBeAg positive	0.04 (0.01-0.13)	<0.001	0.02 (0.01-0.10)	<0.001
anti-HBe antibody positive	36.80 (10.65-127.18)	<0.001		
HBV DNA				
per $\log_{10}$ copies/mL	0.68 (0.54-0.86)	0.001		
>7.0 $\log_{10}$ copies/mL	0.22 (0.09-0.55)	0.001		
qHBsAg per $\log_{10}$ IU/mL	1.00 (0.69-1.46)	0.9		
AST per ULN	1.39 (0.83-2.32)	0.2		
ALT per ULN	1.23 (0.81-1.85)	0.3		
AST/ALT > 40 IU/mL	1.39 (0.58-3.35)	0.5		
BCP mutations	9.50 (2.04-44.19)	0.004	28.94 (3.43-478.91)	<0.001

[†]In the multivariable model, variables on HBV-DNA viral load and anti-HBe antibody status were not included as they were highly collinear with HBeAg-serology. Due to the limited numbers of patient groups, exact logistic regression was used to model parameter estimates.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCP, basal core promoter; BMI, body mass index; CI, confidence interval; HBeAg, hepatitis B “e” antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; OR, odds ratio; qHBsAg, hepatitis B surface antigen quantification; WHO, World Health Organization.

Table 4. Determinants of HBsAg-seroclearance

	No HBsAg-seroclearance (*n*=74)	HBsAg-seroclearance (*n*=12)	*p**
Baseline characteristics			
Gender, male/female (% males)	22/52 (29.7)	5/7 (41.7)	0.5
Age, years†	35 (31-39)	36 (30-43)	0.5
BMI, kg/m²†	21.6 (19.5-23.7)	20.3 (19.0-21.6)	0.13
WHO clinical stage‡			0.5
Stage I/II	47 (63.5)	9 (75.0)	
Stage III/IV	27 (36.5)	3 (25.0)	
HIV-RNA, $\log_{10}$ copies/mL†	5.03 (4.52-5.68)	5.31 (4.98-5.73)	0.3
CD4+ cell count, /mm³†	287 (206-369)	257 (157-287)	0.13
Anti-HBV ART component‡			0.8
LAM	45 (60.8)	8 (66.7)	
TDF/FTC	29 (39.2)	4 (33.3)	
HBV-DNA, $\log_{10}$ copies/mL†	6.04 (3.59-8.05)	6.55 (4.54-7.86)	0.7
HBV-DNA >7.0 $\log_{10}$ copies/mL‡	32 (43.2)	6 (50.0)	0.7
ALT, IU/mL† [N=76]	30 (21-47)	25 (22-51)	0.7
AST, IU/mL† [N=52]	39 (29-60)	45 (32-60)	0.7
ALT or AST >40 IU/mL‡	30 (40.5)	6 (50.0)	0.5
HBeAg-positive‡	25 (33.8)	10 (83.3)	0.003
qHBeAg, PEI U/mL†	173.1 (56.0, 349.3)	98.0 (4.6, 404.6)	0.6
qHBsAg, $\log_{10}$ IU/mL†	4.07 (3.52-4.39)	2.45 (-0.29, 4.08)	0.005
pcG1896A mutation‡	51 (69.9)	0 (0)	<0.001
During follow-up (12 months)			
ΔCD4+ cell count, /mm³†	133 (37, 254)	139 (40, 310)	0.8
ΔHBV-DNA, $\log_{10}$ copies/mL†	-3.22 (-5.43, -2.04)	-3.94 (-6.88, -3.44)	0.11
ΔqHBeAg, PEI U/mL†	-88.0 (-238.4, -22.2)	(-1140.1, -754.4)§	*ntp*
ΔqHBsAg, $\log_{10}$ IU/mL†	-0.03 (-0.23, 0.08)	-3.05 (-3.94, -0.53)	<0.001

†Median (IQR). ‡Number (%).

*Significance between HBsAg-seroclearance groups determined using Kruskal-Wallis test for continuous variables and Pearson χ^2 test or Fisher's exact test for categorical variables. *ntp* – no test performed

§Only two values were available as the other 8 patients were at undetectable levels on the 12-month visit.

FIGURE LEGENDS

Figure 1. HBV DNA viral loads during treatment and *precore* G1896A mutation

HBV-DNA viral loads (**A**) and cumulative probability of achieving undetectable HBV DNA viral load (**B**) are compared between patients with and without the *precore* G1896A mutation at treatment initiation. Individual levels of HBV DNA are expressed as gray lines, whereas median levels are given as dots. HBV DNA was imputed at detection thresholds (12 copies/mL) when undetectable.

Figure 2. HBV serological parameters during treatment and *precore* G1896A mutation

The following end-points were compared between patients harboring the *precore* G1896A mutation at treatment initiation: (**A**) hepatitis B “e” antigen quantification (qHBeAg), (**B**) cumulative probability of hepatitis B “e” antigen seroclearance for patients with HBeAg-positive serology at baseline, (**C**) hepatitis B surface antigen quantification (qHBsAg), and (**D**) cumulative probability of hepatitis B surface antigen seroclearance. Individual levels of qHBeAg and qHBsAg are expressed as gray lines, whereas median levels are given as dots. Levels of these markers were imputed at detection thresholds (0.05 IU/mL or PEI U/mL) when their corresponding serological result was negative.

Figure 1.

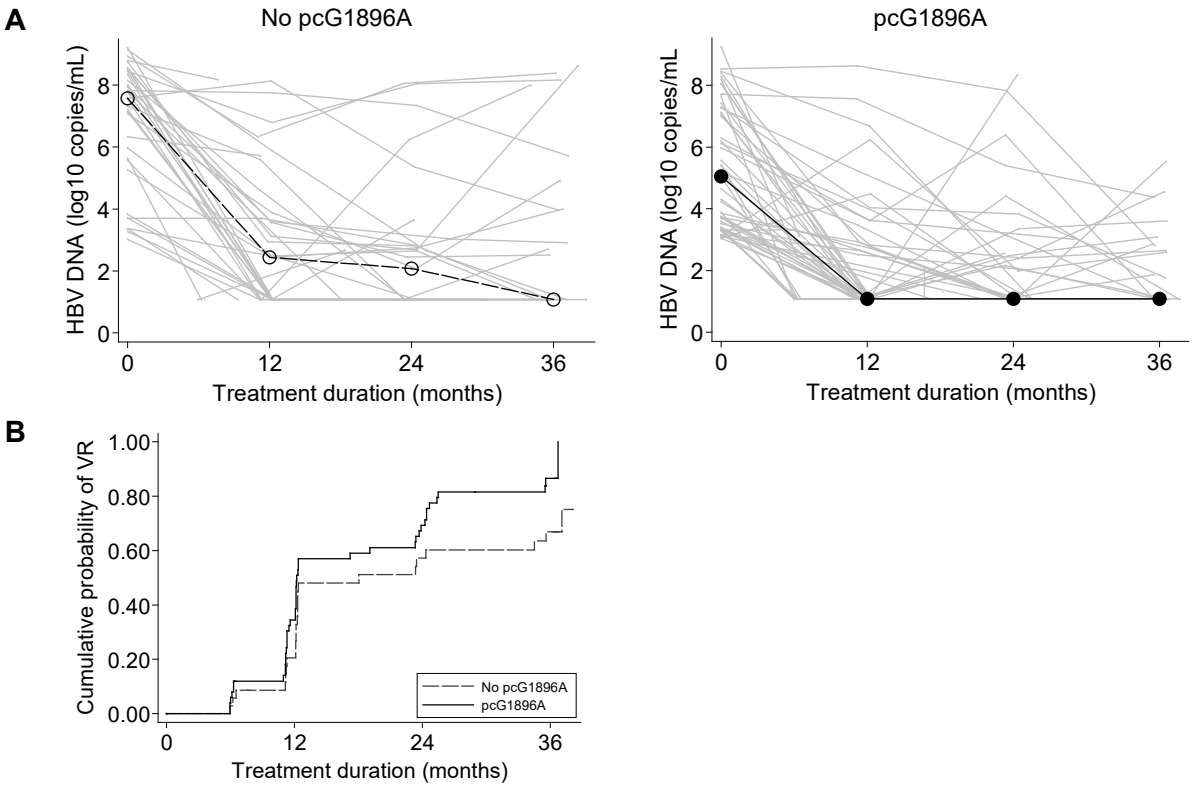
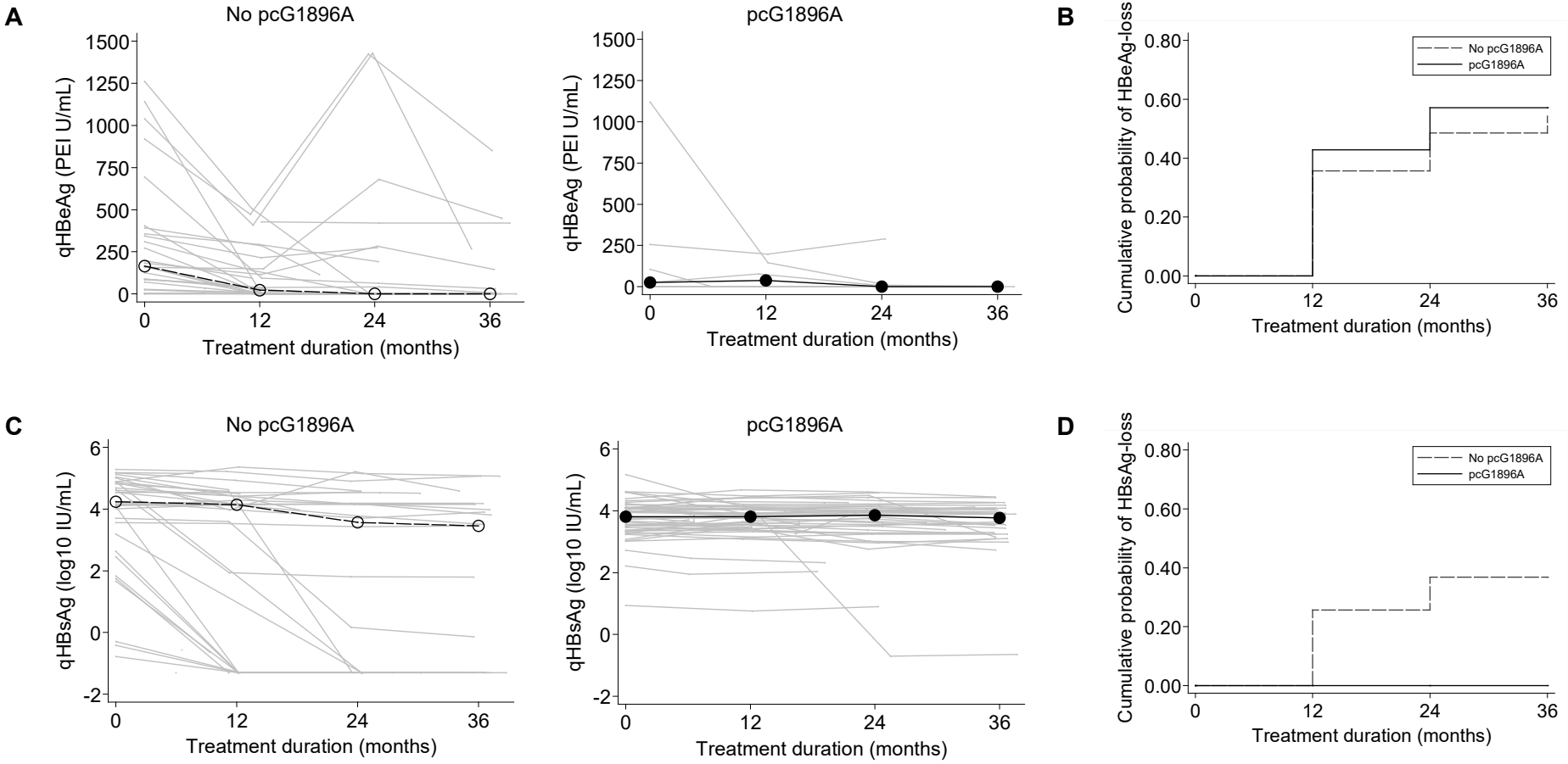

A
No pcG1896A
pcG1896A
HBV DNA (log10 copies/mL)
0
2
4
6
8
0
12
24
36
Treatment duration (months)
B
Cumulative probability of VR
0.00
0.20
0.40
0.60
0.80
1.00
No pcG1896A
pcG1896A

Figure 2.


A
No pcG1896A
pcG1896A
qHBeAg (PEI U/mL)
1500
1250
1000
750
500
250
0
0
12
24
36
Treatment duration (months)
B
Cumulative probability of HBeAg-loss
0.80
0.60
0.40
0.20
0.00
No pcG1896A
pcG1896A
C
qHBsAg (log10 IU/mL)
6
4
2
0
-2
D
Cumulative probability of HBsAg-loss

SUPPLMENTARY MATERIALS

Supplement to: Boyd A, Moh R, Maylin S, et al. *Precore* G1896A mutation is associated with reduced rates of HBsAg-seroclearance in treated HIV-hepatitis B virus co-infected patients from Western Africa.

TABLE OF CONTENTS

SUPPLEMENTARY METHODS

Non-inclusion criteria for the ANRS Trivican and Temprano studies

The study non-inclusion criteria were as follows – both studies: residence outside of Abidjan; unwillingness to participate; pregnancy; severe renal or hepatic disease; severe psychiatric disorder; or any ongoing severe clinical features of undiagnosed origin; *Trivacan*: severe hematological disorder or Karnofsky score <50; *Temprano*: breastfeeding, ongoing tuberculosis disease, or severe cardiac disorder.

Antiretroviral treatment

In the Trivacan trial, all patients started ART at inclusion, receiving zidovudine/lamivudine (LAM) in combination with either efavirenz or ritonavir-boosted lopinavir ("LAM-containing ART"). After a 6 to 18 months phase of continuous ART, those who fulfilled randomization criteria (CD4 >350 /mm^3, plasma HIV-1 RNA <300 copies/mL) were randomized to one of three arms: continuous-ART, CD4-guided ART interruptions (reintroduction when CD4 <250/mm^3, interruption when CD4 >350/mm^3), or fixed-schedule ART interruptions (2-months-off and 4-months-on). Those who did not reach randomization criteria underwent continuous-ART.

In the Temprano trial, patients were randomized at inclusion to either start ART immediately or defer ART until WHO ART-initiation criteria were met. In both strategies, the first-line ART regimen was TDF/emtricitabine (FTC) in combination with one other antiretroviral agent: efavirenz, zidovudine, or ritonavir-boosted lopinavir ("TDF/FTC-containing ART").

SUPPLEMENTARY TABLES

Supplementary Table 1. GenBank referent hepatitis B virus sequences used in phylogenetic analysis

Genotype	Subtype	GenBank accession number
A	A1	HM535200 (1); AF090842 (2).
	A2	AF090838 (2); AB014370 (3).
	A3	AB194950, AB194951 (4).
	A4	AM180623 (5).
	A5	FJ692594, FJ692596 (6).
B	-	D00329, D00330 (7).
C	C1	AB697502, AB697510 (8).
	C2	AB642095, AB642097 (9).
	C3	X75656, X75665 (10).
	C4	AB048704, AB048705 (11).
	C5	EU410080, EU410081 (12).
	C6	GQ358157 (13).
	C7	EU670263 (12).
	C8	AP011104, AP011105 (14).
	C9	AP011108 (14).
	C10	AB540583 (15).
	C11	AB554019, AB554020 (16).
	C12	AB560662 (17); AB554025 (16).
	C13	AB644280, AB644281 (18).
	C14	AB644283, AB644284 (18).
	C15	AB644286 (18).
	C16	AB644287 (18).
D	-	L27106 (19); AY090452 (20).
	D1	AF151735 (21); AF280817 (*NR*).
	D2	AB090268, AB090269 (22).
	D3	AJ132335 (*NR*); AJ131956 (23).
	D4	AB048701 (11); AJ627219 (*NR*).
	D5	AB033558 (7); DQ315779 (24).
	D6	AB493846, AB493848 (25).
	D7	FJ904436, FJ904439 (26).
	D8	FN594769, FN594771 (27).
E	-	AB091255 (*NR*); DQ060830 (28); AB205191 (29); AB106564 (*NR*); FN594763, FN594749 (27).
F	-	AB036905, AB036906 (30).
G	-	AB056514, AB056515 (31).
H	-	AB298362 (32); AB266536 (33).

NR, no reference.

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Supplementary Table 2. Patient characteristics between source studies at ART-initiation

	Source study		
	Temprano *n*=34	Trivacan *n*=52	*p*[¶]
Demographic characteristics			
Gender, male/female (% males)	11/23 (32.4)	16/36 (30.8)	0.9
Age, years[†]	36 (30-40)	35 (31-39)	0.6
BMI, kg/m²[†]	21.0 (19.0-23.8)	22.0 (19.4-23.2)	0.5
Current smoker[‡] [N=85]	6 (17.7)	3 (5.9)	0.15
HIV characteristics			
WHO clinical stage III/VI[‡]	6 (17.7)	24 (46.2)	0.007
HIV-RNA >300 copies/mL[‡] [N=85]	34 (100)	50 (98.0)	0.9
HIV-RNA $\log_{10}$ copies/mL[†§]	5.00 (4.48-5.72)	5.14 (4.64-5.68)	0.4
CD4+ cell count, /mm^{3}[†]	348 (275-467)	226 (169-292)	<0.001
CD4+ cell count[†]	19.9 (14.5-22.9)	12.9 (9.4-18.5)	<0.001
CD4+ count >350 cells/mm^{3}[‡]	17 (50.0)	6 (11.5)	<0.001
HBV characteristics			
HBeAg-positive[‡]	13 (38.2)	22 (42.3)	0.7
Anti-HBe antibody positive[‡]	20 (58.8)	33 (63.5)	0.7
HBV DNA $\log_{10}$ copies/mL[†]	5.60 (3.59-8.20)	6.16 (3.71-7.80)	0.7
HBV genotype[‡]			0.5
A	0 (0)	2 (3.9)	
E	34 (100)	50 (96.2)	
pcG1896A mutation[‡]	20 (58.8)	31 (59.6)	0.9
ALT, IU/L[†] [N=76]	28 (20-47)	30 (21-51)	0.7
AST, IU/L[†] [N=52]	--	40 (29-60)	*ntp*
ALT or AST >40 IU/L[‡]	10 (29.4)	26 (50.0)	0.08

[†]Median (IQR). [‡]Number (%).

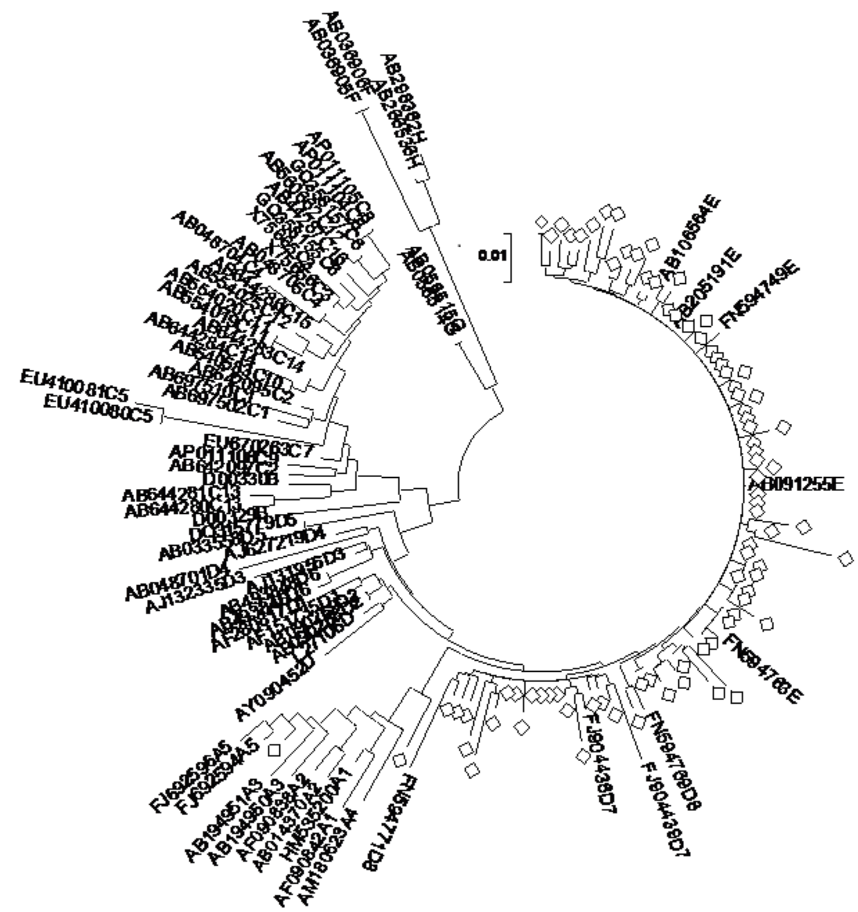
[§]Only among patients with detectable HIV viremia.

[¶]Significance between source studies determined using Kruskal-Wallis test for continuous variables and Pearson χ^2 test or Fisher's exact test for categorical variables. *ntp* – no test performed

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HBeAg, hepatitis B "e" antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; WHO, World Health Organization.

SUPPLEMENTARY FIGURES

Supplementary Figure 1. Hepatitis B virus *preC/C* gene phylogenetic tree for baseline sequences



Individual hepatitis B virus (HBV) sequences from patient samples taken prior to antiviral therapy (hollow diamonds) are compared with complete *preC/C* sequences from HBV referent strains of genotypes A-H (GenBank ascension numbers provided in Supplementary Table 1). Since D7 and D8 HBV subtypes have close evolutionary distances with genotype E and these subtypes have been identified as possible genotype E/D recombinants (1), all patients with phylogenies close to these referent strains were considered to have HBV genotype E. In total, two patients harbored genotype A and 84 harbored genotype E.

Reference:

1. Abdou Chekaraou M, Brichler S, Mansour W, Le Gal F, Garba A, Dény P, et al. A novel hepatitis B virus (HBV) subgenotype D (D8) strain, resulting from recombination between

genotypes D and E, is circulating in Niger along with HBV/E strains. J Gen Virol. 2010 Jun;91(Pt 6):1609–20.