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Abstract word count—250

Word count—5000

Number of tables—1

Number of figures—3

List of abbreviations: HBV**—**hepatitis B virus; *S***—***surface*; MUPIQH**—**mutations possibly influencing quantification of HBV replication markers; HIV**—**human immunodeficiency virus; a.a.**—**amino acid; qHBsAg**—**hepatitis B surface antigen quantification; HBe**—**hepatitis B "e"; SSA**—**Sub-Saharan Africa; ART**—**antiretroviral therapy; TDF**—**tenofovir; HBsAg**—**hepatitis B surface antigen; MHR**—**major hydrophilic region; WHO**—**World Health Organization; LAM**—**lamivudine; ALT**—**alanine aminotransferase; AST**—**aspartate aminotransferase; HBeAg**—**hepatitis B "e" antigen; anti-HBeAb**—**anti-HBe antibodies; rt**—**reverse transcriptase; WT**—**wildtype; PKA**—**cyclic AMP-dependent protein kinase A.

Conflict of interest—The authors report no conflicts of interest.

Financial support—This study was supported by the Agence Nationale de Recherche sur le Sida et les Hépatites (ANRS 12240). A.B. was awarded a post-doctoral fellowship from the ANRS and SIDACTION.

Trial registration number—NCT00158405, NCT00495651

ABSTRACT

Background & Aims: Replication markers exhibit substantial variation during chronic hepatitis B virus (HBV) infection, part of which can be explained by mutations on the *surface* (*S*) gene. We aimed to identify *S*-gene mutations possibly influencing the quantification of HBV replication markers (MUPIQH) in HBV genotype E infection, common to Western Africa.

Methods: 73 antiretroviral treatment (ART)-naïve human immunodeficiency virus (HIV)-HBV co-infected patients from Côte d'Ivoire, initiating anti-HBV-containing ART, had available HBV *S*-gene sequences. *S*-gene MUPIQHs were screened at ART-initiation based on lower HBV-DNA or HBsAg quantification (qHBsAg) compared to wildtype. Their association with HBV virological response and qHBsAg slope during treatment was evaluated.

Results: Genotype E was predominant (95.9%). At ART-initiation, median HBV-DNA was 7.27 $\log_{10}$copies/mL (IQR=5.26-8.33) and qHBsAg 4.08 $\log_{10}$IU/mL (IQR=3.49-4.61). Twelve *S*-gene MUPIQHs were identified among 21 patients (28.8%): sS140L (*n*=4), sD144A (*n*=1), sS167L (*n*=2), sS174N (*n*=6), sP178Q (*n*=2), sG185L (*n*=2), sW191L (*n*=2), sP203Q/R (*n*=2), sS204N/I/R/K/T/G (*n*=7), sN207T (*n*=2), sF212C (*n*=1) and sV224A/Y (*n*=7). MUPIQHs at positions s185+s191+s224 and s178+s204 were within highly-covarying networks of *S*-gene mutations. Older age (*p*=0.02), elevated transaminases (*p*=0.03), and anti-hepatitis B "e" antibody-positive serology (*p*=0.009) were significantly associated with prevalent MUPIQHs at ART-initiation. During treatment, baseline MUPIQHs were not associated with time-to-undetectable HBV-DNA (*p*=0.7) and qHBsAg levels decreased at similar rates between those with versus without MUPIQHs (*p*=0.5).

Conclusion: Several novel *S*-gene mutations were associated with reductions in replication markers among West African co-infected patients. These mutations, however, do not affect response during antiviral treatment. Their diagnostic and clinical consequences need clarification.

Keywords: hepatitis B surface antigen; genetic variability; surface gene; immunosuppression.

LAY SUMMARY:

Certain mutations on a gene of the hepatitis B virus (HBV) can make it difficult to quantify levels of biomarkers that are helpful in following the clinical progression of HBV. We found some of these mutations in a rarely studied group of individuals from Sub-Saharan Africa with a specific genotype, yet further confirmation from laboratories is needed.

INTRODUCTION

Among individuals infected with human immunodeficiency virus (HIV) living in Sub-Saharan Africa (SSA), roughly ten percent are also infected with hepatitis B virus (HBV).[1] Increased rates of morbidity/mortality have been observed in co-infected patients on the continent, yet are mostly attributed to higher or uncontrolled HBV replication.[2–5] However, HBV replication alone might not explain these results and other indicators of viral activity could help elucidate the pathogenic mechanisms giving rise to increased rates of morbidity/mortality during HBV-infection.[6]

One such marker is hepatitis B surface antigen quantification (qHBsAg), which has been useful in predicting more active phases of chronic HBV infection and severe liver-related disease.[7] In patients with HIV-HBV co-infection, serum qHBsAg levels have been strongly correlated with intrahepatic HBV replication during the hepatitis B “e” antigen (HBeAg)-positive phase and implies its use as a proxy for viral activity in the liver.[8] In co-infected patients undergoing antiretroviral therapy (ART) with the potent anti-HBV agent tenofovir (TDF), qHBsAg has given insight on the probability of attaining hepatitis B surface antigen (HBsAg) seroclearance[9], which is the hallmark of reduced risk in liver-related morbidity/mortality.[10]

Nevertheless, certain genetic characteristics of the *S*-gene have called into question the usefulness of this marker. *S*-gene mutations found within the “a” determinant of the major hydrophilic region are associated with discrepancies between qHBsAg assays[11], influencing its correlation with HBV-DNA.[12,13] A recent study has also uncovered mutations outside this region with similar *in vivo* and *in vitro* reductions in qHBsAg.[14] It remains to be determined whether *S*-gene mutations observed in isolates from SSA share similar characteristics in decreased levels of replication markers, especially as few studies have involved HBV genotype E.[15] It is also unknown if harboring these mutant variants at baseline affects monitoring treatment response.

In the study herein, we used unique data among ART-naïve HIV-HBV co-infected patients from two randomized-control studies conducted in Côte d'Ivoire. Our first aim was to examine the distribution of mutations on the *S*-gene, compared to consensus sequences, and the covarying nature of these mutations prior to ART-exposure. From identified mutations at this time-point, we intended to screen for *S*-gene mutations possibly influencing the quantification of HBV replication markers (MUPIQH) and analyze their determinants. Finally, we examined whether patients harboring MUPIQHs exhibited any differences in HBV-DNA or qHBsAg decline during nucleoside/nucleotide analogue (NA)-based treatment.

PATIENTS AND METHODS

Study design

The VarBVA study[16] is an observational cohort including patients from two prospective, randomized trials in 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 concurrent World Health Organization (WHO) recommendations. Study procedures have been detailed elsewhere.[17,18]

Inclusion criteria were as follows: both studies—age ≥18 years, HIV-1 or mixed HIV-1/2 infection, and ART-naïve (except for short-course treatment to prevent mother-to-child HIV transmission); *Trivacan*—CD4 cell count 150-350/mm^3 or 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 study protocols were obtained by the Ministry of Health of Côte d'Ivoire and 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 lamivudine (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.

Markers of HBV-replication

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 (Light Cycler 480, Roche, Boulogne-Billancourt, France) with a detection limit of 12 copies/mL.[16] HBsAg was detected using the HBsAg Qual II test (Architect, Abbott Laboratories, Rungis, France) at baseline and during follow-up. HBe antigen (HBeAg) and anti-HBe antibodies (anti-HBeAb) were detected using the Elecsys assay (Roche Diagnostics, Meylan, France) for the same visits. For HBsAg-positive samples, qHBsAg was quantified using the Elecsys assay with Modular E170 analyzer (Roche Diagnostics, Meylan, France). *HBsAg-seroclearance* was defined as loss of HBsAg from the previous visit.

Determining HBV genotype

Genotypic analysis was performed at baseline for all patients with an HBV-VL >1000 copies/mL.[16] A.a. sequences of the *pol* and *S*-genes were examined by direct sequencing after nested-PCR amplification of the reverse transcriptase (rt) and surface antigen (s) encoding regions (between rt107-rt385 and s99-s226, respectively). Individual a.a. sequences were aligned using the ClustalW full multiple alignment program with 1000 bootstraps in BioEdit (v7.0.5.3, Carlsbad, CA).

HBV genotypes were determined via phylogenetic analysis in which aligned a.a. sequences of the *S*-gene (s99-s226) were compared to referent sequences of HBV genotypes A-H (GenBank accession numbers listed in Supplementary Table 1). A maximum likelihood method based on the Jones-Taylor-Thornton matrix-based model was used, in which a rate variation model was incorporated allowing for some sites to be evolutionarily invariable (+I). Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of model-derived pairwise distances with 1000 bootstrap replications. The resulting phylogenetic tree was constructed using MEGA6 software[20] and is provided in Supplementary Figure 1. Evidence for recombination was examined using BootScan, GroupingScan, and TreeOrderScan of the Simple Sequence Editor package (v1.3) (supplementary methods).

Detection of HBV mutations

Individual a.a. sequences were compared to a genotype-specific consensus sequence of the small hepatitis B surface protein retrieved from the HBVdb[21] (defined in the Supplementary materials). If an a.a. at a given position was different than the consensus sequence, a mutation was considered present at this position. Antiviral resistance mutations on the *pol*-gene were

defined in function of associated agent: LAM (rtV173L,rtL180M,rtM204V/I); adefovir (rtA181T/V,rtN236T); entecavir (rtT184S/A/I/L/G/C/M,rtS202C/G/I,rtM250I/V).

Covariation and network analysis of HBV *S*-gene genetic variability

From aligned sequences, covariation between pairs of *S*-gene a.a. was calculated using McLauchlan-Based Substitution Correlation (McBASC) method from the "Bios2cor" package in R (v3.4.1, Vienna, Austria). This method was chosen over other covariation estimation methods due to the suspected low connectivity of a.a. pairs and lack of a central residue.[22] Highly covarying a.a. pairs were identified as a having a McBASC ≥|1| and were further used to examine networks of mutations using Cytoscape v3.6.1.[23] Clusters of highly-correlated mutation networks were derived using the clusterMaker2 app v1.2.1 available in Cytoscape.

Predicting phosphorylation sites

Generic predictions for serine, threonine and tyrosine phosphorylation sites and kinase-specific predictions of phosphorylation sites were examined using NetPhos v3.1 (http://www.cbs.dtu.dk/services/NetPhos/). This program uses neural networks from empirically-validated phosphorylation sites to predict phosphorylation sites and protein kinases involved in phosphorylation events from a given a.a. sequence.

Statistical analysis

Using a screening approach, we identified *S*-gene mutations that were associated with differences in HBV-DNA or qHBsAg levels compared to strains without any identified *S*-gene mutations [defined herein as wildtype (WT)]. Since mutation prevalence could be low and result

in excess Type II error, we selected mutations with an associated $p<0.1$. During analysis, all mutations associated with lower levels of HBV-DNA or qHBsAg were grouped together as MUPIQHs. HBV mutations from previous studies were not considered when defining MUPIQH.

Comparisons between patients with versus without baseline MUPIQH were conducted using Kruskal-Wallis test for continuous variables and Pearson χ^2 test or Fisher's exact test for categorical variables. During treatment, rates of time-to-undetectable HBV-DNA were compared between MUPIQH groups using Cox proportional hazards regression. Change in qHBsAg $\log_{10}$IU/mL/year was estimated using mixed-effect linear regression with random-intercept. Slopes were compared between mutation groups by testing a time-MUPIQH group interaction term. Multivariable adjustments included *a priori* anti-HBV treatment and baseline HBV-DNA or qHBsAg (depending on end-point).

Analyses were performed using STATA (v12.1, College Station, TX) and a p-value of <0.05 was considered significant. As this analysis was largely exploratory, no adjustments were made for multiple hypothesis testing.[24]

RESULTS

Description of the study population

At inclusion, 259 (10.5%) of 2465 patients (840 and 1625 from the Trivacan and Temprano studies, respectively) were confirmed HBsAg-positive. Among them, 186 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), or had missing HBV viral loads (*n*=8) or *S*-gene sequences (*n*=95). In total, 73 patients were included in analysis (Table 1). HBV genotypes E and A were identified in 70 and 3 patients, respectively, with no evidence of between-genotype recombination.

Description of *S*-gene mutations and covariance between mutations prior to ART

Baseline prevalence of *S*-gene mutations were as follows: sP120A/L/T (*n*=3), sT123D/P/S (*n*=3), sS140L/T (*n*=7), sS143L/T (*n*=5), sS154P/L (*n*=3), sK160N/R or mixed S/F (*n*=8), sE164G/V (*n*=8), sS174N or mixed S/N (*n*=6), sL175S or mixed L/S (*n*=5), sQ181R or mixed Q/R (*n*=4), sT189I/V (*n*=3), sS204G/N/T or mixed S/N or I/K/R/S (*n*=7), sS210R or mixed K/N/R/S (*n*=4), sL216*/Y or mixed L/* (*n*=4), sF220C/L or mixed C/Y or F/C (*n*=4), sV224A/Y (*n*=7), sI226S (*n*=4). All other mutations observed in ≤2 patients are described in Supplementary Table 2. Besides position s216, only two other stop codon mutations were detected at position sW182* (*n*=1) and sL215* (*n*=1). Several highly-covarying networks between mutations were observed (Figure 1), with pairs of a.a. positions demonstrating the highest similarity as: s221+s217 (McBASC=14.0), s206+s130 (McBASC=14.0), s200+s195 (McBASC=14.0), s161+s159 (McBASC=13.4), and s145+s133 (McBASC=14.0).

Several HBV *S*-gene mutations prior to ART were identified to possibly influence quantification of HBV replication markers

During screening for *S*-gene MUPIQHs, markedly lower HBV-DNA and qHBsAg levels were observed with mutations at positions listed in Figure 2A compared to WT. MUPIQHs observed within the same highly-correlated networks were at positions s185, s191, and s224 as well as s204 and s178 (Figure 1). In total, roughly one-third of patients (*n*=21/73) were observed to

harbor a MUPIQH. As screening for these mutations depended on replication markers, patients harboring MUPIQHs had significantly lower HBV-DNA and qHBsAg levels at treatment initiation (Figure 2B).

Clinical and virological determinants of HBV *S*-gene MUPIQHs prior to ART

As shown in Table 1, patients harboring *S*-gene MUPIQHs at ART-initiation were significantly older (p=0.02) and more likely to have elevated ALT/AST levels (p=0.03). These patients were also significantly more likely to have anti-HBe antibodies (p=0.009) compared to those without MUPIQHs. No HIV-related factors were associated with MUPIQHs.

With respect to virological factors, no *pol*-gene mutations were observed at overlapping positions of *S*-gene MUPIQHs (Supplementary Table 3) and no antiviral resistance *pol*-gene mutations were found. Kinase-specific predictions revealed that all 6 MUPIQHs at position s204 lacked cyclic-AMP dependent protein kinase (PKA) activity, whereas all strains without mutations at this position had PKA activity (p<0.001).

HBV *S*-gene MUPIQHs prior to ART did not affect HBV treatment response

LAM- or TDF/emtricitabine-containing ART was administered to 49 (67.1%) and 24 (32.9%) patients, respectively. Of those undergoing LAM-containing ART, 4 were randomized to continuous-ART, 16 to CD4-guided ART-interruptions, 19 to fixed-schedule ART-interruptions, and 10 non-randomized while taking continuous-LAM.

Patients were followed for a median 36 months (IQR=24-36). Median HBV-DNA levels during treatment were similar between patients with and without *S*-gene MUPIQHs (Figure 3A).

Undetectable HBV-DNA was achieved in 50 patients (cumulative rate=81.4%), with no difference in cumulative rates between patients with or without MUPIQHs (*p*=0.7), even after adjustment for anti-HBV treatment and baseline HBV-DNA (*p*=0.9). Two patients developed LAM-resistant mutations (rtV173L+rtL180M+rtM204V) during LAM-containing ART, none of whom harbored a MUPIQH.

As shown in Figure 3B, qHBsAg levels remained mostly stable during treatment (average change from baseline/year=-0.37 $\log_{10}$IU/mL, 95%CI=-0.48,-0.26). No difference in overall slopes between patients with or without *S*-gene MUPIQHs was observed (*p* for interaction=0.5), even after adjusting for anti-HBV treatment and baseline qHBsAg (*p* for interaction=0.4). HBsAg-seroclearance was also observed in 9 patients (cumulative rate=14.7%), while 5 (55.6%) of these patients harbored an MUPIQH at baseline: sS167L (*n*=1), sS174N+sP178Q+sG185L+sS204N+sV224A (*n*=1), sS167L+sP178Q+sW191L+sN207T (*n*=1), sD144A (*n*=1), and sF212C (*n*=1). Of the 9 patients with HBsAg-seroclearance, median qHBsAg levels were 4.89 $\log_{10}$ IU/mL (range=4.08-5.12) and 2.45 $\log_{10}$ IU/mL (IQR=1.75-2.64) in those without and with MUPIQHs, respectively.

Low incidence of *S*-gene MUPIQH during ART

Two incident *S*-gene MUPIQHs were observed during follow-up at positions sS174S/N (*n*=1) and sF212S (*n*=1), giving a cumulative incidence of 1.7/100 person-years. Clinical description of patients with incident MUPIQHs is provided in Supplementary Table 4, with no noteworthy characteristics differing from the overall study population.

DISCUSSION

In this large collection of isolates from Côte d'Ivoire, we were able to characterize *S*-gene mutations in HIV-HBV co-infected patients prior to ART-initiation. Among these mutations, several *S*-gene mutant variants possibly influencing the quantification of HBV replication makers were identified and observed in 29% of our study population. Previous assessments of MUPIQHs estimate their prevalence at 7-30% of HBV mono-infected and HIV-HBV co-infected patients[15,25,26], while these estimates likely vary according to mutation types, algorithms considered or exposure to less effective NAs. Nevertheless, these findings underscore the sizeable extent of mutations bearing diagnostic and therapeutic concern.

The most frequently identified *S*-gene MUPIQHs were at positions s204 and s224 (mostly sV224A), while covariation analysis demonstrated that these mutations were strongly linked to other identified MUPIQHs at positions s178, s185, and s191. Pathological mechanisms explaining altered HBsAg levels have been suggested for mutations at position s204. For example, sS204N mutant variants have demonstrated lower reactivity to monoclonal antibodies directed to various epitopes of the HBsAg.[25] Reduced antibody binding capacity has been observed for other *S*-gene mutations[11,27], which have been implicated in lower qHBsAg levels.[28] Interestingly, MUPIQHs at position s204 were linked to lack of cyclic-AMP-dependent PKA. This enzyme can induce phosphorylation via cyclic-AMP-response element-binding protein[29] and upregulate HBsAg-expression.[30] However, more specific *in vitro* evidence would be required to confirm if this pathway is involved with the HBsAg reductions observed here. No research has examined the effect of sV224A mutations on capacity of antibody binding or viral particle secretion.

Other *S*-gene mutations have been associated with altered markers of replication. Mutations at position sW172* or sW182* are known to truncate S proteins and can trigger an unfolded protein

response, leading to ER stress-induced oxidative DNA damage.[14,28,31] HBV genomic instability ensues and causes reductions in HBV-DNA levels. Moreover, *S*-gene mutations on the "a" determinant (i.e. sP120T, sG145R) and regions overlapping LAM-resistance mutations (i.e. sE164D, sI195M, sW196S) are also fairly common in HIV-HBV co-infected patients[26] and demonstrate reduced binding to anti-HBs antibodies.[27] Mutations at these positions were in fact rarely observed at baseline or during follow-up[16], possibly owing to the low levels of circulating HBV and predominance of HBV genotype E infection.[32] With such low prevalence, our data serve as reassurance that these mutations are not widely circulating in Western Africa.

Alternatively, these mutations could be independent of any biological pathway. Some MUPIQHs (i.e. sP203R, sS204N/R, sV224A) appeared in 2-4% of referent sequences (Supplementary data file), suggesting that they could simply be polymorphisms. Other mutations might arise during later phases of HBV-infection. Anti-HBe antibody-positive serology was indeed a significant determinant for *S*-gene MUPIQHs. HBeAg-negative co-infected patients have been shown to harbor HBV strains with increased mutation frequency across the entire *S*-gene.[33] An explanation for these findings could be greater immune reactivity in patients able to clear HBeAg, thereby inducing the potential immune selective pressures that more broadly evoke mutations on the *S*-gene.[34]

HIV-related immunosuppression could have an additional effect on *S*-gene variability. HBV sequences from HIV-HBV co-infected patients have demonstrated more extensive a.a. conservation of the HBsAg "a" determinant versus those with HBV mono-infection[35], probably because of decreased immune selective pressure in general during co-infection. We did not, however, find any relationship between *S*-gene MUPIQHs and HIV-RNA or CD4+ T cell count; similar to what was observed in overall *S*-gene mutation frequency in a previous study.[33] The

specific immunological pressures in HBV versus HIV-HBV infected patients that give rise to these MUPIQH variants should be honed in further studies.

One unresolved question in the literature is whether *S*-gene MUPIQHs affect HBV treatment response. Patients with versus without baseline MUPIQHs did not exhibit significantly different virological response rates during NA-based treatment. Since antiviral therapy is able to suppress replication of many *S*-gene mutant variants[36], this result would be rather expected. This high degree of potency would also explain the low incidence of MUPIQH over time. In contrast, qHBsAg levels remained consistently lower during antiviral therapy in patients with MUPIQHs prior to ART, while the on-treatment slopes of qHBsAg ran mostly parallel between MUPIQH groups. Any underlying mechanism for reduced serum qHBsAg levels could be considered constant during NA-treatment.

One noteworthy feature of this cohort was the high rate of HBsAg-seroclearance, at 5% per year, compared to other studies in TDF-treated HIV-HBV co-infected patients.[9] This could be partly due to rapid immunorestoration of anti-HBV immune responses after ART-initiation.[37] On the other hand, five of the nine patients with HBsAg-seroclearance harbored MUPIQHs. Whether it contributed to the increased rate of this therapeutic end-point is questionable. In NA-treated HBV mono-infected patients harboring genotype D, network analysis showed a variety of mutations of the *S*-gene, particularly in the transmembrane domain-3/4, common to patients with persistent HBsAg-positive serology.[38] Most were linked to mutations at positions sT125M and sP127T, which were not observed in our study. Furthermore, higher genomic variability of the *S*-gene, as determined by ultra-deep sequencing, is actually more conducive to HBsAg-persistence during treatment.[38] As MUPIQHs covaried with several other *S*-gene mutations, suggesting higher overall variation on the *S*-gene, their importance in HBsAg-seroclearance is likely restrained.

Some limitations of our study need to be addressed. First, certain selection biases likely occurred. Patients with severely elevated transaminases or clinical signs of severe liver disease at inclusion were excluded, while those with insufficient HBV-DNA viral loads for sequencing could not be included. This resulted in study population with a limited spectrum of HBV-related disease. Second, the majority of patients undergoing LAM interrupted treatment as indicated in the clinical trial protocol. The antiviral selective pressures in these patients might not fully reflect those with continuous treatment. Third, the numbers of patients with data on *S*-gene genetic variability were comparable to, if not higher than, other studies on the continent[39], yet were still low enough to question whether we had adequate power to detect other *S*-gene MUPIQHs and their risk-factors. Finally, we did not have complete data on the *S*-gene or any data on the *preS* gene, which prohibits us from evaluating their effect. Mutations at positions sL21R, sL85W and sL98V have been shown *in vitro* to affect extracellular and intracellular HBsAg levels by two serological methods (ELISA and Western blot) in HBV mono-infected patients harboring genotype C[14] and deletions in the *preS1* region and *preS2* start codon are known to reduce virion production and HBsAg secretion both *in vivo* and *in vitro*.[28] It should be determined if genetic variability in these regions is linked to the MUPIQHs identified herein.

In conclusion, we found several novel mutations, particularly at positions s204 and s224 on the *S*-gene, associated with reductions in replication markers among HIV-HBV co-infected patients harboring genotype E. Considering that most of these mutations laid outside the “a” determinant of the HBsAg protein, further research is needed to explain the potential mechanisms of these findings. The diagnostic implications of these MUPIQHs, which could require more attention when evaluating qHBsAg, would be particularly helpful.

ACKNOWLEDGEMENTS

We thank all patients who participated in both trials. We also gratefully acknowledge the valuable contributions of the SMIT, CeDReS, CEPREF, USAC, CIRBA, CNTS, La Pierre Angulaire, Hôpital Général Abobo, Formation Sanitaire Anonkoua Kouté, Centre de santé El Rapha, the Programme PACCI team, as well as the INSERM exU593 and U897 teams; and Bristol-Myers Squibb for providing Zerit and Videx; Gilead Sciences, for the donation of Truvada; and Merck Sharp & Dohme, for the donation of Stocrin.

REFERENCES

1. Matthews PC, Geretti AM, Goulder PJR, Klenerman P. Epidemiology and impact of HIV coinfection with hepatitis B and hepatitis C viruses in Sub-Saharan Africa. *J Clin Virol*. 2014;61:20–33.

2. Matthews GV, Manzini P, Hu Z, et al. Impact of lamivudine on HIV and hepatitis B virus-related outcomes in HIV/hepatitis B virus individuals in a randomized clinical trial of antiretroviral therapy in southern Africa. *AIDS*. 2011;25:1727–1735.

3. Hawkins C, Christian B, Ye J, et al. Prevalence of hepatitis B co-infection and response to antiretroviral therapy among HIV-infected patients in Tanzania. *AIDS*. 2013;27:919–927.

4. Boyd A, Houghtaling L, Moh R, et al. Clinical outcomes during treatment interruptions in HIV-hepatitis B virus co-infected patients from Sub-Saharan Africa. *Am J Trop Med Hyg*. 2017;97:1936-1942.

5. Kouamé G-M, Boyd A, Moh R, et al. Higher mortality despite early ART in HIV and hepatitis B virus coinfected patients with high HBV replication. *Clin Infect Dis*. 2018;66:112-120.

6. Chen C-J, Yang H-I, Iloeje UH, REVEAL-HBV Study Group. Hepatitis B virus DNA levels and outcomes in chronic hepatitis B. *Hepatology*. 2009;49:S72-84.

7. Cornberg M, Wong VW-S, Locarnini S, Brunetto M, Janssen HLA, Chan HL-Y. The role of quantitative hepatitis B surface antigen revisited. *J Hepatol*. 2017;66:398–411.

8. Boyd A, Lacombe K, Lavocat F, et al. Decay of ccc-DNA marks persistence of intrahepatic viral DNA synthesis under tenofovir in HIV-HBV co-infected patients. *J Hepatol*. 2016;65:683–691.

9. Boyd A, Maylin S, Gozlan J, et al. Use of hepatitis B surface and “e” antigen quantification during extensive treatment with tenofovir in patients co-infected with HIV-HBV. *Liver Int*. 2015;35:795–804.

10. European Association For The Study Of The Liver. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol*. 2012;57:167–185.

11. Verheyen J, Neumann-Fraune M, Berg T, Kaiser R, Obermeier M. The detection of HBsAg mutants expressed in vitro using two different quantitative HBsAg assays. *J Clin Virol*. 2012;54:279–281.

12. Tian Y, Xu Y, Zhang Z, et al. The amino Acid residues at positions 120 to 123 are crucial for the antigenicity of hepatitis B surface antigen. *J Clin Microbiol*. 2007;45:2971–2978.

13. Servant-Delmas A, Mercier-Darty M, Ly TD, et al. Variable capacity of 13 hepatitis B virus surface antigen assays for the detection of HBsAg mutants in blood samples. *J Clin Virol*. 2012;53:338–345.

14. Xiang K-H, Michailidis E, Ding H, et al. Effects of amino acid substitutions in hepatitis B virus surface protein on virion secretion, antigenicity, HBsAg and viral DNA. *J Hepatol*. 2017;66:288–296.

15. Simon B, Kundi M, Puchhammer E. Analysis of mutations in the S gene of hepatitis B virus strains in patients with chronic infection by online bioinformatics tools. *J Clin Microbiol*. 2013;51:163–168.

16. Boyd A, Moh R, Gabillard D, et al. Low risk of lamivudine-resistant HBV and hepatic flares in treated HIV-HBV-coinfected patients from Côte d'Ivoire. *Antivir Ther*. 2015;20:643–654.
17. Danel C, Moh R, Minga A, et al. CD4-guided structured antiretroviral treatment interruption strategy in HIV-infected adults in west Africa (Trivacan ANRS 1269 trial): a randomised trial. *Lancet*. 2006;367:1981–1989.
18. TEMPRANO ANRS 12136 Study Group, Danel C, Moh R, et al. A Trial of Early Antiretrovirals and Isoniazid Preventive Therapy in Africa. *N Engl J Med*. 2015;373:808–822.
19. 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. *J Viral Hepat*. 2018. doi:10.1111/jvh.12914
20. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol Biol Evol*. 2013;30:2725–2729.
21. Hayer J, Jadeau F, Deléage G, Kay A, Zoulim F, Combet C. HBVdb: a knowledge database for Hepatitis B Virus. *Nucleic Acids Res*. 2013;41:D566-570.
22. Pelé J, Moreau M, Abdi H, Rodien P, Castel H, Chabbert M. Comparative analysis of sequence covariation methods to mine evolutionary hubs: examples from selected GPCR families. *Proteins*. 2014;82:2141–2156.
23. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003;13:2498–2504.
24. Rothman KJ. No adjustments are needed for multiple comparisons. *Epidemiology*. 1990;1:43–46.
25. Andersson MI, Maponga TG, Ijaz S, et al. The epidemiology of hepatitis B virus infection in HIV-infected and HIV-uninfected pregnant women in the Western Cape, South Africa. *Vaccine*. 2013;31:5579–5584.

26. Lacombe K, Boyd A, Lavocat F, et al. High incidence of treatment-induced and vaccine-escape hepatitis B virus mutants among human immunodeficiency virus/hepatitis B-infected patients. *Hepatology*. 2013;58:912–922.

27. Torresi J. The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. *J Clin Virol*. 2002;25:97–106.

28. Pollicino T, Amaddeo G, Restuccia A, et al. Impact of hepatitis B virus (HBV) preS/S genomic variability on HBV surface antigen and HBV DNA serum levels. *Hepatology*. 2012;56:434–443.

29. Thiel G, Al Sarraj J, Stefano L. cAMP response element binding protein (CREB) activates transcription via two distinct genetic elements of the human glucose-6-phosphatase gene. *BMC Mol Biol*. 2005;6:2.

30. Tacke F, Liedtke C, Bocklage S, Manns MP, Trautwein C. CREB/PKA sensitive signalling pathways activate and maintain expression levels of the hepatitis B virus pre-S2/S promoter. *Gut*. 2005;54:1309–1317.

31. Warner N, Locarnini S. The antiviral drug selected hepatitis B virus rtA181T/sW172* mutant has a dominant negative secretion defect and alters the typical profile of viral rebound. *Hepatology*. 2008;48:88–98.

32. Ma Q, Wang Y. Comprehensive analysis of the prevalence of hepatitis B virus escape mutations in the major hydrophilic region of surface antigen. *J Med Virol*. 2012;84:198–206.

33. Audsley J, Littlejohn M, Yuen L, et al. HBV mutations in untreated HIV-HBV co-infection using genomic length sequencing. *Virology*. 2010;405:539–547.

34. Lim SG, Cheng Y, Guindon S, et al. Viral quasi-species evolution during hepatitis Be antigen seroconversion. *Gastroenterology*. 2007;133:951–958.

35. Cento V, Mirabelli C, Dimonte S, et al. Overlapping structure of hepatitis B virus (HBV) genome and immune selection pressure are critical forces modulating HBV evolution. *J Gen Virol*. 2013;94:143–149.

36. Amini-Bavil-Olyaee S, Vucur M, Luedde T, Trautwein C, Tacke F. Differential impact of immune escape mutations G145R and P120T on the replication of lamivudine-resistant hepatitis B virus e antigen-positive and -negative strains. *J Virol*. 2010;84:1026–1033.

37. Singh KP, Crane M, Audsley J, Avihingsanon A, Sasadeusz J, Lewin SR. HIV-hepatitis B virus coinfection: epidemiology, pathogenesis, and treatment. *AIDS*. 2017;31:2035–2052.

38. Velay A, Jeulin H, Eschlimann M, et al. Characterization of hepatitis B virus surface antigen variability and impact on HBs antigen clearance under nucleos(t)ide analogue therapy. *J Viral Hepat*. 2016;23:387–398.

39. Bell TG, Makondo E, Martinson NA, Kramvis A. Hepatitis B virus infection in human immunodeficiency virus infected southern African adults: occult or overt--that is the question. *PloS One*. 2012;7:e45750.

FIGURE LEGENDS

Figure 1. Network analysis of HBV *S*-gene genetic variability

Highly covarying networks of a.a. on the *S*-gene (from positions s99-s226) prior to antiretroviral treatment initiation.

Figure 2. HBV replication markers at baseline in function of harboring *S*-gene MUPIQH

In (**A**), median (IQR) levels of hepatitis B virus (HBV) DNA and hepatitis B surface quantification (qHBsAg) are represented among patients harboring HBV strains without any identified *S*-gene mutations, defined herein as wildtype (WT), compared to strains with *S*-gene mutations possibly influencing the quantification of HBV replication markers (MUPIQH) (exhibiting a difference in HBV replication markers with WT at $p<0.1$). In (**B**), median levels of HBV-DNA and qHBsAg were compared between patents harboring HBV strains with versus without MUPIQHs. Significance determined using the Kruskal-Wallis rank test: *$p<0.1$ **$p<0.05$.

Figure 3. HBV replication markers during treatment and presence of *S*-gene MUPIQH

Individual trajectories of HBV-DNA (**A**) and hepatitis B surface antigen quantification (qHBsAg) (**B**) are given in gray lines along with median-band spline plots in black lines, stratified on patients harboring HBV with versus without *S*-gene mutations possibly influencing the quantification of HBV replication markers (MUPIQH). Two patients were missing data on qHBsAg.

TABLES

Table 1. Characteristics of patients with and without *S*-gene mutations possibly influencing the quantification of HBV replication markers (MUPIQH) at treatment initiation

	Total (*n*=73)	MUPIQH[a] Absent (*n*=52)	MUPIQH[a] Present (*n*=21)	*p*[b]
Demographic characteristics				
Gender, male/female (% males)	23/50 (31.5)	13/39 (25.0)	10/11 (47.6)	0.06
Age, years*	35 (29-38)	35 (29-37)	37 (34-44)	0.02
BMI, kg/m²*	21.2 (19.1-23.2)	21.2 (18.8-23.5)	21.3 (19.3-22.1)	0.8
Current smoker** [N=72]	7 (9.7)	3 (5.9)	4 (19.1)	0.18
HIV characteristics				
WHO clinical stage III/VI	30 (41.1)	19 (36.5)	11 (52.4)	0.3
HIV-RNA >300 copies/mL** [N=72]	71 (98.6)	50 (98.0)	21 (100)	0.9
HIV-RNA $\log_{10}$ copies/mL*[c]	5.24 (4.59-5.71)	5.21 (4.56-5.68)	5.35 (4.78-5.80)	0.3
CD4+ cell count, /mm^3*	275 (174-340)	283 (179-338)	248 (166-380)	0.7
CD4+ count >350 cells/mm^3*	18 (24.7)	12 (23.1)	6 (28.6)	0.8
Initial antiretroviral regimen**				0.17
LAM-based	49 (67.1)	32 (61.5)	17 (81.0)	
TDF-based	24 (32.9)	20 (38.5)	4 (19.1)	
HBV characteristics				
HBeAg-positive**	37 (50.7)	30 (57.7)	7 (33.3)	0.06
Anti-HBe antibody positive**	38 (52.1)	22 (42.3)	16 (76.2)	0.009
HBV genotype**				0.6
A	3 (4.1)	3 (5.8)	0 (0)	
E	70 (95.9)	49 (94.2)	21 (100)	
ALT, IU/L* [N=67]	29 (22-53)	28 (21-46)	32 (24-69)	0.4
AST, IU/L* [N=48]	41 (31-73)	37 (27-86)	51 (39-69)	0.2
ALT or AST >40 IU/L**	34 (46.6)	20 (38.5)	14 (66.7)	0.03

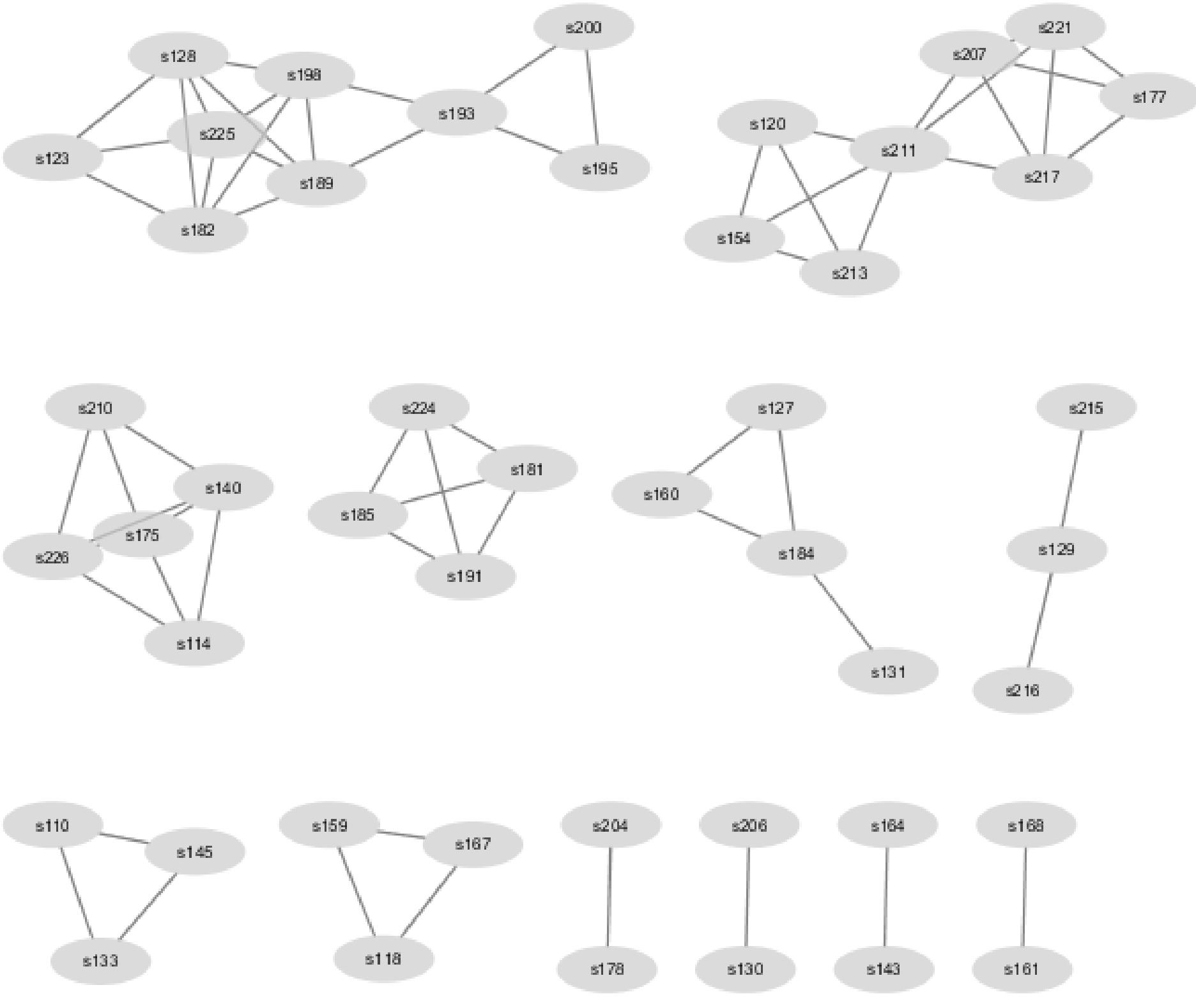
*Median (IQR). **Number (%).

[a]Any patient harboring a mutation at one of the following positions of the HBV *S*-gene: s140, s144, s167, s174, s178, s185, s191, s203, s204, s207, s212, or s224.

[b]Significance between treatment 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

[c]Only among patients with detectable HIV-RNA viral loads.

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; MUPIQH, mutations possibly influencing the quantification of HBV replication markers; TDF, tenofovir; WHO, World Health Organization.


s200
s128
s198
s193
s225
s123
s195
s189
s182
s221
s207
s177
s120
s211
s217
s154
s213
s210
s224
s127
s215
s140
s181
s160
s185
s175
s184
s129
s226
s191
s114
s131
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s167
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s118
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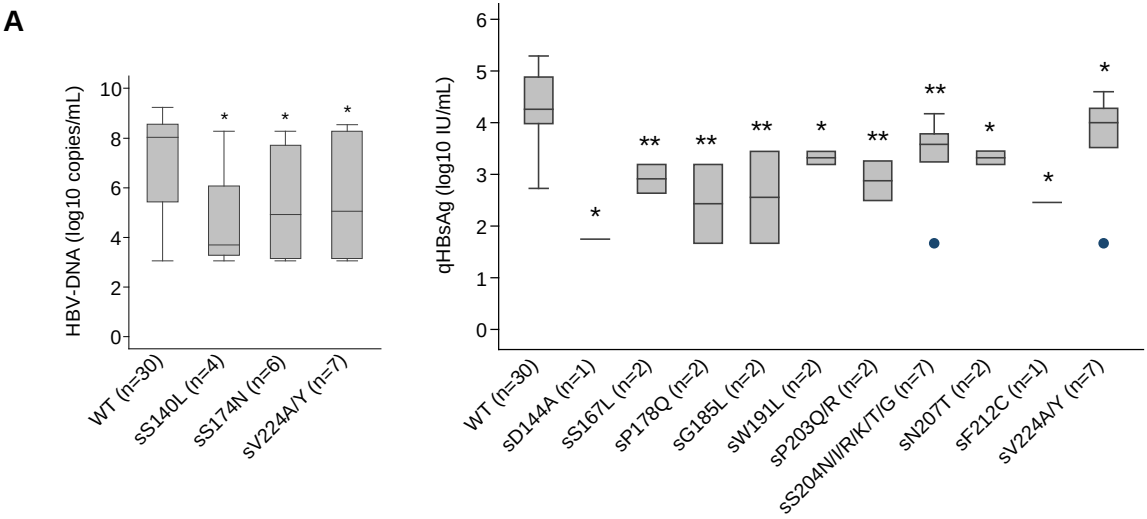
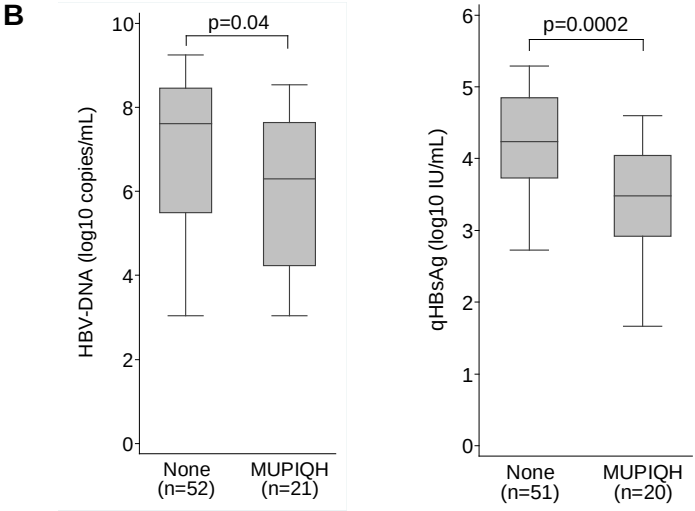
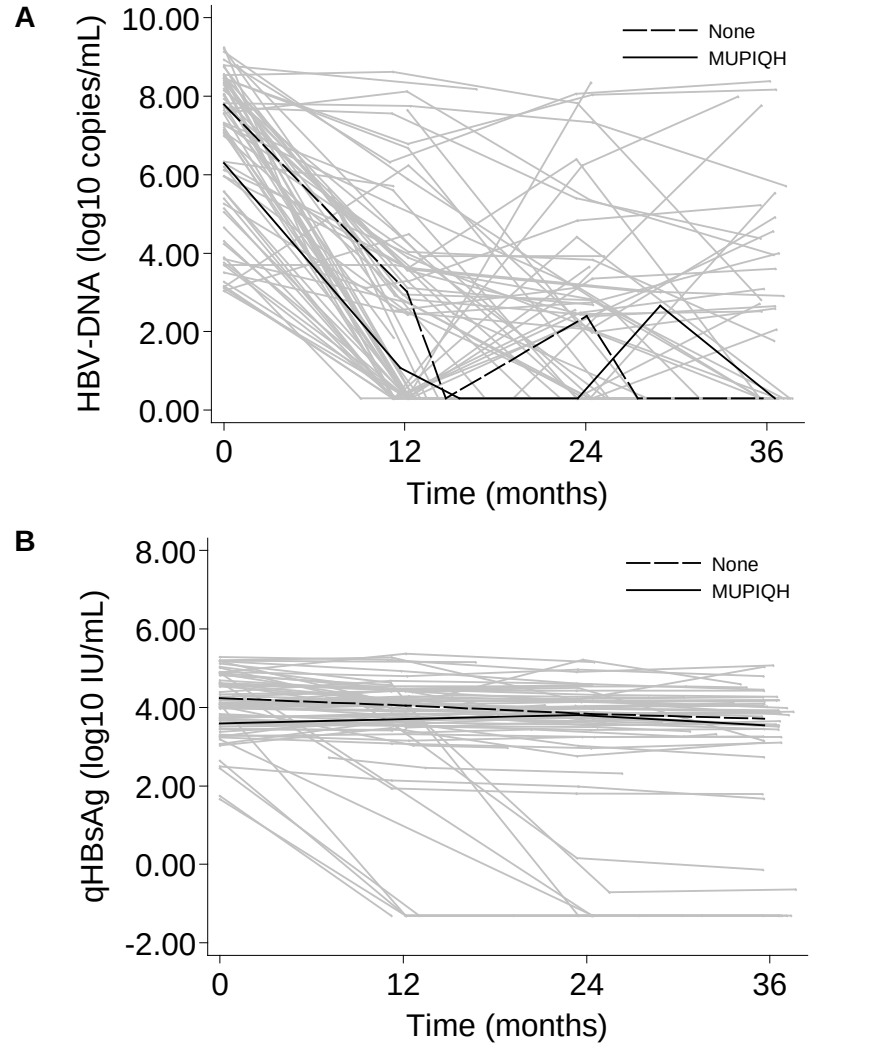
s130
s143
s161


A
HBV-DNA (log10 copies/mL)
WT (n=30)
sS140L (n=4)
sS174N (n=6)
sV224A/Y (n=7)
qHBsAg (log10 IU/mL)
WT (n=30)
sD144A (n=1)
sS167L (n=2)
sP178Q (n=2)
sG185L (n=2)
sW191L (n=2)
sP203Q/R (n=2)
sS204N/I/R/K/T/G (n=7)
sN207T (n=2)
sF212C (n=1)
sV224A/Y (n=7)


B
HBV-DNA (log10 copies/mL)
p=0.04
None (n=52)
MUPIQH (n=21)
qHBsAg (log10 IU/mL)
p=0.0002
None (n=51)
MUPIQH (n=20)


A
10.00
8.00
6.00
4.00
2.00
0.00
HBV-DNA (log10 copies/mL)
None
MUPIQH
0
12
24
36
Time (months)
B
8.00
6.00
4.00
2.00
0.00
-2.00
qHBsAg (log10 IU/mL)
None
MUPIQH
0
12
24
36
Time (months)

SUPPLMENTARY MATERIALS

Supplement to: Boyd A, Moh R, Maylin S, et al. Effect of hepatitis B virus (HBV) S-gene variability on markers of replication during treated HIV-HBV infection in Western Africa.

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SUPPLEMENTARY METHODS

Non-inclusion criteria for the 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").

Determining HBV consensus sequences

As only HBV genotypes A and E were identified (Supplementary Figure 1), consensus sequences were based on these genotypes. All HBV genotype A and E sequences of the small hepatitis B surface protein were retrieved from the HBVdb[1] (accessed: 13 July 2018). The distribution of amino acids (a.a.) at *S*-gene positions s99-s226 was calculated (Supplementary data file). A.a. with a population prevalence >5% were defined as belonging to the consensus sequence.

Recombination analysis

The simple sequence editor (SSE)[2] version 1.3 was used to evaluate evidence of genetic recombination across positions s99-s226 of the *S*-gene. Study sequences were compared to the referent sequences given in Supplementary Table 1 (which were used to define clades).

A.a. alignments of study sequences were initially scanned using the BootScan program on SSE, allowing to detect changes in phylogeny that indicate recombination events. The Kimura two-parameter model was used with 1000 bootstrap replicates along with a consensus threshold of 70% and a fragment length of 90 a.a. and increment of 9 a.a.[3] Any BootScan suggestive of recombination was then re-evaluated using the GroupingScan program on SSE. This method uses a tree scoring method, based on a grouping score, that is more robust compared to the BootScan program, permitting to determine the embeddedness of a study sequence within pre-defined clade of sequences. The Kimura two-parameter model was used with 100 bootstrap replicates along with a consensus threshold of 70% and a fragment length of 90 a.a. and increment of 9 a.a.

If the GroupingScan suggests recombination, a final evaluation of recombination was conducted using the TreeOrder scan program on SSE. A figure is produced whereby changes in sequence orders resulting from changes in phylogeny can be determined along with their breakpoint positions. Alterations in tree order of study sequences are defined by a 70% threshold from 100 bootstraps using a fragment length of 90 a.a. and increment of 9 a.a.

References:

1. Hayer J, Jadeau F, Deléage G, et al. HBVdb: a knowledge database for Hepatitis B Virus. *Nucleic Acids Res.*, 2013;41:D566-D570.
2. Simmonds P. SSE: a nucleotide and amino acid sequence analysis platform. *BMC Res Notes.*, 2012;5:50.
3. Simmonds P, Midgley S. Recombination in the genesis and evolution of hepatitis B virus genotypes. *J Virol*. 2005 Dec;79(24):15467-76.

SUPPLEMENTARY TABLES

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

Genotype	GenBank accession number
A	AB014370[1]; AB194950, AB194951[2]; AB205118[*NR*]; AF090838, AF090842[3]; AM180623, AM180624[4]; FJ692596[5]; HM535205[6]; JN182318, JN182319, JN182320, JN182321, JN182322, JN182323[7]; KF170754[8]; KF922406, KF922407, KF922408, KF922409, KF922414[*NR*]; KJ010776[*NR*].
B	D00329, D00330[9].
C	AB642095, AB642097[10]; AB697502, AB697510[11]; AP011101, AP011104[12]; EU410080, EU410081, EU670263[13]; GQ358157[14].
D	AB048701[15]; AB090268, AB090269[16]; AB493846, AF151735[17]; AF280817[NR]; AJ131956[18]; AJ627219[NR]; AY090452[19]; AY233291, AY233292, AY233294, AY233296[20]; AY738891, AY738912[21]; DQ315778[22]; FJ692502, FJ692505, FJ692506, FJ692507, FJ692508[5]; FJ904435, FJ904439[23]; GQ167301, GQ167302, GQ184322[*NR*]; JQ927384[*NR*]; KF170772, KF170778[8]; L27106[24].
E	AB205188, AB205189, AB205190, AB205191, AB205192, AB205323[25]; AY935700[26]; DQ060822, DQ060823, DQ060824, DQ060825, DQ060826[27]; FJ692540, FJ692542, FJ692543, FJ692544, FJ692545[5].
F	AB036905, AB036906[28].
G	AB056514, AB056515[29].
H	AB266536[30]; AB275308[NR].

NR, no reference.

Sequence references:

1. Ogawa M, Yamaguchi T, Setiyono A, et al. Some characteristics of a cellular receptor for virulent infectious bursal disease virus by using flow cytometry. *Arch Virol*. 1998;143:2327–2341.

2. Kurbanov F, Tanaka Y, Fujiwara K, et al. A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. *J Gen Virol*. 2005;86:2047–2056.
3. Stuyver L, De Gendt S, Cadranel JF, et al. Three cases of severe subfulminant hepatitis in heart-transplanted patients after nosocomial transmission of a mutant hepatitis B virus. *Hepatology*. 1999;29:1876–1883.
4. Olinger CM, Venard V, Njayou M, et al. Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J Gen Virol*. 2006;87:1163–1173.
5. Andernach IE, Nolte C, Pape JW, Muller CP. Slave trade and hepatitis B virus genotypes and subgenotypes in Haiti and Africa. *Emerg Infect Dis*. 2009;15:1222–1228.
6. Gulube Z, Chirara M, Kew M, Tanaka Y, Mizokami M, Kramvis A. Molecular characterization of hepatitis B virus isolates from Zimbabwean blood donors. *J Med Virol*. 2011;83:235–244.
7. Vermeulen M, Dickens C, Lelie N, et al. Hepatitis B virus transmission by blood transfusion during 4 years of individual-donation nucleic acid testing in South Africa: estimated and observed window period risk. *Transfusion*. 2012;52:880–892.
8. Yousif M, Mudawi H, Bakhiet S, Glebe D, Kramvis A. Molecular characterization of hepatitis B virus in liver disease patients and asymptomatic carriers of the virus in Sudan. *BMC Infect Dis*. 2013;13:328.
9. Okamoto H, Tsuda F, Sakugawa H, et al. Typing Hepatitis B Virus by Homology in Nucleotide Sequence: Comparison of Surface Antigen Subtypes. *J Gen Virol*. 1988;69:2575–2583.
10. Inoue J, Ueno Y, Kawamura K, et al. Association between S21 substitution in the core protein of hepatitis B virus and fulminant hepatitis. *J Clin Virol.* 2012;55:147–152.
11. Fujisaki S, Yokomaku Y, Shiino T, et al. Outbreak of infections by hepatitis B virus genotype A and transmission of genetic drug resistance in patients coinfected with HIV-1 in Japan. *J Clin Microbiol*. 2011;49:1017–1024.
12. Mulyanto, Depamede SN, Surayah K, et al. A nationwide molecular epidemiological study on hepatitis B virus in Indonesia: identification of two novel subgenotypes, B8 and C7. *Arch Virol*. 2009;154:1047–1059.
13. Cavinta L, Sun J, May A, et al. A new isolate of hepatitis B virus from the Philippines possibly representing a new subgenotype C6. *J Med Virol*. 2009;81:983–987.
14. Thedja MD, Muljono DH, Nurainy N, Sukowati CHC, Verhoef J, Marzuki S. Ethnogeographical structure of hepatitis B virus genotype distribution in Indonesia and discovery of a new subgenotype, B9. *Arch Virol*. 2011;156:855–868.

15. Butterworth LA, Suzuki S, Ueda R, et al. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol*. 2001;82:883–892.
16. Duong TN, Horiike N, Michitaka K, et al. Comparison of genotypes C and D of the hepatitis B virus in Japan: a clinical and molecular biological study. *J Med Virol*. 2004;72:551–557.
17. Gerner P, Lausch E, Friedt M, Tratzmöller R, Spangenberg C, Wirth S. Hepatitis B virus core promoter mutations in children with multiple anti-HBe/HBeAg reactivations result in enhanced promoter activity. *J Med Virol*. 1999;59:415–423.
18. Petzold DR, Tautz B, Wolf F, Drescher J. Infection chains and evolution rates of hepatitis B virus in cardiac transplant recipients infected nosocomially. *J Med Virol*. 1999;58:1–10.
19. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol*. 2002;83:2059–2073.
20. Kimbi GC, Kramvis A, Kew MC. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol*. 2004;85:1211–1220.
21. Norder H, Couroucé A-M, Coursaget P, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology*. 2004;47:289–309.
22. Banerjee A, Kurbanov F, Datta S, et al. Phylogenetic relatedness and genetic diversity of hepatitis B virus isolates in Eastern India. *J Med Virol*. 2006;78:1164–1174.
23. Meldal BHM, Moula NM, Barnes IHA, Boukef K, Allain J-P. A novel hepatitis B virus subgenotype, D7, in Tunisian blood donors. *J Gen Virol*. 2009;90:1622–1628.
24. Hasegawa K, Huang J, Rogers SA, Blum HE, Liang TJ. Enhanced replication of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *J Virol*. 1994;68:1951–9.
25. Huy TTT, Ishikawa K, Ampofo W, et al. Characteristics of hepatitis B virus in Ghana: full length genome sequences indicate the endemicity of genotype E in West Africa. *J Med Virol*. 2006;78:178–184.
26. Meuleman P, Libbrecht L, Wieland S, et al. Immune suppression uncovers endogenous cytopathic effects of the hepatitis B virus. *J Virol*. 2006;80:2797–2807.
27. Kramvis A, Restorp K, Norder H, Botha JF, Magnius LO, Kew MC. Full genome analysis of hepatitis B virus genotype E strains from South-Western Africa and Madagascar reveals low genetic variability. *J Med Virol*. 2005;77:47–52.
28. Nakano T, Lu L, Hu X, et al. Characterization of hepatitis B virus genotypes among Yucpa Indians in Venezuela. *J Gen Virol*. 2001;82:359–365.

29. Kato H, Orito E, Sugauchi F, et al. Determination of hepatitis B virus genotype G by polymerase chain reaction with hemi-nested primers. *J Virol Methods*. 2001;98:153–159.
30. Kumagai I, Abe K, Oikawa T, et al. A male patient with severe acute hepatitis who was domestically infected with a genotype H hepatitis B virus in Iwate, Japan. *J Gastroenterol*. 2007;42:168–175.

Supplementary Table 2. Hepatitis B virus *S*-gene mutations observed in ≤2 patients at treatment initiation

Position	Mutation
s109	sL109M (*n*=1)
s118	sT118M (*n*=1)
	sT118X (*n*=1)
s121	sC121M (*n*=1)
s126	sT126K (*n*=1)
s127	sL/P127I (*n*=1)
	sL/P127S (*n*=1)
s128	sA128V (*n*=1)
s129	sQ129H (*n*=1)
	sQ129P (*n*=1)
s130	sG130R (*n*=1)
s131	sT/N131I/T (*n*=1)
s133	sM133X (*n*=1)
s135	sP135H (*n*=1)
s143	sS/T143L (*n*=2)
s144	sD144A (*n*=1)
s145	sG145X (*n*=1)
s159	sG/A159X (*n*=1)
s161	sF/Y161X (*n*=1)
s167	sS167L (*n*=2)
s177	sV177A (*n*=2)
s178	sP178Q (*n*=2)
s182	sW182* (*n*=1)
s183	sF183A (*n*=1)
s184	sA/V184G (*n*=1)
s185	sG185L (*n*=2)
s186	sL186S (*n*=1)
s187	sS187P (*n*=1)
s188	sP188T (*n*=1)
s190	sV190W (*n*=1)
s191	sW191L (*n*=2)
s193	sS193L (*n*=1)
s195	sI195X (*n*=1)
s198	sM198T (*n*=1)
s200	sY200X (*n*=1)
s203	sP203Q (*n*=1)
	sP203R (*n*=1)
s206	sY206C (*n*=1)
s207	sN207T (*n*=2)
s211	sP211H (*n*=1)
	sP211P/R (*n*=1)
s212	sF212C (*n*=1)
…	…
s213	sI213T (*n*=1)
	sI213X (*n*=1)
s215	sL215* (*n*=1)
s217	sP217I (*n*=1)
	sP217L (*n*=1)
s218	sI218F (*n*=1)
s219	sF219X (*n*=1)
s221	sC221L (*n*=1)
	sC221Y (*n*=1)
s222	sL222W (*n*=1)
s223	sW223V (*n*=1)
s225	sY225I (*n*=1)
	sY225Y/F (*n*=1)

Abbreviations: HBV, hepatitis B virus; *S*, surface. Mutations labeled “X” represent highly mixed mutant quasi-species.

Supplementary Table 3. Characteristics of patients with *S*-gene MUPIQHs at treatment initiation

Patient	Age (yrs)	Sex	CD4+ (/mm^3)	HBV DNA (log_{10} copies/mL)	Genotypic findings	
					S-gene	Corresponding *pol*-gene
AB001	53	F	286	7.20	sS167L	None
AB158	43	F	405	4.23	sN207T	None
CF112	29	F	467	5.04	sV224A/Y	None
CN138	44	F	166	5.96	sS174N, sP178Q, sG185L, sS204N, sV224A	None
CN142	52	M	401	7.78	sS167L, sP178Q, sW191L, sN207T	None
PF052	36	M	195	4.31	sS204I/K/R/S	None
PF069	46	F	144	7.71	sS174N	None
PF078	34	M	380	3.11	sP203Q	None
RB023	32	F	384	8.53	sV224A	None
RB035	27	F	298	8.28	sS140L, sS174N, sV224A	None
RB051	34	F	255	7.12	sP203R	None
RB065	45	M	237	3.04	sS140L, sS174N, sV224A	None
RB087	53	F	139	3.86	sS140L, sS174N, sV224A	None
RB126	36	F	225	7.32	sS204T	None
RB158	32	F	113	5.57	sD144A	None
SM008	34	M	139	8.49	sS204G	None
SM038	44	M	279	6.30	sS204S/N	None
UC063	37	M	248	7.14	sF212C	None
US022	28	M	202	3.14	sS174S/N, sV224Y	None
US125	42	M	155	7.64	sG185L, sW191L, sS204G	None
US139	37	M	473	3.51	sS140L, sS204N	None

Abbreviations: F, female; HBV, hepatitis B virus; M, male; MUPIQH, mutations possibly influencing the quantification of HBV replication markers.

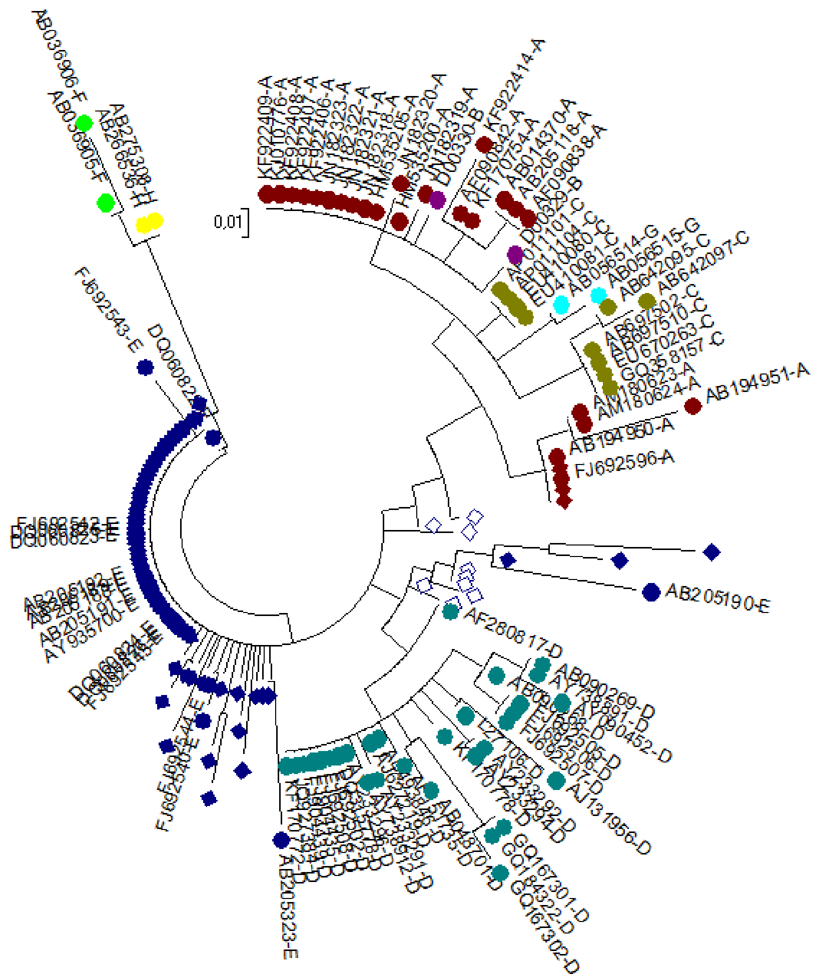
Supplementary Table 4. Description of the two patients with incident *S*-gene MUPIQHs

	Patient PF162	Patient RB092
Age, years	43	35
Sex	Male	Female
HBV genotype	E	E
Anti-HBV therapy at ART-initiation	LAM	LAM
At inclusion		
HIV-RNA, log_{10} copies/mL	5.18	N/A
CD4+ T-cell count, /mm^3	306	227
HBV-DNA, log_{10} copies/mL	8.33	5.97
qHBsAg, log_{10} IU/mL	4.91	3.37
HBeAg status	Pos	Neg
ALT, IU/L	77	53
At incident mutation		
Follow-up time, years	36	24
S-gene MUPIQH	sS174S/N	sS210R+sF212S
Antiviral resistance mutations	None	None
Change in CD4+ cell count, /mm^3	+168	-6
Change in HBV-DNA, log_{10} copies/mL	-3.11	+0.43
Change in qHBsAg, log_{10} IU/mL	-0.12	+0.37
HBeAg status	Pos	Neg
HBsAg status	Pos	Pos

Abbreviations: ALT, alanine aminotransaminase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; LAM, lamivudine; MUPIQH, mutations possibly influencing the quantification of HBV replication markers; N/A, not applicable; Neg, negative; Pos, positive; qHBsAg, hepatitis B surface antigen quantification.

SUPPLEMENTARY FIGURES

Supplementary Figure 1. Phylogenetic tree for baseline amino acid (a.a.) sequences based on the small hepatitis B surface protein of the hepatitis B virus (HBV)



Individual a.a. sequences from patient samples taken prior to antiviral therapy are compared with small hepatitis B surface protein (*S*-gene) sequences from HBV genotype A-H referent strains. Referent strains are labeled with GenBank ascension number (Supplementary Table 1) followed by a "-" and established genotype. Referent strains are represented as circles and study samples as diamonds, while colors are used to differentiate genotypes. Full diamonds indicate that study genotype could be established by the *S*-gene phylogenetic tree alone. Outlined diamonds indicate that study genotypes were ambiguous and had to be determined from a phylogenetic tree based on HBV *precore* sequences from a previous study.[1] The tree

with the highest log likelihood (-1049.93) after 1000 bootstrapped samples is shown. In total, three patients harbored genotype A and 70 harbored genotype E.

References:

1. 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. *J Viral Hepat.* 2018 [in press]