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Kinetics of hepatitis B virus replication in anti-HBc positive/HBsAg-negative people with HIV switching to tenofovir sparing therapy

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ABSTRACT

Objectives: To unravel the still unexplored HBV-replicative kinetics in anti-HBc-positive/HBsAg-negative people-with-HIV (PWH) suspending tenofovir disoproxil-fumarate/tenofovir-alafenamide (TDF/TAF).
Methods: A total of 101 anti-HBc-positive/HBsAg-negative PWH switching to TDF/TAF-sparing therapy were included. Serum HBV-DNA and HBV-RNA were quantified by droplet-digital-PCR at switching (T0), within 12 months (T1) and 12-24 months postswitch (T2).
Results: At T0, 33.7% had cryptic HBV-DNA (undetected by commercial assays, median [interquartile range (IQR)]: 2 [1-5] IU/mL) and 22% were positive to HBV-RNA alone (median [IQR]: 4 [3-4] IU/mL), indicating an active HBV-reservoir despite HBsAg-negativity and TDF/TAF-pressure. Notably, anti-HBs-titer <100 mIU/mL independently correlated with cryptic HBV-DNA at T0 (OR [95% CI]: 2.6 [1.02-6.5], $P = 0.04$). After TDF/TAF-withdrawal, the rate of PWH achieving HBV-DNA >10 IU/mL increased from 12.9% at T1 to 42.6% at T2 ($P < 0.0001$). Likewise, a rise from 2 to 11% was observed for HBV-DNA >100 IU/mL ($P = 0.02$); median (IQR) HBV-DNA: 579 (425-770) IU/mL. Notably, HBV-DNA >10 IU/mL at T2 occurred in 70% of PWH with cryptic HBV-DNA, in 38.5% with HBV-RNA alone and in 25% negative to both HBV-markers at T0 ($P = 0.01$). Cryptic HBV-DNA at T0 and lower nadir CD4+ T-cell-count independently predicted HBV-DNA >10 IU/mL at T2 (OR [95% CI]: 8.2 [1.7-40.6], $P = 0.01$; OR [95% CI]: 8.1 [1.3-52.1], $P = 0.03$). Lastly, persistent HBV-DNA positivity was independently associated with a reduced CD4+ T-cell recovery at T2 (OR [95% CI]: 0.07 [0.01-0.77], $P = 0.03$).
Conclusion: This study underlines the importance to regularly monitor anti-HBc-positive/HBsAg-negative PWH undergoing TDF/TAF-sparing regimen and the role of highly-sensitive HBV markers in optimizing their management.
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Introduction

Worldwide, 15-30% of people-with-HIV (PWH) is estimated to harbor a serological profile characterized by the positivity to antibodies against HBV core-protein (anti-HBc) and negativity to HBV surface-antigen (HBsAg) [1,2]. This serological profile can reflect the presence of an occult hepatitis B infection, characterized by intrahepatic persistence of replication-competent HBV-DNA (HBV circular covalently-closed DNA [cccDNA]), whose transcriptional activity is controlled by immune-response [3]. This condition is particularly relevant in the setting of HIV-infection since HIV-related immune-dysfunction can reduce the immune-control of cccDNA activity thus promoting a persistent (albeit limited) HBV-replication that can predispose to the re-uptake of HBV-replication under sub-optimal pharmacological pressure that can contribute to promote liver disease progression [4,5]. In PWH, anti-HBc positivity/HBsAg-negativity has been associated with higher risk to develop end-stage liver diseases [6,7] and can represent a cofactor promoting persistent immune-activation and systemic inflammation, thus accelerating HIV-related disease progression [8,9]. More recently, anti-HBc positivity/HBsAg-negativity has been also associated with an incomplete HIV-suppression coupled with a delayed immune-recovery under combined antiretroviral treatment (cART) [10,11].

So far, tenofovir disoproxil-fumarate/tenofovir-alafenamide (TDF/TAF)-sparing regimens are increasingly used for optimizing or simplifying antiretroviral strategies [12]. Nevertheless, the current guidelines for PWH management do not provide clear recommendations for anti-HBc positive/HBsAg-negative candidates to suspending TDF or TAF [12]. Indeed, this issue has been analyzed by few studies [5], thus deserving further investigation in larger populations with longitudinal follow-up.

Recently, novel HBV-biomarkers have been proposed to reflect cccDNA amount and its transcriptional activity [13,14], thus representing useful tools to optimize the clinical management of HBV-infection. Among them, serum HBV-RNA measures the virions containing pregenomic RNA lacking the reverse-transcriptase process [14]. This biomarker has been proposed as a minimally invasive parameter to track cccDNA-activity under antiviral therapy [14].

Furthermore, highly-sensitive assays for HBV-DNA quantification have been recently developed to reveal minimal levels of HBV-replication, not detected by commercial assays [13,15]. This issue is critical since there is increasing evidence that residual HBV replicative-activity may promote liver fibrosis progression and increase the risk of HCC development [16,17].

So far, no studies have extensively investigated the above-mentioned markers among anti-HBc positive/HBsAg-negative PWH under TDF/TAF-including ART and after TDF/TAF-suspension. In this light, by applying novel highly-sensitive HBV-biomarkers, this study aims at assessing HBV replicative kinetics in anti-HBc positive/HBsAg-negative PWH switching to TDF/TAF-sparing therapy and to define factors correlated with the re-uptake/enhancement of HBV replication after TDF/TAF withdrawal.

Methods

Study population

This study included 101 anti-HBc positive/HBsAg-negative PWH from ICoNA cohort, a multicenter Italian cohort including PWH from 60 Infectious Diseases centers [18].

All PWH were receiving TDF/TAF-containing ART for ≥12 months and then switched to a TDF/TAF-sparing ART. No PWH experienced HIV-rebound (2 consecutive serum HIV-RNA >50 copies/mL) during the 12 months before TDF/TAF-withdrawal.

For all individuals, 2 serum samples were available: the former at the time of TDF/TAF-withdrawal (T0, collected within 6 months before the therapeutic switch) and the latter collected during the 12 months after TDF/TAF-withdrawal (T1). For 54/101, a serum sample, collected between 12 and 24 months after the therapeutic switch (T2), was also available.

Notably, all the 101 included patients had at least 2 HBsAg-negative measurements: at T0, tested by a chemiluminescence-based immunoassay (LIAISON-XL Murex HBsAg Quant assay [DiaSorin, Saluggia], cut-off for HBsAg detection: 0.05 IU/mL) and at a previous time-point available at a median (interquartile range [IQR]) time of 3.1 (1.7-5.3) years before T0, tested by the commercially available immunoassays, routinely used in the laboratories included in ICONA cohort (cut-off for HBsAg detection: 0.05 IU/mL).

The following information was collected: sex, age, country of origin, transmission route, HIV-diagnosis date, TDF/TAF duration, TDF/TAF-sparing therapy, ART-start date, nadir CD4+ T-cell count, HIV-RNA, CD4+ T-cell count and ALT levels at each time-point.

For all individuals, we quantified serum HBV-DNA and HBV-RNA by highly-sensitive droplet-digital PCR (ddPCR) assays at each time-point, while anti-HBs titers were quantified at T0.

Serum HBV-DNA levels ranging from 1 to 10 IU/mL were defined as "cryptic viremia," as they were quantified by the highly-sensitive ddPCR assay but not by the classical commercial Real-Time PCR assays [15,19].

Serum HBV-DNA levels >10 IU/mL were used as cut-off to define the re-uptake of an HBV active replication, according to the lower limit of quantification of the classical commercial Real-Time PCR assays, most widely used in clinical practice. This is in compliance with the APASL, AASLD and AGA guidelines defining the reuptake of active HBV replication for HBsAg-negative, anti-HBc+ as the development of HBV-DNA at levels detectable by routinely used assays [20].

Highly-sensitive quantification of serum HBV-DNA by ddPCR

As reported in an our previous study [13], the highly-sensitive ddPCR HBV-DNA assay targets a 102 nt sequence of S gene region and is performed using all DNA extracted from 1 mL of patient's serum. Briefly, HBV-DNA was extracted from 1 mL of serum with the Nucleic Acid Extraction System eMAG (Biomerieux, France) in 50 µL of elution buffer, in duplicate. The extract was dried by using Savant DNA SpeedVac Concentrators at low temperature for 1 hour (Thermo Fisher Scientific, USA), and then resuspended in 10 µL of nuclease-free water.

According to the manufacturer's instructions, 5 µL of sample were added to 15 µL of ddPCR Supermix for Probes (No dUTP) (Bio-Rad, USA) mastermix and, after droplet generation, amplified in a thermocycler according to the following protocol: 10 minutes at 95°C, 40 cycles of 30 seconds at 95°C and 1 minute at 55°C, 10 minutes at 98°C and 30 minutes at 4°C, with a ramping rate of 2°C/s. After amplification, HBV-DNA was quantified by QX200 Droplet Reader (Bio-Rad, USA) and the results were expressed in copies and normalized on 1 mL of serum. In order to finally express HBV-DNA ddPCR results as IU/mL favoring the comparison with commercial assays, we calculated the conversion factor between HBV copies/mL and HBV IU/mL by analyzing 5 serial dilutions at least in duplicates (from 4 log to 1 IU/mL) of the WHO International Reference Standard for HBV-DNA testing (expressed as IU/mL). In line with literature [21], the conversion factor, calculated as mean value of the conversion factors obtained at the different tested HBV-DNA loads, resulted 5.6 copies/IU. This conversion factor was then applied to all HBV-DNA values obtained by ddPCR (in copies/mL) in order to report all values in IU/mL.

This assay for HBV-DNA quantification is characterized by a very good linearity ($R^2 = 0.996$ in the range from 4 log to 1 IU/mL), a high accuracy and reproducibility (the differences between the duplicate values in the same run never exceeded 0.1 log IU/mL, and 0.2 log IU/mL considering duplicates from 3 different runs). The limit of detection/quantification (LLoQ) of the ddPCR assay was 1 IU/mL, according to Probit analysis [13], highlighting a very high sensitivity.

Highly-sensitive quantification of serum HBV-RNA by ddPCR

In order to quantify serum HBV-RNA, 1 mL of serum was ultracentrifuged at 23,000 g for 90 minutes to pull down virions. After ultracentrifugation, viral pellet was used for RNA extraction by using the QIAamp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer instructions and eluted in 50 µL of elution buffer.

To increase the sensitivity of HBV-RNA quantification, eluted RNA was dried by Savant DNA SpeedVac Concentrator (Thermo Fisher Scientific, USA) at low temperature for 1 hour and then re-suspended in a volume of 10 µL Nuclease-free water. All the 10 µL of sample were tested by ddPCR assay optimized by ad-hoc primers and probe, designed on the ORF C of HBV genome, specifically targeting pregenomic RNA. The sequences of primers and probe used for HBV-RNA quantification are reported in Table S1. According to the manufacturer's instructions, 5 µL of sample were added to 15 µL of One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, USA) mastermix and, after droplet generation, amplified in a thermocycler according to the following protocol: 1 hour at 45°C, 40 cycles of 30 seconds at 95°C and 1 minute at 51°C, 10 minutes at 98°C and 30 minutes at 4°C, with a ramping rate of 2°C/s. After amplification, HBV-RNA was quantified by QX200 Droplet Reader (Bio-Rad, USA) and the results were expressed in copies and normalized on 1 mL of serum. The conversion factor of 5.6 copies/IU was then applied to all HBV-RNA values obtained by ddPCR (in copies/mL) in order to express all HBV-RNA results as IU/mL. This assay for HBV-RNA quantification is characterized by an excellent linearity ($R^2 = 0.997$ in the range from 4 log to 2 HBV-RNA IU/mL), a high accuracy (the differences between the mean observed values and expected values range from 0.1 to 0.3 log IU/mL of HBV-RNA) by using as reference assay the Cobas HBV-RNA (Roche). Furthermore, the assay demonstrated a very high sensitivity showing a lower limit of detection of 2 IU/mL as defined by Probit analysis [22].

Quantification of anti-HBs titers

Anti-HBs titer was quantified by DiaSorin Anti-HBsIICLIA assay on the fully-automated platform LIAISONXL (DiaSorin, Italy). The cut-off for anti-HBs positivity was 10 mIU/mL.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 9 and IBM-SPSS Statistics23.0 (Armonk, NY).

Data were expressed as median (IQR) for quantitative variables and as counts and percentages for qualitative variables. Chi-squared test based on contingency table 2 × 2 and 3 × 2 were used for qualitative data, while Mann–Whitney unpaired *t*-test and Kruskal–Wallis *t*-test for continuous data ($P < 0.05$ as cut-off for statistical significance).

Uni- and multi-variable logistic regression analyses were performed to assess factors correlated with cryptic HBV viremia at T0, achievement of HBV-DNA >10 IU/mL at T2 and CD4+ T-cell count change at T2. After stepwise elimination for optimized Akaike information criterion, variables with $P < 0.2$ in univariable analysis were included in multivariable model.

Table 1
Characteristics of the study population.

Variables	*N* = 101
Male, *N* (%)	87 (86.1%)
Age in years, median (IQR)	51 (46-58)
Italian, *N* (%)	91 (90%)
HIV risk factor	
Heterosexual, *N* (%)	33 (32.7%)
MSM, *N* (%)	49 (48.5%)
IDU, *N* (%)	19 (18.8%)
Duration of HIV infection in years, median (IQR)	6.7 (3.4-13.7)
Nadir CD4+ T-cell count in cells/mm^3, median (IQR)	233 (98-331)
ART duration in years, median (IQR)	4.8 (2.2-10.8)
TDF/TAF duration in years, median (IQR)	4.3 (2.3-6.6)
Monitoring from TDF/TAF suspension in months, median (IQR)	13.1 (7.1-15.4)
Switch to 3TC, *N* (%)	73 (72.3%)
Parameters at TDF/TAF suspension (T0)	
Anti-HBs Ab positivity *N* (%)[a]	63 (67%)
Anti-HBs Ab titer, median (IQR) mIU/mL[a]	69 (10-932)
CD4+ T-cell count in cells/mm^3, median (IQR)	648 (463-809)
HIV-RNA <200 copies/mL, *N* (%)[b]	101 (100%)
ALT levels, median (IQR) U/L	29 (19-40)

Ab, antibodies; ALT, alanine transaminases; Anti-HBs, antibodies anti hepatitis B surface antigen; ART, antiretroviral therapy; IDU, injecting drug users; MSM, men who have sex with men; TAF, tenofovir alafenamide fumarate; TDF, tenofovir disoproxil fumarate.

[a] Datum available for 94/101 individuals.

[b] 99 PWH showed plasma HIV-RNA <50 copies/mL while the remaining 2 PWH had a single blip of plasma HIV-RNA equal to 83 and 199 copies/mL.

Results

Study population

This study included 101 anti-HBc positive/HBsAg-negative PWH, under virological success for ≥12 months and switching to TDF/TAF-sparing ART. Characteristics of study population are depicted in Table 1. PWH were predominantly male (86.1%) with a median (IQR) age of 51 (46-58) years and were treated with TDF/TAF-based ART for a median (IQR) time of 4.3 (2.3-6.6) years (Table 1).

At the time of TDF/TAF-suspension (defined as T0), median (IQR) CD4+ T-cell count was 648 (463-809) cells/mm^3 and median (IQR) ALT was 29 (19-40) U/L (Table 1). Furthermore, 67% PWH were anti-HBs positive with median titers of 69 (10-932) mIU/mL (Table 1). After TDF/TAF-suspension, PWH were monitored for a median (IQR) time of 13.1 (7.1-15.4) months. Among them, 72.3% switched to a 3TC-based ART (Table 1).

Markers of HBV replicative activity at TDF/TAF-suspension (T0)

The first step of this study was to evaluate the markers of HBV replicative-activity at T0 (still under TDF/TAF pharmacological pressure). Notably, by highly-sensitive ddPCR, a cryptic serum HBV-DNA was detected in 33.7% (34/101) of anti-HBc positive/HBsAg-negative PWH (median [IQR]: 2 [1–5] IU/mL) (Figure 1a). Notably, the concomitant positivity to serum HBV-DNA and HBV-RNA at baseline was observed in 9.9% (10/101) (median [IQR] levels: median [IQR] levels: 1 [1–3] IU/mL for HBV-DNA and 4 [3–4] IU/mL for HBV-RNA (Figure 1a). By multivariable analysis, the only factor significantly correlated with the detection of HBV-DNA at T0 was an anti-HBs titer <100 mIU/mL (OR [95% CI]: 2.6 [1-6.5], $P = 0.04$) (Table 2).

Notably, 21.8% (22/101) of PWH was positive to HBV-RNA [median (IQR): 4 (3-6) IU/mL], despite HBV-DNA undetectability by ddPCR, indicating a residual HBV transcriptional activity (Figure 1a). Lastly, 44.5% (45/101) of PWH was characterized by

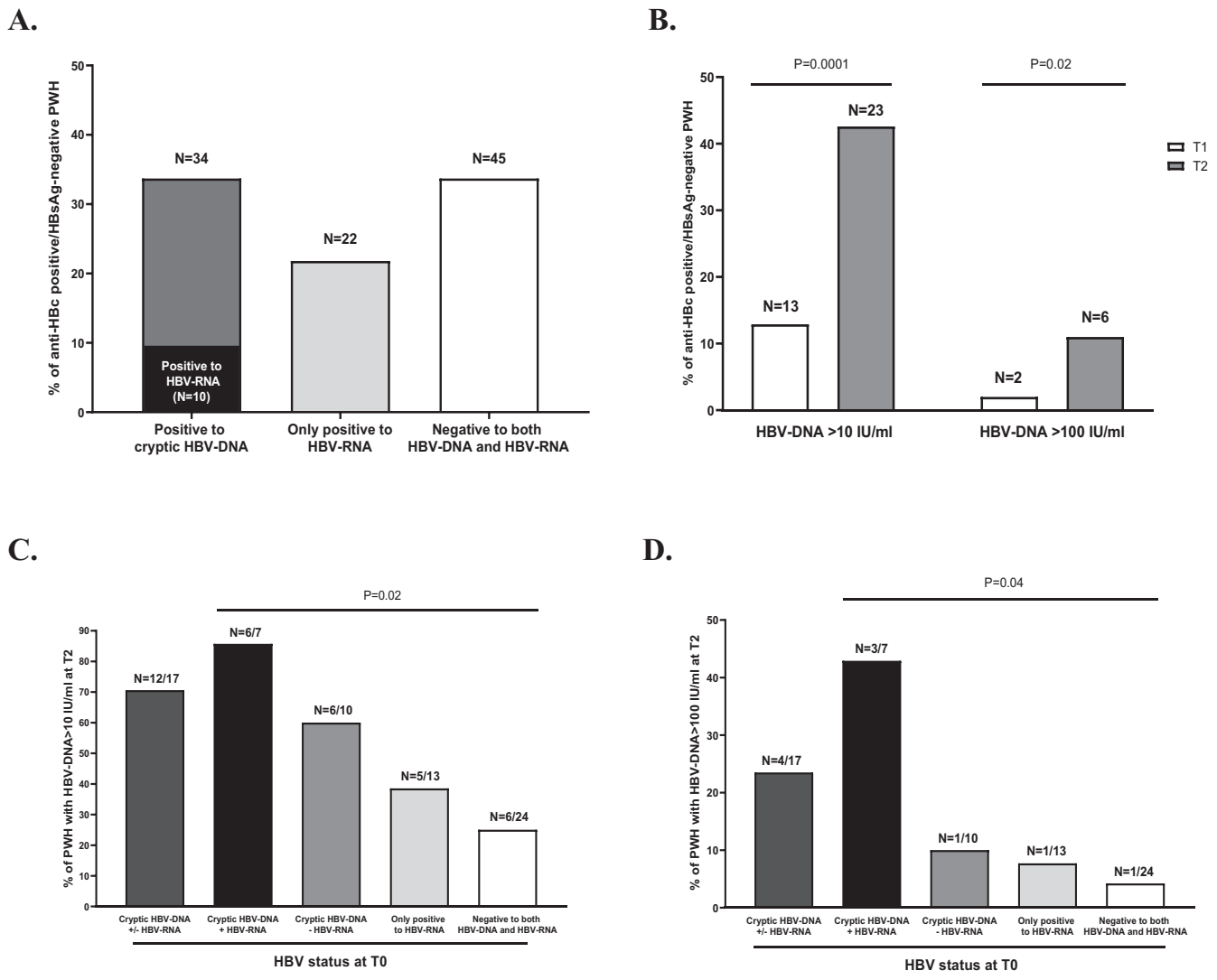

Figure 1. (A) The histogram reports the percentages of anti-HBc positive/HBsAg-negative PWH positive to cryptic HBV-DNA ($N = 34$), positive to HBV-RNA alone ($N = 22$) and negative to both HBV-DNA and HBV-RNA at T0 ($N = 45$). The black bar shows the percentage of PWH positive to both HBV-RNA and HBV-DNA ($N = 10$). (B) The histogram reports the percentages of individuals achieving HBV-DNA >10 IU/mL at T1 ($N = 13$) and at T2 ($N = 23$) and >100 IU/mL at T1 ($N = 2$) and at T2 ($N = 6$) after TAF/TDF withdrawal. Statistically significant differences (*P*-values) were assessed by Chi-squared test. (C) The histogram reports the percentages of individuals achieving HBV-DNA >10 IU/mL at T2 after TAF/TDF withdrawal, stratified according to positivity to HBV markers at T0. For each bar, the absolute number of PWH achieving HBV-DNA >10 IU/mL at T2 on the overall number of patients for each category is reported. Statistically significant differences (*P*-values) were assessed by Chi-squared test for trend. (D) The histogram reports the percentages of individuals achieving HBV-DNA >100 IU/mL at T2 after TAF/TDF withdrawal, stratified according to positivity to HBV markers at T0. For each bar, the absolute number of PWH achieving HBV-DNA >100 IU/mL at T2 on the overall number of patients for each category is reported. Statistically significant differences (*P*-values) were assessed by Chi-squared test for trend.

Table 2
Uni- and multivariable logistic regression analysis of factors associated with cryptic serum HBV-DNA at T0.

Variables	Univariable analysis[a] crude OR (95% CI)	*P*-value	Multivariable analysis[a] adjusted OR (95% CI)	*P*-value
Sex (female vs male[b])	2.3 (0.7-7.8)	0.18	2.02 (0.6-7.2)	0.28
Age (per year increase)	1 (0.95-1.05)	0.91		
Nationality (non-Italian vs Italian[b])	0.5 (0.1-2.4)	0.37		
HIV risk factor				
IDU	1.9 (0.6-6.6)	0.28		
MSM	0.9 (0.3-2.4)	0.82		
Heterosexual[b]	1			
TDF/TAF duration (per year increase)	0.9 (0.8-1.1)	0.8		
Nadir CD4+ T cell count <100 cells/mm^3 (yes vs no[b])	0.5 (0.2-1.5)	0.2	0.5 (0.2-1.3)	0.15
CD4+ T cells/mm^3 at T0 (per 100 cells increase)	0.9 (0.8-1.1)	0.42		
Anti-HBs <100 mIU/mL (yes vs no[b])	**2.5 (1-6.1)**	**0.05**	**2.6 (1-6.5)**	**0.04**

Anti-HBs, antibodies anti hepatitis B surface antigen; CI, confidence interval; IDU, injecting drug users; MSM, men who have sex with men; OR, odds ratio; TAF, tenofovir alafenamide fumarate; TDF, tenofovir disoproxil fumarate.
Variables significantly associated with the cryptic HBV-DNA at T0 are reported in bold.
[a] Univariable and multivariable logistic regression analysis was performed on 94 HBsAg negative/anti-HBc positive PLWH with an available anti-HBs titer.
[b] Reference group.

the complete negativity to both HBV-DNA and HBV-RNA by ddPCR, suggesting a silent HBV activity (Figure 1a).

Kinetics of HBV-replicative activity after switching to TDF/TAF-sparing regimens

In a time-window of 12 months after TDF/TAF-withdrawal (T1), 12.9% (13/101) anti-HBc positive/HBsAg-negative PWH achieved a serum HBV-DNA >10 IU/mL (Figure 1b) (corresponding to the LLoQ of the quantitative assays mostly used in clinical practice and considered as cut-off for defining HBV-DNA re-uptake [20]) with median (IQR) levels of 31 (15-73) IU/mL. Among them, two achieved a serum HBV-DNA >100 IU/mL (Figure 1b): the former (receiving no HBV-active drug) achieved HBV-DNA of 1050 IU/mL and the latter (receiving 3TC) of 274 IU/mL at T1. Despite a comparable rate of HBV-DNA >10 IU/mL in PWH receiving or not 3TC (13.7% vs 10.7%, $P = 1.0$), HBV-DNA levels tended to be lower in the subset of PWH receiving 3TC than those receiving no anti-HBV active drug (median [IQR]: 28 [16-57] vs 96 [54-573] IU/mL), suggesting that 3TC usage could constrain HBV replication after TDF/TAF withdrawal.

Slightly altered ALT (<1.5 ULN) was observed in 3 out of the 13 individuals with HBV-DNA >10 IU/mL (23.1%).

Notably, in the second analyzed time-window, including follow-up samples collected from 12 to 24 months post TDF/TAF-withdrawal (T2, available for 54/101), a remarkable increase in the percentage of PWH achieving HBV-DNA >10 IU/mL was observed respect to T1 (42.6% at T2 vs 12.9% at T1, $P < 0.0001$) (Figure 1b). Superimposable results were observed by focusing on PWH achieving HBV-DNA >100 IU/mL (11% at T2 vs 2% at T1, $P = 0.02$) (Figure 1b). Among them, median (IQR) levels of HBV-DNA at T2 were 579 (425-770) IU/mL, remarking the relevance of long-term surveillance of HBV-DNA levels after TDF/TAF-withdrawal.

Likewise T1, despite a comparable rate of HBV-DNA >10 IU/mL in PWH receiving or not 3TC (42.9% vs 42.1%, $P = 1.0$), HBV-DNA levels tended to be lower in PWH receiving 3TC (median [IQR]: 25 [20-56] vs 60 [27-195] IU/mL).

Slightly altered ALT (<1.5 ULN) was observed in 2 out of the 23 individuals with HBV-DNA >10 IU/mL at T2 (8.7%), while one patient achieved 159 U/L. Nevertheless, no severe adverse events at hepatic level were observed in patients experiencing HBV-DNA >10 IU/mL during the analyzed study period. Furthermore, the re-introduction of TDF/TAF occurred in 8 patients after a median (IQR) time of 2.5 (1.7-4.1) years: 2 out of 13 patients (15.4%) experiencing HBV-DNA >10 IU/mL at T1 and 6 out of 23 patients (26.1%) experiencing HBV-DNA >10 IU/mL at T2. Median (IQR) HBV-DNA was 173 (122-223) IU/mL while ALT flares before the re-introduction of TDF/TAF were observed in 2 patients (87 and 71 U/L).

Viro-immunological factors correlated with HBV-DNA >10 IU/mL at T2

As further step of our analysis, we evaluated the viro-immunological factors predicting the achievement of HBV-DNA >10 IU/mL at T2, the time-point characterized by the highest rate of HBV-DNA reuptake after TDF/TAF withdrawal.

Notably, the achievement of HBV-DNA >10 IU/mL at T2 was more frequently observed in PWH with cryptic HBV-DNA at T0 (70.6% [12/17]). This percentage was even higher in the subset of PWH positive to both cryptic HBV-DNA and HBV-RNA at T0 (85.7% [6/7] positive to both cryptic HBV-DNA and HBV-RNA vs 60% [6/10] positive to HBV-DNA and negative to HBV-RNA) (Figure 1c). Conversely, lower rates of HBV >10 IU/mL were observed in PWH positive only to HBV-RNA at T0 (5/13, 38.5%) and in those negative to both HBV markers at T0 (25% [6/24]), P for trend = 0.02 (Figure 1c). Superimposable results were observed in PWH achieving HBV-DNA >100 IU/mL at T2 (P for trend = 0.04) (Figure 1d).

Furthermore, positivity to both cryptic HBV-DNA and HBV-RNA at T0 significantly correlated with higher HBV-DNA levels at T2 (median [IQR] HBV-DNA: 78 [37-345] with vs 27 [23-69] IU/mL without double positivity to cryptic HBV-DNA and HBV-RNA at T0, $P = 0.02$).

By multivariable analysis, the positivity to cryptic HBV-DNA at T0 (independently from HBV-RNA status) was confirmed to be significantly correlated with a higher risk to achieve HBV-DNA >10 IU/mL post 24 months after TDF/TAF-withdrawal (OR [95% CI]: 8.2 [1.7-40.6], $P = 0.01$) (Table 3). Another factor positively associated with HBV-DNA >10 IU/mL at T2 was a nadir CD4+ T-cell count <100 cells/mm^3 (OR [95% CI]: 8.1 [1.3-52.1], $P = 0.03$), confirming the role of immune impairment in promoting the re-uptake of HBV replication (Table 3).

Table 3
Uni- and multivariable logistic regression analysis of factors associated with the achievement of HBV-DNA >10 IU/mL at T2.

Variables	Univariable analysis crude OR (95% CI)	*P*-value	Multivariable analysis adjusted OR (95% CI)	*P*-value
Sex (female vs male[a])	4 (0.7-23)	0.12	4 (0.4-36.6)	0.22
Age (per year increase)	1 (0.9-1.1)	0.28		
Nationality (Non Italian vs Italian[a])	1.4 (0.1-23)	0.83		
HIV risk factor				
IDU	1.4 (0.3-7.5)	0.67		
MSM	0.7 (0.2-2.3)	0.52		
Heterosexual[a]	1			
ART duration (per year increase)	1 (0.9-1.2)	0.15	0.9 (0.6-1.2)	0.37
TDF/TAF duration (per year increase)	1 (0.9-1.2)	0.77		
Switch to 3TC (yes vs no[a])	1 (0.3-3.2)	0.96		
Duration of HIV infection (per year increase)	1 (0.9-1.2)	0.1	1.2 (0.9-1.5)	0.17
Zenith HIV-RNA (per $\log_{10}$ copies/mL increase)	0.8 (0.4-1.9)	0.65		
Nadir CD4+ T-cell count <100 cells/mm^3 (yes vs no[a])	**3.6 (0.9-14)**	**0.06**	**8.1 (1.3-52.1)**	**0.03**
CD4+ T cells/mm^3 at T0 (per 100 cells increase)	0.9 (0.8-1.1)	0.34		
Anti-HBs <100 mIU/mL (yes vs no[a])	2 (0.6-6.5)	0.25		
HBV markers at T0				
Positive to cryptic HBV-DNA	7.2 (1.8-29)	0.005	8.2 (1.7-40.6)	0.01
Only positive to HBV-RNA	1.9 (0.4-8)	0.4		
Negative to both HBV markers[a]	1			

Variables significantly associated with the achievement of HBV-DNA >10 IU/mL at T2 are reported in bold.
Anti-HBs, antibodies anti hepatitis B surface antigen; ART, antiretroviral therapy; CI, confidence interval; IDU, injecting drug users; MSM, men who have sex with men; OR, odds ratio; TAF, tenofovir alafenamide fumarate; TDF, tenofovir disoproxil fumarate.
[a] Reference group.

Table 4
Uni- and multivariable logistic regression analysis of factors associated with CD4+ T-cell count recovery of at least 50 cells/mm^3.

Variables	Univariable analysis crude OR (95% CI)	*P*-value	Multivariable analysis Adjusted OR (95% CI)	*P*-value
Sex (female vs male[a])	**5.8 (0.6-51.5)**	**0.12**	**20.3 (1.2-357.6)**	**0.04**
Age (per year increase)	1 (0.9-1.1)	0.53		
HIV risk factor				
IDU	0.8 (0.2-4.4)	0.83		
MSM	0.8 (0.2-2.7)	0.67		
Heterosexual[a]	1			
ART duration (per year increase)	1 (0.9-1.1)	0.45		
Switch to 3TC (yes vs no[a])	1.2 (0.4-3.7)	0.75		
Duration of HIV infection (per year increase)	1 (0.9-1.1)	0.76		
Zenith HIV-RNA (per $\log_{10}$ copies/mL increase)	1 (0.4-2.4)	0.95		
Nadir CD4+ T-cell count <100 cells/mm^3 (yes vs no[a])	0.3 (0.1-1.2)	0.09	0.3 (0.1-1.2)	0.08
CD4+ T cells/mm^3 at T0 (per 100 cells increase)	1 (0.8-1.1)	0.7		
Monitoring time (per month increase)	0.9 (0.8-1)	0.08		
HBV-DNA >10 IU/mL at T2 (yes vs no[a])	0.8 (0.3-2.3)	0.67		
Positivity to HBV-DNA at T0 and at T1				
Persistent positive at both time-points	**0.2 (0.02-1)**	0.05	**0.1 (0.01-0.8)**	**0.03**
Intermittent positive at only one time-point	0.6 (0.2-2)	0.38		
Negative at both time-points	1			

Variables significantly associated with CD4+ T-cell count recovery of at least 50 cells/mm^3 are reported in bold.
ART, antiretroviral therapy; CI, confidence interval; IDU, injecting drug users; MSM, men who have sex with men; OR, odds ratio.
[a] Reference group.

Impact of the positivity to HBV markers over time on HBV reuptake and immunological recovery at T2

We then evaluated the impact of a persistent or intermittent positivity to HBV-DNA at T0 and T1 on the achievement of serum HBV-DNA >10 IU/mL at T2.

Notably, the highest risk to experience HBV-DNA >10 IU/mL at T2 was observed in PWH with a persistent HBV-DNA positivity at both T0 (still under TDF/TAF therapy) and at T1 (within 12 months after TDF/TAF suspension) (75% [6/8]), followed by those with an intermittent positivity to HBV-DNA at only one of these two time-points (59.3% [16/27]). Conversely, PWH negative to HBV-DNA at both T0 and at T1 had a significantly lower risk to achieve HBV-DNA >10 IU/mL at T2 (5.3% [1/19]) than PWH with persistent and intermittent HBV-DNA positivity ($P \leq 0.001$ for both).

Then, immunological recovery after TDF/TAF withdrawal was explored according to the presence of a persistent or intermittent HBV-DNA positivity at T0 and T1.

Overall, after TDF/TAF-withdrawal, the median (IQR) change of CD4+ T-cell count at T2 was +76 (−6 to +204) cells/mm^3. Notably, PWH with persistent HBV-DNA positivity showed a lower recovery in CD4+ T-cell count than PWH with intermittent HBV-DNA positivity and those completely negative to HBV-DNA at all time-points (median [IQR] change: +6 [−11 to +51] vs +97 [−57 to +198] vs +93 [+45 to +194] cells/mm^3, *P* for trend = 0.08).

By multivariable analysis, the presence of a persistent HBV-DNA positivity at T0 and T1 resulted an independent factor significantly associated with a reduced probability to achieve CD4+ T-cell count recovery ≥50 cells at T2 (OR [95% CI]: 0.1 [0.01-0.8], $P = 0.03$) (Table 4).

Discussion

By analyzing the kinetics of HBV biomarkers in the first 2 years after TDF/TAF withdrawal, this study shows that the proportion of anti-HBc positive/HBsAg-negative PWH achieving HBV-DNA >10 IU/mL progressively rises with the length of TDF/TAF-sparing ART. Indeed, this proportion increases from 12.9% at T1 to 42.6% at T2 ($P < 0.0001$). A superimposable result was observed for PWH achieving HBV-DNA >100 IU/mL (rising from 2 to 11%, $P = 0.02$).

The issue of HBV re-uptake after TDF/TAF withdrawal has been poorly investigated in literature and most previous studies were based on a limited sample size or anecdotal cases. In particular, in line with our data, previous findings from Cameroon, showed that 10% of anti-HBc positive PWH developed overt HBV-reactivation following TDF/TAF-suspension [23]. Another study described 2 cases of anti-HBc positive/HBsAg-negative PWH in whom serum HBV-DNA from undetectable increased to 3.4 and 7 log IU/mL after TDF/TAF-suspension [24]. More recently, studies on larger multicenter cohorts of PWH suspending TDF/TAF have reported rate of HBV-reactivation close to 1-2% [25,26], percentages lower than that observed in our study. These discrepancies can be attributable to the fact that in these studies, HBV-reuptake has been defined as HBV-DNA detectability according to different Real-Time assays. It is plausible that, since these studies have enrolled patients starting from 2001, the assays used for HBV-DNA quantification were characterized by lower sensitivity (LLoQ up to 2 or even 3 log IU/mL). Differently, in our study, all samples have been re-tested for HBV-DNA by a highly sensitive assay based on ddPCR (LLoQ: 1 IU/mL) and HBV-reuptake has been uniformly defined as the achievement of HBV-DNA >10 IU/mL. Nevertheless, it should be mentioned that also in our study, the rate of HBV-reuptake lowers to 2% and 11% within 12 and 24 months after TDF/TAF withdrawal by considering the achievement of HBV-DNA >100 IU/mL and decreases drastically to 1% and 1.8% for HBV-DNA >1000 IU/mL, data in line with the afore-mentioned cohort studies [25,26].

Overall findings support that the withdrawal of TDF/TAF pharmacological pressure can facilitate the re-uptake/enhancement of viral replication. Despite this replication is initially moderate and uncapable to exert a remarkable pro-inflammatory stimulus (as attested by the limited ALT elevations and the lack of severe hepatic events), it is plausible to hypothesize that the long-term persistence of viral replication can trigger liver disease progression in a longer follow-up.

This highlights the importance to set-up a regular monitor of serum HBV-DNA over-time, in order to reduce the long-term risk to face events of clinically relevant HBV reactivation, characterized by high-level viremia and severe hepatitis flares, as well as to limit the establishment of a new chronic active phase of HBV infection, that could require a long-life course of anti-HBV therapy.

Notably, the usage of 3TC tended to be associated with lower levels of HBV-DNA at both T1 and T2 (median [IQR]: 28 [16-57]

vs 96 [54-573] IU/mL at T1 and 25 [19-56] vs 60 [27-195] IU/mL at T2), reinforcing the protective role of 3TC in constraining the extent of viral replication.

Nevertheless, the rate of HBV-DNA >10 IU/mL (albeit at lower HBV replication level) was not negligible even in the setting of 3TC usage, supporting the importance to regularly monitor serum HBV-DNA also PWH undergoing a 3TC-based simplification strategy. This can be potentially explained by the presence of archived drug-resistance mutations following long-term 3TC usage in previous cART. This hypothesis is supported by previous findings revealing residual HBV-replication in anti-HBc positive/HBsAg-negative PWH under long-term 3TC-including ART, playing a role in the emergence of 3TC-resistant strains [27]. In this light, the potential emergence/re-emergence of 3TC-resistant strains after TDF/TAF withdrawal is a relevant issue to consider in the setting of switch strategy, particularly for anti-HBc positive/HBsAg-negative PWH with a long-term 3TC usage in the past.

The analysis of HBV-biomarkers at T0 (still under TDF/TAF pressure) by highly sensitive molecular assays, revealed the presence of cryptic HBV replicative-activity in one third of anti-HBc positive PWH despite HBsAg-negativity and TDF/TAF-usage. Even more, an additional 22% of PWH presented a positivity to HBV-RNA alone. These data are in keeping with previous studies demonstrating the presence of cryptic HBV activity in conspicuous fractions (from 30 to 78%) of anti-HBc positive/HBsAg-negative PWH receiving HBV-active ART [15,28]. Recent studies have highlighted that PWH with chronic HBV infection tend to achieve HBsAg loss under treatment more frequently than patients with HBV mono-infection [29]. Under this assumption, it is plausible that in PWH, achieving HBsAg loss after chronicity, a residual HBV activity can persist over time. In our study, 4 out of 57 (7.0%) PWH with additional previous HBsAg tests available during their clinical history, resulted HBsAg positive and thus experienced HBsAg loss after chronicity. Notably, one of them presented a persistent positivity to both HBV-DNA and HBV-RNA in all the analyzed time-points, achieving a HBV-DNA of 1465 IU/mL within 24 months after TDF/TAF withdrawal.

Interestingly, in our study a low anti-HBs titer (<100 mIU/mL) was the only factor positively correlated with the presence of cryptic HBV-replication under TDF/TAF-containing ART. This is in line with previous studies showing higher rates of HBV-DNA detection in individuals with isolated anti-HBc [5], reinforcing the key role of an effective anti-HBV immune-response in controlling cccDNA activity. In keeping with this concept, it is noteworthy to mention that, among the 34 PWH with anti-HBs negativity at T0, 9 had lost anti-HBs during clinical monitoring before T0 (median [IQR] time to anti-HBs loss: 3.4 [2.7-10.2] years), reflecting a weakening of anti-HBV immunity. Among them, a remarkable rate of PWH (33%) achieved HBV-DNA >10 IU/mL already within 12 months after TDF/TAF suspension.

Overall data support that anti-HBV vaccination in anti-HBc positive/HBsAg-negative could help enhancing immune response against the virus and in turn limiting HBV replicative-activity. This is even more critical considering that the positivity to cryptic HBV-DNA at T0 was an independent factor strongly correlated with the achievement of HBV >10 IU/mL after TDF/TAF withdrawal (OR [95% CI]: 8.2 [1.7-40.6], $P = 0.01$). In particular, 70% of PWH with detectable cryptic viremia achieved HBV-DNA >10 IU/mL at T2, supporting the role of a still active HBV reservoir at T0 in enhancing viral replication after TDF/TAF-withdrawal. Conversely, PWH characterized by the negativity to HBV-DNA not only at T0 (still under TDF/TAF pharmacological pressure) but also at T1 (within 12 months after TDF/TAF withdrawal), showed a very limited risk to develop HBV-DNA >10 IU/mL after a more prolonged TDF/TAF suspension. In particular, only one out of the 19 PWH (5.3%) with persistent negativity to HBV-DNA experienced HBV-DNA >10 IU/mL at T2.

Furthermore, it is noteworthy to mention that PWH positive to both cryptic HBV-DNA and HBV-RNA at T0 have the highest risk to achieve HBV-DNA >10 IU/mL and >100 IU/mL at T2, supporting that the integration of both HBV markers can provide a more comprehensive estimate of the burden of HBV intrahepatic reservoir. This is in line with a previous study, showing that the quantification of HBV-DNA plus RNA better correlated with intrahepatic cccDNA levels [30].

In this light, the detection of a residual HBV-activity, by novel highly-sensitive markers, can help identifying those patients deserving a stricter monitoring of HBV replicative-activity after TDF/TAF withdrawal, thus optimizing the management of anti-HBc positive/HBsAg-negative PWH undergoing therapeutic simplification.

Notably, lower nadir CD4+ T-cell count (<100 cells/mm^3) was another factor independently associated with an increased risk (>8-fold) to develop HBV-DNA >10 IU/mL, again reinforcing the pivotal role of immune-response in limiting viral rebounds, especially in the setting of no/limited pharmacological pressure [5,31]. Further studies with longer follow-ups are necessary to trace the long-term kinetics of HBV-replication after TDF/TAF-suspension and their long-term impact on liver inflammation.

By evaluating the impact of HBV-DNA over-time on HIV immunological markers, the persistent detection of HBV-DNA at both T0 and T1 was an independent factor significantly associated with a reduced CD4+ T-cell recovery at T2. This is in line with previous studies showing an impaired CD4+ T-cell recovery in both HBsAg-positive and anti-HBc positive/HBsAg-negative PWH despite successful cART [9,31]. Further studies are necessary to finely unravel the pathobiological mechanisms underlying HIV/HBV interplay.

Notably, the risk of HBV-reuptake/enhancement after TDF/TAF-suspension is also critical in light of the increasing usage of long-acting injectable cabotegravir/rilpivirine (LAI-CAB/RPV). A recent study reported HBV-reactivation in 7.9% of anti-HBc positive/HBsAg-negative PWH switching to LAI-CAB/RPV [32]. Similarly, a recent study on 7 anti-HBc positive/HBsAg-negative PWH switching to LAI-CAB/RPV, revealed one HBV-reactivation with high HBV-DNA levels (>10^7 IU/mL) and elevated ALT (>20 ULN) after switching to LAI-CAB/RPV. Notably, HBV-RNA was detected at very low-level before starting LAI-CAB/RPV, suggesting an active HBV intrahepatic reservoir that could have promoted the reuptake of HBV replication [33].

In conclusion, this study shows that a not negligible fraction of anti-HBc positive/HBsAg-negative PWH experiences a re-uptake of HBV replication (albeit moderate) after suspending TDF/TAF, supporting the relevance of a regular virological monitoring of these patients. The detection of a cryptic HBV activity at TDF/TAF withdrawal can contribute to identify anti-HBc positive/HBsAg-negative PWH more prone to the re-uptake of HBV replication, thus optimizing the diagnostic management of PWH undergoing TDF/TAF-sparing ART.

Ethical statement

Approval by the Ethics Committee was deemed unnecessary because, under Italian law, biomedical research is subjected to previous approval only in the hypothesis of clinical trials on medicinal products for clinical use (art. 6 and art. 9, leg. decree 211/2003 D.L.196/2003). The research was conducted on data previously anonymized, according to the Italian Data Protection Code requirements (D.L.101/2018 and 139/2021). Data were collected in full compliance with the Italian law on personal data protection, and

each patient signed an informed consent for participation in the anonymous use of their clinical data for scientific purposes.

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Declarations of competing interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2024.107294.

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