Correlation of serum hepatitis B core-related antigen with hepatitis B virus total intrahepatic DNA and covalently closed circular-DNA viral load in HIV–hepatitis B coinfection

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TITLE PAGE

Correlation of serum hepatitis B core-related antigen with HBV total intrahepatic DNA and ccc-DNA viral load in HIV-hepatitis B coinfection

Running head: HBcrAg and intrahepatic HBV-DNA viral load

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Abstract

Objective: To assess whether quantified hepatitis B core-related antigen (qHBcrAg) is a surrogate marker of intrahepatic replication in HIV and hepatitis B virus (HBV) co-infection.

Design: Cross-sectional study of 31 HIV-HBV-infected patients (total liver biopsies, *n*=38) from a well-defined cohort.

Methods: Spearman's rank correlation coefficients were calculated between qHBcrAg and intrahepatic markers of HBV replication [total intrahepatic (IH)-DNA, covalently-closed circular (ccc) DNA, cccDNA:total IH-DNA ratio].

Results: At biopsy, 22 (71.0%) patients were hepatitis B "e" antigen (HBeAg)-positive, 22 (71.0%) had detectable plasma HBV-DNA and 17 (54.8%) were treated with tenofovir. Median levels (interquartile range) of intrahepatic markers were as follows: HBV cccDNA (*n*=34), 0.26 copies/cell (0.4-2.89); total IH-DNA (*n*=38), 2.38 copies/cell (0.58–207.9), and cccDNA:total IH-DNA ratio (*n*=34), 0.05 (IQR=0.01-0.12). There was a significantly strong correlation between qHBcrAg and cccDNA in all patients (Rho=0.65, $p<0.001$), while a moderate correlation was observed between qHBcrAg and total IH-DNA (Rho=0.57, $p<0.001$) or cccDNA:total IH-DNA ratio (Rho=-0.45, $p=0.01$). Similar findings were observed for HBeAg-positive patients and those with detectable HBV-DNA, with the exception of qHBcrAg and cccDNA or cccDNA:total IH-DNA ratio. In contrast, no significant correlation between qHBcrAg and any intrahepatic marker was observed in HBeAg-negative patients

or those with undetectable HBV-DNA. No significant difference was observed in median qHBcrAg levels across liver fibrosis stages (p=0.5).

Conclusions: qHBcrAg is a potential surrogate marker of cccDNA in HIV-HBV co-infected patients, yet might be less useful with undetectable serum HBV-DNA or HBeAg-negative status. Whether qHBcrAg provides further clinical utility compared to other serological markers remains debatable.

Key words: biomarker; cccDNA; intrahepatic DNA; hepatitis B core-related antigen; hepatitis B virus; HIV.

Introduction

In human immunodeficiency virus (HIV)-positive individuals, untreated co-infection with hepatitis B virus (HBV) leads to accelerated liver fibrosis and higher rates of hepatocellular carcinoma and end-stage liver disease[1]. The recommended treatment for chronic HBV in HIV-positive individuals is currently antiretroviral therapy (ART) including a potent nucleos(-t)ide analogue (NA), such as tenofovir (TDF) or tenofovir alafenamide, both of which have dual activity against circulating HBV and HIV[2]. The ultimate goal of treating HBV infection is the clearance of hepatitis B surface antigen (HBsAg), which is associated with histological improvement, reduced risk of hepatocellular carcinoma and prolonged survival[3]. Nevertheless, very few treated HIV-HBV-co-infected individuals are expected to achieve HBsAg-seroclearance[4,5].

In patients with serologic evidence of HBsAg-seroclearance and acquiring HBs antibodies, covalently closed circular (ccc)DNA can still be detected in infected hepatocytes[6], suggesting continued viral activity despite achieving this endpoint. The formation of cccDNA, an episomal minichromosome that serves as a transcriptional template for the production of new HBV progeny, is a crucial step in the HBV life cycle. As such, its presence indicates active HBV replication in the liver and is responsible for viral persistence during chronic hepatitis B[6–8]. However, as the assessment of cccDNA requires an invasive liver biopsy, the development of novel serum biomarkers that

accurately assess the size of the intrahepatic cccDNA pool and intrahepatic transcriptional activity are needed.

Lower levels of quantified hepatitis B core-related antigen (qHBcrAg) have been shown to bear a strong association with HBeAg-seroclearance for both HBV-mono-infected and HIV-HBV-co-infected patients undergoing NA treatment[9,10]. This novel surrogate marker has also been found to strongly correlate with the size of the cccDNA pool[11–17]. Recent research has shown that qHBcrAg reflects cccDNA transcriptional activity more strongly than quantified HBsAg (qHBsAg)[18]. Nevertheless, these studies were conducted in HBV-mono-infected patients, mainly from Asian countries, where HBV genotypes B and C predominate. Considering that immunological control of intrahepatic HBV is impaired in HIV-HBV-co-infection and stronger degrees of immunosuppression are associated with higher cccDNA levels[1,8], the correlation between qHBcrAg and intrahepatic replication could be different in co-infected individuals.

Unfortunately, no previous study has investigated to what extent qHBcrAg is able to reflect intrahepatic HBV replication for HIV-HBV-co-infection. The aim of this study was then to examine the correlation of qHBcrAg and intrahepatic HBV viral loads, including total intrahepatic (IH)-DNA, cccDNA and the ratio between cccDNA and total IH-DNA, in HIV-positive patients co-infected with chronic HBV.

Methods

Using data from the French HIV-HBV Cohort Study[19,20], we selected patients included in a sub-study on novel markers of HBV replication[9]. Inclusion criteria were as follows: HIV-positive serology confirmed by western blot, HBsAg-positive serology for at least six months, and available quantification of serum HBcrAg and HBV intrahepatic markers from at least one liver biopsy. All patients provided written informed consent and the protocol was approved by the Hôpital Pitié-Salpêtrière and Hôpital Saint-Antoine Ethics Committees (Paris, France) in accordance with the Helsinki Declaration.

Serum HBV-DNA was quantified using a real-time PCR assay (COBAS® AmpliPrep/COBAS TaqMan®, detection limit: 12 IU/mL; or COBAS® Amplicor HBV Monitor, detection limit: 60 IU/mL; Roche Diagnostics, Meylan, France). qHBsAg was performed using the ARCHITECT HBsAg assay (Abbott Laboratories, Rungis, France)[5]. qHBcrAg (U/mL) was measured using a commercially-available, automated HBcrAg chemiluminescence enzyme immunoassay (Lumipulse® G System, FujiRebio Europe, Gent, Belgium)[21]. Liver biopsies were obtained based on concomitant guidelines from the European Association for the Study of the Liver [3]. DNA was extracted from snap-frozen biopsy specimens using the MasterPure DNA purification kit (Epicentre, Le-Perray-en-Yvelines, France). cccDNA and

total IH-DNA levels were quantified by real-time PCR using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) as described previously[6,8].

In statistical analysis, HBV-DNA, qHBcrAg, qHBsAg, total IH-DNA and cccDNA were $\log_{10}$ transformed. Spearman's rank correlation coefficients were calculated comparing each intrahepatic marker of HBV replication (total IH-DNA, cccDNA, or cccDNA:total IH-DNA ratio) to each serum marker (HBV-DNA, qHBcrAg, or qHBsAg). Analysis was stratified on HBeAg-status and detection of serum HBV-DNA ($\geq$60 IU/mL, <60 IU/mL). The Kruskal-Wallis test was used to compare median levels of qHBcrAg at different stages of liver fibrosis (Metavir F0-F1, F2, and F3-F4). Scatterplots were used to illustrate the decline of qHBcrAg, cccDNA and total IH-DNA in individuals with paired biopsies during TDF-containing-ART. All statistical analysis was performed using STATA statistical software (v15.1, College Station, TX, USA) and significance was defined as a *p*-value <0.05.

Results

In total, 31 patients (with 38 liver biopsies) were included. Patients were predominately male (90.3%) with median age of 42 years (IQR=37-53). Only two patients were ART-naïve and six (19.4%) had HIV-RNA >50 copies/mL. Median CD4+ cell count was fairly high at 448/mm^3 (IQR=331-641), yet 8 patients (25.8%) ever had an AIDS-defining illness and nadir CD4+ cell count was a median 262/mm^3 (IQR=150-326). 22 (71.0%) patients were

HBeAg-positive and 22 (71.0%) had detectable plasma HBV-DNA (median=3.1 $\log_{10}$ IU/ml, IQR=2.7-7.1). At biopsy, 23 (74.2%) patients were on an anti-HBV-containing-ART regimen: lamivudine (LAM), *n*=4 (17.4%); TDF, *n*=2 (8.7%); LAM+TDF, *n*=15 (65.2%); LAM+adefovir (ADV), *n*=2 (8.7%). Previous exposure to an active anti-HBV treatment was observed in 28 (90.3%) patients, with a cumulative median months as follows: LAM, 73.2 (IQR=50.0-91.7); ADV, 11.7 (range=8.4-33.6); TDF, 24.7 (IQR=9.1-31.0); interferon, 6.5 (IQR=3.1-14.3); and pegylated-interferon, 23.2 (range=13.2-33.2). Of those with previous exposure to LAM, 7/28 (25%) patients had developed resistance. Approximately 30% of participants (*N*=9) had advanced liver fibrosis or cirrhosis (Metavir F3-F4). A complete description of the study population at the time of liver biopsy is provided in Supplemental Digital Content Table S1.

In the samples taken at the time of biopsy, median qHBcrAg was 5.5 $\log_{10}$ U/mL (IQR=3.1-7.0, *n*=38) and median qHBsAg 4.0 $\log_{10}$ IU/mL (IQR=3.2-4.5; *n*=30). HBV cccDNA was available for 27 patients (in 34 liver biopsies) and was a median 0.26 copies/cell (IQR=0.04, 2.89) or -0.59 $\log_{10}$ copies/cell (IQR=-1.46, 0.46). Total IH-DNA was available for all 31 patients (in 38 liver biopsies) and was a median 2.38 copies/cell (IQR=0.58, 207.9) or 0.38 $\log_{10}$ copies/cell (IQR=-0.24, 2.32). Median cccDNA:total IH-DNA ratio was 0.05 (IQR=0.01, 0.12, *n*=34).

As shown in Table 1, there was a significant and strong correlation between qHBcrAg and cccDNA in all patients (Rho=0.65, $p<0.001$; Figure 1A), while a moderate correlation was observed between qHBcrAg and total IH-DNA (Rho=0.57; $p<0.001$; Figure 1B) or cccDNA:total IH-DNA ratio (Rho=-0.45; $p=0.012$; Figure 1C). Similar findings were observed for HBeAg-positive patients and those with detectable HBV-DNA, with the exception of no significant correlation between qHBcrAg and cccDNA or cccDNA:total IH-DNA ratio. In contrast, qHBcrAg had no significant correlation with any intrahepatic marker when HBeAg was negative or plasma HBV-DNA was undetectable.

qHBsAg was also significantly and strongly correlated with cccDNA (Rho=0.74, $p<0.001$), total IH-DNA (Rho=0.68, $p<0.001$) and cccDNA:total IH-DNA ratio (Rho=-0.49, $p=0.009$) in all patients (Table 1). In contrast, the correlation between qHBsAg and total IH-DNA remained moderate for HBeAg-positive patients, and strong between qHBsAg and total IH-DNA as well as cccDNA for those who were HBeAg-negative. Moreover, in analysis stratified on plasma HBV-DNA, qHBsAg was only strongly correlated with total IH-DNA (Rho=0.80, $p=0.002$) when plasma HBV-DNA was undetectable.

In 5 of the 7 patients who had two liver biopsies during TDF-containing-ART, a moderately faster rate of qHBcrAg decline was observed in the first three years of treatment and became remarkably slower thereafter (Figure 1D). Similarly, median cccDNA and total IH-DNA declined from 2.89 copies/cell (range=0.02-8.36) and 45.08 copies/cell (range=0.58-

198 918.95) to 0.04 copies/cell (range=0.01-0.305) and 1.40 copies/cell (range=0.32-2.44),
199 respectively, during roughly the first three years of TDF (median 40.2 months, IQR=34.9-
200 41.6).
201
202 Although a lower median level of qHBcrAg was observed in patients with none or mild
203 liver fibrosis at biopsy (Figure 1E), no significant difference in median qHBcrAg levels was
204 observed across liver fibrosis stages (F0-F1: 4.3 U/ml, IQR=2.6-7.1; F2: 6.4 U/ml, IQR=4.6-
205 7; F3-F4: 5.2 U/ml, IQR=5-7.5; *p*=0.5).
206
207 **Discussion**
208
209 In our study, we demonstrated a significant and strong correlation overall between
210 qHBcrAg and intrahepatocellular replication, namely levels of cccDNA and total IH-DNA.
211 This would suggest the usefulness of qHBcrAg as a surrogate marker to assess the size of
212 the cccDNA pool and transcriptional activity in HIV-HBV-co-infected patients. This result
213 also corroborates previous findings in HBV-mono-infected population, either
214 untreated[17,18] or during treatment with pegylated-interferon[13,14] or
215 NAs[11,12,15,16,22].
216
217 Nevertheless, other studies in HBV-mono-infected patients have observed significant
218 correlations between qHBcrAg and intrahepatic replication during low-active phases of

HBV infection, that is, when HBV-DNA is undetectable and/or HBeAg is negative[12,17,18,22]. We were unable to confirm these findings in our cohort of HIV-HBV-co-infected patients with HBeAg-negative serology or undetectable serum HBV-DNA. The reasons for these discrepancies are unclear. The majority of studies within the context of HBV-mono-infection were conducted in Asia, where there are substantial differences in viral sequences, replication levels, and disease activity compared to patients from Europe or Africa[23]. HBV genotypes have also been reported to influence the correlation between many markers of HBV replication[15,24] and since our study included mostly patients harboring HBV genotypes A, D and E (as opposed to B and C in many of the Asian, HBV-mono-infection studies), it could be the reason for lack of strong correlation. Alternatively, the lack of correlation could simply be due the small numbers of patients analyzed, contributing to the failure of detecting a significant correlation. It should be noted, however, that the correlations, being between 0.05 to 0.22, were still quite low.

Interestingly, declines in qHBcrAg appeared to tightly coincide with declines in both cccDNA and total IH-DNA during the first three years of TDF-containing-ART. Although we did not have data on intracellular replication thereafter, no further decline in qHBcrAg was noted. This finding is similar to other markers of replication, such as qHBsAg, and assuming that qHBcrAg remains significantly correlated with cccDNA over time, reinforces that very few patients undergoing long-term treatment with anti-HBV NAs are expected to clear intracellular HBV replication[8].

Our study has some limitations. The cross-sectional design makes it difficult to infer on correlation of these markers over time and the small sample sizes prohibits further stratification, especially with respect to genotype, *precore* mutations[25], and levels of CD4+ cell count. Large and multi-center prospective studies would help confirm the present findings, yet as liver biopsies are becoming increasingly rarer in clinical practice, may be unfeasible. In addition, HBcrAg is a composite biomarker whose assessment may be biased by HBeAg positivity and limited sensitivity, especially at low levels of viral replication. Although qHBcrAg is mainly correlated with cccDNA transcriptional activity[18], our study could not analyze other intrahepatic viral RNAs because of the lack of samples. Notwithstanding these limitations, we conclude that serum qHBcrAg could be useful in assessing levels of cccDNA, a marker denoting HBV persistence and stability. Nevertheless, the low correlations of this novel surrogate marker with cccDNA levels when serum HBV-DNA is undetectable or HBeAg is negative could limit its clinical practicality. Since the correlations with qHBcrAg observed herein do not seem to surpass those with qHBsAg, it remains debatable whether qHBcrAg provides further clinical utility over qHBsAg.

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Role of each author. L.D. was responsible for the statistical analysis, interpretation of the data, and drafting the manuscript. S.M. was responsible for HBcrAg, HBsAg and HBeAg quantification, interpretation of the data, and drafting the manuscript. A.G. and C.D. were responsible for HBcrAg, HBsAg and HBeAg quantification and drafted parts of the manuscript. H.R., P.M., C. L-C., and J.C. acquired data for the cohort, assisted in interpreting data, and gave critical revisions of the manuscript. F.Z. gave technical support and provided all biological measurements from liver biopsies, drafted parts of the manuscript, and provided critical revision of the manuscript. P.-M.G. and K.L. helped design, conceptualize, and obtain funding for the French HIV-HBV cohort study, coordinated data collection, and drafted the manuscript. A.B. coordinated data analysis, gave important comments on data interpretation, drafted the manuscript, and provided critical revisions of the manuscript. All authors approved the final version.

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List of Supplemental Digital Content

Supplemental Digital Content Table S1.docx

Figure Subtitle.

Figure 1.

Hepatitis B core-related antigen (qHBcrAg) according to (A) HBV covalently-closed circular (ccc)-DNA levels, (B) total intrahepatic (IH)-DNA, (C) cccDNA:total IH-DNA ratio, (D) decline of cccDNA and IH-DNA levels for individuals with paired biopsies, and (E) liver fibrosis stages (F0-F1, F2 and F3-F4).

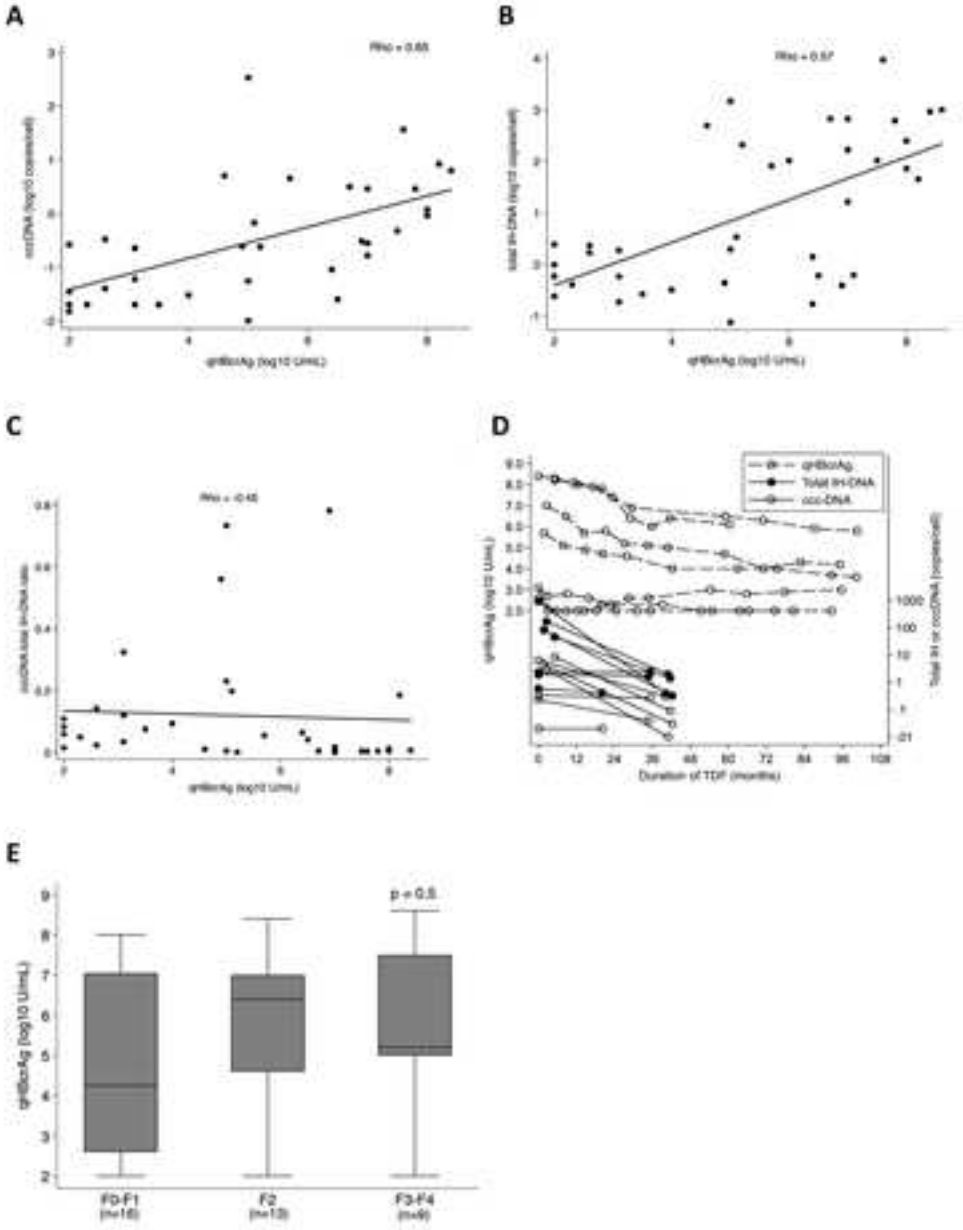

Figure 1.

Table 1. Correlations between qHBcrAg and intrahepatic viral markers

	total IH-DNA			cccDNA			cccDNA:total IH-DNA		
	n*	Rho†	*p* value	n*	Rho†	*p* value	n*	Rho†	*p* value
All liver biopsies (*N*=38)									
HBV-DNA	38	0.65	<0.001	34‡	0.53	0.001	34‡	-0.53	0.001
qHBcrAg	38	0.57	<0.001	34‡	0.65	<0.001	34‡	-0.45	0.01
qHBsAg	30¶	0.74	<0.001	27@	0.68	<0.001	27@	-0.49	0.009
HBeAg-positive (*N*=26)									
HBV-DNA	26	0.73	<0.001	22‡	0.58	0.005	22‡	-0.64	0.001
qHBcrAg	26	0.46	0.019	22‡	0.40	0.07	22‡	-0.48	0.02
qHBsAg	20¶	0.59	0.006	17@	0.42	0.10	17@	-0.40	0.11
HBeAg-negative (*N*=12)									
HBV-DNA	12	0.32	0.3	12	0.13	0.7	12	-0.06	0.9
qHBcrAg	12	0.05	0.9	12	0.22	0.5	12	-0.04	0.9
qHBsAg	10¶	0.78	0.008	10¶	0.68	0.03	10¶	0.08	0.8
HBV-DNA ≥60 IU/mL (*N*=23)									
qHBcrAg	23	0.51	0.01	21‡	0.52	0.02	21‡	-0.37	0.10
qHBsAg	18¶	0.42	0.08	17@	0.44	0.08	17@	-0.21	0.4
HBV-DNA <60 IU/mL (*N*=15)									
qHBcrAg	15	0.08	0.8	13‡	0.15	0.6	13‡	0.02	0.9
qHBsAg	12¶	0.80	0.002	10@	0.24	0.5	10@	-0.28	0.4

Data from 38 biopsies: *n*=17 HBeAg-positive with HBV-DNA ≥60 IU/mL; *n*=9 HBeAg-positive with HBV-DNA <60 IU/mL; *n*=6 HBeAg-negative with HBV-DNA ≥60 IU/mL; and *n*=6 HBeAg-negative with HBV-DNA <60 IU/mL.

cccDNA, covalently-closed circular DNA; HBV, hepatitis B virus; HBeAg, hepatitis B "e" antigen; qHBcrAg, quantified hepatitis B core-related antigen; qHBsAg, quantified hepatitis B surface antigen; total IH-DNA, total intra-hepatic-DNA. *Number of available samples in total. †Spearman's rank correlation coefficient. ‡Probes used for ccc-DNA quantification could perform less efficiently with certain strains of HBV genotype G and were hence considered missing. This concerned 4 liver biopsies: 4 HBeAg-positive (0 HBeAg-negative) and 2 with HBV-DNA ≥60 IU/mL (2 with HBV-DNA <60 IU/mL).

¶No samples were available to quantify qHBsAg and data were hence missing. This concerned 8 liver biopsies: 6 HBeAg-positive (2 HBeAg-negative) and 5 with HBV-DNA ≥60 IU/mL (3 with HBV-DNA <60 IU/mL).

@Missing data due to HBV genotype G and/or missing sample for qHBsAg.