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Quantification of circulating HBV RNA expressed from intrahepatic cccDNA in untreated and NUC treated patients with chronic hepatitis B

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ABSTRACT

Objective A convenient, reproducible biomarker of hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) transcriptional activity is lacking. We measured circulating HBV RNA (cirB-RNA) in untreated and nucleos(t)ide analogues (NUC) treated chronic hepatitis B (CHB) patients to define its correlation with intrahepatic viral markers and HBV core-related antigen (HBcrAg).

Design Paired liver biopsy and serum samples were collected from 122 untreated and 30 NUC-treated CHB patients. We measured cirB-RNA, HBV DNA, hepatitis B surface antigen (HBsAg), HBcrAg and alanine aminotransferase levels. cirB-RNA was quantified using an investigational HBV RNA assay for use on the cobas 6800 system. The test detects a region spanning the HBV canonical polyadenylation site. cccDNA and 3.5 kb RNA in liver tissue were assessed by quantitative PCR and droplet digital PCR.

Results cirB-RNA was detectable in 100% of HBeAg(+) chronic hepatitis (CH), 57% and 14% of HBeAg(–) CH and chronic infection untreated patients and 47% of NUC-treated patients. cirB-RNA undetectability was associated with lower intrahepatic cccDNA transcriptional activity, as well as serum HBcrAg, but no significant differences in HBsAg, in both untreated and treated patients. In untreated HBeAg(–) patients, cirB-RNA correlated with intrahepatic 3.5 kb RNA and cccDNA transcriptional activity, serum HBV DNA and HBcrAg, but not with HBsAg or total cccDNA levels. Combined undetectability of both cirB-RNA and HBcrAg detection in untreated HBeAg(–) patients identified a subgroup with the lowest levels of intrahepatic transcriptionally active cccDNA.

Conclusion Our results support the usefulness of quantification of circulating HBV RNA expressed from cccDNA as an indicator of intrahepatic active viral reservoir in both untreated and NUC-treated CHB patients.

Trial registration number NCT02602847.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Measurement of serum hepatitis B virus (HBV) RNA concentration has been proposed has a promising surrogate marker for intrahepatic HBV replication.

⇒ The lack of a commercially available, standardised and automated assay hampers the implementation of serum HBV RNA quantification in clinical practice.

WHAT THIS STUDY ADDS

⇒ Serum HBV RNA levels quantified by using a new automated prototype assay significantly correlated with intrahepatic covalently closed circular DNA (cccDNA) transcriptional activity in both untreated and nucleos(t)ide analogues-treated chronic hepatitis B patients.

⇒ The prototype assay, whose target region includes the canonical HBV polyadenylation site, preferentially detects RNA derived from cccDNA vs transcripts originating from integrated sequences.

⇒ The distribution of serum HBV RNA and HBV core-related antigens detection shows an incomplete overlap in HBeAg(–) patients.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Measurement of serum HBV RNA levels with the automated prototype assay might represent a new non-invasive, robust and reproducible diagnostic tool to guide treatment cessation and monitor intrahepatic viral clearance.

INTRODUCTION

It is estimated that 296 million people were infected with hepatitis B virus (HBV) globally in 2015.[1] HBV causes chronic hepatitis (CH) and is associated with serious liver disease and cancer. In infected hepatocytes the virus replicates through a covalently closed circular DNA (cccDNA) intermediate, which serves as the template for transcription of viral RNA for protein production and generation of new viral genomes through reverse transcription.[2] HBV-infected patients are managed clinically according to disease stage based on detection and measurement of several host and viral biomarkers in serum including alanine aminotransferase (ALT) levels, the degree of liver inflammation, hepatitis B

surface antigen (HBsAg) and viral DNA.[3 4] Antiviral therapy is based on one or more inhibitors of the viral reverse transcriptase (nucleos(t)ide analogues, NUCs). Response to antiviral therapy is monitored through measurement of the different biomarkers.[3 4] However, complete clearance of HBV cannot be achieved with current treatments because of the persistence of residual cccDNA in hepatocytes. This can lead to resurgence of viraemia in patients who stop antiviral therapy prematurely. Alternative treatment strategies aimed at HBV cure[5] must eliminate the cccDNA reservoir or silence it, the measurement of which is challenging since it requires invasive sampling methods. A diagnostic tool that provides information to guide treatment cessation and monitor intrahepatic viral clearance and that is non-invasive (eg, uses convenient specimen types such as peripheral blood), standardised, amenable to automation, robust and reproducible, is needed. The advantages and disadvantages of several candidate biomarkers have been reviewed elsewhere.[6 7] Briefly, HBeAg and serum HBV DNA concentrations lose their correlation with intrahepatic cccDNA levels after HBeAg seroconversion or NUC treatment, respectively. The fact that HBsAg can be derived from viral sequences integrated in the host genome also undermines its value in predicting cccDNA levels and transcriptional activity, particularly in HBeAg(−) patients.[8 9]

Circulating HBV RNA (cirB-RNA) concentration is a relatively novel biomarker that may be useful for antiviral treatment monitoring. Pregenomic RNA (pgRNA) concentration in blood has been proposed to reflect transcription from cccDNA in infected hepatocytes.[10–13] Serum HBV RNA concentrations, measured mainly with research laboratory developed assays, have good predictive power for both on-treatment serological response and off-treatment durability.[14–18] The combination of undetectable cirB-RNA and HBV core-related antigen (HBcrAg) at the end of treatment is more predictive for sustained suppression of replication off-treatment compared with either biomarker alone.[14]

Here, we measured cirB-RNA levels by using a new automated, prototype quantitative HBV RNA assay in both untreated and NUC-treated patients to assess the relationship between cirB-RNA concentration and other viral markers in blood and in hepatocytes that are associated with cccDNA transcriptional activity.

METHODS

Patients and sampling

Serum samples and core liver biopsies were collected at the same time in 122 untreated chronic hepatitis B (CHB) patients enrolled at the

Table 1 Untreated patient characteristics

Variable		Total cohort (n=122)	HBeAg(+) cirB RNA(+) (n=32)	HBeAg(−) cirB RNA(+) (n=51)	HBeAg(−) cirB RNA(−) (n=39)	P value*
Age†	Years	40.2	32.4	44.2	43.3	ns‡
		(28.6–50.1)	(24–43.8)	(30.4–52.5)	(33.8–54.4)	
Sex	M/F (% M)	86/36 (70%)	25/7 (78%)	36/15 (70%)	25/14 (64%)	ns§
Origin	Europe	42 (34%)	12 (38%)	18 (35%)	12 (31%)	–
	Asia	26 (21%)	10 (32%)	9 (18%)	7 (18%)	
	Sub-Saharan Africa	25 (20%)	3 (9%)	11 (21%)	11 (28%)	
	North Africa	16 (13%)	4 (13%)	7 (14%)	5 (13%)	
	Middle East	13 (11%)	3 (9%)	6 (12%)	4 (10%)	
Viral genotype	A	20 (16%)	9 (28%)	5 (10%)	6 (15.4%)	ns§
	B	3 (2%)	1 (3%)	0 (0%)	2 (5%)	
	C	11 (9%)	7 (23%)	3 (6%)	1 (3%)	
	D	48 (39%)	9 (28%)	29 (57%)	10 (26%)	
	E	14 (11%)	1 (3%)	6 (12%)	7 (18%)	
	F	3 (2%)	2 (6%)	0 (0%)	1 (3%)	
	ND	23 (9%)	3 (9%)	8 (15%)	12 (31%)	
Viral load†	$\log_{10}$ IU/mL	4.4 (2.9–7.2)	8.0 (7.4–8.6)	4.5 (3.5–6.1)	3.0 (2.4–5.3)	<0.0001‡
ALT†¶	U/L	52.5 (34–79.2)	96.5 (67–203.3)	50 (35.5–65.5)	39 (26.5–65.0)	0.007‡
HBsAg†	$\log_{10}$ IU/mL	3.9 (3.4–4.3)	4.6 (3.9–5.2)	3.8 (3.1–4.0)	4.1(3.4–4.5)	ns‡
HBcrAg**	N detectable (%)	90 (75%)	31 (97%)	41 (80%)	19 (49%)	0.001§
	$\log_{10}$ U/mL†,**	4.8 (3.8–7.5)	8 (7.3–8.4)	4.1 (3.7–5.2)	3.5 (3.1–4)	0.004‡
cirB RNA†	$\log_{10}$ copies/mL	3.9 (2–5.4)	5.4 (4.8–6.11)	2.3 (1.5–3.7)	–	–
Fibrosis score	N (%) with score ≥2	55 (44%)	17 (53%)	28 (55%)	10 (26%)	0.009§
Activity score	N (%) with score ≥2	43 (35%)	17 (53%)	19 (37%)	7 (18%)	ns‡
CHB phase††	HBeAg(+) CH	32	32	0	0	
	HBeAg(−) CI	29	0	6	23	<0.0001‡
	HBeAg(−) CH	61	0	45	16	

*Comparing cirB RNA(+) versus cirB RNA(−) in HBeAg(−) patients.
†Data are expressed as median (1st–3rd quartile) of valid results .
‡Mann-Whitney U test, α threshold=0.5.
§Fischer exact test or χ^2 test.
¶Data available for 119 patients.
**Data available for 120 patients.
††According to the European Association for the Study of the Liver (EASL) 2017 guidelines.
–, insufficient data to calculate; CH, chronic hepatitis; CHB, chronic hepatitis B; CI, chronic infection; HBcrAg, HBV core-related antigen; ns, not significant.

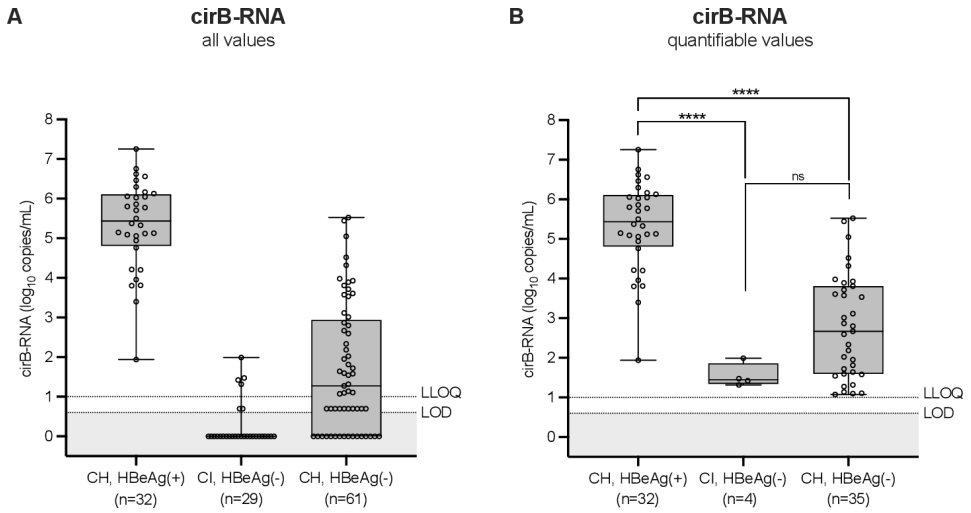

Figure 1 cirB-RNA distribution according to disease status (untreated patients). Boxes span the 25th–75th percentile, whiskers span the range and the horizontal bar in the box represents the median. Assay LOD and LLOQ are marked with dotted lines; the shaded area represents undetectability. ****p<0.001. CH, chronic hepatitis; CI, chronic infection; LLOQ, lower limit of quantification; LOD, limit of detection.

Hepatology Unit of the 'Hospices Civils de Lyon' and at the Gastroenterology and Hepatology Division of the 'Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico' in Milan. Part of the liver tissue was snap frozen and stored at −80°C. Patients were serologically negative for hepatitis C virus, HIV and hepatitis D virus.

Thirty NUC-treated patients who were enrolled in the ECOGR-EFFE French prospective cohort[19] and underwent liver transplantation were also enrolled in this study. All received NUC therapy (90% with entecavir or tenofovir) for a median of 2.6 years. Part of the explanted liver collected from an hepatocellular carcinoma (HCC)-free region surrounding the lesion was snaped frozen, stored at −80°C and available for further virologic investigations.

Patient and public involvement

Patients were not involved in the design and conduct of this research, since not appropriate to the retrospective nature of the study.

Laboratory assays for viral biomarkers in serum and liver

cirB-RNA concentrations in serum were quantified by real-time PCR using an investigational HBV RNA assay for use on the cobas 6800/8800 Systems (cobas HBV RNA, Roche Diagnostics, Pleasanton, California, USA). cobas HBV RNA is a sensitive nucleic acid test for detection and quantification of HBV RNA in EDTA plasma or serum, with limit of detection (LOD) and lower limit of quantification (LLOQ) of 5 and 10 copies/mL, respectively. The difference between DNA and RNA concentrations in untreated patients ranged from 1.5 to 3.4 log10 copies/mL.[20] LOD was confirmed in clinical samples representing HBV genotypes A, B, C, D, E. Assay specificity for RNA versus DNA is enhanced with a blocking oligo that binds to the HBV DNA genome in competition with an assay primer. The assay is calibrated in units of copies/mL, based on a synthetic armoured (arRNA) Roche internal standard spanning 435 bp derived from the 3′ end of HBV RNA and quantitated by droplet digital PCR (ddPCR). The dynamic range of quantification using arRNA was 10–10^9 copies/mL. Results from clinical samples were linear between 10 and 10^7 RNA copies/mL for all genotypes tested. Detectable but not quantifiable results (above the LOD but below the LLOQ) and undetectable results were assigned arbitrary values of 5 and 1 copies/mL, respectively, for illustrative purposes. This prototype assay amplifies HBV transcripts containing the canonical polyadenylation signal[21 22] and thus preferentially quantifies RNA derived from cccDNA. All tests were performed by trained operators in accordance with the manufacturers' specifications using the cobas 6800 system.

Serum HBV DNA viral load was quantified using either the Roche (Roche Diagnostics, Mannheim, Germany) or Abbott quantitative assay (Abbott Diagnostic, Chicago, USA). The LOD for serum HBV DNA was 10 IU/mL for the Roche assay and 20 IU/mL for the Abbott assay. HBsAg quantification was carried out by conventional methods using either Roche (Roche Diagnostics, Mannheim, Germany) or Abbott Architect assay (Abbott Diagnostic, Chicago, USA) with a LLOQ set at 0.05 IU/mL.

Serum HBcrAg concentrations were measured in serum using the Lumipulse G HBcrAg assay on the Lumipulse G600II Analyzer (Fujirebio Europe, Gent, Belgium) according to the manufacturer's instructions (LOD 2.8 $\log_{10}$ U/mL).[23] Samples for which the HBcrAg concentration was≥7.0 $\log_{10}$ U/mL were diluted with a manufacturer-supplied dilution reagent and retested. Undetectable results were assigned arbitrary values of 2.0 U/mL for illustrative purposes.

cccDNA and 3.5 kb RNA concentrations in liver biopsy samples were assessed by quantitative PCR and ddPCR as previously described.[24 25] 3.5 kb RNA primers and probes recognise both pgRNA and pre-Core RNA, thus we collectively refer the identified transcripts as '3.5 kb RNA'. cccDNA transcriptional activity was estimated as the 3.5 kb RNA/cccDNA ratio. Beta-globin DNA and β-glucuronidase (*GUSB*) RNA quantification served as internal references for cccDNA copies/cell and 3.5 kb RNA relative quantity expression, respectively. The cccDNA assay LOD is 3×10^{-6} copies/cell. Undetectable cccDNA and 3.5 kb RNA results were assigned arbitrary values of 3×10^{-6} copies/cell and 10^{-4} units, respectively, for illustrative purposes.

Histological analysis

Fibrosis and necroinflammatory activity were quantified using the METAVIR classification[26] by the pathology services of Hospices Civils de Lyon and 'Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico' in Milan. Patients were divided into two groups for both necroinflammatory activity and fibrosis scores: none or mild (below or equal to 1) versus moderate to severe

(above 2), according to the European Association for the Study of the Liver (EASL) practice guidelines for HBV treatment.[3]

HBV integration harbouring hepatoma cell culture

Hep3B cells were cultured in Dulbecco's modified Eagle's medium (DMEM, ThermoFischer Scientific, Waltham, Massachusetts, USA) supplemented with L-Glutamine (ThermoFischer Scientific) sodium pyruvate (ThermoFischer Scientific), 5% fetal calf serum (Fetalclone II, ThermoFischer Scientific), 100 U/mL penicillin (ThermoFischer Scientific) and 100 µg/mL streptomycin (ThermoFischer Scientific) at 37°C and 5% CO_2. For PLC/PRF/5 cells, Eagle's Minimum Essential Medium (ATCC) was used as a base medium with 10% heat-inactivated FBS (ThermoFischer Scientific). RNAs from cellular extracts of both cell lines were extracted using the Roche manual workflow[20] and quantified by the cobas HBV RNA assay and by an in-house ddPCR assay (see online supplemental material).

Statistical analysis

All data were analysed using Prism V.9.3.0 (GraphPad Software, San Diego, USA). Statistical correlations were tested using the Pearson test for normal variables and the Spearman test for other variables. Categorical variables were compared using the χ^2 or Fischer's exact tests and quantitative variables were compared using the Student's t-test or non-parametric tests (Mann-Whitney or Kruskall-Wallis), as appropriate. Principal component analysis (PCA) was conducted on all viral replication markers in the serum (HBV DNA, qHBsAg and HBcrAg concentrations) and in the liver (HBV-DNA, cccDNA, 3.5 kb RNA concentrations and cccDNA transcriptional activity), as well as on parameters of liver injury (ALT concentration, fibrosis and necroinflammatory activity score). PCA was computed on the first five dimensions using Qlucore Omics Explorer software (Qlucore, Sweden).

RESULTS

Untreated CHB patients

The untreated cohort was composed of 122 patients, 32 HBeAg(+) and 90 HBeAg(−), and was predominantly male; the median age was 40 years (table 1). Six HBV genotypes were represented, with the majority (55.5%) belonging to genotype A or D. Patients had mild levels of fibrosis and necroinflammatory activity (35 to 44% had scores of 2 or more). While all 32 HBeAg(+) patients had detectable cirB-RNA, we defined two groups of HBeAg(−) patients based on detection of cirB-RNA (table 1). HBeAg(−) patients with detectable cirB-RNA (n=51) had higher serum HBV DNA, HBcrAg, and ALT levels, percentage with detectable HBcrAg, and fibrosis score of 2 or more, compared with HBeAg(−) patients with undetectable cirB-RNA (n=39; table 1).

cirB-RNA concentrations and CHB disease phases

Detectability and concentrations of cirB-RNA were assessed in patients classified as having CH or chronic infection (CI).[3] All HBeAg(+) CH patients had quantifiable (>LLOQ) cirB-RNA, compared with only 57% of HBeAg(−) CH and 14% of HBeAg(−) CI patients (figure 1). The median of quantifiable cirB-RNA concentrations was much higher in CH HBeAg(+) patients compared with CH or CI HBeAg(−) patients. In the subgroup of six CI HBeAg(−) patients with quantifiable cirB-RNA, levels of serum HBV DNA, HBsAg, HBcrAg and intrahepatic cccDNA were similar to those with undetectable cirB-RNA (Mann-Whitney U test, p values >0.1; data not shown).

The pattern of differences in serum HBV DNA and HBcrAg concentrations between the three groups was similar to that observed for cirB-RNA (online supplemental figure 1). However, HBsAg concentrations in HBeAg(−) CI patients were about 10-fold lower than HBeAg(+) CH patients and were similar to HBeAg(−) CH patients (online supplemental figure 1). cirB-RNA levels were higher in patients with high versus low ALT levels, necroinflammatory activity or fibrosis scores (online supplemental figure 2). No differences were observed according to HBV genotype (online supplemental figure 3).

cirB-RNA and intrahepatic viral markers

Intrahepatic markers of viral replication were compared in patients with detectable versus undetectable cirB-RNA. Patients

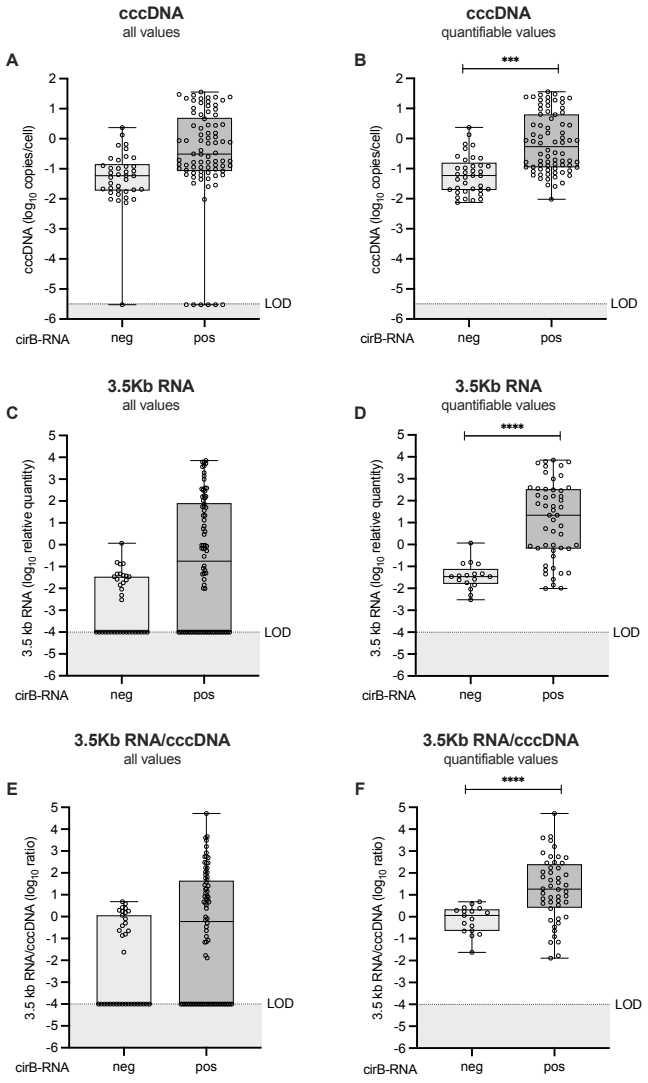

Figure 2 Intrahepatic viral markers in patients with detectable versus undetectable cirB-RNA (all untreated patients). Intrahepatic cccDNA (A, B) and 3.5 kb RNA (C, D) results were available for 120 patients. The 3.5 kb RNA/cccDNA ratio (E, F) could be calculated for 113 patients and was different from 0 in 66 patients (49 cirB-RNA(+) and 17 cirB-RNA(−) patients). cccDNA was undetectable in seven patients (six cirB-RNA(+) and one cirB-RNA(−)), while 3.5 kb RNA was undetectable in 54 patients (34 cirB-RNA(+) and 20 cirB-RNA(−)). Assay detection limits are marked with a dotted line; the shaded area represents undetectability. cccDNA, covalently closed circular DNA. ***p<0.001, ****p<0.0001.

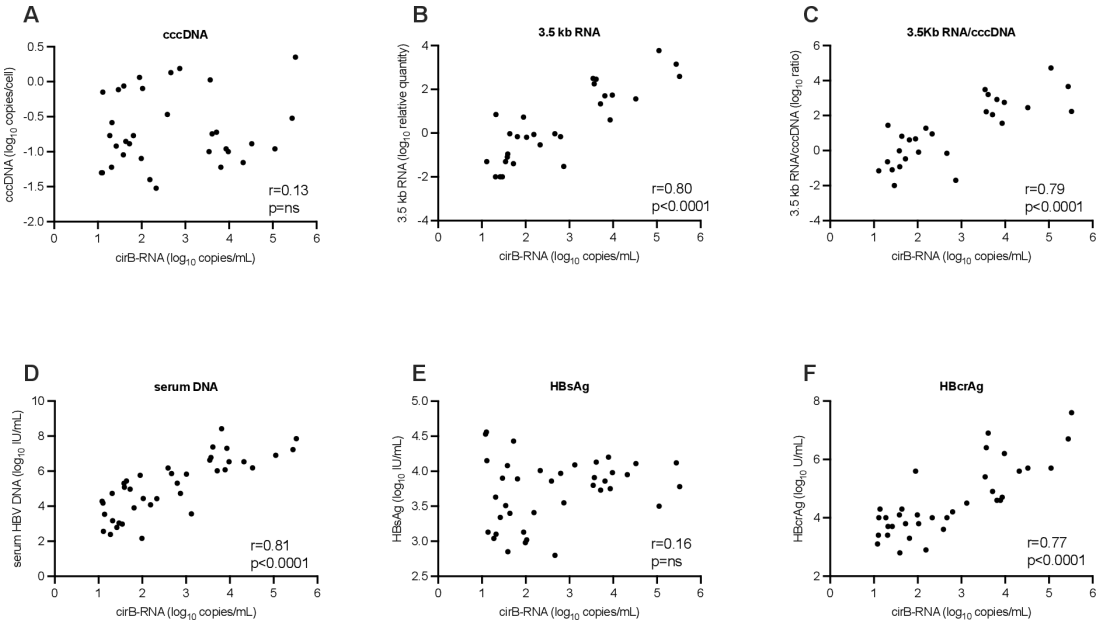

Figure 3 Correlations between cirB-RNA and intrahepatic and serum viral markers (HBeAg(–) untreated patients). Intrahepatic cccDNA (A) and 3.5 kb RNA (B) results were available for 88 HBeAg(–) patients. 3.5 kb RNA/cccDNA ratio (C) could be calculated for 82 patients. Only samples having quantifiable values for both intrahepatic HBV markers and cirB-RNA are shown (n=33 for cccDNA, n=29 for 3.5 kb RNA, n=28 for 3.5 kb RNA/cccDNA). cccDNA, covalently closed circular DNA.

with undetectable cirB-RNA had lower levels of cccDNA (median 0.06 vs 0.56 copies/cell), 3.5 kb RNA (median 0.03 vs 21.9) and cccDNA transcriptional activity (3.5 kb RNA/cccDNA ratio 1.1 vs 17.7), compared with patients with detectable cirB-RNA (figure 2). Similar results were obtained in the HBeAg(−) subset of patients (online supplemental figure 4). HBcrAg and HBV DNA concentrations in serum were significantly higher in HBeAg(−) patients with detectable versus undetectable cirB-RNA, while no significant difference was found in HBsAg levels (see table 1).

In HBeAg(−) patients, cirB-RNA concentrations were positively correlated with levels of intrahepatic 3.5 kb RNA (r=0.77, p<0.001), cccDNA transcriptional activity (r=0.78, p<0.0001), serum HBV DNA (r=0.81, p<0.0001) and serum HBcrAg (r=0.73, p<0.0001), but not with intrahepatic cccDNA or serum HBsAg levels (figure 3), contrary to HBeAg(+) group, where cirB-RNA was significantly correlated with both cccDNA and serum HBsAg levels (online supplemental figure 5), as previously observed with in-house assays.[27 28] In the whole cohort, cirB-RNA concentration positively and significantly correlated with all viral intrahepatic and serum markers (online supplemental figure 6).

In HBeAg(−) patients, the predominant source of HBsAg is HBV DNA integrated in the host cell genome.[8 9] Therefore, the lack of correlation between cirB-RNA and serum HBsAg suggests that the cobas HBV RNA assay preferentially detects viral RNAs produced by cccDNA. This is supported by in vitro data showing that the assay does not detect intracellular HBV RNA in cell lines with integrated HBV DNA but devoid of cccDNA (PLC/PRF/5 and Hep3B cells; online supplemental figure 7).

Two groups of HBeAg(–) CH patients defined by cirB RNA levels

In CH HBeAg(−) patients, there appeared to be a grouping of patients with cirB-RNA above or below approximately 3 $\log_{10}$ copies/mL (eg, figure 3). Intrahepatic 3.5 kb RNA levels, 3.5 kb RNA/cccDNA ratio, liver fibrosis scores and necro-inflammatory activity were significantly higher in the patients with cirB-RNA concentration over 3 $\log_{10}$ copies/mL, while cccDNA quantity and HBsAg levels were not different (online supplemental figure 8).

cirB-RNA distribution according to HBcrAg levels

cirB-RNA and HBcrAg levels in serum have both been proposed as markers of intrahepatic viral replication or transcription, and are correlated with each other. As shown in figure 4, median cirB-RNA levels and the proportion with detectable cirB-RNA both increased in groups of patients with increasing HBcrAg

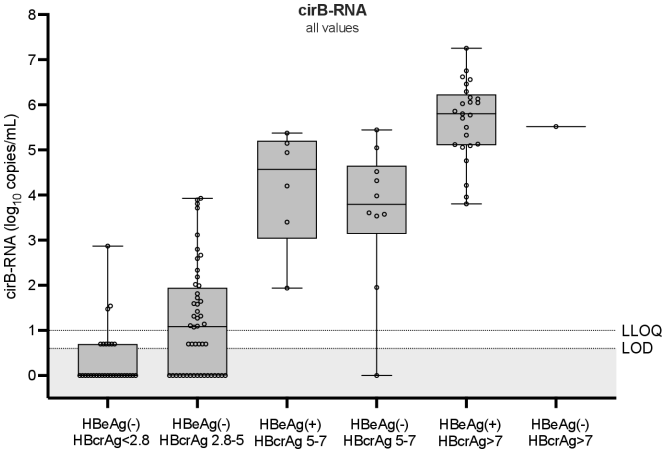

Figure 4 cirB-RNA distribution according to HBcrAg levels (untreated patients). Boxes span the 25th–75th percentile, whiskers span the range, and the horizontal bar in the box represents the median. Assay LOD and LLOQ are marked with dotted lines; the shaded area represents undetectability. HBcrAg, HBV core-related antigen; HBV, hepatitis B virus; LLOQ, lower limit of quantification; LOD, limit of detection.

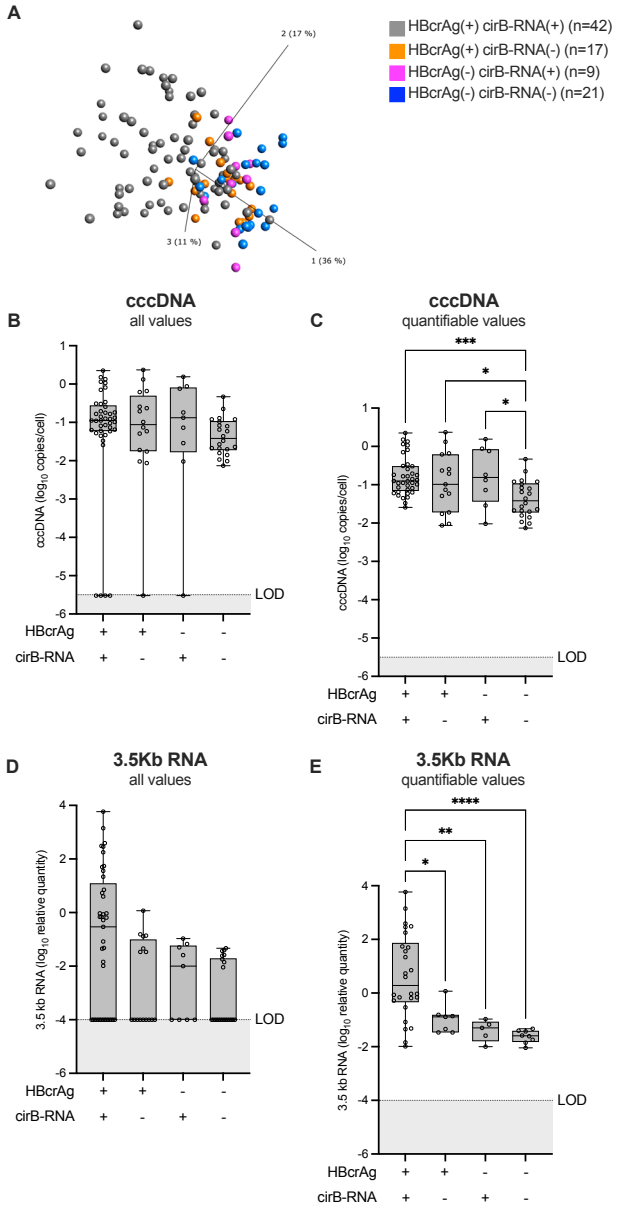

Figure 5 Integrative analysis of serum HBcrAg and cirB-RNA reveals subsets of HBeAg(–) patients with discordant quantification. Principal component analysis (PCA) score plot, where each dot represents a patient. Colours distinguish distribution of HBcrAg(–) cirB-RNA(–) (blue dots), HBcrAg(–) cirB-RNA(+) (fuchsia dots), HBcrAg(+) cirB-RNA(–) (orange dots) and HBcrAg(+) cirB-RNA(+) (grey dots). Distribution of cccDNA (B, C) and 3.5 kb RNA (D, E) levels according to the patients' groups derived from the combination of HBcrAg and cirB-RNA positivity and showed in (A). cccDNA, covalently closed circular DNA; HBcrAg, HBV core-related antigen; HBV, hepatitis B virus; LOD, limit of detection.

levels, irrespective of their HBeAg status. To further investigate the relationship between HBcrAg, cirB-RNA and the viral and clinical parameters, we performed PCA in HBeAg(−) patients. Four profiles are recognisable in the PCA scatterplot in figure 5A, where each point represents a sample from one patient. The HBcrAg(+) cirB-RNA(+) group (grey dots) is clearly spatially differentiated from the other three groups. This can be explained by the higher serum HBV DNA levels in these samples (table 2). Nine out of 30 (30%) patients with undetectable HBcrAg had detectable cirB-RNA (fuchsia dots). This subgroup of patients was mainly composed of HBeAg(−) CH patients (7 out of 9) and had increased intrahepatic cccDNA levels (median 0.16 vs 0.04 copies/cell, p=0.04) compared with the 21 patients with undetectable cirB-RNA and HBcrAg (blue dots in panel A) (figure 5B,C). 3.5 kb RNA levels were also higher, but the difference was not statistically significant (median 0.05 vs 0.03, p=0.1) (figure 5E). Conversely, 17 of 39 (44%) cirB-RNA(−) patients had detectable HBcrAg (orange dots in panel A). Most of them (11 out of 17) were HBeAg(−) CI patients, and showed higher cccDNA amount (0.10 vs 0.04 copies/cell, p=0.02), compared with those with undetectable cirB-RNA and HBcrAg (figure 5B,C). Also, median of 3.5 kb RNA (median 0.13 vs 0.03, p=0.5) was higher, but did not reach statistical significance (figure 5E).

NUC-treated patients

Characteristics of the 25 NUC-treated patients at the time of liver transplantation are summarised in table 3. At the time of sampling, 18 of the 25 NUC-treated patients had hepatocellular carcinoma. Treatment with entecavir, tenofovir or lamivudine for a median of 29 months preceded liver transplant and tissue sampling (table 3).

All treated patients had detectable HBsAg, 19 of 25 (76%) had detectable HBcrAg and 11 of 25 (44%) had detectable cirB-RNA (table 3). No baseline clinical characteristics were associated with cirB-RNA positivity. While only three (12%) treated patients had detectable HBV DNA in serum, most had detectable cccDNA (19 of 25, 76%) and 3.5 kb RNA (17 of 25, 68%) in their liver biopsy sample (table 3). Quantities of intrahepatic HBV DNA and RNA were low (less than 0.05 copies per cell), consistent with other data from patients receiving long term NUC therapy.[24]

Compared with the cirB-RNA(+) NUC-treated patients, the 14 cirB-RNA(−) patients had similar cccDNA levels but lower 3.5 kb RNA levels (median 0.001 vs 0.04, p=0.04; figure 6C,D). The 3.5 kb RNA/cccDNA ratio was also lower (median 0.16 vs 15.8), although the difference was not statistically significant, possibly due to the small sample size. Two of five patients negative for both cirB-RNA and HBcrAg had detectable intrahepatic cccDNA and 3.5 kb RNA by ddPCR).

HBsAg (median 4.9 vs 6.1 $\log_{10}$ IU/mL, p=0.002) and HBcrAg levels (3.8 vs 4.9 $\log_{10}$ U/mL, p=0.005) were also higher in cirB-RNA(+) patients, but cirB-RNA levels were not correlated with serum HBsAg (R=0.3, p=ns) (data not shown).

DISCUSSION

Emerging treatment strategies aimed at HBV cure must eliminate or, at least, permanently inactivate the intrahepatic cccDNA reservoir.[29] The size of the cccDNA pool, as well as its capacity to produce HBV RNA, varies across CHB phases and during NUC treatment.[9 24 30] Therefore, accurate assessment of the intrahepatic HBV reservoir must include quantification of cccDNA levels and transcriptional activity. Evaluation of this reservoir requires sampling of the liver, which is hindered by the invasiveness of the procedure and the potential for sampling bias and suboptimal storage procedures.[31] Measurement of serum biomarkers that reflect cccDNA activity based on its transcriptional output, the viral RNAs, would represent an invaluable diagnostic tool to guide treatment cessation and monitor intrahepatic viral clearance, especially if it was standardised, automated, robust and reproducible. Here, using matched liver biopsies and serum samples, we demonstrate that results from an automated,

Table 2 Serum and intrahepatic viral markers of HBeAg(–) patients according to combined HBcrAg and cirB-RNA analysis

	HBcrAg(+) cirB-RNA(+) (n=42)	HBcrAg(+) cirB-RNA(–) (n=17)	HBcrAg(–) cirB-RNA(+) (n=9)	HBcrAg(–) cirB-RNA(–) (n=22)
Serum HBV DNA (log_{10} IU/mL)	4.85 (3.91–6.28)	2.96 (2.35–3.77)	3.21 (2.93–4.48)	2.69 (2.21–3.03)
Serum HBsAg (log_{10} IU/mL)	3.86 (3.28–4.09)	4.05 (3.15–4.49)	3.5 (2.90–3.77)	3.64 (3.15–4.15)
cccDNA positive/total (copies/cell)	37/42 0.13 (0.07–0.31)	15/17 0.10 (0.02–0.62)	8/9 0.16 (0.04–0.84)	22/22 0.04 (0.02–0.11)
3.5 kb RNA positive/total (units)	26/42 2.46 (0.47–85.7)	7/17 0.13 (0.03–0.15)	5/9 0.05 (0.02–0.09)	8/22 0.03 (0.02–0.04)

cccDNA, covalently closed circular DNA; HBcrAg, HBV core-related antigens; HBV, hepatitis B virus.

prototype quantitative assay that detects preferentially HBV RNAs expressed from cccDNA[20] are able to reflect the intrahepatic pool of transcriptionally active cccDNA in untreated and NUC-treated CHB patients.

Our results indicate that cirB-RNA concentrations in serum were significantly correlated with other markers of viral replication in the serum and in the liver (figures 2 and 3). Moreover, in HBeAg(−) patients, where the transcriptional activity of cccDNA is decreased and, potentially, not all cccDNA molecules in the liver are transcriptionally active,[9 30] cirB-RNA concentration was highly correlated with intrahepatic 3.5 Kb HBV RNA levels and cccDNA transcriptional activity. Interestingly,

Table 3 NUC-treated patient characteristics

Variable		Total cohort (n=25)	cirB-RNA(+) (n=11)	cirB-RNA(–) (n=14)	P value*
Age at LT†	Years	57.4 (47.5–64.2)	58.7 (50.5–64.3)	56.5 (46.2–64.6)	ns
Sex	M/F (%M)	21/4 (84%)	8/3 (73%)	13/1 (93%)	
Origin	Sub-Saharan Africa	9 (36%)	4 (36%)	5 (36%)	–
	Europe	9 (36%)	6 (55%)	3 (21%)	
	Asia	4 (16%)	0	4 (29%)	
	North Africa	1 (4%)	0	1 (7%)	
	Other	2 (8%)	1 (9%))	1 (7%)	
BMI†	kg/m^2	24 (22–26.8)	26.5 (25.4–38.7)	23 (21.2–24)	ns
Time between HBV diagnosis and LT†	Months	6.4 (2.8–12.3)	5.4 (2.5–10.2)	6.7 (3.2–15.9)	ns
MELD at LT registration†		10.6 (6–20.9)	10.6 (6–20)	10.5 (6–21.8)	ns
CHILD-PUGH score at LT registration	Class A	10 (40%)	4 (36%)	6 (42%)	ns
	Class B	8 (32%)	4 (36%)	4 (29%)	
	Class C	7 (28%)	3 (28%)	4 (29%)	
Hepatocellular carcinoma (HCC)	No (%)	18 (72%)	9 (82%)	9 (64%)	ns
Active HCC at LT	No (%)	3/18 (17%)	2/9 (22%)	1/9 (11%)	ns
Serum AFP levels1		5 (3–15)	5.5 (3–18.6)	4 (2.5–13.5)	ns
HBV treatment before LT	Duration (months)†	29 (11–65)	29 (15–33)	34.5 (10.3–72.5)	ns
	Entecavir	10 (40%)	5 (45%)	5 (36%)	ns
	Tenofovir	14 (56%)	6 (55%)	8 (57%)	ns
	Lamivudine	1 (4%)	0	1 (7%)	ns
Virological status at LT					
HBsAg	N detectable (%)	25 (100%)	11 (100%)	14 (100%)	0.002
	log_{10} IU/mL†	5.8 (4.7–6.1)	6.1 (5.8–6.3)	4.9 (4.2–5.8)	
HBeAg	N detectable (%)	2 (7%)	1 (9%)	1 (7%)	–
HBcrAg	N detectable (%)	19 (76%)	10 (91%)	9 (64%)	0.005
	log_{10} U/mL†	4.2 (3.7–5.1)	4.9 (4.3-5.3)	3.8 (3.5-4.1)	
Serum HBV DNA	N detectable (%)	3 (12%)	2 (18%)	1 (7%)	–
	log_{10} IU/mL†	3 (2.3-3.7)			
cirB-RNA	N detectable (%)	11 (44%)	11 (100%)	0	–
	log_{10} copies/mL†	1.6 (1.2–2.6)	1.6 (1.2–2.6)	–	
Intrahepatic cccDNA	N detectable (%)	19 (76%)	9 (82%)	10 (71%)	ns
	copies/cell†	0.002 (0.0009–0.009)	0.002 (0.001–0.02)	0.002 (0.0007–0.007)	
Intrahepatic 3.5 kb RNA	N detectable (%)	17 (68%)	8 (73%)	9 (64%)	0.04
	units†	0.008 (0.002–0.06)	0.04 (0.02–68.4)	(0.001–0.006)	
Intrahepatic 3.5 kb RNA/cccDNA	N detectable (%)	16 (64%)	8 (73%)	8 (57%)	ns
	units†	4 (0.5–48.3)	27.6 (4.2–68.4)	1.2 (0.35–4.7)	

*Comparing cirB RNA(+) versus cirB RNA(–).
†Data are expressed as median (1st–3rd quartile) of valid results.
–, insufficient data to calculate; BMI, body mass index; cccDNA, covalently closed circular DNA; HBcrAg, HBV core-related antigens; HBV, hepatitis B virus; LT, liver transplantation; MELD, model for end-stage liver disease score; ns, not significant; NUC, nucleos(t)ide analogues.

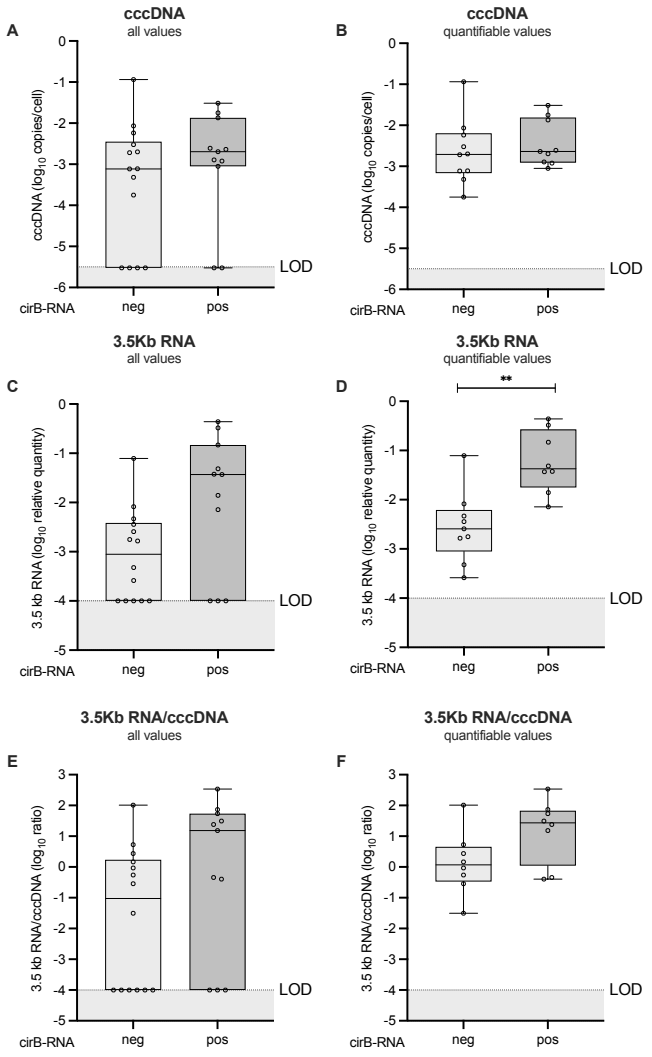

Figure 6 cirB-RNA and intrahepatic viral markers in NUC-treated patients. Intrahepatic cccDNA (A, B), 3.5 kb RNA (C, D) and 3.5 kb RNA/cccDNA ratio (E, F) in NUC-treated patients with undetectable (neg) or detectable (pos) cirB-RNA. Boxes span the 25th–75th percentile, whiskers span the range, and the horizontal bar in the box represents the median. Assay detection limits are marked with dotted lines; the shaded area represents undetectability. cccDNA, covalently closed circular DNA; LOD, limit of detection; NUC, nucleos(t)ide analogues.

in this category of patients, cirB-RNA concentration did not correlate with serum HBsAg levels. Since HBsAg production is mainly ascribed to integrated HBV DNA rather than cccDNA in HBeAg(−) patients,[8 9] this results suggest that the cobas HBV RNA assay preferentially recognises transcripts derived from the cccDNA template. This assumption is consistent with the design of the assay, since its target region includes the canonical HBV polyadenylation site, a region which is lost during integration of viral sequences in the host genome. This is supported by the observation that the assay does not detect HBV RNA produced from cell lines containing only integrated HBV DNA in vitro (online supplemental figure 7). At this stage, we cannot exclude that the cobas assay would not detect cccDNA-derived transcript that would be truncated upstream of the canonical polyA region.[32 33]

HBcrAg is another biomarker in serum that reflects intrahepatic cccDNA transcriptional activity.[34–36] In our cohort of untreated patients, cirB-RNA and HBcrAg concentrations were strongly correlated with each other, in both HBeAg(−) and HBeAg(+) patients. However, PCA helped to highlight subsets of patients having discordant detection of the two biomarkers. Integrative analysis with intrahepatic viral markers demonstrated that patients with detectable HBcrAg or cirB-RNA had higher liver cccDNA and HBV RNA levels compared with those scoring negative for both (table 2). Whether the discrepancy in the results of the two biomarkers is related to technical differences in the assays or to a difference in underlying biological or clinical phenomena remains to be investigated.

Importantly, our study included analysis of difficult to obtain liver samples from NUC-treated patients. We had access to serum and liver samples from patients who were eligible for liver transplantation,[19] and showed correlations between circulating HBV RNA and cccDNA transcriptional activity. Since serum HBV RNA concentration is correlated with transcriptionally active cccDNA in the liver, this surrogate biomarker could play an important role in evaluation of the efficacy of emerging treatment strategies in new proof-of-concept clinical trials, since most of them will be conducted in NUC-suppressed patients. Similar to what we observed for HBcrAg,[34] we identified a positive, and most likely, indirect correlation between cirB-RNA levels and markers of liver damage. Indeed, higher cirB-RNA levels to more severe scores of fibrosis and necro-inflammatory activity and to ALT levels>2N (online supplemental figure 2). It is noteworthy that cirB-RNA levels correlate with cccDNA transcriptional activity even in patients with HBV replication suppressed by NUCs and with severe liver damage undergoing OLT for HCC and/or end-stage liver disease.

Our study has several limitations. Relatively small sample sizes for some analyses may have limited our ability to discern small differences in biomarker concentrations between groups of patients. Given the cross-sectional nature of this study, further investigation is needed to confirm the contribution of the proposed prototype quantitative HBV RNA assay in the context of patient follow-up and prediction of functional cure.

In conclusion, our results represent the first study to correlate intrahepatic viral markers with serum HBV RNA levels, measured with an automated and standardised assay in untreated and NUC-treated CHB patients. These data suggest that measurement of serum HBV RNA represents a non-invasive diagnostic tool to monitor the liver viral reservoir.

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