Research Article
Viral Hepatitis

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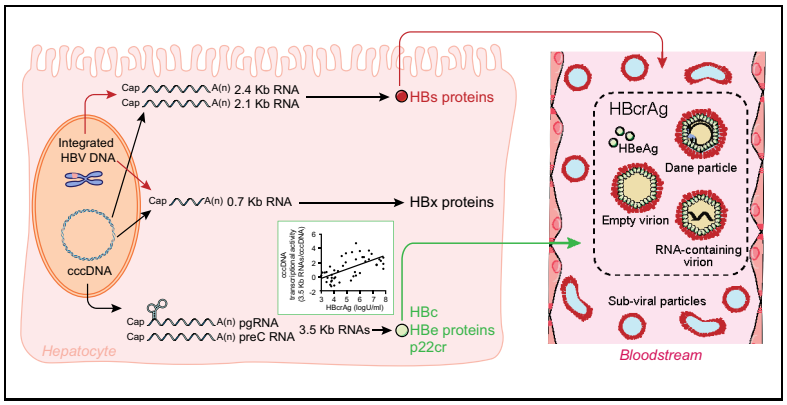
Graphical abstract



Highlights

- Liver HBV cccDNA is responsible for viral persistence despite antiviral treatments.
- cccDNA activity, rather than amount, is correlated with disease progression.
- Serum HBcrAg highly correlates with intrahepatic cccDNA activity.
- Lower levels of HBcrAg are correlated with a more favorable course of the disease.

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Lay summary

Hepatitis B virus causes a chronic infection which develops into severe liver disease and liver cancer. The viral covalently closed circular DNA (cccDNA) is responsible for the persistence of the infection in hepatocytes. To better manage patient treatment and follow-up, and to develop new antiviral treatments directly targeting the intrahepatic pool of cccDNA, serum surrogate markers reflecting the viral activity in the liver are urgently needed. In this work, we demonstrate that quantification of hepatitis B core-related antigen in serum correlates with cccDNA amount and activity and could be used to monitor disease progression.

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Serum hepatitis B core-related antigen (HBcrAg) correlates with covalently closed circular DNA transcriptional activity in chronic hepatitis B patients

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Background & Aims: It has been proposed that serum hepatitis B core-related antigen (HBcrAg) reflects intrahepatic covalently closed circular (ccc)DNA levels. However, the correlation of HBcrAg with serum and intrahepatic viral markers and liver histology has not been comprehensively investigated in a large sample. We aimed to determine if HBcrAg could be a useful therapeutic marker in patients with chronic hepatitis B.

Methods: HBcrAg was measured by chemiluminescent enzyme immunoassay in 130 (36 hepatitis B e antigen [HBeAg]+ and 94 HBeAg−) biopsy proven, untreated, patients with chronic hepatitis B. HBcrAg levels were correlated with: a) serum hepatitis B virus (HBV)-DNA, quantitative hepatitis B surface antigen and alanine aminotransferase levels; b) intrahepatic total (t)HBV-DNA, cccDNA, pregenomic (pg)RNA and cccDNA transcriptional activity (defined as pgRNA/cccDNA ratio); c) fibrosis and necroinflammatory activity scores.

Results: HBcrAg levels were significantly higher in HBeAg+ *vs.* HBeAg− patients and correlated with serum HBV-DNA, intrahepatic tHBV-DNA, pgRNA and cccDNA levels, and transcriptional activity. Patients who were negative for HBcrAg (<3 LogU/ml) had less liver cccDNA and lower cccDNA activity than the HBcrAg+ group. Principal component analysis coupled with unsupervised clustering identified that in a subgroup of HBeAg− patients, higher HBcrAg levels were associated with higher serum HBV-DNA, intrahepatic tHBV-DNA, pgRNA, cccDNA transcriptional activity and with higher fibrosis and necroinflammatory activity scores.

Conclusions: Our results indicate that HBcrAg is a surrogate marker of both intrahepatic cccDNA and its transcriptional activity. HBcrAg could be useful in the evaluation of new antiviral therapies aiming at a functional cure of HBV infection either by directly or indirectly targeting the intrahepatic cccDNA pool.

Lay summary: Hepatitis B virus causes a chronic infection which develops into severe liver disease and liver cancer. The viral covalently closed circular DNA (cccDNA) is responsible for the persistence of the infection in hepatocytes. To better manage patient treatment and follow-up, and to develop new antiviral treatments directly targeting the intrahepatic pool of cccDNA, serum surrogate markers reflecting the viral activity in the liver are urgently needed. In this work, we demonstrate that quantification of hepatitis B core-related antigen in serum correlates with cccDNA amount and activity and could be used to monitor disease progression.

Introduction

The development of novel antiviral agents and immunomodulatory approaches to cure hepatitis B virus (HBV) infection requires new biomarkers capable of reflecting the intrahepatic activity of the virus and chronic HBV (CHB) stages and defining new meaningful treatment endpoints. Indeed, there is an unmet need for standardized assays able to provide mechanistic insights into the effects of the novel antiviral and immunomodulatory agents and to assess treatment efficacy.[1]

HBV covalently closed circular (ccc)DNA constitutes the unique template for pregenomic (pg)RNA transcription and viral genome replication. Its persistence in the nucleus of infected cells is responsible for the chronicity of HBV infection.[2] So far, antiviral therapies have demonstrated a modest effect on the established cccDNA pool.[3–6] Measurement of intrahepatic cccDNA levels and transcriptional activity is therefore crucial for the management of patients with CHB and for treatment individualization. The need for liver biopsy strongly limits the evaluation of cccDNA in routine clinical practice. Currently, serum HBV-DNA, hepatitis B surface antigen (HBsAg) and the quantification of HBsAg (qHBsAg) are the most widely used viral markers to diagnose HBV infection and to monitor antiviral therapy.[7] Nucleos(t)ides analogues (NAs) block viral polymerase and are highly efficient in achieving viral suppression, despite the continuous presence of cccDNA in infected hepatocytes.

Keywords: Hepatitis B virus (HBV); Chronic hepatitis B (CHB); Biomarker; Patient management; Pregenomic RNA (pgRNA); Covalently closed circular DNA (cccDNA).
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Thus, the correlation between intrahepatic cccDNA and serum HBV-DNA is lost and serum HBV-DNA quantification cannot be considered a surrogate marker for cccDNA in NA-treated patients.[4] HBsAg is the hallmark of infection and its clearance is considered to be the most important clinical endpoint[7] because both spontaneous and therapy-induced HBsAg loss are associated with histological improvement, a reduced risk of hepatocellular carcinoma and prolonged survival.[8–11] The degree of correlation between qHBsAg and intrahepatic viral markers, in particular cccDNA levels, varies greatly between studies and is still debated,[12–18] particularly in HBeAg– carriers, where expression from HBV integrants may significantly contribute to HBsAg production, in addition to its expression from the cccDNA template,[19] as has been shown in HBV infected chimpanzees.[20]

Indeed, during NA therapy, the kinetics of qHBsAg decline are much slower in HBeAg– patients than in HBeAg+ patients and slower than those of serum HBV-DNA, reflecting the pool of infected hepatocytes harboring either cccDNA or integrated viral sequences.[21–23] Quantification of serum HBV RNAs may represent a novel option to predict virological response to both NAs and interferon,[24,25] but their correlation with intrahepatic viral parameters needs further investigation with well-defined assays. As yet, no surrogate serum marker satisfactorily reflects the pool of transcriptionally active cccDNA in the liver. The so-called hepatitis B core-related antigen (HBcrAg) assay utilizes a mixture of monoclonal antibodies isolated from HBV core antigen-immunized mice[26] to detect and quantify HBV core antigen (HBcAg), free HBeAg, HBeAg–antibody complex, and the 22 kDa precore protein (p22cr).[26,27] Several reports suggest that HBcrAg levels correlate with serum HBV-DNA in untreated patients with CHB and might be useful to differentiate HBeAg– patients with active and inactive disease.[28–32] A correlation between HBcrAg levels and the size of the intrahepatic cccDNA pool has been suggested in cohorts of Asian patients with genotype B/C CHB, either untreated[33–35] or undergoing NA therapy.[35–37] HBcrAg has been also shown to correlate with intrahepatic viral RNA levels in Asian patients treated with NAs.[37] No studies are available on HBcrAg and intrahepatic viral parameters in HBV genotypes other than B/C and it remains to be defined whether, and to what extent, HBcrAg serum levels reflect the transcriptional activity of cccDNA.

To better characterize the clinical value of HBcrAg detection, we have investigated the relationship between circulating HBcrAg, HBV-DNA, qHBsAg and intrahepatic cccDNA transcriptional activity, in a cohort of untreated patients with CHB infected with several HBV genotypes.

Materials and methods

Patients

Serum samples concomitant to liver biopsy were available for the study in 130 untreated patients with CHB (4 HBeAg+ chronic infection (CI), 32 HBeAg+ chronic hepatitis (CH), 33 HBeAg– CI and 61 HBeAg– CH) (Table S1),[7] who underwent needle liver biopsy for routine histology assessment, enrolled at the Hepatology Unit of the "Hospices Civils de Lyon" and at the Gastroenterology and Hepatology Unit of the "Ospedale Maggiore Policlinico" in Milan. Part of the liver tissue was snap frozen and stored at −80 °C. All patients showed no concomitant serological markers of hepatitis C virus, human immunodeficiency virus and hepatitis D virus infections. The overall cohort was composed of 37 women and 93 men, aged from 16 to 66 years (median of 39 years) (Table 1). All patients were treatment naïve except for 5 patients out of 130: 4 received a course of interferon therapy and 1 was treated with lamivudine for a few months more than 10 years before liver biopsy. All patients provided written informed consent and the protocol was approved by the Lyon and Milan institutional Ethic Committees.

Quantification of serum HBV virological and serological parameters

HBeAg status was determined using Abbott Architect® HBeAg assay (Abbott Diagnostic, Chicago, IL, United States). Serum HBV-DNA quantification was performed by quantitative PCR using Cobas® Ampliprep/Cobas® Taqman® HBV test (Roche Diagnostics, Manheim, Germany). The detection range was between 20 IU/ml and 1.7×10^8 IU/ml. Serum qHBsAg was quantified with Elecsys® HBsAg II kit/Cobas e411® (Roche Diagnostics, Manheim, Germany) according to manufacturer's instructions.

Measurement of serum HBcrAg levels

Quantitative levels of HBcrAg were determined using the Lumipulse G HBcrAg assay using the LUMIPULSE G1200 Analyzer (Fujirebio Europe, Gent, Belgium) according to the manufacturer's instructions. HBcrAg levels are quantified in U/ml and the assay measures simultaneously denatured HBeAg, HBcAg and the precore protein p22cr (aa −28 to aa 150). The assay's measurement linear range spans from 3 to 7 logU/ml. Despite the machine's lowest sensitivity limit being 2 logU/ml, 100% specificity is reached if values are above 3 logU/ml, thus HBcrAg levels between 2 and 3 LogU/ml are considered negative. Samples with HBcrAg above 7 logU/ml were diluted with specific dilution reagent and retested in order to quantify HBcrAg values.

Histologic analysis

Fibrosis and necroinflammatory activity were quantified using METAVIR classification[38] by the pathology services of Hospices Civils de Lyon and "Ospedale Maggiore Policlinico" in Milan. Patients were divided into 2 groups for both necroinflammatory activity and fibrosis scores: none or mild (below or equal to 1) *vs.* moderate to severe (above 2), according to the European Association for the Study of the Liver (EASL) Clinical Practice Guidelines for HBV treatment.[39]

DNA and RNA extraction from liver biopsies

DNA and RNA were extracted from snap frozen human liver needle biopsies as described in Ref. 12. Briefly, liver samples were first homogenized on ice using a TissueRuptor (Qiagen®, Hilden, Germany) in homogenization buffer (Tris HCl pH 8 50 mM, EDTA 1 mM, NaCl 150 mM), then divided in 2 pieces for purification of DNA (MasterPure DNA Purification Kit (Epicentre®, by Illumina, Madison, United States) and RNA (Nucleospin® Total RNA and Protein Isolation Kit, Macherey-Nagel, Düren, Germany), respectively. SuperScript™ III first-Strand Synthesis SuperMix was used for complementary DNA synthesis by qRT-PCR (Invitrogen by Life Technologies©, Carlsbad, United States). Quantity and integrity of the extracted DNA and RNA and synthetized cDNA were assessed by NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

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Table 1. Patients' characteristics, HBcrAg and intrahepatic viral markers quantification.

	Total cohort (n = 130)	HBeAg(+) patients (n = 36)	HBeAg(−) patients (n = 94)	*p* value
Age[1] (years)	39.8 (28.8–49.1)	33.6 (24.2–41.3)	41.8 (32.7–51.3)	0.005[4]
Sex (M/F)	93/37 (71.5/28.5%)	28/8 (78/22%)	65/29 (69/31%)	n.s.[5]
Origin				0.012[5]
Caucasian	55 (42%)	14 (39%)	41 (43.6%)	
Middle East	10 (8%)	4 (11%)	6 (6.4%)	
Asian	22 (17%)	10 (28%)	12 (12. 8%)	
North Africa	17 (13%)	4 (11%)	13 (13.8%)	
Sub-Saharan Africa	24 (18.5%)	3 (8%)	21 (22.2%)	
South America	2 (1.5%)	1 (3%)	1 (1.2%)	
Viral genotype[2]				0.001[5]
A	20 (19%)	9 (27.4%)	11 (15.3%)	
B	4 (4%)	2 (6%)	2 (2.8%)	
C	13 (12.3%)	8 (24.3%)	5 (7%)	
D	51 (48.6%)	11 (33.3%)	40 (55.5%)	
E	14 (13.3%)	1 (3%)	13 (18%)	
F	3 (2.8%)	2 (6%)	1 (1.4%)	
Viral load[1] (LogIU/ml)	4.4 (2.9–7)	8 (7.2–8.5)	3.56 (2.5–4.9)	<0.0001[4]
ALT[1] (IU)	52 (33.2–78.7)	81 (62.5–177)	41.5 (29–64.2)	0.0001[4]
qHBsAg[1] (LogIU/ml)	3.9 (3.4–4.3)	4.61 (4.1–5.2)	3.74 (3.2–4.1)	0.0001[4]
HBcrAg (+/−)	74.6/25.4%	100/0	65/35 %	0.002[5]
HBcrAg[1] (LogU/ml)	5.3 (4–7.6)	8 (7.3–8.3)	4 (3.7–4.9)	0.0001[4]
Total HBV DNA[1] (copies/cell)	11.6 (3.6–744.9)	254.8 (579–4,602.6)	5.7 (2.5–20.6)	0.0001[4]
cccDNA[1] (copies/cell)	0.15 (0.06–1.34)	6.3 (1.4–18.1)	0.09 (0.03–0.2)	0.0001[4]
Total HBV DNA/cccDNA[1] (relative quantity)	105.9 (29.8–463)	314.5 (117.6–1,195.6)	59.9 (22.6–238.9)	0.0001[4]
pgRNA[1,3] (relative to housekeeping gene)	0.69 (0.034–101.9)	150.2 (61.9–1,734.1)	0.075 (0.01–1.11)	0.0001[4]
pgRNA/cccDNA[1,3] (relative quantity)	3.02 (0.23–54.2)	36.2 (8.6–98.9)	1.06 (0.13–8.3)	0.001[4]
Fibrosis (≤1/≧2)	72/58 (55.4/44.6%)	17/19 (47/53%)	55/39 (58.5/41.5%)	n.s.[5]
Activity (≦1/≧2)	87/43 (67/33%)	17/19 (47/53%)	26/68 (27.6/72.4%)	n.s.[5]

HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen.
[1] Data are expressed as median (1st quartile-3rd quartile).
[2] data available for 105 patients (72 HBeAg− and 33 HBeAg+).
[3] data available for 80 patients (58 HBeAg− and 22 HBeAg+).
[4] Mann-Whitney U test between HBeAg+ and HBeAg− groups of patients; alpha threshold = 0.05.
[5] X^2 test between HBeAg+ and HBeAg− groups of patients.

Quantification of total HBV-DNA, cccDNA and pregenomic (pg)RNA in liver samples

Quantification was performed using the Light Cycler® 480 Real Time PCR System (Roche diagnostics, Manheim, Germany) with primers and fluorescence dual hybridization probes specific for total HBV-DNA or cccDNA as described in.[12] Before cccDNA amplification, DNA was treated with 10U of Plasmid-safe DNase (Epicentre® by Illumina) for 45 minutes at 37 °C following the latest update of the international working group on cccDNA standardization (Allweiss *et al.*, 2017 International HBV Meeting, O-45). Serial dilutions of a plasmid containing an HBV monomer (pHBV-EcoR1) served as quantification standard. To normalize the number of viral copies per cell content, the number of cellular genomes was determined using the β-globin gene kit (Roche Diagnostics, Manheim, Germany). Patient samples were independently analyzed in duplicate. The range of quantification was comprised between 10^1 and 10^7 copies of HBV genome/well for both cccDNA and total HBV-DNA assays. cccDNA quantification fell under the limit of detection for 7 out of 130 patients tested. For pgRNA detection, specific primers and Taqman® hybridization probe were used, as described in.[12] Patient samples were independently analyzed in duplicate and pgRNA relative amount was normalized over the expression of housekeeping gene GUSB (Hs99999908_m1, Thermo Fisher Scientific, Waltham, MA, USA).

Determination of HBV genotype

Intrahepatic DNA extracted from liver biopsies was used as a template for the PCR amplification of the RT domain of the HBV polymerase using the primer pair L4 5′-TCACAATACCGCA GAGTCTAGACT-3′ (nucleotide position 231-254) and RV5 5′-GGTTGCGTCAGCAAACACTTG-3′ (1177-1197) and the PrimeSTAR® Max DNA Polymerase according to the manufacturer's instructions (Takara). The PCR products were sequenced by GATC. Sequence analysis was performed using the HBVdb tools to determine HBV genotype (https://hbvdb.ibcp.fr/HBVdb/).[40]

Statistical analysis

Comparison between groups of patients was performed using XLStat and the non-parametric Mann-Whitney *U* Test or Kruskal-Wallis test when necessary. Multiple correlations were calculated with the Spearman's statistic followed by Holm's method for correction. Principal Component Analysis (PCA) was performed on the first 5 dimensions using Qlucore® Omics Explorer software (Qlucore, Sweden). k-means clustering, and heat map generation followed by hierarchical clustering were conducted using Qlucore® Omics Explorer software (Qlucore, Sweden).

For further details regarding the materials used, please refer to the CTAT table.

Results

Patients' characteristics and results of serum and intrahepatic viral marker analysis

Table 1 summarizes patients' demographics and virological parameters in serum and in the liver. Most of the patients were

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of Caucasian (55/130) and African (41/130) origin with a predominance of HBV genotype D (51/105 tested) (Table 1). HBeAg+ patients (HBeAg+ CI and HBeAg+ CH) had the highest HBcrAg levels, with 78% showing HBcrAg levels >7 LogU/ml, while a wider and heterogeneous distribution was found in HBeAg– patients (HBeAg– CI and HBeAg– CH), with HBcrAg levels <3 LogU/ml in 35% of cases (Fig. S1). HBeAg+ patients showed higher values of all viral replication markers (serum viral load, intrahepatic total HBV-DNA [tHBV-DNA], cccDNA, pgRNA and cccDNA transcriptional activity [pgRNA/cccDNA ratio]) compared to HBeAg– patients (Table 1 and Table S1). Mild levels of fibrosis and necroinflammatory activity were found in the majority of patients, with no significant differences between HBeAg+ and HBeAg– groups. HBcrAg values were higher in patients showing fibrosis and necroinflammatory activity values >2xULN and alanine aminotransferase (ALT) levels >2xULN (Fig. S2). No significant differences were observed in HBcrAg distribution according to viral genotypes by Kruskal-Wallis analysis (Fig. S2).

Correlation of HBcrAg with serum and intrahepatic viral markers

HBcrAg levels showed a good correlation with serum HBV-DNA levels in HBeAg+ patients and in HBeAg– CH but not in HBeAg– CI (all patients: R = 0.82, p <0.0001; HBeAg+ CH: R = 0.46, p = 0.005; HBeAg– CH: R = 0.57, p <0.001; HBeAg– CI: R = –0.08, p = n.s.) (Fig. 1, upper panels). Among the HBeAg+ patients, HBeAg+ CI (red dots) could not be distinguished from HBeAg+ CH (black dots) (Fig. 1). Similar results were obtained when viral genotypes A, C and D were analyzed separately (Fig. S3) or when patients were divided according to their ethnicity (Fig. S4). HBcrAg levels also correlated with qHBsAg in HBeAg+ patients but not in HBeAg– patients (all patients: R = 0.44, p <0.0001; HBeAg+ CH: R = 0.48, p = 0.003; HBeAg– CH: R = –0.24, p = n.s.; HBeAg– CI: R = –0.25, p = n.s.) (Fig. 1, lower panels). Similar correlations were observed across genotype A or D and patients of Caucasian origin, whereas the correlation between HBcrAg and qHBs was lost in genotype C HBeAg+ Asian patients (Figs. S5 and S6). Serum HBV-DNA and HBcrAg were positively correlated with all intrahepatic viral replication markers (Table 2), albeit correlation coefficients for HBcrAg were much stronger. On the contrary, qHBsAg showed only a weak correlation with tHBV-DNA, pgRNA and cccDNA transcriptional activity and no significant correlation with cccDNA levels (Table 2). Notably, in HBeAg– CH patients HBcrAg correlated with pgRNA levels and transcriptional activity whereas there was no correlation with qHBsAg (Table 2).

HBcrAg positivity is associated with higher intrahepatic viral markers and cccDNA transcriptional activity in HBeAg– patients

While HBeAg+ patients were all positive for HBcrAg quantification, HBcrAg was considered negative (<3 LogU/ml) in 33/94 HBeAg– patients (Table S2). Thus, we further analyzed the correlates of HBcrAg positivity in HBeAg– patients. HBcrAg– and HBcrAg+ groups showed no significant differences in demographics and ALT levels. In contrast, the HBcrAg+ group presented a higher number of patients with moderate to severe fibrosis and necroinflammatory activity (Table S2). Regarding viral replication markers, serum HBV-DNA levels were higher in HBcrAg+ patients, while qHBsAg levels showed no significant differences according to HBcrAg status. The HBcrAg+ group also had higher cccDNA, tHBV-DNA, pgRNA and cccDNA transcriptional activity levels (Table S2). To further investigate the relationship between HBcrAg and cccDNA activity, we compared HBcrAg– and HBcrAg+ patients with similar amounts of cccDNA. Since HBcrAg– patients had a cccDNA range between 0.01 and 0.87 copies/cell, we applied the same filter to select for HBcrAg+ patients. With the same intrahepatic amount, cccDNA was found more transcriptionally active in HBeAg–

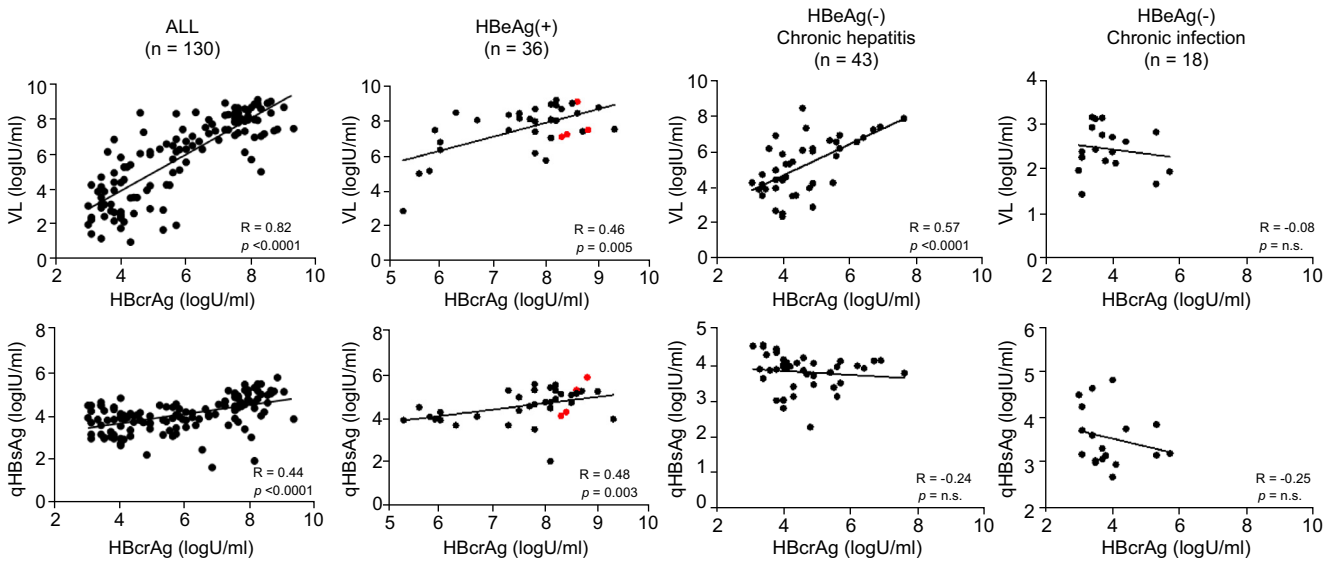

Fig. 1. Correlations between HBcrAg, serum HBV-DNA and qHBsAg levels. Upper panels, correlation between HBcrAg and serum HBV-DNA. Lower panels, correlation between HBcrAg and serum qHBsAg. Only patients having positive HBcrAg values (*i.e.* >3 LogU/ml) were included in the analysis (36 HBeAg+ and 61 HBeAg–, see Table 1 and Table S1). The 4 HBeAg+ CI patients were analyzed together with HBeAg+ CH patients for statistical reasons, but they are highlighted by red dots. The correlation coefficient was calculated using Spearman's correlation test. Two-tailed p-value was calculated for a risk threshold $\alpha = 0.05$. CI, chronic infection; CH, chronic hepatitis; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; qHBsAg, quantitative hepatitis B surface antigen; VL, viral load (serum HBV DNA).

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Table 2. Correlations between HBcrAg, qHBsAg, serum HBV-DNA and intrahepatic viral markers.

	Liver markers			
	tHBV-DNA	cccDNA	pgRNA	cccDNA transcriptional activity (pgRNA/cccDNA)
ALL[1]				
HBcrAg	R = 0.85; p <0.0001	R = 0.74; p <0.0001	R = 0.75; p <0.0001	R = 0.52; p <0.0001
qHBsAg	R = 0.38; p = 0.003	R = 0.26; p = 0.044	R = 0.35; p = 0.006	R = 0.29; p = 0.023
Serum HBV DNA	R = 0.78; p <0.0001	R = 0.57; p <0.0001	R = 0.41; p <0.0001	R = 0.25; p = 0.015
HBeAg+ chronic hepatitis[2] (n = 32)				
HBcrAg	R = 0.79; p <0.0001	R = 0.80; p <0.0001	R = 0.68; p = 0.004	R = −0.02; p = n.s.
qHBsAg	R = 0.49; p = n.s.	R = 0.33; p = 0.01	R = 0.32; p = n.s.	R = 0.26; p = n.s.
Serum HBV DNA	R = 0.50; p = 0.003	R = 0.29; p = n.s.	R = 0.41; p = 0.07	R = 0.18; p = n.s.
HBeAg− chronic hepatitis[1] (n = 43)				
HBcrAg	R = 0.61; p <0.0001	R = 0.25; p = n.s.	R = 0.81; p <0.0001	R = 0.70; p <0.0001
qHBsAg	R = −0.15; p = n.s.	R = −0.4; p = 0.01	R = −0.02; p = n.s.	R = 0.15; p = n.s.
Serum HBV DNA	R = 0.71; p <0.0001	R = 0.19; p = n.s.	R = 0.79; p <0.0001	R = 0.66; p = 0.0002
HBeAg− chronic infection[1] (n = 18)				
HBcrAg	R = 0.34; p = n.s.	R = 0.47; p = 0.05	R = 0.29; p = 0.09	R = 0.11; p = n.s.
qHBsAg	R = 0.24; p = n.s.	R = −0.03; p = n.s.	R = −0.12; p = n.s.	R = 0.08; p = n.s.
Serum HBV DNA	R = −0.02; p = n.s.	R = 0.27; p = n.s.	R = 0.39; p = n.s.	R = 0.28; p = n.s.

HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; pgRNA, pregenomic RNA; qHBsAg, quantitative hepatitis B surface antigen. The correlation coefficient was calculates using Spearman's correlation test. Two-tailed p-value was calculated for a risk threshold $\alpha = 0.05$.
[1] Only patients with positive HBcrAg quantification (*i.e.* >3 LogU/ml) were included in the analysis.
[2] HBeAg+ chronic infection category was composed by only 4 patients (see Table S1), therefore it was not included in the analysis.

patients that are HBcrAg+ compared to HBcrAg− HBeAg− patients (Fig. 2). This is illustrated by the higher levels of pgRNA (median of 0.29 *vs.* 0.03, $p = 0.003$) and pgRNA/cccDNA ratio (median of 1.78 *vs.* 0.23, $p = 0.021$) in HBcrAg+ *vs.* HBcrAg− HBeAg− patients.

HBcrAg distinguishes a subgroup with higher cccDNA levels and disease activity among low viremic HBeAg− patients

HBeAg− CH patients exhibit a different degree of chronic hepatitis, reflected by ALT levels, fibrosis and necroinflammatory activity scores (Table 1). Serum HBV-DNA, together with ALT levels, represent a useful marker to distinguish "more" *vs.* "less" active liver disease in these patients.[7] Since HBcrAg and serum HBV-DNA levels were strongly correlated (Fig. 1), we decided to test if HBcrAg levels could still show a correlation with intrahepatic cccDNA activity and liver disease independently of serum HBV-DNA, in low viremic patients. To this aim, we focused our analysis on a subgroup of 45 HBeAg− patients with serum HBV-DNA <2,000 IU/ml (Table S1). Twenty-two of them had positive HBcrAg measurement (>3 LogU/ml) and receiver operating characteristic analysis revealed that a HBcrAg threshold value of 4 LogU/ml was able to distinguish patients with mild *vs.* minimal liver disease (described as fibrosis and/or necroinflammatory activity scores >2 or <1, respectively) (AUC = 0.74; p <0.0001; 95% confidence interval −0.06–0.55; PPV = 0.44; NPV = 0.92) (Fig. 3). Interestingly, patients with HBcrAg >4 LogU/ml also exhibited higher quantities of cccDNA (median of 0.17 *vs.* 0.06 copies/cell, $p = 0.045$) and cccDNA transcription (median of 1.27 *vs.* 0.08, p = n.s.), but no differences in either HBsAg or ALT levels, with only 4 patients having ALT >ULN (Table S3).

Identification of 2 subgroups of HBeAg− patients with different HBcrAg levels

A principal component analysis (PCA) was conducted on all viral replication markers in the serum (HBV-DNA, qHBsAg and HBcrAg) and in the liver (tHBV-DNA, cccDNA, pgRNA, cccDNA transcriptional activity), as well as on different parameters of liver injury (ALT, fibrosis and necroinflammatory activity score) (Fig. 4). In PCA plots (Fig. 4A–C), each dot represents a patient and colors distinguish either HBeAg+ from HBeAg− patients (Fig. 4A), HBcrAg rank of log values (Fig. 4B) or the 3 clusters

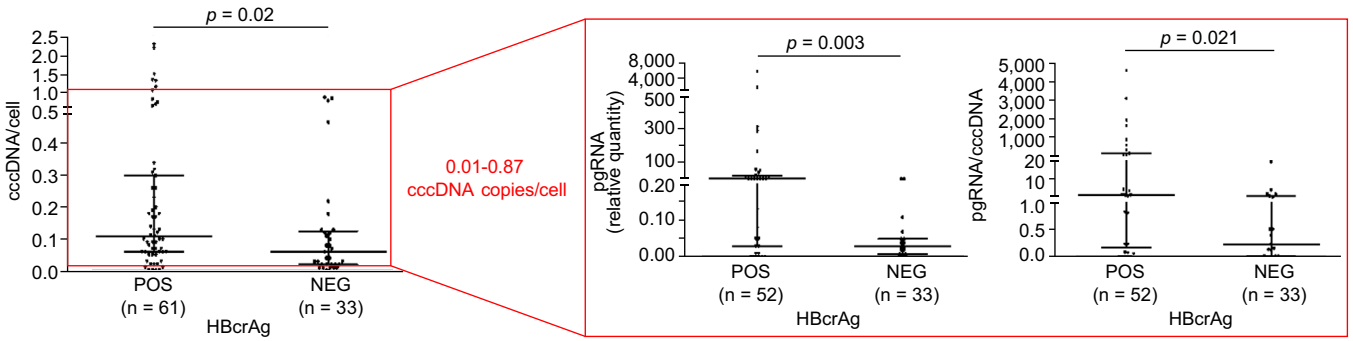

Fig. 2. HBcrAg+ HBeAg− patients show higher cccDNA transcriptional activity than HBcrAg− HBeAg− patients. Dot plots of pgRNA and cccDNA transcriptional activity (pgRNA/cccDNA ratio) levels in HBcrAg+ *vs.* HBcrAg− in 85 HBeAg− patients with cccDNA quantity between 0.01 and 0.87 copies/cell. Lines indicate median with interquartile range. Mann-Whitney *U* test was used to compare HBcrAg+ and HBcrAg− groups, α threshold = 0.05. cccDNA, covalently closed circular DNA; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; pgRNA, pregenomic RNA.

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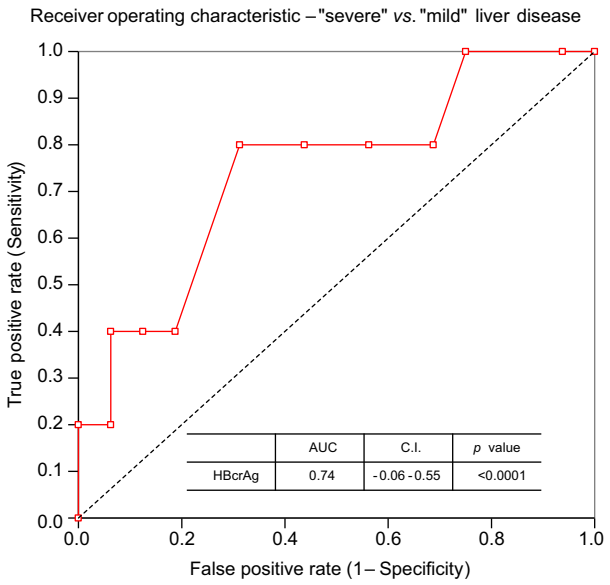

Fig. 3. Receiver operating characteristic curve analysis. An HBcrAg cut-off value of 4 LogU/ml was able to distinguish between HBeAg– HBcrAg+ patients (n = 21) with mild *vs.* minimal liver disease (described as fibrosis and/or necroinflammatory activity scores >2 or <1, respectively) (PPV 0.44; NPV 0.92; p <0.0001; 95% confidence interval −0.06 to 0.55). AUC, area under the curve; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; NPV, negative predictive value; PPV, positive predictive value.

that were identified by k-means analysis (Fig. 4C). By comparing the plots in Fig. 4A and B, most of HBeAg+ patients had the highest HBcrAg values, whereas the HBeAg– group appeared to be more heterogeneous. The analysis based on the k-means unsupervised clustering method identified 3 groups, one of which (Cluster 3) is superposed to the HBeAg+ patients subgroup (compare Fig. 4A and C, violet and blue dots, respectively), while the other 2 clusters identified different subgroups among the HBeAg– patients (compare Fig. 4A and C, pink *vs.* yellow and fuchsia dots). To better characterize the virus and disease associated variables that are distinctive of each cluster, a heat map was built, followed by hierarchical clustering classification (Fig. 4D). Cluster 3 was composed of 31 out of the 36 HBeAg+ patients (Table S4). Cluster 1 and 2 comprised 28 and 64 patients of the 94 HBeAg– patients, respectively. Cluster 1 presented significantly higher levels of serum HBV-DNA, fibrosis, necroinflammatory activity, intrahepatic tHBV-DNA, pgRNA and cccDNA transcriptional activity than Cluster 2 (Table S4). No differences were found in cccDNA and qHBsAg levels. Interestingly, Cluster 1 HBeAg– patients had higher HBcrAg levels (median of 4.9 *vs.* 3.8 LogU/ml, p <0.0001) (Table S4). Thus, higher HBcrAg levels identify a subgroup of HBeAg– patients (Cluster 1) with higher HBV replication (serum and intrahepatic HBV-DNA) and cccDNA transcriptional activity (pgRNA and pgRNA/cccDNA ratio) and disease activity (fibrosis and necroinflammatory scores) that cannot be identified either by cccDNA levels and qHBsAg or a combination of both. The same results were obtained when PCA analysis was applied only to HBcrAg + patients (data not shown).

Next, we asked whether HBcrAg levels might predict the natural course of HBV infection in untreated patients. To this aim, we retrospectively collected clinical follow-up data for 98/130 patients (27/32 patients in Cluster 1, 47/65 patients in Cluster 2 and 24/33 patients in Cluster 3, median follow-up of 8.0 years, interquartile range 6.6–12.3) (Fig. 5). Notably, all the patients in Cluster 1 and 3 fulfilled at a given point of the follow-up the indications for NA treatment according to EASL 2012 Clinical Practice Guidelines.[39] Among the Cluster 2 patients included in the follow-up, 25/47 were treated with NAs (8 classified as HBeAg– CI and 17 HBeAg– CH at the study entry) and 22/47 did not (17 classified as HBeAg– CI and 5 HBeAg– CH at the study entry). Notably, HBcrAg levels did not differ at study entry between the 25 Cluster 2 patients that underwent NA treatment and the 22 patients that did not (3.8 LogU/ml *vs.* 3.75 LogU/ml; p = n.s.). Moreover, the 8 HBeAg– CI patients that underwent NA treatment showed a virological and biochemical reactivation during the follow-up, before the beginning of therapy. Compared to the 17 HBeAg– patients classified as CI at study entry that were not treated, they were older (49.6 *vs.* 36.0; p <0.03), had a slightly higher histologic inflammatory activity and fibrosis but they showed no differences in ALT (28.5 *vs.* 28.0 IU/ml; p = n.s.), HBV-DNA (2.8 *vs.* 2.3, logIU/ml; p = n.s.), qHBsAg (3.3 *vs.* 3.4 logIU/ml; p = n.s.), or HBcrAg (2.4 *vs.* 3.1 logU/ml; p = n.s.) levels. Intrahepatic cccDNA levels were higher (0.13 *vs.* 0.05; p <0.02) whereas cccDNA transcriptional activity was not different in the 2 groups (0.45 *vs.* 0.50; p = n.s.). Focusing on the 22 patients that did not require NA treatment, they all presented, at the last follow-up control, a median of serum HBV-DNA <2,000 IU/ml, all had normal ALT levels with a median qHBsAg <1,000 IU/ml. Thus, low HBcrAg levels (below 4 LogU/ml) are associated with a milder course of the disease in HBeAg– patients (Fig. 3 and Table S3) and this is confirmed in our follow-up analysis (Fig. 5). Altogether, these results confirm that low HBcrAg levels are associated with a lower transcriptional activity of the cccDNA and with a more favorable course of the disease, but do not discriminate between patients that meet the current requirement for NA treatment and those that do not.

HBcrAg levels do not predict HBsAg loss in NUC-treated patients

HBcrAg levels have been shown to be independently associated with response to both NUC monotherapy[41,42] and pegylated-interferon add-on in HBeAg+ patients.[41] Additionally, patterns of HBcrAg changes during treatment are predictive of sustained off-treatment response in this subgroup of patients.[41,42] The relation between HBcrAg levels and HBsAg loss is currently unknown. We sought to determine whether HBcrAg levels might predict HBsAg loss in our followed-up patients. NA-treated Cluster 3 patients had a better response in term of HBsAg loss than Cluster 1 or Cluster 2 patients (8/24, all HBeAg+ *vs.* 1/25 HBeAg– *vs.* 1/26 HBeAg–, respectively) (Fig. 5). Notably, pretreatment HBcrAg levels did not significantly differ in the 8 HBeAg+ Cluster 3 NA-treated patients who lost HBsAg compared to the 16 HBeAg+ Cluster 3 NA-treated patients who did not (median of 7.8 *vs.* 8.1 LogU/ml, p = n.s.). Altogether, pretreatment HBcrAg levels did not correlate with NA-induced HBsAg loss, that appeared to be confined to a subgroup of HBeAg+ CH patients who could not be differentiated by HBcrAg levels. Compared to the pretreatment levels, HBcrAg levels were significantly lower at the time of HBsAg loss in the 8 HBeAg+ Cluster 3 NA-treated patients (3.7 *vs.* 8.0 LogU/ml, p = 0.0002) but most of the HBcrAg decline had already occurred at the time of HBeAg seroconversion (4.2 *vs.* 8.0 LogU/ml, p = n.s.) (Fig. S7, left panel). A similar decline was observed in HBeAg+ Cluster 3 NA-treated patients who did

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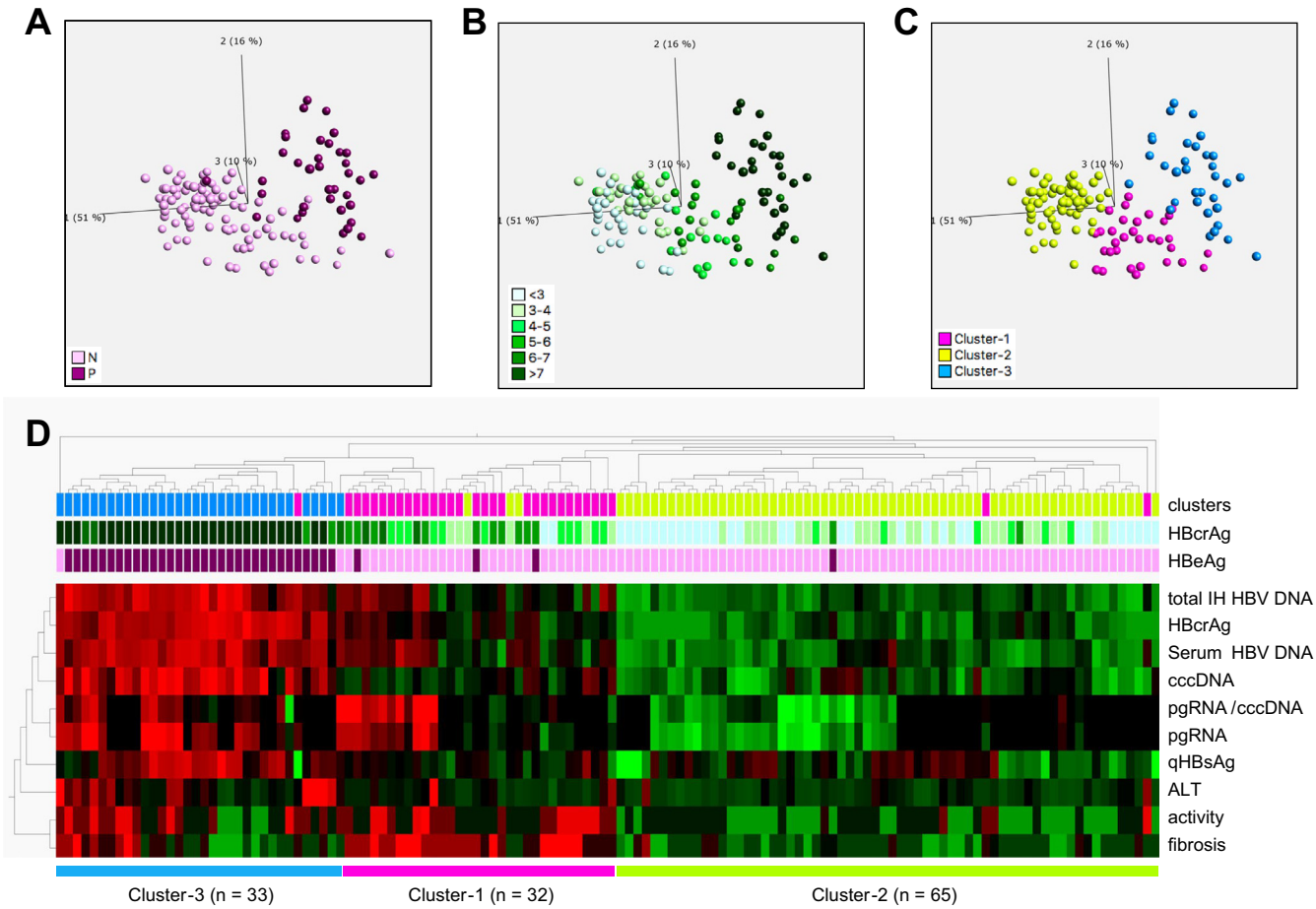

Fig. 4. Principal Component Analysis of serum and intrahepatic viral markers. PCA score plots with highlighted either (A) HBeAg+ and HBeAg– patients (violet and pink dots, respectively); (B) HBcrAg rank values (from light to dark green dots) or (C) clusters derived from k-means unsupervised clustering method. (D) Heat Map representing values of ALT, fibrosis, necroinflammatory activity and serum (HBV-DNA, HBcrAg, qHBsAg) and intrahepatic (tHBV-DNA, cccDNA, pgRNA, pgRNA/cccDNA) viral markers (rows) for each patient (columns). Red color represents values higher than 2 SDs and green color represents values lower than 2 SDs from the mean value for each variable. cccDNA, covalently closed circular DNA; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; pgRNA, pregenomic RNA; qHBsAg, quantitative hepatitis B surface antigen; tHBV, total HBV.

not reach HBsAg loss when HBcrAg levels were compared between pretreatment and the time of HBeAg seroconversion (7.9 *vs.* 4.8 LogU/ml), and no further change in HBcrAg levels occurred during the follow-up (Fig. S7, right panel). Fig. S8 shows the individual HBcrAg follow-up profiles from the 8 HBeAg+ Cluster 3 NA-treated patients who seroconverted for HBeAg and lost HBsAg, 6 of the 16 HBeAg+ Cluster 3 NA-treated patients who seroconverted for HBeAg but remained HBsAg+ and 2 additional Cluster 3 HBeAg+ patients that did not seroconvert for HBeAg under NA treatment.

Discussion

The key determinant of chronicity of HBV infection is the persistence of cccDNA in the infected hepatocytes.[2] An accurate monitoring of intrahepatic cccDNA levels and activity during patient management is limited by the need for invasive liver biopsy procedures. So far, no single serum parameter has been shown to accurately reflect the transcriptional activity of the cccDNA pool in the liver, an important target in the perspective of HBV functional cure.[1] As a consequence, there is an unmet need for the identification and characterization of new non-invasive markers for the evaluation of intrahepatic cccDNA and the prediction of successful therapy.

In this study, we investigated the relationship between HBcrAg serum levels and intrahepatic HBV viral markers in a cohort of untreated patients with CHB, who had concomitant serum and frozen liver biopsy samples. We show that HBcrAg levels reflect viral replication, as they positively correlate with serum HBV-DNA, as well as correlating with serum qHBsAg in HBeAg+ but not in HBeAg– patients. In HBeAg– patients, HBcrAg should theoretically only recognize HBcAg and the p22cr reactivity (although the detection of HBeAg released from antigen/antibody immune complexes is theoretically possible). Viral replication and protein synthesis are not necessarily always linked to each other in the HBV lifecycle.[2,5,43] In HBeAg– patients, viral genome replication and virion production/release are decreased while HBsAg expression is not affected to the same extent.[5,12,18] Whereas transcription from integrated HBV sequences can account for a significant proportion of HBsAg production in these patients, HBcAg and HBeAg necessarily require the full HBV genome template provided by cccDNA.[2,20,43] This can explain why we found only a moderate overall correlation between HBcrAg and qHBsAg, and why this

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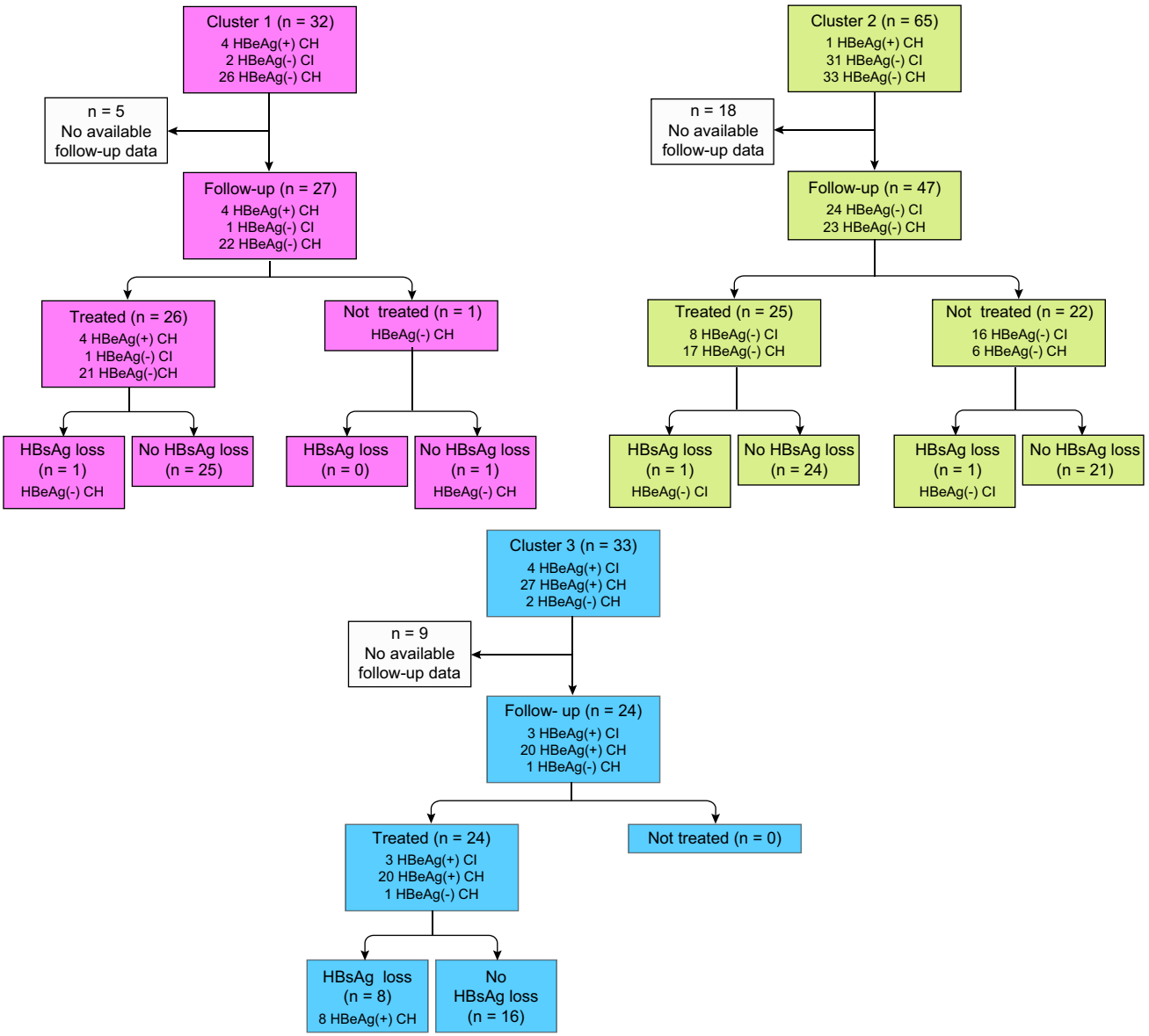

Fig. 5. Patients' clinical follow-up data. Patients are distributed according to the clusters derived from k-means unsupervised clustering analysis described in Fig. 4. Median follow-up time was of 8.0 years (interquartile range 6.6–12.3). CH, chronic hepatitis; CI, chronic infection.[7]

correlation is lost in HBeAg– patients as also suggested in a recent study.[20]

The analysis of the relationship between HBcrAg and intrahepatic viral markers allowed us to further investigate the correlation of HBcrAg with cccDNA quantity and activity. A positive correlation between HBcrAg levels and liver cccDNA has already been reported by Chen *et al.*,[34] but pgRNA levels were not investigated and, therefore, no direct information on cccDNA transcriptional activity could be deduced. Honda *et al.*[37] did not find a correlation between cccDNA and HBcrAg in a small subgroup of patients with available liver biopsies under NA treatment, but showed that NA-treated patients with CHB who were positive for HBcrAg (>3.0 logU/ml) had higher levels of cccDNA and pgRNA in the liver than patients who were negative for HBcrAg (<3.0 logU/ml). Our study provides the first comprehensive evaluation of HBcrAg, qHBsAg and HBV-DNA serum levels and their correlation with all relevant intrahepatic viral markers (cccDNA, pgRNA, tHBV-DNA) in a substantial number of patients with CHB in the different phases of CHB.[7] We found that HBcrAg is strongly correlated with tHBV-DNA, cccDNA and pgRNA levels both in HBeAg+ and HBeAg– patients. Notably, qHBsAg and serum HBV-DNA correlations with the same intrahepatic markers are much weaker. Moreover, cccDNA transcriptional activity, calculated by pgRNA/cccDNA ratio, is only correlated to HBcrAg and not to qHBsAg in HBeAg– patients, suggesting that HBcrAg is a better surrogate marker of cccDNA transcriptional activity than qHBsAg. This notion was reinforced by our PCA followed by unsupervised clustering of patients according to serum and intrahepatic viral markers. Indeed, we could identify 2 subgroups among HBeAg– patients, differing for serum HBV-DNA levels, cccDNA transcriptional activity, HBV-DNA and RNA production, but not for qHBsAg or intrahepatic cccDNA levels. Further information came from the comparison of HBeAg– patients positive or negative for HBcrAg with comparable amounts of cccDNA. The 2 groups present the same levels of qHBsAg but different levels of pgRNA and cccDNA

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transcriptional activity according to HBcrAg status, *i.e.* patients who tested positive for HBcrAg+ had the highest cccDNA transcriptional activity.

The relationship between HBcrAg and ALT levels has been the object of controversial findings.[35,44] Similarly to Maasoumy *et al.*,[29] in our study HBcrAg was only correlated with ALT levels in HBeAg− patients. The evaluation of liver histology in HBeAg− CH patients allowed us to link higher HBcrAg levels to more severe scores of fibrosis and necroinflammatory activity and to ALT levels >2x normal. Similarly, HBcrAg+ HBeAg− patients display more severe fibrosis and inflammatory activity than HBcrAg− patients. Moreover, the analysis of low viremic HBeAg− patients, with serum HBV-DNA <2000 IU/ml, allowed us to highlight a correlation between HBcrAg levels and intrahepatic cccDNA activity and necro-inflammation which was independent from qHBsAg, HBV-DNA and ALT levels. It is important to underline that the relation between HBcrAg and fibrosis or inflammation is indirect. Higher HBcrAg levels reflect a sustained HBV replication driven by the transcriptional activity of the cccDNA, that translates into more inflammation and fibrosis progression over time. Indeed, higher HBcrAg levels have been shown to predict the progression to cirrhosis in HBeAg− patients.[45] Intrahepatic HBV-DNA and cccDNA levels decline in HBeAg− CH and HBeAg− infection compared to HBeAg+ CI and CH patients.[3,12,34] As a consequence HBeAg− patients tend to have lower HBcrAg, independently of their histology, and many untreated HBeAg− patients test negative for HBcrAg because of their low HBV replication in the liver. The correlation between HBcrAg levels, necro-inflammation and fibrosis confirmed in this study, as well as others,[45] does not exclude the possibility that a proportion of HBeAg− CH patients with long duration of disease, significant fibrosis and low replication may test negative for HBcrAg.

Our cohort included only 4 HBeAg+ patients in the chronic infection stage (Table S1), which limited the evaluation of HBcrAg as a surrogate marker of cccDNA activity in this group of patients. In any case, our data suggest that the advantage of HBcrAg measurement would be more significant in untreated HBeAg− patients, where the assay does not detect any confounding HBeAg. In this respect, our results indicate that HBcrAg may represent a useful surrogate marker not only for intrahepatic cccDNA levels, but also for its transcriptional activity. Moreover, we demonstrated that HBcrAg levels continue to reflect liver disease in low viremic patients with ALT levels in the normal range.

Although our study is cross-sectional, we had the opportunity to retrospectively collect the clinical follow-up data for 98/130 (about 75%) of the patients included in the study. The follow-up results showed that low HBcrAg levels alone, measured at a single time point, do not discriminate between patients who do or do not meet the current requirement for NA treatment. Moreover, pretreatment HBcrAg levels did not predict NA-induced HBsAg loss in HBeAg+ patients, while in these patients HBeAg seroconversion represents the major driver of changes in HBcrAg quantification.

The main limitation of the HBcrAg assay is its lower limit of sensitivity. In this study and in contrast to other reports,[29,31,32,46] we used a stringent threshold of 3 LogU/ml according to manufacturer's recommendation to avoid false positivity, but recent unpublished studies suggest that a threshold of 2.5 LogU/ml might be used in the future (personal communication from Fujirebio). However, even with this lower threshold only 5 additional patients could be detected. Overall, one-third of our HBeAg− patients scored negative for HBcrAg despite harboring active cccDNA in their liver, even if at lower levels compared to HBcrAg+ patients. Prospective studies will be interesting to investigate the outcome of these HBeAg− HBcrAg− patients.

In conclusion, our results indicate that HBcrAg may represent a useful surrogate marker not only for intrahepatic cccDNA levels, but also for its transcriptional activity. Currently, HBcrAg represents an interesting novel biomarker for the evaluation of new anti-HBV therapies directly or indirectly targeting cccDNA. In the future, it will have to be compared with other novel biomarkers, such as circulating viral RNAs, when standardized assays become available.

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Conflict of interest

FZ received research grants and consultancy honoraria from Hoffmann-La-Roche, Gilead Sciences, Janssen, Assembly Biosciences, Arbutus, Contravir, Sanofi and Transgene. ML participates to advisory boards of BMS, Assembly Biosciences, Gilead, Arbutus, Janssen, Galapagos, Medimmune. PL participates to advisor and speaker bureau for Gilead, Roche, BMS, GSK, MSD, Arrowhead and Alnylam.

Authors' contributions

BT, PL, ML, FZ conceived the experiments. BT, FL, CM, FB performed experiments and analyzed data. FL, SC, SM, FF, AL collected clinical samples and patients' information. BT, PL, ML, FZ wrote the manuscript.

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Supplementary data

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References

Author names in bold designate shared co-first authorship

[1] **Lok AS**, **Zoulim F**, **Dusheiko G**, Ghany MG. Hepatitis B cure: from discovery to regulatory approval. J Hepatol 2017. https://doi.org/10.1016/j.jhep.2017.05.008.

[2] Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 2015;64:1972–1984. https://doi.org/10.1136/gutjnl-2015-309809.

[3] Werle-Lapostolle B, Bowden S, Locarnini S, Wursthorn K, Petersen J, Lau G, et al. Persistence of cccDNA during the natural history of chronic

Please cite this article in press as: Testoni B et al. Serum hepatitis B core-related antigen (HBcrAg) correlates with covalently closed circular DNA transcriptional activity in chronic hepatitis B patients. J Hepatol (2019), https://doi.org/10.1016/j.jhep.2018.11.030

hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology 2004;126:1750–1758.
[4] Wong DK-H, Seto W-K, Fung J, Ip P, Huang F-Y, Lai C-L, et al. Reduction of hepatitis B surface antigen and covalently closed circular DNA by nucleos(t)ide analogues of different potency. Clin Gastroenterol Hepatol Off Clin Pract J Am Gastroenterol Assoc 2013;11. https://doi.org/10.1016/j.cgh.2013.01.026, 1004–1010e1.
[5] Zhang X, Lu W, Zheng Y, Wang W, Bai L, Chen L, et al. In situ analysis of intrahepatic virological events in chronic hepatitis B virus infection. J Clin Invest 2016;126:1079–1092. https://doi.org/10.1172/JCI83339.
[6] Boyd A, Lacombe K, Lavocat F, Maylin S, Miailhes P, Lascoux-Combe C, et al. Decay of ccc-DNA marks persistence of intrahepatic viral DNA synthesis under tenofovir in HIV-HBV co-infected patients. J Hepatol 2016;65:683–691. https://doi.org/10.1016/j.jhep.2016.05.014.
[7] European Association for the Study of the Liver, Electronic address: easloffice@easloffice.eu, European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol 2017. https://doi.org/10.1016/j.jhep.2017.03.021.
[8] Simonetti J, Bulkow L, McMahon BJ, Homan C, Snowball M, Negus S, et al. Clearance of hepatitis B surface antigen and risk of hepatocellular carcinoma in a cohort chronically infected with hepatitis B virus. Hepatol Baltim Md 2010;51:1531–1537. https://doi.org/10.1002/hep.23464.
[9] Kim G-A, Lim Y-S, An J, Lee D, Shim JH, Kim KM, et al. HBsAg seroclearance after nucleoside analogue therapy in patients with chronic hepatitis B: clinical outcomes and durability. Gut 2014;63:1325–1332. https://doi.org/10.1136/gutjnl-2013-305517.
[10] Brunetto MR, Oliveri F, Colombatto P, Moriconi F, Ciccorossi P, Coco B, et al. Hepatitis B surface antigen serum levels help to distinguish active from inactive hepatitis B virus genotype D carriers. Gastroenterology 2010;139:483–490. https://doi.org/10.1053/j.gastro.2010.04.052.
[11] Nguyen T, Thompson AJV, Bowden S, Croagh C, Bell S, Desmond PV, et al. Hepatitis B surface antigen levels during the natural history of chronic hepatitis B: a perspective on Asia. J Hepatol 2010;52:508–513. https://doi.org/10.1016/j.jhep.2010.01.007.
[12] **Lebossé F**, **Testoni B**, Fresquet J, Facchetti F, Galmozzi E, Fournier M, et al. Intrahepatic innate immune response pathways are downregulated in untreated chronic hepatitis B. J Hepatol 2017;66:897–909. https://doi.org/10.1016/j.jhep.2016.12.024.
[13] Chan HL-Y, Thompson A, Martinot-Peignoux M, Piratvisuth T, Cornberg M, Brunetto MR, et al. Hepatitis B surface antigen quantification: why and how to use it in 2011 – a core group report. J Hepatol 2011;55:1121–1131. https://doi.org/10.1016/j.jhep.2011.06.006.
[14] Wursthorn K, Lutgehetmann M, Dandri M, Volz T, Buggisch P, Zollner B, et al. Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. Hepatol Baltim Md 2006;44:675–684. https://doi.org/10.1002/hep.21282.
[15] Lin LY, Wong VWS, Zhou HJ, Chan HY, Gui HL, Guo SM, et al. Relationship between serum hepatitis B virus DNA and surface antigen with covalently closed circular DNA in HBeAg-negative patients. J Med Virol 2010;82:1494–1500. https://doi.org/10.1002/jmv.21863.
[16] Thompson AJV, Nguyen T, Iser D, Ayres A, Jackson K, Littlejohn M, et al. Serum hepatitis B surface antigen and hepatitis B e antigen titers: disease phase influences correlation with viral load and intrahepatic hepatitis B virus markers. Hepatol Baltim Md 2010;51:1933–1944. https://doi.org/10.1002/hep.23571.
[17] Chuaypen N, Sriprapun M, Praianantathavorn K, Payungporn S, Wisedopas N, Poovorawan Y, et al. Kinetics of serum HBsAg and intrahepatic cccDNA during pegylated interferon therapy in patients with HBeAg-positive and HBeAg-negative chronic hepatitis B. J Med Virol 2017;89:130–138. https://doi.org/10.1002/jmv.24601.
[18] Volz T, Lutgehetmann M, Wachtler P, Jacob A, Quaas A, Murray JM, et al. Impaired intrahepatic hepatitis B virus productivity contributes to low viremia in most HBeAg-negative patients. Gastroenterology 2007;133:843–852. https://doi.org/10.1053/j.gastro.2007.06.057.
[19] Mason WS, Jilbert AR, Summers J. Clonal expansion of hepatocytes during chronic woodchuck hepatitis virus infection. Proc Natl Acad Sci U S A 2005;102:1139–1144. https://doi.org/10.1073/pnas.0409332102.
[20] Wooddell CI, Yuen M-F, Chan HL-Y, Gish RG, Locarnini SA, Chavez D, et al. RNAi-based treatment of chronically infected patients and chimpanzees reveals that integrated hepatitis B virus DNA is a source of HBsAg. Sci Transl Med 2017;9. https://doi.org/10.1126/scitranslmed.aan0241.
[21] Durantel D, Zoulim F. New antiviral targets for innovative treatment concepts for hepatitis B virus and hepatitis delta virus. J Hepatol 2016;64:S117–S131. https://doi.org/10.1016/j.jhep.2016.02.016.
[22] Cornberg M, Wong VW-S, Locarnini S, Brunetto M, Janssen HLA, Chan HL-Y. The role of quantitative hepatitis B surface antigen revisited. J Hepatol 2017;66:398–411. https://doi.org/10.1016/j.jhep.2016.08.009.
[23] Zoulim F, Carosi G, Greenbloom S, Mazur W, Nguyen T, Jeffers L, et al. Quantification of HBsAg in nucleos(t)ide-naïve patients treated for chronic hepatitis B with entecavir with or without tenofovir in the BE-LOW study. J Hepatol 2015;62:56–63. https://doi.org/10.1016/j.jhep.2014.08.031.
[24] van Bömmel F, Bartens A, Mysickova A, Hofmann J, Krüger DH, Berg T, et al. Serum hepatitis B virus RNA levels as an early predictor of hepatitis B envelope antigen seroconversion during treatment with polymerase inhibitors. Hepatol Baltim Md 2015;61:66–76. https://doi.org/10.1002/hep.27381.
[25] Wang J, Shen T, Huang X, Kumar GR, Chen X, Zeng Z, et al. Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. J Hepatol 2016;65:700–710. https://doi.org/10.1016/j.jhep.2016.05.029.
[26] Kimura T, Rokuhara A, Sakamoto Y, Yagi S, Tanaka E, Kiyosawa K, et al. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J Clin Microbiol 2002;40:439–445.
[27] Kimura T, Ohno N, Terada N, Rokuhara A, Matsumoto A, Yagi S, et al. Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. J Biol Chem 2005;280:21713–21719. https://doi.org/10.1074/jbc.M501564200.
[28] Seto W-K, Wong DK-H, Fung J, Huang F-Y, Liu KS-H, Lai C-L, et al. Linearized hepatitis B surface antigen and hepatitis B core-related antigen in the natural history of chronic hepatitis B. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 2014;20:1173–1180. https://doi.org/10.1111/1469-0691.12739.
[29] Maasoumy B, Wiegand SB, Jaroszewicz J, Bremer B, Lehmann P, Deterding K, et al. Hepatitis B core-related antigen (HBcrAg) levels in the natural history of hepatitis B virus infection in a large European cohort predominantly infected with genotypes A and D. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 2015;21. https://doi.org/10.1016/j.cmi.2015.02.010, 606.e1-e10.
[30] Rokuhara A, Sun X, Tanaka E, Kimura T, Matsumoto A, Yao D, et al. Hepatitis B virus core and core-related antigen quantitation in Chinese patients with chronic genotype B and C hepatitis B virus infection. J Gastroenterol Hepatol 2005;20:1726–1730. https://doi.org/10.1111/j.1440-1746.2005.04087.x.
[31] Riveiro-Barciela M, Bes M, Rodríguez-Frías F, Tabernero D, Ruiz A, Casillas R, et al. Serum hepatitis B core-related antigen is more accurate than hepatitis B surface antigen to identify inactive carriers, regardless of hepatitis B virus genotype. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 2017. https://doi.org/10.1016/j.cmi.2017.03.003.
[32] Oliveri F, Surace L, Cavallone D, Colombatto P, Ricco G, Salvati N, et al. Long-term outcome of inactive and active, low viraemic HBeAg-negative-hepatitis B virus infection: Benign course towards HBsAg clearance. Liver Int Off J Int Assoc Study Liver 2017. https://doi.org/10.1111/liv.13416.
[33] Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. J Med Virol 2009;81:27–33. https://doi.org/10.1002/jmv.21339.
[34] Chen E-Q, Feng S, Wang M-L, Liang L-B, Zhou L-Y, Du L-Y, et al. Serum hepatitis B core-related antigen is a satisfactory surrogate marker of intrahepatic covalently closed circular DNA in chronic hepatitis B. Sci Rep 2017;7:173. https://doi.org/10.1038/s41598-017-00111-0.
[35] Wong DK-H, Tanaka Y, Lai C-L, Mizokami M, Fung J, Yuen M-F. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. J Clin Microbiol 2007;45:3942–3947. https://doi.org/10.1128/JCM.00366-07.
[36] Wong DK-H, Seto W-K, Cheung K-S, Chong C-K, Huang F-Y, Fung J, et al. Hepatitis B virus core-related antigen as a surrogate marker for covalently closed circular DNA. Liver Int Off J Int Assoc Study Liver 2017;37:995–1001. https://doi.org/10.1111/liv.13346.
[37] Honda M, Shirasaki T, Terashima T, Kawaguchi K, Nakamura M, Oishi N, et al. Hepatitis B virus (HBV) core-related antigen during nucleos(t)ide analog therapy is related to intra-hepatic HBV replication and development of hepatocellular carcinoma. J Infect Dis 2016;213:1096–1106. https://doi.org/10.1093/infdis/jiv572.
[38] Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatol Baltim Md 1996;24:289–293. https://doi.org/10.1002/hep.510240201.

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[39] European Association For The Study Of The Liver. EASL clinical practice guidelines: management of chronic hepatitis B virus infection. J Hepatol 2012;57:167–185. https://doi.org/10.1016/j.jhep.2012.02.010.

[40] Hayer J, Jadeau F, Deléage G, Kay A, Zoulim F, Combet C. HBVdb: a knowledge database for Hepatitis B Virus. Nucleic Acids Res 2013;41:D566–D570. https://doi.org/10.1093/nar/gks1022.

[41] van Campenhout MJH, Brouwer WP, van Oord GW, Xie Q, Zhang Q, Zhang N, et al. Hepatitis B core-related antigen levels are associated with response to entecavir and peginterferon add-on therapy in hepatitis B e antigen-positive chronic hepatitis B patients. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 2016;22. https://doi.org/10.1016/j.cmi.2016.02.002, 571e5-e9.

[42] Wang B, Carey I, Bruce M, Montague S, Dusheiko G, Agarwal K. HBsAg and HBcrAg as predictors of HBeAg seroconversion in HBeAg-positive patients treated with nucleos(t)ide analogues. J Viral Hepat 2018;25:886–893. https://doi.org/10.1111/jvh.12889.

[43] Tu T, Budzinska MA, Shackel NA, Urban S. HBV DNA integration: molecular mechanisms and clinical implications. Viruses 2017;9. https://doi.org/10.3390/v9040075.

[44] Park Y, Hong DJ, Shin S, Cho Y, Kim H-S. Performance evaluation of new automated hepatitis B viral markers in the clinical laboratory: two quantitative hepatitis B surface antigen assays and an HBV core-related antigen assay. Am J Clin Pathol 2012;137:770–777. https://doi.org/10.1309/AJCP8QDN7NAUXJFJ.

[45] Tada T, Kumada T, Toyoda H, Kobayashi N, Akita T, Tanaka J. Hepatitis B virus core-related antigen levels predict progression to liver cirrhosis in hepatitis B carriers. J Gastroenterol Hepatol 2018;33:918–925. https://doi.org/10.1111/jgh.13989.

[46] Chuaypen N, Posuwan N, Chittmittraprap S, Hirankarn N, Treeprasertsuk S, Tanaka Y, et al. Predictive role of serum HBsAg and HBcrAg kinetics in patients with HBeAg-negative chronic hepatitis B receiving pegylated interferon-based therapy. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis 2017. https://doi.org/10.1016/j.cmi.2017.07.016.

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