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

Testoni Barbara^{1,2*}, Lebossé Fanny^{1,2,3*}, Scholtes Caroline^{1,2,4}, Berby Françoise¹, Miaglia Clothilde^{1,2,3}, Subic Miroslava³, Loglio Alessandro⁵, Facchetti Floriana⁵, Lampertico Pietro⁵, Levrero Massimo^{1,2,3,6} and Zoulim Fabien^{1,2,3,§}

¹INSERM U1052- Cancer Research Center of Lyon (CRCL), 69008 Lyon, France;

²University of Lyon, UMR_S1052, CRCL, 69008 Lyon, France;

³Department of Hepatology, Croix Rouse hospital, Hospices Civils de Lyon, France;

⁴Department of Virology, Croix Rouse Hospital, Hospices Civils de Lyon, France;

⁵Division of Gastroenterology and Hepatology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, Milan, Italy;

⁶Department of Internal Medicine - DMISM and the IIT Center for Life Nanoscience (CLNS), Sapienza University, Rome, Italy;

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*These authors contributed equally to the work

§Corresponding author:

Prof. Fabien Zoulim, INSERM U1052- CRCL, 151 cours Albert Thomas, 69424 Lyon CEDEX 03 - France

Mail: fabien.zoulim@inserm.fr; Phone number: (+33) 4 72 68 19 70; Fax number: (+33) 4 72 68 19 71

Abbreviations:

cccDNA: covalently closed circular DNA; CHB: chronic hepatitis B; CH: chronic hepatitis; CI: chronic infection; HBV: hepatitis B virus; HBeAg: HBV e antigen; HBsAg: HBV s antigen; HCC: hepatocellular carcinoma; HCV: Hepatitis C Virus; HDV: Hepatitis Delta Virus; HIV: Human Immunodeficiency Virus; IFN: interferon; NUC: nucleos(t)ides analogues; pgRNA: pre-genomic RNA; qHBsAg: quantitative HBsAg

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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

Abstract

Background and aims. Serum Hepatitis B core-related antigen (HBcrAg) has been proposed to reflect the intrahepatic cccDNA levels, but a comprehensive investigation of its correlation with serum and intrahepatic viral markers and liver histology in a large number of patients is still lacking.

Methods. HBcrAg was measured by chemiluminescent enzyme immunoassay (Fujirebio Lumipulse® G HBcrAg) in 130 [36 HBeAg(+) and 94 HBeAg(-)] biopsy proven, untreated, CHB patients. HBcrAg levels were correlated with: a) serum HBV-DNA, quantitative (q)HBsAg and alanine aminotransferase (ALT) levels; b) intrahepatic total (t)HBV-DNA, cccDNA, pgRNA 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 scored negative for HBcrAg (< 3 LogU/mL) had less liver cccDNA and lower cccDNA activity as compared to the HBcrAg(+) group. Principal component analysis coupled to unsupervised clustering identified a subgroup of HBeAg(-) patients with higher HBcrAg levels associated to higher serum HBV-DNA, intrahepatic tHBV-DNA, pgRNA, cccDNA transcriptional activity and to higher scores of fibrosis and necro-inflammatory activity.

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

Lay summary

Hepatitis B virus causes a chronic infection which develops in 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 HBcrAg in serum correlates with cccDNA amount and activity and could be used to monitor of disease progression.

Introduction

The development of novel antiviral agents and immunomodulatory approaches to cure chronic HBV infection requires new biomarkers capable to reflect the intrahepatic activity of the virus and CHB stages and define 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 immuno-modulatory agents and to assess treatment efficacy¹.

HBV covalently closed circular (ccc)DNA constitutes the unique template for pgRNA transcription and viral genome replication. Its persistence in the nucleus of infected cells is responsible for the chronicity of HBV infection². So far, antiviral therapies have demonstrated a modest effect on the established cccDNA pool³⁻⁶. Measurement of intrahepatic cccDNA levels and transcriptional activity is therefore crucial for the management of CHB patients and treatment individualization. The need for liver biopsy strongly limits the evaluation of cccDNA as a routine 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 therapy⁷. 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. 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 patients⁴. HBsAg is the hallmark of infection and its clearance is considered to be the most important clinical endpoint⁷ because both spontaneous and therapy-induced HBsAg loss are associated with histological improvement, a reduced risk of HCC and prolonged survival⁸⁻¹¹. The degree of correlation between qHBsAg and intrahepatic viral markers, in particular cccDNA levels, varies greatly between studies and is still debated¹²⁻¹⁸, particularly in HBeAg(-) carriers, where expression from HBV integrants may significantly contribute to HBsAg production, in addition to its expression from the cccDNA template¹⁹, as it has been shown in HBV infected chimpanzees²⁰.

Indeed, during NAs therapy, 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 sequences²¹⁻²³. Quantification of serum HBV RNAs may represent a novel option to predict virological response to both NAs and IFN^{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 mice²⁶ 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 to serum HBV-DNA in untreated CHB patients and might be useful to differentiate HBeAg(-) patients with active and inactive disease²⁸⁻³². A correlation between HBcrAg levels and the size of the intrahepatic cccDNA pool has been suggested in cohorts of Asian genotype B/C CHB patients, either untreated³³⁻³⁵ or undergoing NAs therapy³⁵⁻³⁷. HBcrAg has been also shown to correlate with intrahepatic viral RNAs levels in Asian NAs-treated patients³⁷. 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 CHB patients infected with several HBV genotypes.

Materials and Methods

Patients

Serum samples concomitant to liver biopsy were available for the study in 130 untreated CHB patients [4 HBeAg(+) chronic infection (CI), 32 HBeAg(+) chronic hepatitis (CH), 33 HBeAg(-) CI and 61 HBeAg(-) CH patients (Supplementary Table 1)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 (HCV), human immunodeficiency virus (HIV) and hepatitis D virus (HDV) 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 five patients out of 130: four received a course of interferon therapy and one 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 by 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, Mannheim, Germany). The detection range was between 20 IU/mL and 1.7×10^8 IU/mL. Serum quantitative HBsAg (qHBsAg) was quantified with Elecsys® HBsAg II kit / Cobas e411® (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions.

Measurement of serum HBcrAg levels

Quantitative levels of HBV core-related antigen (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 log U/mL. Despite machine lowest sensitivity limit is 2 log U/mL, 100% specificity is reached if values are above 3 logU/ml, thus HBcrAg levels between 2 and 3 LogIU/ml are considered negative. Samples with HBcrAg above 7 log U/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 classification³⁸ by the pathology services of Hospices Civils de Lyon and "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 EASL practice guidelines for HBV treatment³⁹.

DNA and RNA Extraction from Liver Biopsies

DNA and RNA were extracted from snap frozen human liver needle biopsies as described in¹². Briefly, liver samples were first homogenized on ice using a TissueRuptor (Qiagen®, Hilden, Germany) in homogenization buffer (Tris HCl pH 8 50mM, EDTA 1mM, NaCl 150mM), then divided in two 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 synthesized cDNA were assessed by NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Quantification of total HBV DNA, cccDNA and pre-genomic (pg)RNA in Liver Samples

Quantification was performed using the Light Cycler® 480 Real Time PCR System (Roche diagnostics, Mannheim, Germany) with primers and fluorescence dual hybridization probes specific for total HBV DNA or covalently closed circular (ccc)DNA as described in¹². 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 by using the β -globin gene kit (Roche Diagnostics, Mannheim, Germany). Patients' 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 in¹². Patients' 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'-TCACAATACCGCAGAGTCTAGACT-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 five 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).

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 of Caucasian (55/130) and African (41/130) origin with a predominance of HBV genotype D (51/105 tested) (Table 1). HBeAg(+) patients [HBeAg(+) chronic infection (CI) and HBeAg(+) chronic hepatitis (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 (Supplementary Figure 1). 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)] as compared to HBeAg(-) patients (Table 1 and Supplementary Table 1). 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 > 2 ULN and ALT levels >2 ULN (Supplementary Fig. 2). No significant differences were observed in HBcrAg distribution according to viral genotypes by Kruskal Wallis analysis (Supplementary Fig. 2).

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: $R=-0.08$, $p=ns$] (Figure 1, upper panels). Among the HBeAg(+) patients, HBeAg(+) CI (red dots) did not separate from HBeAg(+) CH (black dots) (Figure 1). Similar results were obtained when viral genotypes A, C and D were analyzed separately (Supplementary Figure 3) or when patients were divided according to their ethnicity (Supplementary Figure 4). 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: $R=-0.24$, $p=ns$; HBeAg(-) CI: $R=-0.25$, $p=ns$] (Figure 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 (Supplementary Figures 5 and 6). 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 weaker 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 as negative (<3 LogU/ml) in 33 / 94 HBeAg(-) patients (Supplementary Table 2). 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 (Supplementary Table 2). Regarding viral replication markers, serum HBV-DNA levels were higher in HBcrAg(+) patients, while qHBsAg levels showed no significant differences according to HBcrAg status. HBcrAg(+) group also had higher cccDNA, tHBV-DNA, pgRNA and cccDNA transcriptional activity levels (Supplementary Table 2). To further investigate the relationship between HBcrAg and cccDNA activity, we compared HBcrAg(-) and HBcrAg(+) patients having similar amounts of cccDNA. Since HBcrAg(-) patients had a cccDNA range comprised between 0.01 and 0.87 copies/cell, we applied the same filter to select for HBcrAg(+) patients. As shown in Figure 2, with the same intrahepatic amount, cccDNA was found more transcriptionally active in HBeAg(-) patients that are HBcrAg(+) as compared to HBcrAg(-) HBeAg(-) patients. 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 show 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 patients⁷. Since HBcrAg and serum HBV DNA levels were strongly correlated (Figure 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 <2000 IU/ml (Supplementary Table 1). Twenty-two of them had positive HBcrAg measurement (>3 LogU/ml) and Receiver operating characteristic (ROC) 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-value<0.0001; C.I.]-0.06; 0.55[; VPP=0.44; VPN=0.92) (Figure 3). Interestingly, patients with HBcrAg>4 LogU/ml showed also higher cccDNA amount (median of 0.17 vs 0.06 copies/cell, p=0.045) and cccDNA transcription (median of 1.27 vs 0.08, p=ns), but no differences in either HBsAg or ALT levels, with only four patients having ALT>ULN (Supplementary Table 3).

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) (Figure 4). In PCA plots (Figure 4A-C), each

dot represents a patient and colors distinguish either HBeAg(+) from HBeAg(-) patients (Figure 4A), HBcrAg rank of log values (Figure 4B) or the 3 clusters that were identified by K-means analysis (Figure 4C). By comparison of the plots in Figure 4A and 4B, most of HBeAg(+) patients showed 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 Figure 4A and 4C, violet and blue dots, respectively), while the other two clusters identified different subgroups among the HBeAg(-) patients (compare Figure 4A and 4C, 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 (Figure 4D). Cluster 3 was composed of 31 out of the 36 HBeAg(+) patients (Supplementary Table 4). 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, necro-inflammatory activity, intrahepatic tHBV-DNA, pgRNA and cccDNA transcriptional activity as compared to the Cluster 2 HBeAg(-) patients (Supplementary Table 4). 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, $p < 0.0001$) (Supplementary Table 4). 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 necro-inflammatory scores) that cannot be identified either by cccDNA levels and qHBsAg or a combination of the two. Same results were obtained when PCA analysis was applied only to HBeAg(+) 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) (Figure 5). Notably, all the patients in Cluster 1 and 3 fulfilled at a given point of the follow up the indications for NUC

treatment according to EASL 2012 CPGs³⁹. Among the Cluster 2 patients included in the follow-up, 25/47 were treated with NUCs (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 NUC treatment and the 22 patients that did not (3,8 LogU/ml vs 3,75 LogU/ml; p=ns). Moreover, the 8 HBeAg(-) CI patient that underwent NUC treatment showed a virological and biochemical reactivation during the follow-up before 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=ns), HBV-DNA (2.8 vs 2.3, logIU/ml; p=ns), qHBsAg (3,3 vs 3,4 logIU/ml; p=ns), HBcrAg (2,4 vs 3,1 logU/ml; p=ns) 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=ns). Focusing on the 22 patients that did not require NUC treatment, they all presented, at the last follow-up control, a median of serum HBV DNA <2000 IU/ml, all had normal ALT levels with a median qHBsAg <1000 IU/ml. Thus, low HBcrAg levels (below 4 LogU/ml) are overall associated with a milder course of the disease in HBeAg(-) patients (Figure 3 and Supplementary Table 3) and this is confirmed in our follow-up analysis (Figure 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 NUC 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 PEG-IFN add-on in HBeAg(+) patients⁴¹ and patterns of HBcrAg changes during treatment are predictive of sustained off-treatment response in this subgroups 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. NUC-treated Cluster 3 patients had a better response in term of HBsAg loss as compared to Cluster 1 or Cluster 2 patients (8/24, all HBeAg(+) vs 1/25 HBeAg(-) vs 1/26 HBeAg(-), respectively) (Figure 5). Notably, pretreatment HBcrAg levels did not significantly differ in the 8 HBeAg(+) Cluster 3 NUC-treated patients who lost HBsAg as compared to the 16 HBeAg(+) Cluster 3 NUC-treated patients who did not (median of 7.8 vs 8.1 LogU/ml, p=ns). Altogether, pre-treatment HBcrAg levels did not correlate with NUC induced HBsAg loss, that appeared to be confined to a subgroup of HBeAg(+) CH patients who could not be differentiated by HBcrAg levels. HBcrAg levels were significantly lower at the time of HBsAg loss as compared to the pre-treatment levels in the 8 HBeAg(+) Cluster 3 NUC-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=ns) (Supplementary Figure 7, left panel). A similar decline was observed in HBeAg(+) Cluster 3 NUC-treated patients who did 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 (Supplementary Figure 7, right panel). In Supplementary Figure 8 are shown the individual HBcrAg follow-up profiles from the 8 HBeAg(+) Cluster 3 NUC-treated patients who seroconverted for HBeAg and lost HBsAg, 6 of the 16 HBeAg(+) Cluster 3 NUC-treated patients who seroconverted for HBeAg but remained HBsAg(+) and 2 additional Cluster 3 HBeAg(+) patients that did not seroconverted for HBeAg under NUC treatment.

Discussion

The key determinant of chronicity of HBV infection is the persistence of cccDNA in the infected hepatocytes². An accurate monitoring of intrahepatic cccDNA levels and activity during patients' management is limited by the need of invasive liver biopsy procedures. So far, no single serum parameter has proven to accurately reflect the transcriptional activity of the cccDNA pool in the liver, an important target in the perspective of HBV functional cure¹. 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 CHB patients with concomitant serum and frozen liver biopsy samples. We show that HBcrAg levels reflect viral replication, since they positively correlate with serum HBV-DNA, and correlated 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 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 correlation is lost in HBeAg(-) patients as also suggested in a recent study²⁰.

The analysis of the relationship between HBcrAg and intrahepatic viral markers allowed us to further investigate the correlation of HBcrAg with cccDNA amount and activity. A positive correlation between HBcrAg levels and liver cccDNA has been already reported by Chen and coll³⁴, but pgRNA levels were not investigated and, therefore, no direct information on cccDNA transcriptional activity could be deduced. Honda and coll.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 CHB patients positive for HBcrAg (> 3.0 log U/ml) had higher levels of cccDNA and pgRNA in the liver as compared to the patients negative for HBcrAg (< 3.0 log U/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, total HBV-DNA) in a substantial number of CHB patients in the different phases of CHB⁷. We found that HBcrAg is strongly correlated to 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 Principal Component Analysis followed by unsupervised clustering of patients according to serum and intrahepatic viral markers. Indeed, we could identify two 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 two groups present the same levels of qHBsAg but different levels of pgRNA and cccDNA transcriptional activity according to HBcrAg status, i.e. patients being tested positive for HBcrAg(+) having the highest cccDNA transcriptional activity.

The relationship between HBcrAg and ALT levels has been the object of controversial findings^{35,44}. Similarly to Maasoumy and colleagues²⁹, in our study HBcrAg was correlated to ALT levels only 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 >2N. Similarly, HBcrAg(+) HBeAg(-) patients display more severe fibrosis and inflammatory activity than HBcrAg(-) ones. 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(-) patients⁴⁵. Intrahepatic HBV DNA and cccDNA levels decline in HBeAg(-) CH and HBeAg(-) infection as compared to HBeAg(+) CI and CH patients^{3,12,34}. As a consequence HBeAg(-) patients tend to have lower HBcrAg, independently from 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 as well as other studies⁴⁵ 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(+) patient in the chronic infection stage (Supplementary Table 1) and, therefore, 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 also 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 meet or not the current requirement for NUC treatment. Moreover, pre-treatment HBcrAg levels did not predict NUC-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 targeting directly or indirectly the cccDNA. In the future, it will have to be compared with other novel biomarkers, such as circulating viral RNAs, when standardized assays will become available for the later.

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Author names in bold designate shared co-first authorship.

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Figure legends

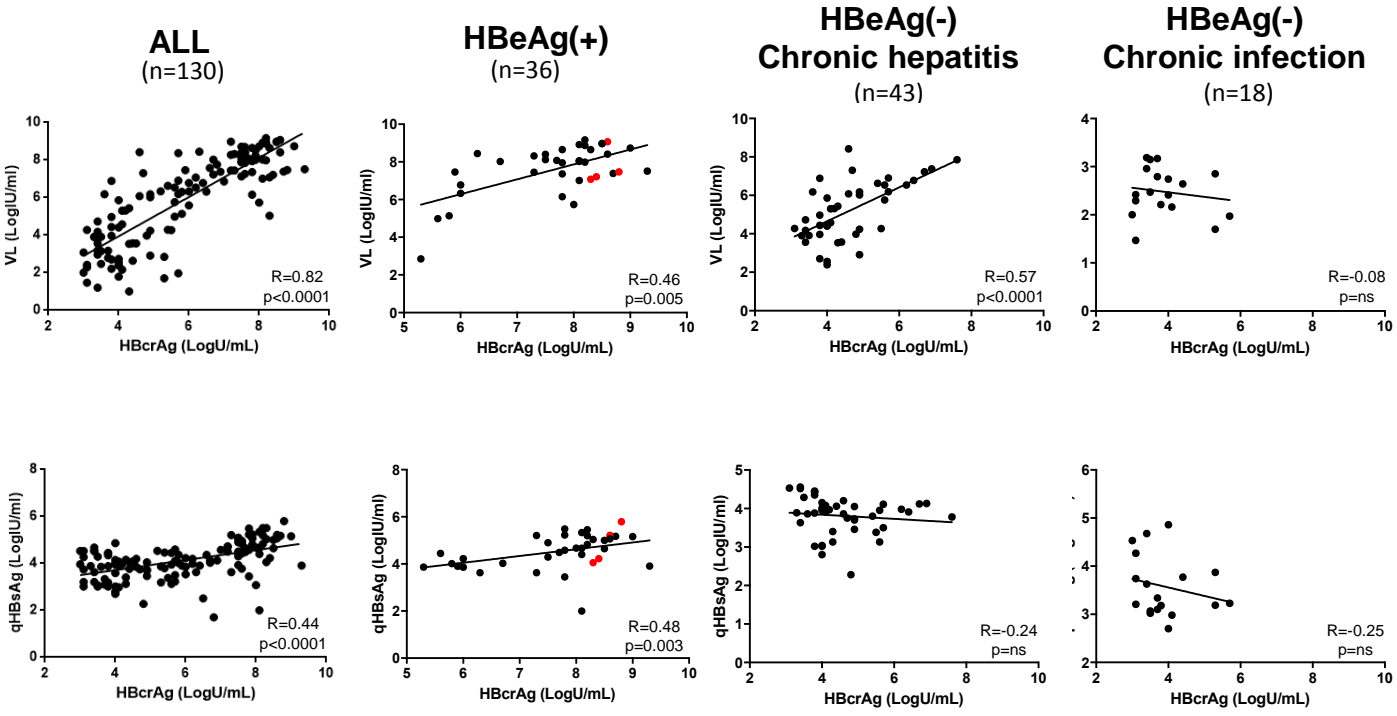
Figure 1. Correlations between HBcrAg, serum HBV-DNA and qHBsAg levels. Upper panels, correlation between HBcrAg and serum HBV-DNA. Lower panels, correlation between HBcrAg and serum qHBsAg. Only patients having positive HBcrAg values (i.e. >3 LogU/ml) were included in the analysis (36 HBeAg(+) and 61 HBeAg(-), see Table 1 and Supplementary Table 1). The four HBeAg(+) CI patients were analyzed together with HBeAg(+) CH patients for statistical reasons, but they are highlighted by red dots. Correlation coefficient was calculated using Spearman's correlation test. Two-tailed p-value was calculated for a risk threshold $\alpha=0.05$.

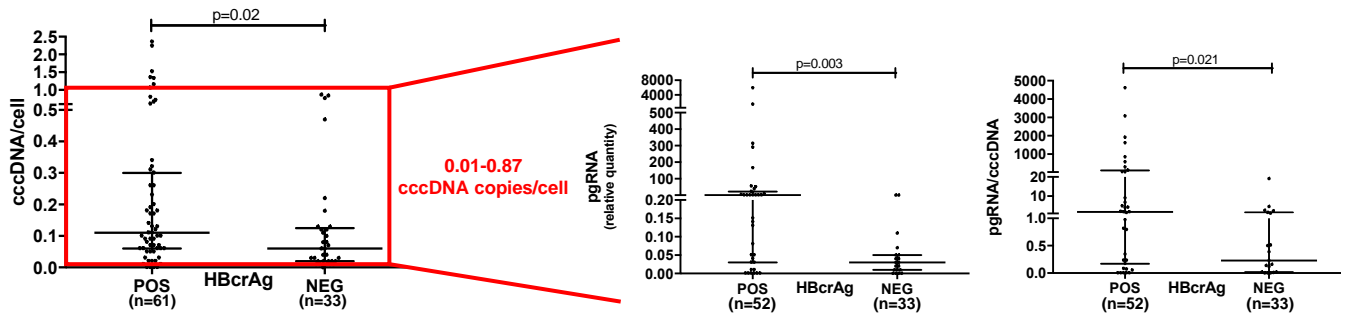
Figure 2. HBcrAg(+) HBeAg(-) patients show higher cccDNA transcriptional activity than HBcrAg(-) HBeAg(-) ones. Dot plots of pgRNA and cccDNA transcriptional activity (pgRNA/cccDNA ratio) levels in HBcrAg(+) vs HBcrAg(-) in 85 HBeAg(-) patients with cccDNA quantity comprised between 0.01 and 0.87 copies/cell. Lines indicate median with interquartile range. Mann-Whitney test was used to compare HBcrAg(+) and HBcrAg(-) groups, α threshold=0.05.

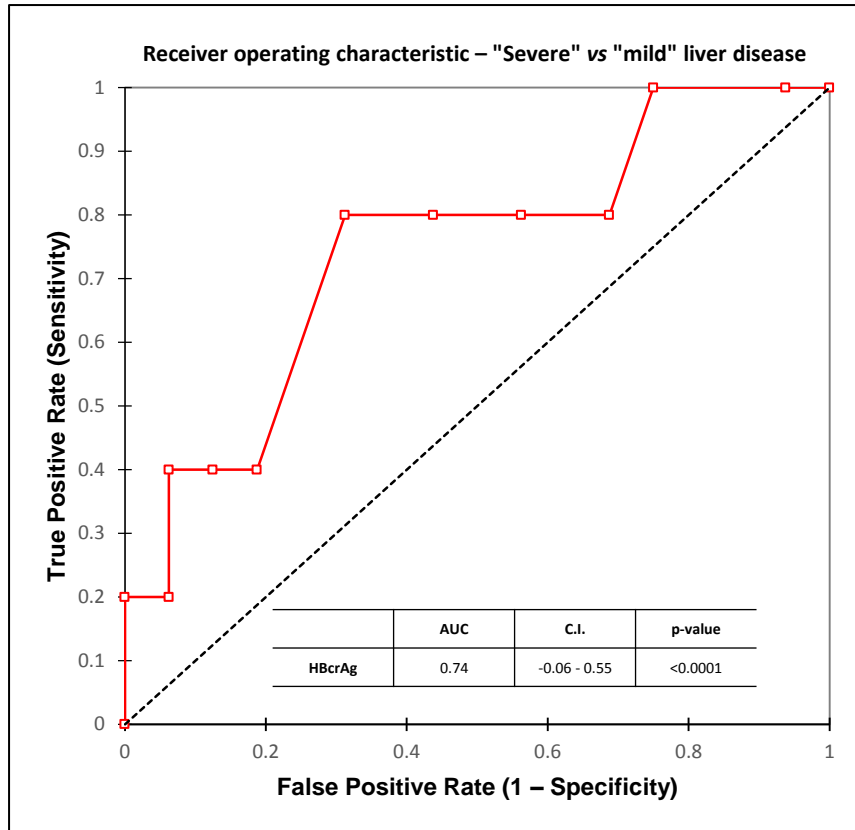
Figure 3. Receiver operating characteristic curve analysis. HBeAg(-) HBcrAg(+) patients (n=21) with mild vs minimal liver disease (described as fibrosis and/or necroinflammatory activity scores >2 or <1, respectively) were significantly distinguished by a HBcrAg cut-off value of 4 LogU/ml (PPV 0.44; NPV 0.92; p-value<0.0001; C.I.]-0.06; 0.55]). AUC = area under the curve; C.I. = confidence interval; PPV = positive predictive value; NPV = negative predictive value.

Figure 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 standard deviations (SD) and green color represents values lower than 2 SD from the mean value for each variable.

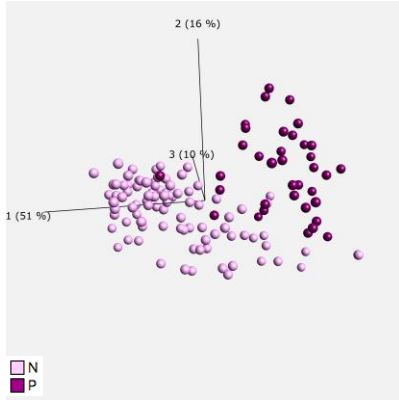
Figure 5. Patients' Clinical follow-up data. Patients are distributed according to the clusters derived from k-means unsupervised clustering analysis described in Figure 4. Median follow-up time was of 8.0 years (interquartile range 6.6-12.3). CH, chronic hepatitis; CI, chronic infection⁷.



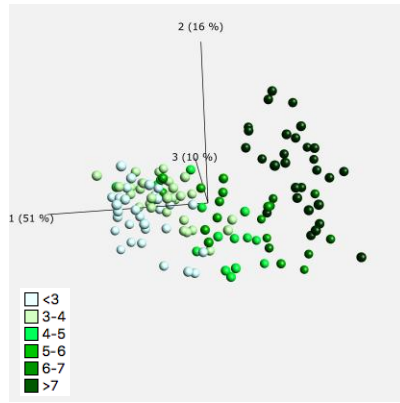




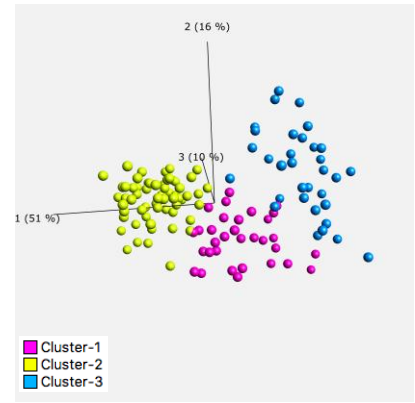
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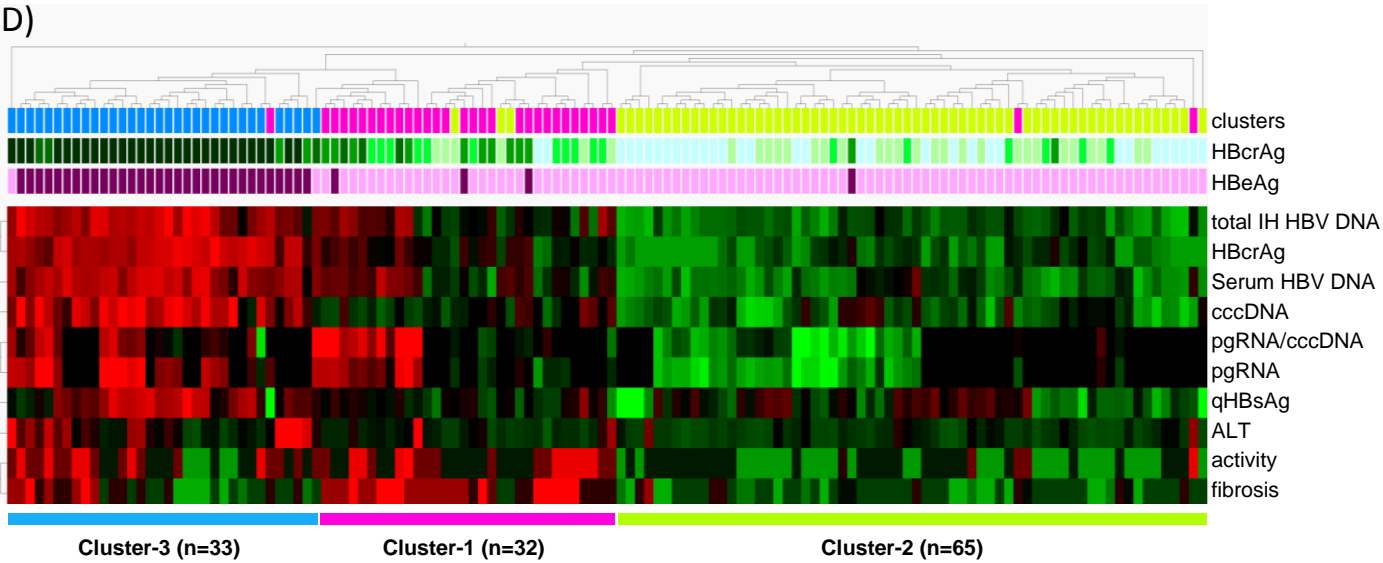
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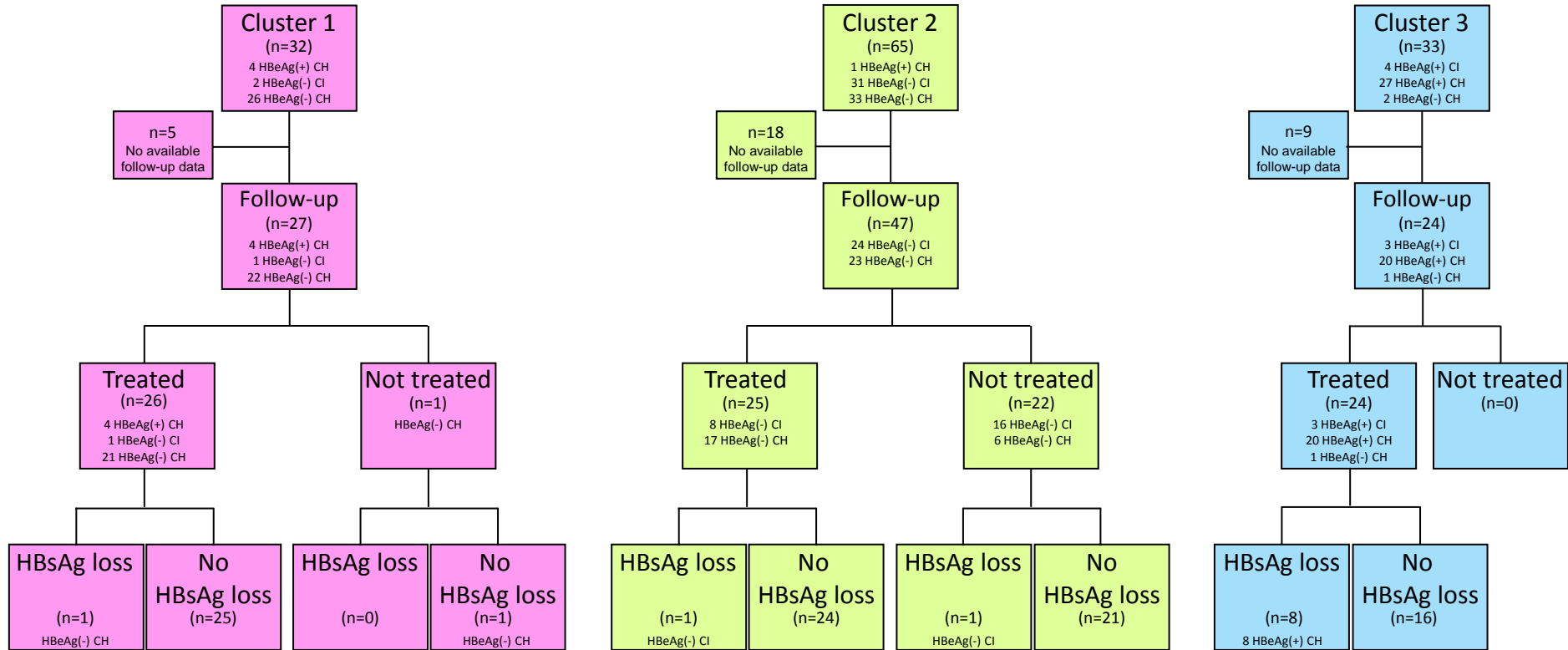


Table 1.
Patients' characteristics, HBcrAg and intrahepatic viral markers quantification

	Total cohort (n=130)	HBeAg(+) patients (n=36)	HBeAg(-) patients (n=94)	p-value
Age¹ (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%)	ns 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-saharian Africa	24 (18.5%)	3 (8%)	21 (22.2%)	
South America	2 (1.5%)	1 (3%)	1 (1.2%)	
Viral genotype²				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 load¹ (LogIU/mL)	4.4 (2.9 – 7)	8 (7.2-8.5)	3.56 (2.5-4.9)	<0.0001 ⁴
ALT¹ (IU)	52 (33.2 – 78.7)	81 (62.5 – 177)	41.5 (29 – 64.2)	0.0001 ⁴
qHBsAg¹ (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
HBcrAg¹ (LogIU/ml)	5.3 (4 – 7.6)	8 (7.3 - 8.3)	4 (3.7 – 4.9)	0.0001 ⁴
Total HBV DNA¹ (copies/cell)	11.6 (3.6 – 744.9)	254.8 (579 – 4602.6)	5.7 (2.5 – 20.6)	0.0001 ⁴
cccDNA¹ (copies/cell)	0.15 (0.06 – 1.34)	6.3 (1.4 – 18.1)	0.09 (0.03 – 0.2)	0.0001 ⁴
totHBV DNA/cccDNA¹ (relative quantity)	105.9 (29.8 – 463)	314.5 (117.6 – 1195.6)	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 – 1734.1)	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 ($\leq 1/\geq 2$)	72/58 (55.4/44.6%)	17/19 (47/53%)	55/39 (58.5/41.5%)	ns ⁵
Activity ($\leq 1/\geq 2$)	87/43 (67/33%)	17/19 (47/53%)	26/68 (27.6/72.4%)	ns ⁵

¹Data are expressed as median (1st quartile-3rd quartile)

²data available for 105 patients (72 HBeAg(-) and 33 HBeAg(+))

³data available for 80 patients (58 HBeAg(-) and 22 HBeAg(+))

⁴Mann-Whitney U test between HBeAg(+) and HBeAg(-) groups of patients; alpha threshold= 0.05

⁵X² test between HBeAg(+) and HBeAg(-) groups of patients

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=ns
	qHBsAg	R=0.49; p=ns	R=0.33; p=0.01	R=0.32; p=ns	R=0.26; p=ns
	Serum HBV DNA	R=0.50; p=0.003	R=0.29; p=ns	R=0.41; p=0.07	R=0.18; p=ns
HBeAg(-) chronic hepatitis ¹ (n=43)	HBcrAg	R= 0.61; p<0.0001	R= 0.25; p=ns	R= 0.81; p<0.0001	R=0.70; p<0.0001
	qHBsAg	R=-0.15; p=ns	R=-0.4; p=0.01	R=-0.02; p=ns	R=0.15; p=ns
	Serum HBV DNA	R=0.71; p<0.0001	R=0.19; p=ns	R=0.79; p<0.0001	R=0.66; p=0.0002
HBeAg(-) chronic infection ¹ (n=18)	HBcrAg	R= 0.34; p=ns	R=0.47; p=0.05	R=0.29; p=0.09	R=0.11; p=ns
	qHBsAg	R=0.24; p=ns	R=-0.03; p=ns	R= -0.12; p=ns	R=0.08; p=ns
	Serum HBV DNA	R=-0.02; p=ns	R=0.27; p=ns	R=0.39; p=ns	R=0.28; p=ns

¹Only patients with positive HBcrAg quantification (i.e. ≥ 3 LogU/ml) were included in the analysis

²HBeAg(+) chronic infection category was composed by only four patients (see Supplementary Table1), therefore it was not included in the analysis

