Incremental value of Hepatitis B core-related antigen to classify HBeAg-negative individuals into chronic infection or hepatitis. A multicenter data analysis.

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Abbreviations:

ALT : alanine aminotransferase ; cccDNA: covalently closed circular DNA; CHB: chronic hepatitis B; ENI: eAg-negative infection (inactive carriers); GZ : Grey Zone, eAg_negative infection with low viremia (<20,000 IU/mL) ; HBV: hepatitis B virus; HBcrAg : HBV core-related antigen ; HBeAg: HBV e antigen; HBsAg: HBV s antigen; HCC: hepatocellular carcinoma; HCV: Hepatitis C Virus; HDV: Hepatitis Delta Virus; HIV: Human Immunodeficiency Virus ; NUC: nucleos(t)ides analogues; qHBsAg: quantitative HBsAg

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MR.Brunetto and M.Buti conceived the study protocol, led the project and wrote the article. Other authors collected biomarker and patient 's data. All calculations regarding the number and position of co-authors were based on numbers of cases included by each center.

Keywords:

hepatitis B virus (HBV); chronic hepatitis B (CHB); biomarker; Hepatitis B core-related antigen (HBcrAg); patient classification

SUMMARY

Background

An accurate, single-point differential diagnosis between HBeAg-negative infection (ENI) and chronic hepatitis B (CHB) is an unmet need.

Aims

To assess the diagnostic value of the new hepatitis B core-related antigen (HBcrAg) assay.

Methods

A retrospective anonymised data analysis was performed in a multicentre European (nine centres and six countries) cohort of 1582 consecutive HBsAg-positive/HBeAg-negative subjects

classified according to EASL guidelines as: 550-CHB, 710-ENI and 322-GZ (grey-zone, HBV-DNA <20 000 IU/mL).

Results

Mean age was 44 (±13.2 y), 59% were men; HBV genotypes were 15% A, 2% B, 2% C, 45% D, 9% E, 1% F and 26% unknown. Median HBV-DNA serum levels were 2.2 (1.5-2.7), 3.5 (3.2-3.8) and 5.6 (4.8-6.6) logIU/mL in ENI, GZ and CHB, $P < 0.0001$. HBsAg serum levels (HBsAgsl) were comparable in CHB and GZ, but lower in ENI (2.9 [2.1-3.6] logIU/mL), $P < 0.0001$. HBcrAg serum levels (HBcrAgsl) were <3 logU/mL in 90.7% (644/710) ENI, 75.2% (242/322) GZ and 4.7% (26/550) CHB ($P < 0.0001$). Median HBcrAgsl were 4.8 (3.9-5.7), 2.5 (2.0-2.9) and 2.0 (2.0-2.5) logU/mL in CHB, GZ and ENI, ($P < 0.0001$). ROC-AUCs for HBcrAg and HBsAg were 0.968 (95% CI, 0.958-0.977) and 0.732 (95% CI, 0.704-0.760) respectively. The optimal HBcrAgsl cut-off to distinguish CHB from ENI was 3.14 logU/mL (95% CI, 3.02-3.25, 91% SE, 93% SP and 92.4% DA). HBcrAgsl were associated with HBV genotypes ($P < 0.001$, one-way ANOVA) but using genotype-specific cut-offs, HBcrAg DA remained unchanged with overlapping 95% CI.

Conclusion

The HBcrAg assay showed high diagnostic performance in the accurate single-point identification of patients with HBeAg-negative CHB, independently of HBV genotype. This should prompt future prospective studies to confirm its diagnostic role in clinical practice.

INTRODUCTION

Patients positive for hepatitis B surface antigen (HBsAg) and negative for hepatitis e antigen (HBeAg) currently account for the largest subgroup of individuals with hepatitis B virus (HBV) infection worldwide.[1] HBeAg-negative phase of chronic HBV infection includes a spectrum of conditions that vary in terms of natural history, severity of liver damage and need for anti-viral treatment: the two polar conditions being now termed chronic infection and chronic hepatitis B (CHB).[1] Unlike patients with CHB, those with HBeAg-negative infection—previously known as inactive carriers—have a favourable long-term outcome, with low risk of cirrhosis or hepatocellular carcinoma and do not require anti-viral treatment.[1-4] To warrant an appropriate clinical management, it is therefore important to correctly and timely diagnose HBeAg-negative carriers as having chronic infection or hepatitis. However, despite careful initial assessment, serial measurements of hepatitis B virus (HBV) DNA and serum alanine aminotransferase (ALT) levels over at least 1 year are required because their fluctuations over time often preclude straightforward classification of patients at a single-point evaluation at least in individuals with

low viraemia (<20 000 IU/mL) and normal ALT at the first observation.[1, 5, 6] Quantification of HBsAg serum levels helps to distinguish HBeAg-negative infection from hepatitis; however, HBsAg serum levels are influenced by HBV genotype, making cumbersome its use in populations with a high heterogeneity of HBV genotypes.[1, 6-8] In addition, recently, it has been shown that, mainly in HBeAg-negative carriers, HBsAg can be produced from HBV-DNA sequences integrated in the host genome.[9] Therefore, an unmet need to improve the management of HBeAg-negative individuals is the availability of new biomarkers fostering an accurate and possibly single-point differential diagnosis between carriers of HBeAg-negative infection and patients with HBeAg-negative chronic hepatitis B. Recently, a standardised assay for the detection of circulating viral proteins with HBeAg and HBcAg antigenicity,[10] the hepatitis B core-related antigen (HBcrAg) has become available. HBcrAg serum levels in untreated patients show high correlation with the levels of viraemia and intrahepatic covalently closed circular DNA (cccDNA)[11-13] and with the transcriptional activity of cccDNA.[13] Therefore, HBcrAg has been proposed as a new diagnostic tool to improve the management of HBV carriers.[3, 5, 6, 14-16] The aim of our study was to assess whether HBcrAg could improve the characterisation of HBsAg-positive, HBeAg-negative individuals using data from a large database collected from multiple European centres.

MATERIALS AND METHODS

2.1Patients and study design

The study was a retrospective analysis of data from nine hepatology centres in six European countries (Italy, UK, Germany, Spain, the Netherlands and France). A common database was prepared to collect all the demographic, virologic, biochemical and imaging information that were obtained from subjects with HBsAg-positive/HBeAg-negative infection or CHB before treatment, prospectively seen in consultation at the nine centres.

The study included all consecutive untreated HBsAg-positive, HBeAg-negative carriers, who were assessed from October 2012 to October 2017 and with at least one baseline serum sample. For HBeAg-negative individuals with HBV DNA ≤20 000 IU/mL and normal ALT (<40 IU/L) at the first observation, data on HBV DNA and ALT (at least three time points) were collected during 12-18 months of follow-up to accurately define, according to European Association for the Study of the Liver, Clinical Practice Guidelines (EASL CPG), their clinical category. For patients with CHB (HB V DNA >20 000 IU/mL and elevated ALT), only baseline data were considered. Exclusion criteria were: HBV DNA ≤2000 IU/mL and persistently or intermittently elevated ALT; viral coinfection (HCV, HDV or HIV); pregnancy; presence of cirrhosis; presence of concomitant alcoholic, autoimmune, metabolic liver disease; previous or ongoing anti-viral treatment.

A serum sample was obtained at baseline from all HBsAg-positive/HBeAg-negative individuals for the quantification of HBV DNA, HBsAg and HBcrAg. A proportion (478 of 1032, 46.3%) of cases without CHB had a second time point measured at an average interval of 43.6 months (minimum 12-maximum 157 months): 303 of 710 individuals meeting the criteria of inactive carriers (HBV DNA <2000 IU/mL and normal ALT) and 175 of 322 with fluctuating HBV DNA ≤20 000 IU/mL and normal ALT (Grey Zone, GZ).

Data originally stored in the databases from each centre were anonymised and shared with an independent statistician (IDDI, Belgium) using ShareFile, a secure, 21 Code of Federal Regulation Part 11-compliant, cloud-based platform. Single centre data were collected in the common database from October 2017 to March 2018. Individual centres only had access to their data, but not to those of other centres.

The study was conducted according to Good Clinical Practice guidelines, and the protocol was approved by the EC of each participating centre. All patients provided written informed consent for further use of their collected samples for research purposes.

2.1.1 Patient categorisation

Patients were categorised into one of three groups or clinical categories, following EASL[1] guidelines: (a) chronic HBeAg-negative infection, those with serum HBV DNA persistently ≤2000 IU/mL and persistently normal ALT levels (<40 IU/L); (b) chronic hepatitis B patients, those with a serum HBV DNA level above 20 000 IU/mL and elevated ALT levels (persistently or intermittently) (CHB group), and (c) chronic hepatitis B infection with fluctuating HBV DNA between 2000 and 20 000 IU/mL and normal ALT levels during the 12-18 months follow-up (Grey Zone, GZ).

2.2 Assays

All measurements on serum samples were performed independently at each clinical centre: ALT serum levels were measured, at baseline and during the follow-up, on fresh serum samples by routine procedures. Qualitative antibody to hepatitis B core antigen (anti-HBc), HBeAg and anti-HBe and antibodies to HCV, HDV and HIV were detected by commercially available immunoassays.

HBV-DNA, HBsAg and HBcrAg serum levels were quantified on different aliquots of the same serum sample obtained from each individual at the first observation (baseline). Serum HBV DNA levels were quantified by COBAS TaqMan assay, sensitivity 6 IU/mL and dynamic range 6-1.10×10^8 IU/mL (Roche Diagnostic Systems Inc). Serum HBsAg was quantified using commercially available assays: Architect HBsAg assay, dynamic range 0.05-250.0 IU/mL (Abbott Laboratories) or Elecsys HBsAg II quant, dynamic range 0.05-52 000 IU/mL (Roche Diagnostic

Systems Inc). The two assays show a high coefficient of correlation as previously reported.[17] Serum HBcrAg levels were measured using LUMIPULSE® G HBcrAg assay (Fujirebio Europe NV), according to the instructions of the manufacturer. The concentration of HBcrAg was calculated by a standard curve generated using recombinant pro-HBeAg (amino acids 10-183) and was expressed in arbitrary unit by the LUMIPULSE® G system. The lower detection limit was 2.0 logU/mL (0.1 kU/mL), and the dynamic range from 3.0 logU/mL to 7.0 logU/mL (1.0-10 000 kU/mL).[18] HBcrAg values in between the lower limit of detection (2.0 logU/mL) and the lower limit of the dynamic range (2.0-3.0 logU/mL) were reported, but as diagnostic cut-off we used the 3.0 logU/mL threshold, as suggested by the manufacturer.
HBV genotype was determined by direct sequencing when the DNA level allowed it.

2.3 Statistical analysis

Quantitative data for viral biomarkers were transformed for analysis using their $\log_{10}$ values. Normally distributed quantitative variables were summarised by the mean and standard deviation (SD) and compared between groups using Student's *t* test. Quantitative variables with non-normal distribution were summarised by the median and interquartile range (IQR) and compared between groups using the Mann-Whitney *U* test. Categorical variables were summarised by percentages and 95% confidence intervals (CI) and compared between groups using the Chi-square test. Pearson's correlation coefficients between HBcrAg, HBsAg, HBV DNA and ALT serum levels were estimated overall and by clinical category. ANOVA models were used to study the unadjusted (one-way) and adjusted (two-way) associations between genotype and HBcrAg levels. Receiver operating characteristic (ROC) curves were constructed, and the areas under these curves (AUC) were computed with a 95% CI based on bootstrapping. ROC curves were used to discriminate between HBeAg-negative infection and CHB patients, whereas Grey Zone patients were removed from these analyses. ROC curves were constructed for HBsAg and HBcrAg and for their combination, with cut-off values to differentiate between HBeAg-negative infection and CHB taking into account the highest Youden index (sensitivity + specificity − 1). The diagnostic performance of HBcrAg and HBsAg levels was evaluated by computing their sensitivity, specificity, positive (PPV) and negative (NPV) predictive values and accuracy. Logistic regression models were used to explore the associations between candidate predictive variables and the presence of CHB; the variables genotype, HBsAg, HBcrAg, ALT, platelets and liver stiffness were included in multivariate models with backward selection. Odds ratios (ORs) and corresponding 95% CIs were used to quantify the strength of associations. The statistical analysis was done using R v3.4.3 and Analyse-it software and *P*-values < 0.05 were considered significant (with the exception of pairwise comparisons of genotypes for HBcrAg levels within clinical categories, other *P*-values were not adjusted for multiple testing).

RESULTS

3.1 Patient characteristics

The main demographic, serological, virological and clinical features of the 1582 HBeAg-negative individuals who were included in the study are reported in Table 1: 663 were from Italy, and others were from the UK (N = 307), Germany (N = 256), Spain (N = 151), the Netherlands (N = 146) and France (N = 59). Overall, 937 HBsAg-positive/HBeAg-negative carriers, who were observed at the clinical centres during the study period, were excluded for the following reasons: elevated ALT in spite of HBV-DNA <2000 IU/mL in 40; co-infection with HDV or HCV or HIV in 60; pregnancy in 7; cirrhosis in 165; concomitant alcoholic, autoimmune or metabolic liver disease in 228. In addition, baseline serum sample was missing in 189 subjects and the follow-up was not adequate in 248.

After a median follow-up of 16.2 months (6-18), the 1582 HBsAg-positive/HBeAg-negative carriers were classified: 710 (44.9%) as chronic HBV infection; 322 (20.4%) with viraemia fluctuating above 2000 IU/mL, but persistently ≤20 000 IU/mL (Grey Zone, GZ) and 550 (34.8%) with chronic hepatitis B (CHB). Overall, 59% of the carriers were males, who were more frequently represented in CHB than in GZ and HBeAg-negative infection (73% vs 47% and 53%, respectively, $P < 0.001$). The mean age was 44 ± 13.2 years. The level of HBV DNA allowed genotyping in 1171 of 1582 (74.0%) cases: the most frequent was genotype D (713 of 1171, 60.9%), which showed a higher prevalence in CHB patients (377/713, 52.9%) than in Grey Zone (113/713, 15.8%) and HBeAg-negative infection (223/713, 31.3%, $P < 0.001$).

Overall, median (25th-75th percentiles) HBV DNA and HBsAg serum levels were 3.2 (2.3-4.9) logIU/mL and 3.4 (2.8-3.9) logIU/mL respectively. As expected, the lowest HBV DNA levels were observed in HBeAg-negative infection (2.2 [1.5-2.7] logIU/mL), the highest in CHB (5.6 [4.8-6.6] logIU/mL), whereas in Grey Zone, the values were intermediate (3.5 [3.2-3.8] logIU/mL). Unlike viraemia, HBsAg serum levels were comparable in CHB and Grey Zone (3.7 [3.3-4.0] and 3.6 [3.1-4.1] logIU/mL, respectively, $P = 0.1584$), but significantly lower in HBeAg-negative infection (2.9 [2.1-3.6] logIU/mL), $P < 0.0001$ (Table 1).

HBcrAg serum levels were <3 logU/mL in 90.7% (644/710) of HBeAg-negative infection, in 75.2% (242/322) of the Grey Zone and in 4.7% (26/550) of CHB patients ($P < 0.0001$ between distribution of all three groups, as well between HBeAg-negative infection and GZ). In the overall cohort, the median HBcrAg serum levels were 2.7 (2.0-4.1) logU/mL: HBcrAg levels were significantly higher in CHB (4.8 [3.9-5.7] logU/mL) as compared to Grey Zone (2.5 [2.0-2.9] logU/mL) and HBeAg-negative infection (2.0 [2.0-2.5] logU/mL) ($P < 0.0001$).

3.2 Correlations between HBcrAg and HBV DNA and HBsAg levels

In the overall cohort of HBcrAg, HBsAg and HBV DNA showed a significant correlation with each other (Figure 1), the strongest being between HBcrAg and HBV DNA (R = 0.80, $P < 0.001$), followed by HBsAg and HBV DNA (R = 0.42, $P < 0.001$) and HBcrAg and HBsAg (R = 0.37, $P < 0.001$). No meaningful correlations were found between ALT and any viral marker. Moreover, the CHB group in whom HBcrAg and HBV DNA strongly correlate, a linear regression fit showed an inter-dependency between the two variables. High viral load (>20 000 IU/mL) was found to be a predictor of HBcrAg level (standardised beta 0.63 ($\log_{10}$ HBV DNA), $P < 0.0001$; $r^2 = 0.393$; N = 533 pairs) in CHB.

3.3 Association between HBcrAg and HBV genotypes

The analysis was run in 1158 of 1582 (73.2%) samples, as genotype was not determinable in 411 (26.0%) cases and genotypes A/E, D/E and F were excluded from the analysis because of the small number of cases (1, 1 and 11 respectively). A significant association was observed between serum HBcrAg levels and HBV genotypes ($P < 0.001$, one-way ANOVA—Figure 2). In HBeAg-negative infection, mean HBcrAg levels were significantly higher (about 7- to 8-fold in U/mL) in genotype B than genotype A, D and E carriers (mean log difference of 0.9, 95% CI: 0.1-1.6). Despite such differences, median HBcrAg level was below 3 logU/mL in all the genotype subgroups. In CHB group, the few patients with HBV genotype C (16 of 394, 4.1%) had a higher (about 20-fold in U/mL) HBcrAg level than patients with HBV genotype E (19 of 494, 3.8%; mean log difference 1.32, 95% CI: 0.24-2.40).

To overcome possible biases due to the low HBcrAg serum levels in Grey Zone and HBeAg-negative infection groups (HBcrAg <3 logU/mL in 75.2% and 90.7% respectively) and the different prevalence of HBV genotypes (Table 1), we analysed the relationship between the HBV genotype and HBcrAg, HBV DNA and HBsAg serum levels only in CHB patients (HBcrAg >3 logU/mL in 95.3% of cases). The median of HBV DNA and HBsAg levels was significantly different according to HBV genotypes ($P < 0.0001$) by converse, the median of HBcrAg levels was less affected ($P = 0.0621$, Kruskal-Wallis test median location). Pairwise comparisons for HBV DNA, HBsAg and HBcrAg levels (Figure 3—Tukey-Kramer all pairs comparisons) confirmed that patients infected by genotype C (N = 16) had higher HBcrAg level than patients with genotype E (N = 19), with a difference of 1.3 logU/mL, $P = 0.0165$. The same holds true for HBV DNA levels (1.2 logIU/mL difference, $P = 0.0339$). Conversely, for HBsAg, genotype E showed levels comparable to genotype C (0.4 logIU/mL difference, $P = 0.1725$).

Furthermore, as genotypes D and A were the most prevalent genotypes in the overall cohort (Gt D 60.9% [713 of 1171] and Gt A 19.7% [231 of 1171] respectively), the differences in HBsAg, HBV DNA and HBcrAg serum levels among all HBsAg-positive/HBeAg-negative individuals

infected by genotypes D and A were further analysed (Table S1). In the overall population and in both individuals with and without chronic hepatitis, a significant difference of HBsAg mean levels was found between the two genotypes. Conversely, HBV DNA mean levels were significantly different between the two genotypes in CHB group (P = 0.0043) and overall (P = 0.0001), but not in carriers without CHB (HBeAg-negative infection or Grey Zone cases; P = 0.1027). No differences were found in HBcrAg mean levels between the two genotypes in CHB patients (P = 0.9284) or in carriers without CHB (P = 0.8814). HBcrAg serum levels resulted significantly different by genotypes (D vs A) when the overall population was considered, because of the reversal of contrast direction for HBcrAg in carriers without CHB (Table S1). The same reversal of contrast direction was observed for HBV DNA.

3.4 Identification of HBeAg negative Infection and CHB

3.4.1 Predictors of CHB.

Univariate analysis was run including CHB and HBeAg-negative infection, overall 867 cases: genotypes D and E, HBsAg and HBcrAg serum levels, platelets and ALT levels and liver stiffness were associated with CHB (Table 2). At multivariate analysis, HBcrAg (OR 15.91, 95% CI 8.59-32.51, P < 0.0001), Gt D (OR 5.44, 95% CI 2.04-15.48, P = 0.001), HBsAg (OR 1.87, 95% CI 1.06-3.57, P = 0.0431) and ALT levels (OR 1.14, 95% CI 1.06-3.57, P = 0.0431) remained independently associated with CHB (Table 2).

3.4.2 Diagnostic performance of individual and combined viral markers

The AUCs of ROC curves for HBcrAg and HBsAg were 0.968 (95% CI, 0.958-0.977) and 0.732 (95% CI, 0.704-0.760) respectively (Figure 4). Combining both the biomarkers did not improve the diagnostic performance of HBcrAg (AUC for the combination, 0.969 [95% CI, 0.960-0.978]).

The optimal cut-off given by the highest Youden index (sum of sensitivity and specificity) to discriminate between HBeAg-negative infection and CHB for HBcrAg was 3.14 logU/mL (95% CI, 3.02-3.25), with sensitivity of 91% (95% CI, 89%-94%), specificity of 93% (95% CI, 90%-95%) and diagnostic accuracy of 92.4%. The optimal cut-off for HBsAg level was 2.96 logIU/mL (95% CI, 2.79-3.35), with corresponding sensitivity of 57% (95% CI, 49%-70%), specificity of 88% (95% CI, 74%-94%) and diagnostic accuracy of 67.6%. (Figure S1). The diagnostic odds ratios were 136 for HBcrAg and 9 for HBsAg.

The consistency of HBcrAg cut-off was verified according to genotype-dependency: the genotype-specific cut-offs for genotypes A, D and E were lower than that identified for the overall cohort (3.02; 3.04; 2.8 vs 3.14 logU/mL). Nevertheless, the diagnostic performance remained in the same range of the overall cohort when genotype-specific cut-off was considered,

or a single cut-off was used for patients with genotypes A, D and E jointly (3.02 vs 3.14 logU/mL, Table S2). The 95% CI of genotype-specific cut-offs was overlapping. Overall, genotype-specific cut-offs did not significantly improve the diagnostic accuracy achieved by using the 3.14 logU/mL cut-off (lower limit 95% CI at 3 logU/mL).

3.5 Association between HBcrAg, HBV DNA and HBsAg levels

The 1582 HBeAg-negative individuals were stratified by combining HBV DNA (< or >2000 IU/mL) and HBcrAg (< or ≥3 logU/mL, considering the lower limit of the 95% CI, 3.02-3.27) serum levels. Accordingly, four groups were identified: 732 (46.3%) cases with HBV DNA ≤2000 IU/mL and HBcrAg <3 logU/mL (group 1); 104 (6.6%) with ≤2000 IU/mL and HBcrAg ≥3 logU/mL (group 2); 180 (11.4%) with HBV DNA >2000 IU/mL and HBcrAg <3 logU/mL (group 3); and 566 (35.8%) with HBV DNA >2000 IU/mL and HBcrAg ≥3 logU/mL (group 4). In Figure 5, we report the distribution of the four groups in HBeAg-negative infection, Grey Zone and chronic hepatitis B. In individuals with HBeAg-negative infection, HBV DNA and HBcrAg were below 2000 IU/mL and 3 logU/mL in 90.7%; HBcrAg ≥3 logU/mL in 9.2% and only one (0.1%) case with both viraemia >2000 IU/mL (3720 IU/mL) and HBcrAg ≥3 logU/mL. Most of CHB patients (92.0%) had both viraemia and HBcrAg above 2000 IU/mL and 3 logU/mL; 4.2% viraemia ≥2000 IU/mL but HBcrAg <3 logU/mL; 3.3% viraemia <2000 IU/mL but HBcrAg ≥3 logU/mL and only three (0.5%) with both HBV DNA and HBcrAg <2000 IU/mL and 3 logU/mL, respectively, at the testing time. Conversely, 48.8% of the Grey Zone individuals had HBV DNA >2000 IU/mL, but HBcrAg <3 logU/mL; 26.4% with both HBV DNA and HBcrAg <2000 IU/mL and 3 logU/mL; 6.5% HBV DNA <2000 IU/mL but HBcrAg ≥3 logU/mL and 18.3% with both HBV DNA and HBcrAg >2000 IU/mL and 3 logU/mL.

3.6 Follow-up analysis

A second sampling, with an average interval of 43.6 months (minimum 12-maximum 157), was available for 303 of 710 (42.7%) HBeAg-negative infection and 175 of 322 (54.3%) Grey Zone individuals. In HBeAg-negative infection, we analysed the paired samples and the shift of the distribution at the second time point, median level of HBV DNA decreased to 2.05 (95% CI, 1.90-2.18) from the baseline values of 2.31 (95% CI, 2.20-2.42) logIU/mL ($P < 0.0001$) and HBsAg decreased to 2.31 (95% CI, 2.08-2.48) from the baseline values of 2.70 (95% CI, 2.50-2.80) logIU/mL ($P < 0.0001$). ALT and HBcrAg distributions at the two time points were similar ($P = 0.432$ and $P = 0.477$ respectively). HBcrAg 75th percentile (third quartile) value was unchanged at 2.3 logU/mL at the two time points (most of HBcrAg values being below the limit of detection). In 97% cases, no change of HBcrAg serum levels was observed according to the threshold of 3 log (</≥) U/mL, whereas in 0.7% of cases (2 of 303 HBeAg-negative infection)

HBcrAg from <3 became ≥3 logU/mL (3.2 and 3.9, respectively, without increase of viraemia levels), and in 2.3% (7 of 303) from ≥3 to <3 logU/mL. Overall, at the second time point, the percentage of HBeAg-negative infection with HBcrAg ≥3 logU/mL decreased from 3.6% (baseline) to 2%.

In the 175 Grey Zone individuals with a second time point, during follow-up, there was a significant decrease of median level for HBV DNA 3.25 (95% CI, 3.15-3.40) vs 3.47 (95% CI, 3.43-3.56) logIU/mL at baseline ($P < 0.0001$) and for HBsAg 3.20 (95% CI, 3.06-3.37) vs 3.39 (95% CI, 3.20-3.58) logIU/mL at baseline ($P < 0.0001$). Similarly to HBeAg-negative infection, no significant differences were observed between baseline and follow-up median levels of ALT 23 (95% CI, 22-25) U/L vs 24 (95% CI, 22-25) U/L ($P = 0.935$) and HBcrAg 2.0 (95% CI, 2.0-2.3) vs 2.0 (95% CI, 2.0-2.3) logU/mL ($P = 0.281$). The HBcrAg status according to </≥3.0 logU/mL showed no change in 92.6% cases, whereas in 2.3% (4 of 175 GZ), HBcrAg from <3 logU/mL became ≥3 (range 3.1-3.4; 3 of these four cases having HBV DNA >2000, ALT normal at same time point) and in 5.1% (9 of 175 GZ) from ≥3 logU/mL to <3 (three of these nine cases having HBV DNA >2000, ALT normal at same time point). At the second time point, the percentage of Grey Zone subjects with HBcrAg ≥3 logU/mL decreased from 14.3% (baseline) to 11.4%.

DISCUSSION

Our study shows in a large multicentre cohort of well-characterised untreated HBsAg-positive, HBeAg-negative individuals that the detection of serum HBcrAg identifies with high diagnostic accuracy HBeAg-negative patients with chronic hepatitis B. Median HBcrAg serum levels were significantly ($P < 0.0001$) higher in 550 CHB patients (4.8 [3.9-5.7] logU/mL) as compared to 710 carriers with HBeAg-negative infection (2.0 [2.0-2.5] logU/mL). At multivariate analysis, HBcrAg resulted as the parameter more strongly associated with CHB (OR 15.91, 95% CI 8.59-32.51, $P < 0.0001$) together with HBV genotype D infection, HBsAg and ALT serum levels, which showed as well a significant independent association with CHB, but with lower strength (OR 5.44, 95% CI 2.04-15.48, $P = 0.001$; OR 1.87, 95% CI 1.06-3.57, $P = 0.0431$ and OR 1.14, 95% CI 1.06-3.57, $P = 0.0431$ respectively). The area under the ROC curve for HBcrAg to discriminate CHB patients from HBeAg-negative infection was 0.968 (95% CI, 0.958-0.977, Figure 4) with an optimal cut-off of 3.14 logU/mL (95% CI, 3.02-3.27) showing 91% sensitivity (95% CI, 89%-94%) and 93% specificity (95% CI, 90%-95%). Interestingly, the diagnostic threshold of the HBcrAg assay, 3 logU/mL, is within the interval confidence of the cut-off given by the highest Youden index. HBcrAg serum levels below 3 logU/mL were found in 90.7% of HBeAg-negative infection, but only in 4.7% of the patients with CHB. Accordingly, HBcrAg serum levels appear to be a robust new viral marker useful to improve the management of untreated HBeAg-negative

carriers enabling an accurate and fast identification of patients who require further evaluation and possibly anti-viral treatment. An additional strength of using HBcrAg in clinical practice is the evidence that the threshold of 3 logU/mL holds true to adequately differentiate CHB from HBeAg-negative infection with high diagnostic accuracy independently from HBV genotype. Accordingly, the addition of HBV genotype to HBcrAg did not improve the diagnostic performance in the identification of HBeAg-negative CHB over the use of HBcrAg alone. In our cohort of HBeAg-negative individuals, who were mainly Caucasian and prevalently infected by genotypes A and D, median HBV DNA and HBsAg serum levels were significantly different by HBV genotype ($P < 0.0001$), whereas the difference between median HBcrAg levels in the different genotype subgroups did not reach the level of significance ($P = 0.0621$). Notably, when only carriers with genotypes A and D infection were studied, a significant difference in HBsAg serum levels was observed according to HBV genotype either in CHB or HBeAg-negative infection or Grey Zone individuals. By contrast, there was not any difference in HBcrAg serum levels according to HBV genotype in both CHB ($P = 0.9284$) or carriers without chronic hepatitis (HBeAg-negative and Grey Zone groups combined; $P = 0.8814$). Therefore, these results, confirming a previous report in a smaller cohort of patients,[5] suggest that at variance with HBsAg, whose serum levels are significantly influenced by HBV genotype, HBcrAg can be consistently used in any clinical setting independently from the need to test for HBV genotype. Furthermore, among HBeAg-negative infection individuals in spite of the higher (about 7- to 8-fold in U/mL) HBcrAg serum levels observed in genotype B as compared to genotypes A, D and E carriers (mean log difference of 0.9, 95% CI: 0.1-1.6), the median HBcrAg levels were below 3 logU/mL in all the genotypes. Accordingly, the use of genotype-specific cut-off did not improve the diagnostic accuracy achieved by the 3.14 logU/mL. Nevertheless, our finding and the proposed cut-off need to be confirmed in future studies including higher number of HBsAg carriers infected by genotypes B, C and F that were underrepresented in our cohort of European subjects.

At the single-point observation, 3.3% of CHB patients, in spite of having viraemia <2000 IU/mL, showed HBcrAg serum levels >3 logU/mL suggesting the presence of an active cccDNA transcription in spite of a transient fluctuation of serum HBV DNA levels below the diagnostic cut-off. This is consistent with the evidence that HBeAg-negative CHB runs mostly asymptomatic and with a highly frequent alternance of remission reactivation phases as previously reported.[19, 20] Interestingly, in the group of HBeAg-negative carriers with viraemia fluctuation above 2000 IU/mL, but persistently ≤20 000 IU/mL, only 24.8% of cases had positive HBcrAg serum levels ≥3 logU/mL, whereas 75.2% had HBcrAg <3 logU/mL. Future prospective studies should confirm whether the combined use of HBV DNA and HBcrAg could contribute to identify at the single time point among individuals with viraemia fluctuations up to 20 000 IU/mL, those

at high risk of hepatitis reactivation.[3, 20] This would warrant an optimisation of HBeAg-negative carrier management, taking into account that the current EASL guidelines suggest to monitor this subset of HBeAg-negative individuals and consider treatment only in case of overt recurrence of liver damage.[1, 21] Actually, their hepatitis recurrence rate appears quite variable (ranging from 10% to 2% at 5 years) in the different cohorts.[5, 20] In our cohort, among the 54% of Grey Zone subjects who underwent a second sampling during a median follow-up of about 44 months, only 2.3% of cases had transition of HBcrAg from serum levels <3 to ≥3 logU/mL, in spite of persistence of HBV-DNA levels <20 000 IU/mL. At the same time, in 5% of cases, HBcrAg levels decreased <3 logU/mL. All together, these observations suggest that most of the HBeAg-negative individuals with low viraemia are in a transition phase towards a progressive control of HBV infection, as previously suggested.[1, 3, 4]

Overall, HBcrAg and HBV DNA serum levels showed a significant correlation ($P < 0.001$), with a high coefficient (R = 0.80) and in CHB patients, viraemia levels >20 000 IU/mL were the best predictor of a positive HBcrAg test with a standardised beta 0.63, $P < 0.0001$; $r^2 = 0.393$. Our findings are in agreement with other reports in smaller cohorts of HBsAg-positive, HBeAg-negative individuals,[3, 6, 15] showing a significant correlation between HBcrAg and HBV DNA particularly in HBeAg-negative CHB. This depends most probably on the fact that in HBeAg-negative individuals, the HBcrAg assay should detect mainly DNA-containing particles, because of the lack of HBeAg production by the prevalent HBeAg defective viral quasi species.[22] This is consistent also with previous evidence of a higher correlation between the two viral markers in HBeAg-negative than in HBeAg-positive CHB patients, whereas no statistical correlation was found between HBV DNA and HBcrAg in HBeAg-negative infection.[13, 15] The significant correlation reported between HBcrAg and HBV DNA in Asian inactive carriers can be explained by the different threshold used for the analysis, that included also values below 3 logU/mL.[14] Our proposed cut-off to distinguish HBeAg-negative CHB from HBeAg-negative infection was identified in a very well characterised cohort of HBeAg-negative individuals, in whom a strict and prolonged follow-up ensured an accurate identification of the virologic profile of each subject. However, our study was retrospective and some genotypes were underrepresented. Therefore, prospective studies in cohort of carriers with adequate numbers of all the genotypes are mandatory to validate our findings and confirm HBcrAg as new viral marker to be used in combination with HBV-DNA to identify at a single time point the different clinical categories of the HBeAg-negative phase of HBV infection.

In conclusion, this study proves in a large number of cases the added value of detecting HBcrAg, as marker of CHB, in the HBeAg-negative phase of HBV infection. World health organization launched a strategic plan to eliminate the burden of CHB (that can be controlled by available anti-viral when treated as early as possible in the asymptomatic phase) by 2030.[23] However,

CHB affects about 40% of the estimated 280 million HBsAg carriers worldwide who live mostly (80%) in developing countries with poor economic resources (South-East Europe, Asia, Africa and Latin America).[24] HBcrAg qualifies to be proposed as a key marker for chronic hepatitis identification in combination with HBsAg (maker of HBV infection) and HBeAg (indirect markers of florid HBV replication), to provide a three antigen qualitative assay for the screening of general population in highly endemic areas for the single time point diagnosis of asymptomatic patients with chronic hepatitis B. This approach would overcome the more expensive diagnosis currently provided by quantification of serum HBV DNA which needs repeated tests over a time-consuming follow-up because of the common fluctuations of viraemia levels below and above the 2000-20 000 IU/mL diagnostic Grey Zone.[1, 21] Prospective studies have to be performed to validate this hypothesis.

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AUTHORSHIP

Guarantor of the article: MR Brunetto.

Author contributions: MR. Brunetto and M. Buti conceived the study protocol, led the project and wrote the article. Other authors collected biomarker and patient's data. All calculations regarding the number and position of co-authors were based on numbers of cases included by each centre. All authors approved the final version of the article, including the authorship list.

REFERENCES

1. 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;2017:370-398.
2. Manno M, Cammà C, Schepis F, et al. Natural history of chronic HBV carriers in northern Italy: morbidity and mortality after 30 years. Gastroenterology. 2004;127:756-763.
3. Oliveri F, Surace L, Cavallone D, et al. Long-term outcome of inactive and active, low viraemic HBeAg-negative-hepatitis B virus infection: benign course towards HBsAg clearance. Liver Int. 2017;37:1622-1631.
4. Papatheodoridis GV, Manolakopoulos S, Liaw YF, Lok A. Follow-up and indications for liver biopsy in HBeAg-negative chronic hepatitis B virus infection with persistently normal ALT: a systematic review. J Hepatol. 2012;57:196-202.
5. Brunetto MR, Oliveri F, Colombatto P, 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.
6. Riveiro-Barciela M, Bes M, Rodríguez-Frías F, 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. 2017;23:860-867.
7. Brunetto MR, Moriconi F, Bonino F, et al. Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. Hepatology. 2009;49:1141-1150.
8. . Cornberg M, Wong VW, Locarnini S, Brunetto M, Janssen HLA, Chan HL. The role of quantitative hepatitis B surface antigen revisited. J Hepatol. 2017;66:398-411.
9. Wooddell CI, Yuen M-F, Chan H-Y, 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;9:1-22.
10. Kimura T, Rokuhara A, Sakamoto Y, 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.

11. Wong DK, Seto WK, Cheung KS, et al. Hepatitis B virus core-related antigen as a surrogate marker for covalently closed circular DNA. Liver Int. 2017;37:995-1001.
12. Wang L, Cao X, Wang Z, et al. Correlation of HBcrAg with intrahepatic hepatitis B virus total DNA and covalently closed circular DNA in HBeAg-positive chronic hepatitis B patients. J Clin Microbiol. 2019;57:e01303-18.
13. Testoni B, Lebosse F, Scholtes C, et al. Serum hepatitis B corerelated antigen (HBcrAg) correlates with covalently closed circular DNA transcriptional activity in chronic hepatitis B patients. J Hepatol. 2019;70:615-625.
14. Seto WK, Wong DK, Fung J, et al. Linearized hepatitis B surface antigen and hepatitis B core-related antigen in the natural history of chronic hepatitis B. Clin Microbiol Infect. 2014;20:1173-1180.
15. Maasoumy B, Wiegand SB, Jaroszewicz J, 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. 2015;21:606.e1-606.e10.
16. Zhang ZQ, Wang YB, Lu W, et al. Performance of hepatitis B core-related antigen versus hepatitis B surface antigen and hepatitis B virus DNA in predicting HBeAg-positive and HBeAg-negative chronic hepatitis. Ann Lab Med. 2019;39:67-75.
17. Zacher BJ, Moriconi F, Bowden S, et al. Multicenter evaluation of the Elecsys hepatitis B surface antigen quantitative assay. Clin Vaccine Immunol. 2011;18:1943-1950.
18. van Halewijn GJ, Geurtsvankessel CH, Klaasse J, et al. Diagnostic and analytical performance of the hepatitis B core related antigen immunoassay in hepatitis B patients. J Clin Virol. 2019;114:1-5.
19. Brunetto MR, Oliveri F, Coco B, et al. Outcome of anti-HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study. J Hepatol. 2002;36:263-270.
20. Brouwer WP, Chan H-Y, Brunetto MR, et al. Good Practice in using HBsAg in Chronic Hepatitis B Study Group (GPs-CHB Study Group). Repeated measurements of hepatitis B surface antigen identify carriers of inactive HBV during long-term follow-up. Clin Gastroenterol Hepatol. 2016;14:1481-1489.

21. Terrault NA, Bzowej NH, Chang KM, et al. AASLD guidelines for treatment of chronic hepatitis B. Hepatology. 2016;63:261-283.
22. Brunetto MR, Stemler M, Bonino F, et al. A new hepatitis B virus strain in patients with severe anti-HBe positive chronic hepatitis B. J Hepatol. 1990;10(2):258-261.
23. WHO.Global Health Sector Strategies for viral hepatitis 2016–21. 2016. http://www.who.int/hepatitis/strategy2016-2021/ghss-hep/ en. Accessed July 1, 2016
24. Polaris Observatory Collaborators. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. Lancet Gastroenterol Hepatol. 2018;3:383-403.

TABLE 1. Demographic and virologic characteristics according to the three clinical categories (chronic hepatitis B, CHB; HBV-DNA ≤20 000 IU/mL and normal ALT, Grey Zone and HBV-DNA ≤2000 IU/mL and normal ALT, HBeAg-negative infection, ENI)

Variables	**CHB, n = 550**	**GZ, n = 322**	**ENI, n = 710**	***P* value**
Sex (male), n (%)	399 (73%)	151 (47%)	376 (53%)	<0.0001
Age (y), mean (SD)	47 (13.0)	40 (12.1)	44 (13.4)	0.0002
Ethnicity				
Caucasian	370 (67%)	189 (59%)	351 (49%)	<0.0001
Asian	30 (5%)	23 (7%)	57 (8%)	0.0756
African	36 (7%)	73 (23%)	156 (22%)	<0.0001
Other	15 (3%)	7 (2%)	19 (3%)	0.9556
Unknown	99 (18%)	30 (9%)	127 (18%)	0.9588
Genotype (A/B/C/D/E/F/mixed), n (%)				
A	68 (12%)	59 (18%)	104 (15%)	0.242
B	9 (2%)	6 (2%)	20 (3%)	0.1709
C	16 (3%)	6 (2%)	16 (2%)	0.4644
D	377 (69%)	113 (35%)	223 (31%)	<0.0001
E	19 (3%)	44 (14%)	78 (11%)	<0.0001
F	3 (1%)	4 (1%)	4 (1%)	0.9661
Mixed	2 (0%)	0 (0%)	0 (0%)	0.9709
Unknown	56 (10%)	90 (28%)	265 (37%)	<0.0001
Liver stiffness (kPa), median (IQR)	6.9 (5.4-10.4)	4.8 (3.9-5.6)	4.5 (3.8-5.4)	<0.0001

Variables	CHB, n = 550	GZ, n = 322	ENI, n = 710	*P* value
Platelets ($\times 10^9$/L), median (IQR)	179 (142-230)	219 (192-263)	222 (191-261)	<0.0001
ALT (U/L), median (IQR)	76 (45-144)	24 (18-32)	22 (17-29)	0.0368
HBV-DNA (logIU/mL), median (IQR)	5.6(4.8-6.6)	3.5 (3.2-3.8)	2.2 (1.5-2.7)	<0.0001
HBsAg (logIU/mL), median (IQR)	3.7 (3.3-4.0)	3.6 (3.1-4.1)	2.9 (2.1-3.6)	<0.0001
HBcrAg (logU/mL), median (IQR)	4.8 (3.9-5.7)	2.5 (2.0-2.9)	2.0 (2.0-2.5)	<0.0001
≥3 logU/mL, number (%)	524 (95.3%)	80 (24.8%)	66 (9.3%)	<0.0001

Notes: Data are expressed as median (25th-75th percentile range, IQR) or number (%).

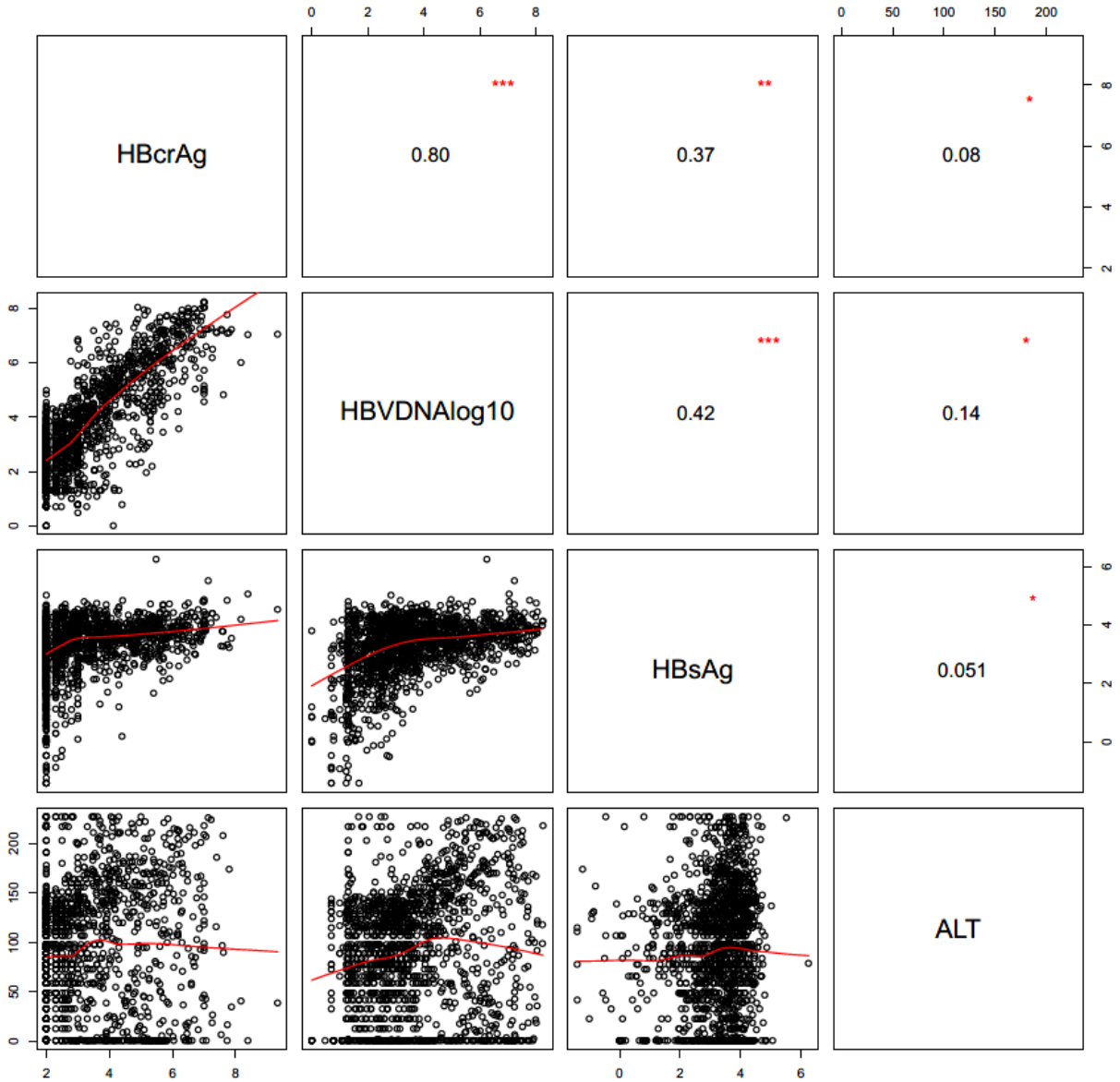
Lower limit of quantification, LLOQ = 3 logU/mL for HBcrAg.

P value: differences between CHB and HBeAg-negative infection (ENI) groups.

TABLE 2. Virologic and biological parameters associated with chronic hepatitis B (CHB): univariate and multivariate analyses by logistic regression model

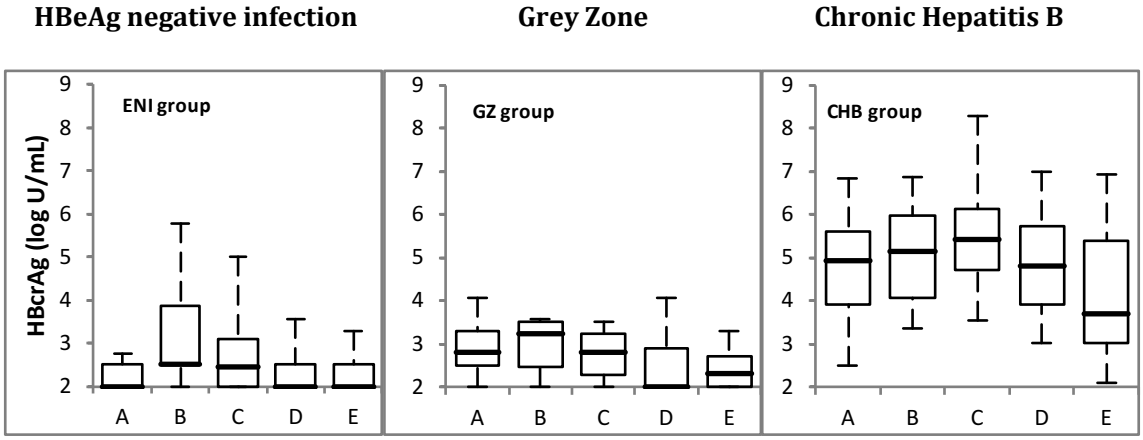
HBV markers	Univariate				Multivariate			
	n	OR	95% CI	*P* value	n	OR	95% CI	*P* value
HBV genotype								
Genotype A	1260	0.82	[0.59-1.14]	0.2420	867	1.03	[0.31-3.24]	0.9645
Genotype B	1260	0.57	[0.25-1.23]	0.1709	867	0.11	[0-8.18]	0.4569
Genotype C	1260	1.30	[0.64-2.64]	0.4644	867	0.38	[0.03-5.95]	0.4775
Genotype D	1260	4.76	[3.75-6.06]	<0.0001	867	5.44	[2.04-15.48]	0.001
Genotype E	1260	0.29	[0.17-0.47]	<0.0001	867	2.73	[0.64-12.86]	0.1847
Liver stiffness (kPa)	919	1.93	[1.75-2.16]	<0.0001	867	1.02	[0.84-1.32]	0.8878
HBsAg (logIU/mL)	1235	3.34	[2.8-4.02]	<0.0001	867	1.87	[1.06-3.57]	0.0431
HBcrAg (logU/mL)	1244	12.45	[9.54-16.67]	<0.0001	867	15.91	[8.59-32.51]	<0.0001
Platelets (log/L)	1098	1.14	[1.1-1.19]	<0.0001	867	0.99	[0.84-1.32]	0.8878
ALT (U/L)	1023	1.15	[1.09-4.02]	<0.0001	867	1.14	[1.06-3.57]	0.0431

Figure 1: Correlations between HBcrAg, HBV-DNA, HBsAg and ALT in the overall cohort. In the lower triangle are reported the scatterplots with overlay of smoothed regression to visualise relationship between pairs of biomarkers. The three viral markers showed a significant correlation ($P < 0.001$), but with a different strength, as shown in the upper triangle by the Pearson correlation coefficient (Pearson correlation coefficient: relative strong (***) if above 0.4; moderate (**) if between 0.2 and 0.4; weak (*) if below 0.2). ALT and the three viral markers did not show any statistical correlation



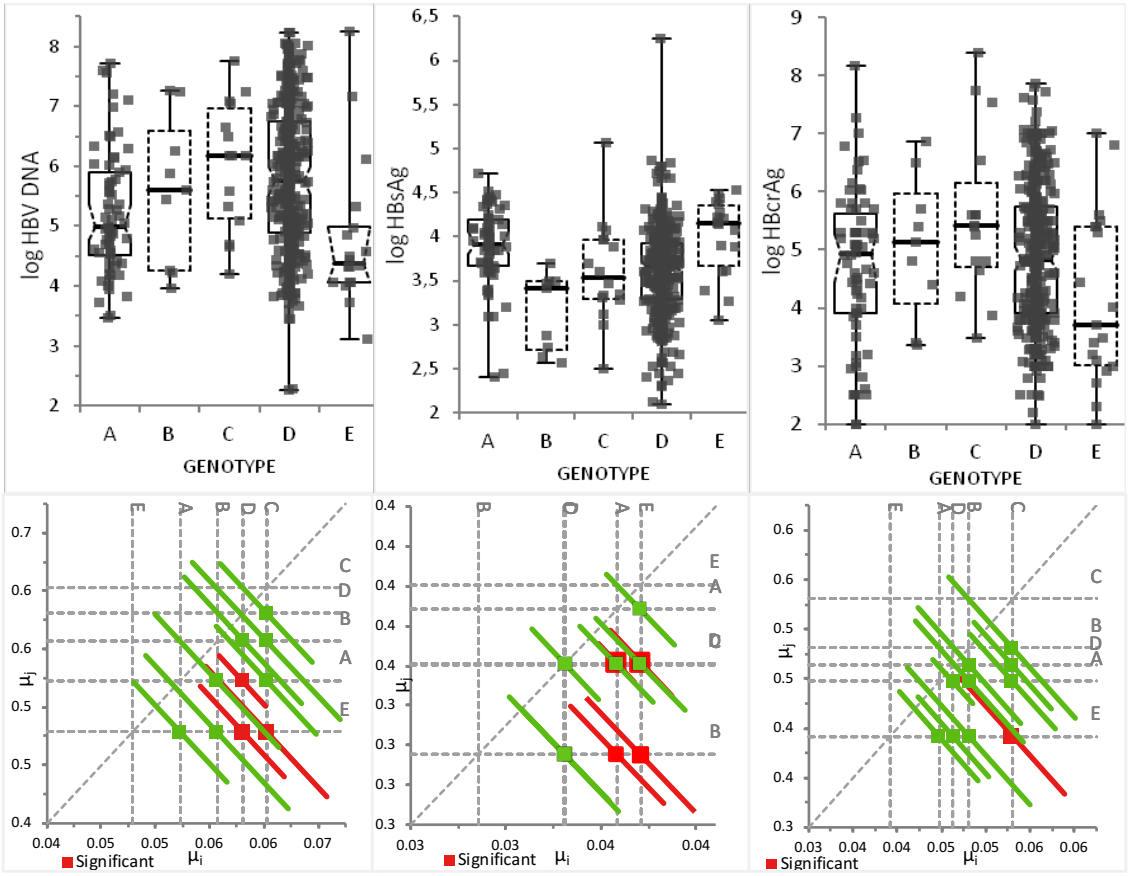
Pearson correlation coefficient: (***) above 0.4, relatively strong; (**) between 0.2 and 0.4, moderate; (*) below 0.2, weak

Figure 2: HBcrAg serum distribution according to HBV genotypes and clinical categories: HBeAg-negative infection (ENI), Grey Zone (GZ) and chronic hepatitis B (CHB). The results are reported as quantile box plot (where median is reported as a line, 1st and 3rd quantiles as a box and 5th and 95th percentiles as end caps). Using Steel-Dwass-Critchlow-Fligner pairwise ranking non-parametric method, in ENI a shift in location of HBcrAg serum levels was observed according to HBV genotype: subjects with genotype B infection showed HBcrAg levels higher than those infected by genotypes D, A and E (B vs D [P = 0.0004], E [P = 0.0008] and A [P = 0.0001]). In the Grey Zone cases, genotype A carriers had higher levels than D and E (A vs D [P = 0.0018] and E [P = 0.0093]). In CHB, genotype C patients showed higher HBcrAg levels than genotype E cases (E vs C, P = 0.001)



Quantile box plot with median as a line, 1st and 3rd quantiles as a box and 5th and 95th percentiles as end caps.

Figure 3: HBV-DNA, HBsAg and HBcrAg serum levels by genotype in chronic hepatitis B patients. The values are reported by Skeletal Notched Boxes (where Plot median is reported as a line, 1st and 3rd quartile as a box; minimum-maximum as end caps). Using Tukey-Kramer all pair comparisons, patients infected by genotype C had higher HBcrAg and HBV-DNA serum level than patients with genotype E, whereas HBsAg serum levels were comparable



Plot median as a line, 1st and 3rd quartile (P25-P50) as a box and minimum – maximum as end caps.

Figure 4: Receiver operating characteristic curve of HBcrAg, HBsAg and HBV-DNA to distinguish HBeAg-negative CHB from HBeAg-negative infection. The AUCs for HBcrAg, HBsAg and HBV-DNA were 0.968 (95% CI, 0.958-0.977), 0.732 (95% CI, 0.704-0.760) and 0.998 (95% CI, 0.995-1.000) respectively; the AUC of combined HBcrAg and HBsAg was 0.969 (95% CI, 0.96-0.978)

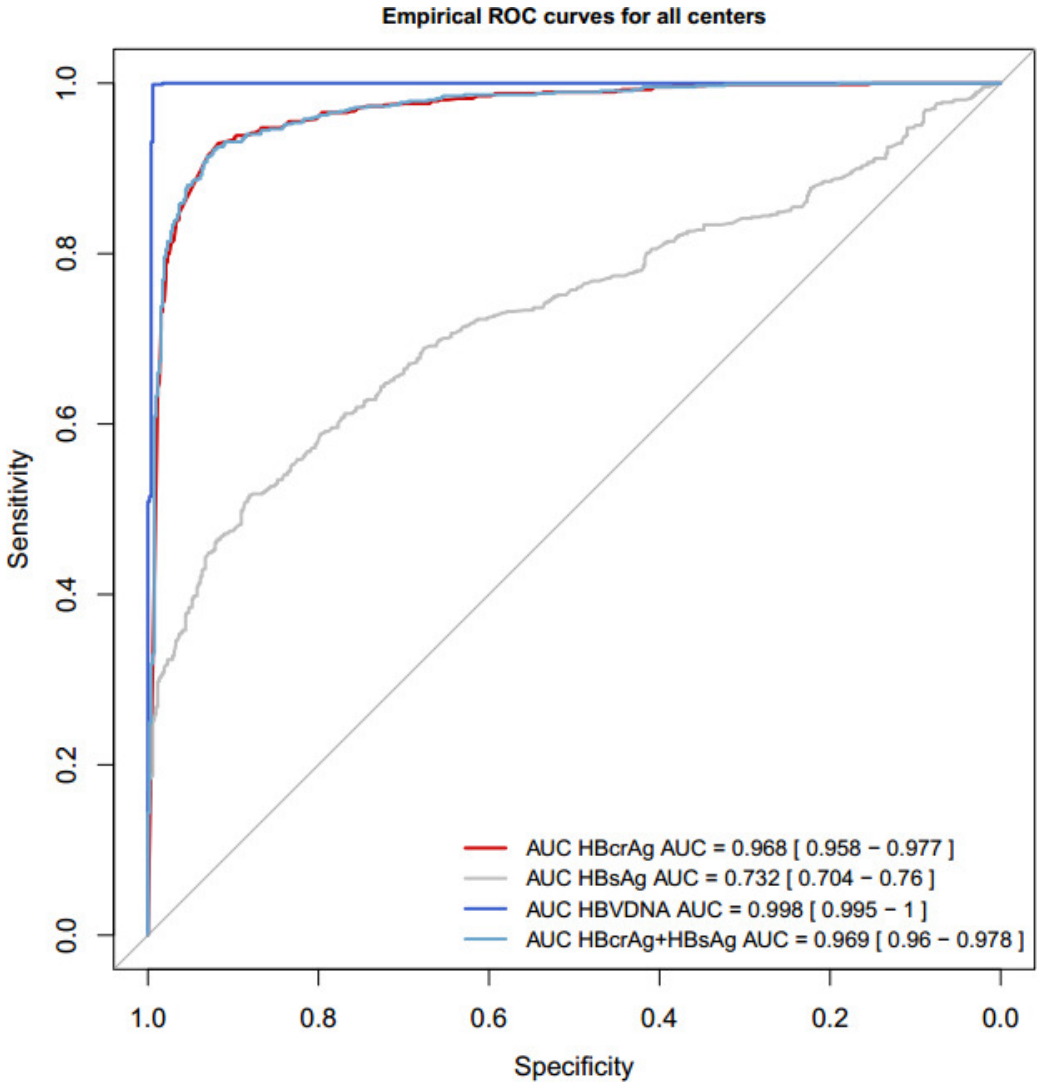


Figure 5: Distribution of the different combination of HBV-DNA (≤ or >2000 IU/mL) and HBcrAg (< or ≥3 logU/mL) in the three clinical categories. In the pies, the colours identify the groups defined by HBV DNA and HBcrAg combination: Green: group 1 (HBV-DNA ≤2000 IU/mL and HBcrAg <3 logU/mL), 91%, 26% and 1% in chronic infection, Grey Zone and chronic hepatitis respectively; Salmon: group 2 (HBV-DNA ≤2000 IU/mL and HBcrAg ≥3 logU/mL), 9%, 7% and 3% in chronic infection, Grey Zone and chronic hepatitis respectively; Violet: group 3 (HBV-DNA >2000 IU/mL and HBcrAg <3 logU/mL), 0%, 49% and 4% in chronic infection, Grey Zone and chronic hepatitis respectively; Light Blue: group 4 (HBV-DNA >2000 IU/mL and HBcrAg ≥3 logU/mL), 0%, 18% and 92% in chronic infection, Grey Zone and chronic hepatitis respectively