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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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Authors 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 center.

Keywords:

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

INTRODUCTION

Patients with positive hepatitis B surface antigen (HBsAg) and negative e antigen (HBeAg) currently account for the largest subgroup of individuals with hepatitis B virus (HBV) infection worldwide.[1] HBeAg negative phase of HBV chronic infection includes a spectrum of conditions that vary in terms of natural history, severity of liver damage and need for antiviral treatment: the two polar conditions being now termed chronic infection (ENI) and chronic hepatitis B (CHB).[1] Unlike patients with CHB, those with HBeAg negative infection - previously known as inactive carriers - have a favorable long-term outcome, with low risk of cirrhosis or hepatocellular carcinoma and do not require antiviral treatment[2-3] .To warrant an appropriate clinical management, it is therefore important to correctly and timely diagnose HBeAg negative carriers as having chronic infection or CHB. 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 viremia (< 20,000 IU/ml) and normal ALT at the first observation.[1,4,5] 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.[1,5-7] In addition, recently it has been shown as, mainly in HBeAg negative carriers, HBsAg can be produced from HBV-DNA sequences integrated in the host genome.[8] 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 standardized assay for the detection of circulating viral proteins with HBeAg and HBcAg antigenicity,[9] the Hepatitis B core-related antigen (HBcrAg) has become available. HBcrAg serum levels in untreated patients show high correlation with viremia and intrahepatic covalently closed circular DNA (cccDNA).[10-12] Therefore, HBcrAg has been proposed as a new diagnostic tool to improve the management of HBV carriers.[3,5,13-15] The aim of our study was to assess whether HBcrAg could improve the characterization of HBsAg-positive, HBeAg-negative individuals by using data from a large database collected from multiple European centers.

MATERIALS AND METHODS

Patients and study design

The study was a retrospective analysis of data from nine hepatology centers 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 Centers.

Included individuals were HBsAg positive, HBeAg negative carriers with an adequate follow-up to accurately define, according to European Association for the Study of the Liver, Clinical Practice Guidelines (EASL CPG), their phase of HBeAg-negative infection. For HBeAg negative individuals with HBV-DNA <20,000 IU/ml and normal ALT at the first observation, data on HBV DNA and ALT were collected during a follow-up period of 12-18 months (at least 3 time points) and used to classify them. For patients with CHB (HBV-DNA > 20,000 IU/ml and elevated ALT), only baseline data were considered. Exclusion criteria were HBV-DNA < 2,000 IU/ml, and persistently or intermittently elevated ALT, viral coinfection (HCV, HDV or HIV); pregnancy; the presence of alcoholic, autoimmune, or metabolic liver disease; previous antiviral treatment.

A serum sample was obtained at baseline for the quantification of the 3 viral markers (HBV DNA, HBsAg, and HBcrAg) and ALT. A proportion (478 of 1032, 46.3%) of cases in the HBeAg negative phase without CHB had a second time point measured at an average interval of 43.6 months (minimum 9-maximum 157 months): 303 of 710 individuals meeting the criteria of inactive carriers (HBV DNA <2,000 and normal ALT; ENI) and 175 of 322 with fluctuating HBV DNA < 20, 000 IU/ml (Grey Zone, GZ)].

Data originally stored in the databases from each center were anonymized and shared with an independent statistician (EC) using ShareFile, a secure, 21 Code of Federal Regulation Part 11-compliant, cloud-based platform. Individual centers only had access to their data, but not to those of other centers.

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

Patient categorization

Patients were categorized into one of three groups or clinical categories, following EASL guidelines[1]: (1) chronic HBeAg negative infection (ENI), those with serum HBV DNA persistently <2,000 IU/mL and persistently normal ALT levels <40 IU/mL; (2) 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 (3)chronic hepatitis B infection with fluctuating HBV DNA between 2,000 and 20,000 IU/mL and normal ALT levels during the 12-18 month follow-up (Grey Zone, GZ).

Assays

All measurements on serum samples were performed independently at each clinical center: ALT serum levels were measured on fresh serum samples by routine procedures and values < 40 U/L were defines as normal. Qualitative antibody to hepatitis B core antigen (anti-HBc), HBeAg and anti-HBe, antibodies to HCV, HDV and HIV were detected by commercially available immunoassays.

Serum HBV-DNA levels were quantified by COBAS TaqMan assay, sensitivity 6 IU/mL, dynamic range 6–1.10 × 10^8 IU/mL (Roche Diagnostic Systems Inc, Mannheim, Germany). Serum HBsAg was quantified using commercially available assays: Architect HBsAg assay dynamic range, 0.05–250.0 IU/mL (Abbott Laboratories; Il, USA,) or Elecsys HBsAg II quant, dynamic range 0.05-52000 IU/ml ((Roche Diagnostic Systems Inc, Mannheim, Germany). The 2 assays show a high coefficient of correlation as previously reported[16] .

Serum HBcrAg levels were measured using LUMIPULSE® G HBcrAg assay (Fujirebio Europe, Belgium), according to the instructions of the manufacturer. The concentration of HBcrAg was calculated by a standard curve generated using recombinant pro-HBeAg (aminoacids 10 to 183) and was expressed in arbitrary unit by the LUMIPULSE® G system. The lower detection limit was 2.0 log U/mL (0.1 kU/mL), and the dynamic range from 3.0 log U/mL to 7.0 log U/mL (1.0 to 10,000 kU/mL). HBcrAg measurements in between lower limit of detection and lower limit of the dynamic range (2.0-3.0 log U/mL) were reported, but as diagnostic cut-off we used the 3.0 log U/mL threshold.

HBV genotype was determined by direct sequencing when the DNA level allowed it.

Statistical analysis

Quantitative data for viral biomarkers were transformed for analysis using their $\log_{10}$ values. Normally distributed quantitative variables were summarized by the mean and standard deviation (SD) and compared between groups using Student's *t* test. Quantitative variables with non-normal distribution were summarized by the median and interquartile range (IQR) and compared between groups using the Mann-Whitney *U* test. Categorical variables were summarized 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 characteristics (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 ENI 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 ENI 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 associations between candidate predictive variables and the presence of CHB; the variables genotype, ethnicity, age, 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

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 the other were from the UK (N=307), Germany (N=256), Spain (N=151), the Netherlands (N=146), and France (N=59). Seven hundred and ten (44.9%) had chronic HBV infection (ENI), 322 (20.4%) viremia fluctuating above 2000 IU/ml, but persistently < 20,000 IU/ml (Grey Zone, GZ), and 550 (34.8%) chronic hepatitis B (CHB). Overall, 59% of the carriers were males, who were more frequently represented in CHB than in GZ and ENI (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%) cases: the most frequent was genotype D (713 of 1171, 61%), which showed a higher prevalence in CHB patients (377/713, 52.9%) than in Gray zone (113/713, 15.8%) and ENI (223/713, 31.3%, P<0.001).

Overall, median (25^{th}-75^{th} percentiles) HBV DNA and HBsAg serum levels were 3.2 (2.3-4.9) log10 IU/mL and 3.4 (2.8-3.9) log10 IU/mL, respectively. As expected, the lowest HBV DNA levels were observed in HBeAg negative infection [2.2 (1.5-2.7) log10 IU/ml], the highest in CHB

[5.6 (4.8-6.6) log10 IU/m], whereas in Grey Zone the values were intermediate [3.5 (3.2-3.8) log10 IU/ml]. Unlike viremia, HBsAg serum levels were comparable in CHB and Grey Zone [3.7 (3.3-4.0) and 3.6 (3.1-4.1) log10 IU/ml respectively, P=0.1584], but significantly lower in HBeAg negative infection [2.9 (2.1-3.6) log 10 IU/ml], P<0.0001 (Table 1).

HBcrAg serum levels were <3 log10 U/ml in 90.7% (644/710) of ENI, 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 3 groups, as well between ENI and GZ). In the overall cohort, the median HBcrAg serum levels were 2.7 (2.0-4.1) Log10 U/ml: HBcrAg levels were significantly higher in CHB [4.8 (3.9-5.7) log10 U/ml] as the compared to Grey Zone [2.5 (2.0-2.9) log10 U/ml] and ENI [2.0 (2.0-2.5) log10 U/ml] (P<0.0001).

Correlations between HBcrAg and HBV DNA and HBsAg levels

In the overall cohort of HBcrAg, HBsAg and HBV DNA showed a significant correlation 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. The viral load (>20,000 IU/mL) resulted a predictor of HBcrAg level [Standardized beta 0.63 (Log HBV DNA), p<0.0001; r^2=0.393; N= 533 pairs] in CHB.

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%) 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 ENI, 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). In spite of such differences, median HBcrAg level were below 3 log10 U/ml in all the genotypes within ENI group. In CHB group, the few patients with HBV genotype C (16 of 394, 3.2%) 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 both the low HBcrAg serum levels in Grey Zone and ENI groups (HBcrAg <3 log10 U/ml in 75.2% and 90.7% respectively) and the different prevalence of HBV genotypes (Table 1), we analyzed the relationship between the HBV genotype and HBcrAg, HBV DNA and HBsAg serum levels only in CHB patients (HBcrAg >3 log10 U/mL in 95.3% of cases). The median of HBV DNA and HBsAg levels were significantly different according to HBV

genotypes (p<0.0001) while 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=19) had higher HBcrAg level than patients with genotype E (N=16), with a difference of 1.3 log10 U/mL, p =0.0165. The same holds true for HBV DNA levels (1.2 log10 IU/mL difference, p=0.0339). Conversely for HBsAg, genotype E showed levels comparable to genotype C (0.4 log10 IU/mL difference, p =0.1725).

As genotypes D and A were the most prevalent genotypes in the overall cohort [Gt D 61% (713 of 1171) and Gt A 20% (231 of 1171), respectively], the differences in HBsAg, HBV DNA and HBcrAg serum levels between individual infected by genotype D and A were further analyzed (Supplementary Table 1). In the overall population and in both individuals with and without chronic hepatitis a significant difference of HBsAg mean levels was found between the 2 genotypes. Conversely, HBV DNA mean levels were significantly different between the 2 genotypes in CHB group (p=0.0.0043) and overall (p=0.0001), but not in carriers without CHB (ENI or Grey Zone cases; p=0.1027). No differences were found in HBcrAg mean levels between the 2 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 (in the overall population and CHB patients mean HBcrAg levels were higher in genotype D vs genotype A individuals; on the contrary in HBeAg negative carriers without CHB mean HBcrAg levels were higher in genotype A vs genotype D). The same reversal of contrast direction was observed for HBV DNA.

Identification of HBeAg negative Infection and CHB

Predictors of CHB. Univariate analysis was run including CHB and ENI, 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=0.043) and ALT levels (OR 1.14, 95%CI 1.06-3.57, P=0.043) remained independently associated with CHB (Table 2).

Diagnostic performance of individual and combined viral markers. The AUCs for HBcrAg and HBsAg were 0.968 (95% CI, 0.958 to 0.977) and 0.732 (95% CI, 0.704 to 0.760), respectively (Supplemetary Figure 1). Combining both of these biomarkers did not improve the diagnostic performance of HBcrAg (AUC for the combination, 0.969 [95% CI, 0.960 to 0.978]).

The optimal cut-off given by the highest Youden index (sum of sensitivity and specificity) to discriminate between ENI and CHB was for HBsAg level 2.96 log10 IU/mL (95% CI, 2.79 to 3.35),

with corresponding sensitivity of 57% (95% CI, 49% to 70%) and specificity of 88% (95% CI, 74% to 94%). The optimal cut-off for HBcrAg was 3.14 log10 U/mL (95% CI, 3.02 to 3.25), with sensitivity of 91% (95% CI, 89% to 94%) and specificity of 93% (95% CI, 90% to 95%) (Figure 4). For the current dataset, the diagnostic accuracy of HBcrAg was 92.4%, whereas that of HBsAg was only 67.6%. 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 log10 U/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 genotype A, D and E jointly (3.02 vs 3.14 log10 U/ml, Supplementary Table 2). The 95% CI of genotype-specific cut-offs were overlapping. Overall, genotype-specific cut-offs did not significantly improve the diagnostic accuracy achieved by using the 3.14 log 10 U/ml cut-off (lower limit 95% CI at 3 log10 U/mL).

Association between HBcrAg, HBV DNA, and HBsAg levels

The 1582 HBeAg negative individuals were stratified by combining HBVDNA (< or > 2,000 IU/ml) and HBcrAg (< or > 3 log10 U/ml, considering the lower limit of the 95% CI, 3.02 to 3.25) serum levels. Accordingly 4 sub-groups were identified: 732 (46.3%) cases with HBV DNA <2,000 IU/mL and HBcrAg <3 log10 U/mL (sub-group 1); 104 (6.6%) with <2,000 IU/mL and HBcrAg ≥3 log10 U/mL (sub-group 2); 180 (11.4%) with HBV DNA ≥2000 IU/mL and HBcrAg <3 log10 U/mL (sub-group 3); 566 (35.8%) with HBV DNA ≥2,000 IU/mL and HBcrAg ≥3 log10 U/mL (sub-group 4). In Figure 5 we report the distribution of the 4 subgroups in HBeAg negative infection (ENI), Grey Zone and Chronic hepatitis B. In individuals with HBeAg negative infection HBV DNA and HBcrAg were below 2,000 IU/ml and 3 log10 U/mL in 90.7%; HBcrAg ≥3 log10 U/mL in 9.2% and only one (0.1%) case both viremia > 2,000 IU/ml (3720 IU/l) and HBcrAg ≥3 log10 U/ml Most of CHB patients (92.0%) had both viremia and HBcrAg above 2000 IU/ml and 3 log U/ml; 4.2% viremia ≥ 2,000 IU/ml but HBcrAg <3 log10 U/ml; 3.3% viremia <2,000 IU/ml but HBcrAg ≥ 3 log10 U/ml and only 3 (0.5%) both HBV DNA and HBcrAg <2,000 IU/ml and 3 log10 U/ml, respectively at the testing time. Conversely, 48.8% of the Grey Zone individuals had HBV DNA > 2000 IU/ml, but HBcrAg < 3 log10 U/ml; 26.4% both HBV DNA and HBcrAg < 2,000 IU/ml and 3 Log10 U/ml; 6.5 % HBV-DNA < 2,000 IU/ml but HBcrAg ≥ 3 log10 U/ml and 18.3% with both HBV DNA and HBcrAg ≥2,000 IU/ml and 3 log10 U/ml.

Follow-up analysis

A second sampling, with an average interval of 43.6 months (minimum 9-maximum157), was available for 303 of 710 (42.7%) HBeAg negative infection. We analyzed 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) log IU/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) log IU/mL at baseline, (p<0.0001). ALT and HBcrAg distributions at the 2 time points were similar (p=0.432 and p=0.477 respectively). HBcrAg 75th percentile (3rd quartile) value was unchanged at 2.3 log U/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 log10 (≥/<) U/ml, whereas in 0.7% of cases (2 of 303 ENI) HBcrAg from <3 became ≥3 log10 U/ml (3.2 and 3.9 respectively, but remaining in ENI criteria) and in 2.3% (7 of 303 ENI) from ≥3 to <3 log U/ml. Overall, at the second time point the percentage of ENI with HBcrAg ≥3 Log10 U/mL decreased from 3.6% (baseline) to 2%.

A second time point was also available for 175 of 322 (54.3%) Grey Zone individuals: 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] log IU/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] log IU/L at baseline (P<0.0001). Similarly to ENI, 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] log U/mL (P=0.281). The HBcrAg status according to ≥/< 3.0 log10 U/ml showed no change in 92.6% cases whereas in 2.3% (4 of 175 GZ) HBcrAg from <3 log 10 U/ml became ≥3 (range 3.1 – 3.4; 3 of these 4 cases having HBV DNA >2000, ALT normal at same time-point) and in 5.1% (9 of 175 GZ) from ≥3 log 10 U/ml to <3 (3 of these 9 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 log U/mL decreased from 14.3% (baseline) to 11.4%.

DISCUSSION

Our study shows in a large and multicenter cohort of well characterized 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) log10 U/ml] as compared to 710 carriers with HBeAg negative infection [2.0 (2.0-2.5) log10 U/ml]. At multivariate analysis HBcrAg resulted the parameter more strongly associated with CHB (OR 12.45, 95% CI 9.54-16.67, P<0.0001) together with HBV genotype D infection , HBsAg and ALT (OR 1.15, 95% CI 2.8-4.02, P=0.431) serum levels, which showed as well a significant independent association with CHB, but with lower strength (OR 4.76, 95% CI 3.75-6.06, P=0.001; OR 3.34, 95% CI 2.8-4-02, P=0.0431 and OR 1.15, 95% CI 2.8-4.02, P=0.431, respectively). The Area Under the Curve for HBcrAg to discriminate CHB patients from HBeAg negative infection was 0.968 (95% CI, 0.958 to 0.977) with an optimal cut-off of 3.14 log10 U/mL (95% CI, 3.02 to 3.25) showing 91% sensitivity (95% CI, 89% to 94%) and 93% specificity (95% CI, 90% to 95%). Interestingly, the currently proposed diagnostic threshold of the assay, 3 log10 U/ml is within the interval confidence of the cut off given by the highest Youden index. HBcrAg serum levels below 3 log10 U/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 antiviral treatment. An additional strength of using HBcrAg in clinical practice is the evidence that the threshold of 3 log10 U/mL holds true to adequately differentiate CHB from HBeAg negative infection with high diagnostic accuracy independently from HBV genotype.

The addition of HBV genotype to the HBcrAg did not improve the diagnostic accuracy over the use of HBcrAg alone. In our cohort of HBeAg negative individuals, where Genotype A and D were the most represented ones, while median HBV DNA and HBsAg serum levels were significantly different by HBV genotype (P<0.0001), 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 infection by genotype A and D 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 indicate 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 ENI in spite of the higher (about 7 to 8-fold in U/mL) HBcrAg serum levels observed in genotype B as compared to genotype A, D and E carriers (mean log difference of 0.9, 95%CI: 0.1-1.6), nevertheless the median HBcrAg level were below 3 log10 U/ml in all the genotypes. Accordingly, the use of genotype specific cut-off did not improve the diagnostic accuracy achieved by the 3.14 log10 U/ml. Interestingly, at the single point observation 3.3% of CHB patients, in spite of having viremia <2000 IU/ml showed HBcrAg serum levels > 3 log10 IU/ml suggesting the presence of an active HBV infection 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 [18]

By converse in the group of HBeAg negative carriers with viremia fluctuation above 2,000 IU/ml, but persistently < 20,000 IU/ml, only 24.8% of cases had positive HBcrAg serum levels >3 log10 U/ml), whereas 75.2% had HBcrAg < 3 log10 U/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 viremia fluctuations up to 20,000 IU/mL, those at high risk of hepatitis reactivation. This would warrant an optimization of HBeAg negative carriers management, taking into account that the current guidelines suggest to monitor them and consider treatment only in case of overt recurrence of liver damage. Hepatitis recurrence rate in this subset of HBeAg negative carriers appears quite variable (ranging from 10 to 2% at 5 years) in the different cohorts.[3,19] Interestingly, among the 54% of Grey Zone subjects who underwent a second time point observation during a median follow-up of about 44 months only 2.3% of cases had transition of HBcrAg from serum levels < 3 to > 3 log10 U/mL; whereas in 5% of cases HBcrAg levels decreased <3 log10 U/mL. All together these observations suggest that most of HBeAg negative individuals with low viremia are in a transition phase towards a progressive control of HBV infection.

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, viremia levels > 20,000 IU/ml were the best predictor of a positive HBcrAg test with a standardized 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, [3,5,14] 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. This is consistent also with previous evidence of a higher correlation between the 2 viral markers in European HBeAg negative than in HBeAg positive patients as compared to HBeAg negative

infection, where no statistical correlation was found between HBV-DNA and HBcrAg.[12,14] The significant correlation observed between HBcrAg and HBV-DNA in Asian inactive carriers can be explained by the different threshold that was used for the analysis including also values below 3 log10 IU/ml. [13]

In conclusion this study proves and defines in a large number of cases the added value of detecting HBcrAg in 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 antivirals when treated as early as possible in the asymptomatic phase) by 2030. 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). HBcrAg qualifies to be proposed as a key marker in combination with HBsAg and HBeAg to provide a very interesting three antigen qualitative assay to be tested 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 viremia levels below and above the 2000-20.000 UI diagnostic grey zone. Prospective studies have to be performed to validate this hypothesis.

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Tables

Table 1: Demographic and virologic characteristics according to 3 clinical categories (Chronic Hepatitis B, CHB; HBV-DNA < 20,000 IU/ml and normal ALT, Grey Zone and HBV-DNA < 2,000 IU/ml, HBeAg negative infection, ENI)

Variables	Overall, n=1582	CHB, n=550	GZ, n=322	ENI, n=710	p value
Sex (male), n (%)	926 (59%)	399 (73%)	151 (47%)	376 (53%)	<0,0001
Age (y), mean (SD)	44 (13.2)	47 (13.0)	40 (12.1)	44 (13.4)	0.0002
Ethnicity					
Caucasian	910 (58%)	370 (67%)	189 (59%)	351 (49%)	<0,0001
Asian	110 (7%)	30 (5%)	23 (7%)	57 (8%)	0.0756
African	265 (17%)	36 (7%)	73 (23%)	156 (22%)	<0,0001
Other	41 (3%)	15 (3%)	7 (2%)	19 (3%)	0.9556
Unknown	256 (16%)	99 (18%)	30 (9%)	127 (18%)	0.9588
Genotype (A/B/C/D/E/F/mixed), n (%)					
A	231 (15%)	68 (12%)	59 (18%)	104 (15%)	0.242
B	35 (2%)	9 (2%)	6 (2%)	20 (3%)	0.1709
C	38 (2%)	16 (3%)	6 (2%)	16 (2%)	0.4644
D	713 (45%)	377 (69%)	113 (35%)	223 (31%)	<0,0001
E	141 (9%)	19 (3%)	44 (14%)	78 (11%)	<0,0001
F	11 (1%)	3 (1%)	4 (1%)	4 (1%)	0.9661
mixed	2 (0%)	2 (0%)	0 (0%)	0 (0%)	0.9709
Unknown	411 (26%)	56 (10%)	90 (28%)	265 (37%)	<0,0001
Liver stiffness (kPa), median (IQR)	5.1 (4.1-6.3)	6.9 (5.4-10.4)	4.8 (3.9-5.6)	4.5 (3.8-5.4)	<0,0001
Platelets ($\times 10^9$/L), median (IQR)	211 (174-252)	179 (142-230)	219 (192-263)	222 (191-261)	<0,0001
ALT (U/L), median (IQR)	29 (19-49)	76 (45-144)	24 (18-32)	22 (17-29)	0.0368
HBV- DNA (log10 IU/mL), median (IQR)	3.2 (2.3-4.9)	5.6(4.8-6.6)	3.5 (3.2-3.8)	2.2 (1.5-2.7)	<0,0001
HBsAg (log10 IU/mL), median (IQR)	3.4 (2.8-3.9)	3.7 (3.3-4.0)	3.6 (3.1-4.1)	2.9 (2.1-3.6)	<0,0001
HBcrAg (log10 U/mL), median (IQR)	2.7 (2.0-4.1)	4.8 (3.9-5.7)	2.5 (2.0-2.9)	2.0 (2.0-2.5)	<0,0001
<3 log10 U/mL, number (%)	912 (57.6%)	26 (4.7%)	242 (75.2%)	644 (90.7%)	
≥3 log10 U/mL, number (%)	670 (42.4%)	524 (95.3%)	80 (24.8%)	66 (9.3%)	

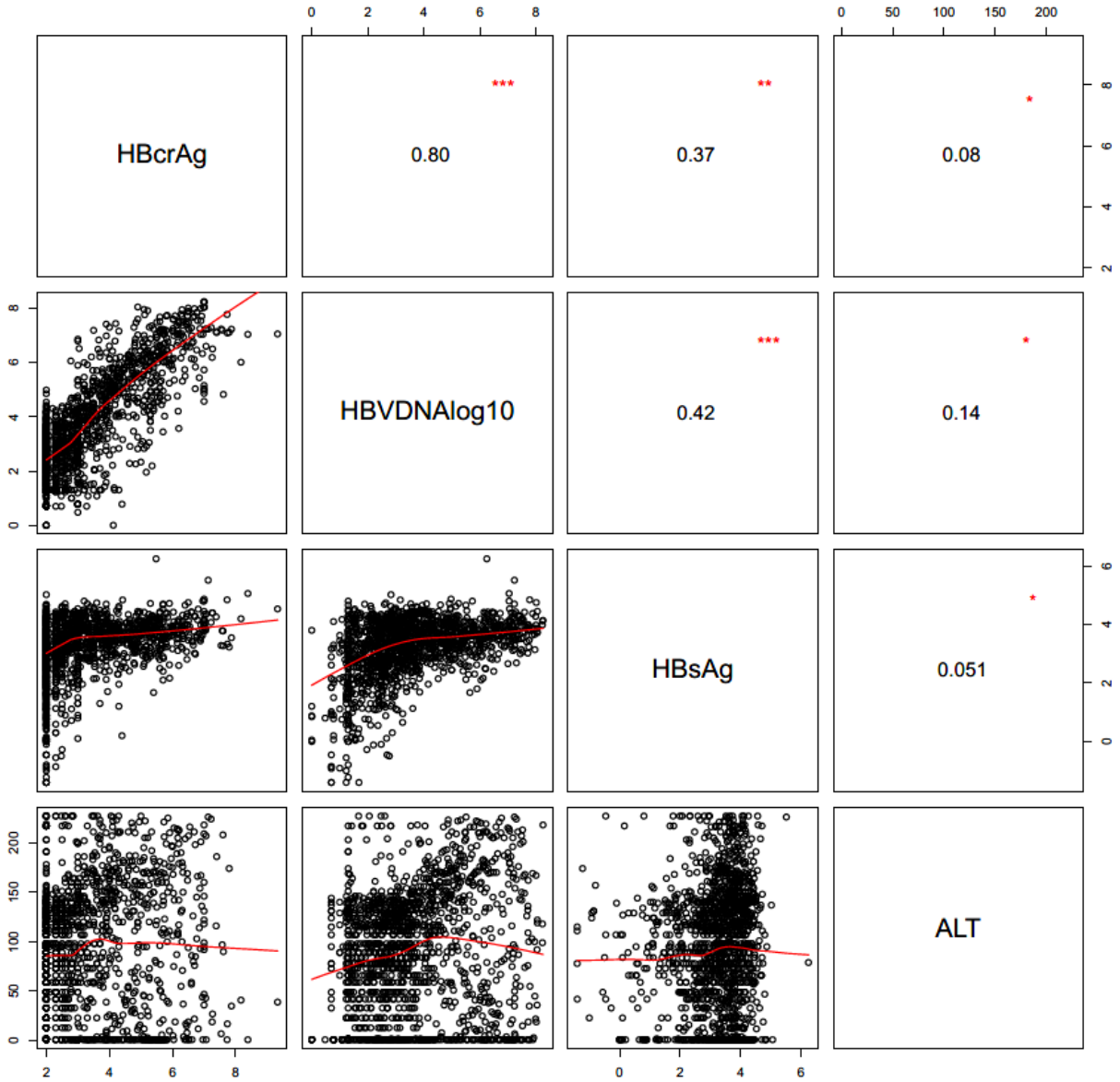
Data are expressed as median (25^{th}-75^{th} percentile range, IQR) or number (%).
Lower limit of quantification, LLOQ = 3 log10 U/ml for HBcrAg.
p value: differences between CHB and HBeAg negative infection (ENI) groups

Table 2: Parameters associated with Chronic Hepatitis B (CHB) according to the logistic regression model

		Univariate				Multivariate		
HBV markers	**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	1.3	[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 Stifness (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/mL)	1023	1.15	[2,8 - 4,02]	<0,0001	867	1.14	[1,06 - 3,57]	0.0431

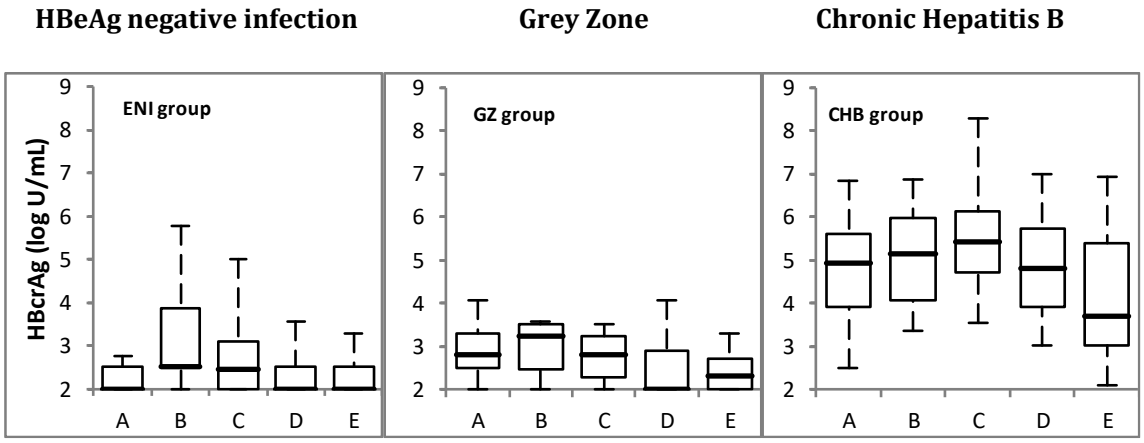
Figures

Figure 1: Scatterplots with overlay of smoothed regression to visualize relationship between pairs of biomarkers (lower triangle) in the overall population; Pearson correlation coefficient (upper-triangle)



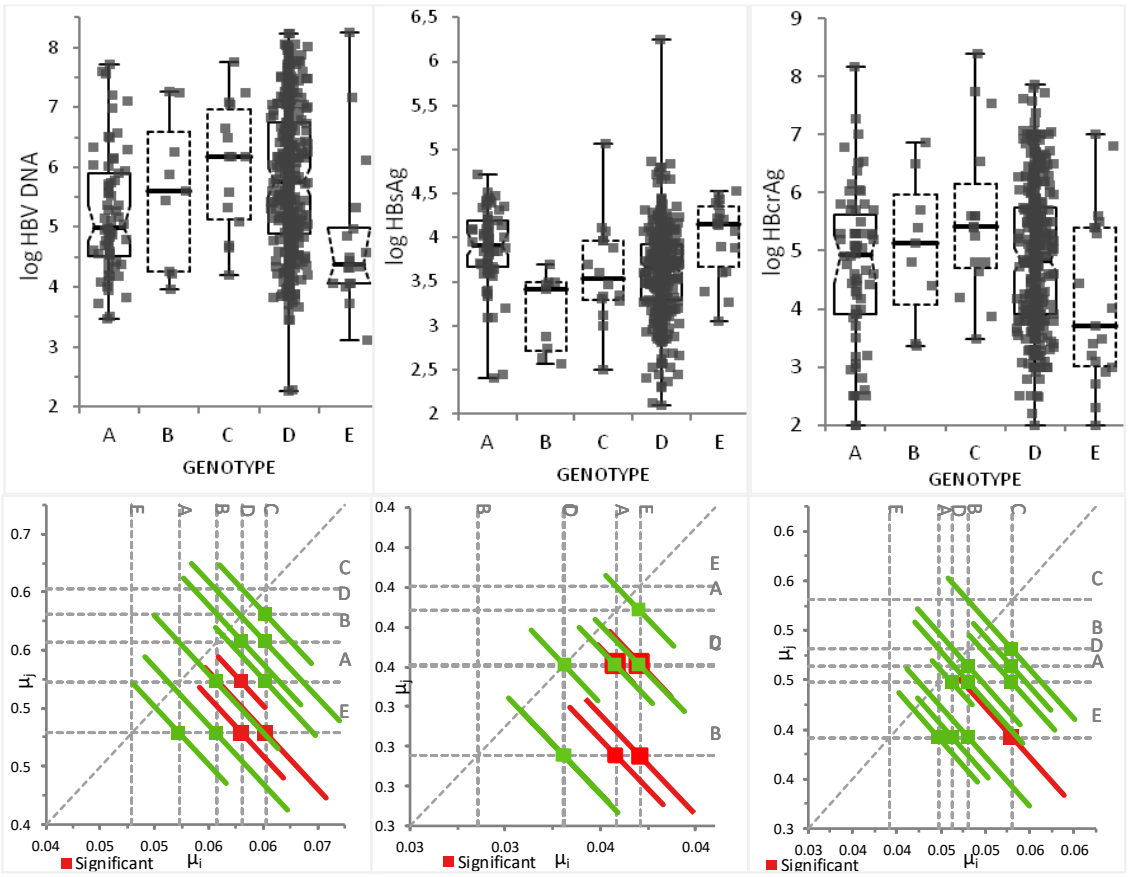
Pearson correlation coefficient: (***) above 0.4, relatively strong; (**) between 0.2 and 0.4, moderate; (*) below 0.2, weak

Figure 2: HBcrAg serum distribution according to HBV genotypes and clinical categories: HBeAg negative infection (ENI), Grey Zone (GZ) and Chronic Hepatitis B (CHB).



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: Quantitative measures of viral markers by genotype - Skeletal Notched Boxes for HBV DNA, HBsAg and HBcrAg in CHB group and Tukey-Kramer all pairs comparisons.



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

Figure 4 : HBsAg and HBcrAg pair scattering and optimal cut-off lines

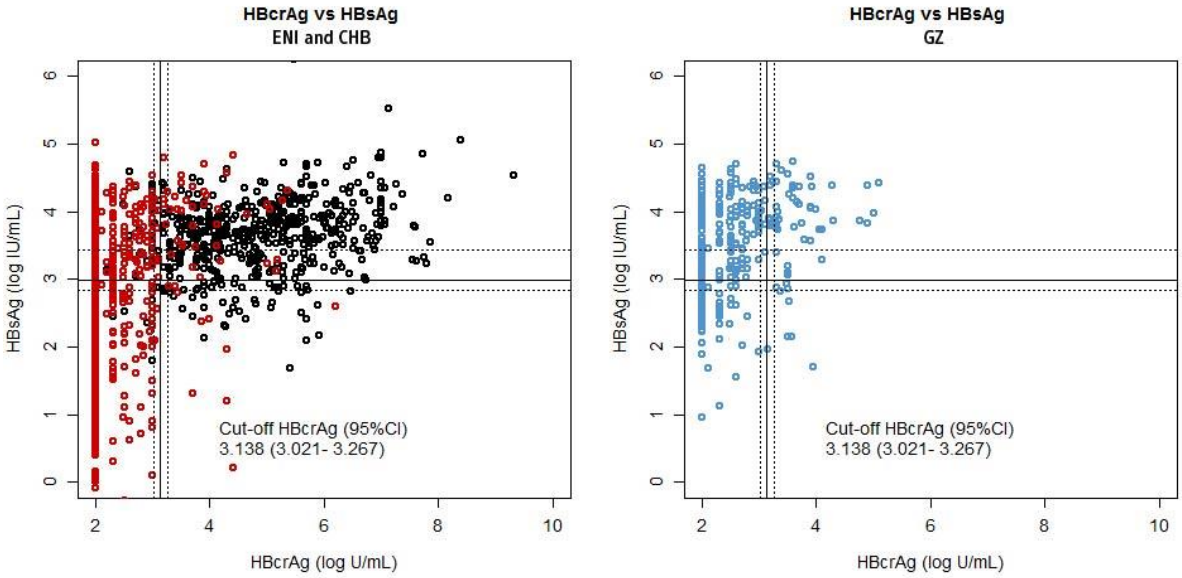
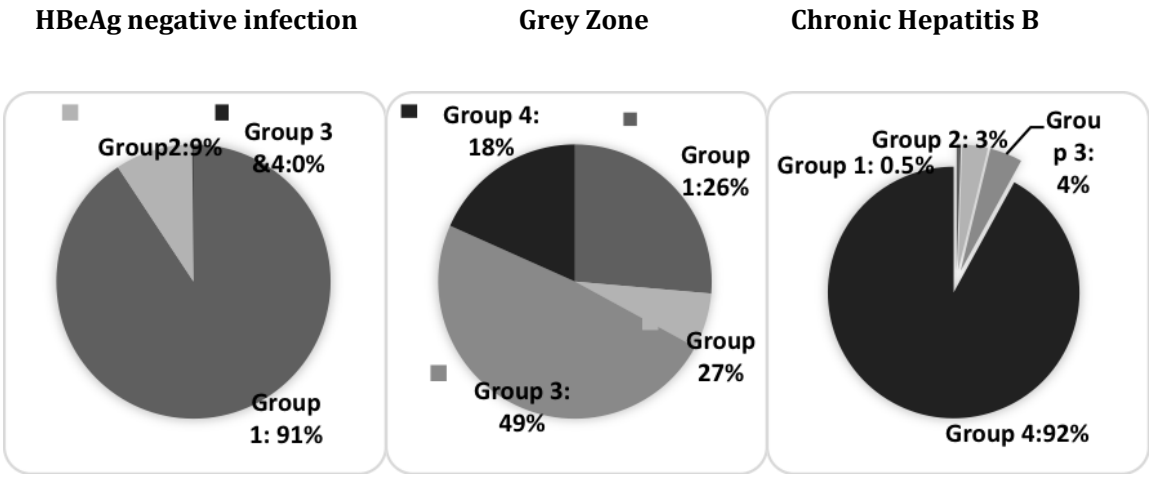


Figure 5 : Distribution of the different combination of HBV-DNA (cut-off 2,000 IU/mL) and HBcrAg (cut-off 3 log10 U/mL) and HBV infection phase



In the pies the numbers refer to the groups defined by HBV-DNA and HBcrAg combination: Group 1: HBV-DNA < 2,000 IU/ml and HBcrAg < 3 log10 U/ml (732 cases, 46.3%); Group 2: HBV-DNA <2,000 IU/ml and HBcrAg ≥ 3 log10 U/ml (104 cases, 6.6%); Group 3: HBV-DNA ≥ 2,000 IU/ml and HBcrAg < 3 log10 U/ml (180 cases, 11.4%); Group 4: HBV-DNA ≥ 2,000 IU/ml and HBcrAg ≥ 3 log10 U/ml (566 cases, 35.8%)

Supplementary Material

Supplementary Table 1: Comparison of viral markers serum levels between genotype D and A

Clinical categories	Contrast	Mean (log10) difference	Simultaneous 95% CI		SE	p-value
		HBV DNA				
CHB group	D - A	0,575	0,121	1,028	0,159	0.0043
ENI and GZ group	A - D	0,205	-0,023	0,433	0,083	0.1027
All samples	D - A	0,778	0,399	1,158	0,129	<0.0001
		HBsAg				
CHB group	A - D	0,268	0,071	0,465	0,069	0.0015
ENI and GZ group	A - D	0,819	0,556	1,081	0,096	<0.0001
All samples	A - D	0,434	0,213	0,655	0,075	<0.0001
		HBcrAg				
CHB group	D - A	0,161	-0,315	0,637	0,166	0.9284
ENI and GZ group	A - D	0,056	-0,107	0,219	0,060	0.8814
All samples	D - A	0,589	0,282	0,897	0,104	<0.0001

Supplementary Table 2: Genotype-specific cut-offs to differentiate CHB from ENI

Patients	N	Cut-off	95% IC	Accuracy
All	1188	3.14	3.02 - 3.27	0.92
Genotypes A+D+E	833	3.02	3.00 - 3.09	0.93
Genotype A	158	3.02	3.02 - 3.51	0.95
Genotype D	581	3.04	3.02 - 3.14	0.89
Genotype E	94	2.8	2.25 - 3.12	0.94

Supplementary Figure 1

ROC-curves for individual biomarkers and combination of HBsAg and HBcrAg