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Anti-HBc IgM associates with acute flare and HBeAg/HBsAg loss in chronic hepatitis B patients with acute exacerbation

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ABSTRACT

Acute exacerbation (AE) is common for patients with chronic hepatitis B (CHB). The aim of the study is to investigate the values of hepatitis B core antibody (anti-HBc) IgM in CHB-AE. Patients were screened from a prospective sub-cohort, 419 CHB patients with AE were enrolled and divided into groups according to antiviral treatment history, treatment naïve, withdrawal above or within 6 months, and on-treatment. The prevalence, clinical characteristics of anti-HBc IgM, and its relationship with the outcomes of CHB were assessed. A total of 157 patients (37.5%) were tested positive for anti-HBc IgM, of which patients with antiviral-withdrawal more than 6 months had the highest prevalence (49.3%). Anti-HBc IgM was significantly associated with HBV DNA and ALT, regarding to its prevalence and serum level. Furthermore, serum anti-HBc IgM values varied in different phases of CHB, of which immune active and HBeAg-negative chronic hepatitis phases were significantly higher than that in inactive carriers ($p = 0.017$ and $p = 0.0097$, respectively). Anti-HBc IgM could distinguish hepatitis from inactive infection phases in HBeAg-negative patients (AUC 0.841). Anti-HBc IgM levels were significantly higher in subgroup who developed ACLF ($p < 0.05$), but had no relationship with short-term mortality. Finally, anti-HBc IgM seropositivity was the only predictor of HBeAg seroclearance (OR 3.18, 95% CI 1.30–7.73) and all patients who achieved HBsAg seroclearance within 1-year had a markedly elevated anti-HBc IgM level. In conclusion, our study shows anti-HBc IgM is highly prevalent in CHB patients with AE and would be a new predictor of HBeAg and HBsAg loss in this population.

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Chronic hepatitis B; acute exacerbation; anti-HBc IgM; hepatitis flare; seroclearance

Introduction

Acute exacerbation (AE) that occurred during the course of chronic hepatitis B virus (HBV) infection is not uncommon, with a cumulative probability of 10–30% every year [1,2]. Some of exacerbations may be mild and self-limited, but notably, it can also lead to hepatic decompensation, liver failure, or even death. AE of chronic hepatitis B (CHB) can occur in a variety of circumstances (e.g. hepatitis B relapse, superimposed infection, alcohol, drugs), in which HBV activation is a major driver of acute liver damage and high short-term mortality in patients with CHB in the Asia-Pacific region [3]. Hence, there is a growing need to discover effective biomarkers for timely recognition and management of HBV-related exacerbation.

As the earliest antibody to develop in response to acute HBV infection, immunoglobulin M antibodies against hepatitis B core antigen (anti-HBc IgM) was also reported to be detected, experience turn-positive or rise significantly during AE or reactivation episodes in CHB patients [4–6]. Furthermore, another 1-year follow-up study with monthly tests found that significant elevation of anti-HBc IgM levels was associated with AE in 96.2% of the cases [7].

It has been investigated that there is a complex interaction among HBV, hepatocytes, and immune cells of the host during hepatitis B flares [8,9]. The induction of anti-HBc IgM indicates a specific humoral immune response to hepatitis B core antigen (HBcAg), while whether it can function as a non-invasive marker for flare-ups of HBV and subsequent immune activation related to hepatocellular damage or viral clearance during the course of chronic HBV infection remains unclear. In the current study, we investigated the prevalence, the relationships with HBV replication and liver inflammation of anti-HBc IgM in CHB patients with different antiviral treatment histories and hepatitis B e antigen (HBeAg) status, as well as its effect on

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acute-on-chronic liver failure (ACLF) and short-term mortality. Furthermore, the levels of anti-HBc IgM in different disease phases of HBV infection were also assessed. Finally, the cumulative incidences of HBeAg and hepatitis B surface antigen (HBsAg) seroclearance were calculated to evaluate whether the baseline anti-HBc IgM level could function as a predictive immune determinant of responses to nucleos(t)ide analog (NA) treatment.

Materials and methods

Patients

Patients were screened from Southwest Hospital (a subcohort of prospective multicenter cohorts in CATCH-LIFE study [10,11] in Chongqing, China from January 2015 to December 2016 and July 2018 to January 2019, respectively. In total, there were 526 patients with acute exacerbation of chronic liver disease in the cohort, of which 71 without HBV infection and 36 with no blood samples available at admission were excluded. Finally, 419 CHB patients with acute exacerbation were included in this study (Figure 1). Data including demographic characteristics, coexisting disorders, laboratory measurements, imaging tests, hospitalization records at admission and follow-up information were collected by an electronic case report form (CRF). Survival time and information regarding liver transplantation within 1 year were obtained from medical records, telephone contact, and/or clinic visiting. The study was approved by the Ethics Committee of the Southwest Hospital of Third Military Medical University (KY2021053). Written informed consent was obtained from every participant or their legal surrogate.

Definitions

Acute exacerbation of CHB was defined as non-malignant CHB with acute decompensation (development of at least one of the bacterial infection, overt ascites, gastrointestinal hemorrhage, hepatic encephalopathy, or total bilirubin [TB] >5 mg/dL within 1 month) or acute liver injury (serum level of alanine aminotransferase [ALT] or aspartate aminotransferase [AST] >3 × the upper limit of normal [ULN], or TB > 2 × ULN within 1 week), which was described in detail previously [12]. Cirrhosis was diagnosed based on liver biopsy or clinical presentation with typical ultrasound or computed tomography imaging. HBV-related ACLF

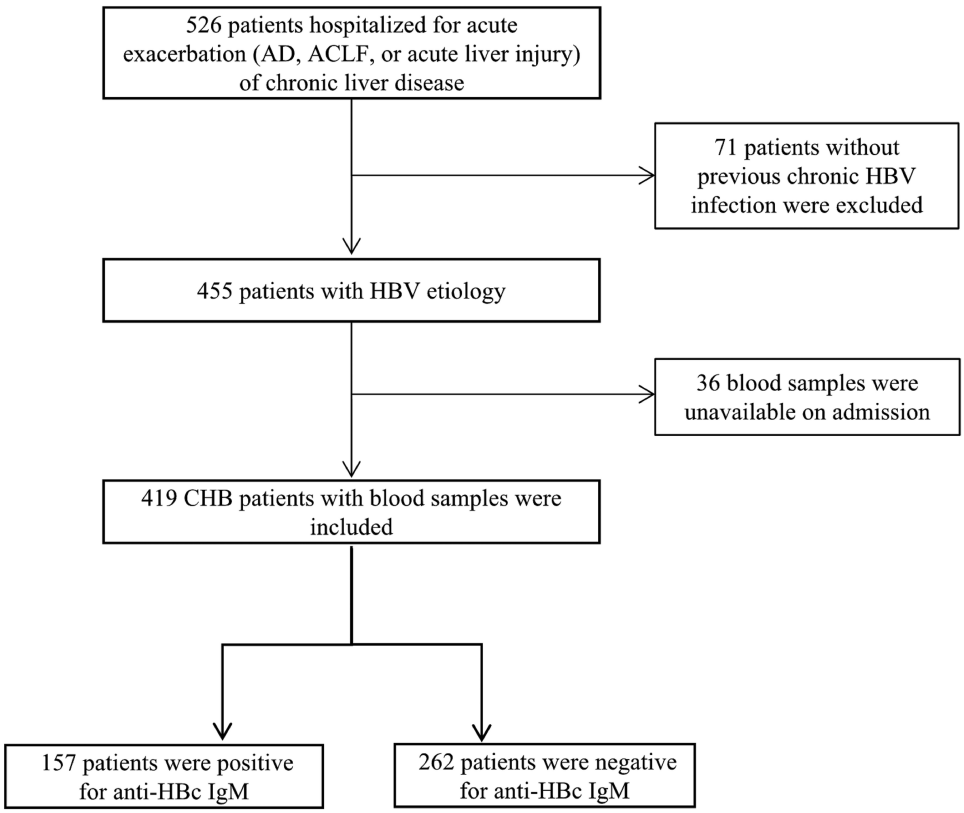


Figure 1. Study flowchart. AD, acute decompensation; ACLF, acute-on-chronic liver failure; HBV, hepatitis B virus; CHB, chronic hepatitis B; anti-HBc IgM, immunoglobulin M antibodies against hepatitis B core antigen.

was defined by the COSSH criteria, which was characterized as an increase in serum total bilirubin ≥12 mg/dL and international normalized ratio (INR) ≥1.5 in patients with chronic hepatitis B [13]. At admission, patients were evaluated for the presence of ACLF. In the non-ACLF patients, the development of ACLF was recorded during their hospital stay. Patients who never received antiviral therapy were classified into different clinical phases according to the guidelines for the management of CHB [14] as IA (immune active, described as HBeAg-positive chronic hepatitis), IC (inactive carrier, described as HBeAg-negative chronic infection), or ENH (HBeAg-negative chronic hepatitis) based on their serum HBV DNA, ALT levels, and HBeAg status on admission. The ULN of ALT was 42 IU/L in this study. Chronic hepatitis phases, IA and ENH, were determined using a threshold of ULN for ALT and 2000 IU/mL for HBV DNA, alongside the HBeAg status (positive and negative, respectively). Hepatitis flare was defined as an event with abrupt rise of ALT levels to >5 times the ULN during chronic HBV infection [9].

Laboratory tests

Blood samples were obtained from 419 CHB patients and the biochemical, serological, and virological parameters were measured using standard laboratory procedures by the laboratory department. Anti-HBc IgM was quantified using a chemiluminescent immunoassay on the Abbott Architect (Abbott GmbH, Wiesbaden Delkenheim, Germany). The serum levels of anti-HBc IgM evaluated by the signal to cutoff (S/CO) value, which is the ratio of the signal strength of sample to the signal strength of an internal cutoff, were used for quantitative analysis. An S/CO value ≥1.0 was considered to be positive for anti-HBc IgM according to the instruction manual. HBsAg, HBeAg, anti-HBe, and anti-HBs were detected quantitatively by chemiluminescent immunoassay (Abbott Laboratory, Chicago, IL) following the instruction. Values exceeding 0.05 IU/mL and 10 mIU/mL were considered positive for HBsAg and anti-HBs, respectively. The serum HBV DNA level was determined by COBAS Amplicor monitor test (Roche Molecular Systems, Branchburg, NJ), which below the detection limit (20 IU/mL) was regarded as negative.

Statistical analysis

The normality of the datasets was tested by the Kolmogorov–Smirnov test. Continuous variables were compared by the Mann–Whitney U test, while categorical variables by Pearson's χ^2 test or Fisher's exact test where required between groups. Spearman's rank correlation coefficient test was used to describe the quantitative association between variables. Receiver operating characteristic (ROC) curves and areas under the curve (AUC) were used to analyze the utility of anti-HBc IgM in discriminating disease phases and predicting HBeAg seroclearance. Univariate and multivariate analysis were performed by logistic regression model and adjusted by stepwise (forward likelihood ratio) to identify independent factors of HBeAg clearance. Liver transplant-free survival was analyzed in groups with different anti-HBc IgM status. The data were analyzed with SPSS v27.0 or GraphPad Prism v9.5.1 software and tests with p value < 0.05 (2-side) were considered statistically significant.

Results

Clinical characteristics of patients and prevalence of anti-HBc IgM

A total of 419 CHB patients with AE were included in this analysis and were tested for anti-HBc IgM. Overall, the median (interquartile range [IQR]) age was 46 (-39.3–52.7) years, 83.3% were male. Among them, 157 (37.5%) subjects were positive for anti-HBc IgM. The clinical characteristics in anti-HBc IgM positive and negative groups are shown in Table 1. Compared to the patients who were negative for anti-HBc IgM, patients with positive anti-HBc IgM had higher levels of HBV DNA, AST, TB, alkaline phosphatase, lymphocyte count, as well as higher rates of HBV DNA positive and infection cases (both $p < 0.05$, Table 1). Further, patients were divided into four groups according to their antiviral treatment and withdrawal histories: treatment naïve ($n = 205$), withdrawal above 6 months ($n = 71$), withdrawal within 6 months ($n = 34$), and on-treatment ($n = 109$). Patients who had a treatment-free interval of >6 months showed the highest prevalence of anti-HBc IgM (49.3% [35/71]), which was significantly higher than that in patients who were receiving antiviral therapy (33.9% [37/109], $p = 0.040$) and treatment naïve (35.1% [72/205], $p = 0.035$, Figure 2(a)). No significant difference was observed in serum anti-HBc IgM levels among groups (Figure 2(b)).

The affection of precipitating factors on anti-HBc IgM prevalence

Since acute exacerbation (AE) of CHB can occur spontaneously or be triggered by some specific causes, we analyzed the precipitating factors among these CHB patients and investigated their

Table 1. Baseline characteristics according to the anti-HBc IgM status.

Variables	Total ($n = 419$)	Anti-HBc IgM (+) ($n = 157$)	Anti-HBc IgM (-) ($n = 262$)	P value
Gender, male, n (%)	349 (83.3%)	124 (79.0%)	225 (85.9%)	0.067
Age, year, median (IQR)[a]	46 (39.3–52.7)	45 (39.2–53.3)	46 (39.4–52.3)	0.853
HBV parameters				
HBV DNA, $\log_{10}$ IU/mL, median (IQR)[a]	4.4 (2.4–6.0)	4.6 (3.0–6.1)	4.1 (0.0–6.0)	**0.030**
HBV DNA positive, n (%)	330 (78.8%)	141 (89.8%)	189 (72.1%)	**< 0.001**
HBeAg positive, n (%)	201 (48.0%)	83 (52.9%)	118 (45.0%)	0.121
Anti-HBc IgM, S/CO, median (IQR)[a]	0.7 (0.3–1.5)	1.9 (1.4–3.6)	0.4 (0.2–0.7)	**< 0.001**
Laboratory tests, median (IQR)[a]				
Alanine aminotransferase (IU/L)	286.0 (79.0–782.1)	310.0 (95.0–808.0)	265.8 (63.2–740.7)	0.136
Aspartate aminotransferase (IU/L)	229.0 (96.0–550.0)	253.0 (139.7–589.6)	202.2 (78.0–507.3)	**0.025**
Alkaline phosphatase (IU/L)	127.0 (104.0–162.0)	135.0 (107.7–170.0)	121.3 (99.5–156.0)	**0.015**
γ-glutamyltransferase (IU/L)	102.0 (55.0–165.0)	105.5 (57.0–166.0)	99.0 (52.5–164.5)	0.484
Total bilirubin (mg/dL)	8.9 (2.8–17.3)	10.8 (3.3–19.1)	7.2 (2.6–15.7)	**0.026**
Albumin (g/L)	32.1 (27.9–36.3)	31.5 (26.8–36.1)	32.3 (28.2–36.6)	0.333
International normalized ratio	1.3 (1.1–1.7)	1.4 (1.1–1.7)	1.3 (1.1–1.6)	0.342
Creatinine (mg/dL)	0.7 (0.6–0.9)	0.7 (0.6–0.9)	0.7 (0.6–0.8)	0.613
Blood urea nitrogen (mg/dL)	4.5 (3.5–5.9)	4.5 (3.5–5.7)	4.5 (3.5–6.0)	0.900
White blood cell, $\times 10^9$ /L	5.2 (3.8–6.8)	5.1 (3.9–7.2)	5.2 (3.7–6.7)	0.537
Neutrophil, $\times 10^9$ cells/L	3.3 (2.3–4.8)	3.2 (2.3–5.0)	3.4 (2.2–4.8)	0.713
Lymphocyte, $\times 10^9$ cells/L	1.2 (0.9–1.6)	1.3 (1.0–1.7)	1.2 (0.8–1.6)	**0.009**
Neutrophil-to-Lymphocyte ratio	2.7 (1.7–4.6)	2.6 (1.6–4.5)	2.7 (1.8–4.7)	0.253
Platelet, $\times 10^9$ cells/L	95.0 (62.0–134.0)	100.0 (70.0–130.5)	92.0 (57.0–135.5)	0.219
Hemoglobin (g/L)	128.0 (113.0–142.0)	126.0 (111.5–138.0)	130.0 (114.0–143.0)	0.225
Potassium (mmol/L)	4.0 (3.6–4.4)	4.0 (3.7–4.4)	4.0 (3.6–4.4)	0.760
Sodium (mmol/L)	138.0 (135.0–140.1)	137.4 (134.2–140.0)	138.0 (135.0–140.4)	0.422
Cirrhosis, n (%)	246 (58.7%)	95 (60.5%)	151 (57.6%)	0.563
Hepatic encephalopathy	17 (4.1%)	5 (3.2%)	12 (4.6%)	0.483
Ascites	183 (43.7%)	77 (49.0%)	106 (40.5%)	0.086
Gastrointestinal bleeding	8 (1.9%)	2 (1.3%)	6 (2.3%)	0.714
Bacterial infection	110 (26.3%)	50 (31.8%)	60 (22.9%)	**0.044**
MELD score[a]	14.0 (10.0–19.4)	15.7 (9.5–19.8)	13.6 (9.8–18.9)	0.153
Mortality, n (%)				
28-day	32 (7.6%)	13 (8.2%)	19 (7.2%)	0.701
90-day	55 (13.1%)	23 (14.6%)	32 (12.2%)	0.475
365-day	68 (16.2%)	29 (18.5%)	39 (14.9%)	0.335
Liver transplantation, n (%)	12 (2.9%)	5 (3.2%)	7 (2.7%)	0.998
Hepatitis flare, n (%)	193 (46.1%)	67 (42.7%)	126 (48.1%)	0.282
ACLF, n (%)	141 (33.7%)	61 (38.9%)	80 (30.5%)	0.081

IQR, interquartile range. S/CO, signal to cutoff. MELD, model for end-stage liver disease. ACLF, acute-on-chronic liver failure. Hepatitis B flare was defined as an event with abrupt rise of ALT levels to > 5 times the upper limit of normal (Chang, M.L. et al., 9).
[a]Statistical tests were calculated with the Mann-Whitney U test. The rest: Pearson χ2 test.

relationships with the prevalence of anti-HBc IgM. The majority of CHB patients included in our study experienced a spontaneous acute exacerbation without a definite precipitating factor ($n = 177$, 42.2%). Bacterial infection ($n = 89$, 21.2%), withdrawal of the antiviral treatment ($n = 76$, 18.1%), recent hepatotoxic drug use ($n = 29$, 6.9%), and active alcohol intake ($n = 28$, 6.7%), NAs resistance ($n = 11$, 2.6%), upper gastrointestinal bleeding ($n = 6$, 1.4%), and co-infection with other hepatitis viruses ($n = 3$, 0.7%) were suspect of causing AE in the remaining patients. The prevalence of anti-HBc IgM in CHB patients with different precipitating factors is shown in Supplementary Figure S1, of which the highest prevalence was in patients with NAs resistance (54.5%) and withdrawal of the antiviral treatment (53.9%). Notably, all patients who had gastrointestinal bleeding and co-infection with HEV were negative for anti-HBc IgM. Patients who had antiviral treatment cessation had significantly higher prevalence of anti-HBc IgM than those who had bacterial infection, recent hepatotoxic drug use and gastrointestinal bleeding ($p < 0.05$).

Association of anti-HBc IgM with HBV DNA in CHB subjects

Since AE of CHB attributed to reactivated virus infection is accompanied by an upsurge of HBV DNA in most cases, we then investigated the consistency between anti-HBc IgM and HBV DNA. In total, the prevalence of anti-HBc IgM was higher in HBV DNA-positive patients than that in HBV DNA-negative groups regardless of whether they were currently treated or not (HBV DNA+ vs. HBV DNA-: total: 42.7% [141/330] vs. 18.0% [16/89], $p < 0.001$; treatment naïve: 39.2% [69/176] vs. 10.3% [3/29], $p = 0.0030$; withdrawal above 6 months: 53.2% [33/62] vs. 22.2% [2/9], $p = 0.17$; withdrawal within 6 months: 38.7% [12/31] vs. 33.3% [1/3], $p = 1.00$; on-treatment: 44.3% [27/61] vs. 20.8% [10/48], $p = 0.010$) (Figure 3(a)). Only 3 out of 72 patients who were anti-HBc IgM positive were negative

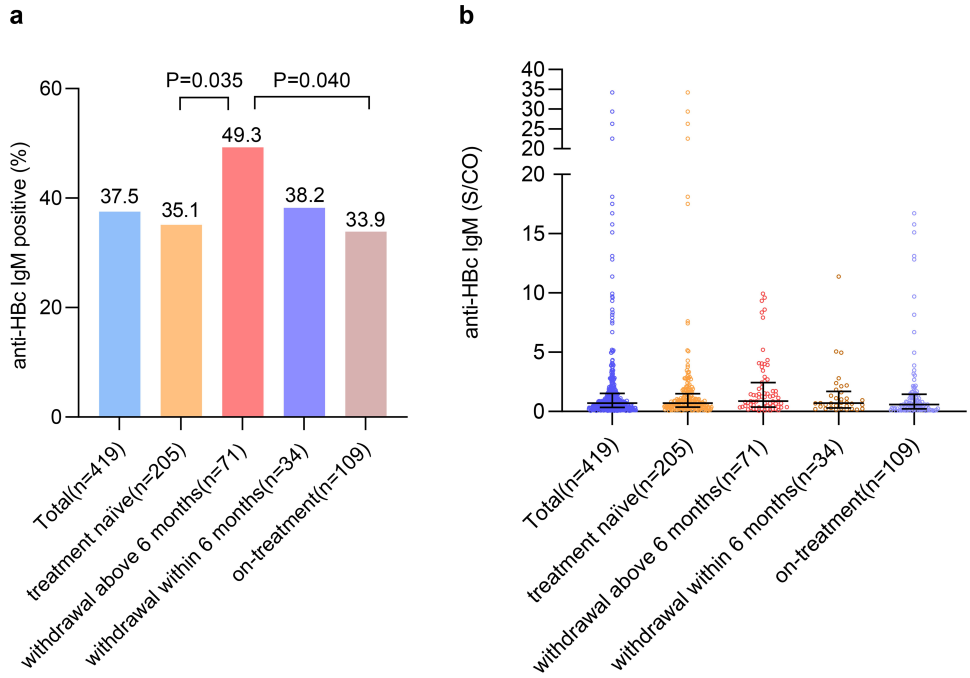


Figure 2. The prevalence (a) and serum levels (b) of anti-HBc IgM among patients with different antiviral treatment histories. Bars represent the median with interquartile range. S/CO, signal to cutoff; anti-HBc IgM, immunoglobulin M antibodies against hepatitis B core antigen.

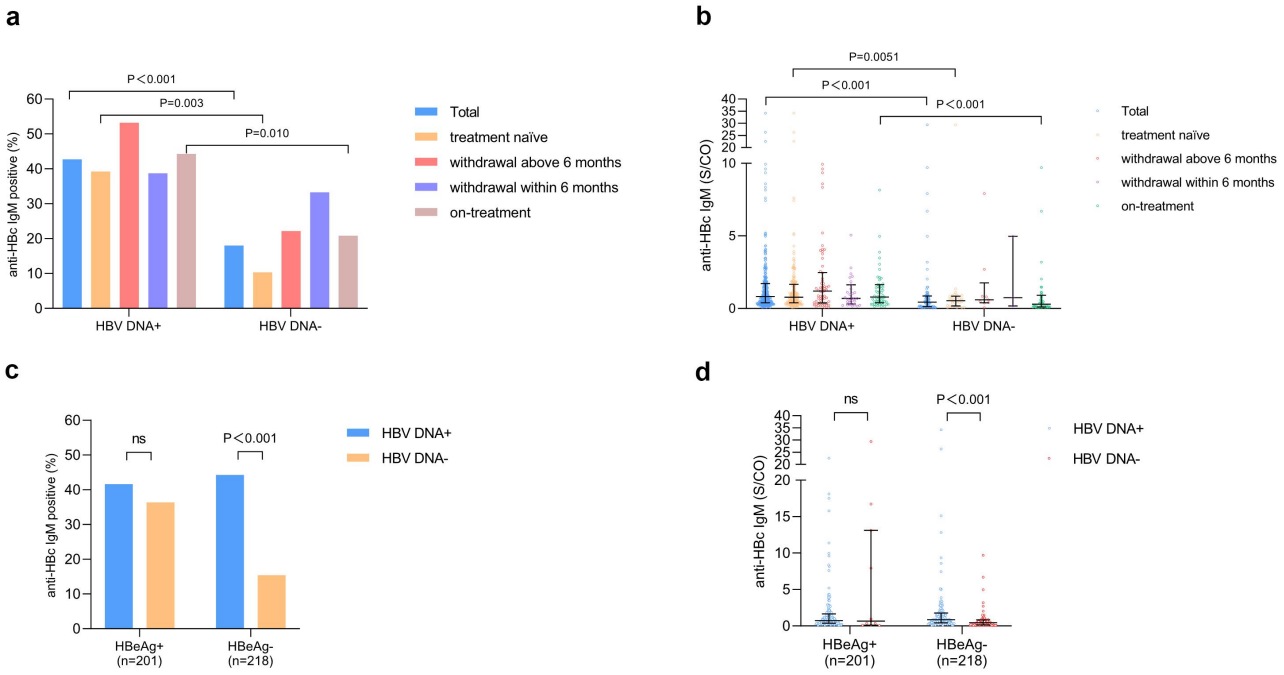


Figure 3. Relationship of anti-HBc IgM with HBV DNA in CHB subjects. The prevalence (a) and serum levels (b) of anti-HBc IgM in HBV DNA positive and negative group stratified by antiviral treatment history. The prevalence (c) and serum levels (d) of anti-HBc IgM in HBV DNA positive and negative group with different HBeAg status. Bars represent the median with interquartile range. Anti-HBc IgM, immunoglobulin M antibodies against hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; S/CO, signal to cutoff.

for HBV DNA in the treatment naïve group. Meanwhile, serum anti-HBc IgM levels were significantly higher in HBV DNA-positive subjects than that in HBV DNA-negative subjects in total (HBV DNA+ vs. HBV DNA-: 0.820 [0.390–1.708], $n = 330$ vs. 0.440 [0.130–0.855], $n = 89$, $p < 0.001$), treatment naïve (HBV DNA+ vs. HBV DNA-: 0.775 [0.393–1.668], $n = 176$ vs. 0.530 [0.180–0.850], $n = 29$, $p = 0.0051$) and on-treatment groups (HBV DNA+ vs. HBV DNA-: 0.790 [0.395–1.640], $n = 61$ vs. 0.295 [0.103–0.910], $n = 48$, $p < 0.001$) (Figure 3(b)). Similarly, HBV DNA-positive subjects had higher prevalence and serum levels of anti-HBc IgM in HBeAg-negative patients (Figure 3(c,d)).

To further analyze the quantitative relationship between immune activation and virus replication, we studied the correlation between the levels of anti-HBc IgM and HBV DNA. First, a threshold of HBV DNA at 2×10^7 IU/mL was used according to the cutoff value of HBV DNA set to differentiate immune tolerant phase from immune active phase [15]. Then, those HBeAg-positive and HBeAg-negative individuals were further sub-grouped into HBV DNA high (HBV DNA $> 2 \times 10^7$ IU/mL) and low (HBV DNA $\leq 2 \times 10^7$ IU/mL) groups, respectively. As shown in Supplementary Table S1, the level of serum anti-HBc IgM correlated positively with HBV DNA in total (rho = 0.140, $p = 0.0040$) and HBeAg-negative groups (rho = 0.263, $p < 0.001$), of which higher correlation coefficients were shown in patients with HBV DNA $\leq 2 \times 10^7$ IU/mL (rho = 0.221, $p < 0.001$ for total subjects and rho = 0.317, $p < 0.001$ for HBeAg-negatives). However, no significant relationship was shown in HBeAg-positive groups or patients with high HBV DNA levels. In view of the inhibitory effects of antiviral drugs on viral replication, we specially explored the relationships in treatment naïve patients and patients who were receiving antiviral treatment for at least 6 months. We found that the correlation between anti-HBc IgM and HBV DNA weakened or disappeared in the former group (Supplementary Table S1) while it turned to be much stronger (rho = 0.626, $p < 0.001$) in the latter group (Supplementary Figure S3 (a)). Altogether, these results indicate that anti-HBc IgM is closely related to viral replication, especially in NA-treated HBeAg-negative patients with HBV DNA $\leq 2 \times 10^7$ IU/mL.

Association of anti-HBc IgM with ALT in CHB subjects

Higher prevalence and serum levels of anti-HBc IgM were found in patients with elevated ALT levels, irrespective of the HBeAg status (Figure 4(a,b)). Since hepatitis B flare is considered to be the result of immune response against HBV and HBcAg-specific B cells were temporarily augmented during hepatitis flares [9,16], we made a further exploration in the relationship between anti-HBc IgM and hepatitis flares. Despite the prevalence of anti-HBc IgM was comparable in two groups (Figure 4(c)), patients with hepatitis flare displayed significantly higher levels of anti-HBc IgM in the total and HBeAg-negative groups (Figure 4(d)). Similar to the results presented in the relationship with HBV DNA, there was also a positive correlation between anti-HBc IgM and ALT in total population and HBeAg-negative group (rho = 0.176 and 0.253, $p < 0.001$), especially in patients with HBV DNA $\leq 2 \times 10^7$ IU/mL (rho = 0.229 for total and rho = 0.291 for HBeAg-negative group, $p < 0.001$, Supplementary Figure S2 and Supplementary Table S1). The correlation coefficients also greatly enhanced in patients who were receiving antiviral treatment for more than 6 months (rho = 0.533, $p < 0.001$, Supplementary Figure S3(b)).

Considering there was no significant correlation between anti-HBc IgM and ALT in patients with HBV DNA $> 2 \times 10^7$ IU/mL, we further investigated the relationship, respectively, in the hepatitis flare and non-flare groups with HBV DNA $\leq 2 \times 10^7$ IU/mL. Anti-HBc IgM was positively correlated with ALT in patients without flares regardless of HBeAg status (Figure 4 (e,f)).

Association of anti-HBc IgM with ACLF and short-term outcomes

Hepatitis B relapse was found to be the predominant factor of CHB-related ACLF in studies conducted in China and could result in higher short-term mortality [13,17]. Therefore, early indicators for HBV-related ACLF are urgently needed. As shown in Supplementary Figure S4a,b, a significantly higher level of anti-HBc IgM was found in ACLF group in comparison with that in CHB group regardless of HBeAg status (ACLF vs. non-ACLF: total: 0.850 [0.465,1.670] vs. 0.645 [0.290,1.475], $p = 0.0030$; HBeAg-positive group: 0.955 [0.495,2.308] vs. 0.690 [0.310,1.530], $p = 0.020$; HBeAg-negative group: 0.820 [0.450,1.470] vs. 0.570 [0.250,1.440], $p = 0.039$) despite comparable prevalence of anti-HBc IgM between the two groups, which may suggest that higher anti-HBc IgM levels indicate concomitantly serious liver injury in these patients.

Nevertheless, within patients with ACLF, no significant difference was observed for the 28-day and 90-day mortality between patients who were positive and negative for anti-HBc IgM ($p > 0.05$, Supplementary Figure S4(c,d)). Likewise, there was also no significant

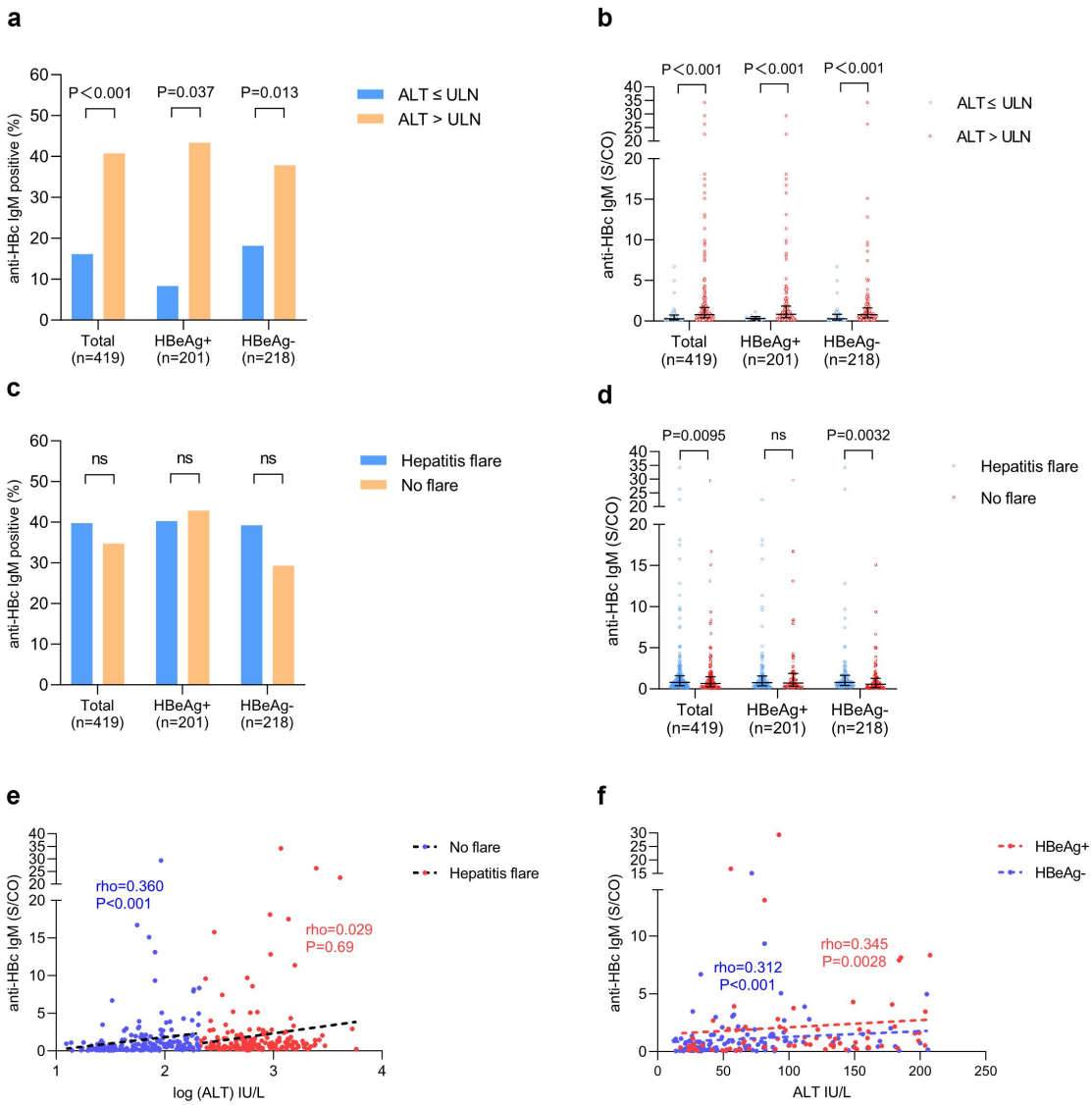


Figure 4. Relationship of anti-HBc IgM with ALT in CHB subjects. The prevalence (a) and serum levels (b) of anti-HBc IgM in groups with normal and abnormal ALT. The prevalence (c) and serum levels (d) of anti-HBc IgM in patients with and without hepatitis flare. Bars represent the median with interquartile range. (e) Scatter plots between serum anti-HBc IgM and ALT of CHB patients with HBV DNA $\leq 2 \times 10^7$ IU/mL. Note: red dots represented CHB patients with hepatitis flare; blue dots represented CHB patients without hepatitis flare. (f) Scatter plots between serum anti-HBc IgM and ALT of CHB patients with HBV DNA $\leq 2 \times 10^7$ IU/mL and without hepatitis flare. Note: red dots represented HBeAg-positive patients; blue dots represented HBeAg-negative patients. Anti-HBc IgM, immunoglobulin M antibodies against hepatitis B core antigen; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; CHB, chronic hepatitis B; S/CO, signal to cutoff; ULN, upper limit of normal.

difference shown in the overall population (Supplementary Figure S5).

Anti-HBc IgM levels during different phases of CHB infection

As shown in Figure 5(a), CHB patients were categorized into three distinct phases based on the virological and serological indicators tested at admission: IA (HBeAg positive, elevated ALT levels and serum HBV DNA > 2000 IU/mL), IC (HBeAg negative, normal ALT levels and serum HBV DNA < 2000 IU/mL) and ENH (HBeAg negative, elevated ALT levels and serum HBV DNA > 2000 IU/mL). Serum levels of anti-HBc IgM varied in different phases of CHB, of which the median levels in each phase were: IA (0.740 [0.390, 1.580], n = 83), IC (0.280 [0.130, 0.490], n = 7) and ENH (0.820 [0.440, 1.730], n = 61). Serum levels of anti-HBc IgM in IA and ENH were significantly higher than IC ($p < 0.05$), and notably, all individuals in HBeAg-negative infection phase were negative for anti-HBc IgM. In HBeAg-negative patients, ROC analysis was performed to distinguish hepatitis from inactive carrier state by anti-HBc IgM level (AUC = 0.841, 95%

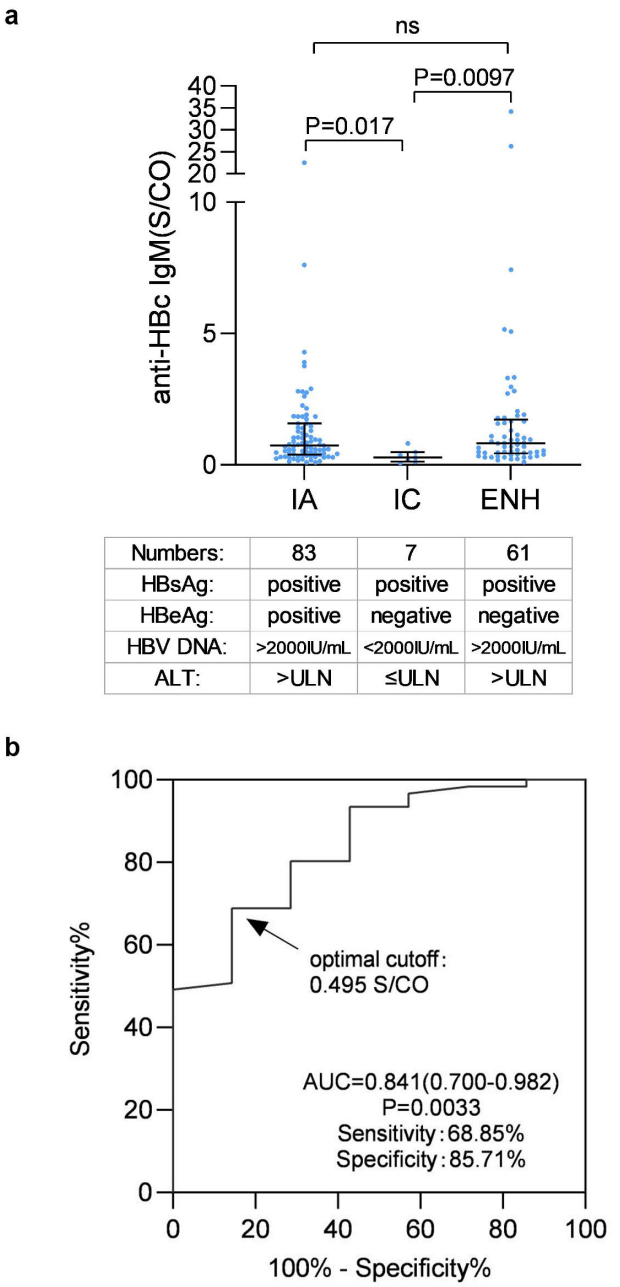


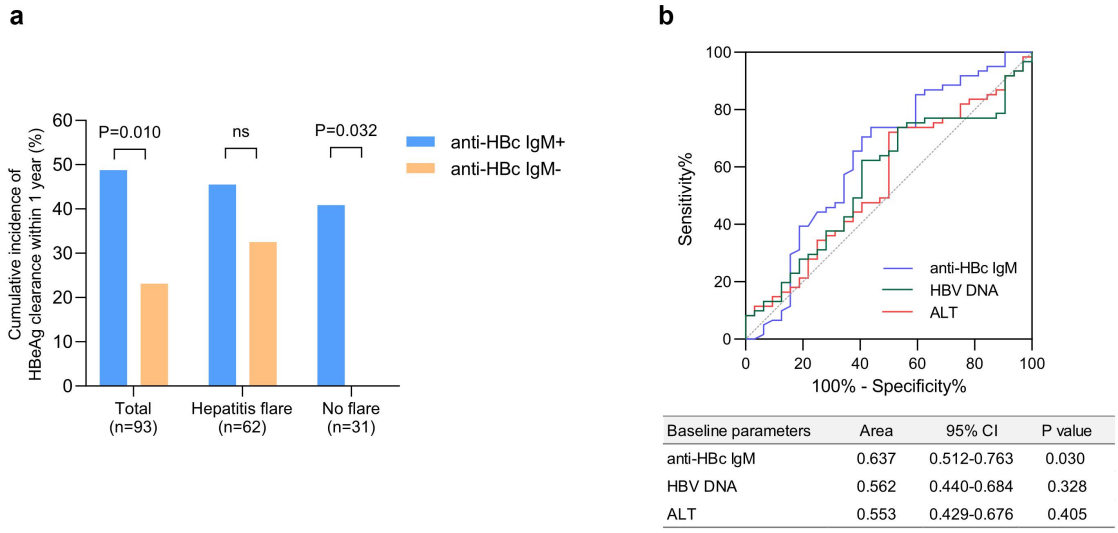
Figure 5. Distribution of serum anti-HBc levels during different phases of chronic HBV infection. (a) Distribution of anti-HBc IgM levels during different phases of chronic HBV infection. Bars represent the median with interquartile range. IA, immune active phase ($n = 83$); IC, inactive carrier phase ($n = 7$); ENH, HBeAg-negative hepatitis phase ($n = 61$). (b) Receiver operating characteristic analysis of anti-HBc IgM to differentiate between the HBeAg-negative chronic hepatitis phase and the HBeAg-negative chronic infection phase. Anti-HBc IgM, immunoglobulin M antibodies against hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; ALT, alanine aminotransferase; ULN, upper limit of normal; S/CO, signal to cutoff; AUC, area under the curve.

confidence interval [CI], 0.700–0.982; $p = 0.0033$, Figure 5(b)), and the optimal cutoff was 0.495 S/CO, with a sensitivity of 68.9% and a specificity of 85.7%.

Prediction of anti-viral therapy-induced HBeAg and HBsAg seroclearance

Seroclearance of HBeAg and HBsAg is associated with host immune responses against HBV and indicates a more favorable outcome. Of the 201 HBeAg-positive patients included, the HBeAgstatus was available for 93 patients after a 1-year follow-up. Patients who were positive for anti-HBc IgM at baseline had a higher cumulative rate of HBeAg clearance within 1 year after enrollment compared to anti-HBc IgM negative individuals (48.8% [20/41] vs. 23.1% [12/52], $p = 0.010$). Considering that hepatitis flare was reported to be associated with a greater incidence of virus clearance, subgroup analyses based on baseline ALT strata were performed to compare the cumulative incidence of HBeAg clearance in patients with different anti-HBc IgM status (Figure 6(a)). Patients with positive anti-HBc IgM had higher 1-year HBeAg clearance rates than those with negative anti-HBc IgM in the non-flare group (40.9% [9/22] vs. 0% [0/9], $p = 0.032$) but no significant difference was found in the flare group (45.5% [10/22] vs. 32.5% [13/40], $p = 0.31$). To further compare the prediction performance of baseline anti-HBc IgM, HBV DNA and ALT levels, we examined the AUC and found that only high levels of anti-HBc IgM at baseline alone could predict the HBeAg seroclearance after NA treatment with an optimal cutoff value of 1.135 S/CO ($p = 0.030$, Figure 6(b)). The univariate and multivariate analysis also showed that only anti-HBc IgM seropositivity (OR 3.18, 95% CI 1.30–7.73; $p = 0.011$) could independently predict HBeAg clearance (Supplementary Table S2).

In total, after a 1-year follow-up, HBsAg status was available in 183 patients, of which only 4 patients (2.2%) achieved HBsAg loss. Similarly, individuals were sub-grouped based on their ALT levels to control confounding factors (Figure 7(a)). Patients with strong anti-HBc IgM responses (> 10 S/CO) had an absolutely higher rate of HBsAg seroclearance within 1-year (4/5, 80%), while all of the patients with anti-HBc IgM ≤10 S/CO were failed to achieve HBsAg loss (0/178, 0%) whether they had hepatitis flare or not ($p < 0.001$). Another patient who had a strong anti-HBc IgM response (22.53 S/CO) experienced an HBsAg decline greater than 3 $\log_{10}$ within 8 weeks



Baseline parameters	Area	95% CI	P value
anti-HBc IgM	0.637	0.512-0.763	0.030
HBV DNA	0.562	0.440-0.684	0.328
ALT	0.553	0.429-0.676	0.405

Figure 6. Prediction of anti-viral therapy-induced HBeAg seroclearance using the baseline anti-HBc IgM in CHB patients with acute exacerbation. (a) Patients were divided into hepatitis flare group and non-flare group according to their baseline ALT levels. Cumulative incidence of HBeAg seroclearance within 1 year was analyzed in subgroups with different anti-HBc IgM status. (b) AUCs of baseline parameters in predicting HBeAg seroclearance within 1 year. Anti-HBc IgM, immunoglobulin M antibodies against hepatitis B core antigen; HBeAg, hepatitis B e antigen; AUC, area under the curve; HBV, hepatitis B virus; ALT, alanine aminotransferase.

even though she was lost to follow-up at 1 year. The kinetics of parameters in these five responders showed that the anti-HBc IgM peak occurred after the HBV DNA and ALT peak in three of the cases and the anti-HBc IgM levels gradually decreased along with HBsAg seroclearance in all cases (Figure 7(b-f)).

Discussion

Previous studies have widely investigated the value of quantitation of anti-HBc as an immunological biomarker in solving different diagnostic dilemmas [18]. However, the total anti-HBc comprises both IgM and IgG types and their respective roles are warrant to be elucidated. A previous study found that anti-HBc IgM was associated with hepatitis B exacerbations [7]. Hence, in the present study, we focused on anti-HBc IgM alone to clarify its clinical use among patients with acute exacerbation of CHB.

The prevalence of anti-HBc IgM was highest in those who had an antiviral treatment-free interval of more than 6 months, suggesting a high risk of viral rebound after cessation of antiviral therapy. Notably, a proportion of HBV DNA-negative patients who were currently receiving NA treatment still had positive results of anti-HBc IgM (20.8% [10/48]), of which two patients had been receiving antiviral treatment above 6 months and were negative for HBeAg with normal ALT levels. These results remind clinicians to take caution when managing these clinically silent patients since the presence of anti-HBc IgM in the serum permits the diagnosis of ongoing HBV infection even in cases with undetectable HBsAg [19].

HBV activation usually starts with viral replication and is followed by liver injury that results from immune responses against virus. In our present data, anti-HBc IgM coincided well with HBV DNA and ALT, demonstrating the possible mechanism that B cells were activated by naked particulate HBcAg produced during ongoing virus replication and released from damaged hepatocytes into the circulation to secrete the corresponding IgM antibody. However, no significant difference was observed between anti-HBc IgM and HBV DNA among HBeAg-positive group, which may be explained by the distinct virological and immunological dynamics. HBeAg-positive patients usually have high viral loads. On the one hand, HBeAg was reported to suppress the cellular and humoral immune response during CHB infection [20], thus even when HBV DNA is positive, the immune response against core antigen is limited. On the other hand, despite HBV DNA may be transiently suppressed by antiviral therapy, residual viral replication and covalently closed circular DNA (cccDNA) transcription in the liver can sustain HBcAg expression to induce the production of anti-HBc IgM. These together result in no significant difference in anti-HBc IgM between HBV DNA-

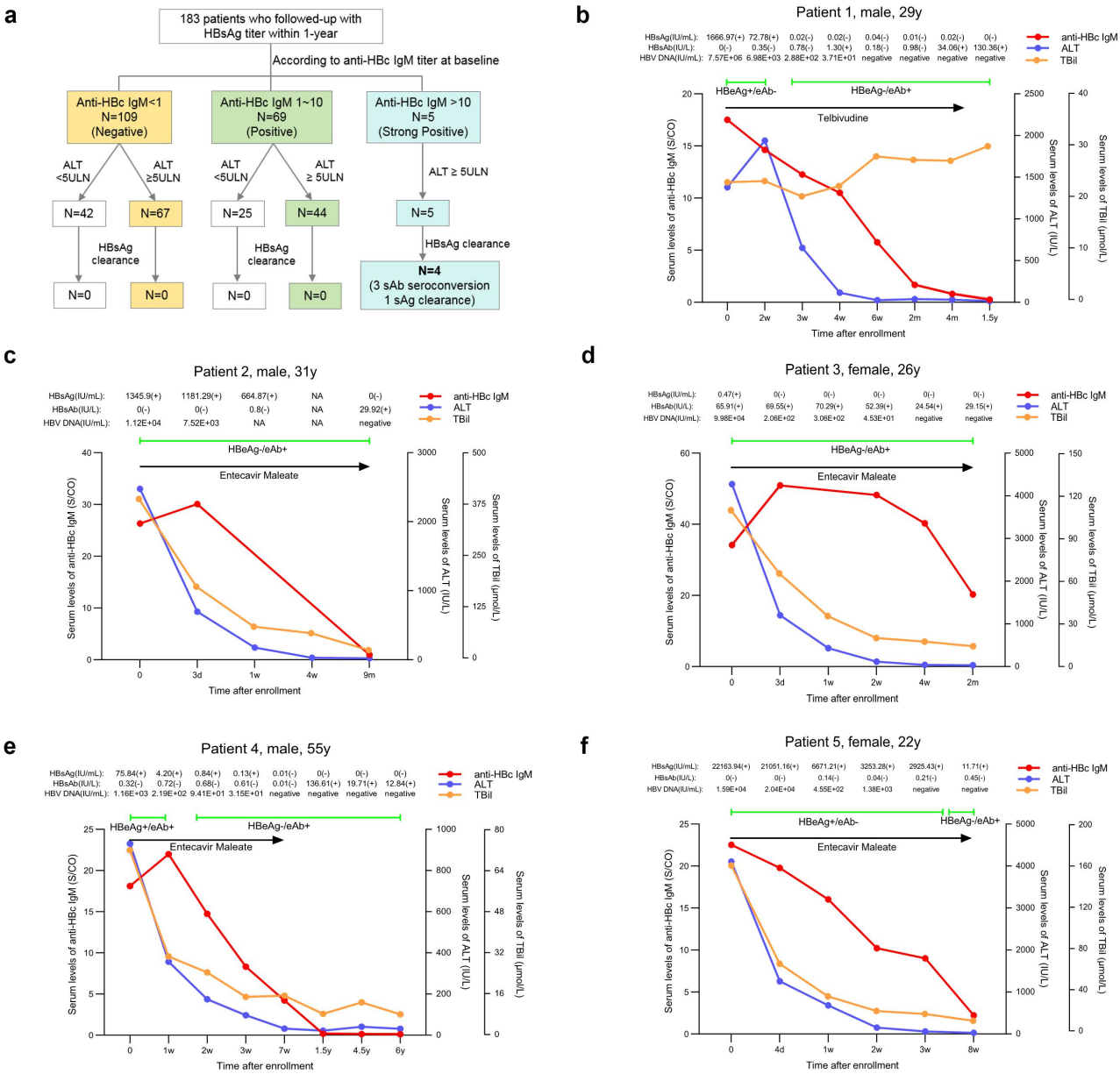


Figure 7. Prediction of HBsAg clearance using the baseline anti-HBc IgM level in CHB patients with acute exacerbation. (a) Cumulative incidence of HBsAg clearance within 1 year in patients who were classified with baseline ALT and anti-HBc IgM strata. (b-f) Kinetics of HBV-related serological and virological parameters in 5 CHB patients with HBsAg seroclearance or markedly reduction of HBsAg. The green segments in the upper part of the figure represent the period of serum HBeAg and anti-HBe. Black arrows indicate the administration of antiviral drugs against HBV. CHB, chronic hepatitis B; ALT, alanine aminotransferase; TBil, total bilirubin; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; anti-HBc IgM, immunoglobulin M antibodies against hepatitis B core antigen; S/CO, signal to cutoff; ULN, upper limit of normal; NA, not available; Log_{10}, logarithm base 10; d,day; w, week; m, month; y, year.

positive and negative groups among HBeAg-positive individuals. While for HBeAg-negative patients who remain in an immunologically active state with relatively effective HBV control, the reduction in serum viral load seems to primarily result from decreased transcriptional activity of cccDNA [21]. Detectable HBV DNA reflects active viral replication and then can trigger the production of anti-HBc IgM in the absence of HBeAg-mediated immune tolerance. Based on this, combining anti-HBc IgM with HBV DNA could help risk stratification and CHB management. HBeAg-negative patients with positive HBV DNA and anti-HBc IgM may be a subgroup with subclinical activity or higher risk of progression, requiring more aggressive antiviral therapy and close monitoring. Furthermore, there were also weak quantitative

relationships of anti-HBc IgM with HBV DNA and ALT, and even no correlation was found between these variables in patients with HBV DNA > 2×10^7 IU/mL or ALT > 5 × ULN, which were similar to the findings by Mels et al. that HBV DNA, ALT, and anti-HBc IgM in hepatitis B exacerbations were chronologically but not quantitatively correlated [22]. These may be explained by the intricate interactions among active virus replication, hepatocytolysis, and antiviral immunoresponses. It has been supposed that prolonged exposure to very high quantities of viral antigens could lead to an accentuated adaptive immune exhaustion status, and ALT flares in these patients may be due to non-virus-specific immune subsets, which were unable to selectively target infected hepatocytes containing HBcAg but caused extensive hepatolysis and ALT release [23,24]. Virus encoding protein such as HBeAg was also reported to impair the proliferation of CD4 T cells while activate macrophages to accelerate liver injury by production of multiple inflammatory factors [25], providing an explanation of some non-significant results for the relationships between anti-HBc IgM and viral replication or hepatitis in HBeAg-positive groups.

Although the clinical phases of CHB used to be described by the concept of immunology, there still lacks direct immunological evidence and indicators, and the current virological, biochemical, and histological indicators are difficult to determine the definite stage of all infected people. In the current study, significantly higher anti-HBc IgM levels were observed in hepatitis phases compared to infection phase, which was consistent with higher HBcAg-directed circulating B cell responses found in HBV clinical phases with elevated serum ALT levels, irrespective of the HBeAg status [26]. High levels of anti-HBc IgM may help recognize HBeAg-negative patients who actually had more advanced disease activities and improve clinical management.

HBeAg seroclearance is an important event that marks the natural history of CHB and a prerequisite for HBsAg loss. In this study, anti-HBc IgM was able to independently predict NA-induced HBeAg seroclearance rather than hepatitis flare, which was consistent with the findings that both higher baseline levels of anti-HBc and anti-HBc IgG could independently predict HBeAg loss in previous studies [27,28], and supplemented the current knowledge about the clinical utility of anti-HBc IgM in CHB patients with AE. Since multiple non-virus related causes could lead to hepatocellular damage, anti-HBc IgM seems to be a more reliable biomarker than ALT for reflecting HBV-specific immune responses. However, anti-HBc IgM shows a suboptimal predictive performance (AUROC 0.637) as a standalone biomarker for HBeAg loss. This underscores the necessity of expanding the sample size and combining other novel virological and serological indicators such as HBcrAg to improve predictive accuracy. As the clinical marker of functional cure, HBsAg seroclearance was only observed in 2% of the patients with NA treatment within 1 year, while all the responders had an obviously high level of anti-HBc IgM for more than 15 S/CO. Since the activation of the adaptive immune response marked by increased HBcAg-specific B cells and helper CD4 T cells with cytotoxic or effector-like signatures has been reported in patients achieving functional cure [29,30], high levels of anti-HBc IgM may indicate the activation of B lymphocytes with the ability to produce antibodies and present HBcAg to T cells that ultimately contributes to HBV clearance. The relationship between this immune biomarker and the phenotype and function of both peripheral and intrahepatic HBV-specific lymphocytes needs to be further elucidated. However, on the contrary, another two follow-up studies found that lower levels of anti-HBc and anti-HBc IgG at baseline were associated with HBsAg seroclearance in HBeAg-seronegative patients [31,32]. Thus, HBeAg status should be taken into consideration when investigating the predicting value of anti-HBc IgM on HBsAg clearance in the future study.

There are also some limitations in the present study. First, we only test the baseline anti-HBc IgM levels, characterizing the dynamic changes of anti-HBc IgM levels along with other HBV-related serological and virological parameters during AE could better understand the disease progression. Second, anti-HBc IgM can be permanently or intermittently absent in immunocompromised individuals with HBV infection, as evidenced by the results of a study in Ghana that none of the 18 patients co-infected with HIV and HBV tested positive for anti-HBc IgM [33]. Therefore, testing for anti-HBc IgM may not be applicable to CHB patients who are immunocompromised. Lastly, the cumulative incidence of HBsAg clearance was extremely low in this cohort from a single center, which limits the evaluation of the relationship between baseline anti-HBc IgM and HBsAg loss. Thus, prospective investigations with longer follow-up time are necessary in multicentre trials involving larger populations.

In summary, our results suggest a relatively high prevalence of anti-HBc IgM in CHB patients with AE, and highlight the potential role of anti-HBc IgM as a new biomarker for determining the clinical phases of CHB

infection and predicting treatment responses from the perspective of HBV-specific B cell responses, helping clinicians to stratify patients and optimize treatment regimens.

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Author contributions

GD and WT: study design. YZ, YZhou, YZhu, and XZ: patient enrollment and data collection. ZT and YD: laboratory examination. YZ: statistical analysis, interpretation of data and drafting of the manuscript. WT: critical revision of the manuscript and study supervision. All authors have read and approved the final work. The corresponding author had full access to the data in the study and had final responsibility for the decision to submit for publication.

Disclosure statement

No potential conflict of interest was reported by the authors.

Data availability statement

The raw data of this study have been deposited at Science Data Bank (ScienceDB, https://www.scidb.cn/en) and are available under the accession DOI 10.57760/sciencedb.19606.

Ethics statement

This study involving human participants was reviewed and approved by Ethics Committee of the Southwest Hospital of Third Military Medical University (KY2021053), and was conducted according to the principles of the Declaration of Helsinki. The patients provided their written informed consent to participate in this study.

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