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Research Letter

Longitudinal Profile of HBsAg Isoforms and Circulating Immune Complexes in the Natural History of Chronic Hepatitis B Infection

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Chronic hepatitis B (CHB) infection is characterized by ongoing viral replication in infected hepatocytes, leading to persistent production of viral nucleic acids and expression of viral antigens. Hepatitis B surface antigen (HBsAg) is one of the most important viral antigens, secreted at levels at least 1,000-fold higher than infectious virions in the bloodstream.[1] HBsAg consists of three isoforms: large (LHBs), middle (MHBs), and small (SHBs) surface proteins, transcribed and translated from the open reading frame S of the HBV genome. The profile of HBsAg isoforms has been described in the hepatitis B e antigen (HBeAg)-negative phase in the natural history of CHB and among patients receiving antiviral treatment.[2,3] In the era of pursuing a functional cure for CHB, identifying biomarkers that can predict HBsAg seroclearance is a priority. Baseline MHBs have been shown to predict HBsAg seroclearance with nucleoside analogue treatment.[4] The profile of HBsAg isoforms has also been described in CHB patients receiving novel therapy with nucleic acid polymers.[5] Circulating HBsAg-antibody immune complex (CIC) is another novel HBV biomarker that has been studied in patients treated with novel therapies.[6] CICs in CHB are known to be associated with disease severity and extrahepatic manifestations,[7,8] but their role in the natural history of CHB remains unclear. The aim of this study is to describe the profiles of HBsAg isoforms and CICs during the natural history of CHB, with a focus on dynamic changes in relation to HBeAg and HBsAg seroclearance.

This retrospective study included treatment-naïve patients with CHB aged ≥18 years from the Department of Medicine, Queen Mary Hospital, The University of Hong Kong. Patients were persistently seropositive for HBsAg for at least six months and had a well-documented timepoint of HBeAg seroclearance (ESC) and/or HBsAg seroclearance, along with retrievable serial plasma samples (defined as ≥ three timepoints) from ≥ five years before ESC and/or HBsAg seroclearance. Patients were excluded if they had hepatocellular carcinoma, cirrhosis, concomitant etiologies of chronic liver disease, pregnancy, liver transplantation, or lacked retrievable plasma samples. Consent was waived, as only de-identified archived blood samples were used. The use of samples was approved by the Institutional Review Board of The University of Hong Kong/Hong Kong West Cluster (UW 24-470). Clinical variables including age and gender were recorded. For the ESC cohort, alanine aminotransferase (ALT) levels after ESC were recorded as a surrogate marker of hepatic necroinflammation. For the HBsAg seroclearance cohort, defined by sustained quantitative HBsAg (qHBsAg) below the detection limit, age- and gender-matched HBeAg-negative CHB patients with normal ALT but without HBsAg seroclearance (CHB controls) were recruited for comparison. Isoforms of LHBs, MHBs, SHBs, and CICs were measured (see Supplementary Appendix for details). In the HBsAg seroclearance cohort, hepatitis B core-related antigen (HBcrAg) and quantitative antibody to hepatitis B core antigen (anti-HBc) were also measured (see Supplementary Appendix).

In the ESC cohort, plasma samples were available at ≥3 of five timepoints (−5 years, −3 years, time of ESC, +1 year, and +3 years) in 40 patients. SHBs were the most abundant isoform at all timepoints, followed by MHBs and LHBs. Before ESC, SHBs showed the strongest correlation with qHBsAg (r = 0.957–0.990), followed by MHBs (r = 0.812–0.843) and LHBs (r = 0.563–0.795). At ESC, all HBsAg isoforms lost their linear correlation with qHBsAg. After ESC, the strongest linear correlation was again observed with SHBs (r = 0.906–0.950), followed by MHBs (r = 0.804–0.892), while LHBs showed only weak correlation (r = 0.302–0.403) (Table 1A). Among these 40 patients, 20 had persistently elevated ALT after ESC. The levels and relative proportions of HBsAg isoforms remained unchanged before and after ESC (Friedman test, $p > 0.05$ across timepoints for all three HBsAg isoforms), regardless

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Table 1. Pearson correlation coefficients at various timepoints with respect to the index event (ESC or HBsAg seroclearance)

A. ESC cohort: correlation with qHBsAg					
	−5 years	**−3 years**	**ESC**	**+1 year**	**+3 years**
LHBs	0.795*	0.563*	−0.275	0.403*	0.302
MHBs	0.843*	0.812*	−0.220	0.804*	0.892*
SHBs	0.990*	0.957*	−0.227	0.906*	0.950*

B. HBsAg seroclearance cohort: correlation with qHBsAg				
	5 to 10 years before HBsAg SC	**3 to 5 years before HBsAg SC**	**1 to 3 years before HBsAg SC**	**0 to 1 year before HBsAg SC**
LHBs	0.609*	0.731*	0.766*	0.787*
MHBs	0.944*	0.953*	0.980*	0.994*
SHBs	0.989*	0.967*	0.991*	0.996*
Anti-HBc	−0.552*	0.098	−0.258	0.392
CICs	0.124	0.245	0.423*	0.278
iTACT-HBcrAg	0.487*	0.556*	0.580*	0.629*

C. ESC cohort: correlation with CICs					
	−5 years	**−3 years**	**ESC**	**+1 years**	**+3 years**
LHBs	0.468*	0.352*	0.199	−0.051	−0.113
MHBs	0.595*	0.661*	0.395*	−0.181	−0.057
SHBs	0.368*	0.432*	0.339*	−0.161	0.089

*Indicates statistical significance ($p < 0.05$). Anti-HBc, antibody to hepatitis B core antigen; CICs, circulating immune complexes; ESC, hepatitis B e antigen seroclearance; HBcrAg, hepatitis B core-related antigen; HBsAg SC, hepatitis B surface antigen seroclearance; qHBsAg, quantitative hepatitis B surface antigen.

of ALT status (Fig. 1A).

In the HBsAg seroclearance cohort, plasma samples were available at ≥3 of five timepoints (−5 to −10 years, −3 to −5 years, −1 to −3 years, and 0 to −1 year before HBsAg seroclearance) in 20 patients, with matched index timepoints in 20 CHB controls. The hierarchy SHBs>MHBs>LHBs was consistently observed at all timepoints. Compared to CHB controls, patients who subsequently achieved HBsAg seroclearance had significantly lower levels of all three isoforms at all timepoints (all $p < 0.001$). In patients with HBsAg seroclearance, all three isoforms declined progressively in the years leading up to seroclearance, while CHB controls showed no significant change (Fig. 1B). In HBsAg seroclearance patients, HBcrAg declined progressively (3.70, 3.09, 3.00, and 2.88 log U/mL; $p = 0.002$), as did anti-HBc (61.77, 57.30, 52.12, and 37.42; $p = 0.001$) at −5 to −10 years, −3 to −5 years, −1 to −3 years, and 0 to −1 year, respectively. In CHB controls, HBcrAg levels (3.97, 3.63, 3.45, and 3.43 log U/mL; $p < 0.001$) similarly declined over time, but anti-HBc levels (57.95, 60.49, 65.57, and 64.47 COI; $p = 0.241$) remained stable. Linear correlation with qHBsAg was strongest for SHBs (r = 0.967–0.996), followed by MHBs (r = 0.944–0.994), LHBs (r = 0.609–0.787), and HBcrAg (r = 0.487–0.629) (Table 1B). At five to ten years before HBsAg seroclearance, qHBsAg was the strongest predictor of subsequent seroclearance (AUROC 0.864), outperforming HBsAg isoforms or iTACT-HBcrAg. In the subgroup with qHBsAg > 1,000 IU/mL at −5 to −10 years, the proportion of LHBs+MHBs was the best predictor (AUROC 0.854), superior to qHBsAg (AUROC 0.799). In the subgroup with qHBsAg ≤ 1,000 IU/mL at −5 to −10 years, LHBs levels were the most predictive (AUROC 1.000), again superior to qHBsAg (AUROC 0.800) (Fig. 1C). HBcrAg (AUROC 0.567, $p > 0.05$), anti-HBc (AUROC 0.671, $p > 0.05$), and CICs (AUROC 0.376, $p > 0.05$) did not predict subsequent HBsAg seroclearance.

Plasma samples were available at the timepoints corresponding to HBsAg isoform measurements detailed above for the measurement of CICs. A linear correlation between CICs and HBsAg isoforms was observed prior to ESC but was lost afterwards (Table 1C). CICs declined gradually leading up to ESC and continued to decline thereafter. ALT levels after ESC were not associated with significant differences in the CIC profile (Fig. 1D). CIC levels were similar regardless of subsequent HBsAg seroclearance (Fig. 1E). Anti-HBc levels did not correlate with CICs.

Our study described the longitudinal profile of HBsAg isoforms and CICs in the natural history of 80 well-characterized CHB patients. SHBs were the most abundant isoform, a finding consistent with other studies.[2,3] The relative abundance of HBsAg isoforms was maintained after ESC, irrespective of ALT levels, and was useful in predicting subsequent HBsAg seroclearance among the HBeAg-negative population at least five years in advance. LHBs levels and the proportion of L+M HBs were superior to qHBsAg measured by conventional assays in the subgroups with qHBsAg ≤ 1,000 IU/mL and > 1,000 IU/mL, respectively. LHBs were the least abundant in blood and the earliest to decline prior to HBsAg seroclearance. After ESC and in the HBsAg seroclearance cohort, which primarily comprised patients with HBeAg-negative chronic HBV infection, excellent correlations were observed between qHBsAg and the HBsAg isoforms SHBs and MHBs (r > 0.8), but not with LHBs. This suggests preservation of SHBs and MHBs synthesis in the HBeAg-negative phase, with less contribution from LHBs. Similarly, HBcrAg showed only modest cor-

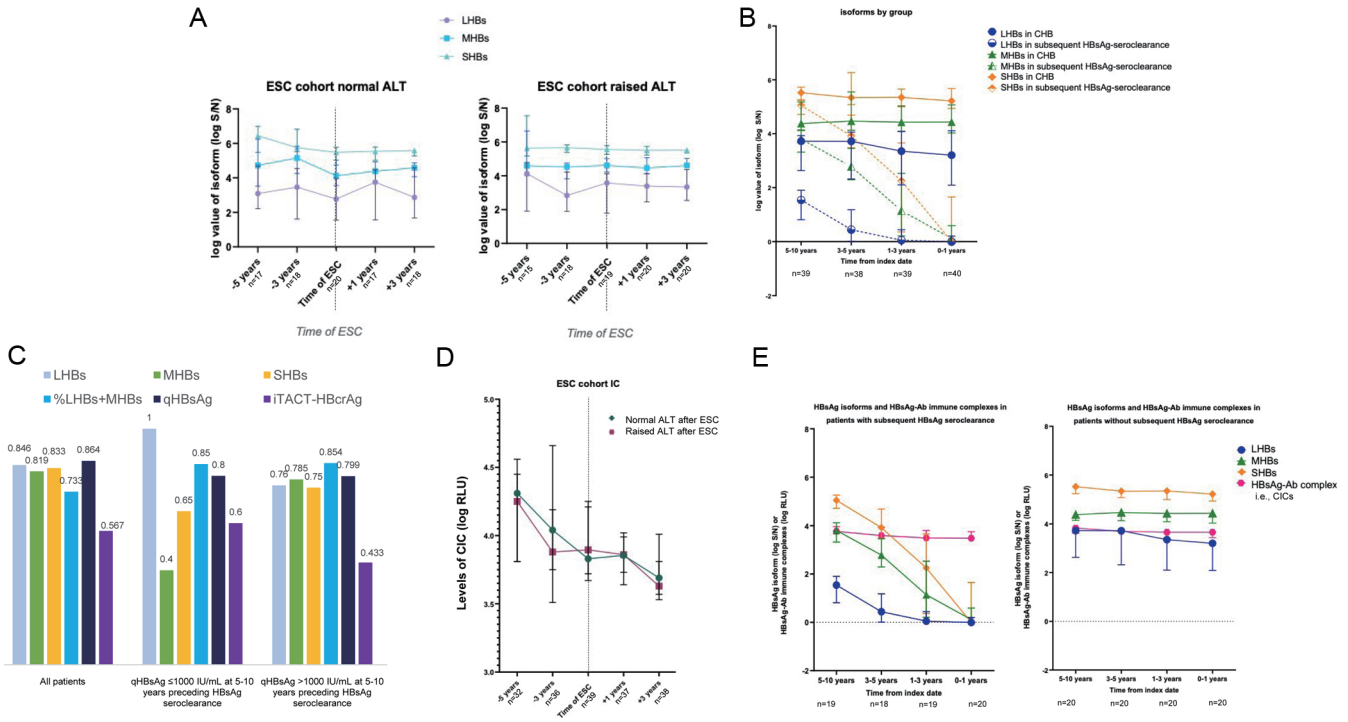

Fig. 1. Serum levels of HBsAg isoforms in patients with chronic hepatitis B at different timepoints (A) before and after HBeAg seroclearance (B) with or without subsequent HBsAg seroclearance. (C) Values of area under the receiver-operating characteristic curve for HBV biomarkers five to ten years prior to HBsAg seroclearance, predicting subsequent seroclearance in all patients, in patients with qHBsAg ≤ 1,000 IU/mL, and in patients with qHBsAg > 1,000 IU/mL. (D) Levels of circulating immune complexes in chronic hepatitis B patients before and after HBeAg seroclearance. (E) Levels of HBsAg isoforms and circulating immune complexes in chronic hepatitis B patients with or without subsequent HBsAg seroclearance. ALT, alanine aminotransferase; CIC, circulating immune complexes; ESC, hepatitis B e antigen seroclearance; HBsAg, hepatitis B surface antigen; qHBsAg, quantitative hepatitis B surface antigen.

relation with qHBsAg. This is consistent with the fact that the source of HBsAg in the HBeAg-negative phase derives not only from covalently closed circular DNA, reflected by HBcrAg, but also from integrated HBV DNA.[9] The potential utility of LHBs and HBcrAg in assessing transcriptional silencing during the HBeAg-negative phase warrants further investigation. CICs declined with the natural progression of disease phase, although the decline slowed after ESC. In addition, the linear correlation between CICs and HBsAg isoforms was lost after ESC. This discrepancy, stable isoform levels but declining CICs, supports the hypothesis that immune complexes may form only between anti-HBs and HBsAg derived from covalently closed circular DNA, but not from integrated DNA, leading to reduced detectable complexes in the HBeAg-negative phase. After a period of time in the HBeAg-negative phase, CIC levels plateaued and remained stable regardless of subsequent HBsAg seroclearance. As CICs have been reported to be much lower in patients with acute hepatitis B infection than in those with CHB,[10] one may postulate that CICs are merely markers of chronicity, with no role in viral control or HBsAg seroclearance. The lack of increased CICs in patients with subsequent HBsAg seroclearance further suggests the involvement of alternative, non-humoral (i.e., cellular) mechanisms in HBsAg seroclearance. Our findings also support the definition of treatment endpoints that do not require anti-HBs seroconversion as a prerequisite for defining functional cure.[11] This study is limited by its retrospective design, which relied on archived samples. Although the effects of long-term storage on assay accuracy are unknown, previous studies evaluating HBsAg isoforms have used samples stored since as early as 1998, in both treatment-naïve[2] and treatment-experienced patients.[3,4] Additional limitations include the relatively small sample size and the focus exclusively on treatment-naïve patients. Nevertheless, our study provides valuable insights into the roles of HBsAg isoforms and CICs in the natural history of CHB. Studies with larger cohorts and diverse clinical phenotypes, including both treatment-naïve and treatment-experienced patients (with nucleoside analogues or novel therapies), will be needed to validate these findings and to further explore the potential of these biomarkers.

In summary, HBsAg isoforms, but not CICs, demonstrate distinct profiles in the HBeAg-negative phase of CHB, with predictive value for subsequent HBsAg seroclearance up to 10 years in advance.

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None to declare.

Conflict of interest

LYM received research funding from Gilead Sciences and Roche Diagnostics, and has been an Editorial Board Member of *Journal of Clinical and Translational Hepatology* since 2025. WKS received speaker's fees from AstraZeneca, is an advisory board member and received speaker's fees from Abbott, received research funding from Alexion Pharmaceuticals, Boehringer Ingelheim, Pfizer, and Ribo Life Science, and is an advisory board member, and received speaker's fees and research funding from Gilead Sciences. Mark Anderson, Tiffany Fortney, and Gavin Cloherty are

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Author contributions

Clinical care for participants (LYM, WKS), study design (LYM, MFY), data analysis (LYM, DKHW, RWHH, MFY), data interpretation (LYM, MA, TF, GC, MFY), drafting of the manuscript (LYM), data curation (MA, TF, GC), critical review of the manuscript (MA, TF, GC, DKHW, RWHH, WKS), data acquisition (WKS), study conception, critical revision of the manuscript, and overall study supervision (MFY). All authors have participated in the preparation of the manuscript and have read and approved the final version.

Ethical statement

The use of samples was approved by the Institutional Review Board of The University of Hong Kong/Hong Kong West Cluster (UW 24-470), in accordance with the Helsinki Declaration as revised in 2024. The written informed consent was waived, as only de-identified archived blood samples were used.

Data sharing statement

The data that support the findings of this study are available on request from the corresponding author, Prof. Man-Fung Yuen, at mfyuen@hkucc.hku.hk.

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