ORIGINAL ARTICLE

Quantification of large and middle proteins of hepatitis B virus surface antigen (HBsAg) as a novel tool for the identification of inactive HBV carriers

Maria Pfefferkorn,[1] Stephan Böhm,[2] Tina Schott,[1] Danilo Deichsel,[1] Corinna M Bremer,[3] Kathrin Schröder,[3] Wolfram H Gerlich,[3] Dieter Glebe,[3] Thomas Berg,[1] Florian van Bömmel[1]

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[1]Section of Hepatology, Clinic for Gastroenterology and Rheumatology, University Hospital Leipzig, Leipzig, Germany
[2]Max von Pettenkofer—Institute for Hygiene and Clinical Microbiology, Ludwig-Maximilians-Universität, Munich, Germany
[3]National Reference Center for Hepatitis B and D Viruses, Institute for Medical Virology, German Centre for Infection Research (DZIF), Justus Liebig University Giessen, Giessen, Germany

Correspondence to
Dr. Florian van Bömmel, Section of Hepatology, Clinic for Gastroenterology and Rheumatology, University Hospital Leipzig AöR, Liebigstraße 20, 04103 Leipzig, Germany; florian.vanboemmel@medizin.uni-leipzig.de

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ABSTRACT

Objective Among individuals with chronic hepatitis B, those with hepatitis B e-antigen (HBeAg)-negative chronic hepatitis (CHB) can be difficult to distinguish from those with HBeAg-negative chronic HBV infection, also referred to as inactive HBV carriers (ICs), but both require different medical management. The level of HBV surface antigen (HBsAg) has been proposed as a marker to discriminate between chronic infection and hepatitis stages. HBsAg consists of large, middle and small HBs. The aim of this study was to determine whether the composition of HBsAg improved the identification of ICs among HBsAg-positive subjects with different phases of HBV infections.

Design HBV large surface proteins (LHBs) and HBV middle surface proteins (MHBs) were quantified in serum samples from 183 clinically well-characterised untreated patients with acute (n=14) HBV infection, ICs (n=44), CHBs (n=46), chronic HBeAg-positive phase (n=68) and hepatitis delta coinfection (n=11) using an ELISA, with well-defined monoclonal antibodies against the preS1 domain (LHBs) and the preS2-domain (MHBs). A Western blot analysis was used to verify the quantitation of the components of HBsAg. Total HBsAg was quantified using a modified commercially available assay (HBsAg V.6.0, Enzygnost, Siemens, Erlangen).

Results The composition of HBsAg showed specific patterns across different phases of hepatitis B. Individuals in the IC phase had significantly lower proportions of LHBs and MHBs than patients in acute or chronic phases irrespective of their HBV e-antigen status ($p<0.0001$) or HBsAg level. Both LHBs and MHBs ratios better predicted the IC phase than total HBsAg levels.

Conclusion Quantification of MHBs, particularly LHBs represents a novel tool for the identification of the IC stage.

Significance of this study

What is already known on this subject?

- Differentiating between patients being in a stable 'inactive carrier' state as compared with those with disease activity or risk of disease reactivation (the inactive HBV carrier (IC) and active phases of HBV infections) is often difficult in clinical practice.
- Serum levels of HBV surface antigen (HBsAg) can be used to identify individuals in the IC phase.
- The HBsAg of infectious virions and non-infectious subviral particles consists predominantly of HBV small surface proteins, with HBV large surface proteins (LHBs) and HBV middle surface proteins (MHBs) as minor components.

What are the new findings?

- The composition of HBsAg was distinct in the different phases of HBV infections.
- The ratio of the different components of HBsAg in a 'real-life' cohort was a better predictor of the IC phase than serum HBV DNA or total HBsAg levels.
- In hepatitis B e-antigen-negative individuals with HBsAg levels>1000 IU/mL, the percentages of LHBs and MHBs were significantly lower in individuals with IC than in patients with chronic hepatitis B ($p=9.12\times10^{-10}$ and $p=0.008136$, respectively).

How might it impact on clinical practice in the foreseeable future?

- Quantification of HBsAg components might be a novel tool for individualised long-term management of chronic HBV infections.

INTRODUCTION

After seroconversion of the HBV e antigen (HBeAg) to its antibody (anti-HBe), chronic HBV infections may subsequently exist as HBeAg-negative chronic infection, also referred to as inactive carrier (IC) phase, with minimal HBV replication and no inflammation or result in HBeAg-negative chronic hepatitis B (CHB), with persistent viral replication and hepatic inflammation. The clinical status of ICs and patients with CHB differs greatly, including their risk of developing end-stage liver disease and hepatocellular carcinoma (HCC). In two longitudinal studies of clinically well-characterised ICs followed for a median of 3.2 and 5.3 years, respectively, none of 263 patients in the first study or 85 in the second study developed cirrhosis or HCC.[1 2] Thus, in the IC phase, the risk of developing cirrhosis or HCC was similar to that of non-infected individuals. In contrast, in patients

with HBeAg-negative CHB, many studies reported an elevated risk of HCC, and the rate of progression to cirrhosis ranged from 3% to 10% per year.[3–8]

Current guidelines in the USA and Europe suggest the use of serum HBV DNA and alanine aminotransferase (ALT) levels to identify ICs.[9 10] ICs typically have HBV DNA levels <2000 IU/mL and normal ALT levels, whereas individuals with HBeAg-negative CHB have HBV DNA levels >2000 IU/mL, with fluctuations that can reach >20 000 IU/mL, accompanied by intermittent or persistent elevations in ALT levels. However, it is difficult to reliably identify ICs using HBV DNA levels because 10%–20% individuals seemingly in the IC phase may experience reactivation of HBV replication and 4%–20% may have one or more reversions back to HBeAg-positive CHB.[1]

Serum levels of HBV surface antigen (HBsAg), the protein antigen of the HBV envelope, can also be used to identify individuals in the IC phase. HBsAg, in addition to being located on virions, circulates in serum as non-infectious subviral particles (SVPs) in 1000–100 000-fold excess relative to the number of virions.[11] Several cross-sectional studies demonstrated significant differences in the HBsAg serum level of ICs and patients with active CHB, revealing its potential usefulness in discriminating between active and inactive disease.[12–18]

HBsAg consists of the three variably glycosilated proteins: large (L), middle (M) and small (S)HBs, which are encoded within one open reading frame in the HBV genome by the alternate use of three translational start codons. All three proteins contain the S domain. MHBs has a 55-amino acid long N-terminal extension, the preS2 domain, and HBV large surface protein (LHBs) has an additional 108 or 119-amino acid N-terminal extension, the preS1 domain. The HBsAg of infectious virions and SVPs consists predominantly of HBV small surface proteins (SHBs), with LHBs and MHBs as minor components.[19] In vitro studies showed that the preS1 region of LHBs is the high affinity attachment site to the HBV receptor NTCP (sodium dependent taurocholate cotransporting polypeptide) and essential for viral entry, whereas MHBs and major parts of preS2 are not necessary for HBV replication.[20–22] Studies also demonstrated that the proportion of MHBs and LHBs in SVPs was highly variable and that it seemed to depend on the stage of HBV infection.[23–25] However, the medical significance of this variability is not understood.

We optimised and validated a previously described relatively simple serological test to quantify the L and M components of HBsAg and assessed the association between the composition of HBsAg and the different phases of HBV infections in an attempt to find new markers for staging of HBV infections.[23]

MATERIAL AND METHODS

Patients and samples

We conducted a retrospective analysis of 416 individuals with HBV monoinfections not receiving antiviral treatment and 17 HBV and hepatitis delta (HDV) coinfected individuals referred to the outpatient clinics of the University Clinic of Leipzig and the Charité University Hospital, Berlin, Germany between 1998 and 2015. For inclusion in the study, a serum sample stored at −20° C taken at the start of the observation period had to be available. From patients with acute HBV infection, the first available serum sample was chosen. Further inclusion criteria were confirmation of active stages of HBV infection ≥6 months apart and the confirmation of the IC stage at least at three time points ≥12 months apart after the start of observation according to criteria proposed in recent guidelines.[9 10] The final study included 183 individuals, with a mean observation time of 38.5±23.9 (6–168) months. The patients were classified as follows: early acute phase, represented by the first available serum sample after admission to hospital (n=14), HBeAg-negative chronic infection or IC phase (n=44), HBeAg-negative chronic hepatitis (n=46), HBeAg-positive (n=68, including 38 with chronic infection and 30 with chronic hepatitis) phase or HBV/HDV coinfected (n=11) (table 1). To investigate changes after early acute infection, serial samples representing month 2 and 9 after admission for acute HBV infection were included according to availability (n=12). To assess the comparability of our assay with an established test for quantitative HBsAg detection, we used an independent confirmatory cohort, which consisted of 100 consecutive HBV-infected individuals, with a wide distribution of HBsAg levels (mean, 4.0±4.2, range −1.2–4.9 $\log_{10}$ IU/mL).

Standard laboratory assessments

Serum levels of HBV DNA were quantified using the COBAS AmpliPrep/COBAS TaqMan HBV kit (lower detection limit: 35 copies/mL; Roche Diagnostics, Mannheim, Germany). HBV genotypes were determined in samples containing >1000 copies/mL of HBV DNA by direct sequencing of the HBV polymerase gene and Blast analysis (HepSeq.org).

Assays for HBsAg quantification

Total HBsAg levels were quantified using the Enzygnost HBsAg 6.0 assay on the BEP III system (both Siemens Healthcare, Erlangen, Germany). As this assay gives very reproducible signals over a wide range of concentrations (Gerlich, unpublished results), independently of the HBV genotype,[26] HBsAg concentrations (total HBsAg) were obtained by calibration with the PEI-Standard (Paul-Ehrlich-Institut, Langen, Germany). In the confirmatory cohort, HBsAg levels were measured in parallel by the HBsAg Architect immune assay (Abbott Diagnostics, Germany).

Quantification of the LHBs and MHBs components of HBsAg

The components of HBsAg were detected using a semiautomatic in-house sandwich ELISA on a BEP III system using MA18/7 and Q19/10 antibodies and by Western blot (WB; for details see online supplementary file 1).

Statistical analysis

Statistical analysis was performed using SPSS V.24 (IBM). Depending on the normal distribution of the groups, independent t-tests, the Mann–Whitney U test or Pearson's correlation coefficient (r) were performed for comparison of means. A two-tailed p value of <0.05 was deemed statistically significant. Results beyond the defined lower limit of detection in the different assays for HBsAg components were set '0'. SHBs values in ng/mL were derived by subtracting LHBs and MHBs values from total HBsAg values. The predictive value of total HBsAg and ratios of LHBs and MHBs in the detection of individuals in the IC phase were summarised using receiver operating characteristic (ROC) curves. To generate the ROC curves, the data were 10-fold cross-validated using random samples. Those in the IC phase according to the ROC curves were further divided and analysed using a cut-off HBsAg level of 1000 IU/mL, which was recently reported to be associated with disease progression.[16]

RESULTS

Characterisation and verification of the ELISA for quantification of HBsAg components

For selective detection of MHBs or LHBs, we used a modification of the Enzygnost HBsAg V.6.0 assay which is licensed for

Table 1 Clinical and virological characteristics of the different patient groups

	(a) Acute phase	(b) IC phase	(c) HBeAg-negative CHB	(d) HBeAg-positive phase	(e) HBV/HDV coinfection	p Value						
						a vs b	a vs c	a vs d	a vs e	b vs c	b vs d	c vs d
Male/female	8/6	23/21	40/6	51/17	7/4							
Age*	44.7±18.9 (20–76)	47.7±14.7 (20–75)	48.3±15.3 (22–78)	41.9±15.2 (16–83)	39.0±12.6 (22–63)	0.457	0.433	0.730	0.501	0.957	0.041	0.031
HBV genotype†												
A (%)	8 (57.2)	16 (36.4)	9 (19.6)	19 (27.9)	0 (0)							
B (%)	0 (0)	4 (9.1)	2 (4.3)	5 (7.4)	0 (0)							
C (%)	0 (0)	0 (0)	2 (4.3)	5 (7.4)	0 (0)							
D (%)	4 (28.6)	14 (31.8)	28 (60.9)	26 (38.2)	5 (45.5)							
E (%)	1 (7.1)	0 (0)	3 (6.6)	3 (4.4)	0 (0)							
NA (%)	1 (7.1)	10 (22.7)	2 (4.3)	10 (14.7)	6 (54.5)							
ALT (ULN)* (n=145)	11.8±39.1 (7.4–120.3)	0.6±0.2 (0.2–1.13)	3.6±7.1 (0.3–35.6)	1.5±1.4 (0.2–8.1)	1.7±0.6 (0.7–2.35)	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	0.334
HBsAg (log_{10}IU/mL)*	3.4±1.2 (0.4–5.3)	3.0±1.3 (0.2–4.4)	3.6±0.6 (2.2–4.7)	4.1±0.7 (2.4–5.5)	4.2±0.6 (2.6–4.8)	0.073	0.507	0.052	0.647	0.051	<0.001	<0.001
HBV DNA (log_{10} cp/mL)*	7.0 (3.3–7.9)	3.3 (1.1–3.9; three nd)	7.8 (2.5–8.9)	9.0 (2.9–10.5)	2.9 (2.1–3.5; three nd)	<0.001	0.024	<0.001	<0.001	<0.001	<0.001	0.004
Observation time (months)*	12.5±17.4 (6–60)	33.2±21.9 (24–72)	45.0±23.0 (6–79)	42.2±23.4 (6–84)	35.2±22.2 (15–66)							

*Mean±SD (range).
†nd.
ALT, alanine aminotransferase; HBeAg, hepatitis B e-antigen; HBsAg, HBV surface antigen; HDV, hepatitis delta; IC, inactive HBV carrier; nd, undetectable.

Figure 1 Validation and correlation data of quantification of total HBsAg. (A) Correlation scatter plot showing HBsAg quantification by the Abbott Architect and Siemens Healthcare BEP III system, respectively, on the basis of the PEI reference sample for HBsAg or (B) on the basis of the ID1 reference plasma from the National Reference Centre for HBV and HDV. Pearson's correlation coefficient (r) and p values are provided. HBsAg, HBsAg, HBV surface antigen; HDV, hepatitis delta; PEI, Paul-Ehrlich-Institut.

qualitative HBsAg detection. We used either the original microplates coated with anti-SHBs antibody or microplates coated in-house with MAb Q19/10 against MHBs or MA18/7 against LHBs. The Enzygnost assay showed an accurate linear correlation between the HBsAg concentration and the signal to cut-off ratio. Thus, after calibration with HBsAg reference samples, the assay was used for quantitation of total HBsAg and in modified form of MHBs and LHBs. The validity of the calibration was determined by comparison of the results of the Enzygnost assay with those obtained using the well-established quantitative HBsAg Architect test. Using the cell culture-derived PEI HBsAg standard with 1000 IU/mL for calibration, there was a strong correlation between the HBsAg quantification results of both assays (r=0.809, p<0.0001; figure 1A). The correlation between the quantitative results of the Enzygnost and Architect assays was even better when a well-characterised internal reference plasma, ID1, with a biochemically determined amount of HBsAg protein was used for calibration (r=0.930; p=0.0001; figure 1B).[27]

To determine the intra-assay and interassay precision of the quantification of LHBs, MHBs and total HBsAg in nanogram/millilitre, four serum samples with different concentrations of HBsAg (ng/mL) were tested using threefold replicates in three runs (see online supplementary tables 1 and 2). The coefficient of variation in the midrange of quantitation was between 5.7% and 14.4%, whereas it was 21.3% in the lower end of quantification for MHBs.

Using the results of 20 independent runs of the ID1 reference plasma, the limit of detection for the MHBs assay was 0.47 ng/mL, 0.03 ng/mL for the LHBs assay and 0.08 ng/mL for total HBsAg, respectively. To confirm the ratio of HBsAg components derived by the ELISA, all the samples were analysed by WB. Owing to sensitivity, HBsAg components could be semiquantified only in 89 of 183 samples. The distribution of the HBsAg components in the WB is shown for representative samples of each phase of HBV infection in figure 2A. In addition to HB1, which detects all HBsAg components, MAb MA18/7 was used to detect the amount of LHBs in each sample (figure 2A, upper blot). HBeAg-positive and HBeAg-negative CHB, as well as HBV/HDV coinfection, showed bands at the expected apparent molecular weights of approximately 39 kDa and 42 kDa, which

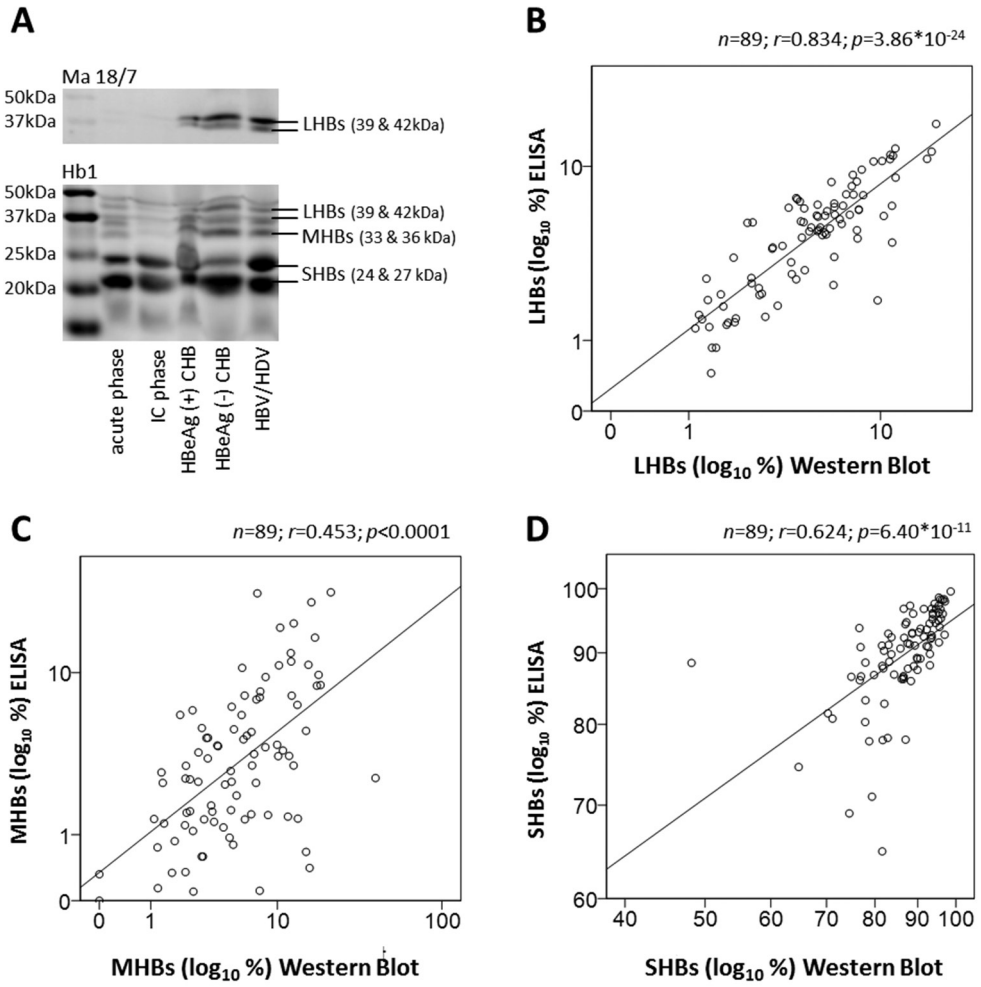

Figure 2 Validation and correlation data of the ELISA and WB (n=89). The lanes show exemplary WBs for one sample from each phase of HBV infection (A). The upper blot shows staining of LHBs (39 and 42 kDa) with MA18/7, and the lower blot depicts staining with HB1. Scatter plots for 89 samples from various phases of HBV infection show the correlation of WB and ELISA results for LHBs (B), MHBs (C) and SHBs (D). (-), negative; (+), positive; CHB, chronic hepatitis B; HBeAg, hepatitis B e-antigen; HDV, hepatitis delta; IC, inactive HBV carrier; LHB, HBV large surface protein; MHB, HBV middle surface protein; SHB, HBV small surface protein; WB, Western blot.

have been reported for P39 and GP42 of LHBs, respectively.[19] The LHBs quantities determined by the ELISA and WB were highly correlated (r=0.834; $p=3.86\times10^{-24}$). The ELISA and WB results showed a weaker correlation for MHBs (r=0.453; p<0.0001) and a moderate correlation for SHBs (r=0.624; $p=6.40\times10^{-11}$; figure 2B–D). From the IC group, 23 of 44 samples could be analysed by WB showing a slightly weaker correlation of LHBs and MHBs with ELISA results as the overall cohort (r=0.522 and r=0.304 vs r=0.834 and r=0.453, respectively).

HBsAg levels in different phases of HBV infections

The mean total HBsAg levels of individuals in the IC phase (mean, 3.0±1.3 (0.2–4.4) $\log_{10}$ IU/mL) and those with HBeAg-negative CHB (mean, 3.6±0.6 (2.2–4.7) $\log_{10}$ IU/mL; p=0.051) and acute HBV infection (mean, 3.4±1.2 (0.4–5.3) $\log_{10}$ IU/mL, p=0.073) were similar. The HBsAg levels were lower in ICs than in individuals in HBeAg-positive phases (mean, 4.1±0.7 $\log_{10}$ IU/mL, $p=1.52\times10^{-7}$) or in those with HBV/HDV coinfection (mean, 4.0±0.6 $\log_{10}$ IU/mL, p<0.0001) (figure 3B). HBeAg-positive individuals had higher HBsAg levels compared with those with HBeAg-negative CHB (p<0.001, table 1).

HBsAg composition in different phases of HBV infections

Individuals in the IC phase had significantly lower quantities of both LHBs and MHBs as compared with those of the other groups (p<0.0001; table 2). In addition, the mean ratios of LHBs (2.3%) and MHBs (1.8%) were significantly lower in individuals in the IC phase as compared with those of the other groups (p<0.0001, figure 3C–D), whereas the mean ratio of SHBs was significantly higher in the ICs (p<0.0001; table 2). The HBV/HDV coinfected individuals showed the highest ratios of LHBs and MHBs as compared with the other groups (p<0.0001; table 2).

HBsAg composition in individuals with low total HBsAg

To further investigate the possible influence of total HBsAg levels on the observed differences in HBsAg composition between individuals in the IC and HBeAg-negative CHB phases, we compared HBsAg components in the individuals in the IC phase with total HBsAg levels >1000 IU/mL (n=26) with those of individuals with HBeAg-negative CHB (n=46). The mean total HBsAg levels were identical in both cohorts (3.8±0.4 (range, 3.0–4.5) and 3.8±0.8 (range, 2.1–4.8) $\log_{10}$ ng/mL, p=0.1295; figure 4B). In contrast, the mean ratios of LHBs and MHBs in the

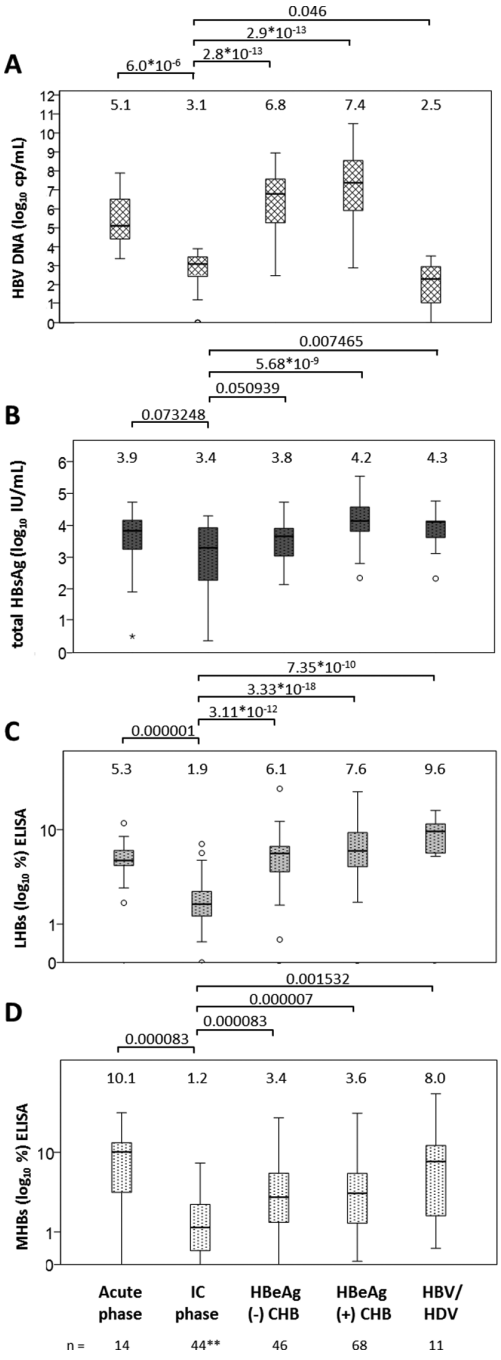

Figure 3 Box plots showing levels of HBV DNA (A), total HBsAg (B), proportion of LHBs (C) and MHBs (D) in different phases of hepatitis B. Median values are given above the plots. pValues derived by a comparison of two groups are indicated above the graphic. Circles and asterisks indicate outliers and extreme values, respectively. Numbers of the patients in the different groups of infection are indicated below the graphic. **=41/44 individuals in the IC phase had detectable HBV DNA (D). (-), negative; (+), positive; CHB, chronic hepatitis B; HBeAg, hepatitis B e-antigen; HDV, hepatitis delta; IC, inactive HBV carrier; LHB, HBV large surface protein; MHB, HBV middle surface protein; SHB, HBV small surface protein.

individuals in the IC phase were significantly lower than those of the individuals with HBeAg-negative CHB (1.8±0.8 (range, 0.9–4.3%) vs 5.7±3.3 (range, 0.5–21.5%), p=8.16–10^{-13} and 2.0±1.5 (range, 0.3–6.1%) vs 4.7±4.3 (range, 0.1–22.0%), p=0.0045, respectively; figure 4C-D). Mean HBV DNA levels were significantly lower in IC with total HBsAg levels >1000 IU/

Table 2 Mean quantities and ratios of L, M and SHBs in the different patient groups and comparison of IC to other disease stages

						p Value			
	(a) ICs	(b) Acute infections	(c) HBeAg-negative CHB	(d) HBeAg-positive phase	(e) HBV/HDV coinfection	(a) vs (b)	(a) vs (c)	(a) vs (d)	(a) vs (e)
LHBs (log_{10} ng/mL)*	1.9±0.5 (−1.9–2.92; 1 nd)	2.6±0.8 (−0.9.4–3.5)	2.5±0.6 (0.7–3.6)	3.1±0.6 (1.4–4.3)	2.9±0.7 (1.7–3.8)	0.0006	3.2×10^{-7}	4.0×10^{-8}	4.0×10^{-5}
MHBs (log_{10} ng/mL)*	1.8±0.6 (0.8–2.9; 10 nd)	2.6±1.3 (−0.9–3.9; one nd)	2.1±0.8 (0.1–3.5; 1nd)	2.6±0.8 (0.8–4.4)	2.7±0.9 (2.1–4.9)	0.0003	0.0003	1.8×10^{-10}	3.0×10^{-4}
SHBs (log_{10} ng/mL)*	3.1±1.1 (0.5–4.5)	3.5±1.3 (0.3–4.9)	3.6±0.5 (2.1–4.8)	4.1±0.7 (2.3–5.6)	3.9±0.7 (2.5–4.8)	0.1999	0.0777	2.8×10^{-8}	0.0111
total HBsAg (log_{10} ng/mL)*	3.1±1.1 (0.5–4.5)	3.6±1.2 (0.3–4.9)	3.7±0.6 (2.1–4.8)	4.2±0.7 (2.4–5.7)	4.0±0.6 (2.6–4.8)	0.1084	0.4809	7.6×10^{-9}	0.0051
LHBs (%)*	2.3±1.6 (0.0–7.5)	5.7±2.4 (1.9–11.3)	6.0±3.3 (0.5–22.0)	7.6±4.0 (2.0–20.8)	9.2±3.0 (5.8–14.5)	1.0×10^{-5}	3.1×10^{-12}	3.3×10^{-18}	7.3×10^{-10}
MHBs (%)*	1.8±1.9 (0.0–7.7)	9.1±6.8 (0.0–24.6)	4.4±4.3 (0.0–22.0)	4.3±4.0 (0.1–24.2)	9.9±10.8 (0.4–37.3)	8.3×10^{-4}	8.3×10^{-4}	7.0×10^{-5}	0.0015
SHBs (%)*	95.9–2.6 (89.6–100.0)	85.2±7.8 (69.1–98.0)	89.6±5.8 (65.7–99.0)	88.1±6.7 (64.9–97.3)	80.9±12.9 48.1–93.7	4.1×10^{-7}	4.1×10^{-11}	7.3×10^{-15}	8.3×10^{-9}

*Mean±SD (range).
CHB, chronic hepatitis B; HBeAg, hepatitis B e-antigen; HBsAg, HBV surface antigen; IC, inactive HBV carrier; LHB, HBV large surface proteins; MHB, HBV middle surface proteins; SHB, HBV small surface protein.

mL compared with those with HBeAg-negative CHB (3.3±0.5 (range, 2.3–3.9) vs 6.5±1.3 (range, 2.5–8.9) $\log_{10}$ cp/mL, p=4.8*10^{-17}; figure 4A).

Prediction of the IC phase by HBsAg components

As shown by the ROC curve analysis, the proportion of HBsAg components better predicted the patient's classification status (ie, IC or HBeAg-negative CHB phase) than total HBsAg levels (figure 5). Thus, the ratio of LHBs was a strong predictor of the IC phase (area under the receiver operating characteristic (AUROC)=0.89; p=2.18×10^{-9}), followed by the ratio of MHBs (AUROC=0.73; p=0.00114) and total HBsAg (AUROC=0.62; p=0.04322). HBV DNA levels were also a strong predictor of IC phase (AUROC=0.98; p=2.8*10^{-13}).

HBsAg composition in acute hepatitis B

The total HBsAg and HBV DNA levels of individuals with early acute hepatitis B were similar to those in HBeAg-positive phases or HBeAg-negative CHB (p=0.9302 and p=0.3429, respectively). LHBs and SHBs quantities were also similar in acute and chronic stages (p=0.712; p=0.938). However, the proportion of MHBs was highest in the individuals with early acute hepatitis B as compared with that of all the individuals with chronic HBV stages (p=0.0038; see online supplementary figure 1E). Besides ALT levels (AUROC=0.99, p=6.39*10^{-8}), the ratio of MHBs served as the best marker for distinction of acute and chronic stages (AUROC=0.84; p=0.0038), followed by the ratios of LHBs (AUROC=0.58; p=0.6679), total HBsAg (AUROC=0.51; p=0.9760) and HBV DNA (AUROC=0.46; p=0.6496; see online supplementary figure 1F). At month two after admission, there was a significant decrease of LHBs and MHBs ratios (see online supplementary figure 2).

HBsAg composition in chronic HBV/HDV coinfection

The mean levels of total HBsAg of the individuals with chronic HBV/HDV coinfection were similar to those of individuals with HBeAg-positive stages or HBeAg-negative CHB (mean 4.2 vs 4.1 $\log_{10}$ ng/mL, p=0.888; figure 3B, table 1). However, the mean ratio of LHBs was higher in individuals with HBV/HDV coinfection (9.2 vs 6.7%, p=0.0206). The ratio of MHBs was also higher for the HDV carriers but the difference was not significant due to the larger SD of the mean values (9.9% vs 4.2%, p=0.0685).

HBV DNA levels and HBsAg composition

Independent of the phase of infection the quantity of HBV DNA showed a weak correlation with total HBsAg levels (r=0.267, p=0.0002), the ratio of LHBs (r=0.017, p=0.821) or of MHBs (r=0.017, p=0.824). The correlation of HBV DNA levels and

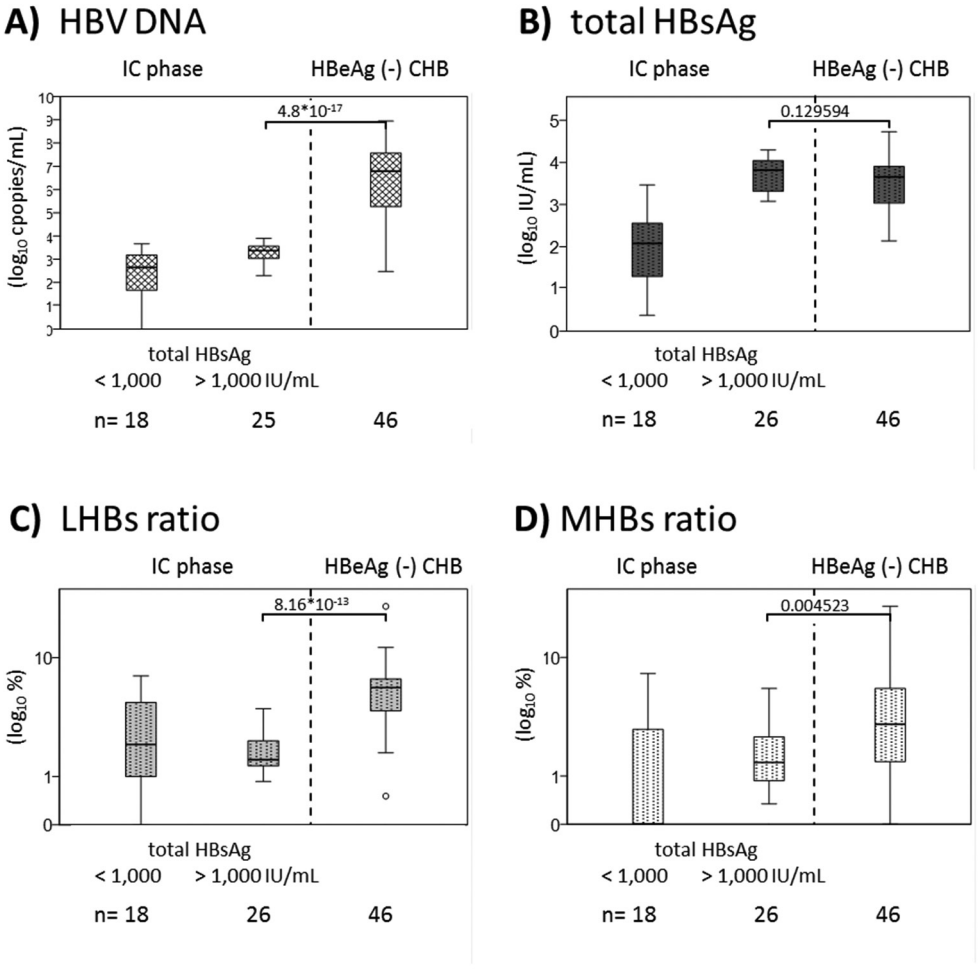

Figure 4 Box plots showing HBV DNA (A), total HBsAg levels (B) and HBsAg components LHBs (C) or MHBs (D) in individuals in the IC phase with HBsAg levels below or above 1000 IU/mL and in individuals with HBeAg-negative CHB. Numbers of individuals in different groups are indicated above the box plots. (-), negative; (+), positive; CHB, chronic hepatitis B; HBeAg, hepatitis B e-antigen; IC, inactive HBV carrier; LHB, HBV large surface protein; MHB, HBV middle surface protein.

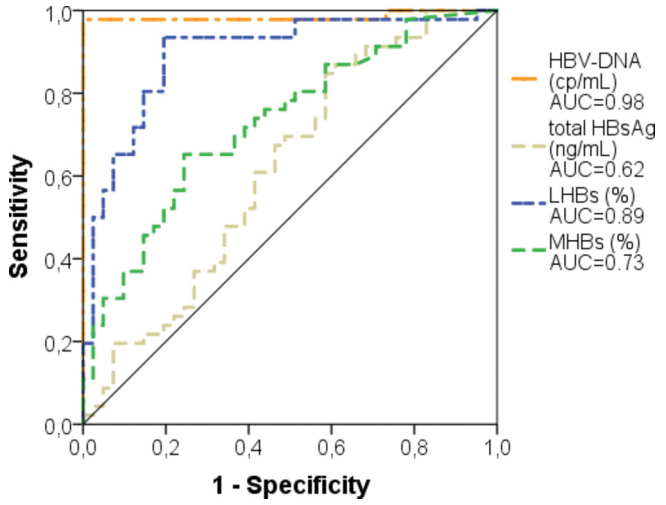

Figure 5 ROC curves showing the ability of a linear model to differentiate individuals in the IC or HBeAg-negative CHB phase by HBV DNA (orange line), ratios of LHBs (blue line), ratios of MHBs (green line) and quantities of total HBsAg (yellow line). AUC, area under the curve; CHB, chronic hepatitis B; IC, inactive HBV carrier; HBeAg, hepatitis B e-antigen; HBsAg, HBV surface antigen; LHB, HBV large surface proteins; MHB, HBV middle surface protein; ROC, receiver operating characteristic.

total HBsAg or HBsAg components in different stages of HBV infections was similar (see online supplementary figure 3A–C).

HBV genotypes and HBsAg composition

The distribution of HBV genotype (GT) in the present study showed that HBV GT A and D were most frequent in all groups of HBV infection (table 1). Interestingly, in individuals of the CHB groups, the ratios of MHBs were 2.4±1.7 (range, 0.1–6.7)% vs 3.1±2.2 (range, 0.2–7.7)% in GT A and D, respectively (p=0.004). The ratios of LHBs were 5.4±2.4 (range, 1.8–12.4)% vs 7.1±3.5 (range, 0.5–20.8)% in GT A and D, respectively (p=0.010).

DISCUSSION

The aim of the present study was to investigate the association of the composition of serum HBsAg with the stage of HBV infections. To this end, we first established and validated an ELISA with monoclonal antibodies against the different components of HBsAg and then used it to quantify the HBsAg components LHBs and MHBs in serum samples from clinically well-characterised patients in different phases of HBV infections. Variability in the protein composition of the HBsAg protein was first reported in 1980, and early studies suggested that the composition might be correlated with specific phases of HBV infection.[23–25 28–30] In the present study, we demonstrated for the first time that the composition of HBsAg was distinct in different clinically well-defined phases of HBV infections. We also showed that the ratios of LHBs and MHBs were better predictors of ICs and HBeAg-negative CHB than total HBsAg levels.

The ELISA used to detect the HBsAg components (LHBs and MHBs) employs monoclonal antibodies. These have selective specificity for LHBs and MHBs and have been used successfully in experimental and clinical studies.[25 31 32] For reasons of practicability, we calibrated the quantitation of SHBs, MHBs and LHBs in nanogram/millilitre of HBsAg protein of a typical reference sample from a highly viraemic, HBeAg-positive HBV carrier. We are aware that the ELISA results do not exactly represent the true nanogram amount of LHBs or MHBs in the sample because the final quantitation is based on the amount of labelled anti-SHBs bound to the SVPs in the microplate. Although WBs are not hampered by this shortcoming, they are more laborious, less sensitive and less accurate overall in terms of interassay reproducibility. However, the comparison of the WB findings with those obtained using the ELISA confirmed that the LHBs and MHBs in the ELISA reacted preferably with samples containing these components, thus generating meaningful results. The agreement between both methods was particularly good for LHBs. The relatively weak correlation between the WB and ELISA for MHBs is not surprising because the preS2 sequence 3–17 is known to bind to so-called polymerised human serum albumin.[33] Although the latter is an artificial substance, a natural form of monomeric modified human serum albumin present in very low amounts also binds to preS2 in vivo and can block the binding of MAb Q19/10 if the preS2 level is not too high.[34] In the WB, denaturation of the HBsAg proteins dissociated the albumin from the preS2 epitope detected by MAb Q19/10. With regard to SHBs, the blocking of the SHBs epitope in vivo by anti-HBs antibodies and release by the denaturation required for WB may also partly explain the suboptimal correlation of the WB and ELISA results. As reported previously, some immune complexes are found in virtually all HBsAg samples from HBV carriers.[35]

Differentiating between patients being in a stable inactive 'carrier' state as compared with those with disease activity or risk of disease reactivation (the IC and active phases of HBV infections) is of paramount importance in clinical practice. In a recent landmark study, Brunetto *et al* demonstrated that in individuals with HBV genotype D, a combined cut-off of ≤2000 IU/mL for HBV DNA levels and <1000 IU/mL for HBsAg levels could identify the IC state, with 91.1% sensitivity and 95.4% specificity.[16] Liu *et al* used the same cut-offs in a large cohort of Asian patients with HBV genotypes B and C and identified ICs, with a sensitivity and specificity of 71% and 85%, respectively.[36] In the present study, the absence of reactivations in our IC cohort could be confirmed during a mean period of 33.2±21.9 (range, 24–72) months. There was also a strong association between HBsAg levels and the HBV infection phase (figure 3B), but there were greater differences in the ratios of LHBs and MHBs than total HBsAg levels across disease stages (figure 3C-D). In particular, low LHBs ratios were a stronger predictor of the IC phase than low total HBsAg levels (figure 5). Furthermore, in ICs with HBsAg levels >1000 IU/mL, in which mean HBsAg levels were similar to those of HBeAg-negative CHB individuals, the percentages of LHBs and MHBs showed a significant association with IC status ($p=9.12\times10^{-10}$ and p=0.008136, respectively; figure 4). Therefore, we suggest that the ratios of LHBs and MHBs may be more useful markers than total HBsAg levels for differentiating between inactive and active forms of CHB.

The reasons for the differences in HBsAg composition in the different phases of HBV infections remain unclear. LHBs is essential for attachment and entry of HBV to its target cell, and a high level of LHBs, as observed in the active stages of HBV infections in our cohort, will allow more effective infection of new hepatocytes, thus maintaining chronicity.[25 31 32] LHBs is more abundant on virions and filamentous SVPs than on spherical SVPs.[19] Thus, a decline in the ratio of virions versus spherical SVPs could contribute to the observed relative decrease of LHBs in low viraemic phases of infection (ie, the IC phase) but the effect is probably very small because even in highly viraemic patients, the amount of small SVP exceeds that of virions by at least the factor 1:1000. Another reason may be that both humoral and cellular immune reactions counter-select against preS1-containing cells

and particles.[37 38] LHBs has its own messenger RNA, different from that encoding MHBs and/or SHBs, allowing independent translation. Furthermore, the two smaller HBs proteins may be expressed from integrated HBV DNA lacking the start codon for LHBs.[39 40] The biological role of MHBs, which is conserved in all orthohepadnaviruses, is unknown, but its N- and O-glycosylation and binding of host serum albumin in the case of HBV may modulate immune responses.[34 41] In phases of immune elimination, there may be counter-selection against this non-essential component, as suggested by the existence of MHBs-negative or preS2 deletion mutants in severe CHB.[42 43]

Interestingly, we found significantly higher ratios of MHBs in individuals in the early phase of acute hepatitis B as compared with those in chronic phases, which potentially provides support for the hypothesis of an immunomodulatory role of MHBs in the early phases of HBV infection, with this role rendered useless once a strong immune response is initiated (see online supplementary figure 1). In addition, the number of integrated HBV DNA fragments expressing only SHBs is probably much lower in the acute phase than during chronic HBV infection. All patients cleared HBsAg at month 9 of observation, and indeed, already at month 2, a strong decrease of the ratios of L and MHBs was detectable (see online supplementary figure 2). On a cautionary note, the samples available for this small cohort likely had very heterogeneous delays after infection, which might have influences on our observation.

The present study also included a small cohort of patients with HBV/HDV coinfection. Although the mean HBsAg levels of these individuals were similar to those with acute or HBeAg-positive CHB, the ratios of LHBs were highest in individuals with HDV/HBV coinfection (figure 4). HDV may upregulate the expression of LHBs, as it requires preS1 for its entry into hepatocytes.[22] Our observation of high LHBs ratios in HDV-infected individuals is consistent with the strong infectivity and prevalence of HDV, even in patients with comparatively low HBV replication.

In conclusion, we showed that the composition of HBsAg was distinct in the different phases of hepatitis B and that the ratio of the different components of HBsAg in a 'real-life' cohort was a better predictor of the IC phase than serum HBV DNA or total HBsAg levels. The origin of the differences in the composition of HBsAg during HBV infections and the role of these differences in the natural course of HBV infections need to be investigated, and the clinical value of the proposed marker, for example for treatment start or stopping rules, needs to be validated prospectively in larger cohorts.

Correction notice This article has been corrected since it published Online First. Figure 1 has been updated.

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Contributors MP designed the study, collected data, established and validated the ELISA, measured samples, analysed results and drafted the manuscript. SB contributed to establishing the ELISA. TS contributed to measuring HBsAg components. DD quantified HBV DNA and determined HBV genotypes. CMB and KS developed the WB for the HBsAg components and measured samples. TS, CMB, DD, KS, WHG, DG and TB critically revised the manuscript. FvB initiated, designed and supervised the study and drafted the manuscript. All authors have approved the final version for publication.

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