HBsAg protein composition and clinical outcomes in chronic hepatitis D and variations across HBeAg-negative chronic HBsAg carriers

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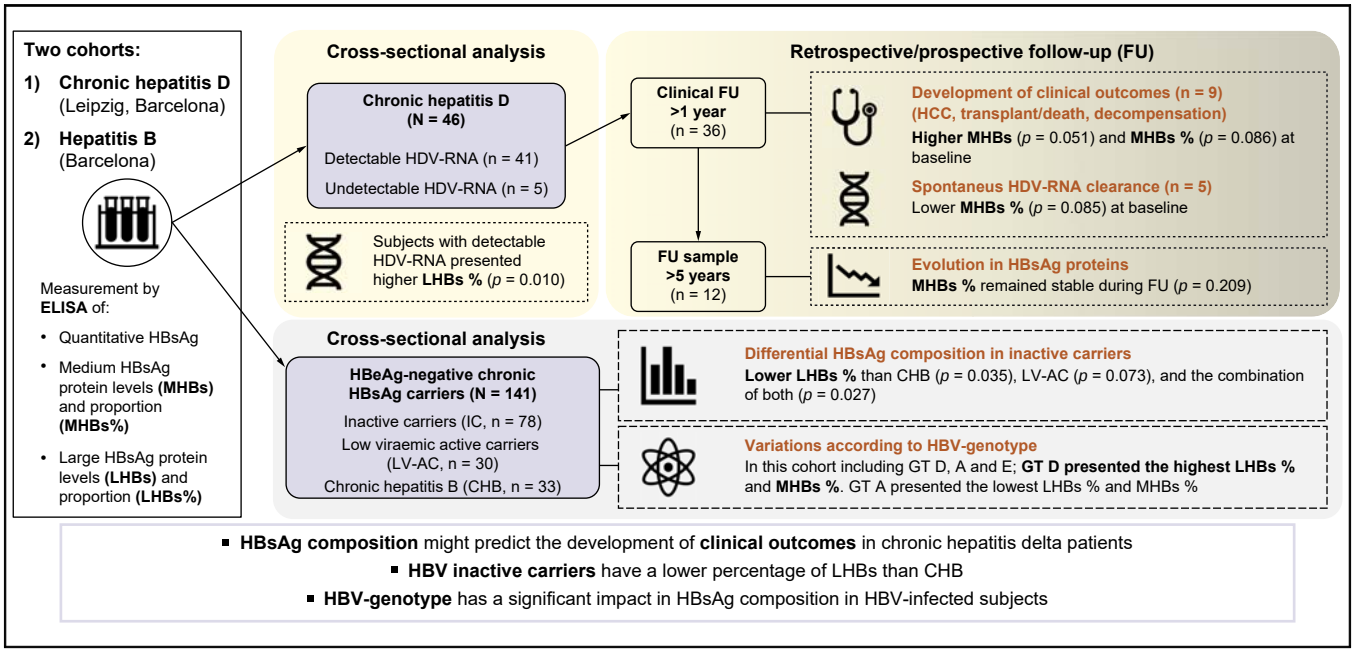
Graphical abstract



Highlights

- Chronic hepatitis D with detectable HDV-RNA showed higher HBsAg and LHBs% than did that with undetectable viraemia.
- In chronic hepatitis D, a trend toward higher baseline MHBs% was observed in patients who developed clinical outcomes.
- A different HBsAg composition, with lower LHBs%, has been validated for HBV HBeAg-negative inactive carriers.
- HBV genotype has shown a significant impact in HBsAg composition in HBV-infected patients.

Impact and implications

The composition of HBsAg in chronic hepatitis D differs in patients with detectable and undetectable HDV viral load and may help predict the likelihood of achieving undetectable HDV viraemia and the development of clinical events such as decompensation. The composition of the surface antigen is also useful to distinguish inactive carriers of HBV, and it varies according to HBV genotype.

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Research article

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HBsAg protein composition and clinical outcomes in chronic hepatitis D and variations across HBeAg-negative chronic HBsAg carriers

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Background & Aims: HBsAg proteins are useful to identify HBV inactive carriers (ICs), but data on chronic hepatitis D (CHD) are scarce. This study aimed to describe HBsAg composition in CHD, its changes during the evolution, and the potential association with clinical outcomes. In addition, we assess the composition of HBsAg across different HBV genotypes and validate previous results on HBsAg proteins in an independent HBV cohort.

Methods: Quantitative HBsAg, medium HBsAg proteins (MHBs), and large HBsAg proteins (LHBs) were measured in two cohorts. The first cohort consisted of patients with CHD. A cross-sectional study of samples from two European institutions (N = 46) was conducted. Outcomes were assessed in a retrospective–prospective study of those patients with a follow-up of >1 year (n = 36), and the longitudinal evolution of HBsAg proteins in those with samples >5 years apart (n = 12) was analysed. The second cohort consisted of patients with HBeAg-negative HBV, and a cross-sectional study was performed (N = 141).

Results: Forty-one (89%) patients with CHD had detectable HDV-RNA, and the presence of HDV-RNA was associated with higher LHBs proportion (p = 0.010). Baseline MHBs (p = 0.051) and MHBs proportion (p = 0.086) tended to be higher in those developing clinical outcomes (9/36, 25%) after a median follow-up of 5.9 years. Patients in which HDV-RNA became spontaneously undetectable during follow-up (5/31, 16.1%) tended to present lower MHBs proportion (p = 0.085). In the longitudinal study, changes in LHBs proportion were observed (p = 0.041), whereas MHBs proportion remained stable (p = 0.209). Regarding HBV, ICs showed lower LHBs proportion (p = 0.027). LHBs and MHBs differed significantly according to HBV genotype, regardless of the HBV phase.

Conclusions: Patients with CHD with detectable HDV-RNA presented higher LHBs proportion than those with undetectable HDV-RNA. A trend toward having higher baseline MHBs proportion was observed in patients who developed clinical outcomes or remained with detectable HDV-RNA. This study validates the different HBsAg composition in HBV ICs and reveals the HBV-genotype influence in HBsAg composition.

Impact and implications: The composition of HBsAg in chronic hepatitis D differs in patients with detectable and undetectable HDV viral load and may help predict the likelihood of achieving undetectable HDV viraemia and the development of clinical events such as decompensation. The composition of the surface antigen is also useful to distinguish inactive carriers of HBV, and it varies according to HBV genotype.

Introduction

HDV is an RNA virus that requires HBsAg to form its envelope and complete the viral particle.[1] Approximately 5% of HBV carriers are living with chronic hepatitis D (CHD), accounting for 12 million people worldwide.[2] Currently, CHD represents a severe form of liver disease with a rapid progression to cirrhosis and a high risk of hepatocellular carcinoma (HCC) development.[3,4] Similar to chronic hepatitis B (CHB), CHD entails an excess of HBsAg as a result of the presence of noninfectious subviral

Keywords: Hepatitis D; Hepatitis B; HBV; Surface antigen; HBsAg proteins; Genotype; Inactive carrier; HDV.

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particles.[5] HBsAg is formed by three glycosylated proteins (small, medium, and large surface proteins [SHBs, MHBs, and LHBs, respectively]), which share a common S domain and their detection and measurement have been recently optimised in a nationwide German study.[6,7] Differential roles of these proteins in viral replication and immunomodulation have been proposed in HBV infection.[6] HBsAg components have also been suggested as a potential tool to differentiate stages of HBV infection, as well as to predict HBsAg loss in HBeAg-positive individuals during different treatments for CHB.[8] However, there are very limited data on HBsAg components in patients with CHD, and their potential role in the natural history and clinical outcomes has not been explored.[7]

The aim of this study was first to describe the HBsAg composition in a cohort of patients with CHD, the dynamics during the natural history, and its potential association with clinical outcomes. Second, we aimed to assess the potential impact of HBV genotype (GT) in HBsAg protein composition and to confirm the differential composition pattern of HBsAg in HBV inactive carriers (ICs) in an independent cohort of well-characterised HBeAg-negative patients.

Patients and methods

CHD cohort

A cross-sectional study of samples from two European academic hospitals in Barcelona (Spain) and Leipzig (Leipzig University Medical Center, Germany) was carried out (n = 46) to describe the composition of HBsAg proteins. Patients with a minimum follow-up of 1 year (n = 36) were included in a retrospective–prospective longitudinal study to assess the potential impact of HBsAg protein composition in the development of clinical events. Moreover, changes in HBsAg protein compositions were explored in those with samples >5 years apart (n = 12). The flow chart of the study is shown in Fig. 1.

CHD was defined by the presence of HBsAg and anti-HDV antibodies for more than 6 months. Patients with undetectable HDV-RNA were included if evidence of previous detectable HDV-RNA was available.

Patients were excluded if they were coinfected with HIV and/or HCV, if they had HCC, or if they received interferon (IFN) in the last 12 months before and during the study. Patients who received treatment with IFN during follow-up were excluded from the longitudinal study.

Hepatitis B cohort

In patients with HBV (n = 141), a cross-sectional analysis was carried out in patients with HBeAg-negative chronic HBV infection or hepatitis from the Spanish institution to validate the role of LHBs and MHBs for identification of HBV ICs.

CHB infection was defined by the presence of detectable HBsAg for more than 6 months. Patients with HBV with prior history of antiviral treatment were excluded. HBeAg-negative patients were categorised in a similar way to those in the previous study for a better comparison of the results.[7] These patients belonged to either the group with HBeAg-negative chronic infection or ICs (those with HBV-DNA <2,000 IU/ml and persistently normal alanine aminotransferase [ALT] and normal liver ultrasound) or the group with HBeAg-negative CHB (those with HBV-DNA >2,000 IU/ml and elevated ALT and/or significant fibrosis at liver biopsy). HBeAg-negative patients who did not meet these criteria were included in a group named low viraemic active carriers (LV-AC) (those with HBV-DNA between 2,000 and

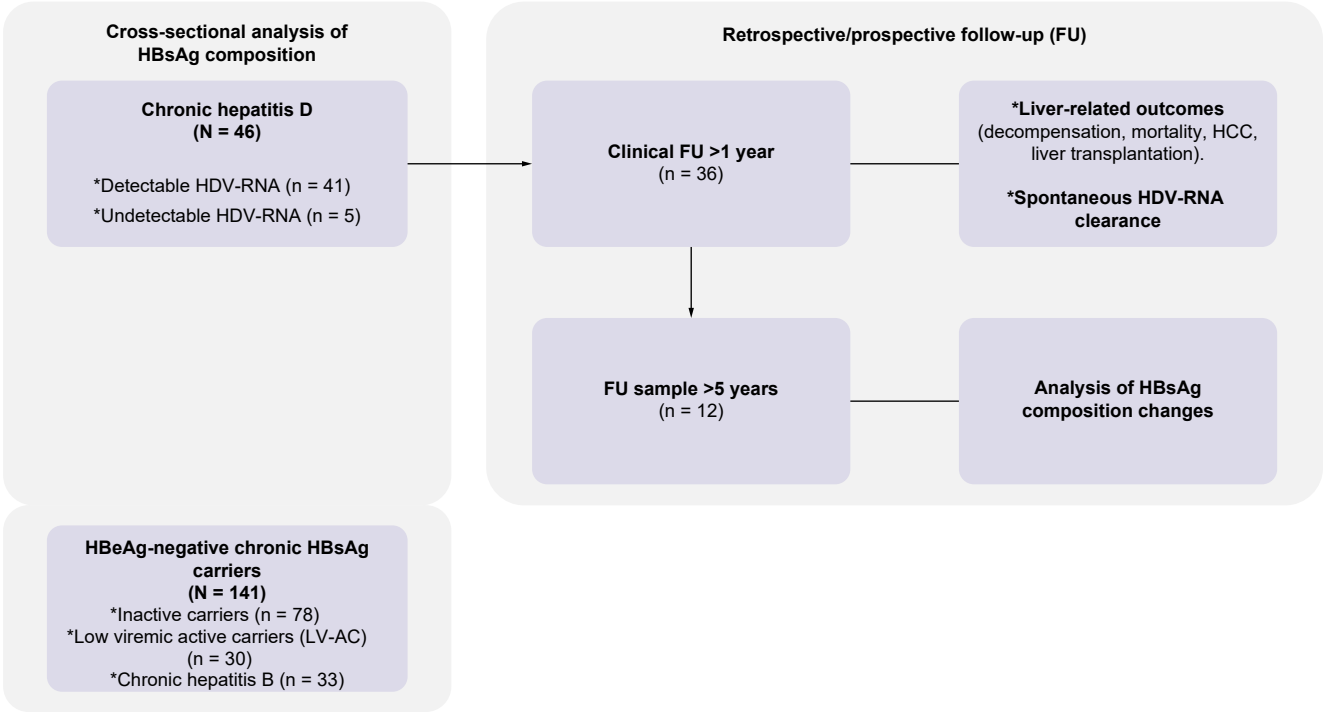

Fig. 1. Flow chart summarising study design. FU, follow-up; HCC, hepatocellular carcinoma.

20,000 IU/ml and persistently normal ALT in the absence of significant fibrosis in liver biopsy).[9]

This study was approved by the Vall d'Hebron Hospital (PR(AG) 247/2018) and the Leipzig University (AZ 112/18-ek) ethics committee, and it was conducted in compliance with the principles of the Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements. Informed consent forms were provided to all included participants, and all data were anonymised.

Clinical and demographic variables

Demographic and clinical features were recorded in all patients. Demographic information included sex, age, and ethnicity. Clinical information and accumulated history of antiviral treatment were collected retrospectively through medical records. The presence of liver cirrhosis was defined according to imaging (signs of portal hypertension and/or abnormal liver) or transient elastography (liver stiffness measurement above 13 kPa), liver biopsy (Ishak fibrosis score of 5–6), or clinical data (previous history of decompensation). Longitudinal follow-up included clinical data regarding liver-related decompensation (ascites, liver encephalopathy, and variceal bleeding), HCC, liver transplantation, and all-cause mortality. All these clinical events were analysed as a combined variable owing to the limited number of patients.

Laboratory methods

Laboratory parameters included platelet count, biochemical panel with liver enzymes, and serological and virological tests. The ALT upper limit of normality was established following the reference laboratory thresholds (35 IU/ml for women and 40 IU/ml for men). HBV serological markers (HBsAg and HBeAg) were tested using a commercial electrochemiluminescence immunoassay (COBAS 8000, Roche Diagnostics, Rotkreuz, Switzerland). Anti-HDV antibodies were determined using an HDV Ab kit (Dia.Pro Diagnostic Bioprobes, Sesto San Giovanni, Italy). Serum HDV-RNA was measured by an in-house quantitative PCR with linearity ranging from 575×10^2 to 575×10^5 IU/ml and a lower limit of detection (LLD) of 5.75×10^1 IU/ml. Serum HBV-DNA was measured by a commercial PCR with an LLD of 10 IU/ml and a lower limit of quantification of 20 IU/ml (COBAS 6800, Roche Diagnostics, Manheim, Germany). HBV genotyping was carried out by Sanger sequencing after amplification of two different viral regions (PreC/Core and PreS/Surface), as previously published.[10,11] MHBs and LHBs were measured by ELISA in a reference laboratory in Leipzig (Leipzig University Medical Center, Germany) in serum samples stored at -80 °C. LHBs and MHBs were quantified in triplicates using well-defined monoclonal antibodies against the preS1-domain (Ma18/7) and *N*-glycosylated preS2-domain (Q19/10), respectively, as previously reported.[7] The LLD was 0.07 ng/ml for the MHBs assay, 0.03 ng/ml for the LHBs assay, and 0.08 ng/ml for the total HBsAg/SHBs assay. SHBs values (ng/ml) were obtained after subtracting LHBs and MHBs values from total quantitative HBsAg (qHBsAg) (ng/ml).

Statistical analysis

All statistical analyses were performed using IBM SPSS, version 26.0 (SPSS Inc., Armonk, NY, USA). Normally distributed quantitative variables were expressed as mean and SD and compared using Student's *t* test. Non-normally distributed quantitative variables were expressed as median and IQR and analysed using the Mann–Whitney *U* test. Categorical variables were expressed as frequency and percentage and compared using the Chi-square test or Fisher's exact test, when frequencies were less than 5%. The results were considered statistically significant when the *p* value was lower than 0.05. Patients who were ICs were stratified and analysed using a clinically significant cut-off of HBsAg of 1,000 IU/ml.[12] Regarding the impact of HBV GT in the composition of HBsAg proteins, data from LV-AC and patients with CHB were analysed all together to increase the number of patients with available GT.

Results

Baseline HBsAg composition in patients with CHD

Forty-six patients with CHD were included. Baseline features of patients with HDV are displayed in Table 1. In brief, 63.0% were male, 87.0% were HBeAg negative, 37.0% presented liver cirrhosis, and 37.0% were under nucleos(t)ide analogues (NAs).

Liver cirrhosis was associated with higher ALT (99 *vs.* 49 IU/ml, $p = 0.027$), lower platelet count ($93 \times 10^9/mm^3$ *vs.* $176 \times 10^9/mm^3$, $p = 0.004$), and a trend toward higher LHBs proportion (6.4 *vs.* 4.5%, $p = 0.055$). No differences were found in either HBsAg levels ($p = 0.764$) or HBsAg composition between patients with and without NA therapy ($p = 0.434$ and $p = 0.450$ for LHBs and MHBs proportions, respectively).

At the time of cross-sectional evaluation, HDV-RNA was detectable in all patients except five: two of them had achieved undetectability after IFN treatment, whereas the remaining three achieved spontaneous clearance of HDV-RNA. Despite the limited number of anti-HDV-positive patients with undetectable HDV-RNA, significant differences were observed regarding HBsAg composition according to the presence of HDV-RNA (Table 2). Absolute levels of qHBsAg were higher in patients with detectable HDV-RNA ($p = 0.056$), as well as absolute values of each HBsAg protein ($p = 0.006$, $p = 0.060$, and $p = 0.056$, for LHBs, MHBs, and SHBs, respectively). Furthermore, patients with detectable HDV-RNA presented a statistically significant higher proportion of LHBs than did those with undetectable HDV-RNA ($p = 0.010$), without differences in the proportion of MHBs and SHBs.

Patients with CHD with positive HBeAg showed higher HBV-DNA (2.24 *vs.* 1.30 log IU/ml, $p = 0.007$), total HBsAg levels (4.56 *vs.* 4.05 log IU/ml, $p = 0.002$), and HBsAg proteins (LHBs: 3.04 *vs.* 2.60 log ng/ml, $p = 0.124$; MHBs: 3.42 *vs.* 2.55 log ng/ml, $p = 0.021$) compared with HBeAg-negative CHD. However, HBsAg protein proportions were similar (LHBs: 2.81 *vs.* 5.00%, $p = 0.385$; MHBs: 6.33 *vs.* 4.77%, $p = 0.707$) in both groups, regardless of HBeAg.

Impact of HBsAg composition in liver-related outcomes in CHD

Data on clinical outcomes were available in 36 patients with CHD with a minimum follow-up of 1 year: 23 (63.9%) were male, 31 (86.1%) had detectable HDV-RNA, 13 (36.1%) presented baseline liver cirrhosis, and 30 (83.3%) were HBeAg negative. During a median follow-up of 5.9 years (IQR 1.6–13.1 years), nine (25.0%) patients had progression of liver disease, with ascites being the most common (eight, 22.2%); three (8.3%) developed HCC; four (11.1%) required liver transplantation; and three (8.3%) died. The main predictor of decompensation during follow-up was the presence of liver cirrhosis (53.8 *vs.* 8.7% among patients without cirrhosis, $p = 0.005$). A higher frequency of clinical outcomes was

Table 1. Baseline characteristics of the two cohorts of patients.

	Chronic hepatitis D (N = 46)	Chronic HBeAg-negative HBsAg carriers (N = 141)
Male	29 (63.0%)	75 (53.2%)
Age (years)	43 ± 11	44 ± 13
Ethnic group		
White	36 (78.3%)	83 (58.9%)
Black	5 (10.9%)	37 (26.2%)
Asian	4 (8.7%)	19 (13.5%)
Hispanic	1 (2.2%)	2 (1.4%)
AST (IU/L)	80 ± 61	32 ± 23
ALT (IU/L)	86 ± 66	33 ± 23
Platelets (× 10^9/mm^3)	163 ± 78	215 ± 53
Liver cirrhosis	17 (37.0%)	10 (7.1%)
NA treatment history	17 (37.0%)	–
IFN treatment history	20 (50%)	–
HBV genotype		
D		40 (28.4%)
A	–	33 (23.4%)
E	–	28 (19.9%)
B or C	–	7 (5.0%)
F or H	–	6 (4.3%)
Mixed	–	3 (2.1%)
N/a	46 (100%)	24 (17.0%)
HBeAg negative	40 (87.0%)	141 (100%)
HBV-DNA (log IU/ml)	1.56 ± 1.20	3.2 ± 1.0
HBsAg (log IU/ml)	4.02 ± 0.55	3.6 ± 0.9
HDV genotype		
1	13 (28.3%)	–
2	1 (2.2%)	–
HDV-RNA detectable	41 (89.1%)	–
HDV-RNA (log IU/ml)	5.6 ± 1.5	–

Qualitative variables are expressed in absolute and relative frequency (%). Quantitative variables are expressed in median and IQR.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; IFN, interferon; LHBs, large hepatitis B surface protein; MHBs, medium hepatitis B surface protein; NA, nucleos(t)ide analogue; SHBs, small hepatitis B surface protein.

observed in those with baseline detectable HDV-RNA than in those with undetectable HDV-RNA, without reaching statistical significance (29.0 *vs.* 0%, p = 0.214). Baseline absolute qHBsAg was similar regardless of the development of liver decompensation (p = 0.494), as summarised in Table 3. However, both the absolute values and proportion of the MHBs tended to be higher in patients who presented decompensation during follow-up (p = 0.051 and p = 0.086, respectively). No significant differences were found in baseline HBsAg composition in the three patients who developed HCC in either protein absolute levels (p = 0.681 for LHBs and p = 0.809 for MHBs) or proportions (p = 0.789 for LHBs and p = 0.351 for MHBs).

During follow-up, HDV-RNA became spontaneously undetectable in five (16.1%) of 31 patients with CHD with detectable HDV-RNA at baseline. Baseline total qHBsAg (p = 0.011) and HBsAg proteins were lower among those in which HDV-RNA became undetectable during the follow-up (p = 0.011, p <0.001, p = 0.007, and p = 0.029, for qHBsAg, LHBs, MHBs, and SHBs, respectively). However, when the proportions were evaluated, the percentage of MHBs tended to be lower among those who reached spontaneous undetectable HDV-RNA during follow-up (p = 0.085). Lower percentage of LHBs was also observed in these patients, although it did not reach statistical significance. Eight (34.8%) of the 23 individuals without baseline cirrhosis progressed to cirrhosis during follow-up. No differences were observed in the absolute values or HBsAg protein proportions according to the later development of liver cirrhosis.

Evolution of HBsAg protein composition during follow-up

Evolution of HBsAg proteins was analysed in 12 patients with an available follow-up sample taken at least 5 years from baseline. Baseline characteristics of this subset of patients were as follows: 75% were male, 66.7% were HBeAg negative, 16.7% presented baseline liver cirrhosis, and 83.3% had detectable HDV-RNA. Median time from the baseline sample to the control sample was 10.3 years (IQR 6.4–11.7 years). A decline in median levels of qHBsAg (4.31 *vs.* 4.12 log ng/ml, p = 0.015) and MHBs (2.84 *vs.* 2.52 log ng/ml, p = 0.019) was observed during follow-up, whereas median LHBs levels did not show significant variations (2.53 *vs.* 2.56 log ng/ml, p = 0.638). The proportion of LHBs showed an increasing trend over time (p = 0.050), whereas MHBs proportion remained stable (p = 0.480) (Fig. 2A and B).

HBsAg protein composition in patients with HBeAg-negative hepatitis B

One hundred forty-one HBeAg-negative HBsAg carriers were included for the cross-sectional analysis. Seventy-eight patients (55.3%) were classified as ICs, 30 (21.3%) as LV-AC, and 33 (23.4%) as patients with CHB. Demographic, biochemical, and virologic baseline characteristics of these patients are summarised in Table 1. Significant variations were found in total qHBsAg values (p = 0.002), LHBs (p = 0.002), MHBs (p = 0.002), and SHBs (p = 0.0025) across different HBeAg-negative phases. HBsAg levels were similar in the LV-AC (3.83 *vs.* 4.00 log ng/ml, p = 0.363) and CHB (4.07 *vs.* 4.00 log ng/ml, p = 0.874) groups compared with the IC group. No differences were found in HBsAg protein levels between the IC and non-IC groups (p = 0.509 for LHBs and p = 0.914 for MHBs). However, when comparing proportions of HBsAg components, ICs presented lower proportion of LHBs than patients with CHB (2.71 *vs.* 5.21%, p = 0.035), patients with LV-AC

Table 2. HBsAg protein composition in the chronic hepatitis D cohort.

	Chronic hepatitis D		
	HDV-RNA (+) (n = 41)	HDV-RNA (-) (n = 5)	*p* value
Total HBsAg (log ng/ml)	4.15 [3.79–4.39]	3.71[2.41–4.07]	0.056
LHBs (log ng/ml)	2.77 [2.42–3.20]	1.89 [0.22–2.47]	0.006
MHBs (log ng/ml)	2.71 [2.14–3.20]	1.52 [1.09–2.89]	0.060
SHBs (log ng/ml)	4.10 [3.65–4.36]	3.70 [2.37–3.97]	0.056
LHBs (%)	5.48 [3.18–8.90]	1.46 [0.77–3.20]	0.010
MHBs (%)	5.18 [2.20–11.90]	6.05 [0.79–21.38]	0.985
SHBs (%)	87.60 [80.40–93.47]	93.67 [77.27–96.02]	0.427

Variables are expressed in median and IQR, and compared using Mann–Whitney *U* test.
LHBs, large hepatitis B surface protein; MHBs, medium hepatitis B surface protein; SHBs, small hepatitis B surface protein.

Table 3. Outcomes of patients with HDV according to the baseline HBsAg levels and their protein proportions.

	HDV-RNA negativisation* (n = 31)			Clinical outcomes (n = 36)		
	Yes (n = 5)	No (n = 26)	*p* value	Yes (n = 9)	No (n = 27)	*p* value
Total HBsAg (log ng/ml)	3.66 [3.30–4.04]	4.22 [4.00–4.42]	0.011	4.08 [3.91–4.56]	4.14 [3.71–4.40]	0.494
LHBs (log ng/ml)	2.34 [1.54–2.46]	2.88 [2.59–3.26]	<0.001	2.86 [2.65–3.11]	2.56 [2.41–3.08]	0.180
MHBs (log ng/ml)	2.09 [1.35–2.32]	3.02 [2.47–3.52]	0.007	2.95 [2.58–3.87]	2.47 [1.80–3.18]	0.051
SHBs (log ng/ml)	3.62 [3.27–4.02]	4.17 [3.91–4.42]	0.029	4.20 [3.82–4.67]	3.94 [3.51–4.30]	0.251
LHBs (%)	4.13 [0.91–6.94]	5.68 [3.14–8.76]	0.214	5.30 [3.26–8.04]	4.50 [1.51–8.60]	0.472
MHBs (%)	2.46 [0.46–5.33]	6.70 [1.82–15.94]	0.085	6.85 [4.54–25.14]	3.74 [1.18–10.94]	0.086
SHBs (%)	92.67 [88.11–98.63]	85.79 [75.97–93.27]	0.057	84.30 [69.09–91.10]	89.25 [83.10–95.08]	0.110

Variables are expressed in median and IQR, and compared using (*Mann–Whitney U* test) Development of clinical outcomes included any of the following: liver decompensation (ascites, hepatic encephalopathy, or variceal haemorrhage), liver-related death, hepatocarcinoma, and liver transplantation.
LHBs, large hepatitis B surface protein; MHBs, medium hepatitis B surface protein; SHBs, small hepatitis B surface protein.
* HDV-RNA negativisation was assessed in the 31 patients with baseline detectable viral load.

(2.71 *vs.* 4.35%, p = 0.073), and the combination of both (p = 0.027).

Compared with patients with CHD, HBeAg-negative HBsAg carriers presented lower qHBsAg and lower absolute levels of all components: LHBs (p <0.001), MHBs (p = 0.020), and SHBs (p = 0.021). However, protein proportions were similar in both groups (p = 0.714 for LHBs, p = 0.421 for MHBs, and p = 0.071 for SHBs). Patients with CHD with detectable HDV-RNA presented significantly higher values of LHBs than ICs (p = 0.025), patients with LV-AC (p = 0.007), and patients with CHB (p = 0.003), despite similar total HBsAg levels (p = 0.007). In addition, a greater percentage of LHBs was observed in patients with CHD with detectable HDV-RNA than in ICs (5.48 *vs.* 2.71%, p = 0.009).

HBsAg protein composition according to HBV GT

A significant variation in HBsAg levels was found in relation to HBV GT in ICs (GT A 3.80 log IU/ml, GT D 3.59 log IU/ml, and GT E 4.14 log IU/ml, p = 0.010) and in the non-IC group (LV-AC + CHB) (GT A 3.71 log IU/ml, GT D 3.75 log IU/ml, and GT E 4.27 log IU/ml, p = 0.047). The highest HBsAg levels were observed in patients infected with GT E in both groups.

HBV GT also showed a significant impact in absolute levels of HBsAg proteins. LHBs levels differed significantly according to HBV GT in ICs with HBsAg <1,000 IU/ml (p = 0.012), whereas MHBs levels differed in all ICs, regardless of total HBsAg levels (ICs with qHBsAg <1,000 IU/ml, p = 0.013; and ICs with qHBsAg ≥1,000 IU/ml, p = 0.003), and in the non-IC group (p = 0.003). Again, patients infected with GT E presented higher absolute median levels of all three HBsAg components, regardless of the phase of HBV infection.

Concerning the proportion of HBsAg components, LHBs proportion showed significant variation according to HBV GT in the IC group regardless of HBsAg levels, whereas MHBs proportion presented significant GT-dependent variations in all groups. LHBs and MHBs proportions in the different phases according to HBV GTs are displayed in Fig. 3A and B, respectively. Patients infected with GT D presented the highest proportion of both LHBs and MHBs in all groups of patients, whereas GT A presented the lowest proportion of both proteins.

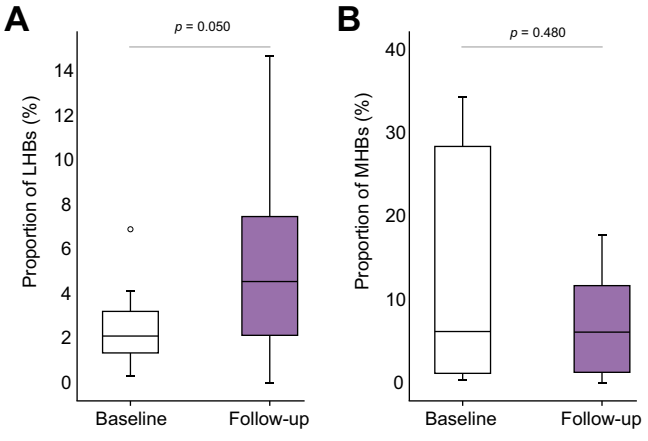

Fig. 2. Evolution of LHBs and MHBs proportions in patients with chronic hepatitis D with longitudinal samples (n = 12). (A) Proportion of LHBs in baseline and follow-up samples. Level of significance p = 0.050 (Mann–Whitney *U* test). (B) Proportion of MHBs in baseline and follow-up samples. Level of significance p = 0.480 (Mann–Whitney *U* test). Bars represent IQR, lines inside the boxplot represent median, and whiskers represent the minimum and maximum values. Outliers are represented as circles. LHBs, large hepatitis B surface protein; MHBs, medium hepatitis B surface protein.

Discussion

This is the first cohort exploring the HBsAg composition in well-characterised patients with CHD, showing that patients with HDV viraemia have a higher HBsAg level and LHBs proportion than those with undetectable viraemia (5.48 *vs.* 1.46%, p = 0.010). A higher proportion of LHBs was reported previously in only 11 patients with CHD, although the relation with HDV-RNA was not assessed.[7] The PreS1 domain located in L proteins is important for HBV and HDV entry into the hepatocytes. A higher proportion of LHBs in patients with active CHD could explain the infectivity that has been shown in *in vitro* models.[13,14] By contrast, MHBs do not appear to be needed for HDV replication, as has been suggested *in vitro* studies.[15]

A novel result of our study is the trend of having higher LHBs proportion among patients with CHD and liver cirrhosis (6.4 *vs.* 4.5%, p = 0.055). In our cohort, HBsAg composition did not differ according to NA exposure, in line with previous studies that reported the limited efficacy of NAs on decreasing HBsAg values.[16] Patients with HBeAg-positive CHD showed higher total HBsAg values, consistently with previous data in patients infected with HBV.[17] Interestingly, the composition (HBsAg protein proportion) was similar regardless of HBeAg status.

In the longitudinal follow-up of our patients with CHD, lower levels of baseline total HBsAg and all three proteins as well as a

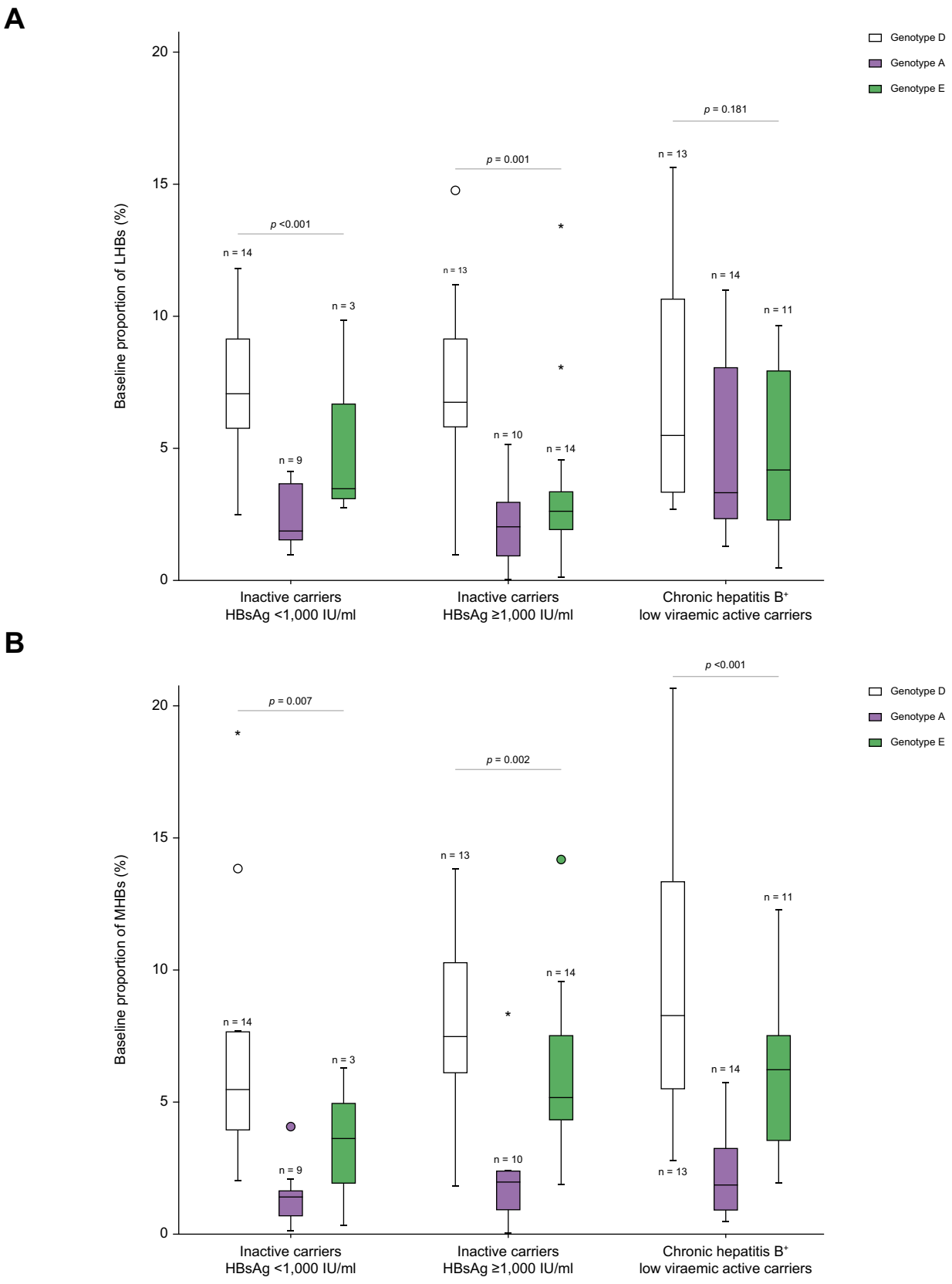

Fig. 3. LHBs and MHBs proportion in HBV-infection phases according to HBV genotype. (A) LHBs proportion in HBV infection phases for HBV genotypes D, A, and E. Levels of significance (ANOVA): $p = 0.001$ (inactive carriers HBsAg <1,000 IU/ml), $p = 0.001$ (inactive carriers HBsAg ≥1,000 IU/ml), and $p = 0.181$ (chronic hepatitis B + low viraemia). (B) MHBs proportion in HBV infection phases for HBV genotypes D, A, and E. Levels of significance (ANOVA): $p = 0.007$ (inactive carriers HBsAg <1,000 IU/ml), $p = 0.002$ (inactive carriers HBsAg ≥1,000 IU/ml), and $p < 0.001$ (chronic hepatitis B + low viraemia). Bars represent IQR, lines inside the boxplot represent median, and whiskers represent minimum and maximum values. Outliers are represented as circles, and extreme outliers as stars. LHBs, large hepatitis B surface protein; MHBs, medium hepatitis B surface protein.

lower proportion of MHBs (2.46 *vs.* 6.70%, p = 0.085) were associated with later HDV-RNA undetectability. The clinical relevance of this fact relies on the documentation of a worse long-term prognosis in patients with persistent HDV-RNA replication.[3,18,19] Concerning the development of unfavourable clinical outcomes (including liver decompensation, HCC, and mortality), higher baseline absolute MHBs levels were observed in patients with CHD who presented any clinical outcome during follow-up (2.95 *vs.* 2.47 log ng/ml, p = 0.051), despite similar total HBsAg levels. These patients also tended to show a higher baseline MHBs proportion (6.85 *vs.* 3.74%, p = 0.086). Remarkably, MHBs proportion remained stable during follow-up, which may allow us to explore its use as a baseline prognosis marker in view of its impact both on the development of complications and on spontaneous negativisation of HDV-RNA. The role of MHBs remains unclear even in HBV infection. Some studies have suggested that MHBs can play an immunomodulatory role similar to that of HBeAg, as high MHBs levels have been observed in early phases of HBV infection.[7,20] A trend toward presenting a higher proportion of MHBs in patients with CHD with unfavourable outcomes might reinforce the pathogenic implications of immunomodulation in CHD.[19] However, MHBs has also been proposed to be involved in carcinogenesis, both as a direct stimulus to oncogenic pathways and as an expression of integrated DNA.[21–24]

Previous studies in HBV cohorts proposed lower levels and proportions of MHBs and LHBs as predictors of treatment response and HBsAg clearance, mostly in HBeAg-positive populations.[8,25–27] A recent study in Asian ICs treated with pegylated IFN showed that the small proportion of patients who cleared HBsAg had lower absolute levels of MHBs and LHBs at baseline and during treatment.[27] However, the absence of consensus on measuring methodologies hinders the comparison of absolute levels among studies.[28] In this scenario, the measurement of relative levels used in this and previous studies might overcome some limitations.[7,8] Despite these data in HBV populations, there is scarce information exploring the HBsAg composition and the incidence of clinical outcomes in CHD populations. An Italian study including 30 Caucasian patients with cirrhosis (11 with chronic HBV monoinfection and 19 with CHD) who had achieved HBV virological suppression under NA treatment described an increase in MHBs proportion associated with the onset of HCC.[24] No differences were found in baseline protein proportions in patients with or without later development of HCC, although results were not analysed according to CHD status.[24] In our cohort, the three patients with CHD who developed HCC during follow-up did not present significant differences in terms of baseline HBsAg composition. The methodological differences between both studies and the limited number of patients who developed HCC limit the comparison of these findings.

Our study also partially validates the previous findings described in the German cohort showing significantly lower LHBs proportions in the IC phase.[7] This finding aligns with the biological role attributed to LHBs. The lack of differences in MHBs proportion in our cohort might be justified owing to a significant discrepancy in the HBV GT distribution in both cohorts and the greater heterogenicity of our cohort in terms of HBV GTs.

Our study also describes the significant impact of HBV GT in the HBsAg protein levels and proportions. Variations of HBsAg levels according to HBV GT have been well described.[11,29] However, differences in HBsAg composition are not as well documented. In our cohort including HBV GTs A, D, and E, we observed that GT E showed higher levels of total HBsAg and its three components, whereas GT D presented higher proportions of both middle and large proteins. A significant impact of HBV GT was previously reported in a limited number of patients with CHB, in which HBV GT D showed higher MHBs and LHBs proportions than GT A.[7] Rinker *et al.*[26] also reported higher absolute levels of MHBs and LHBs in GT B than in GT C in patients with HBeAg-positive CHB. In a different German cohort restricted to HBeAg-positive individuals, HBV GT impacted significantly in HBsAg composition, with GT B presenting a higher LHBs proportion than GTs A and D.[8] Similar findings were observed in HBeAg-negative individuals in a nationwide multicentric study, in which higher MHBs and LHBs proportions were observed in GTs B and D than in GTs A, C, and E.[30] It should be noted that in our cohort, MHBs proportions showed significant variations according to HBV GT in all HBeAg-negative infection phases, whereas LHBs proportions only showed significant variations according to HBV GT in patients who were ICs, which might be explained owing to the limited sample size.

Our study has some limitations. The limited number of patients with CHD and with liver-related events hinders the statistical power of our findings. In addition, HBV GT is not available in all cases and high-prevalent HBV GTs worldwide, such as GTs B and C, are under-represented. The inclusion of mostly HBV ICs with low HBV-DNA hampers the obtention of results for HBV genotyping. Finally, the partially retrospective design might have introduced reporting bias in our analysis.

Despite these limitations, this is, to our knowledge, the first study exploring the association between HBsAg composition and clinical outcomes in patients with CHD, which provides a real-world clinical approach to an experimental hypothesis and further explores the application of MHBs proportion as a prognostic marker. Larger multicentre prospective studies should be designed to validate our findings. Meanwhile, we also provide valuable information supporting a differential HBsAg composition in HBV ICs in a real-world cohort and expand the evidence of a GT-dependent HBsAg structure in HBeAg-negative patients, regardless of the phase of infection. An effort should be made to include under-represented GTs in multicentric ethnically diverse cohorts to generalise our findings.

In summary, baseline HBsAg composition differs in patients with CHD who present negativisation of HDV-RNA and/or clinical outcomes during follow-up, with baseline MHBs proportion having a promising role as a potential prognosis marker. Our work also validates relative levels of LHBs as a differential marker in HBV ICs and reinforces the impact of HBV GT in HBsAg configuration.

Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHB, chronic hepatitis B; CHD, chronic hepatitis D; GT, genotype; HCC, hepatocellular carcinoma; IC, inactive carrier; IFN, interferon; LHBs, large hepatitis B surface protein; LLD, lower limit of detection; LV-AC, low viraemic active carriers; MHBs, medium hepatitis B surface protein; NA, nucleos(t)ide analogue; qHBsAg, quantitative HBsAg; SHBs, small hepatitis B surface protein.

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Conflicts of interest
RE and MBu have served as speakers for Gilead. MR, RE, and M Bu have received grants from Gilead. RE and M Bu have performed as consultants for Gilead, Abbvie, and GSK, and served as speakers for Gilead. MR has served as an advisory board member for GSK. FVB has served as a speaker for and provided consulting services to Gilead, Roche, Janssen, Ipsen, MSD, Esai, and AstraZeneca, and has served as an advisory board member of Janssen. TB has received grants from Abbvie, BMS, Gilead, MSD/Merck, Humedics, Intercept, Merz, Norgine, Novartis, Orphalan, and Sequana Medical, and provided consulting services to Abbvie, Alexion, Bayer, Gilead, GSK, Eisai, Enyo Pharma, HepaRegeniX GmbH, Humedics, Intercept, Ipsen, Janssen, MSD/Merck, Novartis, Orphalan, Roche, Sequana Medical, SIRTEX, SOBI, and Shionogi. TB has served as a speaker for Abbvie, Alexion, Bayer, Gilead, Eisai, Falk Foundation, Intercept, Ipsen, Janssen, MedUpdate GmbH, MSD/Merck, Novartis, Orphalan, Sequana Medica, SIRTEX, and SOBI and served as an advisory board member for Gilead, Assembly, and GSK.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions
Conceptualisation: LR, MR, MB, TB, FVB. Data curation: LR, MR, MP, SS. Funding acquisition: MR, MBu. Investigation: LR, AP, MP. Methodology: MR, MP, SS, MBe, AR, RC, DT, FR. Supervision: MR, MBu. Writing – original draft: LR, MR, MBu. Writing – review and editing: MBu, MP, SS, AP, MBe, AR, RC, DT, FR, TB, RE, FVB.

Data availability statement
The data that support the findings of this study are available from the corresponding author, MR, upon reasonable request.

Supplementary data
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