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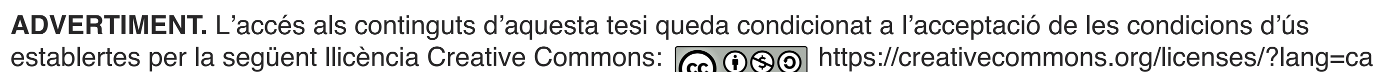
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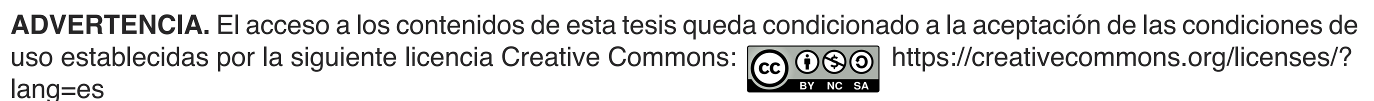
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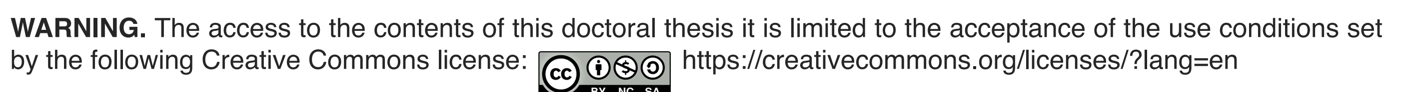
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DOCTORAL THESIS

CLINICAL APPLICATION OF NEW BIOMARKERS IN CHRONIC HBV AND HDV INFECTIONS

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ABBREVIATIONS

A

AASLD American Association for the Study of Liver Diseases
ALT alanine aminotransferase
APASL Asian-Pacific Association for the study of the liver
APRI AST-to-platelet ratio
AST aspartate aminotransferase
AUROC area under the receiver operating characteristics

B

BLV bulevirtide

C

CAMs capsid assembly modulators
cccDNA covalently closed circular DNA
CHB chronic hepatitis B
CHD chronic hepatitis D
CI confidence interval
CLEIA chemiluminescent enzyme immunoassay

E

EASL European Association for the study of the liver
ELISA enzyme-linked immunoassay
EMA European Medicines Agency
ETV entecavir

F

FIB-4 fibrosis-4 index

G

GZ grey zone
GT genotype

H

HR hazard ratio
HBc hepatitis B core protein
HBcAg hepatitis B core antigen
HBcrAg hepatitis B core-related antigen
HBeAg hepatitis B e antigen
HBsAg hepatitis B surface antigen
HBV hepatitis B virus
HCC hepatocellular carcinoma
HCV hepatitis C virus
HDAg hepatitis delta antigen
HDAg-L large hepatitis delta antigen isoform
HDAg-S small hepatitis delta antigen isoform
HDV hepatitis D virus
HIV human immunodeficiency virus

I

IFN interferon

L

LHBs large hepatitis B surface protein
LLD lower limit of detection
LLQ lower limit of quantification
LV-AC low viraemic active carriers
LSM liver stiffness measurement

M

mRNA messenger RNA
MHBs medium hepatitis B surface protein

N

NA nucleos(t)ides analogue
NTCP sodium taurocholate cotransporting polypeptide

O

ORF open reading frame

P

PCR polymerase chain reaction
Peg-IFN pegylated interferon
pgRNA pregenomic RNA
PLHIV persons living with HIV
PPV positive predictive value
PWID persons who inject drugs

Q

qHBsAg quantitative hepatitis B surface antigen

R

rcDNA relaxed circular DNA
RT reverse transcriptase

S

SHBs small hepatitis B surface protein
siRNA small interfering RNA
SVP subviral particle

T

TAF tenofovir alafenamide
TDF tenofovir disoproxil fumarate

W

WHO World Health Organization

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Summary

The natural history of chronic hepatitis B virus (HBV) encompasses different and not always consecutive infection phases with a completely divergent prognosis in the long term. The correct categorization of these phases in the clinical practice is essential to define the indication of treatment and the follow-up strategy. This categorization requires the periodical monitoring of biochemical and virological parameters, and occasionally the performance of liver biopsy. New serum markers have been proposed in the last years to accurately identify the phases of infection in subjects with chronic HBV.

The first study approaches the identification of HBV inactive carriers (IC) using non-invasive markers in a diverse cohort of HBeAg-negative chronic HBsAg carriers. It seeks to develop a combining score for the identification of this phase in a single-point assessment. In this retrospective-prospective study, baseline HBV-DNA levels, hepatitis B core-related antigen (HBcrAg) and liver stiffness measurement (LSM) are identified as independently associated with the IC state. The ACE score constructed with these variables shows a good performance for the identification of IC in a single-point in time, regardless of HBV genotype.

HBsAg constitutes the envelope of HBV and hepatitis Delta virus (HDV), a small RNA satellite virus that relies on HBsAg for virion assembly and infectivity. Different biological roles for the three HBsAg proteins (small, middle and large [SHBs, MHBs, LHBs, respectively]) have been postulated. The absolute and relative levels of these isoforms, whose measurement was recently optimized, have been lately studied as a potential tool for the identification of HBV-IC. However, limited information on the

impact of HBV genotype in HBsAg protein composition is available. The second study explores the distinct composition of HBsAg in the IC group. Our results support the differential absolute and relative composition of HBsAg in subjects in the IC phase, showing lower proportion of LHBs. The study also evidences a significant impact of HBV genotype in HBsAg composition.

The second part of the study describes for the first time the composition of HBsAg in a well-characterized group of subjects with chronic HDV. An association between HBsAg composition and HDV-RNA was found, since subjects with detectable HDV-RNA showed higher absolute levels of the three proteins and higher LHBs proportion. In the longitudinal follow-up, our findings suggest that the baseline proportion of HBsAg proteins are related with spontaneous clearance of HDV-RNA and with the development of liver-related clinical events.

RESUMEN

La historia natural del virus de la infección crónica por el virus hepatitis B (VHB) engloba fases diferenciadas y no siempre consecutivas, con importantes implicaciones pronósticas. La correcta categorización de estas fases en la práctica clínica es esencial para definir la indicación de tratamiento y la estrategia de seguimiento. Esta categorización requiere controles analíticos periódicos, con monitorización de parámetros bioquímicos y virológicos, y ocasionalmente la realización de biopsia hepática. En los últimos años, nuevos biomarcadores séricos se han propuesto para la correcta clasificación de las fases de infección crónica por VHB.

El primer estudio aborda la identificación de portadores inactivos del VHB mediante marcadores no invasivos en una cohorte diversa de portadores crónicos de HBsAg HBeAg negativos, de cara al desarrollo de un sistema de puntos para la identificación de portadores inactivos del VHB en una única evaluación. En esta cohorte retrospectiva-prospectiva, los niveles basales de DNA-VHB, HBcrAg y elastografía hepática se asocian de manera independiente con el estado de portador inactivo. El score ACE, diseñado a partir de estas variables, presenta un adecuado rendimiento para la identificación de los portadores inactivos en una única evaluación, independientemente del genotipo.

HBsAg constituye el envoltorio tanto del VHB como del virus de la hepatitis D (VHD), un virus de RNA de carácter defectivo que depende del HBsAg para el ensamblaje y la infectividad de sus viriones. Se han propuesto diferentes roles biológicos para las tres proteínas que forman HBsAg (pequeña, mediana y larga [SHBs, MHBs, LHBs, respectivamente por sus siglas en inglés]). Los niveles absolutos y relativos de estas

proteínas, cuya determinación en suero ha sido recientemente optimizada, se han propuesto como potencial herramienta para la identificación de portadores inactivos del VHB. Sin embargo, existe información limitada en cuanto al impacto del genotipo del VHB en la composición del HBsAg. El segundo estudio aborda la composición de HBsAg en los portadores inactivos del VHB. Nuestros resultados apoyan las diferencias en la composición absoluta y relativa del HBsAg en portadores inactivos del VHB, describiendo una menor proporción de LHBs en estos sujetos. El estudio también pone en evidencia el impacto del genotipo del VHB en la composición del HBsAg.

La segunda parte del del estudio describe por primera vez las isoformas del HBsAg en un grupo bien caracterizado de pacientes con infección crónica por VHD. Los resultados muestran una asociación entre la presencia de RNA del VHD y las isoformas del HBsAg, describiendo niveles más elevados de las tres proteínas, así como una mayor proporción de LHBs en sujetos virémicos. En el estudio longitudinal de los pacientes, la composición del HBsAg se relaciona con la negativización espontánea de la viremia del VHD y con el desarrollo de eventos clínicos.

1 INTRODUCTION

1.1 HEPATITIS B VIRUS

1.1.1 History

In early and mid-40's cases of jaundice and hepatitis were described after transfusion of blood-related products from asymptomatic donors to previously healthy individuals as part of immunization programs(1). The viral aetiology of what it was initially called *homologous serum jaundice* was suggested by Frederick MacCallum in 1946. MacCallum also proposed the term hepatitis B, opposed to the epidemic forms of faecal/oral transmitted hepatitis in the community named hepatitis A (1,2). Twenty years later, Baruch S. Blumberg described a new serum protein in the blood of a Native Australian man (3). The Australia antigen, known today as hepatitis B surface antigen (HBsAg), became the first serological marker for viral hepatitis. The hepatitis B viral particle was not described until 1970, when David Dane observed 42nm particles in the serum of three HBsAg carriers using electronic microscopy. Cloning and sequencing of hepatitis B virus (HBV) DNA was completed in 1979, allowing a rapid development of diagnostic tests and vaccines(4). Later investigations in primates confirmed the replicative capacity of the virus in liver tissue(4).

Despite these relatively recent discoveries, HBV-DNA has been found in Eurasian human remains from around 7,000 years ago, and phylogenetic studies suggest its presence in Native Australian populations around 51,000 years ago(5). Although some authors suggest a zoonotic transmission from non-human primates, the full evolution of HBV remains unclear (6). Nevertheless, HBV history looks so intimately linked to

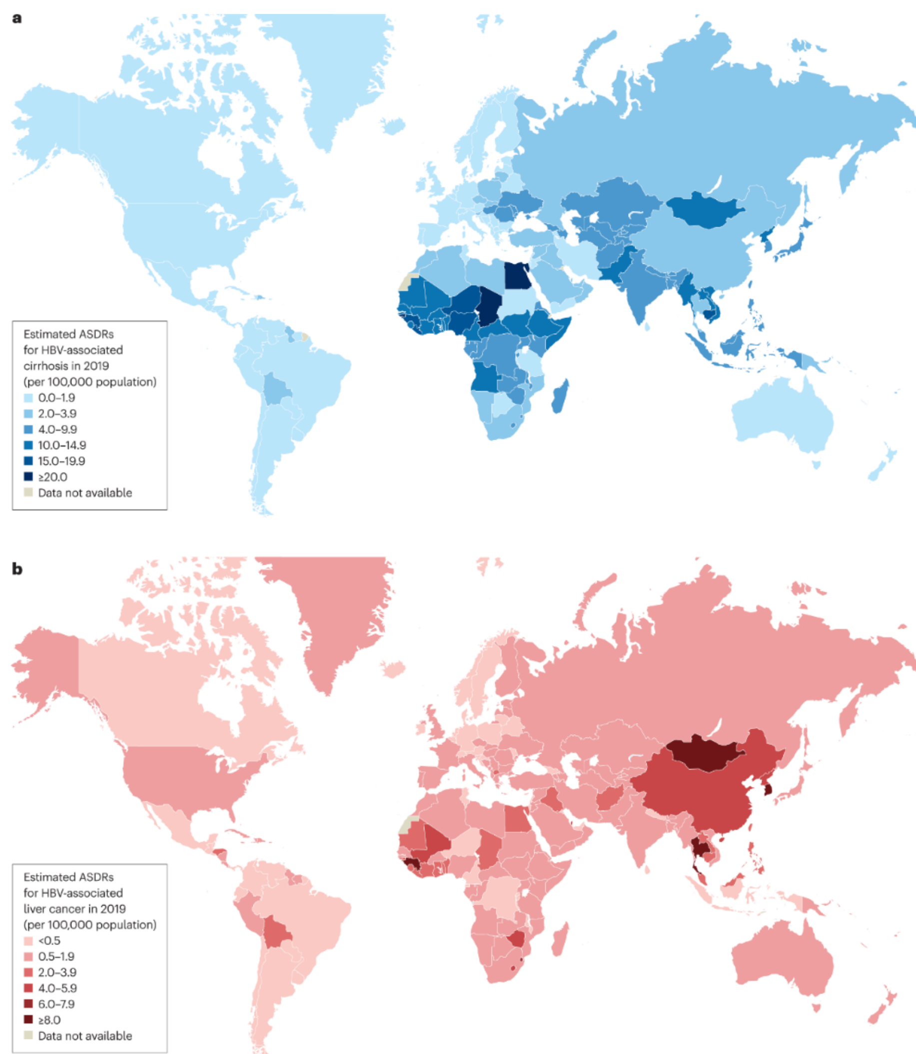
humankind that phylogenetic tracing on HBV has been even used to understand human migrations and interactions in ancient times (7,8).

1.1.2 Epidemiology

Between 217 and 316 million people have been recently estimated to carry HBsAg worldwide(9). The same modelling study estimated global HBsAg seroprevalence in 3.2%(9). Differences in HBsAg seroprevalence at regional and sub-regional level seem to overlap income inequalities, with higher endemicity observed in low and middle resource settings(10). Indigenous communities across the globe show higher HBsAg seroprevalence compared to the overall population(11). The African, Western Pacific and South East Asian World Health Organization (WHO) regions present a higher endemicity, with regional HBsAg seroprevalence reaching 7.5%, 5.9% and 3.0%, respectively (10,12). These regions also face a disproportionate burden in terms of HBV-related mortality, accounting for almost 90% of the 821,000 HBV-related deaths in 2019 (12). Figure 1a and 1b show age-standardized mortality due to HBV-associated cirrhosis (1a) and hepatocellular carcinoma (HCC) (1b) by country(10).

In the European region, the overall HBsAg prevalence was estimated in 1.5%, with significant variations across countries (12,13). A considerably higher HBsAg prevalence was described in high-risk populations such as prisoners, people who inject drugs (PWID), men who have sex with men and migrants, in which robust data are often scarce (13). Higher prevalence in general population and high-risk groups was described in countries from Southern and Eastern Europe.

FIGURE 1. a, Estimated age-standardized death rates for HBV-associated liver cirrhosis per 100,000 population in 2019, by country. b, Estimated age-standardized death rates for HBV-associated liver cancer per 100,000 population in 2019, by country. *Taken by Hsu et al.* (10,14)

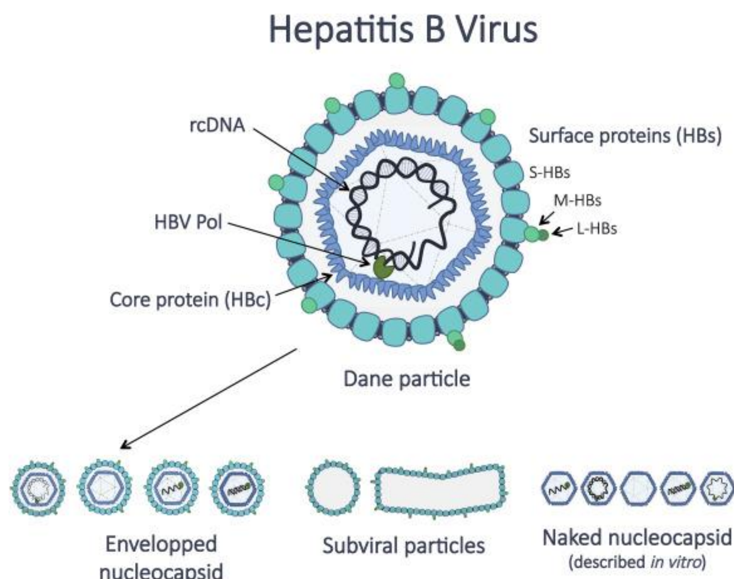


The availability of an effective vaccine against HBV infection endorsed by WHO dramatically impacted HBV and HCC incidence worldwide(15,16). Global coverage of 3-dose vaccination is currently estimated in 84%, still below the 90% target settled by WHO global health sector strategy on viral hepatitis (17,18). Three-dose vaccination coverage shows considerable country-to-country variations. Global coverage drops to 45% in birth-dose administration, with the lowest rates in the African region, where it only reaches 18%. A combination of immense socioeconomic inequalities and cultural aspects justify these disproportions in vaccination coverage(16).

1.1.3 Virology and replication cycle

HBV is a small DNA virus belonging to the *Orthohepadnavirus* genus of the *Hepadnaviridae* family. Full virions correspond to the 42nm particles described by Dr Dane (and named Dane particles after him). These virions are formed by an outer lipid membrane with the three HBsAg proteins (large [LHBs], medium [MHBs] and small [SHBs]), a nucleocapsid constituted by HBV core protein (HBc), the viral polymerase and the viral genome. The HBV genome contains 3.2kb in a partially double-stranded DNA expressing seven viral proteins(19). Besides complete virions, enveloped nucleocapsids containing either none or immature genetic material, as well as nucleocapsid-free subviral particles (SVPs) are also detected in the serum of patients infected with HBV. The structure of virions and viral particles is shown in figure 2(20).

***FIGURE 2.* Representation of HBV particles**. *From Tsukuda et al.* (20)



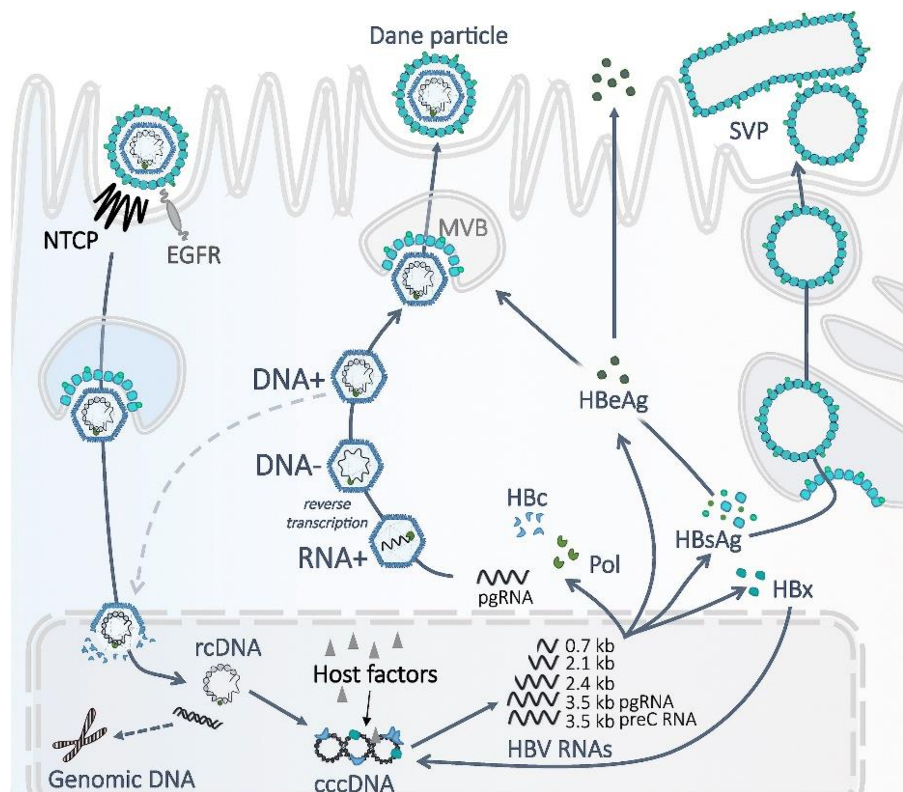
The virus enters the hepatocyte using low-affinity binding receptors and the liver-specific membrane receptor sodium taurocholate cotransporting polypeptide (NTCP),

which binds preS1 region of LHBs (20). Epidermal growth factor receptor seems to assist HBV internalization (21). Viral replication happens in the cell nucleus through reverse transcription. The viral reverse transcriptase (RT) converts the relaxed circular DNA (rcDNA) into an exceptionally compact covalently closed circular DNA (cccDNA) minichromosome with four overlapping open reading frames (ORF), which constitute the transcriptional substrate for 4 classes of messenger RNA (mRNA) that will be exported to cellular cytoplasm: the 3.5kb pregenomic RNA (pgRNA) and preC RNA, the 2.4kb and 2.1kb preS/S mRNAs, and the 0.7kb HBx mRNA (19,22,23). pgRNA results in the expression of core proteins and viral polymerase, and it is considered the only mRNA transcript essential for replication(19). PreC mRNA expresses the precore protein, which will be secreted after post-translational modifications as hepatitis B e antigen (HBeAg). The preS/S region encodes for the three surface proteins (SHBs, MHBs, LHBs), which result from the starting of translation in different starting codons(24). While all three proteins share a common S domain, an extra preS2 region is presented in both MHBs and LHBs and will be transcript from the 2.1kb S mRNA. LHBs shows an additional preS1 extension in the 2.4kb preS mRNA (25). The 0.7kb mRNA transcript will generate HBX protein, that seems to be required for transcription to happen efficiently(24).

HBV-DNA is synthetized from pgRNA, after which virions are either coated by the three envelope proteins and secreted or sent to the nucleus and sustain cccDNA amplification. Simultaneously, filamentous, and spherical SVPs are secreted in much higher amounts than full virions(20). The replication cycle of HBV is shown in figure 3 (20). The lack of proofreading activity of the viral RT results in a high mutation rate due to common replication errors (19,23). This great variability is behind the differentiation of HBV in genotypes (GTs), sub-genotypes and viral quasispecies, and entails

significant clinical implications(11). Variations along the entire HBV genome are present along HBV-GTs(26). The preS/S sequence is considered the most variable region in HBV genome, and it is commonly used for HBV identification. Mutations in this region have shown viral infectivity, replication, and immune recognition(26).

FIGURE 3. ***Hepatitis B replication cycle.*** *Taken from Tsukuda et al.*(20)



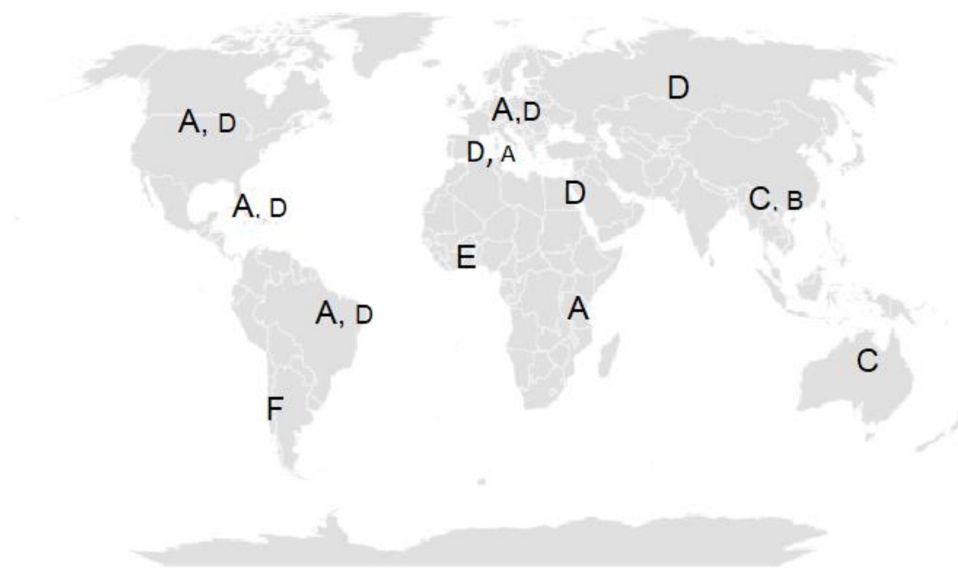
Around 10% of reverse transcription from rcDNA leads to the production of double-stranded linear DNA, the substrate for integration in the hepatocyte genome. Integrated DNA lacks replication capacity, since cccDNA is the only template able to produce pgRNA. However, integrated DNA plays a significant role in the persistence of the infection through the sustained production of viral proteins(27). The maintained production of HBV surface proteins contributes to the persistence of the infection through T-cell and B-cell modulation, especially in HBeAg-negative phases of HBV

infection, where most HBsAg production has been observed to rely on integrated DNA(27,28). An oncogenic potential of DNA integration promoted by oxidative stress, genomic instability and epigenetic changes has been proposed (27,28).

1.1.4 HBV genotype

HBV includes at least 9 different GTs named from A to I and one putative GT (J). The geographic distribution of HBV-GTs and their subtypes varies around the globe(29). The geographic distribution of HBV-GTs was reviewed in a recent metanalysis including data from 17 studies and 93 countries. Results are summarized in figure 4(29).

FIGURE 4. ***Geographic distribution of HBV genotype.*** *Adapted from Liu et al.*(29). *The size of the font represents the relative frequency of HBV genotype in the geographic region.*



HBV-GT A is predominant in Northern and Western Europe and South and East Africa, while B and C co-exist in the South-East Asian and Asia-Pacific regions. GT D presented a global distribution with higher prevalence in Southern Europe, central

Asia, and the Indian subcontinent. GT E is mainly restricted to West Africa. The length of HBV genome differs among GTs, due to nucleotides deletions and insertions. It has been suggested that the existence of multiple subgenotypes would indicate an older origin of a certain GT, while a lower variability would be distinctive from newer strains such as GT E, G and H (19).

HBV variability has shown to be relevant not only for the study of viral phylogenesis, but also for the natural history of HBV infection, disease progression and treatment outcomes(11). HBV-GTs and subgenotypes present differences in HBeAg seroconversion and HBsAg clearance rates, risk of HCC and treatment response. An association with specific transmission patterns has also been described. For instance, GT B and C have been associated to vertical transmission and appeared to be prone to cirrhosis development and HCC (30,31). The role of host-related and environmental factors such as toxins in disease progression is not fully understood(11).

1.1.5 Natural history of chronic HBV infection

Chronic HBV infection is defined by the persistence of HBsAg in serum for more than 6 months. The evolution of chronic hepatitis B infection is represented in virological and clinical phases that result from complex and dynamic interactions between the host immunity and the viral particles (32,33). HBV chronic infection phases are defined by HBeAg status, alanine aminotransferase (ALT), HBV-DNA levels and the presence of liver disease (34) . Proposed terminologies for HBV infection phases and cut-offs lack international consensus (28). The phases of chronic infection according to European Association for the study of the liver (EASL) Clinical Practice Guidelines 2017 are shown in figure 5(34).

***FIGURE 5. Phases of chronic HBV infection, according to EASL Clinical Practice Guidelines 2017**(34).* **Persistently or intermittently. **HBV-DNA levels can be between 2,000 and 20,000 IU/mL in some patients without sings of chronic hepatitis.*

	HBeAg-positive		HBeAg-negative	
	Chronic infection	**Chronic hepatitis**	**Chronic infection**	**Chronic hepatitis**
HBsAg	High	High/Intermediate	Low	Intermediate
HBeAg	+	+	-	-
HBV-DNA	>10^7 IU/mL	10^4-10^7 IU/mL	< 2,000 IU/mL**	>2,000 IU/mL
ALT	Normal	Elevated	Normal	Elevated*
Liver disease	None/minimal	Moderate/severe	None	Moderate/severe
Old terminology	Immune tolerant	Immune reactive HBeAg-positive	Inactive carrier	HBeAg-negative chronic hepatitis

HBeAg-positive chronic infection is characterized by high viral replication (usually HBV-DNA>10^8 IU/mL) and lack of inflammation and cytopathic effect, resulting in normal ALT levels(35). A certain immune tolerance was presumed to happen in this phase, explaining the lack of inflammatory response. However, there is evidence of frequent HBV-DNA integration, higher clonal hepatocyte replacement and specific T-cell mediated immunity in this phase, suggesting a role in oncogenesis and disease progression(36).

The shift to HBeAg-positive chronic hepatitis B (CHB) happens in presence of necroinflammation, which produces oscillating ALT elevations and a progressive development of liver fibrosis. HBV-DNA often falls to 10^4-10^7 IU/mL in this phase. A longer duration of this highly-replicative phase has been associated with an increased risk of cirrhosis, HCC, and mortality(37,38).

Seroconversion to HBeAg-negative stages is estimated to happen in around 15% of cases per year (28). HBeAg-negative stages represent most chronic HBV infections in Western countries (39,40). The risk of cirrhosis, liver cancer, decompensation, and liver-related death dramatically varies throughout the HBeAg-negative infection phases, which are not always consecutive and whose duration may differ among individuals (41). Mutations in precore and core regions, either immunologically-

induced or produced by replicating errors, might lead to HBeAg negativization despite maintained viral replication (42). HBeAg-negative CHB presents with HBV-DNA above 2,000 IU/mL, elevated ALT, or normal values but moderate liver fibrosis or necroinflammation. This phase entails an increased risk for HCC and cirrhosis development.

HBeAg-negative chronic infection (also known as inactive carriers-IC) is characterized by persistently normal ALT levels and low levels of viremia (HBV-DNA<2,000IU/mL) without necroinflammation or fibrosis. Individuals in this phase present a benign long-term prognosis with similar liver-related morbi-mortality than the general non-HBV-infected population (43,44). Despite variations in nomenclature, less intensive management strategies for HBV-ICs are endorsed by EASL, the American Association for the Study of Liver Diseases (AASLD) and the Asian-Pacific Association for the study of the liver (APASL) (34,45,46). These recommendations are underpinned in the benign outcome observed in subjects in this phase in long-term cohort studies mostly in European countries, in which low risk of progression to liver cirrhosis and a low incidence of HCC was reported (47). Most longitudinal studies involving HBV-IC have been performed in European cohorts with predominance of GT D, and longitudinal data on South America, Asia and Africa are scarce(47). Controversially, an increased incidence of HCC and liver-related mortality in HBV-IC compared to HBsAg-negative controls was reported in a large Taiwanese cohort with subjects infected by GT B and C(35). A multicentric prospective Japanese study reported favorable outcomes in 388 subjects (48% infected by HBV-GT-C) after a 3-year follow-up, supporting a benign prognosis in HBV-IC(48). A larger Japanese cohort supported these findings, reporting similar long-term prognosis than the general population(44). Some subjects in the HBeAg-negative chronic infection phase might present higher HBV-DNA (2,000-20,000 IU/mL), maintaining persistently normal ALT levels and without liver fibrosis. A

benign progression has also been reported in this subgroup, occasionally named low viremic active carriers (LV-AC)(49).

HBsAg loss is estimated to happen in around 1% of HBV-IC per year (50). HBsAg loss is considered the functional cure of HBV infection, since integrated DNA cannot be eliminated from a previously infected hepatocyte. Spontaneous HBsAg clearance has been associated with lower HBV-DNA and HBsAg levels. Globally, the risk of reactivation for subjects in the IC phase is considered low. HBV reactivation after HBsAg clearance might be triggered by chemotherapy, immunosuppressive and immunomodulation therapy, immunobiological treatments and direct-action antivirals against hepatitis C virus (HCV).

After HBsAg clearance, HBV-DNA might persist detectable at low levels during some time known as post-window period. Detectable HBV-DNA in blood and/or liver tissue despite negative HBsAg might also happen due to mutations in HBsAg regions (S, pre-S1 and pre-S2 variants)(51). Both scenarios are considered occult HBV infection, which would remain undiagnosed with standard HBsAg serological testing. A considerable prevalence of occult HBV infection (ranging from 5.5% to 12%) have been reported in high-risk groups (human immunodeficiency virus [HIV] and/or HCV-coinfected subjects, patients in haemodialysis) from low and highly endemic settings. Occult HBV infection is considered relevant from the public health and individual health perspective. Emerging evidence has associated this occult HBV infection with a significantly increased risk of HCC, while it entails risk of reactivation under certain concomitant therapies (52).

1.1.6 Clinical approach and current therapies

In the clinical practice, the assessment of HBV infection by ALT and HBV-DNA levels is often insufficient to categorize the infection stage in HBeAg-negative subjects due to frequent fluctuations of both parameters. As a result, a follow-up of three medical visits within the first year after diagnosis is currently recommended to define the phase of infection (34). A liver biopsy is indicated to define the fibrosis stage in case these markers are inconclusive(34). On the other hand, some subjects fall in a grey zone (GZ) category (persistently normal ALT with HBV-DNA above 20,000 IU/mL and absence of significant fibrosis) that requires individualized management and risk assessment.

Indications for antiviral treatment are not consistent among the available guidelines. The lack of global consensus and simplified strategies has hindered the implementation of a common global approach towards HBV. The EASL Guidelines of Clinical Practice recommend treatment for those subjects in the CHB phases, regardless HBeAg status(34). The aim of antiviral treatment is to avoid disease progression and development of clinical events. This is preferably achieved through functional cure, defined as undetectable HBsAg with a limit of detection of 0.05 IU/ml. Due to the low rates of HBsAg loss with current therapies, virological and biochemical response (HBV-DNA suppression and ALT normalization, respectively) are regarded as more feasible endpoints in the clinical practice(53).

The high barrier to resistance nucleos(t)ides analogues (NAs) tenofovir disoproxil (TDF), tenofovir alafenamide (TAF) and entecavir (ETV) are first-line treatments for CHB. The goal of antiviral therapy is to achieve viral suppression and reduce or reverse fibrosis and inflammation. Achieving viral suppression has shown to reduce mortality and improve quality of life (54). NAs have also shown to reduce sexual or

perinatal transmission, and to prevent HBV reactivation in subjects at risk. NAs present a highly effective treatment option with an excellent safety and tolerability profile. They suppress the viral RT at a late step of the replication cycle, inhibiting the conversion of pgRNA to HBV-DNA and thus, they have no effect in transcriptional activity and/or integrated HBV-DNA. Despite the high rate of virological suppression, patients rarely achieve HBsAg loss under chronic NA treatment, and usually NAs should be maintained lifelong(53). Conversely, HCC risk is not eliminated under treatment with NAs.

Interferon (IFN) alfa is a subcutaneous agent also approved for CHB treatment in patients without or with compensated liver cirrhosis. IFN alfa suppress the transcription from cccDNA to RNA activating a lymphotoxin that degrades cccDNA(28). Combination therapy with NAs has shown a higher rate of HBsAg clearance than NA monotherapy. However, IFN regimes are often disregarded in the clinical practice due to common and severe side effects and contraindications.

Considerable efforts have been made in the recent years with the development of new investigational treatments seeking to achieve real cure of CHB, which would combine eradication of cccDNA, suppression or muting of HBV integrated genome and adjustment of the antigenic immune response (28). New antiviral agents include entry inhibitors, RNA interference agents (small interfering RNA [siRNA] and antisense oligonucleotides), HBsAg assembly agents and capsid assembly modulators (CAMs). Significant HBsAg decline has been shown with siRNA and antisense oligonucleotides-based regimes, promoting HBV functional cure(41). Strategies including antiviral treatments combined with immunomodulatory agents, mainly pegylated IFN (peg-IFN) alfa, aim to restore T-cell and B-cell related immunity. Up to

date, none of these experimental treatments or their combinations have reached phase 3 trials.

1.2 Hepatitis Delta virus

1.2.1 History

The delta antigen (HDAg) was described by Rizzetto *et al.* in 1977, in the hepatic tissue of patients infected by HBV with chronic liver disease (55). This novel antigen was finally linked to a unique new virus after the defective transmission capacity in chimpanzees was proved and a distinct RNA was identified (56,57). The sequencing of the viral RNA was finally completed in 1986 (58). Later studies proposed a significant role of HDV in cases of fulminant hepatitis in the Amazonian region described since the 1930´s (59,60).

Several hypotheses have tried to explain the origin and evolution of HDV. The emergence of the virus from plant viroids or from an aberrant self-replicating RNA of HBV-infected hepatocytes have been postulated (61–63). More recently, the replication capacity of HDV in different species and in non-hepatic tissue has reinforced the hypothesis of an origin independent from HBV (64). Based on the finding of HDV-like agents in reptiles, mammals and birds, the possibility of a zoonotic transmission has also been suggested (61).

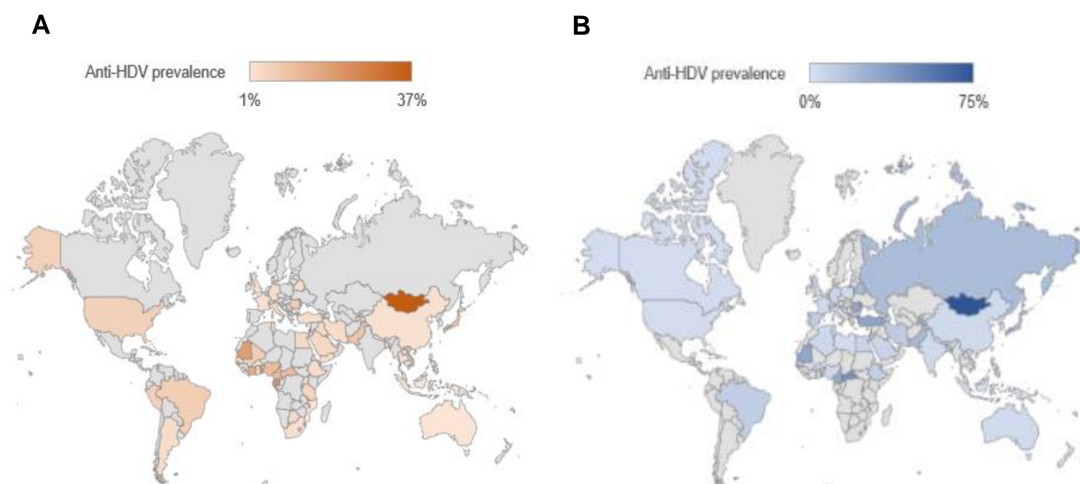
1.2.2 Epidemiology

The global burden of HDV infection is uncertain. Meta-analysis from 2018 and 2019 reported 48 to 72 million cases worldwide (65,66). A more recent meta-analysis from 2020 yielded an estimation of 12 million cases, representing around 4.5% of HBsAg carriers (67). These great discrepancies are explained due to the scarce surveillance, limited access to testing, lack of consensus in diagnostic techniques and inaccuracy of sampling methods for estimates, in which sub-populations at higher risk of HDV are often under-represented.

Epidemiological estimates are often calculated from anti-HDV seropositivity rates among HBV-infected population. However, anti-HDV testing coverage among HBsAg carriers remains poor, even in high-income settings. Despite EASL´s endorsement of universal HDV screening in HBsAg carriers, reported testing coverage in European cohorts ranged from 8% to 40% (68). In the United States, where the AASLD testing recommendations are restricted to high-risk groups, anti-HDV testing coverage was reported in 7% and 19% of HBsAg in two large cohorts (46,69).

Anti-HDV prevalence shows a patchy distribution with geographically-limited high-prevalence areas. These hotspots have been associated with socio-cultural and environmental factors (70). Country-level estimations of anti-HDV prevalence among HBsAg carriers from general population (panel A) and hepatology clinics (panel B) are shown in figure 6 (67).

FIGURE 6. ***Anti-HDV prevalence estimations in (A) general population, (B) hepatology clinics.*** *Modified from Stockdale et al.* (67).



In Europe, the highest prevalence has been observed in Eastern Europe and in vulnerable groups of Russia. Africa and Asia show the highest anti-HDV prevalence.

In Northern Africa, a meta-analysis presented a 5% overall prevalence of anti-HDV, and up to 20% prevalence in patients with liver disease (71). In Sub-Saharan Africa, prevalence in HBsAg carriers was reported close to 8%, with significant variations across countries. West and Central Africa yielded the highest anti-HDV prevalence with 10% and 38% of anti-HDV prevalence in patients with liver disease (72).

Highest prevalence in Asia have been observed in Mongolia (more than 50% of HBsAg-positive subjects) and in Central Asian countries such as Uzbekistan (82%) and Kyrgyzstan (42%). Importantly, reliable nation-wide data in settings with a significant HBV burden such as India, China and Indonesia are currently lacking. In America, exceptionally high anti-HDV seropositivity has been observed in certain isolated populations from the Amazonian region (i.e., 42% in Labrea and 67% in communities from Acre and Purus Rivers in Brazil) or Greenland (Itilleq, 52%).

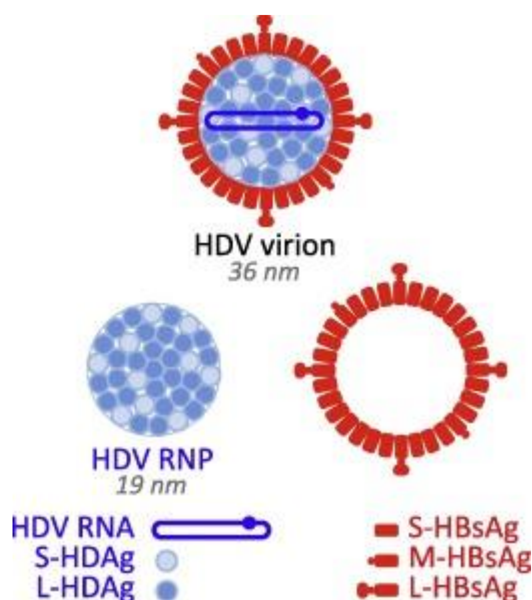
The expansion of immunization against HBV has led to a decrease in HDV prevalence and has altered the epidemiological profile of HDV cases in areas with good vaccination coverage(73). The epidemiological profile of HDV cases in these areas has shifted to a combination of aging subjects with advanced liver disease and high-risk groups such as PWIDs, persons living with HIV (PLHIV) and migrants from highly endemic areas (74).

Although anti-HDV persists as the main epidemiological marker for HDV, a double reflex testing of anti-HDV and HDV-RNA in all HBsAg carriers has been recently proposed as an effective approach to produce more accurate epidemiological estimates of HDV burden in most settings(68). However, further cost-effectiveness evaluation is required for this strategy in HBV endemic settings with low HDV prevalence (75).

1.2.3 Virology and replication cycle

HDV is a satellite viroid-like agent considered the smallest virus affecting animals. It is regarded the only member of the *Deltavirus* genus, reclassified in 2020 by the International Committee in Taxonomy of viruses as part of the *Kolmioviridae* family (76,77). HDV virion is a 36nm particle formed by one copy of around 1.7kb single-stranded circular RNA associated with multiple copies of the non-enzymatic protein HDAg, coated by HBsAg proteins(78). HDV virion structure is represented in figure 7(78).

FIGURE 7. ***Representation of HDV virion***. *Taken from Sureau et al.* (78)



HDV is classified in at least 8 different GTs (1 to 8) that diverge in their RNA size and in up to 35% of their genome sequence (79). These GTs present a differential but changing geographic distribution due to migrations streams. HDV-GT 1 is the most prevalent worldwide, especially in Western Europe and North America, while HDV-GT 2 and 4 are commonly identified in Eastern Europe and East Asia. HDV-GT 3 has

been isolated in the Amazonia, while 5 to 8 GT prevailed in the African continent. HDV-GT might have implications in clinical outcomes(80).

HDV is a defective virus that requires HBV to entry hepatocytes and complete assembly, release, and transmission (61,78). Viral entry is enabled by LHBs binding to NTCP(81). Genomic RNA replication is mediated in the nucleus through the host RNA polymerase II, creating an antigenomic RNA(79). Both genomic and antigenomic RNA have a 100-nucleotide domain that act as a ribozyme with autocatalytic self-cleaving capacity. Genomic RNA encodes for one only protein, HDAg. Post-translational modifications of this protein result in large and small HDAg isoforms (HDAg-L and HDAg-S, respectively) with different roles in the viral cycle. While HDAg-S ensures viral replication enhancing HDV-RNA accumulation, HDAg-L drives HDV assembly with HBV envelope proteins and it is considered essential for HDV entry and secretion(74,79). HBsAg proteins are not required in HDV replication, although they are needed for entry, assembly, and egress of HDV virions. SHBs is sufficient to assist HDV assembly and budding of HDV particles(82). The presence of LHBs seems indispensable for the particles to become infective, since the binding to NTCP is mediated by LHBs preS1 domain (83). Interestingly, envelope proteins produced by integrated HBV-DNA in absence of HBV replication showed to effectively support HDV infectivity (84).

Despite HDV has been exclusively associated with HBV infection in the clinical practice*, in vitro* experiments have shown the capacity of HBV-unrelated enveloped viruses to act as helper viruses for HDV(61,62). Moreover, HCV was observed to spread HDV infection *in vivo* models(64). The clinical implications of these findings remain unexplored.

1.2.4 Natural history of HDV infection

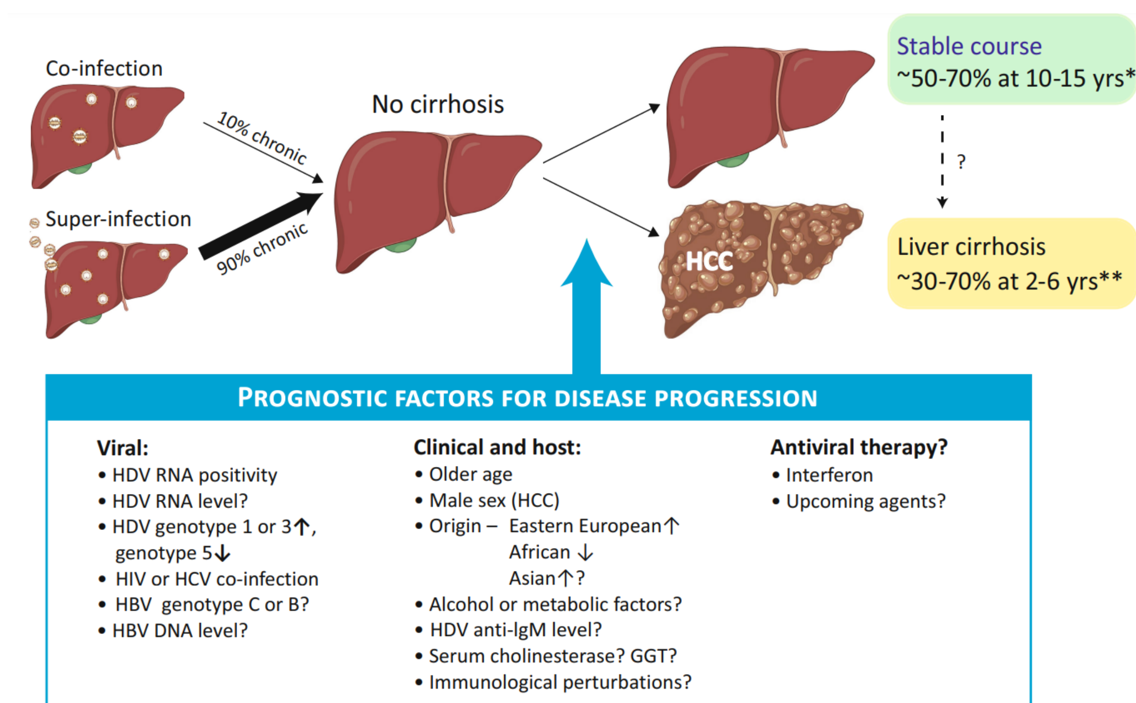
HDV infection can be acquired together with HBV as a coinfection, or as HDV superinfection in subjects previously infected with HBV(80). HDV coinfection leads to a self-limited acute infection in approximately 90% of cases, with low-risk of developing fulminant hepatitis(74,85). In contrast, superinfection with HDV occurs in chronic HBsAg carriers and in a great majority of cases (up to 90%) evolves to chronic hepatitis D (CHD) (66). CHD is associated with a higher risk of cirrhosis and mortality than chronic HBV and HCV monoinfection, and it is still considered the most aggressive form of viral hepatitis (66,86,87).

Large long-term follow-up studies in CHD are scarce and mostly including European cohorts (87–89). It has been argued that these studies, often performed in reference institutions, might exhibit an unaccounted selection bias due to the inclusion of subjects with advanced severe disease that might not represent the outcomes of CHD in the community (85,90). A milder progression of CHD has been documented in some recent studies, probably because of the changing demographic pattern of HDV cohorts and the heterogenicity in terms of risk factors and comorbidities from classic cohorts, in which subjects with other coinfections such as HIV and HCV were more prevalent (85,89).

The contribution of HDV to HCC risk remains controversial. A systematic review and metanalysis with broad inclusion criteria found a higher risk of HCC in subjects with CHD compared to HBV-monoinfection(91). This association was found to be stronger in subjects with HIV coinfection, and in more robust study designs. Further research targeting specific cofounders such as antiviral therapies and underlying liver cirrhosis is required to determine the oncogenic risk of CHD cohorts. Further research is also needed to clarify the impact of HDV-GT in clinical outcomes. HDV-GT 1 has been

linked to severe disease compared to HDV-GT 2 and HDV-GT 5, while HDV-GT 3 has been linked to a fulminant course (92,93). The influence of ethnicity and some unexplored environmental factors might play a role in these divergences(86). HDV natural history is illustrated in figure 8(85).

FIGURE 8. ***HDV natural history.*** *Figure n from Kamal et al.* (85).



1.2.5 Clinical approach and current therapies

The diagnosis of HDV infection relies on the presence of HDV-RNA. The determination of HDV-RNA by polymerase chain reaction (PCR) confirms an active HDV infection. CHD is defined as the persistence of HDV-RNA during 6 months. Due to the documented spontaneous fluctuations of HDV-RNA viremia and the possibility of temporary negativization, a successive determination of HDV-RNA is recommended after 3-6 months to correctly categorize the infection stage (94).

Treatment should be considered in all patients with CHD, regardless fibrosis stage. HDV cure seeks to improve patients´ outcomes through HBsAg clearance and the suppression of HDV replication. In a practical level, the achievement of undetectable HDV-RNA 6 months after treatment has been considered the main treatment goal since it has been associated with a decrease in liver-related events and mortality (95). Antivirals targeting HBV such as NAs have no effect in HDV replication. Off-the-label treatment with IFN alfa was regarded as the only therapeutic alternative in the clinical practice, despite facing significant safety and tolerability challenges and suboptimal efficacy. According to a metanalysis including 13 randomized clinical trials (RCT), monotherapy with peg-IFN alfa only achieved virological response in 29% of subjects, while HBsAg clearance and anti-HBs conversion was only achieved by 1% (96). Late relapse post-IFN alfa treatment even after virological response have been documented in up to around 50% of cases (97,98).

The design of new treatment endpoints for CHD is controversial, and evolves rapidly due to the emergence of new therapeutic agents in the recent years(99). A guideline on treatment endpoints gathering EASL and AASLD experts recommended the extension of HDV-RNA undetectability 1 year after treatment (100). Alternatively, a combination of a decrease of ≥ 2 log in HDV-RNA and ALT normalization was proposed by the expert panel to determine treatment efficacy if HDV-RNA undetectability is not achieved (100). Interestingly, a retrospective multicentric European study including 56 subjects with CHD followed during a mean of 5.6 years reported a spontaneous decline of more than 2 log in a quarter of the cohort, while 20% achieved spontaneous clearance of HDV-RNA(101). Although larger cohorts need to validate these findings, this observation might have implications in the evaluation of HDV therapies.

No specific treatment targeting HDV was approved until 2020, when the European Medicines Agency (European Medicines Agency. conditionally approved a subcutaneous entry inhibitor, bulevirtide (BLV), for treatment of CHD(102). A standard marketing authorization was finally granted in July 2023. Phase II trials have explored BLV efficacy in monotherapy or in combination with IFN and NAs, with promising results in biochemical response and HDV-RNA decline, but no impact in HBsAg titles decline or clearance (103,104). Similar data have emerged from some real-world studies(103,105). Recently published interim results of the open-label phase 3 registration trial showed a higher achievement of the combined endpoint with BLV than with placebo (45-48% against 2%). Differences were also found in undetectable HDV-RNA 48 weeks after treatment, although no subjects presented HBsAg clearance (106).

Other new therapeutic options including drugs targeting either HDV or HBV such as nucleic acid polymers and lonafarnib are currently under research. A phase 3 trial testing the combination of lonafarnib and ritonavir with or without Peg-IFN alpha has recently concluded. Awaiting peer-reviewed analysis, almost 20% of subjects with the three-drug combination achieved the primary combined endpoint of HDV-RNA drop of more than 2log and ALT normalization after 48 weeks, compared to 1.9% of subjects in the placebo arm (107,108).

1.3 CLINICAL USE OF NON-INVASIVE HBV MARKERS

In chronic HBV infection, biomarkers aim to characterized the phase of infection, predict the risk of disease progression, and foresee the response to antiviral treatment. Conventional serum markers in high-income settings include quantitative (q)HBsAg, HBV-DNA levels, ALT and HBeAg, which are used in the clinical practice to define the infection phase. The use of some of these markers has been simplified with point of care tests, with differences in accuracy and availability.

Conventional serum markers are insufficient to express the intrahepatic activity of HBV. Measurement of intrahepatic markers including cccDNA and HBV-RNA has shown to be more accurate for this purpose (109). However, their measurement is not standardized and requires invasive procedures, adding considerable barriers to access in non-specialized facilities or resource-limited settings and a non-negatable risk of complications. Serum HBV-RNA, HBcrAg and HBsAg proteins are considered emerging markers in hepatitis B (109).

1.3.1 HBsAg quantification

While HBsAg quantification in serum was firstly performed more than 40 years ago, automated and affordable essays are accessible since 2010 (110–112). HBsAg originates both from cccDNA and integrated DNA, and constitutes the envelope of HBV infectious virions and non-infectious SVPs (113). Unlike HBV-DNA, the amount of HBsAg seems to express the transcription and translational viral activity, rather than mirroring active replication (114). Besides, the correlation between quantitative HBsAg (qHBsAg) and HBV-DNA seems to be genotype-dependent (115,116). HBsAg quantification assays detect a common S epitope of HBsAg. Whereas they measure HBsAg from both virions and SVP, qHBsAg assays are unable to discriminate the origin of

HBsAg(112). The relative contribution of cccDNA and integrated DNA to qHBsAg is unknown. While HBsAg levels correlate with intrahepatic cccDNA in HBeAg-positive phases, some studies have proposed integrated DNA as the main source of HBsAg in HBeAg-negative subjects based on the lack of correlation between qHBsAg and cccDNA(112,117).

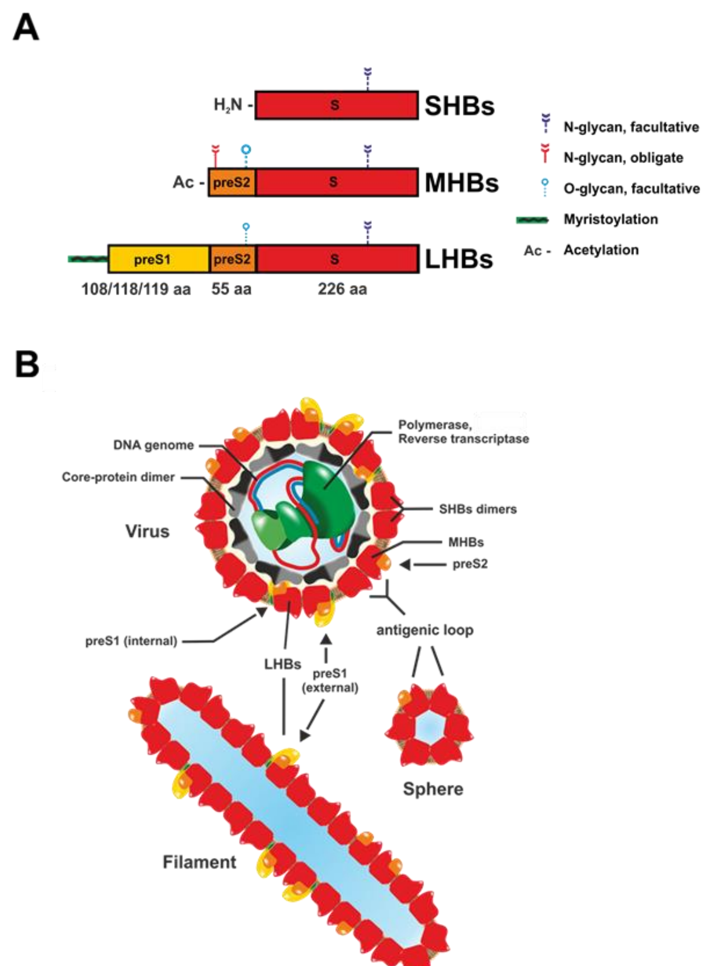
HBsAg levels have been explored as a marker of disease activity and a predictor of treatment response and HCC risk (111). HBeAg-positive subjects exhibit higher levels of HBsAg than HBeAg-negative controls. Similar to HBV-DNA, HBsAg levels have shown to decline along the natural course of chronic HBV, with higher HBsAg/HBV-DNA ratios in HBeAg-negative phases attributed to a greater decline in full virion secretion(111). Declines in HBsAg levels have also been associated with spontaneous HBsAg clearance in untreated HBeAg-negative subjects (118). On the other hand, HBsAg levels have also been postulated as a useful tool to predict and monitor treatment response in subjects under NAs and IFN-based regimes.

Despite these potential applications, qHBsAg significantly differed according to HBV-GT, which considerably hinders the endorsement of universal cut-offs and the widespread of its use(115,119). Hence, clinical models including qHBsAg should be validated in the different HBV-GTs. The distribution of genotype-dependent mutations in precore, basal core promotor and preS region have also been observed to impact in qHBsAg levels (119). On the other hand, the presence of mutations in the preS/S regions and the presence of immune complexes might challenge the measurement of HBsAg(112).

1.3.2 HBsAg proteins

The three HBsAg proteins (SHBs, MHBs and LHBs) are encoded in the S ORF of HBV genome and share a common S domain, to which additional domains are added at N-terminus for MHBs and LHBs (preS2 domain for MHBs and preS2+preS1 domains for LHBs) (figure 9, panel A) (113). All three HBsAg proteins are present in full virions and spheric and filamentous SVPs (Figure 9, panel B) (120). Full virions´ envelope is formed mostly by SHBs in a 4:1:1 ratio compared to MHBs and LHBs(121). In SVPs,

***FIGURE 9*. Structure and location of HBs. (A) Structural differences between the three proteins encoded by the ORF for the HBs proteins. (B) Arrangement of proteins within virions and subviral particles**. *Figure from Rinker et al. (supplementary material)* (113)



the envelope of filamentous particles consists on the three HBsAg proteins, while spherical particles practically lack LHBs and are predominantly formed by SHBs (113,122,123).

Although different proportions of HBsAg proteins in different HBV-GTs have been described *in vitro*, the impact of HBV-GT in HBsAg composition *in vivo* has been poorly studied(124,125). Differences in the levels of HBsAg proteins in HBV-GTs were associated with significant variations in the release of SVP and thus, with variations in secretion ability across GTs(125). Interestingly, in a study with samples from HBeAg-negative subjects, although preS1 and preS2 mutations/deletions altered HBsAg composition *in vitro*, they did not have consequences *in vivo*, supporting a distinct genetic origin of HBsAg composition in SVPs compared to full virions, possibly associated with the integrated genome (126).

The early clearance of preS1 (LHBs) and preS2(MHBs) antigens in subjects with spontaneous HBsAg loss after HBV acute infection was documented by Gerken *et al.* in 1987(127). In the recent years, the measurement of HBsAg components has been postulated as a potential tool to differentiate stages of HBV infection, as well as to predict HBsAg loss under NA and IFN-alfa treatment in HBeAg-positive chronic hepatitis B (128). Lower baseline MHBs and LHBs levels predicted HBsAg clearance in HBeAg-negative IC treated with IFN-alfa(129). In a heterogenous small cohort of HBV and HBV/HDV cirrhotic patients with suppressed HDV-DNA, an early increase in MHBs was observed to identify subjects that developed HCC(130).

Divergent roles of the envelope proteins in the viral cycle have been hypothesized to explain these findings (128). The S domain contains the main antigenic epitope for natural and vaccine-induced immunity(131). LHBs preS1 has been identified as the

attachment site to NTCP, while both preS1 and preS2 regions are thought to be crucial for virion assembly. The over expression of LHBs might cause the intracellular retention of HBsAg, suggesting that specific ratios of the three isoforms are needed for a correct particle secretion(125). The role of MHBs remains uncertain. The presence of T and B cells epitopes have been identified in the preS2 region, and functions related to immunomodulation and particle secretion efficiency have been postulated (132). Viral variants lacking MHBs in CHB support an expendable role of this protein and have been interpreted as an immune adaptation mechanism for the persistence of infection(133). MHBs protein influence in carcinogenesis through modulation of oncogenic genes has been observed *in vitro*(134).

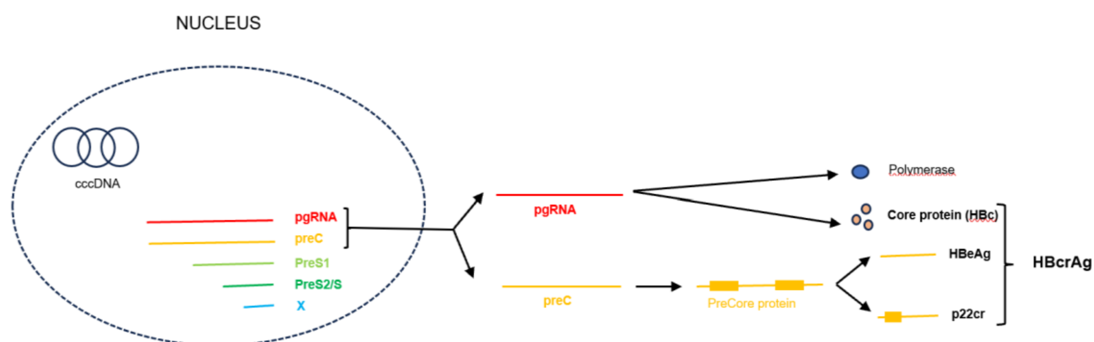
Commercial assays for HBsAg quantification are not able to discriminate and measure the components of HBsAg(112). Due to the common amino acid sequence shared by SHBs, MHBs and LHBs, and the common preS2 domain in MHBs and LHBs, the individual measurement of each protein can only be achieved addressing specific post-translational changes or targeting specific epitopes of preS1(135). Monoclonal antibodies targeting MHBs and LHBs have been used with this purpose. In-house techniques based in enzyme-linked immunoassay (ELISA) or Western Blot using monoclonal antibodies and even mathematical modelling has been used for the estimation of HBV surface proteins(135). The lack of standardization of techniques hampers the comparison and reproducibility of results(135). Pfefferkorn *et al.* characterized an in-house ELISA for the quantification HBsAg proteins using monoclonal antibodies against the previously identified specific epitopes Q19/10 (MHBs) and MA18/7 (LHBs) (136,137). The assay was modified and calibrated from a commercial HBsAg assay, and LHBs and MHBs quantification was validated through Western Blot. MHBs and LHBs were expressed in ng/mL and proportions from total HBsAg. Other approaches

based on establishment of baseline cut-offs of absolute levels of the proteins have been criticized due to the lack of validation of quantification methods(135).

1.3.3 Hepatitis B core-related antigen

The measurement of HBcrAg comprises HBcAg, which results from pgRNA transcription, and two products of the preC mRNA (HBeAg and p22Cr) as represented in figure 10 (138,139). As opposed to HBsAg, which can be originated in integrated genome, HBeAg, HBcAg and p22Cr necessarily stem from cccDNA. The three proteins share a 149 amino acid sequence that can be detected with monoclonal antibodies by chemiluminescence enzyme immunoassay (CLEIA), for which an automated platform is currently available(139). In HBeAg-positive subjects, the vast majority of HBcrAg is formed by HBeAg, while in HBeAg-negative subjects HBcrAg measurement detects p22Cr and HBcAg.

FIGURE 10. Overview of HBcrAg origin. *Modified from Vachon et al.*(138)



HBcrAg levels correlate with serum and intrahepatic HBV-DNA levels, regardless HBeAg status, as well as with intrahepatic cccDNA (139). According to the comprehensive cross-sectional evaluation of serum and intrahepatic markers performed by Testoni *et al.*, serum HBcrAg showed a stronger correlation with intrahepatic HBV-

DNA, cccDNA and pgRNA (140). This study supported the association of HBcrAg levels with transcriptional activity based on the correlation of HBcrAg with pgRNA/cccDNA ratio, not observed with other serum markers. Interestingly, the correlation with intrahepatic cccDNA persists in subjects that achieved viral suppression under antiviral treatment (138,141). Although some studies previously suggested an association between qHBsAg and HBcrAg levels, more recent studies have restricted this association to HBeAg-negative cohorts (139,140,142). This has been explained due to the drop in virion production in the HBeAg-negative phases despite the persistent production of HBsAg from integrated genome.

The determination of HBcrAg in serum has been proposed as a potential tool to characterize the phases of HBV infection (139). HBeAg-positive subjects presented higher HBcrAg, particularly those in the HBeAg-positive CHB phase (140,142–144). In HBeAg-negative subjects, lower levels of HBcrAg have been reported in HBV-IC compared to CHB (139). Some studies have also suggested the use of HBcrAg levels to predict HBeAg seroconversion, while the role in predicting spontaneous HBsAg clearance remains unresolved. HBcrAg levels have also been used to monitor and predict treatment response, both with NA and IFN-based regimes. Regarding HCC risk, higher baseline HBcrAg have been associated with increased risk of disease progression and HCC in HBeAg-negative subjects, even in those under NAs with suppressed viral replication (145–147).

The lower limit of sensitivity of available assays has been postulated as a limitation for HBcrAg measurement (140). Research on performance and validation of specific thresholds in wider and more diverse populations are needed to extend the use of HBcrAg levels in regular clinical practice.

1.3.4 HBV-RNA

Methods for HBV-RNA quantification in serum have mostly targeted pgRNA. In untreated subjects, HBV-RNA levels mirror HBV-DNA(148). Serum HBV-RNA has been explored to determine the phase of HBV infection and to predict response to NAs (149–151). HBV-RNA is postulated as a promising marker for monitoring of response to new agents affecting RNA transcription or stability such as CAMs, siRNA or IFN. Despite these potential clinical applications, HBV-GT, HBeAg status, ALT levels and the presence of mutations in basal core promoter have been reported to impact HBV-RNA correlation with HBV-DNA and HBsAg in a multicentric cohort of untreated subjects with CHB (152). Besides, the detection and measurement of serum HBV-RNA is not well standardized, hindering comparison among studies and its application in the out of the research field.

1.3.5 Non-invasive liver fibrosis markers

Despite serial determinations of conventional serum markers such as ALT, HBV-DNA and HBsAg, a liver biopsy should be performed when the phase of infection cannot be determined. The detection of subjects with significant fibrosis in HBV is crucial, since it implies a direct indication of antiviral treatment, and guides HCC surveillance strategy. Although liver biopsy is still considered the gold standard, the estimation of liver fibrosis has been simplified in the recent years with non-invasive tools, such as the measurement of liver stiffness using transient elastography and non-disease-specific scores like AST-to-platelet ratio (APRI) and fibrosis index-4 (FIB 4)

A systematic review including a comparison between these markers in pooled HCV and HBV cohorts concluded that APRI had a lower performance than FIB-4 and tran-

sient elastography for the determination of fibrosis and cirrhosis (153). The use of Fibrotest (calculated based on alpha-2 macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin and gamma-glutamyl transpeptidase and adjusted by sex and age) was also explored and showed a better performance for the detection of advance fibrosis than transient elastography, without differences in detecting cirrhosis, although faces obvious constraints in terms of a wider application in clinical practice (153).

Transient elastography provides an estimation of the liver fibrosis through a bedside, non-invasive, painless, and highly reproductible measurement of the liver stiffness(154,155). It was developed in 2003, addressing fibrosis estimation in subjects infected by HCV, and it use rapidly spread to other liver conditions(156). Liver stiffness measurement (LSM) has a good correlation with liver fibrosis in chronic HBV. However, the determination of optimal cut-offs has been challenging due to a considerable overlap among consecutive fibrosis stages. On the other hand, elevating ALT values might falsely increase LSM, hampering the interpretation of LSM in subjects with ALT oscillations and flares, common in HBV natural history. A dual cut-off system was proposed by Viganò *et al.* to exclude and identify significant fibrosis (<6.4kPa and >9.4kPa) and cirrhosis (<9.4kPa and >13.1 kPa) in chronic hepatitis B (157). Similar cut-offs for hepatitis B were endorsed by EASL and the Latin-American Association for the study of the Liver in 2015, used in combination with ALT levels and recommending considering liver biopsy for intermediate values (from 6kPa to 9-12kPa according to ALT values)(154).

1.4 CLINICAL USE OF HDV BIOMARKERS

1.4.1 HDV-RNA

HDV-RNA active replication seems to be associated with the risk of clinical events. Detectable HDV-RNA was associated with clinical outcomes in a multicentric Spanish cohort of 118 anti-HDV subjects, after a follow-up of 8 years (94). Subjects with persistent detectable HDV-RNA had a higher risk of liver decompensation, liver transplantation and liver-related mortality compared to those who achieved undetectable HDV-RNA during follow-up (94). The association between detectable HDV-RNA and unfavorable liver-related outcomes have also been observed in HIV coinfected cohorts (158). The association between detectable HDV-RNA and HCC development remains controversial (94,158).

The first international standard for HDV-RNA quantification was developed by WHO in 2013, allowing a better comparison and harmonization of results (159). However, an International External Quality Assessment performed in 2016 including almost all available in-house and commercial tests reported a considerable heterogenicity of results, with less than half of laboratories providing a proper quantification in all samples (160). These results emphasize the need of externally validated, fully automated assays for HDV-RNA quantification.

Few HDV biomarkers other than HDV-RNA have been explored in CHD. The detection of anti-HDV IgM in serum was used before HDV-RNA to diagnose acute HDV infection, although it might remain detectable in CHD cases and even reappear after IFN therapy. Anti-HDV IgM levels were observed to correlate with inflammatory activity and predict treatment response to IFN (161). The presence of anti-HDV IgM was related to fewer clinical events after a median follow-up of 3 years in a multicentric

retrospective study including 78 subjects (162). However, the sensitivity to identify subjects with a benign course of infection was limited, and the use of IgM is currently mostly conceived as a surrogate marker of HDV replication (162).

1.4.2 HBV markers in Chronic Hepatitis D

Due to the defective nature of HDV, some HBV markers have been also examined as markers for hepatitis D infection. HBV replication in CHD is inhibited by the suppression of HBV enhancers and the activation of IFN-alfa related genes, which limits the application of HBV-DNA as a valid marker in CHD (163). HBsAg levels have been proposed as a surrogate marker for HDV replication due to the correlation between qHBsAg and HDV-RNA (164). A retrospective multicentric European cohort of HDV-GT 1 and HBV-GT D studied the association between HBV and HDV markers. In this study, higher HDV-RNA levels were correlated with higher qHBsAg and HBcrAg levels, suggesting that transcriptional activity of HBV cccDNA in CHD is maintained despite the inhibition of HBV replication (165).

Regarding HBsAg proteins in HDV-infected subjects, data on HBsAg components in CHD patients are very limited. The preS1 sequence of LHBs is required for HDV virion infectivity. *In vitro* research has shown that plasmids containing only SHBs-encoding sequence were able to assist HDV assembly particles, concluding that only this isoform is essential for HDV packaging (82). Although HBV-GTs A-J have shown to be able to support HDV assembly and infectivity, HBV-GT-dependent variations in HBsAg proteins excretion seem to also affect HDV cell entry and virion secretion (125,166).

A distinctive envelope composition in HDV particles was suggested in a small group of 11 subjects with HBV/HDV coinfection, in which HDV/HBV presented a higher LHBs

proportion than CHB subjects, while a higher MHBs proportion was also observed but did not reach statistical significancy(137). The potential role of HBsAg proteins in the natural history and clinical outcomes of CHD has not been explored.

2 HYPOTHESIS

This doctoral thesis includes two publications addressing the clinical application of non-invasive markers in chronic hepatitis B and chronic hepatitis D infections.

A proper classification of HBV-infected subjects is essential to identify not only individuals at increased risk of disease progression, but also those in the inactive phases of HBV infection. The accurate identification of these subjects would allow the implementation of less intensive management strategies, avoiding the performance of liver biopsies in certain subjects.

On the other hand, the categorization of emerging HBV markers in HDV infection is crucial for a deeper understanding of the interplay between HDV and HBV. Due to the scarcity of prognosis indicators in natural history of CHD, the role of these markers to predict unfavorable clinical outcomes should be explored.

The following hypothesis are proposed:

- Non-invasive markers could accurately identify HBV-IC among HBeAg-negative chronic HBsAg carriers.
- In CHD, HBsAg protein composition might differ according to HDV-RNA and correlate with liver-related outcomes.

3 AIMS

This doctoral thesis approaches the clinical use of new biomarkers in chronic hepatitis B and D infections. Its main goals are to explore the identification of HBV-IC among chronic HBsAg carriers with non-invasive markers, and to describe HBsAg isoforms as a clinical marker in chronic hepatitis D infection.

The secondary aims are:

- To develop and validate a score that allows an accurate identification of HBV-IC among HBeAg-negative chronic HBsAg carriers in a single point in time.
- To validate a differential HBsAg protein composition in HBV-IC among HBeAg-negative chronic HBV phases.
- To assess the impact of HBV genotype in HBsAg protein composition among HBeAg-negative chronic HBsAg carriers.
- To describe the differences in HBsAg protein composition in untreated subjects with CHD according to the presence of detectable HDV-RNA and its potential association with later development of liver-related outcomes

4 COMPENDIUM OF ARTICLES

4.1 METHODOLOGY SUMMARY

The first study was a retrospective-prospective cohort study performed in a university hospital in Barcelona, Spain. Subjects aged over 16 years with HBsAg documented for at least 6 months who attended the outpatient department between July 2013 and December 2019 were included. Demographic, clinical, and anthropometric variables were collected in the first medical visit. Laboratory data and non-invasive markers of liver fibrosis were recorded at baseline, 6 months, and yearly. Hepatitis B infection parameters included commercial assays for qHBsAg (electrochemiluminescence, COBAS 8000; Roche Diagnostics, Rotkreuz, Switzerland, lower limit of quantification [LLQ] 0.05 IU/mL) and HBV-DNA quantification by PCR (COBAS 6800; Roche Diagnostics, Manheim, Germany; LLQ of 20 IU/mL, lower limit of detection [LLD] of 10 IU/mL). HBcrAg was performed by CLEIA (Lumipulse G HBcrAg assay; Fujirebio, Gent, Belgium) with a LLD of 3 logU/mL. HBV genotyping was performed by Sanger sequencing after amplification of two different viral regions PreC/Core (nucleotides 1,774–2,389, 615 bp) and PreS/Surface (nucleotides 2,828–176,561 bp). Non-invasive fibrosis markers included LSM (FibroScan®) and the serum biomarkers FIB-4 and APRI. According to the one-time assessment at the first visit, subjects were pre-classified in three groups:

- Normal ALT and HBV-DNA <2,000 IU/mL;
- ALT > two-fold upper limit of normal (ULN) and HBV-DNA > 20,000 IU/mL;
- Subjects who did not fulfil any of the above conditions.

Subjects with at least one follow-up visit were reclassified, according to serum ALT, HBV-DNA levels, and liver fibrosis stage by histological sample when needed, following EASL 2017 Clinical Practice Guidelines (34).

The second publication approaches HBsAg protein composition in two sub-studies including a hepatitis B and a hepatitis D cohort, respectively. In the hepatitis B cohort, a cross-sectional analysis was carried out in patients with naïve HBeAg-negative chronic HBV infection or hepatitis from the Spanish institution. HBeAg-negative subjects were categorized according to previous studies in HBeAg-negative chronic infection/ICs (HBV-DNA<2,000 IU/mL and persistently normal ALT with normal liver ultrasound) and HBeAg-negative CHB (HBV-DNA>2,000IU/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 viremic active carriers -LV-AC (HBV-DNA between 2,000 and 20,000 IU/mL and persistently normal ALT in absence of significant fibrosis in liver biopsy).

In the hepatitis delta 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. Patients with a minimum follow-up of 1 year were included in a retrospective-prospective longitudinal study to assess the potential impact of HBsAg proteins composition in development of clinical events. Moreover, changes in HBsAg proteins composition were explored in those subjects with samples more than 5 years apart. CHD was defined by the presence of HBsAg and anti-HDV antibodies for more than 6 months. Subjects with undetectable HDV-RNA were included, if evidence of previous detectable HDV-RNA was available. Patients who received IFN in the last 12 months prior to and during the study were excluded. Longitudinal follow-up included clinical data regarding liver-related decompensation

(ascites, liver encephalopathy, variceal bleeding), HCC, liver transplantation and all-cause mortality.

Demographic and clinical features were recorded in all patients. Clinical information and history of antiviral treatment were collected retrospectively through medical records. Laboratory parameters included platelet count, biochemical panel with liver enzymes, serological (qHBsAg and HBeAg) and virological tests (HBV and HDV viral load). HDV-RNA was measured by in-house quantitative PCR. MHBs and LHBs proteins were measured by ELISA. LHBs and MHBs were quantified in triplicates using well-defined monoclonal antibodies against the preS1-domain (Ma18/7) and the N-glycosylated preS2-domain (Q19/10), respectively, as previously reported(137). SHBs values in ng/mL were obtained after subtracting LHBs and MHBs values from total qHBsAg (ng/mL).

4.2 ARTICLE 1.

ACE Score Identifies HBeAg-negative Inactive Carriers at a Single-point Evaluation, regardless of HBV genotype

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Original Article

ACE Score Identifies HBeAg-negative Inactive Carriers at a Single-point Evaluation, Regardless of HBV Genotype

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Abstract

Background and Aims: Hepatitis B virus (HBV) biomarkers have been used for a better categorization of patients, even though the lack of simple algorithms and the impact of genotypes limit their application. Our aim was to assess the usefulness of noninvasive markers for the identification of HBV inactive carriers (ICs) in a single-point evaluation and to design a predictive model for their identification. ***Methods:*** This retrospective-prospective study included 343 consecutive HBeAg-negative individuals. Clinical, analytical, and virological data were collected, and a liver biopsy was performed if needed. Subjects were classified at the end of follow-up as ICs, chronic hepatitis B and gray zone.A predictive model was constructed, and validated by 1000-bootstrap samples. ***Results:*** After 39 months of follow-up, 298 subjects were ICs, 36 were chronic hepatitis B CHB, and nine were gray zone. Eighty-nine (25.9%) individuals required a liver biopsy. Baseline HBV DNA hazard ratio (HR) 6.0, $p<0.001$), HBV core-related antigen (HBcrAg) (HR 6.5, $p<0.001$), and elastography (HR 4.6, $p<0.001$) were independently associated with the IC stage. The ACE score (HBV DNA, HBcrAg, elastography), obtained by bootstrapping, yielded an area under the receiver operating characteristics (AUROC) of 0.925 (95% CI: 0.880–0.970, $p<0.001$) for identification of ICs. The AUROC for genotype D was 0.95, 0.96 for A, 0.90 for E, and 0.88 for H/F. An ACE score of <1 had a positive predictive value of 99.5%, and a score ≤12 points had a diagnostic accuracy of 93.8%. ***Conclusions:*** Low baseline HBV DNA, HBcrAg, and liver stiffness were independently associated with the IC phase. A score including those variables identified ICs at a single-point evaluation, and might be applied to implement less intensive follow-up strategies.

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Introduction

Chronic hepatitis B infection affects 296 million people worldwide according to the World Health Organization.[1] The course of chronic hepatitis B infection is described in different phases as a result of complex and dynamic interactions between the host immunity and the viral particles.[2] Hepatitis B e antigen (HBeAg)-negative stage represents the vast majority of chronic HBV infections in Western countries.[3,4] The risk of cirrhosis, liver cancer, decompensation, and liver-related death dramatically varies throughout the HBeAg-negative infection phases, which are not always consecutive and whose duration may differ among individuals. Assessment of HBV infection by alanine aminotransferase (ALT) and HBV viral load is not always enough to correctly categorize the disease stage because of frequent fluctuations of both markers. Noninvasive tools such as serum markers and liver stiffness have been tested as complementary information to determine HBV infection

Keywords: Hepatitis B virus; Inactive carrier; Liver stiffness; HBV DNA; Quantitative HBsAg; Core-related antigen.

Abbreviations: ALT, alanine aminotransferase; APRI, AST-to-platelet ratio index; AST, aspartate aminotransferase; AUROC, area under the receiver operating characteristic; CHB, chronic hepatitis B; C-index, concordance index; FIB-4, fibrosis-4 index; GGT, gamma glutamyl transferase; GZ, gray zone; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HR, hazard ratio; IC, inactive carriers; IQR, interquartile range; qHBsAg, quantitative hepatitis B surface antigen; LLD, lower limit of detection; LLQ, lower limit of quantification; LSM, liver stiffness measurement; ULN, upper limit of normal.

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phase.[5,6] However, the accuracy of these markers is not well standardized and some of them, such as quantification of hepatitis B surface antigen (HBsAg), have been shown to be deeply influenced by the HBV genotype.[7,8] These factors make difficult an accurate assessment and prediction of the long-term outcome of HBeAg-negative subjects by a single evaluation. As a result, a follow-up of three medical visits within the first year is currently recommended to define the phase of infection.[9] Although that approach reflects the changing character of the infection, one-point assessments should be developed in real-life cohorts to facilitate decentralized models of care and simplified algorithms. One-point assessments are especially important because of the endemicity of HBV infection in vulnerable populations and in low and middle-resource regions such as Sub-Saharan Africa, the Western Pacific, and Southeast Asia, which account for more than 80% of infections worldwide.[1,10] A proper classification is essential to identify not only individuals at increased risk of disease progression, but also those in an inactive phase of infection who would benefit from less intensive management strategies. The aim of this study was to assess the usefulness of noninvasive markers to identify subjects with HBeAg-negative chronic infection, namely former inactive carriers (ICs), and to develop a predictive model for early identification of these subjects in a single-point evaluation.

Methods

A retrospective-prospective cohort study was performed in a university hospital in Barcelona, Spain. Subjects aged over 16 years of age with HBsAg documented for at least 6 months who attended the outpatient department between July 2013 and December 2019 were included. Subjects who were lost follow-up after the first medical visit were excluded. Subjects who tested positive for HBeAg at the first visit and/or with hepatitis C virus (HCV), hepatitis D virus, and human immunodeficiency virus coinfection defined as positivity for both serology and viral load, history of alcohol abuse and/or evidence of autoimmune liver disease were also excluded. Demographic, clinical, and anthropometric variables were collected in the first medical visit. Laboratory data and noninvasive markers of liver fibrosis were recorded at baseline, 6 months, and yearly. Hepatitis B infection parameters included quantitative HBsAg (qHBsAg). The lower limit of quantification (LLQ) was 0.05 IU/mL, HBeAg, and anti-HBe, all tested by commercially available electrochemiluminescence immunoassays (COBAS 8000; Roche Diagnostics, Rotkreuz, Switzerland). Serum HBV DNA was measured with a commercial PCR assay that had an LLQ of 20 IU/mL and lower limit of detection (LLD) of 10 IU/mL (COBAS 6800; Roche Diagnostics, Manheim, Germany). HBV core-related antigen (HBcrAg) was measured with a chemiluminescent enzyme immunoassay (CLIA, Lumipulse G HBcrAg assay; Fujirebio, Gent, Belgium) that had an LLD of 3 logU/mL. For HBV genotyping, HBV DNA was first enriched by ultracentrifugation of 9.6 mL of serum and Sanger sequencing was carried out after amplification of two different viral regions, PreC/Core (nucleotides 1,774–2,389, 615 bp) and PreS/Surface (nucleotides 2,828–176,561 bp), as previously described.[11] Genotypes H and F were combined because of their phylogenetic proximity and similar geographic distribution.[11,12] Noninvasive fibrosis markers included liver stiffness measurement-LSM (FibroScan) and the serum biomarkers Fibrosis-4 index (FIB-4) and the aspartate aminotransferase (AST)-to-platelet ratio index (APRI).

According to the one-time assessment at the first study visit, HBeAg-negative subjects were preclassified in three groups:

- Normal ALT and HBV DNA <2,000 IU/mL;
- ALT > two-fold upper limit of normal (ULN) and HBV DNA > 20,000 IU/mL;
- Subjects who did not fulfill any of the above conditions.

The ALT ULN was defined by local reference laboratory values of 35 IU/mL for women and 50 IU/mL for men. Subjects were followed by different hepatologists according to the same protocol. Following the guideline recommendations, liver biopsies were performed in subjects with HBV DNA persistently above 2,000 IU/mL and normal ALT or ALT <2-fold the ULN during follow-up.[13] Liver specimens were read by the same pathologist. Significant fibrosis was established in fibrosis stage ≥3 according to the Ishak score.[14] Subjects with at least one follow-up visit were reclassified, taking serum ALT, HBV DNA levels into consideration, and liver fibrosis stage by histological sample when needed, following European Association for the Study of the Liver 2017 Clinical Practice Guidelines.[13,14]

Chronic HBeAg-negative infection ICs was persistently normal ALT and HBV DNA of <2,000 IU/mL or HBV DNA between 2,000–20,000 IU/mL in the absence of significant fibrosis in liver biopsy.

Chronic HBeAg-negative hepatitis (CHB) was elevated ALT and HBV DNA >2,000 IU/mL, and/or significant fibrosis.

Gray zone (GZ) was persistently normal ALT and HBV DNA >20,000 IU/mL in the absence of significant fibrosis.

Liver cirrhosis was diagnosed by either imaging findings (irregular liver surface and direct/indirect signs of portal hypertension) or liver histology with an Ishak fibrosis score of 5–6. Subjects with liver ultrasound and/or histological signs of liver cirrhosis were considered as having CHB infection, regardless of their ALT and HBV DNA levels. Supplementary Figure 1 summarizes the study design. Participant data were anonymized and informed consent was waived because of the study design. The preparation of this manuscript was performed following STROBE guidelines.

Statistical analysis

Quantitative variables with a normal distribution were reported as means and standard deviation. Non-normally distributed quantitative variables were reported as medians and interquartile range (IQR). Comparisons were performed with Student *t*-test and Mann-Whitney U-test. Categorical variables were described as absolute and relative frequencies (percentages, %) and compared with chi-square or Fisher's exact tests in case of relative frequencies below 5%. Baseline variables that had a clinically and statistically significant association to the outcome in univariate analysis (Mantel–Cox test) were selected for the initial models ($p<0.10$). The final models were obtained by a stepwise forward method based on model likelihood ratios (Cox regression). The same significance level ($p<0.05$) was set for including and discarding variables. Quantitative variables included in the models were categorized by clinically significant cutoffs in order to increase the power. The model obtained was calibrated by a 1000-bootstrapping analysis to minimize overfit bias.[15] A weighted semiquantitative score was constructed based on the final model. The score for each variable reflected the risk coefficient obtained after the bootstrapping analysis. The discrimination performance of the obtained predictive models was evaluated with receiver operating characteristic (ROC) curve analysis and the concordance index (C-index). The cutoff values were selected considering the highest Youden's index, and expressed as sensitivity, specificity, and predictive value. The results were considered statistically significant when the *p*-value was <0.05. The statistical analysis

Table 1. Baseline characteristics in the overall cohort and the final classification

	Overall (n=343)	Inactive carriers (n=298)	Gray zone (n=9)	Chronic hepatitis B (n=36)	p-value
Male gender	203 (59.2%)	173 (58.1%)	4 (44.4%)	26 (72.2%)	0.174
Age (years)	44.5±14.6	44.9±14.4	38.2±17.5	43.4±15.1	0.360
Ethnicity					
Caucasian	217 (63.3%)	194 (65.1%)	4 (44.4%)	19 (52.8%)	0.060
Black	79 (23.0%)	63 (21.1%)	5 (55.6%)	11 (30.6%)	0.060
Asian	23 (6.7%)	18 (6.0%)	0 (0%)	5 (13.9%)	0.060
Hispanic	24 (7.0%)	23 (7.7%)	0 (0%)	1 (2.8%)	0.060
Comorbidities					
Obesity	60 (17.5%)	56 (23.0%)	1 (11.1%)	3 (10.7%)	0.240
Dyslipidemia	53 (15.5%)	51 (17.5%	1 (11.1%)	1 (2.8%)	0.075
Arterial hypertension	53 (15.5%)	48 (16.1%)	1 (11.1%)	4 (11.1%)	0.685
Diabetes mellitus	14 (4.1%)	11 (3.7%)	–	3 (8.3%)	0.339
Liver cirrhosis	8 (2.3%)	–	–	8 (22.2%)	<0.001
Platelets ($\times 10^9/mm^3$)	225±58,000	228±57	245±58	198±58	0.009
ALT (IU/L)	28±21	25±11	29±15	56±46	<0.001
HBV DNA (log IU/mL)	2.8±1.2	2.6±1.0	3.4±0.9	4.4±1.3	<0.001
qHBsAg (log IU/mL)	3.1±1.1	3.0±1.1	3.9±0.6	3.7±0.6	<0.001
qHBsAg >1,000 IU/mL[1]	205 (60.5%)	168 (56.9%)	8 (88.9%)	29 (82.9%)	0.003
HBcrAg (log U/mL)[2]					
<3 logU/mL	274 (79.9%)	258 (91.5%)	4 (44.4%)	12 (37.5%)	<0.001
3–4 logU/mL	38 (11.1%)	23 (8.2%)	4 (44.4%)	11(34.4%)	<0.001
4–5 logU/mL	8 (2.3%)	1(0.4%)	1 (11.1%)	6 (18.8%)	<0.001
>5 logU/mL	3 (0.9%)	–	–	3 (9.4%)	<0.001
Genotype[3]					
D	102 (40.8%)	93 (43.1%)	2 (22.2%)	7 (28.0%)	0.209
A	68 (27.2%)	61 (28.2%)	2 (22.2%)	5 (20.0%)	0.209
E	40 (16.0%)	31 (14.4%)	3 (33.3%)	6 (24.0%)	0.209
F/H	26 (10.4%)	20 (9.3%)	2 (22.2%)	4 (16.0%)	0.209
B/C	10 (4.0%)	7 (3.2%)	–	3 (12.0%)	0.209
Mixed	4 (1.6%)	4 (1.9%)	–	–	0.209
Elastography (kPa)	5.6±2.3	5.2±1.7	6.9±1.5	8.2±4.3	<0.001
FIB-4	0.5±0.4	0.5±0.4	0.4±0.2	0.5±0.7	0.617
APRI	0.5±0.4	0.4±0.2	0.5±0.3	0.9±0.9	<0.001

Categorical variables are *n* (%), quantitative variables are means ± SD. [1]qHBsAg was available in 339 subjects of the overall cohort (295 inactive carriers, nine gray zone, 35 chronic hepatitis); [2]HBcrAg was available in 323 subjects; [3]HBV-genotype was available in 250 subjects of the overall cohort (216 inactive carriers, nine gray zone, 25 chronic hepatitis B). ALT, alanine aminotransferase; APRI, ALT to platelet ratio index; FIB-4, fibrosis-4 index; HBV, hepatitis B virus; HBcrAg, hepatitis B core-related antigen; qHBsAg, quantitative hepatitis B surface antigen.

were performed with IBM SPSS, version 26.0 (IBM Corp, Armonk, NY, USA).

Results

Baseline characteristics

Three hundred forty-three consecutive subjects were included (Table 1). Most were male (59.2%), Caucasian (63.3%), and the mean age was 45 years. Black individuals represented a considerable percentage of the overall cohort (23.0%), most of them coming from Western African countries, and 179 subjects (52.1%) were immigrants from non-Western European regions. The HBV genotype was determined in 250 individuals; D and A were the most prevalent, followed by E and H/F. Figure 1 summarizes the country of origin and the most prevalent HBV genotypes among immigrants. At the first visit, most subjects (68.8%) had a

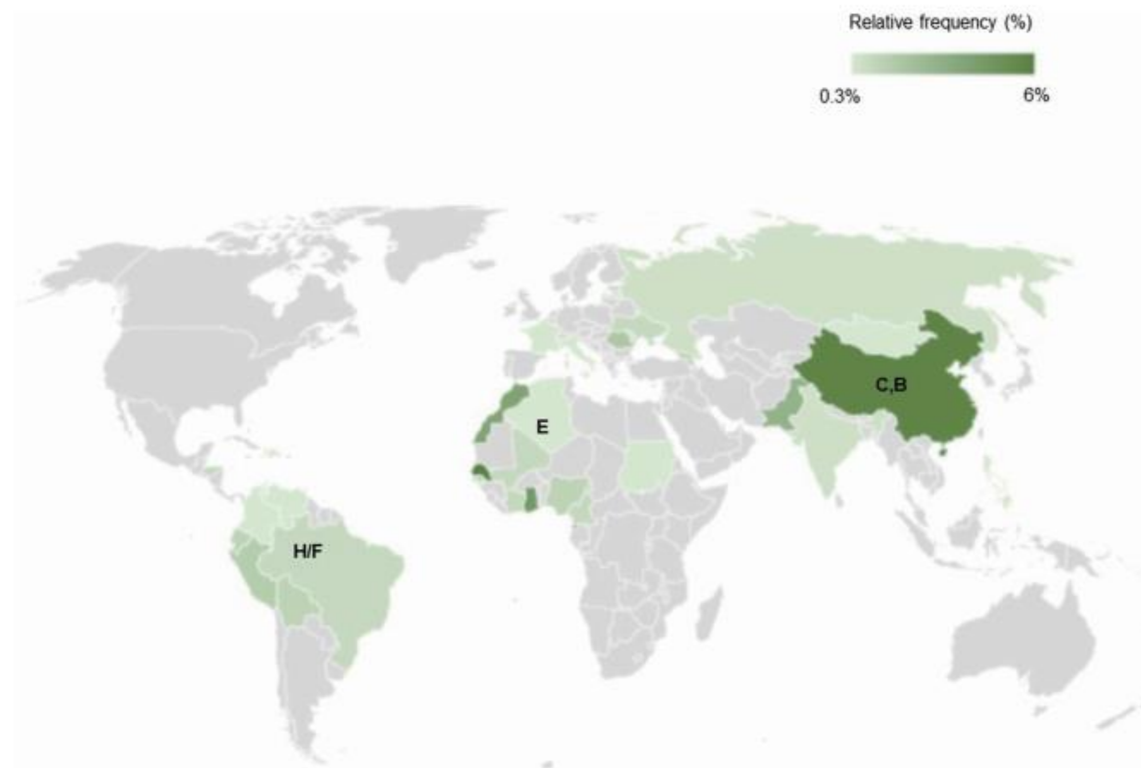

Fig. 1. Country of origin and most prevalent HBV genotype of immigrants in the overall cohort (by relative frequency). HBV, hepatitis B virus.

normal ALT and HBV DNA <2,000 IU/mL, five (1.5%) had an ALT >2× the ULN and HBV DNA >20,000 IU/mL, and 102 (29.7%) did not fulfill the conditions for any of the above categories (Fig. 1).

Clinical follow-up and liver biopsy

Two hundred fifty-four (74.1%) subjects were classified by a noninvasive approach, and liver biopsies were needed in 89 (25.9%) for proper classification of the HBV phase. Twenty-eight (12.1%) of two hundred thirty-one subjects presented with baseline normal ALT and an HBV DNA <2,000 IU/mL, and sixty-one (61.0%) of 100 were initially considered as GZ. The median time from the first visit to liver biopsy was 8.4 months (IQR 2.2–15.3). Nine out of 89 (10.1%) subjects presented significant fibrosis (≥F3), whereas 73 (82.0%) presented Ishak score below F1. Table 2 summarizes baseline features by fibrosis stage, in subjects who underwent invasive management. Significant fibrosis was significantly more frequent in males, whereas no differences were found in baseline ALT, qHBsAg and HBV-DNA levels. However, significantly higher baseline HBcrAg levels were observed in subjects with significant fibrosis ($p<0.001$). LSM tended to be lower among subjects without significant fibrosis ($p=0.085$). However, when categorizing liver stiffness measurements with a double cutoff system (i.e. below 6.5 kPa, between 6.5 and 9 kPa and above 9 kPa), a significant association was found between elastography categories and the presence of significant fibrosis in liver samples ($p=0.05$, Supplementary Fig. 2).[5,16] FIB-4 values did not differ in the presence of significant fibrosis, but those with ≥F3 had higher APRI levels ($p=0.048$). Multivariate analysis found that LSM categories (HR 3.37, 95% CI: 1.08–10.49, $p=0.037$) and baseline HBcrAg (HR 3.615, 95% CI: 1.41–9.25, $p=0.007$) were independently associated with significant fibrosis.

Classification of the HBV phase during follow-up

Subjects were classified after a mean follow-up of 39.0 months. Figure 2 shows the changes from the initial to the final classification. At follow-up, 298 subjects were considered ICs, 36 patients were found to have CHB, and nine subjects remained in in the GZ, all had viral loads persistently over 20,000 UI/mL, normal ALT, and non-significant fibrosis on liver biopsy. Of the 236 subjects with normal ALT and HBV DNA <2,000 IU/mL, 230 (97.5%) were considered ICs during follow-up and six (2.5%) were regarded as CHB after the histological assessment. Of 102 subjects in the GZ group after the one-point assessment at baseline, 68 (66.7%) were finally classified as ICs and 25 (24.5%) as CHB. All five subjects with initial ALT levels >2 times the ULN and HBV DNA >20,000 IU/mL remained a the CHB stage.

Baseline features by final classification are summarized in Table 1. Ethnic distribution tended to differ ($p=0.060$), with a higher proportion of black individuals in the GZ (55.6%). The prevalence of transmission pathways, toxic habits,

Table 2. Baseline features of individuals who required a liver biopsy during follow-up for proper classification of HBV infection

	Non-significant fibrosis (<F3), (n=80)	Significant fibrosis (≥F3), (n=9)	Univariate analysis
			p-value
Male gender	39 (48.8%)	8 (88.9%)	0.023
Age (years)	44±13	41±15	0.510
Ethnicity			
Caucasian	48 (60%)	5 (55.6%)	0.832
Black	21 (26.3%)	3 (33.3%)	0.832
Asian	6 (7.5%)	1 (11.1%)	0.832
Hispanic	5 (6.3%)	0 (0%)	0.832
Platelets (×10^9/mL)	223±56	203±35	0.295
ALT (IU/L)	30±16	39±9	0.130
qHBsAg (log IU/mL)	3.6±0.7	3.5±0.6	0.673
qHBsAg >1,000 IU/mL	63 (79.7%)	8 (88.9%)	0.447
HBcrAg (log U/mL)			
<3 logU/mL	64 (81.0%)	5 (62.5%)	
3–4 logU/mL	11 (13.9%)	–	
4–5 logU/mL	4 (5.1%)	–	
>5 logU/mL	–	3 (37.5%)	
HBV DNA (log IU/mL)	3.6±0.9	4.2±1.3	0.079
Genotype			
D	23 (37.1%)	2 (40%)	0.139
A	16 (25.8%)	0 (0%)	0.139
B/C	3 (4.8%)	0 (0%)	0.139
F/H	10 (16.1%)	0 (0%)	0.139
E	10 (16.1%)	3 (60%)	0.139
Elastography (kPa)	6.1±2.3	7.5±1.9	0.085
FIB-4	0.4±0.2	0.3±0.1	0.092
APRI	0.4±0.2	0.6±0.2	0.048

Categorical variables are n (%), quantitative variables are means±standard deviation. ALT, alanine aminotransferase; APRI, ALT to platelet ratio index; FIB-4, fibrosis-4 index; HBcrAg, hepatitis B core-related antigen; qHBsAg, quantitative hepatitis B surface antigen; HBV, hepatitis B virus.

and comorbidities (i.e. diabetes mellitus, arterial hypertension, and dyslipidemia) was similar in all three phases of infection. Significant differences were found in baseline ALT (p<0.001) HBV DNA (p<0.001), qHBsAg (p<0.001) and HBcrAg levels (p<0.001), as well as LSM and APRI at first visit; no differences were found in FIB-4 score (p=0.617). Genotype distribution was not significantly different in the three phases of infection (p=0.209).

Markers for identification of HBV ICs

Baseline markers associated with IC in the univariate analysis were lower levels of ALT, AST, gamma glutamyl transferase (GGT), higher platelet count, lower HBcrAg, HBV DNA, and LSM. Independent association was confirmed in the multivariate analysis between the IC group and lower HBV DNA, HBcrAg levels and LSM (Table 3). Risk coefficients similar to those obtained by multivariate analysis were obtained by 1000 bootstrapping samples, HR 6.0 (95% CI: 3.0–12.0), p<0.001 for categorized HBV DNA, HR 4.6 (95% CI: 2.3–9.0), p<0.001, for LSM; and HR 6.5 (95% CI: 2.7–15.7, p<0.001) for HBcrAg. An ROC model based on these coefficients yielded an AUROC of 0.925 (95% CI: 0.880–0.970, p<0.001) for the identification of ICs (Fig. 3). The model was validated in most prevalent HBV genotypes and had an area under ROC (AUROC) of 0.95 for genotype D, 0.96 for A, 0.90 for E and 0.88 for H/F (Table 4). An individual-score system, the ACE score, was constructed from simplified coefficients in the bootstrapping analysis (Table 5) and included HBV DNA, HBCore-related antigen, and liver elastography). The ACE score had the highest positive predictive value for identification of ICs for patients with punctuations <1 point, and 12 points was the cutoff with the greatest diagnostic accuracy (93.8%). The accuracy of the different cutoffs of the ACE score are summarized in Table 6.

Discussion

Herein we identified baseline LSM, HBV DNA, and HBcrAg

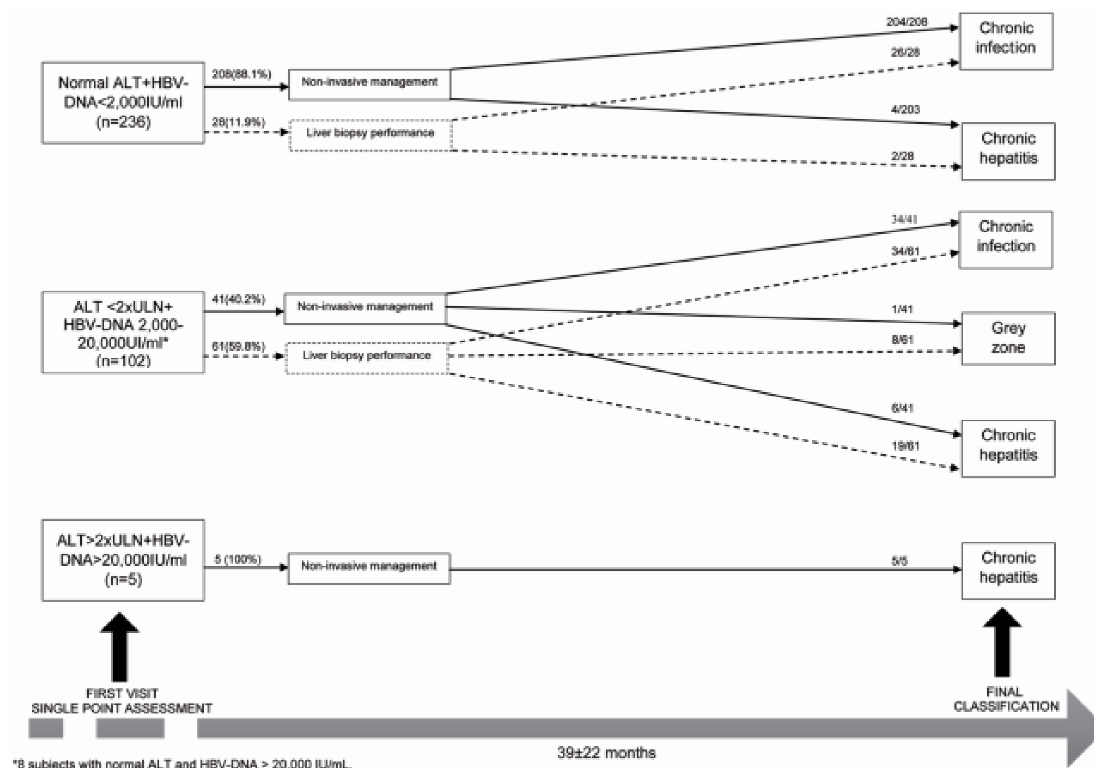

Fig. 2. HBV infection phase evolution and liver biopsy performance during follow-up. *Liver biopsy indication was established by normal ALT plus HBV DNA persistently above 2,000 IU/mL, or ALT <2-fold ULN plus viral load above 2,000 IU/mL during follow-up. **Final classification was carried out according to European Association for the Study of the Liver 2017 Clinical Practice Guidelines.[16] Chronic HBeAg-negative infection-inactive carriers had persistently normal ALT plus HBV DNA <2,000 IU/mL or HBV DNA between 2,000 and 20,000 IU/mL in the absence of significant fibrosis in liver biopsy. Chronic HBeAg negative hepatitis required elevated ALT and HBV DNA >2,000 IU/mL and/or significant fibrosis at liver biopsy. Gray zone required persistently normal ALT and HBV DNA >20,000 IU/mL in the absence of fibrosis in liver biopsy. ALT, alanine aminotransferase; HBV, hepatitis B virus; ULN, upper limit of normal.

Table 3. Univariate and multivariate COX proportional regression analysis of baseline factors associated with the follow-up classification as chronic infection-inactive carriers

n=336	Univariate analysis	Multivariate analysis	
	p-value	HR (95%CI)	*p*-value
Male sex	0.176	–	–
Caucasian ethnicity	0.051	–	0.378
Age	0.288	–	–
ALT (IU/L)	<0.001	–	0.179
AST (IU/L)	<0.001	–	0.323
GGT (IU/L)	<0.001	–	0.269
Platelets (cells/mL)	0.029	–	0.996
qHBsAg (log IU/L)[1]	<0.001	–	0.111
HBV DNA (by category)[2]	<0.001	5.9 (2.9–11.9)	<0.001
HBcrAg (log U/mL)[3]	<0.001	6.3 (2.6–15.1)	<0.001
Elastography (by category)[2]	<0.001	4.0 (2.0–7.9)	<0.001

[1]qHBsAg was available in 339 subjects of the overall cohort; [2]HBcrAg was available in 323 subjects; [3]Categories were introduced according to their most commonly used cutoffs: HBV DNA <2.000 IU/mL, 2,000–20,000 IU/mL, >20.000 IU/mL; liver stiffness: <6.5 kPa, 6.5–9 kPa, >9 kPa. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transferase; HBcrAg, hepatitis B core-related antigen; qHBsAg, quantitative hepatitis B surface antigen; HBV, hepatitis B virus.

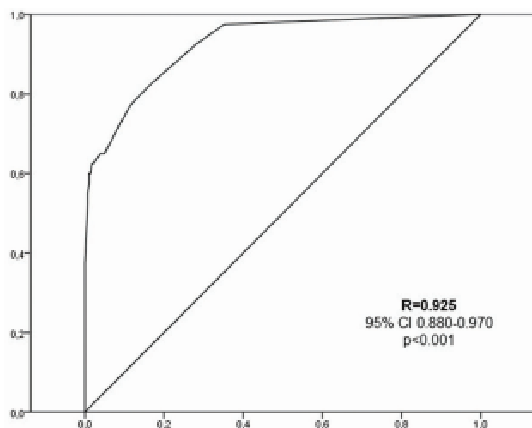

Fig. 3. Area under the receiver operating characteristic (AUROC) of the model for identification of HBeAg-negative chronic infection-inactive carriers subjects.

Table 5. Score system based on the novel model

	Score
HBV DNA (IU/L)	
<2,000 IU/L	0
2,000–20,000 IU/L	6
>20,000 IU/L	12
Liver stiffness (kPa)	
<6.5 kPa	0
6.5–9.0 kPa	5
>9.0 kPa	10
HBcrAg (logU/mL)	
≤3 logU/mL	0
3–4 logU/mL	6
4–5 logU/mL	12
>5 logU/mL	18

HBcrAg, hepatitis B core-related antigen.

values as independent predictors for the identification of HBV ICs. We performed a retrospective-prospective real-life cohort study, with the development of a scoring system (ACE score) that combined the baseline variables. The score had a high specificity and positive predictive value, which implies a trustworthy identification of IC subjects with a low risk of disease progression and good performance regardless of the HBV genotype.

Classically, efforts have been made to identify HBV-infected subjects at increased risk of developing liver-related complications,[17] as antiviral treatment with high-barrier nucleos(t)ides analogues is effective, affordable, and has a great impact on disease progression and survival.[18,19] However, a more recent approach focuses not only on identifying subjects at increased risk, but also those in the inactive phase of the disease who would benefit from less intensive follow-up and management.[20,21] Individuals in this phase have benign outcomes with morbidity and mortality similar to those of the general population.[22] An easy and accurate identification of ICs in a single assessment would facilitate decentralization strategies for HBV follow-up.

Many recent studies have approached the identification of ICs using HBV biomarkers and their combination. HBsAg titles have been proposed as a useful tool to identify IC because of their correlation with HBV DNA levels. An algorithm based on a single-point determination of qHBsAg, ALT, and HBV DNA was described in a large Taiwanese cohort of HBeAg-negative subjects with HBV DNA 2,000 IU/mL (ERADICATE-B cohort). The algorithm proposed the use of qHBsAg <1,000 IU/mL for the identification of subjects at minimal risk of disease progression, but its application was limited to HBV-genotype B and C.[23] The same cutoff was proposed in an Italian cohort of subjects infected with genotype D.[24] However, it is difficult to generalize the results for the overall HBV population, as qHBsAg has been shown to significantly vary among different HBV genotypes, which limits its application in genotype-diverse cohorts.[7,11] HBcrAg was later postulated as a surrogate marker of intrahepatic cccDNA.[25] Significant variation in HBcrAg levels was detected throughout the different HBV infection phases,[11] with the lowest titers detected in the ICs.[26] Interestingly, in another study including HBV genotypes E and H/F, HBcrAg <3 logU/mL combined with HBV DNA <2,000 IU/mL had a diagnostic accuracy of 85% for identification of ICs regardless of HBV genotype.[11] Recently, a multicenter European study including 1,582 HBeAg-negative subjects, HBcrAg <3 logU/mL had an AUROC of 0.968 for identification of ICs.[27] LSM in HBV infection is not as well standardized as in HCV. Double cutoff systems have been proposed to improve performance, although there is no consensus among the different guidelines, which hinders its application in daily practice.[28,29] An Italian study reported a combination of HBsAg, LSM, and HBV DNA with 100% specificity, but no data regarding HBV genotype were available.[30] In fact, to the best of our knowledge, no algorithms including LSM have been developed and validated in all HBV genotypes.

Table 4. Area under the receiver operating characteristic (AUROC) of the model for inactive carrier identification by HBV genotype

Genotype	*n*	AUROC	95% CI	*p*-value
D	98	0.955	0.91–1.0	<0.001
A	67	0.963	0.89–1.0	<0.001
E	37	0.903	0.79–1.0	<0.001
H/F	26	0.883	0.75–1.0	0.005

AUROC, area under the receiver operating characteristic; CI, confidence interval.

On the other hand, the inaccuracy of noninvasive fibrosis markers in the GZ usually leads to the necessity of performing a liver biopsy, which is considered the gold standard for fibrosis assessment. In our study roughly 25% of patients needed a liver biopsy, 10% of whom had significant fibrosis that was independently associated with higher HBcrAg levels and LSM. The relatively high percentage of patients who required a liver biopsy for classification of HBV phase, reinforces the need to optimize the use of noninvasive strategies and to develop pan-genotypic scores.

Our cohort included mainly middle-aged subjects in the IC phase, which is consistent with the current epidemiological profile of the HBV infection in Western countries.[31] On the other hand, almost half of our cohort were non-European migrants, which probably explains the genotype distribution compared with other European cohorts.[26,32] The high proportion of migrants in our cohort should be a reminder of the need of the integration of viral hepatitis management

Table 6. Accuracy of the ACE score cutoffs for identification of subjects who will be considered HBeAg-negative chronic infection (inactive carriers) during follow-up

Total score	<1 point	≤5 points	≤12 points	≤17 points
Sensitivity, % (95% CI)	64.8 (59.0–70.1)	71.9 (66.4–76.8)	98.2 (95.9–99.2)	99.3 (97.5–99.8)
Specificity, % (95% CI)	97.5 (87.1–99.6)	92.5 (80.4–97.4)	62.5 (47.0–75.8)	52.5 (37.5–67.1)
Negative predictive value, % (95% CI)	28.3 (21.4–36.3)	31.9 (24.1–40.9)	83.3 (66.4–92.7)	91.3 (73.2–97.6)
Positive predictive value, % (95% CI)	99.5 (97.0–99.9)	98.5 (95.8–99.5)	94.9 (91.7–96.9)	93.6 (90.3–95.9)
Diagnostic accuracy, % (95% CI)	68.9 (63.6–73.7)	74.5 (66.4–78.9)	93.8 (90.6–95.9)	93.5 (90.2–95.7)

CI, confidence interval.

in the package of care of international health units, as well as of the need of predictive models that include a diverse genotype composition.

Our study has some limitations because of the partially retrospective design, which could lead to bias resulting from missing parameters such as HBcrAg and qHBsAg in some patients. Furthermore, as previously mentioned, the proportion of patients with CHB was relatively low, but was in line with the prevalence shown by other studies carried out in Europe.[11,33] The external validation of our findings is limited by the unicentric character of the study, although patients were followed up by different hepatologists. Also, a limited number of Asian and Hispanic subjects were included in the cohort. Regardless of the limitations, the new model performed well and could be a valuable tool in the clinical practice following validation in large multicenter cohorts.

In summary, in patients with chronic HBV infection, low levels of HBV DNA, HBcrAg, and LSM were independently associated with the inactive carrier state. The ACE score included these variables and accurately identified ICs in a single-point time evaluation, regardless of HBV genotype. Studies in larger cohorts are needed to validate the score.

Acknowledgments

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Conflict of interest

Mar Riveiro-Barciela (MRB) and Rafael Estebal (RE) have served as speakers for AbbVie and Gilead. MRB and María Buti (MB) have received grants from Gilead. MB has served as speaker for Abbvie and Gilead and advisory board member for Gilead, Assembly, GSK.

Author contributions

Guarantors of the article and take responsibility for the integrity of the work (MB, MRB), designed the study (MB, MRB, LR), collected the data (LR, AP, EVA, AR, MB, RC, SS, DT), performed the analysis and interpretation (LR, MRB, MB), drafted the manuscript (LR, MRB, MB), reviewed the manuscript (RE, FRF). All the authors approved the final version.

Ethical statement

Ethical approval was obtained from the Ethical Committee of the University Hospital Vall d'Hebron (Code PR(AG) 247/2018; Date of approval 20 July 2018).

Data sharing statement

The data used to support the findings of this study are available from the corresponding author upon request.

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4.3 ARTICLE 2.

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

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Research article

JHEP|Reports

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

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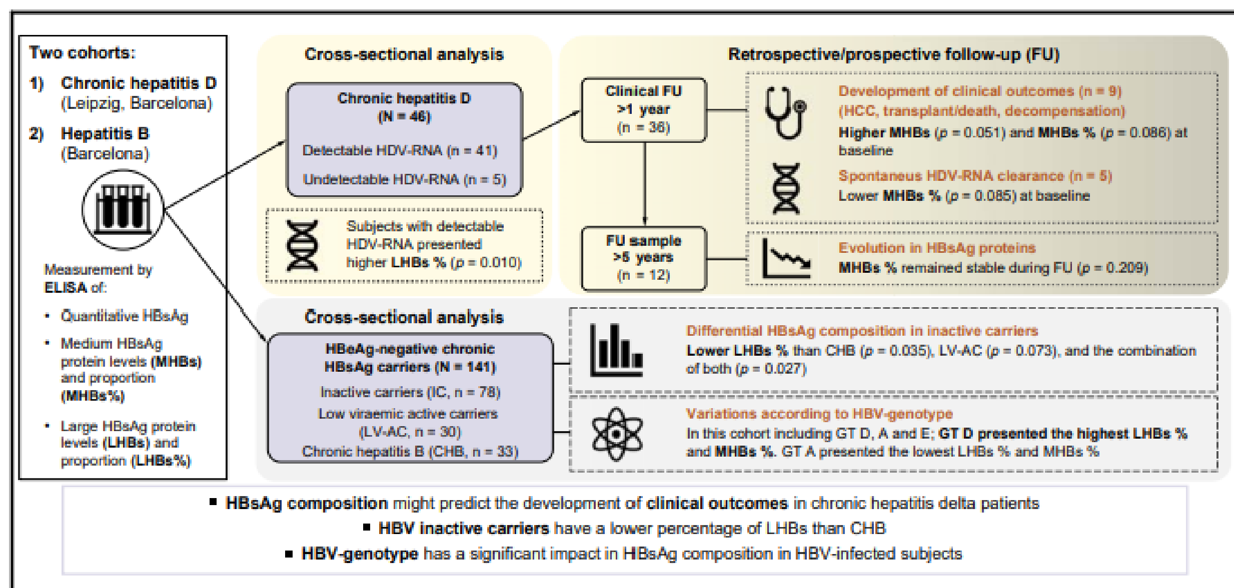
Graphical abstract



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 JHEP|Reports

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.
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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

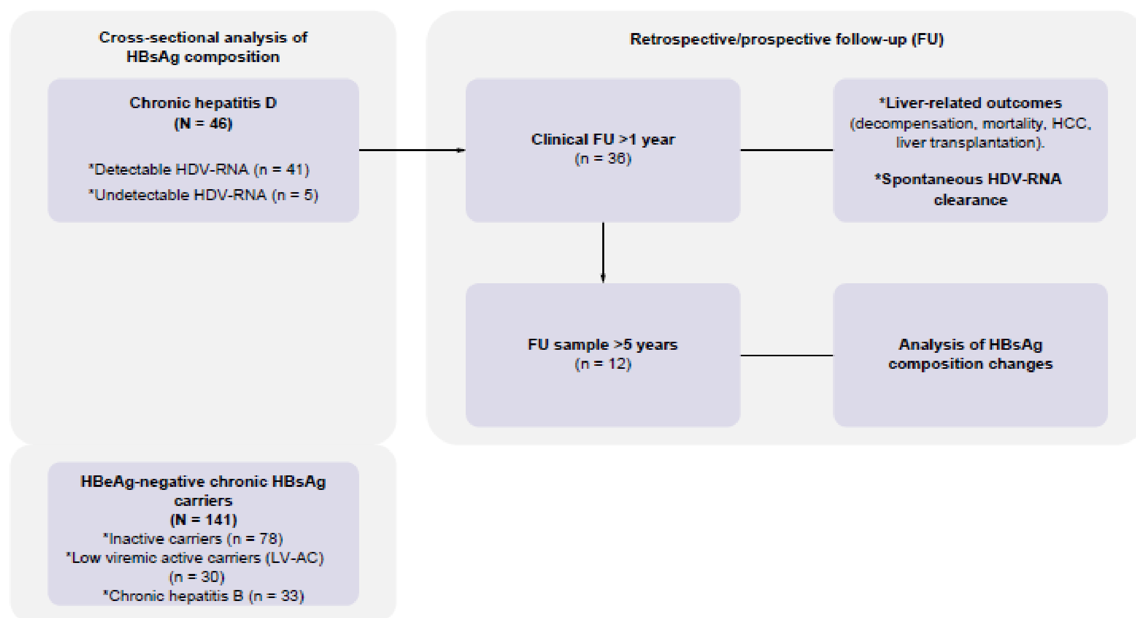

Fig. 1. Flow chart summarising study design. FU, follow-up; HCC, hepatocellular carcinoma.

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 ($\times 10^9/mm^3$)	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.

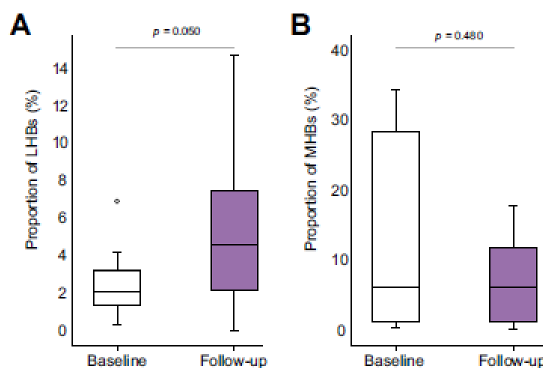

Fig. 2. Evolution of LHBs and MHBs proportions in patients with chronic hepatitis D with longitudinal samples (n = 12). (A) Proportion of LHBs in baseline and follow-up samples. Level of significance p = 0.050 (Mann–Whitney *U* test). (B) Proportion of MHBs in baseline and follow-up samples. Level of 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

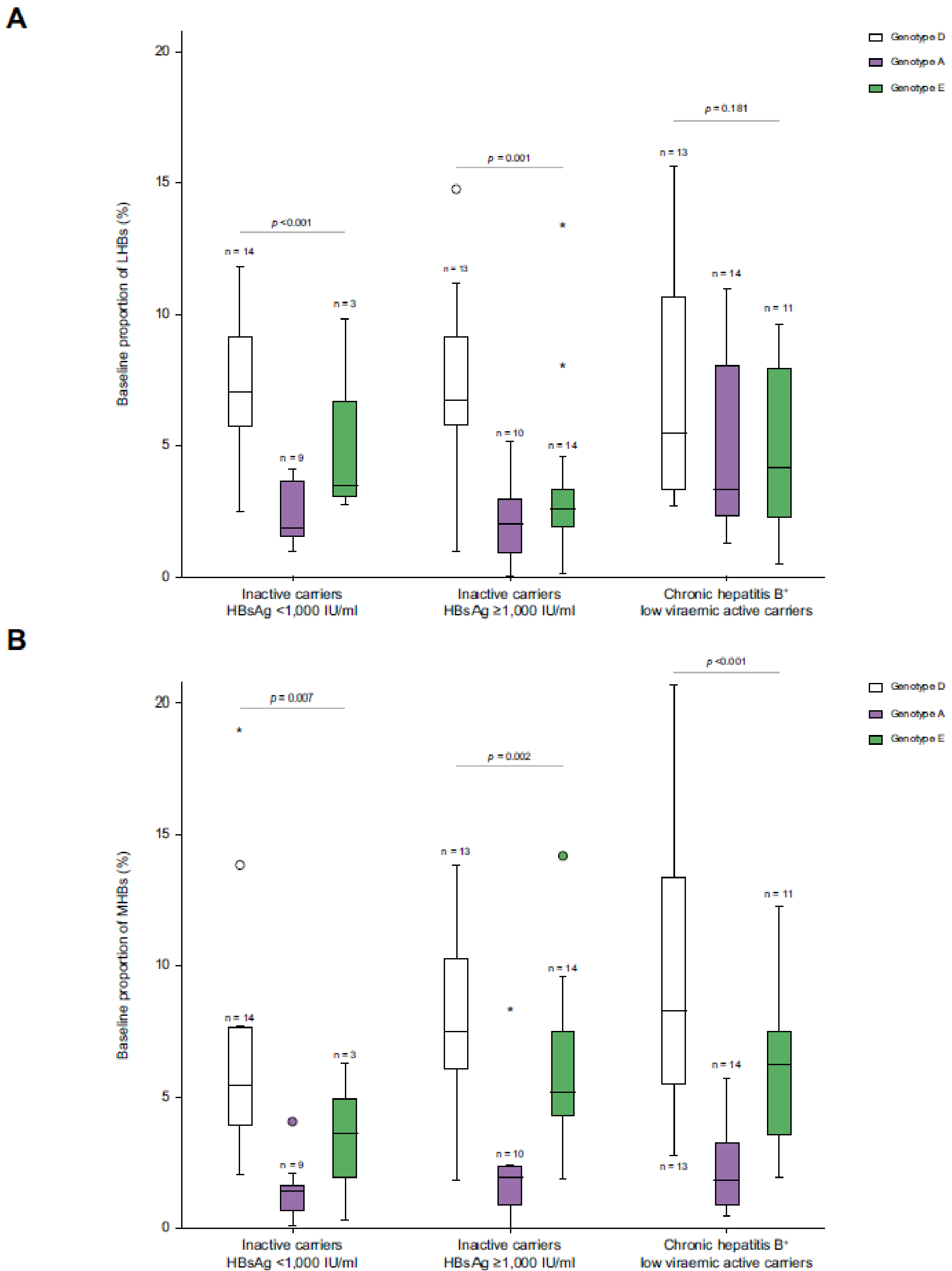

Fig. 3. LHBs and MHBs proportion in HBV-infection phases according to HBV genotype. (A) LHBs proportion in HBV infection phases for HBV genotypes D, A, and E. Levels of significance (ANOVA): $p = 0.001$ (inactive carriers HBsAg <1,000 IU/ml), $p = 0.001$ (inactive carriers HBsAg ≥1,000 IU/ml), and $p = 0.181$ (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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2023.100842.

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Author names in bold designate shared co-first authorship

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5 OVERALL SUMMARY OF RESULTS

The first study includes a cohort of HBeAg-negative chronic HBsAg carriers from a tertiary hospital in Spain. It assessed the usefulness of non-invasive markers to identify HBV-ICs, developing a predictive model for the identification of these subjects in a single-point in time evaluation.

A total of 343 HBeAg-negative chronic HBsAg carriers were consecutively included, 52.1% were migrants. Most were male (59.2%), with a mean age of 45±15 years. Out of the 250 subjects in which HBV-GT was determined, A and D were predominant (27.2% and 40.8%, respectively), followed by HBV-GT E (16.0%).

At the first medical visit, most subjects (236, 68.8%) had a normal ALT and HBV-DNA below 2,000 IU/mL. Five subjects (1.5%) had ALT above two times ULN and HBV-DNA above 20,000 IU/mL. Two-hundred and two subjects (29.7%) did not satisfy the conditions for any of these categories.

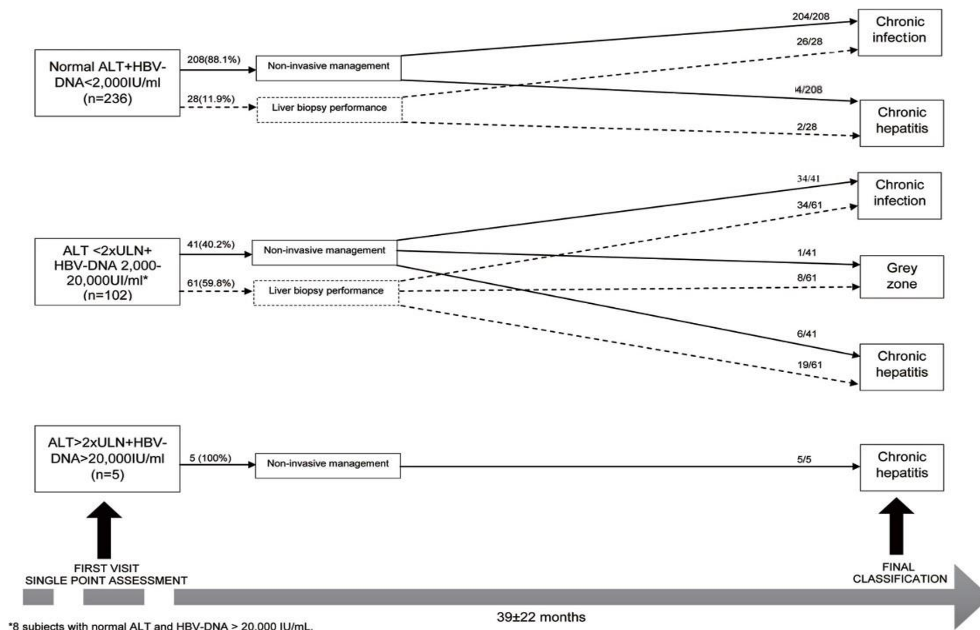
During a mean follow-up of 39 months, 89 subjects (25.9%) required a liver biopsy for a proper classification of HBV infection: 28 (11.9%) out of the 236 subjects with normal ALT and HBV-DNA below 2,000 IU/mL at baseline, and 61 (59.8%) out of the 102 unclassifiable subjects at baseline (figure 11). Liver biopsy indication was established by normal ALT and HBV-DNA persistently above 2,000 IU/mL, or ALT <2-fold ULN and viral load above 2,000 IU/mL during follow-up. Most subjects who underwent a liver biopsy (80, 82.0%) presented an Ishak score below F1, while 9 (10.1%) showed significant fibrosis (≥ F3) in liver sample.

5.1 CLASSIFICATION OF HBV PHASE DURING FOLLOW-UP

At follow-up, 298 subjects were considered ICs and 36 were considered to have CHB. Nine subjects remained as GZ, all of them with viral loads persistently above 20,000 UI/mL, normal ALT, and non-significant fibrosis in liver biopsy. The classification of subjects at baseline and during follow-up is summarized in figure 11.

The multivariate analysis found that a higher LSM category (<6.5Kpa, between 6.5kPa-9Kpa, >9kpa) and higher HBcrAg baseline levels were independently associated with significant fibrosis in liver sample (hazard ratio [HR] 3.37, 95% confidence interval [CI]: 1.08–10.49, *p*=0.037 for categorized LSM and HR 3.62, 95% CI: 1.41-9.25, *p*=0.007 for HBcrAg).

FIGURE 11. HBV infection phase evolution and liver biopsy performance during follow-up.



Baseline ALT, HBV-DNA, qHBsAg and HBcrAg significantly differed among subjects classified during follow-up as ICs, GZ and CHB, as well as baseline

LSM and APRI score (*p*<0.001 for all the variables), while FIB-4 was similar among the three groups (*p*=0.617). HBV-GT distribution was similar in the three phases of infection (*p*=0.209).

5.2 MARKERS FOR IDENTIFICATION OF HBV-ICS

The multivariate analysis identified lower baseline HBV-DNA (HR 5.9 [95% CI: 2.9-11.9], *p*<0.001]), HBcrAg levels (HR 6.3 [95% CI: 2.6-15.1], *p*<0.001) and LSM (HR 4.0 [95% CI: 2.0-7.9], *p*<0.001) as independently associated with the final classification of IC phase. Similar risk coefficients were obtained for these variables by 1000-bootstrapping samples: HR 6.0 (95% CI: 3.0–12.0) for categorized HBV-DNA (*p*<0.001), HR 6.5 (95% CI: 2.7–15.7) for HBcrAg levels (*p*<0.001) and HR 4.6 (95% CI: 2.3–9.0) for categorized LSM (p<0.001).

Based on these coefficients, an area under the receiver operating characteristics (AUROC) of 0.925 (95% CI: 0.880–0.970, *p*<0.001) for the identification of ICs was obtained. The model was validated in most prevalent HBV-GTs yielding an AUROC of 0.95 for GT D, 0.96 for GT A and 0.90 for GT E.

An individual-score system, named the ACE score, was constructed from the simplified coefficients of the bootstrapping analysis including HBV-DN**A**, HB**C**r-Ag, and liver **e**lastography. A score below 1 point at baseline presented the highest positive predictive value (PPV) (99.5% [95% CI: 97.0-99.9]) and specificity (97.5% [95% CI: 87.1–99.6]) for ICs identification, while the 12 points cut-off showed the greatest diagnostic accuracy (93.8% [95% CI: 90.0-95.9]), with 98.2% sensitivity and 62.5% specificity.

The second study focused on the quantification of HBsAg proteins in HBV and HDV chronic infections. It aimed to validate a differential HBsAg composition in HBV-ICs and to evaluate the influence of HBV-GT in HBsAg composition. It also assessed HBsAg composition in subjects with CHD and its association with clinical outcomes.

5.3 HBsAg protein composition in subjects with HBeAg-negative hepatitis B

One hundred forty-one HBeAg-negative chronic HBsAg carriers were included in the cross-sectional analysis. Seventy-eight subjects (55.3%) were classified as ICs, 20 (21.3%) as LV-AC and 33 (23.4%) as CHB. HBV-GT was available in 117 subjects. Predominant HBV-GTs were HBV-GT D (40, 28.4%), GT A (33, 23.4%) and GT E (28, 19.9%). Ten subjects (7.1%) were considered cirrhotic at baseline.

Total qHBsAg and absolute levels of LHBs, MHBs and SHBs proteins significantly differed across the HBeAg-negative phases (p=0.002 for qHBsAg, LHBs and MHBs, and p=0.0025 for SHBs). No differences were found in total qHBsAg comparing IC with LV-AC (p=0.363) and with CHB (p=0.874). Absolute LHBs and MHBs were similar between the IC and the non-IC groups (p=0.509 and p=0.914, respectively). However, when comparing HBsAg isoforms proportions, subjects in the IC phase presented a lower LHBs proportion than the CHB phase (2.71% vs. 5.21%, p=0.035), the LV-AC (2.71% vs. 4.35%, p=0.073) and their combination (p=0.027).

5.4 HBsAg composition according to HBV genotype

Significant variations in qHBsAg according to HBV-GT were evidenced in both the IC (p=0.010) and in the non-IC group (p=0.047). In both groups, the highest qHBsAg was observed in subjects infected with HBV-GT E. HBV-GT also impacted significantly in the absolute levels of HBsAg proteins. LHBs absolute 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 qHBsAg (p=0.013 and p=0.003 in IC with qHBsAg below and above 1,000 IU/mL, respectively) and in the non-IC group (p=0.003). Individuals infected with GT E presented higher absolute median levels of all three HBsAg components in all phases of HBV infection.

Concerning the proportion of HBsAg proteins, LHBs proportion varied significantly according to HBV-GT in the IC group regardless of qHBsAg (p<0.001 in IC with HBsAg< 1,000 IU/mL, p=0.001 in IC with HBsAg ≥1,000 IU/mL). MHBs proportion presented significant variations according to HBV-GT in all groups (p=0.007 in IC with HBsAg<1,000 IU/mL, p=0.002 in IC with HBsAg≥ 1,000 IU/mL and p<0.001 in non-IC). Subjects infected with HBV-GT D presented the highest proportion of both LHBs and MHBs in all groups of patients, whereas HBV-GT A presented the lowest proportion of both isoforms.

5.5 Baseline HBsAg composition in the Hepatitis Delta cohort

Forty-six patients with CHD were included, 40 (87%) were HBeAg-negative and 17(37%) presented liver cirrhosis at baseline. Subjects with liver cirrhosis presented a trend to have higher LHBs proportion (6.4% *vs.* 4.5%, *p*=0.055). Despite HBeAg-positive CHD subjects presented higher qHBsAg, LHBs and MHBs, proportions did not differ according to HBeAg status.

HDV-RNA was detectable 41 subjects. Subjects with detectable HDV-RNA presented higher total HBsAg (4.15 *vs.* 3.71 log ng/mL, *p*=0.056), and higher absolute levels of LHBs (2.77 *vs.* 1.89 log ng/mL, p=0.006), MHBs (2.71 *vs.* 1.52 log ng/mL, *p*=0.60) and SHBs (4.20 *vs.* 3.70 log ng/mL, *p*=0.056). A higher proportion of LHBs was observed in subjects with detectable HDV-RNA (5.48% *vs.* 1.46%, *p*=0.010).

5.6 Impact of HBsAg composition in liver-related outcomes in chronic hepatitis D

Longitudinal data were available in 36 subjects (36.1% with liver cirrhosis at baseline and 86.1% with detectable HDV-RNA). After a median of 5.9 years, 9 patients presented liver-related outcomes. Subjects with detectable HDV-RNA at baseline presented clinical outcomes more frequently, although without statistical significancy (*p*=0.214).

Subjects who presented decompensation during follow-up tended to present higher absolute MHBs (2.95 log ng/mL *vs.* 2.47 ng/mL, *p*=0.051) and MHBs proportion (6.85% *vs.* 3.74%, *p*=0.086) at baseline, despite similar qHBsAg

(*p*=0.494). The development of HCC (n=3) was not associated with significant differences in HBsAg components levels or proportions at baseline.

Five out of 31 subjects presented spontaneous HDV-RNA clearance during follow-up. Significantly lower baseline qHBsAg and lower absolute levels of the three isoforms were found in these subjects. MHBs proportion also tended to be lower among those who reached spontaneous negativization of HDV-RNA (2.46% vs. 6.70%, *p*=0.085).

5.7 Evolution of HBsAg proteins composition during follow-up in chronic hepatitis D

The dynamics of HBsAg proteins was analyzed in 12 subjects with a control sample taken after a median of 10.3 years. A significant decline in median qHBsAg (*p*=0.015) and MHBs (*p*=0.019) was documented. Median absolute LHBs levels did not present significant variations (*p*=0.638). The proportion of LHBs showed an increasing trend over time (*p*=0.050), whereas MHBs proportion remained stable (*p*=0.480).

6 OVERALL SUMMARY OF THE DISCUSSION

Our first study was performed in a real-world cohort of subjects with HBeAg-negative chronic HBV. HBeAg-negative stages represent most chronic HBV infections worldwide, among whom a considerable proportion of subjects are considered HBV-IC (167–169). The demographic features of the cohort are consistent with the current epidemiological profile of HBV infection in Western Europe, with around half of the cohort formed by migrants (169).

We identified baseline HBV-DNA, HBcrAg and LSM as associated with a further classification as HBV-IC phase. The ACE score constructed with these variables showed a high specificity and PPV for the identification of HBV-IC in a single point in time, with a good performance regardless of HBV-GT. The high specificity and PPV of the score would allow a truthful identification of HBV-IC, without exposing subjects at low risk of complications to intensive follow-up.

Many recent studies have approached the identification of HBV using serum biomarkers. Baseline levels of HBcrAg below 3log/mL have been previously correlated with the IC state in longitudinal cohorts. The combination of a single measurement of HBcrAg ≤3 logU/mL and HBV-DNA ≤2000 IU/mL was prospectively evaluated in a Spanish cohort of 202 subjects followed during 12 months, yielding a diagnostic accuracy above 85% in HBV-GT A, D and E with PPV ranging from 86% to 92%(115). Diagnostic accuracy and PPV dropped for HBV-GT H/F. The use of HBcrAg showed a higher diagnostic accuracy than qHBsAg for the identification of HBV-IC in this cohort. Similarly, a retrospective multicentric European study in 1,582 HBeAg-negative subjects showed a good performance of a single-point determination of HBcrAg below 3.14 logU/mL to

identify IC against CHB (170). The proposed HBcrAg cut-off was consistent across HBV-GTs, although non-A, non-D HBV-GTs were underrepresented(170). Notably, a large cross-sectional North-American study including HBV-GT A-D concluded that the determination of single-point in time HBV-RNA and HBcrAg offered no advantages compared to the use of conventional markers (HBeAg, HBV-DNA, qHBsAg) to categorize HBV infection phase (171). The cross-sectional design of this research and the discrepancies in HBV phase definitions hampers the comparison with our results (171).

Single-point in time determinations of qHBsAg have been also proposed to discriminate the phases of HBV infection, with controversies regarding the optimal cut-off and the generalization of the results. HBsAg below 1000 IU/mL has been used to identify subjects in inactive phase of HBV infection in European and Asian cohorts (172–174). In 2010, Brunetto *et al* proposed a single point HBsAg ≤1000 IU/mL combined with HBV≤ 2,000 IU/mL for the identification of HBV-IC in a cohort of chronic HBsAg carriers infected by GT D, achieving a specificity of 98% with 88% PPV. HBsAg above this same cut-off was independently associated with disease progression among subjects with HBV-DNA<2000 IU/mL and normal ALT in a large Taiwanese cohort of subjects infected by HBV-GT B and C (ERADICATE-B cohort) (174). The combination of HBsAg<100 IU/mL and HBV-DNA<2000 IU/mL was proposed for the identification of HBV-IC in a real-life multicentric retrospective study including subjects with HBV-GT A-D, yielding an overall specificity of 98% and 99% PPV, despite low sensitivity (175). Notably, the performance of this combination of biomarkers dropped for HBV-GT B and C (175). Discrepancies in the GT distribution might explain that baseline qHBsAg was not associated with the IC phase in our cohort (p=0.111), and emphasizes the concerns regarding the generalization of algorithms based on qHBsAg.

Regarding liver elastography, the use of LSM in HBV is not as consistent as in other conditions such as chronic hepatitis C infection. An algorithm based on baseline LSM, qHBsAg and HBV-DNA was proposed by Maimone *et al.* in an Italian cohort of HBeAg-negative subjects with 100% specificity and PPV. However, the limited size of the cohort (n=147) and the lack of data regarding HBV-GT represent significant limitations (176). In fact, to our knowledge, no algorithms including LSM have been developed and validated in HBV pan-genotypic cohorts so far.

Despite periodical monitoring, a considerable proportion of subjects still require a liver biopsy due to the inability of serum markers to determine the phase of infection in subjects in the GZ(177). In our cohort, more than a quarter of subjects needed a liver biopsy during follow-up, from which 90% did not present significant fibrosis in the liver sample, reinforcing the need of biopsy-sparing algorithms in the clinical practice. The use of single-point algorithms would contribute to the implementation of decentralized models of care in HBV programs, which are essential for the expansion of HBV care specially in highly-endemic settings (178). In fact, the consecutive evaluations required for the identification of HBV-IC phase threat the retention in care of HBV cohorts, and represents a significant burden of the medical visits in often tertiary institutions (47). A simplified and accurate identification of HBV-IC also aligns with the recently proposed “treat-all-except” policy, endorsing antiviral treatment expansion unless in subjects with minimal risk of disease progression (179,180).

The second study addresses the HBsAg protein composition in HBeAg-negative chronic HBsAg carriers and CHD subjects. In the HBV cohort, a lower LHBs proportion was observed in HBV-IC compared other HBeAg-negative phases. This observation is aligned with the biological role attributed to LHBs, facilitating viral

attachment through the preS1 region. Similarly, a lower LHBs proportion in HBV-IC was reported in a German study using the same assay for HBsAg measurement(137). In this study, Pfefferkorn *et al.* also found significant differences in absolute MHBs and LHBs levels and MHBs proportion, not observed in our cohort. Significant differences in the GT distribution of both cohorts (almost exclusively GT A-D in the German group) may justify these discrepancies. More recently, the measurement of HBsAg isoforms using a novel semi-quantitative assay in 113 individuals did not show any additional benefit for disease staging and treatment monitoring compared to qHBsAg and HBV-DNA(123). Significant methodological differences, together with the unavailability of HBV genotyping and the lack of standardization of the assays for HBsAg proteins measurement impede the comparison of these results.

We also documented a significant impact of HBV-GT in HBsAg protein composition. Variations of qHBsAg across HBV-GTs have been well documented in the literature (115,119,181). These variations are supported by differences in HBsAg secretion according to HBV-GT observed in *in vitro* models(182). However, considerable variations in qHBsAg have also been observed among subjects infected with the same HBV-GT. Factors including viral mutations, host genetics and immune response are suggested to influence HBsAg secretion, impeding the determination of a univocal association between HBV-GT and qHBsAg levels(183).

On the other hand, the impact of HBV-GT in HBsAg isoforms has been scarcely studied. We observed higher absolute LHBs, MHBs and SHBs levels in subjects infected with GT E, while those infected by GT D presented higher MHB and LHBs proportions. Variations in LHBs proportion were only found in the IC group, perhaps due to a limited sample size. In line with our observations, GT D presented higher MHBs (p=0.004) and LHBs (p=0.010) proportions than GT A in a small cohort of

46 HBeAg-negative subjects(137). In a multicenter German study including samples from HBeAg-negative subjects infected with GT A to E, higher relative LHBs and MHBs were observed in GT B and D compared to A/C/E (126). A significant impact of HBV-GT composition has also been observed in chronic HBeAg-positive phases (113,128). The influence of GT in HBsAg composition is also supported by *in vitro* experiments. Hassemer *et al.* have reported a significant influence of HBV-GT in intra and extra-cellular qHBsAg levels and the relative contribution of SHBs, MHBs and LHBs through cell culture(124). A later German study of serum from HBeAg-negative patients evidenced that GT-dependent differences in HBsAg composition impacted the morphology and density of SVPs, hypothesizing implications in immunopathogenesis(126).

Finally, the second study also explores, for the first time to our knowledge in a large study, the HBsAg composition in a well-characterized CHD cohort and its impact in clinical outcomes. Subjects with detectable HDV viremia presented higher qHBsAg and LHBs proportion than those with undetectable HDV-RNA (p=0.010). Prior research has established an association between HDV replication and qHBsAg, probably related to the need of HBV envelope proteins to facilitate HDV assembly and infectivity (164). The impact of HDV viremia in HBsAg composition was not analyzed by Pfefferkorn *et al.* in a small subgroup of 11 subjects with HDV/HBV coinfection, in which a higher LHBs proportion compared to CHB subjects were reported (137). A higher LHBs proportion in subjects with active HDV replication supports the crucial role of LHBs for HDV infectivity observed *in vitro* (81,82).

In the longitudinal follow-up of the HDV cohort, HBsAg composition was associated with clinical endpoints. Lower baseline levels of total qHBsAg, all three isoforms and MHBs proportion (2.46% *vs.* 6.70%, p=0.085) were observed in subjects who

achieved spontaneous HDV-RNA negativization during follow-up. The relevance of this finding relies on the association of detectable and persistent HDV-RNA with unfavorable clinical outcomes in longitudinal cohorts (94,184). On the contrary, subjects developing unfavorable clinical outcomes (decompensation, HCC, liver transplant or death) presented higher baseline MHBs levels (p=0.051), despite similar qHBsAg. A trend to a higher baseline MHBs proportion was also observed (6.85% *vs.* 3.74%, p=0.086) in these subjects. The biological role of MHBs in HDV is poorly understood, and most hypothesis speculate based on its proposed role in HBV infection. Higher levels of MHBs in the early acute HBV compared to chronic infection was interpreted as a possible sign of the immunomodulatory effects of MHBs protein, similar to that proposed in HBeAg (137). Whether differences in MHBs contribution to HBsAg during CHD natural history are related to variations in immunomodulation needs further research. MHBs proportion did not experienced significant variations during follow-up, encouraging further research of MHBs proportion as a potential prognosis marker for CHD.

The involvement of MHBs in carcinogenesis has been suggested *in vitro* (134). We did not observe differences in HBsAg baseline composition in the 3 CHD subjects who developed HCC during follow-up. Prior to our research, only a sub-analysis of 19 CHD cirrhotic subjects with NA-induced HBV-DNA suppression included in a heterogenous cohort provided some input regarding HBsAg composition and HCC (130). In this sub-cohort, baseline relative levels of HBsAg isoforms were not associated with the onset of HCC (130). An increase in absolute levels of MHBs was described prior to HCC diagnosis in the general cohort combining CHD and HBV subjects. Relevant data on IFN treatment history and HBeAg status, as well as disaggregated results according to CHD status were not provided. The extremely small sample size in both cohorts and the significant discrepancies in

methodology and study population do not allow a proper comparison of both studies.

6.1 LIMITATIONS

The first study presents some limitations based on its unicentric and partially retrospective design. The partially retrospective design might lead to information bias due to missing parameters in some cases. Also, the inclusion of HBV-IC with low HBV-DNA hinders the obtention of HBV genotyping in all subjects. The unicentric character of the study might impact the external validity of the results. Subjects were assessed by different hepatologists to partially mitigate this limitation. Despite the limitations, the proposed model showed an adequate performance and could be a valuable tool in the clinical practice following validation in larger multicentric cohorts.

Regarding the second study, the use of a non-standardize technique for HBsAg protein measurement hinders the harmonization and comparison of the results with other studies. However, the measurement of relative levels used in this and previous studies might facilitate comparison of results. Secondly, HBV-GT could not be determined in a considerable proportion of subjects, and the under-representation of highly prevalent HBV-GTs worldwide such as B and C limits the generalization of the results. Efforts should be made to include under-represented GTs in multicentric ethnically diverse cohorts to generalize our findings. HBV-GT was not determined in our CHD cohort due to low levels of HBV replication, and HDV-GT could only be determined in a small proportion of patients. Further research is needed to evaluate the impact of HDV and HBV-GTs and their interplay in HBsAg composition in subjects with CHD. The small size of our cohort, common in studies involving CHD due to the low prevalence of the disease, constitutes an important

limitation and determines the statistical power of our findings. The partially retrospective design could have introduced reporting bias in our results. Larger multicentric prospective studies are needed to validate our results.

7 CONCLUSIONS

1. In HBeAg-negative chronic HBsAg carriers, the combination of serum HBV-DNA, HBcrAg and liver elastography accurately identifies HBV-IC in a single-point evaluation.

2. HBV genotype has a significant impact in the absolute and relative HBsAg protein composition among HBeAg-negative chronic HBV carriers.

3. Patients with Chronic hepatitis D and detectable HDV-RNA present a different HBsAg composition than those with undetectable HDV-RNA, with higher proportion of LHBs in those with detectable viremia.

4. A trend towards having higher baseline MHBs proportion was observed in patients who developed clinical events or remained with detectable HDV-RNA.

5. The composition of HBsAg is also useful to distinguish inactive HBV carriers and it varies according to HBV genotype

8 FUTURE LINES

8.1 VALIDATION OF COMBINING SCORES IN DIVERSE HBV COHORTS

Most emerging biomarkers and combining scores to define HBV infection phases have been assessed in well-resourced and predominantly White Western settings. These tools, such as the ACE score proposed in our research, should be validated in geographically, ethnically, and (sub)genotypically diverse HBV cohorts(109). Efforts should be made to include subjects from highly endemic settings, including non-A/non-D genotypes. Importantly, the application of combining scores should also be explored in special populations such as HCV/HIV-coinfected subjects and children. Notably, the reassessment of these scores using innovative tools such as point-of-care tests and portable platforms would also contribute to the implementation and validation of the emerging markers in the real world, especially in hard-to-reach groups(185).

8.2 HBSAG ISOFORMS AS MARKERS FOR TREATMENT RESPONSE IN HBV AND HDV CHRONIC INFECTIONS

Significant gaps remain in the understanding of the biological roles of HBsAg proteins and their impact in the natural history of HBV and HDV infections. Anyhow, HBsAg loss persists as the cornerstone of treatment endpoints for both infections. In HBV, HBsAg isoforms have been scarcely studied as a marker for HBsAg clearance in subjects under conventional therapies with NA and IFN(123). HBsAg proteins could also be explored to predict reactivation after NA withdrawal and even more, to provide valuable information in treatment monitoring and response of emerging antiviral therapies against HBV such as entry inhibitors, siRNA and antisense oligonucleotides.

A differential HBsAg composition and its association with virological and clinical end-points might be also explored in new therapies against HDV. BLV is a lipopeptide derived from the preS1 region of LHBs. Although no impact has been observed in total HBsAg decline during BLV treatment, the impact of this new therapy in HBsAg composition have not been assessed(106). Similarly, the analysis of HBsAg composition during the treatment with lonafarnib could provide a deeper insight on the interplay of HDV and HBV for particle secretion, and its consequences in pathogenesis. The use of validated assays for quantification of HBsAg isoforms in future research should be prioritized to enable the comparison an understanding of new research.

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