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Research Article

A New Assay for Quantitative Determination of Pres1-Encoded Protein in Patients Infected with Hepatitis B and Delta Viruses

Alessandra Biasiolo[1], Santina Quarta[1], Mariagrazia Ruvoletto[1], Pietro Guerra[1], Andrea Martini[2], Patrizia Pontisso[*, 1]

Abstract

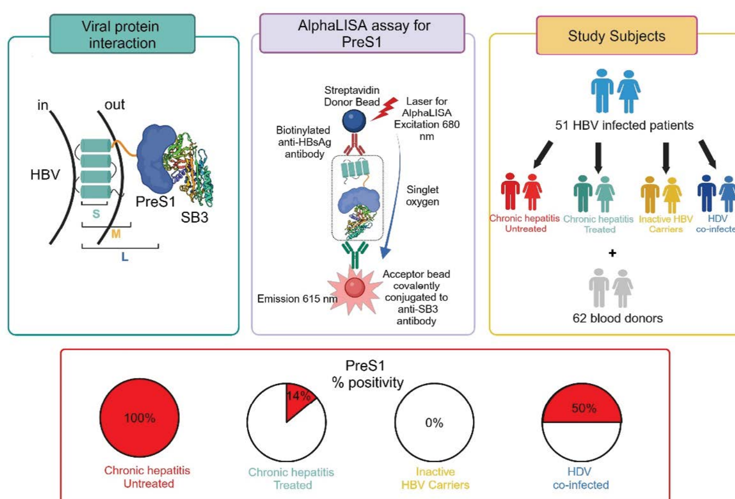
Background & Aims: The Pre-S1 protein of HBV is essential for the infectivity of both B and delta viruses and has been recently proposed as a useful prognostic tool in chronic HBV infection. To date, accurate quantitative methods for PreS1 testing are still lacking. Since PreS1 has a high binding affinity for SerpinB3, the aim of the study was to standardize and validate a new assay based on PreS1-SerpinB3 interaction.

Methods: An AlphaLISA® technology test, based on the interaction between PreS1-encoded protein and SerpinB3, was standardized. Validation was carried out by the assessment of PreS1 levels in serum of 51 HBV-infected patients (5 inactive carriers, 38 with chronic hepatitis, among which 36 were under antiviral treatment, 8 with HDV coinfection). As healthy controls, sera of 62 blood donors were used.

Results: Preliminary set-up experiments allowed us to define the PreS1 linear range of 0.12 to 7.7 µg/mL and the low detection limit of 0.013 µg/mL. The positivity rate and levels of PreS1 were significantly higher in untreated patients with chronic hepatitis B and in HDV patients than in treated patients with chronic hepatitis B, while none of the inactive HBV carriers was positive. No significant correlation between PreS1 levels and viral load was observed.

Conclusion: The developed AlphaLISA® assay is useful for PreS1 detection in serum in HBV and delta-infected patients and correlates with the HBV infection stage.

Graphical Abstract



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Introduction

Hepatitis B virus (HBV) and hepatitis D virus (HDV) are highly prevalent viral diseases, with HBV estimated to infect almost 300 million people, of whom HDV is estimated to infect 12-72 million people worldwide [1,2]. These viruses can be responsible for fulminant hepatitis or cause progressive liver disease, including cirrhosis and hepatocellular carcinoma, representing a remarkable cause of global mortality [3]. HBV is a small enveloped virus, with a partially double-stranded DNA genome of approximately 3.2 kilobases packaged into an icosahedral nucleocapside. The HBV envelope contains three membrane glycoproteins, the large (L), middle (M) and small (S) surface proteins, which form, all in all, the hepatitis B surface antigen (HBsAg) [4,5]. L, M and S proteins share the C-terminal S domain which contains 4 membrane-spanning helices. L domain is the largest protein of the shell's virus and is composed of S protein, Pre-S2 protein and Pre-S1 protein. The N-terminal extension of the L protein, the PreS1 protein, has several functions, the most important being the receptor binding, allowing virus entry [6,7]. One peculiarity of HBV is its ability to produce not only complete viral particles of about 42 nm in diameter [8], but also non-infectious particles, known as filaments (variable length) and spheres with a diameter of 22 nm. The Large protein (LHBs) is a component of the envelope of both the mature viral and filamentous non-infectious particles and is essential for virus infectivity [9,10]. The Pre-S1 domain of the large envelope protein, initially resides in the interior side of virus particles and only after modification by myristic acid and a complex post-translational translocation process is exposed on the virion surface [11,12]. The Pre-S1 domain is considered a key determinant in the multistep process for receptor binding on hepatocyte surface: in the first phase the virus attaches the surface of the host cell by binding to glypican 5, an heparan sulfate proteoglycan [13] then, the N-terminal 1-75 aa region of Pre-S1 domain is involved in the sodium taurocholate co-transporting polypeptide (NTCP) interaction [14]. More specifically, this envelope region binds the 157-165 aa region of the NTCP molecule, which is physiologically involved in the uptake of bile acids and is now recognized as a key entry receptor not only for HBV, but also for HDV. HDV is indeed a small single-stranded RNA "satellite" virus, lacking its own polymerase and requiring HBV for its replication and propagation [14,15]. As reported by Taylor et al.[16], while HDV alone can enter into hepatocytes, initiate RNA replication and the synthesis of HDAg, no secretion of infectious particles occurs in the absence of HBV. The mechanism of viral entry of HDV is similar to that of HBV since the large protein is an important component of the envelope of infectious particles, required for the interaction with NTCP, allowing viral entry into hepatocytes [17–19]. The detection and quantification of LHBs in serum, particularly of PreS1 encoded protein, has recently been proposed as a clinically useful tool in chronic HBV infection. Several authors reported that the composition of HBsAg changes significantly across different stages of HBV infection and have identified the L protein as a novel marker to identify inactive carriers, with an accuracy higher than serum HBV-DNA or total HBsAg levels [20,21]. The amount of LHBs is indeed significantly lower in inactive carriers than in patients with acute or chronic hepatitis, irrespective of their HBV e-antigen status. In addition, the ratio of L, M and S proteins in the envelope of infectious virions (1:1:4) changes progressively in HBeAg-positive patients who achieve HBsAg loss during antiviral treatment. Interestingly, both MHB and LHB proteins decreased earlier than total HBsAg before HBsAg loss, which corresponds clinically with the functional cure, in particular, LHB antigen becomes undetectable about 4.4 ± 2.3 months before total HBsAg loss [22]. In addition, Liu C. et coll. reported that low basal levels of L protein before treatment with PEG-INFα or Entecavir were predictive of a better response to antiviral therapy [23] both in HBeAg positive and negative patients. These data indicate that serum levels of PreS1-encoded L protein may be a reliable candidate marker for the determination of HBV infection stages and that the baseline levels of this protein could predict the virological response to antiviral treatment. Furthermore, as reported by Nishida Y. et al., PreS1 levels were significantly higher in patients with higher grading of liver inflammatory activity and, more interestingly, the PreS1/HBsAg ratio reflected liver fibrosis staging better than HBsAg alone [24]. In addition, since advanced liver cirrhosis is one of the most significant risk factors for hepatocellular carcinoma (HCC) development in patients with chronic HBV infection, the PreS1/HBsAg ratio was found to predict HCC development better than other fibrosis parameters, such as platelet count. The combination of two variables, namely age and PreS1/HBsAg ratio, was even better at accurately predicting the appearance of HCC. These results are in line with previous studies in HBV transgenic mice, where over-production of HBV Large envelope protein in hepatocytes determined severe, prolonged hepatocellular injury, leading to inflammation, regenerative hyperplasia, transcriptional deregulation, aneuploidy and liver tumor progression [25]. In addition, the PreS1-encoded protein facilitates hepatocellular carcinoma development by promoting the appearance and self-renewal of liver cancer stem cells [26]. In agreement with these findings, clinical studies have reported that PreS mutations, such as PreS1 and PreS2 deletions, are significantly correlated with an increased risk of HCC [27,28]. These mutations determine indeed an increase of Large envelope protein production, resulting in its accumulation into the endoplasmic reticulum and causing oxidative stress, with increased liver damage [29].

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To date, ELISA and TRFIA technologies have been applied to the detection of serum PreS1-encoded antigen [30,31]. The literature reports few studies on this topic, most of them describing prototype assays using chemiluminescent immunoassay or ELISA technology, based on "not for sale" monoclonal antibodies against PreS1 [20,23]. Therefore, the aim of our study was to standardize a new test based on AlphaLISA® technology [32,33], exploiting our previous knowledge, based on the fact that the PreS1-encoded portion of the HBV surface antigen has a high binding affinity for the SerpinB3 molecule [34,35]. In fact, by using a synthetic tetravalent PreS1(21-47) peptide, affinity chromatography allowed the isolation from hepatoma cells of a 44 kDa protein, identified as human SerpinB3 (previously known as SCCA1) by sequence similarity search [34]. This newly developed technique has been applied to detect PreS1-encoded protein levels in the serum of HBV-infected patients, with or without HDV coinfection, at different clinical phases of the infection.

Materials and Methods

Patients

Serum samples from 51 HBsAg-positive patients referred to the Regional Referral Center for Liver Diseases of the Padua Teaching Hospital were analysed. The demographic profile of the study population is reported in Table 1. Among the 51 HBV-infected patients, 5 were inactive HBV carriers, among patients with active HBV infection, 36 were on antiviral treatment with nucleoside analogues and 2 were tested before antiviral treatment. In addition, 8 patients had HDV co-infection, defined by the positivity of HDV antigen in serum were also included in the study. As a control group, sera from 62 blood donors were used. Serum samples were obtained from whole blood collected into Vacutainer tubes (BD Diagnostics, USA) after centrifugation for 15 min at 2000 x g. Serum was aliquoted into cryovials and stored at -80°C until use. The study was approved by our institutional Ethics Committee and was performed according to the principles expressed in the Declaration of Helsinki. A signed informed consent for serum sample collection was obtained from all the patients included in the study.

PreS1 AlphaLISA Assay

The PreS1 assay exploited previous knowledge indicating that the PreS1-encoded protein of the HBV surface antigen possesses a high binding affinity for the SerpinB3 molecule. In detail, we have used AlphaLISA® technology (Bielefeld-Sevigny 2009; Cassel JA 2010) where in the solid phase surface the SerpinB3 molecule was captured by the corresponding antibody bound to AlphaScreen® Acceptor beads (Perkin Elmer, MA, USA). These activated beads were able to capture L surface protein containing PreS1 in the analysed sample which was then revealed by the addition of biotinylated anti-HBs antibody that bound streptavidin AlphaScreen® Donors beads (Perkin Elmer, MA, USA) (Figure 1).

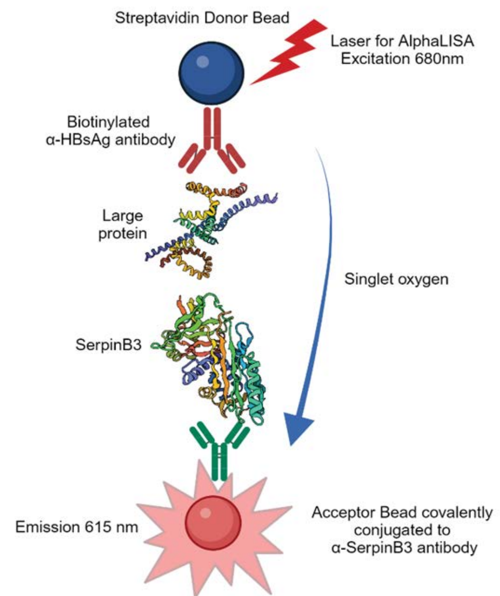

Figure 1: Schematic representation of PreS1 AlphaScreen test. When SerpinB3 and PreS1-encoded protein interact, the Donor beads are brought into proximity of the Acceptor beads and excitation of the Donor beads results in a luminescent signal from the Acceptor beads. Created with Biorender.com

The standardization of the assay was carried out basically in two main steps. First of all, Acceptor beads were covalently conjugated with an anti-SerpinB3 antibody and then preliminary experiments were performed to test different assay components and reaction conditions, in order to optimize the protocol (Suppl. Materials and Methods).

Protocol of PreS1 detection assay. Based on the results of the preliminary experiments, the optimized protocol to detect PreS1 containing HBsAg particles was performed in 1/2 AreaPlate-96 High binding™ (Perkin Elmer, MA, USA) using 20 µL/well of the conjugated AlphaLISA Acceptor beads (Perkin Elmer, MA, USA) at 40 µg/mL in PBS pH 7.4 buffer. The plate was centrifugated for 70 min at 2000 x g at 4°C and left to rest for 1hr at room temperature (RT). As blocking reagent 100 µL/well of a 1% BSA-PBS solution, chosen in set-up method experiments, were incubated for 2 hrs at RT. Then, the blocking solution was removed and 25 µL of PBS (as negative control) or 25 µL of the patient's sample diluted at 1:50 in a solution containing recombinant human r-SerpinB3, obtained in our laboratory as previously described [36], were added at 10 ng/mL concentration. A standard curve, obtained by serial dilutions of a reference HBV-DNA positive serum sample, previously tested positive for PreS1-encoded protein (500 µg/mL) by using as reference calibrator recombinant Large S protein obtained

in yeast (SmithKline Beecham Biologicals S.A., Belgium), was included in each experiment. All patient samples were tested in duplicate. After a quick spin at 2000 x g, followed by an overnight incubation at room temperature, the plate was gently washed 4 times with 100 µL of washing buffer (0.05% Tween20 in PBS pH 7.4). Twenty-five µL/well of 3 nM biotin-conjugated anti-HBs antibody (Meridian Life Science, TN, USA) were added and incubated for 2 hrs at room temperature. Additional 25 µL of streptavidin Donor beads (80 µg/mL, Perkin Elmer, MA, USA) were added under subdued light (< 100 lux) and incubated for 45 min in total darkness, sealing the plate with a protective film (Topseal A plus, Perkin Elmer). The fluorescence intensity was measured using the standard Alpha protocol through of the multimedial plate reader Ensight (Perkin Elmer, MA, USA) and was expressed as Alpha counts.

Serum viral markers

Serological and molecular assays were assessed by routine commercial techniques at the Microbiology Laboratory Unit of the Padua Teaching Hospital. HBsAg was measured by a chemiluminescence-based immunoassay (CLIA) method with a fully automated analyser Liaison XL (DiaSorin, Saluggia, Italy). The HDV antigen was detected by ELISA using the automated processor Analyzer I (Euroimmun, Lübeck, Germany). HBV DNA level was measured using a real-time PCR assay, with a detection range of 1.0-9 Log IU/mL (Alinity m-HBV assay; Abott Laboratories, Abott Park, IL, USA).

Statistical Analysis

Age and gender were categorized for the four groups, identified on the basis of their HBV infection stage, and the difference of each variable was evaluated by the One-Way ANOVA test. PreS1 and HBV-DNA positivity rates were calculated on the basis of their identified cut-off points and the difference of each variable was evaluated by Fisher's test. Mann-Whitney test was applied to analyse the differences of abnormally distributed continuous variables as PreS1, HBsAg and HBV-DNA. The relationship between PreS1 levels and the other HBV markers was explored using Sperman's rank correlation test. All statistical tests were two-sided and the level of statistical significance was set at $p<0.05$. Data were analysed by the GraphPad Prism version 10.2.0 software.

Results

Standardization of the Assay

Before testing PreS1 levels in clinical samples**,** several experiments for the standardization of the protocol conditions were performed. As an initial step, orange Omnibeads at 40 g/mL in PBS pH 7.4 buffer, provided by the company (Perkin Elmer, MA, USA), were used instead of Acceptor beads to assess the stability of the binding which was confirmed by a high mean value of three replicates (19.474 ± 2.694 Alpha counts). This result indicates that the washing step, planned in the protocol, does not affect the binding of the beads to the plate. Regarding the choice of the best incubation time condition, the value of the fluorescence intensity ratio (S/N) between the positive reference serum sample at 1:8 dilution (Signal) and PBS as negative control (Noise) was 6.92, when streptavidin Donor beads were incubated for 45 min and decreased to 5.12 in a prolonged incubation condition (4 hrs). As a consequence, all further assays were conducted using an incubation time of 45 min. Figure 2 shows the different curves of the fluorescence values, expressed as Alpha counts, obtained with serial dilutions of the standard HBV-DNA positive serum sample and two different solutions of the blocking buffer.

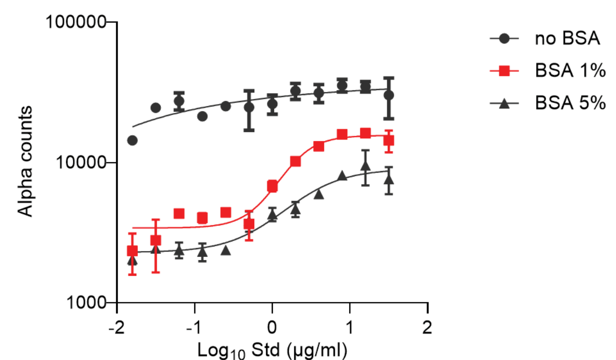

Figure 2: Optimization of the blocking conditions with BSA at 1, 5 % or in absence of BSA (PBS). Serial dilutions of standard serum were determined. The results are expressed as log10 of standard concentration (Log10 Std).

The fluorescence intensity progressively increased with the serum sample concentration. The 1% BSA solution was selected for all subsequent experiments, based on its highest fluorescence values. To assess the lower concentration of biotinylated anti-HBs antibody, able to retain the optimal sensitivity of the assay, four antibody concentrations (from 10 to 0.3 nM) were tested. The reference positive serum sample was tested at 1:8 and 1:128 dilution. As shown in Figure 3, the optimal concentration of anti-HBs antibody was 3nM, while the Hook effect was pointed out at 10 nM. S/N ratio values are reported in Supplementary Table S1.

In order to assess whether the preincubation of the biotinylated anti-HBs antibody with streptavidin could improve the sensitivity of the assay, a variant assay was performed. Donor beads were pre-incubated with anti-HBs antibody in total darkness for 30 min at room temperature and the mixture was then added to each well for 2 hrs in standard conditions. The S/N ratio of the reference positive sample was assessed using four different concentrations of biotinylated anti-HBs antibody (10-3-1-0.3-0 nM). The results obtained documented that the fluorescence values were

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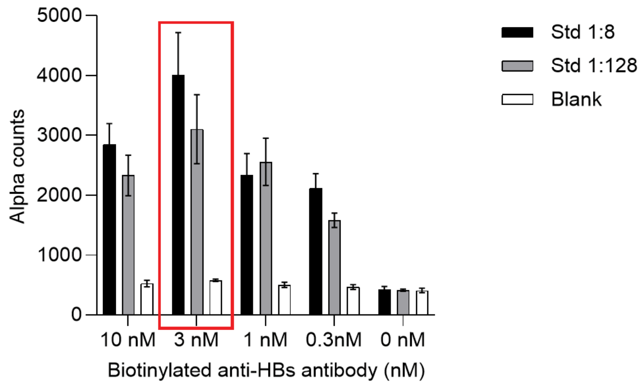

Figure 3: Performance of the AlphaLISA assay using different concentrations of the biotinylated anti-HBs antibody. Fluorescence intensity values of PreS1-encoded protein using the HBV-DNA positive standard sample at 1:8 and 1:128 dilution. PBS buffer was used as negative control (Blank).

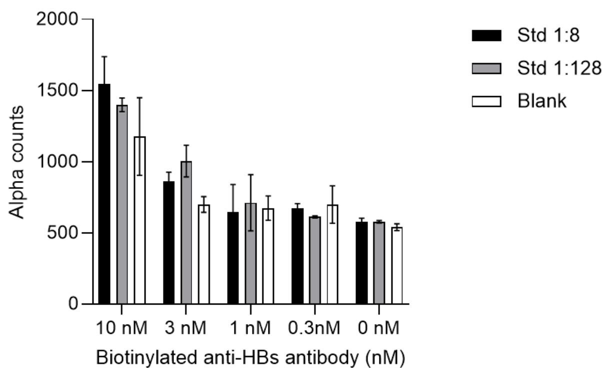

Figure 4: Pre-incubation variant assay using different concentrations of the biotinylated anti-HBs antibody. Fluorescence intensity values of the HBsAg standard sample at 1:8 and 1:128 dilutions and PBS buffer (Blank) was determined after 30 min pre-incubation of donor beads and anti-HBs antibody.

about 50% reduced in comparison with those obtained using the standard protocol. As reported in Figure 4, high values of background were detected in the absence of biotinylated anti-HBs antibody.

The corresponding S/N ratio values are reported in Supplementary Table S2. For these reasons, this protocol was not further considered.

Serum PreS1-encoded protein levels in HBV-positive patients

The AlphaLISA assay was carried out at the optimal conditions detected in the experiments of the preliminary phase. Serum concentrations of PreS1, expressed in µg/mL, were determined by interpolation of Alpha counts of the samples on the calibration curve plotted with the standard reference serum and PreS1 ranged from 62 µg/mL to 0.5 µg/mL. The low detection limit (LDL) of the assay, calculated as the mean of blank replicates +3 standard deviation (SD) was 1,286 Alpha counts, corresponding to 0.013 µg/mL of PreS1. Samples with PreS1 ≥ 11.48 µg/mL were considered positive, on the basis of the mean +3 SD values obtained in sera of 62 blood donors chosen as healthy controls. PreS1-encoded protein levels were quantified in serum of 51 HBsAg-positive patients. All the patients were divided into three groups: patients with chronic hepatitis, HDV-coinfected patients and inactive HBV carriers. The patients with chronic hepatitis were further subgrouped into those who were on antiviral treatment and those who were tested before treatment. Demographic characteristics and rate of positivity for PreS1 and HBV-DNA (considering as cut-off 1 log IU/ml) are summarized in Table 1.

Table 1: Characteristics of the patients included in the study

	Chronic hepatitis B		HBV-HDV coinfection	Inactive	p
	Treatment yes Treatment no		(N=8)	HBV carriers	values
	(N=36)	(N=2)		(N=5)	
Sex n. (%)					
Male	26	2	3	4	ns
Female	10	0	5	1	
Age (years)					
Median	55	41	56	53	ns
(Range)	(27-77)	(34-48)	(49-65)	(30-72)	
Pre-S1 (% positivity)	14%	100%	50%	0	0.005
HBV-DNA (% positivity)	11%	100%	12.50%	80	0.0005

As reported in Figure 5A, median values of PreS1 were significantly higher among patients with chronic HBV infection before treatment, as compared to patients treated with antiviral therapy.

An interesting result was the fact that the HDV co-infected patients presented significantly higher values of PreS1 than patients with antiviral treatment, despite the fact that HBV-DNA was almost undetectable also in this group (Figure 5C). In agreement with these results, HDV-coinfected patients were more frequently positive for PreS1 than treated patients with chronic hepatitis (Table 1). As expected, inactive HBV carriers also showed very low levels of PreS1, since in these patients HBV-DNA was low and HBsAg circulates mainly as 22nm spheres, lacking PreS1-encoded protein [20,37]. No significant differences in HBsAg distribution were observed in

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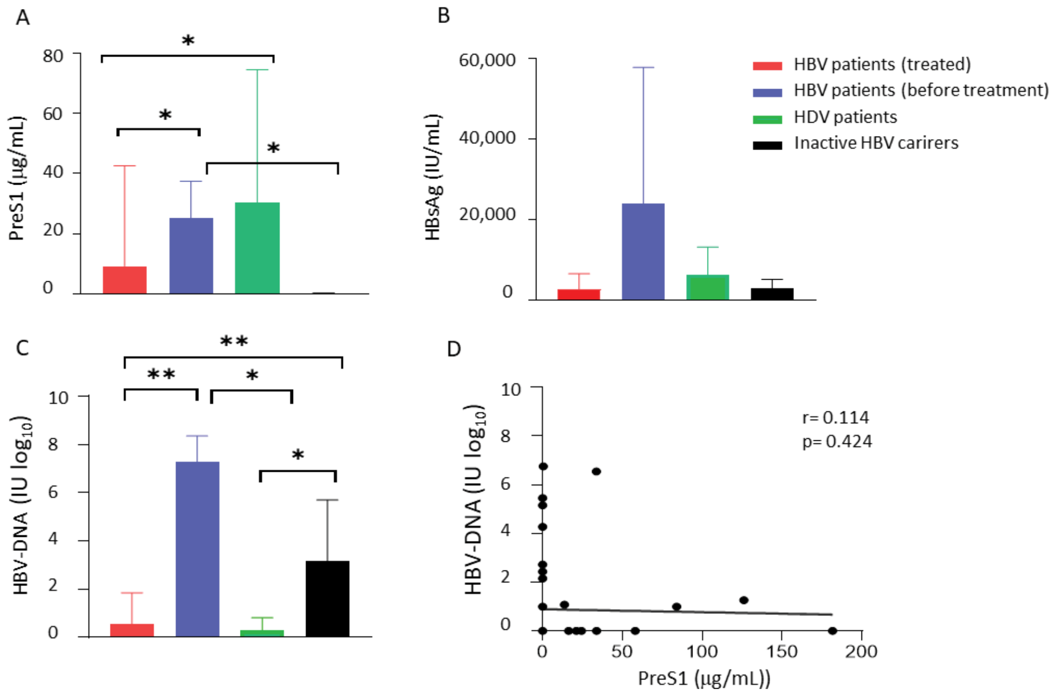

Figure 5. Distribution of PreS1 (panel A), HBsAg (panel B), HBV-DNA (panel C) levels in the different groups of patients. Bars represent median values with standard deviation (SD). **P< 0.005, *P< 0.05 (Mann Whitney test). Panel D: scatter plot showing the comparison between HBV-DNA and PreS1 levels in all the 51 HBsAg positive patients.

all groups of patients. No significant correlation was observed between PreS1 levels and viral load when all patients were considered (Figure 5D). These results support the fact that infected hepatocytes, especially in HDV- infected patients and in HBV patients under antiviral therapy, besides virions, release subviral particles including the filaments containing the Large S protein of the viral envelope, but not HBV-DNA.

The different behaviour of viral biomarkers was further assessed in a single HBV-infected patient which was prospectively followed up in our outpatient clinic for 6 years. The patient was HBeAg positive at presentation and developed anti-HBe seroconversion during follow-up. He was treated with lamivudine and autonomously suspended the treatment at year 5 of follow-up, being HBV-DNA negative for 3 years. After withdrawal HBV-DNA persisted negative, with a progressive decline of HBsAg, while ALT progressively increased, especially at year 6, concurrently with the diagnosis of HCC (Figure 6).

In this patient PreS1 levels progressively increased from presentation, even at 1 year after the starting of antiviral treatment, when HBV-DNA was negative, but ALT was still elevated. At year 3 from presentation, the levels of PreS1 were undetectable and paralleled the behaviour of ALT, maintained normal at year 3 and 4, but progressively increased at year 5 and 6, although their absolute levels were lower than in the HBeAg positive phase. At year 6 a diffuse form of HCC was diagnosed and the patient died within 6 months.

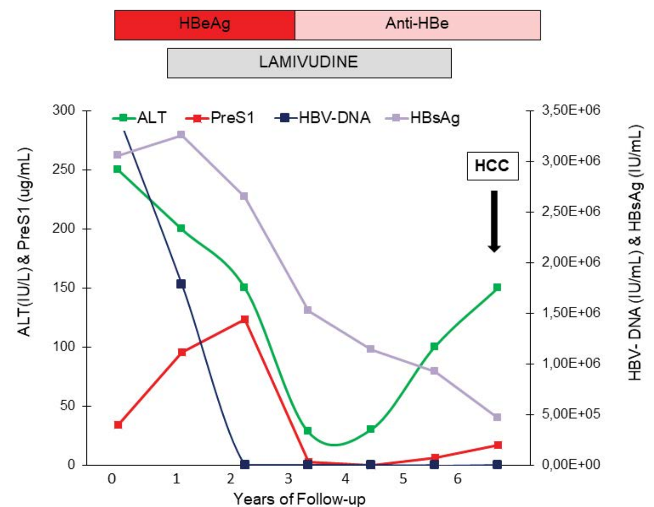

Figure 6. Schematic representation of the behaviour of HBV markers in a patient with chronic HBV infection. The changes overtime of Alanine Amino Transferase (ALT), PreS1, HBV-DNA and HBsAg are reported. HCC, hepatocellular carcinoma.

Discussion

Chronic HBV infection is one of the major global public health problems and long-term consequences include the development of liver cirrhosis and HCC [38,39]. Many mechanisms have been proposed for HBV-mediated hepatocarcinogenesis, however the precise pathogenesis is still not fully understood. Viral integration into the hepatocyte

genome [40,41], increased hepatocyte turnover, chronic inflammatory response towards HBV and the viral protein-induced oncogenesis are all hypothesized mechanisms for the development of HCC [42]. Among these, the expression of the viral surface proteins (HBsAg), plays a considerable role in this process interfering with cell signalling and gene transcription [43–45]. During the infection, mutations of envelope's proteins, due to immune pressure or antiviral treatments are frequent. For instance, the carboxy-terminal truncation of L protein has transactivating properties, thus promoting transcription factors with the resulting increase of proliferation rate of infected hepatocytes. Several studies have shown that PreS mutations such as PreS1 and PreS2 deletions are significantly correlated with an increased risk of HCC. Both PreS1 and PreS2 mutants indeed activate endoplasmic reticulum (ER) stress in hepatocytes with accumulated L protein [27,29,46,47]. Studies conducted on transgenic mice, that overproduce the Large protein, have documented that the accumulation of this protein within the hepatocytes determines oxidative stress, leading to severe, prolonged liver damage [28] and eventually tumor development [25]. Reactive oxygen species can favour carcinogenesis through the activation of VEGF/Akt, NF-kB pathways and calpain cleavage [48]. In addition, PreS1 can lead the production of CD133, well known as a liver cancer stem cell marker in human HCC [49] and of CD117, highly expressed in a wide range of human malignancies, including HCC [50]. More recently, the attention of the scientific community has focused on additional aspects of HBV infection: like other infectious diseases, it is considered a systemic and not an organ-specific disease. The effects of viral-induced alterations affect the entire body, where different organs are connected by blood and lymphatic vessels. Indeed, metabolomic analysis has revealed that HBV induces a metabolism dysregulation both in the liver and in extrahepatic organs, leading to a host's "hypermetabolic" profile [51](Lan W, Metabolites 2022). Using nuclear magnetic resonance (NMR), many changes in the metabolites of nine different tissues were detected in HBV-transgenic, compared to controls, including the enrichment of amino acids in the kidney and intestine and the increase of the metabolites related to tricarboxylic acid cycle (TCA) and glycolysis/gluconeogenesis in the liver, heart, spleen, lung and pancreas. Along these lines, HBV-related HCCs were found to undergo global metabolic reprogramming during tumor growth, both in tumoral and peritumoral tissue [52].

From the clinical point of view, modifications of PreS1 levels over time in HBV-infected patients reflect the significant changes in HBsAg composition across the different stages of the infection. Indeed, early PreS1 reduction during antiviral treatment anticipates total HBsAg decrease [22,23] and PreS1 is very low in serum in inactive carriers, where the majority of HBsAg is composed of 22 nm spheres carrying mainly S protein [20,21]. For all these considerations, the quantification of PreS1 levels in serum appears to be useful in a clinical setting, complementing the information provided by HBV-DNA or total HBsAg levels. On the other hand, Cornberg M. et al recognized a limited clinical use of total HBsAg in the assessment of untreated HBeAg positive or negative patients, suggesting the need to combine HBsAg quantification with HBV-DNA and histologic assessments [53]. Among the tests used in clinical practice to diagnose and classify HBV infection, the quantification of total HBsAg was first standardized about 40 years ago [54], however, it cannot distinguish the three different surface proteins, since it recognizes the S protein of the *carboxyl*-terminus, common to all of them, even in the more recently developed assays showing better performance and higher sensitivity [55]. The assay described in this study allows the specific quantification of the PreS1 protein since only this portion of the envelope protein recognizes the SerpinB3 molecule, attached to the solid phase [34]. Therefore, this assay can accurately identify the presence of mature virions and filaments in case of replication not only of HBV, but also of HDV. Our preliminary results have provided evidence that PreS1 levels, measured with this technique, do not overlap with other parameters, including total HBsAg, transaminases and HBV-DNA levels. In this regard, the described case of the HBV-infected patient who presented a progressive increase of PreS1 levels, even after one year of antiviral treatment, despite HBeAg clearance and HBV-DNA negativization, and eventually developed HCC, represents a challenge and underlines the importance of PreS1 accumulation in liver disease progression. In addition, the high levels of PreS1 detected in HDV-coinfected patients may concur to explain the relevant pathogenicity of this defective virus that requires LHBs for its infectivity [14,19]. In conclusion, the standardized PreS1 assay can become a useful tool to improve the HBV and HDV stage classification. Further studies with a larger number of patients are required to further assess the prognostic potential of PreS1 detection in patients with HBV infection, without and with HDV coinfection.

Abbreviations used in this paper: LHB, large protein; HBV, hepatitis virus B; HDV, hepatitis virus delta; LDL, low detection limit; SD, standard deviation; HBsAg, hepatitis B surface antigen; NTCP, sodium taurocholate co-transporting polypeptide; HDAg, hepatitis delta antigen; HBeAg, hepatitis B envelope antigen; PEG-INFα, polyethylene glycol- interferon alfa; HCC, hepatocellular carcinoma; ELISA, enzyme linked immunoadsorbent assay; TRFIA, time-resolved fluorescent immunoassay; CMO, carboxymethoxylamine; S/N, signal/noise; BSA, bovine serum albumin; PBS, phosphate buffered saline; ALT, alanine aminotransferase; VEGF, vascular endothelial growth factor; Akt ATP-dependent tyrosine kinases; NF-kB, Nuclear factor-κB; CSC, cancer stem cells; NMR, nuclear magnetic resonance; TCA, tricarboxylic acid cycle; AST, aspartate aminotransferase.

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Authors' contribution

AB established and validated the method, analysed results and drafted the manuscript. SQ and MR made laboratory tests, PG and AM provided clinical samples, PP designed the study and critically revised the manuscript. All authors have approved the final version for publication.

Data Transparency Statement: data, analytic methods, and study materials will be made available to other researchers upon request.

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