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Predicting HBsAg clearance in genotype A chronic hepatitis B using HBsAg epitope profiling: a biomarker for functional cure

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Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; NA, nucleos(t)ide analogue; aa, amino acid; TDF, tenofovir; ADV, adefovir; W, weeks; SL, HBsAg loss; VLP, virus-like particles; ALT, alanine aminotransferase; mAbs, monoclonal antibodies; CP, clearance profile; NCP, non-clearance profile; HAV, hepatitis A virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; EBV, Epstein-Barr virus; EIA, enzyme immunoassay; IndCP, induced clearance profile; IndNCP, induced non-clearance profile; RFU, relative fluorescent units; NR, non-responder; EOS, end of study

Lay Summary: New treatments are being developed for patients with chronic hepatitis B (CHB) with the goal of achieving functional cure. The availability of a diagnostic biomarker that would enable the reliable prediction of functional cure would help expand the search for better CHB treatments. This study has identified specific changes in the shape and conformation of the hepatitis B virus envelope protein which can be used as a marker for predicting functional cure in genotype A infected patients, thereby guiding future studies that could lead to improved therapeutic approaches for these patients.

Conflict Of Interest: Thomas Leary and Gavin Cloherty are employees of Abbott Laboratories and own stock and shares in Abbott Laboratories.

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Abstract

Background and Aim: Functional cure is the major goal of CHB therapy though few biomarkers predict this outcome. HBsAg epitope occupancy can be influenced by therapeutic and immune pressure. The aim of this study was to map the HBsAg epitope profiles during long term nucleos(t)ide analogue therapy in patients with genotype A CHB, in the context of HBsAg loss/seroconversion.

Methods: We evaluated 25 genotype A CHB patients in the GS-US-174-0103 trial of HBeAg-positive CHB patients treated with tenofovir or adefovir for 4 years, 14 who achieved HBsAg loss whilst 11 had no change. We epitope mapped the major domains of HBsAg to identify those patients with HBsAg clearance profile (loss of binding at both loops 1 and 2 epitopes of the “a” determinant) versus non-clearance profile (no change in epitope recognition, or loss of epitope binding at one loop only), correlating this to on-treatment HBsAg responses. Complexed anti-HBs was also measured.

Results: Analysis of the HBsAg epitope profiles of the 25 patients at baseline identified no predictive correlation with HBsAg loss. In contrast, analysis at week 48 and end of study (week 192) or prior to HBsAg loss identified significant predictive associations between development of HBsAg clearance profiles and outcome of functional cure. The detection of a clearance profile also correlated with the development of an ALT flare and detection of anti-HBs complexed with HBsAg.

Conclusion: The detection of HBsAg clearance profiles by epitope mapping represents a novel viral biomarker, reflecting an emerging anti-HBs selection pressure prior to functional cure.

Abstract Word Count: 250

Key Words: *antiviral therapy; genotype A infected patients; HBsAg loss; HBsAg epitope mapping; ALT flare; functional cure.*

1. INTRODUCTION

Approximately 2 billion people have been infected with the hepatitis B virus (HBV), of whom 260-350 million have developed chronic disease, resulting in more than 780,000 deaths annually attributed to the clinical outcomes of liver cirrhosis and hepatocellular carcinoma (HCC) [1]. Current antiviral therapies for chronic hepatitis B (CHB) are not curative, and the

available preventative vaccine has no impact on existing infections. Nucleos(t)ide analogue (NA) therapies effectively achieve viral DNA suppression (HBV DNA undetectable), but not the clearance of the HBV surface antigen (HBsAg), which is associated with ongoing risk for developing HCC [2, 3]. Functional cure is the current goal for CHB therapy [4], but is rarely achieved, occurring in only 1-2% of untreated patients annually and only marginal improvement when treated with nucleos(t)ide analogue (NA) therapy [4]. The endpoint of HBsAg loss +/- seroconversion to anti-HBs, with undetectable serum HBV DNA, is considered to be a functional cure for CHB [4], as it allows not only for the cessation of therapy, but is also associated with significant reduction in the rates of liver cirrhosis and the development of HCC, and an overall increased survival rate [2-4].

The mechanism(s) associated with HBsAg loss and/or seroconversion are unknown, but the basis for this clearance is presumably the selective pressure of an effective host antiviral response. An effective B-cell response has been shown to be essential to maintain a functional hepatitis B cure, with B-cell depleting therapies (e.g. rituximab, anti-CD 20) being associated with HBV reactivation leading to liver failure and death [5]. Clinical and diagnostic observations suggest that B-cell clones encoding anti-HBs with low affinity to the homologous HBsAg exist in most patients with CHB [6]. Concurrently expressed, or co-circulating anti-HBs in HBsAg-positive patients with CHB further suggests that the anti-HBs response is quantitatively and/or qualitatively insufficient or inadequate to overcome chronic infection, but does indicate that HBsAg specific antibody production is not completely blocked [7]. During spontaneous resolution of acute hepatitis B infection, a coordinated interaction of HBV-specific T cells and B cells eliminate infected hepatocytes, suppress viral replication via non-cytolytic pathways, and neutralise virions through the production of virus-specific antibodies (anti-HBs). Whether these same or similar events occur during functional cure is not known.

The HBV translates three membrane-associated surface or envelope proteins (L, M, and S) that all share a common C-terminal or S region, which encode the major anti-HBs binding domain, also called the “a” determinant, spanning residues 99 to 160 of HBsAg [8]. The HBsAg “a” determinant is a conformationally dynamic protein domain, incorporating a high density of conserved cysteine and proline residues resulting in the formation of multiple loop domains [8]. The HBsAg is vulnerable to both structural alterations and also to epitope availability from applied immune pressure, which can modify the HBsAg epitope availability profile [9]. HBsAg epitope availability or occupancy are influenced by immune pressure

such as a 'clearing' anti-HBs response targeting the HBsAg antigenic "a" determinant region or major loop domains therein (loop1: amino acids (aa) 107-138; loop2:aa139-149). At present, no biomarkers can reliably predict functional cure. We have previously characterised a unique cohort of NA treated genotype A patients with CHB who have undergone functional hepatitis B cure [10] and also demonstrated that ALT flares can predict HBsAg loss [11]. The availability of this cohort provided the opportunity to study HBV-specific humoral immune responses directed against epitopes within the HBsAg antigenic "a" determinant, and to determine if loss of these epitopes was predictive of a functional cure.

2. METHODS

2.1 Patients: Cohort Selection and Characteristics

Patients were selected from the GS-US-174-0103 (G103) study [10], a randomised double-blind, phase 3 clinical trial of NA treatment-naïve, HBeAg positive, immune clearance phase patients, treated with tenofovir (TDF) or adefovir dipivoxil (ADV) for 48 weeks (W), followed by TDF monotherapy for an additional 144 weeks (Supplemental Figure 1). Complete data to W192 was available for 146 patients, including 42 (29%) genotype A patients. In total, 141 (96%) attained antiviral suppression and 22 (15%) achieved HBsAg loss (SL). In the genotype A subgroup, 33% (14/42) attained HBsAg clearance. We studied a subset of 25 genotype A (HBeAg+) CHB patients from the G103 study, 14 who achieved HBsAg clearance +/- seroconversion, whilst 11 realised no change in HBsAg (<0.5log decline) by W192 (end of study). Demographic and clinical comparison of these two groups of patients revealed they were essentially identical except for HBsAg-loss or not (results not shown [10]).

2.2 Diagnostic Serology

Study samples were serologically analysed for quantitative HBsAg (IU/mL; LLOD 0.05 IU/mL), HBeAg (PE IU/mL), alanine aminotransferase (ALT; U/L), and 'free' anti-HBs antibody (IU/L) using Elecsys kit diagnostic assays on the Roche Cobas platform (Basel, Switzerland).

2.3 HBsAg Epitope Mapping

A 19plex HBsAg epitope mapping assay on a Bioplex®200 platform (Figure 1A), targeting anti-HBs epitopes across 5 HBsAg domains has been described previously [12] (Figure 1B

and 1C). Briefly, epitope display (antigen conformation) and occupancy ('native' anti-HBs recovery) influence the HBsAg profile. HBsAg profile is reported as fold change (95% CI: +/- 0.5-fold) in comparison to the genotype backbone and normalised to the pre-treatment profile (Figure 2 and 3) [12].

2.4 HBsAg Clearance Profile (CP) Analysis

The HBsAg epitope profile was then mapped using this 19plex immunoassay targeting the key epitopes across the HBsAg during the "a" determinant (Figure 1). HBsAg profiles were classified either as a HBsAg Clearance Profile (CP: reduced epitope recognition at both loops 1 and 2 epitopes of the "a" determinant [8]) (Figure 2B) or an HBsAg non-clearance profile (NCP: no profile change, or reduced epitope recognition at one loop only) (Figure 2A). Results were then related to the serum HBsAg level.

2.5 Complexed anti-HBs (with HBsAg): Concurrent anti-HBs and HBsAg

An in-house EIA for detection of anti-HBs complexed with HBsAg (concurrent antibody and antigen), which is not able to be reliably detected using current diagnostic assays, was also developed (see Supplemental Figure 2). In this assay HBsAg is captured by 2plex magnetic bead set conjugated with broadly specific anti-HBs mAbs, and concomitant patient-derived anti-HBs identified indirectly via human IgG Fc domain detection. This assay uses a fluorescent readout based on detection of the complexed anti-HBs Fc domain [13].

2.6 Induced Anti-HBs Clearance Profile (CP) Analysis

The effect of anti-HBs antibody (including patient anti-HBs post-seroclearance) on the HBsAg epitope profile was next examined by pre-incubation of the anti-HBs antibody positive sample, with the wild-type reference HBsAg reagent prior to testing in the 19plex immunoassay for the subsequent HBsAg epitope profile as described above (Supplemental Figure 2). The resulting anti-HBs 'induced' HBsAg profiles were classified either as an Induced Clearance Profile (Ind CP: reduced epitope recognition at both loops 1 and 2 epitopes) or an Induced non-clearance profile (Ind NCP: no profile change, or reduced epitope recognition at one loop only) and the results were related to the clinical outcome of these functional cure patients.

2.7 Peptide Chip Scanning

Pan-genotypic HBsAg (L, M and S domains; 389 residues in total) cyclised and linear peptide chip sets were designed and ordered from PEPperCHIP (Heidelberg, Germany).

Linear peptide chips were printed with a total of 1953 peptides in duplicate (3906 peptides in total), covering HBV genotypes A-H of HBsAg as 13mer peptides, overlapping by 12 residues. Cyclic peptide chips were printed with a total of 1614 cyclised peptides in duplicate (3228 peptides in total), covering HBV genotypes A-D of HBsAg as both 15mer and 10mer peptides, with a shift of 2 residues. Redundant peptides across HBV genotypes were excluded, and the target peptide arrays on both chip sets were framed by internal control Flag (DYKDDDDKGG) and HA (YPYDVDPDYAG) peptides. Peptide chips were blocked with 1% BSA in PBS-T (PBS, pH 7.4 with 0.05% Tween 20), prior to overnight incubation with patient serum diluted at 1 in 1000, and staining with secondary goat anti-human IgG Fc Cy3 antibody (ThermoFisher Scientific, MA, USA). Chips were scanned using a Genepix 4000B scanner with fluorescence intensities quantified by PepSlide Analyzer software and reporting as relative fluorescence units (RFU) signals. Positive binding fluorescence was determined by normalisation of the sample red foreground mean RFU to twice the red background mean RFU, with values >0 RFU indicating positive binding reactivity and values >1000 RFU indicative of strong binding between sample antibody and the chip immobilised peptide spot.

2.8 Statistical Methods

Statistical analyses of categorical outcome variables were conducted using the Fisher exact test, as appropriate for application to small sample sizes. A p-value of 0.05 was considered statistically significant.

3. RESULTS

3.1 HBsAg CP / epitope profile

From the G103 cohort, 181 longitudinal samples (pre-treatment, and time points to HBsAg loss or W192) from the 25 genotype A patients were analysed by mapping each sample timepoint's HBsAg epitope profile. In this subset, 14 patients achieved functional cure and 11 showed no change in qHBsAg by W192. Using the multiplex HBsAg epitope profile immunoassay panel of 19 anti-HBs monoclonal antibodies, divergent antigenic profiles between the 14 HBsAg loss seroconverters and 11 non-responders (NR) were identified. Two patterns were defined: either an HBsAg CP which included a loss of anti-HBs epitope availability at both Loop1 and Loop2 HBsAg "a" determinant, or HBsAg NCP which included no change in anti-HBs epitope recognition, or a loss of recognition at only a single HBsAg epitope loop in the "a" determinant. The HBsAg epitopes were determined across multiple

sample timepoints to HBsAg loss or to W192/EOS, and development of HBsAg CP was established (Figure 3).

An HBsAg CP was identified by W48 of treatment in 10/14 HBsAg loss patients (Table 1). There was strong and statistically significant association (p-value 0.015; PPV 83%) between the development of this HBsAg CP and the outcome of HBsAg clearance/seroconversion (Table 1). The statistically significant association between HBsAg CP and HBsAg loss was enhanced (p-value 0.001) by the end of study (EOS) or the final available sample time point before HBsAg loss and reached a PPV of 92% and an NPV of 77% (Table 1). The baseline HBsAg profile was not predictive of a clinical outcome of HBsAg loss (p-value 0.697). The HBsAg profiles identified within the matched NR patients shared similarity with those profiles obtained with reference cloned escape profiles identified previously in studies of the HBsAg variants sG145R, a vaccine escape variant, and sD144E an HBIg treatment escape variant (data not shown). The development of an HBsAg CP with an outcome of HBsAg loss could also be correlated to the level of HBsAg decline (Table 2), with the association being strengthened with each log IU/ml decline in HBsAg response (p-value 0.003 to 0.0005), as well as to a serum ALT elevation (p-value 0.017) preceding HBsAg decline or HBsAg loss (Table 2) [11]. None of the sera from the specificity sera panel reacted in any of the CP-associated assays (data not shown).

3.2 Complexed anti-HBs development: association with HBsAg CP & HBsAg response

To further understand the development of anti-HBs responses leading up to HBsAg clearance and seroconversion, the complexed anti-HBs assay which measures anti-HBs bound to HBsAg (Supplemental Figure 2) was utilised. This assay identifies the serological profile of concurrent or co-existing anti-HBs antibody with HBsAg during CHB. The development of complexed anti-HBs may represent recovery of the immune response and/or reduction of 'free' HBsAg to increase the ratio of complexed anti-HBs to the HBsAg. Analysis of the G103 genotype A HBsAg loss (n=14) versus NR (n=11) patient subsets determined that complexed anti-HBs was not necessarily associated with HBsAg loss. Complexed anti-HBs with HBsAg was detected prior to HBsAg loss or EOS in 11/14 (79%) patients who achieved HBsAg loss, but also in 7/11 (64%) HBsAg NR subjects. The development of complexed anti-HBs did however, coincide with an HBsAg CP (Table 3), and was significantly associated (p-value 0.004) with an outcome of HBsAg clearance/seroconversion, with a PPV of 91% and an NPV of 71% (Table 3).

In addition, this assay could identify those study patients experiencing incomplete functional cure, where HBsAg persisted beyond the lower detection limit of HBsAg diagnostic assays (<0.05 IU/L), or in a complex with antibody. Complexed anti-HBs with HBsAg was also detected in study patient serum post-seroclearance of HBsAg, indicating incomplete HBsAg clearance. This subset of patients could be in a state of flux or transition to functional cure and could be more prone to reactivation of their CHB status. Analysis of the G103 cohort revealed that HBsAg in complex with anti-HBs persisted in 57% (8/14) patients post- HBsAg loss, reducing to 36% (5/14) by EOS. Thus, 30% to 50% of HBsAg loss patients displayed a serological profile indicative of “diagnostic-assay” clearance (using standard diagnostic serology assays) but not true or complete clearance of HBsAg from the peripheral compartment.

3.3 CP-associated anti-HBs development: Induced HBsAg CP analysis

A third assay was next utilised to measure the ability of the serum samples collected from these functionally cured, seroconverted patients, to induce a clearance profile in the wild-type reference HBsAg reagent used in the Bioplex Platform. In the 14 genotype A HBsAg loss group from the G103 cohort, 12/14 (86%) seroconverted to anti-HBs as determined by the conventional diagnostic assay. Induced CP analysis of multiple sample timepoints between HBsAg loss and W192 from these 12 anti-HBs antibody positive subjects detected a CP-associated ‘clearing’ anti-HBs antibody response or IndCP for 10/12 (83%). The anti-HBs response present in the majority of G103 study patients who achieved HBsAg loss was associated with a HBsAg CP before HBsAg loss. Analysis of the 2/14 anti-HBs negative patients for an Induced CP was also performed on sample timepoints following HBsAg clearance and not surprisingly, these samples did not return a positive result for an IndCP profile of ‘clearing’ anti-HBs antibody.

3.4 Anti-HBs antibody peptide chip screening

The peptide chip screen was designed to comprehensively scan continuous epitopes, both linear and cyclised, spanning the complete HBsAg L, M and S sequence across HBV genotypes A-H (linear peptide chip) or A-D (cyclic peptide chip). All 14 genotype A HBsAg loss subjects from the G103 cohort were screened on the peptide chips using a selected anti-HBs positive sample timepoint post- HBsAg loss for the 12/14 subjects who seroconverted, or a sample between HBsAg clearance and EOS for 2/14 non-seroconverted patients. Only one subject sample (1/14) returned a positive epitope ‘hit’ by peptide chip screening in the cyclic peptide chip, which comprised 15mer cyclic peptides

from genotype A-D. This reactivity was directed to the continuous cyclic epitope within the HBsAg L domain, residues 20-32 (NPLGFFPDHQLDP), associated with the essential domain for NTCP receptor binding [14]. The peptide screening also failed to identify any consistent continuous epitopes within the HBsAg, including the HBsAg S antigenic domain (HBsAg S residues 99-169), which were the target of the anti-HBs response developed post-HBsAg clearance in this cohort of CHB functional cure patients. However, the peptide chips cannot screen for discontinuous epitopes (non-linear, but spatially adjacent residues), which are more applicable to the HBsAg S domain given its highly conformational structure due to multiple cysteine and proline residues. Without an accurate 3D structure for HBsAg (VLPs and virions) design and scanning of discontinuous or conformational HBsAg epitopes cannot be achieved.

3.5 Relationship Between Changes in Serum ALT, Clearance Profile, Complexed Anti-HBs, HBsAg Levels and Functional Cure

The relationship between the development of a serum ALT flare and HBsAg seroclearance in this G103 cohort has been recently published [11]. Table 2 summarises the development of an HBsAg CP with HBsAg decline and ALT flare. A model for the clinical timelines of the virological and diagnostic markers of HBV DNA, HBsAg and ALT together with the development of the HBsAg CP biomarker, along with the detection of complexed anti-HBs with HBsAg and the association of seroconverted anti-HBs response with an Induced CP, is outlined in Figure 4 for the functional cure HBsAg loss patients and Figure 5 for NR CHB patients.

4. DISCUSSION

In this study, three novel assays have successfully been applied to the study of a cohort of patients with CHB [10] in order to monitor HBsAg epitope profile changes for the development of an HBsAg CP, the presence of complexed anti-HBs and finally, the emergence of an antibody response not previously recognised, but capable of inducing a CP leading to functional cure. Collectively, these assays identified quite divergent antigenic profiles and serological responses between patients with HBsAg loss compared to NR patients. The cohort consisted of an important and valuable subset of 14 patients who achieved HBsAg loss/seroconversion (functional cure), and 11 closely matched patients in whom no change in HBsAg ($<0.5 \log_{10}$ IU/ml decline) was observed [10] (Supplemental Figure 1). Epitope mapping of the HBsAg profile resolved this 25 patient cohort into those

who either demonstrated a HBsAg CP or a HBsAg NCP (Figure 1 and 2). By linking these findings to the HBsAg log decline/response on NA therapy, it was possible to demonstrate that an altered epitope availability in the HBsAg antigenic domain is likely due to a developing anti-HBs selection pressure, capable of inducing a CP. Studies of these genotype A CHB patients identified a strong and statistically significant association between the development of this HBsAg CP and the outcome of HBsAg clearance and seroconversion (p -value 0.015 and PPV of 83%) based on HBsAg epitope mapping (Table 1). The development of an HBsAg CP based on loss of specific epitope to the loop 1 and loop 2 regions (due to antibody occupancy or occlusion) were also strongly associated (p -values 0.003 to 0.0005) with a significant decline (>1.0 log IU/ml) or loss in HBsAg (p -value 0.001, PPV 92%) (Table 1 and 2). Indeed, in 9/14 patients who achieved HBsAg loss, an HBsAg CP preceded any HBsAg response, while for 3/14 HBsAg loss patients an HBsAg CP coincided with HBsAg response (>1 log decline), whilst 2/14 HBsAg loss patients an HBsAg CP was not detected. Furthermore, complexed anti-HBs with HBsAg when correlated with an HBsAg CP (Table 3), was strongly associated with HBsAg clearance/seroconversion (p -value 0.004; PPV91%; NPV 71%) . The correlation of the development of an HBsAg CP predictive biomarker with ALT flare (p -value 0.017) (Table 2) further supports the concept that the HBsAg CP is probably driven by the development of a targeted 'clearing' anti-HBs response to clearance-sensitive HBsAg epitopes presented on the surface of hepatocytes. We propose that the HBsAg CP reflects epitope occupancy or availability, particularly within the major loop domains (loop1: aa107-138; loop2: aa 139-149), due to a recovering humoral immune response. Finally, in those 12/14 (86%) HBsAg clearance patients who also seroconverted, the resulting anti-HBs was shown to be capable of inducing an HBsAg CP, and thus can be considered a 'clearing' antibody response or clearance-associated anti-HBs antibody.

Surprisingly, there was a distinct absence of HBsAg S domain epitope reactivity of the 12/14 HBsAg loss with seroconversion subjects when analysed against a comprehensive pan-genotypic peptide chip screening panel of linear and cyclised continuous epitopes across HBsAg L, M and S protein. Not a single positive reactive region within the HBsAg S domain was found in any of the 12 functional cure subjects. These findings strongly indicate that the HBsAg epitopes which need to be targeted to achieve development of a clearing anti-HBs response (and satisfy an HBsAg CP) are discontinuous, spanning fragmented linear sequences displayed in a structurally and spatially dependant manner on or across the HBsAg subunit/s (Figure 1B). The HBsAg is recognised as a highly dynamic

conformational protein subunit forming multi-subunit particles encompassing an unusually large number of highly conserved conformationally influential cysteine and proline residues throughout the “a” determinant antigenic domain [8]. These factors are thought to influence formation of numerous loop structures and contribute to a structural format which is not readily conducive to resolution [12].

This study revealed that the detection of a HBsAg CP preceded or coincided with HBsAg decline and seroconversion. A model linking patients serology responses and biomarker profiles leading up to HBsAg clearance and antibody seroconversion to achieve CHB functional cure (Figure 4) versus HBsAg NR CHB genotype A infected patients (Figure 5) can be proposed. In patients undergoing HBsAg loss/seroconversion a decline in serological markers (HBV DNA, HBeAg and HBsAg) to undetectable levels can coincide with an ALT flare [11] (Figure 4); the HBsAg CP precedes or coincides with an ALT flare (93%) and with complexed anti-HBs (71%) (Figure 4 and Table 3) implying some form of immune reactivity and recovery. In patients who are HBsAg NR, there is no HBsAg decline and a HBsAg CP is not detected (see Figure 5). Serum ALT flares and complexed anti-HBs are inconsistently detected (Figure 5 and Table 3). These profiles could have potential application as biomarkers for predicting functional cure outcomes. Future studies to elucidate the characteristics of this anti-HBs response developed by CHB patients infected with other HBV genotypes could offer a broad based clinically relevant diagnostic for guiding treatment strategies in achieving functional cure.

This study was limited to the analysis of HBV genotype A infected CHB patients, a restriction applied simply by access to available sera from a sufficiently sized cohort of NA treated CHB patients who achieved HBsAg seroclearance +/- seroconversion to antibody. However, future studies will be aimed at expanding this predictive biomarker analysis and validation, and test the model of CP-associated antibody mediated HBsAg clearance culminating in functional cure to additional non-HBV genotype A cohorts of CHB patients. In addition, future studies will involve studies to further characterise the HBsAg CP epitopes targeted by a clearing antibody response and hopefully provide an understanding of the mechanism of antibody mediated CHB clearance that drives functional cure. In conclusion, the development of a HBsAg CP preceded or coincided with decline in HBsAg and was predictive of HBsAg clearance and functional cure. The identification of complexed anti-HBs and induced CP anti-HBs was indicative of an emerging and clearing immune response

developed by patients who achieved functional cure, providing new insights into the events leading to HBsAg loss.

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FIGURE LEGENDS

Figure 1 Diagrammatic representation of the HBsAg epitope profile assay targeting the HBsAg antigenic region, and an assay outcome of an HBsAg CP versus NCP. **A)** 19plex HBsAg epitope profile assay design using the Bioplex200 multiplex platform. Individual fluorescently identified magnetic bead sets which have been pre-conjugated with one of nineteen different anti-HBs mAbs are plexed together and incubated with patient HBsAg-positive sera samples prior to detection via a secondary reported antibody. Interactions are reported in relative fluorescence units (RFU). Raw RFU data is analysed through an *in-house* bioinformatics pipeline and expressed as positive or negative fold change in epitope recognition with respect to HBV genotype backbone and patient baseline pre-treatment sample. **B)** Illustration of the HBsAg S antigenic “a” determinant and C-terminal domains, spanning residues 99-226. Potential loop1 and loop2 domains are indicated, as are the locations of conformationally influential cysteine (orange) and proline (Blue) residues. The

epitope specificity of the 19plex HBsAg epitope mapping mAbs to particular HBsAg domains (N-terminal, Loop1, Loop2, C-terminal, Loop1/2 combinational, conformational) is indicated. **C)** Schematic examples of the HBsAg epitope mapping assay outcome classification of HBsAg profiles as an HBsAg Clearance Profile (CP: reduced epitope recognition at both loops 1 and 2 epitopes) or an HBsAg non-clearance profile (NCP: no profile change, or reduced epitope recognition at one loop only).

Figure 2 Example of HBsAg epitope mapping patient data, illustrating a pre-treatment/baseline HBsAg NCP and conversion to an HBsAg CP prior to HBsAg loss (SL). **A)** Analysis of study patient's pre-treatment or baseline (BL) HBsAg epitope profiles was performed and reported as a +/- fold change with respect to HBV genotype backbone. This patient displays a pre-treatment HBsAg NCP, with NO reduction of epitope recognition (>0.5 fold) detected at either loop1 or loop2 HBsAg epitopes. **B)** During the study the patient developed an HBsAg CP with reduced epitope recognition (>0.5 fold) and both loop1 and loop2 HBsAg epitopes, prior to achieving an outcome of HBsAg loss.

Figure 3 Representative HBsAg epitope profiles (CP and NCP) across study timepoints for and HBsAg SL versus and HBsAg NR patient. **A)** An HBsAg SL patient with a pre-treatment HBsAg NCP developed an HBsAg CP (reduced recognition at BOTH loop1 and loop2 HBsAg epitopes) during the study from week 10, coinciding with HBsAg decline from week 8. An HBsAg CP was maintained from week 10 to week 120 (final HBsAg positive timepoint) prior to SL at week 144. **B)** An HBsAg NR patient with a pre-treatment HBsAg NCP (no change in HBsAg epitope recognition, or reduced binding at only one loop region) that was maintained throughout the study with n=6 timepoints assessed from week 12 to week 192/EOS and no HBsAg decline detected.

Figure 4 HBsAg SL Patient: A model for HBsAg clearance based on clinical serology and developed biomarker profiles. A decline in serological viral markers (HBV DNA, HBsAg and HBeAg) coincided with an ALT flare (week 44-62) indicative of immune activity in the liver. The development of an HBsAg CP predicative of SL from week 12 preceded the viral serology responses. The detection of complexed anti-HBs (with HBsAg) indicative of a developing antibody response coincided with immune re/activation (ALT flare) and also HBsAg CP development. The patient achieved a clinical functional cure outcome of HBsAg clearance and seroconversion at week 96-108.

Figure 5 HBsAg NR Patient: A model for CHB persistence based on clinical serology and developed biomarker profiles. A decline in serological viral markers of HBV DNA and HBeAg followed NA treatment and a brief ALT flare from BL to week 8. The immune event was insufficient or inappropriate and did not result in an HBsAg decline. An HBsAg NCP profile was maintained throughout the study, predictive of CHB persistence. The detection of complexed anti-HBs (with HBsAg) was transient, indicative of an insufficient or weak developing antibody response. To EOS the patient maintained a constant HBsAg load and persistent CHB infection.

Table 1 Development of an HBsAg Clearance Profile (CP) in study patients by week 48 and EOS, or prior to HBsAg loss (SL)

Table 2 Development of an HBsAg Clearance Profile (CP) that coincided with HBsAg decline or ALT flare

Table 3 Detection of complexed anti-HBs antibody (with HBsAg) that coincided with development of an HBsAg Clearance Profile (CP)

SUPPLEMENTARY MATERIAL

Supplemental Figure 1 Schematic representation of the GS-US-174-0103 (G103) phase 3 clinical trial of tenofovir (TDF) +/- adefovir (ADV) nucleoside analogue (NA) treatment study design to week 48 (W48) and week 192 (W192) or end of study (EOS). Viral suppression and HBsAg clearance outcomes are highlighted for the cohort and the genotype A2 subset, from which the study cohort for this study was derived and 25 genotype A2 CHB patients consisting of 14 who achieved HBsAg loss, and 11 HBsAg non-responders.

Supplemental Figure 2 Overview of the complex anti-HBs (with HBsAg) EIA for co-existing antigen and antibody. A 2-plex magnetic bead sets pre-conjugated with two 'capture' anti-HBs mAbs directed to different conserved HBsAg epitopes are incubated with

patient HBsAg-positive sera samples prior to detection of any co-associated or complexed anti-HBs antibody via a secondary IgG Fc reporter antibody. The assay reports in absorbance units at 405nm in comparison to background reference controls.

Supplemental Figure 3 Schematic of the Induced CP HBsAg epitope assay **A)** Based on the 19plex HBsAg epitope profile assay analysis, the induced CP assay introduces analysis of anti-HBs antibody positive patient samples (post-seroclearance) which are pre-incubated with reference HBsAg sample for the detection of an HBsAg CP or NCP directly influenced by the patient anti-HBs **B)** Illustrative explanation of the classification of assay outcome as an Induced CP (reduced epitope recognition at both loops 1 and 2 epitopes) or an Induced NCP (no profile change, or reduced epitope recognition at one loop only).

Table 1

Development of HBsAg CP	At Baseline	By W43 or prior to SL	By EOS or prior to SL
HBsAg Loss (SL), n=14	5	10 (2/10 maintained from BL)	11
Non-Responder (NR), n=11	5	2 (maintained from BL)	1
<i>p-value</i>	0.697	0.015	0.001
Sensitivity	n/a	71%	78%
Specificity	n/a	72%	81%

Table 2

HBsAg CP coinciding with HBsAg decline or ALT flare	0.5 log ₁₀ HBsAg decline	1 log ₁₀ HBsAg decline	2 log ₁₀ HBsAg decline	Coinciding with ALT flare
HBsAg Loss (SL), n=14	6	8	10	12
Non-Responder (NR), n=11	2	0	0	4
<i>p-value</i>	0.234	0.003	0.0005	0.017

Table 3

Association of complexed anti-HBs with an HBsAg CP	Week 192 (EOS) or prior to SL
HBsAg Loss (SL), n=14	10
Non-Responder (NR), n=11	1
<i>p-value</i>	0.004
Sensitivity	91%

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