# The Scientific Basis of Combination Therapy for Chronic Hepatitis B Functional Cure

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***Abstract***

Functional cure of chronic Hepatitis B (CHB) — or HBsAg loss after 24 weeks off therapy — is now the goal of treatment, but rarely achieved with current therapy. Understanding the HBV lifecycle and immunological defects that lead to persistence can identify targets for novel therapy. Broadly, treatments fall into three categories, those that reduce viral replication, those that reduce antigen load and immunotherapies. **[Au: I've removed a sentence here to keep the word count to around 200, ok?]** Profound viral suppression alone does not achieve quantitative (q)HBsAg reduction or HBsAg loss. Combining nucleos(t)ide analogues and immunotherapy reduces qHBsAg levels and induces HBsAg loss in some patients, particularly those with low qHBsAg levels. Even agents that are specifically designed to reduce viral antigen load might not be able to achieve sustained HBsAg loss when used alone. Thus, rationale exists for the use of combinations of all three therapy types **[Au: Edit of this sentence OK, for simplicity?]**. Monitoring during therapy is important not just to predict HBsAg loss but also to understand mechanisms of HBsAg loss using viral and immunological biomarkers, and in selected cases intrahepatic sampling. We consider different paths to functional cure of CHB and the need to individualize therapy of this heterogenous infection until a therapeutic avenue for all patients with CHB is available.

## [H1] Introduction *[Au: 'Introduction' is always our first heading]*

Despite the success of the hepatitis B vaccine, chronic Hepatitis B (CHB) virus infection affects >296 million people, mainly in the Asia-Pacific region, leading to an estimated 820,000 deaths from cirrhosis and hepatocellular carcinoma (HCC) annually[1]. The incidence of **[Au: OK to add 'The incidence'?]** these complications is substantially reduced by loss of hepatitis B surface antigen (HBsAg) — or functional cure[2,3]— which happens rarely in natural history or with current therapy[4]. Complications are also reduced by oral antiviral therapy[5]. The complex natural history of CHB follows four clinical and/or virological phases of infection and ends with a functional cure phase with a decline in quantitative (q)HBsAg and covalently closed circular DNA (cccDNA) levels over time; however, hepatitis B virus (HBV) is never fully eliminated. Viral genome integration is thought to increase with the duration of infection, especially in the hepatitis B e antigen (HBeAg)-negative phase. The persistence of viral cccDNA in the liver might lead to spontaneous or iatrogenic viral reactivation. Thus, depending on the balance between viral replication and immune control, patients can go from one phase to the other, and these phases do not evolve in an obligatory sequential manner.

The pathobiological basis for functional cure involves a sustained shut down of viral replication leading to undetectable viral load and HBsAg in serum, reflecting low levels of cccDNA (with various degrees of epigenetic silencing induced by virus and host cell interactions). The persistence of this viral cccDNA **[Au: OK to change from minichromosome, for consistency?]** reservoir is controlled by innate and adaptive antiviral immune responses[3]. The production of large amounts

of HBsAg by cccDNA and integrated HBV is **[Au: Edit OK?]** a possible contributor to the diversion or exhaustion of immune responses and for the pathobiology of liver disease and its implication for oncogenic events[6].

The goal of any new therapy is to increase the functional cure rate, which is not frequently achieved with currently available treatments[2,7,8]. Drug discovery and development for HBV infection has moved from controlling viraemia to achieving functional cure of CHB[3]. In this context, there are multiple novel agents in early clinical phase development with the objective of functional cure, and are likely to require combination therapy for success. In this review, we provide an overview of the virology and immunology of CHB and how different combinations of these agents are more or less likely to be efficacious based on those that achieve HBsAg loss, as well as surrogate virological and immunological biomarkers. Using this schema we have provided current insights into strategies that are potentially beneficial. Of course, such insights may evolve as more clinical data becomes available.

**[Au: We recommend that you round off the introduction with a guiding paragraph that states clearly what will and will not be discussed in the article.]**

## *[H1] Science of Functional Cure*

### [H2] Virological pathways and mechanisms

HBV is an enveloped hepatotropic DNA virus that uses reverse transcription for its propagation. The HBV genome is a 3.2 kb relaxed circular (rc) partially double stranded DNA. Upon infection, the viral genome is translocated and released into the host cell nucleus where the rcDNA is converted into cccDNA, which serves as

the template for transcription of all viral gene products. The pregenomic RNA (pgRNA), transcribed from the cccDNA, is selectively packaged into nucleocapsids, then reverse transcribed into rcDNA. The capsids can recycle back to the nucleus for cccDNA amplification and/or maintenance or become enveloped and released[9]. An overview of the HBV lifecycle is shown in Fig. 1.

The first step of the HBV cycle is viral cell entry, which is not only important for initiation of HBV infection but also for viral spread and maintenance of chronic HBV[10,11]. Infectious viral particles attach first to heparan sulfate proteoglycans[12] and enter the hepatocyte via the bile acid transporter sodium taurocholate cotransporting polypeptide (NTCP) [13,14].

Following viral entry~~[15]~~ **[Au: Should ref 15 be cited here?]** HBV is uncoated and nucleocapsids move to the nuclear pore complex (NPC) where rcDNA is released into the nucleus and converted into cccDNA[15,16] using recently identified host factors[17,18,19,20,21]. The viral cccDNA associates with cellular histones and non-histone proteins; epigenetic modifications can regulate its transcription[22]. Mechanisms of cccDNA degradation – the ultimate goal of HBV cure — are still only poorly understood[17,23,24,25], although one mechanism is mediated by cytidine deaminases[26,27].

The cccDNA serves as the template for transcription of viral mRNAs and pgRNA. The pgRNA is transcribed into the core and polymerase proteins and at the same time serves as a template for reverse transcription into new rcDNA. This step requires several host factors[28-30]. Viral transcription is regulated by four distinct promoters (preS1, preS2, core and X), two enhancers (Enhancer-I and Enhancer-II)[31] and epigenetic modifications of HBV DNA and cccDNA-associated histones[32,33,33]. These regulatory elements include various transcription factors[34-

[36]. IFN-α and IL-6 have both been shown to decrease acetylation of cccDNA-bound histones and thereby decrease transcriptional activity[37,38,39,40]. This finding is important for understanding the mechanisms of immune-mediated approaches for functional cure. Many of the host chromatin-modifying enzymes are potential drug targets for cccDNA silencing, but adverse effects might be a limitation. HBx protein is an important transcriptional modulator by degrading the structural maintenance **[Au: or structural integrity?]** of chromosomes 5 and 6 complex (Smc5/6) via ubiquitination [41] **[Au: Please reference this statement.]**. Moreover, cccDNA transcription has been shown to be targeted by FXR agonists [42] **[Au: Please reference this statement.]**.

The viral capsid functions as a container for the viral genome allowing reverse transcription and protecting it from host cell nucleases and proteases. The capsid packages pgRNA mediated by HBc-phosphorylation[43]. Reverse transcription occurs only inside the capsid with hepatitis B core protein (HBc) as a co-factor. HBc associated with cccDNA might be involved in transcriptional regulation[44]. This important role has led to the identification of capsid inhibitors as a new class of antiviral agents.

Enveloped virions are essential for spread and their formation requires viral factors (C-terminal of preS1 domain, N-terminal of preS2) and host factors (endosomal sorting complexes required for transport (ESCRT) machinery[45] via the multivesicular body[46]). Subviral particles are non-infectious, and are formed using viral surface proteins as building blocks; the majority of HBsAg in serum is comprised of subviral particles **[Au: Edit OK?]** [47]. This pathway can be inhibited by nucleic acid polymers (NAPs)[48] and S-antigen Transport-inhibiting Oligonucleotide Polymers (STOPS)[49]. Consequently, the HBV life cycle offers

multiple targets for antiviral therapy, some of which are being actively explored for a functional cure as described below (Figure 1).

[H2] Immune pathways and mechanisms

Persistent infection with HBV is thought to result from inadequate immune clearance, including HBV specific CD4 and CD8 T cells that are dysfunctional and depleted by Bcl2-interacting mediator (Bim)-mediated apoptosis and natural killer (NK) cell inhibition[50,51,52,53]. During CHB **[Au:OK to add?]**, T cells have phenotypic, epigenetic and functional features of exhaustion, with upregulated expression of immune checkpoints such as PD-1, CTLA-4, and T cell immunoglobulin domain and mucin domain-3 (Tim-3). Defects extend beyond T cells to multiple components of the immune system, including HBsAg-specific B cells, dendritic cells, NK cells and NK T (NKT) cells [54] **[Au: Please reference this and the previous statement.]**. HBV is thought to be a stealth virus that evades induction of innate immune responses[55], but despite the impaired innate response, robust adaptive immunity is thought to be responsible for clearing infection in most adults infected with HBV. In addition, CHB is also associated with impaired innate immune responses, especially downregulation and dysfunction of Toll-like receptors (TLRs)[56,57]. Moreover, the liver microenvironment is inherently immune suppressive (to protect against severe inflammation), including enzymes such as arginase, IDO[51] and ACAT[58], which impair the functionality of HBV-specific T cells[52].

Immunotherapies can act in tandem with antivirals to clear infected hepatocytes and/or block new infection. The importance of the immune system in clearing HBV is exemplified by resolution of CHB following bone marrow transplantation from an

immune donor[59]. Many immunotherapies attempt to stimulate the patient's own endogenous responses; although immunotherapies are typically considered as either innate or adaptive, many harness both components to some extent, with well-coordinated synergistic activity of the two arms of the immune system likely to be optimal (Figure 2).

Innate immunity mediators lack precise targeting of infected hepatocytes, increasing the risk of untoward consequences; for example, PEG-IFN has direct antiviral effects and expands the NK cell population but suppresses HBV-specific T cells[60,61,62]. Despite its poor tolerability, PEG-IFN has proven capacity for functional cure [63] **[Au: Please reference this statement.]**. Alternative innate immunomodulators include the oral TLR8 agonist selgantolimod, which induces IL-12, thus increasing HBV-specific T cell expansion[64], but might also increase regulatory T ($T_{REG}$) cells, myeloid-derived suppressor cells and regulatory NK cells that might limit T cell boosting[65,66,67] **[Au: Edit OK?]** . MAIT cells, expanded by TLR8 agonists, have the potential to boost the immunogenicity of vaccines[68], and might be a useful bridge linking innate and adaptive activation in the functional cure of CHB.

Adaptive immune responses by T or B cells are a major target of immunotherapeutic strategies, aiming to exploit their precise targeting of HBV to mediate sustained antiviral control[50]. The new generation of therapeutic vaccines might be more successful than past attempts, due to enhanced immunogenicity, inclusion of multiple HBV antigens and careful patient selection. However, existing highly exhausted HBV-specific T cells will probably still be difficult to boost, considering the tolerogenic liver environment. Hence, novel combinations such as the ChAdOx-HBV vaccine[69] in combination with a PD-1 blocking monoclonal

antibody (which is in phase I or IIa trials) might enable better boosting and/or enhanced survival of newly primed T cells[70] **[Au: Are there any references to cite for these trials? The clinical trial website can be cited]** (Figure 2). However, the heterogeneity of checkpoints suggests that individualised immunotherapeutic approaches might be required[71], including alternatives such as IL-2 stimulation[72]. Other potential immunotherapies replace exhausted endogenous immunity with infusions of soluble T cell receptors linked to anti-CD3 antibodies (ImmTavs)[72] or genetically re-directed T cells (TCR-redirected T cells or CAR T)[73].

The contribution of memory B cells to ongoing control of HBV has been highlighted by B-cell depleting therapies (such as rituximab) which cause viral reactivation in CHB infection and those with resolved infection[74]. Boosting endogenous B cell responses could provide the benefit of their full complement of antiviral roles beyond the production of hepatitis B surface antibodies (anti-HBs), including antigen presentation and production of antiviral cytokines[75]. Antibody infusions can reduce excess levels of circulating HBsAg through antibody-dependent cellular phagocytosis, block new hepatocyte infection as well as potentially clear HBsAg-expressing hepatocytes [76] **[Au: Please reference this statement.]**. Bispecific antibodies can simultaneously bind HBsAg on hepatocytes and T cells through an anti-CD3-binding moiety[77], and antibody engineering can create a vaccinal effect, via immune complex–dendritic cell engagement[78].

Understanding the mechanisms of action of immunomodulators can aid rational combinations with antivirals.

## [H1] Strategies for dual combinations

**[Au: Ok to remove the 'Classes of therapeutic agents' heading and combine the info with this paragraph?]** The goal of functional cure has driven a rapid increase in the number of antivirals and immunomodulators that have potent effects in vitro entering clinical development (Table 1). A summary of the various combinations, phase of the study, efficacy and safety can be viewed in Table 2.

### [H2] Combining viral replication inhibitors

An important question is whether combinations of viral replication inhibitors can decrease or silence the pool of cccDNA substantially enough such that functional cure is achieved. The basis for this concept relies on potent suppression of viral replication that decreases the replenishment of the cccDNA pool. One potential impediment to this concept is production of HBsAg through integrated HBV. However, anti-HBs antibody production from HBV-specific B cells might complex any residual HBsAg produced either from cccDNA or integrated sequences.

#### *[H3] Existing antiviral agents: nucleoside analogues in combination*

Current preferred nucleos(t)ide analogues (including entecavir, tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide) offer highly effective suppressive therapy with very low rates of resistance with long-term therapy, but low rates of HBsAg loss (Figure 3). In patients with CHB, low level viremia on treatment is associated with fibrosis progression[79], and patients with decompensated cirrhosis with low level viremia have worse survival **[Au: Worse survival than those without viremia?]** [80]. With new, increasingly sensitive assays, patients who in the past were found to be HBV DNA negative, are now found to have detectable HBV

DNA[81] **[Au: Edit OK? So does this suggest that these existing agents are perhaps not as efficient as previously thought?]**. Intensification of therapy does lead to better HBV DNA suppression particularly in those with high baseline HBV DNA levels, but does not lead to additional benefits in terms of enhanced HBsAg loss or serological outcomes[82].

*[H3] Nucleos(t)ide analogues and capsid assembly modulators (CAMs)*

The combination of nucleos(t)ide analogues and CAMs (11 in clinical trials **[Au: 11 combinations are in clinical trials or 11 CAMs overall in different contexts?]** ) might lead to more potent suppression of viral replication, which could decrease the replenishment of the cccDNA pool and inhibit cccDNA formation in newly infected cells (Figure 3). **[Au: Edit OK?]**

Data are limited on the combination of CAMs with nucleoside analogues. As a monotherapy, most CAMs show 1.4–3.2 log reduction in HBV DNA or pgRNA levels in phase I studies, but little change in quantitative (q)HBsAg [83] **[Au: Please reference this statement.]**. In the phase II Jade study, patients with untreated CHB or who were virologically suppressed, were treated with 250 mg JNJ-56136379 (bersacapavir), once-daily for ≥24 and ≤48 weeks. For the untreated-HBeAg-positive cohort, mean HBV DNA declines at week 24 were 5.88 $\log_{10}$ IU/mL for CAM plus nucleoside analogue versus 5.21 $\log_{10}$IU/mL for placebo plus nucleoside analogue; mean HBV RNA declines were 3.15 versus 1.33 $\log_{10}$copies/mL, respectively, and mean qHBsAg declines were 0.41 $\log_{10}$IU/mL versus 0.25 $\log_{10}$IU/mL, respectively. These findings confirmed the expected target engagement and antiviral potency of bersacapavir in combination with a nucleoside analogue. In untreated-HBeAg-negative patients and virologically

suppressed HBeAg-positive or negative patients, no qHBsAg declines occurred. qHBsAg responders also had HBeAg and Hepatitis B core related antigen (HBcrAg) declines and frequent early on-treatment alanine aminotransferase (ALT) flares. Viral breakthrough occurred with bersacapavir monotherapy, demonstrating that CAMs should be combined with other antivirals to prevent the occurrence of virologic resistance. Overall, bersacapavir in combination with a nucleoside analogue showed good antiviral activity in patients with CHB, but effects on qHBsAg and HBeAg levels were small and mainly observed in untreated HBeAg-positive patients. This finding suggests that either bersacapavir was not potent enough or not administered long enough to deplete the cccDNA pool or that the absence of a significant decline of serum qHBsAg levels was related to HBV integration as a major source of HBsAg expression[84].

In the vebicorvir plus nucleoside analogue phase II study, virologically suppressed patients with CHB treated with vebicorvir plus a nucleoside analogue had greater proportions with HBV DNA <5 IU/ml and undetectable pgRNA, 83% and 59% **[Au: Do 83% and 59% refer to HBV DNA <5 IU/ml levels in the two arms of the study, or do they refer to HBV DNA and undetectable pgRNA in the vebicorvir and NA arm? Please clarify]** versus NA only with 29% and 18% respectively at week 24[85]. In a substudy of the phase II extension study patients with undetectable HBV DNA and HBV RNA, and low or undetectable HBeAg were selected to stop treatment after 72 weeks, but all 42 patients relapsed within 4 months[85], indicating that despite profound viral suppression, silencing of cccDNA transcriptional activity was not achieved.

Together, the results of these two clinical studies suggest that the combination of a nucleoside analogue with the current generation of CAMs is unlikely to achieve

functional cure. Whether more potent CAMs or CAMs with additional modes of action on cccDNA would have the potential to affect the viral reservoir remains to be evaluated.

[H2] Inhibit replication and reduce antigen burden

Viral replication inhibition not only blocks the replication pathway but long-term potent suppression could reduce the cccDNA pool[86]. By adding translational inhibitors the production of viral antigens is also reduced (Figure 4). This approach has further potential to enable exhausted immune cells to recover (Figure 2).

*[H3] Small interfering RNAs (siRNA) and nucleoside analogues*

All four siRNAs in phase II clinical development (JNJ-3989, VIR-2218, RG-6346 and AB-729) achieve predictable on-treatment qHBsAg responses, which seem to be durable for >6 months after the final dose[87,88,89]. These studies were performed in patients with CHB not initially on therapy (JNJ-3989; RG-6346) or already virally suppressed beforehand (JNJ-3989; VIR-2218; RG-6346; AB-729). Monthly dosing of siRNAs results in stepwise reductions in qHBsAg[87,88,89]**[Au: Please reference this statement.]**. ~~Initial modelling suggested that most patients would have undetectable qHBsAg levels after 12 months, potentially restoring host immune responses, but clinical studies showed that this did not pan out because qHBsAg levels plateaued after the first 16-20 weeks despite continued dosing~~ **[Au: Please reference this statement.]**. The preliminary results from the first phase II studies of siRNA **[Au: which one?]** plus a nucleoside analogue show qHBsAg reduction of 2–2.5 log with 50–97% of patients having qHBsAg <100 IU/ml, and a few patients achieving loss of HBsAg[90]. AB-729, an N-acetylgalactosamine (GalNAc)-

siRNA, given alone or with TDF for 8 weeks showed similar reductions in qHBsAg levels (2.03 log IU/ml versus 2.16 log IU/ml[91]), but no patients achieved loss of HBsAg. The limited data available indicate little additional benefit of adding a nucleoside analogue to siRNA for achieving a decrease in qHBsAg **[Au: Edit OK?]**. In the AROHBV1001 study[92], JNJ-3989, an siRNA that targets multiple HBV sites was administered subcutaneously on three occasions (day 0, 28 and 56) at different dosage levels (100-400mg) together with a nucleoside analogue till the end of the study and then followed to day 392. All patients had sustained qHBsAg reduction; responders (defined as >1 log reduction) had -1.9 log reduction, and non-responders (defined as <1 log reduction) had -0.6 log reduction at day 392. Similar sustained reductions in HBV RNA, HBeAg and HBcrAg levels were also seen. This study showed that sustained qHBsAg and other viral biomarker reduction was possible 1 year off-therapy. No major safety concerns were noted and only one patient had a moderate ALT flare.

*[H3] Antisense oligonucleotides and nucleoside analogues*

In a phase Ib study, biweekly 300 mg dosing with a non-GalNAc-conjugated antisense oligonucleotide GSK-3228836 (bepirovirsen) led to 2 log reductions in qHBsAg within 28 days, associated with ALT flares[93]; 4 patients (25%) showed a transient HBsAg loss on treatment and two patients had HBsAg loss lasting almost 6 months off-therapy but eventually relapsed. The rapid and profound qHBsAg reductions with antisense oligonucleotides suggests their efficient uptake even without GalNAc conjugation. In the phase IIb study B-clear final results [94], , bepirovirsen 300mg was dosed weekly for 12 or 24 weeks in untreated patients or patients treated with nucleoside analogues. Results were similar between the two

groups, with 9% (on NA) and 10% (not on NA) achieving undetectable HBsAg and HBV DNA by the end of 24 weeks off-therapy followup, representing approximately half the patients who achieved this at end-of-therapy; the best results were achieved in the bepirovirsen 300mg for 24 weeks arm, and the predictor of response was baseline qHBsAg<3 log IU/ml (up to 25% HBsAg loss). The main safety concerns were local injection site reactions and some grade 3–4 adverse events. ALT elevations up to 3x upper limit of normal were seen in 17% in NA-treated and 41% of not-on NA patients and were mainly related to decreases in HBsAg levels. An important finding from this study was that monotherapy with bepirovirsen had similar results to combination with NA, surmising that bepirovirsen could be the backbone of future combination therapies.

[H2] Inhibit replication and boost immunity **[Au:OK? Ok]**

Given that HBV-specific T cells are functionally impaired in patients with chronic HBV, their stimulation represents a possible strategy for restoration[51] (Figure 5). T cells might be refractory to antigen stimulation but this could be overcome by abrogation of T cell inhibitory mechanisms as a result of the inhibition of viral replication and resolution of liver inflammation[53].

*[H3] Approved therapies*

Various strategies combining a nucleoside analogue and PEG-IFN have been evaluated to increase the rate of HBsAg loss. A meta-analysis found that the combination of a nucleoside analogue and PEG-IFN lead to a 6% higher HBsAg loss than a nucleoside analogue alone[63].

The strategy to add or switch to PEG-IFN in patients receiving nucleoside analogues suggests some benefit of switching although the only randomised controlled trial (RCT) conducted[94] showed no difference in efficacy and a higher rate of ALT flares.

*[H3] Bulevirtide and PEG-IFN*

Bulevirtide is a synthetic myristoylated peptide that shares the identical 47 amino acid sequence of L-HBsAg required for binding to NTCP; it acts via competitive inhibition of the NTCP receptor [95] **[Au: Please reference this statement.]**. In the MYR 203 trial[96] of patients co-infected with HBV and hepatitis D virus, 26.7% of patients (4 of 15 patients) achieved HBsAg loss after receiving buleviritde 2mg plus PEG-IFN by week 72 of treatment. Paradoxically, none of the patients who received a higher buleviritde dose (5mg) plus PEG-IFN demonstrated HBsAg loss. Given that HBsAg loss was only achieved in four co-infected patients, larger trials in mono-HBV infected patients are needed to confirm the results.

*[H3] Nucleoside analogues and TLR agonists*

Another approach to improving immune control in patients with CHB is the stimulation of innate immunity receptors, such as TLRs[97]. In patients with CHB receiving nucleoside analogues, TLR-7 agonist GS-9620 (vesatolimod) add-on was able to boost HBV-specific T cell responses and NK responses with anti-viral cytokine production[98]; however, this treatment had no effect on qHBsAg or HBV DNA reduction. In a phase I multi-ascending dose study of RO7020531 given subcutaneously every 2 weeks, the mean reduction in qHBsAg was 0.3 logIU/ml[99], compared to the mean reduction of -0.01 and 0.002 logIU/ml in untreated and

nucleoside analogue-treated CHB with GS-9620[100]. In a phase II study[101] 24 weeks of TLR8 agonist, selgantolimod, in virally suppressed CHB led to 5% HBsAg loss **[Au: Do you mean loss fo HBsAg in 5% of patients? Yes, 5% (2/39) SLGN-treated patients achieved HBsAg loss]**, 16% HBeAg loss and a mean reduction in qHBsAg of <1 log[102]**.** Dose-proportional changes in levels of peripheral cytokines (IFN-γ, IL-12p40 and IL-RA) were also noted, indicating better efficacy than TLR7 agonists.

*[H3] Nucleoside analogues and checkpoint inhibitors*

Evidence that co-inhibitory molecules are upregulated on HBV-specific T cells suggests that checkpoint inhibitors might restore T cell responsiveness[103-109]. In a pilot study, a single dose of nivolumab, a PD-1 inhibitor, was administered with or without therapeutic HBV vaccination in virally suppressed HBeAg-negative patients with CHB. PD-1-receptor occupancy was 68.9–88.2%, showing sufficient target engagement. The mean reduction at week 24 was −0.48 $\log_{10}$ IU/ml, and one patient (5%) achieved loss of HBsAg[110].

In a late breaking abstract at AASLD 2021[111], ASC22 (envafolimab), a humanized single-domain PD-L1 antibody was tested in a phase IIb study; virally suppressed patients were treated with 1mg/kg every 2 weeks or placebo for 24 weeks (12 doses) with 24 weeks of follow-up. Treated patients had mean qHBsAg reductions of 0.38 log IU/ml versus 0 for placebo, and the reduction was more pronounced in those with baseline qHBsAg <500 IU/ml (-0.7 log). In an update at EASL 2022[112], the three patients who achieved HBsAg loss had baseline qHBsAg <100 IU/ml, and 21% of patients had ALT flares, which were associated with reductions in qHBsAg; however, these flares were grade 1-2 in nature. The study found that

better responses **[Au: do you mean greater reductions in qHBsAg? Yes]** were seen in those with low qHBsAg levels at baseline.

*[H3] Nucleoside analogues and therapeutic vaccines*

A yeast-based therapeutic vaccine containing HBV S, X and core proteins (GS-4774) was administered with nucleoside analogues in patients with CHB[113]. Significantly enhanced antiviral cytokine production by HBV-specific T cells, with a more pronounced impact on CD8 than CD4 T cells was noted, but did not affect serum levels of qHBsAg[114]. The Transgene trial tested TG1050, an adenovirus 5-based therapeutic vaccine expressing HBV polymerase, with domains of HBcAg and HBsAg in virally suppressed patients[115]. The phase Ib study showed stimulation of HBV specific IFN-γ ELISpot responses but little change in qHBsAg levels.

In a study of CVP-NASVAC[116], a therapeutic vaccine comprising HBsAg and HBcAg delivered intranasally, 10 doses every 2 weeks were administered to both untreated and nucleoside analogue-treated patients with CHB in a nonrandomised controlled trial, and were followed up for 30 months post-therapy. The mean qHBsAg reduction was 0.225 log IU/ml in the nucleoside analogue-treated group compared to 0.549 log IU/ml reduction in the untreated group. In the nucleoside analogue-treated group, 2 of 15 (13.3%) patients achieved functional cure, compared to 5 of 18 (27.7%) in the untreated group at 30 months. This report is the first to describe functional cure using a therapeutic vaccine, but the results are counterbalanced by a lack of controls, which cannot discount the possibility that the findings could be natural history events.

**[Au: The other previous sections do not have a summary paragraph. I would suggest removing this here as I think the information has been clearly presented above. I agree]**

~~In summary, the diverse types of immune modulators tested with NA showed variable results. The TLR7 agonist, GS-9620, despite target engagement and cytokine production failed to show clinical efficacy. Clinical results of the other TLR7 agonist RO7020531, are preliminary, hence its efficacy as a class of agent is unclear. The efficacy of TLR7 had been demonstrated in animals but not in humans possibly due to lower doses, based on potential for adverse effects[99]. The TLR8 agonist, selgantolimod, shows small but significant(<1 log) reduction in qHBsAg levels with 5% of patients achieving functional cure. It is unclear whether the addition of NA to TLR agonists were more beneficial with regards to efficacy. The combination of PD-1 or PDL-1 blockage appears to be encouraging with two studies showing evidence of reduction in qHBsAg levels and some patients achieving functional cure. Importantly, repeated dosing of PDL-L1 + NA appears to be safe. Finally, there have been overall disappointing clinical results with therapeutic vaccines while the CVP-NASVAC, due to the lack of controls, raises uncertainty over the findings.~~

~~Overall, this is a promising field with evidence of some efficacy of many immune modulatory agents tested together with NA. There is a suggestion that better qHBsAg responses may be seen in those with low baseline qHBsAg. Dose optimisation, tolerance and safety will guide further studies in the search for improved efficacy.~~

[H2] Reduce antigen burden and boost immunity

Persistent high antigen load is a major factor driving the exhaustion of antiviral adaptive immunity; new antiviral agents that can substantially reduce HBV antigen production (Figure 4) could be rationally combined with direct immunotherapies (Figure 5) in the hope of achieving a synergistic effect. However, given the extensive epigenetic 'scarring' of T cells that is observed in chronic viral infection, T cell exhaustion might not recover[117]. Newly generated T and B cells (for example, via therapeutic vaccination) could be protected from terminal exhaustion if viral antigen load can be lowered sufficiently within hepatocytes[118]. However, if all antigen production is eliminated, cccDNA-containing hepatocytes could effectively become 'invisible' to HBV-specific T cells, limiting ongoing immunosurveillance[119]. Other therapies that block HBsAg release from infected hepatocytes (such as STOPs) or remove it from the circulation (for example, neutralising antibodies) would not be as likely to promote T cell reconstitution given that peptide presentation from the infected hepatocyte can continue **[Au: Please reference this statement.]**. Consistent with this concept, a 2020 study showed that antibody-mediated clearance of serum HBsAg had a negligible impact on CD8 T cell responses in mouse models of HBV infection[120]. However, such approaches would be expected to reduce the antigenic over-stimulation of the anti-HBs antibodies that constitute the cognate receptor on HBsAg-specific B cells. Although the degree of depletion and dysfunction of HBsAg-specific B cells does not correlate with qHBsAg levels in CHB[75,121], it remains important to test whether novel therapies that can produce a rapid and substantial decline in the burden of HBsAg will allow some recovery in humoral immunity that might make it more amenable to boosting by therapeutic vaccines or other immunotherapies.

**[Au: Could the following highlighted sections be replaced by the sentences here (and combined with the paragraph above?) This would be to avoid having 2 very small sections at the end here]**

Thus far, no dual combinations studies have investigated an siRNA or a NAP in combination with PEG-IFN, although triple combinations with a nucleoside analogue have been reported (see later).

~~Small interfering RNA (siRNA) and PEG-IFN~~

~~No dual combination studies but a triple combination with NA has been reported (see triple combination)~~

~~Nucleic Acid Polymers (NAP) + peginterferon~~

~~No dual combination studies but a triple combination with NA has been reported (see triple combination)~~

## [H1] Strategies for triple combinations

In theory, triple combinations of the three categories of therapies (inhibiting viral replication (Figure 3), reducing viral antigen burden (Figure 4) and immune stimulation (Figure 5)) might be considered the ideal combination. However, such combinations need to be investigated in clinical trials, and phase II studies might provide preliminary data on efficacy and insights into their feasibility and potential. A summary can be viewed in Table 2.

**[Au: Character limits for level 2 headings are 39 (including spaces), whereas they are 80 characters for level 3 headings. I propose to make all headings in this 'Strategies for Triple Combinations' section level 3. This means removing a couple of the subheadings below: 'NAPS, NA and PEG-IFN' and 'siRNA, NAs and PEG-IFN'. OK? Please feel free to suggest another solution if possible within our limitations]**

*[H3] Inhibit transcription and replication and boost immunity*

Vonafexor is a new class of agent, an FXR agonist that is proposed to reduce HBV transcription. A phase II study of vonafexor plus PEG-IFN with or without entecavir (dual versus triple therapy) for 16 weeks with 24 weeks of follow-up in HBeAg-negative patients[122], showed a better mean reduction of qHBsAg of -1.1 log IU/ml in the dual arm compared to -0.6 log in the triple arm at the end of therapy, which seemed to be sustained off therapy. HBeAg-positive patients had no reductions in qHBsAg. HBV DNA levels reduced by 3.8 log IU/ml by end of therapy with no difference between dual and triple therapy. The contrasting findings between HBeAg negative and positive patients, the use of PEG-IFN in all arms, as well as the lack of HBV RNA results does not provide clarity with regard to potential target engagement and its impact on viral transcription.

*[H3] Reduce antigen burden, inhibit replication and boost immunity*

In a phase II open label study, HBeAg-negative patients with CHB were treated with TDF for 24 weeks then randomly allocated to receive 24 weeks of a NAP (REP 2139 or REP 2165) plus TDF and PEG-IFN (20 patients) or PEG-IFN plus TDF (20 patients)[123]. Patients in the triple combination arm **[Au:OK?]** had significant reductions in qHBsAg at week 48, with 75% achieving qHBsAg <100

IU/ml and 50% achieving qHBsAg < lower limit of quantitation (LLOQ); 55% achieved anti-HBs seroconversion versus 5%, 0%, and 0% in the dual treatment arm **[Au: What do these other percentages refer to? Which treatment arms?]** respectively in the control arm. Overall, 14 of 40 (35%) patients maintained HBsAg loss after 1 year of treatment-free follow up. Notably, most patients had significant ALT flares during therapy at the time of qHBsAg decline.

In a phase II study[124], VIR-2218 (an siRNA) with or without PEG-IFN was given for 24 weeks in patients with CHB treated with nucleoside analogues; this study had four arms, with one arm without PEG-IFN, and the other arms having increasing duration of PEG-IFN (12, 24 and 48 weeks). The mean reduction in qHBsAg was 2.03 log IU/ml in the VIR-2218/NA arm versus 2.55 log IU/ml in those who had a full 24 weeks of VIR-2218/NA/ PEG-IFN, suggesting synergistic effects, with 95% achieving qHBsAg <100 IU/ml, and 55% achieving qHBsAg <10 IU/ml by week 24. There was increasing efficacy of qHBsAg reduction with increasing duration of PEG-IFN. At week 24, 3 out of 33 patients (9%) had qHBsAg <LLOQ and 2 of these 3 patients achieved detectable anti-HBs, and had an increase in ALT levels. In a week 48 update of this study [125], the best results were seen in cohort 5, which had 13 doses of VIR-2218 and <44 weeks of PEG-IFN with 4/13 (30.8%) of patients achieving HBsAg loss.

[H2] Reduce antigen burden and intensify replication inhibition

The REEF-1 phase IIb study evaluated the efficacy and safety of the siRNA JNJ-3989 and/or CAM JNJ-6379 and a nucleoside analogue for the treatment of CHB. Surprisingly, the results of this 48 week study showed that the double combination

of a nucleoside analogue plus 100 mg dose siRNA induced a more profound reduction in serum qHBsAg levels (2.1 log10) than the triple combination including the CAM (1.8 log10)[126]. This finding remains to be validated in other studies and raises the issue of whether CAMs are necessary to achieve functional cure.

**[Au: As mentioned above, these summary sections seem a little inconsistent. I think you may have added this in response to the peer-review reports, but please consider whether this section is necessary. Thank you. ]**

*The few phase I and II studies show that significant qHBsAg reduction can be achieved with triple combinations. Unsurprisingly, the best reductions in qHBsAg are achieved with agents that reduce viral antigen burden, such as siRNAs or NAPs; the only triple combination study of FXR/NA/ PEG-IFN did not show a mean reduction in qHBsAg>1 logIU/ml. In the REEF-1 study, the reason that the triple combination of NA/CAM/siRNA was less efficacious that NA/siRNA in reducing qHBsAg was unclear. Further studies are needed to determine whether this unanticipated result is a class effect or is drug-specific. The best results seem to combine antigen reduction, NA and immune stimulation but unfortunately, this still relies on PEG-IFN, which is not well tolerated. Nonetheless, in principle this combination is able to achieve qHBsAg<LLOQ during therapy, and in the case of NAP, sustained functional cure.*

[H2] Inhibit viral replication and enhance immune responses

In a phase Ib/IIa study presented at EASL 2022[70], a novel therapeutic vaccine strategy was investigated in virally suppressed patients with CHB receiving nucleoside analogues **[Au: OK to add the bit about receiving NAs?]**; ChAdOx1-

HBV and MVA-HBV were used in a prime–boost strategy (VTB-300) as a therapeutic vaccine with or without low-dose nivolumab (PD1 inhibitor) **[Au: Edit OK, for clarity re the vaccine strategy?]**. A few patients (3 of 18) treated with VTB-300 alone had significant qHBsAg declines of 0.7–1.4 log, and all had baseline qHBsAg <50 IU/ml. Patients (8 of 18) receiving VTB-300 and low-dose nivolumab had significant qHBsAg declines of 1.15 log, which persisted 8 months after the last dose, and one patient achieved loss of HBsAg. Such responders had qHBsAg <1,000 IU/ml **[Au: correct?]**. No major safety issues were reported and only two patients had mild transaminitis. This study shows that potent therapeutic vaccines can work safely and efficaciously with PD1 inhibition as a dual immune stimulation strategy.

## [H1] Strategic issues in combination therapy

### [H2] Timing of immune modulatory therapy **[Au: Change of heading ok? OK]**

T cell exhaustion in chronic HBV infection represents a multifactorial phenomenon sustained by different inhibitory mechanisms, which make its correction difficult to achieve[51,127]. The heterogeneity of the exhausted CD8 T cell population along with the co-existence of CD8 T cell subsets with different functionality and antigenic specificity in individual patients with CHB makes this situation complex[128-131]. In addition, a number of intracellular metabolic processes and signalling pathways are deeply dysregulated in HBV-specific CD8 T cells[64,132,133]~~., and the limited T cell response attributed to the irreversible molecular "scar"~~ **Moreover, a peculiar epigenetic landscape of exhausted T cells has recently been reported in virus-specific CD8 cells in chronic LCMV infection, as a possible irreversible signature of exhaustion, which would explain the limited duration of the T**

**cell reinvigoration effect induced by PD-1/PD-L1 blockade**[134-136]**[Au: I'm not quite sure of the meaning of the last phrase of this sentence, please could you briefly expand or clarify for the benefit of the general reader. Please replace the old sentence with the new one]**. One issue is whether immune therapy is more effective if used during the immune tolerant phase than the immune active phase given that data suggests HBV-specific T cell responses are superior in terms of intensity and quality at this point[137,138]**[Au: Edit of this sentence ok? In my opinion the concept was clearer in the previous version]**. However, this interesting finding has been challenged, with three RCTs showing very low rates of HBeAg loss (around 3–3.8%[139,140,141]) with antiviral therapy (using PEG-IFN and nucleoside analogues) in immune tolerant CHB.

In the absence of accurate immune predictors of response, clinical studies must guide immune modulator therapy. Stopping nucleoside analogue therapy in virally suppressed patients has been shown to increase the rate of HBsAg clearance, particularly in those with low qHBsAg levels (<100 IU/ml;)[142,143,144,145] **[Au: Could this statement be supported by fewer references? I agree, please remove references 141 and 142]** and has been associated with modulation in NK cell and HBV-specific T-cell function[146,147,148]. The additional finding that repeated PDL1 therapy in combination with a nucleoside analogue leads to better **[Au: greater? Ok]** qHBsAg reduction and HBsAg loss in those with low baseline qHBsAg levels[111], together suggest the optimal timing to initiate immune modulator therapy in patients with CHB is when qHBsAg levels are low **[Au: Edit OK? Ok]**. By exploiting the immunomodulatory effects of nucleoside analogue discontinuation to stimulate innate and adaptive immune responses and to modify the liver immune environment, this approach could be a novel therapeutic strategy to achieve a

higher functional cure rate. Therapies that propose to achieve low qHBsAg levels as an end point combined with immune stimulation could be explored; such treatments include siRNAs, antisense oligonucleotides, NAPs and anti-HBs monoclonal antibody therapy.

[H2] Combination or sequential therapy?

The data available to date suggest that achievement of functional cure in CHB requires combination therapy[149]. However, alternatives to combination therapy have not really been explored. The best clinical evidence of benefits of alternative strategies combining nucleoside analogues and PEG-IFN were examined in a meta-analysis[150]. Initial combination therapy versus initial nucleoside analogue monotherapy showed a non-significant increased relative risk (RR) of HBsAg loss of 1.44 **[Au: for combination therapy?]**. PEG-IFN add-on versus nucleoside analogue monotherapy showed a substantially improved RR of 4.52, suggesting add-on PEG-IFN was a useful strategy. However, the best result was a switch from nucleoside analogue to PEG-IFN versus nucleoside analogue monotherapy, which showed an RR of 12.15. This meta-analysis showed that sequential therapy seemed to be better than combination therapy, although these were not head-to-head comparisons. Interestingly, a head-to-head RCT of add-on or switch to PEG-IFN showed no significant difference in rates of HBsAg loss[151]. As discussed in the previous section, stopping nucleoside analogue therapy leads to recovery of immune cell exhaustion and increased rates of HBsAg loss, which is an optimal setting for immune stimulation strategies, such as TLR8 agonists, checkpoint inhibitors or therapeutic vaccines. However, whether other antiviral strategies to reduce qHBsAg levels lead to similar immune states akin to long-term nucleoside

analogue treatment is unclear. There is insufficient information on novel treatments to determine whether denovo combinations or sequential therapy strategies are more likely to be successful.

## *[H1] Biomarkers to monitor treatment efficacy*

### [H2] Serum viral biomarkers

Biomarkers are indicators of biologic, pathogenic processes or pharmacologic responses[152], and in the case of HBV, the utility of biomarkers are best if they are part of the biological pathway. Consequently, virological markers[153] such as HBV DNA, HBV RNA, HBeAg, HBcrAg, quantitative and qualitative HBsAg (qHBsAg), and ultrasensitive HBsAg[154] are the mostly commonly assessed markers. As the HBV lifecycle comprises two interrelated pathways, the secretory (produces HBeAg and HBsAg) and the replicative (measured by HBV DNA and HBV RNA) pathway, agents acting on the replicative pathway (NA, CAMs) may have little impact on the secretory pathway.

**[Au: Is this following paragraph strictly relevant for this section on biomarkers? Please consider shortening or removing it if you agree.]** ~~*The lifecycle of HBV can be broadly divided into two interrelated pathways: the replicative pathway is involved in viral replication, while the secretory pathway leads to production of proteins such as HBeAg and HBsAg (Figure 1). Agents that act on the replicative pathway (such as nucleoside analogues and CAMs) have little efficacy on the secretory pathway directly, although agents that act on the secretory pathway usually have some impact on the replicative pathway. Nucleoside analogues reduce HBV DNA effectively but take many years to reduce HBV RNA[155] whereas CAMs can reduce both. One caveat to shutting down the*~~

~~*secretory pathway in the lifecycle of HBV is that such agents need to also reduce HBsAg that originates from integrated HBV*[156]~~.

In general, qHBsAg levels are good predictors of HBsAg loss during therapy (AUROC 0.77-0.91) [155,156] compared to HBcrAg and HBV RNA. The best predictors of HBeAg-seroconversion are HBV RNA and qHBeAg (AUROC 0.77 and 0.75 respectively)[157]. The best predictors of relapse after stopping therapy are combined HBV RNA and HBcrAg (AUROC 0.7-0.85)[158], but HBcrAg has a 10% false positive rate and hence might be unreliable[159]. A systematic review showed that stopping nucleoside analogue treatment when qHBAg≤100 **[Au: Should this be qHBsAg?]** IU/ml leads to HBsAg loss between 21.1–58.8% and viral relapse rates of 9.1–19.6% [160] **[Au: Which reference here?]**. In the context of functional cure, qHBsAg seems to be the best biomarker and predictor of response. Depending on the mechanism of the drugs a given biomarker might be used as an end point or as a marker of target engagement, but ultimately clinical efficacy is crucial. In evaluation of novel therapies, the extent of log reduction in qHBsAg levels is probably a better and more universal guide in comparing efficacy across regimens, bearing in mind that the proportion of patients with HBsAg loss might be misleading as low baseline qHBsAg levels might lead to high rates of HBsAg loss.

[H2] Immune biomarkers in peripheral blood

The immune Monitoring Working Group of the HBV Forum[161] has provided an overview of the technologies available for assessing immune biomarkers in peripheral blood, their strengths and weaknesses and the priorities of their use. The primary emphasis is on ex vivo measurements of HBV-specific T-cell immunity. The preferred method is overlapping peptide libraries for ELISpot or

fluorospot. This method can detect responses in all patients and covers the full breadth of the response within a patient. This method is also more sensitive than intracellular cytokine staining **[Au: Can you reference this statement, or is all this information in ref 162? This information is in ref 162]** but lacks phenotyping data, making it impossible to identify the cell type responsible for cytokine production and differentiation[161]. Interestingly, in a study of nivolumab (PD1 inhibitor) with a nucleoside analogue with or without a therapeutic vaccine (GS-4774)[110], HBV specific T cells to core, polymerase or S proteins did not increase despite a reduction in qHBsAg levels and HBsAg loss in 4% of patients. However, T cell phenotyping did show an increase in CCR7-CD45RA- effector memory CD4+ T cell frequency at the end of therapy **[Au: Edit OK? It is ok for me]**. Therefore **[Au:OK? It is ok for me]**, although HBV specific T cells are important to measure, their predictive value in functional cure during therapy is questionable.

Phenotypic analyses using flow or mass cytometry of HBV specific CD8 multimers ex vivo is limited by available HLA multimer reagents, hence this technique is more of a research tool. Differential exhaustion and memory markers provide a useful tool for the identification and quantification of dysfunctional HBV-specific T cells and distinct T cell subsets with different degrees of functional impairment. Specifically, co-expression of exhaustion (PD-1, CD39, TOX) and memory (CD127, Bcl-2,TCF1) markers can allow quantification of the relative ratio of different HBV-specific CD8+ T cell subsets and the overall level of T-cell impairment [130] [131]. **[Au: Please reference this statement. Ok, references 129 and 130 can be added]**

Serum and plasma cytokines have yet to predict antiviral responses but can provide insights into potential immunological pathways and mechanisms. Interestingly, a review of patients with HIV–HBV co-infection who start ART therapy[163] found a rate of HBsAg loss of 2.39 per 100 person-years compared to HBV monoinfection of 0.37 per 100 person-years [162] **[Au: 34.5% of patients achieved loss of HBsAg? No, ref changed and revised]**, which is associated with recovery of CD4 responses, often but not completely related to immune re-constitution inflammatory syndrome (IRIS). HBsAg loss was associated with IRIS-hepatic flare, with **[Au: increased levels of]** sCD163 correlating with HBsAg loss **[Au: ref 163? Yes it is correct]**, suggesting macrophage activation[163]. Such analyses provide a global portrait of immune pathways but do not provide sufficient detail on intrahepatic immune activation, or the potential cell types involved, and validation within the intrahepatic microenvironment is needed. Finally innate immune measurements are important, in particular intrahepatic NK cells which are a unique population; their phenotypic profiling, particularly frequencies of CD38, Ki67 and TRAIL can be measured[164], but their role in antiviral responses has not been fully understood.

## [H2] Studying intrahepatic immunology

Recognition that specific immune cells are unique to the liver and not well represented in the blood is increasing. For example tissue-resident subsets of NK cells[165] and T cells[166] have been identified in the HBV-infected human liver, the latter accounting for the intrahepatic retention of the majority of HBV-specific CD8 T cell responses.

Given that HBV-specific T cell frequencies and PD1 expression are both enriched in the liver compared to the peripheral compartment[105,167], intrahepatic sampling is likely to better select individuals for therapies targeting these responses, particularly for liver-targeted therapies.

The intrahepatic compartment can be sampled by biopsy or fine needle aspiration (FNA) and the cells obtained can then be studied using a variety of focused or unbiased techniques[168]. FNA using a 22-guage needle is far less invasive than biopsies, making repetitive longitudinal monitoring of the response to novel combination therapies much more feasible[168]. Although FNA does not provide tissue for histology and assessment of in-situ topology, fine needle aspirates do enable flow cytometric identification of the full complement of intrahepatic innate and adaptive immune populations, including tissue-resident subsets and HBV-specific T cells (which are proportionally represented compared to paired biopsy samples)[169]. Small numbers of live hepatocytes can also be aspirated. However, both biopsy samples and fine needle aspirates run the risk of sampling bias and with fine needle aspirates, variable degrees of blood contamination also need to be adjusted for[169]. State-of-the-art techniques such as single-cell RNA-sequencing [170] or Cite-Seq of FNA will allow unbiased comprehensive monitoring of the response of the whole intrahepatic immune landscape to novel therapies **[Au: Please reference this statement.]**. FNAs are ideally suited to longitudinal sampling particularly at baseline and end-of-therapy, but additional sampling can be performed when antiviral or inflammatory events occur[161].

**[H2] Studying intrahepatic virology**

Access to liver samples in patients with CHB allows direct assessment of the cccDNA pool size[86,171-173], its transcriptional activity[86,172,173] and its epigenetic status[32,86,174] as well as the quantification of HBV integration burden and transcriptionally active HBV integrations[33,175].

Quantification of cccDNA and 3.5kb pgRNA in the liver is performed by PCR-based techniques (quantitative PCR[172,173]and droplet digital (dd)PCR[86,171,172]). The ratio of cccDNA and 3.5kb pgRNA is referred to as cccDNA transcriptional activity. Owing to the need to distinguish between cccDNA (fewer molecules, nuclear) and rcDNA (more abundant, cytoplasmic and in virions in the intrahepatic vascular compartment), cccDNA quantification is intrinsically challenging. Quantification of cccDNA and 3.5Kb pgRNA in fine needle aspirates by ddPCR has proven as accurate as in core liver biopsy samples (Testoni et al, JHepReports, submitted) **[Au: Please reference this statement.]**.

The characterization of cccDNA epigenetic status by chromatin immunoprecipitation (ChIP)-based assays (cccDNA ChIP[86,174] or cccDNA ChIP-Seq[32,176]) is limited to a few expert laboratories and remains confined to research purposes. The applicability of cccDNA-ChIP assays can be hampered by the amount of liver sample available, the need for multiple ChIP reactions using antibodies for different histone post-translational modifications and the difficulty of linking transcriptional activity to one single post-translational modification[32,86]. The advantage of coupling cccDNA-ChIP with high throughput sequencing (cccDNA ChIP-Seq[32,176]) is counterbalanced by the objective complexity of the experimental protocols.

Overall, cccDNA and 3.5kb pgRNA quantification in liver samples remains investigational and further standardization is needed for their introduction in clinical

practice. However, their inclusion will be key to monitor new agents in early phase studies. FNA, which is less invasive than core liver biopsy, enables the simultaneous investigation of the immune microenvironment and intrahepatic viral parameters and is increasingly incorporated in clinical studies.

## *[H1] Conclusions*

Considering the current landscape of novel antiviral drugs or immunotherapeutics in development, it is clear that the first step to achieve significantly higher functional cure rates will require combination therapy. We can glean several insights from existing early phase studies. Intensification of therapy can lead to profound suppression of HBV DNA and HBV RNA and depletion of the cccDNA pool but with little effect on levels of HBsAg, and stopping therapy leads universally to viral rebound[50]. Early phase clinical studies show that antigen reducing agents are not necessary to achieve HBsAg loss, nor are immune modulators. For immune modulators, those affecting both adaptive and innate immunity have evidence of efficacy, indicating that targeting either pathway seems to be effective; however, efficacy seems better in patients with low qHBsAg levels, hence timing of immune modulator introduction is probably optimal when qHBsAg levels are low, with potential immune reconstitution leading to functional cure. Targeting viral transcription and/or translation to decrease qHBsAg levels[177] might also predispose to cccDNA silencing[178], and can have sustained efficacy off therapy, but not all cases of qHBsAg<LLOQ are able to sustain HBsAg loss. The combination of a nucleoside analogue plus NAP plus PEG-IFN was able to achieve functional cure in 35% of patients in a small phase II study[123] but needs confirmation in a larger study **[Au: Please cite the reference again]**; thus, the

strategic approach of inhibiting viral replication, reducing antigen burden and immune modulation warrants further confirmation in large trials. Another promising combination was si-RNA VIR-2218 and PEG-IFN in NA-treated patients for up to 44weeks in a small phase II study [125] with 30.8% HBsAg loss. Surprisingly, bepirovirsen an unconjugated anti-sense oligonucleotide was able to achieve 10% HBsAg loss with undetectable HBV DNA 24 weeks off therapy as monotherapy [94], and combinations with this backbone could an important therapeutic strategy. However, the different agents within these categories might perform differently and the optimal agents and their combinations need to be explored. When assessing new combination therapies, safety will be a major end point given that nucleoside analogues have an excellent safety record[2]. In particular, ALT flares have been seen in the setting of qHBsAg reduction, but not all cases would lead to functional cure. Current studies are of limited duration, ranging from 4 weeks in phase I studies to 12–48 weeks in phase II studies. Most studies have a 24 week off-therapy follow-up. A lack of qHBsAg reduction in an early phase study indicates that such a combination is unlikely to lead to functional cure. Evidence of qHBsAg reductions, particularly of >1 log, show potential for functional cure, but a plateauing of this effect (such as seen in some siRNA studies) suggests that longer duration of therapy might not be successful.

Viral biomarkers, such as qHBsAg (ideal for functional cure), pgRNA and HBcrAg, are better established for monitoring the progress of novel agents and combinations, as immunological monitoring is challenging. A proposal by the Immune Monitoring Working Group of HBV Forum provides much needed guidance on the use of these assays, most of which are still research tools and not suitable for routine clinical trial monitoring[161]. As the liver microenvironment differs

from that of peripheral blood, mechanistic insights for functional cure can be gained by longitudinal FNA, and this technology is generally established as being representative compared to liver biopsy with better safety and acceptability[168,169].

Another consideration is patient selection. In the search for the optimal therapeutic approach, the balance between selecting the best responders to provide proof of principle of efficacy (for example, patients with viral suppression and low levels of qHBsAg) versus the applicability of the strategy in more heterogenous groups of patients including those patients considered difficult to treat (HBeAg-positive, immune tolerant, and with high viral and antigen loads) is challenging, not to mention genotype variability, ethnic diversity and more advanced stages of the disease.

Trial design is crucial, and platform trials with multiple targeted therapies can be investigated in the context of a single disease in a perpetual manner, with therapies allowed to enter or leave the platform on the basis of a decision algorithm. The advantages are the use of a single infrastructure which is ongoing over time with no fixed stopping date, a common control arm to aid in efficiency, and maps out adding and dropping strata[179,180]. Such strategies are already taking place. On the other hand, small focused targeted or exploratory trials could represent an extremely interesting alternative when assessing different strategies in different patient populations according to the phase of the disease, treatment history or other clinical or virological parameters. Investigator initiated trials might study strategies that are not part of the development program of the pharma industry. These studies might generate new insight by including translational studies of novel biomarkers and virologic or immunological responses in the liver compartment that might inform the development of novel cure strategies.

In conclusion, the promise of functional cure is already here, and many different therapeutic strategies and agents have already led to functional cure, albeit mostly in a small proportion of patients. More than one pathway exists for achieving this goal but finding the most successful strategy remains elusive to date. New therapeutics and their clinical outcomes might give us insights into the mechanisms of viral clearance that have not been known previously.

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**Author contributions**

All authors contributed to researching data for the article, discussion of content, writing, and reviewing/editing the manuscript.

**Competing interests**

S.G.L. is on the  Advisory Board for Gilead Sciences, Abbott, Roche, Janssen, GlaxoSmithKline, Grifols, Arbutus, Assembly. He is on the Speakers Bureau for Gilead Sciences, Abbott, Janssen and receives educational/research funding from Abbott, Merck Sharpe and Dohme, Gilead Sciences.

T.F.B. is a founder, shareholder and advisor for Alentis Therapeutics. He receives Institutional grant support from Aligos, Janssen, Alentis, Roche.

C.B. is a Consultant and on the Advisory Board for Gilead Sciences.

E.G. is on the Advisory board for AbbVie, Aligos Therapeutics, Arbutus Biopharma, Arrowhead Pharmaceuticals, Assembly Biosciences, Avalia Immunotherapies, BlueJay Therapeutics, Brii Biosciences, Clear B Therapeutics, Dicerna Pharmaceuticals, Enanta Pharmaceuticals, Finch Therapeutics, Gilead Sciences, GlaxoSmithKline, Immunocore, Janssen, Roche, Silverback, Vaccitech, Benatorx, Virion Therapeutics and Vir Biotechnology. He is on the Speakers Bureau for Gilead Sciences, AbbVie, Abbott Diagnostics, Intellia and Roche.

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**Peer-review information**

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**Key Points**

- Functional cure is defined as loss of Hepatitis B surface antigen (HBsAg) and undetectable HBV DNA after 6 months off therapy; it is associated with improved clinical outcomes and is the optimal goal of therapy for chronic Hepatitis B.
- Novel agents fall into three categories; those that reduce viral replication, those that reduce viral antigen load and immunotherapies; combinations that lead to functional cure are being explored.
- Profound viral suppression alone is unlikely to lead to functional cure or reduction in qualitative (q)HBsAg levels.
- Combining replication inhibition with immunotherapy leads to some reduction in qHBsAg levels (<1 log) and HBsAg loss, usually in patients with low baseline qHBsAg levels
- Reducing viral antigen production reduces qHBsAg levels by up to 2 log, which might be sustained off therapy, and in combination with replication inhibitors or immunotherapy can achieve HBsAg loss in some instances, although not all are sustained.
- More than one path to functional cure is likely to exist, and finding the optimal one for each patient is the challenge.

**[Au: Are you happy for the tables to be included with the main text of the manuscript? I think in your rebuttal you describe one of the tables as being supplementary, but I think they could both be useful in the main manuscript. OK? If so, I would also suggest splitting Table 2 into 2 tables - one for dual combinations and one for triple combinations. OK?]**

***Table 1. Treatments for CHB in clinical trials.***

| ***Type of agent*** | ***Class*** | ***Agents*** |
|---|---|---|
| ***Antiviral agents*** | | |
| ***Replication inhibitors:*** *act on HBV DNA replicative pathway to reduce production of viral particles, HBV DNA and HBV RNA, and the pool size of cccDNA over a longer period.* | ***Nucleoside analogues*** | *Tenofovir disoproxil fumarate (approved)*<br>*Tenofovir alafenamide (approved)*<br>*Entecavir (approved)*<br>*ATI-2173 (phase II)* |
| | ***Capsid Assembly Modulators*** | *GLS-4(Morphothiadin) (phase II)*<br>*RO7049389 (phase I)*<br>*ABI-HB0731 (Vebicorvir) (phase II)*<br>*ABI-H3733 (phase I)*<br>*ALG-000184 (phase I)*<br>*ZM-H1505R (phase I)*<br>*QL-007 (phase II)*<br>*EDP-514 phase I)* |
| | ***FXR agonist*** | *Vonafexor (phase II)* |
| ***Translation inhibitors:***<br>*RNA interference uses homologous nucleotide strands to target post-transcriptional HBV mRNA and downstream viral protein production.* [83] | ***Small interfering RNA*** | *RG 6346 (phase II)*<br>*JNJ 3989(phase II)*<br>*AB-729 (phase II)*<br>*VIR-2218 (ALN-HBV) (phase II)* |
| | ***Antisense oligonucleotides*** | *GSK3228836 (bepirovisen) (phase II)* |
| ***Inhibitors of antigen secretion:*** *target HBsAg secretion pathway through host proteins* | ***Nucleic acid polymers*** | *REP 2139 (phase II)*<br>*REP 2055 (phase II)* |
| ***Inhibitors of viral entry:*** *prevent binding of HBV particles to its receptor, prevent re-circulation of HBV, potentially reducing intracellular viral burden* | ***NTCB receptor blockade*** | *Bulevirtide (approved for HDV)* ***[Au: What is the status for HBV?]*** |
| | ***HBV monoclonal antibodies*** | *VIR-3434 (phase II)*<br>*GC1102 (phase II)* |
| ***Immune modulators*** | | |
| ***Innate immune modulators:*** *stimulate innate immunity through TLR and/or interferon stimulating genes* | ***PEG-IFN*** | *PEG-IFN (approved)* |
| | ***Toll-like Receptor (TLR) 7 and 8 agonists*** | *RO7020531 (phase I)*<br>*GS-9688 (Selgantolimod) (phase II)* |
| ***Adaptive immune modulators:*** *stimulate T cells through reversal of T cell exhaustion or creating new T* | ***Checkpoint inhibitor (PD1 or PDL1 antibody)*** | *Nivolumab (Anti-PD1) (phase II)*<br>*Cemiplimab (Anti-PD1) (phase II)*<br>*ASC22 (Anti-PDL1) (phase II)* |

| | | |
|---|---|---|
| *cell responses* | | *PDL1 LNA (phase II)* |
| | ***Therapeutic vaccines*** | *GenHevac B (phase II)* |
| | | *NASVAC (phase III)* |
| | | *JNJ-64300535 (phase I)* |
| | | *TG1050 (phase I)* |
| | | *VRON-0200 (preclinical)* |
| | | *VBI-2601 (phase II)* |
| | | *VTP-300(ChAdOx-HBV) (phase I)* |
| | | *AIC649 (phase I)* |
| | | *GSK3528869 (phase I)* |
| | ***Other novel immunotherapy approaches*** | *IMC-I109V(ImmTav®) phase I)* |

**Table 2.**

| **Class of combinations** | **Agents** | **Duration** | **Efficacy** | **Safety** | **Phase** |
|---|---|---|---|---|---|
| **DUAL combinations: Combining replication inhibitors** | | | | | |
| NA + NA | ETV vs ETV+ TDF | 96 weeks | Better HBV DNA suppression<br>No increased HBeAg seroconversion<br>No HBsAg loss | No major safety issues | approved |
| NA + CAM | NA vs NA + JNJ-56136379 (bersacapavir) | 24-48 weeks | Similar HBV DNA decline<br>HBV RNA reduction 3.15 for combination vs 1.33 Nuc alone<br>Mean qHBsAg decline 0.41 vs 0.25 | No major safety issues | Phase 2 |
| | ETV vs ETV + verbicovir | 75 weeks | At week 24, , virologically suppressed | There were 3 grade 3 abnormal ALT | Phase 2 |

| | | | | | |
|---|---|---|---|---|---|
| | | | patients on vebicorvir + NA had HBV DNA(-) and pgRNA(-) in 83% and 59% versus ETV only with 29% and 18% respectively | | |
| **DUAL combination: inhibit viral replication and lower viral antigen burden** | | | | | |
| siRNA + NA | NA vs NA + AB-729 | 8 weeks | similar reductions in qHBsAg levels, 2.03 log IU/ml (AB-729) versus 2.16 (Nuc + AB-729) | Grade 1-2 events were injection site reactions and abnormal ALT | Phase 2 |
| | NA + JNJ3989 | 3 doses over 56 days | Mean reduction in qHBsAg was 1.98 log, HBV RNA 1.93 log and HBcrAg was 1.20 log at day 112 | Grade 1-2 events were injection site reactions and abnormal ALT | Phase 2 |
| Anti-sense oligonucleotide + NA | GSK- 3228836 (Bepirovirsen) ± NA | 12-24 doses over 12-24 weeks | Both NA-treated and untreated cohorts had similar response with 9-10% achieving qHBsAg and HBV DNA below quantification by end of followup; responders had qHBsAg<3 log; and the best response was seen in those receiving 300mg for 24 weeks. | Majority of adverse events were injection site reactions, there were a few grade 3-4 abnormal ALT | Phase 2 |
| **DUAL combination: Inhibit viral replication** | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| **and boost immune responses** | | | | | |
| NA + peginterferon | NA + peginterferon (meta-analysis) | 48 weeks | Combination nuc + peginterferon showed 6% higher HBsAg loss than Nucs | Significant adverse events due to interferon toxicity | Approved |
| Entry inhibitor + peginterferon | Bulevirtide + peginterferon in HDV-HBV co-infected patients | 48 weeks | Patients in 2mg bulevirtide + peginterferon arm had 26.7% (4/15) HBsAg loss but the 10mg arm had no HBsAg loss | Other than interferon related adverse events, raised bile acids are seen but do not cause increased itching, and injection site reactions | Phase 2 |
| NA + TLR agonists | NA + TLR 7 agonist (vesatolimod, GS9620) | 24 weeks | Stimulates HBV specific T cell and NK cell responses but little impact on qHBsAg or HBV DNA | More grade 3 adverse events seen in the higher doses, leading to treatment discontinuation in a few cases | Phase 2 |
| | NA + TLR 8 agonist (Selgantolimod) | 24 weeks | mean reduction qHBsAg<1 log 16% HBeAg loss, 5% HBsAg loss, | No major safety issues | Phase 2 |
| NA + checkpoint inhibitors | NA + PD1 inhibitor (nivolumab) | 1 dose given with 24 weeks followup | Mean qHBsAg reduction was 0.48 log and 5% had HBsAg loss | No major safety issues, only 1 patient had grade 3 ALT abnormality | Phase 2 |
| | NA + PDL1 inhibitor (ASC22, envafolimab) | 12 doses over 24 week | Mean qHBsAg reduction 0.38 log but in those with baseline qHBsAg<500 IU/ml, qHBsAg reduction was 0.7log and 3/16 achieved HBsAg loss by end of | Grade 1-2 abnormal ALT seen in 21% of patients | Phase 2 |

| | | | | | |
|---|---|---|---|---|---|
| | | | therapy | | |
| NA + therapeutic vaccine | NA + TG1050 | | Showed HBV specific IFN-g ELISPOT responses but little change in qHBsAg levels | Only grade 1-2 adverse events no abnormal ALT | Phase 1 |
| | NA + NASVAC | 10 doses over 24 weeks | Mean reduction in qHBsAg in Nuc treated group was 0.225 versus 0.549 in the untreated group, however no control group hence findings could be natural history events | No safety issues reported | Phase 2 |
| **TRIPLE combinations** | | | | | |
| Transcriptional inhibition + replication inhibition + immune modulator | FXR agonist (vonafexor) + peginterferon ± ETV | 16 weeks | Responses only seen in HBeAg neg CHB, 1.1 log reduction in qHBsAg in dual arm versus 0.6 log in the triple arm. HBeAg pos patients had no response to therapy | Adverse events due to interferon toxicity | Phase 2 |
| Reducing antigen burden + replication inhibition + immune modulator | Nucleic acid polymers + NA + peginterferon | 48 weeks | 75% achieved HBsAg <100 IU/ml; overall 14/40 (35% achieved HBsAg loss | Adverse events due to interferon toxicity; most patients had significant ALT flares | Phase 2 |
| | siRNA(VIR2218)+ NA + peginterferon | 24 weeks | mean reduction in qHBsAg was 2.03 log IU/ml dual arm (VIR-2218/NA) versus 2.55 log IU/ml triple (VIR-2218/NA/ peginterferon); 95% achieving qHBsAg<100 | Adverse events due to interferon toxicity | Phase 2 |

| | | | | | |
|---|---|---|---|---|---|
| | | | IU/ml, and 55% achieving qHBsAg<10IU/ml by week 24. At week 48, 4/13 (30.8%) achieved HBsAg loss in those receiving 13 doses of VIR-2218 and <44 weeks of PEG-IFN | | |
| Reducing antigen burden + intensifying replication inhibition | JNJ-3989(siRNA) and/or JNJ-6379(CAM) and NA (REEF-1 study) | 48 weeks | Dual combination (NA + 100 mg dose siRNA) showed better qHBsAg reduction(2.1 log) than the triple combination of NA/CAM/siRNA (1.8 log10) | Most adverse events were grade 1-2 | Phase 2 |
| Reduce viral replication and enhance immune responses | NA + ChAdOx1-HBV/MVA-HBV (VTB-300)(Therapeutic vaccine) ± nivolumab (PD1 inhibitor) | Single dose of either MVA-HBV ± ChAdOx1-HBV ± nivolumab over 28 days | Patients with baseline qHBsAg<50 IU/ml had significant qHBsAg reduction given VTB-300 alone, with addition of nivolumab reductions of 1.15 log seen in 8/18 patients who had baseline qHBsAg<1000 IU/ml. One patient lost HBsAg | No major safety issues, two patients had mildly abnormal ALT | Phase 1b/2a |

***Legends to Tables and Figures***

**[Au: I have suggested to our Art Editor to combine Figs 1,3 and 4 as I think this will avoid repetition and provide one clear resource on those two types of treatments. Likewise, I suggest combining Figs 2 and 5, as there is quite a bit of repetition between these 2 figures as well. I will send the figures as soon as they are ready.]**

FIGURE 1 Schematic diagram of HBV lifecycle showing potential targets for antiviral therapy: viral entry, DNA editing, viral transcription, RNA stability, viral translation, capsid formation, viral replication and secretion. There are two overlapping pathways: the secretory pathway (blue) and the replicative pathway (red). “S” antigen can also come from integrated HBV.

FIGURE 2 Schematic diagram of immune pathways and targets for HBV. At high viral DNA/antigen loads, immune cells exhibit the exhausted phenotype; using innate immunity modulators, therapeutic vaccines, metabolic regulation, checkpoint inhibition and novel immunotherapeutics, functional immune phenotypes can be generated leading to functional cure.

FIGURE 3 Schematic diagram of strategies to reduce viral replication in clinical trials or approved. These include entry inhibition, capsid assembly modulators and nucleoside analogues.

FIGURE 4 Schematic diagram of strategies to reduce antigen burden in clinical trials. These include transcriptional inhibitors, interferon, RNA interference (RNAi) comprising siRNA and anti-sense

oligonucleotides(ASO)/Locked Nucleic Acids(LNA) and HBsAg inhibition.

FIGURE 5 Schematic diagram of strategies to activate host immunity in clinical trials. These include TLR7 & 8 agonists, interferon, checkpoint inhibitors, therapeutic vaccines, anti-HBs monoclonal antibodies and novel immunotherapy. **[Au: I don't think the novel immunotherapy IMC-I109V is mentioned in the main text of the review. Please could you discuss this therapy somewhere or perhaps it could be removed from the figure?]**

ToC blurb

In this Review, the authors consider different paths to functional cure of chronic hepatitis B (CHB) and the need to individualize therapy of this heterogenous infection until a therapeutic avenue for all patients with CHB is available.