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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 they fall into three categories, those reducing viral replication, those reducing antigen load and immunotherapies. These therapies are discussed in the context of early phase clinical trials hence represent insights from preliminary data. Profound viral suppression seems unlikely to achieve qHBsAg reduction or HBsAg loss. Combining nucleoside analogues(NA) and immunotherapy reduces qHBsAg levels and induces HBsAg loss in some patients, particularly those with lower qHBsAg levels. Even agents that are specifically designed to reduce viral antigen load may not be able to achieve sustained HBsAg loss when used alone. Thus there is a rationale for the use of combinations of reducing viral replication, antigen reduction and immunotherapy. 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 CHB patients is available.

Key Points

- HBsAg loss after 6 months off therapy with undetectable HBV DNA is defined as functional cure, and is associated with improved clinical outcomes and is the optimal goal of Chronic Hepatitis B therapy.
- Novel agents fall into three categories; those reducing viral replication, those reducing 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 qHBsAg levels.
- Combining replication inhibition with immunotherapy leads to some reduction in qHBsAg levels (<1 log) and HBsAg loss, usually in those with low baseline qHBsAg levels
- Reducing viral antigen production reduces qHBsAg levels by up to 2 log, which may be sustained off therapy, and in combination with replication inhibitors or immunotherapy can achieve HBsAg loss in some instances, although not all were sustained.
- There is likely to be more than one path to functional cure and finding the optimal one for each patient is the challenge.

Abbreviation	Full description
AASLD	American Association for Study of Liver
ALT	Alanine Aminotransferase
ASO	antisense oligonucleotides
AUROC	Area under the receiver operating characteristic curve
Bim	Bcl2-interacting mediator
CAM	Capsid Assembly Modulators
CAR T	Chimeric antigen receptor
cccDNA	covalently closed circular DNA
ChIP	Chromatin Immunoprecipitation
CHB	Chronic Hepatitis B
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic Cells
ddPCR	Digital Droplet PCR
EGF	Epidermal growth factor
ESCRT	Endosomal sorting complex required for transport
ELISpot	enzyme-linked immunosorbent spot
FXR	Farnesoid X Receptor
GALNAC-siRNA	N-acetyl-galactosamine conjugation siRNA
HBcrAg	hepatitis B core-related antigen
HBeAg	hepatitis B "e" antigen
HBV RNA	Hepatitis B Virus Ribonucleic Acid
HCC	Hepatocellular carcinoma
HIV-HBV co-infection	Human Immunodeficiency Virus-Hepatitis B Virus Co-infection
IDO	Indoleamine-2,3-dioxygenase
ImmTavs	Immune mobilizing monoclonal T Cell receptors against virus
IFN	Interferon
IRIS	immune re-constitution inflammatory syndrome
MAIT cells	Mucosal-associated invariant T
MVB	multivesicular body
NA	Nucleos(t)ide analogues
NAP	Nucleic Acid Polymers
NKs/NKTs	Natural Killer/Natural Killer T cells
NPC	nuclear pore complex
NTCP	sodium taurocholate cotransporting polypeptide
PD-1	Programmed cell death protein 1
peginterferon	pegylated-interferon alfa
PMT	histone Post-Translational Modifications
pgRNA	Pregenomic RNA

qHBsAg	Quantitative HBsAg
qPCR	quantitative Polymerase Chain Reaction
Q2W	Dosing every 2 weeks
rcDNA	relaxed circular DNA
RCT	Randomised clinical trial
RT	reverse transcription
siRNAs	small interfering RNAs
Smc5/6	structural maintenance of chromosomes 5 and 6 complex
STOPS	S-antigen Transport-inhibiting Oligonucleotide Polymers
Tim-3	T cell immunoglobulin and mucin domain-containing protein 3
TLR	toll-like receptor
TRAIL	TNF-related apoptosis-inducing ligand

Keywords: HBsAg loss; quantitative HBsAg levels; HBV RNA; HBcrAg; cccDNA; T cell exhaustion; immunotherapy; antiviral therapy; nucleoside analogues; capsid assembly modulators; siRNA; anti-sense oligonucleotides; entry inhibitor; transcriptional inhibitors; nucleic acid polymers, TLR agonists; PD-1 inhibitors;

Abstract	210 words
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1. Introduction: What is functional cure?

Despite the success of the hepatitis B vaccine, chronic Hepatitis B (CHB) virus infection affects more than 296 million people, mainly in the Asia-Pacific region leading to an estimated 820,000 deaths from cirrhosis and hepatocellular carcinoma (HCC) annually¹. Such complications are substantially reduced by loss of hepatitis B surface antigen (HBsAg), or functional cure^{2,3}, which happens rarely in natural history or with current therapy⁴. Such complications are also reduced by oral antiviral therapy⁵. The complex natural history of CHB follows four clinical/virological phases of infection and ends with a functional cure phase with a decline in qHBsAg and covalently closed circular DNA (cccDNA) levels over time, but 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 may 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 with 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 minichromosome reservoir is controlled by innate and adaptive antiviral immune responses³. The viral minichromosome and integrated HBV producing large amounts of HBsAg is also a possible contributor to the diversion or exhaustion of immune responses and for the pathobiology of the liver disease and its implication for oncogenic events⁶.

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 Chronic Hepatitis B³.

2. Science of Functional Cure

2.1 Virological pathways and mechanisms

HBV is an enveloped hepatotropic DNA virus using 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/maintenance or become enveloped and released⁹. 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 proteoglycans¹² and enter the hepatocyte via the bile acid transporter sodium taurocholate cotransporting polypeptide(NTCP)^{13,14}.

Following viral entry¹⁵ HBV is uncoated and nucleocapsids move to the nuclear pore complex (NPC) where rcDNA is released into the nucleus and converted into cccDNA^{16,17} using recently identified host factors^{18,19,20,21,22}. The viral cccDNA associates with cellular histones and non-histone proteins and epigenetic

modifications can regulate its transcription²³. Mechanisms of cccDNA degradation – the ultimate goal of HBV cure are still only poorly understood^{18,24,25,26}. One mechanism contributing to degradation of cccDNA is mediated by cytidine deaminases^{27,28}.

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 template for reverse transcription into new rcDNA. This requires several host factors²⁹⁻³¹. Viral transcription is regulated by four distinct promoters (preS1, preS2, core and X), and two enhancers (Enhancer-I and Enhancer-II)³² and epigenetic modifications of HBV DNA and cccDNA-associated histones^{33,34,34}. These regulatory elements include various transcription factors³⁵⁻³⁷. Interferon-alpha (IFN- α) and interleukin-6 have both been shown to decrease cccDNA-bound histones acetylation and thereby transcriptional activity^{38,39,40,41}. This finding is important to understand the mechanism of immune-mediated antiviral approaches for functional cure. Many of the host chromatin modifying enzymes are potential drug targets for cccDNA silencing but adverse effects may be a limitation. HBx protein is an important transcriptional modulator, by degrading the structural maintenance of chromosomes 5 and 6 complex (Smc5/6) via ubiquitination. Moreover, cccDNA transcription has been shown to be targeted by FXR agonists. The viral capsid functions as a container for the viral genome allowing reverse transcription (RT) and protecting it from host cell nucleases and proteases. The capsid packages pgRNA mediated by HBc-phosphorylation⁴⁵. RT occurs only inside the capsid with hepatitis B core protein (HBc) as a co-factor. HBc associated with cccDNA may be involved in transcriptional regulation⁴⁶. This important role has led to 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) machinery⁴⁷ via the multivesicular body (MVB)⁴⁸}. Subviral particles (SVPs) are non-infectious, and are formed using the viral surface proteins as building blocks, comprising the majority of HBsAg in serum⁴⁹. This pathway can be inhibited by nucleic acid polymers (NAPs)⁵⁰ and S-antigen Transport-inhibiting Oligonucleotide Polymers(STOPS)⁵¹. Consequently, the HBV life cycle offers multiple targets for antiviral therapy of which some are actively explored for a functional cure as described below(Figure 1).

2.2 Immune pathways & 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 NK cell inhibition^{52,53,54,55}. 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, DCs and NKs/NKTs. HBV is thought to be a stealth virus that evades induction of innate immune responses⁵⁶, 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 has also been found to have impaired innate immune responses especially toll-like receptor (TLR) downregulation and dysfunction^{57,58}. Moreover, the liver microenvironment is inherently immune suppressive which protects against severe inflammation,

including enzymes like arginase, IDO⁵³ and ACAT⁵⁹ and, which impair the functionality of HBV-specific T cells⁵⁴.

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 donor⁶⁰. Many immunotherapies attempt to stimulate the patient's own endogenous responses; although 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, e.g., peginterferon- α has direct antiviral effects and expands NK cells but suppresses HBV-specific T cells^{61,62,63}. Despite its poor tolerability, peginterferon- α has proven capacity for functional cure. Alternative innate immunomodulators such as the oral TLR8 agonist selgantolimod induces IL-12, increasing HBV-specific T cell expansion⁶⁴, but also T_{REG}, myeloid-derived suppressor cells and regulatory NK cells that may limit T cell boosting^{65,66,67}. MAIT cells, expanded by TLR8 agonists, have the potential to boost immunogenicity of vaccines⁶⁸; and may 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 control⁵². The new generation of therapeutic vaccines may be more successful than past attempts, due to enhanced immunogenicity, inclusion of multiple HBV antigens and careful patient selection. However, it is likely that existing highly exhausted HBV-specific T cells will still be difficult to boost, considering the

tolerogenic liver environment. Hence novel combinations such as the recently developed ChAdOx-HBV vaccine⁶⁹ in combination with PD-1 blocking mAb in Phase 1/2a might allow better boosting and/or enhanced survival of newly primed T cells (Figure 2), but the heterogeneity of checkpoints suggests individualised immunotherapeutic approaches may be required⁷⁰, including alternatives like IL-2 stimulation⁷¹. 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 Chronic Hepatitis B infection and those with resolved infection⁷⁴. 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 cytokines⁷⁵. Antibody infusions can reduce excess circulating HBsAg, through antibody-dependent cellular phagocytosis, block new hepatocyte infection as well as potentially clear HBsAg-expressing hepatocytes. Bispecific antibodies can simultaneously bind HBsAg on hepatocytes and T cells through an anti-CD3-binding moiety⁷⁶, and antibody engineering can create a vaccinal effect, via immune complex-dendritic cell engagement⁷⁷.

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

3. *Classes of therapeutic agents*

The goal of functional cure has driven a rapid increase in antivirals and immunomodulators with potent effects in vitro entering clinical development. These agents are broadly divided into antiviral agents and immunomodulators and can be viewed in table 1.

4.0 Strategies for dual combinations

A summary of the various combinations, phase of the study, efficacy and safety can be viewed in table 2.

4.1 Combining viral replication inhibitors

One of the important questions is whether combinations of viral replication inhibitors can decrease or silence the pool of cccDNA significantly such that they could achieve a functional cure. The basis for this concept relies on a more potent suppression of viral replication which could decrease the replenishment of the cccDNA pool. One potential impediment to this is production of HBsAg through integrated HBV. It should also be kept in mind that anti-HBs Ab production from HBV-specific B cells may complex any residual HBsAg produced either from cccDNA or integrated sequences.

4.1.1. Existing antiviral agents: nucleoside analogues in combination (NA + NA)

Current preferred nucleos(t)ide analogues, entecavir (ETV), tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF), offer highly effective suppressive therapy with very low rates of resistance with long-term therapy, but low rates of HBsAg loss (Figure 3). In chronic HBV patients, low level viremia on treatment was associated with fibrosis progression⁷⁸, and patients with decompensated cirrhosis with low level viremia had worse survival⁷⁹. With newer assays, patients who were found to be HBV DNA negative had detectable HBV DNA using more sensitive

techniques⁸⁰. Intensification of therapy does lead to better HBV DNA suppression particularly in those with high baseline HBV DNA, but does not lead to additional benefits in terms of enhanced HBsAg loss or serological outcomes⁸¹.

4.1.2 Novel agents: nucleoside analogues (NA) + capsid assembly modulators (CAMs)

A more potent suppression of viral replication which could decrease the replenishment of the cccDNA pool and inhibition of cccDNA formation in newly infected cells using NA+CAM(11 in clinical trials) (Figure 3).

There is limited data of CAMs in combination with NA. As monotherapy, most CAMs show 1.4-3.2 log reduction in HBV DNA or pgRNA in phase 1 studies, but little change in qHBsAg. In the phase 2 Jade study, CHB patients untreated or virologically suppressed, were treated with 250mg JNJ-56136379(bersacapavir), once-daily for ≥ 24 and ≤ 48 weeks. For untreated-HBeAg-positive cohort, mean HBV DNA declines at Week 24 were 5.88 for bersacapavir 250mg+NA versus 5.21 \log_{10} IU/mL for placebo+NA, 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. This confirmed the expected target engagement and antiviral potency of bersacapavir in combination with NA. In untreated-HBeAg-negative patients and virologically suppressed HBeAg-positive/negative patients, no qHBsAg declines occurred. Quantitative HBsAg(qHBsAg) responders also had HBeAg and HBcrAg declines and frequent early on-treatment ALT flares. Viral breakthrough occurred with bersacapavir monotherapy, demonstrating that CAM should be combined with other antivirals to prevent the occurrence of virologic resistance. Overall, bersacapavir in combination with a NA showed good antiviral activity in patients with CHB, but impact on qHBsAg

and HBeAg levels were small and mainly observed in untreated HBeAg-positive patients. This suggests, that either bersacapavir was not potent enough or not administered long enough to deplete the cccDNA pool or that the absence of significant decline of serum qHBsAg was related to HBV integration as a major source of HBsAg expression⁸².

In the vebicorvir plus NA Phase 2 study, virologically suppressed CHB patients treated with vebicorvir plus NA had greater proportions with HBV DNA <5IU/ml and undetectable pgRNA, 83% and 59% versus NA only with 29% and 18% respectively at week 24⁸³. In a substudy of the phase 2 extension study patients with undetectable HBV DNA and HBV RNA, and low/undetectable HBeAg were selected to stop treat after 72 weeks treatment, but all 42 patients relapsed within 4 months⁸³, indicating that despite profound viral suppression, the silencing of cccDNA transcriptional activity was not achieved.

Altogether, the results of these two clinical studies suggest that the combination of NA and the current generation of CAM is unlikely to achieve functional cure. Whether more potent CAM or CAM with additional modes of action on cccDNA would have the potential to affect the viral reservoir remains to be evaluated.

4.2 Inhibit viral replication + lower viral antigen burden

Viral replication inhibition not only blocks the replication pathway but long-term potent suppression could reduce the cccDNA pool⁸⁴. By adding translational inhibitors the reduction of viral antigens is also reduced(Figure 4). This has further potential to allow immune cell exhaustion to recover(Figure 2).

4.2.1 Small interfering RNAs (siRNA) + nucleoside analogues (NA): All 4 siRNAs in phase 2 clinical development(JNJ-3989; VIR-2218; RG-6346; AB-729) achieve predictable on-treatment qHBsAg responses, which appear to be durable for more

than 6 months after the final dose^{85,86,87}. These studies were performed in CHB patients 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. 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. The preliminary results from the first Phase II studies of siRNA plus NA show qHBsAg reduction of 2-2.5 log with 50 – 97% of patients having qHBsAg<100 IU/ml, and a few patients lost HBsAg⁸⁸. AB-729 a 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/ml⁸⁹, but no patients lost HBsAg. The limited data available indicates little additional benefit of adding a NA to siRNA, regarding qHBsAg decrease. In the AROHBV1001 study⁹⁰, JNJ-3989, a siRNA that targets multiple HBV sites was administered Q4W subcutaneously on 3 occasions(day 0, 28 and 56) at different dosage levels(100-400mg) together with NA till end of 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 were also seen. This study showed that sustained qHBsAg and other viral biomarker reduction was possible one year off-therapy. There were no major safety concerns and only one patient had moderate ALT flare.

4.2.2 Antisense oligonucleotides (ASO) + nucleoside analogues (NA): In contrast, in a phase 1b study, biweekly non-GalNac-conjugated ASO GSK- 3228836 300mg (Bepirovirsen) dosing led to 2 log reductions in qHBsAg within 28 days, associated

with ALT flares⁹¹, and 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 ASOs may represent an efficient uptake even without GalNAc-conjugation. In the phase 2b study B-clear, presented at EASL 2022 as a late breaker⁹², bepirovirsen 300mg was dosed weekly for 12 or 24 weeks in NA-treated or untreated patients. Results were similar between the two groups, with 28-29% achieving undetectable HBsAg and HBV DNA by end of therapy, the best results being achieved by the bepirovirsen 300mg for 24 weeks arm, and responders had baseline qHBsAg<3 log IU/ml. The main safety concerns were local injection site reactions, and some grade 3-4 adverse events due to ALT elevations.

4.3 Inhibit viral replication + boost immune responses

Since HBV-specific T cells are functionally impaired in chronic HBV patients, their stimulation represents a possible strategy for restoration⁵³(Figure 5). T cells may be refractory to antigen stimulation but this may be overcome by abrogation of T cell inhibitory mechanisms as a result of the inhibition of viral replication and resolution of liver inflammation⁵³.

4.3.1 Approved therapies

Various strategies combining NA and peginterferon have been evaluated to increase the rate of HBsAg loss. A meta-analysis found that combination of NA and peginterferon had 6% higher HBsAg loss than NA⁹³.

The strategy to add or switch to peginterferon in those on NA suggests some benefit of switching although the only RCT conducted⁹⁴ showed no difference in efficacy and a higher rate of ALT flares.

4.3.2 Novel agents

A. *Bulevirtide + peginterferon*: Bulevirtide (Hepcludex©; Myrcludex B) 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. In the MYR 203 trial⁹⁵ of HBV/HDV co-infected patients, 26.7% of patients(4/15 patients) achieved HBsAg loss after receiving BLV 2mg + PEG-IFN by week 72 of treatment. Paradoxically, none of the patients that received a higher BLV dose(5mg) + PEG-IFN demonstrated HBsAg loss. Since HBsAg loss was only achieved in 4 co-infected patients, larger trials in mono-HBV infected patients are needed to confirm the results.

B. *Nucleoside analogues(NA)+ Toll like receptor(TLR) agonists*: Another possibility to improve immune control in CHB patients, is the stimulation of innate immunity receptors, such as toll like receptors (TLRs)⁹⁶. In CHB patients on NA, TLR-7 agonist GS-9620(vesatolimod) add-on to NA was able to boost HBV-specific T cell responses and NK responses with anti-viral cytokine production⁹⁷, but had no impact on qHBsAg reduction nor HBV DNA. In a phase 1 multi-ascending dose study of RO7020531 given s/c Q2W, the mean reduction in qHBsAg was 0.3 logIU/ml⁹⁸, compared to the mean reduction of -0.01 and 0.002 logIU/ml in untreated and NA-treated CHB with GS-9620⁹⁹. In a phase 2 study¹⁰⁰ 24 weeks of TLR8 agonist, Selgantolimod, in virally suppressed CHB led to 5% HBsAg loss, 16% HBeAg loss and a mean reduction in qHBsAg of <1 log¹⁰¹. There were dose-proportional changes in peripheral cytokines(IFN- γ , IL-12p40 and IL-RA), indicating better efficacy than TLR7 agonists.

C. *Nucleoside analogues (NA) +Checkpoint inhibitors*: Checkpoint inhibitors may restore T cell responsiveness, based on the evidence that co-inhibitory molecules are up-regulated on HBV-specific T cells¹⁰²⁻¹⁰⁸. A single dose of Nivolumab, a PD-1 inhibitor, was administered in a pilot study ± therapeutic HBV vaccination, in virally suppressed HBeAg-negative CHB patients. 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, one patient (5%) lost HBsAg¹⁰⁹. In a late breaking abstract at AASLD 2021¹¹⁰, ASC22(Envafolimab), a humanized single- domain PD-L1 antibody was tested in a phase 2b study; virally suppressed patients were treated with 1mg/kg Q2W 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 2022¹¹¹, the 3 patients who achieved HBsAg loss had baseline qHBsAg<100 IU/ml, and that 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 were seen in those with low qHBsAg at baseline.

D. *Nucleoside analogues (NA)+ Therapeutic vaccines*: a yeast-based therapeutic vaccine containing HBV S, X, and core proteins (GS-4774) was given with NA in CHB patients¹¹². Significantly enhanced antiviral cytokine production by HBV-specific T cells, with a more pronounced impact on CD8 than CD4 T-cells was found but did not affect serum qHBsAg levels¹¹³. The Transgene trial tested TG1050, an adenovirus 5-based therapeutic vaccine expressing HBV polymerase, with domains of HBcAg and HBsAg in virally suppressed patients¹¹⁴. The phase 1b

study showed stimulation of HBV specific IFN-g ELISPOT responses but little change in qHBsAg levels.

In a study of CVP-NASVAC¹¹⁵, a therapeutic vaccine comprising HBsAg and HBcAg delivered intranasally, 10 doses every Q2W were administered to both untreated and NA-treated CHB patients in a non-RCT, and were followed up for 30 months post-therapy. The mean qHBsAg reduction was 0.225 log IU/ml in the NA-treated group compared to 0.549 log IU/ml reduction in the untreated group. In the NA-treated group 2/15(13.3%) achieved functional cure, compared to 5/18 (27.7%) in the untreated group at 30months. This is the first report of 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.

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 effects⁹⁹. 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.

4.4 lower viral antigen burden + boost immune responses

Persistent high antigen load is a major factor driving the exhaustion of antiviral adaptive immunity; new antiviral agents that can substantially lower 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 observed in chronic viral infection, T cell exhaustion may not recover¹¹⁶. Newly generated T and B cells (eg by therapeutic vaccination) could be protected from terminal exhaustion if viral antigen can be lowered sufficiently within hepatocytes¹¹⁷. However, if all antigen production is eliminated, cccDNA-containing hepatocytes could effectively become ‘invisible’ to HBV-specific T cells, limiting ongoing immunosurveillance¹¹⁸. Other therapies that block HBsAg release from infected hepatocytes (e.g. STOPs) or remove it from the circulation (e.g. neutralising antibodies) would not be as likely to promote T cell reconstitution since peptide presentation from the infected hepatocyte can continue. Consistent with this, a recent study showed that antibody-mediated clearance of serum HBsAg had a negligible impact on CD8 T cell responses in mouse models of HBV infection¹¹⁹. 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^{75,120}, 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 may make it more amenable to boosting by therapeutic vaccines or other immunotherapies.

4.4.1 Small interfering RNA (siRNA) + peginterferon

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

4.4.2 Nucleic Acid Polymers (NAP) + peginterferon

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

5. Strategies for triple combinations

Triple combinations of the three categories of therapies, inhibiting viral replication(Figure 3), reducing viral antigen burden (Figure 4) and immune stimulation(Figure 5) may be considered the ideal combination in theory. Such combinations need to be demonstrated in clinical trials, and phase 2 studies may provide preliminary data of efficacy and insights into their feasibility and potential. A summary can be viewed in table 2.

5.1 Transcriptional inhibition + replication inhibition + immune modulator

Vonafexor is a new class of agent, a FXR agonist which is proposed to reduce HBV transcription. A phase 2 study of Vonafexor+peginterferon ± entecavir (dual vs triple therapy) for 16 weeks with 24 weeks of follow-up in HBeAg-negative patients¹²¹, 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 end of therapy, which appeared to be

sustained off therapy. HBeAg-positive patients had no reductions in qHBsAg. HBV DNA 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 peginterferon in all arms, as well as the lack of HBV RNA results does not provide clarity with potential target engagement and its impact on viral transcription.

5.2 Reducing antigen burden + replication inhibition + immune modulator

5.2.1. Nucleic Acid Polymers (NAP) + nucleoside analogues (NA) + peginterferon

In a phase 2 open label, study, HBeAg-negative CHB patients were treated with tenofovir(TDF) for 24 weeks then randomised to receive 24 weeks of REP 2139/REP 2165+TDF, and peginterferon¹²², or peginterferon+TDF. Patients in the TDF/NAP/peginterferon arm at week 48 had significant reductions in qHBsAg with 75% (15/20) achieving qHBsAg<100IU/ml and 50%(10/20) achieving qHBsAg <LLOQ, 55%(11/20) achieved anti-HBs seroconversion versus 5% (1/20), 0%, and 0% respectively in the control arm. Overall, 14/40(35%) patients maintained HBsAg loss after 1 year of treatment-free follow up. Noteworthy, most patients had significant ALT flares during therapy at the time of qHBsAg decline.

5.2.2 Small interfering RNA (siRNA) +nucleoside analogues (NA) +peginterferon

In a phase 2 study¹²³, VIR-2218± peginterferon was given for 24 weeks in NA-treated CHB patients, in a 4-arm study, with one arm without peginterferon, and the other arms having increasing duration of peginterferon(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/peginterferon, suggesting synergistic effects, with 95% achieving qHBsAg<100 IU/ml, and 55% achieving qHBsAg<10IU/ml by week 24. There was increasing efficacy on qHBsAg

reduction with increasing duration of peginterferon. At week 24, 3/33(9%) had qHBsAg<LLOQ and 2/3 achieved detectable anti-HBs, and showed an ALT elevation.

5.3 Reducing antigen burden + intensifying replication inhibition

The REEF-1 phase 2b study has evaluated the efficacy and safety of the siRNA JNJ-3989 and/or CAM JNJ-6379 and NA for the treatment of CHB. Surprisingly, the results of this 48 week study showed that the double combination of NA + 100 mg dose siRNA induced a more profound reduction in serum qHBsAg(2.1 log₁₀) than the triple combination of NA/CAM/siRNA(1.8 log₁₀)¹²⁴. This remains to be validated in other studies and raises the issue of whether CAMs are necessary to achieve functional cure.

The few phase 1 and 2 studies show that significant qHBsAg reduction can be achieved with triple combinations. Not surprisingly, the best reductions in qHBsAg are achieved with agents that reduce viral antigen burden, siRNA or NAPs; the only triple combination study of FXR/NA/peginterferon did not show 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 than 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 peginterferon 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.

5.4 Inhibit viral replication + enhancing immune responses

In a phase 1b/2a study in virally suppressed CHB patients presented at EASL 2022¹²⁵, a novel therapeutic vaccine, ChAdOx1-HBV/MVA-HBV was used in a prime-boost strategy (VTB-300) with and without low dose nivolumab(PD1 inhibitor). A few patients(3/18) treated with VTB-300 alone had significant qHBsAg declines of 0.7-1.4 log, and all had baseline qHBsAg<50 IU/ml. Patients(8/18) receiving VTB-300 and low dose nivolumab had significant qHBsAg declines of 1.15 log, which persisted 8months after the last dose, and one patient lost HBsAg. Such responders had qHBsAg<1000 IU/ml. No major safety issues were reported and only two patients had mild transaminitis. This study showed that potent therapeutic vaccines can work safely and efficaciously with PD1 inhibition as a dual immune stimulation strategy.

6. Strategic issues in combination therapy

6.1 When should you boost immune responses?

T cell exhaustion in chronic HBV infection represents a multifactorial phenomenon sustained by different inhibitory mechanisms, which make its correction difficult to achieve^{53,126}. The heterogeneity of the exhausted CD8 T cell population with the co-existence of CD8 T cell subsets with different functionality and antigenic specificity in individual chronically infected patients makes this situation complex¹²⁷⁻¹³⁰. In addition, a number of intracellular metabolic processes and signalling pathways are deeply dysregulated in HBV-specific CD8 T cells^{64,131,132}, and the limited T cell response attributed to the irreversible molecular “scar”¹³³⁻¹³⁵. One debated issue is whether immune therapy is more effective if used during the immune tolerant phase as recent data suggests HBV-specific T cell responses in these patients are superior in terms of intensity and quality than those in the immune active phase^{136,137}.

However this interesting finding has been challenged with three randomised control trials showing very low rates of HBeAg loss, 3-3.8%^{138,139,140} with antiviral therapy using peginterferon and NA in immune tolerant CHB.

In the absence of accurate immune predictors of response, we have to rely on clinical studies to guide immune modulator therapy. Recently, stopping NA therapy in virally suppressed patients has been shown to increase the rate of HBsAg clearance, particularly in those with low qHBsAg levels(<100IU/ml;)^{141,142,143,144,145,146} and has been associated with modulation in NK cell and HBV-specific T-cell function^{147,148,149}. The additional finding that repeated PDL1 therapy with NA leads to better qHBsAg reduction and HBsAg loss in those with low baseline qHBsAg levels¹¹⁰, together suggest the optimal timing to initiate immune modulator therapy is CHB patients with low qHBsAg levels. By exploiting the immunomodulatory effects of NA discontinuation to stimulate innate and adaptive immune responses and to modify the liver immune environment, this could be a novel therapeutic strategy to achieve a higher functional cure rate. Therapies that propose to achieve low qHBsAg levels as an endpoint combined with immune stimulation could be explored; these include siRNA, ASO, NAPs, and recently anti-HBs monoclonal antibody therapy.

6.2 Combining therapy or sequential therapy?

Data available to date suggest achievement of functional cure in CHB requires combination therapy¹⁵⁰. However, alternatives to combination therapy have not really been explored. The best clinical evidence of benefits of alternative strategies combining NA and peginterferon were examined in a meta analysis¹⁵¹. Initial combination therapy versus initial NA monotherapy showed a non-significant increased RR of HBsAg loss of 1.44. Peginterferon add-on to NA versus NA

monotherapy showed a significant improved RR 4.52 suggesting add-on peginterferon was a useful strategy. The best result however was NA switch to peginterferon versus NA monotherapy which showed RR 12.15. This meta-analysis showed that sequential therapy seemed to be better than combination therapy, although these were not head-to-head comparisons. A head-to-head of add-on or switch peginterferon RCT showed no significant difference in HBsAg loss rates⁹⁴. As shown in the previous section, stopping NA therapy leads to recovery of immune cell exhaustion and increased rates of HBsAg loss, an optimal setting for immune stimulation strategies, such as TLR8 agonists, checkpoint inhibitors or therapeutic vaccines but whether non-NA antiviral strategies to reduce qHBsAg levels lead to similar immune states akin to long term NA is unclear.

7.0 *Biomarkers to monitor treatment efficacy*

7.1 Serum Viral Biomarkers

Biomarkers are indicators of biologic, pathogenic process or pharmacologic responses¹⁵², and in the case of HBV, its utility is best if it is part of the biological pathway. Consequently virological markers¹⁵³ such as HBV DNA, HBV RNA, HBeAg, HBcrAg, quantitative and qualitative HBsAg(qHBsAg), ultrasensitive HBsAg¹⁵⁴ are most assessed.

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 (subviral particles, 1000x in excess of infective virus) as secreted products(Figure 1). Agents that act on replicative pathway such as NA 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. NAs reduce HBV DNA effectively but take many years to reduce HBV RNA¹⁵⁵ while CAMs can reduce both. One caveat to shutting down the secretory pathway in the lifecycle of HBV such agents need to also reduce HBsAg that originates from integrated HBV¹⁵⁶.

In general, qHBsAg levels are good predictors of HBsAg loss during therapy(AUROC 0.77-0.91)^{157,158} compared to HBcrAg and HBV RNA. The best predictors of HBeAg-seroconversion are HBV RNA and qHBeAg(AUROC 0.77 & 0.75 respectively)¹⁵⁹. The best predictors of relapse after stopping therapy are combined HBV RNA and HBcrAg(AUROC 0.7-0.85)¹⁶⁰, but HBcrAg has a 10% false positive rate hence maybe unreliable¹⁶¹. A systematic review showed stopping NA when qHBAg≤100 IU/ml leads to HBsAg loss between 21.1%-58.8% and viral relapse rates of 9.1%-19.6%. In the context of functional cure, qHBsAg appears to be the best biomarker and predictor of response. Depending on the mechanism of the drugs a given biomarker may be used as an endpoint or as marker for target engagement, but ultimately clinically 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 may be misleading as low baseline qHBsAg levels may lead to high rates of HBsAg loss.

7.2 Immune Biomarkers in peripheral blood

The immune Monitoring Working Group of the HBV Forum¹⁶² has provided an overview of the technologies available, 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/fluorospot. This can detect responses in all patients and covers the full breadth of the response within a patient, are more sensitive than intracellular cytokine staining(ICS) but lacks phenotyping data, making it impossible to identify the cell type responsible for the cytokine production and differentiation. However, in a study of the PD1 inhibitor, nivolumab/NA±therapeutic vaccine(GS-4774)¹⁰⁹ there was a reduction in qHBsAg levels and 4%(1/24) HBsAg loss, but during therapy no increase in HBV specific T cells were found to core, polymerase or "S", although T cell phenotyping showed an increase in CCR7-CD45RA- effector memory CD4+ T cell frequency at end of therapy. While 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 is more a research tool. Differential exhaustion and memory markers provides 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.

Serum and plasma cytokines have yet to predict antiviral responses but can provide insights into potential immunological pathways and mechanisms.

Interestingly, patients with HIV-HBV co-infection who start ART therapy¹⁶³ achieve 34.5% HBsAg loss, and this 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 sCD163 correlating with

HBsAg loss, suggesting macrophage activation. 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 have shown to be important, in particular intrahepatic NK cells which are a unique population; their phenotypic profiling, particularly frequencies of CD38, Ki67 and TRAIL can be measured¹⁶⁴, but their role in antiviral responses has not been fully understood.

7.3 Studying intrahepatic immunology

There is increasing recognition that specific immune cells are unique to the liver and not well represented in blood. For example tissue-resident subsets of NK cells¹⁶⁵ and T cells¹⁶⁶ 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.

Since HBV-specific T cell frequencies and PD-1 expression are both enriched in the liver compared to peripheral compartment^{104,167} 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 and the cells obtained can then be studied by a variety of focused or unbiased techniques¹⁶⁸. Fine needle aspirates(FNA) using a 22-gauge needle are far less invasive than biopsies, making repetitive longitudinal monitoring of the response to novel combination therapies much more feasible¹⁶⁸. Although they do not provide tissue for histology and assessment of in-situ topology, they do allow flow cytometric

identification of the full complement of intrahepatic innate and adaptive immune populations, including tissue-resident subsets and HBV-specific T cells (that are proportionally represented compared to paired biopsies)¹⁶⁹. Small numbers of live hepatocytes can also be aspirated. However both biopsy and FNA run the risk of sampling bias and with FNA, variable degrees of blood contamination also need to be adjusted for¹⁶⁹. State-of-the-art techniques such as single-cell RNA-sequencing or Cite-Seq of FNA will allow unbiased comprehensive monitoring of the response of the whole intrahepatic immune landscape to novel therapies. 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 occur¹⁶².

7.4 Studying intrahepatic virology

Access to liver samples in CHB patients allows direct assessment of the cccDNA pool size^{84,170-172}, its transcriptional activity^{84,171,172} and its epigenetic status^{33,84,173} as well as the quantification of HBV integration burden and transcriptionally active HBV integrations^{34,174}.

Quantification of cccDNA and 3.5kb/pgRNA in the liver is performed by PCR-based techniques (qPCR^{171,172} and more recently ddPCR^{84,170,171}). Their ratio is referred to as cccDNA transcriptional activity. Due 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 FNAs by ddPCR has proven as accurate as in core liver biopsy samples.

The characterization of cccDNA epigenetic status by chromatin immunoprecipitation(ChIP)-based assays(cccDNA ChIP^{84,173}, cccDNA ChIP-Seq^{33,175}) is limited to few expert laboratories and remains confined to research purposes. cccDNA-ChIP assays applicability can be hampered by the amount of liver sample available, the need of multiple ChIP reactions using antibodies for different histone PTMs and the difficulty to link transcriptional activity to one single PTM^{33,84}. The advantage of coupling cccDNA-ChIP with high throughput sequencing(cccDNA ChIP-Seq^{33,175}) is counterbalanced by the objective complexity of the experimental protocols.

Overall, cccDNA and 3.5kb/pgRNA quantification in liver samples remain investigational and further standardization is needed for their introduction in clinical practice. Conversely, their inclusion will be key to monitor new agents in early phase studies. FNAs, less invasive than core liver biopsies, allow the simultaneous investigation of the immune microenvironment and intrahepatic viral parameters and are increasingly incorporated in clinical studies.

8. The path forward for combination therapy

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. There are several insights we can glean 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 little impact on HBsAg, and stopping therapy leads universally to viral rebound⁵². It is not necessary to have antigen reducing agents to achieve HBsAg loss, but immune modulators will be needed. For immune modulators, both those affecting adaptive

and innate immunity have evidence of efficacy, informing that targeting either pathway seems to be effective in early stage studies, however the quantum of qHBsAg reduction seems less than 1 log, and efficacy seems better in those with lower qHBsAg level, 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 levels¹⁷⁶ may also predispose to cccDNA silencing¹⁷⁷, and can have sustained efficacy off therapy, but not all cases of qHBsAg<LLOQ are able to sustain HBsAg loss. The combination of NA/NAP/peginterferon was able to achieve functional cure in 35% of patients in a small phase 2 study, hence the strategic approach of inhibiting viral replication, reducing antigen burden and immune modulation warrants further confirmation in large trials. However, the efficacy of different agents within these categories may perform differently and the optimal agents and their combinations need to be explored. When assessing new combination therapies, safety will be a major endpoint since NA have an excellent safety record². In particular, ALT flares have been seen in the setting of qHBsAg reduction, but not all cases could lead to functional cure. Current studies are of limited duration, ranging from 4 weeks in phase 1 studies to 12-48 weeks in phase 2 studies. Most studies have a 24 week off-therapy followup. Lack of qHBsAg reduction in such early phase studies indicate that such combinations are 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 suggest that longer duration of therapy may not be successful. Monitoring progress of novel agents and combinations using biomarkers are better established with viral biomarkers such as qHBsAg (ideal for functional cure), pgRNA

and HBcrAg but immunological monitoring is challenging. A recent proposal by the Immune Monitoring Working Group of HBV Forum provides much needed guidance of the use of these assays most of which are still research tools and not suitable for routine clinical trial monitoring¹⁶². As the liver microenvironment differs from that of peripheral blood, mechanistic insights for functional cure can be gained by longitudinal FNA, and it is established that this technology is generally representative compared to liver biopsies yet is more acceptable and safer^{168,169}.

Another consideration is patient selection. In the search for the optimal therapeutic approach, the balance between selecting the best responders (virally suppressed, lower levels of qHBsAg) provides proof of principle of efficacy, versus the applicability of the strategy in more heterogeneous groups of patients considered difficult to treat (HBeAg-positive, immune tolerant, high viral and antigen load), not to mention genotype variability, ethnic diversity and more advanced stages of the disease, will be challenging.

Trial design will be 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^{178,179}. Such strategies are already taking place. On the other hand, small focused targeted/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/virological parameters. Investigator initiated trials may study strategies that are not part of the development program of pharma industry. These studies may

generate new insight by including translational studies of novel biomarkers and virologic/immunological responses in the liver compartment that may 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. There is indeed more than one pathway to achieve this goal but finding the most successful strategy remains elusive today. New therapeutics and their clinical outcomes may give us insights into the mechanisms of viral clearance that may not have been known previously.

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References:

- 1 World Health Organisation. *Hepatitis B Fact Sheet*, <<https://www.who.int/news-room/fact-sheets/detail/hepatitis-b>> (2021).
- 2 Cornberg, M., Lok, A. S., Terrault, N. A., Zoulim, F. et al. Guidance for design and endpoints of clinical trials in chronic hepatitis B - Report from the 2019 EASL-AASLD HBV Treatment Endpoints Conference. *Hepatology*, doi:10.1002/hep.31030 (2019).
- 3 Fanning, G. C., Zoulim, F., Hou, J. & Bertolotti, A. Therapeutic strategies for hepatitis B virus infection: towards a cure. *Nat Rev Drug Discov* **18**, 827-844, doi:10.1038/s41573-019-0037-0 (2019).
- 4 Anderson, R. T., Choi, H. S. J., Lenz, O. et al. Association Between Seroclearance of Hepatitis B Surface Antigen and Long-term Clinical Outcomes of Patients With Chronic Hepatitis B Virus Infection: Systematic Review and Meta-analysis. *Clin Gastroenterol Hepatol* **19**, 463-472, doi:10.1016/j.cgh.2020.05.041 (2021).
- 5 Lok, A. S., McMahon, B. J., Brown, R. S., Jr. et al. Antiviral therapy for chronic hepatitis B viral infection in adults: A systematic review and meta-analysis. *Hepatology* **63**, 284-306, doi:10.1002/hep.28280 (2016).
- 6 Pollicino, T. & Caminiti, G. HBV-Integration Studies in the Clinic: Role in the Natural History of Infection. *Viruses* **13**, doi:10.3390/v13030368 (2021).
- 7 Revill, P. A., Chisari, F. V., Block, J. M. et al. A global scientific strategy to cure hepatitis B. *Lancet Gastroenterol Hepatol* **4**, 545-558, doi:10.1016/S2468-1253(19)30119-0 (2019).
- 8 Testoni, B., Levrero, M. & Zoulim, F. Challenges to a Cure for HBV Infection. *Semin Liver Dis* **37**, 231-242, doi:10.1055/s-0037-1606212 (2017).
- 9 Beck, J. & Nassal, M. Hepatitis B virus replication. *World J Gastroenterol* **13**, 48-64, doi:10.3748/wjg.v13.i1.48 (2007).
- 10 Colpitts, C. C., Verrier, E. R. & Baumert, T. F. Targeting Viral Entry for Treatment of Hepatitis B and C Virus Infections. *ACS Infect Dis* **1**, 420-427, doi:10.1021/acsinfecdis.5b00039 (2015).
- 11 Verrier, E. R., Colpitts, C. C., Sureau, C. & Baumert, T. F. Hepatitis B virus receptors and molecular drug targets. *Hepatol Int* **10**, 567-573, doi:10.1007/s12072-016-9718-5 (2016).
- 12 Verrier, E. R., Colpitts, C. C., Bach, C. et al. A targeted functional RNA interference screen uncovers glypican 5 as an entry factor for hepatitis B and D viruses. *Hepatology* **63**, 35-48, doi:10.1002/hep.28013 (2016).
- 13 Ni, Y., Lempp, F. A., Mehrle, S. et al. Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. *Gastroenterology* **146**, 1070-1083, doi:10.1053/j.gastro.2013.12.024 (2014).
- 14 Yan, H., Zhong, G., Xu, G. et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* **1**, e00049, doi:10.7554/eLife.00049 (2012).
- 15 Kann, M., Schmitz, A. & Rabe, B. Intracellular transport of hepatitis B virus. *World J Gastroenterol* **13**, 39-47, doi:10.3748/wjg.v13.i1.39 (2007).
- 16 Beck, J., Seitz, S., Lauber, C. & Nassal, M. Conservation of the HBV RNA element epsilon in nakednaviruses reveals ancient origin of protein-primed reverse transcription. *Proc Natl Acad Sci U S A* **118**, doi:10.1073/pnas.2022373118 (2021).

- 17 Nassal, M. Hepatitis B viruses: reverse transcription a different way. *Virus Res* **134**, 235-249, doi:10.1016/j.virusres.2007.12.024 (2008).
- 18 Ligat, G., Verrier, E. R., Nassal, M. & Baumert, T. F. Hepatitis B virus-host interactions and novel targets for viral cure. *Curr Opin Virol* **49**, 41-51, doi:10.1016/j.coviro.2021.04.009 (2021).
- 19 Koniger, C., Wingert, I., Marsmann, M. *et al.* Involvement of the host DNA-repair enzyme TDP2 in formation of the covalently closed circular DNA persistence reservoir of hepatitis B viruses. *Proc Natl Acad Sci U S A* **111**, E4244-4253, doi:10.1073/pnas.1409986111 (2014).
- 20 Qi, Y., Gao, Z., Xu, G. *et al.* DNA Polymerase kappa Is a Key Cellular Factor for the Formation of Covalently Closed Circular DNA of Hepatitis B Virus. *PLoS Pathog* **12**, e1005893, doi:10.1371/journal.ppat.1005893 (2016).
- 21 Long, Q., Yan, R., Hu, J. *et al.* The role of host DNA ligases in hepadnavirus covalently closed circular DNA formation. *PLoS Pathog* **13**, e1006784, doi:10.1371/journal.ppat.1006784 (2017).
- 22 Wei, L. & Ploss, A. Core components of DNA lagging strand synthesis machinery are essential for hepatitis B virus cccDNA formation. *Nat Microbiol* **5**, 715-726, doi:10.1038/s41564-020-0678-0 (2020).
- 23 Dandri, M. Epigenetic modulation in chronic hepatitis B virus infection. *Semin Immunopathol* **42**, 173-185, doi:10.1007/s00281-020-00780-6 (2020).
- 24 Ligat, G., Goto, K., Verrier, E. & Baumert, T. F. Targeting Viral cccDNA for Cure of Chronic Hepatitis B. *Curr Hepatology Rep* **19**, 235–244 (2020).
- 25 Zoulim, F. Inhibition of hepatitis B virus gene expression: A step towards functional cure. *J Hepatol* **68**, 386-388, doi:10.1016/j.jhep.2017.11.036 (2018).
- 26 Martinez, M. G., Boyd, A., Combe, E., Testoni, B. & Zoulim, F. Covalently closed circular DNA: The ultimate therapeutic target for curing HBV infections. *J Hepatol* **75**, 706-717, doi:10.1016/j.jhep.2021.05.013 (2021).
- 27 Lucifora, J., Xia, Y., Reisinger, F. *et al.* Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science* **343**, 1221-1228, doi:10.1126/science.1243462 (2014).
- 28 Riedl, T., Faure-Dupuy, S., Rolland, M. *et al.* Hypoxia-Inducible Factor 1 Alpha-Mediated RelB/APOBEC3B Down-regulation Allows Hepatitis B Virus Persistence. *Hepatology* **74**, 1766-1781, doi:10.1002/hep.31902 (2021).
- 29 Kim, S., Wang, H. & Ryu, W. S. Incorporation of eukaryotic translation initiation factor eIF4E into viral nucleocapsids via interaction with hepatitis B virus polymerase. *J Virol* **84**, 52-58, doi:10.1128/JVI.01232-09 (2010).
- 30 Nguyen, D. H. & Hu, J. Reverse transcriptase- and RNA packaging signal-dependent incorporation of APOBEC3G into hepatitis B virus nucleocapsids. *J Virol* **82**, 6852-6861, doi:10.1128/JVI.00465-08 (2008).
- 31 Wang, H., Kim, S. & Ryu, W. S. DDX3 DEAD-Box RNA helicase inhibits hepatitis B virus reverse transcription by incorporation into nucleocapsids. *J Virol* **83**, 5815-5824, doi:10.1128/JVI.00011-09 (2009).
- 32 Tu, T., Zhang, H. & Urban, S. Hepatitis B Virus DNA Integration: In Vitro Models for Investigating Viral Pathogenesis and Persistence. *Viruses* **13**, doi:10.3390/v13020180 (2021).
- 33 Flecken, T., Meier, M. A., Skewes-Cox, P. *et al.* Mapping the Heterogeneity of Histone Modifications on Hepatitis B Virus DNA Using Liver Needle Biopsies

- Obtained from Chronically Infected Patients. *J Virol* **93**, doi:10.1128/JVI.02036-18 (2019).
- 34 Hsu, Y. C., Suri, V., Nguyen, M. H. *et al.* Inhibition of Viral Replication Reduces Transcriptionally Active Distinct Hepatitis B Virus Integrations with Implications on Host Gene Dysregulation. *Gastroenterology*, doi:10.1053/j.gastro.2021.12.286 (2022).
- 35 Bock, C. T., Schwinn, S., Locarnini, S. *et al.* Structural organization of the hepatitis B virus minichromosome. *J Mol Biol* **307**, 183-196, doi:10.1006/jmbi.2000.4481 (2001).
- 36 Oropeza, C. E., Tarnow, G., Sridhar, A. *et al.* The Regulation of HBV Transcription and Replication. *Adv Exp Med Biol* **1179**, 39-69, doi:10.1007/978-981-13-9151-4_3 (2020).
- 37 Quasdorff, M. & Protzer, U. Control of hepatitis B virus at the level of transcription. *J Viral Hepat* **17**, 527-536, doi:10.1111/j.1365-2893.2010.01315.x (2010).
- 38 Riviere, L., Gerossier, L., Ducroux, A. *et al.* HBx relieves chromatin-mediated transcriptional repression of hepatitis B viral cccDNA involving SETDB1 histone methyltransferase. *J Hepatol* **63**, 1093-1102, doi:10.1016/j.jhep.2015.06.023 (2015).
- 39 Wang, Y., Li, Y., Zai, W. *et al.* HBV covalently closed circular DNA minichromosomes in distinct epigenetic transcriptional states differ in their vulnerability to damage. *Hepatology*, doi:10.1002/hep.32245 (2021).
- 40 Floriot, O., Pascucci, G. R., Pallocca, M. *et al.* Identification of chromatin-accessible domains on the host genome and hepatitis B virus mini-chromosome in infected primary human hepatocytes. in European Association for Study of Liver. 83 (2019).
- 41 Testoni, B., Scholtes, C., M.L., P. *et al.* Circulating HBV RNA correlates with intrahepatic covalently closed circular DNA (cccDNA) transcriptional activity in untreated and NUC-treated chronic hepatitis B (CHB) patients. 358 (2021).
- 42 Decorsiere, A., Mueller, H., van Breugel, P. C. *et al.* Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor. *Nature* **531**, 386-389, doi:10.1038/nature17170 (2016).
- 43 Murphy, C. M., Xu, Y., Li, F. *et al.* Hepatitis B Virus X Protein Promotes Degradation of SMC5/6 to Enhance HBV Replication. *Cell Rep* **16**, 2846-2854, doi:10.1016/j.celrep.2016.08.026 (2016).
- 44 Erken, R., Andre, P., Roy, E. *et al.* Farnesoid X receptor agonist for the treatment of chronic hepatitis B: A safety study. *J Viral Hepat* **28**, 1690-1698, doi:10.1111/jvh.13608 (2021).
- 45 Heger-Stevic, J., Zimmermann, P., Lecoq, L., Bottcher, B. & Nassal, M. Hepatitis B virus core protein phosphorylation: Identification of the SRPK1 target sites and impact of their occupancy on RNA binding and capsid structure. *PLoS Pathog* **14**, e1007488, doi:10.1371/journal.ppat.1007488 (2018).
- 46 Tu, T., Zehnder, B., Qu, B. & Urban, S. D e novo synthesis of hepatitis B virus nucleocapsids is dispensable for the maintenance and transcriptional regulation of cccDNA. *JHEP Rep* **3**, 100195, doi:10.1016/j.jhepr.2020.100195 (2021).
- 47 Schittl, B. & Bruss, V. Mutational profiling of the variability of individual amino acid positions in the hepatitis B virus matrix domain. *Virology* **458-459**, 183-189, doi:10.1016/j.virol.2014.04.030 (2014).

- 48 Zeyen, L. & Prange, R. Host Cell Rab GTPases in Hepatitis B Virus Infection. *Front Cell Dev Biol* **6**, 154, doi:10.3389/fcell.2018.00154 (2018).
- 49 Hu, J. & Liu, K. Complete and Incomplete Hepatitis B Virus Particles: Formation, Function, and Application. *Viruses* **9**, doi:10.3390/v9030056 (2017).
- 50 Vaillant, A. Nucleic acid polymers: Broad spectrum antiviral activity, antiviral mechanisms and optimization for the treatment of hepatitis B and hepatitis D infection. *Antiviral Res* **133**, 32-40, doi:10.1016/j.antiviral.2016.07.004 (2016).
- 51 Kao, C. C., Nie, Y., Ren, S. *et al.* Mechanism of action of hepatitis B virus S antigen transport-inhibiting oligonucleotide polymer, STOPS, molecules. *Mol Ther Nucleic Acids* **27**, 335-348, doi:10.1016/j.omtn.2021.12.013 (2022).
- 52 Maini, M. K. & Burton, A. R. Restoring, releasing or replacing adaptive immunity in chronic hepatitis B. *Nat Rev Gastroenterol Hepatol* **16**, 662-675, doi:10.1038/s41575-019-0196-9 (2019).
- 53 Fiscaro, P., Barili, V., Rossi, M. *et al.* Pathogenetic Mechanisms of T Cell Dysfunction in Chronic HBV Infection and Related Therapeutic Approaches. *Front Immunol* **11**, 849, doi:10.3389/fimmu.2020.00849 (2020).
- 54 Meng, Z., Chen, Y. & Lu, M. Advances in Targeting the Innate and Adaptive Immune Systems to Cure Chronic Hepatitis B Virus Infection. *Front Immunol* **10**, 3127, doi:10.3389/fimmu.2019.03127 (2019).
- 55 Lopes, A. R., Kellam, P., Das, A. *et al.* Bim-mediated deletion of antigen-specific CD8 T cells in patients unable to control HBV infection. *J Clin Invest* **118**, 1835-1845, doi:10.1172/JCI33402 (2008).
- 56 Wieland, S., Thimme, R., Purcell, R. H. & Chisari, F. V. Genomic analysis of the host response to hepatitis B virus infection. *Proc Natl Acad Sci U S A* **101**, 6669-6674, doi:10.1073/pnas.0401771101 (2004).
- 57 Maini, M. K. & Gehring, A. J. The role of innate immunity in the immunopathology and treatment of HBV infection. *J Hepatol* **64**, S60-S70, doi:10.1016/j.jhep.2016.01.028 (2016).
- 58 Dandri, M., Bertoletti, A. & Lutgehetmann, M. Innate immunity in hepatitis B and D virus infection: consequences for viral persistence, inflammation, and T cell recognition. *Semin Immunopathol* **43**, 535-548, doi:10.1007/s00281-021-00864-x (2021).
- 59 Schmidt, N. M., Wing, P. A. C., Diniz, M. O. *et al.* Targeting human Acyl-CoA:cholesterol acyltransferase as a dual viral and T cell metabolic checkpoint. *Nat Commun* **12**, 2814, doi:10.1038/s41467-021-22967-7 (2021).
- 60 Lau, G. K., Lok, A. S., Liang, R. H. *et al.* Clearance of hepatitis B surface antigen after bone marrow transplantation: role of adoptive immunity transfer. *Hepatology* **25**, 1497-1501 (1997).
- 61 Gill, U. S., Peppas, D., Micco, L. *et al.* Interferon Alpha Induces Sustained Changes in NK Cell Responsiveness to Hepatitis B Viral Load Suppression In Vivo. *PLoS Pathog* **12**, e1005788, doi:10.1371/journal.ppat.1005788 (2016).
- 62 Micco, L., Peppas, D., Loggi, E. *et al.* Differential boosting of innate and adaptive antiviral responses during pegylated-interferon-alpha therapy of chronic hepatitis B. *J Hepatol* **58**, 225-233, doi:10.1016/j.jhep.2012.09.029 (2013).
- 63 Penna, A., Laccabue, D., Libri, I. *et al.* Peginterferon-alpha does not improve early peripheral blood HBV-specific T-cell responses in HBeAg-negative chronic hepatitis. *J Hepatol* **56**, 1239-1246, doi:10.1016/j.jhep.2011.12.032 (2012).

- 64 Schurich, A., Pallett, L. J., Jajbhay, D. *et al.* Distinct Metabolic Requirements of Exhausted and Functional Virus-Specific CD8 T Cells in the Same Host. *Cell Rep* **16**, 1243-1252, doi:10.1016/j.celrep.2016.06.078 (2016).
- 65 Amin, O. E., Colbeck, E. J., Daffis, S. *et al.* Therapeutic Potential of TLR8 Agonist GS-9688 (Selgantolimod) in Chronic Hepatitis B: Remodeling of Antiviral and Regulatory Mediators. *Hepatology* **74**, 55-71, doi:10.1002/hep.31695 (2021).
- 66 Huang, W. C., Easom, N. J., Tang, X. Z. *et al.* T Cells Infiltrating Diseased Liver Express Ligands for the NKG2D Stress Surveillance System. *J Immunol* **198**, 1172-1182, doi:10.4049/jimmunol.1601313 (2017).
- 67 Peppas, D., Gill, U. S., Reynolds, G. *et al.* Up-regulation of a death receptor renders antiviral T cells susceptible to NK cell-mediated deletion. *J Exp Med* **210**, 99-114, doi:10.1084/jem.20121172 (2013).
- 68 Provine, N. M., Amini, A., Garner, L. C. *et al.* MAIT cell activation augments adenovirus vector vaccine immunogenicity. *Science* **371**, 521-526, doi:10.1126/science.aax8819 (2021).
- 69 Chinnakannan, S. K., Cargill, T. N., Donnison, T. A. *et al.* The Design and Development of a Multi-HBV Antigen Encoded in Chimpanzee Adenoviral and Modified Vaccinia Ankara Viral Vectors; A Novel Therapeutic Vaccine Strategy against HBV. *Vaccines (Basel)* **8**, doi:10.3390/vaccines8020184 (2020).
- 70 Maini, M. K. & Pallett, L. J. Defective T-cell immunity in hepatitis B virus infection: why therapeutic vaccination needs a helping hand. *Lancet Gastroenterol Hepatol* **3**, 192-202, doi:10.1016/S2468-1253(18)30007-4 (2018).
- 71 Benechet, A. P., De Simone, G., Di Lucia, P. *et al.* Dynamics and genomic landscape of CD8(+) T cells undergoing hepatic priming. *Nature* **574**, 200-205, doi:10.1038/s41586-019-1620-6 (2019).
- 72 Fergusson, J. R., Wallace, Z., Connolly, M. M. *et al.* Immune-Mobilizing Monoclonal T Cell Receptors Mediate Specific and Rapid Elimination of Hepatitis B-Infected Cells. *Hepatology* **72**, 1528-1540, doi:10.1002/hep.31503 (2020).
- 73 Bertoletti, A. & Tan, A. T. HBV as a target for CAR or TCR-T cell therapy. *Curr Opin Immunol* **66**, 35-41, doi:10.1016/j.coi.2020.04.003 (2020).
- 74 Loomba, R. & Liang, T. J. Hepatitis B Reactivation Associated With Immune Suppressive and Biological Modifier Therapies: Current Concepts, Management Strategies, and Future Directions. *Gastroenterology* **152**, 1297-1309, doi:10.1053/j.gastro.2017.02.009 (2017).
- 75 Burton, A. R., Pallett, L. J., McCoy, L. E. *et al.* Circulating and intrahepatic antiviral B cells are defective in hepatitis B. *J Clin Invest* **128**, 4588-4603, doi:10.1172/JCI121960 (2018).
- 76 Quitt, O., Luo, S., Meyer, M. *et al.* T cell engager antibodies enable T cells to control HBV infection and to target HBsAg-positive hepatoma in mice. *J Hepatol*, doi:10.1016/j.jhep.2021.06.022 (2021).
- 77 Bournazos, S. & Ravetch, J. V. Fcγ Receptor Function and the Design of Vaccination Strategies. *Immunity* **47**, 224-233, doi:10.1016/j.immuni.2017.07.009 (2017).
- 78 Sun, Y., Wu, X., Zhou, J. *et al.* Persistent Low Level of Hepatitis B Virus Promotes Fibrosis Progression During Therapy. *Clin Gastroenterol Hepatol* **18**, 2582-2591 e2586, doi:10.1016/j.cgh.2020.03.001 (2020).

- 79 Jang, J. W., Choi, J. Y., Kim, Y. S. *et al.* Effects of Virologic Response to Treatment on Short- and Long-term Outcomes of Patients With Chronic Hepatitis B Virus Infection and Decompensated Cirrhosis. *Clin Gastroenterol Hepatol* **16**, 1954-1963 e1953, doi:10.1016/j.cgh.2018.04.063 (2018).
- 80 Liu, Y. P. & Yao, C. Y. Rapid and quantitative detection of hepatitis B virus. *World J Gastroenterol* **21**, 11954-11963, doi:10.3748/wjg.v21.i42.11954 (2015).
- 81 Chen, J., Zhao, S. S., Liu, X. X., Huang, Z. B. & Huang, Y. Comparison of the Efficacy of Tenofovir Versus Tenofovir plus Entecavir in the Treatment of Chronic Hepatitis B in Patients With Poor Efficacy of Entecavir: A Systematic Review and Meta-analysis. *Clin Ther* **39**, 1870-1880, doi:10.1016/j.clinthera.2017.07.015 (2017).
- 82 Janssen, H. L. A., Hou, J., Asselah, T. *et al.* Efficacy and Safety Results of the Phase 2 JNJ-56136379 JADE Study in Patients with Chronic Hepatitis B: Interim Week 24 Data. in European Association for Study of Liver. 361 (2020).
- 83 Yuen, M. F., Ma, X., Hassanein, T. I. *et al.* HBV pgRNA and DNA both rebound immediately following discontinuation of the core inhibitor vebicorvir despite continued NrtI treatment in patients with HBeAg positive chronic hepatitis B virus infection: findings from a phase 2 open-label study. in American Association for Study of Liver Disease. 359 (2021).
- 84 Lebosse, F., Inchauspe, A., Locatelli, M. *et al.* Quantification and epigenetic evaluation of the residual pool of hepatitis B covalently closed circular DNA in long-term nucleoside analogue-treated patients. *Sci Rep* **10**, 21097, doi:10.1038/s41598-020-78001-1 (2020).
- 85 Gane, E. J., Locarnini, S., Lim, T. H. *et al.* Dose response with the RNA interference (RNAi) therapy JNJ-3989 combined with nucleos(t)ide analogue (NA) treatment in expanded cohorts of patients (pts) with chronic hepatitis B (CHB). in AASLD. 300 (2019).
- 86 Gane, E., Lim, Y. S., Tangkijvanich, P. *et al.* Preliminary safety and antiviral activity of VIR-2218, an X-targeting RNAi therapeutic, in chronic hepatitis B patients. in Digital International Liver Congress (EASL - European Association for Study of Liver). 298 (2020).
- 87 Yuen, M. F., Lim, T. H., Kim, W. *et al.* HBV RNAi inhibitor RG6346 in Phase 1b-2a trial was safe, well-tolerated, and resulted in substantial and durable reductions in serum HBsAg levels in AASLD Digital 351 (2020).
- 88 Hui, R. W., Mak, L. Y., Seto, W. K. & Yuen, M. F. RNA interference as a novel treatment strategy for chronic hepatitis B infection. *Clin Mol Hepatol*, doi:10.3350/cmh.2022.0012 (2022).
- 89 Yuen, M. F., Berliba, E., Sukeepaisarnjaroen, P. *et al.* Low HBsAg Levels Maintained Following Cessation Of The GALNAC-siRNA, Ab-729, In Chronic Hepatitis B Subjects On Nucleos(T)ide Analogue Therapy. in AASLD. 396 (2021).
- 90 Gane, E., Locarnini, S., Lim, T. H. *et al.* Short-term treatment with RNA interference therapy, JNJ-3989, results in sustained hepatitisB surface antigen suppression in patients with chronic hepatitis B receiving nucleos(t)ideanalogue treatment. in EASL 430 (2020).
- 91 Yuen, M. F., Heo, J., Jang, J. W. *et al.* Safety, tolerability and antiviral activity of the antisense oligonucleotide bepirovirsen in patients with chronic hepatitis B: a phase 2 randomized controlled trial. *Nat Med* **27**, 1725-1734, doi:10.1038/s41591-021-01513-4 (2021).

- 92 Yuen, M. F., Andreone, P., Arbune, M. *et al.* Efficacy and safety of bepirovirsen in patients with chronic hepatitis B virus infection: interim results from the randomised Phase 2b B-Clear Study. in European Association for Study of Liver 433 (2022).
- 93 Fonseca, M. A., Ling, J. Z. J., Al-Siyabi, O. *et al.* The efficacy of hepatitis B treatments in achieving HBsAg seroclearance: A systematic review and meta-analysis. *J Viral Hepat* **27**, 650-662, doi:10.1111/jvh.13283 (2020).
- 94 Lim, S. G., Yang, W. L., Ngu, J. H. *et al.* Switching to or Add-on Peginterferon in Patients on Nucleos(t)ide Analogues for Chronic Hepatitis B: The SWAP RCT. *Clin Gastroenterol Hepatol* **20**, e228-e250, doi:10.1016/j.cgh.2021.04.031 (2022).
- 95 Wedemeyer, H., Schöneweis, K., Bogomolov, P. *et al.* Final results of a multicenter, open-label phase 2 clinical trial (MYR203) to assess safety and efficacy of myrcludex B in cwith PEG-interferon Alpha 2a in patients with chronic HBV/HDV co-infection. in EASL 426 (2019).
- 96 Suslov, A., Wieland, S. & Menne, S. Modulators of innate immunity as novel therapeutics for treatment of chronic hepatitis B. *Curr Opin Virol* **30**, 9-17, doi:10.1016/j.coviro.2018.01.008 (2018).
- 97 Boni, C., Vecchi, A., Rossi, M. *et al.* TLR7 Agonist Increases Responses of Hepatitis B Virus-Specific T Cells and Natural Killer Cells in Patients With Chronic Hepatitis B Treated With Nucleos(T)ide Analogues. *Gastroenterology* **154**, 1764-1777.e1767, doi:10.1053/j.gastro.2018.01.030 (2018).
- 98 Balabanska, R., Yuen, M. F., Cottreel, E. *et al.* Safety, PK, PD and antiviral activity of TLR7 agonist RO7020531 in patients with chronic hepatitis B not receiving antiviral treatment: a phase 1 study. in AASLD. 387 (2021).
- 99 Gane, E. J., Lim, Y. S., Gordon, S. C. *et al.* The oral toll-like receptor-7 agonist GS-9620 in patients with chronic hepatitis B virus infection. *J Hepatol* **63**, 320-328, doi:10.1016/j.jhep.2015.02.037 (2015).
- 100 Gane, E., Dubar, R. D., Brooks, A. E. *et al.* Efficacy and Safety of 24 Weeks Treatment with Oral TLR8 Agonist Selgantolimod (GS-9688, SLGN) in Virologically Suppressed Adult Patients with Chronic Hepatitis B: A Phase 2 Study. in EASL 415 (2020).
- 101 Gane, E. J., Kim, H. J., Visvanathan, K. *et al.* Safety, pharmacokinetics, and pharmacodynamics of the oral TLR8 agonist selgantolimod in chronic hepatitis B. *Hepatology*, doi:10.1002/hep.31795 (2021).
- 102 Bensch, B., Martin, B. & Thimme, R. Restoration of HBV-specific CD8+ T cell function by PD-1 blockade in inactive carrier patients is linked to T cell differentiation. *J Hepatol* **61**, 1212-1219, doi:10.1016/j.jhep.2014.07.005 (2014).
- 103 Boni, C., Fficaro, P., Valdatta, C. *et al.* Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* **81**, 4215-4225, doi:10.1128/JVI.02844-06 (2007).
- 104 Fficaro, P., Valdatta, C., Massari, M. *et al.* Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. *Gastroenterology* **138**, 682-693, 693.e681-684, doi:10.1053/j.gastro.2009.09.052 (2010).
- 105 Fficaro, P., Valdatta, C., Massari, M. *et al.* Combined blockade of programmed death-1 and activation of CD137 increase responses of human liver T cells against HBV, but not HCV. *Gastroenterology* **143**, 1576-1585.e1574, doi:10.1053/j.gastro.2012.08.041 (2012).

- 106 Nebbia, G., Peppia, D., Schurich, A. *et al.* Upregulation of the Tim-3/galectin-9 pathway of T cell exhaustion in chronic hepatitis B virus infection. *PLoS One* **7**, e47648, doi:10.1371/journal.pone.0047648 (2012).
- 107 Raziorrouh, B., Schraut, W., Gerlach, T. *et al.* The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function. *Hepatology* **52**, 1934-1947, doi:10.1002/hep.23936 (2010).
- 108 Schurich, A., Khanna, P., Lopes, A. R. *et al.* Role of the coinhibitory receptor cytotoxic T lymphocyte antigen-4 on apoptosis-prone CD8 T cells in persistent hepatitis B virus infection. *Hepatology* **53**, 1494-1503, doi:10.1002/hep.24249 (2011).
- 109 Gane, E., Verdon, D. J., Brooks, A. E. *et al.* Anti-PD-1 blockade with nivolumab with and without therapeutic vaccination for virally suppressed chronic hepatitis B: A pilot study. *J Hepatol* **71**, 900-907, doi:10.1016/j.jhep.2019.06.028 (2019).
- 110 Wang, G., Cui, Y., Xie, Y. *et al.* HBsAg loss in Chronic Hepatitis B patients with Subcutaneous PD-L1 Antibody ASC22 (Envafolelimab) plus Nucleos(t)ide Analogs Treatment: Interim Results from a Phase IIb Clinical Trial. in AASLD. 389 (2021).
- 111 Wang, G., Cui, Y., Xie, Y. *et al.* ALT flares were linked to HBsAg reduction, seroclearance and seroconversion: interim results from a phase IIb study in chronic hepatitis B patients with 24-week treatment of subcutaneous PDL1 Ab ASC22 (Envafolelimab) plus nucleos (t)ide analogs. in European Association for Study of Liver. 432 (2022).
- 112 Boni, C., Janssen, H. L. A., Rossi, M. *et al.* Combined GS-4774 and Tenofovir Therapy Can Improve HBV-Specific T-Cell Responses in Patients With Chronic Hepatitis. *Gastroenterology* **157**, 227-241.e227, doi:10.1053/j.gastro.2019.03.044 (2019).
- 113 Lok, A. S., Pan, C. Q., Han, S. H. *et al.* Randomized phase II study of GS-4774 as a therapeutic vaccine in virally suppressed patients with chronic hepatitis B. *J Hepatol* **65**, 509-516, doi:10.1016/j.jhep.2016.05.016 (2016).
- 114 Zoulim, F., Fournier, C., Habersetzer, F. *et al.* Safety and immunogenicity of the therapeutic vaccine TG1050 in chronic hepatitis B patients: a phase 1b placebo-controlled trial. *Hum Vaccin Immunother* **16**, 388-399, doi:10.1080/21645515.2019.1651141 (2020).
- 115 Yoshida, O., Imai, Y., Shiraishi, K. *et al.* Long Term HBsAg Reduction By A Nasal Administrative Therapeutic Vaccine Containing HBsAg And HBcAg Mixed With Mucoadhesive CVP (CVP-NASVAC) In Patients With Chronic HBV Infection: The Results Of 30 Months Follow Up. in AASLD. 390 (2021).
- 116 Hensel, N., Gu, Z., Sagar *et al.* Memory-like HCV-specific CD8(+) T cells retain a molecular scar after cure of chronic HCV infection. *Nat Immunol* **22**, 229-239, doi:10.1038/s41590-020-00817-w (2021).
- 117 Michler, T., Kosinska, A. D., Festag, J. *et al.* Knockdown of Virus Antigen Expression Increases Therapeutic Vaccine Efficacy in High-Titer Hepatitis B Virus Carrier Mice. *Gastroenterology* **158**, 1762-1775.e1769, doi:10.1053/j.gastro.2020.01.032 (2020).
- 118 Kuiper, A., Sanchez Vasquez, J. D., Mehrotra, A. *et al.* Immunomodulation and RNA interference alter hepatitis B virus-specific CD8 T-cell recognition of infected HepG2-NTCP. *Hepatology*, doi:10.1002/hep.32230 (2021).
- 119 Fumagalli, V., Di Lucia, P., Venzin, V. *et al.* Serum HBsAg clearance has minimal impact on CD8+ T cell responses in mouse models of HBV infection. *J Exp Med* **217**, doi:10.1084/jem.20200298 (2020).

- 120 Salimzadeh, L., Le Bert, N., Dutertre, C. A. *et al.* PD-1 blockade partially recovers dysfunctional virus-specific B cells in chronic hepatitis B infection. *J Clin Invest* **128**, 4573-4587, doi:10.1172/jci121957 (2018).
- 121 Scalfaro, P., Odoul, M., Vonderscher, J. *et al.* Vofanefexor combined with peg-IFN in HBeAg- treatment naive CHB patients. in EASL. 394 (2021).
- 122 Bazinet, M., Pantea, V., Placinta, G. *et al.* Safety and Efficacy of 48 Weeks REP 2139 or REP 2165, Tenofovir Disoproxil, and Pegylated Interferon Alfa-2a in Patients With Chronic HBV Infection Naive to Nucleos(t)ide Therapy. *Gastroenterology* **158**, 2180-2194, doi:10.1053/j.gastro.2020.02.058 (2020).
- 123 Yuen, M. F., Lim, Y.-S., Cloutier, D. *et al.* Preliminary Results From a Phase 2 Study Evaluating VIR-2218 Alone and in Combination With Pegylated Interferon Alfa-2a in Participants With Chronic Hepatitis B Infection. in AASLD. 397 (2020).
- 124 Yuen, M. F., Asselah, T., Jacobson, I. M. *et al.* Efficacy and Safety of the siRNA JNJ-3989 and/or the Capsid Assembly Modulator JNJ-6379 for the Treatment of Chronic Hepatitis B Virus Infection : Results From the Phase 2b REEF-1 Study. in American Association for Study of Liver Disease. 360 (2021).
- 125 Evans, T., Bussey, L., Teo, S. L. *et al.* Phase 1b/2a study of heterologous ChAdOx1-HBV/MVA-HBV therapeutic vaccination (VTP-300) as monotherapy and combined with low-dose nivolumab in virally-suppressed patients with CHB on nucleos(t)ide analogues. in European Association for Study of Liver. 431 (2022).
- 126 McLane, L. M., Abdel-Hakeem, M. S. & Wherry, E. J. CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer. *Annu Rev Immunol* **37**, 457-495, doi:10.1146/annurev-immunol-041015-055318 (2019).
- 127 Cheng, Y., Zhu, Y. O., Becht, E. *et al.* Multifactorial heterogeneity of virus-specific T cells and association with the progression of human chronic hepatitis B infection. *Sci Immunol* **4**, doi:10.1126/sciimmunol.aau6905 (2019).
- 128 Heim, K., Binder, B., Sagar *et al.* TOX defines the degree of CD8+ T cell dysfunction in distinct phases of chronic HBV infection. *Gut*, doi:10.1136/gutjnl-2020-322404 (2020).
- 129 Hoogeveen, R. C., Robidoux, M. P., Schwarz, T. *et al.* Phenotype and function of HBV-specific T cells is determined by the targeted epitope in addition to the stage of infection. *Gut* **68**, 893-904, doi:10.1136/gutjnl-2018-316644 (2019).
- 130 Schuch, A., Salimi Alizej, E., Heim, K. *et al.* Phenotypic and functional differences of HBV core-specific versus HBV polymerase-specific CD8+ T cells in chronically HBV-infected patients with low viral load. *Gut* **68**, 905-915, doi:10.1136/gutjnl-2018-316641 (2019).
- 131 Acerbi, G., Montali, I., Ferrigno, G. D. *et al.* Functional reconstitution of HBV-specific CD8 T cells by in vitro polyphenol treatment in chronic hepatitis B. *J Hepatol* **74**, 783-793, doi:10.1016/j.jhep.2020.10.034 (2021).
- 132 Fisicaro, P., Barili, V., Montanini, B. *et al.* Targeting mitochondrial dysfunction can restore antiviral activity of exhausted HBV-specific CD8 T cells in chronic hepatitis B. *Nat Med* **23**, 327-336, doi:10.1038/nm.4275 (2017).
- 133 Ghoneim, H. E., Fan, Y., Moustaki, A. *et al.* De Novo Epigenetic Programs Inhibit PD-1 Blockade-Mediated T Cell Rejuvenation. *Cell* **170**, 142-157 e119, doi:10.1016/j.cell.2017.06.007 (2017).

- 134 Pauken, K. E., Sammons, M. A., Odorizzi, P. M. *et al.* Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. *Science* **354**, 1160-1165, doi:10.1126/science.aaf2807 (2016).
- 135 Sen, D. R., Kaminski, J., Barnitz, R. A. *et al.* The epigenetic landscape of T cell exhaustion. *Science* **354**, 1165-1169, doi:10.1126/science.aae0491 (2016).
- 136 Kennedy, P. T. F., Sandalova, E., Jo, J. *et al.* Preserved T-cell function in children and young adults with immune-tolerant chronic hepatitis B. *Gastroenterology* **143**, 637-645, doi:10.1053/j.gastro.2012.06.009 (2012).
- 137 Le Bert, N., Gill, U. S., Hong, M. *et al.* Effects of Hepatitis B Surface Antigen on Virus-Specific and Global T Cells in Patients With Chronic Hepatitis B Virus infection. *Gastroenterology* **159**, 652-664, doi:10.1053/j.gastro.2020.04.019 (2020).
- 138 Mieli-Vergani, G., Bansal, S., Daniel, J. F. *et al.* Peginterferon Alfa-2a (40KD) Plus Lamivudine or Entecavir in Children With Immune-Tolerant Chronic Hepatitis B. *J Pediatr Gastroenterol Nutr* **73**, 156-160, doi:10.1097/MPG.0000000000003118 (2021).
- 139 Rosenthal, P., Ling, S. C., Belle, S. H. *et al.* Combination of Entecavir/Peginterferon Alfa-2a in Children With Hepatitis B e Antigen-Positive Immune Tolerant Chronic Hepatitis B Virus Infection. *Hepatology* **69**, 2326-2337, doi:10.1002/hep.30312 (2019).
- 140 Feld, J. J., Terrault, N. A., Lin, H. S. *et al.* Entecavir and Peginterferon Alfa-2a in Adults With Hepatitis B e Antigen-Positive Immune-Tolerant Chronic Hepatitis B Virus Infection. *Hepatology* **69**, 2338-2348, doi:10.1002/hep.30417 (2019).
- 141 Lampertico, P. & Berg, T. Less can be more: A finite treatment approach for HBeAg-negative chronic hepatitis B. *Hepatology* **68**, 397-400, doi:10.1002/hep.29821 (2018).
- 142 Chong, C. H. & Lim, S. G. When can we stop nucleoside analogues in patients with chronic hepatitis B? *Liver Int* **37 Suppl 1**, 52-58, doi:10.1111/liv.13314 (2017).
- 143 Papatheodoridi, M. & Papatheodoridis, G. Can we stop nucleoside analogues before HBsAg loss? *J Viral Hepat* **26**, 936-941, doi:10.1111/jvh.13091 (2019).
- 144 Cornberg, M., Lok, A. S., Terrault, N. A., Zoulim, F. & Faculty, E.-A. H. T. E. C. Guidance for design and endpoints of clinical trials in chronic hepatitis B - Report from the 2019 EASL-AASLD HBV Treatment Endpoints Conference(double dagger). *J Hepatol* **72**, 539-557, doi:10.1016/j.jhep.2019.11.003 (2020).
- 145 Hirode, G., Choi, H. S. J., Chen, C. H. *et al.* Off-Therapy Response After Nucleos(t)ide Analogue Withdrawal in Patients With Chronic Hepatitis B: An International, Multicenter, Multiethnic Cohort (RETRACT-B Study). *Gastroenterology*, doi:10.1053/j.gastro.2021.11.002 (2021).
- 146 Sonneveld, M. J., Chiu, S. M., Park, J. Y. *et al.* Probability of HBsAg loss after nucleo(s)tide analogue withdrawal depends on HBV genotype and viral antigen levels. *J Hepatol*, doi:10.1016/j.jhep.2022.01.007 (2022).
- 147 Zimmer, C. L., Rinker, F., Honer Zu Siederdisen, C. *et al.* Increased NK Cell Function After Cessation of Long-Term Nucleos(t)ide Analogue Treatment in Chronic Hepatitis B Is Associated With Liver Damage and HBsAg Loss. *J Infect Dis* **217**, 1656-1666, doi:10.1093/infdis/jiy097 (2018).
- 148 Rinker, F., Zimmer, C. L., Honer Zu Siederdisen, C. *et al.* Hepatitis B virus-specific T cell responses after stopping nucleos(t)ide analogue therapy in HBeAg-negative chronic hepatitis B. *J Hepatol* **69**, 584-593, doi:10.1016/j.jhep.2018.05.004 (2018).

- 149 Rivino, L., Le Bert, N., Gill, U. S. *et al.* Hepatitis B virus-specific T cells associate with viral control upon nucleos(t)ide-analogue therapy discontinuation. *J Clin Invest* **128**, 668-681, doi:10.1172/JCI92812 (2018).
- 150 Anderson, R. T., Lim, S. G., Mishra, P. *et al.* Challenges, Considerations, and Principles to Guide Trials of Combination Therapies for Chronic Hepatitis B Virus. *Gastroenterology* **156**, 529-533 e524, doi:10.1053/j.gastro.2018.11.062 (2019).
- 151 Liu, J., Wang, T., Zhang, W. *et al.* Effect of combination treatment based on interferon and nucleos(t)ide analogues on functional cure of chronic hepatitis B: a systematic review and meta-analysis. *Hepatol Int* **14**, 958-972, doi:10.1007/s12072-020-10099-x (2020).
- 152 Buyse, M., Sargent, D. J., Grothey, A., Matheson, A. & de Gramont, A. Biomarkers and surrogate end points--the challenge of statistical validation. *Nat Rev Clin Oncol* **7**, 309-317, doi:10.1038/nrclinonc.2010.43 (2010).
- 153 Buti, M., Riveiro-Barciela, M., Rodriguez-Frias, F., Tabernero, D. & Esteban, R. Role of Biomarkers in Guiding Cure of Viral Hepatitis B. *Semin Liver Dis* **40**, 49-60, doi:10.1055/s-0039-3401031 (2020).
- 154 Vachon, A. & Osioy, C. Novel Biomarkers of Hepatitis B Virus and Their Use in Chronic Hepatitis B Patient Management. *Viruses* **13**, doi:10.3390/v13060951 (2021).
- 155 Carey, I., Gersch, J., Wang, B. *et al.* Pregenomic HBV RNA and Hepatitis B Core-Related Antigen Predict Outcomes in Hepatitis B e Antigen-Negative Chronic Hepatitis B Patients Suppressed on Nucleos(T)ide Analogue Therapy. *Hepatology* **72**, 42-57, doi:10.1002/hep.31026 (2020).
- 156 Lindh, M., Rydell, G. E. & Larsson, S. B. Impact of integrated viral DNA on the goal to clear hepatitis B surface antigen with different therapeutic strategies. *Curr Opin Virol* **30**, 24-31, doi:10.1016/j.coviro.2018.01.011 (2018).
- 157 Martinot-Peignoux, M., Lapalus, M., Maylin, S. *et al.* Baseline HBsAg and HBcrAg titres allow peginterferon-based 'precision medicine' in HBeAg-negative chronic hepatitis B patients. *J Viral Hepat* **23**, 905-911, doi:10.1111/jvh.12565 (2016).
- 158 Lim, S. G., Phyo, W. W., Ling, J. Z. J. *et al.* Comparative biomarkers for HBsAg loss with antiviral therapy shows dominant influence of quantitative HBsAg (qHBsAg). *Aliment Pharmacol Ther* **53**, 172-182, doi:10.1111/apt.16149 (2021).
- 159 Zhang, M., Li, G., Shang, J. *et al.* Rapidly decreased HBV RNA predicts responses of pegylated interferons in HBeAg-positive patients: a longitudinal cohort study. *Hepatol Int* **14**, 212-224, doi:10.1007/s12072-020-10015-3 (2020).
- 160 Fan, R., Peng, J., Xie, Q. *et al.* Combining Hepatitis B Virus RNA and Hepatitis B Core-Related Antigen: Guidance for Safely Stopping Nucleos(t)ide Analogues in Hepatitis B e Antigen-Positive Patients With Chronic Hepatitis B. *J Infect Dis* **222**, 611-618, doi:10.1093/infdis/jiaa136 (2020).
- 161 van Halewijn, G. J., Geurtsvankessel, C. H., Klaasse, J. *et al.* Diagnostic and analytical performance of the hepatitis B core related antigen immunoassay in hepatitis B patients. *J Clin Virol* **114**, 1-5, doi:10.1016/j.jcv.2019.03.003 (2019).
- 162 Gehring, A. J., Mendez, P., Richter, K. *et al.* Immunological Biomarker Discovery in Cure Regimens for Chronic Hepatitis B Virus Infection. *J Hepatol*, doi:10.1016/j.jhep.2022.02.020 (2022).
- 163 Yoshikawa, S., Yoshio, S., Yoshida, Y. *et al.* Impact of Immune Reconstitution-Induced Hepatic Flare on Hepatitis B Surface Antigen Loss in Hepatitis B

- Virus/Human Immunodeficiency Virus-1 Coinfected Patients. *J Infect Dis* **223**, 2080-2089, doi:10.1093/infdis/jiaa662 (2021).
- 164 Boni, C., Lampertico, P., Talamona, L. *et al.* Natural killer cell phenotype modulation and natural killer/T-cell interplay in nucleos(t)ide analogue-treated hepatitis e antigen-negative patients with chronic hepatitis B. *Hepatology* **62**, 1697-1709, doi:10.1002/hep.28155 (2015).
- 165 Stegmann, K. A., Robertson, F., Hansi, N. *et al.* CXCR6 marks a novel subset of T-bet(lo)Eomes(hi) natural killer cells residing in human liver. *Sci Rep* **6**, 26157, doi:10.1038/srep26157 (2016).
- 166 Pallett, L. J., Burton, A. R., Amin, O. E. *et al.* Longevity and replenishment of human liver-resident memory T cells and mononuclear phagocytes. *J Exp Med* **217**, doi:10.1084/jem.20200050 (2020).
- 167 Pallett, L. J., Davies, J., Colbeck, E. J. *et al.* IL-2(high) tissue-resident T cells in the human liver: Sentinels for hepatotropic infection. *J Exp Med* **214**, 1567-1580, doi:10.1084/jem.20162115 (2017).
- 168 Gill, U. S., Pallett, L. J., Kennedy, P. T. F. & Maini, M. K. Liver sampling: a vital window into HBV pathogenesis on the path to functional cure. *Gut* **67**, 767-775, doi:10.1136/gutjnl-2017-314873 (2018).
- 169 Gill, U. S., Pallett, L. J., Thomas, N. *et al.* Fine needle aspirates comprehensively sample intrahepatic immunity. *Gut* **68**, 1493-1503, doi:10.1136/gutjnl-2018-317071 (2019).
- 170 Caviglia, G. P., Abate, M. L., Tandoi, F. *et al.* Quantitation of HBV cccDNA in anti-HBc-positive liver donors by droplet digital PCR: A new tool to detect occult infection. *J Hepatol* **69**, 301-307, doi:10.1016/j.jhep.2018.03.021 (2018).
- 171 Charre, C., Levrero, M., Zoulim, F. & Scholtes, C. Non-invasive biomarkers for chronic hepatitis B virus infection management. *Antiviral Res* **169**, 104553, doi:10.1016/j.antiviral.2019.104553 (2019).
- 172 Testoni, B., Lebosse, F., Scholtes, C. *et al.* Serum hepatitis B core-related antigen (HBcrAg) correlates with covalently closed circular DNA transcriptional activity in chronic hepatitis B patients. *J Hepatol* **70**, 615-625, doi:10.1016/j.jhep.2018.11.030 (2019).
- 173 Pollicino, T., Belloni, L., Raffa, G. *et al.* Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. *Gastroenterology* **130**, 823-837, doi:10.1053/j.gastro.2006.01.001 (2006).
- 174 Chow, N., Wong, D. K. H., Mak, L. Y. *et al.* Long term nucleos(t)ide analogue therapy reduced the extent of HBV DNA integration in chronic hepatitis B patients. in American Association for Study of Liver Disease. 77 (2020).
- 175 Tropberger, P., Mercier, A., Robinson, M. *et al.* Mapping of histone modifications in episomal HBV cccDNA uncovers an unusual chromatin organization amenable to epigenetic manipulation. *Proc Natl Acad Sci U S A* **112**, E5715-5724, doi:10.1073/pnas.1518090112 (2015).
- 176 Yuen, M. F., Wong, D. K., Schlupe, T. *et al.* Long-term serological, virological and histological responses to RNA inhibition by ARC-520 in Chinese chronic hepatitis B patients on entecavir treatment. *Gut*, doi:10.1136/gutjnl-2020-323445 (2021).
- 177 Allweiss, L., Giersch, K., Piroso, A. *et al.* Therapeutic shutdown of HBV transcripts promotes reappearance of the SMC5/6 complex and silencing of the viral genome in vivo. *Gut* **71**, 372-381, doi:10.1136/gutjnl-2020-322571 (2022).

- 178 Woodcock, J. & LaVange, L. M. Master Protocols to Study Multiple Therapies, Multiple Diseases, or Both. *N Engl J Med* **377**, 62-70, doi:10.1056/NEJMra1510062 (2017).
- 179 Adaptive Platform Trials, Coalition. Adaptive platform trials: definition, design, conduct and reporting considerations. *Nat Rev Drug Discov* **18**, 797-807, doi:10.1038/s41573-019-0034-3 (2019).

Legends to Tables and Figures

- TABLE 1 Therapeutic agents for CHB being tested in clinical trials. Divided into antiviral agents and immune modulatory agents showing class, mechanism of action, and list of individual agents.
- 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, secretory pathway(blue) and 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.

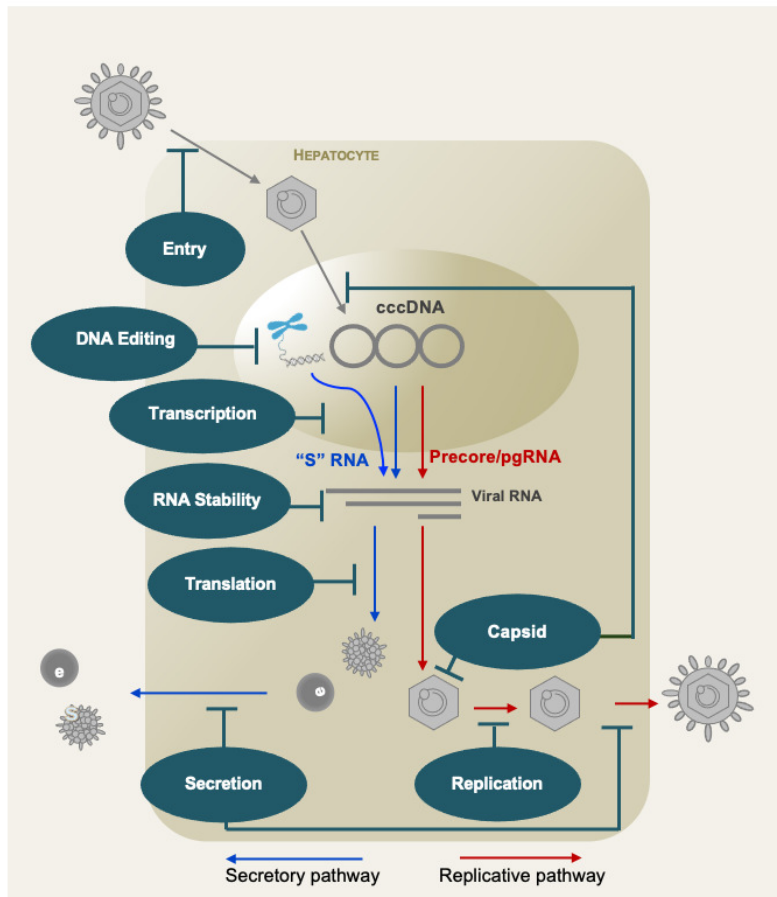
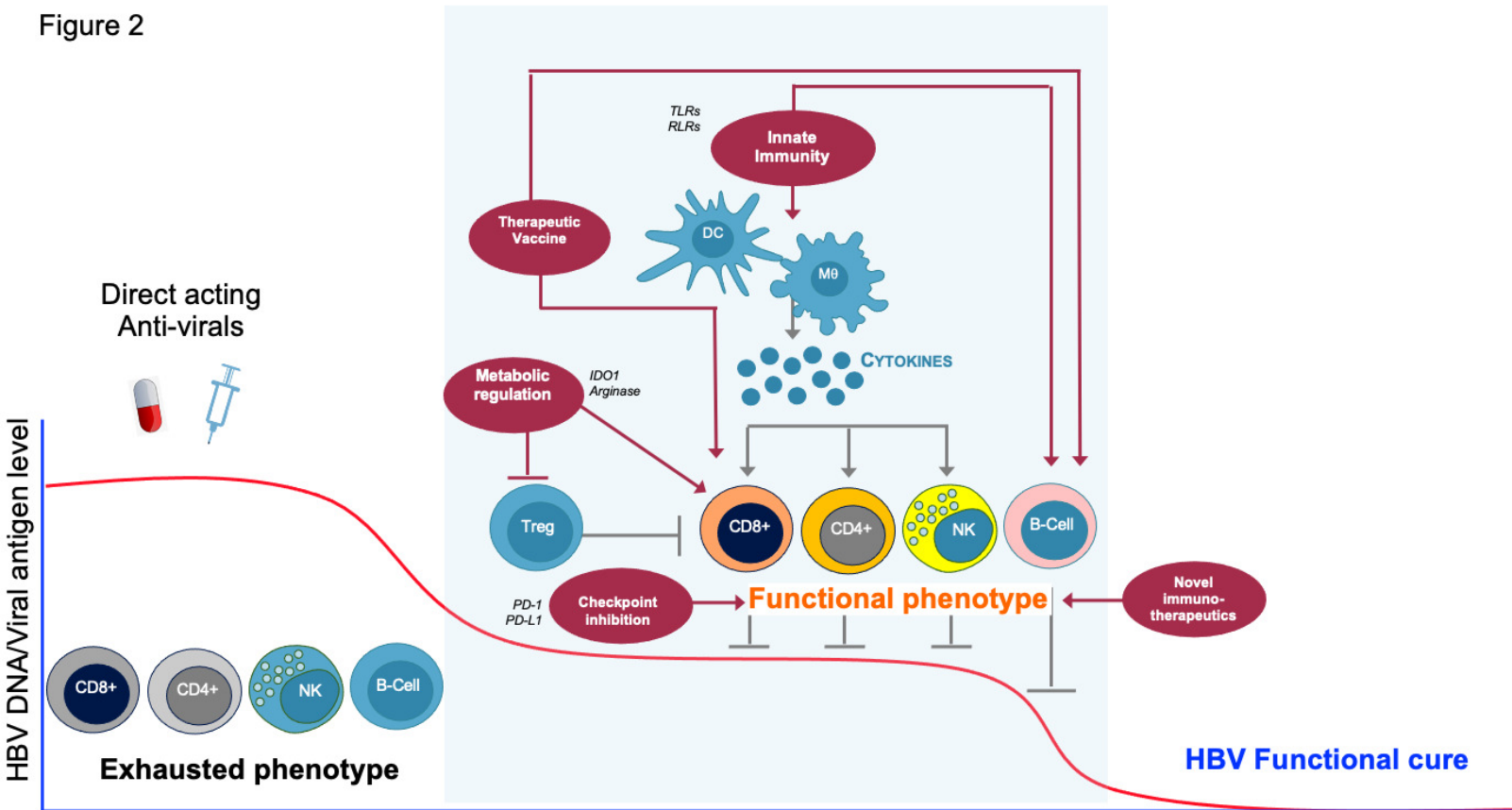


Figure 1

Figure 2



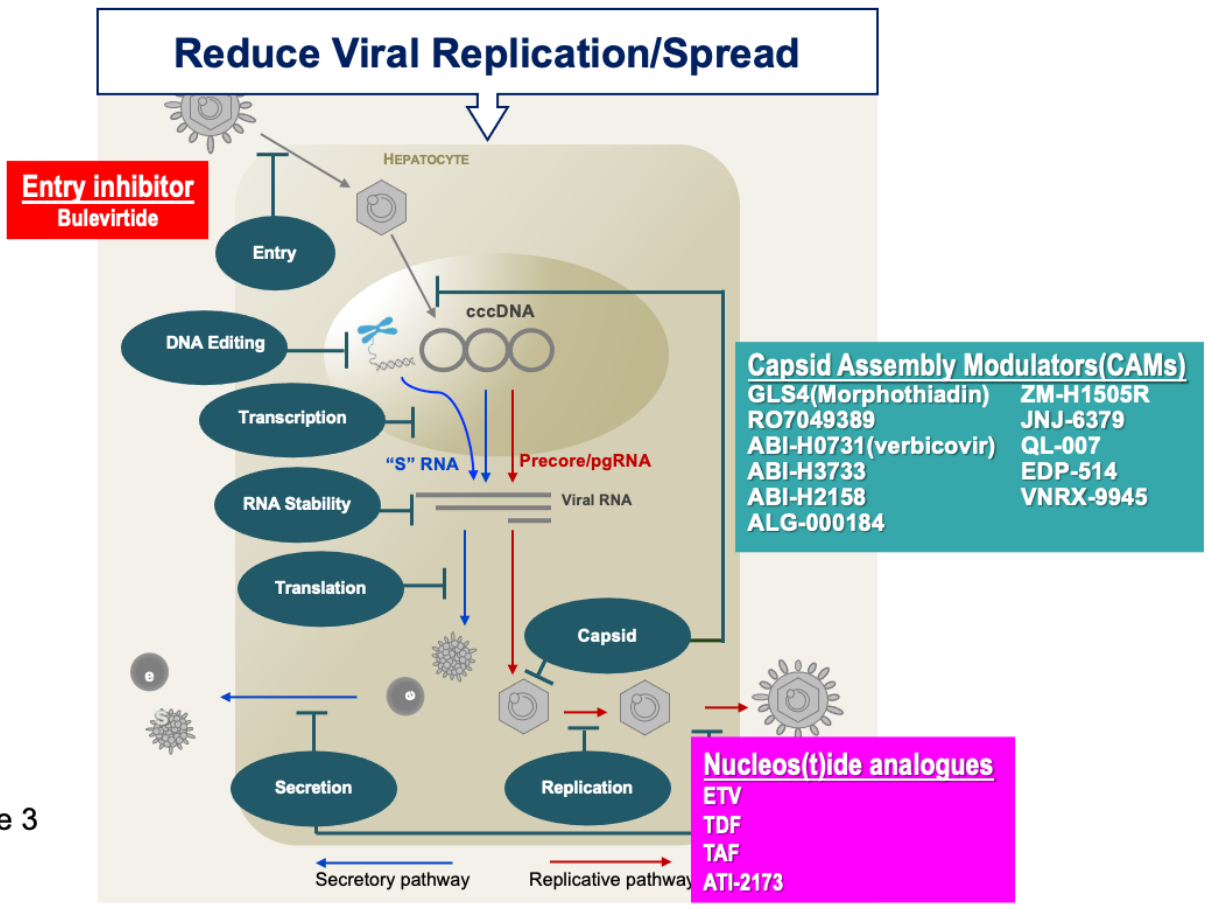


Figure 3

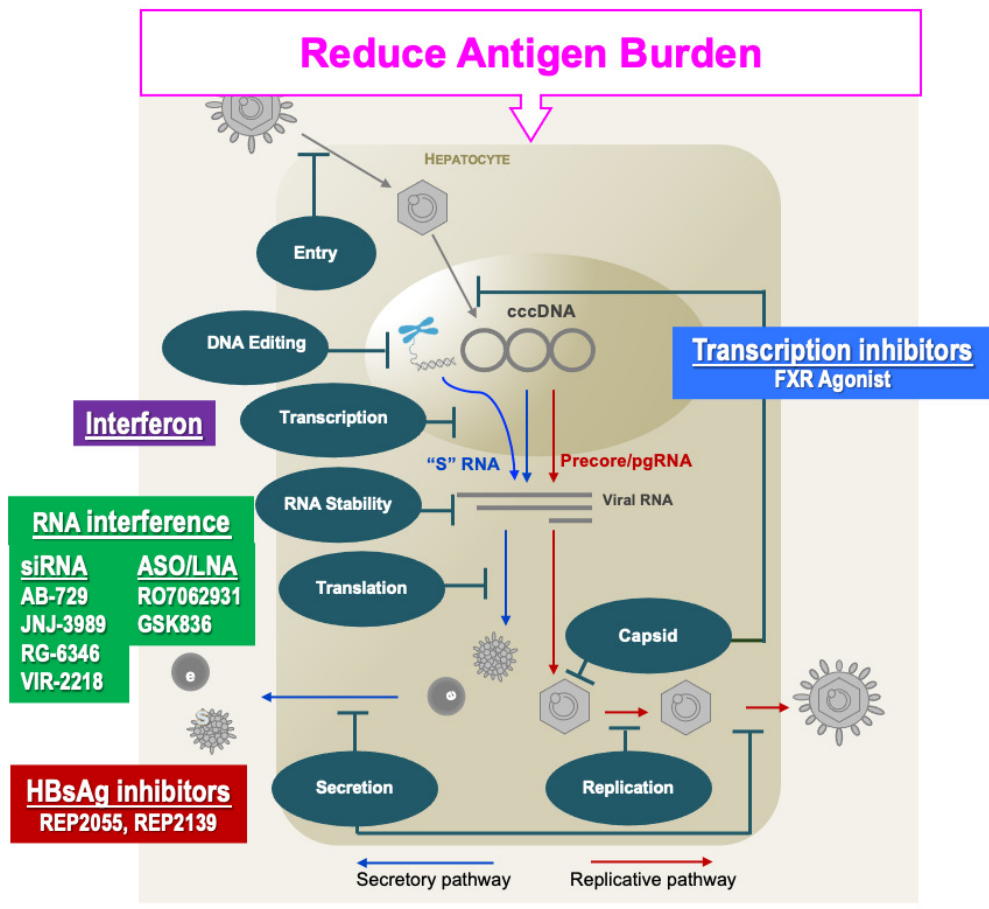


Figure 4

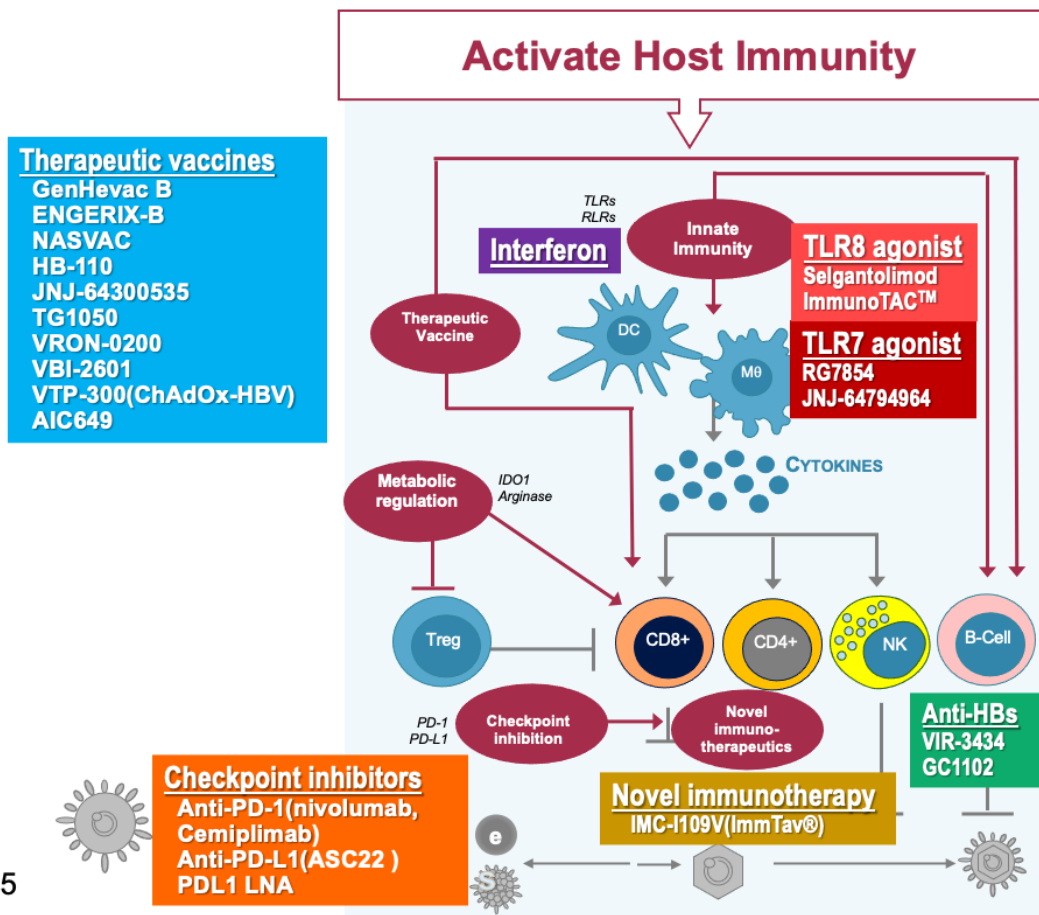


Figure 5