Investigating the role of novel Hepatitis B markers in predicting clinical outcomes in Chronic Hepatitis B patients

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Investigating the role of novel Hepatitis B markers in predicting clinical outcomes in Chronic Hepatitis B patients.

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By

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Declaration

The work herein presented is my own and all experiments, except where acknowledged, were performed by myself.

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Abbreviations

Abbreviations	Meaning
aa	Amino Acid
AASLD	American Association for the study of the Liver
ADV	Adefovir
ALP	Alkaline phosphatase
ALIX	Apoptosis-linked gene 2 (ALG-2)-Interacting protein X
ALT	Alanine Aminotransaminase
APRI	Aspartate Aminotransferase to platelet Ratio Index
APOBEC3	Apolipoprotein B Editing Complex 3
AST	Aspartate Aminotransferase
BCP	Basal Core Promoter
bp	Base pair
cccDNA	Covalently Closed Circular DNA
CHB	Chronic Hepatitis B
CLEIA	Chemiluminescent enzyme immunoassay
CMIA	Chemiluminescent microparticle immunoassay
dAMP	Damage Associated Molecular Patterns
DHEAS	Dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid
DR	Direct Repeat
dsDNA	Double Stranded DNA
EASL	European Association for the study of the Liver
eIF4E	Eukaryotic Translation Initiation Factor 4E
ENH	Hepatitis B e Antigen Negative Hepatitis
ENQ	Hepatitis B e Antigen Negative Quiescent
ESCRT	Endosomal Sorting Complex Needed for Transport
ETV	Entecavir
FBS	Fetal Bovine Serum
FIB4	Fibrosis Score 4

FXR	Farnesoid X Receptor
GGT	Gamma-glutamyl Transferase
HBcAg	Hepatitis B core Antigen
HBcrAg	Hepatitis B core-related Antigen
HBeAg	Hepatitis B envelope Antigen
HBIG	Hepatitis B Immune Globulin
HBsAg	Hepatitis B surface Antigen
HBV	Hepatitis B Virus
HBx	Hepatitis B x protein
HCC	Hepatocellular Carcinoma
HNF	Hepatocyte Nuclear Factor
Hsc	Heat Stress Cognate
Hsp	Heat Shock Protein
HSPG	Heparan Sulphate Proteoglycan
IC	Immune clearance
IFN	Interferon
IFNα	Interferon Alpha
IFNγ	Interferon Gamma
INR	International Normalised Ration
IT	Immune Tolerant
LAM	Lamivudine
LHBs	Large Hepatitis B surface protein
LTβ	Lymphotoxin Beta
MAB	Monoclonal antibody
MHBs	Medium Hepatitis B surface protein
mRNA	Messenger Ribonucleic acid
MVB	Multivesicular Body
NA	Nucleos(t)ide Analogue
NAT	Nucleic acid testing
NT	Nucleotide
NTCP	Sodium taurocholate co-transporting polypeptide
ORF	Overlapping reading frame

PCR	Polymerase Chain Reaction
pgRNA	Pregenomic Ribonucleic acid
PLT	Platelet
Pol	Polymerase
PPARα	Peroxisome Proliferator-activated Receptor Alpha
rcDNA	Relaxed Circular DNA
RNA	Ribonucleic acid
RNaseH	Ribonuclease H
rpm	Revolutions per minute
RXRα	Retinoid X Receptor Alpha
SHBs	Small Hepatitis B surface protein
Smc	Structural Maintenance of Chromosomes
SRD5A2	steroid 5-alpha reductase type II
SVP	Subviral Particles
TBV	Telbivudine
TDF	Tenofovir
Th	Helper T cells
TNAI	Total Nucleic Acid Isolation
TNF	Tumour Necrosis Factor
TNFα	Tumour Necrosis Factor Alpha
TP	Terminal Protein
WHO	World Health Organization
xg	Gravitational acceleration
ZAP	Zinc Finger Antiviral Protein

Abstract

Chronic hepatitis B (CHB) infection is a disease affecting approximately 300 million people worldwide. Chronic hepatitis B is caused by infection of liver cells with hepatitis B virus (HBV) and kills more people than HIV, malaria and tuberculosis put together. HBV infection is predominantly contracted at birth from infected mothers. Although HBV infection is silent in childhood and causes no or minimal liver damage, it is a persistent viral infection, which leads to progressive liver disease in 40% of patients and accounts for 1 million deaths every year. Screening for HBV during pregnancy is widely available to reduce the risk of perinatal transmission, along with other precautions such as antiviral therapy during pregnancy, birth dose vaccination and application of hepatitis B immunoglobulin are also available. However, even with these preventative methods in place in most communities, still 10% of mothers with highly active virus levels pass the infection to their infants.

This thesis investigates the role of serological and virological markers in predicting long term clinical outcomes in patients suffering from chronic hepatitis B virus infection who acquired it perinatally. I will particularly focus on novel markers: Hepatitis B core-related antigen (HBcrAg) and pregenomic Ribonucleic acid (pgRNA) which may be beneficial in HBV management and grant us a step towards curing HBV. Overall, I aim to evaluate the predictive nature of these markers in relation to liver damage progression and to identify their place in chronic HBV management and treatment.

In my first study I investigated whether pgRNA and HBcrAg could predict clinical outcomes in HBeAg negative chronic hepatitis B patients suppressed on nucleos(t)ide analogue therapy. Serum samples from patients who were diagnosed in childhood and followed up through adulthood were collected. Serological markers HBeAg, HBsAg, and HBcrAg and virological markers HBV DNA, and pgRNA were determined, along with liver function tests including ALT, APRI and FIB4 scores. Patients were divided into three cohorts based on their treatment status. The first cohort consisted of patients on maintenance suppressive therapy with tenofovir (TDF); the second cohort consisted of patients on long-term Nucleos(t)ide analogue (NA)

suppressive therapy for at least three years, and in whom NA treatment was withdrawn before HBsAg loss. The third cohort consisted of patients with HBsAg loss on long-term suppressive NA therapy, in whom therapy was withdrawn after HBsAg loss. Results from the first cohort demonstrated that out of 66 patients treatment baseline HBcrAg could be detected in 71% (n=47) of patients and pgRNA could be detected in 83% (n=55) of patients. After 3 years of antiviral therapy 33% (n=22) of patients still had detectable HBcrAg in their serum and 30% (n=20) still had detectable pgRNA in their serum even after HBV DNA could no longer be detected. After 5 years of antiviral therapy HBcrAg could still be detected in 27% (n=18) of patients and pgRNA could be detected in 14% (n=9) of patients. In the second cohort (n=23), detectable levels of HBcrAg was observed in 17% (n=4) of patient serum and pgRNA was observed in 13% (n=3) of patient serum after nucleos(t)ide (NA) withdrawal. Additionally, pgRNA detection at the time of NA withdrawal correlated with ALT flares after stopping NA therapy. In the third cohort (n=19), at the follow up appointment HBsAg was undetectable in all patients after NA withdrawal. However, 11% (n=2) of patients had relapsed as they had detectable HBV DNA in their serum and had increased ALT activity but not detectable HBsAg levels. This group re-commenced NA therapy.

The aim of my second project was to determine whether HBcrAg and HBV pgRNA could be used to assess virological response and liver disease progression in patients who achieve HBeAg seroconversion versus those who do not while on Nucleos(t)ide analogue (NA) therapy. Other markers associated with CHB – Interferon-γ-inducible protein 10 (IP-10 or CXCL10) and Programmed cell death protein 1 (PD-1), were also determined at baseline, and at follow ups. 58 patient samples were collected at 3 different time points (at therapy baseline, at 5 years and 10 years of antiviral therapy). Results demonstrated that DNA suppression and ALT normalization improved steadily with NA therapy. HBcrAg and HBV pgRNA could still be detected in patients even after HBV DNA suppression and achieving anti-HBe status. This suggested that cccDNA was still active in hepatocytes and liver disease could still occur if antiviral therapy is discontinued. High levels of IP-10 and PD1 were associated with increased levels of liver fibrosis.

In my third study I investigated whether markers of cccDNA transcriptional activity: HBcrAg and pgRNA could help to predict future disease progression (need for therapy) in patients with perinatally acquired Chronic Hepatitis B infection followed from childhood to adulthood. Serological markers including HBeAg, HBsAg and HBcrAg and virological markers HBV

DNA and pgRNA were compared between diagnosis and their last clinical visit (median follow-up duration 6 years, range 3-12 years). Liver function markers including ALT, AST, ALP, GGT, bilirubin and albumin were also compared. This cohort included 29 paediatric patients (range: 2-18 years old). It was found that pgRNA and HBcrAg were present in all patient serum at baseline (before the antiviral therapy initiation) and in particularly high concentrations in the patients that were placed on antiviral therapy. During antiviral therapy HBV DNA reduced significantly and markers of cccDNA transcriptional activity – HBcrAg and pgRNA also decreased substantially. Overall, the novel markers of cccDNA (HBcrAg and pgRNA) could be utilised with existing markers for predictive purposes to determine which patients are more likely to require antiviral therapy.

In my fourth study I investigated the clinical relevance of ultrasensitive HBsAg assay. Serum samples from 203 chronic hepatitis B (CHB) patients, all HBeAg negative were tested on the Abbott Architect and then tested using the CLEIA HBsAg-HQ assay (Fujirebio, Europe, Ghent, Belgium) on the Fujirebio Lumipulse platform. Results showed that the Lumipulse HBsAg-HQ assay could consistently determine HBsAg in all HBV genotypes tested and results were very similar to the Abbott assay (median HBsAg levels by Fujirebio :17.7, range 0.0001-150 IU/ml vs Abbott: 18.6, range 0.1-164 IU/mL). There was also a strong bi-variate correlation observed between the two assays ($r=0.977$, $p<0.001$).

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Chapter 1: Introduction

1.1 Hepatitis B Virus Background

Hepatitis B virus (HBV) belongs to the *Hepadnaviridae* family, and it is the smallest DNA virus that infects human beings. It is also the only hepatotropic virus which exists in DNA form(1,2).

HBV infects human liver cells and exerts necroinflammatory, fibrotic and carcinogenic effects which can be deadly. In the serum of infected HBV patients at least three types of HBV particles can be identified: spherical structures of 42nm and 22nm in diameter and filament structures of carriable length that are 22nm in diameter(2). The 42nm particles are the Dane particles which are infectious virions containing a lipid membrane with three viral surface antigens (HBs): Large (LHBs), middle (MHBs) and small (SHBs) that surround a nucleocapsid composed of hepatitis B core protein (HBc), viral polymerase (pol) and viral genome DNA. The 22nm particle is more abundant in patient serum and it contains subviral particles (SVPs) that lack the nucleocapsid and are therefore non-infectious(2–4). Furthermore, there are other non-infectious particles that are produced during infection, they include enveloped particles lacking viral genomic content, those that contain viral RNA, and naked nucleocapsids(3,4).

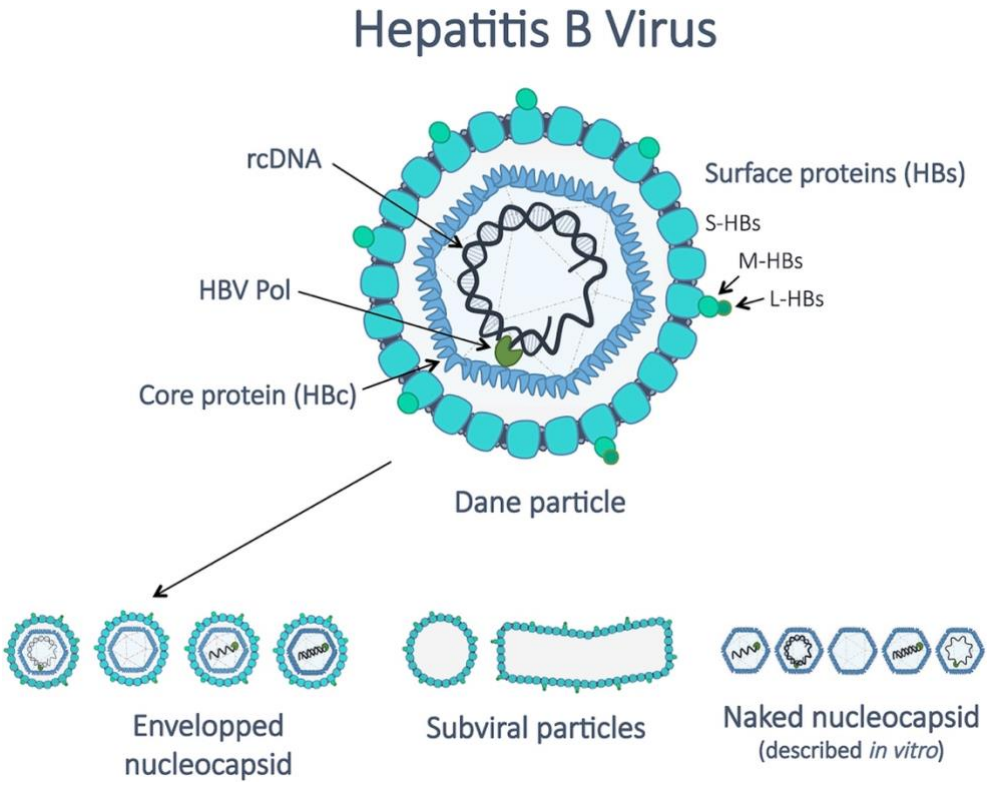

Figure 1. A schematic representation of HBV particles, consisting of the Dane particle (upper), the enveloped capsids with immature DNA/RNA, subviral particles and naked nucleocapsid (lower)(2).

HBV genome: The genome of the Hepatitis B virus is unique because it consists of circular double stranded DNA (dsDNA), with one strand that is only partially complete (Figure 1). The partially double-stranded, circular DNA genome consists of ~3,200 base pairs, with four overlapping reading frames (ORFs) C, P, S and X from which functional viral proteins are formed (Figure 2). HBc and its relatives including Hepatitis E antigen (HBeAg) and the 22-KDa precore protein (p22cr) derive from C, pol derives from P; three types of surface antigens LHBs, MHBs and SHBs derive from S; and HBV X protein (HBx) from X(1–4). The viral genome initially consists of relaxed circular DNA (rcDNA) which is then converted into covalently closed circular DNA (cccDNA) in infected hepatocytes. The cccDNA produces HBV RNAs of different lengths: 3.5Kb, 2.4Kb, 2.1Kb, and 0.7Kb(1,2). The 3.5Kb RNA produces the protein product from C and P(2). The 2.4Kb RNA is translated into LHBs; the 2.1Kb RNA is translated into MHBs and SHBs. The 0.7Kb RNA is responsible for HBx synthesis(1,2). When HBc is synthesised it initially associates to yield a dimer through its N-terminal domain, and then it self-assembles to form an icosahedral capsid containing 90 or 120

dimers. These dimers then incorporated the 3.5Kb pregenomic RNA (pgRNA) associated with pol(1,2). The synthesis of HBe occurs through the translation of the 3.5Kb preC mRNA, having an extended 5' upstream region of the C gene and subsequent cleavage of the protein product at its c-terminus(2). The largest HBV protein is pol, which is made up of four domains with three discrete enzymatic functions. The first is the terminal protein (TP) domain, which is vital for binding to pgRNA and serves as a protein primer in the process of minus strand DNA synthesis; the second is the spacer domain, whose function is unclear. The third is the reverse transcription (RT) domain, which is involved in DNA elongation for both reverse transcription and DNA-dependent DNA polymerisation; and the fourth is the ribonuclease H (RNaseH) domain, which is responsible for pgRNA digestion aster reverse transcription(1,2,5). LHBs, MHBs, and SHBs all share the common C-terminal S region. In addition, LHBs and MHBs have an extended region known as the PreS2 region, located at the N-terminus of the S region. LHBs further contain an extension, which is used for receptor binding during HBV entry, at the N-terminus of the PreS2 and S regions(1,2). The HBx protein is multifunctional; it promotes viral production at multiple stages, including transcription and replication. Furthermore, it is involved in the development of HBV-related hepatocellular carcinoma (Figure 2).

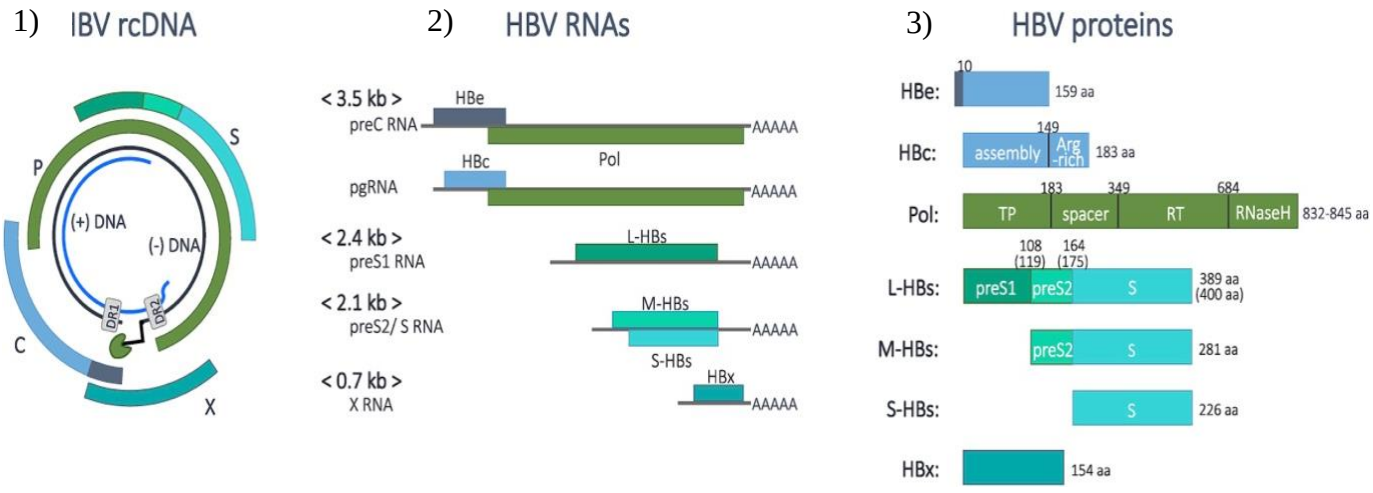

Figure 2. A schematic representation of the structure of HBV genomic DNA, RNAs and proteins. 1) rcDNA and the encoded ORFs - C, P,S and X, 2) HBV RNAs produced by cccDNA transcription and proteins synthesised from RNAs. 3) HBV proteins and domain structures(2).

HBV Replication: HBV replication is complex and involves the process of reverse transcription through an RNA intermediate. Replication is catalysed by the viral polymerase which lacks proof reading abilities. Therefore, sequence heterogeneity is a feature of the virus, which makes its existence within the host as a quasispecies of mixed viral strains.

HBV Replication- Entry: The Hepatitis B virus undergoes a series of complex processes upon infection and entry into host hepatocytes. Firstly, HBV binds to the host cell surface via binding to factors including heparan sulphate proteoglycans (HSPGs) such as glypican 5 in a non-specific and low affinity manner, and then interacts with its receptors with increased specificity and affinity(2,6–8). The virus then binds to Sodium taurocholate co-transporting polypeptide (NTCP) and enters the cell cytoplasm through endocytosis. The NTCP receptor is specifically expressed in the liver for the uptake of bile salts into hepatocytes; it was identified in 2012 as an HBV and HDV entry receptor. The NTCP was shown to be a factor that bound to amino acid (aa) 2-48 of the preS1 region which is a region essential for receptor binding(1,9–11). After entry the virus undergoes uncoating and core disassembly, and its genome is imported into the cell nucleus via the nuclear pore complex in an importin-dependent manner(1,12,13).

HBV Replication- cccDNA formation/maintenance: The viral genome consists of relaxed circular DNA (rcDNA) which is repaired inside the host nucleus to form the more stable form known as covalently closed circular DNA (cccDNA). To form ccccDNA, the Pol-linked terminal redundant sequence in the 5'-end of the minus strand DNA and the RNA oligonucleotide attached at the 5' end of the plus strand DNA are removed from the rcDNA. This leaves a gap in both stands which are filled and ligated to generate cccDNA(1,14,15). The formation of cccDNA is a crucial part of viral replication because it becomes the transcriptional template for viral RNAs: pregenomic RNA (pgRNA), precore RNA, HBsAg RNAs and HBx RNA. It remains unclear how and where cccDNA is maintained inside the nucleus; cccDNA resides episomally but it is inherently stable and is difficult to eliminate throughout HBV infection. The host immune response and cytokine stimulation have been reported to be major factors that affect cccDNA maintenance(16). The processes related to the regulation of cccDNA maintenance/ stability remain unclear; however, a cytidine deaminase, apolipoprotein B editing complex 3 (APOBEC3) is an example of a protein that modulates cccDNA stability. Stimulation by cytokines including interferon α and γ (IFNα, IFNγ), tumor necrosis factor α

(TNFα) and lymphotoxin β (LTβ), leads to the induction of APOBEC3A and/or APOBEC3B and the destabilisation of cccDNA(17,18). Next the ubiquitin conjugating enzyme E2L3 (UBE2L3) and male-specific lethal 2 (MSL2) modulate cccDNA stability by the degradation of APOBEC3A and APOBEC3B respectively(2,15).

In addition to cccDNA synthesis and maintenance, a part of HBV DNA is integrated into the host genome. Research using cell cultures have shown that integration of viral DNA can occur as quick as within a week after infection(19). The integrated HBV DNA is replication-incompetent; however, it can act as a template for HBsAg production, which has been suggested to be related to HBV-specific immune tolerance and the progression of HBV- related pathogenesis(1).

HBV Replication- Transcription: The cccDNA is the main engine behind HBV transcription within the hepatocyte. It becomes a template for four different lengths of RNAs: 3.5, 2.4, 2.1, and 0.7 kb to be transcribed. The transcription of viral RNAs are regulated by four promoters for preS1, preS2, core and X, and two enhancers (Enhancer I and II), which are mediated by the hosts RNA polymerase II machinery-dependent transcription(2,5). The transcription is regulated at the epigenetic level, the transcription factor level and the restriction level. (Figure 3). Epigenetic modification involves histone modification enzymes that positively and negatively regulate transcription. HBV cccDNA exists as a mini-chromosome that interacts with viral proteins and host factors. As it assembles with histones, the post translational modification status of histones directs the transcriptional activity of cccDNA(2,20).

Along with epigenetic control, the recruitment of cellular transcription factors to the viral promoter regions in cccDNA governs the transcription process. The viral promoter regions consist of binding sites for several transcription factors. These transcription factors include liver-enriched hepatocyte nuclear factor 3 and 4 (HNF3, HNF4), retinoid X receptor alpha (RXRα), peroxisome proliferator-activated receptor alpha (PPARα), and farnesoid X receptor (FXR) as nuclear receptors(2,21–24).

Regulation at the restriction level involves HBx activity. HBx is understood to be important for HBV replication after infection(25). It is also associated with the cccDNA mini-chromosome in close parallel to the kinetics of cccDNA-bound H3 acetylation(26). HBx is responsible for modulating the recruitment of chromatin-modifying enzymes (P300. HDAC, SIRT1) and controlling the epigenetic status of cccDNA-associated histones for active transcription(26). Furthermore, HBx has been demonstrated to affect acetylation, methylation and phosphorylation of cccDNA- associated histones in HepG2 cells(2,27). Without HBx,

cccDNA is silenced by a reduction in H3 acetylation and H3K4me3 and an increase in H3K9me2/3 that leads to the recruitment of heterochromatin protein 1 (HP1) and chromatin condensation. While HBx expression relieves this transcriptional silencing by increasing H3K4me3 and interrupting HP1 recruitment on cccDNA(28). Therefore, HBx is active on the epigenetic profile of cccDNA-associated histones to regulate HBV transcription. Moreover, HBx activity has been reported to affect the structural maintenance of chromosomes (Smc) family, which has ATPases that regulate higher-order chromosome organisation(29). It has been reported that the Smc5/6 complex interacts with an episomal HBV DNA reporter and suppresses its transcriptional activity(29). HBx activity leads to the recruitment of DDB1-containing E3 ubiquitin ligase to Smc5/6 and causes degradation to relieve Smc5/6-mediated transcriptional silencing. Genetic knockdown of Smc5/6 brings on the replication of HBx-deficient HBV, indicating a significant role for Smc5/6 antagonism in HBx's function to support viral replication(30). This function is associated with a CCCH motif (motif with 3 cysteines and 1 histidine) in the HBx sequence which is highly conserved among strains(2,31).

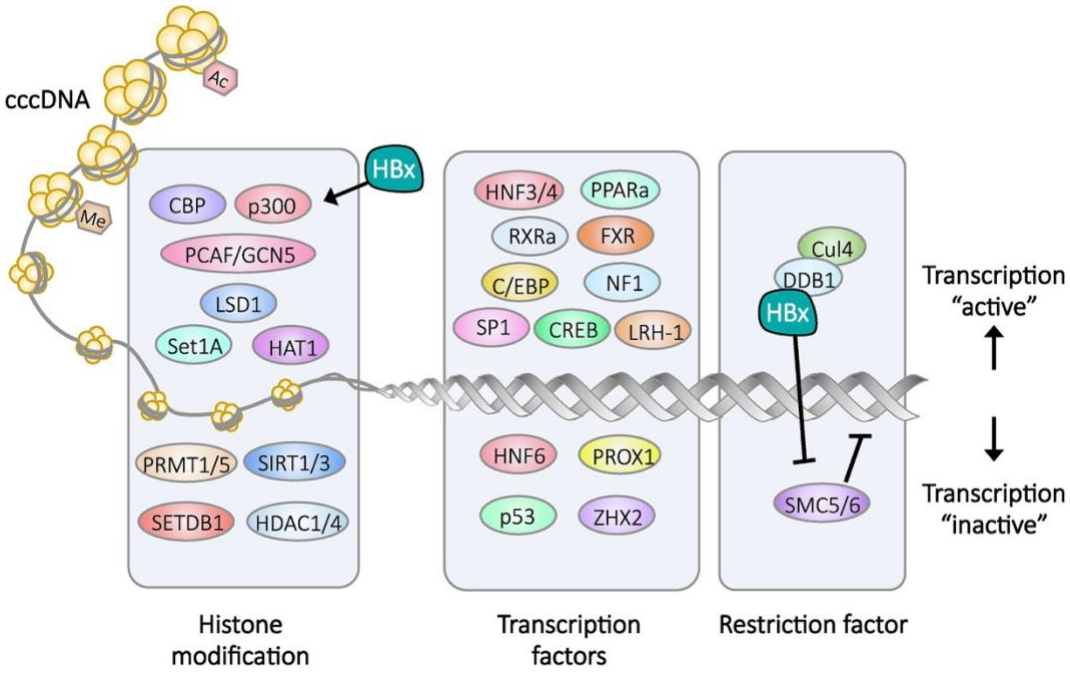

Figure 3. A schematic representation of HBV transcription regulation. Regulation occurs at the epigenetic (left), transcription factor (centre), and the restriction (right) levels(2).

HBV Replication- RNAs stability: HBV RNA stabilisation is a key step in HBV life cycle that has been reported to limit the viral replication level. The HBV pgRNA structure contains a stem loop, known as epsilon, at both the 3' and 5' termini (Figure 4). Epsilon is needed for RNA packaging into capsids and modulating the stability of HBV RNAs. The Epsilon region

on the HBV RNA interacts with Zinc finger antiviral protein (ZAP), causing its decay in the nucleus. This mechanism is observed with IFN treatment(2,32). An ENA helicase known as the super-killer viralicidic activity 2-like (SKIV2L) interacts with HBV RNAs, in particular X-mRNA (0.7KB RNA) and causes their destruction through non-stop-mediated RNA decay pathway(33). HBV RNA degradation is promoted by IFN-stimulated exoribonuclease gene of 20KDa (ISG20) which binds to the lower stem section of the epsilon(34,35).

HBV Replication- encapsidation and DNA synthesis: The next step in HBV replication is RNA encapsidation which requires HBc, pol and viral RNA. Synthesised HBc monomers form into dimers, then 90-120 dimers self-assemble to form an icosahedral capsid(36). Simultaneously with encapsidation, pol interacts with the epsilon stem loop within pgRNA at the 5' terminus to create a ribonucleoprotein complex, which then incorporates into the capsid(37,38). This activity is facilitated by host chaperones, heat shock protein 90 (Hsp90), Hsp40 and heat stress cognate 70 (Hsc70), via interactions with pol and its conformational optimization(2,39,40). Both pol and epsilon RNA interact with the RNA-binding motif protein 24 (RBM24) to mediate encapsidation(41). Host factors are incorporated into the capsid, which include eukaryotic translation initiation factor 4E (eIF4E), DEAD-box RNA helicase DDX3, and APOBEC3G(42–44). Following RNA incorporation, viral genome synthesis occurs within the nucleocapsid. The epsilon region of pgRNA possesses the sequence 5'-UUC-3' which becomes a template for priming. A tyrosine residue at aa 63 in the terminal protein (TP) domain of pol functions as a protein primer. Interactions between the first dGTP residue and the hydroxyl residue in Y63 and dAMPs produce 5'-dGAA-3'(45–47). The pol-dGAA complex created is then translocated to the complementary direct repeat 1 (DR1) sequence at the 3' terminus of pgRNA for the synthesis of minus strand DNA(2,46,48). This strand is extended to the 3'end of pgRNA, forming a unit length minus strand DNA with an additional terminal redundancy. The pgRNA template is degraded at the same time as the minus strand DNA synthesis by the RNase H domain digestion and eventually leaves a short RNA fragment consisting of the capped 5' terminal region including DR1(2). Translocation of this RNA fragment to DR2 occurs at the 3' terminus and is extended to the 5' terminus of the minus strand DNA. The elongation process continues with extending the 3' terminus of the plus strand DNA switching to the 3' redundant sequence on the minus strand DNA, this allows further elongation of the plus strand DNA to eventually create rcDNA(2) (Figure 4).

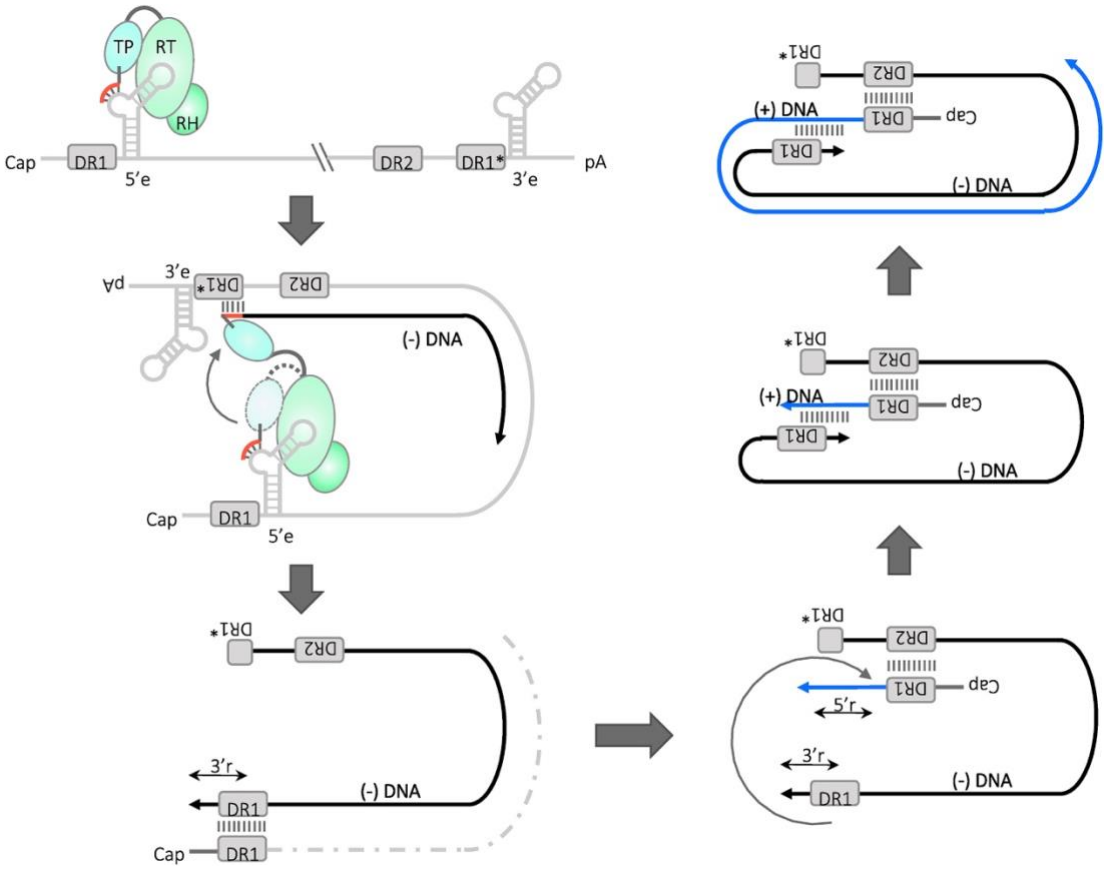

Figure 4. A schematic representation of reverse transcription, RNA degradation, and DNA synthesis in nucleocapsids.

HBV Replication- Morphogenesis: In the final stages the nucleocapsids (containing viral genome) are either shuttled to the nucleus for amplification and maintaining the cccDNA pool (recycled) or assembled with mature envelope proteins to be secreted out of the hepatocytes (viral egress). The capsids which contain replication intermediates, pgRNA, and are without DNA or RNA are also secreted from the cells but using different secretion mechanisms. The secretion process for infectious virions is associated with multivesicular body (MVB)-associated endosomal sorting complex needed for transport (ESCRT) machinery, and it involves gamma 2-adaptin, CHMP3/4, Vps4, Nedd4 and α-taxilin(2,49–51). In contrast, the ESCRT mechanism is not required in the egress of naked capsids, it instead utilises other factors such as apoptosis-linked gene 2 (ALG-2)-interacting protein X (Alix) and HGS(52,53). Moreover, subviral components in the spherical structure that mostly consists of SHBs are released from the endoplasmic reticulum via the general secretory pathway. Filamentous subviral particles that also consist of LHBs are secreted through the ESCRT-dependant

pathway. Thus, the virus secretion process is closely associated with the cellular membrane sorting mechanism(2,54,55).

Epidemiology of HBV

According to the World Health Organization's (WHO) current estimation for the prevalence of HBV infection, there are nearly 300 million people with chronic infection, with 1.5million new cases each year(56). Approximately 820,000 people will die every year because of acute or chronic complications of the virus(56) mostly due to cirrhosis and hepatocellular carcinoma (HCC)(56). The rates of chronic carriage of HBsAg can be as high as 20% in certain countries, and the proportion of patients with serological results linked to previous exposure to HBV can be as high as 70-95% in endemic regions(57,58). In highly endemic regions such as Southeast Asia, China, Sub-Saharan Africa and the Amazon Basin, it is estimated that at least 8% of the population are HBV chronic carriers. Amongst these regions, Asia and the Western Pacific have the largest proportion of Chronic Hepatitis B (CHB) with 75% of the world's CHB cases being concentrated in these countries(57,58). It is estimated that globally, 30% of cirrhosis cases and 53% of HCC cases can be attributable to HBV infection(58,59). The prevalence of HBV infection can vary in different regions of the world. Countries can either be of low, medium, or high prevalence depending on factors including predominant age at infection and mode of transmission and (shown in Table 1).

The Hepatitis B virus is transmitted through contact with infected body fluids such as blood, semen, and saliva (with blood being the most common vehicle for transmission)(57,60,61). There are three modes of transmission currently recognised: perinatal, sexual, and parenteral/percutaneous transmission.

Perinatal transmission of HBV is the most common route of transmission; whereby babies are infected through their carrier mothers. It is the most important factor in determining chronic infection prevalence particularly in high endemic regions. Routine HBV vaccination and the administration of hepatitis B immune globulin (HBIG) to new-borns of infected mothers, greatly reduces mother to child transmission and babies becoming HBV carriers. Before HBV vaccination was integrated into the routine immunization program, the percentage of infants who were HBV carriers was 10-30% for mothers who were HBsAg positive but HBeAg negative. However, when mothers are both HBsAg positive and HBeAg positive, the incidence of perinatal infection is extremely high, up to 90%(57,62,63). HBV can be transmitted from mother to child via three routes; these include transplacental transmission of HBV in utero;

natal transmission during delivery; and post transmission during care or through breast milk(57). Unfortunately, the HBV vaccine and HBIG cannot prevent transplacental transmission because it occurs antenatally(57). For neonates and children under the age of 1year, who acquired HBV perinatally, the risk of the infection becoming chronic is 90%. This may be due to their immature immune systems(57,64). The high rate of chronicity could also be due to the transplacental passage of HBeAg causing an immunological tolerance to HBV in the foetus(57).

PREVALENCE	REGION	PERCENTAGE OF POPULATION HBSAG POSITIVE	PREDOMINANT AGE AT INFECTION	PREDOMINANT MODE OF TRANSMISSION
HIGH (≥ 8%)	Southeast Asia, China, Pacific Island, Sub-Saharan Africa	8%-20%	Perinatal and early childhood	Maternal-infant, percutaneous
INTERMEDIATE (2%-7%)	Eastern Europe, the Mediterranean basin, Middle East, Central and South Asia, Japan, Central and South America	2%-7%	Early childhood/adolescence	Percutaneous sexual
LOW (<2%)	United States and Canada, Western Europe, Australia, and New Zealand	0.2%-0.5%	Adult	Percutaneous sexual

Table 1: Prevalence of HBV infection including regions of high, intermediate, and low prevalence, the percentage of the population that are HBsAg positive and the predominant age at infection and mode of transmission.

HBV is also considered to be a sexually transmitted disease (STD). Sexual transmission is a common source of infection; particularly observed in low endemic regions e.g., North America. Furthermore, the factors that are associated with increased risk of HBV infection include: duration of sexual activity, number of sexual partners, history of STDs and testing positive for syphilis(57).

HBV transmission through the parenteral route includes injection drug use, blood transfusions and dialysis, acupuncture, working in a health care settings, cosmetic procedures, and household contact(57). All blood required for transfusion are now screened for HBV markers and donors who engage in high-risk activities are excluded; this has immensely reduced the risk of transfusion-associated HBV infection. However, transmission is still possible when blood donors are asymptomatic carriers and negative for HBsAg(57,65). The nosocomial spread of HBV infection is mainly observed in hospitals, especially dialysis units and dental units(66). The risk of developing CHB is less than 5% when transmission is through sexual contact, intravenous drug use, acupuncture, and transfusion(64).

HBV Genotypes: Mutations are commonly observed in HBV, accounting genetic heterogeneity of the virus. This is because the viral polymerase lacks proofreading abilities during reverse transcription of the pgRNA. The mutation rate of the hepadnaviral genome is approximately $2x10^4$ base substitutions/site/year. This is 100 times higher than that observed in other DNA viruses, but 100-1000 times lower than that seen in other RNA viruses(67,68). According to phylogenetic analysis, HBV can be classified into 10 genotypes – genotypes A-J. The genotypes are grouped based on an intergroup divergence of 8% or more in the complete nucleotide sequence and they can be further subdivided into subgenotypes with a nucleotide divergence greater than 4%(68–71). Several studies have indicated that HBV genotypes may play a significant role in influencing clinical outcome, HBeAg seroconversion rates, mutational patterns seen in the precore and core promoter regions and response to interferon therapy(72–74). It is well documented that HBV genotypes have specific geographical distributions (this is summarised in Figure 5).

The different HBV genotypes vary in their clinical consequences including the natural course of infection, disease progression and response to treatment(72,75). For example, genotypes B, C and I are often associated with vertical transmission from mother to child, and genotypes A, D and G have been associated with transmission during sexual contact or the injection of drugs(75–78). Additionally, a higher chronicity rate after infection has been linked to

genotypes A and C(78). Furthermore, a faster rate of disease progression to liver cirrhosis and HCC have been observed in patients with genotypes C, D and F(75,78). In relation to antiviral treatment, all genotypes respond in a similar manna with reverse transcriptase inhibitors, however, when treated with IFNα, genotypes A and B demonstrate an increased virological response and higher anti-HBe seroconversion compared to the other HBV genotypes(78,79).

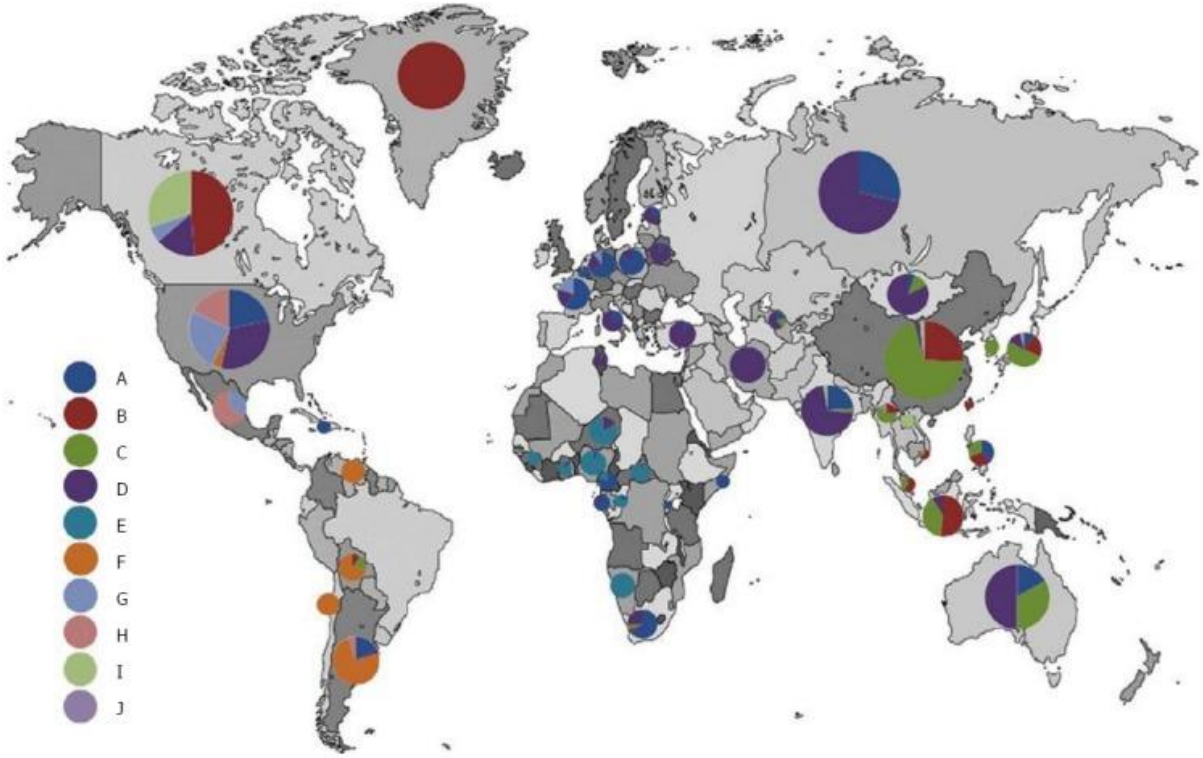

Figure 5: The geographical distribution of HBV including genotypes A-J(72).

1.2 The Natural History of HBV

The natural history of CHB evolves through several clinical phases, reflecting different stages in the host-virus immune relationship. These phases characterise different patterns of HBV viral load, HBeAg status and serum transaminase levels. Understanding the different phases of CHB is an important aspect of disease management as they can be used to determine time and mode of infection, fibrosis progression and appropriate course of treatment.

The host immune response plays a significant role in the natural history of HBV, including at the time of acute infection (in determining whether viral clearance ensues) and the progression into chronic infection (where liver damage is caused mainly by the host immune cells rather than from direct viral activity)(80). The natural history of HBV infection is heavily dependent on the age of the host at the time of infection. There is an inverse relationship between the risk of developing acute hepatitis and its progression into chronic hepatitis and the age of the patient(81). Infants infected with HBV generally remain asymptomatic and have more than 90% chance of the disease progressing into chronic infection. Children up till the age of 5 years, will have a 20% chance of developing chronic infection. At the age of 5 years and above (especially in adulthood) more than 90% develop acute hepatitis and clear the virus within 6 months. The main characteristic of acute HBV is elevated concentrations of liver enzymes including alkaline phosphatase (ALP), alkaline aminotransaminase (ALT), aspartate aminotransaminase (AST) and gamma-glutamyl transferase (GGT). With chronic HBV, patients will remain asymptomatic and will not clear the virus. Furthermore, patients with acute HBV infection may remain asymptomatic, or develop symptoms of acute viral hepatitis or develop acute liver failure. However, in a CHB patient, their liver is continuously exposed to virus-induced injury. Virus-induced liver injury could continue for decades, followed by natural healing with fibrosis; eventually the condition may progress to liver cirrhosis. In cases where cirrhosis is left unchecked for prolonged periods of time, patients may develop complications of cirrhosis e.g. ascites, variceal bleed and hepatic encephalopathy (decompensation)(81).

Another serious complication of CHB is the development of hepatocellular carcinoma (HCC), which can occur even without cirrhosis. This is due to HBV being a DNA virus and it being able to integrate into the human genome. It can cause HCC through replication and mutant types(81–83). Interestingly, HBV is not a cytotoxic virus, meaning it does not cause any injury

to the hepatocyte. Liver injury is primarily mediated by the host's immune system in attempts to clear the virus. The host's immune cells and cytokines target infected hepatocytes and kill them. If the host immune system is tolerant to the virus, then there will be no liver damage, despite a high viral load. On the other hand, if the host immune system attacks the virus, liver injury will occur albeit the viral load will be lower(81,84,85). The healing process following cell damage involves cellular regeneration and fibrosis. The repeated cycle of liver injury, healing eventually may result in liver cirrhosis.

Phases of Chronic Hepatitis B: The phases in the natural history of CHB infection include: 1) HBeAg positive chronic infection, 2) HBeAg positive chronic hepatitis B, 3) HBeAg negative chronic HBV infection 4) HBeAg negative chronic hepatitis B, 5) occult infection. It is not uncommon for patients to experience a backwards shift in phases and reactivation of disease even when they have achieved immune control of the disease. The stages of infection are typically seen in children who acquired the disease perinatally in a sequential manner and will be monitored regularly from birth. While with adult patients, on diagnosis, they might be in any one of the phases. Observations and serology tests will be done for 6-12 months to determine which phase the patient is in(58,81).

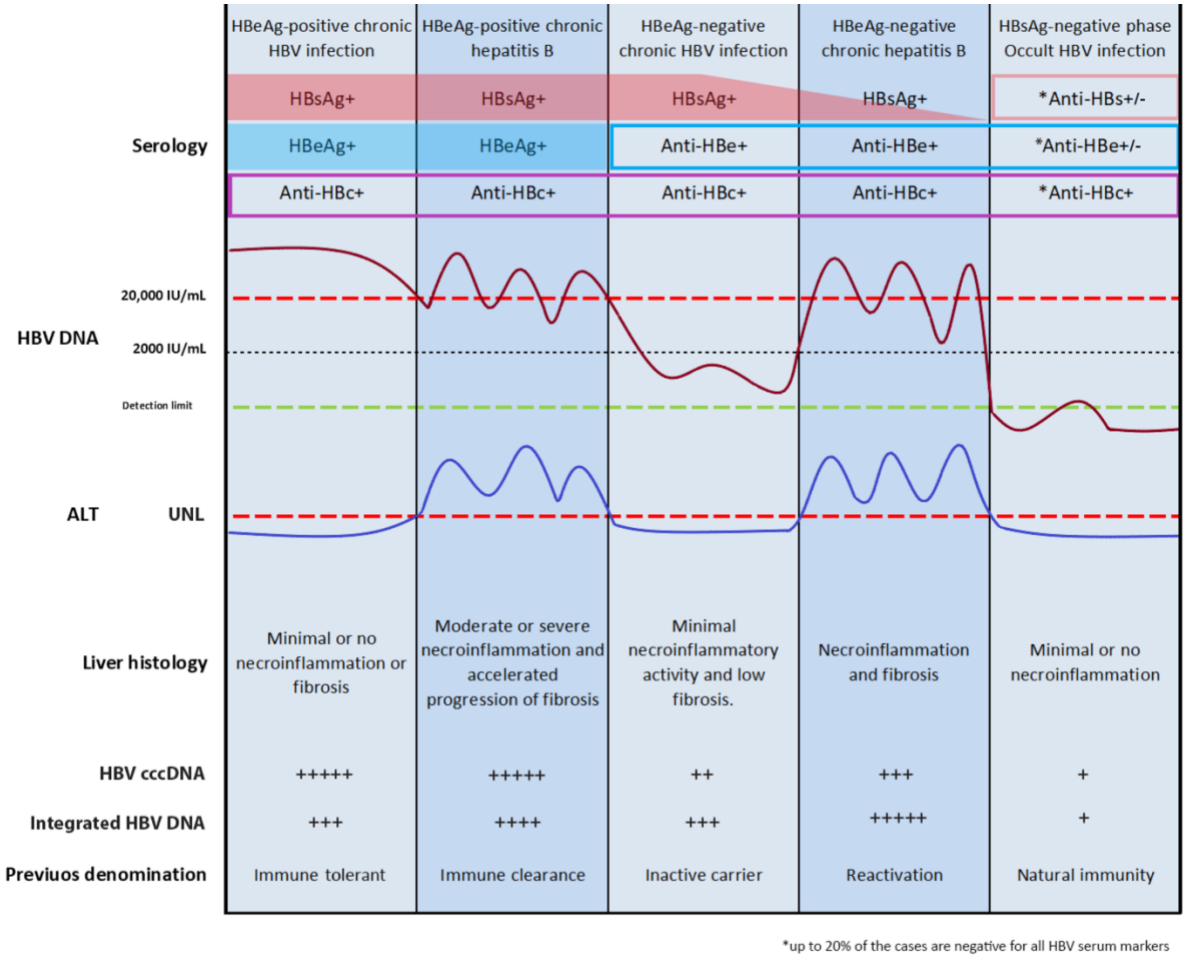

Figure 6: The natural history of Hepatitis B virus. There are five clinical/ virological phases of CHB (according to the European Association for the study of the liver -EASL). += rarely detected; ++ = occasionally detected; +++ = often detected; ++++ = frequently detected; +++++ = almost always detected(86,87).

HBeAg positive chronic infection: The HBeAg positive chronic infection phase is characterised by the detection of HBeAg in serum, very high concentrations of HBV DNA and ALT persistently within the normal range according to traditional cut-off values (upper limit of normal – ULN) approximately 40 IU/L(87,88). At this stage there is minimal or no liver necroinflammation or fibrosis, but high levels of DNA integration and clonal hepatocyte expansion is observed, indicating that hepatocarcinogenesis could occur even in this early phase of infection(87–89). In perinatally acquired CHB patients this phase is more frequent and prolonged and is linked to preserved HBV specific T cell function up on till adulthood. Moreover, the rate of spontaneous HBeAg loss is extremely low in this phase and these patients are highly contagious due to the high concentrations of HBV DNA.

HBeAg positive chronic Hepatitis B: The HBeAg positive chronic hepatitis B phase is marked by the presence of serum HBeAg, high concentrations of HBV DNA albeit lower than that observed in the HBeAg positive chronic infection phase and elevated ALT. Also, moderate to severe liver necroinflammation is observed in these patients along with accelerated progression of fibrosis(87,88). It may take several years after the first phase for the HBeAg positive chronic hepatitis B phase to occur and it is more often and /or rapidly reached in individuals infected during adulthood(87,90). The outcome of this phase is variable. Most patients will achieve HBeAg seroconversion and HBV DNA suppression, leading them into the HBeAg negative infection phase. Other patients may fail to control HBV and these individuals enter the HBeAg negative CHB phase which may last for several years(87).

HBeAg negative chronic HBV infection: The HBeAg negative chronic HBV infection phase is characterised by the presence of serum antibodies against HBeAg (anti-HBe), undetectable or very low levels of HBV DNA (<2000 IU/ml) and normal ALT levels(87,88). There are some patients in this phase who may present with HBV DNA levels of >2000 IU/ml (but usually <20,000 IU/ml) accompanied by persistently normal ALT and very low hepatic necroinflammation and low fibrosis(87,88). If patients remain in this phase, the risk of developing cirrhosis or HCC is very low. Individuals in this phase may also experience spontaneous HBsAg loss and/or seroconversion, however, this occurs in only 1-3% of cases per year(87,88).

HBeAg negative chronic hepatitis B: The HBeAg negative chronic hepatitis B phase is characterised by absence of serum HBeAg along with detectable anti-HBe and persistent HBV DNA (usually in lower levels than that seen in HBeAg positive patients) and fluctuating or persistently elevated ALT levels(87,88). There is necroinflammation and fibrosis present in these patients' livers. Additionally, many of these patients harbour HBV variants in the precore and/or the basal core promoter regions that disrupt HBeAg expression. This phase is also linked to low rates of spontaneous disease remission(87,88).

Occult HBV infection: This phase is also known as the HBsAg negative phase. It is marked by serum negative HBsAg and positive antibodies to HBcAg (anti-HBc), with either the presence or absence of antibodies to HBsAg (anti-HBs). Also, patients in this phase present with normal ALT values and undetectable HBV DNA. However, cccDNA can be detected frequently in the liver(87,88). Early HBsAg loss particularly before the onset of cirrhosis is linked to a reduced risk of cirrhosis, decompensation and HCC, and an overall improvement on survival. However, if liver cirrhosis develops before HBsAg loss, individuals in this phase remain at the risk of HCC. Additionally, immunosuppression can lead to HBV reactivation in these patients(87,88).

Factors associated to disease progression: The risk of developing HBV related cirrhosis and HCC is variable, and it is affected by the host's immune response. In untreated cases the five-year cumulative incidence of cirrhosis ranges from 8% to 20%, and amongst those with cirrhosis, the five-year cumulative risk of hepatic decompensation is 20%(87,88). The annual risk of developing HCC in cirrhosis patients is 2-5%(87,91). In diagnosed CHB patients, the development of HCC is of major concern especially because it can still develop in individuals who have been effectively treated. Disease progression into HCC is higher in cases with one or more factors that relate to the host. These include the presence of cirrhosis, chronic hepatic necroinflammation, older age, being male, alcohol abuse, co-infection with other hepatitis viruses, human immunodeficiency virus (HIV), diabetes, smoking and a positive family history. Other factors that increase the risk of developing HCC are those relating to HBV properties, such as high HBV DNA and/or HBsAg concentrations, genotype C>B and specific mutations(87,92).

The frequent follow up of CHB patients is important in HCC diagnosis. Several tools to help predict HCC in CHB patients have been developed which focus on risk scores. The most common tools include GAGHCC, CU-HCC, REACH-B, and m-REACH which have been validated in Asian untreated CHB patients(93). Albeit research have shown them to have poor predictability in most studies involving CHB patients of other ethnicities(87,94,95). A newly developed and validated score / tool called PAGE-B has been shown to offer good predictability for HCC in CHB patients in more diverse communities during antiviral therapy(94,96). The PAGE-B score is calculated using widely available parameters such as platelet levels, age and gender. Additionally, PAGE-B score can predict HCC development in untreated CHB patients(87,94,96).

1.3 Treatment and Management of chronic Hepatitis B infection

Goal of therapy: Chronic hepatitis B cannot be cured; however, control of the virus can be achieved, referred to as a functional cure often through antiviral treatment. Currently the most important goal for therapy for CHB patients is to improve survival and the quality of life by preventing disease progression and developing HCC. Other goals of antiviral therapy include preventing mother to child transmission, HBV reactivation and preventing HBV-associated extrahepatic manifestations(87). The possibility of achieving these goals depend on several factors, including the timing of therapy during the natural course of the infection; the stage of the disease and the age of the patient when treatment is started. Regression of fibrosis and cirrhosis could also be another aim for treatment within patients with advanced fibrosis or cirrhosis(87). Treatment strategies for the prevention of disease progression to HCC may differ from those adopted to prevent fibrosis. Individuals with HBV-induced HCC are treated with nucleos(t)ide analogues (NA) firstly to suppress HBV replication which prevents disease progression and secondly to reduce the risk of HCC recurrence after potentially curative HCC therapies(87).

The level of HBV replication is a strong predictive measure of disease progression and the long-term outcome of CHB infection. Antiviral therapy with NAs inhibits viral replication and have shown the ability to eliminate CHB-induced necroinflammation and progressive fibrotic liver processes, therefore lowering the risk of HCC in CHB patients(87,97–99). For this reason, it represents the cornerstone endpoint of current therapeutic attempts. The exact level of HBV DNA suppression required to achieve the above goals is still unclear, however it is widely accepted that the lower the level of viral replication the better it is for patients(97–100).
Furthermore, treatment-induced HBeAg loss and seroconversion to anti-HBe demonstrates partial immune control and it is often associated with low replicative phase of CHB infection. However, the durability of this phase can only be determined after discontinuing treatment. After antiviral cessation, HBeAg sero-reversion, as well as the development of HBeAg negative CHB could occur, therefore making this endpoint risky and less reliable(101,102). The alternative is for patients to continue oral antiviral therapy irrespective of HBeAg response until HBsAg loss is achieved.

Suppression of HBV DNA to undetectable concentrations is usually linked with normalisation of ALT levels. When ALT is persistently high in patients with complete suppression of HBV DNA this is associated with a reduced likelihood of fibrosis regression and can cause histologic disease progression(87,103). The loss of HBsAg is considered the optimal treatment endpoint, termed "functional cure." A functional cure is however rarely achieved in CHB patients with current antiviral armamentarium. After HBsAg loss, spontaneous HBsAg sero-reversion with reactivation of inflammatory activity is rare and may only be seen in patients with a serious impairment to their immune system(104–107). HBsAg loss has many clinical benefits, the main advantage being the safe discontinuation of antiviral therapy. As CHB infection cannot be completely eliminated due to the persistence of cccDNA and integrated HBV DNA, it is uncertain whether HBsAg loss actually adds to the prevention of long-term complications of CHB beyond what is achievable by the suppression of viral replication alone. There is still a risk of developing HCC even after spontaneous HBsAg loss with an annual rate of about 0.55%(87,108). However, the risk is lower if HBsAg loss occurs at a younger age and/or without significant fibrosis(87,109).

The need for antiviral therapy is mainly based on patients' serum HBV DNA concentration, ALT levels and the severity of liver disease and treatment is normally the same for both HBeAg positive and HBeAg negative CHB (table 2). Chronic hepatitis B patients without cirrhosis can be considered for antiviral treatment if they have HBV DNA concentrations of >2,000 IU/ml, ALT levels above 40IU/L and moderate necroinflammation and/or fibrosis (assessed by liver biopsy)(87). CHB patients who do not meet the criteria for treatment should be monitored with regular assessments of serum ALT, HBV DNA and checks for liver fibrosis using non-invasive markers (table 2).

Indications for antiviral therapy	Treatment required (Yes or NO)
Patients with HBeAg positive/ negative CHB, defined by HBV DNA >2,000 IU/ml, ALT>ULN and with liver necroinflammation or fibrosis.	Yes
Patients with compensated or decompensated cirrhosis with detectable HBV DNA concentrations at any level.	Yes - regardless of ALT concentration
Patients who have HBV DNA of >20,000 IU/ml and ALT > 2xULN.	Yes - regardless of the amount of fibrosis.
Individuals with HBeAg positive chronic HBV infection, defined by persistently normal ALT and high levels of HBV DNA.	Yes - if older than 30 years
Individuals with HBeAg positive/ negative chronic HBV infection, a family history of HCC or cirrhosis and extrahepatic manifestations.	Yes
Patients younger than 30 years with HBeAg positive chronic HBV infection.	No but should be followed up every 3-6 months
Patients with HBeAg negative chronic HBV infection with HBV DNA levels of <2,000 IU/ml.	No but should be followed up every 6-12 months

Table 2: Recommendation for antiviral treatment(87).

Treatment strategies: There are currently two main therapeutic options for CHB patients; treatment with NA or IFNα, currently pegylated (pegIFNα). The NAs that have been approved for treatment in Europe include lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (TBV), tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide (TAF). These antivirals can be classified into those associated with low barrier against HBV resistance (LAM, ADV, TBV) and those with high barrier to HBV resistance (ETV, TDF, TAF)(87,110). Choosing to treat patients with a more potent NA with high barrier to resistance carries the advantage of predictable high long-term antiviral efficacy which in turn leads to undetectable HBV DNA concentrations in most patients as well as its favourable safety profile(87,88). These antivirals can be safely administered to any HBV patient and represent the only treatment option for many patient subgroups including those with decompensated liver disease, liver transplants, extrahepatic manifestations, etc. Additionally, NAs are the only treatment option available for the prevention of HBV reactivation in patients under immunosuppression(111–113).

Treatment with Pegylated interion alpha (pegIFNα) has its own advantages, the main one being to induce long term immunological control with finite duration treatment. However, major

disadvantages of using pegIFNα is the high variability of response, its unfavourable safety profile, and side effects, making a significant proportion of CHB patients ineligible for treatment(87,114). Patient selection for therapy including disease activity, HBV genotype, stage of disease, concentrations of HBV DNA and HBsAg and HBeAg status can be useful predictors of patient response probability.

CHB patients are placed on antiviral therapy with the aim of preventing, halting or even reversing liver injury that could lead to cirrhosis or HCC so monitoring their response to treatment is very important. Response to therapy may vary and can be categorised into virological, serological, biochemical, and histological. All responses can be estimated at several time points during and after therapy. Virological responses are associated with the timing of therapy and the type of antiviral used (NA or pegIFNα). A positive response to NA therapy is defined as undetectable HBV DNA using a PCR assay with a limit of detection of 10 IU/ml(87). Primary nonresponse is marked by a <1 log10 decrease of serum HBV DNA after 3 months of therapy. Patients who experience partial virological response show a decrease in HBV DNA of >1 log10 IU/ml but detectable HBV DNA after 12 months of therapy(87). Some patients experience virological breakthrough which is specified by an increase in HBV DNA levels of >1 log10 IU/ml compared to the nadir (lowest value) HBV DNA level on-therapy; it may precede a biochemical breakthrough, which is associated with an increase in ALT levels(87). In cases where HBV is resistant to NA, a selection of HBV variants with amino acid substitutions that confer reduced susceptibility to the administered NA. In cases where NA therapy is discontinued, sustained off-therapy virological response is associated with serum HBV DNA levels <2000 IU/ml for at least 12 months after stopping treatment(87).

With pegIFNα, virological response is defined as serum HBV DNA levels of <2000 IU/ml, at a 6 months evaluation and at the end of therapy(87). Sustained off-therapy virological response is marked by HBV DNA concentrations being <2000 IU/ml for at least 12 months after stopping therapy. Sustained off-therapy virological response is achieved where HBV DNA concentration is <2000 IU/ml for at least 12 months after the end of treatment(87).

Serological responses to treatment involve patient's HBeAg and HBsAg status. HBeAg serological response is where patients experience HBeAg loss and develop anti-HBe (HBeAg seroconversion). HBsAg serological response is where patients show HBsAg loss and develop anti-HBs (HBsAg seroconversion)(87).

Biochemical response to treatment mainly relates to ALT levels. It is marked by the normalisation of ALT based on the traditional ULN (40 IU/L). Biochemical response can be

difficult to maintain because ALT often fluctuates overtime, therefore a minimum follow-up of at least 1 year after treatment with ALT determinations at least 3 months is needed to confirm sustained off-treatment biochemical response(87). Additionally, transient ALT elevations before long-term biochemical remission may occur in some CHB individuals within the first year after therapy cessation. In such cases, additional close ALT monitoring is required with follow-up of at least 2 years after Alt elevation to confirm sustained off-therapy biochemical remission.

Histological response is specified by a reduction in necroinflammatory activity without worsening in fibrosis compared to pre-treatment histological findings(87).

1.3.1 Antiviral therapy for Chronic Hepatitis B infection

Chronic hepatitis B (CHB) is the major cause of liver cirrhosis and hepatocellular carcinoma (HCC). HBV is problematic because it cannot be cured and there are no powerful drugs available to eradicate it. Currently, the goal of antiviral therapy is to alleviate liver inflammation and necrosis, also to lower the risk of cirrhosis, HCC and other implications related to the disease through long-term suppression of HBV replication. Current anti-HBV drugs include:

Lamivudine: Lamivudine [(-)β-L-2',3'-dideoxy-3'-thiacytidine (3TC)] contains anti-HIV and anti-HBV properties. CHB patients are instructed to take LAM daily at an oral dose of 100mg(115). its mode of action involves incorporating into the growing HBV DNA chain and terminating the chain. It also acts as a competitive inhibitor of deoxycytidine triphosphate (dCTP) at the level of the DNA polymerase(115). LAM inhibits viral DNA synthesis, but not mitochondrial DNA synthesis. The effects of LAM treatment can be seen during the first months of treatment where a 3-4 log drop in circulating HBV DNA is generally reported(116,117). Simultaneously, HBeAg is cleared more rapidly from circulation and ALT levels may normalise(116,117). The virological and biochemical response may show a reduction of up to 74% and 66%, respectively(118). The drug is normally well tolerated by CHB patients, but monotherapy can quickly cause resistance development. Resistance increases with long term use; approximately 20% of HBeAg-positive patients develop resistance after one year, which increases up to 70% after five years(119,120). The most

common mutation is observed in the catalytic YMDD motif of the viral reverse transcription (RT) polymerase. The primary lamivudine-resistance mutation is M204V/I/S, located in the highly conserved YMDD motif(121). This is often combined with the L180M mutation(115,121).

Adefovir dipivoxil: Adefovir is an acyclic nucleoside phosphonate. It should be taken daily at an oral dose of 10mg (115). When Adefovir is administered it is incorporated into the growing HBV DNA chain, where it behaves as an obligatory chain terminator and/or a competitive inhibitor of the substrate dATP(122). Treatment with Adefovir dipivoxil for an average of 48 weeks has been reported to reduce both cccDNA activity and HBsAg levels in a cohort of HBeAg positive CHB patients. It has been predicted that treatment with Adefovir dipivoxil may take approximately 14.5 years to clear infected hepatocyte from cccDNA(115,123). There are very few side effects associated with adefovir dipivoxil especially at a dose of 10mg per day. However, at a higher dosage i.e >30mg per day, the compound may nephrotoxic(124,125). The rate of developing adefovir resistance is approximately 6% after 3 years and up to 18% after 4 years(126,127). After 5 years of therapy, 29% of patients harbour Adefovir-resistant HBV strains, which is significantly lower than lamivudine at 70%(128). Resistance to adefovir is most associated with the rtN236T and rtA181V/T mutation(129,130).

Entecavir: the metabolism of Entecavir (ETV) is comparable with that of other NAs. When in the body it is phosphorylated three times into its active form ETV-TP and its half-life is about 15 hours(131). ETV interacts with HBV DNA polymerase in several ways (i) it inhibits the priming of the polymerase, (ii) it has a high affinity for the HBV polymerase, (iii) it acts as a competitive inhibitor of dGTP (natural substrate) and (iv) it acts as a chain terminator two or three nucleotides downstream from its incorporation(115,132,133). In patients where treatment with LAM fails, the alternative is to take ETV at a higher dose, as ETV resistance may develop in 10% of these patients after two years(133). Entecavir should be taken orally at a dosage of 0.5mg daily for nucleoside naïve patients. It was recommended that ETV could be taken at the higher dose of 1.0mg daily for Lam-resistant patients; however due to the high rates of ETV resistance in such patients ETV is no longer recommended. The mutations associated with ETV resistance include: rtT184G, rtS202I, and rtM250V(134,135). Two additional mutations responsible for resistance to both LAM and ETV are rtM204V and rTL180M(134). Research has shown that ETV resistance of HBV in nucleoside naïve patients is rare through a 5 year period, thus proving a high genetic barrier of HBV drug resistance to ETV(115,136).

Tenofovir disoproxil fumarate (TDF): Treatment of CHB with TDF was approved by the FDA in 2008. In its active form TDF functions as a chain terminator and a poor substrate for cellular DNA polymerase α, β and ε. It is recommended that TDF is taken daily at 300mg orally. It is safe to use with a superior antiviral efficacy than adefovir dipivoxil following 48 weeks of therapy. TDF resistance is associated with the rtA194T mutation(137).

Interferon alpha (INF-α): INF-α was the first antiviral drug licensed to treat CHB virus infections. A positive response to the drug was seen in only 30% of patients with loss of HBeAg, reduced HBV DNA and ALT normalisation(138). The side effects reported were similar to influenza. The mode of action of interferon is first to establish antiviral activity then immunomodulatory activity (e.g., increased expression of MHC I, and stimulation of CTLs)(27). INF-α can be given daily at 5 × 106 units or ten 2 × 106 units given three times a week subcutaneously for 4-6 months(138). The two types of interferons approved for CHB treatment are INF-α-2b and pegylated INF-α2a.

Novel Anti-HBV Agents

Tenofovir alafenamide: Tenofovir alafenamide (TAF) is administered to adult CHB patients with compensated liver diseases. It is taken orally at a dose of 25mg/day. TAF is a more stable prodrug in the plasma than TDF, leading to decreased plasma exposure of tenofovir. Having reduced exposure to tenofovir lowers the risk of long term TDF toxicities including nephrotoxicity and decreased bone mineral density(139). TAF is a nucleotide reverse transcriptase inhibitor and has the same mechanism of action as TDF. After phase III primary trials TAF was shown to be noninferior to TDF at suppressing the HBV DNA in treatment-naive and treatment-experienced HBeAg-negative and HBeAg-positive patients at 48 weeks, 96 weeks, and 144 weeks of therapy(139). The main adverse events were headache, abdominal pain, fatigue, cough, nausea, and back pain. TAF has been reported to be safe in patients with a creatinine clearance (Cl_{cr}) above 15 ml/min; however, TAF is not currently recommended in patients with an estimated Cl_{cr} below this threshold(139). TAF is also safe in patients with mild

hepatic impairment but is not currently recommended in patients with moderate or severe hepatic impairment (Child-Pugh class B or C)(139).

Clevudine: Clevudine is not registered to be used in the United Kingdom (UK) however it has been approved in South Korea and the Philippines for CHB treatment(140). It has been shown to significantly reduce serum viral load. Serum HBV DNA levels were undetectable at the end of treatment in 59% of HBeAg positive patients and in 92% of HBeAg negative patients(141). An advantageous characteristic of clevudine is the durability of viral suppression, which has been reported to persist for up to 20 weeks after treatment discontinuation. Nonetheless, it has not been reported to increase the rate of HBeAg seroconversion when compared to the placebo controls(141).

1.4 Chronic Hepatitis B and Pregnancy

Antiviral therapy during pregnancy can be complicated, thus family planning should always be discussed with CHB infected women of childbearing age before starting treatment regarding safety of the drugs. PegIFNα is contraindicated during pregnancy. Unfortunately, there are no well controlled studies of LAM, ADV and ETV in pregnant women. Reproductive studies using animals and humans with TDF have demonstrated that these drugs pose no harm to the foetus(87,142–144). TDF is mostly recommended because it contains a better resistance profile and a more extensive safety data on pregnant HBV positive women(87,142–144). Furthermore, in CHB infected women planning a pregnancy, it may be prudent to delay therapy until the child is born. In cases where a patient becomes unexpectedly pregnant while on antiviral therapy, then treatment indications should be re-evaluated. Therapy with tenofovir is nearly always maintained during pregnancy in patients who had started prior to pregnancy to prevent flares related to discontinuation. Pregnant CHB patients who are co-infected with HIV could be prescribed TAF and there is increasing evidence that TAF is safe during pregnancy. The same treatment indications apply to patients who are first diagnosed with CHB during pregnancy. CHB patients with advanced fibrosis or cirrhosis should continue treatment with TDF(87).

In pregnant patients, the prevention of HBV perinatal transmission is crucial and could be lifesaving. Perinatal transmission is considered to occur mainly at delivery, and it is responsible for the majority of CHB infection. To reduce the risk of transmission, a combination of HBIG

and vaccination is given within 12 hours of birth. This prophylaxis reduced the rate of perinatal transmission from >90% to <10%(87). However, in some cases HBIG and vaccine failures can occur, and this is almost exclusively observed in HBeAg positive women with high HBV DNA concentrations (>200,000 IU/ml) and/or HBsAg concentrations above 4 log10 IU/ml(145–147). Moreover, NA prophylaxis can be effective in the few HBeAg negative mothers with high concentrations of viremia but normal ALT levels(145–147). The utilisation of NA (especially TDF) to reduce viremia levels increases the effectiveness of the HBIG and vaccination. In a randomised study with a cohort of HBsAg positive pregnant patients with high HBV DNA concentrations (>2000,000 IU/ml) the rate of mother to child HBV transmission at post-partum week 28 was 0% in patients treated with TDF compared to 7% in the placebo control group(143). When NA is administered as prophylaxis, its duration is unclear. One potential advantage of ending treatment with NA at delivery is that there will be no interference with breast feeding; especially as the safety of NA therapy during lactation is uncertain. HBsAg can be detected in breastmilk, but breast feeding may not be considered a contraindication in HBsAg positive mothers(148). In women treated with TDF, tenofovir levels have been reported in breastmilk; however, its oral bioavailability is limited, and thus infant exposure is minimal(87,148).

1.5 Paediatric Chronic Hepatitis B infection

Chronic hepatitis B virus (CHB) infection in endemic regions normally begins in infancy and early childhood and persists lifelong(149). Though the clinical course of disease may differ amongst different patients, most CHB infected children present with immune tolerant status initially; followed by the immune clearance phase, whereby various degrees of liver damage is common during or beyond puberty. Lastly, paediatric patients enter the inactive phase after HBeAg seroconversion. Some patients may experience HBV DNA elevation with hepatitis flare after HBeAg seroconversion, known as the HBeAg negative hepatitis flare(149). This may lead to the development of cirrhosis and HCC.

The course of chronic HBV infection is complex and is often associated with age/ route of viral acquisition, host factors e.g., immune, and endocrine factors, viral factors, and host-virus interactions(149). The adrenarche and puberty onset modulate the start of the immune clearance phase and may have an impact on the severity of liver inflammation. Other factors that have been linked to the onset of immune clearance of HBV include the genotype and phenotype of human cytokines, innate immunity, and human leukocyte antigens. These factors also affect the severity of liver inflammation. Additionally, immune escape HBV mutant strains may emerge during this phase under host immune surveillance. The presence of these strains may affect viral biosynthesis, host immune responses and clinical course.

Endocrine factors: Spontaneous HBeAg seroconversion in paediatric patients is essential and require better understanding. A long-term study following infants and young children with CHB into adulthood reported low rates of HBeAg seroconversion before the age of 10 years and increased into the teenage years onwards(150). The annual spontaneous HBeAg seroconversion rate was reported at 1.70% before the age of 10; 3.78% between the ages of 10 and 20 years old and 4.02% between the ages of 20 and 30 years old in genotypes B and C CHB(150).

Most infected children experience the immune clearance phase with liver inflammation after their puberty onset(150,151). It has been reported that earlier onset of puberty and increased steroid 5-alpha reductase type II (SRD5A2) enzyme activity are linked to earlier HBeAg seroconversion in male CHB patients(152). Earlier menarche has also been linked with earlier HBeAg seroconversion in female cases with CHB infection(153).

There are clinical differences observed between male and female CHB patients, which are thought to result from cross-talk between different sex steroids and immune effectors. In males the main sex steroid present at puberty is testosterone and in females' oestradiol. Research using animal models has demonstrated that the androgen pathway can increase HBV transcription and suppress the tumour suppressor gene in early hepatocarcinogenesis(154,155). Also, the oestrogen pathway can repress HBV genes transcription(156). Hence, the suggestion that puberty onset in both genders triggers the immune clearance phase may not be answered by sex steroids alone. Other events during the peri-puberty period may contribute to the initiation of the immune clearance stage. An adrenarche marker known as Dehydroepiandrosterone sulphate (DHEAS) if elevated 2-3 years before puberty is strongly associated with the age of HBeAg seroconversion in both genders(157). DHEAS is a potent immune modulator in the human immune response to several infectious pathogens(158). Furthermore, higher DHEAS levels at mid-puberty has been associated with higher decay rates of HBV viral load and HBsAg concentration from min-puberty into adulthood. Therefore, endocrine factors, especially the DHEAS could partly be responsible for triggering immune clearance and liver injury in immune-tolerant CHB patients(157–160).

Immune factors: During HBV infection, the host immune response and its interactions with viral particles play an important role. Human T-lymphocytes identify HBV viral peptides presented by human leukocyte antigen (HLA) on antigen presenting cells. Differences in host immune response are often due to polymorphisms of HLA antigens(161). Several studies have shown that major histocompatibility complex (MHC) class II alleles HLA-DRB1*1301-02 are associated with protection against persistent HBV infection(149,162–164). Additionally, HLA-DP has been shown to have a protective role against CHB and viral clearance in a cohort of Japanese and Korean patients(165). Another long term study demonstrated that HLA class I antigen B61 and class II antigen DQB1*0503 are associated with earlier HBeAg seroconversion in Taiwanese children with CHB(166).

Cytokines are essential in the host immune response to pathogens. Cytokines can directly inhibit viral replication and can indirectly influence the predominant pattern of host immune response. Interferon-γ and tumour necrosis factor-α (TNF-α) may be involved in cell mediated anti-HBV response in children with CHB infection entering the immune clearance phase(167,168).

Furthermore, innate immune responses are also vital in the defence mechanisms relating to HBV infection. The toll-like receptor (TLR) signalling pathway especially has been shown to

activate downstream inflammation cascades such as nuclear factor κB, interferon regulatory factor, mitogen activated protein kinase and proinflammatory cytokines(169,170). CHB infected patients with TLR5 rs5744174 and C allele at TLR9 rs5743836 promoter area polymorphism have been observed to experience earlier spontaneous HBeAg seroconversion(170).

Viral factors: HBV genotypes have been documented to be predictors of the clinical course of CHB infection in paediatric patients. Genotype D has been associated with more violent liver damage than genotype A and more often led to HCC in cohort of Indian patients(171). Genotype C has also been reported to cause more severe liver injury and delayed spontaneous HBeAg seroconversion than genotype B in adults and children(172,173). Additionally, genotype Ba has been associated with the development of HCC in non-cirrhotic paediatric patients in Taiwan(172,173).

A long-term study where paediatric CHB patients where followed up long-term since entering the immune tolerant phase, found that mutations of core promoter at nucleotide position 1752, 1775 and 1799 was associated with HBeAg seroconversion; precore 1896 mutation is found on 50% of children who seroconvert to anti-HBe. Also, genotype C is linked to basal core promoter (BCP) 1762 and 1764 mutations during HBeAg seroconversion(149,174). The prevalence of HBV precore/core mutant strains has been shown to increase significantly in the immune clearance stage compared to the immune tolerant stage(174).

CHB infected children who experience HBeAg seroconversion have been observed to have decreased viral loads, persistent normal ALT levels and a milder course of disease after HBeAg seroconversion(175). However, patients who seroconvert in adulthood particularly after 40 years old show an increase in HBV viral load, HBeAg negative hepatitis flare, liver cirrhosis and HCC(175,176).

These findings suggest that host immune mechanisms along with HBV mutant selection during the immune clearance phase play a pivotal role in paediatric patients who experience HBeAg seroconversion. These factors ultimately may result in multiple clinical outcomes for CHB patients.

Host-virus interaction: The interactions between the host and the hepatitis B virus is important as it determines the course and severity of disease. The host viral relationship is strongly associated with the outcomes of the immune tolerant and the immune clearance phase

of infection and HBeAg seroconversion in paediatric patients. Furin a proprotein peptidase located at the endoplasmic reticulum membrane in hepatocytes, is utilised by HBV to drive the biosynthesis and maturation of HBeAg from 25-KDa protein to 17-KDa mature HBeAg(149,177). The inhibition of furin has been shown to inhibit the biosynthesis of mature HBeAg both *in vivo* and *in vitro*(177,178). Markers of immunologic tolerance PD-1 and PD-L1 have been linked to HBeAg seroconversion in CHB. It has been reported that blocking PD-1 and PD-L1 enhances the reactivation of HBV specific cytotoxic T lymphocytes (CTLs) and the secretion of interferon-γ by circulating intrahepatic lymphocytes linked to CHB infection and HBeAg seroconversion(149,179,180). The up regulation of the PD-1/PD-L1 pathway could result in mitigating pathogenic T-cell responses, reduce liver injury, avoid hepatocyte damage, and fulminate hepatic failure in CHB patients(179,181). Moreover, the up regulation of the PD-1/PD-L1 pathway and the down regulation of furin, could be vital in the process of transitioning from cytolytic to non-cytolytic HBV suppression inside the liver to avoid severe liver injury and failure(182).

Viral mutations in the immune clearance phase involving the precore/ core genes lead to host immune selection pressures. These mutations may alter the amino acid sequence, protein structure, antigenicity of HBeAg and HBcAg. The changes in HBcAg sequence and structure affects the stability of HBV nucleocapsid, pgRNA packaging and the efficacy and accuracy of viral replication(149). Research has shown that IL-10-1082 polymorphism site G/G genotype is linked to higher HBV C2189A mutations in the immune clearance phase and results in HBV DNA reduction(183). Additionally, the core protein P135Q and the precore 1896 mutants have been reported to be the most prevalent mutants present before HBeAg seroconversion in genotypes B and C CHB patients(149,183,184). Furthermore, the P135Q mutant strain has been reported to have active involvement in HBV capsid assembly, HBeAg biosynthesis and the reduction of host immune responses following HBeAg seroconversion(149,184).

The age at which HBeAg seroconversion occurs in paediatric patients and the severity of liver injury during the immune clearance stage are important factors in determining the clinical outcome of CHB infection. Extremely early HBeAg seroconversion before the age of 3 years along with severe liver injury increases the risk of childhood HCC(185–187). On the other hand, HBeAg seroconversion during childhood without severe liver injury has been linked to a relatively uneventful course with low viremia, reduced incidence of HBV reactivation after HBeAg seroconversion and even a higher chance of spontaneous HBsAg seroconversion(149,186,188). Research has shown that earlier breakthrough of immune

tolerance and earlier HBeAg seroconversion in CHB children are both significant predictors of spontaneous HBsAg seroconversion(189).

In conclusion early virological, serological, and biochemical activities that occur in childhood during chronic HBV infection may serve as important predictors for clinical outcome in adulthood. Gaining a deeper understanding of the mechanisms that trigger liver damage and their long-term effects may enhance the development of better therapeutic strategies and earlier intervention for patients with chronic CHB infection.

1.6 HBV diagnosis - Biomarkers in Hepatitis B infection

1.6.1 Existing biomarkers of HBV infection: The biomarkers used to identify HBV infection and its stages reflect its life cycle. The key molecule in HBV life cycle is cccDNA, which is first generated from incoming virons and exists as a stable minichromosome in non-dividing hepatocytes(190–192). Viral cccDNA is the template for transcription of all HBV RNAs and is responsible for the production of virions and subviral particles, which may be infectious or non-infectious. Serum biomarkers currently used in clinical practice to manage CHB and identify disease stages include quantitative HBsAg, HBeAg, HBcAb, HBV DNA and ALT (table 3). However, the classification and use of these biomarkers do not fully reflect the complexity of CHB or HBV intrahepatic activity.

Intrahepatic measurement of cccDNA and viral RNAs has the potential to improve CHB classification but involves using liver biopsy samples. Liver biopsies are invasive and are not part of routine CHB care. Also, only a small section of the liver is sampled so a biopsy may not represent infection in the whole liver, especially since HBV is unevenly distributed in the liver(190,193). There has been some recent development in producing quantitative polymerase chain reaction (PCRs) assays for cccDNA, but the coexistence of HBV replicative DNA intermediates in infected cells e.g. rcDNA and integrated HBV DNA molecules, interferes with the accurate quantification of cccDNA(190,194,195).

Biochemical markers:

HBV infection may alter the serum levels of certain hepatic enzymes and compounds such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, and albumin(196,197). These biomarkers have no direct link to liver fibrosis but they reflect liver dysfunction. During HBV infection elevated levels of these liver markers are observed apart from serum albumin which normally falls below its reference limit(196). ALT is the most common enzyme used in liver disease assessment. Elevated ALT paired with acute flare up may be seen in CHB patients and up to 2-4 times higher in infected pregnant women(198). Several studies done in Europe and Asia have shown that ALT levels higher than the normal limits are strongly associated with an increased risk of liver cirrhosis in CHB patients(199–202). In HBeAg negative patients, a high level of ALT greater than 0.5x to the upper limit of normal (ULN) is linked to advanced fibrosis(199).

Source	Clinical relevance during natural infection	Clinical relevance following treatment
HBV DNA		
Derived from reverse transcription of HBV pgRNA, exclusively transcribed from cccDNA; found intracellularly in capsids and extracellularly in infectious virions	Hallmark of infection; elevated concentrations are seen in high replicative and low inflammatory phase and a reduction is seen in the immune clearance phase.	Treatment-induced suppression correlates with lower risk of liver disease progression and HCC. HBV DNA suppression may not be sustained when treatment with NA is stopped as cccDNA persistence can lead to relapse. Resistance to NAs can lead to high concentrations of serum HBV DNA.
HBeAg and anti-HBe		
Immunomodulatory protein translated from preC mRNA. HBeAg appears early during infection, with anti-HBe antibodies appearing later	HBeAg status (positive or negative) is used to define CHB phases. HBeAg positive status is linked to high viremia, but negative status does not eliminate liver injury. Changing to HBeAg negative CHB is linked to the reduction of cccDNA activity. HBeAg loss is associated with reduced disease activity and higher titres of anti-HBe, with anti-HBc IgG, might reflect cccDNA activity in HBsAg-negative CHB	HBeAg loss and anti-HBe seroconversion can be indicative of virological remission, but the development of basal core promoter and/or precore mutations can lead to HBeAg-negativity with high viral load. HBeAg to anti-HBe seroconversion identifies a primary endpoint in peg-IFNα and NA therapy with cessation in patients who are HBeAg positive.
HBsAg		

Expressed from both cccDNA and integrated HBV DNA and embedded into the envelope of the infectious virions. It also forms non- infectious spherical and filamentous subviral particles.	CHB diagnosis is based on a positive repeat test for HBsAg. Highest HBsAg levels are linked to HBeAg-positivity and high replicative CHB phase. HBsAg titres modestly correlate with cccDNA levels and only in HBeAg positive CHB. HBsAg seroclearance occurs annually in 0.1– 2% of patients with CHB. HBsAg concentration can be a good predictor for HBsAg clearance. HBsAg loss is associated with better clinical outcomes in patients who are HBeAg−negative with low viral loads, higher HBsAg levels independently predict HCC development.	HBsAg is used as a stable endpoint for off-treatment sustained response for NAs and pegIFNα. HBsAg loss is rare but indicates immune control of viral replication associated with histological improvement.
Anti-HBs		
Neutralizing antibodies directed against HBsAg levels can be underestimated in CHB due to antigen/antibody complexes with high circulating HBsAg	Serum anti-HBs Indicates immunity to HBV following natural infection or vaccination. It is associated with ALT flares and achieving a functional cure during therapy.	May or may not be detected after HBV DNA suppression or HBsAg loss after antiviral therapy.
Anti-HBc (HBcAb)		
Non-neutralizing antibodies directed against the viral capsid.	Biomarker of natural exposure to HBV. Total anti-HBc is a marker of acute, chronic, and resolved HBV infection, or occult hepatitis B. It can be used with HBsAg to predict liver inflammation.	It can be used to predict HBV reactivation after immunosuppression. It is recommended to measure anti-HBc levels in patients suspected to have an acute exacerbation of CHB or to decide if patients who are planning to undergo immunosuppressive therapy will require prophylactic antiviral therapy

Table 3: Serological markers for chronic hepatitis B diagnosis and management(190).

Liver Biopsy: Liver biopsy remains the gold standard method for determining liver damage and identifying cirrhosis(203–206). It is a useful diagnostic tool particularly in assessing the severity of liver damage including inflammation, fibrosis stage and grade. Additionally, it can be used to identify precursor lesions of HCC. The histologic staging and grading systems used for chronic hepatitis prognosis and management include the Knodell(207), Ishak(208) and METAVIR(208) systems, with the METAVIR score being the most accepted globally. Current

guidelines do not recommend universal liver biopsy in all CHB patients as the procedure is invasive, painful, costly and may cause complications such as severe bleeding (especially intraperitoneal bleeding)(206).

When looking at the histology of CHB, there is a varying degree of mostly lymphocytic portal inflammation with interface hepatitis and spotty lobular inflammation (Figure 7). Patients in the immune tolerant and inactive carrier phase present with minimal inflammation, but inflammation is prominent in the immune reactive phase. Chronic Hepatitis B patients may also show bridging necrosis, which is severe inflammation connecting portal tracts to each other or to central veins (Figure 8)(209). Additionally, confluent necrosis may be seen where multiple hepatocytes are affected (Figure 9)(209). Liver inflammation is generally associated with scarring which might involve mild portal expansions, periportal fibrous strands, bridging fibrosis and cirrhosis (Figure 10)(209). A higher fibrosis stage is usually seen in livers with central to portal bridging necrosis or confluent necrosis. Hepatocytes with high levels of HBsAg may have “ground-glass” cytoplasm which can be seen using immunohistochemical stains (Figure 11)(209).

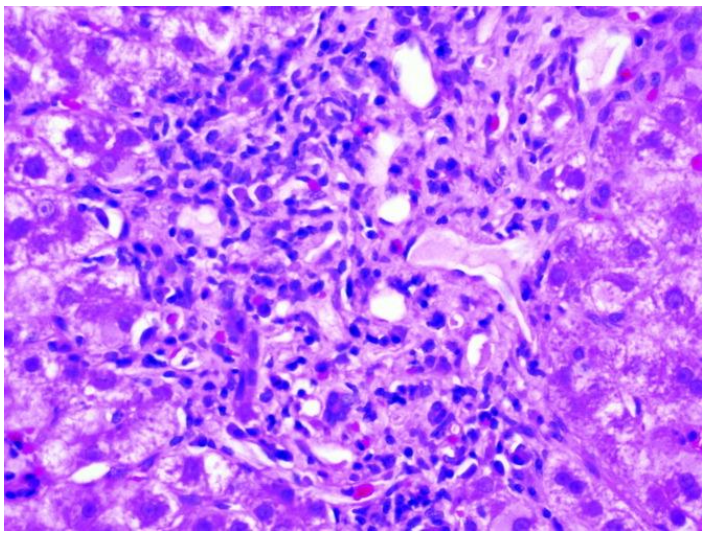

Figure 7: Biopsy of CHB patient liver. Cells are stained with Haematoxylin and eosin. There is mild inflammation with focal interface hepatitis. Eosinophils and plasma cells are present.

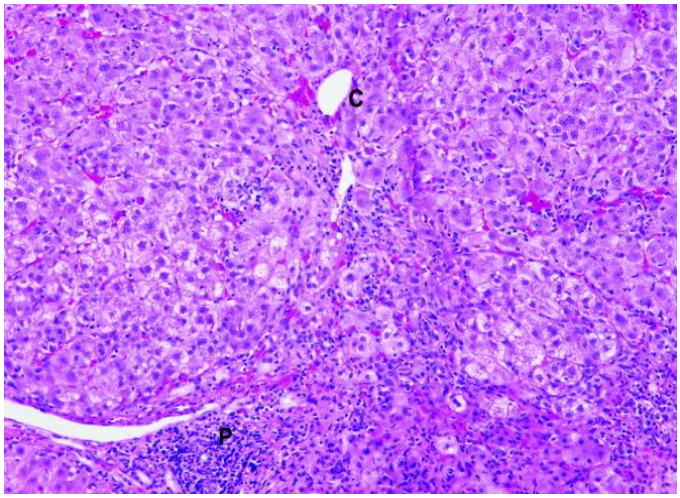

Figure 8: Biopsy of CHB patient liver. Cells are stained with Haematoxylin and eosin. There is severe inflammation, with portal (P) to central (C) bridging necrosis.

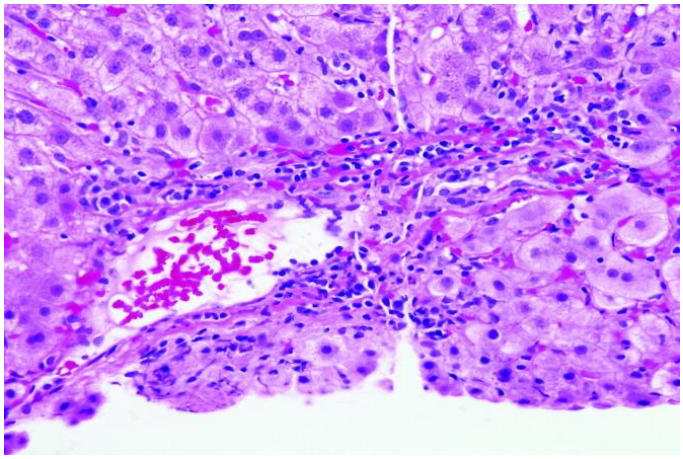

Figure 9: Biopsy of CHB patient liver. Cells are stained with Haematoxylin and eosin. Confluent necrosis may appear as zonal dropout of hepatocytes with residual inflammation.

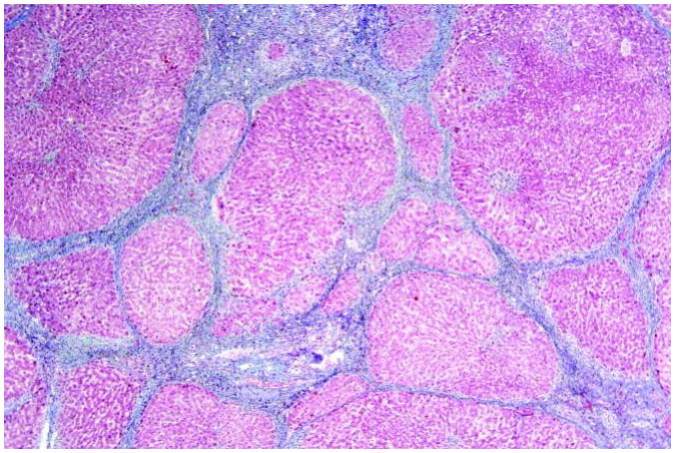

Figure 10: Biopsy of CHB patient liver. Cells are stained with Masson's Trichrome. Liver cirrhosis, fibrotic bands with loss of architecture of nodule formation.

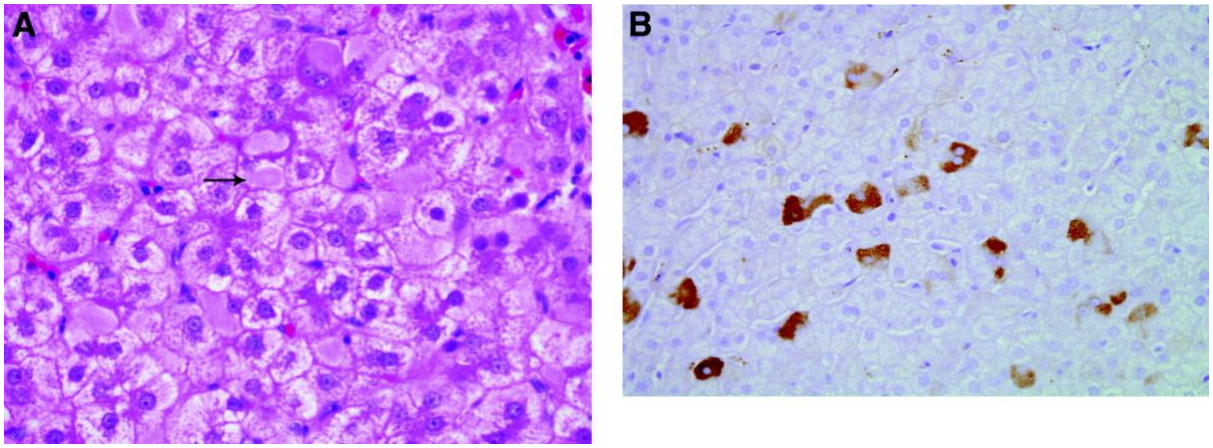

Figure 11: Biopsy of CHB patient liver. Cells are stained with A) Haematoxylin and eosin, B) HBsAg immunostain. Cells rich in HBsAg have a ground-glass appearance.

1.6.2 Novel biomarkers of HBV infection: Viral cccDNA is the key genomic form that is responsible for persistent infection. It persists in hepatocytes of infected patients even after long-term NA treatment and after HBsAg loss and seroconversion. The regulation of the intrahepatic pool of cccDNA is complex and depends on the dynamics of infection in the liver and the intrahepatic antiviral immune response(87,210). Moreover, cccDNA transcriptional activity is controlled by specific epigenetic regulation involving viral and host interactions. Besides the need for standardised assays, the major limitation of cccDNA studies is the

requirement of liver biopsy; thus, the urgency for surrogate biomarkers(87,211). It is noteworthy that not all transcripts are expressed from cccDNA but can also be expressed from viral sequences integrated in the host genome(87). Viral genome replication however cannot result from integration, but HBsAg expression can occur. HBsAg then possesses the advantage of being expressed either from the envelope gene in cccDNA and/or in viral integration, which evidently makes quantification of HBsAg an unreliable biomarker for intrahepatic cccDNA(87,212). Quantification of cccDNA and its transcriptional activity is at the epicentre of novel HBV treatment and potentially finding a cure for HBV infection.

Hepatitis B core-related antigen (HBcrAg) is a composite biomarker consisting of antigens expressed from the precore/core gene: HBcAg, HBeAg and prec22. The HBcrAg associated peptides can also be detected in circulating hepatitis B virions (Dane particles) and in HBV DNA negative Dane particles containing the 22KDa precore protein(213). The concentration of these HBcrAg proteins have been reported to exceed Dane particles by 100 fold and may be present in pregenomic RNA containing virions(87,212). HBcrAg does not overlap with HBsAg quantification, and importantly it is not affected by translation from integrated viral sequences. Therefore, HBcrAg may be a useful marker in providing additional information relating to the translational activity of cccDNA beyond HBsAg quantification and potentially be utilised in the management of CHB infected patients. Several studies have demonstrated that serum HBcrAg levels reflect intrahepatic DNA and cccDNA activity in hepatocytes particularly in HBeAg positive cases(214,215). It could also be useful in defining the phase of CHB infection, especially in HBeAg negative cases, as well as predicting the long-term HCC risk(215,216). Additionally, some research suggests that HBcrAg levels can be used to monitor antiviral treatment response and predicting therapeutic efficacy including the risk of relapse after discontinuing NA therapy(87,217–219).

Another novel biomarker that has been associated with cccDNA activity is HBV RNA. Circulating HBV RNA was first reported in 1996 and was later identified as a potential marker for monitoring CHB patients on NA therapy. HBV RNA can exist in the form of enveloped pregenomic RNA (pgRNA) containing virions and has been reported to have a strong correlation with intrahepatic cccDNA. For this reason, HBV RNA is an interesting biomarker for quantifying cccDNA activity(87,220,221). HBV RNA quantification may be useful in predicting viral rebound after stopping treatment with NAs(87,221).

Further research into novel biomarkers for HBV infection is required. The simultaneous testing of different replicative, transcriptional and translational HBV biomarkers will allow for a better

understanding of CHB infection including the different phases and the possibility of predicting long-term treatment outcomes.

1.6.3 Biomarkers of liver cancer

Chronic HBV infection can progress to liver cancer, which is the sixth most common cancer and the third leading cause of cancer related deaths in the world. Hepatocellular carcinoma (HCC) has poor prognosis, with the incidence and mortality rates being similar. It is more common in men (up to 4 times higher incidence than in women). The median survival of patients with early HCC is >5years but <1 year when detected at an advanced stage(190,222). Unfortunately, most HCC cases are detected at advanced stages, mainly due to limited biomarkers for its detection(222). This significantly reduced treatment options.

HCC surveillance relies on the limited amount of serum biomarkers and/or imaging of the liver. Cancer markers are generally tested for in infected blood, urine and other bodily fluids to indicate the presence of cancer or to predict the risk of developing HCC(190). Considering the severity of HCC, better detection is required. The ideal marker should enable early detection of HCC through screening healthy or high-risk individuals, predict prognosis, monitor response to therapy and detect early recurrence(190,222).

The serum marker mostly used for HCC surveillance is alpha-fetoprotein (AFP). It is a useful marker for diagnostic and prognostic purposes. Additionally, it has been utilised to assess the risk of cirrhotic patients developing HCC, as well as a screening tool for early detection of HCC(190,223). In addition to AFP, other novel biomarkers for HCC surveillance are being investigated including osteopontin, midikine, dikkopf1 and squamous cell carcinoma antigen which are all in phase 2 and need validating(190,224,225). Novel markers beyond phase 2 trials include *Lens culinaris* agglutinin fraction of AFP (AFP-L3) and des-γ-carboxy prothrombin (DCP)(222). Genetic and cellular biomarkers are also promising such as circulating tumour cells, circulating tumour DNA, microRNA and long non-coding RNA(226). Furthermore, novel markers HBcrAg and pgRNA have been associated with predicting HCC in CHB patients. Research has shown that elevated levels of HBcrAg increases the risk of developing HCC in CHB patients without NA therapy (227,228). Additionally, detectable pgRNA in patient serum is associated with disease progression to HCC(229,230).

Although the detection of AFP in serum is the most common biomarker used for HCC surveillance, its effectiveness is limited as not all HCCs secrete AFP. Using AFP alone could

result in under/ missed diagnosis. Also, AFP levels can be elevated in patients with CHB or cirrhosis(231). However, with the use of highly effective NAs for treatment of CHB, high concentrations of AFP in patients on treatment have been demonstrated in a large retrospective-prospective study to be a specific biomarker for HCC as falsely elevated AFP levels in 1,531 patients receiving entecavir were minimised compared to 424 cases without treatment, indicating that in this cohort, a lower AFP cut off value may be used(190,232). Elevated on-treatment AFP is a specific tumour marker for HCC in CHB patients on entecavir treatment(190,232). Another limitation of AFP as a biomarker for HCC is its suboptimal sensitivity (39-65%) and specificity (76-97%)(190,233). On the other hand, several studies have shown that combining AFP with ultrasonography is beneficial(234).

1.6.4 Immunological biomarkers - Interferon-γ-inducible protein and Programmed Cell Death Protein-1

Interferon-γ-inducible protein 10 (IP-10)

Interferon-γ-inducible protein 10 (IP-10), also known as chemokine C-X-C motif ligand (CXCL) 10, is associated with inflammation(235). It is secreted from leukocytes, neutrophils, eosinophiles, monocytes, epithelia, endothelial and stromal cells and keratinocytes in response to IFN-γ(235–237). CXCL10 binds to CXCR3 receptor which is mainly expressed on activated T, B lymphocytes, natural killer, dendritic and macrophage cells to induce chemotaxis, apoptosis, cell growth and angiostasis(235,238,239). Abnormal concentrations of IP-10 have been observed in different bodily fluids of patients infected with viruses, bacteria, fungi and parasites, suggesting an important role in the pathogenesis of these diseases(239–243).

CXCR4 and chemotaxis

There are three CXCR3 receptors ligands (CXCL9, CXCL10, and CXCL11) and they have all shown to induce chemotaxis in a variety of cell types of the immune system(235,244). CXCL10 performs “homing” functions to chemoattract CXCR3-positive cells (macrophages, dendritic cells, NK cells and activated T lymphocytes) toward inflamed, infected and/or neoplastic sites(235). CD4+ T helper (Th) cells can either be type 1(Th1) or type 2 (Th2)(235,245). Th1 cells produce cytokines, including IL-2, IFN-γ, and lymphotoxin-α, which activates

macrophages and the process of opsonization and cytotoxicity. Whereas Th2 cells are considered to play a regulatory rather than protective role, since cytokines produced by these cells (IL-4 and IL-13) inhibit the production of Th1 cytokines and activation of macrophages. Since TH1 cells produce IFN-γ which induces the production by several cell types of CXCL10, enables CXCL10 to attract and recruit TH1 cells, indicating the process of a positive feedback loop between IFN-γ producing TH1 cells and resident cells producing CXCL10(246). The chemotactic action of CXCL10 on activated lymphocytes enable it to modulate both the innate and adaptive immune systems, causing tissue damage and modulating tumour formation(244,247).

CLCL10 mediated apoptosis

Apoptosis mediated by CXCL10 occurs under varied conditions. For example, neuronal apoptosis is mediated by over expression of CXCL10 in simian human immunodeficiency virus encephalitis through the activation of caspase-3(248). CXCL10 may also be involved in apoptosis during development of the nervous system and myeloma, but the mechanisms are not clearly understood. CXCR3 has two isoforms (CXCR3A and CXCR3B) and they have been shown in different cell lines to determine the selectivity of CXCL10 for cellular proliferation or apoptosis depending on the ratio at which they appear(235,249).

CXCL10 regulation of cell growth and proliferation

CXCL10 has dual effects on cell growth. The effect that CXCL1 has on proliferation or anti-proliferation seems to be cell-type dependant, meaning it may rely on the subtype of its receptor CXCR3(235,250). Different CXCR3 variants (CXCR3 – A, B, and alt) are expressed by different cell types, resulting in divergent effects on proliferation marker cytokeratin 17 (K17) in tumour cells(235,250). Its effects on cell growth are cell cycle dependant. CXCR3-A is the most common isoform found in most cells and it codes for a protein of 368aa and joins with Gαi protein to activate ERK1/2, p38/MAPK, JNK and PI3-kinase/Akt signalling pathways, leading to the induction of intracellular calcium influx, DNA synthesis, and cell proliferation or chemotaxis(250,251). Unfortunately, studies on the interactions of CXCL10 and CXCR3-A in infectious disease are underdeveloped. The anti-proliferative function of CXCL10 is regulated by CXCR3-B(235). However, the mechanisms mediating CXCR3-B effects in the context of infectious diseases are not well understood.

CXCL10 and regulation of angiostasis

CXCL10 has dual effects on angiogenesis, depending on the presence or absence of the Glu–Leu–Arg (ELR) motif. ELR-negative CXCL10 is an angiostatic chemokine, which inhibits angiogenesis(235,243). CXCL10 also exerts important effects in anti-tumour effects. For example, in xenograft models of lymphoma, squamous cell carcinoma (SCCA) and adenocarcinoma of lung, the production of CXCL10 has been reported to inversely correlated with tumour growth, which resulted in marked reduction in tumour-associated angiogenesis(252,253). Additionally, CXCL10 induces angiostatic action in a T-, NK-cells or macrophage independent manner.

Programmed Cell Death Protein 1

Programmed cell death protein 1 (PD-1), also referred to as CD279 is found on T cells. PD-1 is necessary for the inhibition of immune responses and the promotion of self-tolerance via modulating T cell activity, activating apoptosis of antigen-specific T cells and inhibiting apoptosis of regulatory T cells(254,255). Programmed cell death ligand 1(PD-L1) is a trans-membrane protein that is a co-inhibitory factor of the immune response. When PD-1 binds to PD-L1 it prevents T cells from killing other cells including cancer cells. Thus, the combination of PD-1 and PD-L1 reduces the proliferation of PD-1 positive cells, inhibits their cytokine secretion, and induces apoptosis(255,256).

PD-1 can inhibit both the innate and adaptive immune responses, and is expressed on activated T, NK and B lymphocytes, macrophages, dendritic cells, and monocytes. The transcription of PD-1 can be triggered by transcription factors such as the nuclear factor of activated T cells (NFAT), NOTCH, Forkhead box protein (FOX) O1 and interferon regulatory factor 9 (IRF9)(257,258).

The conserved upstream regulatory regions B and C (CR-B and COR-C) are vital for the expression of the PD-1 gene. The CR-C region contains a binding site that is connected to NFATc1 (NFAT2) in TCD4 and TCD8 units. Alternatively, c-FOS binds to sites in the CR-B region and intensifies PD-1 expression when it stimulates T-cell receptors upon antigen detection in naive T cells(255). NFATc is activated and binds to the promoter region of the

pdcd1 gene. Moreover, IFN-α combining with IRF9 may lead to PD-1 expression through binding to the promoter of the pdcd1 gene in exhausted T cells. During chronic infections, PD-1 is expressed in exhausted TCD8 cells due to its demethylated promoter(259). The FOXO1 transcription factor binds to the PD-1 promoter and amplifies its expression(259). Interestingly, PD-1 can play opposing roles, as it can be both beneficial and harmful. Its beneficial role involves lowering the regulation of ineffective or harmful immune responses and maintaining immune tolerance. However, PD-1 can cause the dilation of malignant cells by interfering with the protective immune response(257).

HBV infection and PD-1

Programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) are receptors of the CD28 family of co-stimulatory molecules that provide inhibitory signals to T-cells(254). In chronic HBV, the upregulation of PD-1 and CTLA-4 is associated with T cell exhaustion and persistent viral infection. Additionally, intrahepatic T cells upregulate BTLA and produce IL-10 which can inhibit effective T cell function(254,260). Furthermore, HBsAg-specific B cells that fail to mature *in vitro* into antibody secreting cells and also display a high expression of PD-1, could be partially boosted by the addition of anti-PD-1(261). Research by Martinez et al demonstrated for the first time that the upregulation of the PD-1: PD-L1 axis in patients with CHB is not normalised in patients treated with NAs with undetectable viremia for long periods of time(262). Additionally, anti-PD-L1 blockade increased both the number of IFN-γ-producing T-cells and the number of IFN-γ produced per cell in all patients with detectable HBV reactivity, irrespective of their clinical and treatment status. Moreover, *ex vivo* studies using blood from individuals with CHB infection have demonstrated that inhibition of PD-1, CTLA-4 and TIM3 results in enhanced HBV-specific CD8+ T-cell function(261,263).

1.7 The role of covalently closed circular DNA in HBV infection

Chronic hepatitis B infection is characterised by the persistence of the episomal viral genome, the covalently closed circular DNA (cccDNA), which forms a stable mini-chromosome in the nuclei of infected hepatocytes(264). It is impossible to complexly clear the virus and many patients relapse after discontinuing antiviral therapy. This is due to the longevity of the cccDNA and the inability of the immune system to create effective immune responses against the virus(264). Thus, the single most important way to achieve a true cure for CHB infection is to eliminate cccDNA from infected hepatocytes. The route to curing CHB would require better understanding of the role of cccDNA in HBV maintenance, fully understanding cccDNA formation, its transcriptional regulation, turnover and intracellular stability.

HBV cccDNA Establishment

The HBV virus is a small blood-born pathogen transmitted by percutaneous exposure to infected blood or body fluids. Upon transmissions the virus travels through the bloodstream and navigates to the liver to infect hepatocytes. Hepatocytes are the only target cells susceptible for infection(264). Thus, HVB is associated with high tissue and species specificity, as well as a unique genomic organisation and replication cycle. Entry into hepatocytes involves an irreversible binding of the virion to Na^+-taurocholate cotransporting polypeptide (NTCP), which is a hepatocyte- specific receptor(264,265). The viral genome is then transported to the nucleus of the hepatocyte, this ensures a productive infection. The processes following viral entry are still poorly characterised, but in vitro studies have reported the involvement of endocytosis, and microtubule mediated transport of the nucleocapsids to the nuclear envelope(264,266). Interactions with nuclear transport receptors and adaptor proteins of the nuclear pore complex, leads to the capsids disintegrating permitting the release of both core capsid subunits and the relaxed circular DNA (rcDNa) genome(267).

Although the mechanisms responsible for the conversion of the incoming rcDNA form the supercoilded cccDNA molecule are not fully understood, they seem to take place via a muti-step process, and they require the cellular DNA repair machinery(264,268). First, the covalently attached viral polymerase is removed, leading to the formation of a protein-free rcDNA (PF-rcDNA) intermediate, which may persist in infected cells(269). Furthermore, cccDNA synthesis needs the removal of an RNA primer from the positive strand, removal of

terminally redundant sequences from the negative strand, finally the repair of the incomplete positive strand before both DNA strands are ligated(211,264). Therefore, the complex establishment of the episomal, plasmid-like form of the HBV genome heavily relies on the specific interactions between cellular components such as the DNA polymerase κ which has been shown to contribute substantially to the completion of the positive strand DNA in rcDNA(264,270). Additionally, the cellular DNA repair enzyme tyrosyl-DNA-Phosphodiesterease 2 (TDP2) has been shown to release the covalently bound viral polymerase from HBV and duck HBV (DHBV) rcDNA in vitro, and a physiological role in cccDNA synthesis has been suggested because human cells with stable knockdown of TDP2 expression significantly slowed down the rate at which DHBV rcDNA was converted to cccDNA. This phenotype was identified in TDP2 gene knockout cells(271). However, human HBV still had the ability to infect some of the knockout cells, indicating that TDP2 is at least not essential for HBV cccDNA formation(264,272). It is important to identify all host factors that affect the mechanism of cccDNA synthesis as well as viral entry. These will be crucial to the progress in the development of curing CHB.

HBV cccDNA activity and pool size

Within the nucleus, the cccDNA interacts with histone and non-histone proteins for the transcription process(273). Transcription is controlled by two enhancer elements and four distinct promoters, and it is dependent on the activity and dynamic interactions of several transcription factors, co-activators, co-repressors and chromatin modifying enzymes(210,264). The cccDNA contains several binding sites for ubiquitous and liver specific transcription factors which have been reported to be actively involved in the transcription of viral RNAs(274). Nevertheless, the molecular mechanisms that regulate cccDNA activity in infected primary hepatocytes is still not fully understood. Research using hepatoma cell lines, suggest that cccDNA transcription is regulated by the acetylation status of cccDNA-bound H3 and H4 histones and indeed, in HBV-infected patients, histone hypoacetylation and histone deacetylases 1 recruitment onto the cccDNA correlates with reduced viremia(20,264). A chromatin immunoprecipitation sequencing tool was used by Tropberger et al to map post-translational histone modifications across the entire HBV genome in HBV infected HepG2-NTCP cells, primary human hepatocytes and liver biopsies revealing and atypical chromatin structure(275). There are similarities in the distribution and levels of active histone modifications to cellular chromatin and they are enriched at HBV promoters, but there is an underrepresentation of repressive marks even at silent promoters. Given the remarkably

different format of the HBV genome, such as its circular conformation, its small size and compact organisation of transcripts and regulatory components, it is not surprising that there are also distinct differences in its epigenetic regulation(264). The precise nature of these differences and if the epigenetic regulations will be amenable to future antiviral therapies is unknown. In addition to cellular factors, viral proteins also play a role in cccDNA activity. The viral core protein is a vital structural component of cccDNA mini-chromosome responsible for the reduced nucleosomal spacing on the cccDNA compared to cellular chromatin(276). Thus, core proteins may play a role in regulating viral transcription.

Research has also shown the non-structural regulatory hepatitis B X protein (HBx) to be recruited to the cccDNA mini-chromosome, and it is needed to initiate cccDNA-driven transcription of the viral RNAs(25,26,264). Furthermore, studies have shown that HBx mediates the degradation of the structural maintenance of chromosomes (Smc) complex Smc5/6(29,30). When HBx binds to the damaged DNA binding protein (DDB1), it triggers the interaction between smc5/6 with a component of the ubiquitn proteasome system, the E3 ubiquitin ligase known as Cul4, to activate ubiquitination and degradation of the Smc5/6 complex(264). Since the Smc5/6 is involved in chromosome organisation and DNA repair, it may bind to cccDNA and act as a host restriction factor suppressing cccDNA transcription. These finding indicate that during cccDNA regulation, the cellular transcriptional machinery and the non-structural HBx protein play an active role.

When the cccDNA mini-chromosome is formed in infected liver cells, viral replication may initially take place without raising the attention of intrinsic antiviral defences(277–279). The cccDNA is the template of transcription for five viral RNAs required for antigen production and viral replication. Viral replication occurs in the cytoplasm after reverse transcription producing an over-length pregenomic RNA (pgRNA) in the newly formed nucleocapsids(280). The mature rcDNA-containing nucleocapsids are enveloped and secreted into the bloodstream as progeny viruses. HBV have developed advantageous traits to enable it to survive in human livers without raising an alarm to the host immune system. The virus camouflages its genome as a mini-chromosome, hijacking the cellular transcriptional machinery to aid in its replication, and it conceals the production of new virions inside the nucleocapsids, therefore reducing the possibilities for the host to recognise the infection(264).

The transcriptional activity of cccDNA can be determined by measuring pgRNA concentration in infected livers. To avoid the need for live biopsies, circulating antigens such as HBcrAg can also be utilised as serum markers of cccDNA activity to assess disease progression or response to antiviral therapy(4). In addition to DNA containing viral capsids, HBV RNA is present in the serum of CHB infected individuals in the form of pregenomic RNA encapsidated and enveloped in virus-like particles(264). Serum pgRNA has been reported to reflect the amount of pgRNA present in the whole liver. Hence, quantification of serum pgRNA is crucial as it may serve as a surrogate marker to accurately determine the presence of transcriptionally active cccDNA(220,264,281).

Impact of antiviral therapy on cccDNA

The half-life of cccDNA has not been clearly defined. However, in vitro studies suggest that the viral mini-chromosome is very stable in non-dividing human hepatocytes, where it can survive for the life span of the cell(282,283). This makes it extremely difficult to eliminate the cccDNA from the infected liver. Total elimination would require either the destruction of the infected liver cells or the induction of substantial cccDNA destabilisation.

Currently approved treatments for CHB based on nucleos(t)ide analogues (NAs) are effective in disrupting HBV reverse transcription leading to the reduction of viremia even below the detection limits in some cases. However, NAs do not directly target cccDNA, so long term antiviral therapy is required to achieve a significant reduction of the cccDNA pool(123,284–286). In a large cohort of human immunodeficiency virus (HIV)-HBV coinfected patients on long term antiviral therapy with tenofovir, a steady decrease of cccDNA was observed and a half-life of approximately 26 months was reported in HBeAg negative patients and 9 months in HBeAg positive patients(287). The reason for the decline in cccDNA pool is not fully understood, but it could be due to multiple factors including the lack of incoming viruses from the blood and insufficient recycling of viral nucleocapsids to the nucleus because of the strong disruption of viral DNA synthesis in the cytoplasm(264). Despite HBV DNA levels being undetectable, cccDNA persistent within the hepatocytes is the main cause of relapse of viral activity after discontinuation of antiviral therapy in CHB patients. Interestingly, it has been recently shown that the occurrence of a transitory hepatic flare after cessation of long-term therapy was beneficial to treatment outcome in CHB cases(288,289). It seems plausible that the abrupt cessation of therapy in some patients on long term NA therapy along with the concomitant restoration of viral replication may trigger the activation of the immune system

resulting in recognition and destruction of HBV infected liver cells. if so, cell destruction during hepatic flares could significantly contribute to the reduction of cccDNA activity and viral antigen concentrations, which may lead to gaining immunological control.

Other mechanisms that have been associated with cccDNA clearance are cytopathic and non-cytopathic cytokine mediated mechanisms. Cytokines involved in anti-HBV immunity have been reported to inhibit viral replication and may assist in cccDNA destabilization(18,223,290–293). Amongst these, interferon α (IFN-α) has shown promising characteristics such as the ability to accelerate pgRNA degradation and core particle decay in HBV transgenic mice(294–297). Moreover, in vitro studies and studies in HBV infected humanised mice demonstrated that IFN-α can lower the concentrations of both pregenomic and subgenomic HBV RNA by inducing epigenetic modifications of the histones bound to the cccDNA mini-chromosome(298). In line with these findings, chromatin immunoprecipitation-Sequencing (CHIP-Seq) experiments have shown a depleting of active histone marks upon IFN-α administration, which could be recapitulated with a small molecule inhibitor of the responsible histone acetyltransferase suggesting that lower rates of HBV replication and low levels of active histone marks are functionally associated(264,275). These studies indicate that by focusing on cccDNA transcription, IFN-α can directly aid in the reduction of viral antigen levels (HBeAg, HBsAg). Furthermore, IFN-α administration was also shown to promote partial cccDNA depletion via the up regulation of cytidine deaminases and nuclear factor κ-light-chain-enhancer of active B cells (NFκB) pathways(290). It has been shown in CHB patients that one year of combination therapy with polymerase inhibitor and IFN-α can induce a stronger cccDNA reduction up to 2-logs than using monotherapy with polymerase inhibitors alone.(264,299,300). Unfortunately, even with such promising findings, interferon treatment induces immune clearance in only a minority of CHB patients. Consequently, the rate of HBsAg seroconversion remain very low, and the infection typically relapses following treatment discontinuation. Furthermore, a great disadvantage of IFN-based therapy is its association. with systemic side effects and contraindications. (290,291,301).

The elimination of cccDNA from infected liver cells remains a challenge, thus more research is required to develop methods of determining cccDNA activity and novel therapeutic approaches boosting HBV-specific immune responses or agents directly targeting cccDNA. Immune factors such as IFNs and tumour necrosis factor α are crucial in promoting non-cytolytic inhibition of HBV replication and contribute to cccDNA destabilization(18). It is interesting that even after acute infection low concentrations of cccDNA can still be detected

in the liver, this means that not the entire intrahepatic cccDNA reservoir needs to be eliminated to gain immunological control and resolve the infection(302).

1.8 Liver Cirrhosis and Hepatocellular Carcinoma

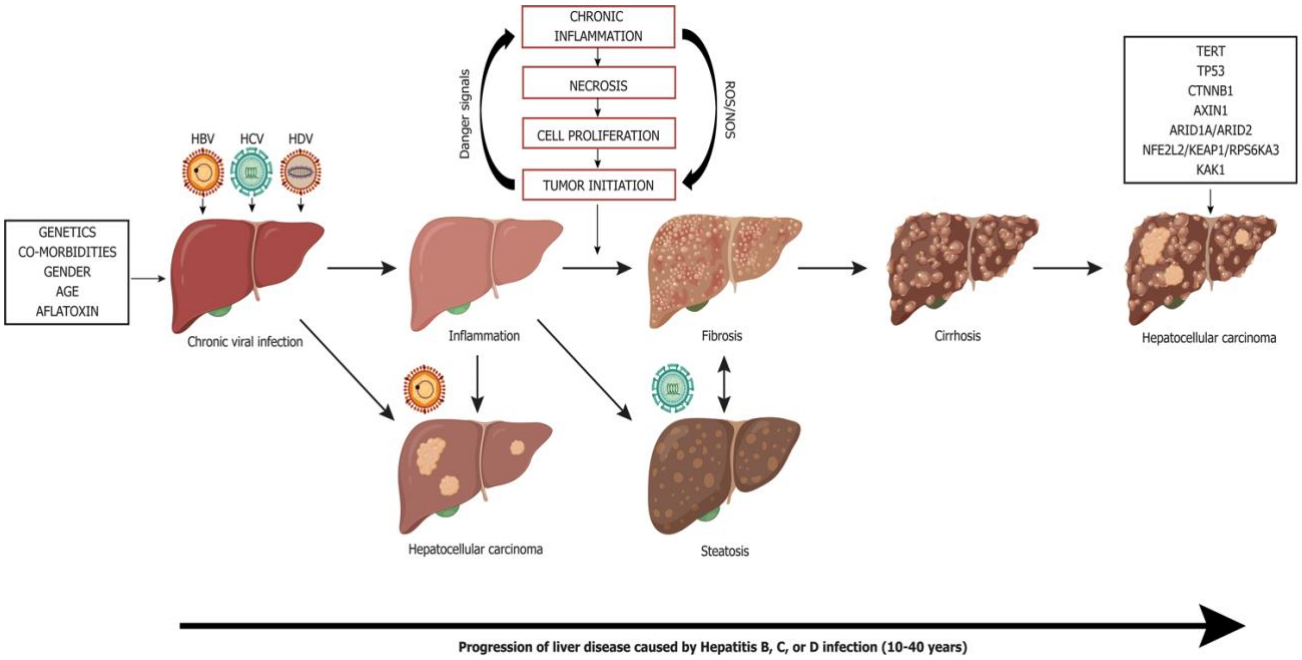

Figure 12: A schematic diagram of disease progression from Chronic HBV infection to cirrhosis and hepatocellular carcinoma.

HBV is an oncogenic virus that increases the risk of developing hepatocellular carcinoma (HCC) directly via viral mechanisms and indirectly via liver inflammation and cirrhosis. The development of cirrhosis and HCC are critical milestones in the natural history of CHB infection. When CHB individuals with cirrhosis decompensate, their quality of life and prognosis becomes significantly poorer.

Hepatitis B virus (HBV) can cause HCC through direct and indirect mechanisms. HBV DNA integration into the host genome occurs early on in infection during clonal tumour expansion and induces both genomic instability and direct insertional mutagenesis of diverse cancer related genes(303). Prolonged expression of the viral regulatory protein HBx and/or mutants of the preS/S envelop protein dysregulates cell transcription and proliferation control and

sensitizes liver cells to carcinogenic factors(303). Hepatocellular transformation can occur when preS1 envelope proteins and/or preS2/S mutant proteins accumulate triggering the unfold proteins response. Epigenetic alterations targeting the expression of tumour suppressor genes happens early in the development of HCC. The HBx protein plays a critical role via being recruited on cellular chromatin and modulates chromatin dynamics at specific gene loci(303). Compared with tumours linked with other risk factors, HBV associated tumours have an increased rate of chromosomal alterations p53 inactivation by mutations and overexpression of foetal liver/hepatic progenitor cells genes(303). Furthermore, the WNT/β-catenin pathway is often activated but HBV associated tumours show a low rate of activating β-catenin mutations(303). HBV-related HCCs may arise on non-cirrhotic livers, further promoting the idea that HBV is directly linked to liver transformation by triggering both common and etiologic specific oncogenic pathways as well as stimulating the host immune response and driving liver chronic necro-inflammation.

Several key factors are involved in hepatocyte transformation, HCC development and progression including; chronic inflammation, DNA damage, epigenetic modifications, senescence and telomerase reactivation, chromosomal instability and early neo-angiogenesis. Majority of HCCs develop in the context of liver cirrhosis, which is classed as a pro-carcinogenic field, but HCC can also develop in non-cirrhotic livers(303). Etiologic factors seem to act through similar mechanisms that converge to affect common pathways. This normally involves point mutations, chromosomal aberrations and epigenetic changes. Notably, mutations and chromosomal aberrations are mostly found in benign and malignant tumour tissues whereas the dysregulation of signalling pathways and epigenetic changes have been detected earlier in the natural history of HCC development, at the stage of cirrhosis(303).

Viral factors are also associated with HCC development. Results from a large Taiwanese cohort with HBV related HCC demonstrated that HBsAg concentrations were positively correlated with incidences of HCC in patients with lower levels of HBV DNA(304). The HBV genotypes have also been associated with the risk of developing HCC. HBV genotype C has been associated with an increased risk of cirrhosis and HCC(305) Moreover, other studies have suggested that genotype B is linked to a predisposition to HCC within children and non-cirrhotic patients(173,306). A high viral load and HBeAg seropositivity also confer increased HCC risk. In a large Taiwanese study where CHB patients were followed for 10 years HBeAg seropositivity was an independent predictor of HCC(307). The emergence of the double

mutation T1762/A1764 in HBV genotypes B and C has also been associated with an increased risk of developing HCC.

Persistently high viral load and ALT are strong independent indicators of HBV related HCC. The significance of HBV replication in HCC pathogenesis was demonstrated in the REVEAL study done in Taiwan. A significant increase in the risk of developing cirrhosis and HCC was associated with high concentration of HBV DNA in CHB patients(308). Chronic replication of HBV increases the risk of progression to cirrhosis and HCC(309). The incidence of developing HCC in non-cirrhotic CHB patients is increased compared to the general population and varies depending on geographical region: ranging from <0.2% per year in European countries to 0.4-0.6% per year in Asian countries(310). There is a further increased risk of HCC in CHB individuals with cirrhosis by over 10-fold. One-third of CHB patients with cirrhosis develop HCC in their lifetime. Among CHB cirrhotic patients, the annual incidence of HCC is 2–3% in western countries and 6–11% in Asian countries(233,311). Regarding gender the incidence rate of HCC are 2-3 times greater in men than in women worldwide(312).

Chapter 2: Research objectives

I am investigating the role of viral and serological markers in predicting liver disease progression. I am focusing on new markers of HBV transcriptional activity of cccDNA including HBcrAg and HBV RNA (pgRNA). The main objective of my research is to demonstrate that novel biomarkers HBV RNA and HBcrAg can be utilised in CHB management, and the quantification of these markers can help predict the progression of liver disease in patients who acquired HBV perinatally and were followed up to adulthood.

The power of predictability is a wonderful thing, to be able to predict the status of disease in 5 years, 10 years or more could really influence how it is managed. Simply having an idea of what could happen will help in the planning of how to manage or cure a disease. HBV pgRNA, and HBcrAg are game changing biomarkers as they have been directly linked to cccDNA transcriptional activity. Utilising these markers could help identify the risk of HBV reactivation in HBsAg negative patients and in HBeAg negative patients as well as determine the safest time to initiate and discontinue antiviral therapy. These novel markers could also help distinguish between truly HBeAg negative infection and other hepatitis phases that cannot be clearly defined (i.e. grey zones). Additionally, these markers could be useful in identifying which patients are likely to develop liver damage, in the future despite their HBV DNA being <20IU/mL and their HBsAg reported as undetected, or even when seroconversion had occurred. These markers can be used to create a pool of patients that are closely monitored based on their HBV RNA, and HBcrAg. It has been reported that even after HBsAg seroconversion has occurred HBV RNA and HBcrAg can still be detected(313,314).

These markers could provide insight into the serological activities that occur in chronic hepatitis B patients from childhood into adulthood. Investigating these new markers alongside well-known HBV markers (serology and virology markers: HBsAg, HBeAg, HBV DNA, HBV genotype and liver markers: ALT, AST, biopsy) could answer the question of why approximately 40% of children born with HBV with little/no liver damage go on to develop liver disease (cirrhosis etc) in adolescent and adulthood. Can these markers help predict which individuals are more susceptible to developing liver damage as young adults? Are these

markers present during the initial diagnosis in childhood of patients with no progression of liver disease in adulthood? Are there any differences in the concentration of markers detected at diagnosis (in childhood) between patients with little/no liver disease in adulthood compared to those patients who develop liver disease (moderate or severe fibrosis) after 10 years of diagnosis. Developing assays to detect and quantify HBcrAg, and HBV RNA in serum at different stages of HBV infection would have tremendous benefits in understanding liver disease progression. It raises the question of whether measuring these serological markers will help predict those HBV paediatric patients who will go on to develop liver disease; and therefore, could be given treatment earlier or even during childhood. Can antiviral therapy in childhood affect the concentration of serological and virological markers detected after 5years and after 10 years of diagnosis. Could the liver disease outcome be totally changed with childhood antiviral therapy.

Chapter 3: Research Methods

3.1 Samples:

All my cohorts consist of human patients living in the United Kingdom (UK), who have visited the hepatitis clinic at King's College Hospital (KCH). All patients or their parents/ guardians provided informed consent including consent for King's College liver biobank and all principles of the Declaration of Helsinki were adhered to.

Samples were taken from the Hepatitis testing services (HTS) frozen serum/plasma archives, which is part of the Liver pathology department at KCH. The samples were from patients who acquired HBV perinatally and have had long-term follow ups and their care transferred to the adult hepatitis clinic. All my cohorts consisted of patients from different regions of Southern England representing a diverse demographic.

3.2 Quantification of HBsAg:

Detection of HBsAg was done using the automated commercially available Abbott ArchitectTM. The Architect HBsAg assay is a chemiluminescent microparticle immunoassay (CMIA) which uses microparticles coated with monoclonal anti-HBs for the detection of HBsAg. The Architect HBsAg assay has a detection limit of >0.05 IU/mL.

The reactants required for this technology includes:

- Paramagnetic microparticles coated with monoclonal anti-HBs.
- Acridinium-labelled conjugate
- Pre-Trigger solution and Trigger solution

Method

1. Anti-analyte microparticles with monoclonal anti-HBs are dispensed into the sample in the reaction vessel.
2. The reaction mixture is mixed using a vortex.
3. The reaction mixture is incubated and any analyte (HBsAg) present in the sample binds to the capture molecule on the microparticles forming an immune complex.
4. A magnet attracts the antigen-antibody complex to a wall of the reaction vessel.

5. Washing step 1: the wash zone manifold washes the reaction mixture, and any unbound material is discarded.
6. The chemiluminescent acridinium-labelled conjugate is transferred into the sample. The conjugate binds to the antigen-antibody complex to complete the reaction mixture.
7. The reaction mixture is incubated.
8. Washing step 2: the wash zone manifold washes the reaction mixture, and any unbound material is discarded.
9. Pre-trigger solution (hydrogen peroxide) is added to the reaction mixture and the CMIA optical system takes a background reading. The purpose of the pre-trigger solution is to create an acidic environment to prevent early light emission; to keep microparticles from clumping; and to split acridinium dye off the conjugate bound to the microparticle complex.
10. The trigger nozzle dispenses Trigger solution (sodium hydroxide) to the reaction mixture. Oxidation of acridinium occurs when exposed to peroxide and an alkaline solution. This reaction activates the chemiluminescent reaction to take place. N-methylacridone forms and releases light energy as it returns to its ground state.
11. The CMIA optical system measures the chemiluminescent emission over a predefined time period to quantify the analyte concentration or to determine qualitative interpretations.

Measurement of HBsAg by Lumipulse HBsAg-HQ assay

To determine HBsAg levels in HBsAg negative patients (determined by the Abbott Architect) the Lumipulse G600II HBsAg-HQ assay (Fujirebio Europe, Ghent, Belgium) was used. The Lumipulse G600II HBsAg-HQ assay was also used to assess if the Fujirebio Lumipulse (Fujirebio, Tokyo, Japan) could quantify HBsAg consistently across HBV genotypes.

The HBsAg-HQ assay kit provided by Fujirebio Europe was used, which has a LLQ of 0.005IU/mL.

Serum samples were placed on the fully automated chemiluminescent enzyme immunoassay (CLEIA) system, the Lumipulse G600II which utilises a two-step sandwich method. This platform has a diagnostic sensitivity of 99.8 and specificity of 99.7 according to the manufactures guide.

250µL of neat patient serum (or plasma) or serum diluted with FBS were transferred into Hitachi cups and placed on the Lumipulse G600II analyser. HBsAg molecules were detected using monoclonal antibodies against external structural regions. Determinant "a", and the internal epitope as a capture reagent was detected by two monoclonal antibodies coupled to alkaline phosphatase as the detector. The relative intensity of chemiluminescence was determined and the HBsAg concentration was quantified from comparison with a standard curve. The limits of detection were 0.005 – 150,000mIU/mL and the samples above this range were retested with a dilution factor of 200 with FBS.

3.3 HBV DNA detection and quantification:

HBV DNA levels were determined using the Cobas Ampliprep/Taqman by Roche. The Ampliprep first captures nucleic acids in the samples, purifies it and transfers it into the Taqman for PCR analysis. The taqman then automates the amplification and detection process for nucleic acid testing with the use of 5' nuclease assay PCR.

DNA detection

Reagents and consumables are loaded on to the COBAS TAqman first:

- Reagent cassette CS1, CS2, CS3, CS
- SPUs
- Amplification tips (K-tips)
- Amplification tubes (K-tubes)
- Sample tubes

Sample preparation

1. As the dynamic range of the assay is so broad, samples can be tested neat.
2. Mix/vortex serum sample well.
3. In a microbiological safety cabinet transfer 720µL of samples in to sample tubes
4. Load samples onto the Ampliprep COBAS TAqman

HBV DNA extraction

5. The HBV virus particles are lysed by being incubated at 94ºC with the addition of protease and chaotropic lysis/binding buffer which releases nucleic acids and protects the released HBV DNA from DNases present in serum and plasma.
6. Protease and HBV QS DNA molecules are added to each sample along with the lysis reagent and magnetic glass particles.
7. The reaction mixture is incubated, and the DNA and HBV QS DNA are bound to the surface of the glass particles.
8. Washing step: upbound particles are removed by washing the magnetic glass particles.
9. The adsorbed nucleic acids are eluted at high temperature with an aqueous solution.
10. The processed sample consisting of the magnetic glass particles and both the released HBV DNA and HBV QS DNA is added to the amplification mixture and transferred to the COBAS TaqMan Analyzer for PCR amplification.

PCR product amplification

11. The PCR amplification reaction is performed with the thermostable recombinant enzyme Thermus specie Z05 DNA Polymerase (Z05) and Primers 1581 and 3131AH_2O.
12. The processed samples are added to the amplification mixture in K-tubes.
13. Manganese (Mn2+) and deoxynucleotide triphosphates (dNTPs) are added to allow Z05 DNA Polymerase to extend the annealed primers forming a DNA strand.
14. The reaction is heated at 94ºC to denature the double stranded DNA and expose the specific primer target sequences on the HBV circular DNA genome and the HBV QS DNA.
15. The reaction is cooled down to 60ºC and the primers anneal to the target DNA.
16. The Z05 DNA Polymerase extends the annealed primers along the target template to produce a double stranded DNA molecule at 70ºC.
17. This process is repeated with each cycle intended to double the number of DNA molecules.
18. Amplification occurs only in the region of the HBV genome between the primers; the entire HBV genome is not amplified.

Detection of PCR products

19. The detection of the PCR products is done using dual-labelled fluorescent probes, which allows detection in real time. It monitors the emission intensity of fluorescent reporter dyes released during the amplification process. There are HBV and HBV QS-specific oligonucleotide probes with a reporter dye and a quencher dye.
20. Then the probes are intact, the fluorescence of the reporter dye is suppressed by the proximity of the quencher dye due to Förster-type energy transfer effects.
21. The probe hybridizes to a target sequence and is cleaved by the 5' $\rightarrow$ 3' nuclease activity of the Z05 DNA polymerase.
22. Once the dyes are released, quenching stops and the fluorescent activity of the reporter dye is elevated.
23. HBV DNA and HBV QS DNA amplification are measured independently at different wavelengths.
24. This process is repeated for each cycle.

Quantification

The Ampliprep COBAS TaqMan assay is quantitative over a broad dynamic range as the detection of the DNA product is performed during the exponential phase of amplification. The higher the HBV titre of a sample, the earlier the fluorescence of the reporter dye of the HBV probe rises above the baseline fluorescence level. The concentration of HBV QS DNA is constant in every sample, so the fluorescence of the reporter dye of the HBV QS probe should appear at the same cycle for every sample. The presence of the specific fluorescent signal is reported as a critical threshold value (Ct). The Ct is defined as the fractional cycle number where reporter dye fluorescence exceeds predetermined threshold and starts the exponential growth phase of this signal. The higher the CT value the lower the titre of initial HBV target material.

Detection range of the assay

- Not detected – no HBV DNA detected in serum sample.
- <2.00E1 IU/mL – HBV DNA detected below the range of the assay.
- ≥2.00E1 and ≤1.70E8 – HBV DNA detected between the linear range of the assay.
- >1.70E8 IU/mL - HBV DNA detected above the range of the assay.

3.4 Quantification of HBcrAg:

The highly sensitive Lumipulse HBcrAg-HQ assay (Fujirebio Europe, Ghent, Belgium) was used to detect serum HBcrAg on the Fujirebio Lumipulse G600II CLEIA analyser (Fujirebio, Tokyo, Japan). This assay measures the antigenic reactivity of 3 proteins: HBeAg, HBV core antigen (HBcAg) and core-related protein p22cr.

The first step is the pre-treatment of serum samples. 170μL of serum samples were added to 170μL of pre-treatment solution and incubated at 60°C for 30 minutes to enable the dissociation of epitopes from immunocomplexes that bind to the circulating antigens. After pre-treatment incubate the samples at room temperature for 5 minutes. Dispense the pre-treatment samples into Hatachi sample cups and place them on the analyser. The freely circulating HBcrAg in the samples bind to the anti-HBcr antibodies that are enclosed in the ferrite beads, and an antigen-antibody complex is formed. The samples are incubated at 37°C for 10 minutes then a washing step occurs to discard any unbound molecules. Alkaline phosphatase (ALP) labelled anti-HBcr antibodies bind specifically to the antibody-antigen complexes, to become enzyme-labelled. The samples are incubated at 37°C for 10 minutes then a washing step occurs to discard any unbound molecules.

Luminescent substrate solution is added to the samples triggering the dephosphorylation of 3-(2'-spiroadamantyl)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD). The transformed AMPPD is cleaved generating luminescence that can be read at a wavelength of 477 nm. The intensity luminescent signal generated will be positively correlated to the concentration of HBcrAg present in the sample originally.

The assay has a linear measurement range of 3-7 $\log_{10}$ U/ml. Samples with HBcrAg >7 $\log_{10}$ U/mL can be diluted and retested to calculate the concentration of HBcrAg. The accuracy of the HBcrAg test is determined by assay values (calculated in $\log_{10}$ U/mL) for 3 in-house controls range within ± 5% of their control values. The coefficient of variation for specimens (calculated in $\log_{10}$ U/mL) is less than 5% when subjected to the assay 6 replicates. The analytical sensitivity of the reagent is 3.0 $\log_{10}$ U/mL. Serum-plasma equivalence is reflected by correlation coefficient of 0.999.

3.5 Quantification of pgRNA:

HBV pgRNA concentrations were determined using a newly developed quantification assay by Abbott diagnostic laboratories in Chicago, USA. The isolation and amplification of HBV RNA as described by Butler et al(315) using the Abbott m2000 system was adopted.

First, primers and probes were designed to conserved regions within the 5′ end of the X gene and the 3′ end of the core gene. To detect the different targets, probes were created with 5′ FAM fluorophore and 3′ BHQ1 quencher (X target) and 5′ Q670 fluorophore and BHQ2 quencher (core target) (BioSearch, Novato, CA). Using Integrated DNA technology, the primers were synthesized. The reaction mastermix was made using Proprietary reagents (Abbott Molecular, Des Plaines, IL) and SSIII reverse transcriptase enzyme (Sigma, St. Louis, MO). The isolation of HBV RNA was done through RNA-selective extraction chemistry (Abbott mSample Preparation System, Abbott Molecular). This was followed by a multiplex real-time quantitative PCR for detecting amplicons in the HBV X and core targets using the m2000 machine (Abbott Molecular). Next RNA extraction and amplification efficiency was validated in each sample by the identification of an armoured RNA internal control added during the lysis step. 10μL of eluate were added to 50μL total reaction volume. The conditions for thermocycling were: 50 °C for 25 minutes; 95 °C for 10 minutes; and 50X (95°C, 15 seconds; 62°C, 30 seconds; 57°C, 85 seconds). Calibration of the HBV RNA assay was established using DNA-extracted HBV DNA secondary standards according to the World Health Organization's HBV DNA standard such that a unit (U) reported for RNA in this method is equivalent to 1 IU of HBV DNA.

3.6 HBV genotype determination:

The genotypes of the samples were determined using an inhouse sequencing method developed by the HTS staff. The HBV genotyping test is an *in vitro* nucleic acid amplification and direct sequencing test for the determination of HBV genotypes A-H in human serum or plasma (650µL of serum or plasma required). The test utilises the Roche COBAS Ampliprep instrument for automated Total Nucleic Acid Isolation (TNAI), Bioer LifeTouch and ABI GeneAmp 9700 thermocyclers for HBV DNA PCR amplification and cycle sequencing. Additionally, the ABI 3130cl genetic analyser is used for direct sequencing.

The HBV genotyping test is based on four major processes:

- HBV DNA isolation from specimen
- Nested PCR amplification of target DNA
- Cycle sequencing of target DNA
- Direct sequencing of target DNA by fluorescence-based capillary electrophoresis technology.

The HBV genotyping test targets the entire surface gene of the HBV genome which is then used to determine HBV genotype, classification of which can be defined as nucleotide (nt) sequence divergence of greater than four percent of the surface gene

The assay limit of detection is $2.00x10^2$ IU/mL HBV DNA viral load. It has a sensitivity of 98.7% (95% CI 95.0-99.8), an accuracy of 100% (95% CI 94.0-100.0), cross reactivity of 0%. The repeatability and reproducibility were both 100%.

Materials required:

Genetic analyser

Cobas Ampliprep/TaqMan Docking System

Lifetouch PCR System

GeneAmp PCR System 9700

Electrophoresis Power Supply and tank

Class II Microbiologigy Safety Cabinet

Centrifuge (Mikro 200. Sorvall Legend T and microcentrifuge)

Vortex

Plate incubator

1.5mL microcentrifuge tubes

15mL and 50mL centrifuge tubes

0.2mL 96-well PCR plate

Microwave oven

500ml Erlenmeyer flask

NanoDrop Lite

Deionised water (dH2O)

Transilluminator, camera box, camera, Genesnap software

TNAI kit

Incubate CS4 at 37°C for 1 hour

From the TNAI kit take all four CS reagents out and place onto the reagent racks in the appropriate positions (positions A-E according to the TNAI kit package insert) and immediately place onto the AmpliPrep.

Load SPUs onto the Ampliprep in positions J-L

Load K-tips onto Ampliprep in positions M-N

Load S-Output tubes onto Ampliprep in positions M-P.

Add barcode clips to sample racks.

Total Nucleic Acid Isolation (TNAI) Extraction

1. Prepare any dilutions of samples needed.
2. Vortex samples and controls and load 650µL of samples/control into S-input tubes.
3. Load sample rack into AmpliPrep in position F-H and start the machine.
4. When TNAI extraction is complete, remove the S-output tubes and label tubes.
5. Insert Amicon ultra-0.5 filters into sample tubes and add 325µL of RNase/RNase free water. Add all TNAI eluate for each sample to be concentrated from S-output tubes to the appropriate filter devices and seal the caps.
6. Load Amicon devices into Mikro 200 centrifuge and spin at 12,000 rpm for 15 minutes.
7. Remove the devices from the centrifuge, transfer the filters upside down to new tubes and discard the used tubes.
8. Place the tubes into Mikro 200 centrifuge and spin at 3400 rpm for 2 minutes.
9. After centrifugation, dispose of filter devices and cap tubes and place them in a rack.

First Round Nested PCR

Reagents	Volume / Reaction (μL)
dH_2O	10
Taq PCR Master Mix: 2xPCR buffer dNTP mix $MgCl_2$ Taq polymerase	12.5 2x in 12.5μL 400μM (each dNTP) 3mM in 12.5μL 250U in 5100μL
Primer 1581	0.5 (10μM)
Primer 3131A	H_2O 1 (10μM)
Total	24

Table 1: reagents and volumes required for the first round nested PCR procedure.

Components of reaction	Total reaction (μL)
Master Mix + Primers	24
Template/ dH_2O (Negative control)	1
Total	25

Table 2: Components required for the first round nested PCR procedure.

10. Place Primers (1581 and 3131AH_2O), Taq PCR Master mix and dH_2O on ice.
11. In a 1.5mL tube transfer the required volume of dH_2O, Taq PCR Master mix and primers and vortex.
12. Demarcate a PCR plate and transfer 24μL of master mix to appropriate wells.
13. Add 1μL of control and sample TNAI elution to the wells. Cover plate with a thermostable optical plate sealer.
14. Briefly vortex and centrifuge at 1000xg.
15. Place on the Bioer Life Touch Thermal Cycler and start the PCR procedure using the conditions in table 3.

PCR/ Thermal cycler conditions			
Steps	**Temperature ^{o}C**	**Time**	
Denature	95	5 min	
Denature	94	30 sec	Repeat cycle steps 2-4 35 times
Anneal Primers	56	45 sec	
Extend Primers	72	60 sec	
Elongate	72	7 min	
cool	4	∞	

Table 3: Thermal cycling conditions for first round nested PCR.

Second Round Nested PCR

Reagents	**Volume / Reaction (μL)**
dH_2O	10.5
Taq PCR Master Mix:	12.5
2xPCR buffer	2x in 12.5μL
dNTP mix	400μM (each dNTP)
$MgCl_2$	3mM in 12.5μL
Taq polymerase	250U in 5100μL
Primer 57	1 (10μM)
Primer 1190	0.5 (10μM)
Total	24.5

Table 4: Reagents and volumes required for the second round nested PCR procedure.

Components of reaction	Total reaction (µL)
Master Mix + Primers	24.5
Template/ dH_2O (Negative control)	0.5
Total	25

Table 5: Components required for the second round nested PCR procedure.

16. Place Primers (57 and 1190), Taq PCR Master mix and dH_2O on ice.
17. In a 1.5mL tube transfer the required volume of dH_2O, Taq PCR Master mix and primers and vortex.
18. Demarcate a PCR plate and transfer 24.5µL of master mix to appropriate wells.
19. Add 0.5µl of first round nested PCR product to the appropriate wells.
20. Cover plate with a thermostable optical plate sealer.
21. Briefly vortex and centrifuge at 1000xg.
22. Place on the GeneAmp PCR system 9700 and use the same PCR cycling conditions as in the first round nested PCR procedure.

Purification of samples and controls

23. In two Amicon tubes insert filters and add 375µl of nuclease free H_2O
24. Add all the second round nested PCR products for purification to the filter devices and seal the caps.
25. Centrifuge the tubes at 12000 rpm for 15 minutes on the Mikro 200 centrifuge.
26. After centrifugation, transfer filter devices (upside down) to new tubes.
27. Centrifuge the tubes at 3400 rpm for 2 minutes then dispose of the filter devices.
28. Measure DNA levels of all samples using the NanoDrop Lite spectrophotometer.
29. Measure the DNA standard (salmon sperm) and record the DNA concentration and A260/A280 ratio.
30. NB. A260/A280: The ratio of absorbance at 260nm and 280nm is used to assess the purity of DNA. A ratio of approximately 1.8 is generally accepted as "pure". If the ratio is appreciably lower it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280nm.

Agarose Gel Electrophoresis

31. Prepare 1.5% agarose gel by adding 3g of agarose to 200mL of TAE buffer in a 500mL flask. Microwave the flask for 3 minutes and check that the contents are well mixed.
32. Add 4µl of the fluorescent stain Nancy-520 into the flask.
33. Set the gel into a casting frame and add the well forming combs.
34. Once set, remove combs, and transfer gel into an electrophoresis tank containing 1x TAE buffer.
35. 8µl of samples (and controls) are added to 2µl 5x loading buffer.
36. 4µl of 200 bp ladder is added to the first well.
37. 10µl of sample/loading dye are transferred into appropriate wells.
38. Conditions for electrophoresis are as follows: 125 volts (V) for 30-40 minutes.
39. The gel is read under UV lamp using the Syngene equipment with Genesnap software (Syngene, Cambridge, UK).

Dilution of PCR product

40. The target concentration of DNA template to be used for the sequencing reaction for this assay is 1ng/µl.
41. Demarcate a PCR plate and add the correct volumes of nuclease free H_2O and samples to dilute all samples to 1ng/µl.
42. Cover plates with a seal, vortex, and centrifuge (allow centrifugation to reach 100 xg then stop).

Sequencing reaction – Post PCR

Reagents	**Volume / Reaction (µL)**
dH_2O	8
BigDye sequencing buffer (5x)	4
Ready Reaction premix	2
Primer 57	4 (10µM dil 1/10=1µM, 4µL in 20µL= 0.2µM)

Total	18

Table 6: Master mix for sequencing reaction – primer 57

Components of reaction	**Total reaction (μL)**
Master Mix	18
Template DNA	2
Total	20

Table 7: Components for sequencing reaction – primer 57

Reagents	**Volume / Reaction (μL)**
dH_2O	8
BigDye sequencing buffer (5x)	4
Ready Reaction premix	2
Primer 460	4 (10μM dil 1/10=1μM, 4μL in 20μL= 0.2μM)
Total	18

Table 8: Master mix for sequencing reaction – primer 460

Components of reaction	**Total reaction (μL)**
Master Mix	18
Template DNA	2
Total	20

Table 9: Components for sequencing reaction – primer 460

Thermal cycler conditions			
Steps	**Temperature ^{o}C**	**Time**	
Denature	94	60 sec	
Denature	94	30 sec	Repeat cycle steps 2-4 35 times
Anneal Primers	59	45 sec	
Extend Primers	72	60 sec	
Elongate	72	7 min	
cool	4	∞	

Table 10: Thermal cycler conditions for sequencing

43. In sterile 1.5ml tubes transfer primers 57 and 460, then add dH_2O, BigDye sequencing buffer(5x) and ready reaction premix into each tube.
44. Demarcate a PCR plate and transfer 18µl of mastermix into wells.
45. Add 2µl of diluted nested PCR product to the wells.
46. Seal plates and briefly centrifuge at 1000 xg.
47. Place the plate on the GeneAmp PCR system 9700 thermocycler and use the sequencing conditions as shown in Table 10.

XTerminator purification

48. Prepare XTerminator premix by combining XTerminator solution with SAM solution using a 1:4.5 ratio.
49. To the sequencing reaction add 110µl of XTerminator.
50. Seal the plate with a thermostable optical sealer and place on a digital mixer and vortex for 30 minutes at 1800 rpm.
51. Centrifuge the reaction plate at 1000xg for 2 minutes.

Sequencing analysis

52. Use a genome analysing software to analyse results based on tree panel data collection.
53. Analyse the data on SeqScape and HBVseq
54. Entre sequencing data on HBVseq.
    i. https://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html
55. Create a tree alignment to compare genotype with that attained from the HBVseq as a form of control.
56. Append sequences and compare to the reference sequences for HBV genotypes A-H.
57. Save results onto the software Neighbour-Joining plot (njplot) which will show HBV genotypes in each sample assigned by the phylogenic tree.

3.7 Sequencing of precore (G1896A) and Basal Core promoter (A1762T/G1764A)

PCR Amplification of the HBV S Gene

PCR amplification of the HBV S gene was done using the nested PCR method. The 400bp fragment of the partial S gene region was amplified using an ABI 3130xl genetic analyser The position of the codons in the S gene covered by the PCR ranges from 22 to 170. The PCR primers used were HBV_S1F (5”-CTAGGACCCCTGCTCGTGTT-3’) and HBV_S1R (5′-CGAACCACTGAACAAATGGCACT-3’) for the first round, and the second round primers were HBV_SNF (5′-GTTGACAAGAATCCTCACAATACC-3’) and HBV_SNR (5′-GAGGCCCACTCCCATA-3). Components for the first round PCR are shown in Table 11, and components required for the second round PCR are shown in Table 12. The thermocycling conditions are shown in Table 13. PCR products were resolved on 2% agarose gel stained with ethidium bromide and viewed using a UV transilluminator.

First round PCR	
Reagents	**Volume / Reaction (μL)**
RNase-free water	13
Primer HBV_S1F (5”-CTAGGACCCCTGCTCGTGTT-3’)	1 (20μM)
Primer HBV_S1R (5′-CGAACCACTGAACAAATGGCACT-3’)	1 (20μM)
DNA Ready Reaction premix	5
Total	20

Table 11: Components for first round PCR reaction

Second round PCR	
Reagents	**Volume / Reaction (μL)**
RNase-free water	13
Primer HBV_SNF (5′-GTTGACAAGAATCCTCACAATACC-3’)	1 (20μM)
HBV_SNR (5′-GAGGCCCACTCCCATA-3)	1 (20μM)
Product from first round PCR	5
Total	20

Table 12: Components for second round PCR reaction

Thermal cycler conditions – first and second round PCR			
Steps	**Temperature ºC**	**Time**	
Denature	94	3 min	
Denature	94	30 sec	Repeat cycle 45 times
Anneal	55	60 sec	
Elongate	70	40 sec	
Elongate	72	7 min	
cool	4	∞ (until needed)	

Table 13: Thermocycling conditions for first and second round PCR

PCR amplification of Precore (PC) and Basal Core Promoter (BCP) genome region

The PC and BCP region were amplified by nested PCR targeting a 360bp fragment of the PC/ BCP region. Table 14 shows the components of the first round PCR and Table 15 shows the thermal cycler conditions. Table 16 shows the components of the second round PCR and Table 17 shows the thermal cycler conditions.

First round PCR	
Reagents	**Volume / Reaction (μL)**
RNase-free water	16
Primer BCP_PC F1 (5GCATGGAGACCACCGTGAAC-3)	1 (20μM)
Primer BCP_PC R1 (5GGAAAGAAGTCCGAGGGCAA-3)	1 (20μM)
DNA Ready Reaction premix	2
Total	20

Table 14: Components of the first round PCR

Thermal cycler conditions – first round PCR			
Steps	**Temperature ^{o}C**	**Time**	
Denature	94	30 sec	
Anneal Primers	55	60 sec	
Denature	72	60 sec	Repeat cycle 35 times
Anneal	72	2 min	
Elongate	68	30 sec	
Elongate	68	10 min	

Table 15: Thermal cycler conditions for the first round PCR

Second round PCR	
Reagents	**Volume / Reaction (μL)**
RNase-free water	16
Primer BCP_PC F2 (5- CATAAGAGGACTCTTGGACT-3)	1 (20μM)
Primer BCP_PC R2 (5- GGCAAAAAACAGAGTAACTC-3)	1 (20μM)
DNA template from the first round of PCR	2
Total	20

Table 16: Components of the second round PCR

PCR/ Thermal cycler conditions – second round PCR			
Steps	**Temperature ºC**	**Time**	
Denature	94	3 min	
Denature	94	30 sec	Repeat cycle 30 times
Anneal	55	30 sec	
Elongate	72	60 sec	
Elongate	72	2 min	
cool	4	∞	

Table 17: Thermal cycler conditions for the second round PCR

The PCR products were resolved in 2% agarose gel stained with ethidium bromide and viewed using a UV transilluminator.

Sequencing for precore (G1896A) and basal core promoter (A1762T/G1764A) mutations

Sanger sequencing was performed on the ABI 3130xl genetic analyser (Life Technologies). Sequences were analysed using SeqScape V2.7 software and by GenBank precore region reference sequences for individual genotypes in ClustalX 2.0.

Five microliters of the secondary PCR product from the HBs gene and PC/BCP gene with the expected band size of 400bp and 360bp respectively and codon positions 22-170 and 54-271 respectively were purified with 2μL of ExoSAP at 37 ^{o}C for 15 minutes and inactivated at 80 ^{o}C for 15 minutes. The purified amplicon was added to the sequencing components as shown in Table 18. Table 19 shows the thermal cycler conditions.

Sequencing	
Reagents	**Volume / Reaction (μL)**
Big dye terminator	4
Nuclease- free water	7
HBV_SNF and HBV_SNR	2
BCP_PCF1 and BCP_PCF2	2
purified secondary amplicons	5
Total	20

Table 18: Components for sequencing.

Thermal cycler conditions for sequencing

Steps	Temperature ^{o}C	Time	
Denature	96	60 sec	
Denature	96	10 sec	Repeat cycle 30 times
Anneal	50	5 sec	
Elongate	72	60 sec	
Elongate	70	4min	
cool	4	∞	

Table 19: Thermal cycling conditions for sequencing

3.8 ELISA protocol for PD-1 and CXCL10 (IP-10)

The identification of PD-1 and CXCL10 was done using the Human CXCL10/ IP-10 Duoset ELISA kit by Biotechne.

Plate preparation

1. Coat a 96 well microplate with diluted capture antibody with 100µL per well. Seal the plate and incubate at room temperature overnight.

2. Aspirate each well and wash with wash buffer three times.

3. Block plate by adding 300µL of reagent diluent to each well. Incubate plate at room temperature for at least one hour.

4. Repeat the aspiration/ wash step from step 2. The plates are now ready for the addition of samples.

Assay procedure

5. Prepare seven assay standards by serial dilution. Transfer 100µL of standards in the first row of wells in duplicate ie. well 1A-G and well 2A-G. In well 1H and 2H add 100µL of negative control.

6. Transfer 100µL of samples into the remaining wells. Cover the plate with an adhesive strip and incubate at room temperature for two hours.

7. Repeat the aspiration/ wash step from step 2.

8. Transfer 100µL of the detection antibody to each well. Cover the pate with adhesive strip and incubate at room temperature for two hours.

9. Repeat the aspiration/ wash step from step 2.

10. Transfer 100µL of the working dilution of streptavidin-HRP to each well. Seal the plate and incubate at room temperature for 20 minutes away from direct light.

11. Repeat the aspiration/ wash step from step 2.

12. Transfer 100µL of substrate solution too each well. Incubate the plate at room temperature for 20 minutes away from direct light.

13. Transfer 50µL of stop solution to each well.

14. Determine the optical density of each well immediately using a microplate reader set to 450nm.

Chapter 4

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.

Abstract

A dichotomous separation of Hepatitis B viral DNA and Hepatitis B surface antigen (HBsAg) concentrations occurs during the natural history and treatment of chronic Hepatitis B. We have evaluated the ability of hepatitis B virus (HBV) RNA and hepatitis B core-related antigen (HBcrAg) as surrogates of silencing of covalently closed circular DNA (cccDNA), to characterize this dissociation, and virological outcomes.

Results: Three cohorts of Hepatitis B e antigen (HBeAg)-negative patients were studied: cohort A: 66 HBeAg-negative patients on long term nucleos(t)ide analogue (NA) therapy; cohort B: 23 antibodies against hepatitis B e antigen (anti-HBe)-positive patients who stopped treatment; and Cohort C: 19 anti-HBe-positive patients on long term NA treatment who achieved HBsAg loss and in whom treatment was withdrawn. Concentrations of HBV serological/ virological biomarkers (HBV DNA, HBsAg, HBcrAg, and HBV RNA) were measured in sequential samples at different time points on/off therapy. Cohort A: After 3 years of antiviral therapy, 33% and 30% had detectable HBcrAg and HBV RNA, respectively, despite all being HBV DNA negative. After 5 years' therapy with NA, 27% and 14% had detectable HBcrAg and HBV RNA. Detectable HBcrAg and HBV RNA at the time of treatment withdrawal was only observed in those patients who developed a severe aminotransferase flare. Only those patients with HBV reactivation in cohort C had detectable HBV RNA at treatment withdrawal, but HBcrAg and HBV DNA were not detected.

Conclusions: HBcrAg and HBV RNA are sensitive biomarkers of continued transcription of cccDNA in HBeAg negative patients despite marked HBV DNA suppression by NA. These markers were predictors of severe alanine transaminase flares, after treatment withdrawal, and HBV DNA reactivation. Their measurement during the natural history of Hepatitis B, and on treatment with current and new agents, could characterize residual HBV RNA transcription from cccDNA and assist drug development and disease management.

Patients and Methods

Patients

To investigate the utility of these markers, we have analysed three distinct clinical cohorts of HBeAg negative patients with CHB. All cohorts were retrospectively studied.

Table 1. Baseline characteristics of cohort A (n = 66)

Male patients (n, %)	**n = 48 (73)**
Median fibrosis stage by Ishak (range)	3 (2-3)
Median inflammation grade by Ishak (range)	5 (3-11)
Median age (range)	45 years (19-67)
HBeAg positive at start of therapy (n, %)	n = 0 (0)
Median baseline HBV DNA (IQR; range)	4.12 $\log_{10}$IU/mL (1.0; 2.67-6.78)
Median baseline HBsAg (IQR; range)	3.69 $\log_{10}$IU/mL (0.81; 2.12-4.34)
Median baseline HBcrAg (IQR; range)	3.6 $\log_{10}$U/mL (1.53; 2.0-6.5)
Median baseline HBV pgRNA (IQR; range)	1.87 $\log_{10}$U/mL (0.43; 1.65-3.25)
Median baseline ALT activity (IQR; range)	45 IU/L (20; 33-212)
Ethnicity	No. of Patients (%)

Male patients (n, %)	**n = 48 (73)**
Asian Indian	n = 4 (6)
Asian Chinese/Vietnamese	n = 12 (18)
Black African	n = 35 (53)
Black Caribbean	n = 4 (6)
White	n = 11 (17)
HBV genotype distribution	No. of Patients (%)
A	n = 12 (18)
B	n = 2 (3)
C	n = 10 (15)
D	n = 10 (15)
E	n = 32 (49)
Precore region mutations	No. of Patients (%)
Precore (G1896A)	n = 13 (20)
BCP region (A1762T/G1764A)	n = 7 (11)
No mutation	n = 46 (69)
Proportion of HBcrAg not detected at baseline (<3 log_{10}U/mL), n (%)	n = 19 (29)
Proportion of HBV pgRNA not detected at baseline (<1.65 log_{10}U/mL), n (%)	n = 11 (17)
Type of antiviral therapy TDF vs. ETV (n)	66 vs. 0

Table 2. Baseline characteristics of cohort B at start of NA therapy (n = 23)

	All Cohorts (n = 23)	**No Flare (n = 9)**	**Mild Flare (n = 10)**	**Severe Flare (n = 4)**
Male patients (n, %)	n = 14 (61)	n = 4 (44)	n = 6 (60)	n = 4 (100)
HBeAg positive (n, %)	n = 0 (0)	n = 0 (0)	n = 0 (0)	n = 0 (0)
Median fibrosis stage by Ishak (range)	3 (2-3)	3 (2-3)	3 (2-3)	3 (2-3)
Median fibrosis grade by Ishak (range)*	5 (3-10)	4 (3-6)	4 (3-8)	5 (4-10)
Median age (range)	48 years (24-64)	49 years (34-64)	47 years (31-57)	48 years (24-55)
Median baseline HBV DNA (IQR; range)*	4.53 log_{10}IU/mL (1.5; 2.5-6.6)	3.73 log_{10}IU/mL (0.8; 2.5-4.9)	4.67 log_{10}IU/mL (1.7; 2.9-6.6)	4.92 log_{10}IU/mL (0.8; 4.7-5.6)
Median baseline HBsAg (IQR; range)	3.81 log_{10}IU/mL (0.8; 2.4-4.5)	3.91 log_{10}IU/mL (1.2; 2.4-4.4)	3.68 log_{10}IU/mL (0.7; 3.1-4.4)	4.07 log_{10}IU/mL (0.8; 3.4-4.3)
Median baseline HBcrAg (IQR; range)*	3.3 log_{10}U/mL (3.0; 2.0-6.8)	2.8 log_{10}U/mL (1.3; 2.0-6.0)	3.6 log_{10}U/mL (3.0; 2.0-5.7)	5.6 log_{10}U/mL (1.4; 5.1-6.8)
Median baseline HBV pgRNA (IQR; range)*	2.65 log_{10}U/mL (1.3; 0.0-4.1)	2.19 log_{10}U/mL (0.9; 0.0-3.9)	2.66 log_{10}U/mL (1.4; 0.0-4.1)	3.36 log_{10}U/mL (0.8; 2.8-3.7)
Median baseline ALT activity (IQR; range)*	29 IU/L (26; 17-109)	21 IU/L (20; 17-62)	37 IU/L (25; 20-109)	47 IU/L (24; 25-63)
HBV genotype distribution,	n (%) of patients			
A	n = 4 (17)	n = 3 (33)	n = 1 (10)	n = 0 (0)
B	n = 3 (13)	n = 1 (11)	n = 1 (10)	n = 1 (25)
C	n = 1 (4)	n = 0 (0)	n = 1 (10)	n = 0 (0)

	All Cohorts (n = 23)	No Flare (n = 9)	Mild Flare (n = 10)	Severe Flare (n = 4)
D	n = 5 (22)	n = 2 (22)	n = 2 (20)	n = 1 (25)
E	n = 10 (44)	n = 3 (33)	n = 5 (50)	n = 2 (50)
Ethnicity	n (%) of patients			
Asian Indian	n = 2 (9)	n = 1 (11)	n = 0 (0)	n = 1 (25)
Asian Chinese/Vietnamese	n = 4 (17)	n = 1 (11)	n = 2 (20)	n = 1 (25)
Black African	n = 12 (53)	n = 5 (56)	n = 5 (50)	n = 2 (50)
Black Caribbean	n = 1 (4)	n = 1 (11)	n = 0 (0)	n = 0 (0)
White	n = 4 (17)	n = 1 (11)	n = 3 (30)	n = 0 (0)
Precore region mutations	No. of patients (%)	No. of patients (%)	No. of patients (%)	No. of patients (%)
Precore (G1896A)	n = 8 (35)	n = 2 (22)	n = 3 (30)	n = 3 (75)
BCP region (A1762T/G1764A)	n = 1 (4)	n = 0 (0)	n = 1 (10)	n = 0 (0)
No mutation	n = 14 (61)	n = 7 (78)	n = 6 (60)	n = 1 (25)
Proportion of HBcrAg not detected at baseline (<3 log_{10}U/mL), n (%)	n = 8 (35)	n = 5 (56)	n = 3 (30%)	n = 0 (0)
Proportion of HBV pgRNA not detected at baseline (<1.65 log_{10}U/mL), n (%)	n = 3 (13)	n = 0 (0)	n = 3 (30)	n = 0 (0)

	All Cohorts (n = 23)	No Flare (n = 9)	Mild Flare (n = 10)	Severe Flare (n = 4)
TDF vs. ETV therapy (no. of patients)	19 vs. 4	8 vs. 1	8 vs. 2	3 vs. 1

- * $P < 0.05$
- Abbreviation: IQR, interquartile range.

Table 3. Clinical Characteristics of Cohort B at Time of NA Withdrawal (n = 23)

	All Cohorts (n = 23)	No Flare (n = 9)	Mild Flare (n = 10)	Severe Flare (n = 4)
Male patients (n, %)	n = 14 (61)	n = 4 (44)	n = 6 (60)	n = 4 (100)
Median FibroScan stiffness (IQR; range)	4.7 kPa (2.3; 2.9-8.1)	4.6 kPa (0.6; 3.3-7.8)	5.7 kPa (2.5; 2.9-8.1)	4.9 kPa (3.0; 4.2-7.9)
HBeAg positive (n, %)	N = 0 (0)	N = 0 (0)	N = 0 (0)	N = 0 (0)
Median HBV DNA at time of withdrawal (IQR; range)	0 $\log_{10}$IU/mL (0; 0)	0 $\log_{10}$IU/mL (0; 0)	0 $\log_{10}$IU/mL (0; 0)	0 $\log_{10}$IU/mL (0; 0)
Median HBsAg at time of withdrawal (IQR; range)	3.41 $\log_{10}$IU/mL (1.0; 2.4-4.5)	3.53 $\log_{10}$IU/mL (1.2; 2.4-4.4)	3.28 $\log_{10}$IU/mL (0.7; 3.1-4.4)	3.51 $\log_{10}$IU/mL (0.9; 3.4-4.5)
Median HBcrAg at time of withdrawal (IQR; range)**	2.0 $\log_{10}$U/mL (0; 2.0-5.4)	2.0 $\log_{10}$U/mL (0; 2.0)	2.0 $\log_{10}$U/mL (0; 2.0)	4.2 $\log_{10}$U/mL (0.9; 3.1-5.4)
Median HBV pgRNA at withdrawal (IQR; range)**	0 $\log_{10}$U/mL (0; 0.0-2.2)	0 $\log_{10}$U/mL (0; 0)	0 $\log_{10}$U/mL (0; 0)	1.93 $\log_{10}$U/mL (0.8; 0.0-2.2)
Median ALT activity at time of	24 IU/L (19; 15-53)	24 IU/L (11; 18-42)	26 IU/L (18; 15-53)	22 IU/L (22; 17-52)

	All Cohorts (n = 23)	No Flare (n = 9)	Mild Flare (n = 10)	Severe Flare (n = 4)
withdrawal (IQR; range)				
Proportion of HBcrAg not detected at NA stop (<3 log_{10}U/mL), n (%)	n = 19 (83)	n = 9 (100)	n = 10 (100)	n = 0 (0)
Proportion of HBV pgRNA not detected at NA stop (1.65 log_{10}U/mL), n (%)	n = 20 (87)	n = 9 (100)	n = 10 (100)	n = 1 (25)
Duration of therapy at time of withdrawal (IQR; range)	77 months (53; 38-118)	55 months (42; 38-114)	92 months (41; 58-118)	66 months (44; 54-110)

- * $P < 0.05$;
- ** $P < 0.01$.
- Abbreviation: IQR, interquartile range.

Table 4. Clinical characteristics of cohort C at start of NA therapy (n = 19)

	All Cohorts (n = 19)	No Relapse (n = 17)	Relapser (n = 2)
Male patients (n, %)	n = 11 (58)	n = 10 (59)	n = 1 (50)
Median fibrosis stage by Ishak (range)	2 (2-4)	2 (2-4)	2 (2)
Median inflammation grade by Ishak (range)	5 (3-11)	5 (3-11)	5 (5)
Median age (IQR; range)	36 years (10; 21-57)	36 years (11, 21-57)	36 years (31-41)

	All Cohorts (n = 19)	No Relapse (n = 17)	Relapser (n = 2)
Median therapy baseline HBV DNA (IQR; range)	4.7 log_{10}IU/mL (3.5; 1.5-8.3)	4.7 log_{10}IU/mL (4.1; 1.5-8.3)	4.7 log_{10}IU/mL (4.0-5.4)
Median therapy baseline HBsAg (IQR; range)	1,045 IU/mL (691; 1-19,344)	1,055 IU/mL (757; 1-19,344)	860 IU/mL (676-1,045)
Median therapy baseline ALT activity (IQR; range)	188 IU/L (109; 25-333)	123 IU/L (212; 25-333)	50.5 IU/L (49-52)
Duration of therapy at time of withdrawal (IQR; range)	8.58 years (3.5; 1.6-12.2)	7.96 years (4.3; 1.6-12.2)	9.27 years (8.62-9.92)
Type of therapy TDF vs. ETV (no. of patients)	16:3	14:3	2:0
HBeAg positive at start of therapy (no. of patients)	n = 7 (37%)	n = 7 (41%)	n = 0 (0%)
Previous therapy with IFN (no. of patients)	n = 7 (37%)	n = 7 (41%)	n = 0 (0%)

- Abbreviation: IQR, interquartile range.

Table 5. Clinical characteristics of cohort C at time of therapy withdrawal after HBsAg loss was achieved (n = 19)

	All Cohorts (n = 19)	**No Relapse (n = 17)**	**Relapser (n = 2)**
Median duration on therapy since HBsAg loss (IQR; range)	1.76 years (0.6; 0.5-3.8)	1.76 years (0.9; 0.5-3.6)	2.75 years (1.74-3.80)
Median HBV DNA at time the first HBsAg loss (IQR; range)	0 log_{10}IU/mL (0; 0)	0 log_{10}IU/mL (0; 0)	0 log_{10}IU/mL (0; 0)
Median HBcrAg at time of the first HBsAg loss (IQR; range)	2.0 log_{10}U/mL (0; 2.0)	2.0 log_{10}U/mL (0; 2.0)	2.0 log_{10}U/mL (0; 2.0)
Median anti-HBsAb at time of the first NA loss (IQR; range)	0 mIU/mL (0; 0.0-5.8)	0 mIU/mL (0; 0.2-5.8)	0 mIU/mL (0.0-0.3)
Proportion of anti-HBsAb detected (>10 mIU/mL) at time of the first NA loss (n, %)	n = 0 (0)	n = 0 (0)	n = 0 (0)
HBeAg positive at time of first NA loss (n, %)	n = 0 (0)	n = 0 (0)	n = 0 (0)
Median ALT activity at time of withdrawal (IQR; range)	22 IU/L (9; 14-44)	21 IU/L (11; 14-44)	21 IU/L (16-25)
Median HBV DNA at time of withdrawal (IQR; range)	0 log_{10}IU/mL (0; 0)	0 log_{10}IU/mL (0; 0)	0 log_{10}IU/mL (0; 0)
Median HBsAg-HQ at time of withdrawal (IQR; range)	1.23 mIU/mL (7.3; 0.5-190.0)	0.9 mIU/mL (4.4; 0.5-18.0)	95.7 mIU/mL (1.5-190.0)
Median HBcrAg at time of withdrawal (IQR; range)	2.0 log_{10}U/mL (0; 2.0)	2.0 log_{10}U/mL (0; 2.0)	2.0 log_{10}U/mL (0; 2.0)
Median pg HBV RNA at withdrawal (IQR; range)**	0 log_{10}U/mL (0; 0.0-2.2)	0 log_{10}U/mL (0; 0)	2.1 log_{10}U/mL (2.0-2.2)
Median anti-HBsAb at time of withdrawal (IQR; range)*	3.32 mIU/mL (8; 0-53)	3.6 mIU/mL (7.6; 0.3-53.0)	0.3 mIU/mL (0.0-0.6)
Median ALT activity at time of withdrawal (IQR; range)	21 IU/L (8; 12-43)	21 IU/L (10; 12-43)	21 IU/L (18-24)

	All Cohorts (n = 19)	No Relapse (n = 17)	Relapser (n = 2)
Proportion of anti-HBs-positive patients (>10 mIU/mL), n (%)	n = 4 (21)	n = 4 (24)	n = 0 (0)
Median HBV DNA 12 weeks after withdrawal (IQR; range)**	0 IU/mL (0; 0-252)	0 IU/mL (0; 0)	153 IU/mL (54-252)
Median HBsAg HQ 12 weeks after withdrawal (IQR; range)	2.2 mIU/mL (5.3; 0.5-191.0)	2.2 mIU/mL (4.1; 0.5-21.0)	96.1 mIU/mL (1.5-191.0)
Median HBcrAg 12 weeks after withdrawal (IQR; range)	2.0 log_{10}U/mL (0; 2.0)	2.0 log_{10}U/mL (0; 2.0)	2.0 log_{10}U/mL (0; 2.0)
Median HBV pgRNA 12 weeks after withdrawal (IQR; range)**	0 log_{10} U/mL (0; 0.0-3.0)	0 log_{10} U/mL (0; 0)	2.65 log_{10} U/mL (2.3-3.0)
Median anti-HBsAb 12 weeks after withdrawal (IQR; range)**	2.67 mIU/mL (8.2; 0-24)	3.12 mIU/mL (7.8; 0.3-24.0)	0.7 mIU/mL (0.4-1.1)
Median ALT activity 12 weeks after withdrawal (IQR; range)*	22 IU/L (9; 12-52)	22 IU/L (8; 12-43)	49.5 IU/L (47-52)

- * $P < 0.05$;
- ** $P < 0.01$.
- Abbreviations: IQR, interquartile range; anti-HBsAb, Hepatitis B surface antibody.

Cohort A: patients on maintenance suppressive therapy with tenofovir

Cohort A was made up of 66 HBeAg-negative, tenofovir disoproxil fumarate (TDF)-treated patients without cirrhosis with CHB receiving continuous maintenance therapy (TDF) and who had suppressed HBV DNA for at least 5 years (70% males; median age, 45 years); median treatment duration was 7.5 years (range, 5.5-11.5). All patients in this cohort had a previous liver biopsy (local clinical guidelines). None had cirrhosis. The detailed clinical characteristics of this cohort at the start of NA are presented in Table 1.

Cohort B: patients on long-term NA suppressive therapy for at least 3 years and in whom NA treatment was withdrawn before HBsAg loss

Initially, 25 HBsAg-positive patients without cirrhosis (64% male; median age, 48 years) were treated for a median duration of 6.9 years (range, 3.2-11.2) with TDF or entecavir (ETV) and had undetectable HBV DNA for at least 3 years (all still HBsAg detected), but only 23 patients were HBeAg negative at the start of NA therapy and these were included in the study. NA therapy was withdrawn from patients, and patients were followed for at least 52 weeks (median, 78; range, 52-96). A proportion of patients (17%) developed clinically significant ALT flares and required restarting NA therapy. Changes in serological/virological markers during NA therapy and post-NA withdrawal were assessed for up to 96 weeks after NA cessation. The detailed clinical characteristics of this cohort are shown in Tables 2 and 3.

Cohort C: patients with HBsAg loss on long-term suppressive NA therapy, in whom therapy was withdrawn after HBsAg loss

Nineteen HBeAg-negative patients without cirrhosis (58% male; median age, 36 years) were treated with TDF or ETV for a median duration of 8.5 years (range, 1.6-12.2) and achieved HBsAg loss after a median of 7.5 years. NA therapy was continued for a median of 1 year (range, 0.5-2.7) after HBsAg loss in these patients, and patients were followed every 3-6 months thereafter. The detailed clinical characteristics of this cohort are summarized in Tables 4 and 5.

This observational, single centre study was conducted following the ethical principles of the Declaration of Helsinki and had institutional ethical approval.

Written informed consent was obtained from all participants in the study.

Sequential serum/plasma samples were available for all patients at the inception of NA therapy and on treatment and after NA therapy.

HBV Serological Markers

HBsAg, antibody to HBsAg (anti-HBs), HBeAg, and antibody to HBeAg (anti-HBe) were analysed in serum using commercially available enzyme immunoassays.

HBV DNA Concentrations in plasma

Plasma HBV DNA concentrations were determined by a quantitative real-time PCR assay, COBAS AmpliPrep-COBAS TaqMan HBV test. The results are presented as $\log_{10}$IU/mL.

HBV genotyping

At the time of diagnosis, HBV genotype was determined in all HBsAg-positive patients with an HBV DNA > 50 IU/mL, using the direct population sequencing method within the S-gene region after nucleic acid amplification and using an ABI 3130xl genetic analyser (Life Technologies, Waltham, MA). Multiple sequence alignment of sample and GenBank genotype reference sequences was performed using ClustalX 2.0.

Sequencing for Precore (G1896A) and Basal Core Promoter (A1762T/G1764A) mutations

Sequencing was performed using an ABI 3130xl genetic analyser (Life Technologies), according to the manufacturer's instructions. Sequences were analysed using SeqScape V2.7 software and by GenBank precore region reference sequences for individual genotypes in ClustalX 2.0.

HBsAg quantification assay

HBsAg concentrations in serum were measured using the Abbott ARCHITECT chemiluminescent microparticle immunoassay. The test has a dynamic range of 0.05-250

IU/mL. Samples were initially diluted 1:500, according to the manufacturer's instructions. Samples with HBsAg levels <25 IU/mL were retested neat. The results are expressed as $\log_{10}$IU/mL.

HBsAg Ultrasensitive assay

HBsAg concentrations in patients with HBsAg levels <1 IU/mL by Abbott ARCHITECT were measured by the ultrasensitive chemiluminscence enzyme immunoassay (CLEIA) Hepatitis B surface antigen ultrasensitive assay (HBsAg-HQ; Fujirebio, Gent, Belgium) on a Lumipulse 600 platform (Fujirebio).[10] The detection limit for this assay is 1 mIU/mL, and the assay dynamic range is 1-150,000 mIU/mL. The results of HBsAg concentrations are presented as mIU/mL.[11,12]

Anti-HBs assay

Concentrations of anti-HBs were measured by an automated chemiluminescent assay (CMIA; Abbott ARCHITECT; Abbott Diagnostics) in all patients with HBsAg levels <100 IU/mL. The results are presented as mIU/mL. Levels <10 mIU/mL are not considered positive.

HBcrAg Quantification assay

HBcrAg serum concentrations were measured using a CLEIA (Lumipulse G HBcrAg assay; Fujirebio). This immunoassay measures the antigenic reactivity of three proteins: HBeAg, HBcAg, and core-related protein p22cr (products of the HBV precore/core gene sharing a 149-amino-acid sequence).[13] The assay has a linear measurement range of 3-7 $\log_{10}$U/mL using 3 $\log_{10}$U/mL as a detection limit for this assay (recommended by the manufacturer); dilution was not performed for samples with concentration >7 $\log_{10}$U/mL. The results are presented as $\log_{10}$U/mL.

Pregenomic HBV-RNA assay

HBV RNA was isolated from plasma and amplified, as described by Butler et al., using the m2000 system (Abbott Molecular) in the Department of Infectious Diseases, Abbott

Diagnostics.[4] The lower limit of quantitation for the assay is 1.65 $\log_{10}$U/mL. The results are presented as $\log_{10}$U/mL.

Definition of ALT flares after withdrawal of NA therapy

Severity of ALT flares were classified based on ALT level changes after stopping therapy. Comparisons were made with the upper limits of normal (ULNs) for ALT levels (female, ≤19 IU/L; male, ≤30 IU/L) and with ALT levels at time of NA withdrawal. Patients with no/minimal changes in ALT activity (within 2 times ULN and not exceeding 2 times from ALT levels at time of withdrawal were defined as have no/minimal flare). Patients with ALT activity >2 times ULN, but <5 times ULN, and increased ALT levels >2 times from ALT levels at time of withdrawal had mild flare. A moderate flare was defined as ALT levels >5× ULN, but <10× ULN, and ALT levels increased >5-fold after NA withdrawal. Severe flare was diagnosed in our cohort in patients with ALT levels exceeding 10× ULN and with an increase in ALT levels 10× the ALT levels at time of NA withdrawal.

Definition of post HBsAg clearance relapse

Patients who had achieved HBsAg and stopped therapy were classified as relapsers if they had detectable HBV DNA or their HBsAg could be detected by the qualitative Abbott Architect (Abbott Diagnostics) assay any time after stopping antiviral therapy.

Statistical analysis

Statistical analyses were performed using SPSS software (version 25.0). Continuous variables are shown as median (ranges) and were compared using the Mann-Whitney U test. Categorical variables were compared using chi- squared or Fisher's exact tests. Correlations between two continuous variables were analysed using Spearman's rank test. A P value < 0.05 was considered significant.

Results

Cohort A: patients on maintenance suppressive therapy with TDF

Baseline characteristics at start of NA

Detailed baseline characteristics of cohort A patients are presented in Table 1 and Supporting Table S1. All patients were HBeAg negative at time of initiating NA therapy and did not have liver cirrhosis. All patients underwent a liver biopsy before start of therapy (local HBV guidelines) and met criteria for antiviral therapy based on the European Association for the Study of the Liver 2017 guidelines.[14] All achieved HBV DNA suppression on NA therapy within a median of 6 months (range, 3-9), and all remained suppressed with HBV DNA concentrations <20 IU/mL during NA therapy.

Proportion of patients with detectable plasma pgRNA and HBcrAg after 1, 3 and 5 years of antiviral therapy

At therapy baseline, 19 patients (29%) had undetectable HBcrAg (≥3 $\log_{10}$U/mL) and 11 patients (17%) had pgRNA below the limit of detection. After 1 year of therapy, when all patients had achieved undetectable plasma HBV DNA (median duration to undetectable HBV DNA was 6 months; range, 3-9), 56% of patients still had detectable HBcrAg and 73% had detectable pgRNA. After 3 years of NA therapy, 33% had detectable HBcrAg and we detected pgRNA in serum in 30% (Fig. 1). After 5 years therapy with NA, there were still 18 patients (27%) with detectable HBcrAg (median, 3.0 $\log_{10}$U/mL; range, 2.1-5.3) and 9 patients (14%) with measurable pgRNA in serum (median, 1.71 $\log_{10}$U/mL; range, 1.67-2.89).

Differential changes in HBV markers during antiviral therapy: HBV DNA, HBsAg, HBcrAg, and pgRNA

Whereas HBV DNA rapidly declined, as expected, after a median duration of NA therapy of 6 months in all patients (Fig. 2A), HBsAg levels declined only slowly at a rate of 25 IU/mL per year, but none of the treated patients achieved HBsAg loss (Fig. 2B). There was no significant change in HBsAg concentrations between therapy baseline and after 5 years on NA therapy.

HBcrAg levels did decline significantly during NA therapy (Fig. 2C). Similarly, pgRNA concentrations dropped significantly between treatment baseline and after 3 and 5 years on therapy, respectively (Fig. 2D), but, as noted above, the decline in pgRNA did not mirror the rapid decrease in serum HBV DNA.

Influence of baseline markers on pgRNA and HBcrAg status after 3 years and 5 years therapy

When we compared the concentrations of serological/virological markers at the NA therapy initiation between patients who achieved pgRNA loss after 3 years, it was noted that baseline HBV DNA, HBsAg, HBcrAg, and pgRNA concentrations were all significantly higher in patients with still detectable pgRNA after both 3 and 5 years of NA therapy (Supporting Fig. S2A-D; Supporting Tables S2 and S3). The role of HBV genotypes and its impact on HBV markers and their changes was analysed, but no significant effect of genotypes was noted. Furthermore, the role of mutations in the precore region (precore-G1896A and basal core promoter BCP] A1762T/G1764A) was assessed. Patients with precore (G1896A) mutation were more likely to have detectable HBcrAg and pgRNA after 3 and 5 years of therapy (Supporting Tables S2 and S3; Supporting Fig. S6).

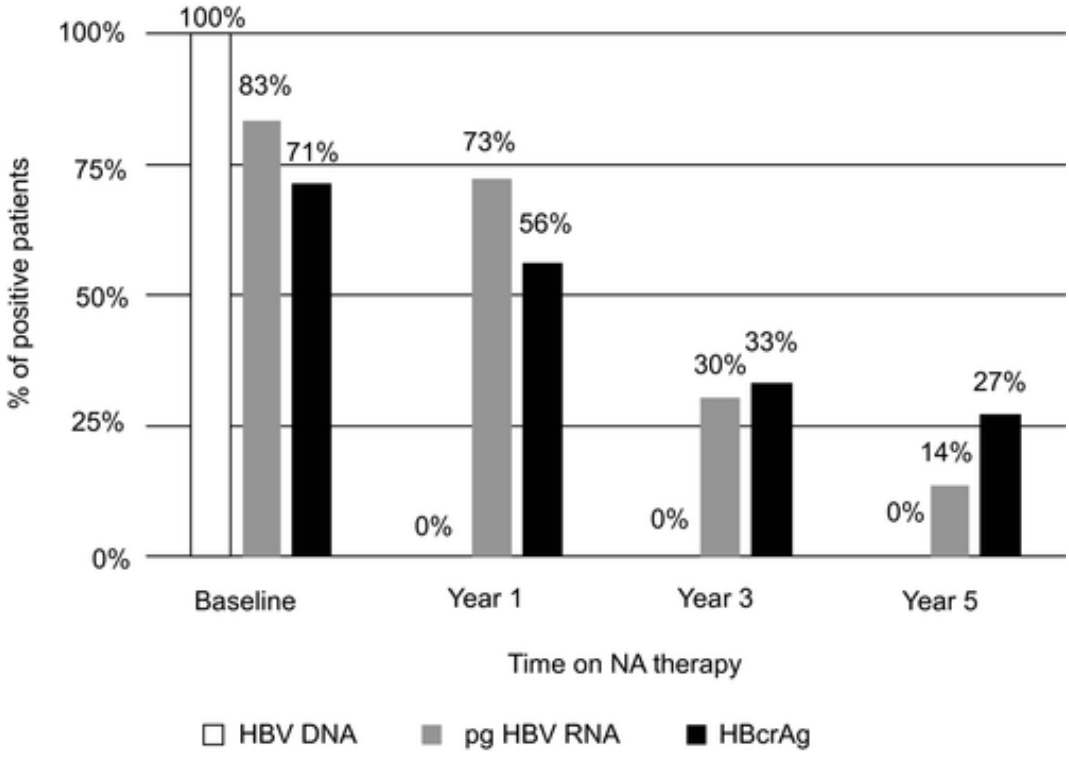

Figure 1

Proportions of patients with detected HBV DNA, pg HBV RNA, and HBcrAg during NA therapy in cohort A.

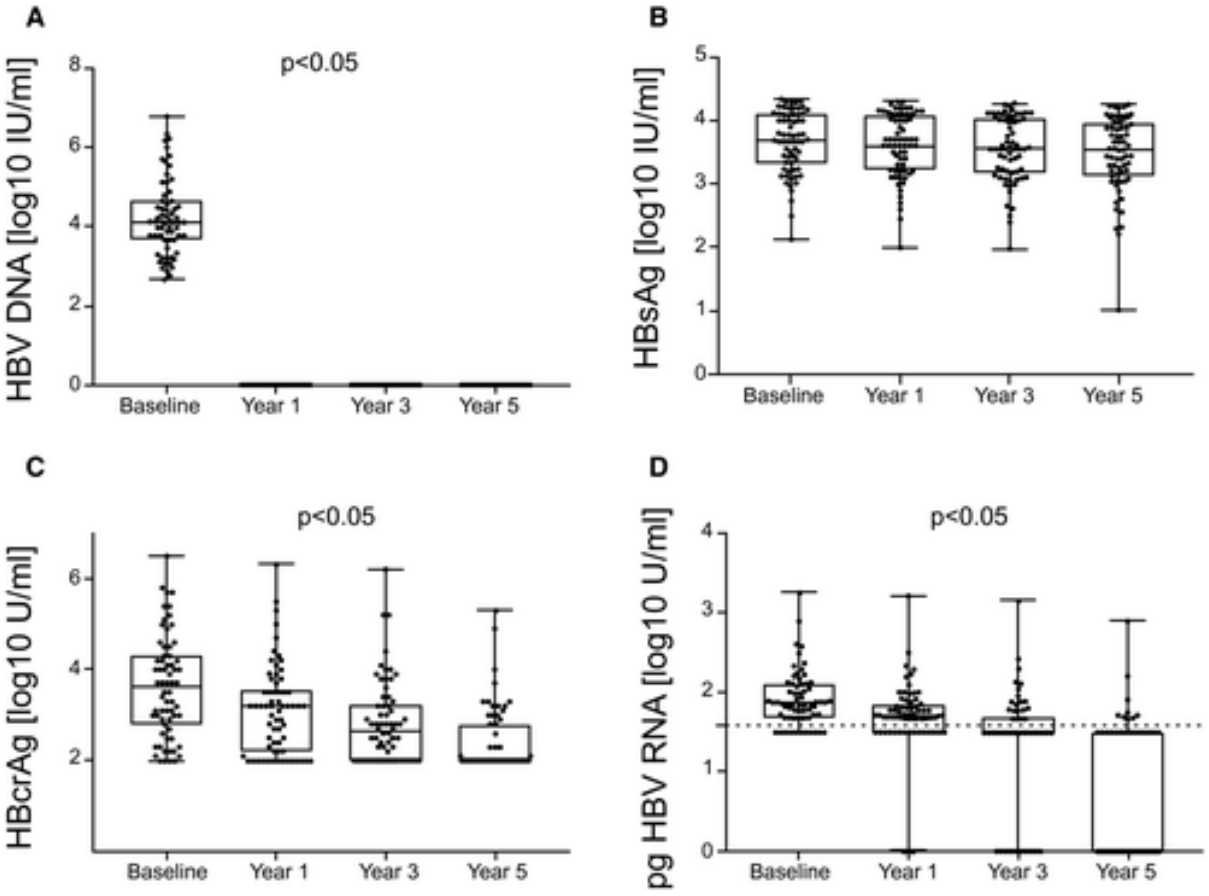

Figure 2
Graphical representation of changes in individual serological/virological markers in different time points (all cohort A)—HBV DNA, HBsAg, HBcrAg, and HBV pg RNA—start of therapy. (A) HBV DNA viral load [log10 IU/ml]. (B) HBsAg levels [log10 IU/ml]. (C) HBcrAg concentrations [log10 U/ml]. (D) HBV pg RNA concentrations [log10 U/ml].

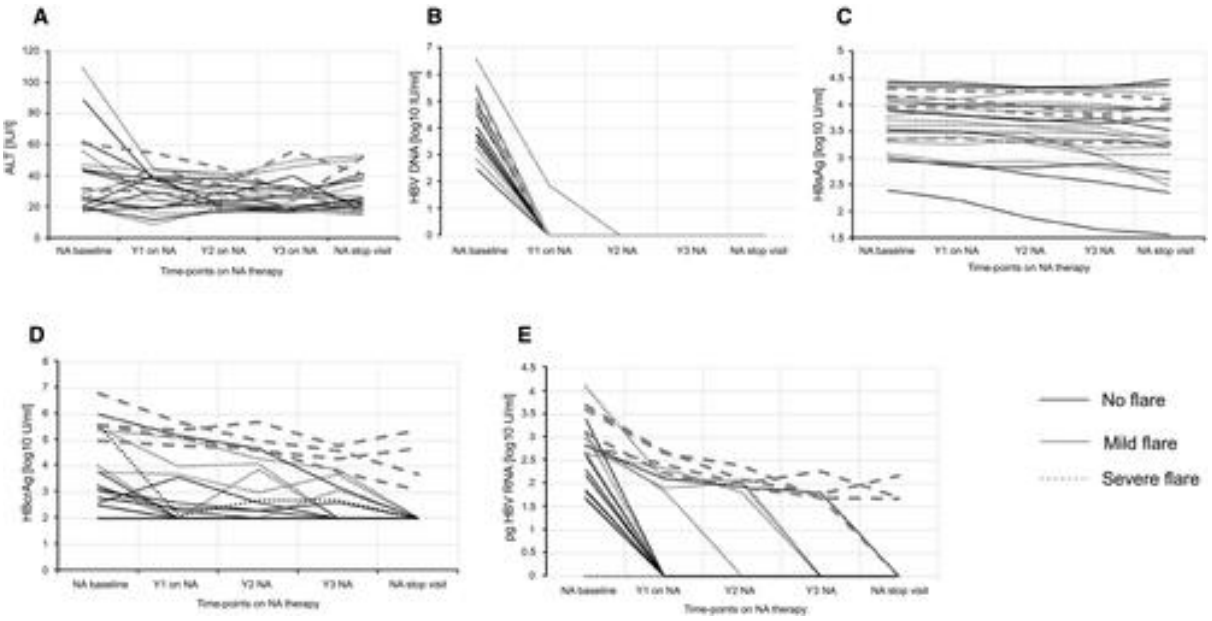

Figure 3
Graphical representation of changes in individual serological/virological markers in different time points during NA therapy (cohort B, n = 23): ALT, HBV DNA, HBsAg, HBcrAg, and HBV pgRNA. (A) ALT [IU/L]. (B) HBV DNA viral load [log10 IU/ml]. (C) HBsAg levels [log10 IU/ml]. (D) HBcrAg concentrations [log10 U/ml]. (E) HBV pgRNA levels [log10 U/ml].

Cohort B: Patients on long- term NA suppressive therapy in whom NA treatment was withdrawn before HBsAg loss

Serological and virological markers at therapy baseline

The detailed summary of the baseline characteristics for HBeAg negative patients (n = 23) is presented in Table 2. Initially, this cohort consisted of 25 patients (Supporting Document S1), but 2 patients were HBeAg positive at the start of NA therapy and, despite achieving HBeAg loss during the therapy, represent a different subgroup of patients[15] and were excluded from the study. All patients enrolled into the study had persistently undetectable HBV DNA for at least 3 years before NA withdrawal.

Dynamic changes in serological and virological markers on NA therapy

All patients achieved improvement in ALT activity (Figure 3A) and HBV DNA suppression within a median of 6 months (range, 3-12; Fig. 3B). HBsAg levels, as expected in a predominantly HBeAg negative cohort, did not show a marked decline on treatment in the majority of patients (Fig. 3C). No patients lost HBsAg during NA therapy. HBcrAg

concentrations declined during NA therapy: 19 of 23 patients (83%) had undetectable HBcrAg at time of NA withdrawal (Fig. 3D). Similarly, pgRNA levels reduced during antiviral therapy with NA and were below the assay detection limit of 1.65 $\log_{10}$U/mL in *20* patients when therapy was withdrawn (Fig. 3E). Similarly to cohort A, whereas HBV DNA was undetectable in all patients after 1 year of therapy, pgRNA was still detected in *9* patients (Supporting Fig. S3). At therapy baseline, HBV-DNA concentrations were relatively higher than pgRNA levels.

Serological/Virological markers at time of NA withdrawal

At the time of NA cessation, all patients were HBeAg negative and had undetectable HBV DNA concentrations (for at least 3 years). Serum HBsAg was detected in all patients in a range from 38 to 30,334 IU/mL, with 5 (22%) patients having HBsAg concentrations >10,000 IU/mL, 12 (52%) patients between 1,000 and 10,000 IU/mL, 5 (22%) patients between 100 and 1,000 IU/mL, and only 1 patient <100 IU/mL, respectively. HBcrAg was detected (≥3 $\log_{10}$U/mL) in 4 patients of this cohort (17%), and pgRNA was detectable (>1.65 $\log_{10}$U/mL) in 3 patients (13%) at time of NA withdrawal. Only patients with detectable HBcrAg at time of NA withdrawal had detectable pgRNA. The clinical characteristics at time of NA withdrawal are shown in Table 3.

ALT flares frequency and ALT kinetics after NA withdrawal

The detailed changes in ALT levels after NA withdrawal are shown in Fig. 4A. The majority of ALT flares occurred between 8 and 24 weeks after stopping NA and persisted for a median of 8 weeks (range, 4-20) before either recovering spontaneously (10 patients) or resolving after recommencing therapy (4 patients). A variable pattern was observed: Nine patients showed no/minimal changes in ALT levels after NA cessation. In contrast, 10 patients had mild ALT flares after stopping NA therapy. Four patients had severe flares. In patients with severe ALT flares, peak of ALT levels coincided with an HBV DNA concentration of >100,000 IU/mL (Fig. 4B), but a rise in DNA concentrations of this order was not observed in patients with mild or no flares. All 4 patients with severe ALT flares were recommenced on NA therapy; ALT flares resolved within 8 weeks on therapy. There was no difference in flares timing between patients with severe flares versus patients with no/mild flares after NA withdrawal.

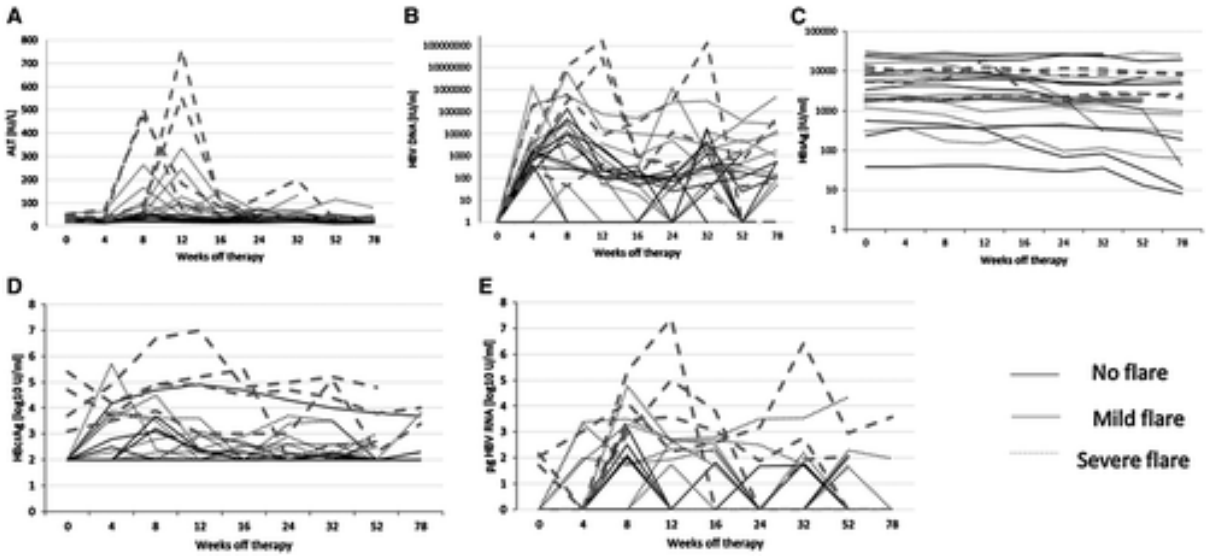

Figure 4
Graphical representation of changes in individual serological/virological markers in different time points after NA therapy cessation (cohort B, n = 23): ALT, HBV DNA, HBsAg, HBcrAg, and HBV pgRNA. (A) ALT [IU/L]. (B) HBV DNA viral load [IU/ml]. (C) HBsAg levels [IU/ml]. (D) HBcrAg concentrations [log10 U/ml]. (E) HBV pgRNA levels [log10 U/ml].

Changes in HBV DNA HBsAg, HBcrAg, and pgRNA after NA withdrawal in patients with ALT flares

All patients had detectable HBV DNA in serum after NA withdrawal. The longitudinal changes are shown in Fig. 4B. HBV DNA was transiently elevated in 5 (22%) of 23 patients (Supporting Fig. S3). In contrast, HBsAg concentrations did not change significantly after NA withdrawal and no patient cleared HBsAg, but 6 (26%) patients (all with HBsAg < 1,000 IU/mL at time of withdrawal; 3 patients with mild flares and 3 with no flares) had a decline in HBsAg of >1 $\log_{10}$IU/mL after NA withdrawal. This decline occurred predominantly beyond 32 weeks after stopping therapy (Fig. 4C). The detailed comparison of markers at time of therapy baseline and time of NA withdrawal between patients with decline in HBsAg > 1 $\log_{10}$IU/mL versus patients with no/minimal HBsAg decline (<1 $\log_{10}$IU/mL) after NA withdrawal is shown in Supporting Table S7. Interestingly, patients with steeper HBsAg decline had lower therapy baseline HBsAg, HBcrAg, and pgRNA levels, respectively. In contrast, only HBsAg levels were lower in sharp HBsAg decline patients at time of NA withdrawal.

The dynamic changes in HBcrAg concentration are shown in Fig. 4D. The majority of patients had transiently elevated HBcrAg, after NA withdrawal, which resolved. However, it is noteworthy that patients with severe ALT flares had detectable HBcrAg at time of NA

withdrawal, the levels of which increased after NA treatment was stopped. HBcrAg concentrations declined in patients with severe flares after recommencing NA therapy.

The majority of patients had transient positivity of pgRNA after NA withdrawal. In 3 patients in whom pgRNA was still detectable in serum at time of withdrawal, severe ALT flares occurred after NA cessation (Fig. 4E). In contrast, in 5 of 23 patients with an undetectable pgRNA after withdrawal, flares were not observed (Supporting Fig. S3). Furthermore, there was no significant influence of genotypes on ALT flares, but the mutations within precore and BCP were more frequent in patients with severe flare (75%) and, although the mechanism is unclear, might contribute to higher HBcrAg concentrations in these patients.

Thus, in summary, both these markers were able, singly and together, to predict severe ALT flares after NA withdrawal. Severe ALT flare after NA cessation occurred exclusively in patients with a detectable HBcrAg in serum at the time of therapy withdrawal (100% specificity and 100% positive predictive value); pgRNA was detected in 3 of 4 patients who subsequently developed a severe ALT flare (100% specificity, 75% sensitivity, and 100% positive predictive value; Supporting Fig. S5).

Cohort C: Patients with HBsAg loss on long-term suppressive NA therapy, in whom therapy was withdrawn.

Serological and virological characteristics of this cohort of patients at initiation of NA therapy are shown in Table 4. Seven patients were initially HBeAg positive and were previous nonresponders to pegylated IFN (Peg-IFN) therapy. The interval between stopping Peg-IFN and starting NA was at least 6 months (median, 15; range, 6-30). All HBeAg-positive patients achieved HBeAg loss within a median of 18 months (range, 6-36) after starting NA and subsequently had HBsAg loss.

Detailed characteristics of serological/virological markers at time of NA withdrawal are shown in Table 5. All patients were treated for a median duration of 8.6 years (range, 1.6-12.2) before HBsAg loss occurred. After HBsAg loss, NA therapy was continued in all patients for at least 6 months (median, 20; range. 6-44).

HBV DNA and HBsAg reactivation after NA withdrawal

All patients were followed regularly every 6 weeks for 6 months and then every 12 weeks after NA cessation. The median duration of follow-up was 60 weeks (range, 36-132). None of the patients had detectable HBsAg by qualitative assay after NA withdrawal, but 2 of 19 patients (11%) had detected HBV DNA after stopping NA. Both patients with HBV DNA reactivation after NA withdrawal were classed as relapsers and had an increase in ALT activity coinciding with detectable HBV DNA (Fig. 5A and Fig. 5B). Both were recommenced on NA and eventually suppressed HBV DNA (Supporting Fig. S7). Mutations within the HBs gene were excluded in these 2 patients.

HBsAg was not detected in any patient by the qualitative Abbott assay after NA withdrawal. However, using an ultrasensitive assay, we were able to detect HBsAg in all patients at time of NA withdrawal and during the follow-up period (12 weeks after stopping therapy). There was no difference between HBsAg levels before and after NA cessation, and the levels of HBsAg did not differ between patients with relapse versus patients who did not relapse (Fig. 5C).

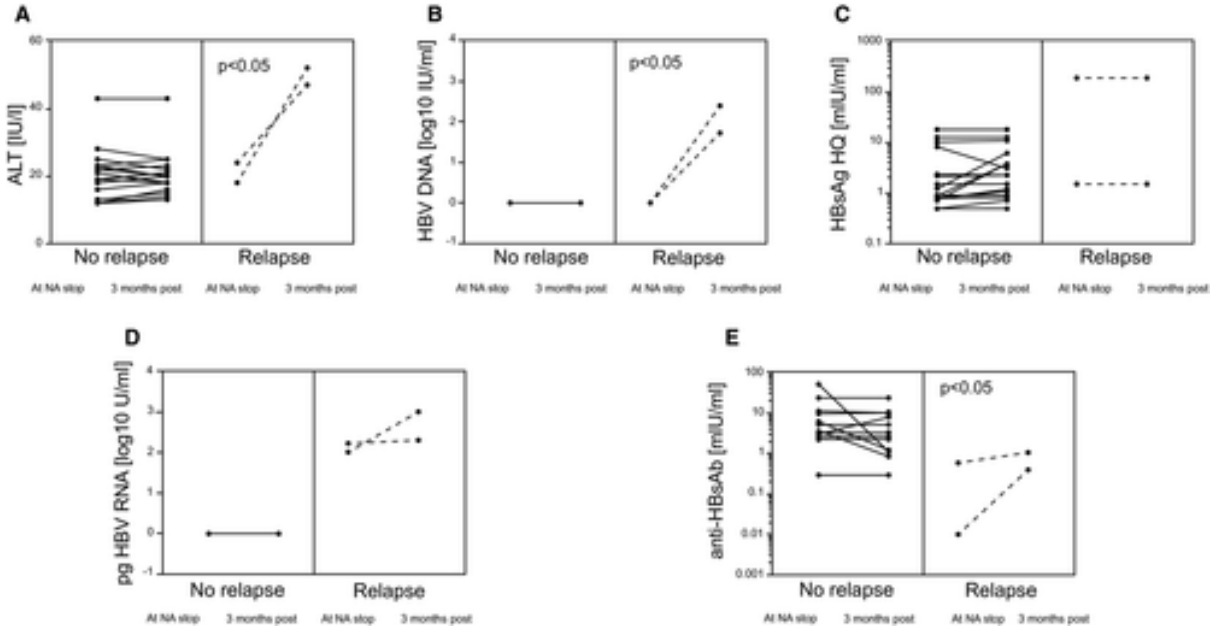

Figure 5
Dynamic changes in serological/virological markers at time of NA withdrawal and during follow-up (ALT, HBV DNA, HBsAg ultrasensitive, HBV pgRNA, and anti-HBs antibody levels) presented based on HBV DNA reactivation after NA cessation (cohort C). (A) ALT [IU/l]. (B) HBV DNA viral load [log10 IU/ml]. (C) HBsAg levels by HQ assay [mIU/ml]. (D) HBV pgRNA levels [log10 U/ml]. (E) anti-HBs antibody levels [mIU/ml].

HBcrAg and pgRNA concentrations

All patients did not have detectable HBcrAg in serum before and after NA cessation. In contrast, 2 patients who developed HBV DNA reactivation after NA withdrawal had detectable pgRNA at time of withdrawal, and their pgRNA levels increased after stopping NA (Fig. 5D). Thus, detectable pgRNA in serum at time of NA withdrawal proved able to predict HBV DNA reactivation after stopping NA. Other serological markers, including ultrasensitive HBsAg levels or anti-HBs antibodies, proved less specific for predicting HBV reactivation.

anti-HBs antibody levels

Serum anti-HBs levels at NA withdrawal were higher in patients without HBV DNA reactivation than in relapse patients, and this was the case after NA withdrawal (Fig. 5E). Levels of anti-HBs antibodies did not increase significantly after stopping NA and was not considered positive in the majority of patients.

Discussion

Current paradigms of management of CHB are complex and require longitudinal serological and virological monitoring during differing phases of the disease. The focus of this observational study was to assess the additional clinical utility of pgRNA, qualitative and quantitative HBsAg concentrations, ultrasensitive HBsAg levels, and HBcrAg in serum to gauge and predict clinical outcomes in three different, but well-characterized and homogenous, cohorts of patients. As apparent from our analyses, serological measurement of HBV DNA and HBsAg are unable to sufficiently inform residual HBV replication in patients or the risk of either reactivation or severe exacerbations of hepatitis after NA withdrawal.

In this study, we have concurrently examined the utility of pgRNA and HBcrAg in patients with ostensibly reduced HBV replication during long-term suppression on antiviral therapy with a potent NA, after NA therapy withdrawal, and during remission of the disease off treatment, following HBsAg loss by a qualitative HBsAg assay and subsequent NA withdrawal.

These perturbations in the natural history and replication of hepatitis B provided an opportunity to study relative alterations in the concentrations of biomarkers of Hepatitis B.

Importantly, we have observed that both pgRNA and HBcrAg remain detectable for years after profound HBV DNA suppression by chain terminating NAs in a cohort of NA treated HBeAg-negative patients. HBsAg concentrations, not unexpectedly, did not decline in this cohort.[16]

Although measurement of HBV RNA in serum has been reported, the dissociation in treated patients is of interest, but requires further characterization in patients.[2,3,17] pgRNA was measured in this study using primers and probes directed to conserved regions within the 5′ end of the X gene and the 3′ end of the core gene without need for DNase pre-treatment. RNA specificity in this assay was confirmed by archiving RNA eluates after DNase I treatment and demonstrated an excellent correlation with the RACE method.[4] The methodology for pgRNA extraction/measurement has been recently standardized, and units were linked to a World Health Organization standard. The sensitivity/ specificity and precision (inter-/intra-) have all been tested for this assay and are equivalent to U.S. Food and Drug Administration/European Commission *in vitro* diagnostic viral load assays. Although pgRNA has been measured before, it was not done with a test that is standardized and has this sensitivity. In addition, the same methodology was used throughout, allowing assessment of the clinical significance of the assay, notwithstanding possible inter-assay variations in pgRNA sensitivity, specificity, and analytical performance. We also studied a sizeable representation of all major HBV genotypes (A-E) reflecting our real-life cohorts. In addition, the role of precore and BCP mutations was cautiously investigated.

The dissociation of serum HBV DNA concentrations, and pgRNA in both cohort A and B patients, during NA therapy indeed suggests that pgRNA in serum is a clinically exploitable surrogate marker indicative of transcriptionally active cccDNA and the persistence of HBV RNA that is not reversely transcribed into HBV DNA. The serological detection of pgRNA circumvents the impractical difficulty of measuring intrahepatic cccDNA.

Our analyses (group of patients infected with variable HBV genotypes) suggest that regardless of profound inhibition of viral replication, cccDNA transcription continues despite chain termination of (–) and (+) strand HBV DNA and may be dependent upon baseline values.[18] Our findings do not, however, establish whether the residual detection of pgRNA in patients

after long-term suppression is a measure of silencing, depletion, or damage to the copy number and pool of cccDNA molecules in treated patients.[9] Rates of disappearance of HBcrAg and pgRNA differed. The reasons for the apparent differences in silencing of cccDNA transcription between patients will require further study, including examination of intrahepatic cccDNA concentrations and, we speculate, perhaps differences in cell turnover, HBV X protein activation, or epigenetic modification of cccDNA. However, such studies were not part of the scope of our analysis.[19]

Measurement of pgRNA provides ancillary information and points to the limitations of HBV DNA and HBsAg measurement to predict remission after NA treatment cessation. The inference is that some patients, despite ostensible suppression of HBV replication, continue to transcribe pgRNA. These observations point to incomplete inhibition of HBV replication in HBeAg-negative patients by NA and may explain high rates of recurrence after NA withdrawal despite apparent effective suppression of HBV DNA.

An insubstantial longitudinal decline in HBsAg was observed in the cohort of HBeAg-negative patients on long-term therapy, probably indicating the derivation of a substantial fraction of HBsAg in serum from integrated viral genomes.[20] HBsAg quantitation is a fallible measure of the risk of reactivation after NA withdrawal and confers predictive value only at low concentrations, which are not observed in the majority of patients.[21-25]

In addition, in these studies, as previously noted, we observed a similar dichotomy in the decline in HBcrAg concentrations, determined using the Lumipulse assay (Fig. 2).[26] Although measurement of HBcrAg in serum could be influenced by genotype and the composition of precore in circulating HBV quasispecies, our data confirm previous findings and suggest that, in HBeAg-negative patients, HBcrAg may be a correlate of pgRNA translation, but could also distinguish a sub- group with higher cccDNA levels and residual HBV replication or cccDNA transcriptional activity.[6,27]

The residual detection of both pgRNA and HBcrAg predicted severe ALT flares after NA withdrawal, given that these were exclusively observed in only patients with detectable HBcrAg and pgRNA (*in 3 of 4* patients) at the time of NA therapy withdrawal. DNA suppression and HBsAg concentrations before withdrawal are not constant or reliable

predictors of a severe flare; there is an important clinical need to predict severe exacerbations, requiring retreatment. Taken together, our data suggest that detectable pgRNA and HBcrAg may together reflect residual cccDNA and transcription that could contraindicate NA withdrawal. Further prospectively designed studies are required to validate this supposition.

This study cohort was heterogeneous, reflecting real-life patients (HBeAg negative at the start of therapy)[15] in our clinic, and was not powered to examine HBsAg loss after NA withdrawal (and indeed no patient cleared HBsAg), but focused on examining the risk of severe exacerbations necessitating retreatment—a clinically important endpoint.[28,29]

Finally, HBV DNA was detected in only 2 patients who cleared HBsAg and stopped NA therapy. Interestingly, HBsAg was detected by an ultrasensitive assay in all patients who had ostensibly become HBsAg negative by a qualitative test; also, pgRNA was detected at the time of withdrawal of these patients, suggesting again that residual pgRNA is a potential marker of regulatable cccDNA transcription by NA therapy.

Our findings complement other recent reports of pgRNA and HBcrAg in serum, but their correlated and comprehensive analysis in these three clinically pertinent phenotypes of CHB patients, for whom NA cessation would be contemplated, could inform and provide measurable endpoints to guide clinical decisions to withdraw NA therapy (and possibly the application and duration of future targeted HBV therapies for functional cure). These markers should be studied in patients treated in clinical trials with newer compounds, which can deepen inhibition of HBV replication, particularly given that these trials are of relatively short and, by definition, finite duration. It is possible that pgRNA and HBcrAg could guide the planning and interpretation of the effectiveness (and safety) of compounds in such studies going forward.[1] We observed a strong positive correlation between pgRNA and HBcrAg in HBsAg-positive patients receiving NA therapy (Supporting Figs. S4 and S5). Automated and high-throughput methods for pgRNA detection will facilitate the expanded use of this marker in clinical practice, in conjunction with other standard serological markers and HBcrAg.[30]

Our data indicate that current NA therapies are able to induce viral suppression in the majority of patients. Lifelong treatment remains the norm but attempts to provide a finite therapy are in progress. Finite therapy may be achieved by NA therapy withdrawal, but our data and that of others signal that residual viral replication and transcription of pgRNA from cccDNA are

detectable years after initiating therapy, is measurable, and can predict severe exacerbations of the disease after NA withdrawal. Other targets to inhibit HBV replication are in development with the goal of achieving a “functional cure” or higher rates of HBsAg loss than is presently possible with current therapy. Many challenges remain. However, the ability to detect residual cccDNA persistence and transcription under treatment with more sensitive assays will be pivotal to the development of therapies.(31)

Chapter 5

Investigating the role of serum HBcrAg and HBV pgRNA in predicting clinical outcomes in CHB patients on antiviral therapy.

Abstract

Antiviral therapy is a vital part of chronic HBV management. Antiviral therapy can dramatically improve the clinical outcome of patients with chronic HBV. Treatment of CHB patients is based on monotherapy with pegylated interferon (Peg-INF) or with Nucleot(s)ide analogues (NAs), especially entecavir, tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide (TAF). It remains unclear when treatment with NAs should be stopped. Though sustained HBV DNA suppression and HBeAg seroconversion are associated with disease remission they do not reflect changes in intrahepatic covalently closed circular DNA (cccDNA). Discontinuing antiviral therapy based on HBV DNA, HBsAg concentration and HBeAg seroconversion could put some patients at risk of reactivation. If cccDNA is still active viral mRNA can still be translated, this in turn may lead to liver damage.

In this study I investigate whether HBcrAg and HBV pgRNA can be useful in predicting clinical outcomes in CHB patients on antiviral therapy who seroconverted to anti-HBe status versus patients who did not seroconvert to anti-HBe. 58 CHB patients were treated and monitored for 10 years. It was found that HBcrAg and HBV pgRNA could still be detected in patient serum after HBeAg seroconversion and HBV DNA suppression. Additionally, HBcrAg and pgRNA could be detected at the point of NA discontinuation in patients who experienced HBV reactivation.

The results of this study indicate that these novel markers could help identify patients who may be at risk of liver damage during antiviral therapy when HBV DNA is suppressed and identify patients who are susceptible for HBV reactivation after HBeAg seroconversion and discontinuation of antiviral therapy.

Aim of study

This study investigates whether HBcrAg and HBV pgRNA can be useful in predicting clinical outcomes in CHB patients on antiviral therapy. A positive association has been previously shown between these novel markers (HBcrAg and pgRNA) and conventional biomarkers (HBsAg, HBV DNA) and their potential in predicting clinical outcomes. The aim of this study is to focus on their role as treatment predictors and whether they can be used to predict virological response and liver disease progression in patients who achieve HBeAg seroconversion versus those who do not while on Nucleos(t)ide (NA) therapy.

Baseline: In this study baseline is described as the time just before patients were placed on antiviral therapy (treatment baseline). Therefore, baseline results represent no antiviral therapy given, the 5 years follow up results represent 5 years of antiviral therapy, and the 10 years follow up results represent 10 years of antiviral therapy.

Study subjects.

This study consists of 58 CHB patients (mean age: 40.5 ± 9.3 years old, male: 66%) who had acquired HBV infection at childhood and were followed up as adults at the hepatitis clinic at King's College Hospital (KCH) as their standard of care. All patients were placed on antiviral therapy with NAs.

The patients were placed into cohorts based on their HBeAg status at treatment baseline (Figure 1). Cohort 1 (n=44) consisted of HBeAg positive patients at baseline who all became HBeAg negative after 5 years of NA therapy. Cohort 1 was divided into 2 sub-cohorts – 1.1 (n=17) and 1.2 (n=27). Sub-cohort 1.1 consisted of patients who became HBeAg negative and acquired anti-HBe antibodies after 10 years of NA therapy. Sub-cohort 1.2 consisted of patients who became HBeAg negative but did not acquire anti-HBe antibodies after 10 years of NA therapy. Cohort 2 (n=14) consisted of HBeAg negative patients at treatment baseline. Cohort 2 was divided into 2 sub-cohorts – 2.1 (n=5) and 2.2 (n-9). Sub-cohort 2.1 consisted of patients who acquired anti-HBe antibodies after 10 years of NA therapy and sub-cohort 2.2 consisted of patients who did not acquire anti-HBe antibodies after 10 years of NA therapy.

All patients provided informed consent for Kings College biobank and all principles of the Declaration of Helsinki were adhered to. Patients were excluded if they were positive for Hepatitis C virus (HCV), coinfected with hepatitis D virus (HDV), human immunodeficiency virus (HIV), or had any severe systemic disease.

Biomarkers measured.

HBcrAg and HBV pgRNA were measured due to their strong association with cccDNA transcriptional activity. These biomarkers were measured at baseline and at follow-ups to determine cccDNA activity and to assess whether they can be utilised to predict clinical outcomes in CHB patients on NA therapy.

Serological markers -HBsAg, HBeAg, HBeAb and virological marker – HBV DNA were measured at baseline and at follow-ups to assess disease progression, viral replication, and activity at the three time points. Additionally, these conventional markers were measured to compare with novel markers (HBcrAg and HBV pgRNA) in their ability to predict clinical outcomes in CHB patients on NA therapy.

Liver function markers and enzymes – ALT, AST, bilirubin, INR and PLT were measured to determine inflammation and ongoing liver damage. Fibrosis scores (FIB-4) were calculated to determine liver fibrosis at the three time points.

The cytokine CXCL10 / IP-10 was measured at baseline, 5 years, and 10 years of CHB management to find out if it is associated with HBeAg seroconversion in CHB patients on NA therapy and to understand its role in liver disease progression. Additionally, to evaluate its association with the serological and virological biomarkers of HBV infection. IP-10 mediates the inflammatory response and is associated with the chronicity of HBV. IP-10 has been found to be mainly produced by hepatocytes in HBV patients and therefore has the potential to be an important cytokine to help predict liver inflammation and disease progression.

PD-1 is an inhibitory regulator of T cell activity and has been implicated in regulating immune responses to HBV infections. Circulating soluble PD-1 (sPD-1) was measured at baseline, 5 years and 10 years follow ups to find out its associated with HBeAg seroconversion in CHB patients on NA therapy and to understand its role in liver disease progression. Furthermore, to evaluate its association with the serological and virological biomarkers of HBV infection.

Altogether these biomarkers could help identify CHB patients who are most at risk of severe liver damage when on NA therapy and are at risk of relapse if NA therapy is discontinued.

Statistical analysis

Serum HBV DNA, HBsAg, HBcrAg and pgRNA levels were expressed in logarithm. Continuous variables were expressed as mean (average) values with standard deviations. The correlation between clinical variables were determined using Pearson correlation. Univariate analysis was done using COX regression for virological response during antiviral therapy. All statistical analysis was performed using SPSS. A two-tailed p value of <0.05 was considered statistically significant. Graphs were created using GraphPad Prism version 9.

Results

A total of 58 CHB patient serum samples were collected (Males = 38) from patients who attended the Hepatitis clinic at King's College Hospital (KCH) in London as part of their standard of care. Informed consent was received from all patients for KCH biobank. Serum samples were collected at baseline (before antiviral therapy administration). All participants were placed on antiviral therapy with Nucleos(t)ide analogue (NA) following their baseline appointment. On follow up appointments patient's blood were taken and sent to HTS for analysis of HBV serum markers. Serum samples were collected for the same patients once they became HBeAg negative, which was approximately at the 5 ± 3 years follow up appointment and finally at the 10 ± 3 years follow up appointment. Serological, virological, and biochemical results were determined at each time point (Table 1). At baseline, 76% (n=44) of patients were HBeAg positive. All patients were either genotypes A, B, C, D or E (Table 2). Patient demographics are shown in Table 1.

Serum HBV DNA was measured in all patients, 93% (n=54) of patients had detectable HBV DNA at baseline with 81% (n=44) being HBeAg positive. Out of the 54 patients 17% (n=9) had DNA levels of <200 IU/mL and 24% (n=13) had very high HBV DNA, which was defined as HBV DNA of >1.1 E8 IU/ml (4.1 $\log_{10}$ IU/mL). Furthermore, serum HBsAg was detected for all (100%) patients at baseline (mean = 3.89 ± 0.60 $\log_{10}$ IU/mL) with 76% (n=44) being HBeAg positive. At baseline all patients had detectable HBcrA (mean =5.51 ± 1.19 $\log_{10}$ U/mL) and 93% (n=54) had detectable pgRNA (mean =3.68 ± 1.15 $\log_{10}$ U/mL). On correlation analysis a positive correlation was observed at baseline between all the serum biomarkers (Table 3).

The patients were placed into cohorts based on their HBeAg status at baseline. The cohorts are described in Figure 1. Cohort 1 consisted of 44 patients and Cohort 2 consisted of 14 patients.

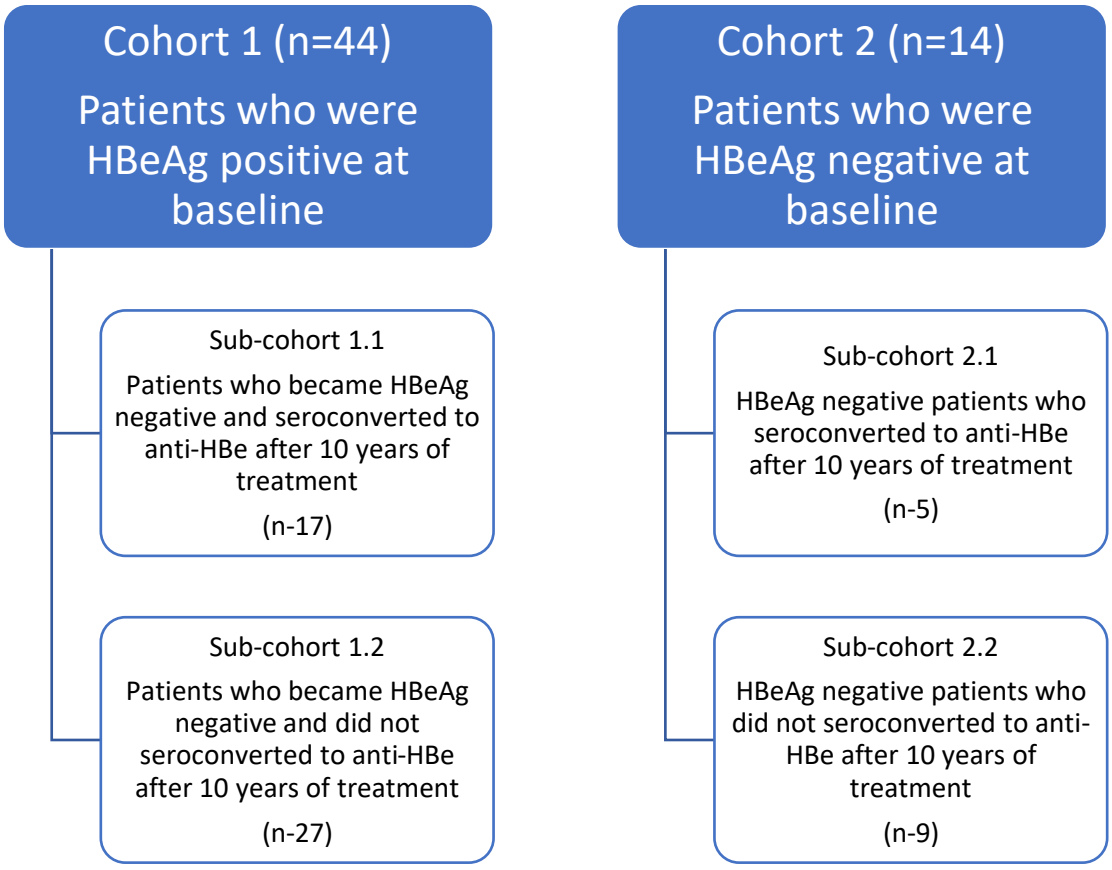

Figure 1: The patient cohorts.

N		**58**	
	Baseline	5 ± 3 years follow up	10 ± 3 years follow up
% male	66 (n=38)		
Mean age	40.5 ± 9.3	46.5 ± 9	52 ± 9
HBeAg positive (%)	76 (n=44)	0	0
HBeAg negative (%)	24 (n=14)	100	100
Anti-HBe positive (%)		29 (n=17)	38 (n=22)
Mean HBV DNA (log_{10} IU/mL)	5.9 ± 2.83	1.15 ± 1.0	0
Mean HBsAg quant (log_{10} IU/mL)	3.89 ± 0.60	2.95 ± 0.79	2.65 ± 0.90
Mean HBcrAg (log_{10} U/mL)	5.51 ± 1.19	4.30 ± 0.73	3.85 ± 0.76
Mean pgRNA (log_{10} U/ml)	3.68 ± 1.15	1.66 ± 0.44	1.18 ± 0.39
Mean ALT (u/L) Normal range = 0-50 u/L	69.24 ± 78.66	31 ± 20	26 ± 20
Mean AST (u/L) Normal range = 10-40 u/L	64.12 ± 73.90	30 ± 18.86	27 ± 12.5
Mean Bilirubin (umol/L) Normal range = <21 umol/L	13.16 ± 7.27	12 ± 5.87	9 ± 5.8
Mean INR Normal range = 0.9-1.2	1.04 ± 0.10	1.05 ± 0.08	1 ± 0.08
Mean PLT (10^9/L) Normal range = 150-400	185.36 ± 70	187.50 ± 69.72	193.5± 60
Mean CXCL10 (pg/mL)	65.48 ± 122	52.38 ± 89.10	48.47 ± 35.79
Mean sPD-1 (pg/mL)	1912.81 ± 624.77	1410.21 ±405	1030.84 ±227.93

Treatment with ETV (%)	70 (n-41)
Treatment with TDF (%)	13 (n=7)
Treatment with LAM (%)	17 (n=10)

Table 1: patient demographic and levels of serum biomarkers at baseline, 5 years, and 10 years of all patients.

HBV Genotypic distribution

N	**58**
Genotype A	25% (n=15)
Genotype B	12% (n=7)
Genotype C	19%(n=11)
Genotype D	23% (n=13)
Genotype E	21% (n=12)

Table 2: Baseline HBV Genotypes of all patients in the study; genotypes A-E.

Correlation analysis at Baseline		
	Pearson's correlation - r	P value
HBsAg and HBV DNA	0.507	<0.001
HBcrAg and HBV DNA	0.540	<0.001
HBcrAg and HBsAg	0.440	<0.001
pgRNA and HBcrAg	0.815	<0.001
pgRNA and HBV DNA	0.622	<0.001
pgRNA and HBsAg	0.500	<0.001
CXCL10 and ALT	0.550	<0.040
CXCL10 and AST	0.400	<0.040
sPD-1 and ALT	0.450	<0.050
sPD-1 and AST	0.410	<0.050

Table 3: Correlation analysis of serum biomarkers at baseline

Cohort 1: HBeAg Positive patients at baseline N= 44			
	Baseline	5 ± 3 years follow up	10 ± 3 years follow up
% male	73 (n=32)	73 (n=32)	73 (n=32)
Mean age (years)	40 ±20	46 ± 9	51 ±10
Anti-HBe positive (%)	0	27 (n=12)	39 (n=17)
Mean HBV DNA (log_{10} IU/mL)	5.54 ±2.6	0.86 ±1.0	0.38 ± 0.73
Mean HBsAg quant (log_{10} IU/mL)	3.90 ±0.5	2.92 ± 0.77	2.60 ±0.85
Mean HBcrAg (log_{10} U/mL)	5.80±0.9	4.38± 0.71	3.77 ±0.78
Mean pgRNA (log_{10} U/ml)	4.09 ±1.80	1.75 ± 1.01	1.32 ±0.93
Mean ALT (u/L)	71.1 ±78.5	31.93 ±12.59	28.52 ± 13.34
Mean AST (u/L)	70.09 ±81.9	29.66 ±6.40	29.14 ± 10.01
Mean Bilirubin (umol/L)	13.07±6.6	12.02 ±6.22	9.89 ± 5.15
Mean INR	1.03 ±0.17	1.04± 0.07	1 ± 0.07
Mean PLT (10E9/mL)	187.50 ±69.3	204.52 ± 71.56	199.36 ± 66.81
Mean CXCL10 (pg/mL)	63.85 ±147.98	49.63 ± 67.22	41.23 ± 28.71
Mean sPD-1 (pg/mL)	1662.20 ±423.01	1366.10 ±315	1000.29 ±211.73
FIB-4 score	2.35 ± 2.60	1.64 ±1.34	1.81 ±1.13

Table 4: patient demographic and levels of serum biomarkers at baseline, 5 years, and 10 years of patients in Cohort 1.

N =44	
Genotype A	18% (n=8)
Genotype B	16% (n=7)
Genotype C	20% (n=9)
Genotype D	20% (n=9)
Genotype E	25% (n=11)

Table 5: The baseline genotypic distribution of patients in cohort1 – HBeAg positive at baseline.

N =17	
Genotype A	41% (n=7)
Genotype B	18% (n=3)
Genotype C	6%(n=1)
Genotype D	29% (n=5)
Genotype E	6% (n=1)

Table 6: The genotypic distribution of 17 patients who acquired anti-HBe after 10 years of monitoring in cohort 1.

N =27	
Genotype A	4% (n=1)
Genotype B	15% (n=4)
Genotype C	29%(n=8)
Genotype D	15% (n=4)
Genotype E	37% (n=10)

Table 7: The genotypic distribution of 27 patients who did not acquire anti-HBe after 10 years of monitoring in cohort 1.

Cohort 2: HBeAg Negative patients at baseline N= 14			
	Baseline	5 ± 3 years follow up	10 ± 3 years follow up
% male	43 (n=6)	43 (n=6)	43 (n=6)
Mean age (years)	43±18	48 ±7	54 ±8
Anti-HBe positive (%)	0	36 (n=5)	36 (n=5)
Mean HBV DNA (log_{10} IU/mL)	3.51 ±3.0	1.20 ±1.02	0.36 ±0.96
Mean HBsAg quant (log_{10} IU/mL)	3.74 ±0.8	2.66 ±0.91	2.37 ±1.10
Mean HBcrAg (log_{10} U/mL)	4.70±1.6	4.13 ±0.83	3.67 ± 0.74
Mean pgRNA (log_{10} U/ml)	2.50 ± 2.28	1.37 ±1.25	1.51 ±1.40
Mean ALT (u/L)	63.36 ± 81.78	45.79 ±32.85	45.07 ±37.29
Mean AST (u/L)	45.36 ±35.27	42.00 ±36.05	33.07 ±18.52
Mean Bilirubin (umol/L)	13.43±9.00	13.43 ±4.65	12.50 ±7.56
Mean INR	1.07 ±0.1	1.09 ±0.13	1.03 ±0.10
Mean PLT (10E9/mL)	178.64 ±77.12	160.50 ±52.84	161.93 ±44.34
Mean CXCL10 (pg/mL)	221.10 ± 972.08	164.23 ±321.44	148.66 ±237.96
Mean sPD-1 (pg/mL)	2134.51 ± 794.45	1466.87 ±711.90	901.45 ±305.12
FIB-4 score	2.26 ±2.27	1.7 ±0.96	1.7 ±0.92

Table 8: patient demographic and levels of serum biomarkers at baseline, 5 years, and 10 years of patients in Cohort 2.

N =14	
Genotype A	50% (n=7)
Genotype B	0
Genotype C	14% (n=2)
Genotype D	29% (n=4)
Genotype E	7% (n=1)

Table 9: The baseline genotypic distribution of patients in cohort 2 – HBeAg negative at baseline.

N =5	
Genotype A	40% (n=2)
Genotype B	0
Genotype C	0
Genotype D	60% (n=3)
Genotype E	0

Table 10: The 5 patients who acquired anti-HBe after 10 years of monitoring in cohort 2.

N =9	
Genotype A	56% (n=5)
Genotype B	0
Genotype C	22% (n=2)
Genotype D	11% (n=1)
Genotype E	11 (n=1)

Table 11: The 9 patients who did not acquired anti-HBe after 10 years of monitoring in cohort 2.

Sub-Cohort 1.1		**N= 17**	
	Baseline	5 ± 3 years follow up	10 ± 3 years follow up
Mean HBV DNA (log_{10} IU/mL)	5.85 ±2.50	0.87 ±1.10	0.44 ± 0.98
Mean HBsAg quant (log_{10} IU/mL)	3.98 ±0.55	2.69 ± 0.80	2.47 ±0.71
Mean HBcrAg (log_{10} U/mL)	6.10 ±1.20	4.37 ± 0.77	3.70 ±1.00
Mean pgRNA (log_{10} U/ml)	4.20 ±2.23	1.65 ± 1.25	1.00 ±0.93
Mean ALT (u/L)	72.29 ±66.24	32.29 ±12.34	27.88 ± 11.54
Mean AST (u/L)	65.71 ±67.59	30.24 ±6.30	28.59 ± 7.54
Mean Bilirubin (umol/L)	12.53 ±5.81	12.94 ±8.46	11.53 ± 7.04
Mean INR	1.02 ±0.11	1.01± 0.06	1 ± 0.08
Mean PLT (10E9/mL)	177.10 ±58.46	190.90 ± 57.46	188.80 ± 53.71
Mean CXCL10 (pg/mL)	44.4 ±12.37	46.11 ± 20.56	43.24 ± 1.77
Mean sPD-1 (pg/mL)	293.6 ±50.41	1549.12 ±2100	4374 ±2331.63
FIB-4 score	2.4 ±2.1	1.52 ±0.64	2.00 ±1.93

Table 12: levels of serum biomarkers at baseline, 5 years, and 10 years of patients in Sub-Cohort 1.1.

Sub-Cohort 1.2		**N= 27**	
	Baseline	5 ± 3 years follow up	10 ± 3 years follow up
Mean HBV DNA (log_{10} IU/mL)	5.64 ±2.68	1.10 ±1.10	0.40 ± 0.54
Mean HBsAg quant (log_{10} IU/mL)	3.99 ±0.50	3.10 ± 1.60	2.73 ±0.96
Mean HBcrAg (log_{10} U/mL)	5.70 ±0.71	4.57 ± 0.44	3.84 ±0.50
Mean pgRNA (log_{10} U/ml)	3.88 ±2.10	1.92 ± 0.9	1.20 ±0.95
Mean ALT (u/L)	73.49 ± 88.03	38.11 ±23.10	31.22 ± 16.19
Mean AST (u/L)	74.26 ±90.41	35.67 ±26.39	32.48 ± 16.21
Mean Bilirubin (umol/L)	13.00 ±7.36	11.39 ±4.22	9.56 ± 4.04
Mean INR	1.02 ±0.10	1.10± 0.07	1 ± 0.07
Mean PLT (10E9/mL)	205.11 ±77.38	202.4 ± 80.03	201.40 ± 74.99
Mean CXCL10 (pg/mL)	82.99 ±124.40	77.80 ± 50.21	42.26 ± 3.76
Mean sPD-1 (pg/mL)	1881 ±1011	1688.12 ±1547	1434 ±2177.63
FIB-4 score	2.27 ±2.80	1.70 ±0.50	1.73 ±1.03

Table 13: levels of serum biomarkers at baseline, 5 years, and 10 years of patients in Sub-Cohort 1.2.

Sub-Cohort 2.1		**N= 5**	
	Baseline	5 ± 3 years follow up	10 ± 3 years follow up
Mean HBV DNA (log_{10} IU/mL)	2.91 ±2.76	1.18 ±1.47	0.64 ± 1.40
Mean HBsAg quant (log_{10} IU/mL)	3.74 ±0.50	2.34 ± 1.13	1.89 ±0.83
Mean HBcrAg (log_{10} U/mL)	4.24 ±1.75	3.68 ± 0.96	3.26 ±0.50
Mean pgRNA (log_{10} U/ml)	2.11 ±1.88	1.05 ± 0.90	1.19 ±0.90
Mean ALT (u/L)	88 ± 94.30	25.00 ±11.51	23.60 ± 7.60
Mean AST (u/L)	61.40 ±54.00	30.67 ±11.56	23.80 ± 6.87
Mean Bilirubin (umol/L)	16.40 ±13.40	12.40 ±4.98	15.2 ± 8.10
Mean INR	1.10 ±0.10	1.10± 0.17	1.00 ± 0.13
Mean PLT (10E9/mL)	153.60 ±91.62	180 ± 61.59	165.20 ± 36.77
Mean CXCL10 (pg/mL)	182.35 ±80.70	66.46 ± 42.91	42.45 ± 0.50
Mean sPD-1 (pg/mL)	977.45 ±980.11	946.30 ±812.55	427.90 ±278.67
FIB-4 score	3.50 ±3.50	2.00 ±1.50	2.10 ±1.20

Table 14: levels of serum biomarkers at baseline, 5 years, and 10 years of patients in Sub-Cohort 2.1.

Sub-Cohort 2.2		**N= 9**	
	Baseline	5 ± 3 years follow up	10 ± 3 years follow up
Mean HBV DNA (log_{10} IU/mL)	2.90 ±2.63	0.57 ±0.68	0.44 ± 0.60
Mean HBsAg quant (log_{10} IU/mL)	3.69 ±0.95	3.00 ± 1.00	2.84 ±1.03
Mean HBcrAg (log_{10} U/mL)	4.59 ±1.13	4.10 ± 0.70	3.81 ±0.80
Mean pgRNA (log_{10} U/ml)	1.86 ±1.30	1.16 ± 1.12	1.20 ±1.72
Mean ALT (u/L)	40.56 ± 15.23	38.11 ±22.61	50.11 ± 44.54
Mean AST (u/L)	32.22 ±7.20	29.44 ±8.44	27.56 ± 9.32
Mean Bilirubin (umol/L)	13.00 ±6.10	12.56 ±5.54	8.90 ± 4.85
Mean INR	1.10 ±0.08	1.10± 0.13	1.00 ± 0.90
Mean PLT (10E9/mL)	159.70 ±48.63	181.60 ± 69.11	174.1 ± 56.87
Mean CXCL10 (pg/mL)	75.90 ±82.72	44.96 ± 5.38	43.00 ± 0.56
Mean sPD-1 (pg/mL)	4002 ± 1398	2643.12 ±988	1903 ±777.63
Fib-4 score	1.40 ±0.83	1.50 ±0.60	1.44 ±0.66

Table 15: levels of serum biomarkers at baseline, 5 years, and 10 years of patients in Sub-Cohort 2.2.

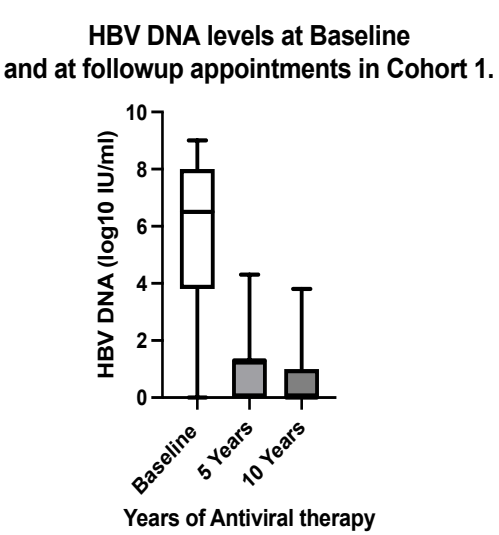

Figure 2: Box plot of mean HBV DNA levels at baseline, after 5 years and 10 years of antiviral therapy in cohort 1- HBeAg positive patients at baseline.

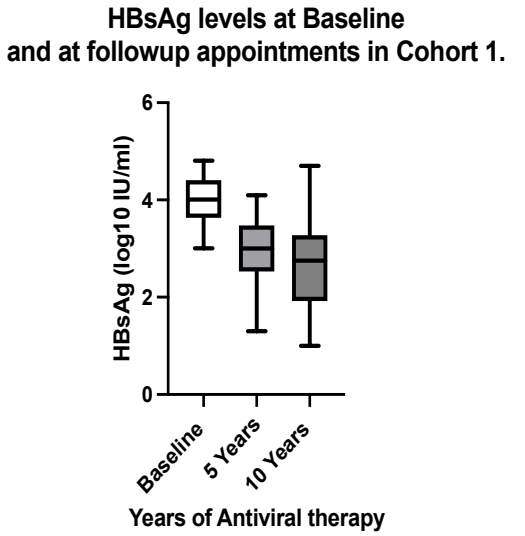

Figure 3: Box plot of mean HBsAg levels at baseline, after 5 years and 10 years of antiviral therapy in cohort 1- HBeAg positive at baseline.
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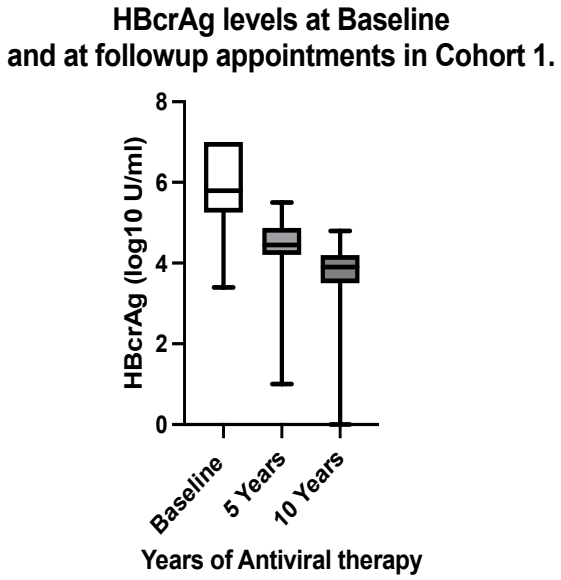


Figure 4: Box plot of mean HBcrAg levels at baseline, after 5 years and 10 years of antiviral therapy in cohort 1- HBeAg positive patients at baseline.

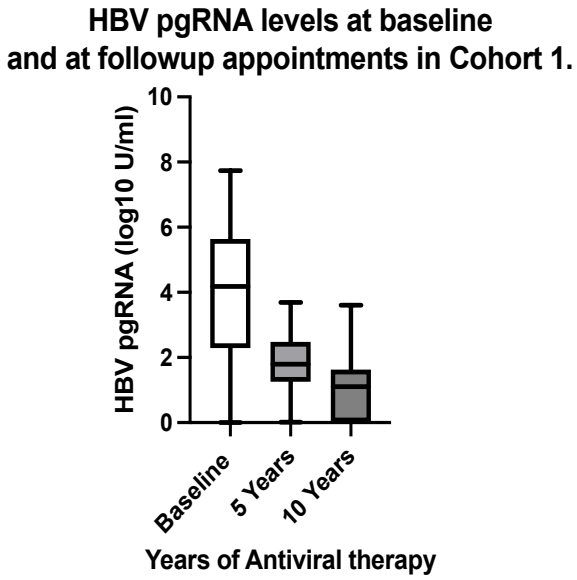


Figure 5: Box plot of mean HBV pgRNA levels at baseline, after 5 years and 10 years of antiviral therapy in cohort 1- HBeAg positive patients at baseline.

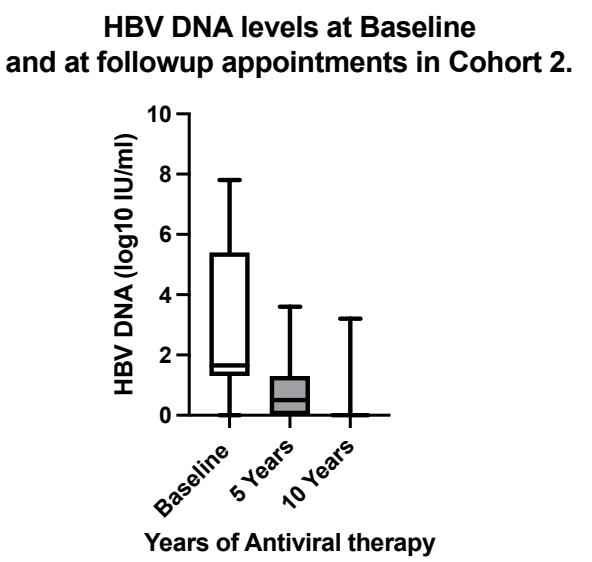


Figure 6: Box plot of mean HBV DNA levels at baseline, after 5 years and 10 years of antiviral therapy in cohort 2- HBeAg negative patients at baseline.

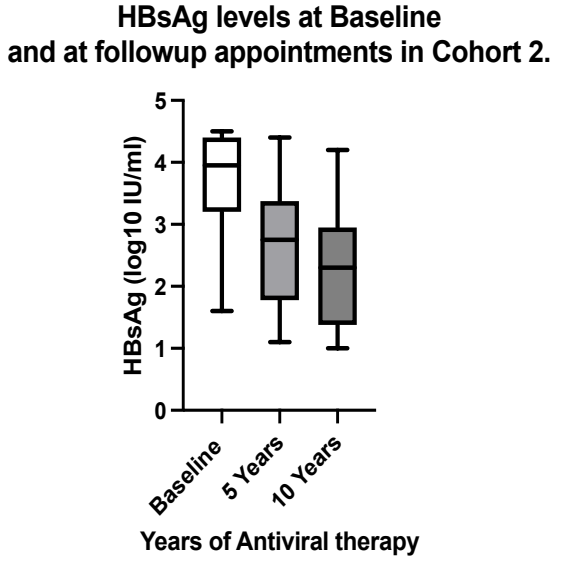


Figure 7: Box plot of mean HBsAg levels at baseline, after 5 years and 10 years of antiviral therapy in cohort 2- HBeAg negative patients at baseline.

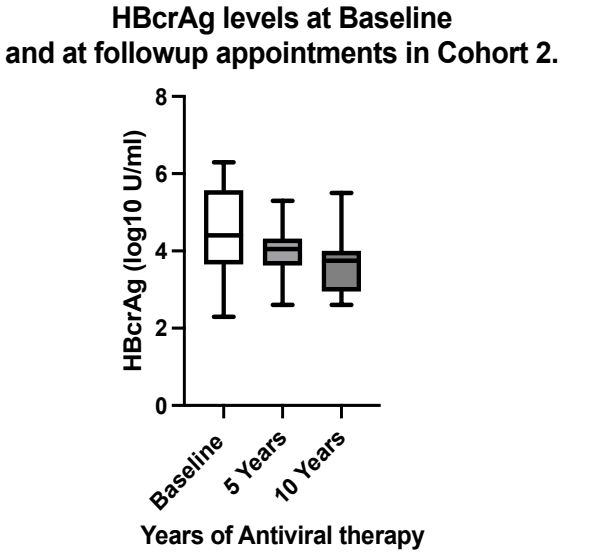


Figure 8: Box plot of mean HBcrAg levels at baseline, after 5 years and 10 years of antiviral therapy in cohort 2- HBeAg negative patients at baseline.

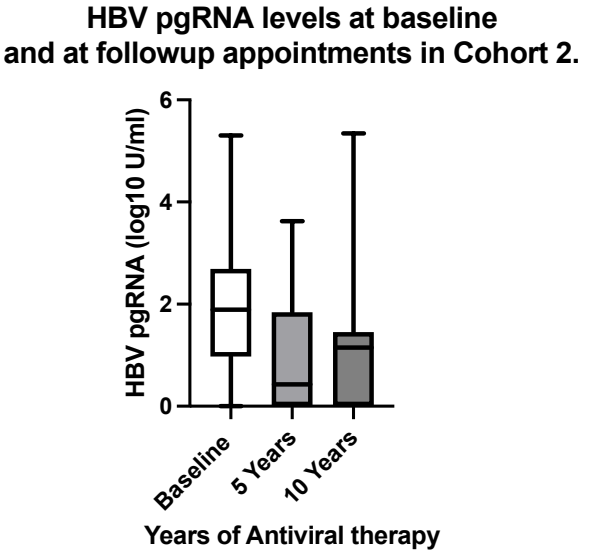
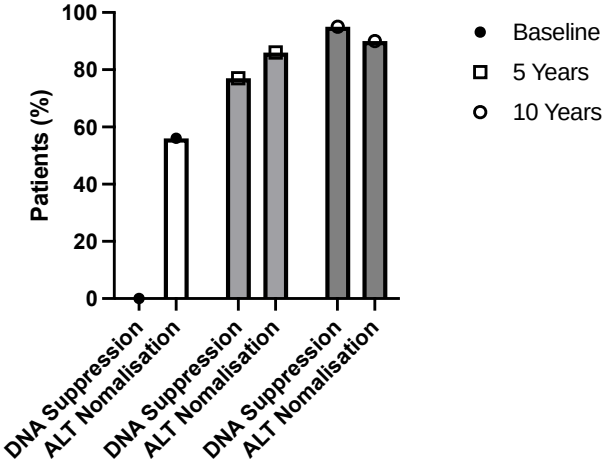


Figure 9: Box plot of mean pgRNA levels at baseline, after 5 years and 10 years of antiviral therapy in cohort 2- HBeAg negative patients at baseline.

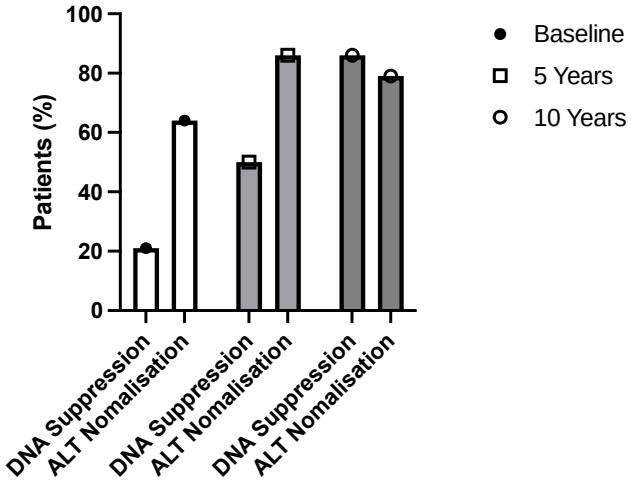
Virological and ALT Normalisation in Cohort 1



N=44	Baseline	5 Years	10 Years
% HBV DNA Suppression	0 (n=0)	77 (n=34)	95 (n=42)
% ALT normalisation	56 (n=25)	86 (n=38)	90 (n=40)

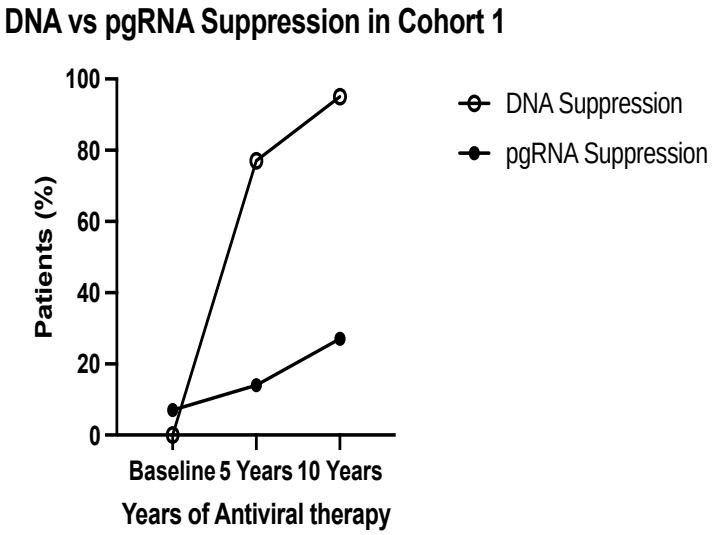
Figure 10: Virological suppression and ALT normalisation at baseline, after 5 years and after 10 years of NA therapy in Cohort 1.

Virological and ALT Normalisation in Cohort 2



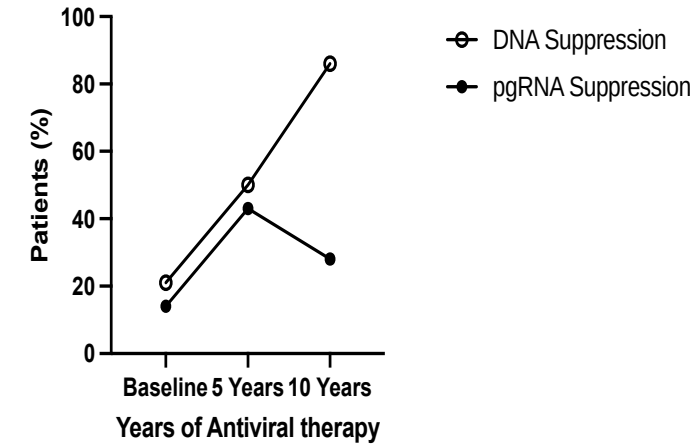
N=14	Baseline	5 Years	10 Years
% HBV DNA Suppression	21 (n=3)	50 (n=7)	86 (n=12)
% ALT normalisation	64 (n=9)	86 (n=12)	79 (n=11)

Figure 11: Virological suppression and ALT normalisation at baseline, after 5 years and after 10 years of NA therapy in Cohort 2.



N=44	Baseline	5 Years	10 Years
% HBV DNA Suppression	0 (n=0)	77 (n=34)	95 (n=42)
% pgRNA Suppression	7 (n=3)	14 (n=6)	27 (n=12)

Figure 12: HBV DNA and pgRNA suppression at baseline, after 5 years and after 10 years of NA therapy in Cohort 1.



N=14	Baseline	5 Years	10 Years
% HBV DNA Suppression	21 (n=3)	50 (n=7)	86 (n=12)
% pgRNA Suppression	14 (n=2)	43 (n=6)	28 (n=4)

Figure 13: HBV DNA and pgRNA suppression at baseline, after 5 years and after 10 years of NA therapy in Cohort 2.

Patient	pgRNA level at 5 years (log_{10} U/mL)	pgRNA level at 10 years (log_{10} U/mL)
1	0	1.23
2	0	1.68
3	0	2.53

Table 16: Patients in Cohort 2 who had suppressed pgRNA after 5 years of treatment but had detectable pgRNA levels after 10 years.

Cohort 1			
Factors	Hazard ratio	95% CI	p value
Age <50 vs ≥50	3.15	1.02 - 11.10	**0.02**
Sex male vs female	2.11	6.99 - 8.2	0.14
Becoming HBeAg negative before 2 years vs after 2 years.	1.60	0.74 - 3.61	**0.05**
Baseline HBsAg <4 vs ≥4 log_{10} IU/mL	0.42	0.1 - 1.3	**0.05**
Baseline HBV DNA <4 vs ≥4 log_{10} IU/mL	1.31	0.1 - 1.0	**0.01**
Baseline HBV pgRNA <5 vs ≥5 log_{10} U/mL	0.92	0.45 - 2.79	**0.04**
Baseline HBcrAg <4 vs ≥4 log_{10} U/mL	0.6	0.3 - 1.4	**0.05**
ALT <55 vs >55 u/L	0.81	0.23 - 1.15	0.3
CXCL10 <200 vs > 200 pg/mL	1.01	1.78-6.55	**0.05**
sPD1 <1500 vs >1500 pg/mL	1.34	1.22- 3.90	0.22

Table 17: Univariate analysis of factors associated with virological response during NA therapy in Cohort 1. Younger age at the start of therapy, becoming HBeAg negative, having HBsAg levels <4 log10 IU/mL, HBV DNA levels <4 log10 IU/mL, HBV pgRNA levels <5 log10 U/mL and HBcrAg levels <4 log 10 U/mL and CXCL10 levels <200 at baseline are linked to better virological response on NA therapy.

Cohort 2			
Factors	Hazard ratio	95% CI	p value
Age <50 vs ≥50	2.87	2.03 – 8.21	**0.05**
Sex male vs female	3.90	5.40 - 9.40	0.55
Baseline HBsAg <3 vs ≥3 log_{10} IU/mL	0.53	0.11 - 1.20	**0.05**
Baseline HBV DNA <6 vs ≥6 log_{10} IU/mL	0.80	0.10 - 1.12	**0.03**
Baseline HBV pgRNA <5 vs ≥5 log_{10} U/mL	1.55	0.61 - 2.19	**0.05**
Baseline HBcrAg <4 vs ≥4 log_{10} U/mL	0.40	0.50 – 2.20	**0.04**
ALT <55 vs >55 u/L	1.67	0.44 - 1.28	0.25
CXCL10 <200 vs > 200 pg/mL	1.22	3.11 - 7.98	**0.05**
sPD1 <1500 vs >1500 pg/mL	1.65	1.04- 4.62	0.39

Table 18: Univariate analysis of factors associated with virological response during NA therapy in Cohort 2. Younger age at the start of therapy, having HBsAg levels <3 log10 IU/mL, HBV DNA levels <6 log10 IU/mL, HBV pgRNA levels <5 log10 U/mL and HBcrAg levels <4 log 10 U/mL and CXCL10 levels <200 at baseline are linked to better virological response on NA therapy.

Cohort 1			
Factors	Hazard ratio	95% CI	p value
Age <50 vs ≥50	2.04	1.55 - 8.30	0.27
Sex male vs female	1.07	5.21 – 7.90	0.14
Baseline HBsAg <4.5 vs ≥4.5 log_{10} IU/mL	1.57	0.8 - 1.77	**0.05**
Baseline HBV DNA positive vs negative	1.15	1.10 - 5.90	0.50
Decline in HBV pgRNA of ≥3 vs <3 log_{10} U/mL after 5 years of treatment	1.95	1.65 - 4.38	**0.05**
Decline in HBcrAg of ≥2 vs <2 og_{10} U/mL in after 5 years of treatment	2.15	1.93 - 7.42	**0.03**
Baseline ALT <55 vs >55 u/L	1.48	1.44 – 9.05	**0.05**

Table 19: Univariate analysis of factors associated with HBeAg seroconversion to anti-HBe in Cohort 1. Having HBsAg levels <4.5 log_{10} IU/mL, a decline of >2 log10 U/mL in pgRNA levels and a decline of >3 log10 U/mL in HBcrAg after 5 years of treatment and baseline ALT levels <55 are associated with HBeAg seroconversion after 10 years of antiviral treatment.

Cohort 1			
Factors	Hazard ratio	95% CI	p value
Age <50 vs ≥50	2.04	1.55 - 8.30	0.27
Sex male vs female	1.07	5.21 – 7.90	0.14
Baseline HBsAg <4.5 vs ≥4.5 log_{10} IU/mL	1.57	0.8 - 1.77	**0.05**
Baseline HBV DNA positive vs negative	1.15	1.10 - 5.90	0.50
Decline in HBV pgRNA of ≥3 vs <3 log_{10} U/mL after 5 years of treatment	1.95	1.65 - 4.38	0.36
Decline in HBcrAg of ≥2 vs <2 og_{10} U/mL in after 5 years of treatment	2.15	1.93 - 7.42	0.32
Baseline ALT <55 vs >55 u/L	1.48	1.44 – 9.05	**0.05**

Table 20: Univariate analysis of factors associated with HBeAg seroconversion to anti-HBe in Cohort 2. Only having HBsAg levels <4.5 log_{10} IU/mL, and baseline ALT levels <55u/L are associated with HBeAg seroconversion after 10 years of antiviral treatment.

	cohort 1.1 (n=17)	Cohort 2.1 (n=5)
Mean HBcrAg log_{10} U/mL	3.70 ±1.00	3.26 ±0.50
Mean pgRNA log_{10} U/mL	1.00 ±0.93 1.1	1.19 ±0.90

Table 21: levels of HBcrAg and pgRNA in patients who seroconverted to anti-HBe and had normal ALT levels. All patients in sub-cohort 1.1and sub-cohort 2.1 had normal ALT levels.

Analysis of results obtained for patients in Cohort 1 and Cohort 2

The mean concentrations of all biomarkers in cohort 1 and cohort 2 are shown in Table 4 and 8. Box plots representing the mean and quartiles of biomarkers HBV DNA, HBsAg, HBcrAg and pgRNA for cohort 1 and 2 are shown in Figures 2 - 9. As all patients in both cohorts were on antiviral therapy with NA, there is a significant reduction in HBV DNA results from baseline to 5 years of therapy and 10 years of therapy. In both cohort 1 and cohort 2, the largest decline of HBV DNA was observed between baseline and 5 years of antiviral therapy. After 5 years of therapy serum HBV DNA appeared to decline at a very slow rate. However, the larger decline in HBV DNA was observed in the HBeAg positive cohort compared to the HBeAg negative cohort (decline of 4.68 vs 2.23 $\log_{10}$ IU/mL respectively) between baseline and 5 years of therapy. Only a small decline was observed in HBsAg levels between baseline and 5 years and between 5 years and 10 years in both cohorts. In cohort 1 a mean HBsAg decline of 0.9 $\log_{10}$ IU/mL was seen between baseline and 5 years and a mean decline of 0.32 $\log_{10}$ IU/mL was observed after 10 years of therapy. In cohort 2 a mean HBsAg decline of 1.08 $\log_{10}$ IU/mL was observed between baseline and 5 years and a mean decline of 0.29 $\log_{10}$ IU/mL was seen after 10 years of therapy. Antiviral therapy with NA appears to have little effect on HBsAg concentration.

HBcrAg and pgRNA concentrations were higher in the HBeAg positive patients (cohort 1) at baseline compared to the HBeAg negative patients (cohort 2). This could possibly be due to HBeAg positive patients experiencing more viral activity as HBeAg promotes viral replication (e.g. by downregulating TLR2, and activating NF- κB). Furthermore, increased viral activity may cause more liver inflammation, which may explain why ALT and AST levels were also higher in HBeAg positive patients compared to HBeAg negative patients.

Additionally, there was a steeper reduction in HBcrAg from baseline to 5 years of therapy in cohort 1 compared to cohort 2 (reduction of 1.42 vs 0.57 $\log_{10}$ IU/mL respectively). However, after 5 years of therapy HBcrAg levels appeared to be of similar levels across both cohorts with very little decline in HBcrAg after 10 years of therapy (Table 4 and 8). A steeper reduction in pgRNA was also observed from baseline to 5 years of therapy in cohort 1 compared to cohort 2 (reduction of 2.34 vs 1.13 $\log_{10}$ IU/mL respectively). After 10 years of antiviral therapy a slight decline in pgRNA was observed in cohort 1 but, a slight increase in pgRNA was observed

in cohort 2 (Table 4 and 8). Since viral activity and replication is likely to be high before antiviral therapy a steep decline in some viral markers is expected once antiviral therapy is initiated.

Genotypic distribution of patients in cohort 1, sub-cohort 1.1 and 1.2 are shown in Tables 5-7. Genotypic distribution of patients in cohort 2, sub-cohort 2.1 and 2.2 are shown in Tables 9-11. In cohort 1 HBeAg seroconversion was more prominent in patients that were genotypes A and D. Patients with genotypes C and E were less likely to seroconvert. In cohort 2 only patients with genotypes A and D seroconverted to anti-HBe. The mutations that occur in genotypes A and D my carry advantageous elements that enhance the rate of HBeAg seroconversion.

The rate of undetectable HBV DNA (DNA suppression) and ALT normalisation are shown in Figures 10 and 11. Normal ranges of biochemical markers are shown in Table 1. At baseline none of the patients in cohort 1 had undetectable HBV DNA, but after 5 years of antiviral therapy with NA 77% (n=34) of patients had undetectable HBV DNA. After 10 years of therapy 95% (n=42) of patients in cohort 1 had undetectable DNA. A steeper decline in serum DNA concentration was observed in patients who had high baseline HBV DNA levels (DNA >5 $\log_{10}$ IU/mL), compared to the patients who had low baseline HBV DNA (DNA <5 $\log_{10}$ IU/mL). In cohort 1 the rate of ALT normalisation increased as the years of antiviral therapy increased. At baseline ALT normalisation was observed in 56% (n=25) of patients, then 86% (n=38) of patients at the 5 years follow up and then 90% (n=40) at the 10 years follow up.

At baseline 21% (n=3) of the patients in cohort 2 had undetectable HBV DNA. After 5 years of antiviral therapy 50% (n=7) of patients in cohort 2 had suppressed HBV DNA and after 10 years the rate of HBV DNA suppression increased to 86% (n=12). ALT normalisation was observed in 64% (n=9) of patients at baseline. After 5 years of antiviral therapy ALT normalisation was observed in 86% (n=12) of patients and after 10 years 79% (n=11) of patients.

ALT normalisation amongst patients with elevated ALT at baseline in cohort 1 (n=20) was 60% after achieving HBeAg loss and approximately 5±3 years of antiviral therapy. In cohort 1 at baseline 24 patients had elevated AST, 10 patients had elevated bilirubin, 10 patients had elevated INR, and 20 patients had abnormal PLT. After patients became HBeAg negative and had been on antiviral therapy for approximately 5±3 years AST, bilirubin, INR and PLT

normalisation were seen amongst 92%, 70%, 50% and 45% respectively. After 10 ±3 years 84% (n=37) of patients had ALT in the normal rage in cohort 1 and out of the group who had elevated ALT at baseline (n=14), 70% of them now had ALT in the normal range.
Out of the patients who had elevated or abnormal levels of AST, bilirubin, INR and PLT in cohort 1, 83%(n=20), 60% (n=7), 50%(n=5) and 50% (n=10) respectively had normal levels of these biochemistry markers after 10 years of antiviral therapy

At baseline 36% (n=5) of patients in cohort 2 had elevated ALT levels. After 5 years of therapy only one out of the five patients still had elevated ALT, and after 10 years only the same patient still had elevated ALT (ALT at baseline, 5 years, and 10 years = 60, 105, 94 u/L respectively). At baseline 29% (n=4) of patients in cohort 2 had elevated AST but, after being on antiviral treatment for 5 years all of the patients had normal levels of AST and after 10 years of therapy all the patients still had normal levels of AST. Abnormal levels of bilirubin, INR and PLT were observed in 21% (n=), 14% (n=2), and 21% (n=3) respectively in patients in cohort 2 at baseline. At the 5 years and 10 years follow up all patients had normal levels of these biomarkers.

Figures 12 and 13 shows the rate of DNA suppression compared to pgRNA suppression within cohort 1 and 2 throughout the years of antiviral therapy. HBV DNA is significantly affected by NA therapy compared to pgRNA. The rate of pgRNA suppression during NA therapy is considerably lower than that of HBV DNA in both cohorts. In cohort 1 the rate of both HBV DNA and pgRNA continue to rise as the years of antiviral therapy increases. In cohort 2, there is an increase in the rate of HBV DNA and pgRNA suppression between baseline and 5 years of therapy. Between 5 years and 10 years the rate of HBV DNA suppression continues to increase exponentially but the rate of pgRNA suppression decreases. Three out of the six patients who had undetectable pgRNA at the 5 years follow up had detectable pgRNA at the 10 years follow up (Table 16). Pregenomic RNA suppression was not as substantial as DNA suppression throughout the 10 years because cccDNA transcriptional activity is not affected by treatment with NA, so pgRNA synthesis continues even when HBV DNA is suppressed.

Univariate analysis of factors associated with virological response during NA therapy in patients who are HBeAg positive at baseline (Cohort 1) reveal that becoming HBeAg negative, having HBsAg levels of <4 $\log_{10}$ IU/mL, HBV DNA levels <4 $\log_{10}$ IU/mL, HBV pgRNA levels <5 $\log_{10}$ U/mL, HBcrAg levels <4 $\log_{10}$ U/mL and CXCL10 levels <200 g/mL at baseline

are linked to better virological response on NA therapy (Table 17). For patients who are HBeAg negative at baseline (cohort 2) factors associated with better virological response during NA therapy included having baseline HBsAg levels of <3 log_{10} IU/mL, HBV DNA levels <6 log_{10} IU/mL, HBV pgRNA levels <5 log_{10} U/mL and HBcrAg levels <4 log_{10} U/mL and CXCL10 levels <200 pg/mL (Table 18).

Analysis of results obtained for patients in Sub - Cohorts 1.1, 1.2, 2.1 and 2.2

The mean concentrations of all biomarkers in patients who seroconverted to anti-HBe and patients who did not seroconvert in both cohort 1 and cohort 2 are shown in Tables 12 - 15 (sub-cohorts 1.1, 1.2, 2.1 and 2.2).

After 10 years of antiviral therapy and monitoring 36% (n=22) of patients in cohort 1 seroconverted to anti-HBe and 36% (n=5) of patients in cohort 2 seroconverted to anti-HBe.

In cohort 1, the patients who seroconverted to anti-HBe (sub-cohort 1.1) had higher concentrations of HBcrAg and pgRNA at baseline than patients who did not seroconvert to anti-HBe (sub-cohort 1.2) (Table 12 and 13). It could be that cccDNA activity is higher in patients who go on to seroconvert to anti-HBe in the future. At baseline HBsAg and HBV DNA levels were similar between sub-cohorts 1.1 and 1.2. All mean biochemistry results (ALT, AST, bilirubin, INR and PLT) were also similar at baseline between sub-cohorts 1.1 and 1.2**.** There was no significant difference found between FIB-4 scores in cohort 1.1 and 1.2 at baseline. Seroconverting to anti-HBe did not have a profound effect on HBsAg, HBV DNA, FIB-4 and biochemistry markers in patients in cohort 1 as the concentrations of theses biomarkers were similar between patients who seroconverted and patients who did not seroconvert after 5 years and after 10 years (Table 12 and 13).

However, with HBcrAg and pgRNA the patients who did seroconvert to anti-HBe had slightly lower levels of these biomarkers than patients who did not seroconvert after 5 years of therapy, suggesting a positive association between markers of cccDNA (HBcrAg and pgRNA) and seroconverting to anti-HBe.

Immunological markers CXCL-10 and sPD-1 remained higher in patients who did not seroconvert to anti-HBe (sub-cohort 1.2) compared to those who did seroconvert (sub-cohort 1.1) from baseline to 10 years of HBV management. Seroconverting to anti-HBe appeared to have a positive effect on CXCL-10 AND sPD-1 levels. It is possible that patients who did not seroconvert had increased immunological activity and more interactions between host and virus. It might be useful to monitor these immunological markers in patients who do not seroconvert to anti-HBe as long term high levels of these markers could cause issues such as T cell exhaustion.

Tables 14 and 15 show patients in cohort 2 who seroconverted to anti-HBe (sub-cohort 2.1) and patients who did not seroconvert to anti-HBe (sub-cohort 2.2). At baseline there was no significant difference in levels of biomarkers – HBsAg, HBV DNA, HBcrAg and pgRNA between patients in sub-cohort 2.1 and 2.2 as the mean concentrations of these biomarkers were very similar between the two sub-cohorts. HBeAg negative patients who seroconverted had more liver fibrosis and inflammation than patients who did not seroconvert as FIB-4, ALT, AST and bilirubin levels were significantly higher in sub-cohort 2.1 compared to cohort 2.2 (FIB-4: 3.50 ±3.50 vs 1.40 ±0.83 respectively; ALT: 88 ±94.30 vs 40.56 ±15.23 respectively, AST: 61.40 ±54 vs 32.22 ±7.20 respectively; Bilirubin: 16.40 ±13.40 vs 13.00 ±6.10 respectively). Furthermore, all patients in sub-cohort 2.1 had ALT, AST and bilirubin levels above the normal range for an adult at baseline. This suggests that HBeAg negative patients who seroconvert to anti-HBe are more likely to have increased liver fibrosis and inflammation years before seroconverting.

After 5 years of antiviral treatment the mean concentrations of biomarkers – HBsAg, HBV DNA, HBcrAg and pgRNA were very similar between sub-cohort 2.1 and 2.2. This was also observed after 10 years. HBV DNA and pgRNA suppression were observed in many of the patients, which is expected for HBeAg negative patients on antiviral therapy with NA. At the 5 years follow up four out of five patients in sub-cohort 2.1 had undetectable HBV DNA and in sub-cohort 2.2 all patients had undetectable HBV DNA. Undetectable pgRNA was observed in three out of five patients in sub-cohort 2.1 and four out of nine patients in sub-cohort 2.2. At the 10 years follow up four out of five patients had undetectable HBV DNA but only one patient had undetectable pgRNA in sub-cohort 2.1. In sub-cohort 2.2 eight out of nine patients had undetectable HBV DNA and three patients had undetectable pgRNA. HBsAg and HBcrAg loss were not observed in any of the patients after 5 years or after 10 years in both groups, indicating that seroconverting to anti-HBe had no effect on HBsAg and HBcrAg loss.

After 5 years the mean biochemistry results were also similar between the two groups. In sub-cohort 2.1 all patients had biochemistry results in the normal range for adults. In sub-cohort 2.2 four out of nine patients had normal ALT results, all patients had AST, bilirubin, INR and PLT in the normal range for adults. The mean FIB-4 score was slightly higher in sub-cohort 2.1 than 2.2 (2.00 ±1.50 vs 1.50 ±0.60) after 5 years. At the 10 years follow up all patients in both groups had biochemistry results in the normal range for adults. The mean FIB-4 score was slightly higher in sub-cohort 2.1 than 2.2 (2.10 ±1.20 vs 1.44 ±0.66) after 10 years.

Immunological marker CXCL-10 was much higher in sub-cohort 2.1 compared to 2.2 at baseline and after 5 years. After 10 years only a small difference in the mean CXCL10 levels was observed in sub-cohort 2.1 and 2.2. It appears that before seroconverting to anti-HBe and years after seroconversion the interaction between CXCL10 and immune cell types in the host is high. It would be of interest to measure the concentration of immune cells associated with CXCL10 such as T cells, NK cells and dendritic cells in patients who seroconvert to anti-HBe versus those who do don't seroconvert. CXCL10 concentrations could be useful in predicting which patients are likely to seroconvert to anti-HBe.

On-the-other hand sPD-1 levels were higher in sub-cohort 2.2 compared to sub-cohort 2.1 at baseline and throughout the 10 years of the study. It might be useful for sPD-1 levels to be monitored in HBeAg negative patients who do not seroconvert to anti-HBe due to the risk of developing HCC. PD-1 can prevent the immune system from killing cancer cells.

Table 19 and 20 show factors associated with HBeAg seroconversion after 10 years of antiviral therapy in cohort 1 and 2. In cohort 1 HBeAg seroconversion after 10 years of therapy was associated with having HBsAg levels < 4.5 $\log_{10}$ IU/mL and ALT levels <55 u/L at baseline. The decline of novel markers pgRNA and HBcrAg also had an association with HBeAg seroconversion. A decline in HBcrAg levels of >2 $\log_{10}$ U/mL after 5 years of antiviral therapy and a decline in pgRNA levels of >3 $\log_{10}$ U/mL after 5 years of therapy was associated with HBeAg seroconversion at the 10 years follow up. In sub-cohort 1.1 where all the patients seroconverted to anti-HBe after 10 years the mean decline in HBcrAg levels at the 5 years follow up was 2.30 $\log_{10}$ U/mL, whereas in sub-cohort 1.2 where no patients seroconverting anti-HBe the mean decline in HBcrAg was 1.01 $\log_{10}$ U/mL. In sub-cohort 1.1 the decline in

pgRNA levels at the 5 years follow up were also higher than that of sub-cohort 1.2 (mean decline of 3.35 vs 2.14 log_{10} U/mL respectively).

In cohort 2 HBeAg seroconversion after 10 years of therapy was also associated with having HBsAg levels < 4.5 log_{10} IU/mL and ALT <55 u/L at baseline. However, biomarkers HBV DNA, HBcrAg and pgRNA had no association with seroconverting to anti-HBe after 10 years in cohort 2.

Table 21 shows the HBcrAg and pgRNA concentrations of patients who seroconverted to anti-HBe and had normal levels of ALT. All patients who seroconverted to anti-HBe had normal levels of ALT. HBcrAg and pgRNA could still be detected in the serum of all the patients.

HBsAg Analysis

All patients had HBsAg levels measured at every follow up. A slow decline of HBsAg was observed over the 10 years (mean HBsAg at baseline, 5 years and 10 years respectively = 3.89 ±0.6, 2.95 ±0.79 and 2.65±0.9 log_{10} IU/mL). The mean decline of HBsAg levels were higher in male patients than female patients after 5 years (1.09 vs 0.09 log_{10} IU/mL, $p<0.05$ decline respectively) and after 10 years of monitoring (0.40 vs 0.16 log_{10} U/mL, $p<0.05$ decline respectively). The mean reduction of HBsAg level was higher in patients who had elevated DNA at baseline (n=32), compared to the group with low baseline DNA (n=26) after 5 years (1.07 vs 0.97 log_{10} IU/mL respectively, $p<0.05$) and after 10 years (0.57 vs 0.04 log_{10} IU/mL respectively, $p<0.05$).

Unfortunately, in this study none of the patients achieved HBsAg seroclearance. After 10 years 24 patients had HBsAg levels of <2.3 log_{10} IU/mL, which is highly predictive of HBsAg seroclearance in the years to come according to some studies(2). Table 22 shows the pgRNA and HBcrAg levels of patients who had HBsAg levels of <2.3 log_{10} IU/mL after 10 years of NA therapy. HBcrAg and pgRNA also appear to be low in these patients.

Other studies such as Moini et al have shown that low baseline HBsAg of <3 log_{10} IU/mL (<1000 IU/mL) and a higher rate of HBsAg decline while on treatment (decline of >0.166 log_{10} IU/mL/year) were predictive of HBsAg seroclearance in the future(3). The chances of achieving HBsAg levels of <2.3 log_{10} IU/mL after 10 years of treatment are shown in Figure

14. In this study the one patient who had baseline HBsAg level of <3 log_{10} IU/mL and on treatment HBsAg reduction of >0.166 log_{10} IU/mL after 5 years had HBsAg levels of <2.3log_{10} IU/mL after 10 years. This finding (albeit for 1 patient) agrees with Moini et al(3).

HBsAg levels of <200 IU/mL (2.3 log_{10} IU/mL) n=24				
Markers	Sub-Cohort 1.1 N=9	Sub-Cohort 1.2 N=7	Sub-Cohort 2.1 N=4	Sub-Cohort 2.2 N=4
Mean pgRNA log_{10} U/mL	0.80 ±0.50	1.33 ±0.90	1.21 ±1.00	0.41 ±0.61
Mean HBcrAg log_{10} U/mL	3.40 ± 1.40	3.41 ± 1.00	3.38 ±1.54	3.23 ±1.65

Table 22: Patients who had HBsAg levels of <2.3 log_{10} IU/mL after 10 years of NA therapy in sub-cohorts 1.1, 1.2, 2.1, and 2.2 and the mean pgRNA and HBcrAg levels.

The rate of patients achieving HBsAg levels of <2.3 log_{10} IU/mL after 10 years of treatment

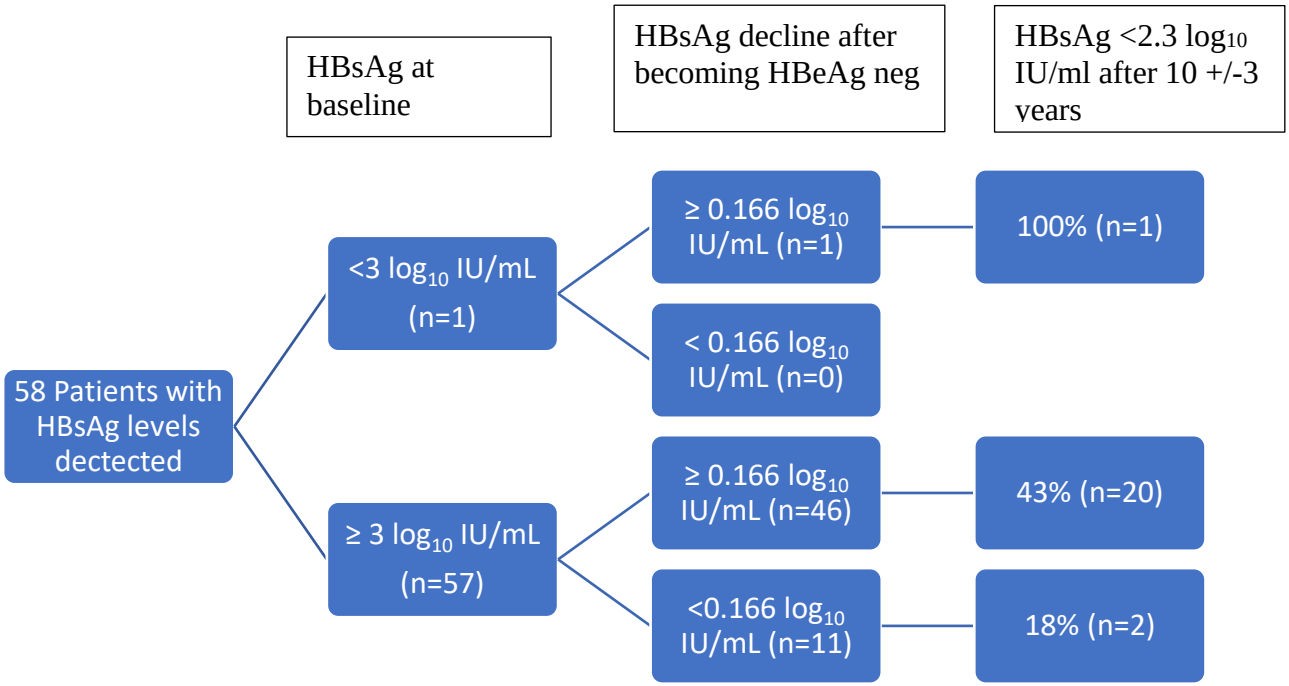


Figure 14 : The number of patients who had HBsAg levels <2.3 log10 IU/mL after 10 years of antiviral therapy and monitoring as stratified by baseline HBsAg level and HBsAg decline after becoming HBeAg negative while on-treatment.

Liver Fibrosis and inflammation analysis

Patients were grouped according to their fibrosis score. Patients who had FIB-4 scores below 3 were placed in the low fibrosis group; patients with FIB-4 scores above 3 were placed into the high fibrosis group. This was done at baseline, 5 years, and 10 years of treatment. The mean levels of biomarkers CXCL10, sPD1, ALT, and AST were calculated at each time point. The results are shown in Tables 23-25. Throughout the 10 years of monitoring patients who presented with low liver fibrosis (FIB-4 score of <3) also had lower levels of immunological markers CXCL10 and sPD1 and had lower levels of liver inflammation markers ALT and AST. Patients with high liver fibrosis (FIB-4 score of >3) had higher levels of CXCL10, sPD1, ALT and AST. FIB-4 correlated positively with CXCL10, sPD1, ALT and AST at baseline and after 5 years of NA treatment (Table 26).

Baseline n=58		
	Low Fibrosis – FIB-4 score <3 (n=34)	High fibrosis – FIB-4 score ≥ 3 (n=24)
Mean CXCL10 (pg/mL)	45.32 ± 28.10	129.15 ±43.71
Mean sPD1 (pg/mL)	884.71 ± 239.24	2305.55 ±654.10
Mean ALT (u/L)	60.02 ±30.11	85.09 ±12.79
Mean AST (u/L)	52.71 ±31.00	71.23 ±15.90

Table 23: Patients grouped according to their fibrosis scores (FIB-4 <3 and FIB-4 ≥3) at baseline and the mean CXCL10, sPD1, ALT and AST results in the two groups.

5 Years n=58		
	Low Fibrosis – FIB-4 score <3 (n=48)	High fibrosis – FIB-4 score ≥3 (n=10)
Mean CXCL10 (pg/mL)	49.84 ±20.17	98.65 ±40.55
Mean sPD1 (pg/mL)	712.33 ± 108.22	2100.16 ±673.19
Mean ALT (u/L)	44.10 ± 9.02	68.19 ±11.47
Mean AST (u/L)	25.21 ±5.44	39.27 ±4.81

Table 24: Patients grouped according to their fibrosis scores (FIB4 <3 and FIB4≥3) after 5 years of treatment and the mean CXCL10, sPD1, ALT and AST results in the two groups.

10 Years n=58		
	Low Fibrosis – FIB-4 score <3 (n=49)	High fibrosis – FIB-4 score ≥3 (n=9)
Mean CXCL10 (pg/mL)	49.11 ±16.82	96.02 ±38.11
Mean sPD1 (pg/mL)	784.85 ±120.99	2066.20 ±713.09
Mean ALT (u/L)	32.33 ±8.42	50.20 ±7.31
Mean AST (u/L)	27.61 ±5.92	31.84 ±5.02

Table 25: Patients grouped according to their fibrosis scores (FIB4 <3 and FIB4≥3) after 10 years of treatment and the mean CXCL10, sPD1, ALT and AST results in the two groups.

	Correlation at Baseline		Correlation at 5 years	
	Pearson's correlation - r	P value	Pearson's correlation - r	P value
FIB4 and CXCL10	0.710	<0.01	0.640	<0.01
FIB4 and sPD1	0.420	<0.05	0.440	<0.02
FIB4 and ALT	0.500	<0.03	0.450	<0.01
FIB4 and AST	0.410	<0.05	0.390	<0.05

Table 26: correlation analysis of FIB-4 against CXCL10, sPD1, ALT and AST at baseline and 5 years of NA treatment.

Reactivation

After 5 years, antiviral therapy was discontinued in five patients (three from cohort 1 and two from cohort 2). However, within 2 years four of the patients were placed back on antiviral therapy with NA (three from cohort 1 and one from cohort 2) as HBV DNA could be detected in their serum (mean: 2.00 ±2.05 $\log_{10}$ IU/mL). At the time of stopping antiviral therapy these patients had seroconverted to anti-HBe status, had undetectable HBV DNA, normal ALT and a mean HBsAg of 1.81 ±1.33 $\log_{10}$ U/mL. Interestingly HBcrAg and pgRNA could still be detected in these patient's serum at the point of stopping antiviral therapy (mean: 3.8 ±2.74 and 1.20 ±2.55 $\log_{10}$ U/mL respectively) and after reactivation their HBcrAg and pgRNA concentrations increased (mean: 4.9 ±2.07 and 2.44 ±1.98 $\log_{10}$ U/mL respectively). HBcrAg and pgRNA could be vital in predicting which patients will experience viral reactivation after discontinuing antiviral therapy if patient's serum are tested for these biomarkers at the point of antiviral discontinuation and future follow up appointments.

Discussion

In this study Chronic Hepatitis B (CHB) patients were placed into cohorts according to their HBeAg status at treatment baseline. Cohort 1 consisted of patients who were HBeAg positive at treatment baseline then became HBeAg negative at the 5 years follow up. Cohort 1 was further split into sub-cohort 1.1 consisting of HBeAg negative patients who seroconverted to anti-HBe after 10 years and sub-cohort 1.2 consisting of HBeAg negative patients who did not seroconvert to anti-HBe after 10 years of treatment. Cohort 2 consisted of patients who were HBeAg negative at treatment baseline. Cohort 2 was also split into sub-cohort 2.1 consisting of HBeAg negative patients who seroconverted to anti-HBe after 10 years and sub-cohort 2.2 consisting of HBeAg negative patients who did not seroconvert to anti-HBe after 10 years of treatment. The effects of antiviral therapy with nucleos(t)ide analogues (NA) were analysed.

This study has demonstrated that NAs are highly effective in reducing the viral load in CHB patients regardless of their HBeAg status at treatment baseline. After 10 years of treatment and monitoring of 58 CHB patients HBV DNA suppression was observed in over 90% (n=52) of patients. In contrast with the excellent suppression of serum HBV DNA, only one patient had undetectable HBcrAg at the end of the follow up and only five patients had undetectable HBV pgRNA. Nucleoside analogues inhibit the HBV reverse transcriptase, thus leading to the reduction of virions containing HBV DNA. However, it has no direct impact on the transcriptional activity of viral messenger RNA from the cccDNA pool. Therefore, there will be continued production of viral proteins including HBcAg and HBeAg for a longer period. The reduction of HBcrAg and HBV pgRNA will only occur when the transcriptional activity becomes lower with natural cell death of infected hepatocytes and reduction of infection of new uninfected hepatocytes.

CHB patients who achieve HBeAg seroconversion should be monitored even when ALT levels are normal. Recently, HBeAg negative viral mutants have been reported to cause persistent HBV DNA replication(4). So, there is a possibility that liver disease will worsen after HBeAg seroconversion. Furthermore, several reports have revealed that HBeAg status is not a predictive tool for HCC, and fulminant hepatitis may occur by the infection of HBV with HBeAg negative(5–7). Therefore, it is necessary for predictive factors for active liver disease in HBeAg negative patients to be identified to aid optimal disease management. In this study

patients who seroconverted to anti-HBe and had normal ALT still had quantifiable HBcrAg and pgRNA in their serum. Even though seroconverting to anti-HBe and having normal ALT levels are associated with positive clinical outcomes, the presence of HBcrAg and pgRNA in serum suggests cccDNA transcriptional activity, so liver damage is still possible.

In CHB patients the HBeAg negative chronic HBV infection phase (also known as the inactive carrier phase) is characterised by negative HBeAg, positive anti-HBe, normal ALT and undetectable or low HBV DNA levels (levels (<2000 IU/mL). Liver biopsy may also show no significant pathological changes in the liver. Furthermore, individuals with low concentrations of HBsAg (<1000 IU/mL) and undetectable HBV DNA are viewed to be the real inactive carriers(8). Studies focusing on liver biopsy show that significant inflammation could still be seen in several HBeAg-negative CHB patients with normal ALT and serum HBV DNA < 2000 IU/mL(9,10). Even though serum ALT is a sensitive marker that reflects liver damage, normal ALT does not exclude the absence of significant inflammation in the liver and does not predict the risk of long-term disease progression(9,11). Other non-invasive techniques to estimate the severity of liver inflammation and fibrosis are currently used in clinical practice including the FibroTest, vibration-controlled transient elastography (VCTE) and FIB-4 score. However, these tests are more useful at excluding confirmed advanced fibrosis or cirrhosis than predicting emerging fibrosis, which is not conducive to early initiation of antiviral therapy(12). Also, the diagnostic accuracy can be influenced by other factors, including hepatic necroinflammation, elevated serum total bilirubin levels, hepatic steatosis etc.

Current international guidelines state that antiviral treatment is not required in CHB patients in the HBeAg negative chronic HBV infection phase(8,12). This is because if patients remain in this phase, they have a low risk of developing cirrhosis or HCC and an annual rate of 1-3% of spontaneous HBsAg loss according to previous research(13–15). However, majority of these reports on the clinical outcomes involved young patients, and the follow-up periods were quite short (approximately 5 years). Therefore, the long-term prognosis of inactive carrier patients requires further investigation. It is unknown whether the favourable outcomes observed can be applied to older patients in the inactive carrier phase identified in different clinical environments(16,17). In this study the patients who were in the HBeAg negative chronic HBV infection phase (sub-cohort 1.1 and 2.1) did have more favourable outcomes of viral activity.

However, some patients did have high FIB-4 scores and markers of cccDNA transcription - HBcrAg and pgRNA could still be detected in their serum at the 10-years follow up.

It is well known that antiviral therapy can improve survival rate in CHB patients and can reduce the complications associated with liver damage. Compared with HBeAg positive patients, HBeAg negative patients have a greater virological response rate to antiviral therapy and a higher chance of achieving functional cure while on treatment. There have been issues in the past with high rates of drug resistance and side effects, with some treatment options including lamivudine (LAM) and adefovir dipivoxil (ADV). Now, first line choice of therapy e.g., entecavir (ETV), is highly effective with low resistance and have a reduced incidence of drug side effects(18,19).

In this study only one patient presented with undetectable HBcrAg at 10 years (treatment with LAM). It is therefore difficult to statistically identify factors that predict undetectable HBcrAg. However, other studies have identified baseline HBeAg negativity, HBcrAg level <3 log10 U/mL and baseline HBsAg <3 log10 IU/mL are all predictive factors of undetectable HBcrAg after 7 years of antiviral treatment with NA(20,21). These predictive factors may be useful in the selection of patients with better outcome and provide clearer guidance for clinical management of CHB. It has been previously demonstrated that serum HBcrAg concentrations correlate highly with intrahepatic cccDNA concentrations, which is a reservoir for viral replication(22–24). Chronic Hepatitis B patients achieving undetectable HBcrAg while on antiviral treatment with NAs indicates low concentrations of cccDNA in their livers. This is according to previous studies that have shown that low concentrations of HBcrAg at the time of cessation of lamivudine is linked to a reduced risk of reactivation of hepatitis after stopping lamivudine therapy(23,25,26). The one patient in this study who had undetectable HBcrAg levels at the 10 years follow-up could be representative of patients with a lower chance of reactivation after discontinuing therapy with NAs. Furthermore, current research has shown that HBcrAg concentrations, but not HBsAg levels, were associated with the risk of developing HCC in CHB patients with undetectable HBV DNA under NA therapy(27,28). It was unfortunate that we could not verify the roles HBcrAg and HBsAg levels have on the development of HCC in our study due to the limited number of patients developing HCC. Therefore, it is of great importance to carry out further work to verify this hypothesis.

The HBsAg seroclearance rate is generally very low among CHB patients, and in this study none of the patients achieved HBsAg seroclearance. The reason for this is because NAs have minimal impact on transcription and translation of HBsAg from cccDNA and secretion of empty HBsAg into the extracellular environment. Multiple studies have indicated that HBsAg of <200 IU/mL (2.3 $\log_{10}$ IU/mL) is predictive of subsequent HBsAg seroclearance in approximately 3 years(29,30). In this study 41% (n=24) of patients did have HBsAg levels of <200 IU/mL (2.3 $\log_{10}$ IU/mL) after 10 years of therapy. Moreover, individuals who had HBsAg levels of <200 IU/mL still had detectable pgRNA and HBcrAg in their serum after 10 years regardless of their anti-HBe status. To improve my study these patients could have been observed for longer to determine whether they did achieve HBsAg seroclearance and if their serum HBV pgRNA and HBcrAg would therefore decrease below the LLOQ. In a study by Mak et al, 15.8% (n=19) of treatment naïve patients with HBsAg loss had detectable HBV RNA and HBcrAg(35,39). These findings indicate that HBsAg loss does not necessarily mean complete cessation of viral transcription and translation as cccDNA is still active(35). This warrant further studies to be done relating to serum HBV pgRNA and HBcrAg kinetics in cohorts of CHB individuals achieving HBsAg loss, as this will improve the role of HBV pgRNA in clinical settings and the possibility of its use as a surrogate marker of cccDNA transcription(35,40).

HBV reactivation is a clinical syndrome that is associated with a sudden increase of serum HBV DNA and an increase of serum ALT levels. Reactivation may occur in patients that are HBsAg negative and possess anti-HBc antibodies(31). The causes of reactivation are generally linked to the inability to gain complete control over HBV replication. Reactivation can occur spontaneously or when a patient becomes immunocompromised. Currently there are no standardized criterion for the level of increase in HBV DNA to define reactivation, however, a 2 log or 100-fold increase in HBV DNA is often used in clinical settings(31,32). Studies have mainly reported a 2 – 3-fold increase in serum ALT above the baseline as a reasonable indicator of HBV reactivation(31,32). HBV reactivation can also be suspected and diagnosed when CHB patients that were previously HBeAg negative or HBsAg negative undergoes seroreversion of these viral markers. Hence the importance of routine evaluation of CHB patients and the utilisation of novel markers such as HBcrAg and pgRNA.

Systematic discontinuation of long-term treatment with NAs is one strategy to increase functional cure rate in CHB individuals that are HBeAg negative. Cessation of treatment with NAs frequently leads to a virologic and biochemical relapse that entails different phases: the lag, reactivation, and consolidation phases(31,33). The abrupt HBV DNA increase normally observed during the reactivation phase is often transient but needs close follow-up evaluation.

In a study by Inoue et al, a HBcrAg assay was developed to monitor CHB patients and to detect HBV reactivation in HBeAg negative patients with persistently undetectable HBV DNA(34). HBcrAg was detected in 97.5% (n=161) of patients with CHB. In the HBV reactivation group (n=13), 9 patients had detectable HBcrAg before and after HBV DNA positivity(34). It was concluded that HBcrAg could indeed be useful in monitoring patient response to antiviral treatment in HBeAg negative patients as well as early detection of HBV reactivation. Insight into disease progression and treatment response could potentially save lives and improve the quality of life for CHB patients.

This study demonstrated that HBcrAg and pgRNA could help determine which patients are at risk of reactivation. Patients in whom antiviral therapy was discontinued and experienced viral reactivation had high levels of HBcrAg and pgRNA in their serum at the time of antiviral cessation. HBcrAg and pgRNA continued to increase after treatment was stopped. Larger studies closely monitoring patients who experience viral reactivation is required to fully understand the role of HBcrAg and pgRNA in predicting clinical outcomes after treatment cessation.

During NA therapy when reverse transcription is inhibited, HBV RNA accumulates and is released in the circulation as HBV pgRNA containing viral particles(35). A study by Wang et al, demonstrated that the concentration of HBV pgRNA virion increased after the administration of entecavir in cell culture models and HBV transgenic mice(36). Goncalves et al developed a mathematical model that predicted a transient increase of HBV RNA levels during the first week of antiviral therapy with tenofovir followed by a slower decline(37). Wu et al also reported that HBV pgRNA increased within 3 months of entecavir treatment and remained elevated than the baseline concentration in the low baseline HBV pgRNA group(35). My study showed that in both HBeAg positive and HBeAg negative patients the level of HBV pgRNA was very high before initiating NA therapy and rapidly decreases in the first 5 years of treatment and then slowly decreases thereafter. HBeAg positive patients had higher pgRNA

at baseline compared to HBeAg negative patients. In patients who achieved HBeAg seroconversion (patients in sub-cohort 1.1 and 2.1) a steeper decline in pgRNA was observed between treatment baseline and 5 years compared to patients who did not seroconvert (sub-cohort 1.2 and 2.2). This study demonstrated that being HBeAg negative at baseline and achieving anti-HBe antibodies was advantageous in reducing the concentration of serum pgRNA during treatment. Furthermore, this study showed that even with long term antiviral therapy HBV pgRNA could still be detected in most patients, albeit in low concentrations. This means that cccDNA is still active and viral particles can still be synthesised, which may lead to liver cirrhosis and HCC. A limitation of this study is not collecting data at earlier timepoints of antiviral therapy e.g. after 6 months of NA therapy; this would have helped to understand the full kinetics of pgRNA during NA therapy. Larger and more detailed kinetic studies are needed to characterize HBV pgRNA kinetics under NA therapy, which would also increase our knowledge of HBV-host interactions and NA mode of action.

Longitudinal analysis of CXCL10 and sPD-1 were done at treatment baseline, 5 years and 10 years of follow-ups. CXCL10 mediates the inflammatory response and is associated with the chronicity of HBV. PD-1 is an immunosuppressive molecule expressed on B cells, T cells, dendritic cells, and natural killer (NK) T cells to suppress anticancer immunity. This study demonstrated that reductions in serum CXCL10 and sPD-1 concentrations 5 years after starting NA treatment correlated with reductions in ALT and AST. This indicates that the levels of CXCL10 and sPD-1 reflected the severity of the damage caused by active inflammation of HBV to hepatocytes. Additionally, the concentrations of CXCL10 were independent of the replicative activity of HBV, which supports other research that the replicative level of HBV is not always positively correlated with the severity of liver inflammation. It has been previously reported that CXCL10 in HBV infected patients was mostly produced by hepatocytes and rarely produced by other organs. Therefore, compared to other cytokines which can be produced by multiple organs, CXCL10 is likely to be a more specific and sensitive indicator to predict inflammatory injury of the liver. Also, this study has demonstrated that CXCL10 and sPD-1 correlates with the course of liver damage by HBV infection. Patients who had high levels of fibrosis and liver inflammation markers – ALT, AST also had higher levels of sPD-1 and CXCL10.

In summary, this study has demonstrated that novel markers HBcrAg and HBV pgRNA can be utilised as markers of cccDNA transcriptional activity, the main reason being that they can be detected during antiviral therapy, when HBV DNA is no longer detected and when patients achieve HBeAg loss and seroconvert. These markers are associated with virological responses during treatment and virological relapse after treatment withdrawal.

Chapter 6

Serum markers of cccDNA transcriptional activity may have a role in predicting long-term outcomes in children with perinatally acquired HBV.

Abstract

Background and aims: Perinatally acquired Chronic Hepatitis B (CHB) has often indolent course in childhood, but persistent viral infection leads to progressive liver disease in 40% of patients in adulthood. The combination of non-invasive serological (HBeAg, HBsAg levels) and virological (HBV DNA) markers together with liver enzyme activity (ALT) and liver damage scores (APRI or FIB-4) aids in monitoring disease stages and progression. Novel serological markers of cccDNA transcriptional activity – HBV core-related antigen (HBcrAg) and pre-genomic HBV RNA (pgRNA) might add more insight and have not been studied in paediatric populations. The aim of this study is to investigate whether markers of cccDNA transcriptional activity in serum: HBcrAg and pgRNA can help to predict future disease progression or need for therapy in patients with perinatally acquired Chronic Hepatitis B infection.

Methods: A retrospective study was carried out with serum samples from 29 CHB paediatric patients diagnosed in childhood with long-term follow up in the same centre. At a follow up appointment (median: 3 years, range 1-5 years) five patients needed to be started on antiviral therapy (median age: 13 years). After initiating antiviral therapy patients were followed up for 6 years (median:6 years, range: 3-12 years) to assess their response to treatment.

Results: The markers of cccDNA transcriptional activity – pgRNA and HBcrAg – combined with the traditional serological/virological markers (HBeAg, HBsAg and HBV DNA levels) were helpful in predicting paediatric patients who might require antiviral therapy and represent a valuable tool to be applied in regular diagnostics. Factors associated with the need to initiate antiviral therapy in paediatric patients include baseline: HBeAg positivity, having HBsAg levels ≥ 4 $\log_{10}$ IU/mL, HBV DNA levels ≥ 6 $\log_{10}$ IU/mL, HBV pgRNA levels ≥ 5 $\log_{10}$ U/mL, HBcrAg levels ≥ 4 $\log_{10}$ U/mL and ALT >40 U/L.

Definitions

Baseline: In this study baseline is described as the time when patients attended the Hepatitis clinic after their diagnosis and assessed whether they should be placed on antiviral therapy or not. Five patients were started on antiviral therapy.

Follow up – The follow up appointment is described as the most recent visit at the Hepatitis clinic at KCH (at the time of this study- median 6 years).

Cohorts – The patients in this study were divided into 2 cohorts. Cohort 1 represented patients who were not placed on antiviral therapy (n=24) and cohort 2 consisted of patients who were placed on antiviral therapy (n=5) at baseline (Figure 1).

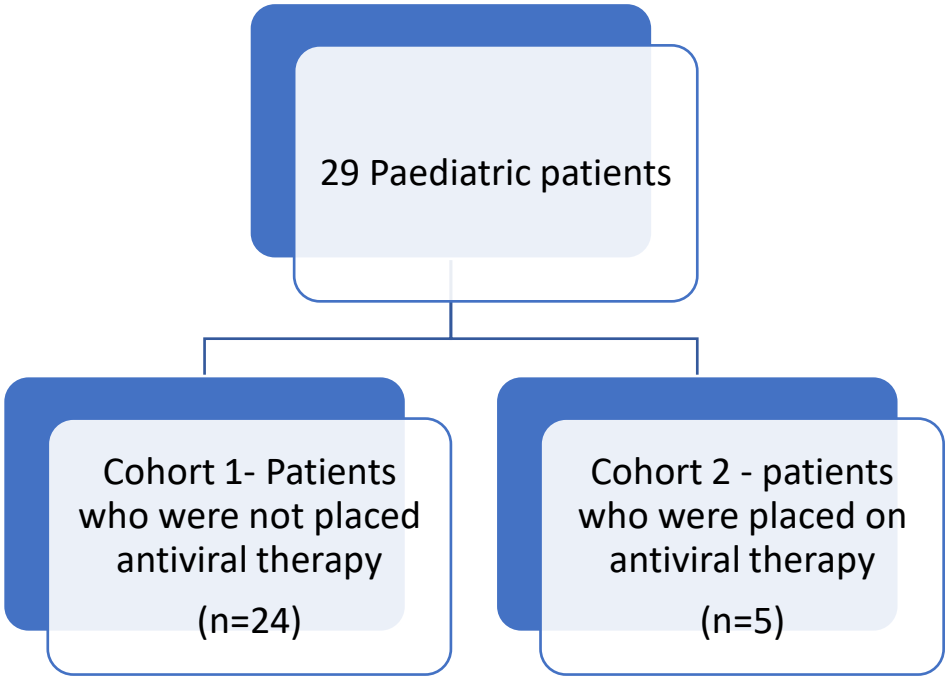


Figure 1: A description of the two cohorts represented in this study.

Study subjects

Serum samples from 29 CHB paediatric patients between the ages of 2 and 18 years old were tested in the study. All patients or their parents/ guardians provided informed consent for King's College liver biobank and all principles of the Declaration of Helsinki were followed. All patients had visited the hepatitis clinic at King's College Hospital (KCH) on multiple occasions and some patients had received antiviral therapy with either NAs or peg-INF. Patients were excluded if they were positive for Hepatitis C virus (HCV), coinfected with hepatitis D virus (HDV), human immunodeficiency virus (HIV), or had any severe systemic disease.

The samples used were from the liver pathology archives in the -20^{o}C freezer at KCH. Five out of the Twenty-nine patients had undergone a liver biopsy with a fibrosis index (F) equal or greater than 2 according to the Ishak scoring system.

Biomarkers measured.

In this study a comparison was made between CHB paediatric patients who received antiviral therapy with NA or Peg-IFNα at their follow-up appointment with untreated patients. Biomarkers at baseline and at follow-up were compared to determine if there is an association with disease progression in adolescence and adulthood.

HBcrAg and HBV pgRNA were measured due to their strong association with cccDNA. These biomarkers were measured at baseline and at follow-up to determine cccDNA activity and to assess whether they are associated with diseases progression from childhood to adolescent and adulthood.

Serological markers -HBsAg, HBeAg, HBeAb and virological marker – HBV DNA were measured at baseline and at follow-up to assess disease progression, viral replication, and activity at the two time points.

Liver function markers/ enzymes – ALT, AST, ALP, bilirubin, GGT and PLT were measured to determine inflammation and ongoing liver damage.

Altogether these biomarkers could help determine which paediatric patients are most at risk of severe liver damage and therefore require antiviral therapy during adolescence.

Statistical analysis

Statistical analysis was performed using the SPSS software. Quantification levels of serum HBV DNA, HBcrAg and pgRNA were log transformed to the same units. The figures were prepared using the prism software. Median levels of the biomarkers were calculated. Degree of correlation was done using the Pearson's correlation analysis with a statistically significant p value of <0.05.

Results – Tables and Graphs

Patient demographic	
n	29
Male	48% (n=14)
Median age	9 (range 2-18)
Median Height (m)	1.3 (range 0.7-1.6)
Median weight (Kg)	29 (range 22-37)
White/ Caucasian/ other White	21% (n=6)
Black African/ Caribbean/ other black	17% (n=5)
Asian	24% (n=7)
South Asian	38% (n=11)
Genotype B	27% (n=8)
Genotype C	17% (n=5)
Genotype D	48% (n=14)
Genotype E	7% (n=2)

Table 1 – Patient demographic at baseline

N	**29**
	Baseline
HBeAg negative	17% (n=5)
HBeAg Positive	83% (n=24)
HBeAb Positive	17% (n=5)
Median HBsAg quant (log_{10} IU/mL)	4.54 (range 2.34-5.35)
Median HBV DNA (log_{10} IU/mL)	8.11 (range 1.1 – 9.12)
Median HBcrAg (log_{10} U/mL)	6.51 (range 3 – 7)
Median pgRNA (log_{10} U/mL)	7.2 (range 1.86 – 7.72)
Median ALT (u/L)	37 (range 14 -626)
Median ALP (u/L)	256 (range 110-348)
Median Bilirubin (umol/L)	8 (range 3-16)
Median Albumin (g/L)	44 (range 14-49)
Median GGT (u/L)	11 (range 6-49)
Median AST (u/L)	46 (range 24-475)
Median PLT (10E9/mL)	295 (range 161 – 534)

Table 2 – Patient results at baseline

N = 29

	Baseline/ Antiviral initiation		Follow up (median: 6 years, range:3-12 years)	
	Untreated (n=24)	Treated (n=5)	Untreated (n=24)	Treated (n=5)
Male %	50 (n=12)	60 (n=3)	50 (n=12)	60 (n=3)
Median age (years)	9 (range 2-18)	13(range 12-17)	14 (range 8-25)	19 (range 18-22)
Median Height (M)	1.3 (range 0.7-1.6)	1.4 (range 1.3-1.5)	1.6 (range 1-1.9)	1.5 (range 1.4-1.8)
Median weight (Kg)	30 (range 22-37)	28 (range 25-32)	48 (range 23-60)	49 (range 45-55)
HBeAg negative (%)	20 (n=5)	0	46 (n=11)	60 (n=3)
HBeAg Positive (%)	80 (n=19)	100 (n=5)	54 (n=13)	40 (n=2)
HBeAb Positive (%)	20 (n=5)	0	45 (n=11)	60 (n=3)
Median HBsAg quant (log_{10} IU/mL)	4.39 (range 2.34 - 5.35)	4.90 (range 4.04 - 5.35)	4.38 (range 2.74 - 5.10)	3.76 (range 3.22 – 4.50)
Anti-HBs (%)	0	0	0	0
Median HBV DNA (log_{10} IU/mL)	8.10 (range 1.10-9.12)	8.68 (range 8.18 - 8.83)	7.10 (range 0 - 8.53)	1.60 (range 1.30 -1.90)
Median HBcrAg (log_{10} U/mL)	4.95 range (2.00-7.00)	6.84 (range 5.56 - 7.00)	4.70 (range 2.10-7.00)	3.50 (range 3.32 -4.21)
Median pgRNA (log_{10} U/mL)	2.65 (range 1.00 - 8.50	7.11 (range 6.23 - 7.44)	3.02 (range 1.65 - 8.02)	1.80 (range 0-2.4)
Median ALT (u/L)	32 (range 14 -626)	80 (range 55-130)	27 (range 11-147)	24 (range 12-46)

Median ALP (u/L)	245 (range 125-348)	283 (range 110-302)	240 (range 70-446)	90 (range
Median Bilirubin (umol/L)	8 (range 3-16)	9 (range 5-15)	10 (range 7-25)	8 (range 5-11)
Median Albumin (g/L)	44 (range 14-49)	45 (range 26-49)	46 (range 41-47)	36 (range 30-39)
Median GGT (u/L)	11 (range 6-49)	13 (range 10-14)	11 (range 7-78)	10 (range 7-15
Median AST (u/L)	44 (range 31-475)	69 (range 24-150)	48 (range 18-122)	30 (range 19-38)
Median PLT (10E9/mL)	302 (range 161 – 534)	220 (range 177-270)	305 (range 111-380)	218 (range 180-240)

Table 3 - Baseline and follow up results of patients who did not receive antiviral treatment (cohort 1) compared to patients who received antiviral treatment (cohort 2).

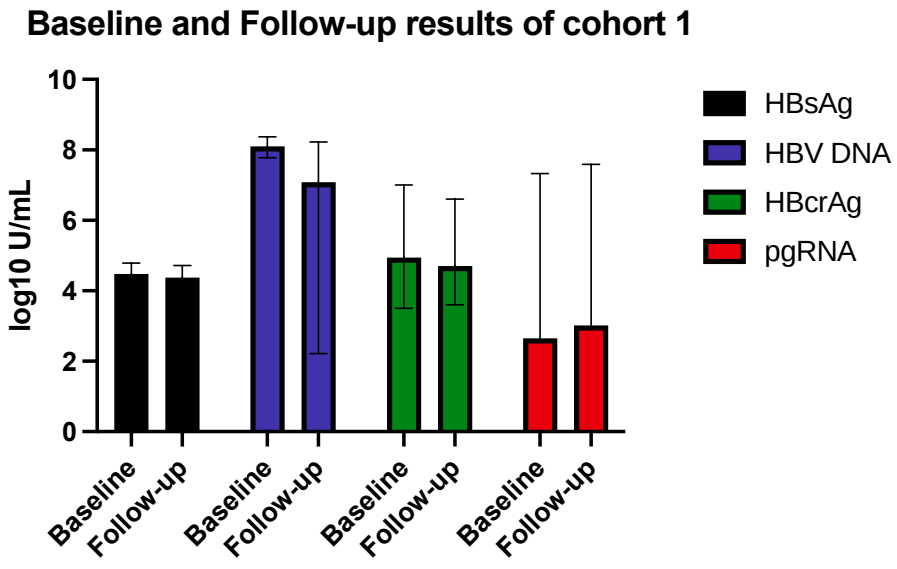


Figure 2: Median levels of HBsAg, HBV DNA, HBcrAg and pgRNA (with 95% CI) at baseline and follow up of patients who were not placed on antiviral therapy (cohort 1).

Biomarkers	95% CI at Baseline	95% CI at Follow up
HBsAg	4.13- 4.66	3.94 - 4.72
HBV DNA	7.78 – 8.37	2.22 – 8.23
HBcrAg	3.50 -7.00	3.60 – 6.60
pgRNA	2.30 – 7.33	2.06 -7.59

Table 4: 95% CI of biomarkers at baseline and follow up of patients in cohort 1.

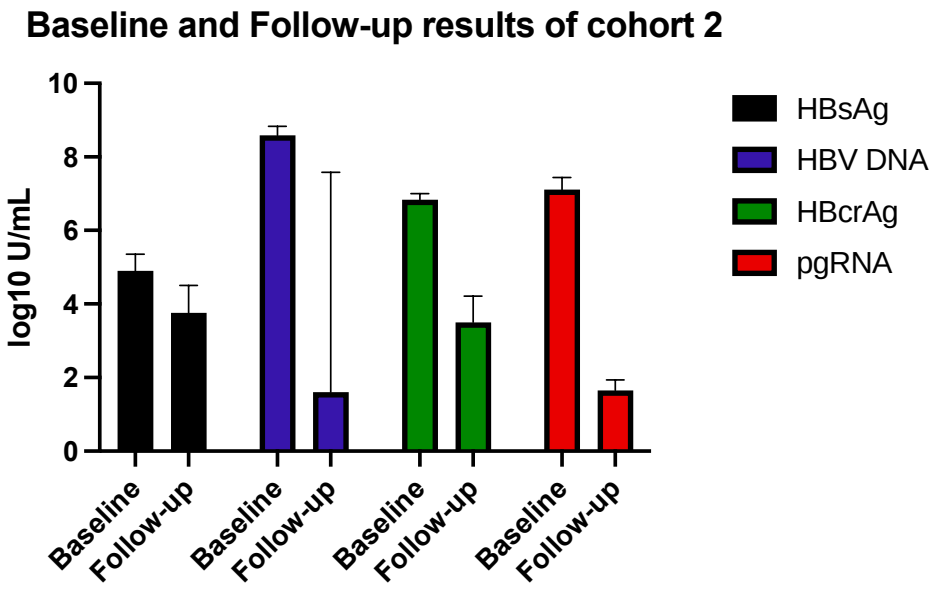


Figure 3: Median levels of HBsAg, HBV DNA, HBcrAg and pgRNA (with 95% CI) at baseline and follow up of patients who were placed on antiviral therapy (cohort 2).

Biomarkers	95% CI at Baseline	95% CI at Follow up
HBsAg	4.04- 5.35	3.22 - 4.50
HBV DNA	8.18 – 8.30	1.30 – 7.58
HBcrAg	5.56 -7.00	3.30 – 4.21
pgRNA	6.23 – 7.44	0 -2.40

Table 5: 95% CI of biomarkers at baseline and follow up of patients in cohort 2.

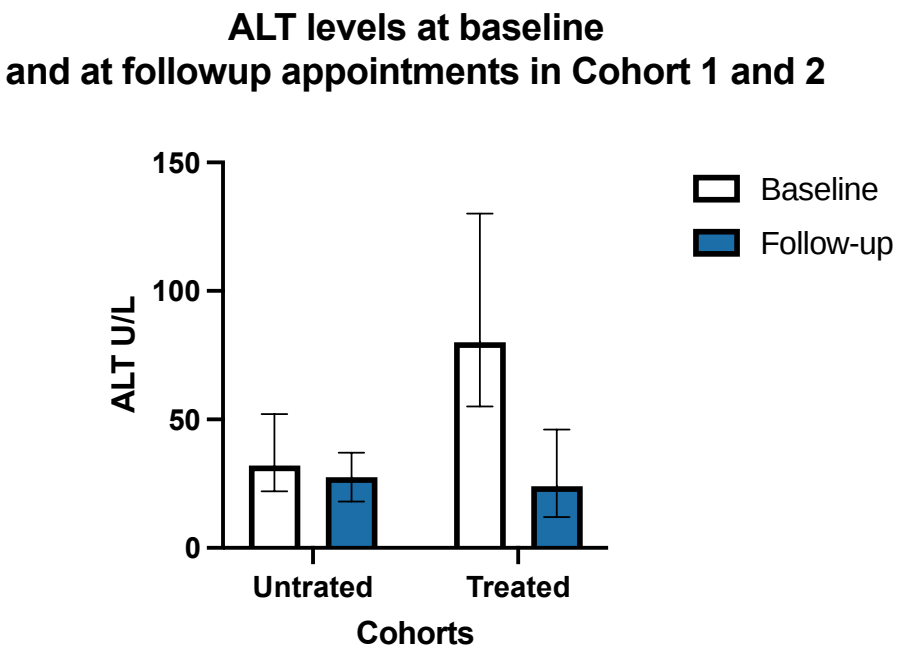


Figure 4: Median levels of ALT (with 95% CI) at baseline and follow up of patients in cohort 1 (untreated) and cohort 2 (treated).

	Untreated	Treated
95% CI at Baseline	22- 52	55- 130
95% CI at Follow up	18 - 37	12 – 46

Table 6: 95% CI of ALT levels at baseline and follow up of patients in cohort 2.

5 patients placed on antiviral therapy with NA – Treatment initiation								
Patient	**age**	**HBeAg status**	**Anti-HBe status**	**HBsAg quant (log10 IU/mL)**	**HBV DNA (log10 IU/mL)**	**HBcrAg (log10 U/mL)**	**pgRNA (log10 U/mL)**	**ALT (u/L)**
1	13	Positive	Negative	4.9	8.18	5.56	6.23	55
2	15	Positive	Negative	5.30	8.83	7	6.74	80
3	12	Positive	Negative	4.04	8.59	7	7.11	130
4	12	Positive	Negative	4.55	8.83	6.84	7.3	123
5	17	Positive	Negative	5.35	8.68	6.34	7.44	57

Table 7 - Baseline results of the 5 patients who received antiviral treatment (cohort 2).

5 patients placed on antiviral therapy with NA – Follow up								
Patient	**age**	**HBeAg status**	**Anti-Hbe status**	**HBsAg quant (log10 IU/mL)**	**HBV DNA (log10 IU/mL)**	**HBcrAg (log10 U/mL)**	**pgRNA (log10 U/mL)**	**ALT (u/L)**
1	19	Positive	Negative	4.5	7.58	4.21	2.4	26
2	18	Negative	Positive	3.62	1.60	3.50	0	24
3	20	Positive	Negative	3.76	1.94	3.3	1.80	46
4	18	Negative	Positive	4.00	1.60	4.00	1.94	12
5	22	Negative	Positive	3.22	1.3	3.30	0	13

Table 8 – Follow up results of the 5 patients who received antiviral treatment (cohort 2).

Correlation analysis at Baseline		
	Pearson's correlation - r	P value
HBsAg and HBV DNA	0.65	<0.001
HBcrAg and HBV DNA	0.50	<0.001
HBcrAg and HBsAg	0.47	<0.001
pgRNA and HBcrAg	0.86	<0.001
pgRNA and HBV DNA	0.82	<0.001
pgRNA and HBsAg	0.74	<0.001

Table 9 : Correlation analysis of serum biomarkers at baseline

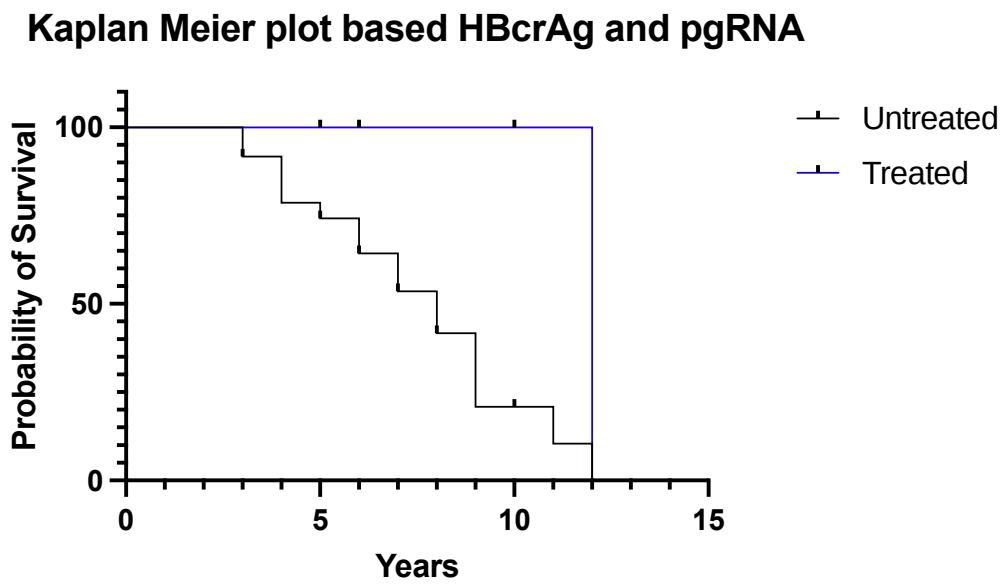


Figure 5: Kaplan Meier plot showing the survival curve of untreated patients compared to treated patients.

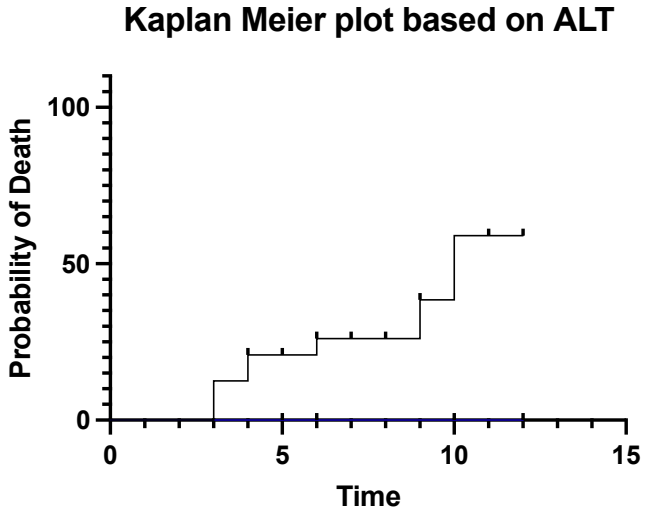
	Untreated / Treated	Treated / Untreated
Hazard Ratio (HR)	4.15	0.24
95% CI	1.12 – 15.30	0.70 - 0.90
P value	0.03	0.03

Table 10: HR, 95% CI and P value of the Kaplan Meir plot above.

Normalisation of Liver function markers (according to the limits for Children)

	Treatment Group % (n=5)		Untreated group % (n=24)	
	Baseline	Follow-up	Baseline	Follow-up
ALT	0 (n=0)	100 (n=5)	58 (n=14)	67 (n=16)
AST	40 (n=2)	60 (n=3)	37 (n=9)	70 (n=17)
Bilirubin	100 (n= 5)	100 (n=5)	95 (n=23)	91 (n=22)
GGT	100 (n=5)	100 (n=5)	100 (n=24)	95 (n=23)
ALP	0	80 (n=4)	8 (n=2)	50 (n=12)
PLT	100 (n=5)	100 (n=5)	87 (n=21)	91 (n=22)

Table 11 – Normalisation of liver function markers the treatment group compared to the untreated group



Cumulative incidence of ALT normalisation	
Untreated	Treated
67% (p<0.01)	100% (p<0.01)

Figure 6: Kaplan Meier plot demonstrating the cumulative incidence of ALT normalisation in the untreated and treated group.

Factors	Hazard ratio	95% CI	p value
age <5 vs ≥5 years	2.30	1.77-6.50	0.15
sex male vs female	2.44	2.46-9.3	0.20
baseline HBeAg positive vs negative	1.1	0.4-2.51	**0.05**
baseline HBsAg ≥4 vs <4 log_{10} IU/mL	0.5	0.11-1.3	**0.05**
baseline HBV DNA ≥6 vs <6 log_{10} IU/mL	0.2	0.1-0.30	**0.001**
baseline HBV pgRNA ≥5 vs <5 log_{10} U/mL	0.54	0.2-1.84	**0.01**
baseline HBcrAg ≥4 vs <4 vs log_{10} U/mL	0.58	0.3-1.99	**0.05**
ALT >40 vs <40 IU/L	0.55	0.35-1.15	**0.05**

Table 12: Univariate analysis of factors associated with disease progression paediatric patients. Baseline HBeAg positivity, having HBsAg levels ≥4 log10 IU/mL, HBV DNA levels ≥6 log10 IU/mL, HBV pgRNA levels ≥5 log10 U/mL, HBcrAg levels ≥4 log10 U/mL and ALT >40 IU/L at baseline are linked to disease progression in paediatric patients.

Factors	Hazard ratio	95% CI	p value
age <5 vs ≥5 years	2.30	1.77-6.50	0.08
sex male vs female	2.44	2.46-9.3	0.60
baseline HBeAg positive vs negative	1.1	0.4-2.51	0.06
baseline HBsAg ≥4 vs <4 log_{10} IU/mL	0.5	0.11-1.3	**0.05**
baseline HBV DNA ≥8 vs <8 log_{10} IU/mL	0.2	0.1-0.30	**0.02**
baseline HBV pgRNA ≥7 vs <7 log_{10} U/mL	0.54	0.2-1.84	**0.02**
baseline HBcrAg ≥6 vs <6 vs log_{10} U/mL	0.58	0.3-1.99	**0.05**
ALT >40 vs <40 IU/L	0.55	0.35-1.15	**0.001**

Table 13: Univariate analysis of factors associated with seroconverting to anti-HBe in adolescence. Baseline HBV DNA <8 log10 IU/ml, HBsAg <4 log10 IU/ml, HBcrAg <6 log10 U/ml, pgRNA <7 log10 U/ml and ALT >40 IU/l, were more likely to seroconvert to anti-HBe in adolescence.

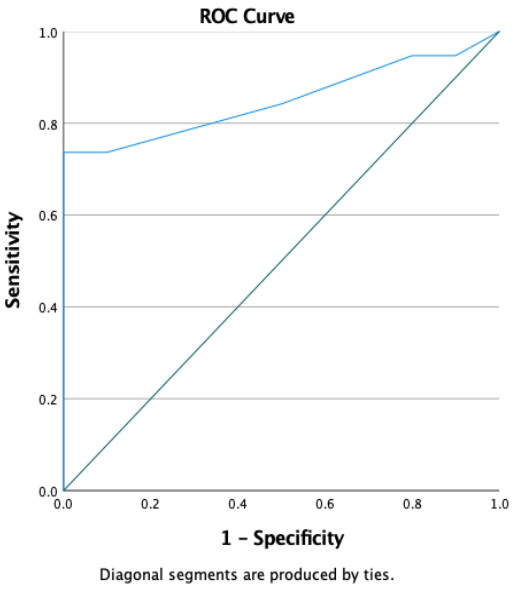


Figure 7-ROC for HBcrAg test. Area = .850

Results analysis

This study consists of 29 paediatric patients who were diagnosed with HBV (median age=2, range 0.7– 5 years). After approximately 3 years five of the patients were placed on antiviral therapy – three patients on Interferon- alpha (IFNα) and two on Entecavir. In this study cohort 1 represented patients who were not placed on antiviral therapy (n=24) after the 3 years and cohort 2 consisted of patients who were placed on antiviral therapy (n=5) after the 3 years (Baseline). Patient demographics are shown in Table 1. Baseline concentrations of biomarkers (median) are shown in Table 2. Baseline and follow up concentrations of biomarkers (median) in cohorts 1 and 2 are shown in Table 3.

In both cohort 1 and cohort 2 baseline concentrations of HBV DNA and HBsAg were very high, however, the patients who were placed on antiviral therapy (cohort 2) had considerably higher levels of ALT compared to patients who were not placed on therapy (median= 32 vs 80 u/L respectively). Furthermore, novel markers – HBcrAg and pgRNA were very high in cohort 2 compared to cohort 1 (HBcrAg: 6.84 vs 4.95 $\log_{10}$ U/mL respectively; pgRNA: 7.11 vs 2.65 $\log_{10}$ U/mL respectively). Particularly pgRNA which was nearly 3 times higher on average at baseline in patients who were placed on antiviral therapy.

At the 6 years follow up, in cohort 2 HBV DNA levels had significantly reduced but HBsAg levels were still high although in lower concentrations than at antiviral initiation. At baseline all patients in cohort 2 had HBV DNA more than 8 $\log_{10}$ IU/ml, but after antiviral therapy four out of the five patients had HBV DNA levels less than 2 $\log_{10}$ IU/ml at the follow up appointment. The cccDNA markers HBcrAg and pgRNA were still quantifiable after 6 years of antiviral therapy although at reduced levels. In cohort 1 biomarkers – HBV DNA, HBsAg, HBcrAg and pgRNA remained high and at similar concentrations to baseline. The median ALT remained within normal range. It appears that paediatric patients who were placed on antiviral therapy showed a much greater decline in HBV DNA, HBcrAg, HBV pgRNA and ALT at follow up compared to patients who were not administered antiviral therapy (Figure 2, 3 and 4).

Results of the five patients who were placed on antiviral therapy (cohort 2) are shown in Table 7 and 8. All patients in cohort 2 were HBeAg positive and anti-HBe negative at baseline. At the follow up appointment 60% (n=3) of the patients had become HBeAg negative and the

same patients acquired anti-HBe antibodies (median duration: 3 years). In cohort 1 80% (n=19) of patients were HBeAg positive at baseline and 20% (n=5) had acquired anti-HBe antibodies. At the follow-up appointment 54% (n=13) of patients were still HBeAg positive with 45% (n=11) achieving anti-HBe status. In cohort 1 one patient had undetectable HBV DNA at the follow up appointment (5 years after their baseline appointment), however cccDNA markers could still be quantified in their serum (HBcrAg = 3.2 $\log_{10}$ U/mL, pgRNA= 3.6 $\log_{10}$ U/mL).

At baseline patients in cohort 2 had higher levels of liver function markers than in cohort 1 (Table 3). Additionally, at the follow-up appointment the normalisation of virological markers and liver function markers were more prominent in the treated group (Table 11). The patients in cohort 2 all demonstrated liver damage/ inflammation at baseline. In this group all patients had fibrosis stage measured using the Ishak scale of 2 or above and necroinflammation measured at 4 or above. Whereas in cohort 1 only 4 patients underwent a liver biopsy and these patients had little or no liver inflammation. HBsAg levels remained high throughout the study for most patients whether in the treatment group or not. In cohort 1 96% (n=24) of patients had HBsAg levels >3 $\log_{10}$ IU/mL at baseline and at follow up 58% (n=14) had HBsAg >3 $\log_{10}$ IU/mL. In cohort 2 all patients had HBsAg levels >3 $\log_{10}$ IU/mL at baseline and at follow up. None of the patients in this study achieved HBsAg loss and seroconverted to anti-HBs antibodies.

Pearson's correlation analysis demonstrated that pgRNA and HBV DNA were well correlated in CHB paediatric patients ($r=0.82$ $p<0.001$). Additionally, a positive correlation was found between pgRNA levels and HBsAg levels ($r=0.74$, $p<0.001$). HBcrAg also correlated positively with HBsAg ($r=0.47$ $p<0.001$) and HBV DNA ($r=0.50$ $p<0.001$). The strongest correlation was found between pgRNA and HBcrAg ($r= 0.86$, $p<0.01$) (Table 9).

Analysis by Kaplan Meier survival model demonstrated that patients on treatment had lower pgRNA levels (<2 $\log_{10}$ U/mL) and lower HBcrAg levels (<3 $\log_{10}$ U/mL) (Figure 5 and Table 10). Lower concentrations of pgRNA and HBcrAg while on antiviral treatment led to better clinical outcomes. Additionally, by Kaplan-Meier analysis, the cumulative incidence of ALT normalisation was higher in the treated group than the untreated group (Table 11 and Figure 6). All the individuals placed on treatment had ALT decreasing below the ULN within 1 year.

Univariate analysis revealed that factors associated with disease progression and possibly the need to initiate antiviral therapy in paediatric patients at the time of adolescence include baseline HBeAg positivity, having HBsAg levels ≥4 log_{10} IU/mL, HBV DNA levels ≥6 log_{10} IU/mL, HBV pgRNA levels ≥5 log_{10} U/mL, HBcrAg levels ≥4 log_{10} U/mL and ALT >40 U/L (Table 12). Also, patients with higher ALT at childhood (>40 U/L), but lower HBV DNA (<8 log_{10} IU/ml), HBsAg (<4 log_{10} IU/ml), HBcrAg (<6 log_{10} U/ml) and pgRNA (<7 log_{10} U/ml) were more likely to seroconvert to anti-HBe in adolescence (Table 13).

At the follow up appointment two patients had undetectable pgRNA, both patients were in cohort 2 (Table 7 and 8). At baseline, both patients had very high pgRNA (6.74 and 7.44 log_{10} U/mL), very high HBV DNA (8.83 and 8.68 log10 IU/mL) and high HBsAg (5.30 and 5.35 log_{10} IU/mL). HBcrAg was also elevated (7.00 and 6.34 log_{10} U/mL). At follow up both patients showed a reduction in HBV DNA concentration (1.60 and 1.30 log_{10} IU/mL), HBsAg levels (3.62 and 3.22 log_{10} IU/mL) and HBcrAg (3.50 and 3.30 log_{10} U/mL). Both patients were HBeAg positive and anti-HBe negative at baseline, however at the follow up both patients achieved HBeAg loss and anti-HBe antibodies. Additionally, all liver function markers including ALT were in normal range at the follow-up appointment. These results suggest that early intervention could lead to pgRNA loss which indicates cccDNA depletion and reduces the likelihood of liver damage.

To find out the sensitivity of the HBcrAg assay for paediatric samples a serial dilution was done on several HBcrAg positive samples ranging from undiluted to a 1 in 1000 dilution. The limit of detection (LOD) was found to be 2 log_{10} U/mL. To find out the specificity of the assay for paediatric patients, known Hepatitis C virus (HCV) positive samples along with known positive HBcrAg serum sample were tested using the assay. The area under the receiver operating characteristic curve (AUROC), sensitivity and sensitivity of serum HBcrAg was 0.85 (95% CI: 0.81-0.91) (Figure 7). This indicates that the assay is very good with the potential of being used in HBV management.

Discussion

The biomarkers pgRNA and HBcrAg are important markers reflecting cccDNA transcriptional activity; they may be helpful biomarkers for assessing disease progression in paediatric patients. Measuring pgRNA and HBcrAg in serum could provide a reference for clinical monitoring of cccDNA transcriptional activity and the selection of appropriate timing for initiating antiviral treatment. Liver biopsies are important in the detection of cccDNA but they cannot be routinely done due to their invasive nature and harm they may cause the patient. Particularly when investigating CHB in paediatric patients, liver biopsies are impractical. Therefore, the standardisation of non-invasive serological and molecular tests that reflect cccDNA transcription are urgently needed.

In this study HBV pgRNA could be detected in all patients at baseline and in most patients (94% n-27) serum at the follow up appointment. Interestingly pgRNA was still present in the sample with undetectable HBV DNA. This is consistent with previous research that indicates that pgRNA synthesis continues even when DNA synthesis is compromised. Compared with HBV DNA, serum pgRNA has an advantage in monitoring changes in sustained viral response and cccDNA levels. The detection of pgRNA in serum of paediatric patients at different stages of infection could help determine which patients are most at risk of liver damage and could help determine the appropriate time to intervene with antiviral therapy. High concentrations of pgRNA in serum is associated with high cccDNA activity and therefore high risk of liver disease progression.

Several studies have shown a positive correlation between markers of cccDNA with HBV DNA and HBsAg concentrations in the serum of CHB patients(2–4). Our results also confirmed that pgRNA, and HBcrAg correlated positively with HBV DNA and HBsAg in CHB paediatric patients. These results suggest that the detection and quantification of pgRNA and HBcrAg in CHB patient serum is comparable with the other traditional diagnostic markers. The strongest correlation was found between pgRNA and HBcrAg. These two markers directly link to cccDNA activity so similar trends in concentrations are expected.

At the end of the study, two patients had undetectable pgRNA at follow-up, both patients were on antiviral therapy. Long term antiviral treatment with NAs may lead to the depletion of cccDNA in some patients and this could explain why patients on antiviral therapy have low concentrations of HBV DNA as well as pgRNA and HBcrAg. However, HBsAg remains detectable though reduced in a minority of cases. Also transitioning to HBeAg negative CHB has been associated with reduced cccDNA transcriptional activity(5,6). Suslov et al reported that the silencing of cccDNA is particularly pronounced in HBeAg negative chronic infection (ENCI) where the concentration of pgRNA could be up to 46 times less per cccDNA compared to HBeAg negative CHB patients(6). Furthermore, some patients with HBeAg negative CHB can be characterised by reduced replication efficiency downstream of pgRNA. In my study patients who had achieved HBeAg loss and anti-HBe antibodies demonstrated lower concentrations of pgRNA and HBcrAg. The reasons for this also require further investigation in a larger cohort.

Complete elimination of HBV is impossible due to the persistence of cccDNA which acts as a viral reservoir. Paediatric patients who do not clear the disease or achieve HBsAg seroclearance (spontaneously or through therapy) are at risk of liver damage and long-term health implications. In this study none of the patients achieved HBsAg seroclearance. HBsAg can be produced through the cccDNA pathway or through the integration of HBV DNA into cellular DNA. These two pathways are the key obstacles in achieving HBsAg seroclearance. Currently there are no markers available for the quantification of HBV DNA integration, but pgRNA and HBcrAg are markers of cccDNA and could provide a breakthrough into curing CHB. Furthermore, from a virological perspective, achieving functional cure does not mean that HBV has been eradicated and therefore it is necessary to evaluate the durability of HBsAg seroclearance and if it can be associated with favourable clinical outcomes. Eliminating the source of HBsAg would be monumental to HBV management and the search for a cure.

Detectable HBV DNA, pgRNA and HBcrAg are associated with the development of cirrhosis and HCC. The development of antivirals that targeted HBV DNA was a vital step in improving CHB patient lives, however complete viral suppression is required to significantly reduce the risk of cirrhosis and HCC which can still occur in patients on long-term therapy. This means that all markers associated with cccDNA transcriptional activity (including HBcrAg and pgRNA) must be monitored in-order to prevent liver damage and HCC. Clearance of cccDNA has been defined as a “cure” for chronic hepatitis B, thus if markers of cccDNA are monitored

from a young age, the patients who need therapy can be identified early and the correct measures can be taken to prevent liver damage. In this study disease progression in paediatric patients was associated with HBV pgRNA levels ≥5 log_{10} U/mL, and HBcrAg levels ≥4 log_{10} U/mL in addition to HBsAg levels ≥4 log_{10} IU/mL, HBV DNA levels ≥6 log_{10} IU/mL, and ALT >40 U/L. Early intervention with antiviral therapy was associated with low/no liver damage, low concentrations of liver markers and virological markers after 6 years (median years). The reduction of these markers was indicative of reduced cccDNA transcriptional activity and will likely reduce the risk of developing cirrhosis and HCC.

Timely intervention with antiviral therapy for HBV can benefit paediatric patients by stopping and disrupting the premalignant tendency. Therefore, if paediatric patients have comparable response to treatment as adults, it may be logical and cost effective to treat HBV infection as early as possible, especially because complications can occur at any point in childhood or in adulthood. Markers of cccDNA (pgRNA and HBcrAg) can play a part in predicting which patients may go on the develop liver damage and therefore need antiviral therapy. My data suggests that paediatric patients treated with NAs and peg-IFNα have more favourable clinical outcomes than untreated patients. Since there is a lack of therapeutic experience for CHB paediatric patients, this study may provide vital reference information. Studies of large cohorts with long term follow-up in adult groups have demonstrated a clear association between high concentrations of HBV DNA, HBcrAg and pgRNA and subsequent progression to liver cirrhosis, and HCC(7–10). Therefore, successful suppression of HBV DNA under treatment with NAs and peg-IFNα while also monitoring HBcrAg and pgRNA concentrations in paediatric patients may hopefully reduce the risk of disease complications and improve quality of life. Sustained HBV virological suppression is an extremely important goal of CHB treatment. Further long-term follow-up in children and adolescents is required to fully evaluate its effect on this goal.

Although my study identifies that the new markers could be clinically useful, the cohort size is relatively small. A larger sample size could provide more data on patients on treatment and more biopsy results, which will allow a more in-depth comparison between the diagnostic techniques. Additionally, comparison of pgRNA and HBcrAg at different treatment time points would give more of an indication of when antiviral treatment can be stopped.

Also, my study was limited in its retrospective nature. Ongoing clinical trials with different antivirals in paediatric patients are needed to fully understand the role of HBcrAg and pgRNA in patient selection for therapy, the response to treatment and possibly reactivation of HBV after discontinuing antiviral therapy.

Chapter 7

Is the performance of ultra-sensitive HBsAg Fujirebio assay consistent across HBV genotypes (comparison between CLEIA HBsAg HQ Fujirebio and Abbott Architect assays)

Abstract

HBsAg quantification plays an important role in the management of Chronic Hepatitis B infection (CHB). Several testing platforms are available, but there is limited data about the assay's performance across HBV genotypes in patients with low HBsAg levels. The aim of this pilot study is to compare HBsAg levels tested on using the Abbott Architect^{TM} assay (standard of care) vs an ultra-sensitive chemiluminescent enzyme immunoassay (CLEIA), HBsAg-HQ on the Fujirebio Lumipulse G600II platform. Samples from CHB patients with a diverse range of HBV genotypes were tested on (genotypes A-E).

197 HBeAg negative patient samples (57% males, median age 49 years) were tested on the Abbott Architect using the HBsAg-QT CMIA assay and then tested on the Fujirebio Lumipulse using the CLEIA HBsAg-HQ assay (Fujirebio, Europe, Ghent, Belgium).

The Lumipulse HBsAg-HQ assay consistently and accurately quantified ultra-low levels of HBsAg (as low as 0.002 IU/mL) across the HBV genotypes tested (genotypes A-E). The two assays displayed excellent linear correlation irrespective of HBV genotypes ($r=0.977$, $p<0.001$), with minor quantification biases observed.

Conclusions: Compared with the Abbott assay, the Lumipulse assay is sensitive and specific for HBsAg quantification. However, the interpretation of the extremely low-level results can be challenging. To eliminate bias the results should be interpreted interchangeably only after conversion to the same units of quantification.

Materials and Methods

Samples: Frozen serum samples were collected between 2017-2018 from 203 Chronic Hepatitis B (CHB) patients (116 males and 87 females) who had attended the Hepatitis outpatient clinic at Kings College Hospital (KCH). All patients provided informed consent for Kings College biobank and all principles of the Declaration of Helsinki were adhered to. All patients were known to be HBeAg negative and were no longer on antiviral treatment.

Methods

(i) Measurement of HBsAg: HBsAg levels in all serum samples were determined by the Abbott Architect HBsAg-QT assay (a two-step sandwich immunoassay) (Abbott Diagnostics USA). The lower detection limit was 0.05IU/ML. This platform uses a chemiluminescent microparticle immunoassay (CMIA) system and calculates HBsAg concentration using a standard curve.

(ii) Measurement of HBsAg by Lumipulse HBsAg-HQ assay: HBsAg quantification was determined using the Lumipulse G600II HBsAg-HQ assay ((Fujirebio Europe, Ghent, Belgium). Serum samples were placed on the fully automated chemiluminescent enzyme immunoassay (CLEIA) system, the Lumipulse G600II (Fujirebio, Tokyo, Japan) which utilises a two-step sandwich method. This platform has a diagnostic sensitivity of 99.8 and specificity of 99.7 according to the manufacturers guide.

(iii) Quantification of HBV DNA: Serum HBV DNA levels were measured by Ampliprep TaqMan (Roche Diagnostic, Burgess Hill, United Kingdom), with a lower detection limit of 20IU/ML.

(iv)HBV genotype: HBV genotypes were determined by in-house direct sequencing. This method detects all HBV genotypes from A – G.

(v) Statistical Analysis: Statistical analysis was done using SPSS and the results illustrated using GraphPad Prism. To compare the two assays, Bi-variate non-parametric correlations and rank tests were done. Statistical significance was defined as $P < 0.05$.

CHARACTERISTICS OF THE TWO HBSAG QUANTITATIVE EASSAYS.

ASSAY	**Analyser**	**Principle**	**Technology (tracer)**	**Pre-treatment**	**Reaction sample volume**	**Assay duration**	**Linear range (analytical sensitivity)**	**On-board dilution**
ABBOTT ARCHITECT HBSAG	Architect c8000	Sandwich principle, capture mAbs and polyclonal detection antibodies	CMIA (acridinium)	**none**	50 µL	29 min	0.05–25000 IU/mL (0.05 IU/mL)	1:500 with recalcified negative human plasma
FUJIREBIO LUMIPULSE G HBSAG-QUANT	Lumipulse G600II	Sandwich principle, two capture mAbs and two detection mAbs	CLEIA (AMPPD)	Yes, to disrupt viral particles and dissociate HBsAg from HBsAg-anti-HBs complexes	100 µL	30 min	0.005–150 IU/mL (0.005 IU/mL)	1:100, 1:200 or 1:1000 with NaCl and Tris buffer

Table 1: Characteristics of the Abbott Architect HBsAg assay and the fujirebio lumipulse HBsAg assay. CMIA, chemiluminescent microparticle immunoassay; ECLIA, electrochemiluminescence immunoassay; CLEIA, chemiluminescent enzyme immunoassay; mAb, monoclonal antibody; AMPPD, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt

Results

Out of 203 serum samples quantified by the Abbott Architect 197 patient samples (57% males, median age: 49 years) had HBsAg levels within the detection limit of the Fujirebio assay (0.005 - 150 IU/mL). The samples where HBsAg was more than 150 IU/mL were diluted with FBS to enable detection by the Lumipulse assay. The results of the 197 samples are analysed below. The patient demographics are shown in Table 2. The genotypic distribution of the patients are shown in Table 3. The samples were stratified into 3 groups based on the HBsAg levels deduced by the Abbott Architect HBsAg-QT assay: HBsAg 0.1-10 IU/mL (n=72), HBsAg 10-100 IU/mL (n=98) and HBsAg 100-1000 IU/mL (n=27) (Table 4).

N=197	
Male (%)	57
Median Age (years)	49 (range 21-68)
White/ Caucasian/ other White	29
Black African/ Caribbean/ other black	55
Asian	70
South Asian	43
HBV DNA (IU/mL)	130 (range 0 - 42700)
HBsAg – Abbott (IU/mL)	18.6 (range: 0 - 337.40 IU/mL)
HBsAg- Lumipulse (IU/mL)	17.7 (range: 0.0001-310.22 IU/mL)
HBeAg Negative (%)	100

Table 2: Patient demographics

Genotype	N	Abbott: Median HBsAg [IU/ml]	Fujirebio: Median HBsAg [IU/ml]	Pearson's Correlation (r)	P value	Median HBV DNA [IU/ml]
A	32	18.19 (range: 0-337.4)	14.39 (range: 0.0002-310.22)	0.974	0.001	25.01 (range: 0-58.45)
B	45	16.2 (range: 0-125.08)	13.2 (range:0.0005-120.98)	0.980	0.001	134.00 (range: 0-260)
C	27	50.55 (range:0-152.26)	68.41 (range:0.0004-141.78)	0.952	0.01	101.00 (range: 0-250.77)
D	41	17.35 (range: 0-164.14)	12.56 (range:0.0003-158.5)	0.969	0.001	36.15 (range: 0-76.11)
E	52	17.23 (range:0-153.39)	26.57 (range:0.0001-161.01)	0.985	0.01	108 (range: 0- 185)

Table 3: A Comparison of HBsAg results from the Abbott Architect HBsAg-QT assay with the Fujirebio HBsAg-HQ assay across the genotypes, including HBV DNA results.

	HBsAg groups		
	HBsAg 0.1-10 IU/mL	HBsAg 10-100 IU/mL	HBsAg 100-1000 IU/mL
N	72	98	27
Median HBsAg detected on the Abbott Architect (IU/mL)	0.400 (range 0-9.800)	24.820 (range: 10.95-95.750)	125.89 (range: 103.27- 337.40)
Median HBsAg detected on the Fujirebio Lumipulse (IU/mL)	0.316 (range 0.0001-9.445)	26.510 (range:11.08-90.883)	130.23 (range:100.565-310.220)

Table 4: Results stratified into HBsAg groups based on results recorded from Abbott HBsAg QT assay.

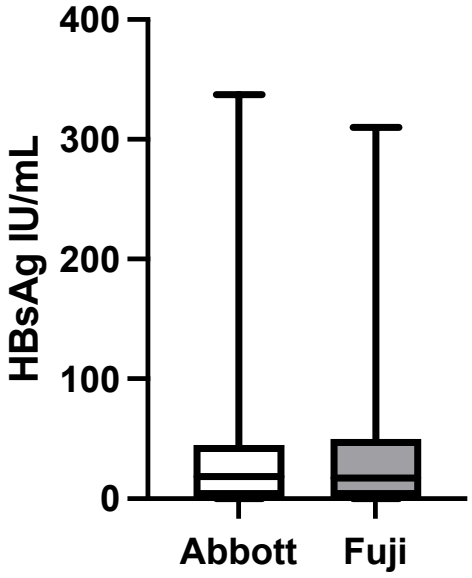


Figure 1: A box plot representing the median HBsAg concentration on the Abbott Architect, 18.6 (range: 0.1-164) vs the Fujirebio Lumipulse, 17.7 (range: 0.0001-839.10 IU/mL).

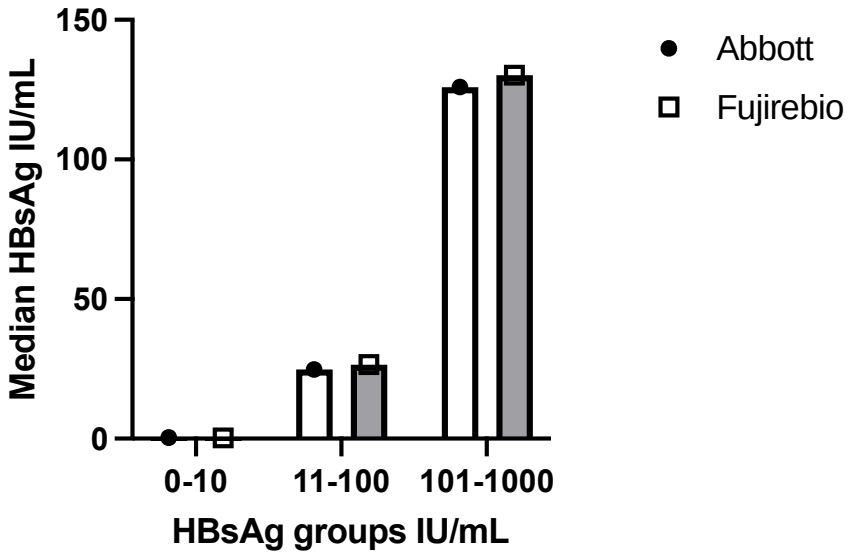


Figure 2: A graph representing the median HBsAg concentration on the Abbott Architect vs the Fujirebio Lumipulse within the 3 groups.

N=20					
Patient	HBsAg result on Abbott (IU/mL)	HBsAg result on Lumipulse (IU/mL)	DNA results (IU/mL)	Genotype	Occult HBV diagnosis
1	0	0.0114	Not Detected	A	
2	0	0.007	4.00E1	A	
3	0	0.0002	Not Detected	A	
4	0	0.0036	Not Detected	A	Yes
5	0	0.03	Not Detected	A	
6	0	0.0005	Not Detected	A	
7	0	0.0005	Not Detected	B	
8	0	0.04	Not Detected	C	Yes
9	0	0.0013	Not Detected	C	Yes
10	0	0.042	2.97E1	C	
11	0	0.009	Not Detected	D	
12	0	0.008	Not Detected	D	
13	0	0.0006	Not Detected	D	
14	0	0.0003	2.46E1	D	

15	0	0.022	Not Detected	D	
16	0	0.0003	Not Detected	E	
17	0	0.047	Not Detected	E	Yes
18	0	0.01	Not Detected	E	
19	0	0.0008	Not Detected	E	
20	0	0.0008	Not Detected	E	

Table 5: Patients who had undetectable HBsAg results on the Abbott Architect but had quantifiable HBsAg on the Lumipulse.

Patients diagnosed with Occult HBV infection N=4	
Median HBsAg on Abbott (IU/mL)	0
Median HBsAg on Lumipulse (IU/mL)	0.0218 (range: 0.0013-0.047)
DNA results (IU/mL)	Not Detected for all 4 patients
Median ALT (U/L)	34 U/L (range 27-38 U/L)
Anti-HBc	Positive for all 4 patients
Anti-HBs	Not present in all 4 patients

Table 6: Biomarker results of patients who were diagnosed with Occult HBV infection.

Sample 1 (Genotype A)	HBsAg level (IU/mL) by Fujirebio	Sample 2 (Genotype D)	HBsAg level (IU/mL) Fujirebio
Original	>150	Original	150
1:10 dilution	>150	1:10 dilution	68.11
1:100 dilution	142.06	1:100 dilution	9.57
1:1000 dilution	61.33	1:1000 dilution	0.03
1:10,000 dilution	15.88	1:10,000 dilution	0
1:100,000 dilution	0.002		
1:100,0000	0		

Table 7: Serial dilution of two serum samples. Samples were diluted in FBS. In sample 1 the Fujirebio assay could identify HBsAg levels as low as 0.002 (IU/mL) and in sample 2 as low as 0.03.

The median HBsAg levels were similar between Abbott (18.6, range 0 - 337.40 IU/mL) and Fujirebio (17.7, 0.0001-310.22 U/mL) (Table 2 and Figure 1) and there was a strong bi-variate correlation observed ($r=0.977$, $p<0.001$). HBV DNA was undetectable in 112 patient serum at the time of this study. In the patients where HBV DNA was present the median concentration was 130 IU/mL (range 20-260 IU/mL). Both assays correlated positively with HBV DNA, however the correlation between the Abbott assay and HBV DNA results had a much stronger correlation compared to the fujirebio ($r=0.95$ $p<0.01$ vs $r=0.20$ $p<0.05$). When the comparison was made between the individual genotypes there was a strong correlation observed in all the genotypes tested (A-E) using the fujirebio assay (Table 3). The Lumipulse assay generally reported lower HBsAg levels for genotypes A, B, and C than the Abbott assay (Table 2). A big difference in median levels was observed between the Abbott assay and Fujirebio assay in genotype C (50.5 vs 68.4 IU/mL respectively, $p<0.01$) and E (17.23 vs. 26.57 IU/mL respectively, $p<0.01$).

The assessment according to HBsAg levels revealed strong correlation between tests at levels 0.1-10 IU/mL ($r=0.967$ $p<0.001$) with gradual decline with increasing HBsAg levels (10-100 IU/mL, $r=0.86$ and for 100-1000 IU/mL, $r=0.545$). Both assays reported very similar HBsAg concentrations with in the three groups (Table 4 and Figure 2).

The Fujirebio HBsAg-HQ assay may be more sensitive towards genotypes C and E as it consistently reported higher HBsAg levels for these genotypes compared to the Abbott HBsAg assay, with overall median levels of HBsAg in genotype C and E being higher when tested on the Fujirebio (Table 2).

It was found that the Fujirebio Lumipulse HBsAg-HQ assay could detect HBsAg in 197 sample and could quantify HBsAg in serum even when the Abbott assay had reported it as 0. There were 20 (10%) samples with results of 0 from the Architect but with positive / quantifiable Lumipulse results (Table 5). The HBsAg levels of these samples were all very low, ranging from 0.0001-0.047 $\log_{10}$ IU/mL measured by the Lumipulse assay. Out of the 20 samples 15% (n=3) also yielded positive HBV DNA results, though at low concentrations (range between 2.00E1 – 4.26E1 IU/mL) (Table 5). According to my findings, 4 out of 197 patients (2%) with occult HBV had low-level serum HBsAg detected by the ultra-sensitive assay (Table 5 and 6).

A serial dilution was done for some of the serum samples using Fetal Bovine Serum (FBS) as a diluent. This was done to find out if the Fujirebio assay could quantify HBsAg at extremely diluted concentrations (Table 7). It was found that the Fujirebio could detect HBsAg as low as 0.002 IU/mL at a 1:100,000 dilution.

Discussion

Even though HBsAg qualitative assays are a good standard in HBV diagnosis, quantitative methods are becoming more popular, and they are proving to be reliable methods in diagnosis and predicting viral efficacy of HBV therapy(1–3). It is necessary to evaluate the HBsAg levels obtained by different immunoassays and the relationships between HBsAg concentration and HBV DNA alongside HBsAg and genotype. In this study a comparison is made between two quantitative assays: the Abbott Architect HBsAg-QT and the new Fujirebio Lumipulse HBsAg-HQ assay. Serum samples tested were from a cohort of HBeAg negative CHB patients who were no longer on antiviral treatment with a diverse genotypic range. The Fujirebio assay could accurately quantify HBsAg levels in serum samples across all the genotypes tested and was consistent with the results reported by the Abbott assay. Additionally, a strong correlation was observed between the two assays. All this indicates that the Fujirebio HBsAg-HQ assay could be clinically useful in HBV diagnosis and management and could be used routinely.

This study has shown that the Fujirebio Lumipulse HBsAg-HQ assay is highly sensitive, and showed a sensitivity of 0.005 IU/mL, which is 10 times more sensitive than the Abbott Architect assay.

When the precision and accuracy was evaluated at the limit of detection of HBsAg the Lumipulse HBsAg-HQ assay showed comparable within-run and total precisions at the level of 0.01 IU/mL and the Architect was at the level of 0.10 IU/mL. Furthermore, by utilising the serially diluted HBsAg reference materials, the Lumipulse assay was able to quantify extremely low levels of HBsAg, as low as 0.002 IU/mL (Table 7). Therefore, this assay is capable of detecting trace amounts of HBsAg in serum and plasma. However, because the Lumipulse HBsAg-HQ assay is 10 times more sensitive than the Abbott HBsAg-QT assay an accurate comparison of both assays is difficult. For a more accurate comparison the Lumipulse assay could be tested alongside another assay that is equally as sensitive.

In clinical settings the association between extremely low concentration of HBsAg and the clinical outcome is largely unknown. It is possible for some CHB patients to achieve HBsAg seroclearance with detectable serum HBsAg, especially those with negative anti-HBs(4,5). Togashi et al reported that extremely low concentrations of HBsAg were observed more frequently in individuals with liver disease compared to the general population(6). My findings suggest that approximately 10% (n=20) more samples will be reported as positive with the

Lumipulse G600II HBsAg-HQ assay (Table5). All the samples that the Abbott assay had reported as negative for HBsAg were quantifiable by the Lumipulse assay (n=20) (Table 5). For these cases, the outcome should be followed up closely, also the potential risk of cirrhosis and HCC should be monitored. Subsequently, serological and nucleic acid testing (NAT) including anti-HBc, anti-HBc IgM, anti-HBs and HBV DNA should be done as well as alanine transaminase (ALT), alpha fetoprotein (AFP) and liver histology. Very low levels of HBsAg could still lead to liver damage as it demonstrates viral activity. HBsAg could be established from the cccDNA pool or from HBV integration. Unfortunately, both assays cannot distinguish between HBsAg derived from cccDNA or HBV integration.

The implementation of the ultra-sensitive HBsAg assay may assist in the strengthening of blood donation security, and the formation of the occult HBV infection (OBI)(7). According to my findings, 4 out of 197 patients (2%) with occult HBV had low-level serum HBsAg detected by the ultra-sensitive assay (Table 5 and 6). As a result, they should not have been diagnosed as OBI any longer.

Due to the similarities in the HBsAg results between Abbott and Fujirebio (median of 18.6 and 17.7 IU/ml respectively), the Fujirebio assay would be a suitable quantitative assay to determine HBsAg levels in serum samples and an alternative assay to Abbott to determine low levels of HBsAg (0-150 IU/ml). The strong bi-variate correlation between the two assays ($r=0.977$, $p<0.001$) also supports this. Other studies have also shown the Lumipulse HBsAg-HQ assay as a consistent and reliable tool in determining HBsAg levels in CHB patients and could be vital in HBV diagnosis (2,3,8).

The Lumipulse is an ultra-sensitive assay and so it is expected to have a higher level of accuracy in detecting samples with very low HBsAg levels. This is demonstrated in this study as the strongest correlation between the Lumipulse and the result groups was found in the 0-10 IU/ml group with gradual decline as HBsAg increased. The Lumipulse assay could be used on samples with very low HBsAg levels and the Abbott Architect for samples with higher HBsAg levels (>150 IU/ml)(3,8).

The Fujirebio Lumipulse could accurately determine HBsAg levels across the HBV genotypes and was as consistent as the Abbott assay. Very strong correlations were found between the two assays across all the genotypes tested (Table 2). Higher HBsAg sensitivity favoured

genotype C and E when tested on the Lumipulse than that of the Abbott assay and the result group 0-10 IU/ml. It can be noted that when investigating patients with very low HBsAg levels especially between 0-10 IU/ml with HBV genotypes C and E it may be advantageous to use the Lumipulse assay.

The protocol for Lumipulse assay includes a pre-treatment process, to eliminate epitopes which may be present in the serum. These epitopes may hide in the antibody-antigen complex or in other particles e.g., Dane particles, and may be exposed to detection, therefore interfering with results(4,9). A study by Zhang et al. reported that co-existing anti-HBs antibodies were reactive to HBsAg but had a lower specific activity than those from an immunized population, suggesting that they might not compete with the capture or detection antibodies used in the HBsAg assays to bind the epitopes. Therefore, the presence of anti-HBs antibodies in the serum may not interfere with the detection of HBsAg using various assays. Moreover, the pre-treatment process included in the Lumipulse assay may not result in a higher HBsAg result.
The measurement of HBsAg concentrations using the ultra-sensitive assay would be particularly well adapted to the treatment of HBeAg negative cases. HBeAg negative hepatitis variants with mutations in the precore or basic promoter regions are common among CHB carriers and HBsAg levels are generally lower in HBeAg negative patients than in HBeAg positive patients. Previous research has demonstrated that the pre-S deletion associated with HBeAg negative HBV infection reduces the synthesis of small surface antigens(10).

In conclusion, as an ultra-sensitive assay, the Lumipulse G600II HBsAg-HQ assay is suitable for the detection of HBsAg across the different HBV genotypes in clinical settings. This study has demonstrated that the Lumipulse HBsAg-HQ assay is consistent across all genotypes, but particularly sensitivity towards genotypes C and E and HBsAg levels within 0.1-10 IU/mL.
This study demonstrated that the Fujirebio Lumipulse HBsAg-HQ assay will detect HBsAg in serum samples even when the Abbott Architect reports it as 0 IU/ml. The Lumipulse HBsAg-HQ assay can detect HBsAg levels as low as 0.002 IU/ml. This very low limit can be advantageous when investigating occult HBV infection(8). It can facilitate the identification of HBV carriers with trace levels of HBsAg. Clinical application of this new assay will have advantages and disadvantages. It provides a tool that could be useful in understanding the significance of extremely low amounts of circulating HBsAg, on the other hand the interpretation of results should be done with caution to prevent unnecessary harm to patients.

As a quantitative assay, the Lumipulse assay strongly correlates with other commonly used assays, therefore making it suitable in monitoring HBsAg kinetics during the natural history of infection and during antiviral therapy. However, minor biases exist in quantifying results among both assays, thus, interpreting results should be done with care, especially in cases where HBsAg concentration is near the cut-off levels predicting the prognosis, or where changes in the HBsAg level is marginal in patients receiving NA therapy or those during the natural history of infection.

Chapter 8: Concluding Remarks and Future Research

8.1 Concluding Remarks

The role of new biomarkers of Chronic Hepatitis B particularly HBcrAg and pgRNA has gained increasing interest over the past few years. However there have remained a lack of in-depth, long-term studies investigating their usefulness in clinical settings and their predictive abilities. The aim of the work presented in this thesis has been to demonstrate that HBcrAg, pgRNA, CXCL10 and soluble PD-1 are clinically relevant biomarkers that can be utilised in the long-term care of CHB patients. Additionally, the predictive value of these novel markers in CHB paediatric patients have been overlooked, hence my research also focuses on CHB patients who acquired HBV perinatally. Even though, the results of each study have been extensively discussed within their relevant chapters, this concluding segment will briefly highlight the main findings of each study and discuss potential future perspectives.

First study: 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.

This observational study was done to assess the clinical relevance of pgRNA, HBcrAg, qualitative and quantitative HBsAg and ultrasensitive HBsAg levels in CHB patient serum and whether they can predict clinical outcomes in three different cohorts. HBV DNA and HBsAg are the main biomarkers used clinically for diagnosis and to assess patient progress throughout CHB infection. However, these markers lack the ability to sufficiently inform residual HBV replication in patients, their risk of reactivation or severe exacerbations of Hepatitis after the discontinuation of NA therapy. In this study it was clear that pgRNA and HBcrAg remain detectable for years after HBV DNA suppression by NA treatment in HBeAg negative individuals. These patients presented with minimal decline in HBsAg levels.

Ultrasensitive HBsAg is also proving to be clinically useful in CHB management. In this study HBsAg was undetectable in all patients by the qualitative method after NA discontinuation. However, HBsAg was detected in all patients after NA withdrawal when using the ultra-sensitive HBsAg assay. So, if the ultrasensitive HBsAg assay is used to monitor patient serum after discontinuing NA therapy it could differentiate between patients who may still have

traceable amounts of HBsAg and are at risk of reactivation and liver damage and patients who are truly HBsAg negative.

In this study two patients experienced HBV DNA reactivation after stopping NA treatment. Interestingly, pgRNA could be detected in the serum of these two patients at the point of NA withdrawal and their pgRNA levels increased after discontinuing NA therapy. Thus, detectable pgRNA in patient serum at the time of NA discontinuation is predictive of HBV DNA reactivation following NA withdrawal.

Furthermore, the quantification of pgRNA and HBcrAg can be predictive of severe ALT flares after NA discontinuation. Although commonly used, HBV DNA suppression and HBsAg levels are not reliable or consistent predictors of ALT flares before antiviral withdrawal. The results of this study strongly indicate that detectable pgRNA and HBcrAg may together reflect residual cccDNA transcription and could identify patients not suitable for NA withdrawal due to higher risk of post therapy cessation flares.

<u>Second study:</u> Investigating the role of serum HBcrAg and HBV pgRNA in predicting clinical outcomes in CHB patients on antiviral therapy.

Active HBV replication is the main driver of liver necroinflammation and disease progression; therefore, the treatment goal of CHB is to suppress HBV replication permanently to stop hepatic decompensation, liver cirrhosis and/or hepatocellular carcinoma and increase survival rates. Long-term therapy is required to maintain HBV suppression but the ultimate goal of HBsAg loss is rarely achieved. HBsAg is generated by the cccDNA pool and by HBV integration. So, while long-term antiviral therapy may affect cccDNA transcription there is no effect on HBV integration, therefore HBsAg persists, and loss is quite rare. Additionally, relapse after discontinuing NUC therapy is common with Hepatitis B flares and an increased risk of decompensation. Therefore, proper monitoring, assessment and retreatment decisions are important to ensure the safety of CHB patients.

The aim of my second study was to determine whether markers of cccDNA transcriptional activity: HBcrAg and pgRNA can be used to predict clinical outcomes in CHB patients on antiviral therapy with NUC and to compare viral activity in patients who seroconvert to anti-

HBe to patients who did not seroconvert to anti-HBe. Quantifying pgRNA levels in CHB patients demonstrated that even when patients are on treatment, have low to undetectable HBV DNA levels, and have anti-HBe antibodies, pgRNA is still present in their serum. The presence of HBV RNA detected at any level in serum indicated that cccDNA was still present and transcriptionally active.

My findings indicate that serum HBV RNA HBcrAg and HBsAg concentrations were generally higher in HBeAg positive patients than in HBeAg negative patients, confirming that these three biomarkers are positively associated with HBeAg status in patients on long-term NA therapy. Factors associated with HBeAg loss included age, HBsAg level at diagnosis and the decline size of HBV RNA and HBcrAg. Older patients with lower HBsAg levels at diagnosis and a greater decline of HBV RNA and HBcrAg were at a significantly greater advantage of achieving HBeAg loss. As biomarkers, HBV RNA and HBcrAg therefore represent an important clinical and virological landmark during CHB infection, resulting in partial immunological control of HBV activity. The findings in this study suggest that HBV RNA and HBcrAg monitoring can be useful in reflecting the attainment of stable virological response in HBeAg negative patients with low/ undetectable DNA concentrations during NA treatment.
My results confirm that although successfully treated patients with NUCs experienced a rapid decline of serum HBV DNA to undetectable concentrations, they manifested a relatively slow decline of HBV RNA and HBcrAg levels and a slow reduction of HBsAg. In patients who became HBeAg negative but did not experience HBeAg seroconversion, the levels of the three serum markers declined slowly and remained at a relatively higher level. In comparison to HBeAg negative/ anti-HBe positive patients the concentration of these three markers also declined but at a relatively faster rate. These findings reinforce the aim of my research that serum HBV RNA and/or HBcrAg levels are useful for monitoring antiviral effects and predicting clinical outcomes in CHB patients.

On the basis of my results combined with data from other publications, I have proposed a schematic diagram detailing the clinical relevance of serum HBV DNA, HBV RNA, HBcrAg and HBsAg (Figure 1). An individual strand of pgRNA is transcribed from cccDNA and packaged into the core during viral nucleocapsid assembly. The RNA is then reverse transcribed to form the first negative strand of HBV DNA. During NA therapy HBV polymerase is inhibited, therefore making HBV DNA the earliest biomarker to become undetectable in patients successfully treated with NA. HBV RNA undetectability is usually an

earlier event as compared to HBcrAg undetectability. So, amongst patients treated with NA, the decline to undetectability of the four serum markers generally proceeds in the order HBV DNA, HBV RNA, HBcrAg and HBsAg. Accordingly, achieving undetectability of each marker reflects approaching a functional cure.

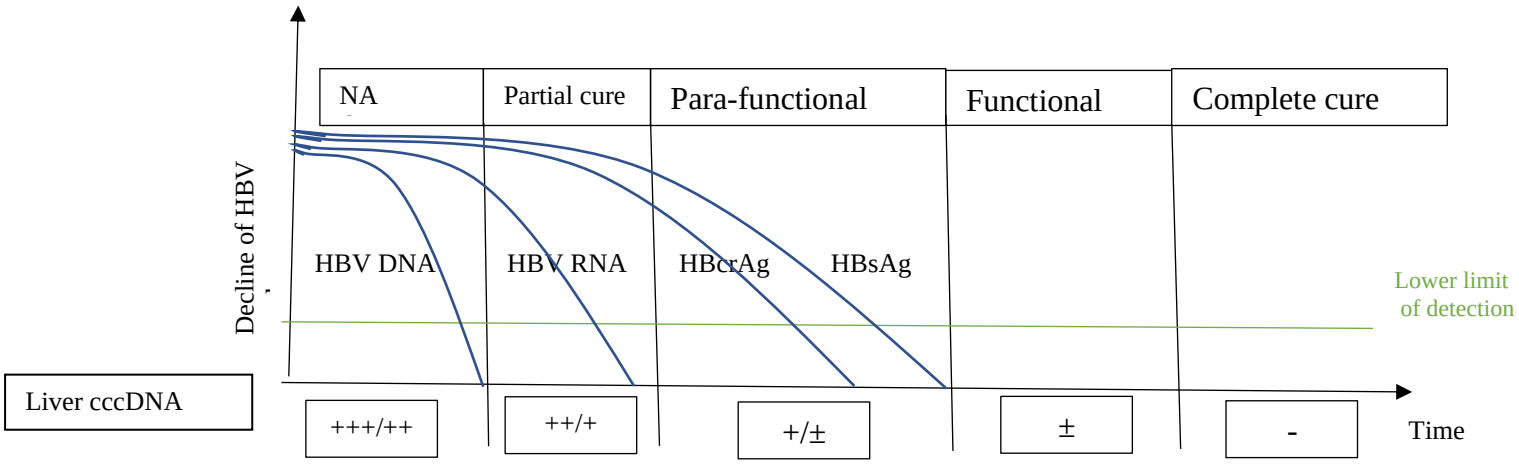


Figure 1: schematic diagram detailing the clinical relevance of serum HBV DNA, HBV RNA, HBcrAg and HBsAg

Another aim of my second project was to assess whether immune markers CXCL10 and sPD-1 can be utilised to predict clinical outcomes in CHB patients. CXCL10 and sPD-1 positively correlated with markers of cccDNA activity – HBcrAg and pgRNA. Additionally, the reduction of these immune markers in patient serum during antiviral therapy was associated with better patient outcomes including less liver fibrosis and less hepatic flares. Therefore, the combination of CXCL10, sPD-1, HBcrAg and pgRNA could be useful in CHB maintenance and possibly in assessing response to treatment. Additionally, these markers could be monitored after discontinuing antiviral therapy to assess the likelihood of reactivation.

<u>Third study:</u> Serum markers of cccDNA transcriptional activity may have a role in predicting long-term outcomes in children with perinatally acquired HBV.

It is difficult to determine when to administer antiviral treatment to paediatric patients who acquire HBV perinatally. The risk of this cohort developing liver disease during adulthood is also difficult to predict. The aim of this tudy was to determine whether markers of cccDNA transcriptional activity in serum: HBcrAg and pgRNA can be utilised to predict future disease progression in patients with perinatally acquired Chronic Hepatitis B infection. It was clear that HBcrAg and pgRNA are markers that are present in paediatric patient serum from an early age. Furthermore, both HBcrAg and pgRNA were consistently positively associated with liver disease markers (ALT and FIB-4) in both HBeAg negative and HBeAg positive patients. At the time that patients in this study were placed on antiviral therapy (cohort 2) HBcrAg and pgRNA were very high in their serum particularly pgRNA which was 3x higher than in the untreated group. Interestingly, a large reduction in HBcrAg and pgRNA was observed during antiviral therapy and 2 patients even suppressed pgRNA.

Measuring these novel markers through the natural course of HBV infection could help accurately assess which paediatric CHB individuals are at risk of liver damage during childhood and in adulthood. Appropriate measures can then be taken including early administration of antiviral therapy. It would therefore be of interest to investigate whether there are links between the markers of cccDNA transcriptional activity and immunological biomarkers of disease progression, such as proinflammatory cytokines, in patients with and without elevated ALT levels.

A limitation of HBcrAg and pgRNA assays is their limited sensitivity: 1.65 $\log_{10}$ U/mL for HBV pgRNA and 3.0 $\log_{10}$ U/mL for HBcrAg. However, in my research HBV pgRNA and HBcrAg could be quantified at lower levels than these limits of quantification with pgRNA detected as low as 0.8 $\log_{10}$ U/mL and concentrations as low as 2 $\log_{10}$ U/mL for HBcrAg.

Since liver biopsies are not ideal for paediatric patients, non-invasive ways of determining the progression of liver disease in these patients could be to measure pgRNA and HBcrAg. Increasing concentrations of these novel markers alongside other markers e.g. HBV DNA and HBsAg are linked to progression of liver disease and could determine the need for antiviral therapy. Paediatric patients could benefit from the utilisation of these markers as they can be used to predict the level of cccDNA transcriptional activity in the liver. High cccDNA activity

along with ALT flares could determine which individuals need antiviral therapy or will need antiviral therapy during adulthood.

Fourth study: Is the performance of ultra-sensitive HBsAg Fujirebio assay consistent across HBV genotypes (comparison between CLEIA HBsAg HQ Fujirebio and Abbott Architect assays)

The Fujirebio Lumipulse HBsAg-HQ assay is highly sensitive and can consistently quantify HBsAg levels in serum and plasma samples of CHB patients from a diverse range of genotypes. Its main advantage is its low limit of detection. Its relevance in clinical settings may include detecting patients with occult CHB and patients who are at risk of HBV reactivation. Additionally, patients who experience HBsAg loss could be closely monitored using this ultra-sensitive assay so that any sign of reactivation is detected early. I have clearly demonstrated that viral activity after seroclearance still needs checking.

Conclusion

To conclude, life-long treatment remains the norm for CHB patients, but huge efforts are being made to provide a finite therapy and to improve the quality of life for CHB patients. Though many challenges remain, the ability to detect residual cccDNA persistence and transcription while patients are undergoing therapy, with more sensitive assays is important in the development of newer therapies.

There are a few limitations to my research. First, all studies were done retrospectively testing stored serum samples. A disadvantage of this is that my data depended on the availability and accuracy of medical records. Additionally, the small sample size of some of the cohorts was a limitation, particularly the paediatric cohort which only had 29 patients. A wider, more in-depth analysis could have been achieved with a larger cohort and analysis of sub-cohorts could have also been done. Third, serum HBcrAg and HBV pgRNA were determined using novel research assays that have not been validated or standardised to use clinically in the UK. The Abbott HBV RNA assay boasts of a low LLOQ of 0.8 $\log_{10}$ U/mL but the HBcrAg assay has a LLOQ of 3 $\log_{10}$ U/mL. Both assays have relatively low sensitivity but there are gradual improvements being made to improve this limitation. Future prospective studies using commercial kits with a higher sensitivity and lower LLOQ in large and diverse population

would provide a more accurate description of HBcrAg and pgRNA kinetics in CHB patients on antiviral therapy.

8.2 Future research

Small, Middle and Large HBsAg proteins

The Hepatitis B Virus encodes three proteins of HBsAg, which form the viral envelope: small (SHB), middle (MHB) and large (LHB)(1). These proteins are formed via protein translation of the pre-S1 mRNA and the pre-S2/S mRNA in the endoplasmic reticulum (ER)(1,2). The virion consists of mostly S proteins and an equal amount of M and L proteins, which approximately makes up one third of the viral envelope. Having an excess amount of S protein is essential for envelopment and secretion of all HBsAg particles(1,2). Several studies have found a link between HBsAg proteins and the different phases of CHB. Low amounts of large and middle proteins have been associated with the HBeAg negative chronic HBV infection phase of CHB(3,4). Pfetterkorn et al demonstrated that HBsAg composition is different across different phases of Hepatitis B. It was recognised that patients in the HBeAg negative chronic HBV infection phase had significantly lower proportions of LHBs and MHBs than patients in the acute or other chronic phases and these findings where regardless of HBeAg status or HBsAg levels(3). Both LHBs and MHBs ratios were deemed as better predictors of the HBeAg negative chronic HBV infection phase than total HBsAg levels(3). However, a lower percentage of LHBs could be linked to deletions in the HBV genome, which leads to the secretion of mutant LHBs. Chang et al found that pre-s deletions in the HBV genome produces viral variants associated with HCC development (5). Therefore, finding low proportions of LHBs and MHBs could be the result of pre-s1 and pres2 deletions.

Quantifying HBsAg proteins could help us understand the role of each HBsAg protein in infectivity and how it can be used in CHB management. Zhu et al reported that quantification of serum LHBs may be a more useful parameter for predicting patient response to peginterferon

alfa-2a than those on entecavir(6). Also combining LHBs, HBsAg and HBV DNA is a more effective method of predicting virological and serological response in patients on treatment(6). Ni et al showed that HBV lacking the M protein is infectious *in vitro*, therefore making the M protein dispensable in infectivity of HBV in hepatocytes(7). The study also showed that HBV carrying the L protein with mostly pre-S2 amino acid (aa) sequences is infectious without the M protein being present(7). Finally, Ni et al also demonstrated that deleting the pre-S2 sequences of more than 20aa disrupts virus assembly and nucleocapsid envelopment(7). These studies show the importance of HBsAg fragments in HBV infectivity.

I will investigate the role of LHB, MHB and SHB proteins in HBV infection and specifically focus on their role in predicting disease progression and asses if there are differences in HBsAg composition in different CHB phases. I will further investigate if HBsAg proteins can be used as surrogate markers to distinguish between HBsAg expression from cccDNA and integrated DNA. This could prove valuable in monitoring future therapeutic approaches(8).

Quantification of large, middle, and small HBsAg proteins:

LHBs, MHBs and SHBs proteins will be determined using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique. Microplates coated with either preS1, preS2, or S specific monoclonal antibodies (mabs) will be created to specifically bind to LHBs, MHBs and SHBs proteins. The procedure will be carried out according to Pfefferlorn et al(3).

The MAb MA18/7 will be generated through immunization with purified HBV particles, followed by western blot analysis with the LHBs protein P39 and its glycosylated form, GP42(3). MAb MA18/7 binds to the peptide sequence (DPXF) of preS1 amino acids 20 to 23 (31–34 in genotype A)(3). MAb Q19/10 binds selectively to single and double glycosylated GP33 and GP36 of MHBs. Its epitope is formed by the N-glycopeptide MQWNST. MAb HB1 binds a linear epitope (PCK/RTCT) in the SHBs amino acids 120–125 is used to detect all three isoforms of HBsAg(3). Quantifying HBsAg components can be achieved using a well-characterized internal standard (ID1), with each set containing a biochemically determined number of LHBs (90.7 ng/mL), MHBs (188.7 ng/mL) and total HBsAg (1,000 ng/mL).

Quantification of the LHBs and MHBs components of HBsAg

MA18/7 or Q19/10 antibodies (2 µg/mL each) will be coated in 0.1 M sodium phosphate buffer at pH 7.4 on 96-well plates. MA18/7 will be incubated for 24 hours and Q19/10 plates for 48 hours. The plates will be washed 4 times with phosphate-buffered saline (PBS) and 0.1% Tween 20. The coated wells will be blocked at 4°C for 24 hours, using Casein Blocker in PBS for Q19/10 and the Blocking Solution for MA18/7. The blocked wells will be washed again and 100µl of serum samples added. Dilutions of 1:2 to 1:120,000 of serum in inactivated FBS Superior will be done to generate signals in the linear range. The Q19/10-coated will be incubated for 2 hours at 37°C then 100 µl of a 1:4 ratio of PBS diluted biotin-conjugated detection antibody will be added. In the MA18/7-coated wells, 25 µl of the biotin-conjugated detection antibody will be directly added after sample loading. Both wells will be incubated for 1 hour at 37°C. The wells will be washed, then peroxidase-conjugated streptavidin will be added.

Western Blot detection

Enrichment of HBsAg in the serum samples will be achieved by precipitation with 12.5% polyethylene glycol 8000 overnight then washed three times with 12.5% PEG and redissolved in 100 µL of PBS. Samples will be loaded and separated (50 ng HBs protein per lane or a maximum of 13 µl of the sample) through 12.5% gels.

The proteins will be blotted onto polyvinylidene difluoride membranes.

For immunostaining of the blotted proteins, the membrane will be blocked in undiluted soymilk and incubated with 1 µg/mL of MAb HB1 in PBS with 50% soymilk. Anti-mouse immune globulin G conjugated with near-infrared fluorescent dye (100 ng/mL) will be mixed with PBS with 50% soymilk, 0.1% Tween 20 and 0.01% SDS. The reaction will be incubated for 1 hour at room temperature, the membrane will be scanned and the ratios of the HBsAg components will be quantified by the LI-COR ODYSSEY® Infrared Imaging System (LI-COR, Bad Homburg, Germany).

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Concluding Remarks and Future Research

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Supplementary documents

Legend for supplementary figures	
Supplementary Figure 1 a-c	Summary of samples time-points for individual cohorts (A-C)
Supplementary Figure 2	Graphical representation of changes in individual serological/ virological markers in different time-points during NA therapy (all cohort A) – HBV DNA, HBsAg, HBcrAg, pg HBV RNA
Supplementary Figure 3	Proportions of patients with detected HBV DNA, HBcrAg and pg HBV RNA during NA therapy (panel A) and post NA withdrawal (panel B) in cohort B
Supplementary Figure 4	Correlations between HBcrAg and pg HBV RNA at different time-points of therapy in cohort A
Supplementary Figure 5	Correlations between HBcrAg and pg HBV RNA during NA therapy, at time of NA withdrawal and after stopping NA in cohort B
Supplementary Figure 6	The impact of Pre-core G1896A mutation and basal core promoter (BCP) mutations (A1762T/G1764A) on HBcrAg and pg HBV RNA concentrations in cohort A

Supplementary Figure 7	Changes in serological and virological markers in 2 patients who had HBV DNA relapse after stopping NA after achieving HBsAg loss

Supplementary Table 1:

Cohort A: Baseline clinical characteristics of all patients at time of commencing tenofovir

Gender	Age	Genotype	Ethnicity	Fibrosis	HBV DNA	HBsAg	ALT
Male	19	D	Caucasian	2	4.01	3.51	89
Female	22	E	BA	2	4.22	4.22	44
Male	23	D	Caucasian	2	4.28	3.69	44
Male	23	C	Oriental	3	3.46	3.67	59
Male	26	A	BC	2	4.12	3.67	97
Female	26	C	Oriental	3	3.33	3.67	59
Male	29	A	BA	2	4.23	4.29	77
Female	29	E	BA	2	5.34	3.9	69
Male	29	E	BC	2	6.34	4.22	59
Male	29	E	BA	2	4.89	3.67	111
Male	29	B	Oriental	2	5.67	3.46	68
Male	29	E	BA	2	3.78	4.1	45
Male	32	E	BA	2	3.22	3.43	62
Male	34	D	Indian	3	5.12	3.09	121
Male	34	E	BA	2	6.01	4.23	200
Male	36	E	BA	2	4.67	3.78	59
Male	37	E	BA	2	5.55	4.29	78
Male	37	D	Caucasian	2	3.77	3.77	67
Male	38	A	BA	2	4.12	3.45	65
Female	38	E	BA	3	4.1	4.01	39
Female	38	C	Oriental	2	4.15	2.5	55
Female	39	D	Indian	3	3.12	3.02	33
Male	39	E	BA	3	3.99	3.67	34
Male	39	A	Caucasian	3	4.79	4.11	59
Male	39	E	BA	2	4.43	3.23	59
Male	41	E	BA	2	4.67	3.78	44
Male	42	E	BA	2	5.78	4.3	110
Male	44	E	BA	2	3.18	3.8	62
Female	44	D	Caucasian	3	4.44	4.01	56
Male	44	E	BA	2	3.67	3.22	55
Female	44	C	Oriental	3	6.22	4.12	69
Male	45	C	Oriental	2	3.22	2.74	56
Female	45	D	Caucasian	2	4.5	3.56	69
Male	45	A	BC	2	4.34	3.12	60
Female	45	A	BA	3	4.18	4.16	49
Male	47	C	Oriental	3	3.09	3.12	45
Male	48	E	BA	3	3.67	3.13	45
Male	48	E	BA	3	6.78	4.02	166
Male	49	D	Caucasian	3	3.78	3.23	55
Female	49	E	BA	3	3.22	2.98	49
Male	49	A	BC	3	4.3	3.56	39
Female	49	A	BA	2	4.11	3.78	46
Male	49	E	BA	3	2.67	2.9	55

Female	51	E	BA	3	5.12	4.2	212
Male	51	E	BA	2	6.18	4.2	133
Female	51	C	Oriental	3	4.12	4.11	49
Male	51	A	Caucasian	2	5.2	4.2	72
Male	52	B	Indian	3	3.88	4.09	79
Male	52	D	Oriental	3	5.7	4.3	56
Male	52	A	Caucasian	2	4.12	4.11	44
Male	52	A	Caucasian	3	4.57	3.33	101
Male	54	E	BA	3	3.3	3.01	53
Male	55	A	Caucasian	3	2.78	3.34	59
Female	55	C	Oriental	3	3.99	3.7	62
Male	55	E	BA	2	3.99	4.12	59
Female	56	E	BA	3	3.67	3.79	67
Male	57	E	BA	3	2.98	3.67	62
Male	57	C	Oriental	3	3.78	3.5	55
Female	57	C	Oriental	3	3.78	2.12	49
Male	58	E	BA	3	4.45	4.01	40
Male	58	E	BA	3	2.89	3.2	60
Male	61	E	BA	2	4.12	4	74
Female	63	D	Indian	3	4.5	4.34	55
Male	66	E	BA	2	2.97	3.12	56
Male	67	E	BA	2	3.89	3.98	64
Male	67	E	BA	3	3.08	3.4	39

Abbreviations: BA: Black African, BC: Black Caribbean

Supplementary Table 2:

Cohort A: Baseline clinical characteristics of the patients with detectable vs. not detectable pg HBV RNA after 3 years of therapy

	pg HBV RNA not detected (n=46)	**pg HBV RNA > 1.65 log_{10}U/ml** (n=20)	**Significance** (p)
Age (median, range) [years]	45.0 (19-67)	45.5 (29-63)	0.856
Gender Male vs. Female (#)	33:13	15:5	0.785
ALT (median, range) [IU/ml]	59.0 (33-121)	68.5 (40-212)	**0.02**
HBV DNA (median, range) [log_{10} IU/ml]	3.99 (2.67-6.22)	4.78 (3.09-6.78)	**<0.001**
HBsAg (median, range) [log_{10} IU/ml]	3.67 (2.12-4.3)	4.01 (2.5-4.34)	**0.024**
HBcrAg (median, range) [log_{10} U/ml]	3.15 (2-5.2)	4.45 (2-6.5)	**0.001**
pg HBV RNA (median, range) [log_{10} U/ml]	1.79 (0-2.33)	2.12 (1.77-3.25)	**<0.001**
Genotypes (# of patients) • A • B • C • D • E	 11 1 8 6 20	 1 1 2 4 12	0.304
Genotype E vs. non genotype E (#)	20:26	12:8	0.217
Mutation in pre-core region vs. no mutation (#)	8:38	12:8	**0.001**

Supplementary Table 3:

Cohort A: Baseline clinical characteristics of the patients with detectable vs. not detectable pg HBV RNA after 5 years of therapy

	pg HBV RNA not detected (n=57)	**pg HBV RNA > 1.65 log_{10}U/ml** (n=9)	**Significance** (p)
Age (median, range) [years]	45.0 (19-67)	44 (29-63)	0.815
Gender Male vs. Female (#)	41:16	7:2	0.714
ALT (median, range) [IU/ml]	59.0 (33-200)	69 (44-212)	0.163
HBV DNA (median, range) [log_{10} IU/ml]	4.11 (2.67-6.78)	5.34 (3.18-6.34)	**0.015**
HBsAg (median, range) [log_{10} IU/ml]	3.67 (2.12-4.3)	4.2 (3.25-4.34)	**0.008**
HBcrAg (median, range) [log_{10} U/ml]	3.5 (2-5.7)	5.0 (2.3-6.5)	**0.05**
pg HBV RNA (median, range) [log_{10} U/ml]	1.83 (0-2.89)	2.31 (1.87-3.25)	**0.001**
Genotypes (# of patients) • A • B • C • D • E	 12 2 10 7 26	 0 0 0 3 6	0.148
Genotype E vs. non genotype E (#)	26:31	6:3	0.24
Mutation in pre-core region vs. no mutation (#)	13:44	7:2	**0.01**

Supplementary Table 4:

Cohort A: Individual patients data comparing the detectable HBcrAg and pg HBV RNA status at time-points

Baseline	Baseline2	Year 3	Year3	Year 5	Year5
HBcrAg	pg HBV RNA	HBcrAg	pg HBV RNA	HBcrAg	pg HBV RNA
Negative	Positive	Negative	Negative	Negative	Negative
Positive	Positive	Negative	Negative	Negative	Negative
Positive	Positive	Positive	Negative	Positive	Negative
Positive	Positive	Negative	Negative	Negative	Negative
Positive	Positive	Positive	Negative	Negative	Negative
Positive	Positive	Negative	Negative	Negative	Negative
Positive	Positive	Positive	Negative	Negative	Negative
Positive	Positive	Positive	Positive	Positive	Positive
Positive	Positive	Positive	Positive	Positive	Positive
Positive	Positive	Positive	Positive	Positive	Negative
Positive	Positive	Negative	Positive	Negative	Negative
Positive	Positive	Negative	Negative	Negative	Negative
Positive	Negative	Positive	Negative	Positive	Negative
Negative	Negative	Negative	Negative	Negative	Negative
Positive	Positive	Positive	Positive	Positive	Negative
Positive	Positive	Negative	Negative	Negative	Negative
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Negative	Positive	Negative	Positive	Negative	Negative
Negative	Positive	Negative	Negative	Negative	Negative
Positive	Positive	Positive	Positive	Positive	Negative
Negative	Positive	Negative	Positive	Negative	Positive
Negative	Negative	Negative	Negative	Negative	Negative
Positive	Positive	Negative	Negative	Negative	Negative
Positive	Positive	Negative	Negative	Negative	Negative

Negative	Negative	Negative	Negative	Negative	Negative
Positive	Positive	Positive	Positive	Positive	Negative
Positive	Positive	Positive	Positive	Positive	Positive
Positive	Positive	Positive	Negative	Positive	Negative
Positive	Positive	Negative	Negative	Negative	Negative
Positive	Negative	Positive	Negative	Negative	Negative
Positive	Positive	Positive	Positive	Positive	Positive
Positive	Positive	Negative	Negative	Negative	Negative
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Negative	Negative	Negative	Negative	Negative	Negative
Positive	Positive	Positive	Positive	Positive	Negative
Positive	Positive	Negative	Negative	Negative	Negative
Positive	Positive	Negative	Negative	Negative	Negative
Positive	Positive	Positive	Positive	Positive	Positive
Positive	Positive	Negative	Negative	Negative	Negative
Positive	Positive	Negative	Negative	Negative	Negative
Negative	Positive	Negative	Negative	Negative	Negative

Spearman correlations between HBcrAg and pg HBV RNA levels at baseline: r=0.596, p<0.01; year 3: r=0.591, p<0 and year 5: r=0.677, p<0.01.

Supplementary Table 5:

Cohort B: Summary Table – the proportion of patients with detected serological/virological markers at time of NA withdrawal (n=23)

	No flare (n=9)	**Mild flare** (n=10)	**Severe flare** (n=4)
HBeAg positive (#, %)	0 (0%)	0 (0%)	0 (0%)
HBV DNA detected (#, %)	0 (0%)	0 (0%)	0 (0%)
HBsAg positive (#, %)	9 (100%)	10 (100%)	4 (100%)
HBcrAg detected (>3 $\log_{10}$ U/ml) (#, %)	0 (0%)	0 (0%)	**4 (100%)**
pg HBV RNA detected (>1.65 $\log_{10}$ U/ml) (#, %)	0 (0%)	0 (0%)	**3 (75%)**

Supplementary Table 6:

Cohort C: Detailed clinical characteristics at antiviral therapy baseline

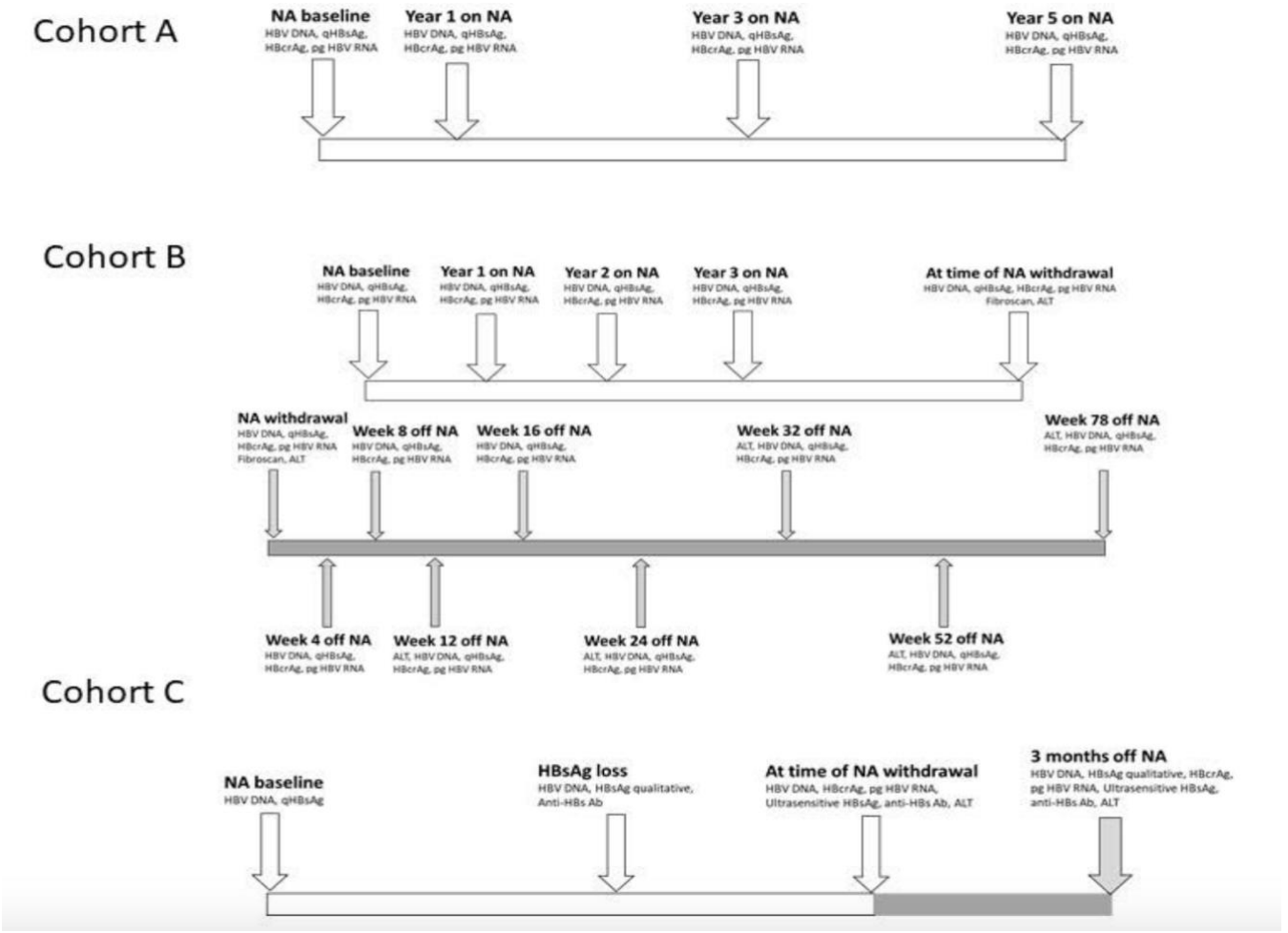
Relapse	Age	Gender	Genotype	Fibrosis	Duration of NA therapy	Type of NA
No	40.19	Female	NK	2	3.21	TDF
No	36.02	Male	B	2	10.7	TDF
No	42.48	Male	D	2	12.21	ETV
No	41.29	Male	A	2	7.96	ETV
No	30.78	Male	B	2	9.85	TDF
No	36.64	Female	E	3	11.89	TDF
No	34.02	Male	A	2	7.08	ETV
No	48.19	Male	A	4	7.08	TDF
No	26.84	Female	E	2	1.6	TDF
No	32.87	Female	C	2	7.71	TDF
No	20.61	Female	E	2	1.91	TDF
No	30.07	Female	A	3	5.59	TDF
No	22.82	Male	C	2	11	TDF
No	32.1	Male	D	2	10.33	TDF
No	35.78	Female	C	3	8.58	TDF
No	57.32	Male	A	2	10.58	TDF
No	44.66	Male	NK	2	7.5	TDF
Yes	40.99	Female	E	2	8.62	TDF
Yes	30.65	Male	C	2	9.92	TDF

Supplementary Table 7:

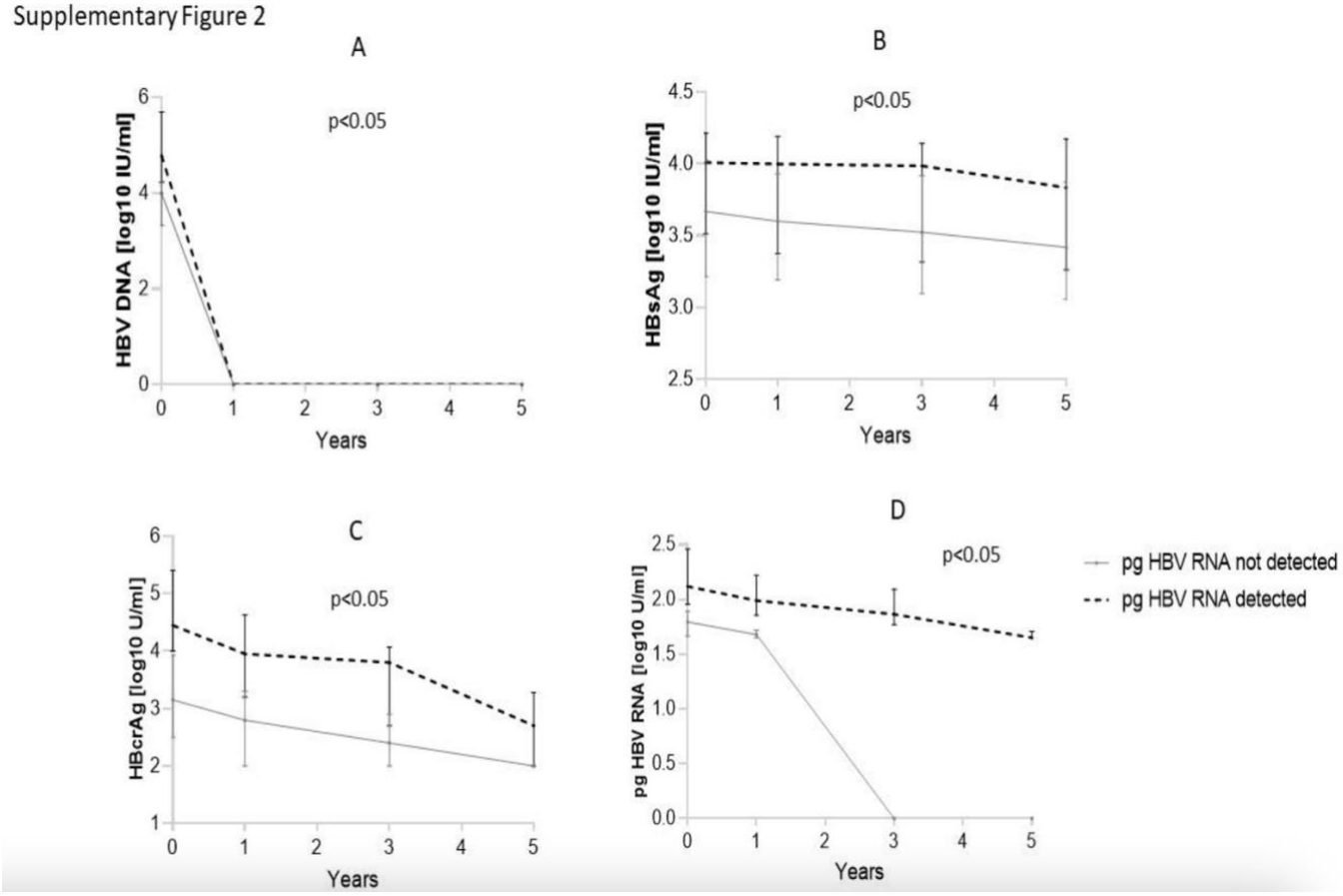
Cohort B: Clinical characteristics of patients at therapy baseline and time of NA withdrawal according to pattern of HBsAg decline post NA withdrawal (n=23)

	HBsAg decline < 1 log_{10} IU/ml (n=17)	**HBsAg decline >1 log_{10} IU/ml** (n=6)	**Significance** (p)
Age (median, range) [years]	47.0 (23 -65)	48 (44-64)	0.319
Gender Male vs. Female (#)	11:6	3:3	0.634
ALT at therapy baseline (median, range) [IU/ml]	32 (17-109)	25 (20-156)	0.303
HBV DNA at therapy baseline (median, range) [log_{10} IU/ml]	4.68 (2.48-6.54)	3.88 (3.49-4.66)	0.256
HBsAg at therapy baseline (median, range) [log_{10} IU/ml]	3.96 (3.32-4.45)	3.06 (2.41-3.63)	**<0.001**
HBcrAg at therapy baseline (median, range) [log_{10} U/ml]	4 (2-6.8)	2.8 (2-3.8)	**0.04**
pg HBV RNA at therapy baseline (median, range) [log_{10} U/ml]	2.82 (0-4.1)	2.01 (0-2.65)	**0.05**
Genotypes (# of patients) • A • B • C • D • E	 2 2 0 4 9	 2 1 1 1 1	0.236
Genotype E vs. non genotype E (#)	9:8	1:5	0.21
Mutation in pre-core region vs. no mutation (#)	9:8	1:5	0.21
HBeAg positive at baseline (#, %)	0 (0%)	0 (0%)	1.0
TDF vs. ETV	14:3	5:1	0.567
Duration of therapy (median, range) [months]	101 (38-117)	59 (45-108)	0.177
ALT at therapy withdrawal (median, range) [IU/ml]	26 (15-53)	22 (16-38)	0.303
HBV DNA at therapy withdrawal (median, range) [log_{10} IU/ml]	0 (0)	0 (0)	1.0
HBsAg at therapy withdrawal (median, range) [log_{10} IU/ml]	3.75 (2.9-4.5)	2.55 (1.59-3.33)	**<0.001**
HBcrAg at therapy withdrawal (median, range) [log_{10} U/ml]	2 (2-5.4)	2 (2)	0.366
pg HBV RNA at therapy withdrawal (median, range) [log_{10} U/ml]	0 (0-2.13)	0 (0)	0.333
Type of flare (# of pts) • No • Mild • Severe	 6 7 4	 3 3 0	0.355

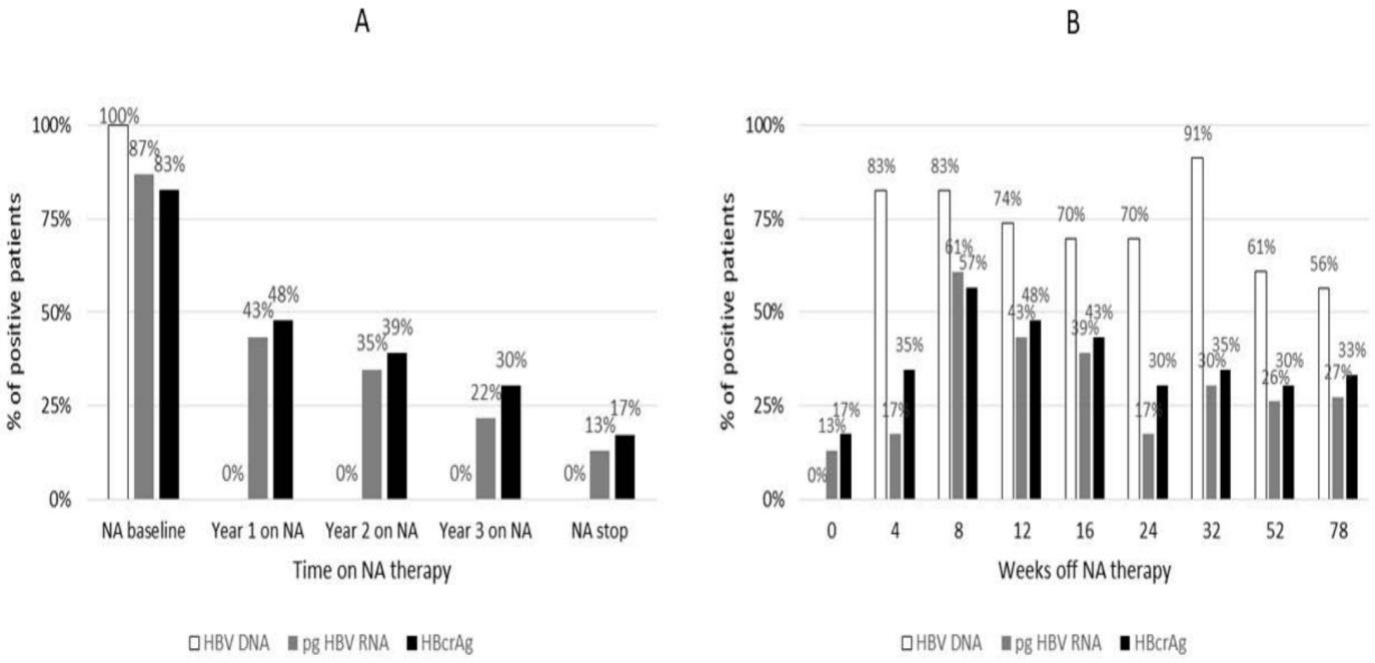
Supplementary Figure 1



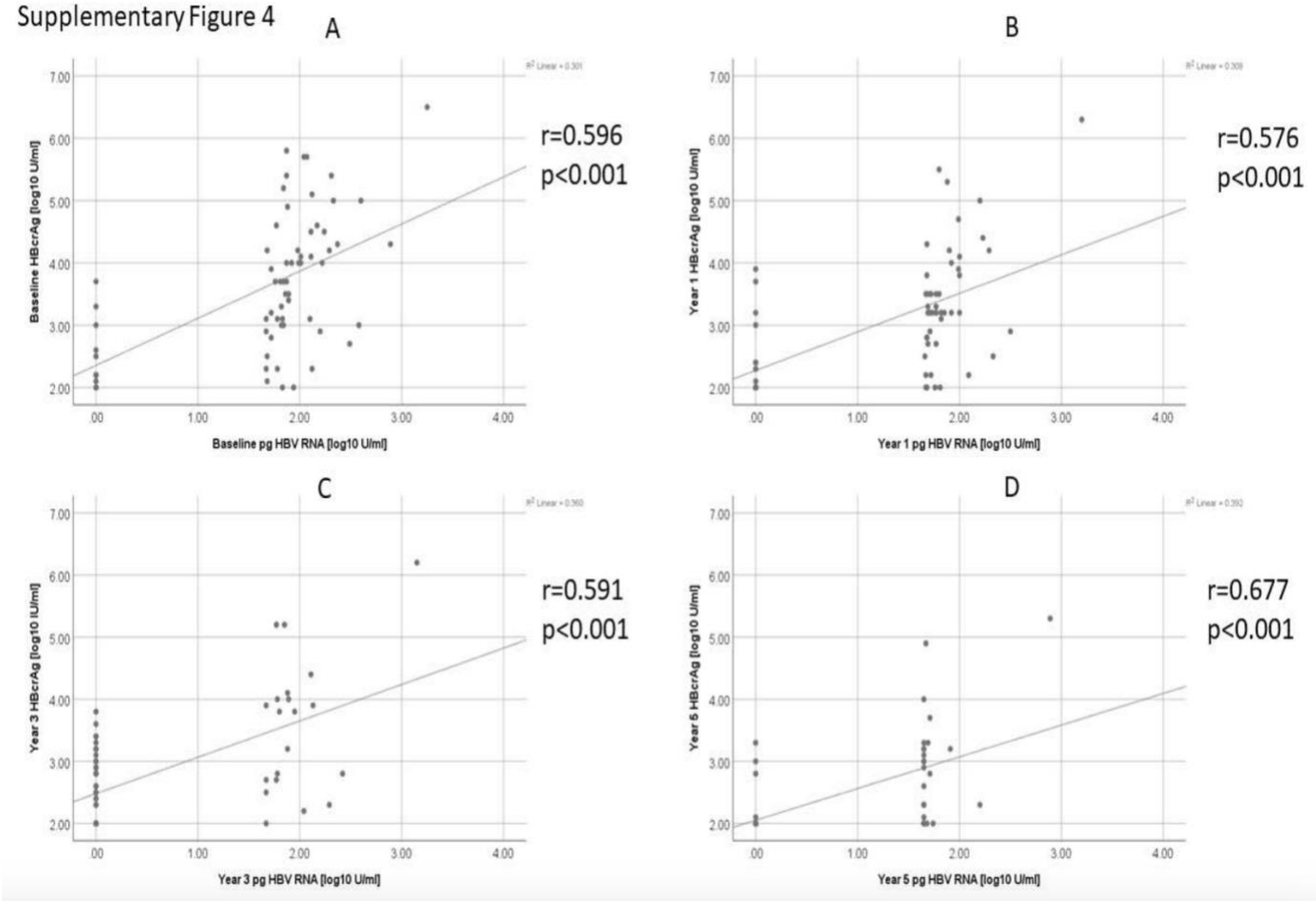
Supplementary Figure 2



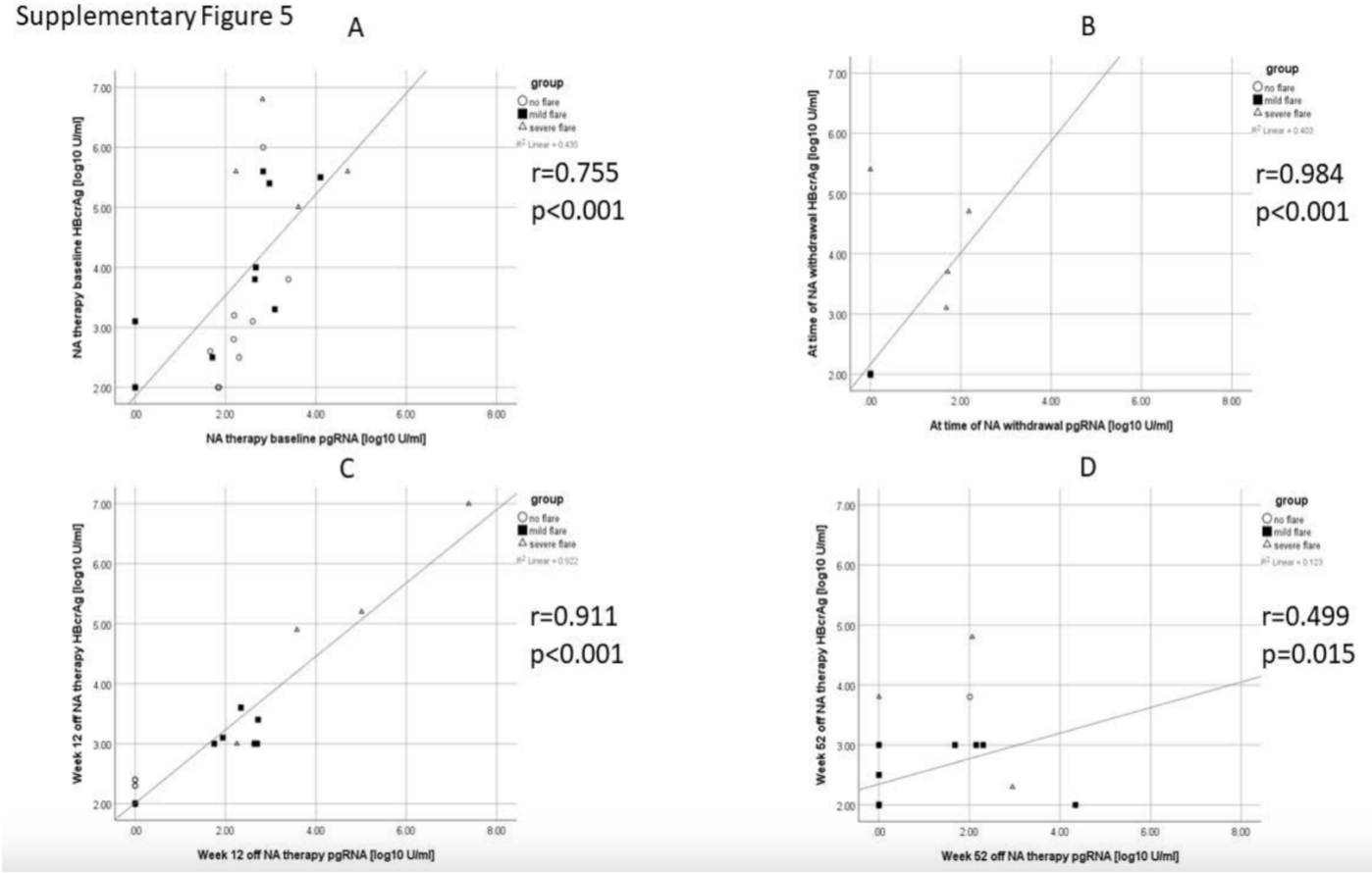
Supplementary Figure 3



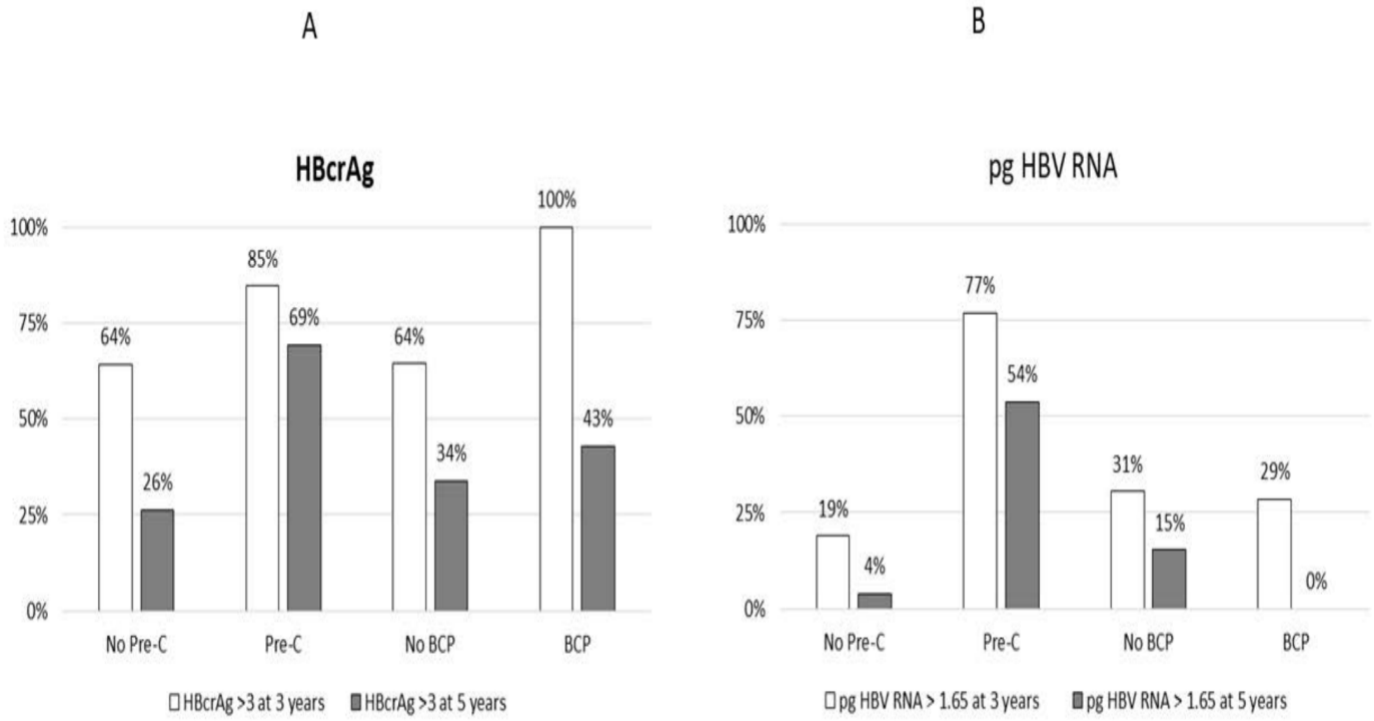
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7