Serological Studies into the Natural History of Chronic Hepatitis B

A thesis submitted in fulfilment of the requirements for
The degree of
Doctor of Medicine

By

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February 2011
Declaration

I declare that this thesis is my own composition and that it is a record of the original work conducted between Jan 2008 and Jan 2010. This thesis contains no material that has been submitted for any degree or diploma at this or any other university. All work described in this thesis was performed and analysed by myself with the following exceptions:

Chapter 3 - A subset of patients with chronic hepatitis B were recruited for a prior study by Dr Alexander Thompson. Peripheral blood mononuclear cell collection for these patients was performed by Ms. Narelle Skinner (Department of Medicine, Monash Medical Centre, Clayton).

Chapter 3 – The measurement of serum HBsAg titres using the Elecsys® platform was performed by Ms Rachel Hammond at VIDRL, Melbourne.

Publications arising from this work:


This thesis is not greater than 100,000 words in length.

Tin Quang Nguyen
February 2010
Acknowledgements

I was jointly supervised in this thesis by Associate Professor Paul Desmond of the Department of Gastroenterology at St. Vincent’s Hospital, Melbourne; and Professor Stephen Locarnini, Victorian Infectious Diseases Reference Laboratory (VIDRL). I would like to thank them for their support, enthusiasm and constant encouragement.

This project would not have been possible without the guidance and technical assistance of my colleagues at VIDRL. Thank you in particular to Dr. Scott Bowden for his words of wisdom, and also for proof reading the thesis. The staff of the Gastroenterology Department at St Vincent’s Hospital have always been supportive, and I would especially like to thank Dr Alexander Thompson and Dr Sally Bell.

This thesis was supported by a Medical Postgraduate Scholarship from The Gastroenterological Society of Australia.

Finally, and most importantly, I would like to thank wife Hong for all of her love and patience, and also to my son Aiden for all the happiness that he has brought into my life.
Abstract

Chronic hepatitis B (CHB) infection represents a global health problem, with an estimated 400 million people affected worldwide. The potential long term sequelae includes cirrhosis, hepatic decompensation and hepatocellular carcinoma.

The paradigm for treatment of chronic hepatitis B virus (HBV) is evolving with the advent of newer medications, improved laboratory assay sensitivity and an increased understanding of the natural history of chronic infection. The natural history of CHB is typically regarded as consisting of four phases which are classified by specific biochemical, serological and virological characteristics, including serum ALT levels, HBeAg serostatus and HBV DNA titre. Whilst serum HBsAg is the serological hallmark of HBV infection, measurement of the serum HBsAg titre is currently not required for the distinction between the different phases of CHB, and is also not routinely assessed during antiviral therapy.

The first aim of this thesis was to perform a detailed cross-sectional examination of the baseline HBsAg titres in the different phases of the natural history of CHB. The cohort of patients with CHB that were evaluated included adult patients attending a tertiary centre, pregnant women and a paediatric group. This study demonstrated that median baseline HBsAg titres differed between the four phases of CHB, with higher titres in HBeAg positive compared to HBeAg negative patients. Furthermore there was an apparent “disconnect” between HBsAg titres and HBV DNA in the different phases of CHB. It was hypothesized that these findings may be due to the expression of HBsAg from integrated viral envelope sequences instead of HBsAg production off mRNA derived from the HBV cccDNA template, or due to differences in the immune regulation of viral replication during different phases of infection.

The second aim of this thesis was to evaluate the changes in serum HBsAg titres during long term therapy with oral nucleos(t)ide agents (NA). HBsAg clearance and seroconversion represent the ultimate endpoint in antiviral in CHB. Although clinical trials have suggested a benefit in monitoring baseline and on-treatment serum HBsAg titres during Peg-IFNα therapy in predicting virological responses, there is little data on the effect of oral NA on HBsAg titres. In this thesis, different patterns of HBsAg decline during oral NA therapy were observed, although overall the on-
treatment reduction in HBsAg titres were modest in comparison to that previously described in the literature with Peg-IFNα therapy. This was attributed to the indirect affect of oral NAs on HBsAg synthesis via inhibition of the intracellular cccDNA conversion pathway, with a subsequent decline in pre-existing cccDNA molecules over time.

Serum anti-HBs is usually only detectable on current commercial assays once HBsAg seroclearance has occurred, and is thought to be due anti-HBs complexing into immune aggregates with the excess envelope proteins. The third aim of this thesis was to test the serum and the B-cell component of peripheral blood mononuclear cells (PBMCs) for anti-HBs in patients who also test positive for serum HBsAg. A minority of patients had detectable anti-HBs by the commercial immunoassay. It was hypothesized that there would be a higher proportion of patients with detectable anti-HBs in the B-cell component of PBMCs. Unfortunately, anti-HBs was not detected in the lysate of B-cells using two commercial immunoassays, and an in-house enzyme linked immunosorbent assay (ELISA) could not be optimised for technical reasons.

In conclusion, the measurement of baseline and on-treatment HBsAg titres has the potential to become the next focus of translational and clinical research in CHB. In the context of the natural history of CHB, monitoring of baseline HBsAg titres may facilitate an improved understanding of the interplay between HBV with the innate and adaptive immune response. Finally, monitoring of HBsAg titres may allow the development of new algorithms to individualise patient therapy, and also encourage further study of novel therapeutic strategies which more directly affect HBsAg levels.
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Chapter 1. Literature Review

1.1 Introduction

In the era of molecular diagnostics, significant progress has been made on our understanding of the natural history and pathogenesis of chronic hepatitis B (CHB). This has been facilitated by the improved sensitivity of hepatitis B viral load assays, development of assays for intrahepatic hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) and other replicative intermediates, and earlier detection of antiviral drug resistant mutants. Recently, sensitive and reliable assays have also been developed to quantify both serum hepatitis B surface antigen (HBsAg) and serum hepatitis B e antigen (HBeAg). Given that HBeAg and HBsAg seroconversion are established therapeutic endpoints, the quantification of HBsAg and HBeAg has emerged as one of the next phases of translational and clinical research in hepatitis B. In the context of the natural history of CHB, baseline HBsAg levels, and to a lesser extent HBeAg levels, have not been well-characterized. Furthermore, the current commercial assays for their corresponding antibodies, anti-HBs and anti-HBe, are qualitative, and are usually only detected after HBsAg and HBeAg have been cleared from the systemic circulation. Further evaluation of both baseline and on-treatment changes in HBsAg and HBeAg titres, and quantification of corresponding antibody in each phase of disease could represent a step forward to better understand the natural history and pathogenesis of CHB. This in turn could lead to the development of new treatment regimens or novel therapeutic strategies.

1.2 HBV Virology

1.2.1 Classification of Hepatitis B Virus

Human hepatitis viruses are those that primarily affect the liver, and include hepatitis viruses A-E. All of these viruses have a single stranded genomic structure, with the exception of HBV, which has a partially double-stranded DNA genome. Other viruses such as Epstein Barr virus, herpes simplex virus, adenoviruses and cytomegalovirus can also affect the liver, although the liver itself is not the primary target of infection.
The focus of this thesis will be based on HBV. Human HBV is the prototype member of the *Hepadnaviridae* family, which can be further divided into the *Orthohepadnaviruses* of mammals and the *Avihepadnaviruses* of birds. Other than humans, HBV is only transmissible to chimpanzees, although all of the higher primates can be infected with their own species specific HBV. For example, gorilla HBV, orang-utan HBV, gibbon HBV\(^1\), etc.

### 1.2.2 HBV Virion Structure and Subviral Particles

The HBV has a double shelled spherical structure with a diameter of 42-45nm. The outer shell is known as the envelope, and is comprised of HBsAg proteins\(^2\). The inner shell is known as the core particle or capsid, and is formed by the hepatitis B core antigen (HBcAg) or core protein. A complex of largely double stranded relaxed circular HBV DNA covalently bound to HBV viral polymerase is located within the inner shell.

There are large amounts of non-infectious subviral particles in the sera of patients infected with HBV. These are composed of excessive envelope proteins which are bound by covalently linked intermolecular disulphide bonds to produce long filamentous and spherical (22nm) subviral particles. The serum concentration of these subviral particles is typically \(10^{12-14}\) particles/mL\(^3\), many orders of magnitude in excess of mature virions.

### 1.2.3 HBV Genome Organisation and Viral Proteins

The HBV genome comprises a partially double-stranded 3.2 kb DNA (dsDNA) which is organised into four overlapping, but frame-shifted open-reading frames (ORFs) (Figure 1.1). These ORFs encode the viral polymerase, surface/envelope, core and X proteins, and are translated into viral polymerase/reverse transcriptase (Pol/RT), envelope (small, medium, large), pre-core/core, and the X proteins, respectively. In addition, encoded within the HBV ORFs are four promoters (preC/C, preS1, S and X) and two enhancers (Enh I and Enh II)\(^3\).
1.2.3.1 Polymerase (Pol) ORF

The HBV Pol ORF contains 834-845 codons, and is the longest of the four HBV ORFs. The polymerase protein (90-kDa) is translated from pre-genomic RNA (pg-RNA) and is found packaged with pg-RNA in the HBV capsid. HBV polymerase is involved in episilon (ε) signal binding with subsequent specific encapsidation of the pre-genome and initiation of reverse transcription. The Pol consists of three functional domains: primase, RT and RNase H. The primase domain is located at the N-terminal end, and is responsible for priming of minus strand DNA synthesis. The RT domain encodes the RNA/DNA dependent polymerase. Finally, the RNase H domain is located at the C-terminus, and is responsible for RNA cleavage of the RNA/DNA hybrids generated during reverse transcription. A non functional domain known as “spacer” is located between the primase and RT domains.

1.2.3.2 Pre-core/C ORF

The Pre-core/C ORF has 212 or 214 codons depending on the genotype, and encodes two proteins: HBcAg and hepatitis B e antigen (HBeAg). Their production is achieved by the presence of two in-frame AUG start codons. Translation of HBcAg is initiated at the second AUG start codon located 29 codons downstream of the first AUG. Translation from the first AUG codon results in the synthesis of the pre-C region followed by the core polypeptide. Despite this, HBcAg and HBeAg are structurally and functionally distinct.
(a) HBcAg / Core Protein

HBcAg (21.5 kDa) is a structural protein which is synthesised in the hepatocyte cytosol, and consists of 183 or 185 amino acids. The first 144 amino acids are the structural component of HBcAg. HBcAg dimerizes via its first 144 amino acids, which are stabilised by disulfide bonds, and subsequently form the viral capsid. Amino acids 145-185 (C-terminal region) of HBcAg are multifunctional, and are critical for viral replication and assembly. This region contains an RNA encapsidation domain, as well as sections important for plus-strand synthesis of HBV DNA. Following capsid formation, RNA encapsidation, and reverse transcription, core particles (also known as replication complexes) are enveloped with HBsAg proteins and are transported through the endoplasmic reticulum and Golgi to bud as HBV.

Thus, HBcAg is essential for the HBV viral replication cycle. It is also important to note that the C-terminal region of HBcAg also contains nuclear localisation signals that mediate transport of mature viral particles into the nucleus of an infected cell, resulting in intranuclear amplification of viral DNA. In addition, the nuclear localisation signals also targets unassembled protein to the nucleus where empty core particles are formed. The function of these empty core particles is unclear, but they might interact with HBV cccDNA to regulate transcription.

(b) HBeAg / Pre Core Protein

HBeAg is a secreted, non-structural protein of HBV. The protein is cytosolic and efficiently secreted through the Golgi complex. HBeAg production follows a different pathway from that of the nucleocapsid (HBcAg) pathway (Figure 1.3). Although the ORF only differs by an additional 29 codons at the 5’ end, the structure and function of these proteins are entirely distinct.

The biological function of HBeAg has been the subject of intensive research, although it is still not fully elucidated. HBeAg is conserved evolutionarily among hepadnaviruses, and is thought to play an immunoregulatory function in the HBV life cycle. Whilst it is not necessary for viral replication or acute infection in vivo, HBeAg appears to be important for the establishment of chronic infection. Clinical studies have demonstrated that acute infection with HBeAg negative variants results in a self limited infection, or a more severe hepatitis. Furthermore, it is thought that HBeAg
may elicit immunological T cell tolerance during perinatal transmission due to its ability to cross the human placenta.

HBcAg and HBeAg share significant primary amino acid homology, and indeed are highly cross-reactive at the T cell level. However, unlike HBcAg which can function as both a T-cell dependent and independent antigen to result in anti-HBc production, anti-HBe production is T-cell dependent only. HBeAg can elicit either a TH₁ or TH₂ like response depending on the cytokine environment. Thus, it may act as either a “toleragen” or “immunogen” depending on the phase of HBV infection.

1.2.3.3 Pre-S/S ORF

HBsAg consists of three structurally related envelope proteins called the small (S), medium (M), and large (L) surface envelope proteins respectively. These are formed from the Pre-S/S ORF, which contains 389-400 codons and is completely overlapped by the Pol ORF. The Pre-S/S ORF consists of three domains which are divided by in-frame start codons: preS1, preS2, and ORF-S (Figure 1.). The HBV viral surface proteins which comprise HBsAg are found both on the surface of the hepatitis B virion, and also on the filamentous and spherical subviral HBsAg particles in the systemic circulation.

Figure 1.2 HBsAg transcription, translation, and physical forms in the systemic circulation
(a) Small hepatitis B surface (SHBs) protein

The small hepatitis B surface protein (22nm) is 225 amino acids in length, and is the most abundant of the three surface proteins, representing 85% of HBsAg. It is encoded by the ORF-S and is highly N-glycosylated. The major antigenic determinant of HBsAg is located within a conformational loop, and is called “determinant a” other minor determinants (d, y, w, and r) have also been identified.

(b) Middle hepatitis B surface (MHBs) / Pre-S2 protein

The middle hepatitis B surface protein containing the Pre-S2 domain consists of SHBs as well as an amino terminal extension of 55 amino acids. It represents 10-20% of HBsAg. It is thought that the Pre-S2 protein does not have a significant role in the viral life cycle. However, a truncated version of Pre-S2 identified in hepatocellular carcinoma (HCC) has been shown to have a trans-activating function. In addition, amino acids 2-16 of Pre-S2 contains a binding site for modified human serum albumin, but the significance of this is currently unknown.

(c) Large hepatitis B surface (LHBs) / Pre-S1 protein

The large hepatitis B surface protein contains an additional 109-119 amino acids, to the Pre-S1 domain, depending on the HBV genotype. The LHBs comprises 10-30% of HBsAg virions and subviral filamentous forms. The Pre-S1 domain of LHBs is essential for viral infection, including mediating attachment of HBV virions to susceptible cells. The Pre-S1 domain interacts with the host immune systems due to the presence of B and T cell epitopes, and it is these antigenic sites which are important for recovery from viral infection or protection from infection following vaccination. Only Pre-S1 is exposed on the particle surface, with the Pre-S2 and S domains hidden by Pre-S1. Furthermore, unlike the other surface proteins, Pre-S1 contains two different topologies, and thus is also located in the cytosol as well as in the endoplasmic reticulum (ER).
1.2.3.4 **X ORF**

The HBV X protein (HBx) is encoded by the smallest open reading frame (ORF) in the HBV genome (Figure 1.1). The HBx ORF is highly conserved among mammalian hepadnaviruses, but seems to be absent in avian hepadnaviridae\(^1\). It encodes a 154 amino acid protein with a molecular weight of 17.5kDa\(^1\). The deduced amino acid sequence of the X protein does not share similar homology with any other known viral protein. The HBx ORF overlaps with the basal core promoter (BCP) region, and the Polymerase ORF.

HBx expression is regulated predominantly at a transcriptional level. It is controlled by Enhancer 1 (Enh1)\(^1\), which not only regulates the HBx promoter, but PreS/S- and PreC-C/pre-genome promoters\(^1\). In addition, HBx expression is also regulated by an auto-regulatory pathway through inflammation driven signalling pathways\(^1\). Whilst little is known about the three dimensional structure of HBx, it is thought to be a non-structural accessory protein of HBV. Most studies have suggested that the distribution of HBx is predominantly cytoplasmic, with a small nuclear fraction\(^2\)\(^3\). However, a few studies have detected HBx only within the nucleus\(^2\)\(^3\). It may be that the distribution of HBx is related to the overall cellular HBx expression level\(^4\).

Despite significant progress in the field of HBV research, the functional mechanisms of HBx have remained relatively elusive. Research into HBx has been limited by its short half-life and poor immunogenicity. It is thought that HBx must have an important role, given that the HBV genome is only 3.2kb in length with four ORFs. HBx has been suggested to be a multifunctional protein. It appears to also be important for viral replication, and modulation of host cellular functions through transcription, signal transduction, cell cycle progression and apoptosis\(^5\). HBx can act as a transcriptional transactivator of a number of viral/cellular promoters by directly interacting with transcriptional factors such as TFIIB, TFIIH, the RPB5 subunit of RNA polymerases and the TATA binding protein\(^6\). Furthermore it is also involved in the activation of signal transduction pathways, such as the Ras/Raf/MAP kinase cascade. Work by Bouchard and colleagues has suggested that HBx may lead to the release of calcium (Ca\(^{2+}\)) from mitochondria into the cytosol, which in turn may stimulate the Pyk2-Src kinase signal transduction pathway that activates HBV reverse transcription and DNA replication\(^7\)\(^8\). HBx has been shown to bind to a
mitochondrial ion-channel protein called HVDAC3\textsuperscript{26}. The observation that HBx affects Ca\textsuperscript{2+} fluxes is an attractive hypothesis which links HBx with multiple viral and cellular processes.

1.3 The HBV Replication Cycle

The HBV life cycle is relatively well characterized, and commences following attachment of HBV to an unknown hepatocyte cell surface receptor\textsuperscript{12}. HBV relaxed circular DNA (RC-DNA) is delivered to the nucleus, where the essential virological step of HBV covalently closed circular DNA (cccDNA) formation from RC-DNA occurs. The HBV cccDNA is arranged in a viral minichromosome, and serves as the transcriptional template for viral mRNAs and pg-RNA transcripts via host RNA polymerase II\textsuperscript{27}.

Genomic mRNA and pg-RNA are exported into the cytoplasm, and from here four separate pathways of protein translation can be identified; nucleocapsid (genomic replication), precore (secretory), envelope and a viral integration pathway (Figure 1.3). These pathways are important when considering the relationships between HBeAg and HBsAg in the context of the natural history of CHB, and during antiviral therapy.
The HBV lifecycle. Four separate pathways of protein translation can be identified including nucleocapsid (core, polymerase), precore, envelope and a viral integration pathway. RC-DNA, relaxed circular DNA; DSL DNA, double stranded linear DNA; cccDNA, covalently closed circular DNA; pgRNA, pregenomic RNA; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen.

1.3.1 Nucleocapsid (genomic replicative) Pathway

The nucleocapsid genomic DNA replication pathway occurs in the cytosol, and involves the translation of pg-RNA into HBV Pol/RT and HBcAg. Following this, the crucial step of reverse transcription of viral pg-RNA into the viral DNA genome occurs. Binding of HBV reverse transcriptase to the encapsidation signal on pg-RNA triggers immature nucleocapsid formation\textsuperscript{28}. Reverse transcription of pg-RNA then occurs within the nucleocapsid, initially with formation of the minus-DNA strand, and then subsequently the positive strand, resulting in circularization of the genome, and formation of the mature HBV nucleocapsid\textsuperscript{12}. The mature HBV nucleocapsid containing RC-DNA can either be recycled back to the nucleus and converted into cccDNA, or can bud into the ER containing envelope protein for eventual extracellular secretion\textsuperscript{12}. The HBx mRNA transcript is also translated in the cytosol.
1.3.2 Precore (secretory) Pathway

HBeAg (precore/secretory) production follows a different pathway from that of the nucleocapsid (HBcAg) (Figure 1.2.3.2). The primary translation product of the precore mRNA is 25kDa in size, and is initially directed to the ER, where the first 19 amino acids are cleaved by a host signal peptidase. The 22kDa protein is subsequently transported to the Golgi, where further cleavage and post-translational modification results in the secretion from the cell of a heterogeneous 15-18kDa protein monomer known as HBeAg. Mature HBeAg is considered an accessory protein of HBV, and is secreted into the circulation. It should be noted that not all HBeAg protein is transported to the Golgi\textsuperscript{3, 29}, and an intracellular precore protein pool has been detected\textsuperscript{30}.

1.3.3 Viral Envelope Pathway

HBsAg synthesis occurs at the ER, and as discussed above the envelope ORF contains three in frame “start” codons which further divide it into preS1, preS2, and S domains. The envelope proteins are generated from two HBV mRNA transcripts, and its subsequent translation results in small (SHBs), medium (Pre-S2+S) and large surface envelope proteins (Pre-S1+Pre-S2+S). These are also known as S, M, and L surface proteins respectively (Figure 1.2). Newly synthesized envelope protein interacts with mature HBV nucleocapsids at the ER prior to secretion from the hepatocyte. However, HBsAg production far exceeds that required for virion assembly (approximately $10^3$-$10^5$ fold)$^3$, and excess surface envelope proteins are also found in the systemic circulation as non-infectious filamentous or spherical subviral particles$^31$.

1.3.4 HBV DNA Integration Pathway

Some viral proteins and products, including HBsAg, may be produced from “integration” of viral DNA into the host genome. Although viral integration is an essential component of the life cycle of retroviruses such as HIV, it is not required for normal productive hepadnaviral infection. Rather, integration of HBV DNA occurs illegitimately through recombination mechanisms using host enzymes from double-stranded linear (DSL) DNA (Figure 1.3)$^{32, 33}$. Viral integration seems to occur early in the HBV infection process. Whilst HBV integration is believed to be a random event, a high preference for integration occurs at the Direct Repeat 1 (DR1) and 2 (DR2)
sequences on the HBV genome\textsuperscript{34}. Integration may either result in inserts with sequences co-linear to the HBV genome, or highly rearranged inserts with both micro and macro deletions\textsuperscript{35}. Integrated sequences cannot provide a template for productive viral replication as a complete genome is not present\textsuperscript{36}. However, given that sequences of the S and X genes of the Enh I elements are often present in integrated segments, HBsAg and HBxAg may be produced\textsuperscript{36}. Whilst HBV integration has been considered to be involved in hepatocarcinogenesis, it is not a specific event in malignant transformation of cells, and so it is likely that additional factors are involved\textsuperscript{37}

### 1.4 Viral Genotype

There are at least eight known HBV genotypes with distinct geographical distribution (Table 1.1). HBV genotypes are defined by a >8% divergence at the nucleotide level over the entire genomic sequence. Genotypes can be further divided into sub-genotypes which differ by at least 4\%\textsuperscript{38}.

**Table 1.1 Geographical Distribution of HBV genotypes\textsuperscript{39,40}**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Predominant Geographic Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>North Western Europe, USA, Central Africa</td>
</tr>
<tr>
<td>B</td>
<td>Taiwan, Japan, China, Vietnam, Indonesia,</td>
</tr>
<tr>
<td>C</td>
<td>Taiwan, China, Japan, Vietnam, Polynesia, Korea, East Asia,</td>
</tr>
<tr>
<td>D</td>
<td>Mediterranean, India</td>
</tr>
<tr>
<td>E</td>
<td>West Africa</td>
</tr>
<tr>
<td>F</td>
<td>Central/South America</td>
</tr>
<tr>
<td>G</td>
<td>France, Germany, North America</td>
</tr>
<tr>
<td>H</td>
<td>Central America</td>
</tr>
</tbody>
</table>

There is mounting evidence which suggests that the virological and clinical manifestations of HBV may differ not only by genotype, but also sub-genotype\textsuperscript{41}. The strongest evidence exists in Asia, where HBV infection with genotypes B and C are prevalent. In comparison to genotype B, genotype C HBV infection is associated with more progressive liver disease, and a decreased response to immuno-modulatory therapy\textsuperscript{40}. The classification of HBV genotypes/sub-genotypes is complex however, and the clinical significance of different genotypes/sub-genotypes requires further study. Consequently current treatment algorithms do not recommend routine baseline genotypic analysis in guiding patient management.
1.5 Variant Viruses

Mutations affecting all of the ORFs of HBV can occur as a result of viral, host (innate and adaptive immune system), and exogenous factors such as antiviral therapy, vaccination and/or use of hepatitis B immunoglobulin (HBIG).

1.5.1 Mutations affecting HBeAg

Mutations which affect HBeAg production have been identified in the precore and basal core promoter (BCP) regions. These mutations can occur either in isolation, or in conjunction with each other depending on the HBV genotype.

The most common precore mutation is a single base substitution (G to A) at nt1896. This mutation results in a translational stop codon (codon 28: TGG; tryptophan to TAG; stop) in the highly conserved stem loop structure, known as the encapsidation signal, epsilon (ε) (see above). The secondary structure of ε is maintained by base pairing. In HBV genotypes containing thymidine (T) at nt1858 (genotypes B, D, E, G, some strains of C), the G1896A mutation is more common because the stop codon mutation helps stabilize the ε structure. In contrast, the mutation is uncommon in other genotypes (A, F, most strains of C) because cytidine (C) is located at nt1858, and so the G-C Watson Crick base pairing is preferred.

Patients chronically infected with the G1896A precore variant as the predominant species lack HBeAg in both serum and hepatocytes. A further mutation at G1899A is frequently found in association with the G1896A mutation, but the reason for this co-selection is not known. Other changes in the precore region which may prevent the translation of HBeAg include non-sense mutations at nt1814-1816, 1817, 1874 and 1897, and frameshift mutations at nt1838-9. Mutations at nt1809-1812, in the so called “Kozak” sequence (involved in ribosomal scanning), may also result in reduced HBeAg expression.

BCP variants typically involve the double mutation of A1762T and G1764A. This results in the transcriptional reduction of Pre-C/C mRNA, with up to a 70% decrease in HBeAg production compared to wild type HBV. The precore protein is thought to have an inhibitory effect on HBV replication, and thus the emergence of BCP variants has been associated with an increase in viral load.
1.5.2 Mutations affecting HBsAg

HBsAg is cleared during recovery from acute infection, and an anti-HBs immune response following vaccination is in general protective against possible subsequent infection. Anti-HBs is directed against the major hydrophilic region of the protein, which comprises amino acids 99-160. This conserved immuno-dominant B cell epitope encompasses the group specific “a” determinant (discussed previously).

Mutations affecting HBsAg have also been identified in liver transplant recipients treated with HBIG\textsuperscript{47}, multi-drug resistant viruses, and vaccine escape mutants can appear under pressure generated from vaccine associated neutralising antibodies\textsuperscript{48}. Vaccine escape isolates often show a substitution from glycine to arginine at residue 145 of HBsAg (sG145R) or aspartate to alanine/glutamine at residue 144 (sD144A/E). It is important to note that variants can be viable and infectious, and that the mutation position can also affect the Pre-S1 and Pre-S2 envelope proteins.

Significant heterogeneity exists in the Pre-S region of the HBV genome\textsuperscript{43}, and Pre-S gene deletions occur frequently in chronic HBV. A higher frequency of Pre-S deletions are seen in patients with CHB and progressive liver disease, as well as in patients with genotype C viral infection (compared to genotype B)\textsuperscript{49}.

1.5.3 Mutations affecting the HBV Polymerase

Mutations affecting the polymerase / reverse transcriptase often occur in the setting of oral antiviral therapy with nucleos(t)ide analogues (NA). In general these mutations are predictable, and are dependent on the chemical class of oral NA. This is discussed further in the section of antiviral resistance below (Chapter 1.13).

1.6 Immuno-pathogenesis of HBV Infection

The immuno-pathogenesis of hepatitis B is dependent on a complex interplay of both host and viral factors. Host factors such as age, gender and immune status are important. Perinatal and childhood infection results in chronic infection in 90-95% and 50% of cases respectively, whereas greater that 95% of immuno-competent adults with acute HBV spontaneously clear the infection. Furthermore, CHB infection is more common in immunocompromised hosts (e.g. HIV infection). Viral factors such as HBeAg status are also important.
The study of HBV immuno-pathogenesis has been limited by the lack of data in humans and cell lines that productively support HBV infection and subsequent replication. Animal models of HBV infection include chimpanzee, transgenic mice, ducks and woodchucks. Whilst these models have all contributed to the understanding of the immunopathogenesis of hepatitis B, all have inherent limitations. For example HBV infection in chimpanzees typically has a milder severity, and typically only results in self limited infection.

### 1.6.1 Early Events in HBV Infection

The HBV is a non-cytopathic virus. Acute infection in humans during the early stages following acute exposure is usually asymptomatic, and consequently little is known about the immunological events during this period. HBV has been described as a “stealth” virus which initially establishes itself without activating the immune system. This is in contrast to acute HCV infection where an immediate immune response is evident. Experimentally infected chimpanzees inoculated with HBV displayed a lag phase of up to five weeks before logarithmic expansion of intrahepatic viral HBV DNA. Furthermore, HBV did not appear to induce any transcriptionally regulated intrahepatic genes during the first few weeks of infection. Whether the results obtained in the chimpanzee model are applicable to acute HBV infection in humans remains unclear.

### 1.6.2 Acute HBV Infection

In the chimpanzee model, HBV DNA levels peak and decline (week 8 & 11 following HBV exposure) prior to the induction of a T-cell mediated response. Serum alanine aminotransferase (ALT) levels during this time may be normal or only mildly elevated. This initial reduction of viral replication is believed to be due to the production of non-cytolytic antiviral cytokines by hepatocytes, as well as cells of the innate and adaptive immune system. These antiviral cytokines include interferon gamma (IFN-γ), tumour necrosis factor (TNF-α), and interferon alpha/beta (IFN-α/β).

Subsequently, recruitment of inflammatory cells such as CD8+ T-cells results in cytolytic destruction of HBV infected hepatocytes, and this is manifested by a rise
in serum ALT levels. Data from animal models supports the concept that both non-cytolytic and cytolytic mechanisms are requisite for successful HBV viral clearance.

1.6.2.1 Coordinated Innate and Adaptive Immune Response

The innate immune system developed early in evolution, and represents an immediate, first line defense against foreign pathogens. It plays a fundamental role in regulating the adaptive immune response. In acute HBV infection, the innate immune response may initially limit the spread of HBV through production of Type-1 IFN (α/β) by infected hepatocytes. This may be mediated by toll-like receptors or other pathogen recognition receptors (PRRs). Type-1 IFN production has both an autocrine and paracrine effect, and may eventually lead to a cascade of events which results in HBV viral clearance.

Type-1 IFN production stimulates antigen presenting cells (APCs) such as dendritic cells, and Kupffer cells (resident intrahepatic macrophages) to produce interleukin-8 (IL-8), interleukin-12 (IL-12), interleukin 18 (IL-18) and other cytokines. IL-12 and IL-18 subsequently induce natural killer (NK) and natural killer T (NKT) cells. NK cells can also be activated by the major histocompatibility complex I (MHC-I) molecules on the surface of infected hepatocytes, or by recognition of stress induced molecules. NK and NKT cells can have a direct cytotoxic effect of infected hepatocytes through cell to cell contact, and can also produce inflammatory cytokines such as IFN-γ and tumour necrosis factor-α (TNF-α). These cytokines can directly inhibit HBV replication, as well as lead to upregulation of MHC-I on hepatocytes for recognition by CD8+ cytotoxic T cells.

A single nucleotide polymorphism near the gene encoding interleukin 28 (IL-28) has recently been demonstrated to be useful in predicting HCV treatment induced viral clearance. IL-28 is one of the interferon lambdas (IFN-λ) which is thought to be involved with the innate control of HCV. In patients with chronic HCV infection, this polymorphism may help account for much of the difference in response rates following pegylated interferon and ribavirin therapy between European-American, African-American and Asian patients. Significant heterogeneity at this allele has not been demonstrated in East Asians. Given that the majority of the burden of CHB infection is in the Asian population, the significance of this polymorphism in the
natural history and therapy of CHB remains to be established. Genetic variants in the HLA-DP locus have recently been suggested to have a strong association with the risk of chronic HBV infection in a cohort of Japanese patients\textsuperscript{61}. The role of this and other genetic polymorphisms require further investigation.

The adaptive immune system response is represented by B and T lymphocytes, and is only activated following recognition of HBV by the innate immune system. Specialised APCs (dendritic and Kupffer cells) present foreign HBV antigen via MHC class I and II molecules to CD8+T cells and CD4+T cells respectively. Ultimately, it is the breadth and strength of this specific adaptive immune response which determines the outcome of acute HBV infection. Animal studies have demonstrated that in self-limited HBV infection, the CD8+T cell and CD4+T cell responses to HBV proteins are strong, polyclonal and multispecific\textsuperscript{62}. Polyclonal and multispecific responses to HBV proteins refer to the presence of several receptors for each viral epitope (polyclonal), and multiple epitopes being recognised by a single T cell (multispecific).

Activated CD8+ cytotoxic T cells represent one of the main effector cells involved in HBV clearance. HBV specific CD8+ T cells responses have been shown to be directed at core, envelope/surface and polymerase\textsuperscript{63}. Other than having a direct cytolytic effect, CD8+ T cells also have a non-cytolytic effect through the production of IFN-\(\gamma\) and TNF-\(\alpha\) (also produced by NK and NKT cells). IFN-\(\gamma\) and TNF-\(\alpha\) have been reported to have an antiviral effect via multiple mechanisms\textsuperscript{64, 65}.

### 1.6.2.2 Humoral Immune Responses to HBV Proteins

Spontaneous resolution from acute HBV infection is also reliant on an effective humoral B lymphocyte response. B lymphocyte activation occurs following interaction with activated T helper II (Th2) CD4+ T cells through interaction at the MHC class II in conjunction with co-stimulatory factors. Activation results in B cell proliferation, antibody production and antibody class switching. In acute HBV infection, serum antibody responses to each of the HBV proteins (core, precore, surface/envelope, polymerase, X) have been detected. Antibody class switching refers to the mechanism that changes the B cell’s production of antibody from one class to another. Early in acute HBV, anti-HBc is predominantly of the IgM class, with IgG antibodies being produced following class switching. The IgG class of
antibodies can be further divided (subclasses IgG1 to IgG4). This IgG subclass profile has been reasonably well characterised for anti-HBc, anti-HBe and anti-HBs, with IgG1 suggested as the most prominent following vaccination, resolved infection and chronic infection (see Discussion Chapter 5)\(^{66-69}\). Whilst specific IgA antibodies directed against hepatitis B antigens have been detected\(^{70}\), their role remains uncertain. However, given that IgA is a class of antibody that has an important role in mucosal immunity (e.g. gastrointestinal tract, respiratory epithelium, genito-urinary tract), it is possible that in hepatitis B infection the production of specific IgA antibodies has a role in controlling extrahepatic reservoirs of HBV. Additionally, it should be noted that HBV has been associated with immune complex diseases including IgA nephropathy\(^{71}\).

It is generally believed that antibodies directed against HBsAg (anti-HBs) are neutralising, and play a key role in containing further spread of infection. Neutralising epitopes of the envelope protein have been mapped to the pre-S and S domains\(^{72}\). Anti-HBs production is the basis for immunity in previously vaccinated individuals. The importance of the B lymphocyte and anti-HBs production in the control of HBV infection is also highlighted by the use of immunosuppressant medications such as Rituximab. Rituximab is a humanised chimeric anti-CD20 monoclonal antibody directed against B lymphocytes, and causes a profound depletion of circulating B cells that persists for at least six months following drug withdrawal\(^{73, 74}\). Rituximab has been independently associated with a significant risk of HBV reactivation\(^{75}\), and there are now several case reports of HBV reactivation with Rituximab therapy (in the absence of antiviral prophylaxis) in patients with prior “resolved” hepatitis B (e.g. HBsAg negative, undetectable HBV DNA, anti-HBs positive, anti-HBc positive)\(^{76, 77}\).

Whilst anti-HBs production is thought to occur early in infection, it cannot usually be identified in serum using current commercial assays due to its complexing into immune aggregates with the excess envelope proteins\(^{10}\). Previous studies have detected anti-HBs using enzyme immunoassay (EIA), and have suggested that IgG1 is the predominant subclass in both recovered individuals, vaccinees, and those with chronic infection\(^{72}\).

Antibodies to the HBV core protein (anti-HBc) and precore protein (anti-HBe) are in general not believed to have a neutralising effect. However, there are reports of anti-HBc and anti-HBe blocking acute infection in chimpanzees\(^{78}\). Anti-HBc is a marker of previous exposure to HBV and can be detected during both acute and
chronic infection. Anti-HBe is detectable on current commercial EIAs after HBeAg has been cleared from the systemic circulation.

Little information is known regarding the role of antibody responses to non-structural proteins such as the hepatitis B X protein and polymerase.

1.6.3 Chronic Hepatitis B Infection

Chronic hepatitis B is defined as the persistence of HBsAg for at least six months. The underlying mechanisms leading to CHB infection have not been fully elucidated, but are likely to be multi-factorial. It is thought that the inability to control HBV infection subsequently results in the relative collapse of virus specific adaptive immunity, presumable via manipulation of the innate immune response.

Chronic HBV infection is characterized immunologically by diminished or narrowly focused CD4+T cell and CD8+T cell responses to corresponding HBV antigens. This may be due to an impairment of the innate immune response, including dendritic cell dysfunction, and reduction in toll like receptor 2 (TLR-2) receptors. Regulatory CD25+CD4+ T cells may also play a role in the development of CHB infection.

Animals models of viral infections demonstrate that the sustained presence of viral antigens may lead to a progressive functional decline of virus specific CD8+ T cell responses. In CHB, HBeAg has been shown to cross the placenta in transgenic mice, and has been suggested to act as a “toleragen”. Furthermore, subviral HBsAg particles have been suggested to facilitate evasion of HBV from the immune response through multiple mechanisms. These include absorbing any neutralising antibodies produced, prevention of viral presentation through saturation of MHC molecules, and depletion of CD4+ and CD8+T cells by a process of clonal exhaustion.
1.7 The Natural History of Chronic Hepatitis B

The natural history of CHB is typically regarded as consisting of four phases: immune tolerant, immune clearance, immune control (low replicative), and HBeAg negative hepatitis (immune escape). These phases have been identified on the basis of specific biochemical, serological and virological characteristics, including serum ALT levels, HBeAg serostatus and HBV DNA titre (Table 1.2). It is important to note that these phases do not occur in all individuals, and do not always necessarily occur sequentially. Typically patients in either the immune-clearance or immune escape phases are potential candidates for the currently approved treatments.

Table 1-2 Phases of CHB infection

<table>
<thead>
<tr>
<th></th>
<th>Immune Tolerant</th>
<th>Immune Clearance</th>
<th>Immune Control</th>
<th>HBeAg negative hepatitis (Immune Escape)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBeAg</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Anti-HBe</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>HBV DNA (IU/mL)</td>
<td>&gt; 20,000</td>
<td>&gt;20,000</td>
<td>&lt;2,000</td>
<td>&gt;2,000</td>
</tr>
<tr>
<td>Serum ALT level (U/L)</td>
<td>Persistently normal</td>
<td>Elevated (1-2X) and fluctuating</td>
<td>Normal</td>
<td>Elevated and fluctuation</td>
</tr>
<tr>
<td>Liver Histology</td>
<td>Normal or mild hepatitis</td>
<td>Moderate to severe hepatitis</td>
<td>Normal to mild hepatitis May have cirrhosis</td>
<td>Moderate to severe hepatitis May have cirrhosis</td>
</tr>
</tbody>
</table>

The immune tolerant phase is characterised by positivity for HBeAg, high levels of HBV DNA, normal serum ALT levels and normal/near normal histological profile. Patients in the immune tolerant phase are usually young, and this period can last for greater than 20 years. Usually patients progressing to chronic infection in adulthood do not undergo a prolonged immune tolerant phase, and may instead enter directly into the immune clearance phase.

When CHB infection is acquired from an early age (perinatal or early horizontal transmission), the immune clearance phase usually occurs between ages 20-40. It is characterised by an elevated serum ALT levels, fluctuating HBV DNA levels, and histologic damage. During this phase, the HBV is under intense immune
pressure, and the emergence of dominant PC and BCP variant quasispecies may occur, with eventual HBeAg seroconversion (HBeAg to anti-HBe) and control of HBV replications, or escape to HBeAg negative CHB.

Following HBeAg seroconversion, the majority of patients enter an immune control or low replicative phase, characterised by low/undetectable viral replication and normal ALT levels. A serum HBV DNA of $5 \log_{10}$ copies/mL (equivalent to 20,000 IU/mL) has been suggested to differentiate the immune control and escape phases. However, there is no absolute cut-off threshold, and thus prospective monitoring is required for determination of the phase of CHB\textsuperscript{88, 89}. A subset of patients can enter into the HBeAg negative hepatitis phase, whereby HBV DNA levels and serum ALT levels rise with consequent further histologic damage.

In general, it is thought that the extent of chronic liver disease is directly linked to the frequency, duration and severity of hepatic flares occurring in either the immune clearance and/or HBeAg negative phases. HBeAg seroconversion predicts for a sustained multi-log reduction in HBV DNA (>3 $\log_{10}$IU/mL), decrease in intrahepatic inflammatory activity and improved prognosis\textsuperscript{90}. For these reasons, HBeAg seroconversion has been traditionally used as a therapeutic endpoint in the management of HBeAg-positive CHB. Not surprisingly then, delayed HBeAg seroconversion, particularly in patients >40 years of age, is associated with higher fibrosis scores on liver biopsy.

In the context of the natural history of CHB, spontaneous HBsAg loss and/or seroconversion is rare with approximately a 1% annual rate\textsuperscript{91, 92}. However, HBsAg seroconversion is associated with a favourable long-term clinical prognosis\textsuperscript{93, 94}.

### 1.8 Extrahepatic Manifestations of HBV

Several extrahepatic manifestations are associated with HBV, and may be associated with significant morbidity and mortality. These manifestations are thought to be related to circulating immune complexes\textsuperscript{95}. The spectrum of HBV-associated extrahepatic manifestations includes: polyarteritis nodosa, glomerulonephritis, the serum sickness-like (arthritis and dermatitis) prodrome of acute HBV infection, mixed essential cryoglobulinaemia and skin manifestations such as palpable purpura\textsuperscript{95}. The treatment of each of these manifestations is different, and may for example include combination antiviral and immunosuppressive therapy in polyarteritis nodosa.
1.9 Serological Diagnosis of HBV Infection

Both acute and chronic HBV infection can be diagnosed by a characteristic serological marker profile (Figure 1.4 & 1.5, Table 1.3).

**Figure 1.4** Typical clinical and laboratory features of acute hepatitis B

![Figure 1.4](image)

**Figure 1.5** Typical clinical and laboratory features of CHB

![Figure 1.5](image)
Table 1.3. Serological Markers for HBV infection and Various Scenarios

<table>
<thead>
<tr>
<th></th>
<th>Incubation period</th>
<th>Acute Infection</th>
<th>Window phase of recovery</th>
<th>Immunity</th>
<th>Vaccine Immunity</th>
<th>Chronic Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+ (6 months)</td>
</tr>
<tr>
<td>Anti-HBs</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HBeAg</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Anti-HBe</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Anti-HBc IgM</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>± (flare)</td>
</tr>
<tr>
<td>Anti-HBc Total</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

1.9.1 Serum HBsAg and Anti-HBs

Serum HBsAg is the serological hallmark of HBV infection, and can usually be detected between 4-10 weeks after exposure. HBsAg can often be detected prior to either the onset of clinical symptoms or a rise in serum aminotransferases. Recovery from acute HBV infection is indicated by the loss of serum HBsAg, which usually occurs after four to six months.

Anti-HBs can be detected following the loss of serum HBsAg from the systemic circulation. Prior to HBsAg clearance, anti-HBs is thought to be bound with HBsAg in immune complexes. Anti-HBs has been detected in the systemic circulation in the presence of HBsAg using sensitive in-house assays. Anti-HBs is also a serological marker of previous vaccination with recombinant HBsAg.

1.9.2 HBCag and Anti-HBc

Hepatitis B core antigen (HBCag) is an intracellular protein and cannot be detected in the systemic circulation. However, antibodies to HBCag (anti-HBc) are detectable during acute hepatitis B, and are predominantly of the IgM class (anti-HBc IgM) (Figure 1.4). Anti-HBc IgM may be the only marker of HBV infection during the window period between loss of HBsAg and subsequent development of anti-HBs. It is also important to note that anti-HBc IgM may also be detected during a flare of CHB. Thus, it may be difficult at times to distinguish between patients with newly acquired HBV from those with chronic infection, as both groups are HBsAg positive, and patients may be clinically well. Although not routinely performed, rapidly rising or falling HBsAg levels are suggestive of acute HBV, in contrast to stable HBsAg levels in two serum samples at four weeks apart in the chronic phase. Newly acquired infection is more likely if the patient has a clear recent risk factor for acquisition,
negative HBsAg in last 1-2 years, and high levels of specific antibody to hepatitis B core protein (anti-HBc IgM) in the absence of prior evidence of HBV infection.

Anti-HBc IgG is an indicator of previous exposure to HBV, and persists in both resolved acute and chronic HBV. In individuals with a negative HBsAg test, and positive anti-HBs, anti-HBc IgG can differentiate between individuals with resolved HBV compared to prior vaccination.

1.9.3 HBeAg and Anti-HBe

During acute HBV infection, HBeAg is detected after HBsAg, and is considered to be an indirect marker of ongoing HBV replication and infectivity. In patients who recover from acute infection, HBeAg seroconversion usually occurs before HBsAg seroconversion.

HBeAg seroconversion may be delayed for many years in patients with CHB. HBeAg seroconversion is considered an important factor in the natural history of HBeAg-positive patients with CHB, as it may be associated with low to undetectable viral replication, remission of liver disease, and an increased likelihood of HBsAg seroconversion. Unfortunately, the current commercial serologic assays for both HBeAg and anti-HBe are qualitative only and thus can diagnose HBeAg seroconversion only after the event has occurred.

1.10 HBV Viral Load Assays

The HBV viral load is a well established key prognostic marker of liver disease progression. A large natural history study in predominantly HBeAg negative patients aged 30-65 years found that baseline HBV DNA viral load was the most important risk factor in the eventual development of cirrhosis and hepatocellular carcinoma (HCC). Accordingly, HBV DNA determination is an important parameter in patient eligibility for antiviral therapy. Furthermore, serial measurement of HBV DNA is a standard of care for on-therapy patient monitoring to assess both for antiviral efficacy, and for the potential development of antiviral resistance.

Multiple commercial HBV DNA assays are currently available, each with their own inherent advantages and disadvantages (Table 1.4). Previous deficiencies in HBV DNA testing have included the lack of standardization between assays, and limitations in dynamic range. The World Health Organisation (WHO) has now
defined an international standard for HBV DNA testing calibrated to IU/mL \(10^6\), and this should ideally be used to ensure comparability between trials, as well as between patients samples if different assays are used.

An ideal assay should have a broad dynamic range. This not only facilitates early detection of virological breakthrough, but also an adequate assessment of an antiviral treatment effect. Real time PCR assays have become recently available. Whilst still expensive, these assays have improved reproducibility compared with other technologies, and exhibit a far broader dynamic range (from \(<10\) up to \(10^9\) IU/mL). It is therefore likely that they will soon become the gold standard for HBV DNA testing, not only in clinical trials but in routine clinical practice as well.

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>BRAND</th>
<th>RANGE (IU/mL)</th>
<th>PROs and CONs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Signal amplification</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digene Capture I</td>
<td>(DIGENE CORP)</td>
<td>No conversion</td>
<td>Generally widely available, and only small sample volume required</td>
</tr>
<tr>
<td>Digene Capture II</td>
<td></td>
<td>1.0×10^5 – 3.4×10^8</td>
<td>Poor lower range of quantification</td>
</tr>
<tr>
<td>Ultrasensitive</td>
<td></td>
<td>1.6×10^3 – 1.0×10^8</td>
<td></td>
</tr>
<tr>
<td>Digene Capture II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Versant HBV 3.0</td>
<td>(Siemens)</td>
<td>3.5×10^2 – 1.8×10^7</td>
<td>No sample extraction required. Reduced risk of sample contamination.</td>
</tr>
<tr>
<td><strong>Target amplification (PCR)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COBAS</td>
<td>AMPLICOR (Roche)</td>
<td>5×10^1 – 4×10^4</td>
<td>Rapid turnaround</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Limited upper range of quantification, may require dilution</td>
</tr>
<tr>
<td><strong>Real time PCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COBAS Taqman</td>
<td>(Rocher)</td>
<td>3×10^1 – 1.1×10^8</td>
<td>Requires larger sample volume Broad dynamic range</td>
</tr>
<tr>
<td>RealTime HBV</td>
<td>(ABBOTT)</td>
<td>1×10^1 – 1.0×10^9</td>
<td></td>
</tr>
<tr>
<td>Real Art</td>
<td>(QIAGEN)</td>
<td>2×10^1 – 1.0×10^8</td>
<td></td>
</tr>
</tbody>
</table>
1.11 Quantitative Serum HBsAg and HBeAg Assays

The current commercial serological assays for both serum HBsAg and HBeAg are qualitative. Recently, sensitive and reliable assays have been developed to quantify both serum HBsAg and HBeAg. The reported diagnostic assays for HBeAg and HBsAg quantification are outlined in Table 1.5.

<table>
<thead>
<tr>
<th>Type of assay</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electro-immunodiffusion (QIE Laurell method)</td>
<td>A combination of two techniques: immunoprecipitation and electrophoresis. Serum samples are loaded onto an agarose gel containing relevant antibody (HBe or HBs). Precipitates formed in electrophoresis can then be quantified against a known standard</td>
</tr>
</tbody>
</table>

**Immunoassays**

- **Sandwich radioimmunoassay**<sup>102</sup> HBeAg from serum can bind to human polyclonal anti-HBe coated beads. Incubation with human polyclonal<sup>125</sup>-anti-HBe can detect and quantify HBeAg.

- **ARCHITECT platform (Abbott Laboratories, IL, USA)**<sup>103</sup> A two step chemiluminescent microparticle immunoassay which initially involves the combination of sample (serum/plasma) with either HBs or HBe coated paramagnetic microparticles. Following washing, the second step involves addition of acridinium labeled antibody conjugate. The resulting chemiluminescent reaction is measured as relative light units.

- **Elecsys® HBsAg II (Roche Diagnostics)**<sup>104, 105</sup> Sandwich complex formed by serum sample with two biotinylated monoclonal HBsAg specific antibodies, a mixture of monoclonal HBsAg-specific antibody, and polyclonal antiHBsAg antibodies labeled with a ruthenium complex. Following the addition of streptavidin-coated microparticles, the complex binds to the solid phase via interaction of biotin and streptavidin. Application of a voltage to the electrode then induces chemiluminescent emission, which is measured by a photomultiplier.

The ARCHITECT platform is the most widely reported of these assays. It is fully automated and has a high throughput capacity. The ARCHITECT quantitative HBsAg assay is internally calibrated using the WHO standard for HBsAg<sup>103</sup>. Quantitative HBsAg levels are reported in IU/mL, with a dynamic range of 0.05-250 IU/ml. Given that most serum HBsAg titres are above this range, samples are usually initially diluted prior to testing. It should be noted that a HBsAg titre 0.1 IU/mL is equivalent to approximately 0.1ng of HBsAg per mL<sup>106</sup>. This in turn is equivalent to $2 \times 10^7$ subviral HBsAg particles or $5 \times 10^6$ virions (based on a virion area which is four times that of a HBsAg subunit particle)<sup>106</sup>.

On the ARCHITECT platform, the HBeAg assay is designed to be semiquantitative, and is reported as sample / cutoff ratio (S/CO). However, it has a reasonable linear range, and can be further modified and optimized for use as a quantitative assay by reference to an external standard, such as the Paul Ehrlich
Institute (Langen, Germany) reference standard for HBeAg (results expressed in PE IU/mL)\textsuperscript{107, 108}.

1.12 The Management of HBV Infection

1.12.1 Acute HBV Infection

The clinical spectrum of acute HBV infection ranges from asymptomatic to fulminant infection (<1% of cases). Prodromal symptoms of acute HBV infection are variable, and may include anorexia, nausea and vomiting, fatigue, malaise, arthralgia, myalgia and headache. These symptoms may precede the onset of clinical jaundice by one to two weeks.

Acute HBV infection results in spontaneous resolution in the majority of immuno-competent adults (>95%). Consequently, antiviral therapy is usually not indicated. Clinical trials evaluating the benefit of oral lamivudine in fulminant acute HBV infection have shown conflicting results\textsuperscript{109, 110}. However, a trial of oral nucleos(t)ide therapy in fulminant HBV infection cases is a reasonable therapeutic option, although this is not addressed in most professional practice guidelines.

1.12.2 Chronic HBV Infection

1.12.2.1 Goals of Treatment for Chronic HBV Infection

The major long term goal of antiviral treatment in the management of CHB is to prevent or delay the onset of complications associated with infection. Given that active viral replication is a well established marker for the development of liver related complications such as cirrhosis and HCC, a cornerstone of HBV therapy is to achieve complete and durable suppression of the HBV DNA viral load\textsuperscript{111, 112}.

1.12.2.2 Indications for Antiviral Therapy

A number of regional consensus HBV treatment guidelines including Asia-Pacific (APASL), European (EASL), and American consensus guidelines (AASLD) have been published\textsuperscript{85, 86, 113, 114}. Serum ALT levels and HBV DNA titres, as well as the degree of histological liver disease are important variables which may influence the treatment algorithm. Subtle differences exist between the regional consensus guidelines, and may be in part due to cost, availability, and possibly the prevalence of
particular HBV genotypes between the different regions. For example, in HBeAg positive patients, the EASL guidelines state that patients should be considered for treatment when the viral load is above 2,000 IU/mL, and/or the serum ALT level is above the upper limit of normal for the laboratory, and the liver biopsy shows moderate inflammation or fibrosis. In contrast, for HBeAg positive patients, the APASL guidelines state that a definite criteria for treatment is when the viral load is above 20,000 IU/mL and the serum ALT level is 2-5 times the upper limit of normal. The AASLD guidelines do not state a viral load threshold at all in HBeAg positive patients, and only require a serum ALT level above two times the upper limit of normal.

The APASL consensus statement for the management of CHB was recently developed by a panel of regional experts, and has incorporated new data on the natural history of CHB (Table 1-6 & 1-7). In general, patient candidates for antiviral therapy may be classified into three groups: HBeAg positive CHB, HBeAg negative CHB, and patients with cirrhosis (may be either HBeAg positive or negative)
### Table 1-6 Asia-Pacific HBV treatment algorithm for patients with compensated disease

<table>
<thead>
<tr>
<th>HBV DNA (IU/mL)</th>
<th>ALT</th>
<th>Treatment strategy</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 20,000</td>
<td>Normal</td>
<td>No treatment Monitor every 3-6 months – HBV DNA, HBeAg, ALT</td>
</tr>
<tr>
<td>&gt; 20,000</td>
<td>Normal</td>
<td>No treatment Monitor every 3-6 months – HBV DNA, HBeAg, ALT Consider biopsy if &gt;40 years – treat if moderate inflammation or fibrosis</td>
</tr>
<tr>
<td>HBeAg Positive</td>
<td>&gt; 20,000</td>
<td>1-2× ULN</td>
</tr>
<tr>
<td></td>
<td>&gt; 20,000</td>
<td>2-5× ULN</td>
</tr>
<tr>
<td></td>
<td>&gt; 20,000</td>
<td>&gt;5× ULN</td>
</tr>
<tr>
<td>HBeAg Negative</td>
<td>&lt; 2,000</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>&gt; 2,000</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>&gt; 2,000</td>
<td>1-2× ULN</td>
</tr>
<tr>
<td></td>
<td>&gt; 2,000</td>
<td>&gt;2× ULN</td>
</tr>
</tbody>
</table>

Patients on treatment should have 3 monthly HBV DNA and ALT monitoring to assess response. Patients at risk of HCC should have 6 monthly screening with alpha fetoprotein (AFP) and ultrasound. Adapted from Liaw et al., with kind permission from Springer Science and Business Media.

<table>
<thead>
<tr>
<th>HBV DNA (IU/mL)</th>
<th>Recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compensated</td>
<td></td>
</tr>
<tr>
<td>&lt; 2,000</td>
<td>No treatment Monitor every 3-6 months – HBV DNA, HBeAg, ALT</td>
</tr>
<tr>
<td>&gt; 2,000</td>
<td>Treatment indicated irrespective of ALT level First line options include ETV, LMV, LdT Adefovir and IFN based therapies can also be considered if no previous hepatic flare</td>
</tr>
<tr>
<td>Decompensated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Consider liver transplantation Treatment indicated First line options include ETV, LMV, LdT, ADV IFN contraindicated</td>
</tr>
</tbody>
</table>

Adapted from Liaw et al., with kind permission from Springer Science and Business Media.

IFN, interferon; ETV, entecavir; LMV, lamivudine; LdT, telbivudine; ADV, adefovir
1.12.2.3 Antiviral Therapy and its Impact on the Natural History of CHB

The two major treatment strategies in CHB include either oral NA therapy, or immune-modulator therapy with pegylated interferon-α (Peg-IFNα) or standard interferon-α.

Currently licensed oral NA therapy for CHB includes lamivudine (LMV), adefovir (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir (TDF). The major licensing trials for these therapies have all demonstrated superior virological, biochemical and histological improvement in comparison to untreated controls or existing standard of care\textsuperscript{111}. Oral antiviral therapy has also been shown to significantly reduce the incidence of hepatic decompensation in patients with advanced fibrosis or cirrhosis\textsuperscript{112}. Furthermore, treatment with either Peg-IFNα or oral NA therapy has been associated with a reduction in the risk of HCC\textsuperscript{115}.

HBeAg positive CHB

The initial therapeutic endpoint in HBeAg positive patients is HBeAg seroconversion, as it is usually associated with suppression of viral replication and an improved prognosis\textsuperscript{90}. The HBeAg seroconversion rate following 48 weeks of therapy is approximately 20% with oral NA, and 32% with Peg-IFNα\textsuperscript{111, 116}. HBeAg seroconversion rates can increase with ongoing oral NA use, and the beneficial effects of Peg-IFNα can persist after treatment. In general, following HBeAg seroconversion with oral NA therapy, an additional consolidation treatment period of six to twelve months is recommended to improve the durability of the virological response. Disease relapse with either reappearance of HBeAg and HBV DNA or evolution into the HBeAg negative hepatitis phase appears to be more common in responders to oral NA therapy than in those responding to immune modulator therapy\textsuperscript{117}.

In HBeAg positive patients treated with 48 weeks of continuous therapy, the major registration trials of LMV, ADV, ETV and TDF have reported HBsAg seroclearance rates of 1%, 0%, 2% and 3.2% respectively\textsuperscript{113, 118, 119}. More prolonged use of oral NA therapy can increase these rates, with ETV and TDF being associated with to 5% and 6% HBsAg seroclearance rates following 96 weeks of continuous use respectively\textsuperscript{120, 121}, and around 8% by week 144 with TDF\textsuperscript{122}. However, in both of these studies involving ETV and TDF, the factors associated with a higher likelihood
of HBsAg loss were Caucasian patients and those with HBV genotype A and D (i.e. “non Asian” HBV genotypes)\textsuperscript{123, 124}.

**HBeAg negative CHB**

In HBeAg negative disease, there is no agreed therapeutic endpoint due to the high rate of relapse following therapeutic drug cessation. Consequently, long term oral NA is currently recommended to effectively suppress HBV viral replication, a strategy which may increase the risk of antiviral resistance emerging over time. Whilst, Peg-IFN\(\alpha\) therapy results in a 63\% undetectable HBV DNA rate at end of therapy, only 19\% of patients continue to have adequate suppression of viral replication six months after treatment cessation\textsuperscript{125}.

In CHB the preferred therapeutic endpoint in both HBeAg positive and negative CHB is HBsAg seroconversion. In clinical practice HBsAg loss/seroclearance is not always accompanied by the detection of anti-HBs on current routine commercial immunoassay. Nevertheless, HBsAg seroclearance is still believed to represent successful immunological control of HBV, and is also associated with an improved long term outcome\textsuperscript{93, 126, 127}. A recent longitudinal study evaluating the clinical outcome of HBsAg seroclearance has identified age as an important factor in terms of the risk of HCC\textsuperscript{128}. This study followed 298 patients, and demonstrated that HBsAg seroclearance before the age of 50 was associated with both a lower risk of HCC development, and a lower risk of significant fibrosis on transient elastography in comparison to later HBsAg seroclearance (>50 years)\textsuperscript{128}. Such results may influence current treatment guidelines for CHB, especially in the Asia Pacific region.

As discussed previously, in CHB the annual rate of spontaneous HBsAg seroclearance is 1-2\%. Treatment with Peg-IFN\(\alpha\) results in HBsAg seroclearance rates of 3\% following 48 weeks of therapy\textsuperscript{129}. Furthermore, in long term virological responders to Peg-IFN\(\alpha\) therapy, HBsAg loss can still occur whilst off therapy, highlighting the ongoing positive immune-modulating effects of interferon\textsuperscript{111, 130}. In comparison to HBeAg positive CHB, HBsAg seroclearance rates with oral NA are much lower in HBeAg negative CHB. The rate of HBsAg seroclearance has been reported as approximately 0.3\% following 48 weeks of ETV\textsuperscript{131}, and 0\% following 96 weeks of TDF\textsuperscript{132}.
Clinical trials have suggested a benefit in monitoring baseline and on-treatment serum HBsAg titres during Peg-IFNα therapy in predicting virological responses\textsuperscript{133-135}. However, the on-treatment changes in HBsAg titre have not been well characterised with oral NA therapy (see Chapter 4).

### 1.12.2.4 Combination Therapy

Combination antiviral therapy is the mainstay of treatment in HIV, where drugs with different mechanisms of action and targets are used to minimise the development of resistance. In chronic HCV infection, combination therapy with Peg-IFNα and ribavirin has a synergistic effect, both in improving sustained virological response rates, and reducing long term relapse rates. The landscape of chronic HCV treatment is further evolving with the recent studies evaluating specific antiviral protease and polymerase inhibitors\textsuperscript{136}. However, although combination therapy in CHB remains a focus of research, there is still insufficient data to support its use as first line therapy.

A number of studies have evaluated the combination of IFNα and LMV \textit{versus} LMV or IFNα monotherapy in CHB \textsuperscript{116, 125, 137, 138}. Whilst the on-treatment viral suppression was greater in the combination group, there was no significant difference in the sustained off-treatment responses when compared to the IFNα monotherapy arm. Furthermore, combination IFNα and LMV has not been proven to be more efficacious in previous IFNα non-responders\textsuperscript{139}.

A potential benefit of combination oral NA therapy is the reduction of drug resistance. However the combination of LMV and telbuvudine (LdT), which are both L-nucleosides and thus compete for the same cell transporters and phosphorylation systems, has been proven to be inferior to LdT monotherapy in all parameters of response\textsuperscript{140}. Another trial comparing LMV \textit{versus} lamivudine/adenosvir combination revealed no improvement in HBeAg seroconversion rates at week 104\textsuperscript{141}. However, the combination of lamivudine/adenosvir was associated with greater suppression of viral replication, and lower rates of resistance to LMV.

More potent antiviral agents with a high genetic barrier to the development of antiviral resistance such as ETV and TDF are now available. Entecavir monotherapy as primary therapy is associated with only 1.2% genotypic resistance following five years of continuous monotherapy\textsuperscript{142}. With the availability of these highly potent
drugs which have a high genetic barrier to resistance, there is currently insufficient need to warrant routine combination therapy as initial first line therapy at present.

### 1.13 Antiviral Drug Resistance

#### 1.13.1 Pathways of Antiviral Drug Resistance

Three structural classes of NAs are currently approved for therapy against HBV: L-nucleosides, acyclic phosphonates, and D-cyclopentane group. Classification of NAs by the chemical structure and/or orientation of the sugar side-chain is useful for predicting the common pathways of antiviral resistance (Table 1.8). Rescue add-on therapy can then be determined based on the cross-resistance profile of the mutations detected in the HBV DNA isolated from the patient’s blood. Eight HBV polymerase substitutions (Table 1.8) have been shown to be involved in the selection of primary antiviral resistance to these NAs. These substitutions are involved in a number of pathways of viral evolution associated with antiviral drug resistance. The annual rates of resistance to oral NA therapy are outlined in Table 1.9.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Resistance Mutations</th>
<th>Associated Resistance</th>
<th>Cross Resistance Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Nucleoside</td>
<td>rtM204V/I</td>
<td>Lamivudine (LMV)</td>
<td>R            S            S            S            I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Emtricitabine (FTC)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Telbivudine (LdT)</td>
<td></td>
</tr>
<tr>
<td>Acyclic Phosphonate</td>
<td>rtN236T</td>
<td>Adefovir (ADV)</td>
<td>S            R            I            S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tenofovir (TDF)</td>
<td></td>
</tr>
<tr>
<td>“Shared”</td>
<td>rtA181T</td>
<td>L-Nucleosides</td>
<td>R            R            I            S</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acyclic phosphonates</td>
<td></td>
</tr>
<tr>
<td>Naive Entecavir</td>
<td>rtL180M + rtM204V</td>
<td>Entecavir (ETV)</td>
<td>R            S            S            S            R</td>
</tr>
<tr>
<td>Resistance</td>
<td>with one of</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rtT184, S202 or M250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multi-drug Resistance</td>
<td>rtA181T + rtL233V +</td>
<td>Multi-drug</td>
<td>*consult reference laboratory</td>
</tr>
<tr>
<td></td>
<td>rtN236T + rtM250L</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* R, resistant; S, sensitive; I, intermediate
Table 1.9 Annual resistance rates for oral antiviral agents in chronic hepatitis B

<table>
<thead>
<tr>
<th>DRUG</th>
<th>Cumulative resistance rate (% of patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 yr</td>
</tr>
<tr>
<td>Lamivudine (LMV)</td>
<td>23</td>
</tr>
<tr>
<td>Adefovir (HBeAg neg)</td>
<td>0</td>
</tr>
<tr>
<td>Entecavir</td>
<td>0.2</td>
</tr>
<tr>
<td>Emtricitabine</td>
<td>13</td>
</tr>
<tr>
<td>Telbivudine (HBeAg pos)</td>
<td>4.4</td>
</tr>
<tr>
<td>(HBeAg neg)</td>
<td>2.7</td>
</tr>
<tr>
<td>*Tenofovir</td>
<td>0</td>
</tr>
</tbody>
</table>

Adofovir (LMV resistant)     | 0-18 | 38.3  | -     | -     | -    |
| Adefovir/LMV (LMV resistant)| 1    | 2     | 4     | 4     | -    |
| Entecavir (LMV resistant)   | 6    | 15    | 35    | 39.5  | -    |

*Week 72 switch to Truvada (Tenofovir 300mg and Emtricitabine 200mg) for all patients viraemic on TDF.

1.13.2 Monitoring and Diagnosis of Antiviral Drug Resistance

Antiviral drug resistance is becoming a major clinical challenge for physicians treating patients with CHB. The emergence of antiviral resistance can be indicated by a number of factors including: an increasing HBV viral load despite compliance ($\geq 1.0 \log_{10} \text{IU/mL}$ increase in viral load from nadir), identification of genotypic markers associated with antiviral resistance, increasing serum ALT levels, and clinical deterioration such as hepatic decompensation.

Patients should have regular three to six monthly assessments to monitor clinical status and compliance with therapy, as well as determination of HBV viral load and serum ALT levels. Renal function should be assessed in patients on adefovir or tenofovir therapy, and HBeAg positive patients should be monitored for HBeAg seroconversion.

In clinical practice, serum HBV DNA testing with sensitive assays is the most effective way to monitor antiviral efficacy and patient compliance. A reduction in serum HBV DNA always precedes biochemical and histologic response, and conversely virologic breakthrough always precede loss of these responses (see Table 1.10 & 1.11 for definitions). Regular HBV DNA testing allows determination of an initial antiviral treatment effect and subsequently durability, and monitoring for the development of virologic breakthrough.
Table 1.10 Definitions of response during treatment of CHB\textsuperscript{111, 152}

<table>
<thead>
<tr>
<th>Response Type</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiviral treatment effect</td>
<td>A $\geq 1 \log_{10}$ IU/mL reduction of HBV DNA from baseline levels during therapy and within 3 months of treatment initiation</td>
</tr>
<tr>
<td>Virologic response</td>
<td>A decrease in serum HBV DNA to undetectable levels by sensitive PCR assays, with HBeAg seroconversion in patients who were initially HBeAg positive</td>
</tr>
<tr>
<td>Biochemical response</td>
<td>Normalisation of serum ALT levels</td>
</tr>
<tr>
<td>Histologic response</td>
<td>A decrease in histologic activity index by at least 2 points, and no worsening of fibrosis score when compared to pre-treatment biopsy.</td>
</tr>
</tbody>
</table>

Table 1.11 Definitions related to antiviral resistance during the treatment of CHB\textsuperscript{111, 152}

<table>
<thead>
<tr>
<th>Response Type</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antiviral treatment failure</td>
<td>The failure of an oral NA to initially reduce HBV DNA by $\geq 1 \log_{10}$ IU/mL within 3 months of treatment initiation</td>
</tr>
<tr>
<td>Virologic breakthrough</td>
<td>An increase in serum HBV DNA by $\geq 1 \log_{10}$ IU/mL above nadir after achieving a virologic response during continued treatment</td>
</tr>
<tr>
<td>Secondary antiviral treatment failure</td>
<td>A rebound of HBV replication of $\geq 1 \log_{10}$ IU/mL in patients with an initial antiviral treatment effect, as confirmed by two consecutive laboratory assessments one month apart</td>
</tr>
<tr>
<td>Genotypic resistance</td>
<td>The \textit{in vitro} detection of mutations shown to confer resistance to NA in question</td>
</tr>
<tr>
<td>Phenotypic resistance</td>
<td>\textit{In vitro} confirmation that the detected mutation decreases susceptibility to the NA in question (indicated by increased inhibitory concentration)</td>
</tr>
</tbody>
</table>

Following commencement of antiviral therapy, a week 12 HBV viral load measurement is important to assess for either an initial antiviral treatment effect, or primary antiviral treatment failure. Consideration of switching drug therapy should be given with primary antiviral treatment failure.

Following this, measurement of viral load at week 24 is a key time-point to reassess therapy and characterise virologic responses. It should be noted that week 48 data may be more useful with drugs of lower potency, such as adefovir, if they have a high genetic barrier to resistance. Nonetheless, patients with suboptimal responses at week 24 may be at increased risk of developing antiviral resistance especially if the agent is a low genetic barrier drug, and consideration should be given to add-on therapy\textsuperscript{114}.

A virological breakthrough is defined by an increase in serum HBV DNA by $>1 \log_{10}$ IU/mL above nadir after achieving a virologic response during continued treatment\textsuperscript{111}. Given virological breakthrough may occur months before a biochemical breakthrough\textsuperscript{111}, vigilant viral load monitoring provides the opportunity for early initiation of salvage therapy. This is particularly important in high risk groups, such
as patients with cirrhosis, in whom a hepatitis flare can be complicated by hepatic decompensation and even death.

Patients compliant with therapy who develop virological breakthrough should ideally be tested for genotypic resistance in order to identify any possible drug resistant viruses. Genotypic resistance is defined as the detection of mutations that have been demonstrated in vitro to confer resistance to the NA in question. The detection of these mutations facilitates appropriate decision making with regards to salvage “add-on” therapy. It is important to note, however, that in clinical trials up to 30-50% of patients with virological breakthrough do not harbour resistant mutations\textsuperscript{153}. This is usually attributable to poor compliance with therapy, and highlights the importance of patient education and counselling both prior and during antiviral therapy.

1.13.3 Management of Antiviral Drug Resistance

There are a number of published consensus guidelines outlining the management of antiviral resistance (Figure 1.6)\textsuperscript{111, 114}. These have focused on recommendations for changing or switching therapy in patients who have developed resistance to monotherapy. Whilst these recommendations are useful, some exceptions to the general rules exist, and consequently clinicians need to understand the patterns of drug resistance with HBV infection so as to be able to determine the most appropriate salvage add-on treatment.

As a general principle, salvage “add-on” or “switch” to drugs with similar cross resistance profiles should not be used (Table 1.8). The advantage of “add-on” therapy is that continuation of the original drug will have an ongoing effect on wild type virus, and that the combination of two drugs may reduce the replication fitness of virus. The potential disadvantage is that secondary compensatory mutants may develop over time.
FLOW CHART FOR MANAGING DRUG RESISTANCE

**Prevention of resistance**
- Avoid unnecessary treatment
- First line therapy should have high potency with low rate of drug resistance
- Adherence to therapy important

↓↓ ↓↓

**Monitoring**
- 3 monthly monitoring of HBV DNA with sensitive assay, ALT, HBeAg status, clinical status
- Alternative therapy in patients with primary antiviral treatment failure
- In patients with virologic breakthrough, check compliance, and confirm resistance with genotypic testing

↓↓ ↓↓

**Timing of salvage therapy**
- Earlier initiation of salvage therapy better

<table>
<thead>
<tr>
<th>LMV</th>
<th>AASLD Guidelines</th>
<th>US Guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add ADV or TDF</td>
<td>Add ADV (preferable to switch)</td>
<td>Switch to ETV (increased risk of subsequent ETV failure)</td>
</tr>
<tr>
<td>Switch to Truvada*</td>
<td>In future, potential add-on TDF or switch to Truvada</td>
<td></td>
</tr>
<tr>
<td>Switch to ETV (pre-existing LMV-R resistance predisposes to ETV-R)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ADV</th>
<th>AASLD Guidelines</th>
<th>US Guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add LMV</td>
<td>Add LMV (preferable to switch)</td>
<td>Switch to ETV (if no prior LMV resistance)</td>
</tr>
<tr>
<td>Switch to Truvada*</td>
<td>In future, potential switch to Truvada</td>
<td></td>
</tr>
<tr>
<td>Switch to or add ETV</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ETV</th>
<th>AASLD Guidelines</th>
<th>US Guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Switch to or add ADV or TDF</td>
<td>Add or switch to ADV or TDF</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LdT</th>
<th>AASLD Guidelines</th>
<th>US Guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add ADV or TDF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Switch to Truvada*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Switch to ETV (pre-existing LdT-R resistance predisposes to ETV)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

LMV, lamivudine; ADV, adefovir; ETV, entecavir; TDF, tenofovir, LdT, telbivudine
* Truvada (combination emtricitabine 200mg and tenofovir 300mg)

**Figure 1.6** Management of antiviral resistant HBV
1.14 Prevention of HBV Infection

HBV infection is a vaccine preventable disease. Target groups for vaccination in Australia are outlined in Table 1.12. The introduction of universal infant hepatitis B vaccination has had a profound impact on reducing the incidence of new hepatitis B cases. However, it is important to note that vaccination will only prevent new cases rather than reduce the current burden of chronic HBV infection. The currently used recombinant HBV vaccine is safe, has a high efficacy, and requires three doses

Table 1.12 Target Groups for Vaccination in Australia

<table>
<thead>
<tr>
<th>Part of Australian National Immunisation Program</th>
<th>Individuals exposed to community groups with a high prevalence of HBV infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Infants</td>
<td>- Injecting drug users</td>
</tr>
<tr>
<td>- Adolescents aged between 10 and 13 years</td>
<td>- Prison inmates</td>
</tr>
<tr>
<td></td>
<td>- Household contacts of people with HBV infection</td>
</tr>
<tr>
<td></td>
<td>- Men who have sex with men</td>
</tr>
<tr>
<td></td>
<td>- Female commercial sex workers</td>
</tr>
<tr>
<td></td>
<td>- Frequent/long term travellers to endemic areas</td>
</tr>
<tr>
<td></td>
<td>- Aboriginal and Torres Strait Islander people</td>
</tr>
</tbody>
</table>

The prevention of HBV infection also includes avoidance of high risk practices which may lead to transmission either sexually or via contaminated blood (e.g. injecting drug use). Patients with CHB should use strict precautions such as barrier protection, and their partner/s should be screened for HBV and vaccinated.

1.15 Conclusion

CHB infection represents an important public health issue both in Australia and worldwide. The HBV is one of the most successful human pathogens, with two billion people globally showing serological evidence of either past or current infection. Whilst serological assays can also help classify the phase of CHB infection, they do not adequately reflect the underlying dynamic changes occurring during the different phases of infection. Commercial assays for HBsAg and HBeAg are traditionally qualitative only, and assays for anti-HBs and anti-HBe generally only become positive once their respective antigens are cleared from the circulation. Recently, sensitive and reliable assays have also been developed to quantify both serum HBsAg and HBeAg.

Although significant advances have been made into the understanding of the HBV pathogenesis and viral life cycle, a number of critical questions warrant further
evaluation. These include the determination of the baseline HBsAg titres in different phases of CHB infection, analysis of the changes in HBsAg titre during oral NA therapy, as well as detection of corresponding anti-HBs and anti-HBe in the presence of co-existing corresponding antigen. This may in future lead to further classification of the natural history of CHB infection, and refinement of clinical management algorithms including individualisation of therapy.

1.16 Aims of this Project

The aims of this project are:

1) To determine the serum HBsAg titres during the different phases of the natural history of CHB (Chapter 3)

2) To evaluate the changes in serum HBsAg titres during long term therapy with oral NAs (Chapter 4)

3) To determine whether the production of anti-HBe and anti-HBs can be detected in the systemic circulation using sensitive assays, and what role this could have determining the current phase of CHB infection (Chapter 5)
Chapter 2. Materials and Methods

2.1 Introduction
General materials and methods are outlined in this Chapter.

2.2 Material Providers

Abbott Laboratories Chicago, IL USA
Abcam Cambridge UK
Siemens Healthcare Diagnostics Tarrytown, NY USA
BD Biosciences North Ryde, NSW Australia
BioMerieux Marcy l'Etoile France
CSL Biotherapies Parkville, VIC Australia
Dako Glostrup Denmark
eBioscience San Diego, CA USA
FujiFilm Tokyo Japan
GlaxoSmithKline (GSK) Rixensart Belgium
Invitrogen Carlsbad, CA USA
Miltenyi Biotec North Ryde, NSW Australia
MO BIO Laboratories Carlsbad, CA USA
QIAGEN Valencia, CA USA
Roche Diagnostics Mannheim Germany
SigmaAldrich St Louis, MO USA
Thermo Scientific Scientific Rochester, NY USA
2.3 Patient Recruitment

The patient cohorts described in this thesis were recruited from three separate clinical sites: 1) St Vincent’s Hospital, Melbourne, Australia (Unit Head – Associate Professor Paul Desmond); 2) Liverpool Hospital, Sydney, Australia (Unit Head – Dr Miriam Levy); and 3) The Royal Children’s Hospital, Melbourne, Australia (Unit Head – Associate Professor Winita Hardikar). This studies were approved by the each respective hospital’s Human Research and Ethics Committee, in compliance with the Declaration of Helsinki.

All patients and individuals tested negative for laboratory markers of infection with the HCV, hepatitis D virus and human immunodeficiency virus (HIV). Markers for co-existent auto-immune or metabolic liver disease were also negative.

Patient demographics, liver biochemistries, qualitative HBsAg and HBeAg status (by standard qualitative EIA), HBV DNA (IU/mL), and HBV genotype were recorded. Biochemical and virological data were obtained from patient serum samples collected on the same day.

2.3.1 St Vincent’s Hospital, Melbourne, Australia

Treatment naïve patients (n=175) with CHB were recruited from the specialist liver clinics of St Vincent’s Hospital, Melbourne. Patients from St Vincent’s Hospital were also recruited at the time of their liver biopsy as part of routine medical care. A second pass liver biopsy was also obtained in these patients as part of a separate clinical study (Chapter 2.4.8). A venepuncture was performed for routine blood tests for haematology, biochemistry, HBV serology and HBV DNA. An additional 40mL of bloods was taken for peripheral blood mononuclear cell (PBMC) collection (Chapter 2.4.9), as well as for quantitative HBsAg and HBeAg testing (Chapter 2.4.3 and 2.4.4), and sequencing of the HBV basal core promoter (BCP) and precore (PC) regions (Chapter 2.4.7).

Patients were clinically stable, with no evidence of decompensated liver disease at baseline. The serum alpha fetoprotein level was normal, and ultrasound examination of the liver demonstrated no evidence of HCC in any patient at the time of study.
2.3.2 Liverpool Hospital, Sydney, Australia

Treatment naïve patients and individuals (n=70) with CHB were recruited from the specialist liver clinics of Liverpool Hospital, Sydney. This was a cohort of treatment naïve pregnant women with CHB. Testing of routine biochemistries, HBV serology and HBV DNA were performed at Liverpool Hospital. Testing of HBsAg titres (Chapter 2.4.3) was performed at The Victorian Infectious Diseases Reference Laboratory (VIDRL) in Melbourne.

2.3.3 The Royal Children’s Hospital, Melbourne, Australia

Virological and biochemical data were retrospectively assessed from a paediatric cohort (<18 years of age) of treatment naïve patients/individuals (n=38) from The Royal Children’s Hospital, Melbourne. This cohort was predominantly HBeAg positive, and data included both cross-sectional and longitudinal follow up samples. The majority of baseline serum ALT levels in this cohort were elevated, suggesting that these patients were in the immune clearance phase of CHB. The HBsAg titres (Chapter 2.4.3) of patients in this cohort were tested at VIDRL, and this data was analysed separately to the adult cohort from St Vincent’s and Liverpool Hospital. This is because there is very little literature on HBsAg titres in the paediatric population, and there may be different factors which affect HBsAg titres in this group.

2.4 Analysis of Clinical Samples

2.4.1 HBV DNA Analysis

The HBV DNA testing for the patients recruited from St. Vincent’s Hospital, and The Royal Children’s Hospital was performed by the Molecular Microbiology Department at VIDRL. Initial HBV DNA viral load testing was performed using the Versant HBV DNA 3.0 (Siemens Healthcare Diagnostics, Tarrytown, NY, USA), according to the manufacturer’s instructions (dynamic range $3.5 \times 10^2$ – $1.8 \times 10^7$ IU/mL). Samples with a viral load above the upper limit of the dynamic range were diluted by 1 in 1000 to obtain a defined titre within the range of the bDNA assay. The ARCHITECT HBsAg Manual Diluent (Abbott Laboratories, IL, USA) was used to
dilute patient sera. In some situations, samples were reflexed into the Abbott RealTime HBV test (Abbott Molecular, IL, USA) when the HBV DNA titre was below the lower limit of detection on the bDNA assay.

The HBV DNA testing for patients recruited from Liverpool Hospital, Sydney between 2002 and 2005 was performed using the COBAS AMPLICOR MONITOR HBV test (Roche Diagnostics, Mannheim, Germany). HBV viral loads above the linear range were determined by dilution, as recommended by the manufacturer. From October 2005, the method of HBV DNA quantitation changed to the COBAS TaqMan HBV test with High Pure extraction (Roche Diagnostics), and from November 2007, the COBAS AmpliPrep–COBAS TaqMan HBV test (Roche Diagnostics) was used.

### 2.4.2 Qualitative Measurement of Serum HBV Markers

Enzyme-linked immunoassay was used to measure HBsAg (VIDAS Ultra, bioMerieux, France), HBeAg and anti-HBe (AxSYM; Abbott Laboratories, IL, USA) according to each manufacturer’s instructions. This testing on the St Vincent’s cohort was performed by the staff of the Serology Department, St. Vincent’s Hospital, Melbourne, Australia.

### 2.4.3 Quantitative Measurement of Serum HBsAg

Serum HBsAg titres were measured by EIA using the ARCHITECT platform (Abbott Laboratories, IL, USA), as per the manufacturer’s instructions. A number of diagnostic assays for HBsAg quantification have previously been reported. The ARCHITECT platform uses a chemiluminescent microparticle immunoassay, and is the most widely reported assay in clinical studies. It is a convenient assay, as it is fully automated and has a high throughput capacity. The ARCHITECT quantitative HBsAg assay is based on a calibration curve standardized to the WHO standard for HBsAg$^{103}$. Quantitative HBsAg levels are reported in IU/mL, with a reactive range of 0.05-250 IU/mL. Given that most undiluted serum HBsAg titres are above the dynamic range of the assay, samples were initially tested at dilutions of 1 in 100 or 1 in 1000. ARCHITECT HBsAg Manual Diluent (Abbott Laboratories) was used to dilute patient sera. This diluent contains recalcified human plasma that is non-reactive.
for HBsAg, HIV-1 RNA or HIV-1 Ag, anti-HIV-1/HIV-2, anti-HCV, or anti-HBs according to the manufacturer.

Roche Diagnostics (Penzberg, Germany) have recently developed a prototype HBsAg quantification assay which can be tested on their Elecsys® platform. A subset of patients also had serum HBsAg titres measured on this platform for comparison with the ARCHITECT results. HBsAg titres using the Elecsys® platform was performed by Ms Rachel Hammond at VIDRL under the supervision of Dr Tin Nguyen and Dr Scott Bowden. The same serum sample for HBsAg quantification was used for both assays, and the samples were initially tested at dilutions of 1 in 1000.

2.4.4 Quantitative Measurement of Serum HBeAg

Serum HBeAg levels were measured by commercial EIA (Abbott Diagnostics) on the ARCHITECT platform. This commercial kit is designed to be qualitative only, but can be modified to be semi-quantitative with a limited linear range. The HBeAg reference preparation was obtained from the Paul Ehrlich (PE) Institute (Paul-Ehrlich Institute, Langen, Germany), and has a defined HBeAg activity of 100 PE IU/mL. The PE reference standards were stored at -20°C.

An in-house working HBeAg standard was prepared from a pool of high-titre HBeAg positive specimens received for routine diagnostic testing, and calibrated against the PE reference preparation. On each day that clinical samples were tested, aliquots of the diluted reference sample were used to generate a standard curve. Linear regression was used to convert the assay result into PE IU/mL for each sample. Patient sera were diluted at 1 in 100 for serum HBeAg quantification. Samples which were non-reactive at this dilution were tested at 1 in 10, and those which were above the upper limit of the assay were tested at 1 in 500. ARCHITECT HBsAg Manual Diluent was used for serum dilutions.

2.4.5 Anti-HBe and Anti-HBs

Qualitative testing of serum anti-HBs and anti-HBe were performed by EIA on the ARCHITECT platform according to the manufacturer’s instructions. The ARCHITECT assay was also used to assess for the presence of anti-HBs and anti-HBe in patient B lymphocytes lysates (see Chapter 5).
2.4.6 HBV Genotyping

Genotyping (HBV genotype A-H) was performed using methods as previously described\textsuperscript{156}. HBV DNA was extracted from 200uL of patient serum using the QIAamp DNA MiniKit (QIAGEN, CA, USA) according to the manufacturer’s instructions. Oligonucleotides were synthesised by Geneworks, Adelaide, Australia. For amplification of the polymerase gene the sense primer was 1877a (nt 1877D1896, CCT GCT GGT GGC TCC AGT TC) and the antisense primer 2996 (nt 2996D3014, GCG TCA GCA AAC ACT TGG C) was used. The amplified HBV DNA representing a portion of the envelope/surface gene was purified using PCR purification columns (MO BIO Laboratories, CA, USA) and directly sequenced using ABI PRISM Terminator Cycle Sequencing Ready Reaction Kit Version 3.1 (PE Applied Biosystems, CA, USA). HBV consensus sequences were constructed using the DNA sequence analysis program Seqscape (Applied Biosystems, USA). HBV genotype was determined using a web-based program, SeqHepB (http://www.seqvirology.com/genome7/index.htm)\textsuperscript{157}. This program analyses HBV DNA to determine HBV genotype, to identify key mutations associated with antiviral resistance as well as other clinically important HBV variants by comparing the input sequence data with known HBV reference sequences.

2.4.7 Sequencing of the BCP and PC regions of the HBV genome

Analysis of the BCP and PC regions of the HBV genome was performed after specimen extraction and HBV DNA amplification. Briefly, HBV DNA was extracted from 200uL of patient serum using the QIAamp DNA MiniKit (QIAGEN, CA, USA) according to the manufacturer’s instructions. DNA was eluted in a final volume of 50ul of supplied elution buffer. The same PCR reaction was used to amplify the basal core promoter (BCP)/ precore (PC) regions of the HBV genome, as previously described\textsuperscript{156}. The BCP/PC region was amplified with the sense primer PC5 5’-TCG CAT GGA GAC CAC CGT GAD3’(nt204D223) and the anti-sense primer PC2 5’-GGC AAA AAC GAG AGT AAC TCD3’(nt540D559). The subsequent hemi-nested round of amplification used the sense primer PC5 and the antisense primer 527 5’-GTA ACT CCA CAG WAG CTC C-3’ (nt 527-546).

HBV sequence was then analyzed using the online HBV genome analysis program SeqHepB as previously described\textsuperscript{157}. The PC variant was defined by the
presence of the G1896A mutation (PC mutation), and the BCP variant by the A1762T/G1764A mutations (BCP mutations).

2.4.8 Liver Biopsy

A two pass liver biopsy was performed on all patients recruited from St. Vincent’s Hospital after 2004. The first biopsy (approximately 1.5 x 14-15 mm) was formalin fixed and sent to the Pathology Department at St. Vincent’s Hospital, where it was embedded in paraffin and processed for routine histology and hematoxylin-eosin staining, and a Masson-trichrome stain for identifying liver fibrosis. The necro-inflammatory activity (A score) and fibrosis stage (F score) were determined by the METAVIR scoring system\textsuperscript{158}.

The second biopsy was immediately divided into two sections. The first section was immediately placed in RPMI-1640 (Gibco-BRL) supplemented with 100 U/mL penicillin G and 100 U/mL streptomycin penicillin (Gibco-BRL), and 5% heat-inactivated FCS, for transport at room temperature to the laboratory where single cell suspensions were performed. To generate a single cell suspension, the biopsy specimen (1.5 × 18 mm) was homogenised in a Dounce homogenizer with a loose pestle to separate approximately 6 × 10\textsuperscript{4} hepatocytes mixed with other cells. The cells were then stained with appropriate antibodies and analyzed by flow cytometry as part of a separate clinical study.

The second section was a small fragment (1.5 x 3-4mm) which was placed in RNAlater stabilization reagent (Ambion, Austin, TX, USA), placed on ice for transport to the laboratory, where it was kept at 4°C overnight before storage at -20°C as per the manufacturer’s instructions. This was later used for covalently closed circulate DNA (cccDNA) and total intrahepatic HBV DNA quantification as part of a separate clinical study.

2.4.9 Peripheral Blood Mononuclear Cell Collection

PBMC collection was performed on a subset of patients recruited from St Vincent’s Hospital, Melbourne (n=120) on the same day as patient blood sample collection for routine laboratory biochemistry and HBV DNA testing. Approximately 40mL of whole blood per patient was collected in either ACD or lithium heparin blood tubes. Whole blood was centrifuged at 1000g for 10 minutes at room
temperature. Plasma was removed to within 5mm from the buffy coat and stored. The buffy coat was then transferred to a 50mL centrifugation tube and diluted with 15mL of phosphate buffered saline (PBS). The buffy coat medium mixture was then carefully overlayed onto either 15mL of Ficoll-Paque (GE Healthcare, NJ, USA) or Lymphoprep (Axis Shield, Oslo, Norway), and centrifuged at 800g for 30 minutes. Lymphocyte bands were transferred to another 50mL centrifugation tube and PBS was added to bring the total volume to 50mL. PBMCs were isolated after centrifugation at 400g for 10 minutes, and stored in a freezing medium contain 10% dimethyl sulphoxide (DMSO) and 90% foetal calf serum (FCS).

Determination of the PBMC number and viability was assessed on a haemocytometer following dilution with Trypan Blue (1 to 1). The PBMCs were stored in cryovials in 1mL aliquots (final PBMC concentration 5-10×10^6 cell/mL). Cryopreservation was initiated using a “Mr Frosty” unit which allows a controlled rate of freezing. PBMCs were then stored in a -70°C freezer overnight, and transferred to liquid nitrogen.

2.4.10 B-Lymphocyte Isolation from Peripheral Blood Mononuclear Cell Collection

The PBMCs were removed from liquid nitrogen and rapidly thawed in a 37°C waterbath. Cellular debris was removed by passing the aliquot of PBMCs through a filter (Miltenyi Biotec, North Ryde, Australia). The cell count and viability of PBMCs was rechecked under the haemocytometer following the addition of 10uL of Trypan Blue to a small aliquot of PBMCs.

B lymphocytes were separated from the remaining PBMCs using a commercially available magnetic isolation kit (BD Biosciences, North Ryde, NSW) according to the manufacturers’ instructions. The buffer used for this experiment was PBS + 2mM EDTA + 0.5% foetal calf serum (pH 7.4). Biotin conjugated antibodies were added to the PBMC sample. Two different antibody kits were used: B cell isolation Kit (CD2, CD14, CD16, CD 36, CD43, CD234a), and Antihuman CD19 beads. The B cell isolation kit isolates B lymphocytes using an “indirect” method, with antibodies binding to all cells except B lymphocytes. CD19 is a cell surface marker of B lymphocytes, and so the use of Antihuman CD19 beads represent a “direct” labelling system. Following the addition of the commercial antibody kits,
anti-biotin microbeads are then added, and the sample then passed through the magnetic field of the MACS Separator (Miltenyi Biotec). The B lymphocyte enriched component was only separated from the PBMCs on the same day that they were required for subsequent testing and experimentation.

### 2.4.11 Determination of B Lymphocyte Purity

Flow cytometry was used to confirm that the magnetic isolation kit successfully isolated the majority of B lymphocytes in the PBMC aliquot. Both the positive and negative fractions following isolation were assessed to confirm that 1) the majority of the positive fraction were B lymphocytes and 2) the negative fraction had minimal B lymphocytes.

This was performed immediately after B lymphocyte isolation as a quality control measure on a subset of samples. This was performed with the kind assistance of Joe Manitta in the VIDRL Haematology section.

### 2.4.12 B lymphocyte Lysis

Following B lymphocyte isolation from the aliquot of PBMCs, B cells were disrupted with two different lysis buffers: a commercial lysis buffer (Roche mammalian lysis buffer, complete lysis M EDTA free, Mannheim, Germany) according to the manufacturer's instructions, and the non-denaturing “core prep” lysis buffer (10M Tris pH 7.5, 1mM EDTA, 50mM NaCl, 0.5% NP40) used at VIDRL for HBV DNA analysis from transfected cells. The B cell lysate was then diluted 1 in 2 with PBS prior to testing.

### 2.4.13 Immunohistochemistry

Immunohistochemistry was performed on samples from patients who had two liver biopsies (baseline biopsy, on-treatment biopsy) to identify and compare the intrahepatic localisation and intensity of HBsAg staining (Chapter 4).

Immunostaining for all liver slides was performed at by Dr Alison Boyd at St Vincents Hospital, Melbourne, Australia using the BenchMark XT automated staining system (Ventana, Tucson, AZ, USA). A standard 30 minute heat retrieval step was used for all slides. The primary antibodies used for HBsAg was M3506 (Dako,
Liver sections were scored by microscopic examination by two independent investigators (Dr John Slavin and Dr David Iser) who were blinded to patient identity and clinical data, using a semi-quantitative scale of 0-4, (modified from 159, 160), as follows: 0 = no staining; 1 = <5% of area; 2 = 5-9% of area; 3 = 10-29% of area; 4 = ≥ 30% of area. The distribution pattern of HBsAg staining was classified according to Chu and Liaw, 161 as follows: S1 = no HBsAg staining; S2 = cytoplasmic HBsAg staining alone; S3 = predominant membranous HBsAg staining.

2.4.14 In-house Enzyme Linked Immunosorbent Assay (ELISA)

An in-house sandwich ELISA was developed in order to increase the sensitivity for detecting the presence of anti-HBs from B lymphocytes in the PBMC compartment. The basic steps involved in the in-house ELISA included (Figure 2.1): adsorb HBsAg onto ELISA plate (solid phase), wash and block, incubate with B cell lysate, incubate with secondary antibody (conjugated to horseradish peroxidase) [HRP], add substrate, and measure absorbance with a luminometer. A number of different sources of HBsAg, positive control anti-HBs and secondary antibodies were used in the assay optimisation process (see below).

**Figure 2.1** In-house ELISA experiment
The initial protocol for the in-house ELISA is described below. A number of modifications were made to this protocol during the optimisation process. These modifications are discussed in more detail in Chapter 5.

- **DAY 1**
  - Dilute HBsAg in PBS to a concentration of 200ng/50uL (this concentration was used based on the VIDRL in-house ELISA protocol).
  - Coat Maxisorb ELISA plate overnight at 4ºC with HBsAg (50uL/well).

- **DAY 2**
  - Wash ×3 with PBS + 0.05% Tween 20 (PBS-T)
  - Block with 300uL/well of 5% Skim Milk Powder in PBS for 2 hours at 4ºC
  - Wash ×3 with PBS-T
  - Incubate for one hour with 50uL/well of primary antibody (positive control, B cell lysate) at room temperature
  - Wash ×3 with PBS-T
  - Incubate for one hour with 50uL/well of secondary antibody-HRP (dilution 1 in 2000 in PBS) at room temperature
  - Wash ×3 with PBS-T
  - Add 100uL/well citrate buffer (0.1M citrate substrate buffer, 200uL of 50× ABTS, 10uL of 30% H₂O₂)
  - Measure absorbance on luminometer at 405nm

**In-House ELISA Test Components**

1. **HBsAg**

Various preparations of HBsAg were used for the development of the in-house ELISA experiments (Table 2.1). The HBsAg titre for each of these sources of HBsAg was also assessed on the ARCHITECT platform (Abbott Laboratories, IL, USA) according to the manufacturer’s instructions (Chapter 2.4.3).
Table 2.1 Sources of HBsAg

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DETAILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine</td>
<td>HB-VAXII (CSL Biotherapies, Aus, 10ug/mL)</td>
</tr>
<tr>
<td></td>
<td>Engerix-B (GSK, Belgium, 20ug/mL).</td>
</tr>
<tr>
<td>Commercially available</td>
<td>Ab73741 (Abcam, UK, 1mg/mL). This is a recombinant full length</td>
</tr>
<tr>
<td>HBsAg</td>
<td>hepatitis B virus surface antigen (subtype:ayw) protein from HBV 320</td>
</tr>
<tr>
<td></td>
<td>genome expressed in <em>Saccharomyces cerevisiae</em> (24kDa)</td>
</tr>
<tr>
<td>In House HBsAg</td>
<td>HBsAg obtained from tissue culture and transient transfection of Huh-</td>
</tr>
<tr>
<td></td>
<td>7 cells was kindly provided by Dr Nadia Warner (Victorian Infectious</td>
</tr>
<tr>
<td></td>
<td>Diseases Reference Laboratory [VIDRL], Victoria, Aus).</td>
</tr>
</tbody>
</table>

2. Anti-HBs: Primary Antibody for In-House ELISA

Anti-HBs from various sources was used as the Positive Control for the inhouse ELISA experiments (Table 2.2). The anti-HBs titre for each of these sources was determined on the ARCHITECT platform according to the manufacturer’s instructions.

Table 2.2 Sources of Anti-HBs

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>DETAILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Sera from healthy controls who received a HBsAg booster vaccination</td>
</tr>
<tr>
<td></td>
<td>(GSK, Belgium).</td>
</tr>
<tr>
<td>Hepatitis B Immunoglobulin</td>
<td>Hepatitis B immunoglobulin (CSL Biotherapies, Australia)</td>
</tr>
<tr>
<td>Ab 20522</td>
<td>Goat polyclonal antibodies to HBsAg (Abcam, UK, 100ug). Immunogen:</td>
</tr>
<tr>
<td></td>
<td>HBsAg subtypes ad and ay. Recommended for ELISA experiments</td>
</tr>
<tr>
<td>Ab 8636</td>
<td>Mouse monoclonal antibodies to HBsAg (Abcam, UK, 100ug). Immunogen:</td>
</tr>
<tr>
<td></td>
<td>Purified serum HBsAg. Recommended for ELISA experiments.</td>
</tr>
</tbody>
</table>

3. Secondary antibodies for In-house ELISA

Secondary antibodies for the In-house ELISA experiments were purchased from Dako (Glostrup, Denmark). This included anti-human IgG-HRP, anti-mouse IgG-HRP, and anti-goat IgG-HRP.
2.5 Statistical Analysis

Statistical analysis was performed using GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com). Specific analytical methods are discussed separately in Chapters 3, 4 and 5.
Chapter 3. Serum HBsAg Titres in the Natural History of Chronic Hepatitis B

3.1 Introduction

The natural history of chronic hepatitis B (CHB) is typically regarded as consisting of four phases\textsuperscript{85, 86}; immune-tolerant (IT), immune-clearance (IC), non / low replicative (LR), and hepatitis B e antigen negative hepatitis (ENH). These phases have been classified by specific biochemical, serological and virological characteristics, including serum alanine transaminase (ALT) levels, hepatitis B e antigen (HBeAg) serostatus and hepatitis B virus DNA (HBV DNA) titre. It is important to note that these phases do not occur in all individuals, and do not always necessarily occur sequentially\textsuperscript{87}.

The understanding of the pathogenesis and natural history of CHB continues to develop. This has been facilitated by the improved sensitivity of HBV DNA viral load assays, and the development of technologies for the detection and measurement of HBV intrahepatic replicating forms of the virus such as covalently closed circular DNA (cccDNA) and other key replicative intermediates\textsuperscript{162-164}. Sensitive and reliable assays have also been developed to quantify serum hepatitis B surface antigen (HBsAg). These assays include the ARCHITECT from Abbott Laboratories, and the Roche Diagnostics HBsAg quantification assay which can be performed on the Elecsys® platform.

The seroclearance of HBsAg represents the preferred endpoint of therapy for CHB, as it is believed to represent successful immunological control of active HBV replication\textsuperscript{128}. Recent data from clinical trials suggest a potential role for on-treatment monitoring of serum HBsAg and HBeAg titres during pegylated interferon-alpha (Peg-IFNα) therapy in predicting virological responses\textsuperscript{134, 165}. However, baseline HBsAg titres have not been well-characterized in each phase of CHB, particularly in the IT and LR phases, nor are they known in the infected paediatric population. The main objectives of this study were to:

1. Determine the serum HBsAg titres during the different phases of the natural history of CHB in a well characterised cohort of patients\textsuperscript{166};
2. Correlate HBsAg titre with HBV DNA and serum ALT levels in each phase of infection;
3. Determine whether baseline serum HBsAg titres are different for the main HBV genotypes

4. Compare the results of HBsAg titres between the ARCHITECT and Elecsys® platforms; and to

5. Evaluate serum HBsAg titres longitudinally in particular populations such as a paediatric cohort
3.2 Methods

3.2.1 Patients

A cross-sectional study was performed on 283 patients with CHB from three tertiary hospitals [St Vincent’s Hospital Melbourne (n=175), Liverpool Hospital Sydney (n=70), and The Royal Children’s Hospital Melbourne (n=38)] (refer to Chapter 2.3.3 for a more detailed description). Patients from Liverpool Hospital included a cohort of pregnant women. The Royal Children’s Hospital cohort was a predominantly HBeAg positive paediatric group (< 18 years of age), and analysis included both cross-sectional and longitudinal follow up data. This cohort was analysed separately to the adult population as there is very little literature on HBsAg titres in the paediatric population, and also because there may be different factors which affect HBsAg titres in this group.

All patients tested negative for laboratory markers of infection with the hepatitis C virus (HCV), hepatitis D virus and human immunodeficiency virus (HIV). Laboratory markers for co-existent auto-immune or metabolic liver disease were also negative.

Patient demographics, liver biochemistry, qualitative HBsAg (VIDAS Ultra, bioMerieux, France) and HBeAg status (AxSYM; Abbott Laboratories, IL, USA), HBV DNA load (Siemens Healthcare Diagnostics, Tarrytown, NY, USA), and HBV genotype\textsuperscript{156} were recorded. Biochemical and virological data were obtained from patient blood samples collected on the same day.

Patients were classified into a phase of CHB at the centres participating in the study after a follow-up period of 3-6 months. The phase of CHB for each patient was determined by HBeAg/anti-HBe serostatus, and measurement of HBV DNA (IU/mL) and serum ALT (U/L) levels according to the recently published European Association for the Study of the Liver (EASL) clinical practice guidelines\textsuperscript{85}. The IT phase was defined as: HBeAg positive, high viral load, serum ALT <2× upper of limit normal (ULN). The IC phase was defined as: HBeAg positive, elevated viral load, serum ALT >2× ULN. The LR phase was defined as; HBeAg negative, HBV DNA <2,000 IU/mL, normal serum ALT level. The ENH phase was defined as: HBeAg negative, HBV DNA >2,000 IU/mL, serum ALT >2× ULN. For this study, the cutoff for a normal ALT level was ≤30 U/L.
The study was conducted according to the guidelines of the Declaration of Helsinki, and was approved by the local institutional ethics research committee.

3.2.2 Quantitative Serum HBsAg Assay

Serum HBsAg was quantified using the ARCHITECT platform (Abbott Laboratories), as per the manufacturer’s instructions (Chapter 2.4.3). Given that most serum HBsAg titres are above the dynamic range of the assay (0.05 to 250 IU/mL), samples were initially tested at dilutions of 1 in 100 or 1 in 1000. ARCHITECT HBsAg Manual Diluent (Abbott Laboratories) was used to dilute patient sera. This diluent contains recalcified human plasma that is non-reactive for HBsAg, HIV-1 RNA or HIV-1 Ag, anti-HIV-1/HIV-2, anti-HCV, or anti-HBs according to the manufacturer.

A subset of patients (n=68) also had serum HBsAg titres measured using the recently developed Roche Diagnostics HBsAg quantification assay (Penzberg Germany) which can be tested on the Elecsys® platform (Chapter 2.4.3). The same serum sample for HBsAg quantification was used for both assays, and the samples were initially tested at a dilution of 1 in 1000. This was performed by Ms Rachel Hammond at VIDRL under the supervision of Dr. Tin Nguyen and Dr Scott Bowden. Comparison of HBsAg titres obtained between both assays with linear regression was performed.

3.2.3 HBV DNA Viral Load

Initial HBV DNA viral load testing was performed using the Versant HBV DNA 3.0 (Siemens Healthcare Diagnostics), according to the manufacturer’s instructions (dynamic range 3.5 x 10^2 – 1.8 x 10^7 IU/mL) (Chapter 2.4.1). Samples with a viral load above the upper limit of the dynamic range were diluted by 1 in 1000 to obtain a defined titre within the range of the bDNA assay. ARCHITECT HBsAg Manual Diluent was used to dilute patient sera. In some situations, samples were reflexed into the Abbott RealTime HBV assay (Abbott Molecular, IL, USA) when the HBV DNA titre was below the lower limit of detection on the bDNA assay.
3.2.4 HBV Genotyping

Genotyping (HBV genotype A-H) was performed in 219/245 (89%) patient samples using methods as previously outlined (see Chapter 2.4.6)\textsuperscript{156}. Genotyping was not possible in 26 samples from patients in the LR phase due to insufficient HBV DNA.

3.2.5 Statistical Analysis

Continuous and categorical variables were compared between groups, using the Mann-Whitney test and Kruskall-Wallis ANOVA for non-parametric continuous data, and Chi square / Fisher’s exact test for categorical data. Linear regression analysis and Pearson’s correlation coefficient ($r$) was used to describe the correlation between two variables. Statistical analysis was performed using GraphPad Software, San Diego California USA, www.graphpad.com.
3.3 Results

The HBsAg titres in the adult and paediatric cohorts were analysed separately. This is because very little is known about HBsAg titres in the paediatric population, and it is possible that different factors affect HBsAg titres in this group of patients.

3.3.1 Adult Cohort (>18 years of age)

Two hundred and forty five treatment naïve patients were recruited. There were 175 patients recruited from St Vincent’s Hospital (Melbourne), and 70 from Liverpool Hospital (Sydney), respectively. Patients from Liverpool Hospital comprised a cohort of pregnant women, and were relatively evenly distributed across all phases of CHB infection. Overall, the classification of patients into respective phases of CHB was: IT (n=32), IC (n=53), LR (n=57), ENH (n=103).

The baseline patient characteristics are presented in Table 3.1. The majority of the study population was infected with HBV genotypes B or C (90%) and were of Asian ethnicity. There were more females in the study group (54%). HBeAg positive patients were younger than HBeAg negative patients (p<0.001). In HBeAg positive patients, there was no significant age difference between those in the IT and IC phases. Patients in the ENH phase were older than those in the LR phase (p=0.02).

<table>
<thead>
<tr>
<th>Table 3.1. Baseline Population Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>HBeAg Status</td>
</tr>
<tr>
<td>Age* (Years)</td>
</tr>
<tr>
<td>Sex M/F</td>
</tr>
<tr>
<td>HBV DNA** Log10 IU/mL</td>
</tr>
<tr>
<td>ALT** IU/mL</td>
</tr>
<tr>
<td>Genotype B / C / D / Other (%)</td>
</tr>
</tbody>
</table>

*Data expressed as the median (IQR) and as percentages, # 10-90% confidence interval
3.3.1.1 Population Distribution of HBsAg Titre

The distribution of serum HBsAg levels across the study population was evaluated (Figure 3.1). HBsAg titres were different between each phase of CHB \((p=0.001)\). The median HBsAg titres in each phase of CHB were: IT \((4.53 \log_{10} \text{ IU/mL})\), IC \((4.03 \log_{10} \text{ IU/mL})\), LR \((2.90 \log_{10} \text{ IU/mL})\), and ENH \((3.36 \log_{10} \text{ IU/mL})\) respectively. There was no difference in median HBsAg titres between pregnant \((n=14)\) and non-pregnant \((n=18)\) IT cohorts \((p=0.055)\). The median HBsAg in the LR and ENH phases were significantly lower than both HBeAg positive phases.

![Figure 3.1 – Distribution of serum HBsAg titres throughout the natural history of CHB. Median values with 95% confidence interval (of median) represented. IT: immune tolerant, IC: immune clearance, LR: low replicative, ENH: HBeAg negative hepatitis.](image)

3.3.1.2 Correlation of Serum HBsAg Titres with Serum ALT Level

There was no observed statistical correlation between HBsAg titres with serum ALT levels in any phase of CHB.

3.3.1.3 Correlation of Serum HBsAg Titres with HBV Viral Load

The correlation between serum HBsAg titres with serum HBV DNA in each phase of CHB is presented in Figure 3.2 a-D. There was a modest correlation observed in the IC phase \((r = 0.77, p=0.0001)\), but weak correlation between serum HBsAg and HBV DNA in either the IT \((r=0.30, p=0.09)\), LR \((r=0.24, p=0.05)\) or ENH phases \((r=0.26, p=0.006)\).
Because of the nature of the HBV replication strategy, where the HBV DNA and HBsAg are produced from different mRNA transcripts (see Figure 1.3 and 3.11), the ratio of HBsAg (log\textsubscript{10} IU/mL) to HBV DNA (log\textsubscript{10} IU/mL) in each phase of CHB was also examined (Figure 3.3). The HBsAg/HBV DNA ratio was significantly higher in the LR phase compared to IT, IC and ENH phases respectively (1.09 vs 0.55, 0.55, 0.69, p<0.0001).
Figure 3.3. Ratio of HBsAg and HBV DNA in each phase of CHB. Median values with 95% confidence interval (of median) represented. IT: immune tolerant, IC: immune clearance, LR: low replicative, ENH: HBeAg negative hepatitis.

3.3.1.4 Serum HBsAg Titres Between Genotypes B and C

HBV genotypes B and C are prevalent in patients of Asian ethnicity. The distribution of serum HBsAg titres in different phases of CHB were evaluated based on these two HBV genotypes (Figure 3.4). Comparison of HBsAg levels between HBV genotypes A to D (prevalent in Caucasians/Mediterraneans) was not possible due to an insufficient number of patients.

The median HBsAg titres in HBV genotype B and C subgroups were similar in the IT, IC and ENH phases. In contrast, the median serum HBsAg titre in the LR phase was lower in genotype B (2.24 log\(_{10}\) IU/mL) compared with genotype C (3.34 log\(_{10}\) IU/mL), p=0.002. However, it should be noted that HBV viral genotype was not available in all patients in this phase (27/57, 47%) as some patients had insufficient HBV DNA to allow for genotype determination by sequencing.

Figure 3.4 a-b. Distribution of serum HBsAg titres in a) genotype B CHB, and b) genotype C CHB. Median values with 95% confidence interval represented.
3.3.2 Comparison of Serum HBsAg Titres Between Abbott ARCHITECT and Roche Elecsys® Assays

Serum HBsAg titres obtained using the ARCHITECT assay then were compared to those obtained using the Roche HBsAg prototype assay on the Elecsys® instrument for a subset of patients (n=68). This subset included a range of samples encompassing HBV genotypes B (n=24), C (n=24) and D (n=20). Other HBV genotypes were not assessed due to inadequate sample size.

A strong correlation between HBsAg titres was found between the two different assays (r=0.98, p<0.0001) (Figure 3.5). This strong correlation was observed in all three HBV genotypes examined (Figure 3.6).

![Figure 3.5](image1.png)

**Figure 3.5.** Correlation between HBsAg titres using the ARCHITECT and Elecsys® platforms (all HBV genotypes).

![Figure 3.6 a-c](image2.png)

**Figure 3.6 a-c.** Correlation between HBsAg titres using the ARCHITECT and Elecsys® platforms by HBV genotype.

3.3.3 Paediatric Cohort (<18 years of age)

Thirty eight children with CHB were recruited from The Royal Children’s Hospital, Melbourne. This cohort was separated into four age groups of 0 to 5, 6 to 10, 11 to 15 and 16 to 18 years of age. The baseline characteristics are presented in Table 3.2. All children in this cohort were HBeAg positive, and greater than 90% had a serum ALT level above 30 U/L (the upper limit of normal for this cohort),
suggesting that the majority of children were in the IC phase of CHB. Males comprised 66% of the overall cohort. The median serum ALT level was 56 U/L and there was a high median viral load of greater than $7.25 \log_{10} \text{IU/mL}$ (upper limit of bDNA assay). Further dilutions of serum samples above the upper limit of the dynamic range of the Versant HBV DNA 3.0 assay was not possible because many serum samples were unavailable.

**Table 3.2. Baseline Population Characteristics of Paediatric Cohort**

<table>
<thead>
<tr>
<th>Number</th>
<th>38</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>25 / 13</td>
</tr>
<tr>
<td>Age at first serum sample</td>
<td></td>
</tr>
<tr>
<td>0 – 5</td>
<td>5 (13%)</td>
</tr>
<tr>
<td>6 – 10</td>
<td>11 (29%)</td>
</tr>
<tr>
<td>11 – 15</td>
<td>14 (37%)</td>
</tr>
<tr>
<td>16 – 18</td>
<td>8 (21%)</td>
</tr>
<tr>
<td>HBeAg positive</td>
<td>100%</td>
</tr>
<tr>
<td>Baseline ALT (median)</td>
<td>56 (range 21 – 216)</td>
</tr>
<tr>
<td>Baseline Viral load (median)</td>
<td>$&gt; 7.25 \log_{10} \text{IU/mL}$</td>
</tr>
<tr>
<td>Ethnicity (%)</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>40</td>
</tr>
<tr>
<td>African</td>
<td>40</td>
</tr>
<tr>
<td>Caucasian</td>
<td>11</td>
</tr>
<tr>
<td>Other</td>
<td>9</td>
</tr>
</tbody>
</table>

The distribution of serum HBsAg levels in this cohort is shown in Figure 3.7. The overall median serum HBsAg titre was $4.54 \log_{10} \text{IU/mL}$. This is higher than the median HBsAg titre in the adult IC group ($4.03 \log_{10} \text{IU/mL}$), $p=0.0014$, and more closely linked to the adult IT group ($4.53 \log_{10} \text{IU/mL}$). The youngest age group of 0 to 5 years had the highest median HBsAg titre ($4.91 \log_{10} \text{IU/mL}$) compared to age group 6 to 10 ($4.37 \log_{10} \text{IU/mL}$, $p=0.70$), age group 11 to 15 ($4.50 \log_{10} \text{IU/mL}$, $p=0.03$) and age group 16 to 18 ($4.19 \log_{10} \text{IU/mL}$, $p=0.045$).
3.3.3.1 Correlation of HBsAg Titre with Serum ALT Level

There was a modest correlation \((r=0.48, p=0.007)\) between HBsAg titre and serum ALT level (Figure 3.8).

3.3.3.2 Correlation of Serum HBsAg Titres with HBV Viral Load

Correlation of HBsAg titre with HBV DNA was not possible as the majority of children had a viral load above the upper limit of detection. Further dilutions of serum samples above the upper limit of the dynamic range of the Versant HBV DNA
3.0 assay was not performed because access to these serum samples was no longer possible.

### 3.3.3.3 Longitudinal Changes in HBsAg Titre

Longitudinal HBsAg titres were evaluated in a subset of 21 children (Figure 3.9). Overall, the median duration of follow up was 30 months (range 3 to 60). HBsAg titres were stable during this period, with a median change between initial and last serum sample of $0.10 \log_{10} \text{IU/mL}$ (range 0.02 to 0.81). The patient who had a reduction in HBsAg titre of $0.81 \log_{10} \text{IU/mL}$ was an 11 year old Asian boy whose last follow up serum sample was at 34 months. The child also had a large reduction in HBeAg titre ($1.62 \log_{10} \text{PE IU/mL}$) (Figure 3.10). Whilst this suggests that this patient may be in the process of achieving spontaneous HBeAg seroconversion, no further serum samples were available for analysis.

![Figure 3.9](image)

**Figure 3.9** – Fluctuations in serum HBsAg titre in a paediatric cohort

![Figure 3.10](image)

**Figure 3.10** – Fluctuation in serum HBsAg titre, HBeAg titre and serum ALT in an 11 year old boy
3.4 Discussion

This study aimed to evaluate the baseline serum HBsAg titres in different phases of CHB infection in several cohorts including adult patients/individuals with CHB attending a major tertiary centre, pregnant women and a paediatric cohort. All patients were treatment naïve, and the majority of the study population were of Asian ethnicity.

Within the adult cohort, this study demonstrated that serum HBsAg titres differed between the four phases of CHB. Median HBsAg titres were not significantly different between non-pregnant and pregnant cohorts. HBsAg titres were higher in HBeAg positive compared to HBeAg negative CHB. The lowest HBsAg titres were evident in the LR phase. The difference between median lowest and highest HBsAg titres in the different phases was less than 2 log\textsubscript{10} IU/mL, far smaller than that observed with changes in HBV DNA levels. There was no observed statistical correlation between HBsAg titres and serum ALT levels in any phase of CHB. However, there was a modest correlation (r=0.77, p=0.0001) between HBsAg levels and HBV DNA in the IC phase. With regards to HBsAg titres between HBV genotypes B and C, there was no difference in median HBsAg titres other than in the LR phase (Genotype B 2.24 log\textsubscript{10} IU/mL, Genotype C 3.34 log\textsubscript{10} IU/mL, p=0.002), although genotyping could not be performed in a subset (27/57) of patients in the LR phase due to insufficient HBV DNA.

HBsAg titres in a paediatric cohort were also examined. The majority of this cohort was in the IC phase of CHB as indicated by HBeAg positivity, high HBV DNA levels, and an elevated serum ALT level. The overall median serum HBsAg titre was higher than the adult IC group (4.54 compared to 4.03 log\textsubscript{10} IU/mL, p=0.0014). Within this cohort, the youngest age group (0 to 5 year) had the highest median HBsAg level. There was a modest correlation (r=0.48, p=0.007) between serum HBsAg levels and serum ALT levels. However, correlation of HBsAg titres with HBV DNA was not possible as most of the paediatric patients had viral loads above the upper limit of the bDNA assay, and serum samples were no longer available for dilution to obtain a defined titre within the range of the assay.

Longitudinal evaluation of serum HBsAg titres in the paediatric cohort demonstrated that HBsAg levels did not fluctuate appreciably over a median follow up duration of 30 months. One patient had a significant reduction in both serum
HBsAg and HBeAg titre, which is consistent with the notion that HBsAg titres are relatively stable until the development of HBeAg seroconversion.

The Abbott ARCHITECT platform is the most frequently used assay for quantification of HBsAg in clinical studies published to date. Serum HBsAg titres obtained using the ARCHITECT assay were compared with the prototype Roche Diagnostics HBsAg quantification assay which uses the elecsys® platform. A strong correlation (r=0.98, p<0.0001) was demonstrated, suggesting that the Roche assay may provide a satisfactory alternative for serum HBsAg quantification.

Interest in quantitative HBsAg serology as a clinical biomarker has been based upon studies which showed a positive association with intrahepatic HBV cccDNA levels\(^{103, 162, 167}\) and serum HBV DNA\(^{103, 168}\). Currently, HBV DNA quantification is the gold standard in selecting patients who are potential candidates for therapy, monitoring response to therapy, and detecting the emergence of drug resistance. Compared to HBV DNA, the assays for HBsAg quantification are far less expensive, and are fully automated with a high throughput capacity. However, the utility of HBsAg titres as a reliable surrogate for both HBV cccDNA and HBV DNA remains unclear, as other studies have also shown a poor correlation with HBV cccDNA\(^{169}\), and only a positive correlation with HBV DNA in HBeAg positive CHB in our collaborative group\(^{164}\).

An understanding of HBsAg titre changes throughout HBV infection may provide some potentially useful insights into hepatitis B pathogenesis and viral life cycle. The mechanisms linking HBsAg and viral replication during different phases of CHB are currently unclear. This study observed a modest correlation of serum HBsAg with HBV DNA in the IC phase of CHB (r = 0.77, p=0.0001). No correlation was observed in the IT, LR or ENH phases. Furthermore, the ratio of HBsAg to HBV DNA was significantly higher in the low replicative phase compared to all other phases (1.09 vs 0.55, 0.55, 0.69, p<0.0001), a finding which is in accordance with previous studies\(^{162}\). The apparent “disconnect” between HBsAg and HBV DNA at different phases may possibly be due to the expression of HBsAg from integrated viral envelope sequences, instead of HBsAg production off mRNA derived from the HBV cccDNA template. A second possible explanation is a difference in the immune regulation of viral replication during different phases of infection, resulting in altered ratios of HBV virion to sub-viral HBsAg particles\(^{31}\). 
HBsAg synthesis during the HBV viral life cycle is complex, and typically occurs at the endoplasmic reticulum (ER) (Figure 1.3 and 3.11). The envelope open reading frame (ORF) contains three in-frame “start” codons which sub-divide it into preS1, preS2, and S domains. Envelope proteins are generated from two HBV mRNA transcripts, with subsequent translation resulting in production of the small (S), medium (Pre-S2+S) and large surface envelope proteins (Pre-S1+Pre-S2+S); these are also known as S, M and L surface proteins, respectively.

Figure 3.11. The two separate pathways of HBsAg and HBVDNA production. RC-DNA, relaxed circular DNA; DSL DNA, double stranded linear DNA; cccDNA, covalently closed circular DNA; mRNA, messenger RNA; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen.

Newly synthesized envelope protein interacts with mature HBV nucleocapsids at the ER prior to secretion from the hepatocyte. However, HBsAg production far exceeds that required for virion assembly, and excess surface envelope proteins are secreted as non infectious filamentous or spherical subviral particles. These subviral particles may play a role in evading or subverting the host immune response, and may also co-exist with anti-HBs as part of circulating immune complexes (see Chapter 5 of thesis). It is important to appreciate that whilst HBsAg quantification detects all three forms of systemic HBsAg (part of HBV virion, spherical, filamentous), differentiation between the relative proportions is currently technically demanding, and not routinely performed, requiring either density gradient
centrifugation or non-denaturing gel electrophoresis for separation and immunoblot for initial detection and subsequent clarification.

HBsAg may also be produced from HBV DNA integrated into the host genome. Although viral integration is an essential component of the life cycle of retroviruses such as HIV, it is not required for normal productive hepadnaviral infection. Rather, integration of HBV DNA occurs illegitimately through recombination mechanisms using host enzymes such as topoisomerase acting on the double-stranded linear (DSL) HBV DNA (Figure 1.3 and 3.11)\textsuperscript{32, 33}. In HBV infection, viral integration does seem to occur early in infection. Whilst HBV integration is believed to be a random event, a high preference for integration occurs at the direct repeat 1 (DR1) and DR2 sequences on the HBV genome\textsuperscript{34}. Such integrated sequences cannot provide a template for productive viral replication as a complete genome is typically not present\textsuperscript{36}. However, given that sequences of the S genes of the Enh I elements are often present in integrated segments, HBsAg may be produced\textsuperscript{36}.

The phase of CHB is currently determined by three main factors; HBeAg/anti-HBe status, HBV DNA titre and serum ALT level. This study demonstrated that HBsAg titres change during the natural history of CHB, and suggests that there may be HBsAg titre “set-points” within each phase. Further evaluation of baseline HBsAg titres in other cohorts of patients with CHB are required to confirm the findings of this study, and may help refine the current definition of the different phases of CHB.

The status of a patient’s HBeAg/anti-HBe, HBV DNA and serum ALT are also the parameters which are currently used to assess the response to antiviral therapy. Sustained suppression of HBV replication as assessed by HBV DNA measurement currently represents the cornerstone of evaluation of antiviral efficacy. In the absence of HBsAg loss, long-term therapy with potent oral NAs is required to maintain effective suppression of HBV DNA. Thus, there is now a paradigm shift towards striving to achieve HBsAg loss and/or seroconversion. HBsAg loss is believed to be associated with both successful immunological control of HBV and durable suppression of viral replication, and consequently may represent an indication to cease oral NA therapy. Evaluation of HBsAg titres may allow determination of baseline levels which may be more predictive of HBsAg loss. Furthermore, assessment of on-treatment changes in HBsAg titres may facilitate new algorithms and future trials which are aimed at achieving this important endpoint.
The role of quantitative HBsAg in predicting response to peginterferon therapy has been the focus of several recent studies. In HBeAg negative patients treated with pegylated interferon, the decline in HBsAg titre at week 12 and 24 has been shown to be a useful predictor of achieving an undetectable viral load at 24 weeks post therapy. The results of the study described in this section of the thesis may have implications for future treatment algorithms evaluating the on-treatment decline of HBsAg titres in both HBeAg positive and negative patients. For example, the median baseline serum HBsAg titre in the LR phase was $2.86 \log_{10}$ IU/mL. Future clinical trials could evaluate whether an on-treatment HBsAg titre decline to these levels is a positive predictor of durable suppression of viral replication.

A limitation of this study was its cross-sectional design, as it would have been useful to follow more patients longitudinally through different phases of infection. However, such longitudinal follow up is difficult given patients can remain in either the IT or LR phases for years, and patients in the IC and ENH phases are potential candidates for antiviral therapy.

In conclusion, this study demonstrated significant differences in the baseline serum HBsAg titres across the different phases of CHB infection. Quantitative HBsAg assays are non-invasive, easy to perform and relatively inexpensive. Understanding the changing HBsAg titres throughout the natural history of CHB represents a step forward in further investigating the HBV viral life cycle and the influence of the host immune response. Baseline and on-treatment HBsAg quantification may help refine future treatment algorithms for both immune-modulator therapy and oral NA therapy. Larger prospective studies are now required to evaluate longitudinal changes in serum HBsAg, and to evaluate their significance in predicting the ultimate goal of antiviral therapy, HBsAg seroconversion.
Chapter 4. Serum HBsAg Decline in Chronic Hepatitis B Patients Treated Long-term with Nucleos(t)ide Analogues

4.1 Introduction

HBsAg clearance represents the ultimate endpoint in antiviral therapy for CHB, as it is believed to represent successful immunological control of viral infection. HBsAg is cleared during recovery with acute infection, and an anti-HBs immune response following vaccination is in general protective against possible subsequent infection. Furthermore, multiple studies have demonstrated that HBsAg seroconversion is associated with a favourable long-term clinical prognosis.\(^\text{93, 94}\)

In the context of the natural history of CHB, spontaneous HBsAg loss is rare with approximately a 1% annual rate.\(^\text{91, 92}\) This can be increased with a finite course of either standard IFN-\(\alpha\) or Pegylated-IFN (Peg-IFN-\(\alpha\)) therapy, with overall reported rates of 7.8%\(^\text{173}\) and 3%\(^\text{113}\) following 10-24 weeks and 48 weeks of therapy, respectively, in HBeAg positive CHB. Furthermore, in long term virologic responders to interferon, HBsAg loss can still occur whilst off therapy, and thus rates do steadily increase over time.\(^\text{111, 130}\)

Clinical trials have suggested a benefit in monitoring baseline and on-treatment serum HBsAg titres during Peg-IFN-\(\alpha\) therapy in predicting virological responses.\(^\text{133-135}\) For example, in HBeAg negative patients treated with Peg-IFN-\(\alpha\), a reduction in HBsAg titre of >0.5 log\(_{10}\) IU/mL at week 12 of therapy had a 89% positive predictive value of achieving an undetectable HBV DNA (<70 copies/mL) 24 weeks after treatment cessation.\(^\text{134}\) A decline in HBsAg titre of >1.0 log\(_{10}\) IU/mL at week 48 of therapy was associated with a 30% rate of HBsAg loss at three years after treatment.\(^\text{165}\)

Conversely, oral nucleos(t)ide analogue (NA) agents have traditionally not been considered to significantly influence HBsAg clearance. It is believed that this is because oral NA primarily target the HBV polymerase to inhibit viral replication, rather than having any direct immuno-modulatory effect.

In HBeAg positive patients treated with 48 weeks of continuous therapy, the major registration trials of LMV, ADV, ETV and TDF have reported HBsAg seroclearance rates of 1%, 0%, 2% and 3.2% respectively.\(^\text{113, 118, 119}\) More prolonged
use of oral NA therapy can increase these rates, with ETV and TDF being associated with 5% and 6% HBsAg seroclearance rates following 96 weeks of continuous use respectively\textsuperscript{120, 121}. In both of these studies involving ETV and TDF, the factors associated with a higher likelihood of HBsAg loss were Caucasian ethnicity and those patients infected with HBV genotype A or D (i.e. “non Asian” HBV genotypes)\textsuperscript{123, 124}.

In HBeAg negative CHB however, HBsAg seroclearance rates with oral NA are much lower, with an approximately 0.3% rate following 48 weeks of ETV\textsuperscript{131}, and 0% following 96 weeks of TDF\textsuperscript{132}.

Although the rates of HBsAg loss whilst on oral NA therapy are not as great as for Peg-IFN\textalpha{} therapy, the changes in HBsAg titre during oral NA treatment have not been clearly evaluated. Therefore, the aims of this study were to:

1. Evaluate longitudinal changes in serum HBsAg titre in patients treated with long term NA therapy
2. Determine whether there are patterns of changes in the HBsAg titre that can be predicted by biochemical, serological and/or virological markers
3. Assess the intrahepatic localisation of HBsAg by immunohistochemistry during oral NA therapy.
4.2 Methods

4.2.1 Patients

A retrospective longitudinal study was conducted on patients from St Vincent’s Hospital, Melbourne, Australia with CHB (n=16) who received long-term oral NA therapy. For this study, long-term therapy was determined as greater than two years duration, although an interim analysis at 12 months was also performed. The frequency of clinic visits and blood samples collected for this cohort was on average three to four times each year.

An additional two patients who had a second liver biopsy following 12 months of therapy were also included in this study. These two patients only had data until month 12 of therapy, and so did not meet the criteria for the long-term therapy study. However, they were included as it was felt that the comparison of intrahepatic HBsAg staining using immunohistochemistry between pre-treatment and on-treatment biopsy specimens was particularly relevant. The results for these two patients were analysed separately.

All patients tested negative for laboratory markers of infection with the hepatitis C virus (HCV), hepatitis D virus and human immunodeficiency virus (HIV). Laboratory markers for co-existent auto-immune or metabolic liver disease were also negative.

Patient demographics, liver biochemistries, qualitative HBsAg and HBeAg status (by standard qualitative enzyme immunoassay), HBV DNA were recorded at baseline, and subsequently every three to six months. A baseline HBV genotype (Chapter 2.4.6) was also determined. These results were obtained from the St Vincent’s Hospital Hepatitis B Database. Biochemical and virological data were obtained from patient blood samples collected on the same day.

4.2.2 Liver Biopsy

A baseline liver biopsy was obtained in all patients prior to commencement of oral NA therapy as part of their routine standard of care management. This biopsy (approximately 1.5 x 15-20 mm) was formalin fixed and sent to the Pathology Department at St. Vincent’s Hospital, where it was embedded in paraffin and processed for routine histology, hematoxylin-eosin staining, and a special stain
(Masson-trichrome) for identifying liver fibrosis. The necro-inflammatory activity (A score) and fibrosis stage (F score) were determined by the METAVIR scoring system.\textsuperscript{158}

### 4.2.3 Immunohistochemistry

There were two patients who underwent a second liver biopsy as part of a separate clinical trial protocol. Immunohistochemistry was performed on both sets of liver biopsy specimens to identify and compare the intrahepatic localisation and intensity of HBsAg (see Chapter 2.4.13).

### 4.2.4 Serum HBsAg Titre

Serum HBsAg was quantified using the ARCHITECT platform (Abbott Laboratories, Chicago, IL, USA) as previously described (Chapter 2.4.3). Serum samples were stored at \(-20^\circ\text{C}\) at the Victorian Infectious Diseases Reference Laboratory until testing.

#### 4.2.4.1 Patterns of HBsAg Titre Changes

**Interim Analysis – Change in HBsAg Titre following 12 months of therapy**

An interim analysis of the changes in HBsAg titre following 12 months of oral therapy was performed. A \textit{Significant Decline (SD)} in HBsAg level was defined as \(>0.5 \log_{10} \text{IU/mL}\) reduction following 12 months of therapy. A \textit{Significant Increase (SI)} in HBsAg level was defined as a \(>0.5 \log_{10} \text{IU/mL}\) increase following 12 months of therapy. A value of \(0.5 \log_{10} \text{IU/mL}\) was chosen as “significant” based on a study which suggested that achieving this reduction at week 12 of Peg-IFN\(\alpha\) therapy was a positive predictor of an effective viral suppression at week 24 after therapy (also see Discussion for further explanation)\textsuperscript{134}. A timeframe of 12 months (rather than at 12 weeks) was chosen on the assumption that the observed changes in HBsAg titre whilst on oral NA therapy would be less compared to treatment with Peg-IFN\(\alpha\).

**Analysis of HBsAg Titre changes at the last Treatment/Study Period time-point**

The change in serum HBsAg from baseline to the last follow-up sample was analysed and classified into three different patterns: \textit{Slow Decline}, \textit{No Change}, or \textit{Slow Increase}. A \textit{Slow Decline} and \textit{Slow Increase} pattern in HBsAg were defined as
a $>0.5 \log_{10}$ IU/mL decrease or $>0.50 \log_{10}$ IU/mL increase in HBsAg titre from first to last follow-up sample respectively. Patients who did not reach the $0.5 \log_{10}$ IU/mL target, but exhibited an overall steady pattern of either HBsAg titre decline or increase were also classified as Slow Decline or Slow Increase patterns, respectively. A No Change pattern was defined as a HBsAg titre between 0-0.5 $\log_{10}$ IU/mL from baseline to last follow up sample.

4.2.5 HBV DNA Measurement

HBV DNA viral load testing was performed using the Versant HBV DNA 3.0 (Siemens Healthcare Diagnostics, Tarrytown, NY, USA), according to the manufacturer’s instructions (dynamic range $3.5 \times 10^2 - 1.8 \times 10^7$ IU/mL). Where possible, values outside this range were diluted 1 in 1000 to obtain a defined titre within the range of the assay. ARCHITECT HBsAg Manual Diluent (Abbott Laboratories) was used to dilute patient sera. This diluent contains recalcified human plasma that is non-reactive for HBsAg, HIV-1 RNA or HIV-1 Ag, anti-HIV-1/HIV-2, anti-HCV, or anti-HBs according to the manufacturer.

4.2.6 HBV Genotype

Genotyping (HBV genotype A-H) was performed in 11/16 (70%) patients using methods as previously outlined in Chapter 2.4.6.

4.2.7 Statistical Analysis

Statistical analysis was performed using GraphPad Software, San Diego California USA, www.graphpad.com. Continuous and categorical variables were compared between groups, using the Mann-Whitney test for non-parametric continuous data, and Chi square / Fisher’s exact test for categorical data.
4.3 Results

The HBsAg titres in the long-term therapy cohort were analysed separately from the two patients with dual liver biopsies. This was because these two patients only had data for treatment until 12 months, and did not meet the criteria for long-term therapy.

4.3.1 Long-term Therapy Cohort

4.3.1.1 Baseline Characteristics

Sixteen patients receiving long-term oral NAs were evaluated. Lamivudine (LMV) was the initial oral NA in 15/16 (94%) patients, with the other being initially commenced on Adefovir (ADV).

The baseline characteristics are presented in Table 4.1. There were 12 males and four females, and five HBeAg positive versus 11 HBeAg negative patients. The median age at commencement of antiviral therapy was 46 years (42 years of age in the HBeAg positive group versus 49 years of age in the HBeAg negative group).

The majority (11/16, 69%) of patients were of Asian ethnicity, with the remainder being of Mediterranean descent (5/16, 31%). Genotype was available in 11 (70%) patients, and was consistent with ethnicity. Genotype B and C were prevalent in Asians (two genotype B, five genotype C), whilst all patients of Mediterranean background were infected with genotype D HBV. The median duration of therapy was 46 months.

The median baseline serum HBV DNA was $7.10 \log_{10} \text{IU/mL}$ (>7.25 $\log_{10} \text{IU/mL}$ for HBeAg positive patients [upper limit of bDNA assay], and 5.8 $\log_{10} \text{IU/mL}$ for HBeAg negative patients. The median serum HBsAg titre was 6556 IU/mL (3.82 $\log_{10} \text{IU/mL}$), with overall titres being higher in the HBeAg positive compared to the HBeAg negative subgroup (median 9,312 versus 4,932 IU/mL, p=0.08)

The median necro-inflammatory and fibrosis scores using the METAVIR scoring system were A2F1 in the HBeAg positive subgroup, versus A2F2 in the HBeAg negative subgroup.
Table 4.1 Baseline Characteristics of the Long-term Treatment Cohort.

<table>
<thead>
<tr>
<th></th>
<th>Entire Cohort</th>
<th>HBeAg Positive CHB</th>
<th>HBeAg Negative CHB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total HBeAg Positive</td>
<td>Asian Ethnicity</td>
</tr>
<tr>
<td>Number</td>
<td>16</td>
<td>5 (31%)</td>
<td>4</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Age (yrs)*</td>
<td>46</td>
<td>42</td>
<td>41</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>11 / 16 (69%)</td>
<td>4</td>
<td>See above</td>
</tr>
<tr>
<td>Mediterranean</td>
<td>5 / 16 (31%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(available in 11/16 patients)</td>
<td>B - 2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C - 5</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>D - 4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Follow up (months)*</td>
<td>46</td>
<td>42</td>
<td>49</td>
</tr>
<tr>
<td>Serum ALT*</td>
<td>92</td>
<td>81</td>
<td>82</td>
</tr>
<tr>
<td>HBV DNA (log_{10} IU/mL)*</td>
<td>7.10</td>
<td>&gt;7.25</td>
<td>&gt;7.25</td>
</tr>
<tr>
<td>Biopsy (METAVIR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HBsAg Titre (IU/mL)*</td>
<td>6,556</td>
<td>9,312</td>
<td>8,012</td>
</tr>
<tr>
<td>HBsAg Titre (log_{10} IU/mL)*</td>
<td>3.82</td>
<td>3.97</td>
<td>3.90</td>
</tr>
</tbody>
</table>

* Median values
4.3.1.2 Interim Analyses Following 12 Months of Therapy

Interim Analysis: HBV DNA

Overall, 56% of patients achieved a virological response, which was defined as an undetectable HBV DNA on bDNA testing at 12 months of therapy. HBeAg positive patients were less likely to achieve a virological response compared to HBeAg negative patients (20% versus 64%, \( p=0.282 \)).

A virological breakthrough (VB) was defined as an increase in serum HBV DNA by \( \geq 1 \log_{10} \text{IU/mL} \) above nadir after achieving a virologic response during continued treatment. A VB was noted in 3/16 (19%, all HBeAg negative) of the patients after 12 months of therapy.

HBV DNA and HBsAg titres were not correlated due to the small sample size (see Chapter 3 for baseline correlations in a larger cohort). The pattern of HBV DNA titres during therapy had no significant influence on changes in HBsAg levels.

Interim Analysis: Serum ALT levels

Normalisation of serum ALT levels occurred in 10/16 (63%) of patients during the first 12 months of therapy; 2/5 (40%) HBeAg positive patients versus 8/11 (73%) HBeAg negative patients.

Interim Analysis: HBsAg Titre

Within the overall cohort of 16 patients, there was one case of a Significant decline (SD) pattern in HBsAg titre during the first 12 months of therapy (Table 4.2). This was a 22 year old male with Genotype C HBV and HBeAg positive CHB, who was initially commenced on LMV therapy. The baseline HBsAg titre was relatively high at 5.08 \( \log_{10} \text{IU/mL} \), with a baseline HBV DNA and serum ALT level of \( >7.25 \log_{10} \text{IU/mL} \) and 182 U/L, respectively (Figure 4.1 & Figure 4.4e). On liver biopsy, the patient had mild/moderate inflammation and mild fibrosis (Metavir score A2F1). Following 12 months of therapy, a 0.88 \( \log_{10} \text{IU/mL} \) reduction in HBsAg was observed (5.08 to 4.2 \( \log_{10} \text{IU/mL} \)). However, the HBV DNA remained relatively high at 6.8 \( \log_{10} \text{IU/mL} \), and the serum ALT had not yet normalised. This patient received “add on” ADV at month 18 in the setting of a likely virological breakthrough (unfortunately the upper limit of HBV DNA level was not obtained for his patient).
Despite achieving a SD pattern for HBsAg, the HBV DNA titre remained detectable by the end of follow up, and the patient did not achieve either a HBsAg or HBeAg seroconversion.

![Graph](image)

**Figure 4.1.** SD in HBsAg Titre at 12 months of therapy in one patient with HBeAg positive CHB.

There were no cases of a SI pattern in HBsAg titre during the first 12 months of therapy. HBsAg titres were noted to increase only in the setting of a virological breakthrough (see below)

### 4.3.1.3 Analyses to the Last Treatment Period Time-point

**HBV DNA**

All patients demonstrated a reduction in HBV DNA whilst on oral NA (Figure 4.2 a-c). Overall, 63% (10/16) of patients had an undetectable HBV DNA by the end of the treatment period. HBeAg positive patients were less likely to have an undetectable HBV DNA (2/5 HBeAg positive patients versus 8/11 HBeAg negative patients, p=0.16). A virological breakthrough occurred in 6/16 (38%) of the patients during therapy.
Figure 4.2 a-c. Individual patient plots of the changes in HBV DNA whilst on oral NA therapy: a) HBeAg Positive cohort, b) HBeAg Negative and Slow Decline HBsAg pattern cohort, c) HBeAg Negative and No Change HBsAg pattern cohort.
Serum ALT levels

A biochemical response with normalisation of serum ALT levels was evident in the majority of patients by the end of the treatment period. The serum ALT level was observed to increase in the setting of a virological breakthrough.

Patterns of HBsAg Titre Changes

Two common patterns of serum HBsAg titre changes were observed between the baseline and final follow up HBsAg level during long-term NA therapy: Slow Decline and No Change (Table 4.2). In the long-term cohort, there were no instances of a Slow Increase pattern, although HBsAg titres did increase in the setting of virological breakthrough.

Table 4.2. Patterns of HBsAg levels during NA therapy

<table>
<thead>
<tr>
<th>Pattern</th>
<th>INTERIM ANALYSIS at 12 months</th>
<th>END OF TREATMENT PERIOD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Significant Decline*</td>
<td>Significant Increase*</td>
</tr>
<tr>
<td>Total n=16</td>
<td>1/16</td>
<td>0</td>
</tr>
<tr>
<td>HBeAg positive n=5</td>
<td>1/5</td>
<td>0</td>
</tr>
<tr>
<td>HBeAg negative n=11</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significant Decline and Increase Pattern – either a >0.5 \( \log_{10} \) IU/mL reduction or increase in HBsAg titre at 12 months respectively
# Slow Decline and Slow Increase Pattern – either a >0.5 \( \log_{10} \) IU/mL reduction or increase in HBsAg titre from baseline to the end of follow-up
^ No Change Pattern – a 0.00 to 0.5 \( \log_{10} \) IU/mL change in HBsAg titre from baseline to the end of follow-up

Slow Decline Pattern in Serum HBsAg Levels

In the five HBeAg positive patients, an overall Slow Decline pattern in HBsAg titre was observed in all cases (Figure 4.3 & Figure 4.4). The magnitude of decline was significantly less than the multi-log reduction in viral load. The median yearly HBsAg decline in HBeAg positive patients was 0.12 \( \log_{10} \) IU/mL (range 0.07-0.21). HBeAg seroconversion was achieved in 3/5 (60%) cases during this period. In these three cases, the baseline and 12 month HBsAg levels were not significantly different from the two patients who did not achieve HBeAg seroconversion. No HBeAg positive patients achieved HBsAg seroclearance during the follow up period.
**Figure 4.3.** Patterns of HBsAg levels during NA therapy in overall HBeAg positive cohort. VB; increase in HBsAg titre associated with virological breakthrough.

**Figure 4.4 a-e.** Individual patient graphs for the Slow Decline HBeAg positive subgroup. HBsAg titre (solid line). HBV DNA (dotted line). Serum ALT level (numbering at top of each graph). Lower limit of HBV DNA; 2.55 log$_{10}$ IU/mL.
In the 11 HBeAg negative patients, a *Slow Decline* pattern was observed in four (36%) cases (Figure 4.5 & 4.6). The median yearly HBsAg decline in HBeAg negative patients was 0.15 log$_{10}$ IU/mL. Of the four HBeAg negative patients with HBV Genotype D, a *Slow Decline* pattern was evident in 2/4 (50%) of cases. It was not possible to compare Slow Decline and No Change patterns with HBV Genotypes B and C due to a lack of sample size.

![Figure 4.5. Patterns of HBsAg levels during NA therapy in the Slow Decline HBeAg negative cohort. VB; increase in HBsAg titre associated with virological breakthrough.](image-url)
Figure 4.6 a-d. Individual patient graphs for the Slow Decline HBeAg negative cohort. HBsAg titre (solid line), HBV DNA (dotted line). Serum ALT (numbering at top of each graph). Lower limit of HBV DNA; 2.55 log\textsubscript{10} IU/mL.

No Change in Serum HBsAg Levels

This pattern was observed in 7/11 (64%) of HBeAg negative patients (Figure 4.7 & 4.8), with a median yearly change in HBsAg titre of 0.03 log\textsubscript{10} IU/mL. This was despite the suppression of HBV DNA to an undetectable level in all cases. There were three patients with a low baseline HBsAg of less than 3 log\textsubscript{10} IU/mL.

There were no HBeAg positive patients (0/5, 0%) who displayed a No Change pattern during the study duration.

Figure 4.7. No Change pattern in HBeAg negative patients. VB; increase in HBsAg titre associated with virological breakthrough.
Figure 4.8 a-g. Individual patient graphs for the No Change Pattern HBeAg negative subgroup. HBsAg titre (solid line). HBV DNA (dotted line). Serum ALT (numbering at top of each graph). Lower limit of HBV DNA; $2.55 \log_{10} \text{IU/mL}$.
4.3.1.4 HBsAg Sero-clearance

Within the cohort of 16 patients, one patient achieved HBsAg seroclearance (Figure 4.6a & 4.9). This patient was a 51 year old man with Genotype B HBV infection. The patient was HBeAg negative, and had a pre-treatment liver biopsy which demonstrated mild inflammation and moderate fibrosis (METAVIR score A1F2). The baseline viral load, HBsAg titre and serum ALT were >7.25 log_{10} IU/mL, 3.69 log_{10} IU/mL and 90 U/L respectively. The patient was on LMV therapy for the entire study duration, and prior to HBsAg loss at month 45, had demonstrated complete suppression of HBV DNA for almost 36 months, and an overall reduction in HBsAg titre of 0.54 log_{10} IU/mL (3.69 to 3.15 log_{10} IU/mL). There was no significant flare in the serum ALT level prior to HBsAg loss.

![Figure 4.9. HBsAg seroclearance at month 45 in a HBeAg negative patient.](image)

4.3.1.5 Prediction of Slow Decline Pattern

Using univariate analyses, the factors associated with a Slow Decline pattern were HBeAg status (positive 5/5 100%, negative 4/11 36%, p=0.03), higher median baseline HBsAg titre (4.00 versus 3.19 log_{10} IU/mL, p=0.04), and younger median age (42 versus 50 years of age, p=0.02). There was no difference in the median baseline HBsAg level between HBeAg positive and negative patients who demonstrated a Slow Decline pattern (Figure 4.10). The No Change group had the lowest median baseline level of HBsAg, which was 3.19 log_{10} IU/mL (p=0.04)

By contrast, ethnicity, gender, baseline ALT, baseline HBV DNA, viral genotype and degree of inflammation or fibrosis were not associated with a Slow Decline in HBsAg titre pattern.
4.3.1.6 Increases in HBsAg titre occur after Virological Breakthrough due to Antiviral Drug Resistance

There were six cases (38%) of VB which were all associated with an increase in HBsAg titre. The HBsAg titre increases were usually between two to three fold in magnitude, rather than being greater than one log_{10} IU/mL. An increase in HBsAg level tended to occur after a lag-phase of between three to six months after a VB. Subsequent therapy included switch to ADV, add-on ADV, or switch to ETV (1.0 mg per day). This led to further suppression of viral replication, and the stabilisation or a further decline in HBsAg level. A representative example of this is shown in Figure 4.11.
4.3.1.7 Estimated Time to HBsAg Seroclearance

The time to HBsAg seroclearance as estimated using an Excel database (Microsoft, Redmond, WA, USA) was greater than 10 years. This was based on the assumption of an ongoing linear reduction in HBsAg titre in those with a Slow Decline pattern (HBeAg positive $0.12 \log_{10} \text{IU/mL}$, HBeAg negative $0.15 \log_{10} \text{IU/mL}$). For example, the median HBsAg level at the end of the study period in HBeAg positive patients was $3.68 \log_{10} \text{IU/mL}$. Assuming a yearly linear reduction $0.12 \log_{10} \text{IU/mL}$, after 10 further years the HBsAg titre would be $2.48 \log_{10} \text{IU/mL}$. Similarly, the median HBsAg level at the end of the study period in HBeAg negative patients with a Slow Decline pattern was $3.40 \log_{10} \text{IU/mL}$. Assuming a yearly linear reduction of $0.15 \log_{10} \text{IU/mL}$, after 10 further years the median HBsAg would be $1.90 \log_{10} \text{IU/mL}$.

However, it is recognised that the reduction in HBsAg titre may not always be linear. Indeed the single patient who achieved HBsAg seroclearance had a HBsAg titre of greater the $3 \log_{10} \text{IU/mL}$ several months prior to HBsAg loss.

Figure 4.11  Lag phase between VB and an increase in HBsAg level. HBV DNA (dotted line), HBsAg (solid line)
4.3.2 Comparison of Intrahepatic HBsAg Staining in Two Patients with a Baseline and Second On-Treatment Liver Biopsy

There were two additional patients who had a second liver biopsy at 12 months as part of a separate clinical trial. These patients had treatment with TDF, and only had data until month 12 of therapy. Although these patients did not meet the criteria for long-term therapy, they were included because it was felt that a comparison of intrahepatic HBsAg staining using immunohistochemistry between pre-treatment and on-treatment biopsy specimens, and serum HBsAg titres could be relevant to the present study.

4.3.2.1 Baseline Characteristics

The baseline characteristics of the two patients are outlined below. There was one HBeAg positive and one HBeAg negative patient. The HBeAg negative patient was older, had a lower baseline HBV DNA, and a higher fibrosis score on liver biopsy.

Table 4.3. Baseline Characteristics and Results

<table>
<thead>
<tr>
<th></th>
<th>Patient One</th>
<th>Patient Two</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td><strong>Age (yrs)</strong></td>
<td>28</td>
<td>55</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td>Caucasian</td>
<td>Mediterranean</td>
</tr>
<tr>
<td><strong>Antiviral Therapy</strong></td>
<td>TDF</td>
<td>TDF</td>
</tr>
<tr>
<td><strong>HBV Genotype</strong></td>
<td>B</td>
<td>D</td>
</tr>
<tr>
<td><strong>HBeAg Status</strong></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td><strong>HBV DNA (log_{10} IU/mL)</strong>*</td>
<td>8.81</td>
<td>7.11</td>
</tr>
<tr>
<td><strong>Serum ALT (U/L)</strong>*</td>
<td>107</td>
<td>532</td>
</tr>
<tr>
<td><strong>Liver Biopsy (METAVIR)</strong>*</td>
<td>A1F1</td>
<td>A3F2</td>
</tr>
<tr>
<td><strong>HBsAg Titre (log_{10} IU/mL)</strong>*</td>
<td>5.12</td>
<td>3.86</td>
</tr>
</tbody>
</table>

* Baseline values

4.3.2.2 Analysis at 12 months of Therapy

The on-treatment response during TDF therapy between baseline and month 12 is demonstrated in Figure 4.12 & Figure 4.13. Both Patient One and Two demonstrated a virological and biochemical response. However, whilst Patient One had a Significant Decline in HBsAg titre following 12 months of therapy (5.12 to 4.08 log_{10} IU/mL), Patient Two had a Significant Increase in HBsAg titre during the same time period (3.86 to 4.37 log_{10} IU/mL)
Figure 4.12 & 4.13. Patient One and Two: Changes in HBsAg titre, HBV DNA and Serum ALT following 12 months of TDF therapy. HBsAg titre (solid line), HBV DNA (dotted line). Serum ALT (numbering at top of each graph). Lower limit of HBV DNA; 2.55 log_{10} IU/mL.

4.3.2.3 Liver Biopsy: Histology

The second liver biopsy from Patient One demonstrated an improvement in fibrosis score, but no change in inflammation. In contrast, the second liver biopsy from Patient Two demonstrated no change in fibrosis, but an improvement in inflammation (Table 4.4).

Table 4.4 METAVIR scores at baseline and second liver biopsy.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Baseline Biopsy</th>
<th>Follow up Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inflammation</td>
<td>Fibrosis</td>
</tr>
<tr>
<td>One</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Two</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>
4.3.2.4 Liver Biopsy: Immunohistochemistry for HBsAg

Immunohistochemistry was performed on both matched sets of liver biopsy specimens to identify and compare the intrahepatic localisation and intensity of HBsAg (Table 4.5, Figure 4.14 – 4.17). The intrahepatic HBsAg staining was low and distributed in the cytoplasm for Patient One in both liver biopsy specimens. In contrast, Patient Two had a higher baseline amount and intensity of staining, with both cytoplasmic as well as a membranous distribution. Furthermore, the follow up biopsy demonstrated increased amount and intensity of staining, but was limited to a membranous distribution only.

Table 4.5 Baseline Characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>Percentage Staining</th>
<th>Intensity</th>
<th>Distribution</th>
<th>Percentage Staining</th>
<th>Intensity</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>1%</td>
<td>1</td>
<td>Cytoplasmic</td>
<td>1%</td>
<td>1</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Two</td>
<td>5%</td>
<td>2</td>
<td>Cytoplasmic/Membranous</td>
<td>10%</td>
<td>3</td>
<td>Membranous</td>
</tr>
</tbody>
</table>
Figure 4.14. Baseline Biopsy for Patient One. Percentage HBsAg staining (1%), Intensity (1), Cytoplasmic distribution

Figure 4.15. Follow up Biopsy for Patient One. Percentage HBsAg staining (1%), Intensity (1), Cytoplasmic distribution
Figure 4.16. Baseline Biopsy for Patient Two. Percentage HBsAg staining (5%), Intensity (2), Cytoplasmic/Membranous distribution

Figure 4.17. Follow Up Biopsy for Patient Two. Percentage HBsAg staining (10%), Intensity (3), Membranous distribution
4.4 Discussion

This study aimed to evaluate the changes in HBsAg titre in a cohort of sixteen patients with CHB receiving long-term oral NA therapy. The majority of these patients were commenced on LMV therapy (94%), with a median study period of 46 months. There were five HBeAg positive and eleven HBeAg negative patients. Consistent with the current understanding of the natural history of CHB, HBeAg negative patients overall tended to be older, with a lower HBV DNA level and higher median fibrosis score. Additionally, there was a mixture of Asian and Mediterranean patients, with HBV genotypes which reflected their ethnicity.

An interim analysis was performed following 12 months of oral NA therapy, and HBsAg titres were assessed for a Significant Decline (SD) or Significant Increase (SI) pattern, which were defined as a >0.5 log10 IU/mL reduction or increase in HBsAg titre respectively. The value of 0.5 log10 IU/mL was based on a study using Peg-IFNα for the treatment of CHB, which suggested achieving this reduction at week 12 was “significant”, and may be a positive predictor of effective and durable suppression of viral replication. A timeframe of 12 months (instead of 12 weeks) was chosen because it was assumed given the HBsAg seroclearance rates on oral NA therapy are lower than for Peg-IFNα, any observed changes in HBsAg titre would also be slower. Within the long-term cohort of 16 patients, there was one HBeAg positive patient who demonstrated a SD pattern (5.08 to 4.2 log10 IU/mL), and no instances of a SI pattern.

At the end of the treatment period, HBsAg titres were assessed for a Slow Decline, No Change or Slow Increase pattern. All five HBeAg positive patients exhibited a Slow Decline pattern, with a median yearly decline of 0.12 log10 IU/mL. HBeAg seroconversion occurred in 3/5 (40%) of cases. Within the HBeAg negative group, a Slow Decline pattern was observed in 4/7 (36%) of cases, with the remaining seven patients (7/11, 64%) being classified as No Change. There were no observed Slow Increase pattern cases. The median yearly HBsAg decline in the “HBeAg negative with Slow Decline” subgroup was 0.15 log10 IU/mL. The factors associated with a Slow Decline pattern were HBeAg status (HBeAg-positive 5/5 100%, HBeAg-negative 4/11 36%, p=0.03), higher median baseline HBsAg titre (4.00 versus 3.19 log10 IU/mL, p=0.04), and younger median age (42 versus 50, p=0.02). The HBV
genotype did not appear to influence the pattern of HBsAg titre decline, however, this may have been limited by the small sample size of this study.

HBsAg titres were observed to increase following the development of a VB as a result of antiviral resistance. This usually occurred 3-6 months following VB, and were between two to three fold in magnitude rather than the >1.0 log_{10} IU/mL increases observed in HBV DNA. Commencement of salvage therapy resulted in stabilisation and/or further reduction in HBsAg titres.

The estimated time to achieve HBsAg loss was determined using the median yearly HBsAg titre decline observed in HBeAg positive and negative patients (0.12 and 0.15 log_{10} IU/mL). Assuming a yearly linear decline in HBsAg, it was estimated that it would take greater than 10 years to achieve HBsAg loss. In this study HBsAg loss occurred in one patient (1/16, 6.3%) over the study period. The HBsAg titres were observed to be declining slowly (3.69 to 3.15 log_{10} IU/mL) in this patient, whereupon HBsAg loss abruptly occurred at month 45. The HBV DNA had been undetectable for almost 36 months, and there was no significant ALT flare prior to HBsAg loss.

Two additional patients who had a second “on-treatment” liver biopsy at month 12 were included in this study, and analysed separately because the criteria for long-term treatment (greater than two years duration) had not been fulfilled. Both patients (one HBeAg positive, one HBeAg negative) were commenced on TDF therapy as part of a separate clinical study. Following 12 months of therapy, both patients demonstrated a virological and biochemical response. However, whilst Patient One was observed to have a SD (5.12 to 4.08 log_{10} IU/mL) pattern in HBsAg titre, Patient Two in contrast had a SI (3.86 to 4.37 log_{10} IU/mL) pattern. In addition, the month 12 liver biopsy from Patient Two demonstrated no improvement in fibrosis score, and immunohistochemistry on the specimen revealed an increased amount and intensity of HBsAg staining, with a slight change in distribution from cytoplasmic/membranous, to membranous only, a pattern previously describe to be associated with high viraemia^{174} (see below).

An understanding of the HBV life-cycle is necessary when assessing the difference in kinetics between HBV-DNA and HBsAg observed in this study. In the context of the HBV life-cycle, NA agents inhibit HBV-DNA replication by blocking reverse transcription (Figure 4.18). This reduces the formation of mature HBV nucleocapsids, resulting not only in viral load reduction, but also reduced recycling of
relaxed circular DNA (RC-DNA) back to the nucleus for conversion into cccDNA. Thus, whilst it is important to note that NAs do not directly inhibit the production of HBsAg, HBsAg synthesis may be indirectly affected by oral NA via inhibition of the intracellular cccDNA conversion pathway, with a subsequent decline in pre-existing cccDNA molecules over time (dependent on hepatocyte turnover). It should also be noted however, that a previous study has demonstrated that viral suppression from LMV therapy is associated with the partial HBV non-specific restoration of CD4+ and CD8+ T cells against HBV epitopes\(^{175}\). The decline in HBsAg titres demonstrated in this study is consistent with the known mechanism of action of oral NAs, and was much smaller than the multi-log reduction observed in HBV DNA. In this study, the magnitude of decline in serum HBsAg titre was small (median yearly 0.12-0.15 log\(_{10}\) IU/mL), and similar to the on-treatment decline in cccDNA previously described\(^{176}\).

The indirect effect of NAs on HBsAg would be expected to result in a lag-phase between HBV DNA and HBsAg titre decline. A delay has previously been described between HBV viral load and quantitative HBeAg decline during potent antiviral therapy\(^{177}\). In contrast, in the sixteen patients describe in this chapter, there did not appear to be any significant delay between viral load reduction and HBsAg titre, although this may be have been limited by type II error. However, a delay in HBV DNA and HBsAg changes was evident with VB (Figure 4.11). Thus, in a clinical setting, quantitation of HBsAg does not appear to offer any advantage over serum viral load in detecting the emergence of antiviral resistance.
Several studies have suggested a potential role for monitoring serum HBsAg titres during Peg-IFNα therapy\textsuperscript{134, 165, 172}. In HBeAg negative patients treated with Peg-IFNα therapy, a $>0.5 \log_{10}$ IU/mL reduction in HBsAg titre at week 12 has a 89% positive predictive value for achieving an undetectable viral load at 24 weeks post therapy\textsuperscript{134}, and a $>1.0 \log_{10}$ IU/mL reduction in HBsAg titre by week 48 is associated with nearly an eleven fold increased chance of HBsAg seroclearance\textsuperscript{165}. Similarly, HBeAg positive patients who demonstrate a reduction in HBsAg titre to $<1500$ IU/mL at week 12 on Peg-IFNα therapy are more likely to achieve HBeAg seroconversion and HBsAg loss\textsuperscript{172}.

In contrast, the significance of HBsAg titre changes during oral NA therapy has not been clearly evaluated. In this study, the defined on-treatment changes in HBsAg titre did not clearly predict a favourable virological response. The single patient who was observed to have a SD pattern in HBsAg titre at 12 months developed a VB at month 18, and continued to have detectable HBV DNA at the end of the study period. Furthermore, five of the nine patients (3/5 HBeAg positive, 2/4 HBeAg negative) who were observed to have a Slow Decline pattern developed a VB whilst on therapy, and required salvage antiviral therapy.
There are a number of possible reasons to explain why the HBsAg decline whilst on oral NA therapy did not represent a marker of effective suppression of viral replication in this study. Firstly, the kinetics of HBsAg decline are much more rapid on Peg-IFNα than oral NA, and it may be that it is only an early (e.g. week 12) decline in HBsAg levels which is more predictive. Furthermore, the immune modulating effect of Peg-IFNα can directly affect viral replication and HBsAg production. Peg-IFNα has a broadly acting non-specific antiviral effect at a transcriptional and translational level. In contrast, oral NAs essentially only target the HBV polymerase. It may be that with oral NA therapy, a reduction in HBsAg titre to a specific threshold level is more helpful than achieving a specific reduction (e.g. >0.5 log₁₀ IU/mL). Finally, the sample size of this study was small, with the majority of patients being initially commenced on LMV which is a low potency drug with a low genetic barrier to antiviral resistance. Future clinical trials using newer and more potent oral NAs could evaluate whether an on-treatment HBsAg titre decline to a specific level is a predictor of durable suppression of viral replication.

The pattern of HBsAg titres was classified as No Change in 7/16 (44%) patients, all of whom were HBeAg negative. Other than HBeAg status, other predictive factors for a No Change pattern were older age, and a lower baseline HBsAg titre. Age may be a confounder with HBeAg status, as the median age of HBeAg positive cases was lower (42 years versus 49 years). Viral integration may possibly explain the association of a lower baseline HBsAg titre with a No Change pattern. Whilst integrated HBV sequences cannot provide a template for productive viral replication, HBsAg synthesis from viral integration may be produced independently of HBV cccDNA, and thus would not be expected to be affected by oral NAs. This is in contrast to the immune-modulating effect of Peg-IFNα which may also result in loss of infected cells, as well as a broad antiviral activity. Another possible explanation is that there may be a baseline “set-point” level of HBsAg production and secretion which is not significantly affected by fluctuations in viral load. This set-point may be a reserve or threshold level of ongoing HBV cccDNA transcription and mRNA translation which is not affected by oral NA therapy.

The rate of HBsAg loss whilst on oral NA therapy is higher than the spontaneous HBsAg loss rate. The HBsAg loss rate following 96 weeks of continuous ETV or TDF therapy is 5% and 6% respectively. In these studies,
HBsAg loss was much more likely in HBeAg positive patients, and those with HBV Genotypes A and D (i.e. “non Asian” HBV genotypes)\textsuperscript{123, 124}. In contrast, the single patient who achieved HBsAg loss in this study was HBeAg negative and was infected with genotype B HBV. Whilst HBsAg loss occurred at month 45, it is also interesting to note that at month 42 the patient had a HBsAg titre of 3.15 log\textsubscript{10} IU/mL and serum ALT of 43U/L. A HBsAg titre of 3.15 log\textsubscript{10} IU/mL is similar to the median baseline HBsAg level in patients in the low replicative phase (2.90 log\textsubscript{10} IU/mL, Chapter 3.3.1.1). Taken together, it is tempting to speculate that HBsAg may only need to decline to a certain level (e.g. approximately 3 log\textsubscript{10} IU/mL) for adequate immunological control of HBV, and possible subsequent HBsAg loss to then occur. Furthermore, the lack of an ALT flare prior to HBsAg loss suggests that HBV may have been cleared by a non immune based mechanism such as apoptosis. These are important hypotheses which should be evaluated in future clinical research studies.

One of the major goals of antiviral therapy for CHB is the effective suppression of viral replication. This has been traditionally assessed by regular monitoring of HBV DNA. However, HBsAg loss/seroconversion is the preferred endpoint of therapy because it is believed to represent successful immunological control of the HBV, is associated with a favourable prognosis, and may also provide the opportunity for cessation of antiviral NA-based therapy. A recent longitudinal study evaluating the clinical outcome of HBsAg seroclearance has identified the age of the patient at which HBsAg seroclearance occurs as an important factor in determining the risk of hepatocellular carcinoma (HCC)\textsuperscript{128}. This study from Hong Kong followed 298 patients, and demonstrated that HBsAg seroclearance before the age of 50 was associated with a lower risk of HCC development in comparison to later HBsAg seroclearance (after 50 years)\textsuperscript{128}. Consequently, there has been a tendency to aim at not only suppressing viral replication, but also achieving increased rates of HBsAg loss. The results of this long-term treatment study strongly support a Peg-IFN\textalpha based approach to the management of CHB. Further studies are required to assess the effect of combination Peg-IFN\textalpha and potent oral NA therapy on the HBsAg decline rate. In the future, algorithms may be developed which individualise patient therapy depending on the rate of HBsAg titre decline. For example, potent oral NA therapy could be “added” to Peg-IFN\textalpha if a slow decline in HBsAg titre is observed at week 12. Alternatively, Peg-IFN\textalpha therapy could be added if a patient on long term
oral NA therapy demonstrates effective suppression of viral replication, but minimal or slow reduction in HBsAg titre.

Intrahepatic demonstration of HBV antigens (HBcAg and HBsAg) using immunohistochemistry is an important diagnostic parameter, particularly when the aetiology of liver disease is unclear. Three patterns of intrahepatic HBsAg staining in liver tissue have been described: membranous, submembranous and cytoplasmic. The immune tolerant phase of CHB infection with its high viraemia is characterised by submembranous HBsAg staining in conjunction with diffuse nuclear HBcAg staining. Membranous staining of HBsAg has been well correlated with HBV DNA and disease activity. Cytoplasmic HBsAg staining has been associated with histologically inactive liver disease and active viral replication.

In this study there were two patients who had a second liver biopsy at month 12 of TDF therapy. The baseline intrahepatic HBsAg staining was cytoplasmic for Patient One (HBeAg positive), and mixed cytoplasmic/membranous for Patient Two (HBeAg negative). Interestingly, whilst Patient Two was observed to suppress viral replication to an undetectable level by month 12, the HBsAg titre increased by 0.51 log10 IU/mL (3.86 to 4.37 log10 IU/mL), and there was an increased amount and intensity of intrahepatic HBsAg staining in a membranous pattern. The reason and significance of these observations are unclear. It is possible that given the high potency of TDF, there may a preferential translation of mRNAs encoding HBsAg, or that a virus variant has been selected that causes intracellular HBsAg retention, with the emergence of substitutions such as rtA181T or rtN236T which are ADV resistance changes that may also affect the antiviral efficacy of TDF. The rtA181T results in a sW172* change in the overlapping S region and causes intracellular retention.

Certainly a Significant Increase in HBsAg titre was not observed in any of the sixteen patients in the long term treatment cohort who were initially commenced on the low potency oral NA LMV. The increase in on-treatment HBsAg titre and intrahepatic HBsAg staining observed in Patient Two merits further evaluation in a larger cohort of patients.

The main limitation of this study was its small sample size. However, the study cohort was well characterised with regular serum samples obtained. Another limitation was that the majority of these patients were initially treated with LMV, which was the only medication available in most cases at the time. LMV is a low potency drug with a low genetic barrier to resistance, and is now no longer a preferred
first-line agent. It will be useful to study HBsAg titre changes using the newer and more potent oral NAs which have demonstrated higher HBsAg loss rates in HBeAg positive patients infected with HBV Genotypes A and D\textsuperscript{123, 124}. Whether the magnitude or patterns of on-therapy changes in HBsAg are different between various NAs has not been evaluated. A focus of future studies should be to incorporate a larger number of patients treated with newer oral NAs to confirm the preliminary findings of this study.

In conclusion, this study demonstrated that the longitudinal changes in HBsAg titre whilst on oral NA therapy are far smaller than the multi-log decreases in HBV DNA. Greater than 0.5 log\textsubscript{10} IU/mL reductions in HBsAg titre are uncommon in the first 12 months, with the common patterns of changes during the course of therapy include overall “Slow Decline”, “No Change” and “Increase with Virological Breakthrough”. Changes in HBsAg titres tend to occur after HBV DNA changes, and thus are unlikely to be more useful than HBV DNA in detecting the emergence of antiviral resistance. However, the patterns of change in HBsAg during oral NAs therapy may help predict the likelihood of achieving effective and durable suppression of viral replication and HBsAg seroclearance. Thus, the patient response “read-outs” should include not only HBV DNA, but also the pattern of HBsAg titre changes over time. It may be safe to trial discontinuation of therapy in HBeAg negative patients once the HBsAg titre has fallen to a threshold level, although this will need to be tested in controlled trials. The kinetics of HBsAg should be incorporated into the design of larger prospective trials involving newer antiviral agents. Finally, given the consensus in favour of achieving HBsAg loss rather than merely suppressing viral replication, this study strongly supports further investigation of the role of combination therapy including both an immune-modulating based approach (e.g. Peg-IFN\textsubscript{α}) as well as oral NA.
Chapter 5. Anti-HBs and Anti-HBe in the Pathogenesis of Chronic Hepatitis B

5.1 Introduction and Aims

The pathogenesis of CHB involves the complex interplay between HBV and the innate as well as the adaptive immune responses (Chapter 1.6). In acute HBV infection, spontaneous resolution is in part dependent on an effective humoral B lymphocyte response. As part of this response, activated B lymphocytes produce antibodies to the HBV antigens, specifically anti-HBs, anti-HBe and anti-HBc (Chapter 1.9). Anti-HBs is directed to the “a” epitope of HBsAg and is regarded as a neutralising antibody, with an important role in limiting further spread of infection. In addition, an anti-HBs immune response following vaccination is regarded as protective against subsequent infection. However, antibodies to the HBV core protein (anti-HBc) and precore protein (anti-HBe) are in general not believed to have any neutralising effect. Anti-HBc is a marker of previous exposure to HBV and can be detected during both acute and chronic infection. It crosses the placenta and provides no protective effect on the neonate following perinatal infection. The detection of anti-HBe in blood indicates the patient has either controlled HBV replication and is in the non-replicating phase, or is infected with a HBeAg escape variant and is in the HBeAg negative phase of CHB.

Both HBsAg and HBeAg are secreted into the systemic circulation, the former as subviral particles (and on virions) and the latter as a soluble protein. HBsAg is produced in excess of that required for virion assembly, and secreted subviral HBsAg particles typically exceed HBV virions by a variable factor of \(10^2\text{-}10^5\) (see discussion Chapter 3). Whilst HBsAg and HBeAg can be easily detected by commercial serological assays, anti-HBs and anti-HBe in contrast are generally only detectable in most patients with current commercial immunoassays after the respective antigens (HBsAg and HBeAg) have been cleared from the systemic circulation. Prior to clearance, it is thought that anti-HBs and anti-HBe may exist in the systemic circulation in the form of immune complexes with their respective antigens.
A previous study has detected serum anti-HBs and anti-HBe in the presence of HBsAg and HBeAg using in house ELISA assays\textsuperscript{66}. This Japanese study evaluated a series of serum samples in 200 patients with HBeAg positive CHB who were seronegative for both anti-HBe and anti-HBs by commercial immunoassay. Using sensitive in-house assays, they suggested that the majority of patients with evidence of liver disease, and 50\% of those without liver disease had demonstrable anti-HBe and anti-HBs\textsuperscript{66}. However, these results have not been confirmed. Antibodies to HBV antigens have also been detected in the lysate of the B lymphocyte component of peripheral blood mononuclear cells (PBMCs)\textsuperscript{186}. Furthermore, it has been suggested that antibodies against HBV antigens in B lymphocytes may be detected prior to their detection in serum\textsuperscript{186}.

The aim of the study described in this section was to test the serum and the B-cell component of PBMCs for anti-HBs and anti-HBe in patients who also test positive for serum HBsAg and HBeAg respectively, and relate the presence of these antibodies to the stage of their CHB.
5.2 Methods

5.2.1 Patient Recruitment

Treatment naïve patients \((n=120, \text{ HBeAg positive}=35, \text{ HBeAg negative}=85)\) with CHB were prospectively recruited from the specialist liver outpatient clinics of St Vincent’s Hospital, Melbourne (Chapter 2.3). Patients were clinically stable, with no evidence of decompensated liver disease at baseline (see Table 5.3 for demographic details). The serum alpha-fetoprotein level was normal, and ultrasound examination demonstrated no evidence of hepatocellular carcinoma (HCC) in any patient at the time of study.

All patients tested negative for laboratory markers of infection with the hepatitis C virus, hepatitis D virus and human immunodeficiency virus (HIV). Laboratory markers for co-existent auto-immune or metabolic liver disease were also negative.

Patient demographics, liver biochemistry results, qualitative HBsAg and HBeAg status (by standard qualitative enzyme immunoassay) and HBV DNA (Chapter 2.4.1) were recorded at baseline, and subsequently every three to six months. Biochemical and virological data were obtained from patient serum samples collected on the same day. Each patient also had 40mL of blood taken for PBMC collection (see below).

5.2.2 HBV Genotyping

Genotyping (HBV genotype A-H) was performed in 107/120 (89%) patient samples using methods as previously outlined (see Chapter 2.4.6)\textsuperscript{156}.

5.2.3 Sequencing: Precore, Basal Core Promoter and S region

Sequencing of the Precore (PC) and Basal Core Promoter (BCP) region of the HBV genome was (Chapter 2.4.7) performed in a subset of HBeAg positive patient serum samples with a low HBeAg titre of less than 100 PE IU/mL. This subset was chosen to determine the emergence of the BCP and PC variants prior to HBeAg seconversion (see Discussion for further explanation).
Sequencing of the S (envelope) region of the HBV genome was performed in patient serum samples with both detectable serum HBsAg and anti-HBs. This subset was chosen as it was thought that the co-existent detection of anti-HBs may reflect an underlying immune pressure on HBV, which may in turn select out possible anti-HBs escape variants.

Briefly, HBV DNA was extracted from 200uL of patient serum using the QIAamp DNA MiniKit (QIAGEN, CA, USA) according to the manufacturer’s instructions. Polymerase chain reaction (PCR) testing was then used to amplify the BCP, PC and S regions of the HBV genome, as previously described\textsuperscript{156}. HBV genes were then analyzed using the online HBV genome analysis program (SeqHepB: www.seqvirology.com)\textsuperscript{157}. This program compares input sequence data with known reference sequences to determine HBV genotype, variants and mutations associated with antiviral resistance. The PC variant was defined by the presence of the G1896A mutation (PC mutation), and the BCP variant by the A1762T/G1764A mutation (BCP mutation).

This study was approved by the St Vincent’s Hospital Human Research and Ethics Committee, in compliance with the Declaration of Helsinki.

5.2.4 Healthy Controls

Blood samples were also taken from four healthy controls, who were negative for markers of infection with HBV. These controls had received hepatitis B vaccination in the past, but were found to have a low (<100 IU/mL) anti-HBs level. Each consented to have a booster vaccination, and also for the collection of a 40mL blood sample for PBMC isolation (Chapter 2.4.9) prior to, and seven days after booster vaccination. A time-frame of seven days was chosen based on a previous study which demonstrated that following vaccination for hepatitis B, anti-HBs could be detected in B cell lysates within seven to fourteen days\textsuperscript{186}. A blood sample was not re-collected at day fourteen post vaccination because each of the four controls demonstrated an adequate rise in serum anti-HBs on commercial immunoassay (AxSYM; Abbott, USA) at day seven post vaccination. An adequate post vaccination response was defined as a greater than ten fold increase in serum anti-HBs titre.
5.2.5 Detection of Serum Anti-HBs and Anti-HBe: Semi-Quantitative Testing

A commercial enzyme immunoassay (AxSYM; Abbott Laboratories, IL, USA) was used to determine the presence of serum anti-HBe and anti-HBs. Semi-quantitative serum anti-HBe analysis was only performed in the cohort of HBeAg positive patients and individuals (n=35). Serum anti-HBs was tested in the entire cohort of HBsAg positive patients (n=120).

5.2.6 Serum HBeAg and HBsAg Titres: Quantitative Testing

The baseline serum HBeAg and HBsAg titres were assessed for each patient using the ARCHITECT platform (Abbott Laboratories) according to the manufacturer’s instructions (Chapter 2.4.3 and 2.4.4). This was performed to determine if there were any differences in baseline HBeAg or HBsAg in patients who had detectable serum anti-HBe or anti-HBs.

5.2.7 Detection of Anti-HBs and Anti-HBe from the B-cell component of PBMCs

5.2.7.1 PBMC collection

The PBMCs were isolated from a 40mL sample of whole blood (Chapter 2.4.9), and collected on the same day as the blood sample for biochemical and virological data. Following isolation, the PBMCs were counted and then aliquoted and stored in a -70°C freezer overnight, and then transferred to liquid nitrogen.

5.2.7.2 Isolation of B lymphocytes from PBMCs

The B lymphocyte enriched component was separated from the PBMCs on the same day that they were required for subsequent testing and experimentation. B lymphocytes were separated from the aliquot of PBMCs using a commercially available magnetic bead isolation kit (BD Biosciences, North Ryde, NSW) according to the manufacturers’ instructions (Chapter 2.4.10). Briefly, the biotin conjugated antibodies were added to the PBMC sample. Two different antibody kits were used: B cell isolation Kit (CD2, CD14, CD16, CD 36, CD43, CD234a), and Antihuman CD19 beads. The B cell isolation kit uses an “indirect” method, with antibodies binding to
all cells except B lymphocytes. CD19 is a cell surface marker of B lymphocytes, and thus the antihuman CD19 beads represent a “direct” labelling system. Following the addition of the commercial antibody from the kit, anti-biotin microbeads were then added, and the sample then passed through the magnetic field of the MACS Separator.

Flow cytometry was used to confirm that the magnetic isolation kit successfully isolated the majority of B lymphocytes in the PBMC aliquot. Both the positive and negative fractions generated following isolation were then assessed to confirm: 1) that the majority of the positive fraction were B lymphocytes and 2) that the negative fraction had minimal B lymphocytes. Assessment was performed immediately after B lymphocyte isolation as a quality control measure on a subset of samples with the kind assistance of Dr Joe Manitta in the VIDRL biochemistry section.

5.2.7.3 Commercial Enzyme-Linked Immunoassay (EIA)

Initially, two commercial EIAs (AxSYM Abbott Laboratories, IL, USA; and Abbott Murex anti-DHBs assay, Wiesbaden, Germany) were used to determine the presence of anti-HBe and anti-HBs in the B-cell specimens from the PBMC aliquot. B lymphocytes were firstly isolated from an aliquot of PBMCs, and then disrupted with two different lysis buffers: a commercial lysis buffer, Roche mammalian lysis buffer, complete lysis M EDTA free, (Roche, Mannheim, Germany) according to the manufacturer’s instructions, and the non-denaturing “core prep” lysis buffer (10M Tris pH 7.5, 1mM EDTA, 50mM NaCl, 0.5% NP40) used at VIDRL for routine HBV DNA analysis from HBV infected cells. The B cell lysate was then diluted 1 in 2 with PBS prior to testing.

5.2.7.4 In-House Enzyme-Linked Immunosorbent Assay (ELISA)

An in-house sandwich ELISA was developed in order to increase the sensitivity for detecting the presence of anti-HBs from B-cells in the PBMCs. The presence of anti-HBe in the B-cells of the PBMCs was not assessed since the in-house EIA for anti-HBs could not be adequately optimised for technical reasons (see results section).

The key steps involved in the in-house ELISA included (Figure 5.1): adsorb HBsAg onto ELISA plate (solid phase), wash and block, incubate with B cell lysate, incubate with secondary antibody (conjugated to horseradish peroxidase [HRP]) , add
substrate, and measure absorbance on luminometer. A number of different sources of HBsAg, positive control anti-HBs and secondary antibodies were trialled in the assay optimisation process (see below).

![ELISA diagram](image)

**Figure 5.1** In-house sandwich ELISA. Both dimeric and monomeric anti-HBs IgG are shown (see Discussion for more detailed description).

**Initial In-house ELISA Protocol**

The initial protocol for the in-house ELISA as well as the in-house ELISA test components are described in more detail in Chapter 2.4.14. A number of modifications were made to this protocol during the optimisation process (see Results section)

**5.2.8 Statistical Analysis**

Continuous and categorical variables were compared between groups, using the Mann-Whitney test and Fisher’s exact test respectively. Statistical analysis was performed using GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com).
5.3 Results

5.3.1 Patients

One hundred and twenty treatment naïve patients with CHB were prospectively recruited. There were 35 HBeAg positive (four in the immune tolerant phase; and 31 in the immune clearance phase), and 85 HBeAg negative (28 in the low replicative phase; and 57 in the HBeAg negative hepatitis phase) patients, respectively. The baseline characteristics of the patients are described in Table 5.3. Greater than 80% of the recruited cohort was of Asian ethnicity, and in keeping with this, the majority of patients were infected with either HBV Genotype B or C. Overall, HBeAg positive patients were younger (p<0.0001), had a higher median viral load (p<0.0001), and also had a higher median ALT level (p=0.003).

5.3.2 Controls

Blood samples were also taken from four healthy controls for serum and PBMC collection both prior to and seven days after booster vaccination against HBV. All controls had a baseline anti-HBs titer of <100 IU/mL, and were confirmed to have a post vaccination anti-HBs response of a greater than ten fold increase in serum anti-HBs titre at day seven post vaccination.
Table 5.3 – Baseline Patient Characteristics

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<td>3</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>*<em>Serum ALT (U/L)</em></td>
<td>53</td>
<td>83</td>
<td>24</td>
<td>91</td>
<td>45</td>
<td>25</td>
</tr>
<tr>
<td><strong>HBV DNA</strong> (Log_{10} IU/mL)*</td>
<td>5.37</td>
<td>7.79</td>
<td>8.25</td>
<td>8.07</td>
<td>4.58</td>
<td>&lt;2.55</td>
</tr>
<tr>
<td><strong>Biopsy (METAVIR)</strong> (n=99)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammation</td>
<td>1</td>
<td>1</td>
<td>no biopsy</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

# Mean values, * Median values
5.3.3 Detection of Serum Anti-HBs and Anti-HBe

5.3.3.1 Serum Anti-HBe in HBeAg Positive Patients

Four HBeAg positive (4/35, 11.4%) patients were found to also have detectable serum anti-HBe on the commercial qualitative EIA (Table 5.4). All four of these patients were in the immune clearance phase of infection (Chapter 3.2.1). Patient 3 had a normal serum ALT level at the time of serum anti-HBe measurement, but was classified as being in the immune clearance phase based on previously elevated serum ALT levels.

The baseline HBeAg titre was less than 100 PE IU/mL in each of these four patients. Their baseline median HBeAg titre was statistically significantly lower compared to the remainder of the HBeAg positive cohort (0.97 versus 3.19 log\textsubscript{10} IU/mL, p = 0.007) (Figure 5.2). However, there were seven other HBeAg positive patients with a HBeAg titre less than 100 PE IU/mL who had an undetectable serum anti-HBe on EIA (Table 5.5). There were no other differences in baseline characteristics detected between these seven patients, and the four patients with detectable anti-HBe. In particular, there was no observed difference in baseline HBsAg titres between the two groups (Table 5.5 & 5.6). However, it should be noted that three (3/7, 43%) of the seven patients (Patient 5, 6 & 7) with “HBeAg <100 PE IU/mL and no detectable serum anti-HBe” also had detectable serum anti-HBs, compared to none (0/4, 0%) of the four HBeAg positive patients with detectable anti-HBe (p=0.07) (also see section 5.3.3.2).

![Figure 5.2](image-url)  
*Figure 5.2* Comparison of baseline HBeAg Titres (Log\textsubscript{10} PE IU/mL) in patients with detectable and not-detectable serum anti-HBe. Median values with 95% confidence interval (of median) represented.
### Table 5.4 – Baseline Characteristics of “HBeAg positive patients with detectable serum anti-HBe”

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Ethnicity</th>
<th>Genotype</th>
<th>ALT (U/L)</th>
<th>HBVDNA (Log_{10} IU/mL)</th>
<th>Anti-HBe</th>
<th>HBeAg Titre (PE IU/mL)</th>
<th>PC” Sequencing</th>
<th>BCP” Sequencing</th>
<th>HBsAg Titre (Log_{10} IU/mL)</th>
<th>Anti-HBs Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>35</td>
<td>Asian</td>
<td>C</td>
<td>68</td>
<td>5.94</td>
<td>REACTIVE</td>
<td>6</td>
<td>Wild Type</td>
<td>A1762, G1764A</td>
<td>3.67</td>
<td>NR</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>25</td>
<td>Asian</td>
<td>B</td>
<td>261</td>
<td>8.44</td>
<td>REACTIVE</td>
<td>4</td>
<td>Wild Type</td>
<td>Wild Type</td>
<td>4.80</td>
<td>NR</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>22</td>
<td>Asian</td>
<td>B</td>
<td>19</td>
<td>3.72</td>
<td>REACTIVE</td>
<td>14</td>
<td>G1896A</td>
<td>Wild Type</td>
<td>2.86</td>
<td>NR</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>27</td>
<td>Asian</td>
<td>B</td>
<td>106</td>
<td>8.07</td>
<td>REACTIVE</td>
<td>60</td>
<td>Wild Type</td>
<td>Wild Type</td>
<td>3.96</td>
<td>NR</td>
</tr>
</tbody>
</table>

# PC; Precore, BCP; Basal Core Promoter regions of the HBV Genome.

### Table 5.5 – Baseline Characteristics of “HBeAg positive patients with HBeAg titre <100 PE IU/mL, and no detectable serum anti-HBe”

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Ethnicity</th>
<th>Genotype</th>
<th>ALT (U/L)</th>
<th>HBVDNA (Log_{10} IU/mL)</th>
<th>Anti-HBe</th>
<th>HBeAg Titre (PE IU/mL)</th>
<th>PC” Sequencing</th>
<th>BCP” Sequencing</th>
<th>HBsAg Titre (Log_{10} IU/mL)</th>
<th>Anti-HBs Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>F</td>
<td>51</td>
<td>Asian</td>
<td>B</td>
<td>94</td>
<td>8.05</td>
<td>NR^</td>
<td>1</td>
<td>mixed</td>
<td>mixed</td>
<td>2.85</td>
<td>115.51</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>49</td>
<td>Asian</td>
<td>C</td>
<td>19</td>
<td>5.36</td>
<td>NR^</td>
<td>54</td>
<td>Wild type</td>
<td>Wild Type</td>
<td>1.78</td>
<td>14.19</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>37</td>
<td>Asian</td>
<td>Mixed</td>
<td>34</td>
<td>5.42</td>
<td>NR^</td>
<td>67</td>
<td>mixed</td>
<td>mixed</td>
<td>3.60</td>
<td>10.16</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>24</td>
<td>Asian</td>
<td>C</td>
<td>44</td>
<td>5.27</td>
<td>NR^</td>
<td>6</td>
<td>Insufficient sample</td>
<td>Insufficient sample</td>
<td>3.37</td>
<td>NR</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>30</td>
<td>Caucasian</td>
<td>B</td>
<td>32</td>
<td>3.24</td>
<td>NR^</td>
<td>41</td>
<td>G1896A</td>
<td>Wild Type</td>
<td>4.08</td>
<td>NR</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>35</td>
<td>Mediterranean</td>
<td>A</td>
<td>2579</td>
<td>4.93</td>
<td>NR^</td>
<td>45</td>
<td>Wild Type</td>
<td>Wild Type</td>
<td>4.52</td>
<td>NR</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>43</td>
<td>Asian</td>
<td>-</td>
<td>60</td>
<td>8.17</td>
<td>NR^</td>
<td>61</td>
<td>Insufficient sample</td>
<td>Insufficient sample</td>
<td>4.53</td>
<td>NR</td>
</tr>
</tbody>
</table>

# PC; Precore, BCP; Basal Core Promoter regions of the HBV Genome. ^ NR; Non reactive.
5.3.3.2 Basal Core Promoter and Pre-Core Sequencing

Sequencing of the BCP and PC regions of the HBV genome was determined in subset of 11 HBeAg positive patients with a HBeAg titre of less than 100 PE IU/mL (Table 5.6). This subset included four (4/11, 36%) patients who had detectable serum anti-HBe (Table 5.4), and seven (7/11, 64%) with no detectable serum anti-HBe (Table 5.5). Serum anti-HBe was detectable in 3/5 (60%) of patients with wild type sequences in the PC region, and 3/6 (50%) patients with wild type sequences in the BCP region. Variants at either the PC or BCP region were only detected in a minority of this group, and did not appear to be related to the presence of anti-HBe on the commercial assay. There was no patient who had virus with changes at both PC and BCP regions of the HBV genome. Taken together, this data set suggests that anti-HBe is not the selection pressure which drives the emergence of variants at the PC and BCP region.

With regards to HBV genotype, both patients who had virus with a G1896A change in the PC region on sequencing had genotype B HBV infection, and had a corresponding thymidine (T) instead of cytidine (C) at nt1858 (see Discussion).

Table 5.6 BCP and PC sequencing in HBeAg positive patients with a HBeAg titre < 100 PE IU/mL

<table>
<thead>
<tr>
<th></th>
<th>Sequencing</th>
<th>Overall n=11</th>
<th>Anti-HBe Positive n=4</th>
<th>Anti-HBe Negative n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Region</td>
<td>Wild Type</td>
<td>5</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>G1896A</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>BCP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Region</td>
<td>Wild Type</td>
<td>6</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>A1762T, G1764A</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Other</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>
5.3.3.3 Serum Anti-HBs in Both HBeAg-positive and HBeAg-negative Patients

Eight (8/120, 6.7%) patients had detectable serum anti-HBs by the commercial EIA (Table 5.7). Of these eight individuals, there were four HBeAg positive, and four HBeAg negative patients respectively.

Serum Anti-HBs: HBeAg positive subgroup

In the HBeAg positive subgroup (n=35), four patients (4/35, 11.4%) had detectable anti-HBs. All of these patients were classified as being in the immune clearance phase (Chapter 3.2.1), with the staging of Patient 6 based on previously elevated serum ALT levels (results not shown). None of the HBeAg positive patients with detectable serum anti-HBs overlapped with the four patients who had detectable serum anti-HBe (i.e there were no patients with both detectable serum anti-HBs and anti-HBe). However, there were three patients with detectable anti-HBs who also had an HBeAg titre <100 PE IU/mL (Patients 5-7, see Table 5.7). However within the “HBeAg positive subgroup with detectable serum anti-HBs”, the median HBeAg titre was not significantly different to the “HBeAg positive subgroup without detectable anti-HBs” (1.8 versus 2.90 log_{10} IU/mL, p=0.2) (Figure 5.3). In the HBeAg positive subgroup, the median HBsAg titre was lower in those with detectable serum anti-HBs (3.23 versus 4.61 log_{10} IU/mL, p=0.01) (Figure 5.4).

Figure 5.3 Comparison of Baseline HBeAg Titres (Log_{10} PE IU/mL) in patients with detectable and not-detectable serum anti-HBs. Median values with 95% confidence interval (of median) represented.
Table 5.7 - Baseline Characteristics patients with detectable serum anti-HBs

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>HBeAg</th>
<th>Ethnicity</th>
<th>Genotype</th>
<th>ALT (U/L)</th>
<th>HBV DNA (Log_{10} IU/mL)</th>
<th>Anti-HBs Titre (IU/mL)</th>
<th>HBsAg Titre (Log_{10} IU/mL)</th>
<th>HBeAg Titre (PE IU/mL)</th>
<th>Anti-HBe (qualitative)</th>
<th>PC* Sequencing</th>
<th>BCP* Sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>5*</td>
<td>F</td>
<td>51</td>
<td>POS</td>
<td>Asian</td>
<td>B</td>
<td>94</td>
<td>8.05</td>
<td>115.51</td>
<td>2.85</td>
<td>1</td>
<td>Non reactive</td>
<td>Mixed</td>
<td>Mixed</td>
</tr>
<tr>
<td>6*</td>
<td>F</td>
<td>49</td>
<td>POS</td>
<td>Asian</td>
<td>C</td>
<td>19</td>
<td>5.36</td>
<td>14.19</td>
<td>1.78</td>
<td>54</td>
<td>Non reactive</td>
<td>Wild Type</td>
<td>Wild Type</td>
</tr>
<tr>
<td>7*</td>
<td>F</td>
<td>37</td>
<td>POS</td>
<td>Asian</td>
<td>Mixed</td>
<td>34</td>
<td>5.42</td>
<td>10.16</td>
<td>3.60</td>
<td>67</td>
<td>Non reactive</td>
<td>Mixed</td>
<td>Mixed</td>
</tr>
<tr>
<td>12</td>
<td>F</td>
<td>24</td>
<td>POS</td>
<td>Asian</td>
<td>C</td>
<td>821</td>
<td>6.81</td>
<td>23.75</td>
<td>4.03</td>
<td>4625</td>
<td>Non reactive</td>
<td>Wild Type</td>
<td>A1762T, G1764A</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>50</td>
<td>NEG</td>
<td>Asian</td>
<td>C</td>
<td>24</td>
<td>2.68</td>
<td>11.55</td>
<td>2.96</td>
<td>NEG</td>
<td>Non reactive</td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>48</td>
<td>NEG</td>
<td>Asian</td>
<td>C</td>
<td>25</td>
<td>2.82</td>
<td>33.84</td>
<td>3.03</td>
<td>NEG</td>
<td>Non reactive</td>
<td>Wild Type</td>
<td>Wild Type</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>26</td>
<td>NEG</td>
<td>Asian</td>
<td>B</td>
<td>51</td>
<td>4.16</td>
<td>31.58</td>
<td>3.57</td>
<td>NEG</td>
<td>Non reactive</td>
<td>Not available</td>
<td>Not available</td>
</tr>
<tr>
<td>16</td>
<td>F</td>
<td>33</td>
<td>NEG</td>
<td>Mediterranean</td>
<td>D</td>
<td>184</td>
<td>6.72</td>
<td>14.06</td>
<td>2.64</td>
<td>NEG</td>
<td>Non reactive</td>
<td>G1896A</td>
<td>A1762T, G1764A</td>
</tr>
</tbody>
</table>

*Patients 5, 6 and 7 are also represented in Table 5.5.

# PC; Precore, BCP; Basal Core Promoter regions of the HBV Genome
Serum Anti-HBs: HBeAg negative subgroup

In the HBeAg negative subgroup of patients (n=85), 4.7% (4/85) had detectable serum anti-HBs on commercial EIA (Table 5.7, Figure 5.4). Two patients were classified in the low replicative (Patients 13 & 14) and HBeAg negative hepatitis (Patients 15 & 16) phases, respectively. There was no observed difference in the median HBsAg titre in the HBeAg negative subgroup with, and without detectable serum anti-HBs (3.0 versus 3.5 log_{10} IU/mL, p=0.25) (Figure 5.4).

Figure 5.4 Comparison of Baseline HBsAg Titres (Log_{10} IU/mL) in patients with detectable and not-detectable serum anti-HBs. Median values with 95% confidence interval (of median) represented.
### Sequecing of the S (Envelope) Region of the HBV Genome

Sequencing of the envelope region of the HBV genome was performed in a subset of patient samples who had detectable serum anti-HBs (Table 5.8). The significance of many of these envelope sequence variations is not fully known, and it may be represent natural polymorphisms. However, the mutation sG145R has been associated with vaccine escape and this variant (sG145R) was detectable in combination with wild-type sequences in Patient 12.

<table>
<thead>
<tr>
<th>Patient</th>
<th>HBeAg</th>
<th>Anti-HBs Titre (IU/mL)</th>
<th>Genotype</th>
<th>Pre S1</th>
<th>Pre S2</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>5*</td>
<td>POS</td>
<td>115.51</td>
<td>B</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>6*</td>
<td>POS</td>
<td>14.19</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>sS53L, sW74S/W, sY100Y/C, sT113K/N/T, sS117S/R, sK160R, sA184V, sS210N, sY221C</td>
</tr>
<tr>
<td>7*</td>
<td>POS</td>
<td>10.16</td>
<td>Mixed</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>POS</td>
<td>23.75</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>sN3S, sS53L, sP62P/L, sF134I/F, <strong>sG145R/G</strong>, sK160R, sA184V, sS210N, sY221C</td>
</tr>
<tr>
<td>13</td>
<td>NEG</td>
<td>11.55</td>
<td>C</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>NEG</td>
<td>33.84</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>sF41S, sG44E/D, sG44E/D, sP46L, sT47M, sP49R, sQ51L, sS55C, sV96A, sQ101K, sI126S, sM133T, sK160R, sA184V, sL213T/S, sF220L, sY221C</td>
</tr>
<tr>
<td>15</td>
<td>NEG</td>
<td>31.58</td>
<td>B</td>
<td>-</td>
<td>G16R</td>
<td>sG7R/G, sV14G/V, sN40N/S, sS59N/S, sI110I/L, sK122R, sI198M, sF200Y</td>
</tr>
<tr>
<td>16</td>
<td>NEG</td>
<td>14.06</td>
<td>D</td>
<td>-</td>
<td>-</td>
<td>sT45T/P, sS207N</td>
</tr>
</tbody>
</table>

* Unable to identify due to mixed populations or insufficient patient sample
5.3.4 Detection of Anti-HBs and Anti-HBe in the B-cells from PBMC Preparations using Commercial EIA

In this clinical cohort, serum anti-HBe was detected in 11.4% of HBeAg positive patients, and serum anti-HBs was detected in 6.7% of patients using commercial EIA, respectively. However, the commercial EIA is designed to detect serum anti-HBe and anti-HBs efficiently only when the respective antigen has been cleared from the systemic circulation. Consequently, the PBMCs and more specifically B lymphocyte lysates were evaluated to determine whether a higher rate of antibody production could be detected by sampling this compartment.

a) Anti-HBs

The presence of anti-HBs in B lymphocyte lysates (using both commercial “Roche” lysis buffer, and the “core prep” lysis buffer) was initially assessed in twelve samples: four healthy controls (known high serum anti-HBs titres), and the eight patients with detectable serum anti-HBs (Table 5.7). Two different commercial EIA assays were used: ARCHITECT Platform anti-HBs assay, Abbott Murex anti-HBs assay. Anti-HBs was not detected in any of the twelve samples tested (data not shown).

b) Anti-HBe

The presence of anti-HBe in B lymphocyte lysates was initially assessed in the four HBeAg positive patients with detectable serum anti-HBe using the ARCHITECT Platform anti-HBe assay. Anti-HBe was not detected in any of the four samples tested (data not shown).

Given these “negative results” in samples which had confirmed detectable serum anti-HBs and anti-HBe, the PBMCs from the remaining patients in the clinical cohort were not assessed using ARCHITECT platform in order to preserve the limited amount of PBMC aliquots for other uses.

A possible explanation for these negative results is that the B cell lysate may contain predominantly immature forms of antibody, such as single heavy/light chain structures which are not yet assembled into a corresponding heavy/light chain via disulphide bonds to form a mature four chain antibody structure (see Discussion for
further explanation). Furthermore, the antibody capture mechanisms of the commercial assays tested may not be designed to adequately detect these precursor forms of immunoglobulin. Consequently, an in-house sandwich ELISA format was developed in an attempt to overcome this. This was initially developed for the detection of anti-HBs.

5.3.5 Detection of Anti-HBs in the PBMC Compartment Using In-House ELISA

HBsAg from commercial vaccine (HB-VAX II and Engerix) was used to coat the ELISA plate in the solid phase. Post vaccination sera from non-HBV controls was used as the Positive Control. Both vaccine and patient sera were assessed on the ARCHITECT platform prior to ELISA experiments (Table 5.9). Abbott ARCHITECT Manual Diluent was used as the Negative Control. HBsAg Manual Diluent contains recalcified human plasma that is non-reactive for HBsAg, HIV-1 RNA or HIV-1 Ag, anti-HIV-1/HIV-2, anti-HCV, and anti-HBs.

<table>
<thead>
<tr>
<th>DETAILS</th>
<th>Titre (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg: Vaccine</td>
<td></td>
</tr>
<tr>
<td>HB-VAXII (CSL Biotherapies, Australia, 10ug/mL)</td>
<td>1.5</td>
</tr>
<tr>
<td>Engerix-B (GSK, Belgium, 20ug/mL)</td>
<td>44</td>
</tr>
<tr>
<td>Anti-HBs: Serum</td>
<td></td>
</tr>
<tr>
<td>Sera from healthy controls who received a HBsAg booster vaccination</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

An initial “checker-board” was designed in order to optimise the amount of HBsAg to adsorb to the ELISA plate, and secondary antibody concentration (Table 5.10). In this experiment, the concentration of unlabelled primary antibody concentration remained fixed at 1 in 100. The absorbance was assessed after an interval of 45 minutes following the addition of substrate.
### Table 5.10 Initial checker-board ELISA experiment

<table>
<thead>
<tr>
<th></th>
<th>1/500</th>
<th>1/1000</th>
<th>1/2000</th>
<th>1/4000</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Positive Control 1/100</td>
<td>2.40</td>
<td>1.37</td>
<td>1.82</td>
<td>1.26</td>
</tr>
<tr>
<td>B</td>
<td>2.44</td>
<td>1.98</td>
<td>2.35</td>
<td>1.67</td>
</tr>
<tr>
<td>C</td>
<td>2.12</td>
<td>2.35</td>
<td>1.98</td>
<td>1.19</td>
</tr>
<tr>
<td>D</td>
<td>2.53</td>
<td>2.47</td>
<td>2.86</td>
<td>1.22</td>
</tr>
<tr>
<td>E Negative Control 1/100</td>
<td>1.22</td>
<td>0.99</td>
<td>0.91</td>
<td>0.74</td>
</tr>
<tr>
<td>F</td>
<td>0.75</td>
<td>1.06</td>
<td>0.91</td>
<td>0.72</td>
</tr>
<tr>
<td>G</td>
<td>1.44</td>
<td>0.86</td>
<td>1.25</td>
<td>0.67</td>
</tr>
<tr>
<td>H</td>
<td>0.89</td>
<td>0.76</td>
<td>0.86</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Positive Control primary antibody: post vaccination sera, Negative Control primary antibody; ARCHITECT Manual Diluent, Secondary antibody; DAKO anti-human IgG-HRP.

Although an optimal coating HBsAg and secondary antibody concentration could not be determined from this experiment, a number of insights were obtained from the results of this ELISA.

a) The amount of HBsAg used to coat each well did not affect the final absorbance reading (e.g. Row A1-A12), suggesting that there was strong non-specific binding in the Positive Control sera to the ELISA plate.

b) The absorbance values in the Positive Control wells between different dilutions did not demonstrate a broad dynamic range (e.g. Column 1A to 1D). A broad dynamic range is desirable with the Positive Control in this experiment as it was expected that the amount of anti-HBs in patient B lymphocyte lysates would be at a relatively lower level and avidity in comparison to the post vaccination lysates from non-HBV controls.

c) The absorbance values in the Negative Control wells (e.g. Row E1-E12) were far too high. This may suggest that the ARCHITECT recalculated plasma Manual Diluent is not suitable as a Negative Control. Even though this Manual diluent is negative for HBsAg and Anti-HBs, there may be other components in this which make it “sticky”.

The in-house ELISA was sequentially modified in an attempt to resolve the issues highlighted above;
a. **ELISA plate**

The Immuno 96 Microwell ELISA Plate (Thermo Fisher Scientific, Rochester, USA) was compared to the Maxisorp ELISA plate (eBioscience, Sandiego, USA). There was no significant difference in absorbance values obtained between the two ELISA plates. Thus, the remainder of the ELISA experiments were performed using the Maxisorp ELISA plates.

b. **Blocking Solution**

Blocking with PBS – 4% (w/v) Bovine Serum Albumin (PBS-4%BSA) was compared against PBS – 5% (w/v) Skim Milk Powder. A commercially concentrated BSA solution was purchased and diluted in PBS to a concentration of 4% (w/v).

When blocking with PBS-4%BSA in the ELISA experiments, it was discovered that an agglutination process would occur in each well following the addition of the sodium citrate substrate buffer [2.58g citric acid, 2.18g Na₂HPO₄ dissolved to 200mL double distilled water (ddH₂O), pH 4.0]. The reasons for this were unclear. However, this was resolved using a different substrate buffer (see below) as an alternative to the citrate substrate buffer.

c. **Substrate Buffer**

OPD substrate (Sigma Aldrich, St Louis, USA) was included in experiments which had PBS-4%BSA as a blocking solution. For each ELISA plate, 10mL of OPD substrate was prepared by adding two OPD tablets (5mg/tablets) to 10mL of Phosphate Citrate Buffer (one sodium perborate capsule dissolved in 100mL of deionised water). This was prepared immediately prior to usage according to the VIDRL protocol.

The use of OPD substrate did result in a slightly broader dynamic range at the lower end of absorbance values. However, this was not significantly greater. It was decided that all future ELISA experiments would revert to the original protocol of blocking PBS-5% (w/v) Skim milk powder, and using sodium citrate substrate buffer.

d. **Incubation of Primary Antibody**:

It is possible that the primary antibody incubation time of one hour may have been insufficient. Increasing the incubation time to overnight did not have a
significant impact on the ELISA results obtained. However, it was decided to continue longer incubation times for subsequent experiments.

e. **Increase Number of PBS-T Washes:**

The amount of washes with PBS-T between incubations was increased from three to five. This modification resulted in no appreciable difference in final absorbance values.

f. **Filter Vaccine**

The HBsAg vaccine is comprised of a sterile suspension of purified HBsAg adsorbed on Al$^{3+}$ as aluminium hydroxide. It was hypothesised that the aluminium component of the vaccine could be interfering with the ELISA results as it is a reasonably large molecule. Consequently, an attempt was made to filter out the aluminium component using a desalting column. Unfortunately, very low HBsAg titres were recorded on the ARCHITECT platform following the filtration process, and so the step was abandoned.

g. **Dilution Buffer and Incubation Temperature**

HBsAg vaccine was diluted in PBS-Bicarbonate (1M, pH 8.2) rather than PBS, and both primary and secondary antibody was incubated at 37°C rather than at room temperature. These changes were made based on a previous article which tested for the presence of anti-HBs in B lymphocyte lysates$^{186}$. Unfortunately, the modifications did not resolve the technical issues in spite of following the protocol used in the published article$^{186}$ (Table 5.11). There was no significant difference between diluting HBsAg in PBS-Bicarbonate compared to PBS alone.

An example of the experiments is demonstrated below in Table 5.11. In this ELISA, 200ng of HBsAg (from HBsAg vaccine) was adsorbed onto the Maxisorp plate. The Positive Controls used were varying concentrations of the positive vaccination sera and B lymphocyte lysates from non-HBV controls, and the Negative Control was the ARCHITECT Manual Diluent.
**Table 5.11** Initial checker-board ELISA experiment

<table>
<thead>
<tr>
<th>Control Dilutions</th>
<th>HBsAg Diluted in PBS-Bicarbonate (200ng HBsAg per well)</th>
<th>HBsAg Diluted in PBS (200ng HBsAg per well)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Neat</td>
<td>2 1 in 10</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

With regards to the Positive Control (sera) results, there was an approximate ~10 fold difference between the lowest (1 in 1000 well e.g. A4) and highest absorbance values (“neat” well, e.g. A1). However, there was no significant gradient observed in the Positive Control (B lymphocyte lysate) wells. Again, it was evident that ARCHITECT Manual diluent was an unsuitable Negative Control.

Following the multiple unsuccessful results obtained from the ELISA protocol modifications listed above, the sources of HBsAg and anti-HBs were broadened (Table 5.12) to provide an improved Positive Control.

**Table 5.12** Source of HBsAg and Anti-HBs.

<table>
<thead>
<tr>
<th>HBsAg</th>
<th>DETAILS</th>
<th>Titre (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine</td>
<td>HB-VAXII (CSL Biotherapies, Australia, 10ug/mL)</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Engerix-B (GSK, Belgium, 20ug/mL).</td>
<td>44</td>
</tr>
<tr>
<td>Commercial</td>
<td>Ab73741 (Abcam, UK, 1mg/mL).</td>
<td>40</td>
</tr>
<tr>
<td>In-House</td>
<td>HBsAg obtained from tissue culture and transient transfection of HuH-7</td>
<td>900</td>
</tr>
<tr>
<td>HBsAg</td>
<td>Sera from healthy controls who received a HBsAg booster vaccination</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Serum</td>
<td>Hepatitis B immunoglobulin (CSL Biotherapies, Australia)</td>
<td>155 (diluted 1 in 10⁴)</td>
</tr>
<tr>
<td>Anti-HBs</td>
<td>AB 20522: Goat polyclonal antibodies to HBsAg (Abcam, UK, 100ug)</td>
<td>435 (diluted 1 in 10⁴)</td>
</tr>
<tr>
<td>Commercial</td>
<td>AB 8636: Mouse monoclonal antibodies to HBsAg (Abcam, UK, 100ug)..</td>
<td>Non-reactive</td>
</tr>
</tbody>
</table>

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A checkerboard ELISA was performed with coating of the ELISA plate with transfected HBsAg (see below, Table 5.13) at different dilutions.

**Table 5.13 Checkerboard ELISA**

<table>
<thead>
<tr>
<th>Transfected HBsAg</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse monoclonal Anti-HBs 1 in 10⁵</td>
<td>0.30</td>
<td>0.29</td>
<td>0.28</td>
<td>0.28</td>
<td>0.32</td>
<td>0.30</td>
<td>0.32</td>
<td>0.28</td>
<td>0.26</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>B 1 in 10⁶</td>
<td>0.32</td>
<td>0.30</td>
<td>0.27</td>
<td>0.28</td>
<td>0.28</td>
<td>0.82</td>
<td>0.28</td>
<td>0.28</td>
<td>0.33</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>C 1 in 10⁷</td>
<td>0.32</td>
<td>0.30</td>
<td>0.39</td>
<td>0.31</td>
<td>0.31</td>
<td>0.29</td>
<td>0.30</td>
<td>0.31</td>
<td>0.29</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>Goat polyclonal Anti-HBs 1 in 10⁵</td>
<td>0.63</td>
<td>0.64</td>
<td>0.59</td>
<td>0.65</td>
<td>0.56</td>
<td>0.63</td>
<td>0.61</td>
<td>0.60</td>
<td>0.62</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>E 1 in 10⁶</td>
<td>0.68</td>
<td>0.69</td>
<td>0.65</td>
<td>0.71</td>
<td>0.59</td>
<td>0.60</td>
<td>0.57</td>
<td>0.61</td>
<td>0.61</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>F 1 in 10⁷</td>
<td>0.67</td>
<td>0.70</td>
<td>0.67</td>
<td>0.68</td>
<td>0.66</td>
<td>0.63</td>
<td>0.61</td>
<td>0.64</td>
<td>0.63</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>HBIG 1 in 10⁵</td>
<td>0.76</td>
<td>0.82</td>
<td>0.69</td>
<td>0.70</td>
<td>0.70</td>
<td>0.64</td>
<td>0.64</td>
<td>0.67</td>
<td>0.65</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>H 1 in 10⁶</td>
<td>0.35</td>
<td>0.35</td>
<td>0.32</td>
<td>0.35</td>
<td>0.33</td>
<td>0.31</td>
<td>0.32</td>
<td>0.32</td>
<td>0.31</td>
<td>0.30</td>
<td></td>
</tr>
</tbody>
</table>

The observations from this ELISA were:

a) The absorbance values for wells using the Mouse monoclonal anti-HBs were relatively lower compared to Goat polyclonal anti-HBs and HBIG wells. This is consistent with the non-detectable anti-HBs titre observed for the Mouse monoclonal antibody on the ARCHITECT assay.

b) The absorbance values in all wells coated with the Goat polyclonal anti-HBs were similar, and did not appear to be affected by the amount of coating HBsAg, or dilution of anti-HBs.

c) The highest absorbance values were evident in the the wells using HBIG at a dilution of 1 in 10⁵.

Similar results to the above experiment were also obtained when using the commercial HBsAg (instead of transfected HBsAg) as the coating antigen (data not shown). Given that the HBsAg titre of the commercial HBsAg was found to be much lower that the in-house HBsAg, this again suggested that there may be substantial non specific binding of the primary antibody.
An ELISA comparing the different coating HBsAg and primary anti-HBs was then performed (Table 5.14). The dilution of secondary antibody used was 1 in 2000.

**Table 5.14** Comparison of different coating HBsAg and Primary anti-HBs.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In-House HBsAg</td>
<td>Commercial HBsAg</td>
<td>Vaccine (CSL)</td>
<td>Vaccine (GSK)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 in 10</td>
<td>1 in 10²</td>
<td>1 in 10</td>
<td>1 in 10²</td>
<td>1 in 10</td>
<td>1 in 10²</td>
<td>1 in 10</td>
<td>1 in 10²</td>
</tr>
<tr>
<td>A Mouse Monoclonal:  1 in 10²</td>
<td>0.24</td>
<td>0.25</td>
<td>0.24</td>
<td>0.26</td>
<td>0.29</td>
<td>0.28</td>
<td>0.27</td>
<td>0.28</td>
</tr>
<tr>
<td>B 1 in 10²</td>
<td>0.25</td>
<td>0.21</td>
<td>0.23</td>
<td>0.22</td>
<td>0.27</td>
<td>0.25</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>C Goat Polyclonal:  1 in 10²</td>
<td>0.44</td>
<td>0.39</td>
<td>0.40</td>
<td>0.48</td>
<td>0.60</td>
<td>0.49</td>
<td>0.70</td>
<td>0.65</td>
</tr>
<tr>
<td>D 1 in 10²</td>
<td>0.43</td>
<td>0.45</td>
<td>0.42</td>
<td>0.39</td>
<td>0.59</td>
<td>0.55</td>
<td>0.59</td>
<td>0.55</td>
</tr>
<tr>
<td>E HBIG:  1 in 10²</td>
<td>3.67</td>
<td>3.68</td>
<td>3.58</td>
<td>3.70</td>
<td>3.64</td>
<td>3.50</td>
<td>3.63</td>
<td>3.52</td>
</tr>
<tr>
<td>F 1 in 10²</td>
<td>1.60</td>
<td>1.70</td>
<td>1.62</td>
<td>1.59</td>
<td>2.28</td>
<td>2.01</td>
<td>1.94</td>
<td>1.80</td>
</tr>
</tbody>
</table>

Despite numerous attempts at optimising the protocol for the sandwich ELISA, a Positive Control sample with adequate dynamic range could not be developed. A final attempt was made using a different ELISA protocol with a different antibody capture mechanism (see below, Table 5.15). Unfortunately, the outcome from this ELISA were also unsuccessful.

- Step 1: Anti-Fc (human) coating onto ELISA Plate
- Step 2: Add B cell lysate
- Step 3: Anti commercial HBsAg-HRP (Murex, Wiesbaden, Germany).

**Table 5.15** Modified ELISA

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-Fc: 1 in 10</td>
<td>Anti-Fc: 1 in 10²</td>
</tr>
<tr>
<td>A B Cell Lysate (neat) HBsAg-HRP 1 in 2000</td>
<td>0.25</td>
<td>0.29</td>
</tr>
<tr>
<td>B B Cell Lysate (neat) HBsAg-HRP 1 in 4000</td>
<td>0.27</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Again, the absorbance values obtained in this ELISA were no different to the “background” values obtained from previous experiments.
5.4 Discussion

This study aimed to evaluate the serum and B-cell component of peripheral blood mononuclear cells (PBMC) for the presence of anti-HBs and anti-HBe production in a well characterised cohort of 120 treatment naïve patients with CHB (35 HBeAg positive patients, 85 HBeAg negative patients). Using a commercial EIA, serum anti-HBe was detected in 11.4% of HBeAg positive patients (4/35). All of these patients were classified as being in the Immune Clearance phase of CHB based on: HBeAg positive, elevated serum ALT level (U/L) and HBV DNA (IU/mL). Detection of serum anti-HBe was more likely in patients with a low HBeAg titre of <100 PE IU/mL (p=0.007). Serum anti-HBs was detected in 11.4% (4/35) and 4.7% (4/85) of HBeAg positive and negative patient subgroups respectively. There were no HBeAg positive patients with both detectable anti-HBe and anti-HBs. All four HBeAg positive patients with detectable serum anti-HBs were in the Immune Clearance phase, whereas there were two HBeAg negative patients each in the Low Replicative and HBeAg negative hepatitis phases, respectively. The “HBeAg positive patients with detectable anti-HBs” had a similar baseline median HBeAg titre compared to the group without detectable anti-HBs. However, a lower median HBsAg titre was evident in the HBeAg positive group with anti-HBs (3.23 versus 4.61 log_{10} IU/mL, p=0.01), whilst in HBeAg negative patients there was no observed difference in baseline HBsAg titre between patients with, and without detectable anti-HBs (3.0 versus 3.5 log_{10} IU/mL, p=0.25).

The B cell component of PBMCs was then assessed for the presence of anti-HBe and anti-HBs. The B lymphocytes from healthy controls (who had received a booster HBsAg vaccination) as well as from patients with detectable serum anti-HBs or anti-HBe were isolated, enriched and disrupted, and the resultant lysate was assessed by commercial EIA (Abbott Laboratories and Abbott Murex assay). Unfortunately, antibody was not detected in any of these samples, and so the PBMCs from the remaining cohort of patients were not assessed in order to preserve the limited store of PBMCs for future investigations of more sensitive and specific ELISAs.

An in-house ELISA was then developed to determine whether anti-HBe and anti-HBs could be detected in B lymphocyte lysates in patients with CHB. Unfortunately, despite multiple attempts at optimising the in-house ELISA, an
adequate Positive Control sample with a broad dynamic range of absorbance values was not developed. A broad dynamic range is highly desirable to allow even a small amount of anti-HBs or anti-HBe (in patient B lymphocyte lysates) to be reliably detected. Given the difficulties with the in-house ELISA experiments, and the “negative results” with samples which would be expected to be positive (lysate from post HBsAg vaccination samples, and samples from patients with detectable serum antibody), the PBMCs from the remaining cohort of patients were not used for further in-house ELISA testing.

HBeAg seroconversion is defined as the detection of an antibody response to HBeAg (anti-HBe), with corresponding loss of serum HBeAg. In the context of the natural history of CHB, HBeAg seroconversion represents a serological marker for the end of the immune clearance phase. It is often correlated with a significant reduction in HBV DNA (often to an undetectable level), and may also be associated with normalisation of liver function tests and histological improvement. Spontaneous HBeAg seroconversion is associated with favourable outcomes\textsuperscript{127}, particularly if this occurs before the age of 30\textsuperscript{187}. Furthermore, HBeAg seroconversion is also an established therapeutic endpoint, as it is associated with a more favourable prognosis\textsuperscript{90}. Similarly HBsAg seroconversion is defined as the detection of anti-HBs with corresponding loss of serum HBsAg. This is the preferred endpoint of therapy in CHB, as it is typically associated with durable and complete suppression of viral replication and improved clinical outcomes\textsuperscript{85}, and is best achieved before the age of 50\textsuperscript{128}.

Both serum HBeAg and HBsAg may exist in the systemic circulation in high titre (Chapter 1.2). Unfortunately, the available commercial EIAs usually detect corresponding anti-HBe and anti-HBs only after their respective antigens have been cleared from the systemic circulation. This is because it is believed that prior to seroconversion, any anti-HBe or anti-HBs which is produced exists in blood in the form of immune complexes with their respective antigens, and is not detected\textsuperscript{10, 66}. Thus, the current commercial assays most likely confirm seroconversion well after the event has occurred, rather than providing any indication of the onset, or likelihood of predicting seroconversion. Whilst the combination of HBV DNA and serum HBsAg titres (Chapter 3) did provide supplementary information on the status of HBV infection, and has been likened to “latitude” and “longitude” in determining a ship’s position in the ocean\textsuperscript{188}, the additional evaluation of co-exisiting antibody levels was
hypothesised to provide a more comprehensive indication of the underlying changes in the innate and humoral immune responses. This would not only facilitate a better understanding of the highly dynamic changes during the natural history of CHB, but also an opportunity to individualise patient therapy.

This study demonstrated that in a subgroup of patients, serum anti-HBe could be detected by commercial EIA in the presence of coexisting HBeAg. In HBeAg positive patients, all of the patients with detectable serum anti-HBe were in the immune clearance phase of CHB, with a low HBeAg titre (<100 PE IU/mL). The detection of coexistent low levels of anti-HBe and HBeAg is consistent with the concept that HBeAg seroconversion is not a “discrete” event, but rather a process which may be developing slowly, with a gradual reduction in HBeAg titre and increase in anti-HBe. It is also important to note however, that the “trigger” for the development of HBeAg seroconversion remains poorly understood, and that there were 7/35 (20%) HBeAg positive patients with a HBeAg titre less than 100 PE IU/mL who did not demonstrate detectable serum anti-HBe.

Sequencing of the HBV BCP and PC regions was performed in a subset of 11 HBeAg positive patients with a HBeAg titre of less than 100 PE IU/mL. A minority (3/11, 27%) of these patients had evidence of escape mutations in these regions, with no patients demonstrating both BCP and PC mutations. Although the sequencing of BCP and PC in only a subset of patients may have introduced an element of bias, these results do provide an insight into the background changes in HBeAg synthesis prior to HBeAg seroconversion. Whilst BCP and PC mutations are associated with a reduction in HBeAg production and titre\(^{189}\), it is thought that only ~10% of HBV containing wild type sequences at the BCP and PC regions is required for the phenotypic detection of HBeAg (unpublished observations from VIDRL laboratory). Thus, the detection of BCP and PC mutations in a subset of patients who remain HBeAg positive serologically is further evidence that HBeAg seroconversion is a gradual event starting several years before the actual final seroconversion rather than a discrete “one off” type event\(^{190}\).

The HBV genotype may also be important in determining the likelihood of specific PC and BCP mutations. The most common PC mutation is a single base substitution (G to A) at nt1896, known as the G1896A mutation. This results in a translational stop codon (codon 28: TGG; tryptophan to TAG; stop) in a highly conserved stem loop structure, known as the \(\varepsilon\) structure. The base of the \(\varepsilon\) structure is
formed by a base pair between G1896 with nt1858. In HBV genotypes containing thymidine (T) at nt1858 (genotypes B, D, E, G, some strains of C), the G1896A mutation is more common because the stop codon mutation helps stabilize the ε structure. In contrast, the mutation is uncommon in genotypes [A, F, some strains of C] containing cytidine (C) at 1858, predominantly because the G-C Watson Crick base pairing is preferred. Other changes in the PC which may prevent the translation of HBeAg include non-sense mutations at nt1814-1816, 1817, 1874, and 1897, and frameshift mutations at 1838-9. BCP variants typically involve the double mutation of A1762T and G1764A. This results in the transcriptional reduction of Pre-C/C mRNA, with up to a 70% decrease in HBeAg production compared to wild type HBV.

This study also demonstrated that in a subgroup of patients, serum anti-HBs could be detected by commercial EIA in the presence of coexisting HBsAg. It is generally believed that antibodies directed against HBsAg (anti-HBs) are neutralising, and play a key role in containing further spread of infection. Anti-HBs production is the basis for immunity in previously vaccinated individuals. The importance of the B lymphocyte and anti-HBs production in the control of HBV infection is highlighted by the use of immunosuppressant medications such as Rituximab. Rituximab is a humanised chimeric anti-CD20 monoclonal antibody against B lymphocytes which is used as either part of combination chemotherapy or monotherapy in the treatment of B cell non-Hodgkins lymphomas and chronic lymphocytic leukaemia. Following the administration of Rituximab, there is a profound and durable depletion of circulating B cells that persists for at least six months following drug withdrawal. Rituximab has been independently associated with the risk of HBV reactivation, and there are now several case reports of HBV reactivation with Rituximab therapy (in the absence of antiviral prophylaxis) in patients with prior “resolved” hepatitis B (e.g. HBsAg negative, anti-HBs positive, anti-HBe positive). This has led to a proposed recommendation that the duration of prophylactic antiviral therapy following cessation of Rituximab based chemotherapeutic regimens is increased.

Sequencing of the envelope region of the HBV genome was performed on patient serum samples with detectable anti-HBs. Viruses are under constant selection pressure, and it was postulated that there may be significant envelope sequence variations in this subset of patients. Following natural infection or after immunisation,
the anti-HBs response comprises antibodies that predominantly recognise the major hydrophilic region of the HBsAg protein. This region consists of amino acids 99-160, which contains the “a” determinant epitope region which a variety of anti-HBs is able to recognise, including vaccine-associated antibody and HBIG. A number of amino acid changes which have been associated with vaccine escape (e.g. sG145R and D144A) are located in this “a” determinant region. In the sequences that were analysed, one patient (Patient 12, Table 5.8) had evidence of sG145R changes in combination with wild type (sG145). A number of other amino acid changes (in comparison to the reference sequence) were identified, although the significance of the changes have not yet been determined. Further longitudinal analyses of these envelope sequences either prior or during antiviral therapy is important to assess the potential significance of envelope sequence variation.

Using in-house assays, a previous study has demonstrated the presence of anti-HBe and/or anti-HBs in patients with CHB who were seronegative for these antibodies using commercial assays. The protocol for antibody detection used in this Japanese study included coating of ELISA plates with HBsAg, and anti-human IgG-HRP as the conjugate. In the study described in this thesis, the major technical problem experienced with the use of “sera” (either patient sera, Abbott ARCHITECT manual diluent, or HBIG), was of non-specific binding to the ELISA plate. Other than the presence of the desired antibody (in this case anti-HBs or anti-HBe), sera contains multiple proteins which make it “sticky” and non-specifically adsorb onto the ELISA plate. This may have also contributed to the narrow dynamic range consistently found between lowest to highest absorbance values. Further independent studies are required to assess the validity of the results obtained in the Japanese study.

Given that the presence of HBeAg and HBsAg within the systemic circulation may obscure the detection of corresponding antibody, B lymphocyte lysates were evaluated for the presence of anti-HBe and anti-HBs. The isolation of B lymphocytes effectively removes all interfering antigen (HBsAg and HBeAg) in the blood, thus allowing for the sensitive detection of intracellular antibody. Unfortunately, all of the initial samples tested (post vaccinations control samples and patients with detectable serum anti-HBe or anti-HBs) were non-reactive on the adapted commercial assays. A possible explanation for this is the antibody capture mechanism of the commercial EIAs. These assays utilise HBsAg on the solid phase (coating of microwell plates),...
and also in the liquid phase as a conjugate for anti-HBs detection (see assay package insert). Thus, they are designed to only detect a completely assembled and secreted antibody, which consists of two identical heavy chains linked by disulphide bonds to two identical light chains (H2L2 antibody molecule), with a hinge region (see Figure 5.1). It is possible that B lymphocyte lysates contain predominantly immature antibodies or antibodies in different stages of assembly, and these are not captured by the kit HBsAg.

The synthesis, assembly and secretion of immunoglobulin has been well characterised192, 193. Heavy chains (H) are synthesised developmentally before light chains (L) in Pre-B cells. These heavy chains initially form homodimers which are retained in the endoplasmic reticulum (ER) via an association with an “immunoglobulin heavy chain binding protein” (BiP)194. The BiP is a chaperone protein which also acts as a surrogate light chain195. Light chain production later occurs separately on different polyribosomes, with subsequent assembly with heavy chains to form a basic H2L2 structure in the ER195, 196. Following correct assembly, the immunoglobulin molecule is transported to the Golgi complex for further post-translational processing197. Incorrectly or incompletely assembled immunoglobulin molecules are not secreted, and are targeted for intracellular degradation195. The sequence in which heavy and light chain subunits assemble is related to the heavy chain isotype. IgM and IgG2b initially assemble as a H1L1 structure, whereas the heavy chains of IgG1 and IgG2a initially form a homodimer, followed by the addition of a single light chain (H2L) before forming the complete H2L2 structure197, 198. Thus, a typical B cell lysate would be expected to contain antibody in different stages of assembly.

An in-house ELISA was developed in an attempt to overcome some of the potential limitations in the antibody capture technical platform of the commercial immunoassays. Rather than using HBsAg in both the solid and liquid phases of the ELISA, HBsAg was only used in the solid phase (microwell plate coating), with anti-human IgG-HRP used as the conjugate. This would allow for the potential detection of any immature forms of antibody within the B lymphocyte lysate. These experiments were unfortunately unsuccessful due to a number of technical issues. Consequently, a second ELISA protocol which involved using anti-human Fc in the solid phase and commercial HBsAg-HRP was also attempted, with unfortunately similar negative results.
The IgG subclass profile in the context of hepatitis B staging and/or pathogenesis has been reasonably well characterised for anti-HBc, anti-HBe and anti-HBs (Table 5.15)\textsuperscript{66-69}. Each IgG subclass (IgG1-4) has different biological activities, which is due to factors such as differences in the affinity of IgG subclass to the Fc receptor on phagocytes, and flexibility in the hinge region of Fab\textsuperscript{199}. IgG3 has the most flexible hinge region, and is thought to confer greater capacity for effector functions. Whilst opsonisation can occur with all IgG subclasses (albeit to different degrees) only IgG1-3 can activate complement\textsuperscript{200}. Evaluation of the specific subclass of IgG is not part of the routine evaluation of CHB, and was not evaluated in this study due to the technical issues encountered with the various ELISA formats. However, the evaluation of IgG subclass restriction may offer further insights into the immunological events which result in transition between different phases of CHB.

<table>
<thead>
<tr>
<th>Predominant IgG subclass</th>
<th>Groups</th>
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<tbody>
<tr>
<td>Anti-HBs</td>
<td>IgG1 &gt; IgG2\textsuperscript{67, 72, 201, 202}</td>
</tr>
<tr>
<td>Anti-HBe\textsuperscript{69}</td>
<td>IgG1 &gt; IgG4</td>
</tr>
<tr>
<td>Anti-HBc</td>
<td>IgG 1-4\textsuperscript{203}</td>
</tr>
<tr>
<td></td>
<td>IgG1 &gt; IgG3\textsuperscript{68, 70}</td>
</tr>
<tr>
<td></td>
<td>IgG3 &gt; IgG1\textsuperscript{203}</td>
</tr>
<tr>
<td></td>
<td>IgG3 &gt; IgG1\textsuperscript{68}</td>
</tr>
</tbody>
</table>

In conclusion, this study demonstrated that serum anti-HBe and anti-HBs could be detected in a subgroup of patients with CHB in whom corresponding antigen level was still positive by commercial immunoassays. The anti-HBs titres detected were low, with detection being more likely if the corresponding antigen titre was also low. Unfortunately, the presence of anti-HBe and anti-HBs in B lymphocyte lysates could not be detected using commercial immunoassays or in-house ELISA techniques. Future evaluation could include using different ELISA protocols and/or other techniques (e.g. Western Blotting). Detection and quantitation of anti-HBs and anti-HBe production in the presence of co-existing antigen may potentially lead to a sub-classification of the current phases of CHB, which may be clinically useful. Furthermore, it may provide an opportunity to refine the current therapeutic algorithms and develop novel therapeutic approaches.
Chapter 6. Conclusions and Future Directions

6.1 Serum HBsAg Titres in the Natural History of Chronic Hepatitis B

The first detailed study of baseline serum HBsAg titres in the context of the natural history of CHB was performed. A cross-sectional study was carried out on a large cohort of 283 patients with CHB from three tertiary hospitals [St Vincent’s Hospital, Melbourne (n=175), Liverpool Hospital, Sydney (n=70), and The Royal Children’s Hospital, Melbourne (n=38)]. The treatment naïve cohorts that were evaluated included adult patients/individuals with CHB attending a major tertiary centre, pregnant women and a paediatric cohort. HBsAg levels were also evaluated longitudinally in the paediatric group.

6.1.1 Adult Cohort

Within the adult cohort, this study demonstrated that baseline serum HBsAg titres differed between the four phases of CHB. The median HBsAg titres in each phase of CHB were: IT (4.53 log_{10} IU/mL), IC (4.03 log_{10} IU/mL), LR (2.90 log_{10} IU/mL), and ENH (3.36 log_{10} IU/mL) respectively. The majority of the adult study population was of Asian ethnicity. There was no significant difference in median HBsAg titre between pregnant and non-pregnant subjects. HBsAg titres were higher in HBeAg positive compared to HBeAg negative patients with CHB. The lowest HBsAg titres were evident in the LR (immune control) phase, and the highest in the IT phase. There was a modest correlation (r=0.77, p=0.0001) between HBsAg levels and HBV DNA in the IC phase only. There was no observed statistical correlation between HBsAg titre and serum ALT level in any phase of CHB.

6.1.2 Paediatric Cohort

The majority of the paediatric cohort was in the IC phase, and the overall median serum HBsAg titre was higher than in the adult IC phase cohort (4.54 log_{10} IU/mL compared to 4.03 log_{10} IU/mL, p=0.0014). The youngest age group (0 to 5 years) had the highest median HBsAg level (4.91 log_{10} IU/mL). Longitudinal
evaluation of serum HBsAg titres demonstrated that HBsAg levels did not fluctuate appreciably over a median follow up duration of 30 months.

6.2 Serum HBsAg Decline in Chronic Hepatitis B Patients Treated Long-term with Nucleos(t)ide Analogues

HBsAg titres in a cohort of sixteen patients with CHB receiving long-term oral NA therapy (mainly LMV therapy 94%) were assessed. The median study period was 46 months, and there were five HBeAg positive, and eleven HBeAg negative patients. As expected, HBeAg negative patients were older, and had a lower HBV DNA level and a higher median liver fibrosis score.

6.2.1 Patterns of HBsAg Titre Changes

Following 12 months of oral NA therapy, an interim analysis of HBsAg titres was performed, and HBsAg titres were assessed for a Significant Decline (SD) or Significant Increase (SI) pattern (Table 6.1). At the last treatment time-point, HBsAg titres were assessed for a Slow Decline, No Change or Slow Increase pattern (Table 6.1).

Table 6.1 Patterns of HBsAg levels during NA therapy

<table>
<thead>
<tr>
<th>Pattern</th>
<th>INTERIM ANALYSIS at 12 months</th>
<th>END OF TREATMENT PERIOD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Significant Decline*</td>
<td>Significant Increase*</td>
</tr>
<tr>
<td>Total n=16</td>
<td>1/16</td>
<td>0</td>
</tr>
<tr>
<td>HBeAg positive n=5</td>
<td>1/5</td>
<td>0</td>
</tr>
<tr>
<td>HBeAg negative n=11</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significant Decline and Increase Pattern – either a >0.5 log_{10} IU/mL reduction or increase in HBsAg titre at 12 months respectively
# Slow Decline and Slow Increase Pattern – either a >0.5 log_{10} IU/mL reduction or increase in HBsAg titre from baseline to the end of follow-up
^ No Change Pattern – a 0.00 to 0.5 log_{10} IU/mL change in HBsAg titre from baseline to the end of follow-up

The factors associated with a Slow Decline pattern were HBeAg status (HBeAg-positive 5/5 100%, HBeAg-negative 4/11 36%, p=0.03), higher median baseline HBsAg titre (4.00 versus 3.19 log_{10} IU/mL, p=0.04), and younger median age (42 versus 50, p=0.02). The HBV genotype did not appear to influence the pattern of HBsAg titre decline, however, this may have been limited by the small sample size of this study. HBsAg titres were observed to increase following the development of a virological breakthrough (VB) as a result of antiviral resistance, and
this usually occurred 3-6 months following VB, and were between two to three fold in magnitude rather than the >1.0 log_{10} IU/mL increases observed in HBV DNA. HBsAg loss occurred in one patient (1/16, 6.3%). This patient demonstrated a *Slow Decline* pattern, and had a HBsAg titre of 3.15 log_{10} IU/mL three months prior to an abrupt HBsAg loss at month 45.

### 6.2.2 Comparison of Intrahepatic HBsAg Staining in Two Patients with a Baseline and Second On-Treatment Liver Biopsy

Two patients (one HBeAg positive, one HBeAg negative) who had a second “on-treatment” liver biopsy at month 12 were included in this study, and analysed separately because the criteria for long-term treatment (greater than 2 years duration) had not been fulfilled. Patient One was observed to have a *Significant Descline* (5.12 to 4.08 log_{10} IU/mL) pattern in HBsAg titre, Patient Two in contrast had a *Significant Increase* (3.86 to 4.37 log_{10} IU/mL) pattern.

The intrahepatic HBsAg staining was low and distributed in the cytoplasm for Patient One in both baseline and on-treatment liver biopsy specimens. Patient Two had a higher baseline amount and intensity of staining, with both cytoplasmic as well as a membranous distribution. The month 12 liver biopsy from Patient Two demonstrated no improvement in fibrosis score, and immunohistochemistry on the matched specimens revealed an increased amount and intensity of HBsAg staining, with a slight change in distribution from cytoplasmic/membranous, to membranous only, a pattern associated with high viraemia^{174}.

### 6.3 Anti-HBe and Anti-HBs in the Pathogenesis of Chronic Hepatitis B

This study aimed to evaluate the serum, and B-cell component responses of peripheral blood mononuclear cells (PBMC) for the presence of anti-HBs and anti-HBe in a well characterised cohort of 120 treatment naïve patients with CHB (35 HBeAg positive patients, 85 HBeAg negative patients).

#### 6.3.1 Serum Anti-HBe and Anti-HBs

Using a commercial EIA, serum anti-HBe was detected in 11.4% of HBeAg positive patients (4/35). All of these patients were classified as being in the IC phase of CHB. Detection of serum anti-HBe was more likely in patients with a low HBeAg
titre of <100 PE IU/mL (p=0.007). Serum anti-HBs was detected in 11.4% (4/35) of HBeAg positive and 4.7% (4/85) of HBeAg negative patient subgroups respectively. There were no HBeAg positive patients with both detectable anti-HBe and anti-HBs. All four HBeAg positive patients with detectable serum anti-HBs were in the IC phase, whereas there were two HBeAg negative patients each in the LR and HBeAg negative hepatitis phases respectively. The “HBeAg positive patients with detectable anti-HBs” had a similar baseline median HBeAg titre compared to the group without detectable anti-HBs. However, a lower median HBsAg titre was evident in the HBeAg positive group with anti-HBs (3.23 versus 4.61 log_{10} IU/mL, p=0.01), whilst in HBeAg negative patients there was no observed difference in baseline HBsAg titre between patients with and without detectable anti-HBs (3.0 versus 3.5 log_{10} IU/mL, p=0.25).

6.3.2 Anti-HBe and Anti-HBs in the B cell Component of PBMCs

6.3.2.1 Commercial Enzyme Immunoassay

Anti-HBe and Anti-HBs were unfortunately not detected in the lysate of B lymphocytes from healthy controls (who had received a booster HBsAg vaccination) as well as from patients with detectable serum anti-HBs or anti-HBe were isolated, enriched and disrupted, and the resultant lysate was assessed on two commercial EIAs (Abbott Laboratories, IL, USA; Abbott Murex, Wiesbaden, Germany).

6.3.2.2 In-House ELISA

Despite multiple attempts at optimising the in-house ELISA, an adequate Positive Control sample with a broad dynamic range of absorbance values was not identified. Modifications to the initial ELISA protocol did not resolve the multiple technical issues encountered.

6.4 Conclusion and Future Directions

The natural history of CHB is typically regarded as consisting of four phases which are classified by specific biochemical, serological and virological characteristics, including serum ALT levels, HBeAg serostatus and HBV DNA titre. Measurement of the serum HBsAg titre is currently not required for the distinction between the different phases of CHB, and is also not routinely assessed during
antiviral therapy. Furthermore, whilst HBsAg and HBeAg can be easily detected by serological assays, anti-HBs and anti-HBe in contrast are only detectable in most patients with current commercial assays after the respective antigens (HBsAg and HBeAg) have been cleared from the systemic circulation.

In this context, this thesis describes the first detailed examination of the baseline HBsAg titres in the different phases of the natural history of CHB. In parallel with changes in HBsAg level, this study also assessed the presence of anti-HBe and anti-HBs in patients who were HBeAg and HBsAg positive, respectively. This study demonstrated that baseline HBsAg levels are indeed different between phases of infection, and also that serum anti-HBs and anti-HBe could be detected in a subset of patients in whom the corresponding antigen was still detectable. Taken together, these results strongly suggest highly dynamic changes in both antigen and antibody production throughout the different phases of CHB, and provide potentially useful insights into the underlying changes in the innate and humoral immune responses. It is envisaged that this data set could lead to future routine use of quantitative HBsAg titres in patient management, and assessment of the changes in antibody production during the natural history of CHB. This should facilitate an improved understanding of the immunopathogenesis of CHB, lead to possible further sub-classification of the different phases in infection, and also provide an opportunity to further individualise patient therapy.

This study also evaluated the changes in HBsAg levels during patients treated long-term with NA therapy. Antiviral therapy for CHB is currently indicated in the IC and immune escape (HBeAg negative hepatitis) phases of infection. Highly potent oral antiviral agents such as entecavir and tenofovir are now able to effectively suppress viral replication, and are associated with very low rates of antiviral resistance. However, since these medications are associated with relatively low rates of HBsAg loss and/or seroconversion, this has essentially created a large cohort of patients on NA therapy with an undetectable HBV DNA and normal serum ALT levels, who remain HBsAg positive. The new challenge for treating clinicians is how to monitor these patients, how to individualise antiviral therapy, and how to increase the rates of HBsAg loss. This has no doubt been spurred by recent evidence which has demonstrated that even in individuals who achieve HBsAg loss, the risk of hepatocellular carcinoma is greater if this endpoint is only achieved after the age of 50 compared with at an earlier age.\textsuperscript{128}
With this in mind, monitoring of HBsAg titres has the potential to become the next focus of translational and clinical research. Monitoring of on-treatment HBsAg titres may allow for the development of algorithms to individualise patient therapy depending on the rate of HBsAg titre decline. In the context of increasing HBsAg loss, the results presented in this thesis support a Peg-IFNα or immune-modulator based approach to the management of CHB. HBsAg quantitation may have future clinical utility in determining the optimal dose and duration of Peg-IFNα therapy (e.g. the role of induction dosing), as well as evaluating whether “add-on” Peg-IFNα therapy confers any additional long-term benefit. Additionally, there remains an urgent need for the development of novel therapeutic approaches towards the management of CHB, and this should be facilitated by evaluation of these therapies for their effects on HBsAg levels.

In the past decade, there has been an explosion of knowledge and research in the field of hepatitis B. It is envisaged that the future understanding of the natural history of CHB will continue to evolve and that management algorithms will become more individualized, incorporating not only quantitative serology but possible genotyping and markers of the innate and adaptive immune responses.
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Author/s: Nguyen, Tin Quang

Title: Serological studies into the natural history of chronic hepatitis B

Date: 2011

Citation: Nguyen, T. Q. (2011). Serological studies into the natural history of chronic hepatitis B. Doctorate, Department of Gastroenterology, St Vincent’s Hospital and Victorian Infectious Diseases Reference Laboratory, The University of Melbourne.

Persistent Link: http://hdl.handle.net/11343/36264

File Description: Serological studies into the natural history of chronic hepatitis B

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