Chronic Hepatitis B in an Australian tertiary hospital cohort: Natural history of disease and the association of viral markers with histological and serological outcomes

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

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Produced on archival quality paper.
Abstract

Chronic Hepatitis B (CHB) is a major worldwide public health problem with an estimated 240 million people chronically infected and at risk of the complications of liver cirrhosis and hepatocellular carcinoma. There are recognized phases of disease in CHB which are usually defined using a combination of HBeAg status, HBV DNA level and ALT level. These can provide a useful framework to conceptualise a patient’s risk of fibrosis and need for treatment. Using the large CHB cohort at our tertiary hospital in Melbourne, we described our patient group in terms of their phase of disease in a cross-sectional analysis in chapter 3. This analysis highlighted some of the limitations of tests used, eg the insensitivity of older generation HBV DNA. Histological liver injury due to hepatitis B virus (HBV) was also able to be examined in a large proportion of our cohort with liver biopsies and in chapter 4 we evaluated the effect of HBV DNA on liver fibrosis. We found that increasing HBV DNA was associated with increasing prevalence of significant fibrosis in HBeAg negative patients, however in HBeAg positive patients, the reverse was true. This once again leads back to the concept of phase of disease in which HBeAg positive patients in the immune tolerant phase have normal ALT and minimal fibrosis despite very high HBV DNA levels. Longitudinal analysis of untreated HBeAg negative patients in chapter 5 brought to light some of the weaknesses of current classification systems for phase of disease, since we showed that low level fluxes in viral load in HBeAg negative patients were common and strict thresholds for HBV DNA to define the immune control phase were often crossed. The effects of HBV genotype and viral variants (precore and basal core promoter) on liver histology were examined in chapter 6. No significant associations were found although there was a trend towards higher prevalence of significant fibrosis in patients with HBV genotype C. Furthermore we explored the role of the novel markers of quantitative HBeAg and HBsAg (qHBeAg and qHBsAg) for classification of patients into phases in the natural history of CHB and found significant differences in qHBsAg levels in patients in different phases of disease in chapter 7. Finally we evaluated the role of qHBeAg and qHBsAg in the setting of Nucleos(t)ide analogue treatment by comparing 2 groups of HBeAg positive patients, one of which was known to lose HBeAg during the 18 months of followup and the other group which did not. Differences in both baseline and on treatment levels of qHBeAg were found between the 2 groups, however no significant differences in qHBsAg levels were noted. Management of CHB frequently involves monitoring patients to decide on the need and optimal time for therapy. A better understanding of the tools used in evaluating patients and the performance of these in different phases of disease and on therapy helps to enhance the clinical care of CHB patients.
Declaration

I declare that this thesis is my own composition and comprises only original work conducted by myself towards the degree of Doctor of Medicine, over the period February 2007 to July 2015, except as indicted in the preface.

Due acknowledgement has been made in the text to all other materials used.

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

All work described in this thesis was performed and analysed by myself with the following exceptions.

Following initial analyses of the data and formulation of specific questions performed by myself, further statistical analyses that involved the use of specialised statistical software programs were performed by Mr James Williams, Department of Gastroenterology St Vincent’s Hospital for the data in chapter 3, by Associate Professor Ian Gordon, Department of Statistics, University of Melbourne for the data in chapter 4 and Mr Paul Bassett of StatsConsultancy Ltd Buckinghamshire UK, for the data in chapters 5, 6, 7 and 8. Mr Bassett also created figures 8.5 and 8.6.

None of this work has been submitted for other qualifications and no part of this body of work was commenced prior to enrolment in the degree of Doctor of Medicine.
Acknowledgements

This thesis was supported by scholarship funding from the University of Melbourne (Melbourne Research Scholarship) and the NHMRC during the period 2007-2010 as well as from the Department of Gastroenterology at St Vincent’s hospital during 2007.

I would like to extend my very grateful thanks to my supervisors Associate Professor Paul Desmond and Dr Sally Bell who have provided great support and encouragement throughout the extended period of my candidature not only of this work but of many other aspects of life including career, travel and family plans. Your accessibility and engagement in this work as well as tremendous patience has been invaluable to me.

I also thank Professor Stephen Locarnini for the opportunity of performing part of this project in his lab at the Victorian infectious Diseases Reference Laboratory (VIDRL). Thank you to Lilly Yuen at VIDRL for supplying me with the list of all St Vincent’s Hospital patients who had HBV genotype testing and sequencing performed at St Vincent’s and for helpful comments on some aspects of chapter 5 in preparation for submission of it as a manuscript for publication. Thank you to Professor Alex Thompson and Dr Tin Nguyen for showing me the technique for performing quantitative HBeAg and HBsAg and to the staff at VIDRL who assisted me with familiarising myself with the lab and troubleshooting problems with the Architect machine. My special gratitude is due to Margaret Littlejohn and Anna Ayres for many trips down to the minus 20 freezers to retrieve serum samples for Quantitative HBeAg and HBsAg testing and for advice on what to wear for the trips.

I acknowledge the work of Dr George Yu Kong for creation of the program which enabled electronic updating of the HBV database. This populated empty fields in seconds doing work in time that would not have been humanly possible and made the database much more complete during 2007 and subsequently.

I also thank the clinicians, Drs Barb Demediuk, Gideon Shaw and Marno Ryan for copying their letters from clinic to the database and particularly to Dr Rob Chen who also faithfully copied all letters from his rooms to the database, and the hepatology nurses and database entry personnel for maintenance of the HBV database.

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I thank my parents for the enormous practical help they have provided us with over all the years, for their unfailing backup and unstinting generosity with their time as well as constant love and care. I am extremely grateful for you allowing me the time to work on this thesis especially over the last stretch by keeping our household and family running. Thank you also to my brothers and sisters-in-law for helping out in many ways at many times with the practical demands of family life. Thank you to my parents in law for longstanding, regular and reliable commitments to looking after our children which have been greatly appreciated.

I would like to thank my husband Daniel for “sharing the journey” with me, over the past 8.5 years of this project and especially in the last few weeks with assistance in formatting the document. This work would not have been possible (and life not nearly as fun), without your good company, sense of humour, calm ability to deal with my intermittent episodes of decompensation and loving encouragement always to continue.

Last but not least, thanks to our children, Emily, Peter, Gerard, Elizabeth and Josephine. I know it has felt at times that I have been “doing this thesis” for your “whole life” (because in many cases I have). I really appreciate your patience and understanding. I am really looking forward to spending more time with you!
Abbreviations

WHO, World Health Organization
HBV, Hepatitis B Virus
HBsAg, Hepatitis B surface antigen
HBeAg, Hepatitis B core antigen
HBcAg, Hepatitis B core antigen
HBeAg, hepatitis B e antigen
anti-HBe, Hepatitis B e antibody
anti-HBC, Hepatitis B core antibody.
Anti-HBs, Hepatitis B surface antibody
CHB, chronic hepatitis B
HIV, Human Immunodeficiency Virus
HCV, Hepatitis C Virus
HBIG, human hepatitis B immunoglobulin
HCC, hepatocellular carcinoma
NTCP, Sodium taurocholate cotransporting polypeptide
rcDNA, relaxed circular DNA
ORF, open reading frame
cccDNA, covalently closed circular DNA
pgRNA, pre-genomic RNA
ER, Endoplasmic reticulum
ALT, Alanine aminotransferase
AIDS, Acquired Immunodeficiency Syndrome
ULN, Upper Limit of Normal
IU/ml, international units/ millilitre;
OR, Odds Ratio
CI, Confidence Intervals
LLD, lower limit of detection
pg, picograms.

AASLD, American Association for the study of liver diseases,

APASL, Asian Pacific Association for the study of the liver

EASL, European Association for the Study of the Liver

REVEAL-HBV: The Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-In HBV

PNALT, persistently normal ALT

IFN, Interferon

NA, nucleoside/nucleotide analogue

BCP, basal core promoter

IFN-γ, interferon gamma,

NF-κB, nuclear factor kappa-B

TNF-α, tumour necrosis factor alpha

IFN α/β, interferon alpha/beta

IL-8, Interleukin 8

IL-12, Interleukin 12

MHC, Major Histocompatibility Complex

HLA, Human Leukocyte antigen

CTL, cytotoxic T lymphocytes

qHBsAg, quantitative HBsAg

qHBeAg, quantitative HBeAg

TDF, Tenofovir disoproxil fumarate
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Chapter 1: Literature Review

Epidemiology

Global epidemiology of HBV

Hepatitis B is a major worldwide public health problem. World Health Organization (WHO) estimates of the global prevalence of hepatitis B infection in 2014 were that 240 million people have chronic infection and that approximately 780,000 people die every year due to the acute or chronic complications of the virus (1). Estimates of chronic infection from around a decade previously were higher, with the figure of 350 million being most commonly quoted and appraisals suggesting that 2 billion people worldwide have been exposed to the Hepatitis B virus (HBV) (2). In the past, Asia and the Western Pacific have been estimated to contribute to almost 75% of the world’s HBV burden (3) however rates of Hepatitis B surface Antigen (HBsAg) prevalence have remained stable over the past 25 years in East Asia (4). In contrast, in Africa, which also has an enormous and somewhat neglected burden of HBV (5), HBsAg prevalence has been increasing in some parts, in particular in south and east SubSaharan countries (4). Overall, 30% of cirrhosis and 53% of Hepatocellular carcinoma worldwide has been calculated to be attributable to Hepatitis B infection and in certain regions of the world eg Africa and China that proportion is even higher (6). The prevalence of HBV infection varies in different countries (7). Traditionally, countries of low (<2%), medium (2-8%) and high (>8%) prevalence have been identified, and these differ in many ways including predominant mode of transmission and age at infection (8) (See Table 1.1). More recent figures have refined the categories of endemicity into low-intermediate (2-4.9%) and higher intermediate (5-7.9%) HBsAg prevalence (9). Certain countries in South East Asia (including Taiwan, Korea, Philippines, Thailand, Vietnam), China, Sub-Saharan Africa, the South Pacific Island nations and the Amazon basin have a prevalence of chronic hepatitis B >8% (10) with some having an estimated prevalence of up to 20% (11). An intermediate prevalence of 2-7% is found in countries around the Mediterranean, in Eastern Europe, the Middle East and South and Central America (12). Central Asian countries including the Indian subcontinent and Indonesia, Malaysia and Singapore also have an intermediate prevalence of HBV (13). The prevalence of chronic hepatitis B (CHB) is low, <2%, in most developed parts of the world including the USA, Australia and Western Europe. However, immigration to these countries of people from areas of high endemicity has increased prevalence rates (14, 15). A safe and effective vaccine against hepatitis B has been available since 1982. In 1992 the WHO passed a resolution recommending
global vaccination against hepatitis B and in 2013 the vaccine was part of the infant vaccination schedule of 183 countries (1). This measure has been shown to have a significant impact on reducing the prevalence of HBsAg, especially in countries of high endemicity in Asia (16, 17). In particular, Taiwan has been a model for other countries with the early introduction in 1984 of a nationwide vaccination program translating into a 91% reduction in HBsAg carriage rate (18). The huge impact of vaccination on reducing the complications of acute and chronic HBV infection are already being realised and reported by many groups eg reduced incidence of fulminant hepatitis, reduced incidence of membranous nephropathy associated with HBV and reduced mortality from chronic liver disease (9). Control of HBV requires a multipronged, organized and systematic approach to various aspects of the disease including control of vertical transmission of HBV with active and passive immunisation (19), screening programs for HCC (20), effective antiviral therapies and public policy. The contribution of all these measures, but especially immunization to a decrease in the incidence of HCC which is the perhaps the deadliest outcome of HBV is also, after a long lead time, coming to fruition with 2013 data from Taiwan confirming an 80% reduction in incidence of HCC in patients <30 years and a decrease in mortality of > 90% (21).

**Modes of transmission**

The HBV may be transmitted by exposure to infectious blood or body fluids. HBV is more infectious than the Human Immunodeficiency Virus (HIV) or Hepatitis C Virus (HCV). It is found in highest concentration in blood but HBsAg has also been demonstrated in semen and in saliva (22). It has been shown to be viable outside the human body for up to 7 days and has also been found on inanimate objects in high concentrations (23). The predominant mode of transmission differs in different parts of the world being largely perinatal in areas of high endemicity, horizontal (child-child) in areas of intermediate prevalence and sexual or percutaneous in developed countries with low rates of endemicity. The different modes of transmission are closely linked to the age at infection which is inversely related to the rates of chronicity.

**Perinatal transmission of HBV**

Perinatal or vertical transmission may occur during or shortly after birth but transplacental passage of HBV is thought to be uncommon. There have been varying reports about this however in a study by Aziz et al (24) in 1973, HBV was found in only 1 of 18 specimens of cord blood in HBV positive mothers. Infants infected perinatally have been thought to have a very high risk, up to 90%, of developing chronic hepatitis B (25), although other data from
Singapore found that overall 43% of babies born to HBsAg positive mothers developed chronic infection in the first year (26). Maternal-infant transmission is thought to account for 40-50% of HBV infections in China (27) and a similar proportion in Taiwan (28). Immunoprophylaxis of infants born to HBsAg positive mothers is recommended within 12 hours of birth, by both active (hepatitis B vaccine) and passive (using human hepatitis B immunoglobulin (HBIG)) means and reduces the risk of transmission in the majority of cases. The risk of perinatal transmission is related to HBeAg status and maternal serum HBV DNA levels (26, 29). In a study at Liverpool hospital in Sydney, 4 cases of transmission were reported in a cohort of 313 HBsAg positive women. The rates were 7% in babies of HBeAg positive mothers despite vaccination of the infant (although one baby inadvertently missed HBIG passive vaccination) (29), 9% from HBeAg positive mothers with HBV DNA (>10^6 copies/ml or 1.7 x 10^7 IU/ml), but 3% overall from HBV DNA positive (HBeAg positive and negative) mothers. Thus in HBsAg positive mothers, the failure rate of full active and passive vaccinations to prevent transmission is approximately 1% (3/313). Lamivudine use in late pregnancy was shown in the past to reduce risk of transmission in mothers who have high viral loads and although there are data suggesting it may be beneficial (30), others have shown that it lacks the potency required to cause sufficient drop in viral load pre-delivery and is associated with the development of drug resistant variants (31). Tenofovir has more recently been shown to be safe, efficacious and more potent than Lamivudine in preventing transmission of HBV (32). Caesarean section is not routinely recommended for HBsAg positive mothers as at present there is insufficient evidence that it alters transmission rates especially when HBIG is administered (33) Paternal transmission of HBV from HBsAg positive fathers to their infants (where the mother is HBsAg negative) has been reported at rates of up to 65% in intrafamilial studies using genotypic and phylogenetic analysis of virus (34). The transmission is thought to occur through close contact which exposes the infant to infectious blood or other body fluids of the father rather than through transfer to the foetus by “infected sperm”. The impact of genotype on perinatal transmission as well as the question of whether different genetic polymorphisms in HLA class II and nuclear factor kappa-B (NF-kB) in Asian versus European populations may account for the difference in rates of chronicity needs further exploration (35).

**Childhood (horizontal) transmission of HBV**

The risk of developing chronic infection is 20-50% in children infected through horizontal spread (36, 37). Horizontal transmission occurs through exposures to the open cuts or skin lesions of an infected person, or through sharing of household items such as toothbrushes, razors or even toys (which presumably have been contaminated by blood) (38). Horizontal
transmission from an HBsAg positive person to household contacts is common and the risk is especially high to children and between siblings (38) thus vaccination of all household contacts of HBsAg positive patients is recommended to prevent transmission. Infection of children who do not live with a HBsAg positive person also occurs presumably through contact in the playground or institutional settings with other infected children (39, 40) although transmission in child care settings is thought to be rare (41).

Percutaneous spread via injections administered using inadequately sterilised needles or other medical equipment, or traditional medicine practices have certainly been another possible method of HBV transmission in children and young adults with chronic Hepatitis B (36).

**Adult transmission of HBV**

Adult acquired hepatitis B is predominantly transmitted sexually - both through heterosexual sex and in men who have sex with men. It may also be transmitted percutaneously through injecting drug use. The vast majority (95-99%) of infections are cleared and the rate of chronicity is very low (42).
### Table 1-1 Geographical variation in Hepatitis B prevalence and patterns of transmission

<table>
<thead>
<tr>
<th>Pattern of Prevalence</th>
<th>Geographical Area</th>
<th>% of population HBsAg positive</th>
<th>% of population anti-HBs positive</th>
<th>Predominant age at infection</th>
<th>Predominant mode of transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>High ≥ 8%</td>
<td>Southeast Asia, China, Pacific Islands, Sub-Saharan Africa</td>
<td>8-20%</td>
<td>70-95%</td>
<td>Perinatal and early childhood</td>
<td>Maternal-infant, percutaneous</td>
</tr>
<tr>
<td>Intermediate 2-7%</td>
<td>Eastern Europe, the Mediterranean basin, Middle East, Central and South Asia, Japan, Central and South America</td>
<td>2-7%</td>
<td>20-55%</td>
<td>Early childhood</td>
<td>Percutaneous</td>
</tr>
<tr>
<td>Low&lt; 2%</td>
<td>United States and Canada, Western Europe, Australia and New Zealand</td>
<td>0.2-0.5%</td>
<td>4-6%</td>
<td>Adult</td>
<td>Sexual, Percutaneous</td>
</tr>
</tbody>
</table>

Epidemiology of Hepatitis B in Australia

Estimates of chronic hepatitis B infection in Australia in the year 2000 ranged from 88,000-163,500 (0.47-0.87%) (44). This data came from a national sero-survey for the period 1996-1999 in 1-59 year olds based on opportunistic testing of sera sent to laboratories for a broad range of diagnostic tests. It also used a second methodology of forming estimates of HBsAg prevalence in specific groups at “higher” and “lower” risk for HBV. For example various published reports of prevalence in higher risk groups eg injecting drug users, men who have sex with men, indigenous Australians and prison inmates were used. Data on the HBsAg prevalence in first time blood donors from the Australian Red Cross Blood service was applied to the rest of the Australian population to estimate prevalence of CHB in lower risk groups (44). A 2010 Victorian serological survey of 3212 samples of convenience from the years 1995, 2000 and 2005 estimated the HBsAg prevalence at 1.1% (95% CI 0.8%-1.6%) (45). Figures in 2013 from the same group suggested that 218,000 Australians are living with CHB with over 95% of newly identified cases being in patients migrating from endemic areas (46). Other cohorts in whom Hepatitis B prevalence has been studied include a cohort of 2115 patients attending an endoscopy unit in central Sydney in whom the prevalence of HBsAg was 2.1% and the prevalence of natural immunity (anti-HBs and anti-HBc) was 9.5% (47). 70% of patients with current infection (HBsAg positive) in this study were born overseas. A previous study from our institution, St Vincent’s Hospital, Melbourne, in 2004 demonstrated that 82% of patients with chronic hepatitis B seen at this tertiary hospital’s liver clinics were born overseas, with 65% from Asia and 14% from the Mediterranean (48). A steadily increasing chronic HBV prevalence due to immigration from the Asia Pacific region was estimated from the period 1960 to 2005 and is predicted to rise further by the year 2025 (49). The projected rise also applies to the attendant complications of CHB in particular hepatocellular carcinoma (HCC) and there have been calls for a national public health strategy for hepatitis B which should include a number of initiatives including screening programs both for CHB and HCC, further epidemiological and virological research into HBV in Australia and improved access to treatment (50).
Hepatitis B Virus Molecular Virology

The HBV is a hepatotropic virus of the family hepadnaviridae. Other animal hepadnaviruses include woodchuck hepatitis viruses and duck hepatitis B virus. Whilst these cannot establish infection in humans they have proven to be valuable models for studying hepatitis B viral replication and for the evaluation of antiviral drugs. HBV infection is only supported in humans and chimpanzees and in addition it has been difficult to culture in cell lines of hepatocytes or HCC although some have been described (51, 52). Establishing a cell line that can support the entire life cycle of HBV however has proven difficult (53) although there have been some recent reports of possible mouse models for HBV immunotolerance (54).

Structure of HBV
The Hepatitis B virion comprises a central icosahedral core protein (HBcAg) which contains the viral DNA and HBV viral polymerase (the nucleopapsid). This core is surrounded by a lipid membrane studded with viral proteins which are the small, medium and large HBV surface proteins. The entire virion is 42 nM and was originally referred to as the Dane particle following its discovery by an English pathologist DS Dane (55). In addition to the virions, electron microscopy of HBV infected blood reveals excess surface proteins (without a core) which form themselves into spherical and filamentous particles. Several hundred to thousand fold more of these empty particles made up of surface protein are secreted by infected cells than are infectious virions. The reason for this is unclear however it is thought that they may play a role in immune evasion (56). The HBV’s DNA is unusual in that rather than being fully double stranded, it is only partially so. Thus there are 2 DNA strands of different lengths. The short length strand (positive sense) is 1700-2800 nucleotides long and the full length strand (negative sense) is 3020-3320 nucleotides long (57). The DNA is in a relaxed circular (rc) configuration. The 5' end of the the full length strand is covalently linked to the DNA polymerase. The genome is able to encode 4 known genes: C (core), S (surface/envelope), P (polymerase) and X by using 4 overlapping, but frame shifted, open reading frames (ORF) (58). An ORF is a sequence of nucleotides in DNA that contains no termination codons and so can potentially translate as a polypeptide chain.
Figure 1-1: Hepatitis B virus structure and particles.

Figure legend: The hepatitis B virion is made up of a central core containing the viral DNA and the viral polymerase which acts as a reverse transcriptase. This core is surrounded by a lipid envelope embedded with HBsAg protein. Excess HBsAg also forms into empty filamentous and spherical particles which are secreted in vast excess of the number of virions.
Genome of HBV
The genome encodes 4 overlapping over reading frames (ORF’s) designated polymerase (Pol), precore/core (C), surface (S) and X (43).

Precore/Core ORF
The HBcAg and HBeAg proteins are both encoded by the Pre-core/Core ORF which has has 212 or 214 codons (varying according to genotype). There are 2 AUG start codons present in the precore/core ORF, the second being 29 codons downstream of the first. HBcAg is coded for by translation from the second AUG start codon. Hepatitis B e antigen (HBeAg) is coded for by translation from the first AUG start codon. The coding region for HBeAg therefore includes this upstream (precore) region plus the core part of the ORF (59). The Pre-C + C protein product is further processed by proteolytic cleaving to form the HBeAg (56). It is then secreted from the cell in a pathway that is separate to the nucleocapsid pathway.

Polymerase (Pol) ORF
The polymerase ORF contains 834-845 codons and encodes the HBV DNA polymerase which functions as the reverse transcriptase for the virus.

Surface (S) ORF
The surface (also known as envelope) ORF has 3 domains initiated by 3 in-phase start codons and all sharing a common stop codon. This divides the Surface ORF into 3 regions, the S, Pre-S1 and Pre-S2. The S region encodes the small surface protein. the Medium is coded for by Pre S2 + S regions and the Large HBsAg by Pre S1 + Pre S2 + S (60). The small surface protein contains the major epitope (antigenic determinant), known as determinant “a” of Hepatitis B, recognized by B and T cells. It is a conformational epitope (ie interacts with the Antibody based on its 3D surface structure) (56). The function of the middle protein is not fully understood.

The large Surface protein plays an important role in attachment and entry of HBV virions to the hepatocyte. The surface protein in fact has multiple functions in addition to the above, for example as a structural protein of the HBV envelope (or outer coat), and as a transporter molecule involved in transportation of the HBV into the nucleus (61)

X ORF
The X ORF is the smallest ORF and encodes the X Protein of 154 amino acids whose function is not clearly known however has been implicated in hepatocarcinogenesis (62).
Figure legend: The genome of HBV is made up of partly double stranded DNA with a complete coding minus strand (−) and an incomplete noncoding positive strand (+). The genome encodes 4 overlapping over reading frames (ORF’s) designated polymerase, precore/core, surface and X.
Life cycle of HBV

Attachment, Entry and translocation to nucleus
The HBV enters the hepatocyte through binding to the recently identified HBV receptor - the Sodium taurocholate cotransporting polypeptide (NTCP) receptor (63). Following the endocytosis of the virion into the cell, the viral capsid is released and transmits its relaxed circular DNA (rcDNA) through nuclear pore complexes into the nucleus (64).

Formation of cccDNA
Once in the nucleus the rcDNA which is only partially double stranded must be transformed into covalently closed circular DNA (cccDNA). This occurs by completion of the plus (positive) strand HBV DNA. The cccDNA persists in the infected nucleus as an episomal “minichromosome” (65) and may remain there for the lifetime of the hepatocyte. The HBV genome can also randomly integrate itself into host chromosomes (66). Such viral integration is thought to be a major potential mechanism underlying the oncogenetic potential of HBV and the development of HCC including in occult HBV (67).

Transcription of viral RNA
The cccDNA created is then transcribed into 5 RNA products (56):

1) 3.5 kb pre-core mRNA which is translated into the precore protein. (68).

2) 3.5 kb pregenomic (pg) RNA which is translated into the core protein and the polymerase protein and is also reverse transcribed into viral DNA which is then exported as virion in newly formed capsids and also partly shunted back into the nucleus to be formed again into cccDNA.

3) 2.4 kb mRNA translated into large (L) envelope (surface) protein.

4) 2.1 kb M and S RNA translated into medium (M) and small (S) envelope or surface proteins.

5) 0.7 kb X mRNA translated into the X Protein.

The mRNA and pgRNA are transferred from the nucleus into the cytosol where translation of proteins and replication occur.

HBeAg and HBsAg secretory pathways
The initial precore protein product is further proteolytically processed into HBeAg in the endoplasmic reticulum and Golgi apparatus and secreted from the cell in a pathway that is separate from the nucleocapsid pathway (69).
HBsAg synthesis occurs from translation of the mRNA transcripts above into small, medium and large surface proteins. Some of the surface protein is incorporated into the mature virion’s coat but HBsAg is produced far in excess (100-100,000 fold) of what is required for virion assembly (70). Excess HBsAg forms into filamentous particles up to 200nm long and spherical particles which are around 20nm in diameter. These empty sub-viral particles are non-infectious and are secreted at significantly higher levels than that of HBV virions. Their role is not fully known but they may contribute to mopping up neutralizing Antibodies and thus assist the virus in establishing infection.

**Viral replication pathway - Synthesis of HBV DNA from pregenomic RNA**
The HBV genome is replicated via an RNA intermediate (the pregenomic RNA) through the action of the polymerase protein which acts as a reverse transcriptase. The polymerase, the pregenomic RNA and the core protein are encapsidated together and reverse transcription occurs within the capsid, first with the synthesis of a new minus strand HBV DNA followed by a new plus strand DNA (71).

**Assembly of virions and secretion**
Endoplasmic reticulum (ER) membrane engulfs the DNA containing capsids and the membrane is then studded with the 3 envelope proteins (S, M and L). Virions generally contain about 100 small HBsAg particles for every 5 medium sized and every 1 large HBsAg particle. While some mature nucleocapsids are thus enveloped and secreted by the hepatocyte, some are shunted back into the nucleus to increase the pool of cccDNA (56).
Figure 1-3: Life Cycle of HBV

Figure legend: Life cycle of HBV showing the HBV virion is uncoated on entering the cell and relaxed circular (rc) DNA enters the nucleus and is converted to cccDNA. cccDNA forms the transcriptional template for viral mRNA. The pregenomic mRNA transcript is translated into the polymerase proteins which serves as the reverse transcriptase for formation of rc DNA. This occurs in the immature nucleocapsid with the core protein and this is then engulfed by the endoplasmic reticulum, studded with HBsAg and the mature virion is secreted. Precore mRNA forms the precore protein which is further translated in the Golgi and ER and secreted as HBeAg. HBsAg is translated from 2 separate mRNA transcripts and secreted in the coat of the virions and excess HBsAg is also secreted as empty filamentous or spherical particles of HBsAg.
Overview of the natural history of Hepatitis B

The natural history of chronic HBV infection is determined by a complex interplay of viral, host and environmental factors. The genotype, replicative status and mutations present in the virus can impact on the various factors including likelihood of progression to chronicity, the duration of phase of disease, and the risk of the clinical outcomes of cirrhosis or HCC. Environmental influences including co-infection with other hepatitis viruses or HIV, coexistent sources of liver injury such as alcoholic or fatty liver disease, and in parts of the developing world, exposure to other infectious agents such as Schistosomiasis or carcinogens like Aflatoxin, also have a bearing on the outcomes of CHB including cirrhosis or HCC.

Host factors such as gender also play a role with male sex being an important risk factor for liver inflammation as measured by Alanine aminotransferase (ALT) (72). The host immunological response to viral infection plays a key role in the natural history of disease both at the time of infection in determining clearance versus persistence of HBV and throughout its course. The HBV is not itself a cytopathic virus in immunocompetent individuals and liver damage is immune mediated. Only in situations of extremely high viral load due to immune suppression, such as was seen for example with AIDS and post transplant in the era prior to antiviral agents, with the development of fibrosing cholestatic hepatitis, are direct cytopathic effects seen (73).

The behaviour of HBV in different parts of the world can be quite distinct and therefore applicability of studies in certain regions to other areas may be limited. The term “natural history” in hepatitis B essentially describes the untreated course of the virus in infected persons. This may be observed from a number of different perspectives, ranging from population based studies of HBV in large cohorts monitored over years for the development of significant clinical outcomes, to documentation of the fluctuations in blood parameters of untreated HBV patients in tertiary hospital clinics, who may be monitored intensively over months. With this in mind I will next seek to outline the natural history of hepatitis B from a holistic perspective with delineation of the differences in the course of disease in various geographical locales and examining the short, medium and longer term courses of infected patients in different phases of disease.
Historical overview of HBV and the phases of disease – 50 years of hepatitis B

Blumberg et al identified Hepatitis B in 1965, reporting that year on their discovery of the “Australia Antigen”. They had noted an antibody in the serum of a transfused haemophilia patient that appeared different to other antibodies they were studying and reacted with only 1 of 24 sera against which it was tested. The reacting serum came from an Australian Aborigine, giving the novel antigen its initial name (74). It was subsequently identified that the Australia Antigen was associated with hepatitis (75) especially “long incubation” viral hepatitis (76) and post transfusion hepatitis (77). Electron microscopy studies helped establish that the Australia Antigen was the coat protein of HBV (78) which was subsequently renamed Hepatitis B Surface Antigen. The complete Hepatitis B virion (Dane particle) was discovered by Dane and colleagues in 1970 (55) and was soon shown to be a DNA virus (79, 80). The hepatitis B e antigen (HBeAg), was discovered in 1972 (81). Further developments in the molecular understanding of hepatitis B were made with the sequencing of the HBV genome in 1979 and the discovery by 2 groups of a single nucleotide change in the precore region, G1896A, which resulted in a premature stop codon that abolished HBeAg production (82, 83). The global distribution of hepatitis B and significant geographical variation in prevalence was also identified early in the course of its discovery (77, 84). It was also noted that patients with HBsAg could fall into 2 clinical categories; those with evidence of liver disease with abnormal ALT and necroinflammation on biopsy – chronic hepatitis B- and those without, who were termed “healthy” HBsAg carriers. (84, 85). The natural history of HBV infection was then observed by groups from different parts of the world and differences in the serological profiles and clinical courses of HBV infected patients from these different regions emerged. For example an early followup study by Hoofnagle of 25 HBsAg and HBeAg positive individuals showed loss of HBeAg and normalisation of ALT in 13, while in the other 12 HBeAg persisted as did elevation of ALT (86). HBeAg to Anti-HBe seroconversion was recognized as a marker of change in the activity of chronic hepatitis B (86, 87). Thus 2 sequential phases of “replicative” and “non-replicative” chronic hepatitis B were proposed. However observation of Asian patients identified an early phase with HBeAg positivity and very high HBV DNA levels but normal ALT and liver histology. This was noted in both children and young adults who presumably had perinatally acquired HBV and therefore an initial phase of “immunotolerance” was integrated into the understanding of HBV’s natural history (88, 89). The fourth phase of CHB, with viral replication seen in patients who were HBeAg negative and Anti-HBe positive, was then described and this phase of reactivation was also added to the schema of natural history (90-92). Over the 30 years since these studies used serological features of disease to
define natural history, further refinement of our understanding of hepatitis B has been made possible. This has been through the development of increasingly sensitive HBV DNA assays, characterisation of the molecular biology of the HBV and insights into host immunological responses to HBV (93). Integration of HBV DNA into the host genome over the course of persistent infection was recognized in 1981 (94), which is an important factor in the pursuit of eradicability of HBV in infected patients and in the pathogenesis of Hepatocellular carcinoma. More recently a resurgence of interest in the role of quantitation of HBsAg in different phases of disease have provided further insight into the molecular workings of the HBV and these assays have been harnessed as tools to guide therapy.

The phases of disease in Chronic Hepatitis B
Identifying the phase of disease in individual CHB patients is a key part of their initial evaluation. The phase of disease gives clues to the likely time and mode of their infection with HBV, it prognosticates about their probable degree of liver fibrosis, their likely short and long term course and it determines the appropriate course of management be it with regular monitoring or anti-viral therapy. The nomenclature for the phases of disease has evolved over time and place and continues to do so. For example the National Institutes of Health (NIH) sponsored workshops in the United States in 2000 and 2006 defined 3 phases of chronic HBV infection: the immune tolerant phase, the immune active phase (encompassing both HBeAg positive and negative patients with elevated ALT, although with different thresholds for HBV DNA elevation) and the inactive hepatitis B phase (95, 96). This followed on partly from the clinic-pathological work of Chu and colleagues in the 1980’s (88). The term “recovery phase”, for resolved hepatitis B (HBsAg negative, anti-HBc positive) was also proposed (97, 98). Meanwhile at a 2006 workshop in Frankfurt of the European Viral Hepatitis Educational Initiative (EVHEI), the terminology of “Immune Tolerant”, “Immune clearance”, “Immune Control” and “Immune Escape” was used for the 4, largely sequential, HBeAg positive (Phases 1 and 2) and HBeAg negative (Phases 3 and 4) CHB disease states (99). Australian guidelines published by the Digestive Health Foundation of the Gastroenterological Society of Australia in 2007 and 2009/10 also use the 4 phase immune based terminology (100). The characteristics of these phases of disease, adapted largely from all the above guidelines, are outlined in Table 1.2. The most recent guidelines for classification into phases of disease from the 3 major international associations for the study of liver diseases, American Association for the study of liver diseases (AASLD) (101), the European Association for the Study of the Liver (EASL) (102) and Asian Pacific Association for the study of the liver (APASL)(103), show more concensus
than previously, although there remain a few differences in particular in nomenclature as outlined in Table 1.2. It must be noted that not all patients with chronic hepatitis B go through all phases. Furthermore the duration of time spent in different phases may vary, indeed transition from one stage to the next may be so fast that distinct phases may not be recognizable. Lastly the phases 1-4 do not necessarily occur in that sequence and reversion from one phase to another may also occur. In addition to the 4 phases outlined in Table 1.2, the EASL 2012 guidelines (102) also define the HBsAg negative phase, after HBsAg loss, when low level HBV replication may persist with HBV DNA detectable in the liver although usually not in the serum. This is referred to in the AASLD guidelines (101) as “resolved hepatitis B” and is defined as:

1. A known history of acute or chronic hepatitis B or the presence of anti-HBc +/- anti HBs,

2. HBsAg negativity

3. Serum HBV DNA undetectable or detectable at very low levels using sensitive PCR assays.

4. Normal ALT levels.

This state is also known as occult HBV.
**Table 1-2: Phases of disease in Chronic Hepatitis B.**

<table>
<thead>
<tr>
<th></th>
<th>Phase 1 Immune Tolerance</th>
<th>Phase 2 Immune Clearance</th>
<th>Phase 3 Immune Control</th>
<th>Phase 4 Immune Escape</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serology</strong></td>
<td>HBeAg positive</td>
<td>HBeAg positive</td>
<td>HBeAg negative</td>
<td>HBeAg negative</td>
</tr>
<tr>
<td></td>
<td>Anti-HBe negative</td>
<td>Anti-HBe negative but spontaneous seroconversion to anti HBe can occur</td>
<td>Anti-HBe positive</td>
<td>Anti-HBe positive</td>
</tr>
<tr>
<td><strong>ALT (IU/ml)</strong></td>
<td>Persistently normal</td>
<td>Persistently or intermittently abnormal</td>
<td>Persistently normal</td>
<td>Persistently or intermittently abnormal</td>
</tr>
<tr>
<td><strong>HBV DNA (IU/ml)</strong></td>
<td>Very high (usually &gt; 2x10^6 - 2x10^7)</td>
<td>High but less than in Immune tolerant phase</td>
<td>&lt;2000 IU/ml (but up to 20,000 IU/ml may be seen)</td>
<td>&gt;2000 IU/ml (fluctuating)</td>
</tr>
<tr>
<td><strong>Liver Histology</strong></td>
<td>Normal or mild hepatitis</td>
<td>Moderate or severe necroinflammation and fibrosis</td>
<td>Normal or mild inflammation.</td>
<td>Moderate to severe inflammation and fibrosis. May have cirrhosis</td>
</tr>
<tr>
<td><strong>Specific features</strong></td>
<td>Age usually young (&lt;20-30 yrs). Phase absent or very short in Mediterranean CHB.</td>
<td>Characterised by intermittent flares or ongoing chronic mild elevation of ALT. Ends with seroconversion to HBeAg negative, anti-HBe positive state.</td>
<td>May have serum HBsAg levels &lt; 1000 IU/ml.</td>
<td>Predominance of HBV with precore/Basal core promoter mutations</td>
</tr>
<tr>
<td><strong>Alternative nomenclature</strong></td>
<td>Immunotolerant</td>
<td>HBeAg positive immune active (AASLD)/ Immune reactive HBeAg positive phase (EASL)</td>
<td>Inactive HBsAg carrier state (EASL/AASLD)/Inactive chronic HBV infection or clinical remission (APASL)</td>
<td>HBeAg negative chronic Hepatitis B</td>
</tr>
</tbody>
</table>
Immune Tolerant phase
The key features of this phase are HBeAg positivity, very high serum HBV DNA levels (usually > \(2 \times 10^6\) but normal ALT levels and minimal or absent inflammation or fibrosis on liver biopsy. Current Australian guidelines include HBV DNA levels of >20,000 IU/ml for definition of this phase. The immune tolerant phase is classically seen in Asian children infected perinatally with HBV and can last for several decades (27). The phase is also seen in childhood acquired, horizontal infection but is thought to be of much shorter duration (104). An early study by Lok and Lai in Chinese children in the immune tolerant phase (43 HBeAg positive children, median age 10, followed for a median of 24 months) showed that HBV DNA levels were highest in the youngest children. They also demonstrated that while ALT levels were similar at the first and last followup visits being 25 and 20 IU/ml, mild transient elevations of ALT, sometimes accompanied by changes in HBV DNA were noted during followup (89). Chang and Beasley published a followup study of 18 Taiwanese children with perinatal infection aged 4-9 years, which also showed some fluctuation of ALT in and out of the normal range over followup, however the normal range of ALT (as determined in 1127 HBsAg negative children) was defined as <14 KU (translates to IU/ml) while in adults it was 35 KU. Thus close observation of patients in the clinic highlights the difficulty of trying to use a rigid classification system for a dynamic disease. The immune tolerant phase is one in which significant liver injury is uncommon as shown in Beasley’s above study where liver biopsies in the 18 children showed non specific histologic change in 15 and chronic persistent hepatitis in 3 (105). A further, more recent study from Hong Kong in 2007 showed little or no histological fibrosis on initial liver biopsy (median fibrosis stage 1) in 57 adult immunotolerant patients. Paired liver biopsies in 48 of these who remained in the immunotolerant phase after a 5 year period showed that the followup fibrosis stage was comparable with the initial fibrosis stage. Histological disease progression however was greater in those 9 patients that developed elevated ALT and transitioned to the immune clearance phase (106). Lai et al recently found that significant histological change could be seen in HBeAg-positive subjects with persistently normal ALT (PNALT) which was defined by them as ALT <40 on at least 2 occasions over at least 6 months. However these authors acknowledge that few of their PNALT patients truly fit the picture of immune tolerance being older or with lower viral loads. They suggest that the term be reserved for young patients with very high HBV DNA levels (>10^8 copies/ml) and low normal ALT (107). Thus a degree of suspicion about the true degree of “immune tolerance” in older HBeAg positive patients especially those with high normal ALT is prudent. APASL recommends liver biopsy in patients with high normal ALT and age>40 (108). Similarly liver biopsy is
recommended in the AASLD guidelines for HBeAg positive patients >40 years with normal ALT and HBV DNA>20,000 IU/ml and treatment recommended if biopsy shows moderate/severe inflammation or significant fibrosis. Keefe et al also advocate for liver biopsy in patients >35-40 even if ALT is normal (109). The “loss” of immune tolerance was noted by Andreani to occur at a median age of 30 yrs. This occurred over followup in 12 of their 40 immune tolerant patients (HBeAg positive patients with normal ALT and HBV DNA> 10⁷ copies/ml). Subsequent rapid transition to the inactive carrier state occurred in two thirds of their patients (9/12) and to chronic hepatitis in one third (3 patients) (110). Studies in children treated during the immune tolerant phase have not shown increased rates of HBeAg seroconversion either with Interferon or Lamivudine therapy (111, 112). Therefore, because of the lack of proven efficacy and since there is thought to be only a low risk of liver injury, treatment has not been advocated during the immune tolerant phase. However a recent study by Poddar et el found that 28 immune tolerant children treated with Lamivudine for 8 weeks followed by Lamivudine + IFN alpha for 44 weeks compared to 34 untreated children found HBeAg seroconversion rates of 39% (compared to 5.9% in controls) and HBsAg loss rates in 21% (compared to 0% in controls) (113).

**Immune clearance phase**

The immune clearance phase is a phase of HBeAg positive chronic hepatitis, characterised by intermittent or persistent elevation of ALT levels and high but fluctuating HBV DNA levels although not as high as in the immune tolerant phase. This is a period of immune mediated liver damage as evidenced by necroinflammation on liver biopsy and varying degrees of fibrosis. Transition from the immune tolerant phase may be recognizable or not and it typically occurs during the second and third decades in patients with perinatally acquired disease with the majority of patients entering the immune clearance phase after the age of 20 years (114).

**Immunoclearance phase active liver disease - flares**

A typical feature of this phase is the occurrence of spontaneous flares which represent an intensification of the immune response to HBV. Flares are often preceded by an increase in the HBV DNA level (115) and thus may be precipitated by the increased levels of replicating wild-type virus (116). The term flare has also been used to describe reactivation of hepatitis B in HBeAg negative patients with sudden episodes of ALT elevation associated with re-emergence of viral replication which may be with mutant virus (116). The discussion here on spontaneous flares will largely be related to HBeAg positive CHB. There is no strict definition of or criteria
for a flare, however in early work done by several Asian groups the studies generally used ALT >5 times the upper limit of normal (ULN) or ALT >300 IU/ml. Histologically there is evidence of acute lobular hepatitis superimposed on changes of chronic viral hepatitis (117). Severe changes of bridging necrosis have also been described. Clinically, most flares are asymptomatic however some may be accompanied by symptoms of acute hepatitis. Hepatic decompensation with jaundice or encephalopathy and rarely fatality can occur in a small number of patients (118). IgM to HBeAg can appear (119) and result in misdiagnosis of a flare in chronic hepatitis B for acute infection. Anti-HBc IgM titres are much higher in acutely acquired HBV with liver failure compared to exacerbation of chronic HBV (120) and titres of >1:10,000 have been reported to have a sensitivity of 96% and specificity of 93% for diagnosing acute HBV (121). An increase in the serum Alpha-fetoprotein level may raise concerns about the diagnosis of HCC however it is a marker of cell turnover and Liaw noted that AFP levels >100ng/ml, which were frequently associated with bridging hepatic necrosis on liver biopsy, had a higher annual HBeAg clearance rate (122). Jeng et al (123) published a study in 2010 of flares in non cirrhotic HBeAg positive CHB patients (some of which were spontaneous and others following resistance to antivirals) and found that 5.1% of flares resulted in decompensation and this could be predicted by HBV DNA levels of > 1.55 x 10^9 copies/ml (roughly 2.88 x 10^7 IU/ml). The authors suggest that such patients therefore should have immediate anti-viral therapy with nucleotide/side analogues and that Interferon is not advisable in such situations.

**Frequency of flares in immune clearance phase**

In a 6 year followup study (mean 24.5 months) of 237 HBeAg positive patients with biopsy verified CHB, 199 episodes of flare (defined as ALT >300 IU/ml) were noted in 148 (62%) patients (124). In another study of 240 HBeAg positive patients (mean age of 27.6 +/- 6.2 years), ALT levels of ≥ 300 IU/ml were seen in 29% of patients and 5% had 2 or more episodes of ALT ≥ 200 IU/ml (125). The mean age at anti-HBe seroconversion was 31.3 +/- 7.0 years. These patients had a normal ALT at the beginning of the study which may account for the lower frequency of flares observed. HBeAg positive patients who are cirrhotic also seem to have a lower incidence of flares as shown in an 8 year followup study in which only 8 of 28 (28.6%) of patients with HBeAg positive cirrhosis experienced flares. ALT flares are more common in men than women which may account for the higher rates of cirrhosis in men (126). Although flares are a well recognized and frequent part of the natural course of hepatitis B, it is certainly however wise also to consider superimposed infections with other hepatotropic viruses (Hepatitis A, Hepatitis C and Hepatitis D) especially in patients with possible risk factors.
Other situations in which flares occur in chronic hepatitis B

Pregnancy
Pregnancy can be a risk factor for flares which may occur during pregnancy (127) or more commonly in the post partum period (128) and are occasionally severe. The frequency of post partum flares depends on the definition used for flare, however in a study by Giles et al (129) which used a threshold of twice the ULN (or twice the baseline value if baseline ALT was normal), was found to be 25% in all HBsAg positive patients (24% HBeAg positive). Further work by this Australian group reported on a cohort of 101 pregnancies (91 patients, all with HBV DNA ≥ log 7 IU/ml and 97% HBeAg positive) the rates of flare were 29% in patients who received no antiviral therapy and no significant reduction in the rates of flares was achieved with the use of antiviral therapy, for even up to 12 weeks post partum (130). The onset of flare was typically at weeks 8-10 post partum. Pregnancy is a state of relative immune suppression, associated with high levels of adrenal corticosteroids and alteration in cytokines involved in the immune response. The delivery of the baby, therefore, is analogous to the situation of withdrawal of steroid medication in which immune reconstitution results in recognition and attack of the HBV infected hepatocytes. Although the question has been raised as to whether increased viral load during pregnancy “sets the scene” for a flare post partum, Giles et al (129) measured HBV DNA and found no significant difference in HBV DNA between trimesters. They also found that HBV DNA was not a predictor of flare and hypothesise that the determinants of flare may be primarily immunologically based and perhaps reactive to some other viral factor not captured by current assays.

Flares associated with HBV therapy
In addition to the spontaneous flares which are part of the natural history of CHB it is also pertinent to briefly mention at this point the significant flares of liver disease that may occur due to HBV in other important settings. During therapy for CHB, for example, Interferon (IFN) alpha induced flares may occur in 1/3 of treated patients and usually occur during the second or third month of therapy. Decompensated cirrhosis and Genotype A are particularly associated with IFN alpha induced flares (131). Although the flares in genotype A also translate into higher rates of HBsAg loss in these patients, Interferon is not recommended for the treatment of CHB patients with decompensated liver disease as it can precipitate fatal consequences. ALT flares may also occur during the course of treatment with nucleoside/nucleotide analogues (NA) such as lamivudine and adefovir, eg if these antivirals are withdrawn (when there is a resurgence of wild type HBV) or due to the emergence of lamivudine resistance (YMDD mutant virus) while therapy is ongoing. The risk of flares has
been shown to increase with time from the initial detection of lamivudine resistance and is 29% at 1-2 years but 61% at >4 years (132). Thus, shifting to other therapies, which are fortunately now available, is the recommended course of action once anti-viral resistance has been detected. Given the relative lack of resistance to entecavir and tenofovir, flares that occur in the setting of these newer agents are more likely to represent non compliance or cessation of medication.

**Flares associated with immunosuppression**

Flares of ALT in hepatitis B may also occur in situations where immunosuppressant therapies have been used and are then withdrawn. These may include steroids, immunosuppressives used post organ transplant to prevent rejection and in autoimmune diseases such as rheumatoid arthritis and IBD, and also in cancer chemotherapy regimes. The immune suppression allows unchecked replication of the HBV (this may occur both in patients who are HBsAg positive or in those who had prior occult HBV with anti-HBc positivity who may develop HBsAg reversion). On cessation of the immunosuppressant medication, reconstitution of the immune system results in brisk destruction of the HBV infected hepatocytes with subsequent liver enzyme elevation and damage (133). Chemotherapy regimes which include glucocorticoids have a particularly high risk of causing reactivation of hepatitis B and subsequent flares (134). The monoclonal antibody Rituximab directed against the CD20 antigen on B-lymphocytes and used in the treatment of Lymphomas, Chronic Lymphocytic Leukaemia and several other conditions, has been associated with HBV flares in several reports including in patients who were HBsAg negative but anti-HBc positive (135). Reactivation of HBV has also been noted following transplantation, including bone marrow transplantation and solid organ (eg renal) transplants. CHB reactivation with ALT flares has also been observed in patients treated with biological response modifiers including anti-tumour necrosis factor antibodies (Infliximab) used in Crohn’s disease or rheumatoid arthritis, and Methotrexate - especially following withdrawal (136). In patients co-infected with HIV and HBV the use of anti-retroviral therapy results in restitution of the immune system. Flares have been described following the institution of HBV inactive anti-retroviral therapy and it is thought this is due to immune restoration. Elevated HBV DNA and ALT prior to the initiation of therapy have been identified as risk factors (137).

**Outcomes of flares during the natural history of HBeAg positive immune clearance phase CHB**

In 2003 Yuen et al (138) looked at 1215 of their HBeAg positive patients followed up for a median of 29 months with HBeAg seroconversion in 40.6% at median age of 34.5 years. They
showed that the chance of HBeAg seroconversion within 3 months of a flare in patients with peak ALT levels > 5 x ULN was 46.4% compared with 27.2% and 35.6% in patients with peak ALT levels 1.5-2xULN and >2-5XULN. Those with peak ALT>5 ULN as well as peak AFP levels >100 ng/ml had the highest chance of HBeAg seroconversion (57%) within three months of the exacerbation. Thus the traditional definition of a flare as used in early studies, including a significant elevation of ALT (>200-300IU/ml) appears to have prognostic significance for short term seroconversion. Yuen et al (139) also showed in a further study that patients with the highest elevation of ALT >6X ULN had a lower rate of complications than those with ALT 1-2xULN. The authors speculated that acute flares may result in acute injuries to the liver (as in acute Hepatitis B) which may resolve completely as is the case in acute Hepatitis A or B. These features may identify patients who may not require therapy since they have a high chance of spontaneous seroconversion following an acute exacerbation or flare. However severe acute exacerbation complicated by subacute hepatic failure carries an increased risk of cirrhosis (140). A flare of the immune clearance phase of CHB may also be accompanied by a marked reduction in HBV DNA but with HBeAg remaining positive. In other patients there may be several abortive attempts at seroconversion associated with flares, with loss of HBeAg but then seroreversion being seen, especially in patients who have not yet developed anti-HBe (141).

Other patterns of ALT abnormalities in the immune clearance phase.

"Uneventful" HBeAg seroconversion
Other patients in the immune clearance phase may not have documented episodes of flare (ALT >300 IU/ml) prior to HBeAg to anti-HBe seroconversion. In a group of HBeAg positive patients followed for 60 months, Liaw et al (142) found that while 65% (13/20) patients with spontaneous seroconversion had documented episodes of flare prior, the other 35% didn’t. A few years later however, the same group showed that in patients with apparently “uneventful” seroconversion, an acute exacerbation with high levels of HBV DNA was documented 6-27 months prior to the seroconversion (143). The authors showed that the HBV DNA leading up to the “uneventful” seroconversion was low but what is not clear is whether the ALT remained abnormal (suggesting ongoing residual hepatocyte damage) or whether this was also normal and for unclear reasons the seroconversion was delayed following a previous ALT flare. Certainly it seems that in some patients, a classical flare with very high ALT and seroconversion can be over in a few short weeks, whereas in others the process of viral control is slower.
Low level ALT elevation

Yuen et al (138), in 2003 provided data on the duration of acute exacerbations (defined by them as an increase in ALT to more than 1.5 times the ULN) and noted that they were shorter in HBeAg positive patients who did not have subsequent HBeAg seroconversion than in those that did (median of 17 weeks compared to 24 weeks). The definition of exacerbation used in this study however was quite different from the conventional understanding of the term flare and these data perhaps suggests recurrent episodes of, or indeed persistent low grade inflammation may also be seen in CHB. The deleterious effect of persistent low level inflammation in CHB was also shown by Yuen et al (139) who found that the cumulative risk of developing complications of cirrhosis (ascites, SBP, oesophageal varices, or encephalopathy) or HCC was highest in patients with baseline ALT levels of 1-2x ULN. When the patients were grouped into ALT categories according to the median of at least 3 ALT levels, once again those with ALT levels >1-2 x ULN had the highest risk for the development of complications. The risk of complications was in fact lower in patients with ALT levels >6x ULN at presentation than in those with normal baseline ALT. They also found that number of flare episodes, duration and peak ALT levels of acute exacerbations were not associated with an increased risk of complications. The median age at initial presentation of this cohort was 38 years (range 1-85) and 39% of patients were HBeAg positive, thus it is possible that the lower level inflammation noted in Yuen’s study may have been more representative of HBeAg negative chronic hepatitis B however they reported no significant difference in the cumulative risk for the development of complications between HBeAg positive and anti-HBe positive patients. A large prospective cohort study in Korea of 94,533 men and 47,522 women showed a positive association between the aminotransferase concentration and mortality from liver disease, even within the normal range (35-40 IU/l). The thresholds for increased liver related death began at an ALT >20 IU/ml in women and >30 IU/ml in men. The viral hepatitis status of the participants was not known, however, in a country of high HBV endemicity such as Korea, the prevalence of CHB in the cohort could be assumed to be significant (144). This again suggests that low level inflammation over time can contribute to risk of significant liver injury.

Thus liver injury during the immune clearance phase of chronic hepatitis B may come about by different mechanisms, episodic acute resolving inflammation or chronic persistent inflammation. While older guidelines for treatment (eg APASL and AASLD) suggest that HBeAg positive patients with minimally elevated ALT (<2x ULN) should not be treated but observed the 2012 EASL guidelines allow that treatment may be considered in patients with ALT above ULN if they have a liver biopsy showing at least moderate necroinflammation and/or fibrosis
Thus we may be moving more towards recognition of the need to prevent persistent low level inflammation. Thresholds for the upper limit of normal or ALT may need to be re-evaluated \((145)\), for example Shlomai et al showed that ALT values of greater than 10 IU/L in women and 22 IU/L in men may predict Diabetes Mellitus \((146)\). Other studies also suggest that upper limits of normal for ALT and AST should be decreased to 30 IU/ml for men and 19 IU/ml for women \((147)\).

**HBeAg to Anti-HBe Seroconversion**
Loss of HBeAg and the development of anti-HBe is the event known as HBeAg seroconversion \((86, 87)\). It is important to note that although it may have been conceptualised as such in the past, partly due to limitations of the assays used to detect HBeAg and antiHBe, HBeAg seroconversion is not an acute or sudden immunological event \((148)\) and that using sensitive immunoassays, anti-HBe may be detected even in the presence of excess antigen, a considerable time, even years before the loss of HBeAg \((149)\). HBeAg seroconversion and the period on either side of it represents a gradual process of viral diversification with immune pressure resulting in the selection of quasispecies that produce little or no HBeAg. These viral variants trade their persistence in the host for reduced replicative fitness \((148)\). In some patients sero-reversion to HBeAg positive disease may be seen such as in the study by Lok et al \((141)\) of 512 Chinese patients, followed over 3 years, in whom reversion from HBeAg negativity to HBeAg positivity was seen in 7.8% of patients who were HBeAg negative at presentation and 32.3% of HBeAg positive patients who cleared HBeAg over the course of followup. It is also worth noting that the significance of HBeAg seroconversion may vary. For example, Sanchez Tapias et al \((150)\) in their Spanish cohort for genotype A, D and F patients, demonstrated that although there was no significant difference in timing of HBeAg seroconversion, it was more commonly associated with sustained biochemical and virological remission with higher rates of subsequent HBsAg loss in genotype A compared to genotype D patients. Thus while in genotype A HBeAg seroconversion signals entry into the immune control phase of disease, in genotype D it may signal transition to precore mutant HBeAg negative CHB.

**Factors impacting on HBeAg seroconversion**
As described above the severity of flares in the immune clearance phase may predict the likelihood of HBeAg seroconversion, however numerous other factors, many of which are inter-related also have a bearing on the timing of HBeAg seroconversion.
Age

Age, which in many cases is a marker of duration of infection and immune activity, influences the likelihood of HBeAg seroconversion having taken place. In perinatally infected Asian patients there is a prolonged period of immunotolerance and low rate of clearance of HBeAg until later life. Fewer than 2% of patients per year clear HBeAg by age 3 and the rate is 4-5% per year in older children (151).

Loss of HBeAg increases with increasing age (152) and the prevalence of HBeAg positivity in 0-15 year olds is 90%, 60-70% in 15-19 year olds and 10% by age 40 (153). Data from Hong Kong in 2011 shows that in a cohort of 1400 Chinese patients, approximately 20% of 352 in the 36-45 year age bracket were HBeAg positive and 15% of 441 patients in the 46-55 year age bracket were still HBeAg positive (154).

In the Mediterranean area there are differences in the age of seroconversion reported from centres in Italy versus Greece. For example in a long term followup study of 70 Caucasian patients at Verona Italy, the mean age at seroconversion was 30 years (13-65 range) (155). However in Bortlotti’s study of 91 Italian HBeAg positive horizontally infected children, 95% were inactive carriers before reaching adulthood (156). Numerous Greek studies have shown that seroconversion has occurred in almost 80% of patients by the second decade of life (114). Whether these slight discrepancies in age of seroconversion from patients from similar geographic regions reflect differences in age at infection or possibly the effect of subgenotypes of genotype D is not clear (157).

Genotype

Many studies have demonstrated that Genotype C is associated with delayed seroconversion. In a retrospective study of 273 Chinese patients (122 with genotype B and 147 with genotype C) it was found that HBeAg seroconversion in genotype B patients occurred approximately 1 decade earlier than in genotype C (158). Multivariate analysis showed that high ALT (baseline and during followup), age > 30 years and genotype B were independent factors associated with spontaneous HBeAg seroconversion. Chu et al (159) performed a longitudinal study comparing the course of 202 patients (150 Genotype B and 52 Genotype C) who began followup in the immunotolerant phase. Although they found no difference in the hepatitis activity during the HBeAg positive phase between genotypes B and C, once again in this study genotype B was associated with significantly earlier and higher rates of HBeAg seroconversion. Baseline ALT in genotype C and age at entry into study in genotype B patients were correlated with seroconversion (159). Livingston’s study of an Alaskan native population with representation
from genotypes A,B,C,D and F also confirmed that Genotype C was associated with late seroconversion. They reported that the age at which 50% of patients cleared HBeAg was <20 years for genotypes A, B, D and F but 47.8 yrs for genotype C (160).

**Other factors**

Other possible factors predicting HBeAg seroconversion have included the results of a 2009 Korean study of 151 HBeAg positive Genotype C patients which found that seroconversion within 6 months of an acute exacerbation (ALT >5xULN) was predicted by non vertical transmission and low serum HBV DNA (<10^7 copies/ml). Some of this cohort received antiviral therapies (including lamivudine and IFN) however the early HBeAg seroconversion rate was no different between those patients who did not receive antiviral therapy at acute exacerbation (14/34; 41%) and those that did start antiviral therapy within 6 months of acute exacerbation (25/76; 33%) (P =0.518) (161).

Others have raised the potential impact of better nutrition and living conditions (less viral co-infection) on immune response including the authors of a Canadian study of 70 adopted, Asian born children who were recruited at average age of 2, who found that 75% had seroconverted over next 13 years (162). It is not stated what genotype of HBV these children had however and the lack of a non adopted control group also makes it difficult to be certain of the conclusions drawn by the authors.

**Factors related to HBeAg seroconversion – impact on clinical outcomes.**

Although it may have varying significance, HBeAg seroconversion remains for the most part a useful marker of change in disease activity. The timing of the HBeAg seroconversion event has been shown in certain studies to have clinical implications. Chu et al (125) showed that the rate of development of cirrhosis in a group of 240 HBeAg patients followed up from the immunotolerant phase (mean age at study entry 27.6 years) through to Anti-HBe seroconversion was 5%. The annual incidence of cirrhosis was 0.5% and the factors predictive of the development of cirrhosis were age at anti-HBe seroconversion and relapse of hepatitis which was seen in 15% of patients (annual rate of 2.2%) following HBeAg to anti-HBe seroconversion.

Chen and Liaw et al (163) followed 483 patients from the time of confirmed anti-HBe seroconversion and found that their prognosis was age specific. Over a mean period of followup of 11.7 yrs, HBeAg negative hepatitis developed in 34%, cirrhosis in 10% and HCC in 2.5%. However the rates of HBeAg negative hepatitis and cirrhosis were significantly higher (at
67% and 43% respectively) in patients whose seroconversion occurred after age 40. The lowest risk was in patients who seroconverted before the age of 30. Following seroconversion, the duration of remission, prior to relapse with HBeAg negative chronic hepatitis B, was significantly shorter in those whose seroconversion occurred after the age of 40 compared to those with seroconversion before age 30 yrs (6.2 yrs v/s 9.5 yrs p=0.004).

**Immune control phase (Inactive HBsAg carrier state)**
The immune control phase is entered following immune clearance and is characterised by loss of HBeAg, development of anti-HBe, normalization of ALT and reduction of HBV DNA levels to undetectable or very low levels. Histological regression of inflammation and fibrosis is also usually seen and together these events signify clinical remission of CHB. This phase of disease has had varying names attached to it as well as definitions including Inactive HBsAg carrier state (EASL/AASLD) (101, 102), inactive chronic HBV infection or clinical remission (APASL 2008) (108) (Table 1.2). In this thesis the terms “immune control phase” and “inactive HBsAg carrier” or “inactive carrier” state are used interchangeably.

**ALT levels during the immune control phase**
Persistently normal ALT, signifying lack of inflammatory activity remains the mainstay of defining the immune control phase. Distinguishing between inactive carriers and those with chronic HBeAg negative hepatitis B can at times prove challenging since ALT levels may be intermittently normal in the latter. Rather than evaluation at a single time point alone, it is important that repeat testing should show that ALT is persistently in the normal range prior to classifying a patient into this phase of disease. Tai et al (164) suggest that at least 3 years of followup are needed to definitively decide that a patient has persistently normal ALT. In their large study of 4376 HBeAg negative patients followed for a mean period of 13.4 (+/- 5.2) years, they showed that in 3673 patients who began followup with a normal baseline ALT, 46.7% subsequently developed abnormal ALT. However for those who maintained persistently normal ALT (ULN < 36 U/L and measured 6 monthly), there was a lower risk of cirrhosis, HCC and mortality compared to those whose maximal ALT over followup was at least 2 x ULN (164). Similarly in a systematic review of 6 studies of HBeAg negative CHB patients (335 patients) with persistently normal ALT only a small proportion of patients had liver biopsy finding of significant inflammatory activity (10%) or fibrosis (8%) (165). It should be noted however that these 6 studies were done in quite diverse ethnic populations (2 Caucasian/Mediterranean cohorts, 1 Japanese, 1 Indian, and the other 2 mixed cohorts with overall a low number of Asian patients). The authors provide a useful algorithm for optimal followup of patients with
HBeAg negative CHB with normal ALT and suggest that it should be with ALT every 3-4 months and once again over a 3 year period and 6 monthly thereafter. Thresholds for normal values of ALT are likely important in the immune control phase as in the immune tolerant phase, for example Lin et al (166) followed 414 HBeAg negative patients with persistently normal ALT (defined as <40 IU/ml for men and <30 IU/ml for women) over 2 years and found that 65% had an HBV DNA level greater than $10^4$ copies/ml (approximately 1785 IU/ml) but in particular those with “high-normal” ALT (0.5 -1 X ULN) had a greater proportion of patients with HBV DNA > $10^4$ copies/ml and higher prevalence of BCP mutation. Zacharakis et al (167) also reported that in their group of 195 inactive carriers, patients with undetectable HBV DNA were more likely to have low normal ALT levels (86%) while those with detectable HBV DNA were more likely to have high normal ALT levels (67%) (p<0.005). There are other data from mixed (HBeAg positive and HBeAg negative) groups supporting the finding that “high normal” levels of ALT (0.5-1 x ULN and 1-2 x ULN) as opposed to low normal (<0.5 x ULN) has an increased risk of complications including the previously discussed study by Yuen et al (139). Lai and colleagues (107) also found in a group of 59 patients with normal ALT (approximately 45% HBeAg negative) that 46% of patients with high normal ALT had either significant inflammation or fibrosis compared to 20% of patients with low normal ALT. In contrast Tai et al (164) found that there was no significant difference in outcomes between HBeAg negative patients with persistently normal ALT in the low-normal or high-normal range. Thus as in HBeAg positive CHB, re-evaluation of normal range for ALT also has relevance to the HBeAg negative immune control phase of CHB.

HBV DNA levels during the immune control phase

The level of HBV DNA used to define the inactive carrier state has varied over time. Initially a level of 100,000 copies/ml (approximately 17857 IU/ml) (95) was suggested although subsequent data suggested it should be revised down to 30,000 copies/ml (approximately 5357 IU/ml) (168). The findings of the “Risk Evaluation of Viral Load Elevation and Associated Liver Disease/Cancer-in HBV” (REVEAL-HBV) studies (169) that the risk of cirrhosis and HCC increased with increasing HBV DNA levels, beginning at the level of >$10^4$ copies/ml (1785 IU/ml) led to a focus on the threshold of 2000 IU/ml as part of the definition for the inactive carrier state. However, the practicability of using this low threshold has been questioned. For instance, Chen et al (170) studied 62 patients with persistently normal ALT (<36 U/L) over a followup period of at least 10 years and found that serum HBV DNA was always <$10^5$ copies/ml (17857 IU/ml) in 44 (71%) of patients but always < $10^4$ copies/ml (1785 IU/ml) in only 13 (21%) of patients. In the small subset of 9 patients whom they biopsied, most had minimal fibrosis,
however the authors had excluded any patient with evidence of cirrhosis or HCC till last followup from their cohort. This unfortunately weakens their conclusions that a cut off level of $10^5$ copies/ml (17857 IU/ml) can “safely define” the inactive carrier state. Feld et al (171) however also reported that while only 1 patient with normal ALT and HBV DNA $>10^4$ copies/ml (1785 IU/ml) developed ALT elevation within the next year, 67% of patients with HBV DNA $>10^5$ copies/ml (17857 IU/ml) did. They concluded that patients with HBV DNA levels between $10^4$ and $10^5$ copies/ml could be safely followed at 6 monthly intervals, while HBV DNA levels $>10^5$ copies/ml were highly predictive of future ALT elevation. Paptheodoridis et al (172) studied 85 patients in the inactive carrier state, defined as persistently normal ALT over 12 months and baseline HBV DNA $<20,000$ IU/ml and reported that HBV DNA levels were $\geq 80$ IU/ml and $\geq 2000$ IU/ml in 81% and 23% of patients. Zacharakis et al (167) followed 195 inactive carriers (HBeAg negative and persistently normal ALT for >12 months) and found that 97 (50%) had undetectable HBV DNA while the other 98 (50%) had positive HBV DNA, although only 4 of these were in the range $>2000$ IU/ml. In this cohort the mode of transmission was interfamilial in 49.5%, transfusion in 12%, percutaneous exposure in 3% and unknown (presumed sexual) in 35.5%. Thus the comparability of this cohort to Asian cohorts of perinatally transmitted disease is not clear. These studies however do demonstrate that fluctuations in HBV DNA occur underlining the fact that in CHB, immune control of the virus is in a dynamic state of play.

**Fibrosis on liver biopsy in immune control state**

In a smaller group of HBeAg negative inactive carriers from Greece (defined as persistently normal ALT over at least 1 year and HBV DNA 2000-20,000 IU/ml), liver biopsies showed minimal or absent fibrosis in 83% (29/35) and moderate fibrosis (Ishak stage 2-3) in 17%. No patient had cirrhosis (173). Similarly Zacharakis showed that 90/95 (95%) of biopsied inactive carriers who had detectable HBV DNA showed no progression in liver histology over a 4 year period (167).

**Risk of relapse from immune control phase**

Hsu et al showed that after HBeAg seroconversion in 283 patients, 189 (66.8%) showed sustained remission over a median followup period of 8.6 years, while the other 94 (33.2%) experienced ALT elevation $>2x$ ULN, 12 (4.2%) with HBeAg sero-reversion. The HBV DNA assay used was the Digene Hybridization assay with a lower limit of detection of 0.5 picograms/ml (approximately 25,000 IU/ml) and 14 of the 94 (4.9%) were not HBV DNA positive and the cause of the hepatitis was considered undetermined however it seems likely that reactivation
of HBeAg negative CHB with levels of HBV DNA below the limit of detection of the Digene assay were the likely cause (174). The same group reported on the ongoing followup of their patients 9 years later, in 2010 and found a similar rate of HBeAg negative hepatitis occurring in 165/483 (34.2%) patients over a median followup of 11.7 years and at a mean age of 39 years (163). The definition for HBeAg negative CHB they used was ALT elevation >2 x ULN and serum HBV DNA >2 x 10^4 IU/ml. The 15 year cumulative incidence of HBeAg negative hepatitis was 38.6% overall, but was 66% in those who underwent HBeAg seroconversion after age 40, compared to 31% and 37% in those who seroconverted before the age of 30 and in the age group 31-40, respectively (163). The relapse rate with HBeAg negative CHB reported by the same Taiwanese group in 2007, of 1241 “asymptomatic HBsAg carriers” was 19.3% at 15 years and was 26.9% for males and only 12.5% for females at 20 years (175). HBV DNA was measured using hybridization assay but ALT >2x ULN was again used for definition of chronic hepatitis B. In this study Chu also reported that the proportional increase in risk of relapse was lower with longer followup, eg the cumulative incidence of relapse was 10.2% at 5 years, 17.4% at 10 years,19.3% after 15 years, and 20.2% at 20 years (representing only a 1.9% and 0.9% increase respectively for the 5 year blocks during the second decade of followup.) (175). It was reported to be negligible after 20 years of follow up. The discrepancy in rates of CHB relapse between the 2 aforementioned studies could be related to the fact that followup in Chen’s study (163) began at time of documented seroconversion while in Chu’s (175) it began during the inactive carrier (immune control) phase which may have been at a point already some time distant from seroconversion, thus with better immune control established which is more likely to be durable. In keeping with this Chu and Liaw et al (176) also reported on a cohort of 133 patients followed from the HBeAg positive phase in whom the annual reactivation rate following HBeAg seroconversion was 3.3%. Factors that predicted reactivation following seroconversion in this cohort included genotype C, male sex and also seroconversion after the age of 40 years and a more vigorous immune response during the HBeAg positive immune clearance phase (as measured by peak ALT of >5 x ULN and possibly reflecting a more difficult to clear HBV) (176). In an Indian study of 217 HBeAg negative patients followed for 69 months, the annual rate of relapse with spontaneous increase in ALT to > 2 x ULN was 4.3% (177). In contrast the relapse rate in Zacharakis’ cohort was lower with only 4 of 195 (2.6%) showing reactivation with HBV DNA >2000 IU/ml over followup (167). Recent studies have suggested that using a combination of quantitative HBsAg levels and HBV DNA levels can help predict which HBeAg negative patients are likely to develop reactivation and this is explored further in the section on quantitative HBsAg (178, 179).
**Chronic HBeAg negative CHB**

Chronic HBeAg negative hepatitis B was originally described in patients from around the Mediterranean basin (91). It is now known to be globally prevalent (180) and in fact is the more common form of chronic hepatitis B (181, 182). This is most likely because of the ageing of the overall cohort of CHB patients. The molecular basis for HBeAg negative CHB are strains of HBV that produce HBeAg in reduced quantities or not at all due to mutations in the basal core promoter (BCP) or precore region. Chronic HBeAg negative hepatitis B may develop directly from HBeAg positive chronic hepatitis B or as is more usual, as a reactivation following a period of immune control at annual rates alluded to above. There is a male preponderance with the reported ratio of males to females ranging from 4.6 to 17 (183). Patients with HBeAg negative CHB have been shown to be significantly older than those with HBeAg positive disease in the same region, ranging from 40-55 years in most studies (182, 183). This most likely reflects the fact that reactivation with precore/BCP mutant strains usually takes place after a period of some years of relative immune control. These mutations will be discussed in more detail subsequently. The development of the precore mutation depends on the genotype and therefore the prevalence of HBeAg negative CHB varies geographically (183). In particular the precore mutation rarely forms in genotype A (184) but may develop in particular in genotypes D and B.

**ALT abnormalities in HBeAg negative CHB**

2 main patterns of disease activity are described in HBeAg negative CHB. Firstly, in 30-40% of cases there is a pattern of persistently elevated ALT with an average increase to 3-4 times the ULN (185). A second pattern is that of intermittent elevation of ALT or so called flares in 45-65% of cases (183) (186) which may have normal ALT in between. Flares may be severe with ALT levels over 1000 U/L and the development of IgM anti-HBc (183). The HBV DNA level usually increases significantly prior to flares (187) and may start to decrease before the ALT spikes (183). In severe exacerbations of liver disease serum HBV DNA and/or liver HBcAg may be undetectable therefore serial monitoring is required to identify virus (183). In other situations there may be uncertainty about whether an ALT rise represents acute infection or a flare in a chronically infected patient. Detection of precore and BCP mutations can help distinguish acute from chronic flares as these mutants are more commonly found in patients with acute exacerbation of CHB than in those with acute infection (81% vs 19% respectively for basal core promoter and 58% vs 6% for precore mutants) (188). It is also important to ensure that temporary quiescence between flares in chronic HBeAg negative hepatitis B is not mistaken for the inactive carrier state. HBV DNA levels can usually give a clue to this, as higher
HBV DNA levels (> $10^5$ copies/ml or 17857 IU/ml) predict for ALT elevation in the subsequent year as previously discussed (171). ALT normalization was seen in 40-50% of HBeAg negative patients who had HBV DNA levels ranging from >$10^4$ to > $2 \times 10^5$ copies/ml (approximately 1785 – 35714 IU/ml) and factors predictive of this were older age and previous ALT normalization (171). However, although intervening periods of inactivity may be prolonged, sustained spontaneous remission of HBeAg negative chronic Hepatitis B has only been reported in 6-15% of patients (189).

**HBV DNA levels in HBeAg negative chronic hepatitis B**

HBeAg negative CHB is associated with lower levels of HBV DNA than in HBeAg positive disease and but there is evidence of slow increase in HBV DNA levels with increasing age. For instance, Fung et al (154) showed that in HBeAg negative patients aged ≤ 25, 26-35, 36-45,46-55 and >55 years the HBV DNA levels were 3.7, 4.4, 4.7, 4.9 and 5.2 log copies/ml. Fluctuations of HBV DNA in the HBeAg negative phase have been observed although not described in great detail (185).

**Histological findings in HBeAg negative chronic hepatitis B**

HBeAg negative chronic hepatitis B is frequently associated with significant liver disease. In an early French study significant rates of cirrhosis were reported in HBeAg negative patients and this was thought to relate in part to longer duration of disease (190). Similarly in an Italian followup study (median 6 years), 63 untreated anti-HBe positive patients had followup liver biopsy and 38% showed no change, 12.7% showed higher degree of necroinflammation and 49.2% showed progression to cirrhosis. The progression rate was particularly high in patients older than 36 years (185). In an Asian cohort followed from the time of HBeAg seroconversion, 21 of 269 (7.8%) patients without evidence of cirrhosis at the time of seroconversion, developed cirrhosis with the risk being significantly higher in those that developed chronic HBeAg negative hepatitis (174).

**Resolved Hepatitis B**

The rates of HBsAg clearance were found to be 19/984 (1.9%) in patients with chronic hepatitis B (HBeAg positive and negative) and 35/1598 (2.2%) in chronic HBV “carriers” with normal ALT (Immune control phase) followed up for 4 +/-2.3 yrs and 2.7 +/-1.4 yrs respectively (191). This translates to an annual rate of 0.5% in CHB patients and 0.8% in inactive HBsAg carriers. HBsAg clearance in this study always occurred after HBeAg seroconversion and the rate of HBsAg loss described is slightly higher than in a previous small Asian study on HBsAg carriers.
clearance in patients followed for 6 months in which only 1 of 323 chronic carriers cleared HBsAg (192). However both rates are lower than in older Caucasian group studies (155).
Figure 1-4: Natural History and Phases of Chronic Hepatitis B

Figure Legend: This figure represents the phases in the natural history of chronic hepatitis B with the red line representing HBV DNA and the black line representing ALT (adapted from Sherlock's Diseases of the liver and biliary system 12th Ed Edited by James Dooley, Anna Lok, Andrew Burroughs and Jenny Heathcote, Chapter18 Page 378 Wiley Blackwell press). (43).
The natural history of acute, adult acquired hepatitis B

Acute hepatitis B infection in adults is often acquired sexually or parenterally (especially through injecting drug use). The incubation period is 4-16 weeks and there may be a prodromal period of malaise, anorexia, right upper quadrant discomfort and nausea. Jaundice is seen in only approximately 30% of cases. The symptoms may take 1-3 months to resolve and some patients report fatigue for sometime thereafter (43). ALT/AST levels may increase to 1000-2000 U/L and also may take up to 4 months to completely normalise. HBV DNA is detectable first and levels are high early on, then wane (43). HBsAg may be detectable for weeks before the onset of any symptoms from around 1 week post infection. HBeAg also appears early usually around 4-10 weeks. Anti-HBe develops after about 4 months and HBsAb after about 6-7 months typically. Anti HBc IgM appears shortly before the onset of clinical illness and as it declines anti-HBc IgG appears. Fulminant hepatitis is seen in <1% and because viral clearance is especially brisk in this setting, HBV DNA may be negative. Thus anti-HBc IgM may be the only serological marker of Hepatitis B infection in the window period between loss of HBsAg and development of HBsAb (193). Although it has been held that the rate of chronicity from adult acquired hepatitis B is rare, the actual rates reported are variable. A recent study of 215 patients from Japan, (mean age 31.8 years) with acute hepatitis B, none of whom received antiviral therapy found that 21 (9.8%) developed chronic infection defined as persistent HBsAg for >6 months. However all but 6 (2.8%) had cleared HBsAg by 12 months and 5 of the 6 were genotype A. The rates of chronicity at 6 months were 12.4% in genotype A, 3.8% in genotype B and 8.2% in genotype C.. The authors propose that criteria for chronic HBV infection be changed to HBsAg persistence at 12 months rather than 6 months. They report that higher HBV DNA levels and HBsAg levels early in the course of infection correlated with likelihood of chronicity (194).In general, management of acute hepatitis B need only be supportive as the majority of patients resolve the infection and develop long lasting immunity. A 2007 randomized controlled trial from New Delhi of treatment of acute hepatitis B, (including 60-72% patients with severe acute hepatitis B), failed to show any benefit from Lamivudine treatment in terms of improvement of INR/bilirubin and ALT. Although in Lamivudine treated patients there was a greater initial (week 4) reduction in viral load there was no difference in viral loads subsequently (195). Others however have shown that early treatment with Lamivudine has been shown to result in improvement in mortality although with a lower HBsAg seroconversion rate(196) A study from Japan in 2014 however suggested that early initiation of NA treatment (within 8 weeks of onset of acute hepatitis B) shortened the duration of HBsAg positivity and HBV genotype A patients in this group never progressed to
chronicity (197). Thus early treatment with NAs in acute hepatitis B may have in a role in preventing progression to chronicity as well as in the management of severe cases, (with elevated bilirubin or prolonged prothrombin time), when oral antivirals, may have been advisable to prevent fulminant liver failure (198).
The immunopathogenesis of hepatitis B infection

Immunopathogenesis of acute HBV infection

Acute Hepatitis B infection in immunocompetent adults is contained and cleared by a series of complex, inter-weaving processes of both the innate and adaptive immune systems. The lack, until recently, of a good small animal model for HBV infection and a useful cell line that supports HBV growth and replication has made study of its immunopathogenesis difficult. Nevertheless, numerous important observations have been made that show the complex interplay between the lines of defence in the human immune system. Early in acute hepatitis B, infected hepatocytes produce anti-viral cytokines including interferon gamma (IFN – γ), tumour necrosis factor (TNF-α) and interferon alpha/beta (α/β). Clearance of virus is through non cytolytic mechanisms of the innate immune system as evidenced in a chimpanzee model of acute hepatitis B in which the HBV was largely undetectable in the serum and liver well before the peak of the T cell infiltration and the onset of the hepatitis (199). Further events in part of a cascade are then triggered, eg Interferon α/β stimulates the production of Interleukin-8 (IL-8) and Interleukin 12 (IL-12) by antigen presenting cells, which in turn induce natural killer and natural killer T cells which can be cytotoxic to infected hepatocytes (200, 201). The emergence of a strong adaptive immune response with cytotoxic T cells (CD4+ and CD8+ T cells), which are directed at multiple viral epitopes (the viral envelope, nucleocapsid and polymerase proteins) and which are long lasting eventually results in long term control of HBV infection (202). The humoral immune response to HBV proteins with the production of neutralizing Antibodies by activated B lymphocytes is also important in both resolution of the acute infection and in the maintenance of immunity to further infection. The clinical importance of B lymphocytes and anti-HBe production in the control of hepatitis B infection has been highlighted by reactivation of apparently resolved HBV infection in patients treated with Rituximab (a mono-clonal antibody therapy for haematological malignancies which causes profound depletion of circulating B cells) (135). It is now thought that a negative feedback loop in which traces of virus may maintain the HBV specific cytotoxic T lymphocyte response, which in turn keeps the virus in check, operates perhaps for life after acute infection with HBV (203). It is of interest to note that histological evidence of inflammation and fibrosis may be noted up to a decade after apparent clearance of HBsAg from the serum in patients with acute hepatitis B (204). The clinical syndrome of acute hepatitis B can range from asymptomatic to symptomatic with jaundice and in a small proportion (up to 1%) fulminant hepatitis resulting in transplantation or death (205). By and large however the adverse outcomes of infection with hepatitis B are related to the establishment of a chronic infection.
Immunopathogenesis of chronic hepatitis B

**Immunopathogenesis - Immune tolerance**

As alluded to in the section on transmission, the age at infection and the mode of transmission determine the progression to chronicity. It is held that the presence of wildtype HBV, i.e., a strain producing HBeAg, is the *sine qua non* for the transition of an acute infection to a chronic one (104, 189). HBeAg is a non-structural protein and is not known to have a function in viral replication or assembly. Key experimental work performed by Milich et al (206) in 1990 found that HBeAg expressing transgenic mice were tolerant to both HBeAg and the nonsecreted nucleocapsid (Hepatitis B core antigen/HBcAg) at the T cell level. Since HBeAg and HBcAg were highly cross-reactive at the T cell level, deletion of the helper T cell response to HBeAg resulted in an ineffective cytotoxic T lymphocyte response to HBcAg and therefore ineffective lysis of infected hepatocytes. They also showed that maintenance of T cell tolerance to HBcAg/HBeAg required continued secretion of HBeAg and in its absence persisted for less than 16 weeks. This led them to postulate that the function of HBeAg may be to act as a “tolerogen” and since it could cross the placenta it could contribute to the viral persistence following perinatal infection (206, 207). Hsu et al found that the peripheral or cord blood mononuclear cells of neonates born to HBeAg positive mothers were unresponsive when exposed to HBcAg in vitro. They postulated, in accordance with Milich’s findings that in utero exposure to HBeAg resulted in immunological tolerance to HBeAg/HBcAg epitopes at the T Cell level in neonates (208). In this study it must be noted that the T cells of neonates born to HBeAg negative mothers also did not react to HBcAg although an infant who contracted HBV at 2.5 months which was presumed to be from its HBeAg negative mother showed a significant proliferative response to HBcAg. Transmission rates of 32% in infants born to HBeAg negative mothers are reported (compared to 85-90% in infants born to HBeAg positive mothers) (209). In a 2011 cohort of 2356 Taiwanese children born to HBsAg positive mothers, the prevalence of HBsAg and HBcAb in neonates born to HBeAg positive mothers was 9.26% and 16.76% respectively which was significantly higher than the rates in infants born to HBeAg negative mothers (0.29% and 1.57%). All had received active HBV vaccination at birth and most also had HBIG. Among the HBV infected children the rates of progression to chronicity were 17% in children born to HBeAg negative mothers (compared to 54% in those born to HBeAg positive mothers) (210). Hadziyannis in a review reasons that although HBeAg may be not measurable in anti-HBe positive patients, mixtures of wildtype virus often co-circulate with pre-core mutant virus, thus HBeAg may still be present and cross the placenta and induce the tolerance required for chronicity in infants contracting HBV from their HBeAg negative mother (104). As well as viral
factors, the other pre-requisite for chronicity is that the host must be able to be “tolerised” and in simple terms it seems attractive to deduce that the immunological immaturity of neonates predisposes them particularly to chronic infection, whilst older children and adults are much less susceptible. This can certainly explain the inverse correlation between chronicity and age at infection. Some studies of adult acquired hepatitis B in fact suggest that after exclusion of exacerbations of unrecognized pre-existing chronic hepatitis B, the risk of progression to chronicity in adults is <1%. However others put the risk at about 10% and suggest that this may be because of a reduced capacity to produce alpha- and gamma-interferon (211).

It is likely that the progression to chronic infection is controlled by more than just one factor and that the highly complex mechanisms involved are yet to be elucidated. Other recent clues include identification of a subset of “regulatory” T lymphocytes which are CD4+ CD25+. It has been speculated that their role may be to inhibit HBV specific CD8+ T cells which could limit the cytotoxic hepatocyte injury caused by this immune response but at the cost of reduced viral clearance (212). There is also some evidence that the immune responses elicited by different HBV genotypes may vary, for example genotype B has been shown to induce a stronger T Helper 1 and weaker T Helper 2 response than genotype C (213). It has been suggested that increased immunogenicity and susceptibility to host immune response of genotype B may account in part for earlier seroconversion seen with this genotype compared to genotype C (214). A role for toll-like receptor 2 in chronic HBV infection has recently been demonstrated by a Melbourne collaboration (215). They showed that the HBeAg suppresses the TLR pathway of the innate immune system thereby assisting the virus in establishment of chronic infection. Other investigators have suggested that some of the inherent immunological features of the liver eg the low level of expression of Major Histocompatibility Complex (MHC) class 1 molecules on the surface of hepatocytes in contrast to that seen on other antigen presenting cells may help HBV evade detection especially when it is replicating at low levels (212). Others have also suggested that the ability of MHC 1 to present viral epitopes is inhibited by the fact that some HBV surface proteins which are formed within the endoplasmonic reticulum fail to be degraded enough to be presented to cytotoxic T lymphocytes and induce the necessary response (216). The influence of different Human Leukocyte antigen (HLA) alleles and polymorphisms on the clinical outcome of HBV has also begun to be investigated (217) and different HLA haplotypes which are protective or increase
risk of various outcomes of HBV infection including chronicity have been identified in Asian populations (218).

**Immunopathogenesis - Immune clearance**
The factors which lead to the loss of immunological tolerance and the onset of immune clearance are also incompletely understood. HBeAg negative HBV begins to accumulate during immune clearance. It is possible that emergence of precore mutant (HBeAg negative) HBV can lead to a reduced level of HBeAg and thus trigger the onset of loss of tolerance (219). The immune mechanisms of the classical flares of the immune clearance phase, with the rise in HBV DNA followed by abrupt increase in ALT, are thought to be the result of cytotoxic T lymphocyte mediated immune response to HBV antigens. Hepatocytes show strong expression of class 1 HLA and intercellular adhesion molecule type 1 (ICAM-1) on their surface and HBcAg and HBeAg are shifted to the cytoplasm from their usual place in the nucleus (220). The rise in HBV DNA appears to trigger the ALT flare through increased T cell responses to HBcAg and HBeAg (221). A role for IFN-gamma inducible chemokines CXCL-9 and CXCL-10 has also recently been suggested in the pathogenesis of flares with differential expression in acute versus chronic hepatitis B patients (222).

**Immunopathogenesis - HBeAg negative CHB**
The immune pathogenesis of liver injury in HBeAg negative CHB are thought to be similar to those operating in HBeAg positive immune active disease. It is thought that host immune pressure selects out certain HBV sequences over time and that precore and BCP mutant HBV emerges as a result of selection pressure on wildtype virus. There are many postulates as to why virus defective in the production of HBeAg may have a survival advantage which were summarised by a review by Hadziyannis in 2001(189). The HBeAg is potentially a target for humoral-mediated immune responses (antibodies to Hepatitis B e epitopes are present in some cases years before HBeAg loss) (149). In addition, it has been shown that cells expressing both HBcAg and HBeAg may elicit a stronger CD4(+) T cell response than those expressing HBcAg alone (ie those infected with mutant virus) (223). The same may be the case for cytotoxic T lymphocytes (CTL) although the CTL epitopes of the precore/core (HBe) and core (HBC) proteins are shared, thus this may only be of relevance in situations where levels of viral replication are low (224). Ongoing viral adaptation due to continued host immune pressure, particularly in HBeAg negative disease has been shown by looking at the relationship between HBV sequence variation and HLA type (225). A putative replicative advantage for HBeAg negative HBV has also been suggested since nucleotide 1896 is important in the formation of a
stem-loop structure (epsilon) which is required for the encapsidation of pregenomic RNA into the nucleocapsid in order for HBV to complete its life cycle. The change of the nucleotide at position 1896 from G to A leads to the development of both a stop codon and stabilisation of the Epsilon signal (226). The factors that precede reactivation of HBeAg negative hepatitis B from the inactive carrier state are still however largely unclear.

**Clinical manifestations of Chronic Hepatitis B**
Chronic hepatitis B is generally clinically silent with no symptoms discernible until the development of complications of cirrhosis or HCC. Some patients may occasionally complain of right upper quadrant discomfort or fatigue. During the course of chronic infection severe flares may be accompanied by jaundice with some of the symptomatology of acute hepatitis including nausea, anorexia, fatigue and occasionally hepatic decompensation with ascites or encephalopathy (210).

**Cirrhosis: Incidence and sequelae (decompensation and liver related death).**
The development of cirrhosis is one of the most significant sequelae of CHB because of its attendant risks of liver decompensation with ascites, encephalopathy, or variceal bleeding. The reported risk of the development of cirrhosis in CHB has been quite variable. The REVEAL-HBV study was a population based study of 3582 untreated HBsAg positive Taiwanese villagers aged 30-65 with a mean followup period of 11 years. Those with clinical or ultrasound diagnosed cirrhosis at the outset were excluded and a total of 365 patients (10%) were diagnosed with cirrhosis on ultrasound, during followup. The study population was predominantly male (61%), 84% were HBeAg negative at baseline and 81% overall were both HBeAg negative and had an ALT<45 at baseline thus the results largely represent the risk of cirrhosis as a result of HBeAg negative CHB (169). The incidence of cirrhosis increased with increasing baseline HBV DNA levels, rising from 34/869 (3.9%) in those with undetectable HBV DNA, to 4.9% in those with HBV DNA < 10⁴ copies per ml (approx. 1750 IU/ml), to 8.8% in those with HBV DNA < 10⁵ copies/ml l (17,678 IU/ml) to 19.5% in those with HBV DNA < 10⁶ copies/ml (176,785 IU/ml) and 25.5% in those with HBV DNA ≥ 1,000,000 copies/ml (> 178,571 IU/ml).

Other cohorts in developed countries eg French Canada and Austria have reported lower rates of liver related morbidity and a long term (mean 30 yrs) followup study of 296 HBsAg positive patients in northern Italy reported the rates of liver related death were not different to that of a control group (227). “Unequivocal” liver disease was found in only 4 carriers and 32% cleared HBsAg over followup. However a recent study, also from Italy reports the development of cirrhosis in 32/105 (30.4%) HBsAg positive blood donors followed for approximately 30 years.
with a cumulative probability of 21% and 33% at 10 and 25 years respectively (228). The mean age at study entry in this cohort was 30 years. Cirrhosis was defined either by ultrasound or histologically. Cirrhosis developed in 10% of inactive carriers compared to 52% of patients with chronic active hepatitis (HBeAg positive or HBeAg negative and HBV DNA positive). Liver related death occurred in 7/105 patients (6.6%), 6 due to HCC and 1 due to liver failure. All but 1 of these patients had cirrhosis. The 25 year cumulative incidence of liver related mortality was 20% in cirrhotic patients compared to 2% in non-cirrhotics.

Fattovich et al have previously calculated summary measures of the incidence rates of cirrhosis, HCC and hepatic decompensation from pooled results of Asian and European studies in their 2008 paper (229). They report 5 yr cumulative incidence of cirrhosis of 8% and 17% in HBeAg positive patients from East Asian and European countries respectively and 13% and 38% in HBeAg negative CHB from East Asia and Europe respectively. They reason that inclusion of immunotolerant patients in the Asian cohorts account for the lower rates of cirrhosis in HBeAg positive patients and inclusion of patients with more advanced liver disease (possibly due to the nature of clinic based cohorts in Europe) account for the discrepancy in HBeAg negative patients.

**Risk of decompensated cirrhosis.**

Liaw estimated the rate of hepatic decompensation and variceal bleeding to be approximately 2.3% per year in an Asian cohort of untreated hepatitis B cirrhosis in the late 1980’s (230). The same group also reported in 2007 of an annual risk of decompensation of 1.5% in their cirrhotic cohort (231). The 5 year survival rate of compensated cirrhosis was estimated at approximately 80%. In a European cohort of untreated HBsAg positive cirrhotics, the 5 year risk of decompensation unrelated to HCC reported by Fattovich in 1995 was 23% (232) and in 2002 was 16% (compared to 28% in HCV cirrhotics) (233). Mortality following an episode of decompensation was high, being estimated at 65% at 5 years. In 2008, Fattovich reported summary incidence of liver decompensation in cirrhotic patients from 4 studies to be 15% at 5 years. The mortality rate following decompensation was 70-85% at 5 years (229), being lower in those presenting with ascites alone than in those with more than one symptom of liver decompensation (233). Treatment with oral NA’s has been shown to significantly delay disease progression in cirrhotic patients and is therefore advocated in viraemic CHB patients (234).

**Hepatocellular carcinoma**

The risk of HCC in untreated HBsAg positive individuals was also examined in the REVEAL-HBV study cohort which found 164 patients (4.4%) developed HCC over a mean followup period of
As was the case with the outcome of cirrhosis, the cumulative incidence of HCC at the end of the 13th year of followup, increased in strata of increasing baseline HBV DNA levels. Approximately 15% of patients with HBV DNA >1 million copies/ml (>1.78x10^5 IU/ml) at study entry developed HCC by the end of followup, as did 12.7% of those with HBV DNA 10^5 to 10^6 copies/ml (1.78x10^4 to 1.78x10^5 IU/ml) but only 1.3% of those with undetectable HBV DNA. HBeAg positivity, reflecting HBV replication, was also been shown to be a risk factor for the development of HCC in Yang’s study (a forerunner of the REVEAL-HBV study) with a relative risk of 60.2 in those CHB patients (aged 30-65 years) who were HBeAg positive compared to 9.6 in those who were HBeAg negative (236).

Fattovich et al (229) also studied HCC incidence rates reported in 42 independent cohort studies and found geographic variation and as would be expected and higher incidences among those with more advanced liver disease. The summary 5 year cumulative incidence rates of HCC in patients within the following groups 1) inactive carrier, 2) CHB without cirrhosis and 3) compensated CHB cirrhosis were 1%, 3% and 17% respectively in East Asian studies and were lower at 0.1%, 1% and 10% in Europe and the US. It was thought that perhaps the longer duration of infection may account for the higher rates of HCC seen in Asian patients. In the most recent European followup study by Fattovich (228), HCC developed in 8 of 105 (7.6%) Italian patients (7 of 8 were cirrhotic) with a cumulative incidence of 1% and 9% at 10 and 25 years respectively in the whole cohort but 18% and 38% at these time points in cirrhotic patients. Cirrhosis thus increased the risk of HCC by 20 fold.

**Extrahepatic manifestations of hepatitis B**

Extra hepatic manifestations of acute and chronic HBV include arthritis, dermatitis, polyarteritis nodosa and glomerulonephritis. These syndromes are thought to be mediated by circulating immune complexes containing viral proteins (193, 237). A serum sickness type arthritis/dermatitis with fever, arthralgias and rash may occur in the prodromal phase of acute hepatitis B (43). Erythematous, maculopapular skin lesions occur on the face and extremities (papular acrodermatitis) and the syndrome is also known as Gianotti Crosti syndrome and is a distinct manifestation of acute hepatitis B of childhood (238). The lesions may last about 15-20 days and are thought to be due to activation of the complement system by HBsAg-HBsAb complexes.

**Polyarteritis Nodosa.**

Polyarteritis Nodosa (PAN) is a relatively rare complication of HBV occurring in less than 1% of patients with either acute or more often chronic infection. Prior to mass vaccination programs
it was estimated that 30% of patients with PAN had CHB but subsequently the rate has been revised down to about 7% (237). The vasculitis develops from the deposition of circulating immune complexes containing the HBsAg, in medium to small arteries and arterioles of various organs. The organ systems that may be affected include the renal tract (renal impairment, haematuria), gastrointestinal tract (causing abdominal pain, PR bleeding), central and peripheral nervous system (polyneuropathy/ mononeuritis multiplex, stroke/confusion), cardiovascular (cardiomyopathy, pericarditis), respiratory (infiltrates, nodules) and systemic symptoms as well as arthralgias and rash such as purpura and livedo reticularis (193). Treatment of PAN associated with HBV can involve antiviral therapy with or without plasmapharesis (239).

**Glomerulonephritis**
Membranous and less often memranoproliferative glomerulonephritis (GN) can occur in chronic hepatitis B, again due to deposition of circulating immune complexes. HBeAg/Anti-HBe complexes have been noted in membranous GN. It is seen more frequently in children and clinical presentation is with nephrotic syndrome. It typically resolves with HBeAg seroconversion. In adults, spontaneous resolution is less frequent and there may be slow progression to renal failure (240). Treatment of HBV with either oral antivirals or Interferon is recommended and there is a limited role for immunosuppression.

**Cryoglobulinaemia**
The association of hepatitis B with cryoglobulinemia is much less common than that of hepatitis C. Type 2 cryoglobulins are polyclonal IgG and monoclonal IgM whereas Type 3 cryoglobulins contain polyclonal IgG and rheumatoid factor (239). The manifestations may be minor but cryoglobulinemia can also cause symptoms of vasculitis with purpura, arthralgias, and glomerulonephritis (241).
Genotypes in Hepatitis B

HBV was initially divided into subgroups based on the antigenic determinants of the HBsAg and 4 serotypes or subtypes were known, adr, adw, ayr and ayw (242). In 1988 it was first suggested by Okamoto et al that HBV could be divided into 4 genotypes based on a divergence of ≥ 8% in the complete genomic sequence and genotypes A, B, C and D were identified (243). The relationship between serotypes and genotypes is not clearly known and the same serotype may be classified into different genotypes (243). Genotyping may be performed by a number of different techniques including restriction fragment length polymorphism, line probe assay, Enzyme Linked Immunosorbent Assay (ELISA) or genotype specific PCR (244). Direct sequencing can also be used and for commercial purposes, genotype can usually be determined through a partial sequence especially of the S gene since it is usually more conserved than other parts of the HBV genome. Following the initial description of genotypes A-D, Norder et al (245) also proposed genotypes E and F which differed by more than 4% in the S gene from the other genotype groups and this has become an alternative criterion for classification of distinct genotypes. Genotype G is the least common of the genotypes and was reported in 2000 from samples of French and American patients (246) but its geographic origin is still unknown (247). The precore and core regions of genotype G are aberrant with a 36-nucleotide insertion within the core gene making it the longest of the HBV genotypes (248). Stop codons in the precore region are also present and some have suggested it is not able to produce HBeAg (248) although others report high HBeAg levels in HIV/HBV coinfected patients with genotype G (249). However, this may be due to coinfection with genotype A (250). It is thought that genotype G requires the presence of another genotype, most commonly genotype A2 to enhance its viral replication (251) however monoinfection has been reported although this was transient (252).

Genotype H has been shown to be prevalent in Central America and the Amazon region and is closely related to genotype F HBV. It is thought to perhaps have split off from genotype F within the “New World” (253). It is particularly common in Mexico (254).

Genotype I described in Vietnam (255) may not meet the criteria for a novel genotype since the diversity in its complete genome sequence is only 7% from that of its closest neighbour, genotype C (256). Genotype J is a novel variant described in a Japanese patient who had previously travelled to Borneo and was thought to be phylogenetically positioned between human and primate HBV variants being close to strains which had been previously found in orang-utans and gibbons (257). This genetic variability in the HBV may come about through
natural mutation or by recombination. The HBV is error prone since its reverse transcriptase lacks proof reading ability and it is estimated that the rate of nucleotide substitution per site per year is approximately $1.4-3.2 \times 10^{-5}$ (258) which is 10 times higher than other DNA viruses and 100 times higher than the human genome (259). Recombination, in which DNA exchange or cross over of parts of a gene sequence occurs between two different viruses, is thought to be another potential mechanism for the development of divergent strains of HBV (260) and has been described between numerous different genotypes (261, 262). Recent evidence suggests that the core gene may be a preferred site for recombination to take place as noted in West African patients with A/E recombinant strains of HBV (263).

Subgenotypes
Subgenotypes are also described if there is a divergence of > 4% (but less than 7.5%) of the nucleotide sequence in the complete genomic sequence (259). There have been numerous (up to 40) subgenotypes reported amongst genotypes A-D and F. Thus genotype A is further typed into A1-A3, B into two major groups Bj found in Japan (including B1 and B6) and Ba (including B2,B3,B4 and B5) mainly found in Asia, C into C1-5, D into D1-4 and F into F1-4 (259). Geographic distribution varies for subgenotypes and certain clinical outcomes have also been attributed to some of them however confounding factors are difficult to control for in many of these studies. Divergence of <4% between subgenotypes are referred to as “clades” (264). There has been concern raised by experts in the field about the accuracy of classification of some of the newly reported subgenotypes (265). Pourkarim et al suggest that applying phylogenetic analysis over a full length genome sequence rather than partial sequence only is critical to avoid misclassification. They also suggest that recombinant strains should not necessarily be introduced as independent subgenotypes and propose the term “recombino-subgenotype” to identify HBV strains that show strong evidence of recombination in their nucleotide divergence (265).

Geographical distribution of genotypes
The geographical distribution of the different genotypes is quite varied and for most of the older known genotypes has been documented by a number of researchers. It is worth bearing in mind that most reports are from hospital based cohorts and furthermore since genotyping requires detectable HBV DNA in serum, there may be a bias in the studies towards patients with more active disease. Many parts of the world have dominant genotypes and most often there are at least 2 major HBV genotypes present in a given region. Patients may occasionally be coinfected with the 2 prevalent genotypes of the region eg B and C in Asia (266). Genotype
G also as mentioned appears to require the presence of genotype A (250) or H for chronic infection (267). The distribution of HBV genotypes in different continents are detailed in Table 1.3.

**Africa**

In East Africa (including Malawi, Madagascar and Tanzania) genotype A is found in 93% of patients (268). In South Africa the prevalence of genotype A is up to 75% (269). Genotype A is less common in central Africa, accounting for about 30% of cases (270). In other parts of central Africa and also in western Africa Genotype E makes up the majority (50-60%) of variants. (266). There has been some work suggesting that the introduction and expansion of genotype E HBV in West Africa has been a relatively recent phenomenon (271, 272). In contrast, in sub-Saharan (or Northern) Africa including Egypt, Algeria and Libya which forms part of the Mediterranean Basin, genotype D predominates in up to 80% of patients (273, 274).

**Europe**

In the European countries of the Mediterranean basin, in particular Greece (104), Italy (275), and Spain, the predominant genotype is Genotype D (276). Genotype D is also found in about 50% of cases in Eastern Europe, with genotype A in approximately 30% (266). Although some countries have a higher proportion of genotype A eg 86% in Poland and 67% in the Czech republic, Genotype D is found in the majority of Russian (93%), Romanian (67%) and Croatian (80%) patients (277, 278). The proportions of genotypes A and D are similar at about 30-40% in the remainder of Europe (the European Union and northern Europe) with smaller contributions from other genotypes including B and C, most likely due to migration (279).

**Asia**

In Western Asia (the Middle East including Iran, Yemen, Saudi Arabia, and Turkey the prevalent genotype is D in almost 95% of cases (280). Central Asian countries of Uzbekistan and Tajikistan similarly, have a preponderance of genotype D infection of up to 88% (281, 282). Southern Asian countries also show predominantly genotype D infection eg 95% of cases in Afghanistan (283) and a majority of Indian patients (284-286) and 65% of Pakistani patients (287). However genotype A is also seen in India (288) and a recent study from eastern India highlights a shift in the prevalences of genotypes with an increase in Genotypes A and C along with a decrease in that of genotype D in east India (289).

Moving further east into South East Asia genotypes B and C start to predominate. The relative prevalences of genotypes in many countries of South East Asia is set out in Table 1.4 (287, 290-
In brief however, genotype C is seen in the majority of patients of Cambodian, Thai, Laotian and Myanmar ethnicity. Genotype B is predominant in Vietnamese cohorts and some parts of Indonesia and Malaysia. In China, genotype C is prevalent in most areas although in
Table 1-3: Geographic distribution of genotypes by continent.

<table>
<thead>
<tr>
<th>Region</th>
<th>Genotype Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subsaharan Africa (Egypt, Algeria, Libya)</td>
<td>Genotype D</td>
</tr>
<tr>
<td>West Africa (Guinea Bissau, Ghana, Cameroon)</td>
<td>Genotype E and also A</td>
</tr>
<tr>
<td>Central Africa</td>
<td>Genotype E and also A</td>
</tr>
<tr>
<td>East Africa (Malawi, Madagascar, Tanzania)</td>
<td>Genotype A1 (95%)</td>
</tr>
<tr>
<td>South Africa</td>
<td>Genotype A</td>
</tr>
<tr>
<td>Mediterranean Basin (Greece, Italy, Spain)</td>
<td>Genotype D in majority. Gotype A also seen in Spain.</td>
</tr>
<tr>
<td>Western Europe</td>
<td>Mixtures of A-D from various migrant groups.</td>
</tr>
<tr>
<td>Eastern Europe</td>
<td>Genotype A (Czech republic, Poland) and D (Russia, Croatia, Romania)</td>
</tr>
<tr>
<td>North America</td>
<td>Mixtures of genotypes A-D from various migrant populations. Genotype F and B in Alaskan natives.</td>
</tr>
<tr>
<td>Central America</td>
<td>Genotype H (Mexico) Genotype F (Costa Rica)</td>
</tr>
<tr>
<td>South America</td>
<td>Genotype F predominant &amp; Genotypes A and D in Brazil/Argentina</td>
</tr>
<tr>
<td>Western Asia (Iran, Yemen, Saudi Arabia, Turkey)</td>
<td>Genotype D (95%)</td>
</tr>
<tr>
<td>Central Asia (Uzbekistan, Tajikistan, Afghanistan, Pakistan)</td>
<td>Genotype D</td>
</tr>
<tr>
<td>South Asia (India and Pakistan)</td>
<td>Genotype D in India but also Genotype A.</td>
</tr>
</tbody>
</table>
other parts, genotype B is seen frequently. Japan has a predominance of genotype C (82% in a study of 1271 patients) (313). This study also reported an increase in the prevalence of genotype A from 1.7% to 3.5% from the period 2001-2006 which is thought to be due to persistence of sexually acquired acute HBV in adulthood. In Korea genotype C2 predominates (314).

**The Americas**
Among Indigenous populations living in the Arctic, and northern Canada and Greenland, genotype B (subgenotype B6) has been found to be prevalent (315). Genotype F has also been found in Alaskan native CHB patients and appears to be associated with HCC development (316). Genotype F is also found in South and central American countries accounting for about 35% of infections there (317) although in Brazil, and Argentina Genotypes A and D are also seen (318). In Central America, genotype H HBV is prevalent, being found in approximately 75% of patients in a small study in Mexico (319). In the USA, chronic hepatitis is found primarily in migrant populations and there tends to be a mix of different genotypes reflecting the various immigrant groups in that country. A large study of 694 patients in the USA identified a strong correlation between ethnicity and genotype and found that in patients of Asian background, genotypes B and C were most common and in those of white or African American background who usually acquired hepatitis B in adulthood through sexual transmission, genotype A was most common (320).

**Australia and the Pacific**
In Australia, Bell et al showed in 2005 that in the cohort at St Vincent’s Hospital, Melbourne, 8% had genotype A, 29% B, 41% C and 22% D reflecting the multicultural nature of Australian society and the patterns of migration from the Mediterranean region and more recently South East Asia (48). There are few studies from the Pacific Island nations however one from the Solomon Islands where Hepatitis B is hyperendemic (prevalence of 21%), found a predominance of genotypes C and D which appeared ethnicity specific (321)
Table 1-4: Distribution of genotypes in countries of South East Asia.

<table>
<thead>
<tr>
<th>Country</th>
<th>Number</th>
<th>Genotype Distribution</th>
<th>Notes</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cambodia</td>
<td>12</td>
<td>67% C, 33% B (subtype 4)</td>
<td></td>
<td>(290)</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>72% C, 28% B</td>
<td></td>
<td>(291)</td>
</tr>
<tr>
<td>Vietnam</td>
<td>115</td>
<td>74% B</td>
<td>Based in Hanoi.</td>
<td>(292)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>63% B, 18% C, 2.5% B+C, 5% not determined</td>
<td></td>
<td>(293)</td>
</tr>
<tr>
<td>Indonesia</td>
<td>54</td>
<td>76% B, 24% C</td>
<td>West Indonesia (Java), East Indonesia Islands (Nusa Tenggara)</td>
<td>(294)</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>100% B</td>
<td>Surbaya</td>
<td>(295)</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>85% C, 7.4% B, 7.4% D</td>
<td>Papua</td>
<td>(296)</td>
</tr>
<tr>
<td>Malaysia</td>
<td>83</td>
<td>62% B, 35% C, 2% D</td>
<td>Genotype B 80% in ethnic Chinese. Genotypes B &amp; C equal prevalence in Ethnic Malays. Genotype D in Indian patients.</td>
<td>(297)</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>56.9% B, 31.4% C, 7.8% B+C coinfection 2% each D and E</td>
<td></td>
<td>(298)</td>
</tr>
<tr>
<td>Thailand</td>
<td>224</td>
<td>86.6% C (Subgeno C1), 11.2% B, 0.44% each of A and D, 3 suspected recombinations</td>
<td>Myanmar ethnicity 97.5% genotype C, Laos ethnicity 71% C, 26% B, Cambodia 84% C, 12% B. Study in migrant workers to Thailand</td>
<td>(299)</td>
</tr>
<tr>
<td></td>
<td>216</td>
<td>89.3% C, 7.4% B, 1.9% B+C, 0.5% A</td>
<td>Northern Thailand adult voluntary blood donors</td>
<td>(300)</td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>90.6% C, 7.5% B, 1.9% B+C</td>
<td>Children in Chiang Mai (perinatal transmission)</td>
<td>(301)</td>
</tr>
<tr>
<td></td>
<td>332</td>
<td>73.2% C, 20.8% B, 3.3% A, 2.7% unclassified.</td>
<td>Cohort included CHB and HCC patients and found that genotype B was not associated with HCC in younger patients</td>
<td>(302)</td>
</tr>
<tr>
<td>----------------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>Philippines</td>
<td>100</td>
<td>51% A</td>
<td>22% B</td>
<td>27% C</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>28% A</td>
<td>12% B</td>
<td>26% C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>101</td>
<td>36% B</td>
<td>64% C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>121</td>
<td>33% B</td>
<td>63.6% C 1.7% B/C 1.7% D</td>
<td>From Beijing China 304</td>
</tr>
<tr>
<td></td>
<td>126</td>
<td>38.1% B 54.8% C 0.8% D 1.6% B/C, 1.6% A/C 3.2% unknown</td>
<td>Yunnan China 305</td>
<td></td>
</tr>
<tr>
<td></td>
<td>142</td>
<td>9.2% B 88% C 2.8% D</td>
<td>Northern China (Harbin University China) 306</td>
<td></td>
</tr>
<tr>
<td></td>
<td>142</td>
<td>4.2% A 14.1% B 78.9% C 1.4% D</td>
<td>Southern China (Nanning) 307</td>
<td></td>
</tr>
<tr>
<td></td>
<td>786</td>
<td>0.89% A 63.23% B 34.99% C 0.89% D</td>
<td>Southern China (Guizhou) 308</td>
<td></td>
</tr>
<tr>
<td></td>
<td>220</td>
<td>1.4% A 17.2% B 81.4% C</td>
<td>Shanghai China 309</td>
<td></td>
</tr>
<tr>
<td>Tibet</td>
<td>26</td>
<td>96% C/D recombinant 4% C</td>
<td>Sequences based on surface Ag gene showed that 25 clustered with genotype D and 1 clustered with genotype C. However based on core gene all clustered with genotype C. 310</td>
<td></td>
</tr>
<tr>
<td>China (Hong Kong)</td>
<td>776</td>
<td>1.5% A 32.5% B 62.6% C 3.4% Mixed.</td>
<td>Hong Kong 311</td>
<td></td>
</tr>
</tbody>
</table>
Genotype and clinical Outcomes
There are a number of studies documenting the effect of genotype on various clinical outcomes. Many of these predominantly provide comparisons of 2 prevalent genotypes in a region, eg A vs D, or B vs C. Table 1.5 sets out some of what is known about the different genotypes and clinical associations. There is a paucity of information about genotype E and its clinical outcomes and more work is needed to further elucidate its impact on HBV related liver disease.

Genotype and risk of chronicity
As has already been discussed there is evidence that genotype A has the highest risk of progression to chronicity following acute adult acquired Hepatitis B and resolution of acute hepatitis B is often prolonged in genotype A (197). In a cohort of Asian patients, genotype C2 was independently associated with progression to chronicity, compared to genotype B (322). Acute infection with genotype D appears to be more commonly associated with acute liver failure than other genotypes (323).

Genotype and HBeAg and HBsAg seroclearance
Many studies have documented the more prolonged HBeAg positive phase and delayed HBeAg seroclearance of genotype C in comparison to Genotype B (324, 325). As previously mentioned HBeAg seroconversion occurs about 10 years earlier in genotype B compared to genotype C (158). A study of 1158 Alaskan natives which also looked at timing of seroconversion found that HBeAg seroconversion in genotype C (Subgenotype C2) patients lagged behind that other genotypes by approximately 3 decades. Age at HBeAg seroconversion of the 75th percentile of patients was 32 years in genotype A2, 27.5 years in B6, 27.3 years in D, 24.5 in F1 but 58.1 years in C2 (160). In particular genotype C patients are more prone to repeated episodes of acute exacerbation with failure of HBeAg seroconversion (326) and HBeAg seroreversion after HBeAg loss (160). Genotype C (and also genotype F) has been shown to have a higher risk of relapse with HBeAg negative CHB. Rates of spontaneous HBsAg clearance are higher in genotype B compared to C (327). Sustained remission following HBeAg seroconversion has been reported to be more commonly seen in genotype A than D as was HBsAg clearance (150).
### Table 1-5: Clinical associations with HBV Genotypes

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progression to Chronicity</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological inflammation</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological Fibrosis</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Association with advanced liver disease</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Association with HCC</td>
<td>+ (Possibly subgenotype A1)</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++ (F2)</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early HBeAg Seroconversion</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sustained remission after HBeAg Seroconversion</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg clearance</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td></td>
<td>+++</td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Response to IFN Treatment</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Association with PreCore mutations</td>
<td>+/-</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+++ (F1 but not F2)</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Association with BCP mutation</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>++</td>
</tr>
</tbody>
</table>

**Genotype and HBV DNA levels**

Genotype C has been reported to have a significantly higher viral load than genotype B (328), while viral load in genotype D has also been shown to be significantly higher than in Genotype A (329). Genotype E is reported as being more likely to be associated with HBeAg positive disease and higher HBV DNA levels than genotype D thus perinatal infection of infants from infected mothers is likely to be an important factor in transmission for African people infected with genotype E (330).
**Genotype and liver disease**

Genotype C patients are more prone to the complications of advanced fibrosis and cirrhosis (159, 331, 332) than Genotype B patients. Some small studies have shown that histological inflammation is more significant in genotype C than genotype B patients (305, 333). Genotype F in studies of Arctic, South American and Spanish populations appears to be associated with worse liver disease (150, 334, 335). Genotype A appears to have a more favourable prognosis than genotype D with one small Indian study of 52 patients (46% Genotype A and 48% genotype D) showing more severe histological disease in genotype D (336). The high rates of cirrhosis reported in association with HBeAg negative CHB from Mediterranean countries are now thought possibly attributable to genotype D (114). A Spanish study showed that sustained remission following HBeAg seroconversion was more commonly seen in genotype A than D (55% vs 32% p<0.01) as was HBsAg clearance (267). Genotype H CHB in Mexican patients is often adult acquired and thus is frequently associated with low viral loads and low risk of chronic liver disease and HCC (337). Occult HBV infection is reported to be commonly seen in genotype H patients however this may be partly due to the suboptimal sensitivity of HBsAg assays used (338). Furthermore the contribution of contribution of HBV genotype H to liver disease in Mexican populations is difficult to establish as alcohol, HCV coinfection and obesity are common cofactors (254).

**Genotype and HCC**

Genotype C has been shown to convey an increased risk for the development of HCC in the REVEAL-HBV study cohort, with an adjusted hazard ratio of 1.76 compared to 0.34 for genotype B (339). In addition there is data to suggest that HCC in genotype C is associated with a higher tumour recurrence rate (48, 324, 340-344). Genotype B on the other hand may be more likely to be associated with HCC in non cirrhotic patients and features of higher rates of solitary tumours and more satellite nodules have also been reported more often in genotype B than genotype C (345). Genotype B has been reported to be more prevalent in patients with HCC developing at a younger age compared to age matched inactive carriers (80% vs 52% in those <50 yrs and 90% in those <35 yrs) (346). Genotype A in Africans has been shown to be associated with HCC and at a much younger age than in other groups. Subgenotype A1 which is the most prevalent type in sub-Saharan Africa appears to be the main factor associated with this increased risk (269). However the contribution of aflatoxin, HIV coinfection and dietary iron overload are also factors to be considered (347). Genotype F has also been shown to be a risk factor for HCC especially in young Alaskan natives in a case control study (316) while the rates of HCC in genotype H affected populations in Mexico are low (337).
**Genotype and Treatment for HBV**
Response rates to treatment with Peg IFN differ by genotype being highest in genotype A and lowest in genotype D in a study comparing genotypes A-D (348). A small study of Peg IFN treatment in Genotype E patients also showed poor responsiveness (349) Limited data currently available on genotype F patients response to IFN suggests similar response to genotype A (335). Genotype G in a small number of patients treated with standard IFN showed poor responsiveness (350). HBV genotype does not appear to influence response rates to nucleoside analogue therapy, however the patterns of drug resistant mutations that develop have been reported to be different in different genotypes (351).

**Variant Viruses**
HBV replicates at a high rate through the reverse transcription of an RNA intermediate resulting in a high error rate (352). Thus HBV exists as a quasispecies, ie a heterogeneous viral population comprised of closely related but non identical genomes (353). The predominant strain selected out is determined by factors such as host immune response, viral replication fitness and exogenous pressures such as due to antiviral therapy (354). The most frequently occurring natural HBV variants are the precore and the basal core promoter mutations resulting in a reduction or abolition of HBeAg production. During the immunotolerant phase, these mutations in the core or precore region are uncommonly seen, but they emerge during the immune clearance phase as a result of immune selective pressures (355).

**HBeAg variants**

**Precore mutation**
A point mutation at nucleotide 1896 in the HBV with substitution of G (guanine) for A (Adenine) results in a stop codon at this point thus preventing production of HBeAg although without affecting replication and HBcAg production (83). The formation of the “precocene” mutation is essentially precluded in certain genotypes in particular A, F and H and it occurs most frequently in genotype D and to a lesser extent in Genotypes B, C and E (264). The reason for this relates to the fact that nucleotide 1896 is important in maintaining a stem loop structure (epsilon) which is necessary for enclosure of the pregenomic RNA into the nucleocapsid. Nucleotide 1896 is opposite nucleotide 1858 in the stem loop structure and thus genotypes with a T (thymine) at nucleotide position 1858 (eg genotype D) are more likely to predispose to the development of the G1896A mutation since T-A pairing is more stable than T-G pairing (226). Likewise genotypes with C (cytosine) at position 1858 (genotype A) are much less prone to development of the precore mutant virus since C-A pairing is weaker than
than C-G pairing (184). Subgenotype F2, similarly codes for C at position 1858 but F1 does not, so precore mutation may occur in some but not other genotype F CHB patients (356).

It has been suggested that stabilisation of the epsilon encapsidation signal may increase the replicative fitness of HBV which may be one reason for it being selected out (355). Although HBV DNA levels are lower in HBeAg negative disease, this impairment in virion productivity is not thought to be related to precore and BCP mutants but instead be the result of an independent process (357). The HBeAg and its relationship to the host immune response is complex. It has been shown to be able to tolerize T cells and cause immunomodulation of Toll-like receptor mediated signalling pathways to evade immune responses and thus is thought to contribute to viral persistence (358). However its presence in the cytosol also acts as a target for the inflammatory response (207). Thus it has been suggested that HBeAg may act as a tolerogen or an immunogen in different circumstances and that loss of HBeAg may be a favourable biological characteristic that renders the HBV less vulnerable to immune attack (359). Numerous other mutations have been described in the precore region including a point mutation at G1899A (commonly seen in association with G1896A) (360). Although the traditional conceptualisation was that the precore mutant developed at the point of HBeAg seroconversion (226), it has been shown that viral mutation rates increase in the immune clearance phase when compared to the immune tolerant phase (361) and in fact there is significant viral sequence diversity present in the months/years leading up to HBeAg seroconversion (362). Furthermore, the high rates of nucleotide substitution continue post seroconversion, with ongoing immune selection pressure being applied in the immune control phase although at lower viral loads (148).

Clinically, the G1896A precore mutation is a major cause of HBeAg negative chronic hepatitis and although at one point, it was thought to be a cause of fulminant hepatitis, especially in acute adult acquired HBV (363) although not in fulminant cases of CHB (344). It was shown to be associated with a slightly lower risk of HCC than wildtype virus in a subanalysis of the REVEAL-HBV study cohort (339) however, also recently published is a meta-analysis of 85 case control studies of 16,745 patients with 5781 cases of HCC who report that the precore mutations G1896A, G1899A as well as deletions in Pre-S region were associated with an increased risk of HCC (364).

**Basal Core promoter (BCP) region mutations**

Mutations in the core promoter region occur most commonly at nucleotides 1762 (adenine (A) to thymine (T)) and 1764 (guanine (G) to adenine (A)) and are usually found together (365, 366). Once again this is thought to possibly have some compensatory advantage and Tacke et
al showed that BCP mutations increased viral replication levels to above those of wildtype virus, including in strains with Lamivudine resistant mutations also present (367). Other mutations in the basal core promoter region have also been described, including at positions 1653, 1753-57, 1766, 1768 and these are usually seen in addition to the A1762T and G1764A variants. The association with HBeAg negativity overall is less strong in patients with BCP mutations than in those with precore mutations. The BCP mutation reduces the production of HBeAg by approximately 70% (368) but this may be even further reduced in HBV variants with the additional mutations in position 1753 and 1766 (368). The BCP mutation has been found to occur more frequently in genotype C than B (312, 369) and one possible reason for this may be because genotype C is more likely to have a C at nucleotide position 1858, which largely precludes formation of the precore mutation (370) however this varies between subgenotypes of genotype C. The prevalence of the BCP does appear to vary between genotypes being reported at 41%, 27%, 60% and 42% in genotypes A, B, C and D (320). The lower prevalence in genotype A compared to C suggests that other factors apart from the nucleotide at position 1858 are also important. The BCP mutations have been implicated quite strongly in more advanced liver disease and in the development of HCC (371). Yuen et al (312) reported an association with higher ALT levels in patients with BCP mutations compared to those with wildtype. They also subsequently looked at 66 patients with liver biopsies, 71% of whom had the BCP mutations and found they had more severe necroinflammation than those without (372). Lin et al, (373) also showed that the BCP mutation was associated with the development of cirrhosis and HCC although this was restricted to males. Increasing prevalence of the BCP mutation was reported by Kao et al (369) in patients with more significant liver disease, being 3% in inactive carriers and 64% in patients with HCC. Orito et al (374) also described a significant association between BCP and more advanced liver disease (OR 4.1 CI 1.6-10.2). Recently a study by Tseng et al (375) of 251 spontaneous seroconverters (Genotypes B and C) showed that a higher proportion of BCP mutation (>45%) was associated with an OR of 2.81 for the risk of cirrhosis. However, whether the BCP mutant HBV is causative or simply a reflection of more significant immune pressure and thus immune mediated inflammation and fibrosis is not known.

The link between the BCP mutation and HCC has been documented by a number of groups who reported the BCP mutation was significantly associated with HCC in both genotypes B and C (369, 376) and by Baptista who found a high prevalence of the mutations in black African patients with HCC (377). More recently the REVEAL-HBV study group also reported an
increased risk of HCC in patients with BCP A1762T/G1764A mutant compared to wildtype virus (Hazard ratio 1.73 95% CI =1.13 to 2.67). The highest risk was amongst those with the BCP mutations, Genotype C and wildtype for the 1896 precore variant site (adjusted hazard ratio 2.99, 95% CI=1.57 to 5.7) (339). There is no strong evidence that the presence or absence of the precore or BCP mutations affects the response to Interferon or nucleos(t)ide analogue treatment although some studies have shown that the proportion of precore and BCP mutant virus present prior to treatment correlates with the chance of HBeAg seroconversion and HBV DNA suppression with an approximately 2% increase in HBeAg seroconversion rates per 1% increase of precore and BCP mutant percentages (378).

**HBsAg variants**

**Vaccine escape mutants**

Variations in the HBsAg protein can result in viral infection developing in a vaccinated subject. Anti-HBs is directed towards a highly conserved region of the surface protein (amino acids 99-160) which includes the major “a” determinant of the surface protein. HBsAg mutations resulting in amino acid substitutions in the region 137-147 of the surface protein can change the conformational epitope in the “a” determinant so that it is not recognized by the neutralizing anti-HBs antibodies. In particular the G145R vaccine escape mutant is known to be stable and replication competent (379, 380). Although the infectivity of these variants is currently thought to be low, another problem is their lack of detectability by serological tests (381). The development of vaccine escape mutants has been thought in some parts to be related to the emergence of anti-viral drug resistant mutants because of the overlap of the polymerase gene (where nucleot(s)ide analogue associated resistant mutations occur) with the surface antigen domains recognized by anti-HBs (382). The sG145R vaccine escape mutant has also been detected in 2 of 65 patients in an Australian indigenous cohort, despite wild type polymerase gene sequences in all (383). There is evidence however that emergence of the vaccine escape mutants predates mass vaccination programs (384). It is thought possible that immune pressure exerted on HBV due to expanding vaccination programs will result in an increasing problem of vaccine escape mutants (381). Graft infection with HBV following liver transplant in patients who received hepatitis B Immune globulin (HBIG) therapy post transplant has also been shown to be due to the development of S gene mutant HBV (385).
**S escape mutants**

Other mutations in the S protein, eg due to missense mutations in the S gene have also been described and are a particular concern for screening of blood donors since they result in false negatives on HBsAg serological testing (386). The prevalence of 8 mutations associated with HBsAg diagnostic failure, including P120T, T126S, Q128H, G130N, S143L, D144A, and G145R was found to be approximately 1% in a study of 11,221 HBV sequences encompassing genotypes A-H (387).

A study of 4.4 million Dutch blood donations identified 23 HBsAg negative but HBV DNA positive persons and also reported the presence of multiple escape mutations in the S gene especially in Genotype D patients with occult HBV (388). Other reasons for HBsAg negativity in the setting of HBV DNA positivity in this study were early acute HBV infection (prior to development of HBsAg), occult HBV infection, genotype G HBV with decreased HBsAg production and suppressed infection after vaccination.

**Pre-s deletions**

Mutations in the pre-S region, including Pre-S2 deletions, pre-S1-S2 deletions and Pre-S2 start codon mutations have been described (389, 390). Pre-S mutants have been shown to be associated with decreased synthesis and secretion of HBsAg (391). It has been also been shown that Pre-S2 deletions are associated with more advanced liver disease (392-394) and possibly with the development of HCC especially in younger patients (395).

**HBV DNA assays.**

Accurate quantification of HBV DNA levels is an integral part of the initial assessment and ongoing monitoring of patients with CHB both on and off treatment. In 2001, as a result of a collaborative effort by WHO, an international standard for HBV DNA reporting was established with reporting in International Units per ml (IU/ml) (396). Prior to this reporting was in first in picograms/ml and then copies/ml. Different commercial assays use different technologies and there are varying thresholds for upper and lower limits of detection. Signal amplification type assays in which nucleic acid is quantified had the advantages of more direct measurement of target, simpler technique in terms of preparation of the sample and less variation from sample to sample (397). However the 1st and 2nd generation bDNA assays (eg Digene Capture 1 Digene Coro and Digene capture II) had poor lower range of quantification, which was 1.6 x 10^3 IU/ml for the ultrasensitive Digene Capture II. The Versant HBV 3.0 (Seimens) signal amplification assay has a dynamic range of 3.5 x 10^2 to 1.8x10^7 IU/ml.
Target amplification (or PCR) assays used to measure HBV DNA include the COBAS AMPLICOR HBV DNA assay by Roche. Although it had the benefit of a rapid turnaround, there was limited quantification in the upper ranges (range approx. $5 \times 10^1 – 4 \times 10^4$ IU/ml).

More recently real time PCR (in which the amplification is measured by fluorescence detected with a camera during each PCR cycle) techniques have been developed and new assays with very broad dynamic ranges (approximately $1-3 \times 10^1 – 10^8-9$ IU/ml) have been developed by Roche (Cobas Taqman), Abbott (RealTime HBV) and Qiagen (Real Art). For ease of comparison all DNA quantification in this thesis has been standardized to IU/ml.
Quantitative HBsAg in Chronic Hepatitis B

There has been a resurgence of interest over the past few years in the use of quantitative HBsAg (qHBsAg) levels both for evaluation of patients during the natural history of CHB and also during treatment. Quantitation of HBsAg was first described in the 1970’s (398). Correlation with clinical events e.g. an early reduction in serum qHBsAg in patients with acute hepatitis B who cleared the virus (399) and use of HBsAg in treatment response to IFN therapy (400) were also described early on. The interest has been particularly fuelled by the possible finding that HBsAg levels may be a surrogate marker of cccDNA (401). Since cccDNA is the intranucleic HBV DNA reservoir, clearance of this is an important therapeutic aim. cccDNA is not substantially reduced in nucleoside analogue treatment, since these agents inhibit the reverse transcriptase step of viral replication and do not directly act on the intranucleic cccDNA pool. However in IFN therapy, reductions of HBsAg may be seen as hepatocytes may be cleared of cccDNA, through non cytoplastic mechanisms (402). The relationship between HBsAg levels and cccDNA however are complex and not fully understood, in particular in HBeAg negative disease (403) where there appears to be a disconnect between regulation of virus production and HBsAg production. HBsAg loss however is the best approximation of a cure that can be reached in HBV and there has been much work done on the clinical utility of HBsAg levels, including in predicting HBsAg loss. There are now several commercially available assays for the quantitation of HBsAg including Architect HBsAg QT assay (Abbott Diagnostics, Abbott Park, IL, USA) and the Elecsys HBsAg II assay (Roche Diagnostics, Indianapolis, IN, USA) with results expressed in IU/ml. These assays are fully automated and capable of high throughput.

Quantitative HBsAg in Natural History of CHB

HBsAg levels have been shown to correlate with different phases of disease with the highest levels being seen in HBeAg positive immune tolerant patients, followed by those in the Immune clearance phase (404). In HBeAg positive patients, qHBsAg correlates with serum HBV DNA and intrahepatic DNA whereas in HBeAg negative CHB there is no such correlation (405). It has also been shown to be useful for differentiating patients in the inactive carrier state from those with HBeAg negative CHB with fluctuating ALT (406) and in this study by Brunetto, a combined HBV DNA ≤2000 IU/ml and qHBsAg levels of <1000 IU/ml identified inactive carriers with 95.4% specificity and 91.1% sensitivity. Baseline measurements of HBV DNA ≥200 IU/ml and HBsAg ≥ 1000 IU/ml have also been shown to predict reactivation with a negative predictive value of 96% (178).
HBsAg levels may also help predict future loss of HBsAg, both following HBeAg seroconversion (407) and especially in HBeAg negative patients with undetectable viral load (408). Tseng et al for example showed that in a group of spontaneous HBeAg seroconverters, HBsAg levels of <100 IU/ml 1 year after this event, was predictive of subsequent HBsAg loss (409). This same group also showed that in 688 HBeAg negative patients with low HBV DNA levels (<2000 IU/ml), an annual HBsAg clearance rate of up to 7% was seen in those with HBsAg <10 IU/ml compared to those with HBsAg >1000 IU/ml (410). Seto et al (411) did a case control study of 203 patients with spontaneous HBsAg clearance and reported that a cut-off HBsAg level of <200 IU/ml and/or HBsAg reduction of 0.5 log IU/ml/year predicted HBsAg clearance with a relatively high sensitivity and specificity. The clearance of HBV DNA precedes HBsAg decline and levels of HBsAg may change little over years, with reduction in titres in HBeAg negative patients being gradual and slow (412).

HBsAg levels have been shown to correlate with histology, for example in HBeAg positive patients higher levels of HBsAg correlate with lower risk of fibrosis (413), although there was no such correlation in HBeAg negative patients (414). HCC risk has also been shown to be predicted by HBsAg levels (415), especially in HBeAg negative patients with HBV DNA <2000 IU/ml in whom a HBsAg of ≥1000 IU/ml (compared to <1000 IU/ml) gave a hazard ratio of Hazard ratio of 5.4 for risk of HCC, while patients in this group with HBsAg <1000 had HCC rates similar to those of non-infected patients (416). HBsAg levels ≥1000 IU/ml have also been shown to be associated with increased progression to HBeAg negative hepatitis and cirrhosis (417). Much of the data on the relationship of quantitative HBsAg and clinical outcomes is from Asian cohorts and further work in genotypes A, D, E and F needs to be done however it may indeed prove a useful tool for inclusion in risk assessments of CHB patients.

The use of HBsAg during treatment will be discussed in the treatment section.

**Quantitative HBeAg levels in Chronic Hepatitis B**

HBeAg may be quantified using an assay developed by Abbot (Abbott Dianostics, Abbott Park,IL,USA). The assay for quantification of HBeAg using the Abbot Architect platform is semiquantitative and reported in terms of sample/cut off ratio (S/CO) which is then validated in house using reference standards from the Paul Ehrlich Institute (Langen Germany) and expressed as PEIU/ml.

There is very little reported on quantitative HBeAg (qHBeAg) levels in the natural history of CHB, apart from a report by Thompson et al from our group in 2010 (405). They found that in a
group of untreated HBeAg positive patients in the immuneclearance phase, the distribution of qHBeAg was skewed with almost 50% of patients having levels < 1000 PEIU/ml. A positive correlation between qHBeAg and HBV DNA was also found but not with intrahepatic cccDNA. The emergence of BCP/Preceore variants significantly reduced HBeAg titres therefore clinical use of HBeAg titres should take into account the phase of disease and likely viral subpopulations present (405).

Monitoring of HBeAg levels to predict HBeAg seroconversion has been done in both therapy with Peginterferon and Lamivudine therapy as far back as the early 1990’s when Perrillo measured qHBeAg around seroconversion and compared it to HBV DNA (418). Fried et al (419) showed that in Peg IFN treated patients who achieved HBeAg seroconversion, levels of HBeAg fell consistently during therapy and remained low for 24 weeks post treatment. Levels of qHBeAg both at baseline and at Week 24 of treatment correlated with likelihood of HBeAg seroconversion with the highest rates of seroconversion being seen in those with the lowest baseline HBeAg levels. Quantitative HBeAg has also been looked at in patients treated with Nucleoside analogues and a continuous decrease to more than 90% of pre treatment values of HBeAg was associated with increased likelihood of HBeAg seroconversion (419-421). Use of qHBeAg in peg IFN and NA therapy is discussed further in the treatment sections.

Further evaluation of other serum markers have recently been described, including quantitative anti-HBc evaluation which has shown possible efficacy in predicting HBeAg seroconversion in both peg-IFN and NA based therapy (422) and serum hepatitis B RNA levels as early predictors of HBeAg seroconversion during treatment with polymerase inhibitors (423).
Management of HBV Infection

Goals of treatment
Reduction in the complications of chronic HBV infection, namely cirrhosis, decompensated liver disease, hepatocellular carcinoma and liver related death are the main goals of treatment of HBV. A cure of the disease is currently not possible due to the persistence of cccDNA in the nucleus of infected liver cells and moreover the integration of the viral genome into that of the host, however sustained viral suppression is a necessary step in achieving reduction in the risk of complications. Important endpoints of CHB treatment response include virological (HBV DNA undetectability), biochemical (ALT normalisation), histological (regression of liver fibrosis) and serological (HBeAg seroconversion - if applicable) response and ultimately HBsAg clearance with development of anti-HBs. There are 2 major categories of therapeutic agents available; immunodulatory agents ie Interferon and anti-viral agents which include a number of oral nucleos(t)ide analogues.(103)

Indications for treatment
The indications for and timing of therapy depend on a number of clinical features, including the phase of disease, the degree of ALT and HBV DNA elevation and the degree of hepatic inflammation and/or fibrosis present.

There are different treatment guidelines set out by the 3 international societies for the study of liver disease AASLD (101), EASL (102) and APASL (103) which are largely concordant. In Table 1.6 they are detailed in rows according to previously described phase of disease as defined by Thomas et al (99) although there is some variation in the definitions used between guidelines.

Choice of treatment
The choice of therapy is determined by the likelihood of sustained response, patient tolerability to certain side effects and in certain parts of the world, cost and availability of different medications. There have been significant advances in the available treatments for CHB over the past 3 decades with the advent of pegylated interferon which also now has useful on treatment predictors of response (qHBsAg) and high efficacy in certain populations (eg genotype A patients), including the possibility of HBsAg clearance. Oral nucleos(t)ide analogues now available have high potency and very low rates of resistance in comparison to the early NA’s such as lamivudine, the main drawback to using them being the need for indefinite therapy in HBeAg negative patients as viral rebound commonly occurs with cessation. There are no current recommendations made by any of the international societies about the choice of therapy to be used initially and this is largely left up to individual clinicians,
however clinical guidelines published in the National Health Service in Britain recommend offering Peg IFN initially to patients with compensated liver disease for both HBeAg positive and negative patients (424).
Table 1-6: Recommendations for initiation of treatment by international societies.

<table>
<thead>
<tr>
<th>Immune tolerant</th>
<th>AASLD 2009</th>
<th>APASL 2012</th>
<th>EASL 2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBeAg positive, ALT ≤ 2 X ULN</td>
<td>Observe. Consider tx when ALT becomes elevated (degree of elevation not mentioned). Consider biopsy if &gt;40 yrs/ALT high normal/FHx HCC. Consider treatment if HBV DNA &gt;20,000 and biopsy shows significant inflammation/fibrosis</td>
<td>HBeAg Positive, ALT normal (regardless of HBV DNA) – monitor HBV DNA/ALT/HBeAg 3-6 monthly</td>
<td>HBeAg Positive, PNALT and high HBV DNA. If &lt;30 yrs and no FHx of HCC or evidence of liver disease – 3 monthly followup. No treatment required. Consider liver biopsy if &gt;30yrs and/or FHx HCC/cirrhosis.</td>
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| Immune clearance | HBeAg positive HBV DNA ≥ 20,000 IU/ml ALT > 1-2 X ULN. Observe for 3-6 months. If no spontaneous HBeAg loss, HBV DNA≥20,000 IU/ml and ALT >2x ULN – treat. If HBV DNA>20,000. ALT <2xULN or >/=40 years Consider liver biopsy and treat if mod/severe inflammation/fibrosis. | HBV DNA ≥ 20,000: and ALT 2-5 x ULN - Treat if persistent over 3-6 months or if concerns about decompensation. Or ALT > 5 X ULN . Treat, although if HBV DNA < 2 x 10^5 may choose to observe for 3 months for spontaneous seroconversion, if no risk of decompensation. | Obviously active CHB (HBeAg positive, ALT >2xULN and HBV DNA>20,000) - start tx (no liver biopsy required but fibroscan useful). Any patient with elevated HBV DNA>2000, ALT >ULN and mod-severe fibrosis can be considered for treatment. |

<p>| Immune control | HBeAg negative, HBV DNA ≤2000 IU/ml, ALT ≤ ULN Observe. | HBV DNA &lt;2000 IU/ml and ALT normal Observe with ALT/DNA 6-12 monthly. | HBeAg negative, PNALT, HBV DNA&lt;2000 – no treatment. 3/12ly ALT and HBV DNA 6-12 monthly for at least 3 years |</p>
<table>
<thead>
<tr>
<th>Immune Escape</th>
<th>HBeAg negative, HBV DNA &gt;2000 IU/ml, ALT &gt; 2x ULN → Treat.</th>
<th>HBV DNA ≥ 2000 IU/ml and ALT &lt; 2x ULN → No tx. If age &gt; 40 yrs liver biopsy or fibroscan and treatment if moderate/severe inflammation.</th>
<th>Obviously active CHB (HBeAg positive, ALT &gt;2xULN and HBV DNA&gt;20,000) - start treatment (no liver biopsy required but fibroscan useful). Any patient with elevated HBV DNA&gt;2000, ALT &gt;ULN and mod-severe fibrosis can be considered for treatment.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBeAg negative, HBV DNA &gt;2000 IU/ml and ALT 1-2 X ULN → Consider liver biopsy and treat if moderate/severe inflammation or fibrosis.</td>
<td>HBV DNA ≥ 2000 IU/ml and ALT &gt; 2 X ULN → treat if persistent for 3-6 months or concern re decompensation.</td>
<td></td>
</tr>
</tbody>
</table>

| Cirrhosis – compensated | HBV DNA > 2000 IU/ml → treat. HBV DNA <2000 IU/ml consider tx if ALT elevated. | HBV DNA <2000 IU/ml → No treatment. Monitor ALT/HBeAg or HBV DNA 3 monthly. HBV DNA ≥ 2000 IU/ml and ALT elevated → treat. Can consider IFN only if ALT not ≥ 5 X ULN. | Consider starting treatment if detectable HBV DNA even if ALT normal. |

| Cirrhosis - decompensated | If HBV DNA detectable coordinate tx with NA with transplant centre. | Urgent treatment with NAs. (HBV DNA level not mentioned). | Urgent commencement of NA treatment if any detectable HBV DNA. Consider transplant |
Peg Interferon for HBeAg positive CHB

Peg IFN is available for use as a once weekly injection and has modest antiviral effect but also has an immunomodulatory effect. The optimal dose and duration of Peg IFN α-2a is 180 mcg weekly for 48 weeks (425). In a European trial of 52 weeks of Peg IFN α-2b in HBeAg positive patients, (n=307), HBeAg seroconversion rates 26 weeks post therapy were 36% (348). HBeAg loss varied with genotype, being 47% in genotype A, 44% in Genotype B, 28% in genotype C and 25% in D. A large trial of 814 Asian patients with 3 arms (Peg IFN monotherapy, Lamivudine monotherapy and Peg IFN/Lamivudine combination therapy) showed that at followup 24 weeks following 48 weeks of Peg IFN α-2a, 29% of patients had achieved HBeAg seroconversion (compared to 19% receiving Lamivudine alone), 41% had ALT normalisation, 32% had HBV DNA < 10^5 Copies/ml and 14% had HBV DNA <400 copies/ml (426). The combined response rate of HBeAg seroconversion, normalisation of ALT and HBV DNA < 100,000 copies/ml was 10% at end of treatment and rose to 23% 24 weeks post end of treatment. These patients were predominantly Asian (87%) and genotype C (60%) (426). The HBsAg loss rate at 6 months post end of therapy was 3-5% in these studies. HBeAg seroconversion, especially if achieved during treatment or early post treatment is durable in the majority (83%), as is HBV DNA suppression and ALT normalisation (427). In a European cohort, overall sustained HBeAg and HBsAg loss at 3 years following Peg IFN treatment has been reported in 37% and 11 % respectively with even higher rates in genotype A patients and in initial responders (428). In patients with HBeAg loss before 32 weeks of therapy, the rates of HBV DNA suppression < 400 copies/ml and HBsAg loss were enhanced at 47% and 36% at 3 yrs (429).

The use of Peg IFN α-2b in HBeAg positive CHB patients with advanced fibrosis and well compensated cirrhosis has been shown to be safe and effective in a study by Buster et al (430) which included 70 patients with advanced fibrosis (including 24 cirrhotic patients), compared with 169 patients without advanced fibrosis. They in fact reported higher response rates (HBeAg seroconversion and HBV DNA < 10,000 copies/ml at week 78) in those with advanced fibrosis compared to those without (25% v/s 12%), however genotype A was more prevalent in the former groups than the latter (57% vs 24%)

Buster et al of (431) also analysed the pooled data of the 2 largest global trials of Peg IFN in HBeAg positive patients (n=712) and reported on predictors of sustained response (defined as HBeAg loss and HBV-DNA level less than 2.0 x 10^{-3} IU/mL 6 months after treatment). They report that HBV genotype, ALT ≥ 2 x ULN, HBV DNA < 2 x 10^8 IU/ml, female sex, older patients
(average age those with response was 34 compared to 32 in non response) and lack of previous IFN exposure were associated with a higher chance of sustained response (Table 1.7). They chose a predicted response rate of 30% or greater on which to base recommendations for use of Peg Interferon and recommend it be used in all genotype A patients, in genotype B and C patients with a high ALT and a low HBV DNA and not at all in genotype D patients. They also include useful nomograms in genotypes A-D to predict % chance of SVR based on individual patient characteristics. Others have suggested that Interferon should be first line therapy in Genotype A and in HBeAg positive genotype B patients (432). There has also been interest in whether some host genetic variants, including single nucleotide polymorphisms may play a role in the response to Peg IFN treatment. Unlike the situation in Hepatitis C, IL28B does not appear to predict responsiveness to IFN as evidenced in a study of largely Asian patients, the majority of whom had the good response (CC) IL28 B genotype (433). There are a few studies which have looked at host IFN pathway genes, or certain HLA locus genes which have shown a possible improvement in response in patients with certain polymorphisms (434, 435), although there is not enough evidence to include these factors in clinical decision making at present.

Table 1-7: Pre-treatment predictors of response to Peg IFN therapy

<table>
<thead>
<tr>
<th>Predictors of response to Peg IFN in HBeAg positive patients:</th>
</tr>
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<tbody>
<tr>
<td>• Genotype A&gt;B&gt;C&gt;D</td>
</tr>
<tr>
<td>• ALT ≥ 2 x ULN</td>
</tr>
<tr>
<td>• HBV DNA &lt; 2 x 10^8 IU/ml</td>
</tr>
<tr>
<td>• Female sex</td>
</tr>
<tr>
<td>• Absence of previous IFN therapy</td>
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</tbody>
</table>

Quantitative HBsAg and HBeAg levels in Peg IFN therapy for HBeAg positive CHB.

Serum qHBsAg levels have been considered possible markers of the transcriptional activity of cccDNA (402). Baseline qHBsAg levels have been shown to be a possible predictor of sustained response in HBeAg positive patients treated with Peg IFN (436, 437). HBsAg levels at weeks 12 and 24 of Peg IFN therapy may be used to identify HBeAg positive patients with a low chance of response. In genotype A and D, absence of any decline in qHBsAg at week 12 has a negative predictive value (NPV) of 97-100% for response. In genotypes B and C, week 12 qHBsAg levels of >20,000 IU/ml is predictive of lack of response. Week 24 HBsg levels of >20,000 IU/ml have
a NPV for response of 99% and therefore are a stopping rule for all genotypes (A-D) (438). qHBsAg level of <1500 IU/ml at week 12 on the other hand is associated with a good response with 45% of patient showing a response and 17% of patients achieving HBsAg clearance at 24 weeks post treatment (438). Chan et al have also shown that a >1 log reduction in qHBsAg at month 6 combined with qHBsAg ≤ 300 IU/ml at this point had a positive predictive value of 75% for sustained response (defined as HBeAg seroconversion and HBV DNA <10 000 copies/mL until 12 months post-treatment) (436).

Quantitative HBeAg levels may also be of use in response guided therapy for peg IFN (Table 1.8). A qHBeAg level of ≥ 100 PEIU/ml at week 24 is a predictor of poor response with only 4% of patients with this level achieving HBeAg seroconversion 6 months post therapy while in contrast a qHBeAg level of <10 PEIU/ml at week 24 is associated with HBeAg seroconversion in over 50% of Peg IFN treated patients (419).

**Peg Interferon for HBeAg negative CHB**
Peg IFN α in the treatment of HBeAg negative CHB was evaluated in over 500 patients by Marcellin et al (439) who compared 48 weeks Peg-IFN alone, to Peg-IFN + Lamivudine and Lamivudine alone. Six months post therapy, normalisation of ALT was seen in 59% and HBV DNA < 20,000 copies/ml was seen in 43% of Peg IFN monotherapy patients and these rates were significantly higher than in the lamivudine group. Combined biochemical and virological response was seen in 36% of patients but this dropped to 25% at 3 years post followup (440). HBsAg clearance occurred in 6% of the Peg IFN group (compared to 0% in the Lamivudine group) at 24 weeks post treatment and this increased to 8.7% at 3 years.

Similar to the case in HBeAg positive CHB, predictors of response to Peg IFN (defined as ALT normalisation and HBV DNA < 20,000 copies/ml 24 weeks post treatment) include high baseline ALT, lower HBV DNA at baseline and female gender (441). Younger age and genotype (B and C better than D) were also significant predictors. There have been conflicting reports on the effect of IL28 B genotype on Interferon treatment responsiveness in HBeAg negative patients. In a study of predominantly genotype D patients (92%), Lampertico reported higher sustained response rates (31% vs 13%) and higher HBsAg clearance rates (29% vs 13%), in those with the IL28 B genotype CC (at position rs12979860) compared to non CC patients (442). However other studies have not shown any difference (443). A number of the large studies in HBeAg negative chronic hepatitis B have been done in cohorts of largely genotype D patients and because of the overall poorer response, strategies to improve response rates have been tried, e.g extension of Peg IFN treatment to 96 weeks from 48 weeks. This results in
viral suppression rates (HBV DNA < 2000 IU/ml) at 6 months post treatment, of 28.8% compared to 11.8% in the 48 week treatment group (444). Trials of combination Peg IFN with Tenofovir/ Entecavir may show the optimal way to use a combination of NA’s and Peg IFN, whether at the same time, or after a “lead in” of several weeks of NA.

*Quantitative HBsAg levels in Peg IFN therapy for HBeAg negative CHB.*

Early decrease in HBsAg levels in HBeAg negative patients treated with Peg IFN has been shown to predict sustained virological response. Moucari et al (445) showed that decrease of 0.5 $\log_{10}$ at week 12 and a 1 $\log_{10}$ at week 24 had a high positive predictive value (89% and 92% respectively) for sustained response (defined as undetectable serum HBV DNA <70 copies/ml 24 weeks post treatment cessation). Changes in HBsAg levels during Peg IFN were shown to be genotype specific in a study of 230 HBeAg negative CHB patients. The authors suggest different end of treatment cutoff values for genotypes A-D to predict long term virological response (defined as HBV DNA <10,000 copies/ml at 5 years post treatment). They found that positive predictive values of 75%, 47%, 71% and 75% could be obtained using end of treatment cut off values for qHBsAg of <400 IU/ml (genotype A), <50 IU/ml (genotype B), <75 IU/ml (genotype C) and <1000 IU/ml (genotype D) (446). End of treatment HBsAg levels also correlate with HBV DNA suppression to ≤400 copies/ml 6 months post treatment. Furthermore, long term HBsAg clearance (at 3 years) has been shown to be strongly predicted by an HBsAg decline of >1 $\log_{10}$ IU/ml together with HBsAg <10 IU/ml at week 48 (447) however the number of patients in whom this is achieved is small. Stopping rules in genotype D HBeAg negative CHB based on no decline in HBsAg and < 2 $\log_{10}$ drop in HBV DNA at week 12 of therapy has also become part of recent guidelines (102) based on a very high negative predictive value for sustained response (448, 449).
Table 1-8: On treatment predictors of response to Peg IFN treatment

<table>
<thead>
<tr>
<th></th>
<th>HBeAg positive</th>
<th>HBeAg negative</th>
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<tbody>
<tr>
<td><strong>qHBsAg</strong></td>
<td>• qHBsAg level of $&lt;1500$ IU/ml at week 12</td>
<td>• HBsAg decline of $&gt;1 \log_{10}$ IU/ml and HBsAg $&lt;10$ IU/ml at week 48 (53% HBsAg loss at 3 years post tx).</td>
</tr>
<tr>
<td></td>
<td>• qHBsAg decrease of $&gt;1 \log_{10}$ and qHBsAg $&lt;300$ IU/ml at week 24 of treatment</td>
<td>• 0.5 $\log_{10}$ decrease in qHBsAg at week 12 (89% PPV of sustained response).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• 1 $\log_{10}$ decrease in qHBsAg at week 24 (92 % PPV of sustained response)</td>
</tr>
<tr>
<td><strong>qHBeAg</strong></td>
<td>• qHBeAg level of $&lt;10$ PEIU/ml at week 24 (associated with HBeAg seroconversion in over 50%)</td>
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Nucleoside/Nucleotide analogues (NA’s) for CHB
There are 5 oral drugs which have been used for the treatment of CHB, all belonging to the class of HBV Polymerase inhibitors. Lamivudine, Entecavir and Telbivudine are nucleoside analogues and Adefovir dipivoxil and Tenofovir disoproxil fumarate (TDF) are nucleotide analogues. Due to the significant issues with resistance encountered with Lamivudine, Adefovir and Telbivudine, only the 3rd generation NA’s, Entecavir and TDF are recommended as first line choices for CHB. For patients with prior resistance to lamivudine, adefovir or telbivudine – a second line nucleos(t)ide analogues without cross resistance should be chosen. The main advantages of NA’s over Peg IFN are their oral administration, tolerability and safety and the high rates of virological suppression achieved.

Entecavir
Entecavir is a cyclopentyl guanosine analogue that selectively inhibits the HBV polymerase in its DNA synthesis and reverse transcription functions. It has a high genetic barrier to resistance, requiring at least 3 codon substitutions for resistance to develop. Entecavir resistance in treatment naïve patients is very rare (1.2% after 5 years of tx). However in patients with prior Lamivudine resistance, the presence of the rtL180M and rtM2041I/V codon substitutions means that only one further substitution is required and the development of genotypic resistance is 51% at 5 years (450).

Serological, virological, biochemical, histological response
In the initial phase 3 trial of Entecavir versus Lamivudine in 715 HBeAg positive patients, at 48 weeks the rates of undetectable HBV DNA were 67%, ALT normalisation 68%, and histologic improvement 72% all of which were significantly better than in the Lamivudine group. HBeAg seroconversion occurred in 21% of the entecavir group compared to 18% Lamivudine group (451).

In the HBeAg negative cohort of 648 patients, at 48 weeks 90% had undetectable HBV DNA, 78% normalisation of ALT and 70% had histologic improvement and again all rates were higher than in Lamivudine treated patients (452).

There is a progressive increase in HBV DNA undetectability with Entecavir treatment over time and 5 year followup of the HBeAg positive group (n=183) treated with 1mg Entecavir found that 94% had HBV DNA <300 copies/ml, 80% had normal ALT and a further 23% (in addition to those achieving it is the 1st year) achieved HBeAg seroconversion (340). Numerous other studies have also shown high rates of HBV DNA suppression and ALT normalisation in HBeAg negative patients with progressive increases with increased duration of therapy although it is
interesting to note that ALT normalisation rates are usually about 10% lower than rates of HBV DNA undetectability, In Yuen’s Hong Kong study 98% had undetectable HBV DNA and 86% normal ALT at 3 years (453) and in a Japanese study 100% had undetectable HBV DNA and 91% normalised ALT after 4 years of Entecavir (454).

Histologic improvement with reduction in necroinflammation was seen in 96% of patients and regression of fibrosis by ≥1 point was seen in 88% of patients treated with Entecavir for at least 3 years (455). HBsAg loss is uncommon occurring in 5% of HBeAg positive patients after 96 weeks of treatment and a further 1.4% after 5 years of Entecavir treatment (340). In HBeAg negative patients the rates are lower being approximately 0.3% after 48 weeks of Entecavir (452).

**Tenofovir disoproxil fumarate (TDF)**

TDF is an acyclic adenine nucleotide analogue used in the treatment of both HBV and HIV. Tenofovir at a daily dose of 300 mg daily for 48 weeks resulted in viral suppression (<69 IU/ml) in 76% of HBeAg positive and 93% of HBeAg negative patients (both significantly more than in Adefovir treated patients) (456). ALT normalisation was seen in 68% and loss of HBsAg in 3%. At 3 year followup, HBeAg seroconversion was seen in 26% of HBeAg positive patients and HBsAg clearance in 8% of HBeAg positive patients but no HBeAg negative patients in a European cohort (457). At 5 year followup, sustained viral suppression was seen in 97% of patients and there were no cases of resistance. Furthermore regression of cirrhosis was seen in 74% of patients who had biopsy proven cirrhosis at the the outset of treatment (458). HBsAg loss in Tenofovir treatment is 3.2% at week 48 (456) and 6% following 96 weeks of therapy (459) although the rates in HBeAg negative patients are lower than in HBeAg positive patients.

**Safety/Tolerability of NA’s**

Entecavir has been shown to have a very good safety profile with a long term study (184 weeks) showing that the most common side effects (seen in ≥10%) were upper respiratory tract infections, headache, nasopharyngitis, cough and fatigue (460) however discontinuation was uncommon. The medication does need to be taken on an empty stomach, at least 2 hours after a meal and 2 hours before the next one. Lactic acidosis has been reported in patients with decompensated cirrhosis on Entecavir (461) although other reports have not confirmed this (462).

TDF has been associated with adverse effects of renal dysfunction and phosphate wasting as well as decrease in bone mineral density and osteomalacia however most initial reports of
these were from HIV patients receiving TDF containing regimens. In 6 year followup data of initial TDF registration trials the rates of renal events (defined as ≥0.5 mg/dl increase in serum creatinine from baseline) or eGFR <50 ml/min or phosphorus < 2mg/dl were ≤1.5% overall. Dose reduction may be required in patients who have some underlying renal impairment but TDF has been shown to be relatively safe even in elderly populations and those with decompensated liver disease (463). Monitoring of eGFR and serum phosphate is recommended for those receiving TDF every 3 months for 1 year and 6 monthly thereafter (102). Tenofovir alafenamide is a newly formulated version of Tenofovir and has been shown to have significantly better bone and renal safety than TDF (464) and its availability for HBV is eagerly anticipated.

**HCC risk and treatment with NA’s**
Recent attention has been focused on whether the risk of HCC is reduced in CHB patients treated with entecavir or TDF. A Japanese study showed that compared to a historical cohort of untreated patients, entecavir treatment reduced the HCC risk but this was only in cirrhotic patients (7% vs 39% p<0.001) (465). Similarly, in a study by Wong comparing entecavir treated patients to historical untreated controls, although no difference overall in hepatic events (defined as complications of cirrhosis, HCC or liver related mortality) was seen between the 2 groups, there was a reduction in all these outcomes in the cirrhotic subgroup (466). The reason these studies fail to demonstrate HCC risk reduction in non cirrhotic patients may relate to the low baseline risk in these patients (467). A 2014 Taiwanese paper which was a retrospective nationwide cohort study of over 20,000 CHB patients treated for at least 90 days with nucleoside analogue therapy showed a reduced risk of HCC in this group compared to a cohort of similar size given “hepatoprotective” therapy alone, (which included Silymarin, liver hydrolyste and choline bitartrate) (468). Nevertheless, since HCC risk is not eliminated in patients on long term treatment with NA’s, ongoing surveillance is required.
Partial Virological Response to NA's
Detectable HBV DNA after 48 weeks of NA therapy is considered a partial virological response. Higher proportions of HBeAg negative patients achieve full virological response and in the VIRGIL study group virological response defined as HBV DNA <80 IU/ml was seen at weeks 48, 96 and 144 in 48%, 76% and 90% of HBeAg positive and 89%, 98% and 99% of HBeAg negative patients respectively (469). The recommended management of partial virological response is debated however if the level of virus is ≤1000 IU/ml or there is a continuous decrease in levels, the same NA may be continued. However in patients with viral loads >1000 IU/ml with no ongoing reduction or with underlying cirrhosis, switch to a non cross resistant NA is recommended (469).

Cessation of NA's
Current recommendations from the international societies is that in HBeAg positive patients, NA therapy may be stopped 12 months after the development of Anti-HBe and undetectable HBV DNA (EASL) (102) or 6 months (AASLD, APASL) (101, 103). Cessation of NA therapy is not recommended in HBeAg negative patients according to EASL or AASLD guidelines except for situations where HBsAg is cleared. APASL suggests that following a minimum of 2 years with HBV DNA undetectable on at least 3 occasions at least 6 months apart, NA treatment may be stopped (103). However in a study of 95 HBeAg negative patients who met this criteria, 45% of patients experienced a recurrence of their disease (ALT>2x ULN and HBV DNA >2000 IU/ml) within 1 year of discontinuation of Entecavir (470).

Quantitative HBeAg and HBsAg during NA therapy
In comparison to IFN based therapy, the changes in HBsAg levels during NA treatment of CHB are slower and less pronounced (447, 471). Significant changes in HBsAg levels were noted in HBeAg positive patients treated with entecavir who had an elevated baseline ALT and who subsequently went on to lose HBeAg but no significant change was seen at all in HBeAg negative patients treated with entecavir (472). Others also report only a slow decline in HBsAg levels with NA treatment (473). Baseline qHBsAg levels that can predict virological response, although not necessarily serological response, have been reported by some (474, 475). Some studies have suggested that HBsAg loss may be predicted by a steep decline in HBsAg levels, especially early in the course of NA treatment however other studies have produced conflicting results (402). Use of end of treatment HBsAg levels to predict long term viral suppression have also been studied but further, longer term and larger studies are required to further elucidate the role of quantitative HBsAg in NA therapy. Major interest also centres on
whether qHBsAg can predict which patients can stop NA therapy long term and remain in remission (476).

There have been only a few studies on quantitative HBeAg in NA therapy with mixed results. Early work in older generation NAs including lamivudine, identified baseline qHBeAg levels and certain patterns of qHBeAg decline (descrescendo) that correlated with HBeAg loss (420). Other studies with newer more potent NA’s seem to suggest that on treatment thresholds at 24 or 48 weeks best predict HBeAg seroconversion (474)

**New targets for CHB therapy**

New targets for CHB therapies are the subject of ongoing research with multiple steps in the HBV replication cycle being investigated as potential targets including viral entry, cccDNA (both its formation and regulation by epigenetic mechanisms), nucleocapsid assembly and the RNAse H activity of HBV polymerase. The identification recently of the entry receptor for hepatitis B (and Hepatitis D), sodium taurocholate cotransporting polypeptide (NTCP) has been a particularly exciting advance (63). Inhibition of viral entry into cultured hepatocytes has been shown to occur with Cyclosporin A and this represents an exciting avenue for future drug research into novel therapies for hepatitis B (477). There has also been some in vitro evidence that IFN-α can inhibit established cccDNA(478). Other compounds that can interfere with RNA encapsidation have also been studied with some early promise (479). In addition, targets in the innate and adaptive immune system have been studied, for example there has been evidence of interferon stimulated gene expression and reduction in viral load in chimpanzees treated with an agonist of Toll-like receptor 7 (480). Interest has also been focused on antivirals that target HBsAg levels since it seems intuitive that with a rapid reduction in HBsAg an excess of free anti-HBs may be able to contribute to humoral control of the virus(481). The trends in research are towards finite treatments that will clear cccDNA and HBsAg and restore immune control (482). Proposed definitions of “cure” have also been put forward, with a “functional cure” being a combination of undetectability of viral load, HBsAg and cccDNA, off treatment, with or without HBsAb, although it is recognised that the risk of death from liver disease may only be brought down to that of a person with naturally resolved infection, as opposed to one who has never been infected (483).
Conclusion
Since its discovery 50 years ago, great progress has been made in the understanding of hepatitis B and its natural history, which may be very diverse in different individuals. Contributions from regions around the world have helped elucidate the various phases of hepatitis B infection, with their distinctive risk of liver damage and need for treatment. Yet the tools we have available for establishing the phase of hepatitis B and the likely short to medium term course for an individual patient, remain somewhat blunt (eg ALT or a single viral load). In addition, the constantly dynamic interaction between virus and host leads to difficulty at times in knowing where the balance lies (eg immune control or immune escape phase). More detailed examination of the tests currently available including HBV DNA, should help guide our understanding of their utility and limitations in clinical practice. Further understanding of the impact of genotypes on clinical questions is also critical as it can add information on risk in different clinical contexts (eg likely responsiveness to Interferon treatment, likely time of HBeAg seroconversion). Ongoing study of viral variants and mutations is also important since the evolution of the HBV is likely to be ongoing and likely to proceed in ways that are not easily predictable. The exploration of new ways of measuring viral products and the relevance of these in both the natural history of CHB and during treatment will also enable better characterisation of an individual patient’s likely course. Widespread implementation of a complete infant vaccination schedule will remain the key to future control of the disease. In those who have chronic infection, therapeutic agents that will mimic the immunological prowess of the adult human’s immune system and bring the virus fully under control with HBsAg loss and reduction of risk of HCC and cirrhosis to that of uninfected patients is the ultimate endpoint toward which we must strive.
**Aims of this project**

The aims of this project were:

1. To characterise the cohort of CHB patients at St Vincent’s hospital including using HBV DNA and HBeAg status/ALT to claffigy patients into a phase of disease with the hypothesis that ALT is an imperfect surrogate marker of HBV viraemia and in CHB (Chapter 3).

2. To examine the relationship between HBV DNA and risk of fibrosis in HBeAg positive and negative CHB patients with the hypothesis that the relationship of HBV DNA to fibrosis differs in HBeAg positive and negative disease and that phase of disease, rather than HBV DNA alone is an important predictor of risk of fibrosis (Chapter 4).

3. To examine fluctuations in HBV DNA levels around the threshold of 2000 IU/ml over time in HBeAg negative patients with the hypothesis that this arbitrary threshold is often crossed in HBeAg negative patients but that such fluctuations are minor and may not necessarily reflect a transition to a different phase of CHB (Chapter 5).

4. To characterise the virological profile of the St Vincent’s CHB cohort and in particular to examine differences between the 4 represented genotypes and those with common viral variants and their effect on histological outcomes with the hypothesis that important clinical differences would be evident (Chapter 6).

5. To examine the utility of quantitative HBeAg and HBsAg in defining phases of CHB with the hypothesis that there are differences in the levels of these markers in different phases of the natural history of CHB which may in future prove useful for defining them (Chapter 7).

6. To examine the changes in quantitative HBeAg and HBsAg levels in HBeAg positive patients receiving NA antiviral therapy with the hypothesis that there were differences in the baseline and on treatment changes of these markers in patients who underwent HBeAg seroconversion on therapy compared to those who did not (Chapter 8).
Chapter 2: Materials and Methods

Introduction
General comments on methods used in this thesis are made in this section and the quantitative HBsAg and HBeAg methods are outlined in detail. Other relevant methods are discussed in each chapter in which they are utilised.

Patients - HBV Database:
The majority of the research reported in this thesis, chapters 3-6 inclusive, came about through interrogation of the St Vincent’s hospital Chronic Hepatitis B database. This database is a Microsoft Access database that was first established at St Vincent’s in the mid 1990’s. Clinicians are requested to copy the letters they write on all CHB patients seen through St Vincent’s hospital liver and hepatitis clinic to either the hepatitis nurse consultants, database managers or hepatology fellows. A small number of clinicians also forward letters on patients seen through their private rooms. Data maintained on the database on each patient include demographics, biochemical data (liver function tests), virological information including HBV DNA levels, genotype and mutational status if performed, histology and treatment related data including dates of therapies used and on treatment blood parameters including HBV DNA. This is done both initially and longitudinally with entry of recent test results every time a patient is seen in clinic as notified by the letters to the above mentioned staff who are responsible for the upkeep of the data. Until about 2008, the database was largely maintained manually however in that year an automated program was developed which enabled linking of the HBV database to the pathology system on which the vast majority of blood testing was done and downloading of results was possible. This enabled the database to be updated in a much more time efficient manner, although manual data entry is still also occasionally required.

Quantitative measurement of serum HBsAg
Following HBV DNA testing done in the course of routine clinical care, specimens were stored in the minus 20 freezers at VIDRL for a period of years. After identification of appropriate patients from the database for inclusion in the qHBsAg and qHBeAg studies, the relevant samples were retrieved from the freezers for testing.

Quantitative measurement of serum HBsAg titres were measured by Enzyme Immunoassay (EIA) using the Architect platform (Abbott Laboratories, IL, USA) as per the manufacturer’s instructions. The architect platform involves a chemiluminescent microparticle immunoassay
which is fully automated and has a high throughput capability. A calibration curve standardized to the WHO standard for HBsAg is used and levels of HBsAg are reported in IU/ml with a reactive range of 0.05-250 IU/ml. Serum was tested at dilutions of 1 in 100 or 1 in 1000 with Architect HBsAg Manual Diluent (Abbott Laboratories) which consists of recalcified human plasma that is non reactive for HBsAg, anti HBs and other markers of blood borne viruses. Dilutions were performed prior to testing since most HBsAg titres were above the range of the assay in undiluted form.

**Quantitative measurement of serum HBeAg**

Serum HBeAg levels were measured by commercial EIA (Abbott Diagnostics) on the Architect platform. Modification of Abbott’s commercial kit for qualitative HBeAg detection can be achieved to produce semiquantitative results with a limited range based on the method described by Perillo et al (418). A reference preparation was obtained from the Paul Ehrlich (PE) Institute (Paul –Ehrlich Institute, Lange, Germany), with a defined HBeAg activity of 100 PEIU/ml. An in house working HBeAg standard was then prepared from a pool of known high titre HBeAg positive specimens and calibrated against the PE reference preparation. The HBeAg titre of the working standard was used to generate a calibration curve within the linear range of the assay. Dilutions of the in house working standard (diluted twofold from 1 in 2 to 1 in 4096 were prepared using the Architect HBsAg Manual diluent (Abbott Diagnostics, Sligo, Ireland). On every day that clinical samples were tested, a standard curve was generated with aliquots of the diluted reference sample (1 in 16 – 1 in 2048). The assay result for each sample was converted to PEIU/ml by linear regression. Samples were tested at dilutions of 1 in 100 using the ARCHITECT HBsAg Manual Diluent (Abbott Diagnostics) for serum HBeAg quantification and those which were non reactive at this dilution were tested at 1 in 10 or 1 in 2. Non-reactive human serum was used as the negative control.
Chapter 3: Cross sectional description of the chronic hepatitis B cohort in an Australia tertiary referral centre.

Introduction
Chronic hepatitis B (CHB) is an important health problem in Australia with an estimated prevalence of 90,000-160,000 (44). The majority of CHB patients in Australia are migrants who have acquired the virus in the perinatal or early childhood period by vertical or horizontal transmission in their overseas country of birth. Routine screening with Hepatitis B surface antigen (HBsAg) and hepatitis B surface antibody (anti-HBs) of individuals from areas of high and intermediate prevalence rates including South Asia, the Mediterranean and Africa is recommended (98). An increased burden of disease, in particular from the complication of hepatocellular carcinoma (HCC) is predicted over the next 2 decades (49) and the health economic implications of chronic hepatitis B are significant (484). Data from the USA suggests that only 28% of HBsAg positive patients receive further laboratory evaluation of their HBV infection (485). HBV DNA testing has been listed for a Medicare rebate since April 2008 and the sensitivity of assays used has increased over time. When used alongside HBeAg status and ALT, HBV DNA testing allows a patient to be classified into a phase of chronic hepatitis B. These phases are the HBeAg positive immune tolerant and immune clearance phases and the HBeAg negative immune control and immune escape phases (99) and they have implications for monitoring and treatment of patients. Prior to the widespread accessibility of HBV DNA testing, ALT was used as a surrogate marker for hepatitis B disease activity, however this has many limitations because not all patients with CHB have persistently elevated aminotransferases (486), notably those in the HBeAg positive immunotolerant phase and some of those with HBeAg negative CHB. HBV DNA has also been shown to be a strong predictor of the risk of cirrhosis (169). Thus HBV DNA testing is crucial in the evaluation of a patient with CHB; to classify the patient into a phase of disease, to establish a causal relationship between HBV infection and the liver disease, as a guide to the need for therapy, and to predict the risk of complications such as cirrhosis and HCC. In 2005, a description of the CHB cohort of the St Vincent’s Hospital was reported by Bell et al which used either HBV DNA or elevated ALT to define active replication (48). We hypothesised that sensitive HBV DNA tests are important for detecting current viraemia and enabling classification into a phase of disease and furthermore that ALT may not be a good surrogate marker of HBV viral replication as measured by serum HBV DNA.
Aims

1. To classify patients into a phase of CHB based on HBV DNA, HBeAg status and ALT level and to examine differences between the groups.

2. To evaluate the differences in proportions of patients found to be replicating by 2 different HBV DNA assays.

3. To examine the relationship between HBV DNA and ALT in HBeAg positive and negative CHB cross sectionally.
Methods

Patients
Demographic details, including date of birth, sex and ethnic origin of chronic hepatitis B patients seen through the St Vincent’s Hospital’s liver clinics are recorded in a Microsoft Access database. HBV serology including Hepatitis B e Antigen (HBeAg) and Hepatitis B e Antibody (anti-HBe) are recorded as well as HBV DNA levels and serum Alanine Aminotransferase (ALT) levels.

For the purposes of examining patients based on the phase of disease for this study, the following classification used was:

Immune tolerant (Phase 1): HBeAg positive, HBV DNA detected and ALT ≤ Upper Limit of Normal (ULN) which was defined as 35 IU/ml.

Immune clearance (Phase 2): HBeAg positive, HBV DNA detected and ALT > ULN.

Immune control (Phase 3): HBeAg negative, HBV DNA not detected by bDNA assay.

Immune escape (Phase 4): HBeAg negative, HBV DNA detectable by bDNA assay.

Hepatitis B Virus Serological and DNA testing
HBsAg is measured using a commercially available immunoassay (Abbott Laboratories, North Chicago, IL, USA) and HBeAg and Anti-HBe by an immunoassay produced by BioMerieux Clinical Diagnostics (Marcy l’Etoile, France). Since April 2004 HBV DNA levels have been measured by the Bayer Versant HBV DNA 3.0 assay (bDNA signal amplification probe method). The dynamic range is 351 to 17,543,900 IU/ml. Patients who were above or below the dynamic range of the assay were assigned the upper and lower limit as their HBV DNA value respectively. One IU/ml is equivalent to 5.6 copies/ml. Between May 2000 and April 2004 HBV DNA levels were performed using a capture hybridisation assay (Digene Hybrid Capture, Digene Diagnostics Inc, Beltsville USA) with a lower limit of detection of 0.5 pg/ml (approximately 25,000 IU/ml) and rates of HBV detectability on a separate cohort of patients tested with the Digene assay were compared to rates using the Bayer Versant assay. All tests pertaining to HBV DNA were performed at the Victorian Infectious Diseases Reference Laboratory (VIDRL).

Statistical Analysis
Statistical analysis was performed with the statistical package SPSS (version 17.0, SPSS Inc, Chicago, IL). The corrected chi square test or two sided Fisher’s exact test was used to compare
categorical data, while the student’s t-test or one way ANOVA was used for group comparisons of parametric quantitative data and the Mann-Whitney or Kruskal-Wallis test for similar comparisons of non-parametric data. Results were presented as mean or median whenever appropriate. In all cases tests of significance were two-tailed with a level at <0.05.
Results

Patient characteristics
There were 348 patients with a complete baseline dataset of HBV DNA performed using the Bayer Versant assay, HBeAg status, ALT and demographic features available for analysis. They were examined for differences in their characteristics based on the phase of disease (Table 3.1) as defined in the Methods. The patient age differed significantly between the four groups (p<.001); HBeAg positive Immune tolerant (mean age 32) and immune clearance (mean age 35) groups were significantly younger than the HBeAg negative immune control and immune escape groups (mean ages 42 and 45 respectively). Post hoc testing showed that the 2 HBeAg positive groups did not differ significantly in age from each other, nor did the 2 HBeAg negative groups. The proportion of patients under 30 years of age was 25% overall. Gender makeup of the 4 phase groups also differed (p=0.019) with the immune tolerant group having the lowest proportion of males (40%) and the immune escape having the highest proportion at 68%. The ethnic makeup of the total group was Asian 75%, Non Mediterranean Caucasian 7%, Mediterranean 8%, African 3% and other ethnicities 7%. The ethnic makeup of the phase groups differed significantly (p=0.001) with fewer Asians in the immune control groups (55%) compared to the other groups and proportionally more Africans in this group although the numbers of Africans overall was small.

Distribution of baseline HBV DNA and ALT.
All patients with detectable HBV DNA by the Bayer Versant assay (n=271/348) were examined for the distribution of their HBV DNA in HBeAg positive (n=96) and negative (n=175) groups (Table 3.2). In the HBeAg positive replicating group the majority of patients (72%) had an HBV DNA of ≥ 10^{7} IU/ml and only 11% of patients had an HBV DNA of <10^{5} IU/ml. In the HBeAg negative replicating group the majority (61%) of patients had an HBV DNA of < 10^{5} IU/ml. Of the 348 patients tested with the Bayer Versant assay, 77 (22%) had HBV DNA < 357 IU/ml. All except 1 of these patients were HBeAg negative. 38% of the patients with undetectable HBV DNA had an abnormal ALT (>35 IU/ml), including 13% with an ALT 2-3 x ULN suggesting secondary liver pathology.

The distribution of baseline ALT was also examined in the group of 348 patients (Table 3.3). The proportion of HBeAg positive replicating patients who had a normal (≤35 IU/ml) baseline ALT was 20%. In the HBeAg negative replicating group, 35% of patients who had a detectable HBV DNA had a concomitant ALT that was in the normal range. The degree of elevation of ALT
was modest overall with only 34% and 18% of the HBeAg positive and HBeAg negative replicator groups respectively having an ALT of >3 times the upper limit of normal.

**Correlation between baseline HBV DNA and ALT in HBeAg positive and HBeAg negative replicating groups.**
Correlations between ALT and HBV DNA performed on the Bayer Versant HBV DNA tested cohort showed a small positive relationship between ALT and HBV DNA in the HBeAg negative replicating group (Pearson correlation coefficient= 0.240, p=0.01) but no relationship between them in HBeAg positive replicating patients (p=0.976).

**Comparison of HBV DNA detectability rates between Bayer Versant and Digene HBV DNA Assays**
The overall rate of HBV DNA detectability in the main cohort of patients tested with the Bayer Versant HBV DNA assay was 78% (271/348). A further 235 patients had been seen in clinics and had had HBV DNA performed prior to April 2004 on the Digene II assay and in this group the proportion of patients with detectable HBV DNA was 48% (113/235). Figure 2.1 shows HBV DNA detectability by the 2 different HBV DNA assays across different age brackets revealing approximately 30% more patients have detectable HBV DNA using the more sensitive Bayer Versant bDNA assay in all age brackets.

**Discussion**
Our study of a large Australian cohort of chronic hepatitis B patients reveals three things which we feel are pertinent to the initial evaluation of CHB. Firstly, CHB should ideally be conceived of as having 4 phases of disease (487) and we outline in an Australian adult cohort some of the demographic, virological and serological differences in patients in these phases. Secondly, the serum ALT and HBV DNA do not correlate strongly with each other. Thus HBV DNA testing to establish the presence of viral replication is important, particularly in HBeAg negative CHB where fluctuating or even persistently normal ALT levels can be seen in patients who have detectable serum HBV DNA. Thirdly, the sensitivity of HBV DNA assays has improved in recent years and many patients previously thought to be non-viraemic may actually be shown to have detectable HBV DNA in their serum and require further assessment.

Chronic hepatitis B can seem a complex condition due to changes in testing, varying nomenclature and definitions for the natural history phases and the evolution in the treatments available. Further evaluation of the HBsAg positive patient should be performed and we believe initial assessment should include HBeAg, HBV DNA and ALT in order that patients may be classified into a phase of disease. Our data highlights some of the ways in
which these phases of disease differ. Firstly the HBeAg positive patients are younger than HBeAg negative patients. HBeAg seroconversion is usually followed by a period of immune control with very low levels of virus. Although ongoing viral replication and chronic HBeAg negative hepatitis B (the immune escape phase) may develop close to the phase of HBeAg loss (183), it usually occurs as a relapse some years after a period of quiescence and this is reflected in the finding that our patients in the immune escape phase are significantly older than the HBeAg positive patients. Male gender is associated with a higher risk of hepatitis relapse in HBeAg negative patients and in our cohort the proportion of males is highest in the immune escape group at 68% (488). The immune escape phase comprises half of our cohort which underlines the increasing problem that HBeAg negative chronic hepatitis is becoming in many parts of the world (180). The ethnic makeup of the cohort of patients at our centre has changed over the past 5 years when compared with a report in 2005 (48). There has been an increase in the number of Asians overall and fewer patients of Mediterranean and non Mediterranean Caucasian origin. This is likely to reflect patterns of migration to Australia. In the Australian literature other reports of HBsAg positive patients have included cohorts from prison settings (489) and injecting drug user groups (490). In these groups adult acquired hepatitis B is more likely to be prevalent than in our cohort of predominantly perinatally or childhood acquired disease. A report of HBsAg positive patients screened at the endoscopy unit of a tertiary hospital showed that 70% of the 45 currently infected patients were born in the Asia Pacific region (47). HBeAg was not tested in these patients therefore their phase of disease was not known. Another report of pregnant women referred to Liverpool hospital's liver clinics in NSW (29) showed that the majority of those were of South East Asian background and 29% were HBeAg positive. Patients were not strictly classified into a phase of disease but were placed into groups of low (<10^5 copies/ml, approximately 17857 IU/ml), high and very high viral loads.

The relationship between ALT and HBV DNA levels is different in HBeAg positive and negative disease. HBeAg positive patients with rare exceptions are all replicating and a normal ALT signifies immune tolerance. In HBeAg negative patients however we show that on a single evaluation an elevated ALT cannot be equated with elevated HBV DNA, since 35% of patients with detectable virus using a sensitive assay, have an ALT below the ULN. The converse finding that 38% of patients with undetectable virus have an abnormal ALT once again underscores the importance of measuring HBV DNA so that other possible causes of liver disease (e.g Non Alcoholic Steatohepatitis or Hepatitis Delta) may not be mistakenly attributed to Hepatitis B.
Thus, as has been shown by others (171) we confirm that there is a correlation between HBV DNA levels and ALT in HBeAg negative patients but it is weak.

Increasingly sensitive HBV DNA assays reveal that active replication is ongoing in the majority of patients with chronic hepatitis B. Patients deemed to be an “inactive carrier” of Hepatitis B (without active viral replication) on the basis of an older HBV DNA assay should be re-tested with a more sensitive assay. Recommendations in the past have been that inactive carriers should be monitored yearly to detect transition to the immune escape phase of disease (229) although more recent algorithms would include incorporation of newer tests such as quantitative HBsAg to determine risk of reactivation.

The REVEAL-HBV study in 2006 identified an HBV DNA level of >=10^4 copies/ml (1.78 x 10^3 IU/ml) as portending an increased risk of HCC (235) and in both our HBeAg positive and negative replicating patients the vast majority are above this threshold. There has been debate about the HBV DNA threshold which should be used to define inactive disease (Phase 3, the immune control phase) with recommendations being reduced over the past few years from 100,000 copies/ml (17,857 IU/ml) (95) to 2000 IU/ml (109). Current American guidelines use an HBV DNA level of 2000 IU/ml to distinguish between HBeAg negative patients with chronic HBeAg negative hepatitis (Immune escape) versus those in the “inactive HBsAg carrier” (Immune control) phase (101). However this definition of “inactive HBsAg carrier” also includes the criteria of persistently normal ALT/AST levels and a liver biopsy showing the absence of significant hepatitis. Strict definitions of the phase groups usually involve serial ALT monitoring to establish a persistently normal or elevated state and liver histology findings. It is important to note that our definitions do not include these criteria but do reflect to a degree, real life practice, in which a clinician will make an early estimation of a patient’s phase of disease based on cross sectional blood testing available. The HBV DNA thresholds used in our definitions (detectability or not) also differed from current international guidelines. This was partly due to the fact that at the original time these analyses were performed, there had only been a recent move away from older, less sensitive HBV DNA assays (with LLD of approximately 25,000IU/ml) and the accepted levels of HBV DNA to define patient risk were still in the process of being translated into clinical practice.

The decision to treat in Hepatitis B is an individual one which needs to incorporate several factors including the risk of development of cirrhosis and HCC, the degree of histological fibrosis and inflammation, HBV DNA and ALT levels, and patient factors such as age and
likelihood of compliance (487). Although usually not candidates for treatment, patients in the HBeAg positive immune tolerant and HBeAg negative immune control phases still require regular monitoring.

**Conclusion**

We describe a uniquely Australian experience of chronic Hepatitis B in a large diverse group of patients using demographic, biochemical and virological characterisation cross sectionally to identify phase of disease as this most often reflects the real life practice in the clinical setting. The ALT is not always a good guide to a patient’s HBV DNA level so should not be relied on exclusively as a surrogate marker of viral replication. In order that CHB patients access and benefit from advances, clinicians must take the steps of screening at risk populations and performing an appropriate initial evaluation of them which should include HBV DNA level. Furthermore, improvements in the sensitivity of tools available for evaluation of CHB, in particular HBV DNA assays should be taken advantage of since they may add new information about clinical state for patients with this usually lifelong disease.
Table 3-1: Patient characteristics by phase of disease (n=348)

<table>
<thead>
<tr>
<th>Total bDNA cohort n=348</th>
<th>Immune tolerant</th>
<th>Immune clearance</th>
<th>Immune control</th>
<th>Immune escape</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbers (%)</td>
<td>20 (6%)</td>
<td>77 (22%)</td>
<td>76 (22%)</td>
<td>175 (50%)</td>
<td></td>
</tr>
<tr>
<td>Average Age years (SD)</td>
<td>32 (11.65)</td>
<td>35 (11.51)</td>
<td>42 (14.20)</td>
<td>45 (12.83)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>Number (%) Males</td>
<td>8 (40%)</td>
<td>40 (52%)</td>
<td>47 (62%)</td>
<td>119 (68%)</td>
<td>P=0.019</td>
</tr>
<tr>
<td>Number (%) Asian</td>
<td>15 (75%)</td>
<td>58 (75%)</td>
<td>42 (55%)</td>
<td>145 (83%)</td>
<td></td>
</tr>
<tr>
<td>Number (%) Non Mediterranean Caucasian</td>
<td>3 (15%)</td>
<td>10 (13%)</td>
<td>7 (9%)</td>
<td>5 (3%)</td>
<td>P=0.001</td>
</tr>
<tr>
<td>Number (%) Mediterranean</td>
<td>0</td>
<td>5 (7%)</td>
<td>11 (15%)</td>
<td>12 (7%)</td>
<td></td>
</tr>
<tr>
<td>Number (%) African</td>
<td>0</td>
<td>1 (1%)</td>
<td>6 (8%)</td>
<td>3 (1.5%)</td>
<td></td>
</tr>
<tr>
<td>Number (%) Other ethnicity</td>
<td>2 (10%)</td>
<td>3 (4%)</td>
<td>10 (13%)</td>
<td>10 (5.5%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3-2: Distribution of baseline HBV DNA in patients with detectable HBV DNA by Bayer Versant Assay (n=271).

<table>
<thead>
<tr>
<th>Baseline HBV DNA (IU/ml)</th>
<th>HBeAg positive replicators (HBV DNA &gt; 357) (n=96)</th>
<th>HBeAg negative replicators (HBV DNA &gt;357) (n=175)</th>
</tr>
</thead>
<tbody>
<tr>
<td>357 to &lt;1000</td>
<td>1 (1%)</td>
<td>20 (11.5%)</td>
</tr>
<tr>
<td>1000-9999</td>
<td>3 (3%)</td>
<td>44 (25%)</td>
</tr>
<tr>
<td>10,000-99,999</td>
<td>7 (7.5%)</td>
<td>42 (24%)</td>
</tr>
<tr>
<td>100,000-999,999</td>
<td>10 (10.5%)</td>
<td>25 (14%)</td>
</tr>
<tr>
<td>1mill-9.99mill</td>
<td>6 (6%)</td>
<td>20 (11.5%)</td>
</tr>
<tr>
<td>&gt;/=10 million</td>
<td>69 (72%)</td>
<td>24 (14%)</td>
</tr>
<tr>
<td>Total</td>
<td>96 (100%)</td>
<td>175 (100%)</td>
</tr>
</tbody>
</table>
Table 3-3: Distribution of baseline ALT in patients tested with Bayer Versant assay.

<table>
<thead>
<tr>
<th>Baseline ALT level (IU/ml)</th>
<th>HBeAg positive replicators (HBV DNA &gt; 357 IU/ml) n=96</th>
<th>HBeAg negative replicators (HBV DNA &gt; 357) n=175</th>
<th>HBV DNA undetectable (bDNA Assay) n=77</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT≤35 (&lt;ULN)</td>
<td>19 (19.8%)</td>
<td>61 (34.9%)</td>
<td>48 (62.3%)</td>
</tr>
<tr>
<td>ALT 36-52 (1 to &lt;1.5 x ULN)</td>
<td>15 (15.6%)</td>
<td>34 (19.4%)</td>
<td>11 (14.3%)</td>
</tr>
<tr>
<td>ALT 53-70 (1.5 to &lt;2 x ULN)</td>
<td>11 (11.4%)</td>
<td>32 (18.3%)</td>
<td>8 (10.4%)</td>
</tr>
<tr>
<td>ALT 71-105 (2 to 3 x ULN)</td>
<td>18 (18.8%)</td>
<td>16 (9.1%)</td>
<td>5 (6.5%)</td>
</tr>
<tr>
<td>ALT &gt;105 (&gt;3 x ULN)</td>
<td>33 (34.4%)</td>
<td>32 (18.3%)</td>
<td>5 (6.5%)</td>
</tr>
<tr>
<td>Grand Total</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>
Figure 3-1: Proportion of patients deemed to be replicating by assay

Figure legend: This figure shows the proportion of patients who had detectable HBV DNA by 2 different HBV DNA assays. Across most age groups, approximately 30% more patients had detectable HBV DNA when tested with the bDNA assay compared to testing by the Digene II assay.
Chapter 4: Increasing hepatitis B viral load is associated with risk of significant liver fibrosis in HBeAg negative but not HBeAg positive chronic hepatitis B.

Introduction
The development of advanced fibrosis or cirrhosis of the liver is one of the most significant sequelae of chronic hepatitis B (CHB) (491). The annual incidence of cirrhosis in patients with CHB has been estimated at 1.3 -2.4% (492). Although the cumulative 5 year survival rate for patients with compensated cirrhosis is 84% (493), in patients with decompensated cirrhosis, this drops to 14-35% (232, 493). The prevalence of significant histological lesions has been shown to be high in biopsied cohorts (173) and active inflammation is thought to be the driving force to fibrosis (494).

A number of studies have examined predictors of progression to advanced fibrosis (229, 495-498) and much attention has been focused on a large, prospective study of the incidence of cirrhosis in CHB published by the REVEAL-HBV group (169). This study followed 3582 subjects for a mean of 11 years and reported that cirrhosis was strongly correlated with the level of circulating virus, with increasing incidence of cirrhosis being found in patients with higher levels of virus at entry into the study. It should be noted that 85% of this cohort was HBeAg negative and the study was restricted to patients over 30 years of age. Cirrhosis was diagnosed on the basis of ultrasound. It is unclear whether this data can be applied to younger, HBeAg positive patients.

The current literature in HBeAg positive patients does not support an association between HBV DNA and fibrosis. Two studies of over 300 predominantly HBeAg positive patients did not find HBV DNA to be a predictor of fibrosis (499, 500) In fact, in HBeAg positive patients it has been suggested that lower HBV DNA, reflecting increased immune response, is correlated with fibrosis. Wang et al studied 28 HBeAg positive, immunotolerant patients and reported that lower serum HBV DNA level, along with age>30 years was independently correlated with stage 2 fibrosis or more on liver biopsy (501).

In recent clinical practice there has been a focus on HBV DNA levels as the trigger for antiviral therapy. We postulated that this is only appropriate in patients with HBeAg antigen negative CHB.
Aims
The aims of our study were:

1. To evaluate the prevalence of significant fibrosis, cirrhosis and inflammation in a well characterised cohort of HBeAg positive and HBeAg negative patients who had undergone liver biopsy, divided into three groups according to phase of disease (see below).
2. To examine the relationship between inflammation and fibrosis on an individual level.
3. To evaluate the interaction between HBeAg status, HBV DNA, ALT and significant histological fibrosis and inflammation.
4. To identify predictors of significant fibrosis, inflammation and cirrhosis in HBeAg positive and negative groups.
Methods

Patients
St Vincent’s Hospital, Melbourne has maintained a database of CHB patients seen in their liver clinics since 1996. CHB patients on whom a full set of baseline demographic (including date of birth, sex, ethnic origin and country of birth), serological, virological and histological details were available as at 1 October 2008 were included for analysis. The baseline clinic recordings of HBV serology including hepatitis B e antigen (HBeAg) and antibody (anti-HBe) as well as HBV DNA levels and serum ALT levels were used. Liver biopsy was performed pre-treatment at the discretion of the clinician and in some situations had been performed prior to the patient being seen at the liver clinic. In Australia, liver biopsy was a requirement for subsidised antiviral therapy until 2008, and therefore most liver biopsies were performed in patients being assessed for treatment. For analyses of patient characteristics and the overall prevalence of significant fibrosis/inflammation and cirrhosis, the patients were divided into the following 3 groups on the basis of HBeAg status and HBV DNA level:

- HBeAg positive/DNA detected (HBeAg positive (EP); immune tolerant and immune clearance phase)
- HBeAg negative/DNA ≤ 25,000 IU/ml (HBeAg negative low replicators (ENLR) or immune control phase)
- HBeAg negative/DNA >25,000 IU/ml (HBeAg negative high replicators or (ENHR); immune escape phase).

For other analyses the total cohort was simply split according to HBeAg status. The prevalence of significant fibrosis and inflammation was examined separately in HBeAg positive and negative groups in strata of HBV DNA increasing in 1 log increments from <10^5 to ≥10^7 IU/ml and in strata of ALT increasing from below the upper limit of normal (ULN) to >3 x ULN. The study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the St Vincent’s Hospital Human Research and Ethics Committee.

Hepatitis B Virus Serological and DNA testing
Hepatitis B surface antigen (HBsAg) was measured using a commercially available immunoassay (Abbott Laboratories, North Chicago, IL, USA) and HBeAg and anti-HBe by an immunoassay produced by BioMerieux Clinical Diagnostics (Marcy l’Etoile, France).
All tests pertaining to HBV DNA were performed at the Victorian Infectious Diseases Reference Laboratory (VIDRL). Prior to April 2004, HBV DNA levels were performed using a capture hybridisation assay (Digene Hybrid Capture, Digene Diagnostics Inc, Beltsville USA) with a lower limit of detection of 0.5 picograms/ml (approximately 25,000 IU/ml). Patients tested using the Digene assay who were below the limit of detection were assigned the value of 25,000 IU/ml. Any patient with an undetectable HBV DNA on Hybridisation assay prior to Jan 2000 was excluded unless a concomitant PCR was also negative to ensure exclusion of older generation, less sensitive hybridisation assays. Subsequent to April 2004 the HBV DNA viral load was performed by a bDNA signal amplification probe method (Bayer Versant HBV DNA 3.0 assay). The dynamic range is 351 to 17,857,140 IU/ml. One IU/ml is equivalent to 5.6 copies/ml. Patients tested using the bDNA assay whose HBV DNA levels were below and above the limits of detection were assigned the values of 351 and 17,857,140 IU/ml respectively for analyses involving HBV DNA.

**Liver biopsy**

Formalin fixed, paraffin embedded liver biopsies were sectioned and stained according to usual histology methods and evaluated by a hospital pathologist. The majority of histological lesions were graded according to the classification proposed by the METAVIR study group (502). Activity is graded applying an algorithm which includes the severity of piecemeal and lobular necrosis resulting in a scale of 0-3 (0=none, 1= mild, 2=moderate and 3=severe activity). Significant inflammation was defined as activity scores of A2/3.

Fibrosis is scored from 0-4 (0 being no fibrosis, 1=enlarged fibrotic portal tracts, 2= periportal or portal-portal septa but intact architecture, 3 = fibrosis with architectural distortion but no obvious cirrhosis, 4 = probable or definite cirrhosis). Significant fibrosis was defined as fibrosis score of F≥2. Additional biopsies in which fibrosis had initially been staged using an alternative staging method (e.g. Scheuer’s) had fibrosis levels revised into Metavir scoring system by one of the St Vincent’s Hospital pathologists.

**Statistical Analysis**

Data entry and statistical analysis were done with the statistical package SPSS (version 17.0, SPSS Inc, Chicago, IL). The corrected chi square test or two sided Fisher’s exact test was used to compare categorical data, while the student’s t- test or one way ANOVA was used for group comparisons of parametric quantitative data and the Mann-Whitney or Kruskal-Wallis test for similar comparisons of non-parametric data. Logistic regression models were used for multivariable analysis. Logistic regression models were fitted separately for HBeAg positive and
negative patients with significant fibrosis, inflammation and cirrhosis as the outcomes and the following as potential explanatory variables: age, gender, ethnicity, HBV DNA and ALT. Results were presented as mean or median whenever appropriate. In all cases tests of significance were two-tailed with a level at <0.05.
Results

Patient characteristics

394 (HBeAg positive n=198 and HBeAg negative n=196) patients were available for analysis of histological fibrosis and 306 (HBeAg positive n= 143 and HBeAg negative n=163) for inflammation. The liver biopsies were done at a median time of 2.92, 8.03 and 3.04 months following baseline HBV DNA testing in the EP, ENLR and ENHR groups respectively. 155 patients were tested with Bayer Versant 3.0 HBV DNA assay and 239 patients with Digene hybridisation capture assay. The distribution between the patients in the 3 HBeAg and DNA based groups showed that 50% of patients were EP, 17% were ENLR and 33% were ENHR (Table 4.1). The ethnic makeup of these groups did not differ significantly (p=0.189) with the majority (>74%) of patients being of Asian ethnicity in all groups. The gender proportions of the 3 groups varied with significantly more males in the ENHR (79%) than the EP group (62%) (p=0.001).

EP patients were the youngest (mean 33 years) and the ENHR were the oldest (mean 47 years) with ENLR patients being intermediate (mean 42 years) (p<0.001). Using post hoc pair wise comparisons, the difference of means between the EP and ENLR (9.1 years), EP and ENHR (13.6 years) as well as ENLR and ENHR (4.6 years) groups were all highly statistically significant (p<0.001).

The serum HBV DNA was significantly higher in EP patients than ENHR patients (p<0.001) and the ALT was also significantly different in the 3 groups (P<0.001) with the EP patients having the highest ALT and the ENLR having the lowest ALT levels (Table 4.1)

Patient characteristics - Genotype

202 patients had genotyping available. 110 (54%) were in the EP group, 72 (36%) in the ENHR and 20 (10%) in the ENLR group (Table 4.2). The most common genotypes were B and C accounting for 37% of the total group each. Genotype D represented 16% of the group and genotype A 6%. A small number of patients had mixed genotypes; B/C in 6 cases and C/D in 1 case. The predominant genotype amongst EP patients was genotype C representing 45% of the group, followed by genotype B with 33%. Genotypes D and A represented 12% and 8% respectively in this group.

70% of the ENLR group was genotype B and 20% genotype D. There was only one genotype C patient in the ENLR group and the only Genotype G patient was also in the ENLR group. Genotypes B and C were the most common in the ENHR group at 35% and 33% respectively.
Patients with genotypes B and C were of Asian ethnicity in over 95% of cases. Genotype A patients were predominantly Caucasian (58%) and 33% were Asian with the remaining 8% being of Mediterranean origin. Genotype D patients were predominantly Mediterranean (73% of cases), with minorities also being Asian and Caucasian.

Prevalence of significant fibrosis and inflammation in HBeAg/ HBV DNA groups
The highest prevalence of F2/3/4 fibrosis was found in the ENHR group at 58% (Figure 4.1). This was significantly higher than that in the EP group of 40% (p=0.002) and in the ENLR group of 36% (p=0.005). The prevalences of F2/3/4 fibrosis in the EP and ENLR groups were not significantly different (p=0.561). The prevalences of cirrhosis in the EP, ENLR and ENHR groups were 11%, 8% and 18% respectively but these differences did not reach statistical significance (p=0.069) (Table 4.1). The prevalence of A2/3 inflammation was also not significantly different amongst the 3 groups being 34%, 24% and 32% in the EP, ENLR and ENHR groups respectively (p=0.382).

Relationship of histological inflammation and fibrosis scores.
Increasing grades of inflammation were strongly associated with increasing degrees of fibrosis on liver biopsy (p<0.001). No patients with F0 fibrosis had moderate or severe inflammation, while 76% of cirrhotic patients had A2 or A3 inflammation (Figure 4.2).

The positive correlation between inflammation and fibrosis was also seen in analyses confined to both the HBeAg positive (n=143) (p<0.001) and negative groups (n=163) (p<0.001). The mean Metavir activity score also increased with increasing fibrosis, being 0.65, 1.05, 1.45, 1.64 and 1.95 in patients with F0, F1, F2, F3 and F4 fibrosis respectively.

Prevalence of significant fibrosis and inflammation in different strata of HBV DNA in HBeAg positive and negative disease
The prevalence of F2/3/4 fibrosis increased from 38% in HBeAg negative patients with HBV DNA of <10^5 IU/ml to 71% in those with HBV DNA of ≥10^7 IU/ml (Table 4.3). In HBeAg positive patients however the pattern was the reverse; 75% of patients in the HBV DNA strata of <10^5 IU/ml had F2/3/4 fibrosis while only 38% in the strata of HBV DNA≥10^7 IU/ml did. Using Logistic regression a significant interaction was found between the HBeAg status and HBV DNA level with F2/3/4 fibrosis as the outcome (p<0.001). In HBeAg negative patients, the odds of F2/3/4 increased by 1.42 (95% CI 1.16- 1.74) for every 1 log increase in HBV DNA (p=0.001). In contrast, in HBeAg positive patients the odds of F2/3/4 decreased; OR 0.71 (0.52-0.97) per 1 log increase in HBV DNA (p=0.03). Thus the association between HBV DNA and F2/3/4 was positive in HBeAg negative disease, but negative in HBeAg positive disease.
The prevalence of A2/3 inflammation also increased from 26% in HBeAg negative patients with HBV DNA of <10^5 IU/ml to 40% in those with HBV DNA of >10^7 IU/ml (Table 4.3). In HBeAg positive patients however the pattern was the reverse; 71% of patients in the HBV DNA strata of <10^5 IU/ml had A2/3 while only 31% in the strata of HBV DNA >10^7 IU/ml did. Using logistic regression a significant interaction was found between the HBeAg status and HBV DNA with A2/3 inflammation as the outcome (P = 0.013). In HBeAg negative patients the odds ratio of A2/3 increased by 1.17 (0.93-1.48) for every 1 log increase in HBV DNA (p=0.18). In HBeAg positive patients the odds of A2/3 decreased 0.69 (0.49 – 0.98) for every 1 log increase in HBV DNA (p=0.035)

Prevalence of significant fibrosis and inflammation in different strata of ALT in HBeAg positive and negative disease.
The interaction between ALT and the presence of either F2/3/4 or A2/3 was not significantly different in HBeAg positive and negative groups (p=0.096 and 0.339 respectively). Therefore the prevalence of F2/3/4 and A2/3 according to strata of ALT was examined in the overall cohort. The prevalence of F2/3/4 was lowest in patients with an ALT below ULN at 33% and highest in patients with ALT > 3 x ULN at 52% (Table 4.4). A2/3 inflammation in the overall cohort was lowest in patients with an ALT below ULN (20%) and highest in patients with ALT > 2 x ULN (38-40%) (Table 4.4). The odds of F2/3/4 fibrosis increased by 2.80 (95% CI 1.6-4.9) for every 10-fold increase in ALT (p<0.001). The odds of A2/3 inflammation increased by 2.90 (95% CI 1.51-5.58) for every 10-fold increase in ALT (p<0.001).

Predictors of significant fibrosis and inflammation in patients in HBeAg positive and negative disease.
Using Logistic regression (see Methods) in the HBeAg positive group, the only predictor of significant fibrosis was age (p<0.001). The odds of F2/3/4 increase by 1.057 for each increase in age of one year or 1.73 for an increase of 10 years. The proportion of HBeAg positive patients with F2/3/4 in groups aged <20, 20-29, 30-39, 40-49, 50-59 and > 60 yrs was 10%, 30%, 41%, 56%, 63% and 80% respectively. The numbers of patients in these groups was 10, 79, 56, 32, 16 and 5 respectively.

In the HBeAg negative group, the predictors of significant fibrosis were HBV DNA which had an Odds Ratio of 1.3 for every 1 log increase in HBV DNA (p=0.03) and ALT with an Odds Ratio of 2.9 for every 10 fold increase in ALT (p=0.02)
In the HBeAg positive group, ALT was an independent predictor of A2/3 (p = 0.003) with an Odds Ratio of 5.33 for every 10 fold increase in ALT. In HBeAg negative disease, there were no statistically significant predictors of A2/3 at the 5% level.

Predictors of cirrhosis in patients with HBeAg positive and negative CHB.
A further logistic regression (see Methods) was fitted for HBeAg positive (n=21) and negative (n=28) patients with cirrhosis (F4 fibrosis) as the outcome. In the HBeAg positive group, once again age was a strong predictor of cirrhosis (p=0.001). Lower HBV DNA was also found to be a predictor with a significant negative association being seen between HBV DNA and cirrhosis (p=0.002). The Odds Ratio for every 1 log increase in HBV DNA was 0.498. In the HBeAg negative group, the only significant variable was age (p=0.03).

Discussion
This study demonstrates that progressive liver disease in CHB is driven by three main factors: age - a surrogate of disease duration; immune activation - as measured by ALT; and viral replication, measured by HBV DNA levels. However, the influence of HBV DNA levels differs in HBeAg positive and negative disease. Increasing HBV DNA decreased the odds of significant fibrosis in the HBeAg positive patients and increased the odds of significant fibrosis in the HBeAg negative group. This highlights the fact that the significance of HBV DNA in terms of fibrosis risk is best considered in the context of the patient’s phase of disease and HBeAg status.

The natural history of perinatally acquired CHB may be classified into four phases (immune tolerant, immune clearance, immune control and immune escape) (99) based on HBeAg status, HBV DNA, ALT level and histology. HBeAg positive patients in the immunotolerant phase usually have very high HBV DNA levels and little hepatic inflammation or fibrosis (105) as was seen in this cohort (see schema for explaining relationship of HBV DNA and fibrosis, Figure 4.3). In the immune clearance phase the host clears virus and removes hepatocytes, resulting in the development of fibrosis (142). Accordingly, the HBeAg positive patients with the lowest HBV DNA (<100,000 IU/ml) had the highest prevalence overall of F2/3/4 and A2/3 at 75% and 71% although the numbers in this group were small. Furthermore, the association between lower HBV DNA and cirrhosis in HBeAg positive patients is also supportive of immune clearance of HBV DNA comes at the cost of liver fibrosis. In the HBeAg negative patients those with a low HBV DNA (ENLR) have a low prevalence of F2/3/4 and may be considered to be in a state of immune control. Liver injury is not marked in these ENLR patients as they may have transitioned to this phase after a short immune clearance phase and also because of the
regression of fibrosis over time in this phase (97). HBeAg negative patients with higher HBV DNA levels (ENHR) may be considered to be in the immune escape phase of disease and it is proposed that the higher prevalence of F2/3/4 in this group is the cumulative effect of injury sustained during the immune clearance phase and the ongoing inflammation in HBeAg negative CHB (Figure 4.3).

This data confirm the results of the REVEAL-HBV study, which showed a clear relationship between increasing HBV DNA and fibrosis in a mainly HBeAg negative CHB cohort over 30. However, our study differs from REVEAL-HBV in that all patients had histological quantification of fibrosis rather than ultrasound based diagnosis of cirrhosis. In addition, over half the patients had HBeAg positive disease, and in these patients high HBV DNA was not associated with fibrosis.

The strong predictive power of age as a marker of significant fibrosis in HBeAg positive disease leads to proposing that older age may signify a protracted immune clearance phase with repeated unsuccessful attempts at viral clearance. This cannot however, be known with certainty from cross sectional data. A longer immune tolerant phase with a severe flare on entry into immune clearance could result in a similar outcome.

Serum ALT was shown to be a useful marker of risk for both significant inflammation and fibrosis. Our findings of relatively low median ALT’s (below 3 x ULN) in all HBeAg/DNA groups support the increasing recognition that even modestly elevated ALT levels can be associated with risk of liver damage (107)

In this study not all four phases of CHB were truly represented. All HBeAg positive patients were considered together since numbers in the immunotolerant group would have been quite small primarily because liver biopsies are not often performed in this group. The definitions used for the different groups were also not strictly concordant with other classification systems for phase of disease in CHB An HBV DNA threshold of 25,000 IU/ml was used to distinguish between HBeAg negative phases of disease because this was the lower limit of detection of the relatively insensitive Digene II assay on which over half the cohort had been tested and we acknowledge that this is a higher cut-off point than recent recommendations (101).

Although this is a cross sectional study, significant differences in ages between patient groups were observed, suggesting a transition from HBeAg positive disease to a HBeAg negative non
or low replicative state (ENLR) over years, followed by relapse to HBeAg negative chronic hepatitis (ENHR) as has been observed in longitudinal studies (174). The ENHR group has a preponderance of males and the high proportion of Asians in this group suggests that HBeAg negative CHB is common in Asian populations as has been increasingly recognized (180).

The high prevalence of F2/3/4 fibrosis in ENHR patients is in keeping with other recent studies which have reported a high prevalence of histological indications for treatment in HBeAg negative patients (173). However it must be acknowledged that the biopsied group presented here is biased towards patients with a higher likelihood of advanced fibrosis because of clinicians using markers of higher ALT and signs of chronic liver disease on examination to guide the decision to biopsy. The finding of moderate and severe inflammation in the majority of patients with F3 or F4 fibrosis highlights that viral suppression with resultant reduction in inflammation is very important in patients with advanced fibrosis (503).

**Conclusions**
Our study of an Australian clinical cohort confirms that CHB is associated with relatively high rates of significant fibrosis especially among ENHR patients, representing the immune escape phase of disease or chronic HBeAg negative Hepatitis B. We confirm that there is a positive correlation between high HBV DNA levels and the presence of significant liver fibrosis in HBeAg negative disease but not in HBeAg positive disease, and outline a potential explanation for this based on the underlying phase of disease. The factors predictive of significant histological disease were age in HBeAg positive patients, and both HBV DNA and ALT in HBeAg negative patients. The risk of liver inflammation and fibrosis in chronic hepatitis B is the complex result of many interacting factors and we argue that it is best considered in the context of the traditional phase of disease model rather than as a simple function of any single factor alone. In particular the HBV DNA level should not be relied on in isolation to predict significant fibrosis/inflammation, but rather needs to be interpreted as a measure of risk in the context of other factors especially HBeAg status.
<table>
<thead>
<tr>
<th></th>
<th>HBeAg positive (EP)</th>
<th>HBeAg negative DNA &lt; 25,000 IU/ml (ENLR)</th>
<th>HBeAg negative DNA &gt; 25,000 IU/ml (ENHR)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (%)</td>
<td>198 (50%)</td>
<td>66 (17%)</td>
<td>130 (33%)</td>
<td></td>
</tr>
<tr>
<td>% Males</td>
<td>123 (62%)</td>
<td>49 (74%)</td>
<td>103 (79%)</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Average age in years (range)</td>
<td>33 (15 – 76)</td>
<td>42 (18 – 69)</td>
<td>47 (22 – 76)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Asian Ethnicity</td>
<td>162 (82%)</td>
<td>49 (74%)</td>
<td>101 (78%)</td>
<td></td>
</tr>
<tr>
<td>Mediterranean ethnicity</td>
<td>14 (7%)</td>
<td>6 (9%)</td>
<td>19 (14%)</td>
<td>p=0.189</td>
</tr>
<tr>
<td>Caucasian ethnicity</td>
<td>17 (9%)</td>
<td>7 (11%)</td>
<td>6 (5%)</td>
<td></td>
</tr>
<tr>
<td>Other ethnicity</td>
<td>5 (2%)</td>
<td>4 (6%)</td>
<td>4 (3%)</td>
<td></td>
</tr>
<tr>
<td>Median Baseline HBV DNA in IU/ml (range)</td>
<td>2 x 10^7 (877 – 3 x 10^6)</td>
<td>Not applicable 1 x 10^6 (30,000 – 3 x 10^6)</td>
<td>p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Median Baseline serum ALT IU/ml (range)</td>
<td>93 (6 – 1463)</td>
<td>51 (12 – 2038)</td>
<td>78 (14 – 2270)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>% (number) patients with F2/3/4 fibrosis</td>
<td>40%(80)</td>
<td>36%(24)</td>
<td>58%(75)</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>% (number) patients with cirrhosis</td>
<td>11% (21)</td>
<td>8% (5)</td>
<td>18% (23)</td>
<td>p=0.069</td>
</tr>
<tr>
<td>% patients with A2/3 inflammation (n=306)</td>
<td>34% (48/143)</td>
<td>24% (14/59)</td>
<td>31% (32/104)</td>
<td>P=0.382</td>
</tr>
</tbody>
</table>
Table 4-2: Patient Characteristics by HBV Genotype.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total n=202 (%)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>G</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>12(6%)</td>
<td>75(37%)</td>
<td>74(37%)</td>
<td>33(16%)</td>
<td>1(0.5%)</td>
<td>7(3%)</td>
</tr>
<tr>
<td>HBeAg/ DNA Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number EP n=110(%)</td>
<td></td>
<td>9(8%)</td>
<td>36(33%)</td>
<td>49(45%)</td>
<td>13(12%)</td>
<td>0</td>
<td>3(2%)</td>
</tr>
<tr>
<td>Number ENLR n=20 (%)</td>
<td></td>
<td>0</td>
<td>14(70%)</td>
<td>1(5%)</td>
<td>4(20%)</td>
<td>1(5%)</td>
<td>0</td>
</tr>
<tr>
<td>Number ENHR n=72(%)</td>
<td></td>
<td>3(4%)</td>
<td>25(35%)</td>
<td>24(33%)</td>
<td>16(22%)</td>
<td>0</td>
<td>4(6%)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian n=161(%)</td>
<td></td>
<td>4(3%)</td>
<td>73(45%)</td>
<td>73(45%)</td>
<td>3(2%)</td>
<td>1(1%)</td>
<td>7(4%)</td>
</tr>
<tr>
<td>Caucasian n=11(%)</td>
<td></td>
<td>7(64%)</td>
<td>1(9%)</td>
<td>0</td>
<td>3(27%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mediterranean n=25 (%)</td>
<td></td>
<td>1(4%)</td>
<td>0</td>
<td>0</td>
<td>24(96%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other n=5(%)</td>
<td></td>
<td>0</td>
<td>1(20%)</td>
<td>1(20%)</td>
<td>3(60%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 4-3: Numbers (%) of patients significant fibrosis and inflammation of strata of HBV DNA from $<10^5$ to $>10^7$ IU/ml according to HBeAg status.

<table>
<thead>
<tr>
<th>Strata of HBV DNA in IU/ml</th>
<th>&lt;100,000</th>
<th>100,000-999,999</th>
<th>1 million - 9.99 million</th>
<th>&gt;10 million</th>
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</tr>
<tr>
<td>F0/1</td>
<td>2 (25%)</td>
<td>7 (64%)</td>
<td>15 (54%)</td>
<td>94 (62%)</td>
<td>118 (60%)</td>
</tr>
<tr>
<td>F2/3/4</td>
<td>6 (75%)</td>
<td>4 (36%)</td>
<td>13 (46%)</td>
<td>57 (38%)</td>
<td>80 (40%)</td>
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<tr>
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<tr>
<td>F0/1</td>
<td>55 (62%)</td>
<td>18 (45%)</td>
<td>15 (42%)</td>
<td>9 (29%)</td>
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<td>2 (29%)</td>
<td>4 (50%)</td>
<td>15 (75%)</td>
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<td>A2/3</td>
<td>5 (71%)</td>
<td>4 (50%)</td>
<td>5 (25%)</td>
<td>34 (31%)</td>
<td>48 (34%)</td>
</tr>
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<td>A0/1</td>
<td>59 (74%)</td>
<td>23 (77%)</td>
<td>19 (68%)</td>
<td>15 (60%)</td>
<td>116 (71%)</td>
</tr>
<tr>
<td>A2/3</td>
<td>21 (26%)</td>
<td>7 (23%)</td>
<td>9 (32%)</td>
<td>10 (40%)</td>
<td>47 (29%)</td>
</tr>
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<td>28</td>
<td>25</td>
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Table 4-4: Significant fibrosis and inflammation in total cohort by strata of ALT in IU/ml.

<table>
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<tr>
<th></th>
<th>&lt;=35 IU/ml (&lt;ULN)</th>
<th>ALT 36-52 IU/ml (1-1.5 x ULN)</th>
<th>ALT 53-70 IU/ml (1.5-2x ULN)</th>
<th>ALT 71-105 IU/ml (2-3x ULN)</th>
<th>ALT &gt;105 IU/ml (&gt;3 x ULN)</th>
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</tr>
</thead>
<tbody>
<tr>
<td><strong>F0/1</strong></td>
<td>41 (67%)</td>
<td>39 (62%)</td>
<td>29 (56%)</td>
<td>36 (50%)</td>
<td>70 (48%)</td>
<td>215 (55%)</td>
</tr>
<tr>
<td><strong>F2/3/4</strong></td>
<td>20 (33%)</td>
<td>24 (38%)</td>
<td>23 (44%)</td>
<td>36 (50%)</td>
<td>76 (52%)</td>
<td>179 (45%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>61</td>
<td>63</td>
<td>52</td>
<td>72</td>
<td>146</td>
<td>394</td>
</tr>
<tr>
<td><strong>A0/1</strong></td>
<td>41 (80%)</td>
<td>36 (78%)</td>
<td>34 (76%)</td>
<td>34 (60%)</td>
<td>66 (62%)</td>
<td>211 (69%)</td>
</tr>
<tr>
<td><strong>A2/3</strong></td>
<td>10 (20%)</td>
<td>10 (22%)</td>
<td>11 (24%)</td>
<td>23 (40%)</td>
<td>41 (38%)</td>
<td>95 (31%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>51</td>
<td>46</td>
<td>45</td>
<td>57</td>
<td>107</td>
<td>306</td>
</tr>
</tbody>
</table>
Figure 4-1: Prevalence of significant fibrosis in HBeAg positive and negative groups.

Figure Legend: This figure shows that the highest proportion of F2/3/4 fibrosis is seen in ENHR at 58% which is significantly higher than that seen in ENLR (36%) and in HBeAg positive patients (40%).
Figure 4-2: Correlation between inflammation and fibrosis scores on liver biopsies in chronic hepatitis B patients (n=306).

Figure Legend: This figure shows the close association between histological inflammation and fibrosis with increasing proportions of severe inflammation (A2/3) seen in patients with higher degrees of fibrosis (F3/F4).
Figure 4-3: Prevalence of F2/3/4 fibrosis in HBeAg positive and negative patients in increasing strata of HBV DNA.

Figure legend: This figure provides a schema for understanding the association between prevalence of F2/3/4 and HBV DNA level in HBeAg positive (triangles solid line) and HBeAg negative (squares dashed line) patients based on the phase of disease.
Chapter 5: Longitudinal observation of viral load changes in untreated HBeAg negative chronic hepatitis B.

Introduction
In the natural history of Chronic Hepatitis B the HBeAg positive phases of immune tolerance and clearance occur first and following seroconversion the patient becomes Hepatitis B e Antigen (HBeAg) negative and Hepatitis B e Antibody (Anti-HBe) positive (89, 105, 141). Usually this serocconversion leads to quiescent disease however HBeAg negative chronic hepatitis with ongoing viral replication and inflammation that is measurable biochemically and histologically may develop directly from the HBeAg positive immune clearance phase (355). HBeAg negative chronic hepatitis may also develop as a reactivation following a period of immune control (156, 174). Distinguishing between the 2 phases of HBeAg negative CHB can be difficult and nomenclature and guidelines on the features to classify patients into either the immune control (Phase 3/inactive carrier state) or immune escape (Phase 4/ Chronic HBeAg negative disease) have evolved over recent times. European (102), American (101) and Asian (108) practice guidelines exist and Australian and New Zealand Chronic Hepatitis B Recommendations have also been published by the Gastroenterological Society of Australia (487).

The inactive carrier (immune control) state is generally defined as HBeAg negative patients with low HBV DNA levels, in addition to persistently normal alanine aminotransferase (ALT) and the absence of significant hepatitis on liver biopsy. The cut-off level of Hepatitis B Virus (HBV) DNA to define this state has been difficult to reach consensus on and has changed over the last few years, decreasing from 100,000 copies/ml (approximately 17857 IU/ml) to 30,000 copies/ml (approximately 5357 IU/ml) (95, 168, 504, 505) and further still to 2000 IU/ml in APASL and AASLD guidelines in 2008 and 2009 respectively (101, 108). In 2009, EASL guidelines on the definition of the inactive carrier state did not use a strict HBV DNA threshold (486) but in 2012 they suggested the threshold of 2000 IU/ml although allowing that some inactive carriers with persistently normal ALT may have HBV DNA levels that are higher than 2000, (usually less than 20,000) (102). The level of 2000 IU/ml has also been recommended as a level for consideration of therapy (101, 109, 486, 487) thus it has become an important threshold used for making decisions regarding monitoring and treatment.

It is recognized that HBeAg negative hepatitis B can run a fluctuating course (185) with variability in HBV DNA and ALT levels over time. There is a reasonable body of literature on
cohorts of Hepatitis B patients with persistently normal ALT (107, 165, 170, 506) however studies focusing on variations in HBV DNA levels are fewer. The published guidelines also focus primarily on frequent documentation of normal ALT levels with less clear emphasis on the the regularity with which HBV DNA levels should be checked in criteria for the inactive carrier state.

**Aims**

We sought to examine:

1. The stability of HBV DNA levels around the threshold of 2000 IU/ml in untreated HBeAg negative patients with the hypothesis that small fluctuations are common and this threshold may often be crossed,

2. The longitudinal correlation between HBV DNA levels and ALT levels with the hypothesis that persistently normal ALT levels may not always correlated with HBV DNA levels <2000 IU/ml.
Methods
The patients were all CHB patients seen through St Vincent’s Hospital, Melbourne on whom demographic details including date of birth, sex, ethnic origin and country of birth were recorded in a Microsoft Access Database as at 1 October 2008. The database was searched for all HBeAg negative patients who had had serial HBV DNA testing done whilst not on any treatment over a period of at least 18 months. The first tests for HBV DNA and ALT tests performed on the St Vincent’s Hospital pathology system were used as baseline values. Patients with a baseline HBV DNA and in addition at least 1 followup HBV DNA test and ALT test in each of two consecutive years following the baseline date were included in the study. HBV DNA and ALT testing were performed at approximately 6 monthly intervals from the baseline date. Normal ALT was defined as ≤35. For all patients with detectable baseline HBV DNA, pharmacy records and patients’ hospital records were searched to ensure that no treatment was used during the period of followup. The patients were divided into 2 groups according to their baseline HBV DNA status:

1. HBeAg negative and HBV DNA < 2000 IU/ml (Group 1).
2. HBeAg negative and HBV DNA ≥ 2000 IU/ml (Group 2).

The study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the St Vincent’s Hospital Human Research and Ethics Committee.

Hepatitis B Virus Serological and DNA testing
Hepatitis B surface antigen (HBsAg) was measured using a commercially available immunoassay (Abbott Laboratories, North Chicago, IL, USA). HBeAg and anti-HBe were measured by an immunoassay produced by BioMerieux Clinical Diagnostics (Marcy l’Etoile, France). All tests pertaining to HBV DNA were performed at the Victorian Infectious Diseases Reference Laboratory (VIDRL). The HBV DNA viral load was performed by a bDNA signal amplification probe method (Bayer Versant HBV DNA 3.0 assay, Bayer Diagnostics, Emeryville, CA, USA). The dynamic range is 357 to 17,857,140 IU/ml. One IU/ml is equivalent to 5.6 copies/ml. Patients tested using the bDNA assay whose HBV DNA levels were below and above the limits of detection were assigned the values of 357 and 17,857,140 IU/ml respectively for analyses involving HBV DNA.

Statistical Analysis
Data entry and statistical analysis were performed with the statistical package Stata (version 9.2, Statcorp). The corrected chi square test or two sided Fisher’s exact test was used to compare categorical data, while the student’s t-test or one way ANOVA was used for group
comparisons of parametric quantitative data and the Mann-Whitney or Kruskal-Wallis test for similar comparisons of non-parametric data. Spearman’s rank correlation was used to examine the association between HBV DNA and ALT values. Multilevel linear regression was used to examine the change in HBV DNA and ALT values over time. Results were presented as median and range whenever appropriate. In all cases tests of significance were two-tailed with a level at <0.05.
Results

Patient Characteristics
47 HBeAg negative patients, 27 with baseline HBV DNA <2000 (Group 1) and 20 with baseline HBV DNA ≥2000 IU/ml (Group 2) were followed up for a median of 24 months (18-48 months). The median baseline HBV DNA of the total group was 1255 IU/ml (range 357-2,133,453) and 34 of 47 (72%) had baseline HBV DNA <20,000 IU/ml. Groups 1 and 2 were similar in gender distribution and in age with an average age of 44 and 48 respectively. They also had similar numbers of HBV DNA and ALT tests performed (Table 5.1). The ethnicities of the groups differed with Group 2 having a higher proportion of Asians 95% vs 67% in group1 (p=0.03). The majority of Asian patients were from South East Asia (especially Vietnam, Cambodia, Laos, Thailand) and China. Group 1 also included patients of Mediterranean (Greek and Italian) (7%) and Caucasian (15%) ethnicity. The baseline ALT levels of Group 1 was significantly lower than those of Group 2 at 29 compared to 48 U/L (p=0.03).

The distribution of HBV DNA in Group 1 was 67% HBV DNA ≤357 IU/ml and 33% 357-1999. The distribution of HBV DNA in Group 2 was 35%, 15%, 35% and 15% in patients with baseline HBV DNA 2000-19,999, 20,000–99,999, 100,000-9.9 x 10^5, ≥10^6 IU/ml. The proportion of patients with baseline ALT ≤ULN, 1-1.5 x ULN, 1.5 to 2 x ULN and >2 x ULN was 67%, 18%, 11% and 4% in Group 1 and 40%, 10%, 25% and 25% in Group 2. A significant correlation was found between ALT and HBV DNA in Group 2 (Correlation coefficient 0.39, p <0.001) but not in Group 1 (Correlation Coefficient -0.17, p=0.06).

Fluctuation of HBV DNA and ALT over time.
The change in DNA and ALT values in individual patients over time was examined using multilevel linear regression, and the results are summarised in the Table 5.2. The regression coefficients presented represent the change in the outcome for an increase in time of one month. When examined in this way there was no evidence of a significant change over time in either ALT or HBV DNA in groups 1 or 2.

Longitudinal followup of HBeAg negative patients with HBV DNA <2000 IU/ml
The 27 HBeAg negative patients with baseline HBV DNA <2000 IU/ml (Group 1) were followed over a median of 24 months (range 18-48). Of these, 16 (59%) had an HBV DNA over followup that was persistently <2000 IU/ml (Group 1A), while the other 11 (41%) had one or more HBV DNA levels over 2000 IU/ml during the followup period (Group 1B). Despite these fluctuations in HBV DNA to above 2000 IU/ml over followup, 45% of Group 1B patients maintained a
The longitudinal HBV DNA levels of the Group 1B patients are shown in Table 5.3. Ten of the eleven Group 1B patients continued to be observed following the rise in HBV DNA above 2000 IU/ml and had an HBV DNA test available 6 months after the test showing the elevation. In the eleventh patient there was a rise in DNA from 1461 IU/ml to 2.77 x 10^6 at which point the patient went onto therapy. In 7 of the 10 observed Group 1B patients the elevated DNA was an isolated one with the subsequent DNA test showing a return to below 2000, although in one patient there was a further transient rise above 2000 IU/ml in the following year. The highest HBV DNA level seen in these patients was 10,697 IU/ml. The other 3 of 10 patients showed a sustained elevation of HBV DNA >2000 over 2 or more tests although the levels remained under 20,000 IU/ml. ALT remained persistently normal in all 3 patients with a sustained elevation of HBV DNA although it was persistently normal in only 2 of 7 patients with a transient elevation (Table 5.3). Of 52 followup HBV DNA tests in all 11 patients, 15 (29%) were elevated to over 2000 IU/ml. The median of all followup HBV DNA’s in these patients was 529 (range 357 – 2,773,754 IU/ml) and the median of the 15 elevated HBV DNA tests was 3644 IU/ml (range 2104 – 2,773,754 IU/ml).

Of the 27 group 1 patients who had a baseline HBV DNA of <2000 IU/ml, over followup only 8 (30%) had both ALT persistently normal and HBV DNA persistently <2000 IU/ml. Of the remaining Group 1 patients, 5 (18%) had a persistently normal ALT but HBV DNA was not persistently below 2000 IU/ml (mean age 34), a further 6 (22%) had neither a persistently normal ALT nor HBV DNA persistently below 2000 IU/ml (mean age 39) and 8 (30%) had an HBV DNA that was persistently <2000 IU/ml but ALT was not persistently normal (mean age 45) (figure 5.1). There were significant differences in the ages of the group 1 patients as set out above (p=0.04) with the subgroup with persistently normal ALT and HBV DNA persistently <2000 IU/ml being significantly older at 54 years than the other 3 subgroups.

**Longitudinal followup of HBeAg negative patients with DNA >2000 IU/ml**

The 20 HBeAg negative patients with a baseline HBV DNA >2000 IU/ml had a median followup of 24 months (range 18-24). 12 of the 20 (60%) of patients had HBV DNA persistently >2000 IU/ml over followup (Group 2A) and 8/20 (40%) had one or more HBV DNA result <2000 IU/ml (Group 2B). The 2 groups were similar in their demographics (age, sex and ethnicity), duration of followup and the number of followup HBV DNA and ALT tests done per patient, however group 2B had a lower median baseline HBV DNA compared to group 2A (8610 v/s 208763, p=0.03) (Table 5.4). The proportion of patients with HBV DNA persistently >2000 IU/ml were
29%, 66% and 80% in those with baseline HBV DNA of 2,000-19,999 (n=7), 20,000-99,999 (n=3) and ≥ 10^5 (n=10) respectively. 20% of Group 2 patients had a persistently normal ALT.

Examination of the viral load changes in Group 2B patients showed that 5 of 8 had a baseline HBV DNA between 2000 and 20,000 IU/ml and 4 of the 5 fluctuated below 20,000 while the 5th patient’s HBV DNA rose to > 2x10^5 after 18 months (Table 5.5). 3 of these 5 had persistently normal ALT. A further Group 2B patient had a baseline HBV DNA level of 52,555 IU/ml and had moderate level fluctuations over 18 months. The last 2 patients in group 2B had a baseline HBV DNA over 10^5 IU/ml and underwent spontaneous reduction in HBV DNA by over 2 logs during follow up, both dropping to <2000 IU/ml. This occurred gradually over the course of 12 months.

By contrast, only 2 of 12 Group 2A patients had a baseline HBV DNA <20,000 IU/ml and only 1 of these had HBV DNA levels that remained <20,000 during followup although this was not accompanied by a persistently normal ALT. A persistently normal ALT was seen in only 1 of 12 Group 2A patients. Although the proportion with persistently normal ALT was 1/12 (8%) in group 2A and 3/8 (38%) in group 2B, this did not reach statistical significance (p=0.26) however the numbers in these subgroups were small.
Discussion

This study observes the virological and biochemical fluctuations in an Australian cohort of HBeAg negative chronic hepatitis B patients, over a period of at least 18 months. It is a retrospective study predominantly of Asian patients, with mean age in the mid forties who have low level viraemia and it focuses on the changes that occur around the threshold of 2000 IU/ml. We show that 40% of patients who begin above or below the arbitrary HBV DNA level of 2000 IU/ml will subsequently cross this threshold. These fluctuations in both groups 1 and 2 were mostly of a low level, around a low baseline and almost all of the Group 1B patients whose HBV DNA rose over the level of 2000 IU/ml remained under 20,000 IU/ml. In our cohort only 8/27(30%) patients with a baseline HBV DNA <2000 IU/ml in fact demonstrated levels of virus persistently below 2000 IU/ml and persistently normal ALT. Furthermore we show that a persistently normal ALT is seen in significant proportions of patients who had HBV DNA levels that fluctuated above 2000 IU/ml (45% in group 1B and 20% in Group 2 overall).

There is a relative paucity of published data on the fluctuations in HBV viral load that occur in chronic hepatitis B or in inactive carriers. Important work done approximately 10 years ago demonstrated that viral load over time in HBeAg negative patients was relatively stable (505, 507). Consistent with this, large fluctuations in HBV DNA or ALT were not observed in our cohorts when measured by regression analysis.

There have since been other studies focusing on changes in HBV DNA and ALT over time during seroconversion (508, 509) and in cohorts with HCC (510, 511) or in cohorts of predominantly HBeAg positive patients (506) and other small studies including one of 14 inactive carriers found that although there was significant fluctuation in HBV DNA below the range of $10^4$ copies/ml (approximately 1785 IU/ml), the ALT remained persistently normal when measured monthly for 12 months (512). HBV DNA even at low levels, is now recognized as a risk factor for the complications of CHB of cirrhosis and HCC. In particular the impact of the REVEAL-HBV study which identified the the threshold of $10^4$ copies/ml (1785 IU/ml) as the level above which the risks of cirrhosis and hepatocellular carcinoma were increased (169, 235) has no doubt partly driven a focus on the level of 2000 IU/ml to define inactive carriers/chronic hepatitis B and also as a threshold for treatment in HBeAg negative patients.

The question of viral load fluctuations in HBeAg negative patients around the level of 2000 IU/ml has been dealt with in part by a couple of groups. In a study by Zecharakis with a median followup of 5.3 years, HBV DNA levels <2000 IU/ml on at least one occasion were seen in 5/12
(42%) HBeAg negative patients with intermittently abnormal ALT and in 8/36 (22%) of patients with persistently abnormal ALT (167). Similarly in our group 2 patients, who had baseline HBV DNA >2000 IU/ml, we demonstrated a drop below 2000 IU/ml in 40% (8/20) (Group 2B). Group 2B patients had a significantly lower baseline HBV DNA than those that didn’t drop below 2000 IU/ml (Group 2A) and the viral load of 4 of the 8 Group 2B remained in the range <20,000 IU/L.

Papatheodoridis also observed 65 HBeAg negative patients with chronic hepatitis and found that 18% had occasional changes in HBV DNA to below the level of 2000 IU/ml (172). They also studied 85 patients in the inactive carrier state, defined as persistently normal ALT over 12 months and baseline HBV DNA <20,000 IU/ml and found no significant change in HBV DNA from baseline to Year 1 (172).

Using the cut off level of 20,000 IU/ml in place of 2000 IU/ml to define the inactive carrier state has gained further support with 2 studies of HBeAg negative patients with PNALT, both of which showed that many of these patients had HBV DNA levels <5 log_{10} copies/ml (approximately 17857 IU/ml) but only a small proportion had HBV DNA < 4 log_{10} copies/ml (approximately 1785 IU/ml) (166, 170). Papatheodoridis et al also recently reviewed 6 studies of HBeAg negative patients with persistently normal ALT and concluded that if persistently normal ALT was based on strict criteria, significant histological liver disease was rarely found on liver biopsy in patients with HBV DNA up to 20,000 IU/ml (165).

Of our group 1 HBeAg negative patients with a baseline HBV DNA <2000 IU/ml, we found only (8/30) 30%, over followup had persistently normal ALT and HBV DNA persistently <2000 IU/ml. It is of interest to note that these patients were the oldest of the Group 1 patients and there appeared to be a gradient of ages in the other subgroups studied here. We speculate that this may reflect the slow re-gaining of immune control over time of HBeAg negative CHB. We hypothesise that the first stage in transition from immune control to HBeAg negative chronic hepatitis B is a slow increase in viral load, although ALT increase lags behind and remains largely normal (mean age of patients 34). ALT then also increases along with HBV DNA being more often >2000 IU/ml (mean age of patients 39). Next, in some patients, with some re-development of immune control HBV DNA levels reduce and are persistently <2000 IU/ml, however the liver inflammation takes some time to resolve (mean age 45) and finally there is regain of immune control over HBeAg negative (precore/BCP mutant) virus and these patients have HBV DNA persistently <2000 IU/ml and persistently normal ALT (mean age 54). Of course
there may be other reasons for the discrepancies between HBV DNA and ALT found in these patients but Feld et al (171) also noted that a common explanation was simply that ALT changes lag behind HBV DNA viral load changes and on liver biopsy specimens in their study, although NAFLD was seen in some, changes of mild active CHB were also found. ALT normalisation is certainly known to lag behind viral load reduction in treatment of CHB with oral NA’s (453). However it must be acknowledged that these suppositions are based on a very small group of patients, in an already selected sub-group of HBeAg negative CHB with low level viraemia and others have reported that spontaneous remission of HBeAg negative CHB is uncommon (189). Further study of the natural history of chronic HBeAg negative hepatitis B would be of interest but may prove ethically unfeasible. The recommended interval for testing of HBV DNA and ALT to more accurately classify patients into the appropriate HBeAg negative phase of disease is currently limited by the fact that in Australia only 1 HBV DNA test per year is rebated by Medicare for those patients not on treatment, We would propose that at least 6 monthly HBV DNA testing be performed, and possibly even 3 monthly in the first year of evaluation. 3 monthly ALT levels should be performed.

Amongst those 11/27 patients that had HBV DNA >2000 IU/ml at some point over followup, the viral load fluctuations were largely under 20,000 and were usually only transiently above 2000 IU/ml. In this group of 11 patients with HBV DNA fluctuations under 20,000 IU/ml, 5 had persistently normal ALT and 6 did not. We thus concur with data from the above groups (165, 170) that a more generous cut off of 20,000 IU/ml for defining inactive carriers may be appropriate. Paptheodoridis et al provide a useful algorithm in their recent paper, drawing attention to the fact that whilst patients with HBV DNA 20,000 IU/ml with truly persistently normal ALT do not require liver biopsy and can be considered inactive carriers, those with abnormal ALT should certainly be considered for liver biopsy. This can help guide the need for therapy in a patient who may have fluctuating low level viraemia, especially if the ALT is only mildly or intermittently abnormal. In such cases, decisions regarding antiviral therapy may need to include factors other than just HBV DNA and ALT, such as family history of HCC.

Our study, like others recently (513) highlights the difficulty of classifying HBeAg negative patients into a phase using very strict criteria. The use of quantification of HBsAg titres may further add to defining inactive carriers in the future (514). There appears to be increased recognition that higher thresholds of HBV DNA (up to 20,000, rather than 2000 IU/ml) may be reasonable for defining the inactive carrier state. The 2012 EASL guidelines still suggest that along with persistently normal ALT, a cut-off level of 2000 IU/ml is used for defining inactive
carriers but concede that a level of up to 20,000 IU/ml may be seen (102). APASL guidelines in 2012 go further, stating that it would be reasonable to define inactive carriers as having persistently normal ALT and HBV DNA <20,000 IU/ml (103). This is a revision from earlier guidelines in 2008 which used the level of HBV DNA 2000 IU/ml (108). We feel that this higher threshold of 20,000 IU/ml is valid and worth emphasizing especially in light of recent data showing that PNALT is often accompanied by levels of HBV DNA up to 20,000 IU/ml and our data which shows that fluctuations around the level of 2000 IU/ml occur commonly but are usually transient and low level. Although it may be reasonable to use the value of up to 20,000 to guide decisions for treatment, the long term effect on disease progression in patients with viral loads of this magnitude is open to speculation. It seems likely that there is a higher chance of progression to chronic HBeAg negative hepatitis B in such patients than in those with undetectable HBV DNA.

Our study has limitations including its retrospective design and relatively small numbers in some patient subgroups. It also did not use the categorizations of inactive carrier and chronic hepatitis B but this was because we did not feel comfortable using the strict guidelines that were advised since our hypothesis was that fluctuations above the level of 2000 IU/ml were common despite persistently normal ALT in some HBeAg negative patients which we were able to demonstrate.

**Conclusions**

This study represents a detailed description of the course, untreated, of a moderate sized cohort of HBeAg negative chronic hepatitis B patients over a period of at least 18 months. We found that fluctuations around the HBV DNA threshold of 2000 IU/ml were common. In particular about 40% of patients who began with HBV DNA <2000 rose transiently to a level under 20,000 IU/ml. Many of these patients had a persistently normal ALT. Similarly, 40% of patients with a viral load above 2000 IU/ml at baseline subsequently fall below this. Our data showing the frequent small fluctuations that occur in viral loads in HBV patients lends support to the recent suggestion that a higher threshold of 20,000 IU/ml for definition of the inactive carrier state may be appropriate.
<table>
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<tr>
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<th>Group 1 (HBeAg neg, DNA &lt;2000)</th>
<th>Group 2 (HBeAg neg, DNA ≥ 2000)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
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<td>Number</td>
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<td>20</td>
<td></td>
</tr>
<tr>
<td>Number (%) Males</td>
<td>18 (67%)</td>
<td>11 (55%)</td>
<td>0.55</td>
</tr>
<tr>
<td>Age (Mean, SD)</td>
<td>44 (14)</td>
<td>48 (14)</td>
<td>0.28</td>
</tr>
<tr>
<td>% Asian</td>
<td>18 (67%)</td>
<td>19 (95%)</td>
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</tr>
<tr>
<td>% Other Ethnicity</td>
<td>9 (33%)</td>
<td>1 (5%)</td>
<td></td>
</tr>
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<td>Baseline DNA IU/ml Median (IQR)</td>
<td>357 (357, 883)</td>
<td>88589 (7570,457232)</td>
<td>&lt;0.001</td>
</tr>
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<td>29 (20,47)</td>
<td>48 (28,74)</td>
<td>0.03</td>
</tr>
<tr>
<td>Number of followup DNA tests/patient (Mean, SD)</td>
<td>3.9 (1.5)</td>
<td>3.4 (0.6)</td>
<td>0.10</td>
</tr>
<tr>
<td>Number of followup ALT tests/patient (Mean, SD)</td>
<td>4.3 (1.7)</td>
<td>3.6 (0.6)</td>
<td>0.06</td>
</tr>
<tr>
<td>Number (%) with normal baseline ALT</td>
<td>18 (67%)</td>
<td>8 (40%)</td>
<td>0.08</td>
</tr>
<tr>
<td>Number (%) with persistently normal ALT</td>
<td>13 (48%)</td>
<td>4 (20%)</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Table 5-2: Multilevel linear regression showing change in ALT and HBV DNA in Group 1 and 2 patients for an increase in time of one month.

<table>
<thead>
<tr>
<th>Group</th>
<th>Regression Coefficient (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALT in U/L</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (HBeAg Neg, DNA&lt;2000 IU/ml)</td>
<td>-0.13 (-0.40, 0.14)</td>
<td>0.35</td>
</tr>
<tr>
<td>Group 2 (HBeAg Neg, DNA&gt;2000 IU/ml)</td>
<td>-0.54 (-1.23, 0.16)</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>HBV DNA in IU/ml</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1 (Neg, DNA&lt;2000 IU/ml)</td>
<td>2817 (-458, 6093)</td>
<td>0.09</td>
</tr>
<tr>
<td>Group 2 (Neg, DNA&gt;2000 IU/ml)</td>
<td>-687 (-13865, 12491)</td>
<td>0.92</td>
</tr>
</tbody>
</table>
Table 5-3: Fluctuations in HBV DNA in IU/ml over followup in patients with initial HBV DNA <2000 IU/ml and subsequent rise to over 2000 IU/ml.

<table>
<thead>
<tr>
<th>Transient rise &gt;2000 IU/ml</th>
<th>DNA 0m</th>
<th>DNA 6m</th>
<th>DNA 12m</th>
<th>DNA 18m</th>
<th>DNA 24m</th>
<th>DNA 30m</th>
<th>DNA 36m</th>
<th>DNA 42m</th>
<th>DNA 48m</th>
<th>PNALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt. 1</td>
<td>357</td>
<td>357</td>
<td>357</td>
<td>4373</td>
<td>357</td>
<td>470</td>
<td>531</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. 2</td>
<td>357</td>
<td>747</td>
<td>6556</td>
<td>357</td>
<td>2298</td>
<td>357</td>
<td>1390</td>
<td>391</td>
<td>357</td>
<td>Yes</td>
</tr>
<tr>
<td>Pt. 3</td>
<td>357</td>
<td>506</td>
<td>357</td>
<td>2957</td>
<td>480</td>
<td>357</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. 4</td>
<td>509</td>
<td>3558</td>
<td>583</td>
<td>527</td>
<td>357</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. 5</td>
<td>357</td>
<td>357</td>
<td>10697</td>
<td>357</td>
<td>357</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. 6</td>
<td>1255</td>
<td>2104</td>
<td>1536</td>
<td>711</td>
<td>1089</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. 7</td>
<td>1260</td>
<td>2250</td>
<td>357</td>
<td>357</td>
<td>357</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sustained rise &gt;2000 IU/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. 8</td>
<td>357</td>
<td>1148</td>
<td>357</td>
<td>2390</td>
<td>5346</td>
<td>357</td>
<td>357</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. 9</td>
<td>357</td>
<td>986</td>
<td>6951</td>
<td>5404</td>
<td></td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not Evaluable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Pt. 10</td>
<td>1045</td>
<td>999</td>
<td>2233</td>
<td>3644</td>
<td></td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pt. 11</td>
<td>357</td>
<td>357</td>
<td>357</td>
<td>357</td>
<td>1461</td>
<td>2.7x10^6</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>
Table 5-4: Patients with baseline HBV DNA >2000 IU/ml, n=20

<table>
<thead>
<tr>
<th></th>
<th>Group 2A</th>
<th>Group 2B</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(HBV DNA persistently &gt;2000 IU/ml)</td>
<td>(HBV DNA not persistently &gt;2000 IU/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number (%)</strong></td>
<td>12 (60%)</td>
<td>8 (40%)</td>
<td></td>
</tr>
<tr>
<td><strong>Number (%) Males</strong></td>
<td>7 (58%)</td>
<td>4 (50%)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Mean Age in years (SD)</strong></td>
<td>47 (13)</td>
<td>49 (7)</td>
<td>0.81</td>
</tr>
<tr>
<td><strong>Number of followup DNA tests done per patient Mean (SD)</strong></td>
<td>3.5 (0.5)</td>
<td>3.3 (0.7)</td>
<td>0.37</td>
</tr>
<tr>
<td><strong>Number of followup ALT tests per patient, Mean (SD)</strong></td>
<td>3.7 (0.7)</td>
<td>3.5 (0.5)</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>Baseline HBV DNA in IU/ml median (IQR)</strong></td>
<td>208,763 (45764, 1051342)</td>
<td>8610 (4861, 87656)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Baseline ALT in IU/ml, median (IQR)</strong></td>
<td>50 (32, 87)</td>
<td>41 (23, 55)</td>
<td>0.33</td>
</tr>
<tr>
<td><strong>Number (%) with persistently normal ALT</strong></td>
<td>1 (8%)</td>
<td>3 (38%)</td>
<td>0.26</td>
</tr>
</tbody>
</table>
Table 5-5: Fluctuations in HBV DNA in IU/ml over followup in Group 2B patients with initial HBV DNA >2000 IU/ml and subsequent drop to <2000 IU/ml.

<table>
<thead>
<tr>
<th>Patient</th>
<th>HBV DNA 0 months</th>
<th>HBV DNA 6 months</th>
<th>HBV DNA 12 months</th>
<th>HBV DNA 18 months</th>
<th>HBV DNA 24 months</th>
<th>HBV DNA persistently &lt;20000 IU/ml</th>
<th>Persistently normal ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>2247</td>
<td>357</td>
<td>2128</td>
<td>473</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>2571</td>
<td>2632</td>
<td>1123</td>
<td>3143</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td>9231</td>
<td>504</td>
<td>6156</td>
<td>2214</td>
<td>357</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Patient 4</td>
<td>7150</td>
<td>17490</td>
<td>930</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Patient 5</td>
<td>7989</td>
<td>1451</td>
<td>376</td>
<td>237571</td>
<td>22115</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Patient 6</td>
<td>52555</td>
<td>1760</td>
<td>14970</td>
<td>941</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Patient 7</td>
<td>122756</td>
<td>41461</td>
<td>396</td>
<td>4489</td>
<td>2458</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Patient 8</td>
<td>326435</td>
<td>15948</td>
<td>35640</td>
<td>1527</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5-1: Distribution of patients with HBV DNA <2000 IU/ml

Figure legend: This figure shows the distribution in terms of followup HBV DNA and ALT levels of 27 HBeAg negative patients who have a baseline HBV DNA of <2000 IU/ml.
Chapter 6: Characterisation of Genotype, precore and Basal Core Promoter mutations and correlations with liver histology: Study of an Australian cohort of Chronic Hepatitis B patients.

Introduction
Genomic variation of the Hepatitis B virus resulting in distinct genotypes has been established in the literature for at least the past 2 decades (515). Nine different genotypes (designated A-I) and a putative tenth genotype “J” isolated from a single patient, have been identified based on a divergence in the nucleotide sequence of 7.5% or more over the entire HBV genome (516). There is considerable geographic variation in the distribution of the HBV genotypes; genotype A is prevalent in North Western Europe and North America, genotypes B and C are mainly found in Asia and Genotype D in the Mediterranean Basin and the Middle East. Genotype E has been reported from West Africa, genotype F from native Americans and Polynesians and genotype H from Central America. A large multi-centre cross sectional study from the United States of 694 patients showed that the prevalence of HBV genotype A was 35%, B 22%, C 31% D 10%, G 1% and E and F both <1% each (320). A strong correlation between HBV genotype and ethnicity was found and the genotypic diversity of the chronic hepatitis B population of the USA is largely contributed to by migration to that country. Similarly in Australia, the prevalent genotypes are those found in countries of origin of distinct migrant groups (48).

A number of clinical differences have been noted between HBV genotypes and although it is not yet part of routine clinical practice, identification of the genotype of an HBV infection may offer prognostic information on clinical outcomes as well as likelihood of response to certain therapies (particularly Interferon). Infection with genotype B has possibly been associated with better clinical outcomes than genotype C infections by some including earlier spontaneous HBeAg seroconversion and slower progression to cirrhosis (331). The evidence linking HBV genotype with HCC is slightly conflicting. Several Asian studies examining the impact of HBV genotype on the development of HCC have shown that patients with genotype C had a higher risk of HCC than other genotypes (328, 339, 517). However other conflicting evidence suggests that HBV genotype B infected patients with advanced fibrosis and HCC were significantly older than those infected with HBV genotype C (331) however there was no difference between the
2 genotypes in terms of overall prevalence of these complications suggesting the lifetime risk of the complications was the same for HBV genotype B and C infections (518). Other studies still have suggested that HBV genotype B is more commonly associated with non-cirrhotic HCC especially in younger patients (341, 346).

Most studies looking at endpoints of liver fibrosis have usually only included 2 predominant HBV genotypes and again results have been inconsistent. While some (333, 372) have showed that histological activity index (HAI) scores were lower in patients with genotype B infections than C, others eg Luo et al (519) in their study of 264 patients with genotypes B and C showed no difference in the histological inflammation or fibrosis scores and Yuen et al (518) also found no difference between these 2 genotypes in terms of cirrhosis related complications. HBV genotype C however has been shown to be associated with more severe liver fibrosis than HBV genotype B on the basis of transient elastography in 1106 patients (332). Similarly, studies comparing liver biopsies in cohorts with predominantly HBV genotypes A and D showed no significant difference in patient histological activity and fibrosis (277, 279, 520). However other data have suggested more advanced liver disease in patients infected with genotype D compared to A (336) whilst still others have suggested the reverse (521). In contrast in HBV HIV coinfected patients, HBV genotype G is a determinant of liver fibrosis (522).

The relationship of HBV genotype to the common mutations seen in the precore and the basal core promoter (BCP) region of the HBV genome has been described, again usually in studies comparing 2 prevalent HBV genotypes. In studies comparing HBV genotypes A and D, genotype D has been found to have a higher prevalence of precore mutations (65-75%) than genotype A (9-18%) (523) (524). Studies comparing HBV genotypes B and C have produced mixed results. The precore mutation was found more commonly in genotype B than C by Watanabe et al (525) but Sumi et al (331) found that although it was more common overall and in HBeAg positive genotype B patients, the prevalence of the G1896A mutation in the precore was similar between HBV genotypes B and C in HBeAg negative patients. Another Japanese study of 50 patients found the prevalence of the precore mutation was similar between HBV genotypes B and C at 60% and 62% respectively (374). An American study with representation from all 4 HBV genotypes found that the prevalence of precore mutation was 55% in D, 44% in B, 22% in C and 3% in A (526). The prevalence of BCP mutations A1762T and G1764A has been shown to be more common in HBV genotype C than B (331, 374, 525).
In terms of the relationship of HBV precore and BCP mutations to the presence of fibrosis and inflammation, reports of the association of the BCP mutation with more significant histological lesions have been made since over a decade ago (527). Yuan et al (528) found that HBV BCP mutations if present were associated with higher histological scores of fibrosis and inflammation in 54 patients. Similarly Yuen et al (372) also looked at 66 patients with HBV genotype B and C infections and found that those with the BCP mutation in the HBV genome had more severe necroinflammation and a Japanese study also found that the frequency of BCP mutations increased with the progression of liver fibrosis (529). However, other data on the relationship of the precore and BCP mutations to degrees of fibrosis or inflammation as measured histologically is scarce.

Thus the data on the relationship of HBV genotype and viral variants to clinical outcomes is somewhat discordant and further study is needed to assist with recommendations about testing for HBV genotype and viral mutations for prognostication in CHB.

**Aims**

1. To describe the prevalence of different HBV genotypes in an Australian cohort of Chronic Hepatitis B.

2. To examine correlations between HBV genotype and other patient characteristics including:
   a. Demographic features (sex, ethnicity)
   b. Biochemical features (ALT)
   c. Virological features (HBeAg status, HBV DNA level).

3. To examine the prevalence of precore and BCP mutations in different HBV genotypic groups and in HBeAg positive and negative groups.

4. To examine the association between different HBV genotypes and the prevalence of significant fibrosis and inflammation on liver biopsy.

5. To examine the association between the presence of precore and BCP mutations and the prevalence of histological injury on liver biopsy.
Methods

Patients
Patients of St Vincent’s Hospital Liver Clinics who had had HBV genotyping +/- sequencing and an associated HBV DNA and HBeAg status performed within a 3 month period were included for analysis. The period of collection of data spanned from 1997 to 2008.

Hepatitis B Virus Serological and DNA testing
Hepatitis B surface antigen (HBsAg) was measured using a commercially available immunoassay (Abbott Laboratories, North Chicago, IL, USA) and HBeAg and anti-HBe by an immunoassay produced by BioMerieux Clinical Diagnostics (Marcy l’Etoile, France). All tests pertaining to HBV DNA were performed at the Victorian Infectious Diseases Reference Laboratory (VIDRL). Two different assays were used for HBV DNA testing, prior to April 2004 a capture hybridisation assay (Digene assay) and after April 2004 a bDNA signal amplification probe method (Bayer Versant HBV DNA 3.0 assay). The Digene capture hybridisation assay had 2 generations, the first being used between 1997 and April 2000 with a lower limit of detection of approximately 200,000 IU/ml. Patients who were undetectable on this assay were excluded because of its lack of sensitivity. The second generation Digene Assay was used between approximately April 2000 and April 2004 and had a lower limit of detection of approximately 25,000 IU/ml (0.5 picogram/ml). Patients who were undetectable on this assay but who also had a concomitant PCR which was positive were assigned the value of 25,000 IU/ml for analyses involving HBV DNA. Patients who were above the upper limit of detection of the Digene Assay were assigned the upper limit as their HBV DNA value (2000 pg or 10^8 IU/ml). Picograms were converted to IU/ml by multiplying by 50,000. The dynamic range of the Bayer versant HBV DNA assay is 351 to 17,857,140 IU/ml. Patients tested using the bDNA assay whose HBV DNA levels were below and above the limits of detection were assigned the values of 351 and 17,857,140 IU/ml respectively for analyses involving HBV DNA. One IU/ml is equivalent to 5.6 copies/ml.

HBV genotyping and sequencing of BCP and PC regions of the HBV genome
All testing for HBV genotype and sequencing was performed at the Victorian Infectious diseases reference laboratory (VIDRL) during the course of clinical care and reported on by VIDRL. Methods were as have been previously described (530) but in brief, this involved extraction of HBV DNA from 200 µL of patient serum using the QIAamp DNA MiniKit (QIAGEN, CA, USA) according to the manufacturer’s instructions. HBV DNA amplification was performed with 2 different PCR reactions for the polymerase and BCP/precore regions of the HBV genome.
according to methods previously described (530). For genotyping, the amplified HBV DNA from part 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). The HBV genes were then analyzed using an online HBV genome analysis program. This program compares input sequence data with known reference sequences to determine HBV genotype, variants and mutations associated with antiviral resistance (531). For purposes of this study the precore variant was defined by the presence of the G1896A mutation, and the BCP variant by the A1762T/G1764A mutation (BCP mutation).

Liver biopsy
99 patients had had a liver biopsy performed within 12 months of the date of their genotype and sequencing testing. In Australia, until 2008, liver biopsy was a requirement for subsidised antiviral therapy, and therefore most liver biopsies were performed in patients being assessed for treatment. Formalin fixed, paraffin embedded liver biopsies were sectioned and stained according to usual histology methods. The histological lesions were graded according to the classification proposed by the METAVIR study group (502). Activity is graded applying an algorithm which includes the severity of piecemeal and lobular necrosis resulting in a scale of 0-3 (0=none, 1=mild, 2=moderate and 3=severe activity). Significant inflammation was defined as activity scores of A2/3.

Fibrosis is scored from 0-4 (0 being no fibrosis, 1=enlarged fibrotic portal tracts, 2=periportal or portal-portal septa but intact architecture, 3=fibrosis with architectural distortion but no obvious cirrhosis, 4 = probable or definite cirrhosis). Significant fibrosis was defined as a fibrosis score of F>2.

Statistical Analysis
Data entry and statistical analysis were performed with the statistical package Stata (version 9.2, Statcorp). The statistical methods used were dependent on the nature of the outcome variables. Fisher’s exact test was used to compare categorical data, while the student’s t-test or one way ANOVA was used for group comparisons of parametric quantitative data and the Kruskal-Wallis test for similar comparisons of non-parametric data. Results were presented as median and range whenever appropriate. In all cases tests of significance were two-tailed with a level at <0.05. Logistic regression was used for analyses examining factors associated with the precore mutation and BCP mutation. Initially the separate effect of each factor upon the
outcomes was examined. Subsequently the joint effect of the factors upon the outcomes was examined in a multivariate analysis. This has the advantage that the effect of each factor upon the outcome is adjusted for all the other factors. A backwards selection procedure was used to retain only the statistically significant variables.
Results

Patient Characteristics
198 patients had HBV sequencing performed along with HBeAg, HBV DNA and ALT level. The prevalences of HBV genotypes in these patients were genotype A 7%, Genotype B 36%, Genotype C 37%, and Genotype D 16%. Mixed genotypes (B/C) were found in 6 patients (3%) and there were 2 further patients, one with HBV genotype E and the other with HBV genotype G. Given the small number of patients in these latter 3 groups they were omitted from all subsequent analyses leaving 190 patients. The proportion of males in each HBV genotype group differed with genotypes A and D having a higher proportion (over 85%) than genotypes B and C who had 68% and 66% each (p=0.03) (Table 6.1). The mean age of patients in each genotype group also varied with HBV genotype D infected patients being the oldest with a mean age of 48 whilst patients with genotype B and C infections were the youngest with a mean age of 39 in both groups (p=0.001). Ethnicity varied markedly with HBV genotype (p<0.001). Genotypes B and C were almost exclusively Asian. Genotypes A had a large proportion of Caucasian (Anglo-Celtic) patients (50%) with the remaining being Asian or of other ethnic groups including African. Genotype D had a majority of patients of Mediterranean ethnicity (65%) with the others being split between Caucasian (Anglo-Celtic) and other ethnic groups. HBV DNA levels were not found to vary significantly between HBV genotypes (p=0.22). Serum ALT levels were however found to differ between the HBV genotype patient groups (p=0.002) being highest in the HBV genotype D group with a median of 135 and lowest in HBV genotype B and C groups with medians of 69 and 66 respectively. The proportion of patients who were HBeAg positive also varied being highest in the genotype A group (79%) and lowest in the genotype D group at 29% (p<0.001). Patients infected with HBV Genotype C had an HBeAg positivity rate of 66%.

Pre core and BCP mutations
Sequencing data of the HBV precore and BCP regions was available in 155 of the 198 genotyped patients (Table 6.2). The classic precore mutation (G1896A) was present in the HBV of 65/155 patients (42%). Of these isolates 30 (46%) had G1896A alone and 35 (54%) had G1896A as well as additional mutations in the precore start codon A1814C and T1815C/T and or the G1899A mutation. There were 10/155 (6.5%) of patients who had no classic precore mutation but had mutations other than G1896A in the precore region including 7 with G1899A (+/- T1815C or C1817T) and 3 with various other mutations including A1814C and G1816A/G.
Sequencing of the BCP region of the HBV detected the A1762A/T + G1764A mutations in 75/155 patients (48%). Of these patients, 7 patients also had a C1766C/T mutation. There were 11 further patients (7%) with BCP mutations other than the classic A1762A/T + G1764A ones including one patient each with a single A1762A/T mutation and G1764A mutation, and others with mutations at C1766C/G, T1768A and combinations of these mutations. Mixed populations (that is a sample with greater than 1 predominant viral variants, one of which had an insertion or deletion in the sequence precluding further sequencing) were found in 6 patients (3.8%).

Prevalence of Pre-core and BCP mutations in HBeAg positive and negative disease.
There was a significant association between patient HBeAg status and the presence of an HBV BCP and or precore mutation (p<0.001). In HBeAg negative patients overall 74/83 (89%) had either the classic precore and/or BCP mutations, while 9/83 (11%) had neither classic mutation in these regions. Of these 9 HBV isolates, 3 had wild type sequences, 2 had “mixed populations” and 4 had a non classic mutation at one or both genomic regions. The non classic mutations found were G1899A in precore + C1766G in BCP region of the viral isolate of patient 1, a T1815C + G1899A in the precore region of the HBV of patient 2, A1814C in the precore region of HBV in patient 3 and G1899A in precore and G1764A/G in the BCP region of the HBV of patient 4. The overall prevalence of the pre core mutation in HBeAg positive disease was 8/72 (11%) with 7/72 patients having precore only and 1/72 patient (1.3%) having both the precore and BCP mutations. The prevalence of HBV BCP mutation was 39% in the HBeAg positive cases and was 57% in HBeAg negative cases. It is of note that the BCP mutation by itself was detected in the isolates of 37.5% of the HBeAg positive patients and 20% of HBeAg negative patients.

Prevalence of Precore and BCP mutation in different genotypes.
A significant association was found between HBV genotype and the presence of the precore and BCP mutations in the viral genome (p<0.001). The prevalence of precore mutations differed between patients in the different genotypic groups being 9% in A, 59% in B, 21% in C, and 62% in D (Table 6.2). The prevalence of BCP mutations also varied in the HBV isolated from patients with different genotype infections being 36.% in genotype A, 22% in B, 75% in C and 58% in D. The prevalence of the precore and BCP mutation combination was similar amongst the isolates of patients infected with HBV genotypes B and C (19% and 13% respectively) and was highest among those infected with HBV genotype D at 42% (Table 6.2). No patient with HBV genotype A had both mutations and the majority (55%) of these patients had neither
mutation types. A large proportion (62%) of HBV genotype C patients had a viral variant with a BCP mutation only.

Logistic regression was used to examine the factors associated with HBV precore and BCP mutations (Table 6.3). When examined individually, patient age, HBeAg status and HBV genotype, were all significantly associated with a precore mutation and the BCP mutation, however patient ethnicity and gender were not. Increasing age was associated with an increasing odds ratio (OR) for the presence of the viral mutations: OR of 1.73 (1.29-2.33) (p<0.001) for precore mutation and 1.57 (1.18-2.09) (p =0.002) for BCP mutation for every 10 year increase in patient age. HBeAg negative CHB was also associated with both the precore and BCP mutations with a stronger effect for the precore compared to the BCP mutation. HBV genotypes B and D were the most likely to have a precore mutation with an OR for precore mutation of 14.2 (1.7, 118) in genotype B and 16 (1.77, 145) in genotype D. The OR for the presence of a BCP mutation was highest in HBV genotype C at 5.25 (1.32, 20.1) (p<0.001).

In a multivariate analysis only HBeAg status (p<0.001) and HBV genotype (p=0.002) were found to be significantly associated with a precore mutation. The OR for the presence of precore mutation was highest in HBV genotype B at 14.9 (1.36, 163) (Table 6.4).

Multivariate analysis with BCP mutation as the outcome found that patient age (p=0.03), HBeAg status (p=0.03) and HBV genotype (p<0.001) were all independent predictors of a BCP mutation (Table 6.4).

**Prevalence of significant fibrosis and inflammation in genotype groups.**

99 patients of the cohort of 198 (50%) with genotyping available had had a liver biopsy performed within a one year period of their sequencing test. 3 biopsied patients of the mixed B/C group and a patient with genotype E were excluded from analyses due to small numbers leaving 95. This comprised 5/14 (36%) of the genotype A group, 42/72 (58%) of genotype B patients, 33/73 (45%) of genotype C, 15/31 (48%) of genotype D (p=0.28) (Table 6.5). The proportions of patients with significant fibrosis (defined as F2/3/4) were 80% in genotype A, 36% in genotype B, 58% in genotype C and 53% in D, however these differences did not reach statistical significance (p=0.12) and the numbers of biopsied patients in genotype A was very small. The prevalence of significant inflammation was 20% in genotype A, 26% in genotype B, 39% in genotype C and 60% in genotype D (p=0.10). 7 patients of the biopsied group (7%) had cirrhosis and the prevalence of cirrhosis by genotype was 0% in Genotype A, 7% in genotype B, 3% in genotype C and 20% in genotype D (p=0.20). Statistical analyses were also performed.
looking only at genotypes B and C and no significant differences were found in the rates of biopsy (p=0.14), cirrhosis (p=0.63), or significant inflammation (p=0.32) between these 2 genotypes alone. The prevalences of F2/3/4 fibrosis was 36% in genotype B and 58% in genotype C however although there was a trend towards significance it did not reach it (p=0.07).

**Prevalence of significant fibrosis and inflammation in patients with precore and BCP mutations.**

There were 75 patients with available BCP/precore sequencing who had also had a liver biopsy collected within a one year period of the sequencing test The prevalence of F2/3/4 stage fibrosis was similar in patients with and without the precore mutation (51% and 53% respectively, p=1.00) and in those with and without the BCP mutation (59% and 47% respectively, p=0.35) (Table 6.6). Similarly, the prevalence of A2/3 grade inflammation was similar in patients with and without the precore mutation (40% and 38% respectively, p=1.00) and with and without the BCP mutation (47% and 33% respectively, p=0.24). Patients with non-classic mutations in the precore and BCP regions were analysed further to assess the effect of non-classical mutations in these regions on fibrosis and inflammation (Table 6.6). In patients without the classic precore mutation (n=40), those with non classic mutations versus no mutation at the precore site had similar prevalences of F2/3/4 (p=0.65). The same was true of patients without the classic BCP mutation (n=43); no significant difference was found in F2/3/4 between those with non-classic mutations versus wildtype sequences at the BCP site (p=0.22). These analyses were repeated with A2/3 grade inflammation as the outcome and no significant differences were found.

The effect on histological outcome of the presence of any mutation (classic or non classic, BCP or precore) versus no mutation was also examined (Table 6.6). Those with HBV mutations had a slightly higher prevalence of A2/3 inflammation (46%) compared to those with no mutational change at all (19%) (p=0.04). No difference in the prevalence of significant fibrosis was found in those with some versus no mutational change (p=0.20).

The group was subsequently divided into 4 categories of patients: precore mutation only, BCP mutation only, precore + BCP mutation and neither mutation present (Table 6.7). When comparing these 4 groups, no statistically significant differences were found in the prevalence of F2/3/4 fibrosis (p=0.15) or A2/3 inflammation (p=0.35). The prevalence of F2/3/4 was highest in patients with viral variants containing the BCP mutation alone at 73% however this result did not reach statistical significance (p=0.15).
Mutations found in patients with cirrhosis.
7 patients with HBV BCP/precore sequencing testing had cirrhosis present on liver biopsy. Of these 7, 4 had the BCP mutation (1 with the BCP mutation alone, 1 with the BCP + precore mutations and 2 with the BCP mutation and non-classic mutations in the precore region). The HBV of 1 further patient had a non classic BCP mutation alone and another had a non-classic BCP and a precore mutation. A final patient had a precore mutation alone.

Discussion
Our cohort is a heterogeneous one with representation from the 4 major groups of HBV genotype infections around the world and presents for the first time to our knowledge detailed genotypic and sequencing analysis of the HBV isolated from an Australian cohort of CHB patients as well as correlation with liver histology. We confirm that different genotypes are associated with varying prevalence of precore and BCP mutations. However, no significant association was found in our cohort between HBV genotype or HBV mutational status and histological measures of inflammation or fibrosis.

CHB in Australia is a disease largely of migrant populations including those of Italian and Greek backgrounds who migrated in the 1930’s and those from South East Asia (predominantly Vietnamese people who arrived in the 1970’s, but also people from other countries of the region including Cambodia, Laos, Thailand and more recently from China). The differing ages of patients in the different HBV genotype groups reflects the fact that the Mediterranean cohort (Genotype D) are older and the Asians (genotypes B and C) are younger. HBeAg positivity rates differed between different HBV genotype groups. ALT levels were lower in the patients infected with genotypes B and C (prevalent in Asia) compared to those with genotypes A and D and this may be in keeping with several Asian studies showing that liver injury can occur at lower ALT levels (125, 138) and perhaps support lower thresholds for defining normal for ALT in Asian patients.

In addition to the classic precore and BCP mutations found in our cohort, numerous other non-classic BCP and precore mutations (6.5% of cases in the precore region and 7% in the BCP region) were also found. These included the G1899A mutation which has been well described (226) and is often seen in association with the precore stop codon mutation G1896A (532). Mutations were also found in the precore start codon (nucleotide positions 1814-1816) which have also been reported to abolish HBeAg production (533). The non classical mutations in nucleotide positions 1766 and 1768 in the core promoter regions have also been described.
previously (360) and have been shown to possibly increase replication in an in vitro system (534).

As would be expected in multivariate analysis, HBeAg status is an independent predictor of the presence of precore and BCP mutations in the HBV genome. This is in keeping with the known abrogation or reduction of HBeAg production seen in patients with HBV that have the precore and BCP mutations (82, 535). While the prevalence of precore and BCP mutations was higher in HBeAg negative patients (89% overall), close to half (49%) of the HBeAg positive patients had either the precore or BCP mutations present although only 1 patient isolate had both mutations. The finding of BCP mutation without precore was in fact more commonly seen in HBeAg positive patients than negative patients (38% v/s 20%). Yuen et al have showed that both precore and BCP mutations are present in the serum of patients in significant quantities (>20%) both before and after HBeAg seroconversion (536). Lim et al (362) showed that during the HBeAg positive immune clearance phase, the HBV often exists as a viral quasispecies containing both wildtype and mutant viruses with high rates of nucleotide substitution in the precore/core gene (especially in those who undergo HBeAg seroconversion). Thus immune selection pressure drives diversification of the viral population and this may be evident up to 3 years before HBeAg seroconversion with viral diversity increasing further after viral load reduction. (148, 362). Furthermore BCP mutation reduces but it does not abolish HBeAg production (366). Of the HBeAg negative patients 9 (11%) were found to have neither the precore nor the BCP mutations on sequencing. The non-classic mutations found in 4 of the patients were presumably responsible for reducing their HBeAg production however no mutations at all were reported in 3 (of 84) HBeAg negative patients and the reason for their HBeAg negativity is unclear but may perhaps reflect low levels of HBV DNA associated with immune control although this was not specifically examined.

With regard to the association of genotype and viral variants, our study found a significant association between genotype and the presence of BCP/Precore mutations on univariate and multivariate analysis. In particular, genotype B is associated with the precore and genotype C with the BCP mutation. Others have reported the association between HBV genotype C and BCP (537) and HBV genotype B and the precore mutation (518) which was confirmed in the present study. The highest OR for precore mutation on multivariate analysis in our cohort was in genotype B which is in contrast to others who have found genotype D to have the strongest association with precore mutation (526) although in concordance with other studies with representation from genotypes A-D which also report a higher prevalence of precore mutation in Genotype B compared to D (538). Earlier HBeAg seroconversion in genotype B compared to C has been reported in many studies (158, 160, 324). The mechanisms underlying the earlier HBeAg
seroconversion seen in HBV genotype B compared to C are not known however Yuen and colleagues have provided some immunologic evidence that this may be because HBV genotype B induces a greater Th1 and lesser Th2 response than genotype C (213). They demonstrated higher numbers of IFN gamma producing cells and lower numbers of IL-10 producing cells in HBeAg positive HBV genotype B patients compared to genotype C during hepatitis flares which may account for the higher chance of HBeAg seroconversion. In this study it was also noted that similar immunological responses (to that seen with HBV genotype B) were also found in those with precore mutation compared to those without. HBV genotype A had the lowest prevalence of precore mutations and of combined BCP/precore mutations which is in keeping with the findings of other groups and the known structural barrier to the development of the precore mutation (184). The HBV precore mutation. G1896A is more likely to occur when the nucleotide opposite it (nucleotide 1858) in the stem loop structure (epsilon encapsidation signal) is a thymine (T) since this double hydrogen bond pairing (T_{1858}-A_{1896}) would generate an epsilon that is more stable than pairing T_{1858} with G_{1896}. In contrast, a virus with C_{1858} and G_{1896} already has a stable epsilon (C-G has triple hydrogen bonding) and is unlikely to select for a G1896A mutation. Genotype A is known to frequently have a C (cytosine) at position 1858 (within codon 15) and therefore precore mutants are rarely associated with HBV genotype A. 1858C is positively associated with HBV genotypes A and H and 1858T with genotypes B, D, and E. Subgenotype C2 is associated with 1858T but C1 is associated with C at position 1858 (264). Chan et al (370), postulated that perhaps in genotypes with C1858, where the precore mutation was essentially precluded, there may be preferential selection of BCP mutation. However the association between HBV genotype C and the BCP mutation is likely to involve factors other than this which are at this stage not yet clear since in our genotyping methodology the reference sequence for HBV-C sequences has a T at 1858 and most of our HBV-C patients are of C2 subgenotype and therefore are likely to have a T at 1858.

Age is not found to be an independent predictor of the presence of precore mutation in HBV but it is for the BCP mutation which is slightly at odds with Chan’s study which reported that this mutation emerged early in their cohort (370). However it is possible that increasing time (and immune selection pressure) results in progressive increase in the prevalence of BCP mutations in patients with age.

In our study we could demonstrate no difference across the 4 genotypes in terms of the presence of significant fibrosis, inflammation or cirrhosis. The small numbers in certain subsets including patients with HBV genotypes A and D and cirrhotic patients may have had a bearing on this. There is little data comparing rates of fibrosis and cirrhosis across all 4 genotypes, however there are a number of studies from Asia showing that patients with genotype C have
more severe liver disease than those with genotype B (159, 346) and this is probably due to delayed HBeAg seroconversion and prolonged active disease (332) or possibly to do with the association with the BCP variant. In the analysis confined to genotypes B and C alone, the prevalences of F2/3/4 fibrosis was higher in genotype C at 58% compared to 36% in genotype B with a trend towards significance (p=0.07). There was a relatively high prevalence of F2/3/4 in our biopsied cohort across genotypes and the lack of difference shown overall may reflect the fact that the biopsied patients were biased towards those with active liver disease.

We did not find any association between the HBV precore or BCP mutations and the presence of significant fibrosis or inflammation on liver biopsy which is out of keeping with emerging literature on the association of the BCP mutation with more significant liver disease. For example, Lapalus et al (539) in a study of 377 CHB patients recently showed that the presence of BCP variants was a predictor of significant fibrosis (≥F2). Although in our study the highest prevalence of F2/3/4 was found in patients with the BCP mutation alone (73%) this result was not significant (p= 0.15). We surmise that reasons for our negative finding may have been small numbers and a slight bias in the cohort toward those with more significant fibrosis overall. Other recent data includes Fang et al (540) and Kao et al (369) who report a higher prevalence of BCP mutations in patients with cirrhosis. Others, including Chen (541) and more recently Chu et al (542) in their case control study have also identified BCP as an independent risk factor for cirrhosis. Tseng et al (543) have also recently shown that higher proportions of BCP (>45%) increases the risk of liver cirrhosis in HBV genotype B or C infection. Li et al (544) showed that the prevalences of many point mutations in the precore and BCP regions of the HBV genome and also deletion mutations mostly in the pre-S1 and preS2 regions increased with progression of disease state, from acute to chronic hepatitis B and on to liver failure. The small number of cirrhotic patients with HBV sequencing performed in our cohort had a number of mutations and we also showed that the prevalence of significant inflammation (A2/3) was significantly higher in those who had some mutation versus no mutation (p=0.04). Others have similarly shown that it is the presence of multiple mutations (in both the precore and BCP regions of HBV) that predict disease progression (394, 545). The BCP mutation as well as genotype C have been shown to be associated with HCC development (371, 546) although this outcome was not examined in our study.

**Conclusion**

Our study documents in detail the distribution of HBV genotypes in an Australian cohort of chronic hepatitis B patients. Reasonable representation from the 4 major genotypes A, B, C
and D is made. In this diverse cohort we found patients with genotype B HBV infection had the highest risk for selecting precore mutation and the highest risk for selecting BCP mutations was in those with HBV genotype C infections. Our study was negative in terms of identifying any of the 4 major HBV genotypes or viral mutants as factors associated with patients’ histological liver fibrosis and inflammation although there was a trend toward the association of HBV genotype C infection with F2/3/4 fibrosis. CHB is a complex condition in which the development of liver disease is determined by the interplay of many factors that are still incompletely understood. Other viral mutations and also many host factors (immunological, genetic and gender related) and the effect of these on the natural history of CHB are also likely to be importance. Our data contribute, in an ethnically diverse Australian CHB population a detailed examination of the relationships between virological profile and histological results. The lack of obvious positive findings in our study underscore the intricacy of this viral infection and its effects on the human liver and further study of other less well characterized viral mutants may provide further insight in time.
Table 6-1: Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>14 (7%)</td>
<td>72 (38%)</td>
<td>73 (38%)</td>
<td>31 (16%)</td>
<td></td>
</tr>
<tr>
<td>Males (%)</td>
<td>12 (86%)</td>
<td>49 (68%)</td>
<td>48 (66%)</td>
<td>29 (90%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Mean age (SD)</td>
<td>43 (12)</td>
<td>39 (11)</td>
<td>39 (11)</td>
<td>48 (14)</td>
<td>0.001</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>3 (21%)</td>
<td>71 (99%)</td>
<td>72 (99%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Caucasian (Anglo-Celtic)</td>
<td>7 (50%)</td>
<td>1 (1%)</td>
<td>0</td>
<td>4 (13%)</td>
<td></td>
</tr>
<tr>
<td>Mediterranean</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20 (65%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Other</td>
<td>4 (29%)</td>
<td>0</td>
<td>1 (1%)</td>
<td>7 (23%)</td>
<td></td>
</tr>
<tr>
<td>HBV DNA (x10^5) IU/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>92 (1,978)</td>
<td>27 (1,175)</td>
<td>43 (3,175)</td>
<td>175 (8,270)</td>
<td>0.22</td>
</tr>
<tr>
<td>ALT IU/ml Median (IQR)</td>
<td>120 (47,202)</td>
<td>69 (46,126)</td>
<td>66 (39,112)</td>
<td>135 (82,192)</td>
<td>0.002</td>
</tr>
<tr>
<td>HBeAg positive (%)</td>
<td>11 (79%)</td>
<td>30 (42%)</td>
<td>48 (66%)</td>
<td>9 (29%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 6-2: Prevalence of HBV pre-core and BCP mutations in patients by HBeAg status and genotype.

<table>
<thead>
<tr>
<th>HBeAg status</th>
<th>Precore mutation alone</th>
<th>BCP mutation alone</th>
<th>BCP + Precore mutation</th>
<th>Neither classic mutation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBeAg positive</td>
<td>7 (9.72%)</td>
<td>27 (37.5%)</td>
<td>1 (1.39%)</td>
<td>37 (51.39%)</td>
<td>72 (100%)</td>
</tr>
<tr>
<td>HBeAg negative</td>
<td>27 (32.53%)</td>
<td>17 (20.48%)</td>
<td>30 (36.14%)</td>
<td>9 (10.84%)</td>
<td>83 (100%)</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1 (9.09%)</td>
<td>4 (36.36%)</td>
<td>0</td>
<td>6 (54.55%)</td>
<td>11 (100%)</td>
</tr>
<tr>
<td></td>
<td>23 (39.66%)</td>
<td>2 (3.45%)</td>
<td>11 (18.97%)</td>
<td>22 (37.93%)</td>
<td>58 (100%)</td>
</tr>
<tr>
<td>B</td>
<td>4 (7.69%)</td>
<td>32 (61.54%)</td>
<td>7 (13.46%)</td>
<td>9 (17.31%)</td>
<td>52 (100%)</td>
</tr>
<tr>
<td>C</td>
<td>5 (19.23%)</td>
<td>4 (15.38%)</td>
<td>11 (42.31%)</td>
<td>6 (23.08%)</td>
<td>26 (100%)</td>
</tr>
</tbody>
</table>
Table 6-3: Logistic regression of individual factors on presence of precore and BCP mutations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Precore mutation N (%)</th>
<th>Odds Ratio (95% CI) for precore Mutation</th>
<th>P-value</th>
<th>BCP Mutation N (%)</th>
<th>Odds Ratio (95% CI) for BCP mutation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (*)</td>
<td>-</td>
<td>1.73 (1.29, 2.33)</td>
<td>&lt;0.001</td>
<td>-</td>
<td>1.57 (1.18, 2.09)</td>
<td>0.002</td>
</tr>
<tr>
<td>Female</td>
<td>16/39 (41%)</td>
<td>1</td>
<td>0.89</td>
<td>15/39 (38%)</td>
<td>1</td>
<td>0.15</td>
</tr>
<tr>
<td>Male</td>
<td>49/116 (42%)</td>
<td>1.05 (0.50, 2.20)</td>
<td></td>
<td>60/116 (52%)</td>
<td>1.71 (0.82, 3.59)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity Asian</td>
<td>46/118 (39%)</td>
<td>1</td>
<td>0.19</td>
<td>57/118 (48%)</td>
<td>1</td>
<td>0.97</td>
</tr>
<tr>
<td>Ethnicity Other</td>
<td>19/37 (51%)</td>
<td>1.65 (0.79, 3.47)</td>
<td></td>
<td>18/37 (49%)</td>
<td>1.01 (0.48, 2.12)</td>
<td></td>
</tr>
<tr>
<td>HBeAg negative</td>
<td>57/83 (69%)</td>
<td>1</td>
<td>&lt;0.001</td>
<td>47/83 (57%)</td>
<td>1</td>
<td>0.03</td>
</tr>
<tr>
<td>HBeAg positive</td>
<td>8/72 (11%)</td>
<td>0.06 (0.02, 0.14)</td>
<td></td>
<td>82/72 (39%)</td>
<td>0.49 (0.26, 0.93)</td>
<td></td>
</tr>
<tr>
<td>Genotype A</td>
<td>1/11 (9%)</td>
<td>1</td>
<td></td>
<td>4/11 (36%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Genotype B</td>
<td>34/58 (59%)</td>
<td>14.2 (1.70, 118)</td>
<td>&lt;0.001</td>
<td>13/58 (22%)</td>
<td>0.51 (0.13, 2.00)</td>
<td></td>
</tr>
<tr>
<td>Genotype C</td>
<td>11/52 (21%)</td>
<td>2.68 (0.31, 23.3)</td>
<td></td>
<td>39/52 (75%)</td>
<td>5.25 (1.32, 20.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Genotype D</td>
<td>16/26 (62%)</td>
<td>16.0 (1.77, 145)</td>
<td></td>
<td>15/26 (58%)</td>
<td>2.39 (0.56, 10.2)</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 6-4: Multivariate analysis of factors significantly associated with precore and BCP mutations

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio (95% CI)</th>
<th>P-value</th>
<th>Odds Ratio (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>For precore mutation</td>
<td></td>
<td>for BCP mutation</td>
<td></td>
</tr>
<tr>
<td>Age (*)</td>
<td>-</td>
<td>NS</td>
<td>1.56 (1.05, 2.34)</td>
<td>0.03</td>
</tr>
<tr>
<td>HBeAg negative</td>
<td>1</td>
<td>&lt;0.001</td>
<td>1</td>
<td>0.03</td>
</tr>
<tr>
<td>HBeAg positive</td>
<td>0.04 (0.01, 0.12)</td>
<td></td>
<td>0.35 (0.13, 0.92)</td>
<td>0.03</td>
</tr>
<tr>
<td>Genotype A</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Genotype B</td>
<td>14.9 (1.36, 163)</td>
<td>0.002</td>
<td>0.41 (0.09, 1.91)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Genotype C</td>
<td>2.19 (0.31, 24.1)</td>
<td></td>
<td>7.65 (1.68, 34.9)</td>
<td></td>
</tr>
<tr>
<td>Genotype D</td>
<td>9.36 (0.79, 111)</td>
<td></td>
<td>1.36 (0.28, 6.66)</td>
<td></td>
</tr>
</tbody>
</table>

(*) Odds ratio given for a 10 year increase in age.
Table 6-5: Prevalence of significant fibrosis, inflammation and cirrhosis among different HBV genotypes.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Genotype A N=14</th>
<th>Genotype B N=72</th>
<th>Genotype C N=73</th>
<th>Genotype D N=31</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not Biopsied</td>
<td>9 (64%)</td>
<td>30 (42%)</td>
<td>40 (55%)</td>
<td>16 (52%)</td>
<td>0.28</td>
</tr>
<tr>
<td>Biopsied</td>
<td>5 (36%)</td>
<td>42 (58%)</td>
<td>33 (45%)</td>
<td>15 (48%)</td>
<td></td>
</tr>
<tr>
<td>F0/1 fibrosis</td>
<td>1 (20%)</td>
<td>27 (64%)</td>
<td>14 (42%)</td>
<td>7 (47%)</td>
<td>0.12</td>
</tr>
<tr>
<td>F2/3/4 fibrosis</td>
<td>4 (80%)</td>
<td>15 (36%)</td>
<td>19 (58%)</td>
<td>8 (53%)</td>
<td></td>
</tr>
<tr>
<td>No Cirrhosis</td>
<td>5 (100%)</td>
<td>39 (93%)</td>
<td>32 (97%)</td>
<td>12 (80%)</td>
<td>0.20</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>0 (0%)</td>
<td>3 (7%)</td>
<td>1 (3%)</td>
<td>3 (20%)</td>
<td></td>
</tr>
<tr>
<td>A0/1 Inflammation</td>
<td>4 (80%)</td>
<td>31 (74%)</td>
<td>20 (61%)</td>
<td>6 (40%)</td>
<td>0.10</td>
</tr>
<tr>
<td>A2/3 Inflammation</td>
<td>1 (20%)</td>
<td>11 (26%)</td>
<td>13 (39%)</td>
<td>9 (60%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 6-6: Prevalence of significant fibrosis and inflammation in patients with precore and BCP mutations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>F0/1 Fibrosis N (%)</th>
<th>F2/3/4 Fibrosis N (%)</th>
<th>P-value</th>
<th>A0/1 Inflammation N (%)</th>
<th>A2/3 Inflammation N (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Precore Mutation</strong></td>
<td>Not detected</td>
<td>19 (47%)</td>
<td>21 (53%)</td>
<td>1.00</td>
<td>25 (62%)</td>
<td>15 (38%)</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Detected</td>
<td>17 (49%)</td>
<td>18 (51%)</td>
<td></td>
<td>21 (60%)</td>
<td>14 (40%)</td>
<td></td>
</tr>
<tr>
<td><strong>Without precore Mutation</strong></td>
<td>Non-classic</td>
<td>3 (60%)</td>
<td>2 (40%)</td>
<td>0.65</td>
<td>1 (20%)</td>
<td>4 (80%)</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>No significant changes</td>
<td>16 (46%)</td>
<td>19 (54%)</td>
<td></td>
<td>24 (69%)</td>
<td>11 (31%)</td>
<td></td>
</tr>
<tr>
<td><strong>BCP Mutation</strong></td>
<td>Not detected</td>
<td>23 (53%)</td>
<td>20 (47%)</td>
<td>0.35</td>
<td>29 (67%)</td>
<td>14 (33%)</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Detected</td>
<td>13 (41%)</td>
<td>19 (59%)</td>
<td></td>
<td>17 (53%)</td>
<td>15 (47%)</td>
<td></td>
</tr>
<tr>
<td><strong>Without BCP Mutation</strong></td>
<td>Non-classic</td>
<td>2 (29%)</td>
<td>5 (71%)</td>
<td>0.22</td>
<td>3 (43%)</td>
<td>4 (57%)</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>No significant changes</td>
<td>21 (58%)</td>
<td>15 (42%)</td>
<td></td>
<td>26 (72%)</td>
<td>10 (28%)</td>
<td></td>
</tr>
<tr>
<td><strong>Combined mutation</strong></td>
<td>No mutations</td>
<td>13 (62%)</td>
<td>8 (38%)</td>
<td>0.20</td>
<td>17 (81%)</td>
<td>4 (19%)</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Some mutation</td>
<td>23 (43%)</td>
<td>31 (57%)</td>
<td></td>
<td>29 (54%)</td>
<td>25 (46%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 6-7: Significant fibrosis and inflammation in patients with sequencing of the precore and BCP region.

<table>
<thead>
<tr>
<th></th>
<th>F0/1</th>
<th>F2/3/4</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neither Mutation (n=27)</td>
<td>17 (63%)</td>
<td>10 (37%)</td>
<td>p=0.15</td>
</tr>
<tr>
<td>Precore mutation alone (n=18)</td>
<td>8 (44%)</td>
<td>10 (56%)</td>
<td></td>
</tr>
<tr>
<td>BCP mutation alone (n=15)</td>
<td>4 (27%)</td>
<td>11 (73%)</td>
<td></td>
</tr>
<tr>
<td>BCP and Precore mutation (n=17)</td>
<td>9 (53%)</td>
<td>8 (47%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A0/1</td>
<td>A2/3</td>
<td></td>
</tr>
<tr>
<td>Neither Mutation (n=27)</td>
<td>20 (74%)</td>
<td>7 (26%)</td>
<td>p=0.35</td>
</tr>
<tr>
<td>Precore mutation alone (n=18)</td>
<td>11 (61%)</td>
<td>7 (39%)</td>
<td></td>
</tr>
<tr>
<td>BCP mutation alone (n=15)</td>
<td>7 (47%)</td>
<td>8 (53%)</td>
<td></td>
</tr>
<tr>
<td>BCP and Precore mutation (n=17)</td>
<td>10 (59%)</td>
<td>7 (41%)</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 7: Quantitative HBeAg and HBsAg in phases of disease of CHB

Introduction
Perinatally acquired hepatitis B has traditionally been conceived of as consisting of 4 largely sequential phases of disease, the HBeAg positive immune tolerant and immune clearance phases and HBeAg negative immune control and immune escape phases. At times these are difficult to define precisely as the dynamic state of balance between the HBV and host immune system leads to fluctuating ALT and HBV DNA levels and indeed occasionally, HBeAg status. ALT levels in the immune clearance phase may be intermittently normal making distinction from the immune tolerant state difficult if monitoring is not particularly intensive. A threshold of HBV DNA to distinguish immune tolerant from immune clearance phases has never been set in guidelines although it is generally accepted that patients with immune tolerance have HBV DNA levels 2-3 logs higher than those in the immune clearance phase. With increased screening for hepatitis B, more patients are referred to clinic at a young age who may remain in the HBeAg positive immune tolerant phase for years. Serum markers that may separate out those who may be approaching immune clearance (and therefore the possible need for treatment) from those who are likely to remain stably immune tolerant for some time would be useful as it would delineate those who require more intensive monitoring. There is almost nothing in the literature about quantitative HBeAg levels in untreated CHB patients apart from a detailed study by Thompson et al (405) from our group. In their paper they report the distribution of qHBeAg levels in 71 HBeAg positive patients (all of whom had elevated ALT and thus were in the immune clearance phase) showing a skewed distribution of qHBeAg with 49% having qHBeAg <1000 PEIU/ml and 18% having qHBeAg < 31 PEIU/ml They also found a positive correlation between HBeAg levels and HBV DNA (including intrahepatic HBV DNA although not cccDNA). The emergence of BCP and precore mutants was found to reduce HBeAg titres independent of viral replication (405).

In HBeAg negative patients also, HBV DNA thresholds (usually of 2000 IU/ml) that are used to differentiate inactive carriers from HBeAg negative CHB may often be crossed and therefore bring about uncertainty about phase of disease. Once again for purposes mainly of quantifying risk of chronic hepatitis, need for treatment and intensity of monitoring, a marker which reliably helps differentiate these 2 HBeAg negative states would be of clinical use.
Aims
We sought to examine the qHBeAg levels in immune tolerant versus immune clearance phases of HBeAg positive CHB and furthermore we aimed to examine the quantitative levels of HBsAg in different phases of CHB to see if they may help to differentiate between patients in the various phases.

Methods

Patients
Patients with Chronic hepatitis B were identified, cross sectionally from the St Vincent’s hospital Hepatitis B database who met the following criteria. They were treatment naïve and had a complete dataset enabling classification into one of the following groups:

1. Immune tolerant: HBeAg positive (qualitative assay) and anti HBe negative, ALT ≤45.

2. Immune clearance: HBeAg positive (qualitative assay) and antiHBe negative, ALT >45.

3. Immune control: HBeAg negative (qualitative assay) and anti-HBe positive, HBV DNA <2000 IU/ml.

4. Immune escape: HBeAg negative (qualitative assay) and anti-HBe positive, ALT >35.

Quantitative HBeAg and HBsAg
Quantitative measurement of HBeAg: Serum HBeAg levels were measured by commercial EIA (Abbott Diagnostics) on the Architect platform. Modification of Abbott’s commercial kit for qualitative HBeAg detection can be achieved to produce semiquantitative results with a limited range based on the method described by Perillo et al as elaborated on in Methods, Chapter 2. Quantitative HBsAg Assay: Serum HBsAg was measured by EIA using the Architect platform (Abbott Laboratories) as per manufacturer’s instructions which expresses HBsAg against an internal WHO reference standard in IU/ml, once again as described in Chapter 2.

HBV DNA Assays
HBV DNA: Samples after April 2004 were tested using the Versant HBV DNA 3.0 assay (bDNA) (Siemens Healthcare Diagnostics, Tarrytown, NY) dynamic range 351-17,500,000 IU/ml. Patients whose HBV DNA level was above or below the range of the bDNA test were assigned the upper or lower limit respectively as their HBV DNA value. Samples before April 2004 were tested using the Digene Hybrid capture II assay (Digene Diagnostics, Beltsville, MD) LLD 25,000 IU/ml. Digene results in picograms converted to IU/ml by approximating 1picogram=50,000 IU/ml.
**Statistical methods.**
Categorical variables were compared using Fisher’s exact test. The continuous outcomes were found to have skewed (non-normal) distributions, and so the Kruskal-Wallis test was used to compare between groups for these measures. Additionally the association between each of qHBeAg and qHBsAg with ALT and HBV DNA was examined. All variables were measured on a continuous scale, and most were found to be not normally distributed. Therefore, Spearman’s rank correlation was used for these analyses.

**Results**
95 patients with a complete cross sectional data set were identified, 16 in the immune tolerant phase, 32 in the immune clearance phase, 20 in the immune control phase and 27 in the immune escape phase of disease. The mean age of patients in the 4 groups differed, with the HBeAg positive patients being younger than the HBeAg negative ones (Table 7.1). HBV DNA levels and ALT levels were also different in the 4 groups as would be expected (Table 7.1).

The distributions of qHBeAg in the immune tolerant and immune clearance group are shown in Figure 7.1. Although median qHBeAg levels were higher in the immune tolerant than the HBeAg positive immune clearance group at 2537 PEIU/ml v/s 1166 PEIU/ml), this did not reach statistical significance (p=0.20). The proportions of patients with qHBeAg <1000 PEIU/ml was 3/16 (18.75%) in the immune tolerant group and 13/32 (41%) in the immune clearance group (p=0.193). The proportion with qHBeAg <100 PEIU/ml was 0 in the immune tolerant and 6/32 (19%) in the immune clearance group which once again was not statistically different (p=0.082).

**qHBsAg levels in phases of chronic hepatitis B.**
The median qHBsAg levels were significantly different between the 4 HBV phase groups, being highest at 39,025 IU/ml in the immune tolerant group and lowest at 1980 IU/ml in the Immune control group (p<0.001) (Table 7.1). The distributions of qHBsAg values in each of the 4 groups are shown in Figure 7.2.

**Correlation of HBV DNA With qHBeAg and qBHsAg**
Spearman’s rank correlation was used to examine the association between the ALT, HBV DNA and qHBeAg/qBHsAg in the whole cohort. The results suggested a significant association between HBV DNA and both qHBeAg and qBHsAg (Table 7.2). The correlation with HBV DNA was stronger with qBHsAg at 0.61 (p=<0.001) than qHBeAg at 0.41 (p=0.006). However the upper limit of the assay (17,857,140 IU/ml) was a limiting factor in this analysis. There was no association between ALT and qBHsAg although there appeared to be a weak negative correlation between ALT and qHBeAg (-0.29) of borderline statistical significance (p=0.05).
**Discussion**

Our data demonstrates that qHBsAg levels differ significantly in different phases of CHB. Similarly, as has been shown by Thompson et al (405), looking at the cohort as a whole, a correlation was demonstrated between qHBeAg and HBV DNA and also between HBsAg levels and HBV DNA. Although there appeared to be a lower median qHBeAg and greater % with qHBeAg <1000 PEIU/ml in Immunotolerant group compared to the immune clearance group, no statistically significant difference was demonstrated in our study. Lower qHBeAg in Immune clearance patients could be expected for a number of reasons. Firstly, given the correlation between HBV DNA and qHBeAg it would follow that immune tolerant patients with very high HBV DNA levels would be expected to have correspondingly high qHBeAg levels. Furthermore, the immune clearance phase precedes HBeAg seroconversion and viral quasispecies diversification increases as patients near the HBeAg seroconversion event. A negative correlation has been shown between viral diversity and HBeAg levels (547). The appearance of increasing proportions of BCP/PC mutant virus which occurs leading up to HBeAg seroconversion (548) would lower HBeAg levels. The failure to demonstrate a difference in qHBeAg levels in our group may partly due to small numbers, especially in the immune tolerant group. It should also be noted that the definition of immune tolerance we used was with a single ALT only and at a relatively high threshold (45 IU/ml for both men and women), which was chosen because of the very small numbers of untreated patients with lower ALT levels. Thus the group may not have represented a truly immune tolerant cohort. It may be possible that a larger cohort with persistently normal ALT (possibly of lower thresholds and different ones for men and women) and including data on liver fibrosis may demonstrate a difference. Establishing cut-offs of qHBeAg to predict immune tolerant versus immune clearance phases could assist clinically with decisions about frequency of monitoring.

HBeAg and HBsAg are translated from separate transcripts derived directly from HBV covalently closed circular DNA (cccDNA) and have been considered possible surrogate markers of the number of infected hepatocytes (549). Thus it has been postulated that HBsAg levels may represent an indirect measure of immunological control of the HBV (and hence phase of disease). Some studies have shown that there is a correlation between HBsAg and cccDNA and (401, 550). Detailed work by Thompson et al showed that in HBeAg positive CHB, HBsAg correlated with serum and intrahepatic total HBV DNA as well as cccDNA but in HBeAg negative patients there was only a weak correlation between HBsAg levels and HBV DNA in the serum and none with intrahepatic total HBV DNA and cccDNA (405). The HBsAg levels in
different phases of disease demonstrated in the current study formed part of the dataset used by Nguyen et al. from our group who reported significant differences in HBsAg levels across different phases of CHB in an Asian cohort in 2010 (551). Differences in HBsAg levels have also been described in a European cohort (404) although overall the values of qHBsAg were lower in the Asian cohort which may be related to differences in genotype (552). Since then others have further refined the utility of qHBsAg levels for defining phases, in particular for differentiating inactive carriers from those with HBeAg negative CHB or predicting reactivation to that state. For example, longitudinally studied cohorts have also confirmed the lower HBsAg levels in inactive carriers compared to HBeAg negative CHB although the difference is HBsAg levels is not large (2.24 vs 2.98 log IU/ml p=0.054)(412). An Italian group who defined 209 patients (HBeAg negative, genotype D) into active or inactive infection following a year of monitoring reported significantly lower serum HBsAg levels in inactive carriers than active HBeAg negative CHB (62 vs 3029 IU/ml) (406). The combination of HBV DNA ≤2000 IU/ml and HBsAg <1000 IU/ml at a single time point was reported to have a sensitivity of 91% and a Positive Predictive value of 87.9% for identifying a 3 year period of inactive carrier status and was recommended as being comparable to long term tight monitoring (406). These same thresholds have also been shown to be predictive of inactive carriers in an Asian cohort of genotype B and C patients (553). Similarly, thresholds for identification of subsequent reactivation to Chronic HBeAg disease from inactive carrier state have been shown by a French group to be HBsAg levels of ≥ 1000 IU/ml and HBV DNA ≥ 200 IU/ml (178).

Other uses for quantitative HBsAg in the natural history of disease include in predicting HBsAg loss (409, 410, 554). Furthermore degrees of decline of ≥ 0.3 log 10 IU/ml/year has also been reported as heralding HBsAg loss (178). Correlations with other clinical features of CHB have been sought and demonstrated in the last few years, including prediction of liver fibrosis by HBsAg levels in HBeAg positive disease (414, 555). HBsAg levels may also play an important role in quantifying HCC risk in HBeAg negative patients with low viral loads (<2000 IU/ml with levels ≥ 1000 IU/ml signifying increased risk (416).

**Conclusions**

Although understanding Chronic hepatitis B in terms of phase of disease is critical and has proven largely very usable, there remain subsets of patients who may be difficult to accurately classify both in terms of their current stage of disease and their medium to longer term risk of active disease and its outcomes. Increasing use of non invasive serum markers such as quantitative HBeAg/HBsAg serology can be helpful to the clinician and qHBsAg being a cheap
and automated assay can fairly easily be incorporated into clinical algorithms. Quantitative HBeAg levels, alone or in combination with HBV DNA, may have a prognostic role but the data that has thus far been reported is very limited and further work remains. Studies, perhaps of larger and better defined immune tolerant cohorts may reveal differences in HBeAg levels, and more detailed analyses of the interaction between levels of qHBeAg and measures of viral diversity and BCP/Precore mutations may further elucidate things. A better understanding of the immune tolerant state (which is likely to include identification of novel immunological markers both host and viral) will provide the key to many aspects of hepatitis B, including reasons for viral persistence in some but not others and factors relating to loss of tolerance. HBeAg is still a fairly poorly understood protein, but likely to play critical roles in the above processes.
### Table 7-1 Patient Characteristics and baseline qHBeAg and qHBsAg in the 4 phases of CHB.

<table>
<thead>
<tr>
<th></th>
<th>Immuno-tolerant</th>
<th>Immune-clearance</th>
<th>Immune control</th>
<th>Chronic HBeAg Negative</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>16</td>
<td>32</td>
<td>20</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td><strong>Age mean (SD)</strong></td>
<td>30.44(9.16)</td>
<td>32.44(10.12)</td>
<td>37.83(13.52)</td>
<td>48.46(12.58)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>ALT Median (range)</strong></td>
<td>32 (10-45)</td>
<td>125 (52-570)</td>
<td>39 (9-2038)</td>
<td>67 (36-492)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td><strong>HBV DNA in IU/ml</strong></td>
<td>6.4 x 10^7 IU/ml (7.2x10^5 - 2.7x10^6)</td>
<td>1.78 x 10^7 (877-1.78 x10^7)</td>
<td>366 (357-546)</td>
<td>3.3 x 10^6 (357-1.78 x 10^7)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td><strong>qHBeAg Median (range)</strong></td>
<td>2537 (352, 5065)</td>
<td>1168 (7, 15908)</td>
<td>-</td>
<td>-</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>qHBsAg Median (range)</strong></td>
<td>39025 (1140, 91390)</td>
<td>11800 (230, 170460)</td>
<td>1980 (9, 37040)</td>
<td>3280 (10, 30730)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 7-2: Spearman’s rank correlation coefficients for HBV DNA, ALT and qHBeAg/qHBsAg.

<table>
<thead>
<tr>
<th>Variable</th>
<th>qHBeAg</th>
<th>qHBsAg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of patients</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>HBV DNA</td>
<td>44</td>
<td>0.41</td>
</tr>
<tr>
<td>ALT</td>
<td>45</td>
<td>-0.29</td>
</tr>
</tbody>
</table>
Figure 7-1: Quantitative HBeAg levels in immunetolerant and immune clearance phases of disease

Figure legend: This figure shows box plots of the distribution of qHBeAg levels in PEIU/ml in patients in the immune tolerant and immune clearance phases showing minimum, 1st quartile, median, 3rd quartile and maximum values. There was no statistically significant difference between the 2 groups (p=0.20)
Figure 7-2: Quantitative HBsAg levels in phases of Chronic hepatitis B.

Figure legend: This figure shows box plots of the distribution of qHBsAg levels in patients in the 4 phases of CHB showing minimum, first quartile, median, 3rd quartile and maximum values in each group. There were significant differences between the groups (p<0.001) with qHBsAg levels being highest in the immune tolerant patients and lowest in those in the phase of immune control.
Chapter 8: Quantitative HBeAg and HBsAg in seroconverters and non seroconverters

Introduction
Recognition of HBsAg led to the discovery of Hepatitis B in the 1960’s and this remains the serological marker used for diagnosis of viral infection with HBV. Loss of HBsAg is uncommon in chronic Hepatitis B but is considered the best outcome for patients whether occurring in the context of treatment or spontaneously. HBeAg is a marker of active replication and is also used clinically to identify patients in certain phases of disease; in particular the immune tolerant and immune clearance phases in which patients are HBeAg positive (236). Loss of HBeAg and the development of anti-HBe has been used as a therapeutic endpoint in HBeAg positive CHB after which cessation of therapy may be considered as patients have usually entered the immune control phase. Although traditionally measured qualitatively, there has been recent interest in quantitation of the serum markers of HBeAg (qHBeAg) and HBsAg (qHBsAg) to further explore the natural history of CHB and to help predict responses to treatment.

The utility of qHBsAg during Peg IFN treatment has been established with lower baseline HBsAg levels being shown to be predictors of sustained response (437). Marcellin et al reported in an AASLD abstract in 2013 that those with baseline HBsAg levels of <20,000 IU/ml had higher rates of treatment response 6 months post treatment compared to those with HBsAg levels >20,000 IU/ml (556). Stopping rules for use in response guided Peg IFN therapy for HBeAg positive CHB have been put forward which are genotype specific at Week 12 but apply to all genotypes at week 24 (qHBsAg >20,000 IU/ml at week 24) (438). Conversely any decline at week 24 is predictive of a good response and therefore an indication to continue therapy to 48 weeks (557). Quantitative HBeAg levels during Peg IFN treatment have also been shown to be good predictors of response. In a study of 30 HBeAg positive patients treated with 48 weeks of Peg IFN, Tangkijvanich et al (558) showed that responders (HBeAg seroconversion and HBV DNA <10,000 copies/ml) had lower baseline HBsAg and HBeAg as well as consistent decrease of HBeAg and HBsAg levels on treatment. There has been less work done with quantitative HBsAg and HBeAg serology in Nucleoside analogue treatment with very little done on qHBeAg. An early report on quantitative HBeAg levels by a Dutch group suggested that an early fall in HBeAg levels (between Weeks 0 and 4) was predictive of HBeAg seroconversion in both IFN and Lamivudine treated patients (559). Subsequent work done by the Ulsan University
Hospital in Korea in lamivudine treatment showed that pre-treatment quantitative HBeAg levels were predictive of HBeAg seroconversion (420). These authors also identified ALT and a pattern of “decrescendo” (continuously decreasing HBeAg levels to more than 90% of pretreatment values) as predictive of seroconversion and a pattern of “decrescendo crescendo” (reduction followed by increase in HBeAg levels) as predictive of virological breakthrough, which apparently preceded the increase in viral load by a mean of 7 months. The same group (Ulsan University hospital) also reported that no change or fluctuating levels of HBeAg predicted lack of HBeAg loss and virological breakthrough (421). Some years later, they looked at the utility of qBHeAg in 82 HBeAg positive CHB patients treated with Entecavir for ≥ 3 years and found that although baseline quantitative HBeAg levels were not predictive of virological response, HBeAg seroconversion (achieved in 32%) was better predicted by on treatment HBeAg levels (best cut off value being 0.62 log₁₀ PEIU/ml at 12 months (560). In a study of 27 HBeAg positive HBV/HIV coinfected Thai patients treated with HBV active anti-retroviral treatment, Matthews et al (475) reported that the baseline qHBeAg and qHBsAg levels of those with HBeAg seroconversion (seen in 44% by end of followup) were significantly lower than those who did not achieve seroconversion. These authors also reported that a reduction in HBsAg of >0.5 logs at week 12 had a 100% sensitivity but was not very specific for HBeAg seroconversion, whereas a >1 log decline at 24 weeks had a high specificity (92%).

Lee et al (474) also looked at 95 patients who were taking Entecavir for 2 years (60% HBeAg positive) and reported that of those achieving HBeAg loss at 24 months there were no baseline predictors of this response however there was a steeper decline in qHBeAg levels in those with HBeAg loss compared to those without. A 1 log reduction in qHBeAg at 6 months of treatment had a sensitivity of 75% and specificity of 90% for predicting serological response (defined as HBeAg loss at 24 months).

With regard to qHBsAg levels in NA treated patients, it is known that HBsAg decline is slower in NA treated patients compared to IFN treated ones (471). Chevaliez and colleagues (473) studied 30 patients (60% HBeAg negative) with a median followup of 8.5 years, who were largely started on Lamivudine treatment (or Lamivudine/Adefovir combination treatment de novo) and were switched to more potent agents (eg add on adefovir, switch to tenofovir) with the development of resistance to Lamivudine. They reported that antiviral therapy was associated with a slow but consistent reduction in the level of HBsAg throughout treatment in the vast majority (27 of 30) with a mean annual HBsAg decline of -0.138 ±0.171 log₁₀ IU/ml. They estimated using mathematical modelling that the median number of years needed to
clear HBsAg after HBV DNA undetectability was 52.2 years (IQR 30.8-142.7). The effect of Lamivudine resistance and virological breakthrough in this study was to modestly increase HBsAg levels that subsequently returned to previous rate of decline following the institution of rescue therapy.

Singh et al (561) studied 121 patients treated with Tenofovir and described HBsAg declines of ≥ 1 log from baseline in 15-16% of patients at 1 year and 20-36% of patients at 5 years with greater reductions seen in HBeAg positive compared to HBeAg negative patients. Approximately 30% of HBeAg positive patients underwent HBeAg seroconversion but HBsAg at baseline did not predict this, nor did HBsAg decline at Week 4 or 12. Fung et al (562) looked at 166 patients treated with 2 years of Entecavir (68 HBeAg positive). There was an overall decline in HBsAg from baseline to year 1 and year 2 (3,377 vs. 2,316 vs. 1,903 IU/ml, respectively, p<0.001) and 37% underwent HBeAg seroconversion. Neither HBsAg at baseline nor early decline at weeks 12 or 24 was predictive of HBeAg seroconversion at 2 years. Lee et al (474) reported that in their Entecavir treated cohort, virological response (HBV DNA 60 copies/ml at 24 months) but not HBeAg seroconversion, was associated with lower baseline qHBsAg levels, and a cutoff level of 9550 IU/ml yielded the highest predictive value. Gish et al (563) evaluated qHBsAg levels over 48 weeks in 95 entecavir treated patients and found that on treatment decline in titres was more pronounced in patients who subsequently lost HBeAg or HBsAg and in genotype A or D patients.

Thus there has been great variability in methodologies of studies examining qHBeAg and qHBsAg levels in treatment as well in the results reported. Although some have shown that pre-treatment qHBeAg levels may predict HBeAg loss, others have not. The timing at which on treatment qHBeAg levels have been done in published studies varies from 4 weeks to 12 months. There is also no conclusive evidence of a particular pattern of decline in qHBeAg that may be predictive of subsequent HBeAg loss. The degrees of change in qHBsAg on treatment reported have also varied between studies but overall appear small in NA treatment reflecting the disconnect between the pathway of NA associated viral suppression and that of HBsAg production. Furthermore the change in HBsAg levels may be genotype specific (as appears to be the case in IFN treatment). Given the lack of clarity of the utility of these tests in NA therapy we felt that further exploration of differences in qHBeAg and qHBsAg in HBeAg positive patients treated with NA’s was warranted.
Aims
We performed a retrospective case control study of HBeAg positive patients who did or did not undergo HBeAg loss during an 18 month period of observation. The aims of the study were:

1. To examine differences in qHBeAg and qHBsAg levels in HBeAg positive patients treated with NA therapy who did and did not undergo HBeAg loss within 18 months of treatment.
2. To examine the on treatment change in HBeAg and HBsAg levels and identify any differences in patterns in those patients who did and did not undergo HBeAg loss during 18 months of observation.

Methods
A search of the St Vincent’s Hospital database was performed to identify HBeAg positive patients with CHB who had been commenced on treatment with a nucleoside analogue and had had followup HBV DNA testing for a period of at least 18 months. These patients were divided into 2 groups, those that did achieve HBeAg loss within the period of 18 months of followup (Group 1) and those that did not (Group 2). The cutoff at 18 months was chosen arbitrarily because of preliminary observations that many of our patients had HBeAg loss fairly early after treatment commencement and also to ensure availability of a reasonable number of patients available for study, since fewer patients had longer followup periods. Patient demographics, liver biochemistries and HBV DNA were recorded at baseline and at intervals on average of 2-4 months thereafter during followup. Qualitative HBeAg and Anti-HBe were measured by an immunoassay produced by BioMerieux Clinical Diagnostics (Marcy l’Etoile, France). HBV DNA testing: Samples after April 2004 were tested using the Versant HBV DNA 3.0 assay (bDNA) (Siemens Healthcare Diagnostics, Tarrytown, NY) dynamic range 351-17,500,000 IU/ml. Patients whose HBV DNA level was above or below the range of the bDNA test were assigned the upper or lower limit respectively as their HBV DNA value. Samples before April 2004 were tested using the Digene Hybrid capture II assay (Digene Diagnostics, Beltsville, MD) LLD 25,000 IU/ml. Digene results in picograms converted to IU/ml by approximating 1picogram=50,000 IU/ml. The blood samples stored at Victorian Infectious Diseases Reference Laboratory (VIDRL) for these patients, following testing for HBV DNA, were retrieved from storage in a -20° freezer at VIDRL and testing on samples for both quantitative HBeAg and quantitative HBsAg was performed. Methods for Quantitative HBeAg and HBsAg testing has been described in detail in Chapter 2.
Statistical Methods
Comparisons between patients with and without HBeAg loss within 18 months of treatment were made. Continuous variables that were found to be normally distributed were compared between groups using the unpaired t-test and those that were not normally distributed were compared using the Mann-Whitney test. Fisher's exact test was used to compare categorical variables between the two groups. Additionally the patients were split into four groups based on the time to HBeAg loss. Analysis of variance (ANOVA) was used to compare normally distributed variables between the four groups, whilst the Kruskal-Wallis test was used for the variables that were not normally distributed. A final set of analyses examined the change in qHBeAg and qHBsAg values over time for patients with and without HBeAg loss within 18 months (Groups 1 and 2). To allow for the repeat measurements, the analysis was performed using multilevel linear regression. Two level models were used with repeat measurements nested within patients. Due to skewed distributions of both qHBeAg and qHBsAg, these were log transformed before analysis. The shape of the relationship between time and these outcomes were examined, and where necessary a more flexible fit between variables was used by including squared and cubic terms for time. The change in values over time was allowed to vary for the two groups by including interaction terms between group and time. Due to complexity the full model is not presented.

Results

Patient characteristics.

Patients with HBeAg loss within 18 months: Group 1
There were 11 patients who achieved HBeAg loss within 18 months of commencing treatment with NA’s. In addition to the 11 patients reported on below, a further 4 patients had been initially identified as having HBeAg positivity with subsequent HBeAg loss on treatment from the St Vincent’s database. However these patients were excluded from the analyses reported here as their baseline quantitative HBeAg test was unreactive which suggests that the qualitative assay may be more sensitive than the quantitative assay. Of the 11 patients, 9 had treatment courses of Lamivudine and 2 of adefovir (1 with prior Lamivudine treatment). The mean followup time was 35 months. The median pre-tx qHBeAg level was 184 PE IU/ml (range 1.12 to 1530) and the median time of HBeAg loss was 5.6 months (range 2.3 to 13.9 months) post commencement of NA treatment.
Patients without HBeAg loss over followup of 18 months: Group 2

There were 17 HBeAg positive patients with followup of at least 18 months on a treatment course during which time no HBeAg loss was noted on qualitative assay as previously reported on pathology system and stored on database. In 9 of these 17 patients, the treatment course was with Lamivudine with no other prior recent treatment. A further 7 patients were followed from the time of commencement of Adefovir and in the majority of these patients there had been prior treatment with Lamivudine, often with a period of overlap of both Lamivudine and Adefovir. One additional patient was treated with Entecavir. In addition, over the course of followup, changes in therapy occurred in some patients especially in those treated with Lamivudine with addition of or switch to Adefovir for management of viral resistance.

The patients of Group 1 and Group 2 were similar in terms of demographics and treatments used (Table 8.1) although there was a trend to those in Group 1 being older at 43.6 years compared to 36.4 years in Group 2 (p=0.09). All patients were Asian apart from one who was Mediterranean. The duration of followup was also similar between the 2 groups at 34 and 36 months (p=0.70) as was the number of followup tests done. Those who underwent HBeAg loss within 18 months had a significantly lower baseline qHBeAg level at 184 PEIU/ml compared to 1407 PEIU/ml in group 2 (p=0.001). There was a trend toward Group 1 also having lower baseline qHBsAg and HBV DNA levels compared to Group 2 but these did not reach significance (Table 8.1).

Transient HBeAg loss and case study of a group 1 patient who underwent HBeAg loss then reappearance.

Of the 11 patients in Group 1, 3 had transient HBeg loss with subsequent reappearance. Original HBeAg loss in these patients had occurred at 4, 13 and 14 months. A case study of one of these patients is included in Figure 8.1. This patient had an initial response to Lamivudine with a fall in HBV DNA and ALT. There was a drop in qHBeAg from 562.39 PE IU/ml to 2.9 PE IU/ml over 3 months and qHBeAg was undetectable at 4 months post treatment commencement. There was subsequent viral rebound at 12 months of Lamivudine treatment with increase in HBV DNA, ALT and reappearance of HBeAg at a level of 3.32 PE IU/ml. Over the next 7 months the patient’s qHBeAg rose to approximately 332 PEIU/ml at 19 months. At this time, Adefovir was added and qHBeAg fell over the next 4 months to being unreactive again. While the changes in qHBeAg seemed to parallel viral load, qHBsAg changes did not appear to change significantly over time however there was an isolated elevation of qHBsAg at
39 months to 17,790 IU/ml. This slight peak did not relate to a change in HBV DNA, ALT or qHBeAg.

**Timing of HBeAg loss in whole cohort.**

Of the group 1 patients, 7 lost HBeAg between 0 and 6 months, 2 lost HBeAg between 6-12 months and 2 patients lost HBeAg at 12.9 and 13.9 months. Although no Group 2 patients underwent HBeAg loss within 18 months (by definition), 5 of these 17 patients had a qHBeAg test that became unreactive during followup, occurring at 18.2, 18.8, 24.4, 26 and 27.7 months. When the whole cohort of 28 patients was compared by timing of HBeAg loss at any time during followup (Table 8.2), once again there was a significant difference in the baseline qHBeAg levels in the 4 groups (p=0.01). These were highest in patients with no HBeAg loss during follow-up, with a median qHBeAg of 1457 PEIU/ml, and lowest in patients with HBeAg loss within six months of treatment, where the median value was 58 pEIU/ml.

**Distribution of qHBeAg levels and cumulative HBeAg loss over time by baseline qHBeAg level.**

The cohort was then divided according to baseline qHBeAg level in 27 patients (Table 8.3) (One patient of the cohort was excluded from this analysis due to a missing baseline qHBeAg value – this patient did not undergo HBeAg loss during followup). There were 6 patients with qHBeAg at baseline <100 PEIU/ml, 9 patients with qHBeAg 100-1000 PEIU/ml and 12 patients with qHBeAg level >1000 PEIU/ml. The proportion of patients who underwent HBeAg loss within 6 months was 66 % in those with qHBeAg <100 PEIU/ml, compared to 22% in the group with baseline levels 101-1000 and 8% in those with qHBeAg >1000 PEIU/ml. There was a cumulative increase in the number of patients undergoing HBeAg loss over time in all tiers of qHBeAg. In those with baseline levels of qHBeAg 101-1000 with the proportion increased from 22% at 6 months, to 55% at 18 months and 77% by the end of followup.

**Longitudinal qHBeAg levels in Groups 1 and 2**

Mean quantitative HBeAg and HBsAg levels in Groups 1 and 2 were calculated at 3 monthly intervals and plotted on a graph (Figures 8.3 and 8.4). qHBeAg levels dropped in group 1 patients in the first 3 months after institution of therapy with further reduction to low levels (and undetectability in many) seen thereafter. The elevation of mean qHBeAg seen in Group 1 at month 15-18 represented the reappearance of HBeAg to moderate levels that occurred in a subset following initial disappearance in the 3 months prior. In group 2 patients, there was a steep drop from high pretreatment levels in the first 3 months following initiation of
treatment. Overall qHBeAg levels remained higher in Group 2 patients than Group 1 patients throughout followup.

**Longitudinal qHBsAg levels in Groups 1 and 2**

The longitudinal quantitative HBsAg levels showed apparently more variability in Group 2 patients compared to Group 1 patients as seen in Figure 8.4. To illustrate this further, the log changes from baseline throughout followup in both groups were examined. There were 3 patients, 1 from group 1 and 2 from group 2 who were excluded from analysis as their pre-treatment HBsAg values were missing.

Of the 10 evaluable group 1 patients, one demonstrated a 1 log decrease in qHBsAg levels occurring within the first 2 months. No other patients demonstrated a reduction of ≥0.5 log during followup and none of the 10 patients had an increase of ≥0.5 log. Thus the vast majority 9/10 (90%) of Group 1 patients had fluctuations only within 0.5 log of the baseline value.

By contrast, in group 2 patients, 6/15 (40%) had a reduction of ≥1 log and 4/15 (27%) had a reduction of between 0.5-1 log. There were 5/15 (33%) who showed no reductions in HBsAg of ≥0.5 log. In terms of increase in HBsAg levels, 2 patients of 15 showed fluctuations of ≥0.5 log in HBsAg levels in both directions (ie increase and decrease) at different times over followup however 3 others had an increase of ≥0.5 log in HBsAg but no decrease of ≥0.5 log. Thus of the group 2 patients with available data only 2/15 (13%), had fluctuations within 0.5 log of the baseline value whilst 87% had fluctuations of greater magnitude, predominantly reductions. HBsAg levels therefore appeared more stable in Group 1 patients whereas there were greater changes noted in the Group 2 patients.

**Longitudinal qHBeAg and qHBsAg levels in Groups 1 and 2 – regression models.**

A regression model was constructed to examine the relationship between qHBeAg and qHBsAg values over time in both Groups 1 and 2. Due to complexity the full model, as analysed by the statistician, is not presented but is briefly described in methods. Firstly the qHBeAg values were considered. In order to best fit the observed data, linear, squared and cubic terms for time were included in the regression model. A significant interaction between group and time (p=0.004) was found. This suggests that the change in qHBeAg values over time varied significantly between Groups 1 and 2. The longitudinal changes in qHBeAg values in Groups 1 and 2 from the regression model are depicted graphically Figure 8.5. The graph suggests that Group 2 had much higher pre-treatment qHBeAg levels and underwent a sharper decline in values over time. The qHBeAg levels also declined in Group 1 patients (those with HBeAg loss.
within 18 months) but at a lesser rate. At the end of follow-up the values in the two groups were similar.

A similar multilevel linear regression analysis was performed to examine changes in qHBsAg over time. Once again linear, squared and cubic terms for time were required to adequately fit the data. When the interaction between group and time was included in the analysis, this interaction was not found to be statistically significant (p=0.13), suggesting that the change in qHBsAg values over time did not vary significantly between the two groups (Figure 8.6). The apparently lower qHBsAg levels in Group 2 may have been due to the skewed distribution of the sample which was given a log transformation for the purposes of the model.
Discussion
These data represent close observation of a cohort of predominantly Asian, HBeAg positive CHB patients following the initiation of nucleoside analogue therapy. We examined differences in quantitative levels of HBeAg and HBsAg in 2 groups defined on the basis of HBeAg loss within 18 months of therapy or not. We found that baseline qHBeAg level, but not qHBsAg levels are lower in those who undergo subsequent HBeAg loss within 18 months. There appears to be a relationship between qHBeAg level and time to HBeAg loss with earlier HBeAg loss in those with lower qHBeAg levels and later loss in those with higher qHBeAg levels. HBsAg levels over time are quite static in those who undergo HBeAg loss and although there appeared to be more variability seen in Group 2 patients, statistical analysis suggested that change of HBsAg levels over time was not significantly different between the 2 groups.

The finding of lower baseline qHBeAg levels in group 1 (with HBeAg loss within 18 months) compared to those that did not lose HBeAg in this time (Group 2) is in keeping with other reports in both Peg IFN and NA treated patients where baseline qHBeAg is a predictor of subsequent HBeAg seroconversion (420, 475). There was also a cumulative increase in the proportion of patients with HBeAg loss over time in patients in all tiers of baseline qHBeAg however the highest rates of early loss was in those with the lowest baseline qHBeAg. This raises the question of whether, although the HBeAg loss observed in Group 1 patients occurred following institution of therapy, these patients were “primed” and a spontaneous immune based HBeAg seroconversion event was already underway. Using the arbitrary pre treatment level of HBeAg <100 PEIU/ml, we were able to identify a high proportion of patients (66%), who had HBeAg loss on treatment within the following 6 months. Fried et al found in Peg IFN treated patients, pretreatment HBeAg concentrations of <31 PEIU/ml was associated with a high number of HBeAg seroconversions. They also reported that qHBeAg levels of ≥ 100 PEIU/ml at Weeks 12 or 24 of therapy was associated with a very low chance of HBeAg seroconversion (419). A threshold of qHBeAg to predict HBeAg loss in the near future could perhaps best be used to identify patients in whom a course of therapy for their HBeAg positive disease is likely to be finite. It is also tempting to speculate on whether a baseline qHBeAg threshold could be of use to identify patients who are about to undergo spontaneous HBeAg seroconversion since there is some evidence that such seroconversions are more likely to be associated with subsequent HBsAg loss (564). However, the applicability of the levels seen in our study to non treatment induced HBeAg loss is unknown. Testing this hypothesis would also be difficult since it would involve looking at qHBeAg levels in untreated HBeAg positive
patients and since the timing of the seroconversion event is difficult to forecast, leaving patients who qualify for treatment untreated, would be ethically fraught. Future studies may be able to further refine predictors of HBeAg loss, eg a combination of qHBeAg and qHBsAg levels or qHBeAg and HBV DNA levels.

In Group 2 patients, a sharp decline in qHBeAg levels following institution of therapy was seen. This decline would be expected, since qHBeAg levels would parallel the reduction in viral load occurring with the institution of NA therapy. The levels of qBHeAg remained higher throughout treatment in group 2 compared to group 1. This may indicate that reduction to the level at which HBeAg loss can occur takes time and may have been seen with longer followup. This was also suggested by the increasing proportions of patients with HBeAg loss over time in various baseline tiers of qHBeAg. Alternatively, higher qHBeAg levels may represent an “immunologically different” patient to those that did not undergo HBeAg loss over the 18 month followup period.

In contrast to those with Lamivudine, studies of the newer more potent NA’s especially Entecavir have reported that HBeAg seroconversion is not predicted by pretreatment qHBeAg levels but by reduction in qHBeAg levels at 6 months (474) or at 12 months (560). The majority of our patients had undergone HBeAg loss by 12 months (9 of the total of 16 who lost HBeAg through to the end of followup). Although the division of our groups into HBeAg loss or not at the 18 month mark was arbitrary, when the whole cohort was evaluated, pretreatment qHBeAg levels remained significantly different across patients undergoing HBeAg loss at different stages, including out to 30 months. It is possible that the kinetics of HBeAg decline are different in Lamivudine/Adefovir therapy versus Entecavir/Tenofovir.

Given the high rates of resistance seen in Lamivudine treated patients, studying HBeAg levels during treatment with these older NA’s is complicated by the problem of virological resistance and rebound as this is often accompanied by increased titres of HBeAg as was illustrated by the case study. A weakness of our study was that we focused only on HBeAg loss and not the development of anti-HBe, nor did we evaluate for longitudinal HBV DNA suppression, due to time constraints. As was shown in the case study, there may be a state of flux in HBeAg levels, especially preceding the development of anti-HBe. Sustained loss of HBeAg accompanied by appearance of anti-HBe and virological suppression is necessary for defining HBeAg seroconversion and sustained response. It is also worth recalling that falling HBeAg levels may in some instances reflect increasing proportions of mutant virus (precore and BCP variants).
that lack capability to produce HBeAg (405). Quantitative analysis of precore and BCP mutants in HBeAg seroconversion has shown that HBeAg seroconversion occurring in patients who had a high proportion of precore mutant virus at the end of IFN treatment tended to be associated with high viraemia after seroconversion (378). This suggests that such an HBeAg seroconversion represents a shift to precore mutant CHB rather than an immune control state. Sonneveld et al have also reported that the presence of detectable precore and BCP mutants influences the relationship between HBeAg and response to Peg IFN therapy and they recommended against using qHBeAg levels as an indication for Peg IFN or for monitoring on therapy (565). Similarly in NA treatment the presence of Precore and BCP mutations increases the probability of HBeAg seroconversions but relapse and insufficient viral suppression is seen more commonly than in patients without these mutations (566). We did not perform quantitative analyses of viral mutants but the impact of these viral variants on qHBeAg levels and therefore its utility in on treatment monitoring needs to be kept in mind. Baseline qHBsAg levels in our cohort were not significantly different between Groups 1 and 2 although there was a trend to lower levels in Group 1 with the mean qHBsAg being 3810 IU/ml in this group compared to 11935 in group 2 (p=0.09). There is conflicting data in the literature with some studies showing baseline qHBsAg can predict virological response in NA treated patients (474, 475), whilst others report no association of either baseline qHBsAg or early decline in qHBsAg levels to subsequent HBeAg seroconversion (562). In our patients, the qHBsAg levels appeared to fall from a higher baseline and also to fluctuate more in the patients of group 2 compared to group 1. Levels in Group 1 patients appeared to change very little. However these differences were not statistically borne out. Most reports suggest that HBsAg levels in NA treated patients remain largely stable in the majority of patients (562) or undergo slow decline over time (471, 473). It has been suggested that NA therapy results in a smaller reduction in qHBsAg levels compared to PegIFN therapy, because while IFN has direct antiviral and immunomodulatory effects with potentially greater effects on cccDNA levels (from which HBsAg is transcribed), the Nucleot(s)ide analogues act by inhibiting the reverse transcription step and therefore only affects HBsAg production slowly over time through the indirect effect of reduced recycling of relaxed circular DNA back into the nucleus to form cccDNA (402). Others have reported significant change in HBsAg levels which they found predicts HBeAg loss, which is quite different to the apparent stability of qHBsAg levels in Group 1 in our study. For example, Gish et al (563) found that on treatment decline (over 48 weeks) in qBHsAg titres was more pronounced in their Entecavir treated patients who subsequently lost HBeAg or HBsAg, however this was noted mainly in genotype A or D patients. Matthews’ et al also report that a
0.5-1 log reduction in HBsAg was predictive of HBeAg seroconversion (475), however it should be remembered that this group was coinfected with HIV and also had an unusually high proportion of patients who underwent HBsAg loss (13%). Our data is more in keeping with that of Chevaliez who reported an annual HBsAg change of \(-0.138 \pm 0.171 \text{ log(10)} \text{ IU/ml}\) in patients treated with Lamivudine (473). We hypothesise that although there was no significant difference over time demonstrated by the regression model, the apparent stability of HBsAg levels in Group 1 patients compared to Group 2 may reflect a degree of immune control which predated the institution of NA therapy. This also fits in with the proposed explanation for the relatively low qHBeAg levels and relatively early HBeAg loss seen in the majority of our patients in Group 1.

**Conclusions**

Pre treatment qHBeAg levels are lower in patients who subsequently undergo NA associated HBeAg loss, than in those who retain HBeAg over >18 months of treatment. Baseline qHBsAg levels are not associated with subsequent HBeAg loss and qHBsAg appears to be very stable in those who undergo HBeAg loss, but fluctuates more in those that do not undergo HBeAg loss although in our small study with older generation NA’s, no statistically significant difference was seen in qHBsAg levels over time in the 2 groups. In our study it is possible that those who underwent HBeAg loss had a degree of immune control already established prior to institution of NA therapy that partly contributed to the HBeAg loss.
Table 8-1: Patient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Patients with HBeAg loss within 18 months of treatment</th>
<th>Patients without HBeAg loss within 18 months of treatment</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>11</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Number (%) of males</td>
<td>7(64%)</td>
<td>10 (59%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Mean Age at time of treatment</td>
<td>43.6 (12.9) [20.7-69.8]</td>
<td>36.4 (8.9)[21.78-50.6]</td>
<td>0.09</td>
</tr>
<tr>
<td>Ethnicity number (%)</td>
<td>Asian 10 (91%)</td>
<td>17 (100%)</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>Other 1 (9%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total followup period in months. Mean (SD)</td>
<td>33.9 (17.2)</td>
<td>36.1 (11.6)</td>
<td>0.70</td>
</tr>
<tr>
<td>Treatment used</td>
<td>Lamivudine 9 (82%)</td>
<td>9 (53%)</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>Adefovir 2 (18%)</td>
<td>7 (41%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Entecavir 0</td>
<td>1(6%)</td>
<td></td>
</tr>
<tr>
<td>Median time at which pre treatment test taken in months (range)</td>
<td>1.2 months pre-treatment (7.5 pre-tx, 0.3 months pre-tx)</td>
<td>1.26 months pre-tx (5.5 months, 0.1 months pre-tx)</td>
<td>0.35</td>
</tr>
<tr>
<td>Mean number of tests during followup period (SD)</td>
<td>8.1 (4.5)</td>
<td>7.6 (2.6)</td>
<td>0.74</td>
</tr>
<tr>
<td>Median baseline qHBeAg in PEIU/ml (range)</td>
<td>184 (1,1531)</td>
<td>1407 (44, 8179)</td>
<td>0.001</td>
</tr>
<tr>
<td>Median baseline qHBsAg in IU/ml (range)</td>
<td>3810 (0,25030)</td>
<td>11925 (200-60120)</td>
<td>0.09</td>
</tr>
<tr>
<td>Median baseline HBV DNA in IU/ml (range)</td>
<td>2,750,000 (25000,47800000)</td>
<td>44,378,550 (50000, 236250000)</td>
<td>0.06</td>
</tr>
<tr>
<td>Median baseline ALT in IU/ml (range)</td>
<td>93 (31,453)</td>
<td>77 (46,436)</td>
<td>0.92</td>
</tr>
</tbody>
</table>
Table 8-2: Baseline levels of qHBeAg, qHBsAg, HBV DNA and ALT in patients with HBeAg loss at different times or not at all during followup.

<table>
<thead>
<tr>
<th>HBeAg loss in &lt; 6 months</th>
<th>HBeAg loss in 6 to &lt;18 months</th>
<th>HBeAg loss in 18 to &lt;30 months</th>
<th>No HBeAg loss during followup</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Mean time of HBeAg loss</td>
<td>4.4 months</td>
<td>9.8 months</td>
<td>23 months</td>
<td>N/A</td>
</tr>
<tr>
<td>Mean length of followup (SD)</td>
<td>36.6 months (18.2)</td>
<td>29.2 months (16.7)</td>
<td>44 months (14)</td>
<td>32.5 months (9.0)</td>
</tr>
<tr>
<td>Median baseline qHBeAg in PEIU/ml (range)</td>
<td>58 (1, 1531)</td>
<td>222 (5, 650)</td>
<td>1356 (107, 6571)</td>
<td>1457 (44, 8179)</td>
</tr>
<tr>
<td>Median baseline qHBsAg in IU/ml</td>
<td>2140 (0, 7150)</td>
<td>6980 (3160, 25030)</td>
<td>13480 (2030, 60120)</td>
<td>11300 (200, 38800)</td>
</tr>
<tr>
<td>Mean baseline HBV DNA in log10 IU/ml (SD)</td>
<td>6.10 (1.21)</td>
<td>6.27 (1.68)</td>
<td>6.94 (1.94)</td>
<td>7.37 (0.77)</td>
</tr>
<tr>
<td>Median baseline ALT in IU/ml (range)</td>
<td>113 (31, 453)</td>
<td>56 (45, 66)</td>
<td>110 (77, 131)</td>
<td>56 (46, 436)</td>
</tr>
</tbody>
</table>
Table 8-3: Pre treatment qHBeAg levels and their relationship to subsequent HBeAg loss within 6 months and between 6-18 months.

<table>
<thead>
<tr>
<th>Pre treatment qHBeAg in PEIU/ml</th>
<th>Cumulative number (%) of patients with HBeAg loss in ≤6 months</th>
<th>Cumulative number (%) of patients with HBeAg loss in ≤18 months</th>
<th>Cumulative number (%) of patients with HBeAg loss in ≤30 months or to end of followup</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;100 (n=6)</td>
<td>4/6 (66%)</td>
<td>5/6 (83%)</td>
<td>5/6 (83%)</td>
</tr>
<tr>
<td>101-1000 (n=9)</td>
<td>2/9 (22%)</td>
<td>5/9 (55%)</td>
<td>7/9 (77%)</td>
</tr>
<tr>
<td>&gt;1000 (n=12)</td>
<td>1/12 (8%)</td>
<td>1/12 (8%)</td>
<td>4/12 (44%)</td>
</tr>
</tbody>
</table>
Figure 8-1: Case study of quantitative HBeAg and HBsAg in a patient treated with Lamivudine who underwent HBeAg loss with subsequent reappearance.

Figure legend: This patient had an early episode of HBeAg loss after commencement of Lamivudine but then had reappearance of HBeAg at approximately 12 months which coincided with virological rebound. There was repeat loss of HBeAg following addition of Adefovir. qHBsAg levels were fairly static over followup apart from an isolated elevation of qHBsAg at 39 months which was not paralleled by changes in qHBeAg or HBV DNA.
Figure 8-2: HBV DNA and ALT levels in the case study patient who had HBeAg loss then reappearance.

Figure legend: The HBV DNA and ALT levels parallel each other and also reflect the changes in qHBeAg (including the recrudescence of qHBeAg (Fig 8.1a) in concert with viral rebound and biochemical flare at approximately 18 months).
Figure 8-3: Mean longitudinal qHBeAg levels in Group 1 and 2

Figure legend: This figure shows the change in mean qHBeAg levels in Groups 1 and 2 over the course of followup with an initial steep reduction in Group 2. The reappearance of HBeAg in 2 patients in Group 1 accounts for the elevation noted in this group at 15-18 months.

Figure 8-4: Mean longitudinal qHBsAg levels in Group 1 and 2.

Figure legend: This figure shows the changes in qHBsAg levels in groups 1 and 2 over time with apparently more variability and higher levels seen in Group 2 compared to Group 1.
Figure 8-5: Regression model of change in qHBeAg levels over time in Groups 1 and 2.

Figures Legend: This graph was generated from the regression model looking at change in qHBeAg levels in Groups 1 and 2 over time, which were found to be significantly different. Group 2 patients have a higher pretreatment qHBeAg level and undergo a steep decline with continued reduction over time. Group 1 patients begin at a lower baseline level of qHBeAg and at the end of followup, the qHBeAg levels in group 2 were similar to those of group 1.
Figure 8-6: Regression model of change in qHBsAg values over time in Groups 1 and 2.

Figure Legend: This figure is generated from the regression model and shows the relationship over time for qHBsAg levels in Groups 1 and 2 with the interaction term for time omitted (since it was not found to be significant) and a constant group difference over time assumed. Although the qHBsAg levels in Group 2 appear slightly lower than in group 1 (which may be due to the log transformation given to the values in the regression model due to skewed distribution), they was not statistically significant from Group 1 values.
Chapter 9: Conclusions and Future Directions

Chronic hepatitis B remains a global public health problem with considerable morbidity and mortality attached to the complications of HBV infection, namely cirrhosis of the liver and hepatocellular carcinoma. However the frequencies with which these outcomes develop varies significantly in different patients and are determined by complex interactions between virological, host and environmental factors. HBV is usually an asymptomatic infection until a late stage and therefore understanding the natural history of CHB is critical since treatment, which is generally prescribed long term, is initiated largely for the purposes of preventing the serious liver related sequelae of the HBV infection. This thesis contains work examining questions about the natural history of chronic hepatitis B as observed and managed in a tertiary referral hospital in Australia, in particular how serum markers can be best used in classifying patients into phases of disease and how HBV DNA, genotype and viral variants correlate to histological outcomes. In addition we explored the relatively novel technique of quantifying serum markers of HBV proteins HBeAg and HBsAg and sought to clarify their role in evaluating a patient’s phase of disease and also in the course of treatment with antiviral agents.

In chapter 3, a description of the St Vincent’s hospital CHB cohort was performed which provided an updated picture of the largest reported clinical CHB cohort in Australia and revealed some changes in the demographics (prevalent ethnicities) of the group from an earlier report in 2005. It also utilised HBV DNA in all cases to better characterise the group into a phase of disease which had previously been done using HBV DNA and/or ALT. This work was done at the time when HBV DNA first became a medicare rebateable item in Australia and helped emphasise the important role of this test in clinical care of CHB by demonstrating the limitations of using ALT as a surrogate marker of liver inflammation. It also highlighted the insensitivity of the older generation HBV DNA assays with the aim of encouraging clinicians to take up the newly funded HBV DNA test to identify further potential CHB treatment candidates. Ongoing development and upkeep of the prospectively collected information on the St Vincent’s hospital CHB database will enable further evaluation of any changes in the profile of our clinical cohort as well as serve as an invaluable resource with which to begin study of different clinical questions as they arise.

The importance of HBV DNA levels in predicting risk of cirrhosis become a major focus of attention of those managing CHB with the publication of the REVEAL-HBV study in 2006, which
showed that increasing levels of HBV DNA were associated with increasing incidence of cirrhosis (169). The analysis in Chapter 4 of almost 400 liver biopsies in CHB patients confirmed that in HBeAg negative patients there was certainly increasing prevalence of significant fibrosis with increasing levels of HBV DNA. However in HBeAg positive disease the correlation between HBV DNA and significant fibrosis was negative, so lower levels of HBV DNA were associated with more fibrosis and in fact lower HBV DNA was an independent predictor of cirrhosis. The explanation we offered for this was that, as has long been known, HBeAg positive immune tolerant patients have the highest HBV DNA levels but no liver inflammation and in the immune clearance phase, patients cleared HBV DNA but at the cost of leaving behind liver scarring. Thus our study helped highlight the fact that while the findings of the REVEAL-HBV study were of great consequence, it is important to be aware that this cohort was predominantly HBeAg negative and all were over the age of 30 years. The findings therefore could not necessarily be generalised to all CHB patients and phase of disease remains an important concept in clinical management. Further work in fibrosis risk in CHB should now involve study of non-invasive markers, in particular liver stiffness measurements by fibroscan. Since liver biopsy is no longer routinely used we should explore in our own CHB populations how best to use transient elastography and other non-invasive methods in defining phase of disease and in decisions regarding monitoring and treatment.

Also stemming from the impact of the REVEAL study, the threshold of HBV DNA of 2000 IU/ml became a focus for defining the inactive carrier state in HBeAg negative disease and for considering therapy. However, in the clinical setting using single HBV DNA measures on which to base decisions about potentially lifelong therapy in HBeAg negative disease, seemed excessively rigid. Guidelines were vague about the frequency of HBV DNA testing required and there was little data on the stability of HBV DNA at low levels of viraemia and also how often a persistently normal ALT correlated with viral loads persistently below 2000 IU/ml. Therefore in chapter 5, we chose to explore the fluxes that occurred in HBV DNA around the threshold of 2000 IU/ml and found that minor fluctuations up to 20,000 IU/ml were common. Furthermore we found that HBV DNA persistently <2000 IU/ml accompanied by persistently normal ALT was seen in only a minority of patients who happened to be the oldest of the studied patients. This data was in concordance with other contemporary reports suggesting that use of a higher threshold of HBV DNA (up to 20,000 IU/ml) for defining the inactive carrier state and for treatment indications may not be unreasonable and this revised figure has also been proposed by some international guidelines including APASL in 2012 although it must be acknowledged
that such guidelines seek to strike a balance between risk of CHB and cost of treatment. Room for further refinement of the phases of disease exists, especially given that many patients will not strictly fit into the currently described categories, for example those HBeAg negative patients with HBV DNA levels that are higher than 20,000 IU/ml but with persistently normal ALT. Exploration of the course and outcomes of these patients, for example with serial transient elastography measures and close monitoring of their HBV DNA and ALT levels would be of interest. Those with low viral load but abnormal ALT could also be further explored eg by auditing the frequency with which we find definite other causes of liver disease (eg Hepatitis delta, NASH), or indeed to explore if very low level HBV can still result in liver inflammation. The level of clinical risk attached to such states is unknown and further characterisation of them would be useful since increased use of HBV DNA testing is showing them to be more common than previously thought. Further exploration of the relationship of age related factors (eg time from known HBeAg seroconversion), which we hypothesise may be a marker of immune control, in the establishment of truly quiescent disease would be of interest and potentially help identify differing monitoring schedules for older patients in the inactive carrier state. This would probably need to incorporate qHBsAg levels. In the patients in our study who began observation with HBV DNA >2000 IU/ml, while many still had moderate viral loads and viral load fluxes of reasonable magnitude, 40% dropped below this threshold during followup. Treatment decisions may need to be individualised in such patients taking into account factors such as family history of HCC. Knowledge of the changes in viral load is pertinent since our data show that variability is common. From a public health perspective, reform to the current arrangements for medicare rebate of HBV DNA testing should be considered. At present, monitoring is only rebated by Medicare once per year for untreated patients, but 4 times a year for patients on NA therapy. With highly potent NA’s the issue of virological resistance is far less relevant than it was with Lamivudine, thus redistribution of HBV DNA testing so that more intensive checks can be done on patients in whom therapy in being considered, and if necessary, less monitoring of Tenofovir/Entecavir treated patients in whom viral load falls and rarely rebounds would seem logical.

Recognition of factors that can predict outcomes of CHB are constantly sought in order to identify patients most at risk from a disease that can have very varying outcomes. The virological diversity of the HBV is an enticing arena to explore, since despite the evolution of HBV into different genotypes and stable naturally occuring mutants the literature is burdened with conflicting accounts of the impact of these on liver disease. The collaboration between
our department at St Vincent’s Hospital and VIDRL enabled us to examine the sequencing data on a subset of our cohort and perform clinical correlation with liver biopsy findings in Chapter 6. Our cohort had representation from the 4 well studied genotypes, in contrast to many other studies which mainly look at 2 prevalent genotypes in a region. We confirm associations between the precore mutation and HBV Genotype B and the BCP mutation and HBV genotype C. There were no significant differences in rates of significant fibrosis or cirrhosis in any of the genotype or mutational groups although there were some results approaching significance including higher rates of F2/3/4 in genotype C compared to genotype B. The negative findings in our study may relate to relatively small numbers, the way that significant fibrosis was defined in our study and the fact that the biopsied cohort may have been biased toward those with more significant fibrosis given that liver biopsy was usually performed in patients who were under consideration for therapy. Other factors such as exploration of host factors, especially immunological makeup, which was not touched on in this research, is also likely to be crucial. However, exploration of other viral factors, especially for example the more recently described S gene mutants may provide further insights into the impact of viral mutation on CHB and exploration of the clinical correlates of these through collaboration with VIDRL could be of value. Further work also needs to be done on the African genotypes (E and also A) and genotype F as there is much less data available on these genotypes and migration patterns to Australia are likely to make these genotypes more prevalent in our local CHB population. Analyses on the influence of genotype and viral variants on liver stiffness measurements could be easily done at our centre and would contribute to knowledge in transient elastography in CHB which we are now using routinely in clinical care. Although not looked at in our study, there are areas in which virological testing is established as having a role, e.g. genotype is known to have an impact on Peg IFN treatment outcomes, and the BCP is a known risk factor for development of HCC. Incorporating this testing into clinical practice however is challenging. Auditing the use by clinicians of genotype in decision making about interferon treatment and also the use of risk scores to predict outcomes of HCC and liver cirrhosis, (one of which currently includes the BCP mutation) (567) may prove revealing. Study could be made of the barriers to using these tools in the clinic and ways to overcome them.

Novel markers to aid in classification of a patient’s phase of disease in the natural history of CHB may prove to be useful clinical tools. In Chapter 7, quantification of HBeAg and HBsAg was explored in the 4 phases of CHB and significant differences in qHBsAg levels across different phases were found. Levels of qHBeAg, although appearing slightly higher in immune
tolerant patients were not significantly different from the levels in immune clearance phase patients. This may have been due to small numbers and the older age of our immune tolerant patients, who thus may not have been truly immune tolerant. Further exploration of differences in an immune tolerant group (defined by strict criteria) may yield significant results in qHBeAg levels when compared to HBeAg positive patients in the immune clearance phase.

Further study of how quantitative HBsAg may be included into definitions of phases of disease, et through a study of longitudinal qHBsAg levels in untreated HBeAg positive and negative cohorts (immune tolerant and immune control phases) may be of value to expand our understanding of the natural history of the virus in these phases and may identify timely endpoints at which attempts at viral eradication may be more successful.

Differences of qHBeAg and qHBsAg in NA treated HBeAg positive patients who do and do not achieve HBeAg loss within a defined period were examined in Chapter 8. We noted lower baseline qHBeAg but not qHBsAg levels in those who responded to treatment with HBeAg loss. Whilst a threshold level of pretreatment qHBeAg which may predict HBeAg seroconversion would be of benefit, the utility of qHBeAg levels during treatment is limited by the fact that reduction in levels may reflect the increasing prevalence of precore and BCP mutants that produce less HBeAg, rather than movement toward HBeAg seroconversion associated with sustained response. Further work looking at correlations of on treatment qHBeAg levels with sustained virological suppression and perhaps quantitative analysis of mutants may help clarify the the potential role of qHBeAg levels in NA treatment. Study of qHBsAg levels in treatment are appealing since HBsAg loss is the ultimate endpoint of currently available HBV treatments and because HBsAg levels are believed to reflect cccDNA levels in the liver. Others have shown that changes in qHBsAg levels are small and occur slowly in NA treatment. We noted that qHBsAg levels over followup appeared to be more stable in those who underwent HBeAg loss than those who did not, but there was no statistically significant difference in the change over time on treatment between the 2 groups in our study. These groups were relatively small and followup may have been too short for major differences to emerge. Furthermore the use of older generation NA’s with low barrier to resistance may have confounded results in this regard. Further exploration of quantitative HBsAg levels in therapeutic regimes with newer NA therapies may reveal this cheap and easily performed test to be of use in treatment algorithms for NA treatment, as is the case in PegIFN. In particular, the utility of qHBsAg as a predictor of the safety of stopping NA treatment could be further explored.
Over the 50 years since its discovery many advances have been made in the field of the HBV, including increased understanding of its natural history and most importantly the development of an effective vaccine and potent antiviral therapy. However many challenges remain in the effective management of patients with this condition. The natural history of CHB is variable, both in terms of disease activity over time in individual patients and in terms of the liver related outcomes between individual patients. A better understanding of the natural history of disease is critical to deliver the personalised care necessary in the management of CHB patients. This work contributed to this firstly by examining cross sectionally the utility of the tests we use regularly in clinic for defining phase of disease (HBeAg, ALT and HBV DNA levels) and finding some of them (eg older generation HBV DNA assays) somewhat insensitive for this purpose. Further longitudinal analyses in HBeAg negative patients also highlighted that HBV infection is a dynamic state and thus strict thresholds of HBV DNA used to define phases of disease may often be crossed, an observation which contributed to an evolving debate about whether higher thresholds for defining the inactive carrier state may be reasonable. Measurement of histological endpoints of fibrosis and inflammation was possible in our cohort because of the relatively large numbers of patients with liver biopsies. Analyses of the associations between viral markers of HBV DNA and ALT and significant fibrosis helped to dissect out the place of HBV DNA in the management of CHB in the wake the REVEAL-HBV study which showed it to be the strongest predictor of cirrhosis. Further analyses of the impact of HBV genotype and BCP and precore mutants on histological outcome was negative however our study may contribute in an eventual meta-analysis on this still somewhat vexed question. The search for novel markers to refine classification of the phases of CHB was successful in the finding of different qHBsAg levels in different phases of disease and work performed in the course of this candidature contributed to the important report in the literature by Nguyen et al (551). Furthermore our finding of lower baseline qHBeAg in those who underwent NA associated HBeAg loss is one that may warrant further exploration as a possible indicator of those in whom treatment is likely to be successful. The field of CHB is vast and complex. For the clinician, a better understanding of the natural history of this disease through interpretation of tests that we already have available to us and the exploration of new tools with possible roles in management algorithms, will help provide optimised care for patients with CHB.
References:
1. WHO. Hepatitis B Fact sheet No 204

2015.


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Chan HL, Hussain M, Lok AS. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology. 1999;29(3):976-84. Epub 1999/03/03.


450. Tenney DJ, Rose RE, Baldick CJ, Pokornowski KA, Eggers BJ, Fang J, et al. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-


464. Sax PE, Wohl D, Yin MT, Post F, DeJesus E, Saag M, et al. Tenofovir alafenamide versus tenofovir disoproxil fumarate, coformulated with elvitegravir,


by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. The
virus (HBV) mutants resistant to nucleoside analogs are susceptible in vitro to non-
2011/08/30.
GS-9620, an oral agonist of Toll-like receptor-7, induces prolonged suppression of
substituted tetrahydro-tetrazolo-pyrimidine is a specific and novel inhibitor of hepatitis
482. Zoulim F. Are novel combination therapies needed for chronic hepatitis B?
Chronic hepatitis B: what should be the goal for new therapies? Antiviral research.
484. Dan YY, Aung MO, Lim SG. The economics of treating chronic hepatitis B in
insufficient evaluation and undertreatment of chronic hepatitis B infection in a
486. European Association For The Study Of The L. EASL Clinical Practice
2008/12/05.
487. Australia DHFGSo. Australian and New Zealand Chronic Hepatitis B (CHB)
488. Chan HL HY, Leung NW, Ching JY, Chan FK, Sung JJ. Risk factors for active
liver disease in HBeAg negative chronic hepatitis B virus-infected patients. American
2004 Australian prison entrants' blood-borne virus and risk behaviour survey. Aust N Z
Hepatitis B virus among injecting drug users in Sydney, Australia: prevalence,
2010/01/06.
Natural history and prognostic factors for chronic hepatitis type B. Gut. 1991;32(3):294-
8. Epub 1991/03/01.
492. Liaw YF, Tai DI, Chu CM, Chen TJ. The development of cirrhosis in patients
1988/05/01.
493. de Jongh FE, Janssen HL, de Man RA, Hop WC, Schalm SW, van Blankensteijn
M. Survival and prognostic indicators in hepatitis B surface antigen-positive cirrhosis


552. Araujo NM, Vianna CO, Moraes MT, Gomes SA. Expression of Hepatitis B virus surface antigen (HBsAg) from genotypes A, D and F and influence of amino acid variations related or not to genotypes on HBsAg detection. The Brazilian journal of


556. Marcellin P, Curescu MG, Peikarska A. Outcome of treatment with peginterferon alfa 2a (40KD) in HBeAg positive and HBeAg negative patients with chronic hepatitis B (CHB) in the real world: interim analysis of data from the large European S-collate cohort. Hepatology. 2013;58(S4):656A.


Appendix:
Publications arising from this thesis include:

1. Croagh CM, Bell SJ, Slavin J, Kong YX, Chen RY, Locarnini S, Desmond PV. Increasing hepatitis B viral load is associated with risk of significant liver fibrosis in HBeAg negative but not HBeAg positive chronic hepatitis B. Liver International 2010 Sep 30 (8):1115-22.


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Author/s:
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