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MOLECULAR CHARACTERISATION OF HEPATITIS B VIRUS ISOLATED FROM HUMAN IMMUNODEFICIENCY VIRUS – INFECTED ADULTS AT VARIOUS TIME POINTS AFTER THE INITIATION OF ANTIRETROVIRAL THERAPY

Lanish Singh

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Master of Science in Medicine

November 2017

DECLARATION

I, Lanish Singh declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine, at the University for the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

_____

_____ day of _____2017

PRESENTATIONS AND PUBLICATIONS

None to declare

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ABSTRACT

Sub-Saharan Africa is a high endemicity region of both Hepatitis B Virus (HBV) and Human Immunodeficiency Virus (HIV) infection. There is a paucity of information in this highly endemic region on molecular evolution of HBV in HIV-infected individuals receiving long-term Lamivudine (Lam) therapy. This study aimed at characterizing the molecular evolution of HBV in HIV-infected black Southern Africans prior-to the initiation of a Lam- containing antiretroviral (ARV) drug regimen, and 3, 6, 12 and 18 months post-initiation. HBV viral-loads were quantified using real-time PCR and used to determine the viral suppression in 39 participants from the Shongwe Hospital in rural Mpmualanga, Republic of South Africa. The study participants included 16 participants who were HBsAg+ and 23 HBsAg- at baseline. Of the HBsAg- participants, 19 remained negative throughout follow-up these were defined as the HBsAg- group. The remaining 20 participants were HBsAg+ at baseline and/or at one time-point during follow-up, are referred to as the HBsAg+ group, nine were HBsAg+ throughout the study. Seven participants sero-converted to HBsAg- at a median of 4.2 months, two participants gained the HBsAg at 18.3 months. Two participants were HBsAg- at baseline, thereafter became sero-positive but had retro-converted to HBsAg- by last time-point. A significant finding between these two HBsAg serological groups, was a higher viral suppression achieved in the HBsAg- group -100%, with the HBsAg+ group achieving 13.54% HBV suppression (p = 0.01). HBV was fully suppressed in ten participants, with no suppression found in the remaining participants 29, of which 10 experienced a virologic breakthrough (VBT). HBsAg-negativity was a predictor of viral suppression, with ten HBsAg-negative participants achieving full suppression of HBV ($p = 0.01$). The NS VBT+ group had a significantly higher percentage of viral suppression, 51,90%, compared to the NS VBT- group 14,35%, despite the VBT events (p = 0.03). Biochemical analysis revealed that baseline alanine transferase (ALT) levels were significantly lower in the full suppression (FS) group indicating that lower ALT levels are a predicator of viral suppression ($p = 0.02$). Participants in the FS group had significantly lower ALT levels (15.5) at baseline compared to the NS group (35) (p=0.02). Another finding of the study was that only participants belonging to the HBsAg-negative group were able to clear the HBV virus whereas HBsAg positivity at any time point precluded clearance of HBV DNA. The Basal Core Promotor/PreCore (BCP/PreC) and complete surface (S) regions were amplified and sequenced to genotype HBV isolated from this cohort, as well as find detection or immune escape mutations. The majority of HBV isolates belonged to subgenotype A1, with the

exception of two baseline isolates that belonged to genotype E and subgenotype D3, respectively. Various mutations were found in the 61 BCP/PreC region sequences (T1753C, A1762T G1764A, Kozak sequence, G1862T, G1896A) that could account for the high prevalence of HBeAg-negative infections observed at the various time-points. These mutations can lead to the down regulation of PreC mRNA transcription or translation, and/or affect post-translational modification of HBeAg. Amplification of the complete S-region and overlapping Polymerase regions yielded 47 sequences. Twenty-three of these sequences were from baseline samples, and the remaining from follow-up time-points. PreS deletions involved in the development of HCC were found in two follow-up isolates. These deletions, and other immune or detection escape mutations found in the S region, may contribute to the HBsAg negativity found in this study. In conclusion ALT levels and HBsAg status at baseline were predictors of the outcome of HBV suppression in response to anti-retroviral therapy. This study adds to the limited information available on the molecular changes observed in HBV isolates in HIV-infected South Africans under selection pressure from Lam.

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ABBREVIATIONS

3TC	Lamivudine
aa	Amino acid
ART	Anti-Retroviral treatment
ARV	Anti-Retroviral
ASHV	Artic squirrel hepatitis virus
BCP	Basic Core Promoter
Bp	base pair
cccDNA	Covalently closed circular DNA
CHB	Chronic HBV infection
CHBV	Crane hepatitis B virus
CHV	Chimpanzee hepatitis virus
D4T	Stavudine
DHBV	Duck hepatitis B virus
DNA	deoxyribonucleic acid
DR	direct repeats
dsDNA	double-stranded DNA
ds-rcDNA	double stranded relaxed circular DNA
EFV	Efavirenz

ER	Endoplasmic reticulum
ESLD	end-stage liver disease
GiHBV	Gibbon hepatitis B virus
GoHBV	Gorilla hepatitis B virus
GSHV	Ground squirrel hepatitis virus
HBV	Hepatitis B Virus
HBcAg	HBV core antigen
HBeAg	HBV e antigen
HBsAb	Hepatitis B surface antibody
HCC	Hepatocellular carcinoma
HHBV	Heron hepatitis B virus
HIV	Human Immunodeficiency Virus
IVD	Intravenous drug
IVDU	Intravenous drug use
kb	kilobase
kd	kilodalton
LHBs	Large hepatitis B surface protein
MHBs	Middle hepatitis B surface protein
mRNA	Messenger RNA

MSM	Men who have sex with men
NAT	Nucleic Acid Testing
NCP	Neviripine
nt	nucleotide
OBI	Occult HBV Infection
ORF	Open reading frames
OuHV	Orangutan hepatitis virus
PCR	Polymerase Chain Reaction
pgRNA	pre-genomic RNA
Pol	Polymerase
PreC	PreCore
SVPs	Sub-viral particles
rcDNA	relaxed circular DNA
RGHBV	Ross's goose hepatitis B virus
RNA	ribonucleic acid
SGHBV	Snow goose hepatitis B virus SHBs Small hepatitis B surface protein
STHBV	Stork hepatitis B virus
WHV	Woodchuck hepatitis virusWHMV Wooley monkey hepatitis virus

DEDICATION

I dedicate this dissertation to my family

CHAPTER 1: INTRODUCTION

With a genome of 3.2 kilobase (kb), hepatitis B virus (HBV) is the smallest DNA virus infecting humans. A vaccine against HBV was developed in the early 1980's, however, three decades later the world's population is still heavily burdened by this hepatotropic pathogen and the diseases associated with it, namely hepatitis, decompensated cirrhosis, end-stage liver disease (ESLD) and hepatocellular carcinoma (HCC) (Lavanchy, 2004; Gerlich, D). These HBV-related complications result in 786 000 deaths annually (Stanaway *et al.*, 2016), posing a major public health problem (Rantala and de Laar, 2008). The public health risk posed by HBV is further exacerbated by the development of drug-resistant and vaccine-escape mutants of the virus (Glebe and Urban, 2007).

For a number of reasons, HBV infection is a major problem in sub-Saharan Africa. Firstly, sub-Saharan Africa unlike other high HBV endemicity regions did not have a vaccine catch-up programme (Spearman *et al.*, 2013). Secondly, theHBV burden is compounded by the high prevalence of Human Immunodeficiency Virus (HIV) infections in this region. Of the 34 million HIV-infected persons globally, approximately two-thirds are found in sub-Saharan Africa, with HBV-HIV co-infections rates of up to10% (Matthews *et al.*, 2014). Thirdly, anti-retroviral treatment (ART) containing lamivudine (3TC), which also acts against HBV, has been widely used in sub-Saharan Africa to treat HIV-infected individuals(Hoffmann *et al.*, 2008).Lamivudine treatment can result in the development of drug resistant mutations in 80% of patients after 5 years of treatment(Fasano *et al.*, 2012) leading to reactivation of asymptomatic HBV. Thus the introduction of ART, has resulted in a changed disease profile, with an increase in HBV-associated ESLD (Thomas, 2006). Therefore it is important that HBV in HIV infected individuals is studied especially after ART.

1.1 Epidemiology

Globally there are an estimated 2 billion people with past or present HBV infection, with 240 million chronic carriers of HBV (Hepatitis B Fact sheet no 204. http://www.who.int/mediacentre/factsheets/fs204/en/ October 2015). Serological evidence of HBVinfection is measured by hepatitis B Surface Antigen (HBsAg) positivity and is used to discern between geographical areas of low (< 2%), intermediate (2 - 8%) and high (> 8%) endemicity (Hou *et al.*, 2005). Areas of low endemicity are the United States of America, Northern Europe, Australia and parts of South America. Intermediate endemicity regions include the Middle East, Eastern Europe and the Mediterranean basin. HBV is most prevalent (>5-10%) in sub-Saharan Africa, southern-eastern Asia, the Balkan regions, Pacific Islands and the Amazon basin (Figure 1.1) (Sunbul, 2014). More than half the world's population reside within highly endemic regions with 5-10% of these populations burdened by chronic hepatitis B (CHB)(Schweitzer *et al.*, 2015; World Health Organisation, 2016). There are approximately 4 million cases of acute infections annually and a low 0.5- 1% fatality rate (Lavanchy, 2004). Acute infection leads to chronic infection in 90% of neonates, 20 - 60% of children under the age of 5 years and < 5% of adults (McMahon, 2004).

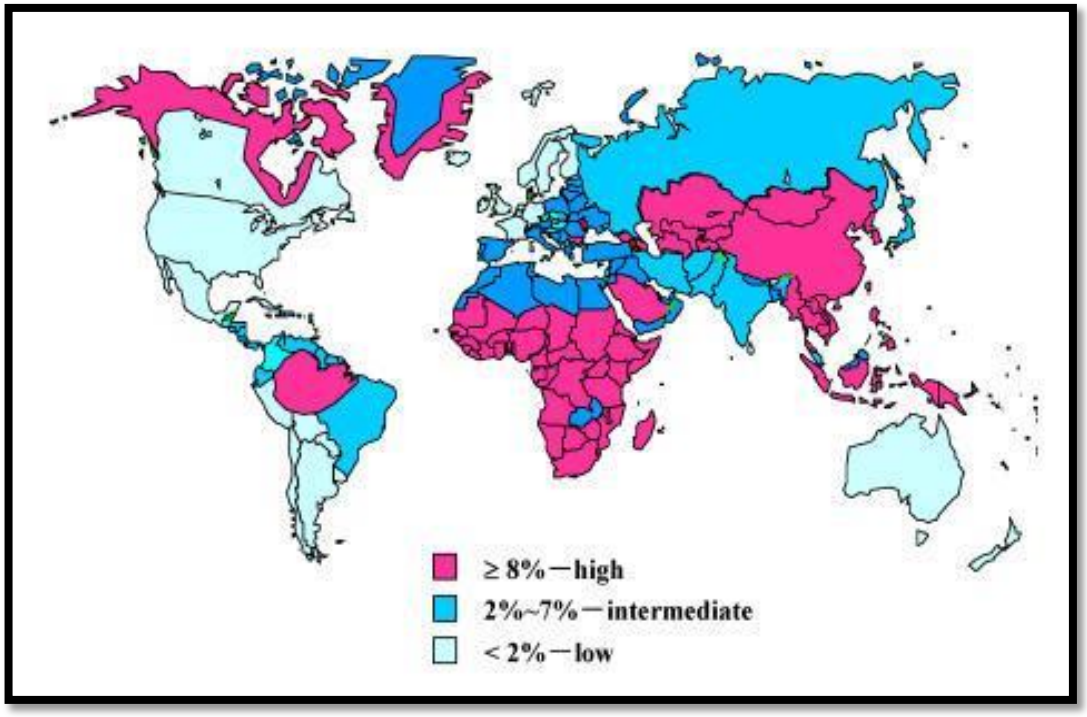

Figure 1.1 Global overview of low, intermediate and high HBV endemicity regions. Areas of high endemicity (*pink*), intermediate endemicity (varying shades of blue, low intermediate-light blue, high intermediate-dark blue), low endemicity (pale blue) (Hou *et al.*, 2005; Lavanchy and Kane, 2016)

1.2 Transmission

HBV is transmitted through various routes such as unprotected sexual contact, percutaneously by intravenous drug (IVD) use, perinatally (vertically), horizontally, during transfusion of blood products, needle-stick injury and hepatic or extra-hepatic organ transplantation (Mauss *et al.*, 2014). Routes of transmission differ, between different geographic locations, also between genotypes (Hou *et al.*, 2005).

Sexual transmission is most common in areas of low prevalence, 25% of these infections are transmitted by men who have sex with men (MSM) (Edmunds *et al.*, 1996). In high endemicity areas, the mode of HBV transmission is either

vertical or horizontal (Davis, Weber and Lemon, 1989). Perinatal transmission is predominant in Asia, occurring either *in utero*, peri-partum or post-partum (Merican *et al.*, 2000). In sub-Saharan Africa horizontal transmission during childhood, accounts for majority of infections through parenteral contact among family members or unrelated children (Kramvis and Kew 2007; Anigilaje and Olutola 2013). Intravenous drug use (IVDU) is the most prevalent type of percutaneous transmission (Hoffmann and Thio, 2007), however, acupuncture, tattooing, scarification, body piercing, sharing of razors or toothbrushes, play a role in transmission. HBV is the most transmitted blood borne virus in the healthcare industry (Mauss *et al.*, 2014). HBV and HIV share transmission routes namely: vertical, parenteral and sexual (Davis, Weber and Lemon, 1989; Dabis and Ekpini, 2002)

1.3 Hepatitis B Virus

1.3.1 Classification of HBV

HBV is a hepatotropic DNA virus belonging to the family*Hepadnaviridae*, of which it is the prototype virus. Members of this family are double-stranded DNA viruses but replicate as pararetroviruses using a single-stranded RNA-intermediate (Robinson, Miller and Marion, 1987). This family consists of two genera: Orthohepadnaviruses (infecting mammals) and Avihepadnaviruses (infecting birds). Orthohepdnaviruses have been found in humans (HBV), bats (BHBV) (Drexler et al. 2013), ground squirrels (GSHV) (Marion *et al.*, 1980), artic squirrels (ASHV) (Testut *et al.*, 1996), woolly monkeys (WMHV) (Lanford *et al.*, 1998), woodchucks (WHV) (Summers, Smolec and Snydert, 1978), chimpanzees (CHV) (Vaudin *et al.*, 1988; Zuckerman *et al.*, 2016), gorillas (GoHBV) (Grethe, Heckel and Rietschel, 2000), gibbons (GiHBV) (Norder *et al.*, 1996; Grethe, Heckel and Rietschel, 2000), baboons (Dickens *et al.*, 2013)and orangutans (OuHV) (Warren, Heeney and Swan, 1999). Avihepadnaviruses have been found in ducks (DHBV) (Mason, Seal and Summers, 1980), herons (HHBV) (Sprengel, Kaleta and Will, 1988), snow geese (SGHBV) (Chang *et al.*, 1999), Ross's geese

(RGHBV) (Pult *et al.*, 2001), storks (STHBV) (Pult *et al.*, 2001; Prassolov *et al.*, 2003) and cranes (CHBV) (Prassolov *et al.*, 2003).

DHBV and WHV have been used to study hepadnaviral replication and chronic liver disease (Seeger and Mason, 2000)whereas WHV and GSHV are the closest relatives of HBV (Marion *et al.*, 1980). HBV solely infects humans and chimpanzees, and chimpanzees are purposely infected and used as a model to study HBV (Maynard *et al.*, 1972; Barker *et al.*, 1973; Sureau, 1993). However, recent findings of HBV in bats indicate that BHBV has the ability to infect humans (Drexler *et al.*, 2013).

1.3.2 HBV Structure and Biology

1.3.2.1 HBV Viral Structure

Hepatitis B virus has three types of structures, the infectious Dane particle and two sub-viral particles (SVPs): the filamentous and spherical particles (Figure 1.2), (Bayer, Blumberg and Werner, 1968; Dane, Cameron and Briggs, 1970). The sub-viral particles do not contain DNA rendering them non-infectious. The spherical particle is ~22 nm in diameter, the filamentous particles shares a similar width but may be of variable length (Glebe and Urban, 2007). The SVPs act as a decoy for the neutralising anti-HBs, allowing immune evasion of the infectious Dane particles (Chen *et al.*, 2005). SVPs vastly outnumber Dane particles in ratios of 1000:1 up to 10 000:1 (Scaglioni, Melegari and Wands, 1996). All three types of HBV particles contain viral surface proteins. The Dane particle is enclosed by an envelope comprised oflipid membranes, which are not acquired from the host plasma-membrane but from the lipid membranes of the host's intracellular compartments (Glebe and Urban, 2007). The mechanism of assembly of SVPs differs from that of Dane particles (Tiollais, Pourcel and Dejean, 1985).

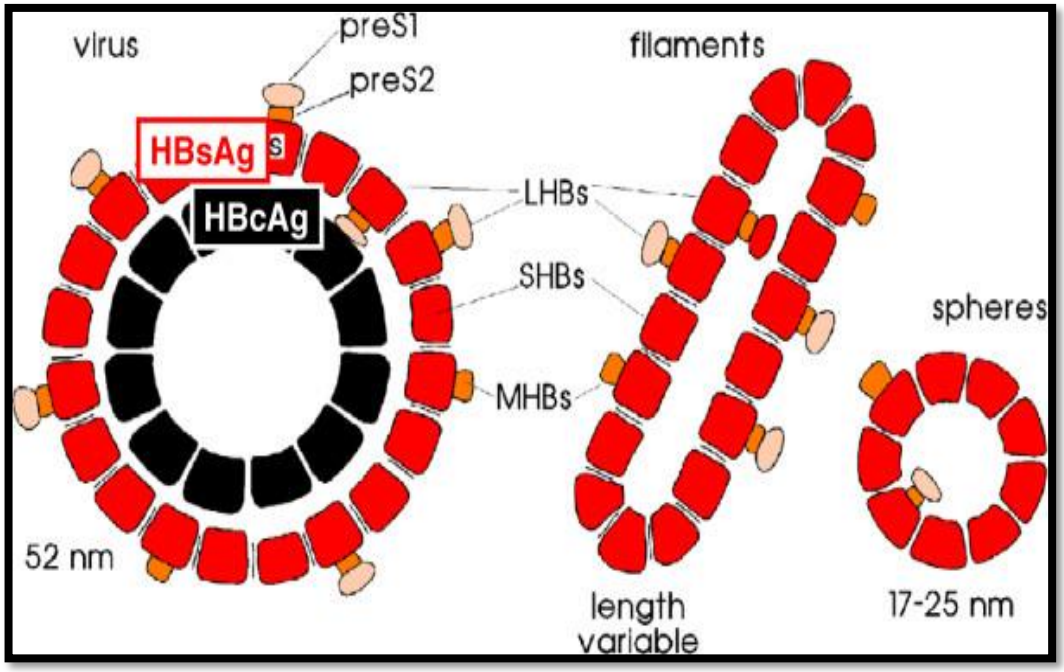

Figure 1.2 Dane Particles, filamentous and spherical sub-viral particles: the black and red double-circular structure represents the Dane particle indicating the Hepatitis B surface Antigen (HBsAg) and the Hepatitis B core Antigen (HBcAg). The PreS1 and PreS2 domains of Large HBV surface Protein (LHBs) embedded in the viral capsid are indicated on the Dane particle. The LHBs, Medium HBV surface protein (MHBs) and Small HBV surface protein (SHBs) demonstrate the surface proteins found on both the Dane and sub-viral particles (Gerlich, 2013). Reproduced in accordance with the Open Data Policy of Biomed Central and the Creative Commons CC1.0 Public Domain Dedication Waiver

Dane particles (Figure 1.3) are spherical, double-layered structures of 40 – 44 nm in diameter (Dane, Cameron and Briggs, 1970). The outer layer is made-up of host-derived lipids and randomly distributed surface proteins. The inner-layer or nucleocapsid is an icosahedral structure of densely packed HBV core proteins, containing a single copy of partially double-stranded relaxed circular DNA (ds rcDNA) (Summers, O'Connell and Millman, 1975; Robinson, Miller and Marion, 1987).

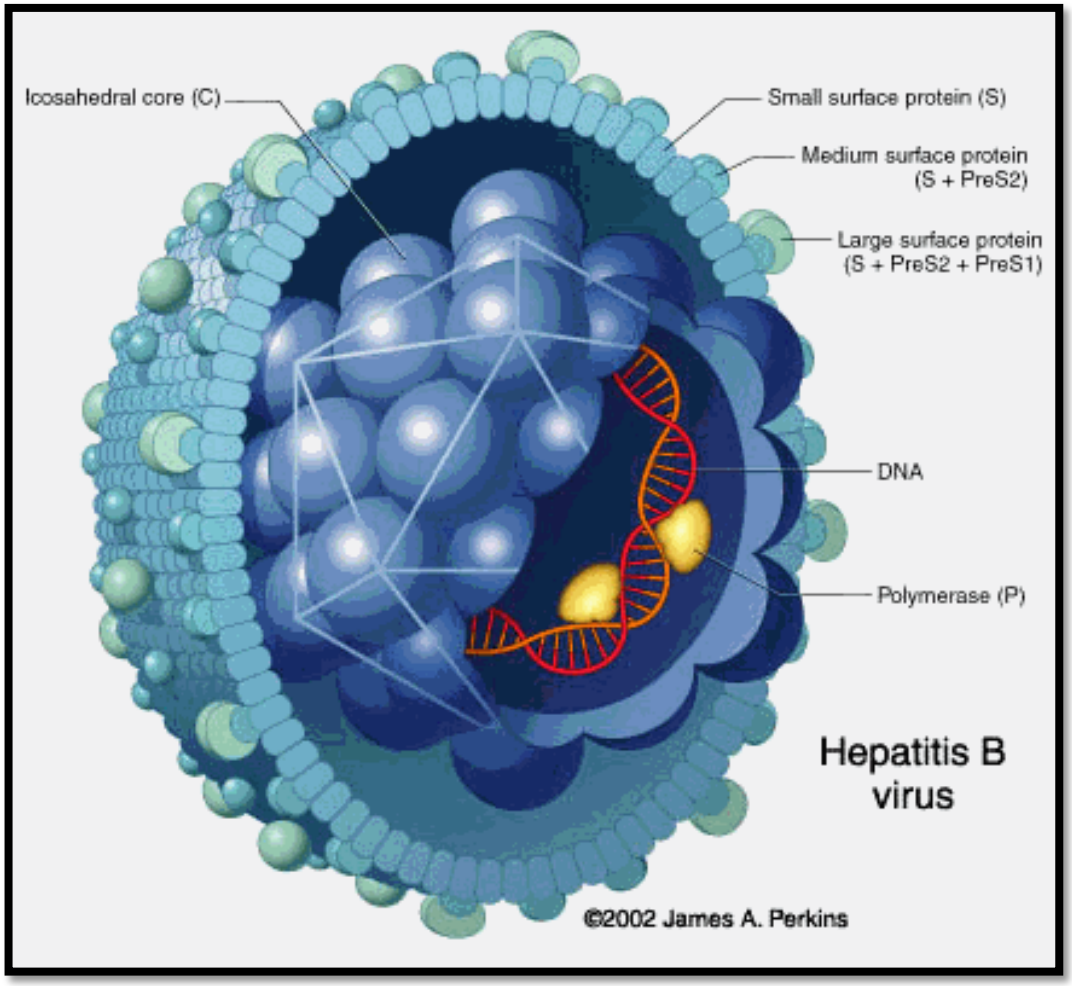

Figure 1.3 HBV Dane particle: Illustration Copyright James A. Perkins. Used with permission.

1.3.2.2 HBV Genome Organisation

The nucleocapsid envelops a 3200 base pair (bp) closed circular DNA (size of genome varies with genotype) comprising of a complete minus-strand and an incomplete (50 - 75% complete) plus-strand (Lutwick and Robinson, 1977; Summers, 1987). Numbering of the HBV genome begins at the *EcoR*1 site, henceforth, this will be the system used when referring to nucleotide positions. A polymerase molecule is covalently bound to the 5'- end of the minus-strand using a phosphotyrosine bond (Gerlich and Robinson, 1980; Nassal, 2008). An 18 nucleotide (nt) ribonucleic acid (RNA) oligomer, the pre-genomic RNA (pgRNA), essential for replication, is found at the 5'-end of the positive strand.

Two redundant DNA sequences of eight nt, essential for DNA replication, are found at both the 5' - and 3'- ends of the minus strand, these direct repeats (DR) are referred to as DR1 and DR2 (Seeger, Ganem and Varmus, 1986; Nassal, 2008). The integrity of the circular DNA structure is maintained by base-pairing between the minus- and plus-strands rather than covalent bonding (Tiollais, Pourcel and Dejean, 1985).

Despite HBV being the smallest DNA virus infecting humans, it makes efficient use of its compact genome by producing seven proteins from four partially overlapping Open Reading Frames (ORFs), with the entire genome being involved in coding (Tiollais, Pourcel and Dejean, 1985). Six start codons, four promoters (Core, PreS1, PreS2/S and X) and two enhancers (EnhI and EnhII) are found within the coding regions (Moolla, Kew and Arbuthnot, 2002). The four ORFs are in the same orientation and use a common polyadenylation signal to terminate transcription (Seeger and Mason, 2000).

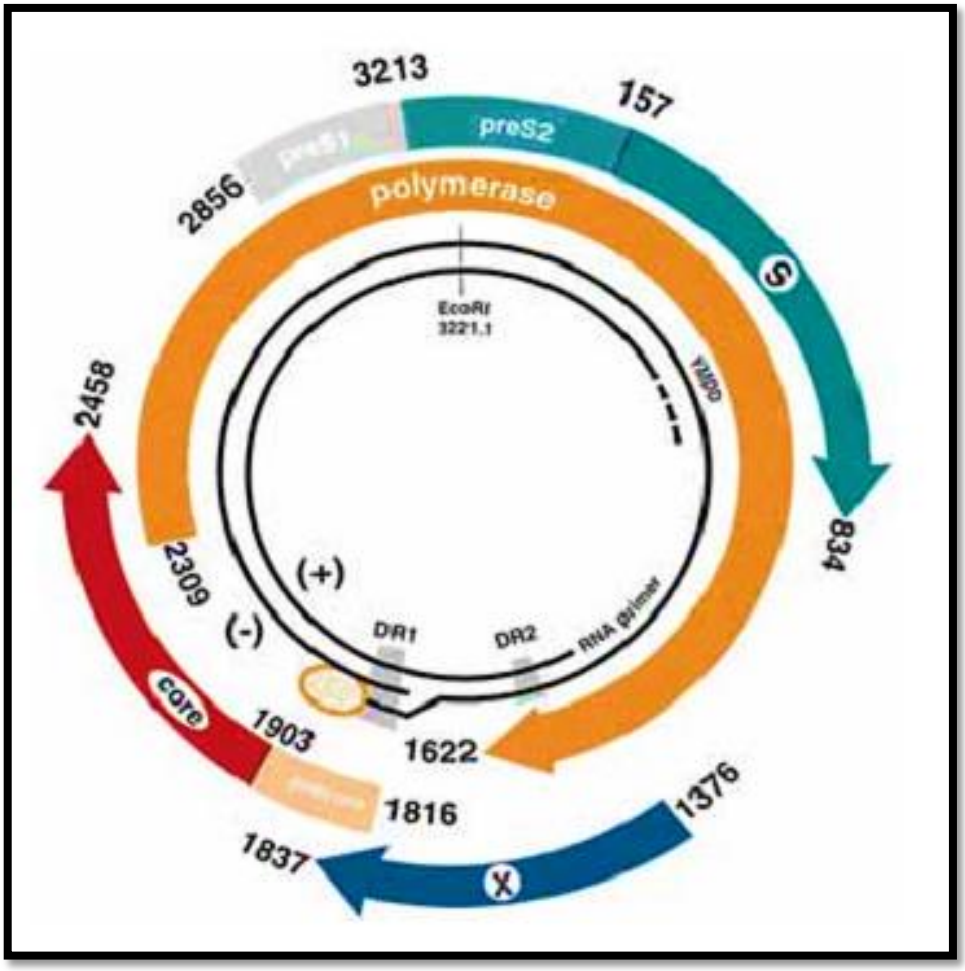

Figure 1.4 HBV genome organisation: Indicating the complete minus-strand and incomplete plus-strand, also represented are the four ORFs which code for all seven HBV proteins. Surface (S), polymerase, PreC/Core and x genes and their co-ordinates are represented by the coloured fragments. YMDD, an important amino-acid sequence within the reverse-transcriptase protein; RNA primer, the 18 nt RNA oligomer found at 5'-end of the plus-strand and the polymerase molecule (orange oval structure) bound to the 5'-end of the minus-strand. Direct repeats (DR1&2).(Hunt *et al.*, 2000) Reproduced with permission from John Wiley and Sons ©, 2001.

1.3.3 Viral Proteins

The PreS/S ORF codes for three structural or envelope proteins, are named according to their size; Large, Medium and Small Hepatitis B Surface proteins (LHBs, MHBs and SHBs, respectively). The PreCore/Core (PreC/C) ORF codes for; the secretory Hepatitis B 'early' (or 'envelope') antigen (HBeAg) and the structural nucleocapsid protein referred to as Hepatitis B core antigen (HBcAg). The polymerase (Pol) and x-ORF encode, the multifunctional polymerase enzyme and x-protein, respectively (Heermann *et al.*, 1984). Four mRNA transcripts, the pregenomic (3.5 kb), PreS1 (2.4 kb), PreS2/S (2.1 kb) and x (0.7 kb) mRNAs are used to produce the seven proteins.

1.3.3.1 Viral Surface Proteins

The S-gene has three start codons and a common stop codon (UAA) at the 3'- end (Seeger and Mason, 2000). The PreS/S ORF codes for three viral surface proteins, LHBs, MHBs and SHBs, found on the membrane of Dane particles and SVPs (Heermann *et al.*, 1984). Surface proteins are synthesised in the Endoplasmic Reticulum (ER) (Le Seyec *et al.*, 1999), vastly outnumbering the amount required for mature virion synthesis (Heermann *et al.*, 1984; Bruss and Ganem, 1991). Surface proteins are glycosylated or unglycosylated (Stibbe and Gerlich, 1983), however all contain SHBs-domains, also known as HBsAg. The crucial antigenic 'a' determinant site is found within the SHBs region of all HBV surface proteins (Sanchez *et al.*, 1981).

1.3.3.1.1 LHBs: coded for by nt 2848 - 835, LHBs (42kDa) uses the 2.4 kb PreS1 mRNA transcript to produce a 389 - 400 amino acid (aa) protein (aa length is dependent on genotype) (Heermann *et al.*, 1984). The PreS1, PreS2 and S-domains constitute the LHBs molecule and the PreS1-domain is unique to this protein. During synthesis the LHBs protein is found either exposed to the cytoplasm of the host-cell or is projected into the lumen of the ER, therefore two topological variations of LHBs occur (Bruss and Ganem, 1991; Glebe and Urban,

2007). Those exposed into the cytoplasm are used to provide an attachment site for the nucleocapsid core protein molecules during virion maturation, allowing the nucleocapsid to bind or anchor to the outer viral capsid (Prange and Streeck, 1995). The LHBs projecting into the ER lumen becomes exposed on the surface of the virion or the SVPs, and are pivotal for HBV attachment to hepatocytes (Neurath *et al.*, 1985). The S-domain occurring within LHBs is glycosylated (Stibbe and Gerlich, 1983) whilst, the PreS2-domain which is always glycosylated in MHBs is unglycosylated in LHBs proteins (Heermann *et al.*, 1984). Myristylation and phosphorylation occur at the LHBs N-terminus, the myristate group is essential for infectivity but not replication (Persing, Varmus and Ganem, 1987; Le Seyec *et al.*, 1999). Transmembrane insertion of LHBs molecules in the lipid bilayer is facilitated by the myristate group (Bruss and Ganem, 1991).

1.3.3.1.2 MHBs (31 kDa) is coded for by nt 3206 – 835, generating a 2.1 kb mRNA transcript to synthesise a 281 aa protein (Heermann *et al.*, 1984). MHBs is a N-glycosylated protein consisting of both PreS2 and S-domains. An O-glycan residue can be found at aa 37 and an acetyl-residue is found at the N-terminus (Heermann *et al.*, 1984). The function of this molecule is not essential for any viral activity, such as infectivity (Fernholz *et al.*, 1993), however some antigenic epitopes are located within the PreS2-domain (Neurath *et al.*, 1985). The Spacer-region of the polymerase molecule overlaps with the PreS2-region and large deletions or mutations in these regions are well tolerated (Fernholz *et al.*, 1993; Pollicino *et al.*, 2014).

1.3.3.1.3 SHBs (or HBsAg) encoded by nt 155 - 835, produces a 226 aa molecule from the PreS/S ORF. Similar to MHBs, SHBs molecules are transcribed from the 2.1kb mRNA transcript (Heermann *et al.*, 1984). SHBs is the most abundant of the surface proteins constituting 80% of the envelope proteins (Robinson, 1977). Glycosylation of SHBs is essential for secretion of virions however not all SHBs are glycosylated. SHBs has a molecular weight of 24 kDa when unglycosylated

and 27 kDa when glycosylated. The role of SHBs in HBV-virion binding to hepatocytes is lesser than that of LHBs, however SHBs is required for transmission of infection (Glebe and Urban, 2007). The 'a' determinant is found within the SHBs molecule, and is the most important HBV antigenic epitope, used to detect active HBV infection and is the target of neutralising anti-HBs, produced by the host (Honorati and Facchini, 1998; Nassal, 2008).

SHBs has five transmembrane α-helices (Helix I to V) which contain both hydrophilic and hydrophobic regions (Tiollais and Wain-Hobson, 1984; Eble, Lingappa and Ganem, 1986). Each helix has 4 - 5 hydrophobic coils traversing the ER membrane, which becomes the virion membrane. Helix I, begins with the N-terminus exposed to the cytoplasm, the helix then translocates the molecule across the ER membrane, terminating with the first major hydrophilic loop (49 aa), either in the cytoplasm of the host or later, the interior of the virion (Prange and Streeck, 1995).

Helix II contains an 18aa long (aa 80 - 98) hydrophobic domain, this helical structure transverses the ER membrane terminating in the lumen of the ER (or Golgi Apparatus (Golgi)), the second major hydrophilic structure of SHBs extends from Helix II (Eble, Lingappa and Ganem, 1986). It is within this hydrophilic region that the 'a' determinant is found. This is a cysteine-rich region, resulting in the formation of disulphide bonds between complimentary amino acids, allowing the molecule to form a loop (Mangold and Streeck, 1993). The 'a' determinant is found between amino-acids 99 and 168 of the SHBs and is considered the hallmark epitope of HBV. Mutations in this region can result in detection escape or immune/vaccine escape mutants (Weinberger *et al.*, 2000). The 'a' determinant is common to all serological subtypes and is characterised by a threonine or isoleucine at position 126 (Kramvis and Kew, 2005), due to the conserved nature of 'a' determinant they are used in HBV vaccines conferring protection to all sub-types.

The ‘a’determinant is used to distinguish between the ten serological sub-types based on the allelic variations of the ‘a’ determinant (Tiollais and Wain-Hobson, 1984). Amino-acids 122 and 160 of SHBs are used to differentiate between serotypes *d/y* and/or *w/r* respectively. At position 122 a lysine or an arginine can be found for serotype *‘d’* or *‘y’*, respectively. Position 160 is characterised by a lysine or arginine, when distinguishing between serological subtypes *‘w’* or *‘r’*, respectively. Recent studies found that positions 134, 140 and 159 are important for *w1* subtypes and position 127 for *w2-w4*. Additionally amino acid 177 is used to distinguish between *adrq+* and *adrq-* whilst position 178 is used to distinguish *adw4q+* and *adw4q-*(Okamoto *et al.*, 1988; Locarnini, 2003; Kramvis, Kew and François, 2005; Purdy *et al.*, 2007). Helices III, IV and V also traverse the ER membrane, notably the order in which the five helices are arranged differs between LHBs, MHBs and SHBs (Locarnini, McMillan and Bartholomeusz, 2003).

1.3.3.2 HBV Polymerase

The Pol gene is located between nt 2307 – 1623, this 90 kDa protein is synthesised from the 3.4 kb pgRNA. The N-terminus of this 845aa protein is bound to the 5’-end of the minus-strand. HBV polymerase is comprised of four domains: the terminal protein, spacer, RNA- and DNA-dependent reverse transcriptase, and RNase H (Seeger and Mason, 2000).

Terminal protein (1 - 183 aa) is used in minus-strand priming (Stuyver *et al.*, 2001). The function of the spacer domain is yet to be elucidated, however large deletions in this domain do not inhibit polymerase function(Beck and Nassal, 2007). The third Pol domain is responsible for reverse transcriptase/polymerase activity whereby the minus-strand is synthesized from the pgRNA template (Nassal, 2008). Synthesis of the plus-strand using the minus-strand as a template is enabled by the reverse transcriptase. The aa sequence YMDD, responsible for

reverse transcriptase activity, is found in the reverse transcriptase domain(Schlicht, Bartenschlager and Schaller, 1991). Lastly the RNAseH domain degrades the pgRNA template during minus-strand synthesis. The polymerase protein is vital in the treatment of HBV infection, as this is a protein targeted by most therapies (Rehermann *et al.*, 1995). The polymerase contains two T-cell epitopes between aa 107 – 115 and 227 – 235 (Locarnini, 2003).

1.3.3.3 HBc

The core gene is located between nt 1901 and 2450, translating the 3.4 kb pgRNA into a 183 aa HBc (183-195 aa in length depending on genotype) with a molecular weight of 21 kDa (Tiollais, Pourcel and Dejean, 1985; Will *et al.*, 1987). The first functional domain involving aa 1 -144 of HBc (the assembly domain), is involved in encapsidation and replication (Ganem and Schneider, 2001). The second domain (nucleic-binding domain) of approximately 43 aa (aa 145 - 187) includes the arginine-, serine- and proline-rich C-terminus, involved in anchoring HBV-DNA to the nucleocapsid. During nucleocapsid formation, the core protein monomers dimerise, using disulphide bonding. The dimers spontaneously form an icosahedral structure of 36 nm consisting of 120 dimers or a smaller structure composed of 90 dimers. These icosahedral structures encapsidate the HBV genome, therefore referred to as nucleocapsids (Seeger and Mason, 2000). The core protein contains four alpha-helices and two exposed regions, the first of these regions is a protrusion at the dimer-binding sites and the other is involved in the assembly of the dimers into the multimer structure (Seeger and Mason, 2000).

1.3.3.4 HBe

Translation of the core gene from the upstream PreCore (nt 1814 - 1900) region results in the production of a non-particulate, soluble protein, HBe (Nassal, Junker-Niepmann and Schaller, 1990). Translation of the second species of 3.5 kb mRNA, the PreC/Core mRNA transcript, produces a HBe precursor protein of 210 aa, p25. The first 19 aa of HBe (p25) contain a signal peptide that directs the

protein to the ER where these 19 aa's are cleaved off by cellular signal peptidase, resulting in another protein intermediate (p22) that is subsequently translocated into the Golgi for further post-translational modification. After approximately 34 aa are cleaved off the C-terminus, the final derivatives are 15 – 18 kDa heterogenous species of HBe, (Tiollais, Pourcel and Dejean, 1985) of which most is secreted into serum whereas 20% is retained in the cytoplasm. A small amount of HBe precursor (p25) remains in the cytoplasm, furthermore this precursor contains nuclear signalling properties transporting it back into the nucleus, therefore HBe and its precursors can be found in almost all cellular compartments (Ganem and Varmus, 1987). HBe is not required for replication and its function remains enigmatic, however it is a highly conserved region, which is postulated to have immune tolerant functions (Milich *et al.*, 1990; Miyakawa, Okamoto and Mayumi, 1997). HBV does require HBe to establish persistent infection but not to sustain infection high levels of HBe have been associated with high HBV viral-loads but normal liver histology and viral replication. Lower levels of HBe have been associated with more rigorous host immune activity against HBV, alluding to the immune-tolerogenic function of HBe. The TLR signalling pathway is antagonised by HBe retained in the cytoplasm further impairing immune function (Lang *et al.*, 2011).

1.3.3.5 HBx

The x-gene is located between nt 1374-1836, coding for the 17kDa x-protein (HBx), using the x-mRNA transcript (Tiollais, Pourcel and Dejean, 1985). Although this 154 aa protein is essential for infectivity, the role played in this process is unknown. HBx is a transactivator of numerous cellular and viral gene promoters namely; RBP5 (a sub-unit of RNA Polymerase II), TATA-binding protein. Various signal transduction pathways such as Ras/Raf/MAP kinase cascade, AP-1 and NF-κB, are also activated by the HBx (Locarnini, McMillan and Bartholomeusz, 2003). HBx binds to and inhibits the tumor suppressor protein p53 implicating a role in HBV associated HCC.

1.3.4 HBV Life Cycle

1.3.4.1 Attachment and Entry Into Hepatocytes

The first step in the life-cycle is specific attachment to the sodium taurocholate receptor found on thehepatocyte surface (Yan *et al.*, 2014), facilitated by the PreS1 domain of LHBs (Urban, 2008). Entry into the cytoplasm occurs either by receptor-mediated endocytosis or cell-membrane fusion. Microtubules are used to transport the nucleocapsid through the cytoplasm to the nuclear-pore (Kann, Schmitz and Rabe, 2007). Prior to the release of HBV rcDNA into the nucleoplasm, the nucleocapsid is disassembled on the cytoplasmic side of the nuclear-pore (Tuttleman, Pourcel and Summers, 1986).

1.3.4.2 cccDNA Formation

Covalently closed circular (ccc)-DNA formation of HBV-DNA from partially ds-rcDNA is essential for HBV replication. The following events need to occur to allow cccDNA formation, removal of the polymerase from the minus-strand, removal of the 18 nt RNA oligomer at the 5'-end of the plus-strand, the incomplete plus-strand is repaired using host-cell enzymes (such as cellular DNA polymerase) and the plus- and minus-strands become covalently bound (Bock *et al.*, 2001). Using, cccDNA as a transcriptional template, host-derived RNA polymerase II, along with liver-specific enzymes and non-specific transcription factors, the four HBV mRNAs are transcribed (Sommer, van Bömmel and Will, 2000). The 3.5 kb overlength pgRNA is used not only to transcribe the core and polymerase proteins, but serves as the template for viral replication later on.

Histological analysis of HBV infected hepatocytes, displays episomal structures, which are mini-chromosomes formed by cccDNA using histones (Bock *et al.*, 2001). A single hepatocyte can contain 50 or more copies of cccDNA (Zhu *et al.*, 2001). The half-life of DHBV cccDNA is 30 – 60 days (HBV is assumed to have a similar half-life) (Beck and Nassal, 2007), which is believed to be longer than

hepatocyte cellular regeneration therefore cccDNA persists in newly synthesized hepatocytes (Zhu *et al.*, 2001). cccDNA is responsible for persistence of chronic HBV infection (Levrero *et al.*, 2009).

1.3.4.3 Formation of Nucleocapsids

The mRNA transcripts generated in the nucleus are delivered to the cytoplasm for translation (Hirsch *et al.*, 1990). The newly synthesized core protein molecules dimerise thereafter, using host-chaperone molecules the dimer sub-units spontaneously assemble, forming nucleocapsids each containing a single copy of pgRNA attached to a polymerase molecule (Hirsch *et al.*, 1991; Wynne, Crowther and Leslie, 1999).

1.3.4.4 Reverse Transcription

HBV is sometimes referred to as a para-retrovirus because it reverse transcribes DNA from an RNA template (Summers, 1987). Within the nucleocapsids, the HBV polymerase binds to a stem-loop structure, the epsilon or encapsidation signal (ε) (Junker-Niepmann, Bartenschlager and Schaller, 1990). The 5'-UUC-3' motif on ε is used to transcribe the first aa (tyrosine) of the minus-strand. The tyrosine molecule is used to prime the pgRNA to initiate reverse transcription and synthesis of the minus-strand (Wang and Seeger, 1993). The Pol-tyrosine complex translocates to DR1 at the 3'-end of the pgRNA, for elongation of the minus-strand (Rothnie, Chapdelaine and Hohn, 1994). During minus-strand synthesis, RNAse H degrades the pgRNA, with the exception of an 18 nt oligomer (including DR1) at the C-terminus of the pgRNA, which remains bound to the 3'-end of the minus-strand (Loeb, Hirsch and Ganem, 1991).

The RNA-oligomer translocates to DR2 whereby it primes plus-strand synthesis. The plus-strand remains incomplete, ceasing synthesis after 50 - 70% of

complementing the minus-strand (Beck and Nassal, 2007). The elongated plus-strand transfers to the 3'-end of the minus-strand. Base-pairing between the plus- and minus-strands results in the formation of partially double-stranded rcDNA. Post reverse-transcription the ds-rcDNA either returns to the nucleus for another round of replication or matures into a Dane particle (Nassal and Rieger, 1996).

1.3.4.5 Maturation of HBV Virion

The nucleocapsid, containing a single copy of rcDNA, enters the ER (Bartenschlager and Schaller, 1992), following which, it attaches to the PreS1 domain of LHBs inserted in the ER membrane (Bruss *et al.*, 1994). During budding-off from the ER the nucleocapsid becomes enveloped with the ER membrane containing HBV surface proteins and host-derived lipids, releasing mature virions into the Golgi, where they are encapsulated in multi-vesicular bodies in preparation for exocytosis (Demirov and Freed, 2004). Following exocytosis the virions either enter the host's circulation or infect neighbouring hepatocytes (Gerelsaikhan, Tavis and Bruss, 1996).

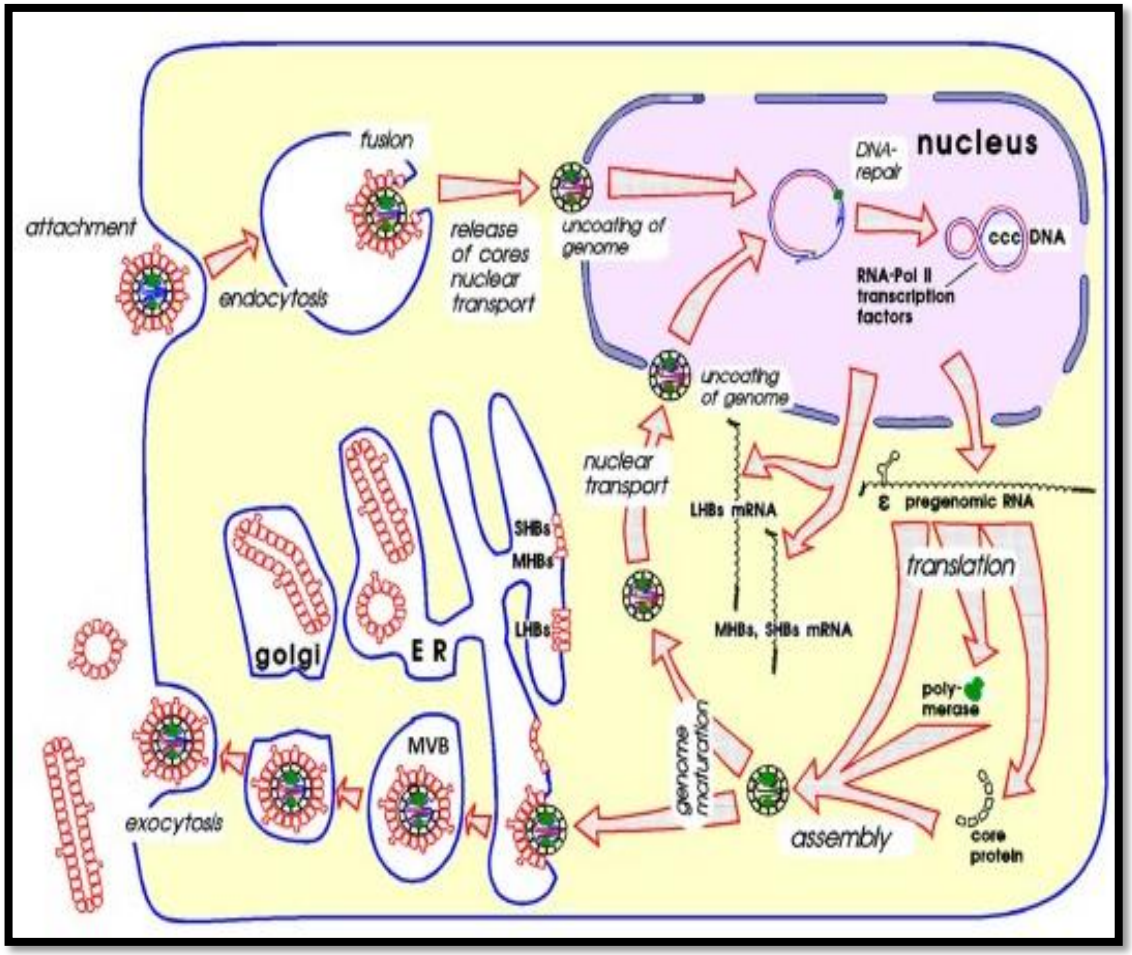

Figure 1.5 HBV Replication Cycle: The process begins with attachment followed by endocytosis, subsequently envelope removal, thereafter releasing the nucleocapsid into the cytoplasm. Microtubules deliver the nucleocapsid to the nuclear-pore, for uncoating and release of rcDNA into the nucleoplasm. cccDNA formation and the production of mRNAs occurs within the nucleus. These are used to synthesize HBV structural and non-structural proteins in the cytoplasm. The proteins produced, are used in reverse-transcription and virion formation. Nucleocapsids are assembled thereafter allowing reverse transcription to occur within the capsid. Nucleocapsids are then transported back to the nucleus for another replication cycle or are delivered to the ER for encapsidation. The enveloped virions thereafter enter the Golgi preparing it for exocytosis(Gerlich, 2013). Reproduced in accordance with the Open Data Policy of Biomed Central and the Creative Commons CC1.0 Public Domain Dedication Waiver

1.3.5 HBV Genotypes and Serological Subtypes

1.3.5.1 Genotypes

Genotypes refer to the genomic constitution of an organism, moreover it can be described as the replication competent sequence variant that has stabilized after a prolonged period of time (Kramvis, Kew and François, 2005). HBV is classified into genotypes and subgenotypes. Many studies have demonstrated genotype or subgenotype specific geographic distributions, mutation selection, clinical manifestations, transmission routes, response to anti-retroviral therapy and progression to severe liver disease(Kramvis and Kew 2007; Pujol *et al.*, 2009). Therefore understanding the differences among the genotypes and subgenotypes is advantageous. Genotypes are variable in genome length, ORF length and mutations (Kramvis, Kew and François, 2005). There are 9 genotypes of HBV (A-I) and a putative tenth, genotype 'J', this based on an intergroup nucleotide divergence of >8% (Okamoto *et al.*, 1988; Norder, Courouce and Magnius, 1992; Kramvis, Kew and François, 2005; Yu *et al.*, 2010). Genotypes are further stratified into subgenotypes based on a nucleotide divergence of 4-8% (Miyakawa and Mizokami, 2003; Tatematsu *et al.*, 2009; Kramvis, 2014).

1.3.5.2 Serological Subtypes

Previously serological-typing was used to distinguish between the various strains of HBV, based on the 'a' determinant in SHBs (Norder, Courouce and Magnius, 1992). However genotyping is more widely used, due to the limitations of serological subtyping (Kao & Chen 2006). The 'a' determinant is used to distinguish between ten serological sub-types based on the allelic variations of this region (Tiollais and Wain-Hobson, 1984). Amino-acids 122 and 160 of SHBs are used to differentiate between serotypes *d/y* and *w/r***,** respectively. At position 122 a lysine or an arginine can be found for serotype *'d'* or *'y'*, respectively. Position 160 is characterised by a lysine or arginine, when distinguishing between

serological subtypes *'w'* or *'r'*, respectively. Recent studies have found that positions 134, 140 and 159 are useful in describing subtypes *w1* and position 127 for *w2-w4*. Additionally nt 177 is used to distinguish between *adrq+* and *adrq-* whilst position 178 is used to distinguish *adw4q+* and *adw4q-*(Okamoto *et al.*, 1988; Locarnini, 2003; Kramvis, Kew and François, 2005; Purdy *et al.*, 2007). A correlation between genotyping and serotyping exists, with *adw* associated with genotypes A, B, F, G and H and *ayw* associated with genotypes D and E however, this is not a rule (Kramvis *et al.*, 2008).

1.3.5.3 Geographic Distribution of Genotypes

Genotype A is widespread across Africa, subgenotype A1 is found predominantly in Africa, specifically sub-Saharan Africa , and Asia (Bowyer *et al.*, 1997; Kimbi, Kramvis and Kew, 2004; Kramvis and Paraskevis, 2013). Subgenotype A3 and A4 are found in Africa and Haiti, with subgenotype A2 found in Europe and North America (Hannoun *et al.*, 2005; Kurbanov *et al.*, 2005). Subgenotypes B1 – B4 predominate in Asian countries with the exception of subgenotype B4, also found in France (Shi *et al.*, 2012; Kramvis, 2014). Subgenotype B5 prevails in Eskimos and Inuits (Osiowy *et al.*, 2006).

Genotype C is the most sub-classified group of the HBV genotypes, having 16 subgenotypes, found mostly in Asia (Shi*et al.*, 2012). Subgenotype C3 however, circulates in New Caledonia and Polynesia whilst subgenotype C4 is predominant in Australian Aborigines (Kato *et al.*, 2003; Shi*et al.*, 2012; Davies *et al.*, 2013). The majority of genotype C subgenotypes occur in the Indonesian and Phillippino populations (Huy *et al.*, 2004; Tanaka *et al.*, 2006; Kramvis *et al.*, 2008). Until recently this genotype was subdivided into 8 subgenotypes, when the work of Yousif and Kramvis 2013, provided a new classification method, whereby Genotype D was divided into six rather than eight genotypes. Genotype D is the most widely distributed genotype (Pujol *et al.*, 2009). Subgenotype D1 is found in the Middle East and Central Asia whilst D2 is found in Europe, the Mediterranean

area, Japan and Lebanon (Norder *et al.*, 2004; Schaefer, 2007). HBV subgenotype D3 is a globally distributed strain (Kramvis, 2014).Subgenotype D4 is found in a variety of populations namely Australian aborigines, Northern and Central Americas, Micronesians, Papua New Guineans and Arctic Denes (Norder *et al.*, 2004; Pujol *et al.*, 2009). Subgenotype D5 is exists predominantly on the Indian subcontinent (Banerjee *et al.*, 2006; Yousif and Kramvis, 2013), whilst Tunisia and Nigeria have the highest number of subgenotype D6 circulating in their respective countries (Kramvis and Kew 2007; Norder et al. 2004).

Genotype E is found in mainly in western and central Africa (Kramvis *et al.*, 2005) . With only four subgenotypes, genotype F prevails in central and South America, with the exception of subgenotype F1, which is also found in Alaska (Huy *et al.*, 2004; Kato *et al.*, 2005). With a wide geographic distribution, genotype G prevails in North America, Mexico, Germany, Italy, France and the UK (Tanaka *et al.*, 2004; Lindh, 2005). Genotype H is predominant in Mexico, Japan, Nicaragua and the USA (Arauz-Ruiz *et al.*, 2002) Genotype I has only two subgenotypes distributed in Laos, Vietnam, China and India (Yu *et al.*, 2010). The putative tenth genotype, "J", exists only in Japan (Tatematsu *et al.*, 2009). Currently no subgenotypes have been reported for genotypes E, G, H or J.

1.3.5.4 Genotypes Prevalent in sub-Saharan Africa

Subgenotype A1 is the dominant strain of HBV in sub-Saharan Africa (Kramvis, Kew and François, 2005). Subgenotypes A1 and A2 are found in sub-Saharan Africa, whilst subgenotypes A3 and A4 are predominant in central and west Africa (Pujol *et al.*, 2009). Genotype E prevails in western Africa, with genotype D found globally (Norder *et al.*, 2004; Schaefer, 2007; Pujol *et al.*, 2009; Yousif and Kramvis, 2013).

Even within subgenotype A1 there are variations depending on the geographical location. The African subgenotype A1 clade has a greater nucleotide divergence than the Asian clade therefore Africa is the probable origin of this subgenotype (Kramvis and Kew, 2007). This is supported by evidence of historical migration patterns indicating that subgenotype A1, may have spread to the Indian subcontinent and South America, from Africa during the slave trade (Kramvis and Paraskevis 2013; Alvarado-Mora *et al.*, 2013). Subgenotype A2 is found predominantly in Europe, but is believed to originate in southern Africa hence it can still be found in this region. European travellers to sub-Saharan Africa in the fifteenth century are supposedly responsible for introducing this strain into the European population (Hannoun *et al.*, 2005). Subgenotype A2 differs from subgenotype A1 in various regions of all HBV genes, resulting in different protein characteristic between these subgenotypes (Kimbi, Kramvis and Kew, 2004).

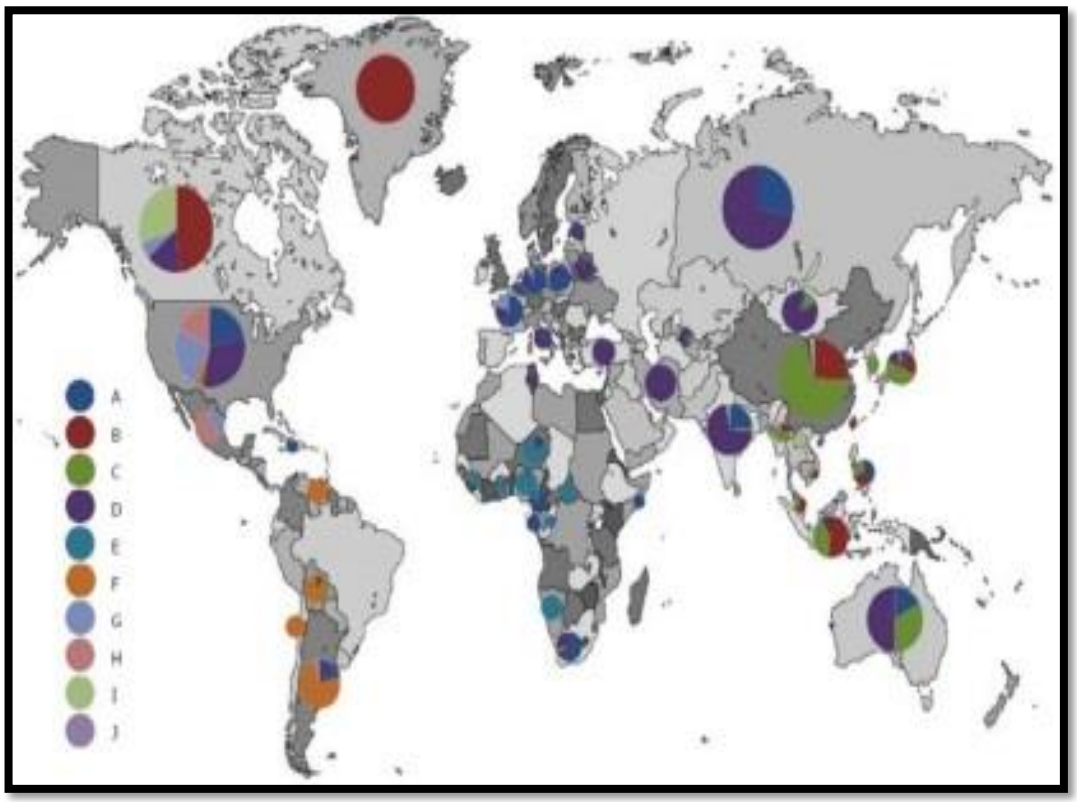

Figure 1.6 Global distribution of HBV genotypes Reprinted from Infections, Genetics and Evolution with permission from Elsevier (Shi *et al.*, 2013)

1.3.5.5 Genetic variations among genotypes

Genotype A contains six additional nucleotides at the carboxyl-end of the core gene and is characterised by an 'A' at position 1888. Genotype D has a 33 nucleotide deletion in the PreS1 region at the amino-terminus (Kramvis, 2014). Characteristic to Genotype E is a three nucleotide deletion at the amino terminus of the PreS1 region. A 36 nucleotide insert at 3' - end of nt 1905, a three nucleotide deletion at the amino terminus of the PreS1 region and two stop codons at amino-acids 2 and 28 of the PreC region, are distinct characteristics of Genotype G. Genotype J has a 33 nucleotide deletion at the N-terminus of the PreS1 region (Norder, Couroucé and Magnius, 1994; Stuyver *et al.*, 2000; Kramvis, Kew and François, 2005; Purdy *et al.*, 2007).

Genotypes B, C, F, H and I have genomes of 3215bp whilst, genotypes D and J are both characterised by genome lengths of 3182bp. Genotypes A, E and G have genome lengths of 3221bp, 3212bp and 3248bp, respectively. Genotypes A, D and E are characterised by a 'CG' at loci 1802-1803 however genotypes B, C and F have a 'TT' at the same positions. Loci 1858 is associated with a, 'C' in genotypes A, F and H, and a 'T' with genotypes B, D and E (Stuyver *et al.*, 2000; Purdy *et al.*, 2007; Kramvis *et al.*, 2008). These distinctions allow easier identification of genotypes and infer information about protein expression and functional differences resulting from the respective insertions or deletions.

Recombination of genotypes has been documented, occurring most frequently in the BCP/PreC, small S and core regions (Simmonds and Midgley, 2005; Shi *et al.*, 2013). Recombinants of genotype D/E have been reported in Africa. Subgenotypes B1 and B5 do not contain recombinant sequences of genotype C in the PreC/C region, as do the rest of the B subgenotypes (Sugauchi *et al.*, 2002; Kramvis, 2014). Aside from recombination, in areas where two or more genotypes coexist, co-infection with different genotypes have been reported (Kramvis, Kew and François, 2005).

1.3.5.6 Clinical differences between genotypes found in Sub-Saharan African

The heterogeneity of HBV subgenotypes contributes to pathogenic and/or therapeutic differences (Kramvis and Kew, 2005; Kao and Chen, 2006). Persons infected with subgenotype A1 demonstrate an increased risk of HCC, than those infected with other non-A genotypes (Kew *et al.*, 2005). Subgenotype A1 infections are associated with increased HBeAg-negativity in all individuals and high HBsAg-negativity in HBV-HIV co-infected individuals (Kramvis and Kew, 2007; Mphahlele *et al*., 2006). Additional associations with subgenotype A1 are, low HBV DNA levels and higher rate of HBeAg-negativity compared to subgenotype A2. Subgenotype A2 has a lower risk of developing serious disease sequelae and clears HBeAg later than subgenotype A1 (Tanaka *et al.*, 2004). In comparison to other genotypes in sub-Saharan Africa, subgenotype A1 infected individuals have a risk of developing HCC, and the onset thereof occurs at a younger age (Kew *et al.*, 2005).

In studies carried out on other continents, where subgenotypes differ from those prevailing in Africa, genotype D is associated with greater risk of HCC development and severity of disease than genotype A (Thakur *et al.*, 2002; Pujol *et al.*, 2009). An Indian study reported early HBeAg seroconversion, as a result of the aggressive nature of Genotype D infections. This early seroconversion could result in an earlier onset of HCC compared to Genotype A (Thakur *et al.*, 2002). Although there are exceptions(Gopalakrishnan *et al.*, 2013) subgenotype A2 is the predominant subgenotype of A prevailing in India. Genotype D does not respond as efficiently to anti-viral treatment, compared to genotype A (Lin and Kao, 2010), supposedly caused by the high number of anti-viral resistance mutations associated with Genotype D (Schaefer, 2007). Genotype E has higher viral-loads and a higher frequency of HBeAg positivity than Genotype D (Cote *et al.*, 2000; Yousif *et al.*, 2013).

1.4. Natural history

The dynamic natural history of HBV begins with an asymptomatic or symptomatic acute phase, lasting 6 months or less. The chronic phase of infection is characterized by varying degrees of severity ranging from an inactive carrier state to liver necro-inflammation progressing to liver cirrhosis, End Stage Liver Disease (ESLD) and HCC. Hepatitis B infections are detected by abnormal liver histology, fluctuating levels of liver transaminases, HBsAg detection and the presence of HBV-DNA in serum (McMahon, 2004; Lok and McMahon, 2009). From a virologic perspective there are replicative and non-replicative phases, during the natural history of HBV infection (Fattovich, 2003).

1.4.1 Acute Hepatitis

Following infection the virus undergoes an incubation period lasting between 1-6 month/s (Ganem and Prince 2004). In subclinical acute infections HBeAg and HBsAg are serologically detectable two weeks post-infection, remaining present up to 8 weeks thereafter. Anti-HBc is present early during infection, whilst anti-HBs is detectable as HBV replication decreases. One-third of acutely infected individuals become symptomatic, initially presenting with: fever; fatigue; anorexia; nausea; jaundice; dark urine; clay or pale-coloured stools and general malaise. Symptomatic acute infections are characterised by HBsAg-positivity, detectable HBV-DNA in serum, elevated ALT and AST levels, liver inflammation and hepatocellular necrosis (Liang, 2009). Acute hepatitis B is self-limiting, resolving after 3-6 months (WHO, 2015). The case fatality rate in acute infections is 0.5-1% (Lavanchy, 2004). Although fulminant hepatic failure is possible it is rare, occurring in 1-2% of acute infections (Sulkowski, 2008), resulting in severe symptoms such as fever, abdominal pain, vomiting, jaundice and disorientation including symptoms of acute infection (Berk and Popper, 1978).

HBsAg positivity for longer than 6 months is considered indicative of progression to chronic HBV infection (CHB), the same applies to increased ALT levels longer than 6 months (Hoofnagle, 1981; Ribeiro, Lo and Perelson, 2002), only 5% of acute infections become chronic if an individual is infected in adulthood (Mahoney, 1999).

1.4.2 Chronic Hepatitis

Most patients are asymptomatic and infection may remain undiagnosed until liver disease has progressed. Chronicity and the severity thereof is dependent on the age at which infection occurred, as well as the route of transmission (Sugiyama *et al.*, 2006). Chronic infection is most prevalent in neonates occurring in 90% of those infected perinatally, in children under the age of five years 20-60% develop chronic infection whilst in adults approximately 5% develop chronic infection (McMahon, 2004; Hoofnagle *et al.*, 2007).

CHB is dynamic, with four phases of infection and variable degrees of sequelae, these phases do not always occur sequentially or do not strictly adhere to their respective characteristics (Bertoletti and Kennedy, 2015). Four phases of infection characterised by host-immune and virological responses these are: high replicative, low inflammatory phase, immune clearance phase, HBeAg-negative chronic hepatitis phase and the low replicative phase.

- **High replicative, low inflammatory phase**

  This asymptomatic phase is characterised by HBeAg and HBsAg positivity, high HBV viral-loads (>2 x 10^5 IU/ml), normal or slightly elevated ALT and aspartate amino transferase (AST) (Chang *et al.*, 1988; Takashima *et al.*, 1992) . The duration of this phase is between 10-30 years occurring most frequently in those infected perinatally or during early childhood these individuals generally are HBsAg-positive. Individuals infected in adulthood

do not typically experience this phase (Shi *et al.*, 2009). Disease progression to fibrosis or HCC is minimal, during this phas
e (Hui *et al.*, 2007).

- **Immune clearance phase**

This immune active phase is initially characterised by fluctuating ALT and HBV DNA levels followed by HBeAg seroconversion, elevated ALT levels, decreased levels of HBV-DNA and signs of hepatic-inflammation resulting in abnormal liver histology. The neutralizing anti-HBc assist in containing infection by preventing infection of uninfected hepatocytes (Gerlich, 2013). This phase lasts a few weeks up to several years, occurring in the second or third decade of life in those infected perinatally or during early childhood (Shi, Shi and Al, 2009). Liver damage begins during the immune clearance phase, due to necroinflammation, especially if an individual experiences this phase for a prolonged period. Some clinical symptoms may start to present during immune clearance (Fattovich, 2003; Ganem and Prince, 2004).

- **HBeAg-negative chronic hepatitis**

Necroinflammation persists during this phase with high or fluctuating ALT levels. HBV DNA levels are high to moderate whilst liver disease is progressive (Gish *et al.*, 2015)

- **Low replicative phase**

This is the low or non-replicative phase of HBV infection showing marked decrease in HBV viremia, sometimes to undetectable levels. HBeAg seroconversion to anti-HBe occurs during this phase, indicative of infection remittance. ALT levels normalise whilst inflammation and fibrosis decrease (Hoofnagle *et al.*, 2007). If severe sequelae of liver disease did not occur by this phase the individual has a good prognosis, whilst the risk of severe liver

disease such as cirrhosis and HCC are drastically reduced. Most chronic carriers of HBV are found to be in this inactive carrier phase. Only 0.5% of inactive carriers clear HBV, annually (Seto *et al.*, 2014).

A reactivation phase has been described which occurs when the immune system becomes compromised for example in HIV-infected patients or those undergoing chemotherapy (WHO, 2015). Individuals who have resolved the infection and are both HBsAg and HBV-DNA negative but anti-HBc positive, may experience reactivation under severe immune-compromise (WHO, 2015).This phase is characterised by anti-HBc positivity, fluctuating HBV-DNA and ALT levels and an increased risk of progression to severe hepatic fibrosis (Fattovich, 2003). Reactivation of infection may lead to fatal acute-on-chronic hepatitis. CHB suffers need to be regularly monitored as fluctuations in HBV-DNA and ALT-levels make diagnosis and staging difficult (Shi, Shi and Al, 2009). HBsAg positivity is maintained throughout chronic infection. However in occult HBV infection (OBI) the HBsAg is not detectable in serum but HBV DNA can be found in the liver (Raimondo *et al.*, 2008).

1.4.3 Long-term effects of CHB

CHB long-term sequelae range from hepatic-fibrosis, decompensated cirrhosis, ESLD to HCC. These outocmes are determined by viral, host and environmental factors. In those adults who contracted HBV during childhood or infancy 15-25% are at risk of fatal liver disease during adulthood (Wong and Wong, 2013). Adults with high HBV DNA levels (> 2000 IU/mL), experience higher rates of cirrhosis and HCC (Chen *et al.*, 2006). Approximately one-third of CHB sufferers will develop long term sequelae. HBsAg seroclearance is not common in chronic infections. When HbsAg loss occurs prognosis is improved although the infection is never eliminated completely (Terrault *et al.*, 2015).

It is well established that HBV-infection leads to fatal liver-cirrhosis or HCC. HCC is not always preceded by cirrhosis (Neuveut, Wei and Buendia, 2010). HCC usually develops as a result of mutations or clonal insertions of truncated or rearranged HBV-DNA. Oncogenes such as *myc* may be activated by HBV enhancers and promoters (Liu *et al.*, 2009). HBV-DNA levels, HBeAg sero-status and cirrhosis are important predictors in the development of HCC.

Chronic hepatitis leads to cirrhosis in 10-20% of cases, compensated cirrhosis progresses to hepatic decompensation and HCC in 30% and 15 % of CHB cases, respectively, within a five year period (Fattovich, Bortolotti and Donato, 2008). HBV-DNA levels, ALT levels and HBeAg sero-status are the most important determinants in progression to cirrhosis (Chen *et al.*, 2006).

1.5 Serology

1.5.1 HBsAg and Anti-HBs

HBsAg is used as an indicator of infection it is also the first serological marker to appear in serum, appearing 1-10 weeks after infection. HBsAg is considered an indication of transcriptional activity of HBV cccDNA (Lok and McMahon, 2009). Anti-HBs appears in individuals who are immune by vaccination against HBV or have recovered from a previous infection. Post-vaccination anti-HBs is detectable in serum. In some areas HBV surface antigen (HBsAg) sero-status is used as the sole indicator of active HBV infection (Krajden, McNabb and Petric, 2005). Persistence of HBsAg-positivity is the principal serological marker of liver-disease risk profiling. HBsAg-negativity seen in OBI infected individuals can either be the result of low HBV viral loads, the formation of immune-complexes or mutations in the region coding for the HBsAg, which can change the immunogenicity of the protein, thus infection is undetected by serological assays i.e. detection escape mutations (Torresi, 2002).

1.5.2 HBeAg, Anti-HBe, HBcAg and Anti-HBc

HBeAg reflects replication of HBV, sero-conversion to anti-HBe, is indicative of HBV replication cessation, which presents at the end of immune clearance phase. Anti-HBc can be either IgG or IgM and are collectively referred to as anti-HBc. IgG can be used to determine previous exposure to HBV even in the absence of anti-HBs whilst anti-HBc IgM appears during acute infection or reactivation. IgM appears during HBsAg seroconversion to anti-HBs (Keeffe *et al.*, 2004; Lok and McMahon, 2009).

An individual is considered to be acutely infected with HBV, if the person is serologically positive for both HBsAg and anti-HBc IgM (Krajden, McNabb and Petric, 2005). Previous studies from sub-Saharan Africa found, individuals test sero-positive for HBV surface antibody (anti-HBs) 'alone' (which is indicative of having resolved a HBV-infection) but are HBV-DNA positive (Owiredu, Kramvis and Kew, 2001; Mphahlele *et al.*, 2006).

1.6 HBV-HIV co-infection

Two thirds of the 34 million HIV-infections in the world, reside in sub-Saharan Africa, which is also high endemicity (15%) region of HBV (Matthews *et al.*, 2014). HBV and HIV share transmission routes (parenteral, vertical and sexual) therefore chronic co-infection with these viruses is frequent occurring in 5-10% of HIV-positive individuals. Barth *et al.*, 2010 found that 15% of HIV-infected individuals in sub-Saharan Africa are chronic carriers of HBV. Prevalence of CHB in co-infected individuals is ten times higher than in HIV-mono-infected individuals (Spradling *et al.*, 2010). Given that sub-Saharan Africa has an estimated 2.5 million persons chronically infected with HBV (McMahon, 2004; Puoti *et al.*, 2006), there is an increased risk of chronicity and OBI in HBV-HIV co-infected persons versus mono-infected (Spearman *et al.*, 2013). There is a

more rapid progression to cirrhosis, ESLD or HCC in HIV-infected CHB sufferers (Cardona *et al.*, 2011).

Five percent of HIV-positive sub-Saharan African adults (15 years and older) are burdened with HBV-HIV co-infection (World Health Organisation, 2016). Only one third of HBV infections are detected in HIV-positive individuals, in a routine clinical laboratory setting, leaving a large majority of HBV-HIV co-infected patients undiagnosed and untreated (Bell *et al.*, 2012). Infection with HBV is not always considered in the management of HIV-infection, although co-infection has been associated with an increased turnover rate of liver morbidity, and complicated HIV pathology (Ayuk, Mphahlele and Bessong, 2013). Whilst increasing the life-expectancy of HIV-positive individuals with Anti Retrovirals (ARV), failure to manage HBV-infection leads to liver disease and HCC in co-infected individuals. HIV-infection results in an earlier onset of HCC (Soriano *et al,*. 2008; Puoti *et al.* 2004). Co-infection increases progression to cirrhosis and the risk to HCC (Hoffmann and Thio, 2007; Sulkowski, 2008).

The World Gastroenterology Organisation (WGO) explains that a principle objective in the suppression of HBV-induced liver disease in HIV-positive individuals, is to delay or prevent hepatic-cirrhosis or HCC (WGO, 2015). Additionally, ARV use should be maintained as long-term therapy for sustained viral suppression. HBV-HIV co-infected individuals should strive to sustain normal ALT levels, suppress HBV viral replication and show histologic improvement to prevent liver-disease. Immune reconstitution after ART can cause exacerbations of HBV infection when the immune clearance phase begins, as the immune system mounts a rigorous attack on the liver resulting in severe consequences. Despite the great risk associated with the development of resistance to the ARV's used in treatment of co-infected individuals, there is greater risk involved in a lack of therapy, possibly resulting in fatal liver disease. Therefore combination therapy is required in such individuals.

1.7 Study Background

The baseline studies by Bell *et al.*, 2012and Makondo, Bell and Kramvis, 2012determined the HBV genotypes and subgenotypes of the study population and identified mutations in the BCP/PreC and complete S region mutations. Of the 298 Lam-treatment naïve, HIV-infected adults enrolled into the study, 71 had detectable HBV DNA. The number of lifetime sexual partners was the significantly higher in the participants with detectable HBV DNA compared to those that were HBV DNA negative(Bell *et al.*, 2012). Subgenotype A1 was the predominant strain found in this study with numerous mutations in the BCP/PreC region that could account for the high prevalence of HBeAg negative infection associated with this subgenotype. The G1862T mutation located in the BCP/PreC region was significantly associated with HBsAg positive HBV infection ($p< 0.05$). Deletions and mutations were found on the S region, explaining the HBsAg negativity occurring in this cohort. Three isolates had drug resistance mutations rtV173L, rtL180M, rtM204V and rtV214A at baseline. Thirty-nine participants from the baseline study returned for follow-up, the present study compared the follow-up findings to that of the baseline studies to determine the effect of Lam on HBV infection.

1.8 Rationale and Aims

Whilst increasing the life-expectancy of HIV-positive individuals with ARV's, conversely the situation is exacerbated with regards to liver disease and HCC. Given that only one third of HBV infections are actually detected in HIV-positive patients, in a routine clinical laboratory setting this leaves a large majority of HBV-HIV co-infected patients undiagnosed and untreated. Medical practioners will benefit from this work, on the basis of increased HBV infection diagnosis thus resulting in more favourable therapeutic treatment options for HBV-HIV co-infected patients. A vast amount of studies attribute non-response or viral-breakthroughs in ARV treated patients to the use of 3TC. This study will aim to provide more information on patterns of 3TC resistance in HIV-positive patients

on ART. The possible outcomes of this study will provide significant information to both the scientific and medical research communities in South Africa which is burdened by infection with sub-genotype A1, whereas previous studies outside Africa focus on other strains of HBV.

- Determine HBV-suppression in response to Lamivudine in HIV-infected adults
- Identify risk factors and markers of viral suppression whilst on Lamivudine
- Determine genotypes and genotypic changes during follow-up
- Molecular characterisisation of HBV in HBsAg-positive or HBsAg-negative individuals, pre- and post-initiation of ART

CHAPTER 2: MATERIALS AND METHODS

This study is a laboratory-based follow-up of a study initiated in 2008 (Bell *et al.*, 2012; Makondo, Bell and Kramvis, 2012).

2.1 Study Participants

Ethical approval for the study was granted by the Human Research Ethics Committee of the University of Witwatersrand and the Mpumalanga Department of Health, Republic of South Africa. Inclusion criteria for the study required that participants be over the age of 18, HIV-positive and ART-naïve. Enrolment was undertaken over an 18 month period and participants were followed-up at 3, 6, 12 and 18 months after initiation of ART. The National Health Laboratory Service (NHLS), hospital and TherapyEdge-HIV (TE)™records were used to obtain participant information namely: ALT levels, CD4+ counts, age, sex, height and weight. The 298 treatment-naïve individuals, qualified for ART, based on their CD4+ counts and HIV viral-loads, as stipulated by the South African Department of Health, HIV treatment guidelines at the time (South African National Department of Health, 2004). Thus at baseline, subjects were placed on one of two ART drug regimens referred to as D001 [Stavudine (D4T) + Lamivudine (3TC) and Nevirapine (NCP)] or D002 [Stavudine (D4T) + Lamivudine (3TC) and Efavirenz (EFV)]. The South African, Department of Health's ART guidelines have changed to include Tenofovir (TDF), as a replacement for Stavudine (South African National Department of Health, 2010).

Blood samples were collected from participants at enrolment and at each follow-up time-point at Shongwe Hospital, in rural Mpumalanga province. Of the 298 HIV-positive participants, 23.83% (71/298) tested positive for HBV DNA in at least two of three different genomic regions (Bell *et al.*, 2012; Makondo, Bell and Kramvis, 2012). Only 40 of the 71 participants, who tested positive for HBV (55%) returned for follow-up at a minimum of one time-point, after initiation of

ART. Due to insufficient serum volume, one of the participants in the follow-up group could not be included in the present study. Demographic data, serological and clinical laboratory data for 298 HIV-positive participants enrolled in our previous study were examined (Bell *et al.*, 2012; Makondo, Bell and Kramvis, 2012). Sera from the 39 follow-up patients were processed for downstream analysis of HBV serology and DNA extracted for HBV DNA quantification and molecular characterization of HBV

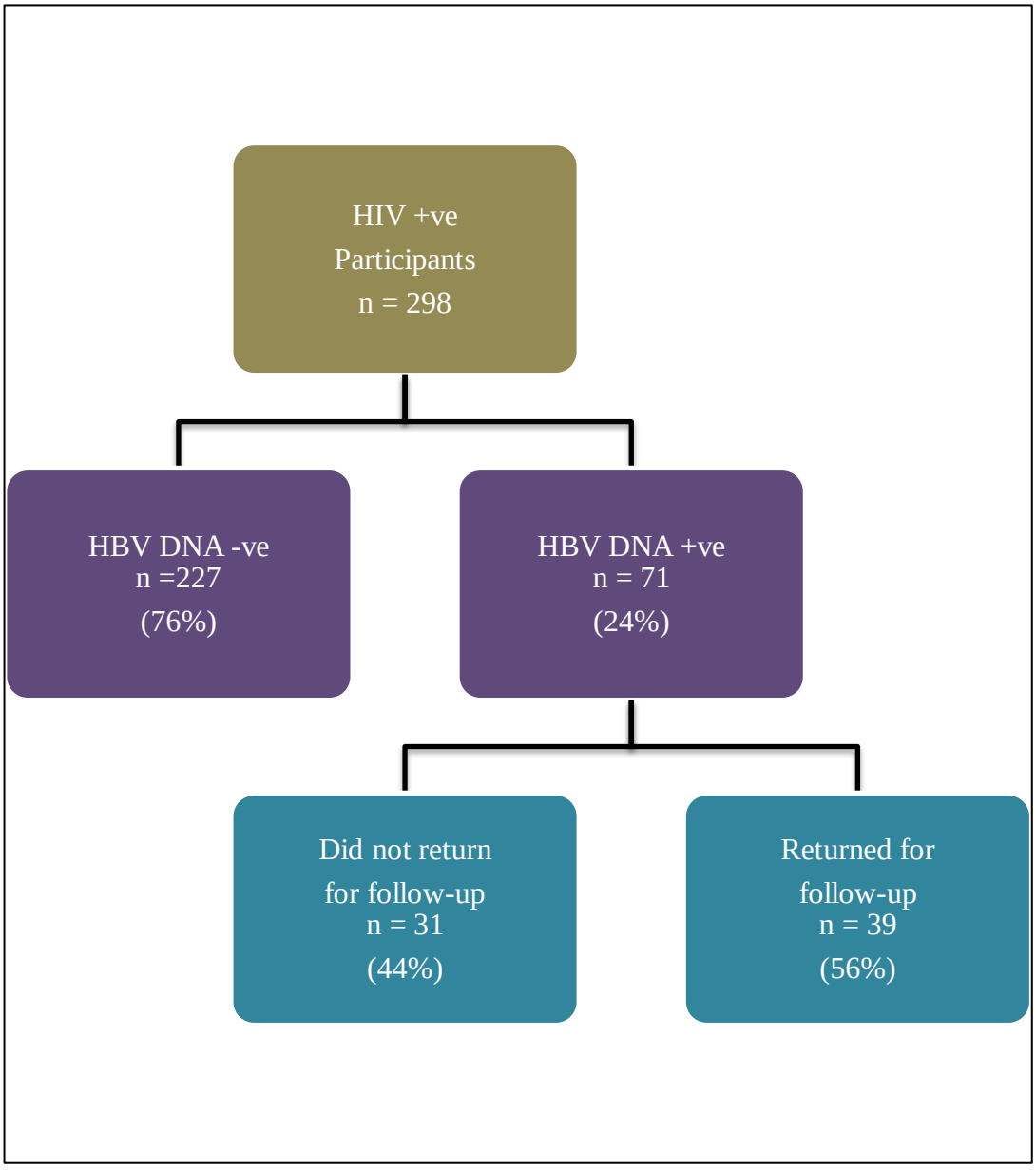

Figure 2.1: Hierarchial division of participants based on HIV-status, HBV-status and return for follow-up

2.2 Serology

Detection of antigens and antibodies were carried out for the baseline samples in the initial study (Bell *et al.*, 2012; Makondo, Bell and Kramvis, 2012) and subsequently for the follow up samples. HBsAg, anti-HBs, anti-HBc, HBeAg, anti-HBe and anti-HBc IgM, were determined using Monolisa™ HBsAg ULTRA, HBsAb ULTRA, HBcAb PLUS ELISA, Monolisa™ HBeAg-Ab PLUS (all the above mention ELISA kits were from BioRad, Hercules, USA) and ARCHITECT® (Peviva AB, Stockholm, Sweden). Antigen and antibody detection, together with viral-loads and ALT levels, were used to determine the natural history of infection as explained in section 1.4 above.

2.3 DNA Extraction

The QIAamp DNA Blood Mini-kit (QIAgen Gmbh, Hilden, Germany) was used to extract DNA from blood, plasma or serum. The first step of the protocol involved adding 20 µl of Proteinase K (provided with the kit), 200 µl of lysis buffer and 200 µl of sample, to sterilised 1.5 ml tubes, which were pulse-vortexed for 15 sec. The tubes were subsequently heated at 56˚C for 15 min, this step allows lysis of cell membranes and degradation of membranous proteins. Following the addition of 200 µl of 100% ethanol to each tube, tubes were pulse-vortexed for 15 sec then incubated for 5 min at room-temperature. Ethanol allows the impurities that remain, to become sufficiently soluble to pass through the filter in the QIAamp Mini-spin Column®. The mixture was then centrifuged at 3000 rpm for 1 min. The entire mixture from each tube was carefully transferred to a QIAamp Mini-spin Column® and centrifuged at 8000 rpm for 1 min, allowing the DNA to adhere to the column membrane. Contents of the collection tubes were discarded and the columns placed into clean collection tubes. Column were then treated with 500 µl of buffer AW1, allowing purification of the DNA in the column. Tubes were centrifuged at 8000 rpm for 1min. The second wash step entailed discarding the contents of the collection tubes and placing the columns into clean collection tubes, 500 µl AW2 was added to each column, after which

the samples were centrifuged at 8000 rpm for 1 min. The use of two different wash buffers ensures added purity of the DNA. Columns were moved to new collection tubes, then centrifuged at 14 000 rpm for 1 min, to get rid of any excess wash buffer. The final steps of DNA extraction involved unbinding DNA from the membrane inside the column. Elution buffer (provided with the kit) is generally used to suspend and store the DNA, however for the sake of reproducibility and comparison the protocol used by Bell *et al.*, 2012 and Makondo, Bell and Kramvis, 2012 was replicated. Therefore Best Quality Water (BQW) was used in place of the elution buffer. Once 75 µl BQW was added to each column, the samples were allowed to incubate at room temperature for 5 min. Columns were placed in 1.5 ml storage tubes and centrifuged at 14 000 rpm for 1 min. Extracted DNA was stored at -20˚C until further use.

2.4 HBV-DNA Quantification

Quantative Polymerase Chain Reaction (qPCR) is based on a similar concept as conventional PCR (Refer to section 2.5) but with the amplicon labelled with fluorescent dyes or probes with fluorescent reporters, these are only detectable once they have hybridised to the complementary sequence, sensors detect the fluorescence and amplicon is counted at the end of each cycle. A standard curve is generated using standards of known concentration, which allows measurement of the unknown sample concentration. The HBV qPCR protocol involves amplification within the S-region (nt 379-402 from the *Eco*RI site)

2.4.1 Preparation of Plasmid Standards

HBV viral-loads were determined using qPCR, the protocol for which, was described by (Weinberger et al. 2000). In-house plasmids consisting of a 1.3 mer HBV-A1 over-length clone were used to develop standards required for qPCR. Plasmids were revived from storage by plating onto an ampicillin containing agar plate. Following overnight incubation at 37˚C, a single colony was picked and

used to inoculate 5 ml of Luria Bertani (LB) broth containing ampicillin and left to incubate overnight at 37 ˚C in a shaking incubator. A 1 ml volume of inoculum was used to inoculate 100 ml of LB broth treated with ampicillin was incubated at 37˚C overnight in a shaking incubator, following which the inoculum was centrifuged at 4˚C for 20 min, the supernatant was discarded and a maxi-prep was carried out using QIAgen Endo-free Maxi Prep Kit (QIAgen Gmbh, Hilden, Germany). A Nanodrop (Thermo Fischer Scientific, USA) was used to measure the DNA concentration, thereafter serial dilutions of the plasmids were made using elution buffer as the diluent.

2.4.2 Quantification PCR

A serial dilution of the plasmids with concentrations ranging from 1×10^3 - 1×10^{10} IU/ ml, was used as standards that were used to generate the standard curve (1×10^1 IU/ML and 1×10^2 IU/ml did not produce stable wavelength readings). The second Eurohep standard (product code 97/750) from National Institute for Biological Standards and Controls (NIBSC); Hertfordshire, UK], with a final concentration of 10^6 IU/ml, was used as the internal standard.

The qPCR reaction mixture consisted of 25 µl 2x EagleTaq Master Mix, 1.5 µl 10 µM of each primer (Taq 1 and Taq 2), 1 µl 10µM Probe [(FAM-TAMRA labelled probe was synthesised by ABI (Applied Biosystems, Carlsbad, California, USA)], 19 µl BQW and 2 µl BQW. PCR was carried out on the CFX96™ Touch Real-Time PCR Detection System (BioRad, Hercules, CA). Please refer to Table 2.1 for probe and primer sequences. PCR cycling involved 45 cycles at 50 ˚C for 2 min, 95 ˚C for 10 and 95 for 0.15 sec. Standards were run in triplicate, whilst controls and samples were run in duplicate. An R-squared value of > 0.95 was achieved for each run. Results were analysed using BioRad' CFX Real-Time PCR Software.

Table 2.1 qPCR and BCP/PreC, PCR and sequencing primers and probe sequences		
	qPCR Primers and Probes	Nucleotide Co-ordinates
Probe	5’FAM-TCC TCC AAT TTG TCC TGG TTA TCG CT-TAMRA3'	349-375
Primer one	5’ -CAA CCT CCA ATC ACT CAC CAA- 3'	321-342
Primer two	5’ -ATA TGATAA AAC GCC GCA GAC AC- 3'	402-379
	BCP/PreC PCR and Sequencing Primers	
BCP 1F	5'-GCA TGG AGA CCA CCG TGA AC-3'	1606-1625
BCP 1R	5'-GGA AAG AAG TCC GAG GGC AA-3'	1955-1974
BCP 2F	5'-CAT AAG AGG ACT CTT GGA CT-3'	1653-1672
BCP 2R	5'-GGC AAA AAA CAG AGT AAC TC-3'	1940-1959
BCP1	5'-GAG GCA TAC TTC AAA GAC TG-3'	1698-1717
BCP2	5'-AGT AAC TCC ACA GTA GCT CC-3'	1928-1947

2.5 Nested PCR and Sequencing

2.5.1 BCP/PreC PCR and Sequencing

This BCP/PreC PCR protocol was developed by Takahashi et al. 1995. The first round PCR mixture included; 13.9 µl BQW, 2.5 µl 10x NH_4, 1.5 µl 3.0 mM $MgCl_2$, 2 µl dNTP's (200 µM of each), 1.25 µl of each primer (BCP1F and BCP1R, refer to Table 2.1 for probe primer sequences), 0.1 µl BIOTAQ enzyme (Bioline, London, UK), 2.5 µl DNA with a total reaction volume of 25 µl. PCR cycling conditions included, denaturation at 95˚C for 1 min, annealing at 55˚C for 1 min and extension at 72˚C for 2 min, carried out on the Mastercycler (Eppendorf, Germany).

The second round amplification mixture consisted of 29 µl BQW, 5 µl 10x NH_4, 1.5 µl 3.0 mM $MgCl_2$, 4 µl dNTP's (200 µM of each), 1.25 µl of each primer (BCP2F and BCP2R, refer to Table 2.1 for primer sequences), 0.1 µl BIOTAQ enzyme (Bioline, London, UK), 2.5 µl DNA with a total reaction volume of 25 µl.1 PCR cycling conditions included, denaturation at 95˚C for 1 min, annealing at 55˚C for 1 min and extension at 72˚C for 2 min, carried out on the Mastercycler (Eppendorf, Germany). The sequencing primers used for this region were BCPS1 and BCPS2.

2.5.2 Complete S PCR and Sequencing

The second region amplified was the 1203 bp, complete S-region (nt 2848-835 from the *EcoR*I site (GAATTC), where the *EcoR1* site is used as the first nucleotide using the PCR protocol of Vermeulen et al. 2012. The first round of PCR consisted of 9.25 µl BQW, 12.5 µl QIAgen Hotstar Master Mix (QIAgen Gmbh, Hilden, Germany), 0.375 µl primers (S1F and S1R, refer to Table 2.1 for primer sequences), 2.5 µl DNA making up a 25 µl reaction mixture. PCR cycling involved 40 cycles of denaturation at 94˚C for 60 sec, annealing at 65˚C for 1 min

and extension at 72˚C for 3 min, carried out on a Mastercycler (Eppendorf, Germany).

The second round reaction mixture was composed of 18.5 µl BQW, 25 µl QIAgen Hotstar Master Mix, 0.75 µl primers (S2F and S2R, refer to Table 2.1 for primer sequences), 5 µl DNA making up a 50 µl reaction mixture. PCR cycling involved 40 cycles of denaturation at 94˚C for 60 sec, annealing at 66˚C for 1 min and extension at 72˚C for 3 min, carried out on a Mastercycler (Eppendorf, Germany). Three sequencing primers; 2497F, 3188F and 591F, all in the forward direction were used in sequencing this region.

The overlapping ORFs of the complete S and Pol regions of the HBV genome, allow for analysis of a portion of the Pol gene that overlaps with the complete S-region. The Pol region spanning from nt 2307 – 1623 (from the *EcoR1* site) overlaps with the complete S-region which spans from nt 2848 – 835 (from the *EcoR1* site) (Lin and Kao, 2010), it is within this region that the Spacer and the first 236 aa's of reverse transcriptase overlap with the complete S-region . A two nucleotide frame-shift change in the alignment of the complete S-region allows for analysis of the Pol region. The complete Pol ORF is not easily amplified due to the large size of this gene.

Table 2.2 Complete S PCR and sequencing primer sequences		
	Complete S PCR and Sequencing Primers	Nucleotide Co-ordinates
S1F	5’ -TCA ATC GCC GCG TCG CAG AAG ATC TCA ATC-3′	2410-2439
S2F	5’ -TCC AGA CCG/T GCT GCG AGC AAA ACA-3′	1314-1291
	Complete S PCR and Sequencing Primers	
S2R	5’ -AAT GTT AGT ATT CCT TGG ACT CAT AAG GTG GG-3′	2451-2482
S2F	5’-AAT GTT AGT ATT CCT TGG ACT CAT AAG GTG GG-3′	1254-1286
2497F	5’ -TTC CTT GGA CTC ATA AGG TG-3′	2461-2480
3188F	5’ -AG TCA GGA AGG CAG CCT AC-3′	3152-3170
591F	5’ -ATT GCA CCT GTA TTC CCA TCC-3′	591-611

2.6 Gel Electrophoresis and Sequencing

All PCR products were electrophoresed on a 1% agarose gel in 1 x TBE solution, at 85 volts for 45 – 90 min, depending on the size of the amplicon of interest. Migration of a 100 bp DNA ladder (Promega, Madison, WI, USA) was used to detect the 300 bp BCP/PreC and 540bp P7/P8 PCR amplicons. A 1 kb ladder (Promega, Madison, WI, USA) was used to detect the 1200 bp complete S-region amplicon. Ultraviolet light was used to visualise samples using the GelDoc Imaging System (BioRad, USA). Samples with positive amplification detected,

were sent to the Central Analytical Facility, Stellenbosch University, South Africa, for PCR clean-up and sequencing. The BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA) was used to prepare amplicons, which were sequenced on the ABI 3130XL Genetic Analyser (Applied Biosystems, Foster City, CA).

2.7 Statistical Tools

The R statistical language was used to perform all statistical test (Team & Computing, 2013). Chi-squared, Wilcoxon and one-way ANOVAs were used to compare continuous variables. Statistical significance was considered at $p < 0.05$, all p-values were two sided. Medians and Interquartile Ranges (IQR) were used to analyse and represent data.

2.8 Phylogenetic and Bioinformatic Analyses

Chromatograms were viewed using FinchTV 1.4.0 (Geospiza, 2009) and manually aligned in GeneDoc (Nicholas and Nicholas, 1997). Sequences of the complete S-region were assembled using the Fragment Merger Tool (Bell and Kramvis, 2013). Alignments were processed through MEGA v6.0 (Tamura *et al.*, 2013) to construct a neighbour-joining phylogenetic tree using the Kimura 2-parameter model (Kimura, 1980). Trees were prepared using Pipeline: TreeMail (Bell and Kramvis 2015), which uses the PHYLIP package (Felsenstain, 1989) and viewed using TreeView 1.6.6. (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Bootstrapping was performed using 1 000 replicates in order to determine the support for the specific nodes. Trees were futher analysed and arranged using FigTree (Rambaut, 2012), TreeGraph (Müller and Müller, 2004)was use to makes layout changes to the two trees constructed. The accession numbers of HBV isolates sequenced in this study have been deposited in GenBank. The Mutation Reporter Tool (Bell and Kramvis

2013b) was used to locate relevant mutations at various regions on the HBV genome.

3. RESULTS:

Figure 3.1 represents an overview of the structure of the present study. This study extended on the baseline work conducted by Bell *et al.*, 2012 and Makondo, Bell and Kramvis, 2012.Figure 3.2 summarizes the results in terms of serological groups, virologic suppression and viral breakthrough (VBT) All values presented in this study were median values and the 25 percentile – 75 percentile Inter Quartile Range [IQR]) values. Significance for this study was set at 0.05.

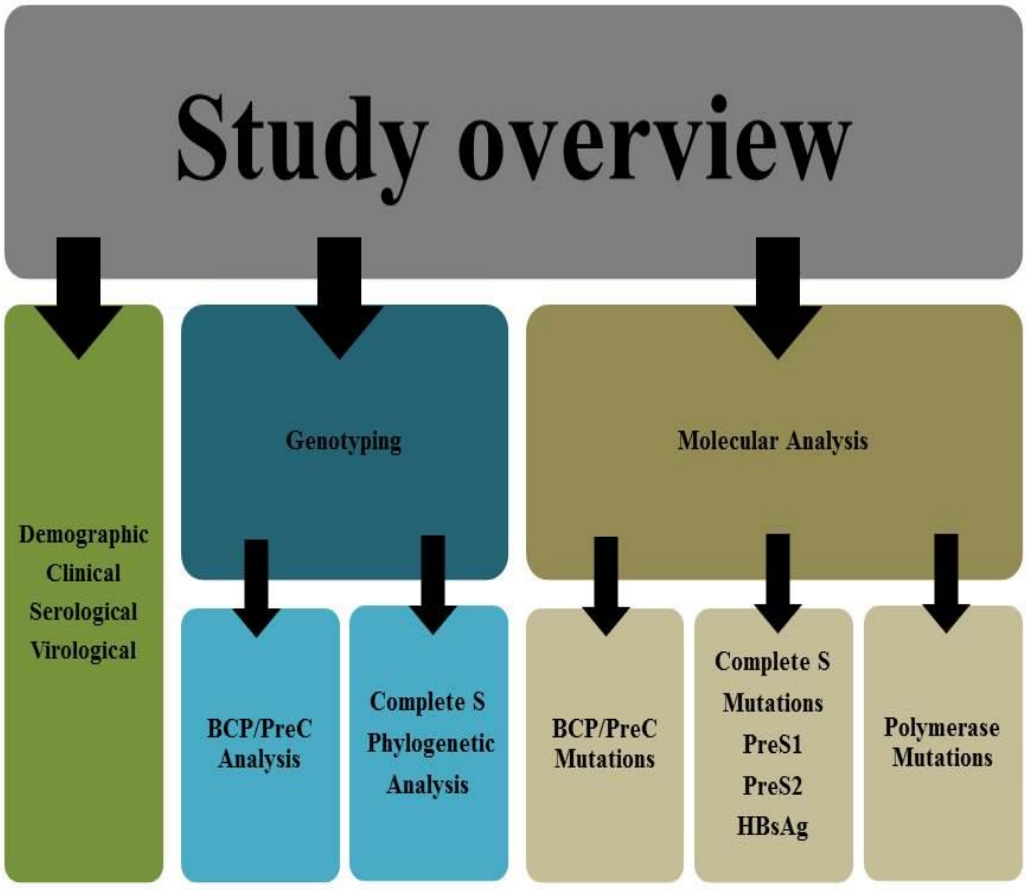

Figure 3.1 Study Overview

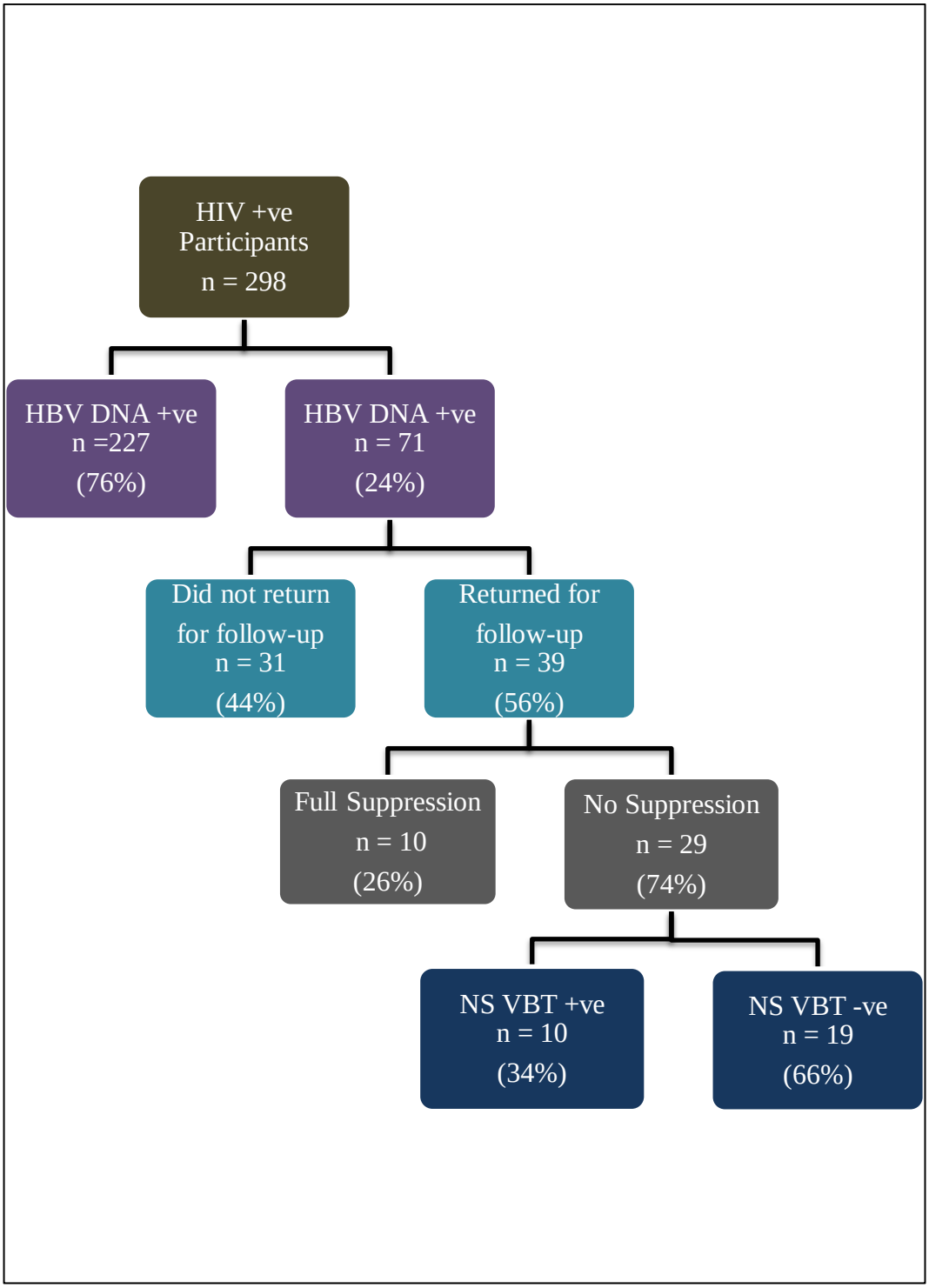

Figure 3.2 Hierarchial Overview of Participant Distribution into study groups

NS: No Suppression

VBT: Virologic Breakthrough

3.1 Follow-up Visit

Of 71 HBV-HIV co-infected participants, only 39 returned for visits after initiation of ART, representing a 45% loss to follow-up. The reason for the loss to follow up included, death, relocation and inability to trace the participant. Table 3.1 summarises participation in the follow-up visits. The prefix SHH (**SH**ongwe **H**ospital) were used in the participant numbers, the suffixes A, B, C, D and E refer to the baseline and four follow-up time-points.

Table 3.1:Summary of participation at each visit

Follow-up visit after baseline	A	B	C	D	E
Median return for follow-up (months)	0	4.2	7.4	12	18.2
No. of participants at each visit	39	31	34	30	29
Percentage return for follow-up	N/A	79.49%	87.18%	76.92%	74.36%

A, B, C, D and E represent baseline and the four follow-up time-points

3.2 Demographic, Clinical, Serological and Virological features of Participants

During the initial study (Bell *et al.*, 2012; Makondo, Bell and Kramvis, 2012), HBV viral-load, genotyping and sequence data were generated for all baseline samples. Serological laboratory tests for baseline and follow-up samples were carried out during the initial study and this data was used in the current study in conjunction with all new data obtained.

The median age of the 39 participants at baseline was 34 years (28.5 - 40), 20 were males. All participants were black southern Africans. The median (IQR)

BMI, weight, ALT and CD4+ counts at baseline were, 21.6 (20.15 - 24.4) kg/m^2; 61.4 (53.35 - 71.6) kg; 25 (18 - 42) IU/ml and 148 (80.5 - 201.5) cells/ml, respectively (Table 3.2, Column 2). The median baseline and final HBV viral-loads for all participants were 7.24 x 10^3 ($2.05x10^3$ -$3.62x10^4$) IU/ml and 9.36 x 10^3 (0 - $1.74x10^3$) IU/ml, respectively. When comparing the percentage change of HBV viral-loads between baseline and the final time point, a median decrease of 47.25% was achieved overall. Refer to the second column in Table 3.2 to find the demographic, clinical, serological and virological features of all study participants.

Table 3.2 Demographic, Clinical and Virological Characteristics of HBsAg+ and HBsAg- Groups at baseline and/or the final time-point				
	All n = 39	**HBsAg+ n = 20**	**HBsAg- n = 19**	**P-value**
Demographic and Clinical Characteristics				
Sex (M/F)	20:19	11:09	08:11	0,27
Age	34 (28,50 - 40)	35 (31 - 41,00)	32 (28 - 39)	0,75
Baseline ALT (normal range 7-55 u/l)	25 (18 - 42)	38 (23,50 - 60,75)	20 (10 - 26)	0,98
Baseline CD4+ count	148 (80,50 - 201,50)	125 (83,75 - 197,25)	151 (84,50 - 224)	0,90
Baseline Weight (kg)	61,4 (53,35 - 71,60)	64,5 (59,30 - 77,83)	56,3 (51,95 - 64,45)	**0,02**
Baseline BMI	21,60 (20,15 - 24,40)	22,5 (20,88 - 25,45)	20,4 (19,90 - 23,70)	0,12
Virologic Characteristics				
Baseline HBV Viral Load (IU/ml)	7,24E+03 (2,05E+03 - 3,62E+04)	5,69E+03 (1,81E+03 - 5,14E+04)	9,47E+03 (4,27E+03 - 2,43E+04)	0,82
Final HBV Viral-Load (IU/ml)	9,36E+01 (0 - 1,74E+03)	1,74E+03 (7,94E+02 - 3,09E+03)	0 (0 - 4,57E+01)	0,29
Difference in HBV viral-loads between baseline and last follow-up (IU/ml)	-5,78E+03 (-2,64E+04 - 6,82E+02)	-2,04E+03 (-3,52E+04 - 1,35E+02)	-9,47E+03 ([-2,43E+04] - [-4,27E+03])	0,82
Percentage of HBV Suppression achieved (%)	-47,75 ([-100] - [-13,54])	-13,54 ([-29,65] - [0,91])	-100 ([-100] - [-59,34])	**0,01**

Chi-Square or Fishers Exact were used to test statistical significance

3.3 Comparative analysis of HBsAg Positive and HBsAg Negative Groups

The data presented by Bell et al. 2012, found in the baseline study that of the 298 Lam treatment-naïve HIV positive study individuals, 71 tested positive for HBV-DNA by Nucleic-Acid testing (NAT), 26 were HBsAg+ and 45 HBsAg- (Table 3.3). The participants that were followed up included 16 participants who were HBsAg+ and 23 HBsAg- at baseline. Of the HBsAg- participants, 19 remained negative throughout follow-up these were defined as the HBsAg- group. The remaining 20 participants were HBsAg+ at baseline and/or at one time-point during follow-up, are referred to as the HBsAg+ group, nine were HBsAg+ throughout the study. Seven participants sero-converted to HBsAg- at a median of 4.2 (3.9 - 5.9) months, two participants gained the HBsAg at 18.3 (18.25 - 18.5) months. Two participants (SHH071 and SHH246) were HBsAg- at baseline, thereafter became sero-positive but had retro-converted to HBsAg- by last time-point (Table 3.3).

Table 3.3 HBsAg serological changes observed in the HBsAg+ group during the study

Participant number	A	B	C	D	E
***HBsAg+** throughout study*					
SHH001	+	+	+	ND	ND
SHH109	+	+	ND	ND	ND
SHH100	+	ND	+	+	+
SHH126	+	+	+	ND	ND
SHH148	+	+	+	+	+
SHH156	+	+	+	+	+
SHH240	+	+	ND	ND	ND
SHH256	+	ND	+	+	+
SHH300	+	+	+	+	+
*Seroconverted from **HBsAg+** to **HBsAg-***					
SHH002	+	-	-	ND	-
SHH016	+	-	-	-	-
SHH042	+	-	-	-	-
SHH070	+	-	-	-	-
SHH159	+	+	+	+	-
SHH180	+	-	-	ND	-
SHH274	+	+	-	-	ND
Median time of seroconversion 4.2 (3.9 - 5.9) months					
Participant number	**A**	**B**	**C**	**D**	**E**
*Seroconverted from **HBsAg-** to **HBsAg+***					
SHH053	-	-	-	-	+
SHH221	-	-	-	-	+
Median time of seroconversion 18.3 (18.25 - 18,50) months					
*Seroconverted from **HBsAg-** to **HBsAg+** to **HBsAg-***					
SHH071	-		+	-	ND
SHH246	-	+	ND	-	ND

Baseline weight was significantly higher in the HBsAg+ group (64.5 [59.30 – 77.83] kg) compared to the HBsAg- group (56.3 [51.95 – 64.45] kg) (p=0.02) (Table 3.2). Another significant finding between these two HBsAg serological groups, was a higher viral suppression achieved in the HBsAg- group (-100% [(-100%)–(-59%)]), with the HBsAg+ group achieving -13.54% ([-29.65%] - [0.91%]) HBV suppression (p = 0.01) (Table 3.2). Although HBV viral-loads at

baseline were indistinguishable between the HBsAg+ (5.69 x 10^3 [1.81 x 10^3 – 5.14 x 10^4])IU/ml and HBsAg- (9.47 x 10^3 [4.27 x 10^3 – 2.43 x 10^3]) IU/ml groups (Figure 3.3), the final HBV viral-load for the HBsAg- group was lower 0 [0 - 4.57x10^1] IU/ml than the final viral-load of the HBsAg+ group which was 1.74 x 10^3 [7.94 x 10^2 - 3.09 x 10^3] IU/ml, however, this was not a significant finding (p = 0.81) (Table 3.2).

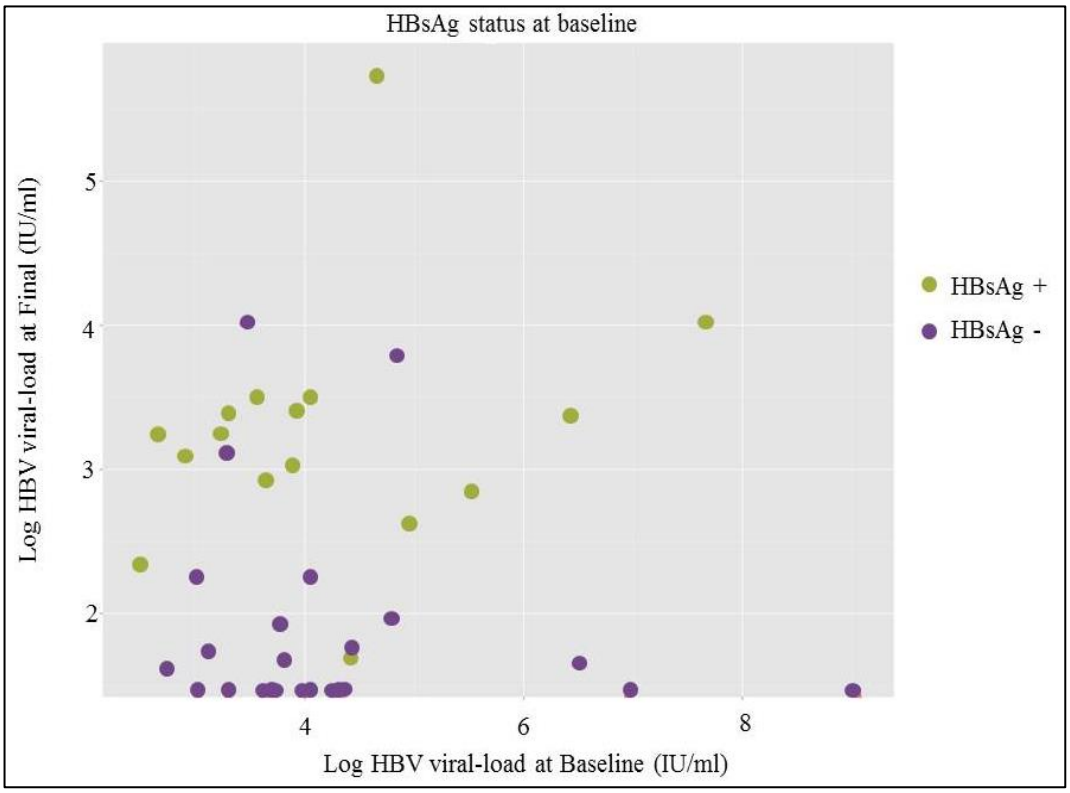

Figure 3.3 Scatter Plot of Baseline and Final HBV viral-loads of participants who were HBsAg + or HBsAg- at baseline

3.4 Comparison of Full Suppression (FS) and No Suppression (NS) Groups

Full suppression was defined as an HBV viral-load equivalent to zero IU/ml by qPCR amplification, and which was maintained throughout the study after clearance of the virus. Participants were classified as NS if they met one of the

following two criteria: *i)* a participant did not fully clear the virus at any time point in the study, *ii)* the participant experienced a full suppression followed by the detection of HBV by qPCR. Ten participants experienced full suppression of HBV, after 17.5 (12.43 - 18.2) months on ART. All participants that experienced full suppression were HBsAg-, which was a significant finding, indicating that HBsAg negativity is a predictor of full suppression of HBV(p = 0.01).

Participants in the FS group had significantly lower ALT levels (15.5, [6 - 23.75]) at baseline compared to the NS group (35, [8 - 55]) (p=0.02) (Figure 3.4). The FS group had a median final HBV viral-load of zero, this was significantly lower than the NS group (8.32 x 10^2 [0 – 2.42 x 10^3]) (p = 0.01) (Figure 3.5). The HBV viral-loads between these two groups were comparable at baseline (FS: (7.32 x 10^3 [4.2 x 10^3 – 1.78 x 10^4]), NS: (7.24 x 10^3 [3.01 x 10^2 – 6.07 10^4])) but not at the final time-point (Figure 3.5) Seven participants in the NS group completely suppressed the HBV virus before experiencing a VBT. A -100% viral suppression was achieved by the FS group whereas the NS group achieved a significantly lower percentage of viral suppression with a median -24.02% ([-100%] - [-5.03%]) (p = 0.01) (Figure 3.6).

Table 3.4 Demographic, Clinical and Virological Characteristics of the Full Suppression and No Suppression Groups at baseline and/or the final time-points

	All n = 39	FS n = 10	NS n = 31	P-value (CI:95%)
Demographic and Clinical Characteristics				
Sex (M/F)	20:19	4/6	15/14	0,79
Age	34 (28,50 - 40)	30,5 (7,25 - 45)	35 (18 - 39)	0,82
Baseline ALT (normal range 7-55 u/l)	25 (18 - 42)	15,5 (6 - 23,75)	35 (8 - 55)	**0,02**
Baseline CD4+ count	148 (80,50 - 201,50)	168,5 (88 - 229)	113 (9 - 196)	0,53
Baseline Weight (kg)	61.4 (53,35 - 71,60)	56 (50,43 - 61,85)	63,3 (37,9 - 72,4)	0,07
Baseline BMI	21,60 (20,15 - 24,40)	20,35 (19,95 - 21,73)	22,4 (13,6-26,2)	0,13
Virologic Characteristics				
Baseline HBV Viral Load (IU/ml)	7,24E+03 (2,05E+03 - 3,62E+04)	7,32E+03 (4.2E+03 - 1.78E+04)	7,24E+03 (3,01E+02 - 6,07E+04)	0,99
Final HBV Viral-Load (IU/ml)	9,36E+01 (0 - 1,74E+03)	0	8,32E+02 (0 - 2,42E+03)	**0,01**
Difference in HBV viral-loads between baseline and last follow-up (IU/ml)	-5,78E+03 (-2,64E+04 - 6,82E+02)	-7,32E+03 ([-1,78E+04] - [4,2E+03])	-5,78E+03 ([-4,74E+07] - [-4,98E+02])	0,54
Percentage of HBV Suppression achieved (%)	-47,75 ([-100] - [-13,54])	-100	-24,02 ([-100,00] - [-5,03])	**0,01**

FS: Full Suppression
NS: No Suppression
P-values were calculated using Chi-Square or Fisher's Exact

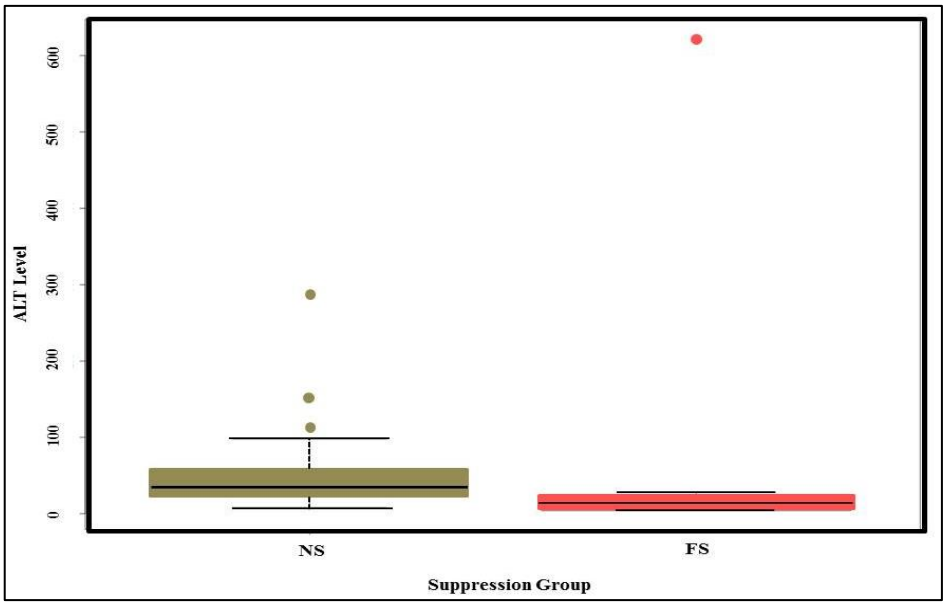

Figure 3.4 A whisker-box plot comparing baseline ALT levels between the Full suppression (FS) and No suppression (NS) groups. Lower baseline ALT levels were observed in the FS group compared to the NS group.

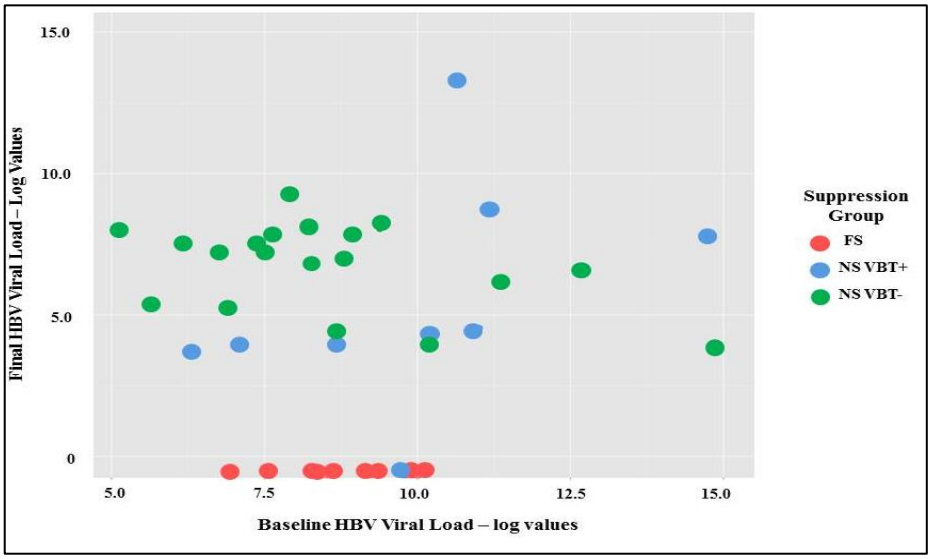

Figure 3.5 Comparison of baseline and final viral-loads among the different suppression groups. This figure shows that the baseline viral-loads were comparable among all groups.

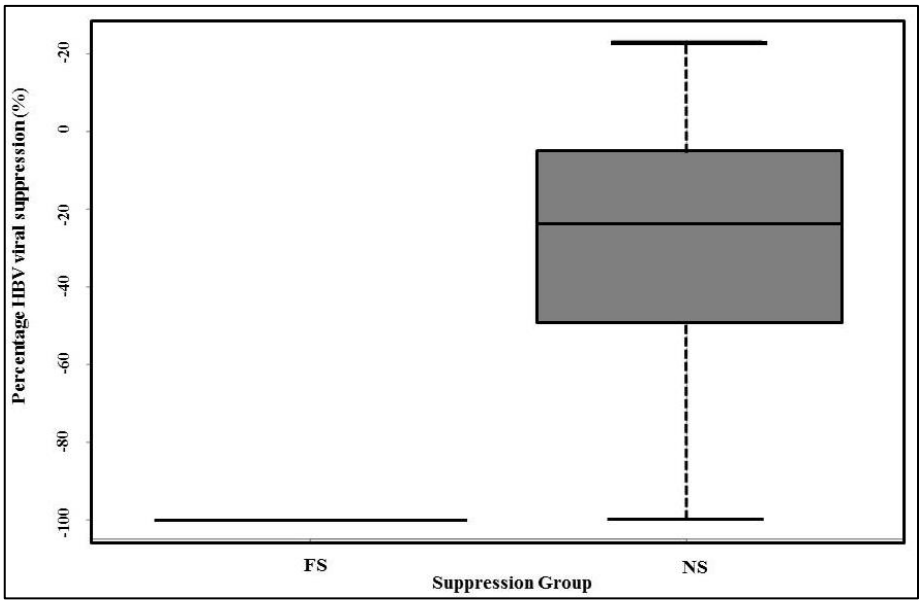

Figure 3.6 A whisker-box plot comparing percentage viral suppression achieved between the FS and NS groups. The NS group had only a 40% viral suppression during the study compared to a 100% viral suppression achieved by the FS group

3.5 Comparison of the NS VBT+ and NS VBT- Groups

A virologic breakthrough (VBT) is defined as a >1 log increase in viral load after nadir (Hongthanakorn *et al.*, 2011). The HBV virus was not adequately suppressed in 19 participants, of which, 10 experienced a VBT, referred to as NS VBT+. A higher frequency of VBT occurred among the HBsAg- participants, which accounted for 7 of the 10 VBTs. Median time between initiation and nadir was 5.1 (3.6-8.2) months. Participants in the NS VBT+ group experienced a VBT 12.35 (6.5-18.23) months after initiating ART and 5.3 (2.3-10.28) months after nadir. Furthermore, all seven HBsAg- participants that fully suppressed HBV and experienced a VBT ultimately showed full suppression. Although the NS VBT+ group had higher baseline ALT levels (38, [22.5-37.75] IU/ml) than the NS VBT- group (35 [20 – 57.5] IU/ml, this was not significant (p = 0.60) (Table 3.5). Interestingly the NS VBT+ group had a significantly higher percentage of viral suppression (Figure 3.7) , (-51,90% [-59,61] -[-41,37]), compared to the NS VBT- group -14,35% ([-47,14%] - [-0,73%]), despite the VBT events (p = 0.03).

Table 3.5 Demographic, Clinical and Virological Characteristics of NS VBT+ and NS VBT- Groups at baseline and/or the final time-points				
	All n = 39	**NS VBT+ n=10**	**NS VBT- n=17**	**P-value (CI:95%)**
Demographic and Clinical Characteristics				
Sex (M/F)	20:19	6/4	15/14	0,8
Age	34 (28,50 - 40)	30 (27,25 - 38,25)	35 (33,5 - 39)	0,21
Baseline ALT (normal range 7-55 u/l)	25 (18 - 42)	25,5 (22,25 - 37,75)	35 (20 - 57,5)	0,6
Baseline CD4+ count	148 (80,50 - 201,50)	130,5 (80 - 166,75)	110 (92,5 - 201,5)	0,57
Baseline Weight (kg)	61,4 (53,35 - 71,60)	65,65 (53,9 - 71,8)	63,2 (56,65 - 80,35)	0,67
Baseline BMI	21,60 (20,15 - 24,40)	21,95 (20,03 - 25,78)	22,60 (20,65 - 26,3)	0,62
Virologic Characteristics				
Baseline HBV Viral Load (IU/ml)	7,24E+03 (2,05E+03 - 3,62E+04)	5,64E+01 (4,37E+01 - 1,75E+03)	1,25E+03 (3,19E+02 - 2,47E+03)	0,16
Final HBV Viral-Load (IU/ml)	9,36E+01 (0 - 1,74E+03)	5,64E+01 (4,37E+01 - 1,75E+03)	1,25E+03 (3,19E+02 - 2,47E+03)	0,09
Difference in HBV viral-loads between baseline and last follow-up (IU/ml)	-5,78E+03 (-2,64E+04 - 6,82E+02)	-2,22E+04 ([-6,16E+04] - [-2,48E+03])	-3,30E+03 ([-1,75E+04] - [-4,76E+00])	0,23
Percentage of HBV Suppression achieved (%)	-47,75 ([-100] - [-13,54])	-51,90 ([-59,61] - [-41,37])	-14,35 ([-47,14] - [-0,73])	**0,03**

NS VBT+: No Suppression Virologic Breakthrough Positive
NS VBT-: No Suppression Virologic Breakthrough Negative
P-values were calculated using Chi-Square or Fisher's Exact
Significant findings are highlighted **in bold**

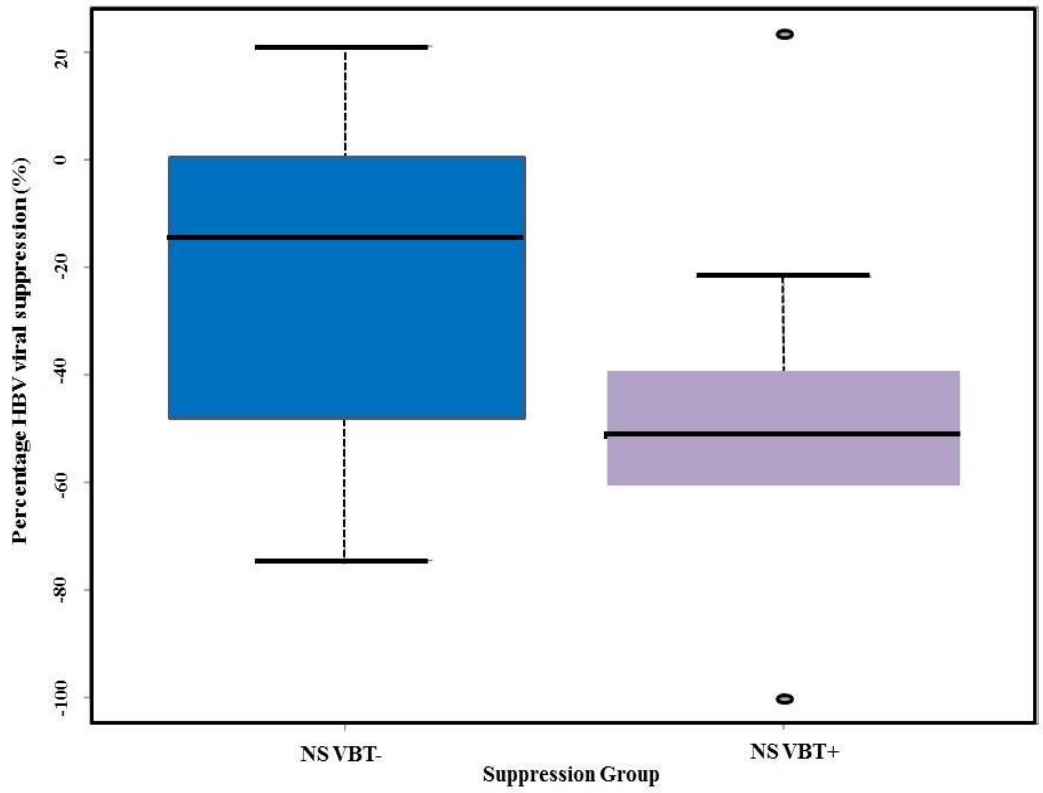

Figure 3.7 Percentage viral suppression achieved in the NS VBT- and NS VBT+ groups. Despite the virological breakthrough event in the NS VBT+ group this group achieved a viral suppression of 40 -60% whilst the NS VBT- group only experienced a viral suppression of approximately 20%

3.6 Occult and Covert Infection

Occult HBV Infection (OBI) is defined by serum HBV-DNA viral-loads of ≤ 200 IU/ml in the absence of HBsAg (Raimondo *et al.*, 2008). Covert (false occult) infection is defined by HBV viral-loads >200 IU/ml in HBsAg- individuals (Bell *et al.*, 2012). Prior to initiation of ART all study participants, who were followed up in the HBsAg- group were coverts. Four participants (SHH053A, SHH071A, SHH221A and SHH246A) in the HBsAg+ group were coverts at baseline as they were HBsAg- at this time-point, the remaining were overts (HBsAg+ with detectable HBV DNA in serum). No occult infection was found at baseline.

At the last follow-up, four HBsAg+ participants, sero-converted to HBsAg- and were now classified as covert infected, as they had viral-loads >200

IU/ml.SHH246 was HBsAg- at both baseline and the final time-points but was HBsAg+ at time-point "B" therefore was included in the HBsAg+ group. In the HBsAg- group, 13 participants completely suppressed the virus at the final time-point of these seven experienced a VBT during the study. Six participants in the HBsAg- group were covert at baseline but occult at the last follow-up.

Table 3.6 Summary of HBV covert and occult infection using the baseline and final viral-loads and HBsAg sero-status

		Baseline HBV Viral Load (IU/ml		Final HBV Viral Load (IU/ml)	
HBsAg+	SHH126	4,78E+02		1,76E+03	
	SHH148	1,65E+03		1,73E+03	
	SHH156	8,33E+04		4,28E+02	
	SHH159	4,74E+07		1,02E+04	covert
	SHH180	7,94E+03		2,53E+03	covert
	SHH221	1,86E+03	covert	1,27E+03	
	SHH240	8,44E+02		1,25E+03	
	SHH246	9,57E+02	covert	1,84E+02	covert
	SHH256	2,64E+04		5,23E+01	
	SHH274	3,11E+05		6,80E+02	covert
	SHH300	3,01E+02		2,10E+02	
HBsAg-	SHH024	2,66E+04	covert	5,96E+01	occult
	SHH029	1,14E+09	covert	0,00E+00	
	SHH032	1,26E+03	covert	5,32E+01	occult
	SHH037	2,97E+06	covert	4,47E+01	occult
	SHH041	6,07E+04	covert	9,36E+01	occult
	SHH045	6,33E+03	covert	4,68E+01	occult
	SHH052	5,41E+02	covert	4,26E+01	occult
	SHH054	5,16E+03	covert	0,00E+00	
	SHH074	9,47E+03	covert	0,00E+00	
	SHH110	1,01E+04	covert	0,00E+00	
	SHH173	5,86E+03	covert	8,30E+01	
	SHH193	4,39E+03	covert	0,00E+00	
	SHH194	1,79E+04	covert	0,00E+00	
	SHH213	2,20E+04	covert	0,00E+00	
	SHH214	1,05E+03	covert	0,00E+00	
	SHH219	1,98E+03	covert	0,00E+00	
	SHH222	2,04E+04	covert	0,00E+00	
	SHH264	9,02E+06	covert	0,00E+00	
	SHH270	4,14E+03	covert	0,00E+00	

Highlighted in green are the covert infections
Highlighted in purple are the occult infections
Some HBsAg+ partipants were HBsAg at certain time-points

3.7 Genotyping

3.7.1 Genotyping using the BCP/PreC region

The baseline study (Bell *et al.*, 2012) found five HBeAg+ participants. Ten participants in the follow-up study were HBeAg+ for at least one time-point during the study (Table 3.9). Mutations in the BCP/PreC region coding for HBeAg were investigated, comparing mutations found in the HBeAg+ group with the HBeAg- group. Analysis of positions 1809-1812, 1858 and 1888 (from the *EcoR1* site) (Figure 3.8) within the BCP/PreC region of HBV was used to distinguish genotypes and subgenotypes.

Table 3.7 HBeAg positive participants and the HBeAg serological changes observed during the study

Participant Number	Time-points				
	A	B	C	D	E
SHH002	-	-	-	-	+
SHH037	-	-	+	ND	ND
SHH100	-	-	+	-	-
SHH110	-	-	ND	-	+
SHH126	-	+	ND	ND	-
SHH159	+	+	+	+	-
SHH180	-	-	+	-	-
SHH193	-	-	-	+	+
SHH214	-	+	ND	+	-
SHH274	+	+	-	-	-

The presence of ^{1809}TCAT^{1812}, C1858 and A1888 ,characteristicof subgenotype A1(Kimbi *et al.*, 2004; Tanaka *et al.*, 2006) was found in 71 of BCP/PreC sequences. Whilst isolate SHH032A, SHH042D and SHH274D had ^{1809}GCAC^{1812}, C1858 and G1888, these are consistent with subgenotype A2. Phylogenetic analysis of the complete S-region sequences with reference sequences of subgenotype A1 and A2 showed that isolates SHH032A,

SHH042Dand SHH274D clustered with subgenotype A1. Discordant results were also found in isolates SHH016A, SHH042A and SHH159D which displayed characteristics of non-A genotype with ^{1809}GCAC^{1812}, T1858 and A1888. However the sequences from isolates of both these isolates clustered with subgenotype A1, during phylogenetic analysis of the S region. HBV isolate SHH053A showed non-A infection by BCP/PreC genotyping, this was confirmed as genotype E infected by complete S region phylogenetic analysis. Additional discordant findings were found in isolate SHH173A, which clustered distinctly with subgenotype D3 upon a phylogenetic analysis of the complete S sequences, however, the BCP/PreC analysis showed subgenotype A1 characteristics. These discordant findings could not be further analysed without cloning or full-lengthsequencing, because this was outside the scope of this study.

Table 3.8 BCP/PreC Loci 1809-1812, 1858 and 1888 used to genotype HBV sequences, including Non-A1 isolates from this study

	BCP/PreC Loci (numbered from the *EcoR1* site)		
	1809 - 18012	1858	1888
HBV Subgenotype A1	TCAT	C	A
HBV Subgenotype A2	GCAC	C	G
HBV Genotype Non-A	GCAC	T	G

3.7.2 Phylogenetic Analysis

Amplification of the complete S-region (nt 2854 – 835 from the *EcoR*1 site) yielded 47 sequences. Fourteen of these sequences were from baseline samples, and the remaining from follow-up time-points. Fifteen baseline sequences and 24 follow-up sequences were from the HBsAg+ group. These sequences were used to construct a neighbour-joining phylogenetic tree (Figure 3.9). Bootstrapping of 1000 replicates was used to test the statistical support of relatedness of isolates in the tree. The complete S region sequences are preferred for use of HBV phylogenetic analyses as the region is sufficient in length allowing for statistical

robustness and the region is small enough to produce more amplicon than full genome amplification.

Analysis of the tree constructed with genotypes and subgenotype A-H, revealed that isolate SHH053Abelonged to genotype E at baseline. This isolate clustered with subgenotype A1 at the first follow-up. Genotyping of the third, fourth and fifth follow-ups was completed using the BCP/PreC sequences as no further complete S amplicon were generated for HBV isolated from isolate SHH053. BCP/PreC loci indicated that isolate SHH053 was infected with HBV subgenotype A1 at time-points 'A', 'B' and 'C' (Figure 3.10 BCP Genotyping). Isolate SHH173A clustered with subgenotype D3 at baseline. No further S region sequences were available for phylogenetic analysis. However at the 6 monthfollow-up point, from the BCP/PreC sequences available the HBV isolated belonged to subgenotype A1. The 45 remaining sequences clustered with subgenotype A1. In summation, other than isolates SHH053A and SHH173A all other isolates belonged to sub-genotype A1.

Isolates; SHH002, and SHH070 complete S region sequences from follow-up clustered together but the baseline sequences belonged to different clades. This was more evident in some isolates (SHH159, SHH256 and SHH300), in which the follow-up sequences clustered together but distantly from the baseline sequences. This could be caused by selection pressure under which the wild-type is susceptible to Lam, therefore quasispecies diversity post-treatment appears to be highly divergent compared to baseline pretherapy sequences.

HBV sequences isolated from SHH300 at baseline and last follow-up show more homogeneity whilst isolates SHH300B+D, (which clustered separately from SHH300A+E, *see* Figure 3.), showed more homogeneity. Upon mutational analysis of the BCP/PreC, complete S and polymerase regions, isolates SHH300A+E showed distinct similarities in sequences indicating that at the last

follow-up this isolate was reverting to a baseline sequence indicating that treatment was no longer able to efficiently suppress HBV (SHH300 belonged to NS VBT- group). This adds to the interpretation that the wild-type (pre-treatment) HBV strains in this study may have a susceptibility to Lam, causing the emergence of a strain that can withstand Lam treatment. However without further amplification, cloning and sequencing it is difficult to ascertain the quasispecies evolutionary diversity and complexity.

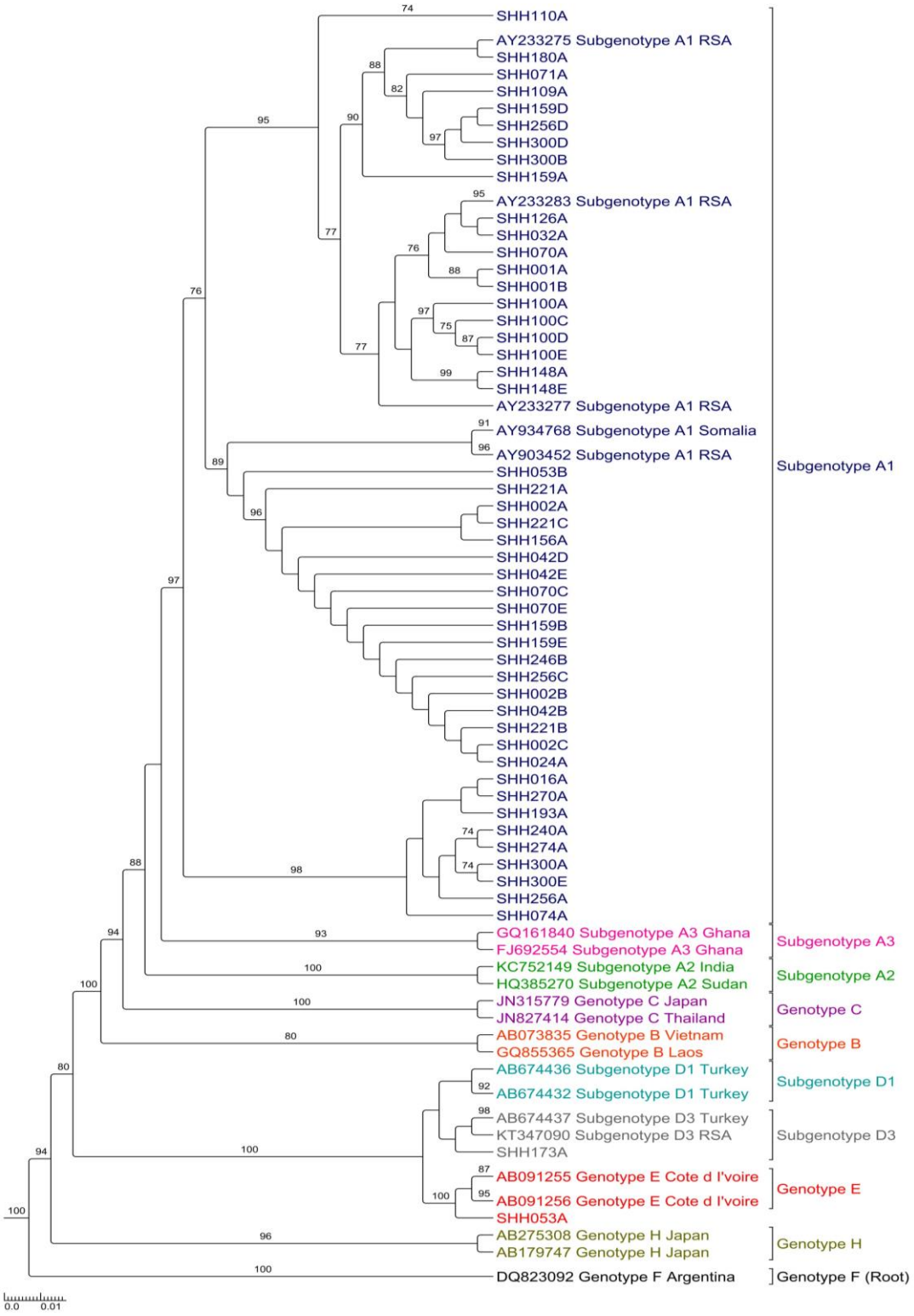

Figure 3.8 A neighbour-joining phylogenetic tree with 1000 bootstrap replicates was constructed to determine the genotypes or subgenotypes of participants in this study. The majority of HBV isolates belonged to Subgenotype A1 except SHH173A and SHH053A which belonged to genotypes D and E, respectively

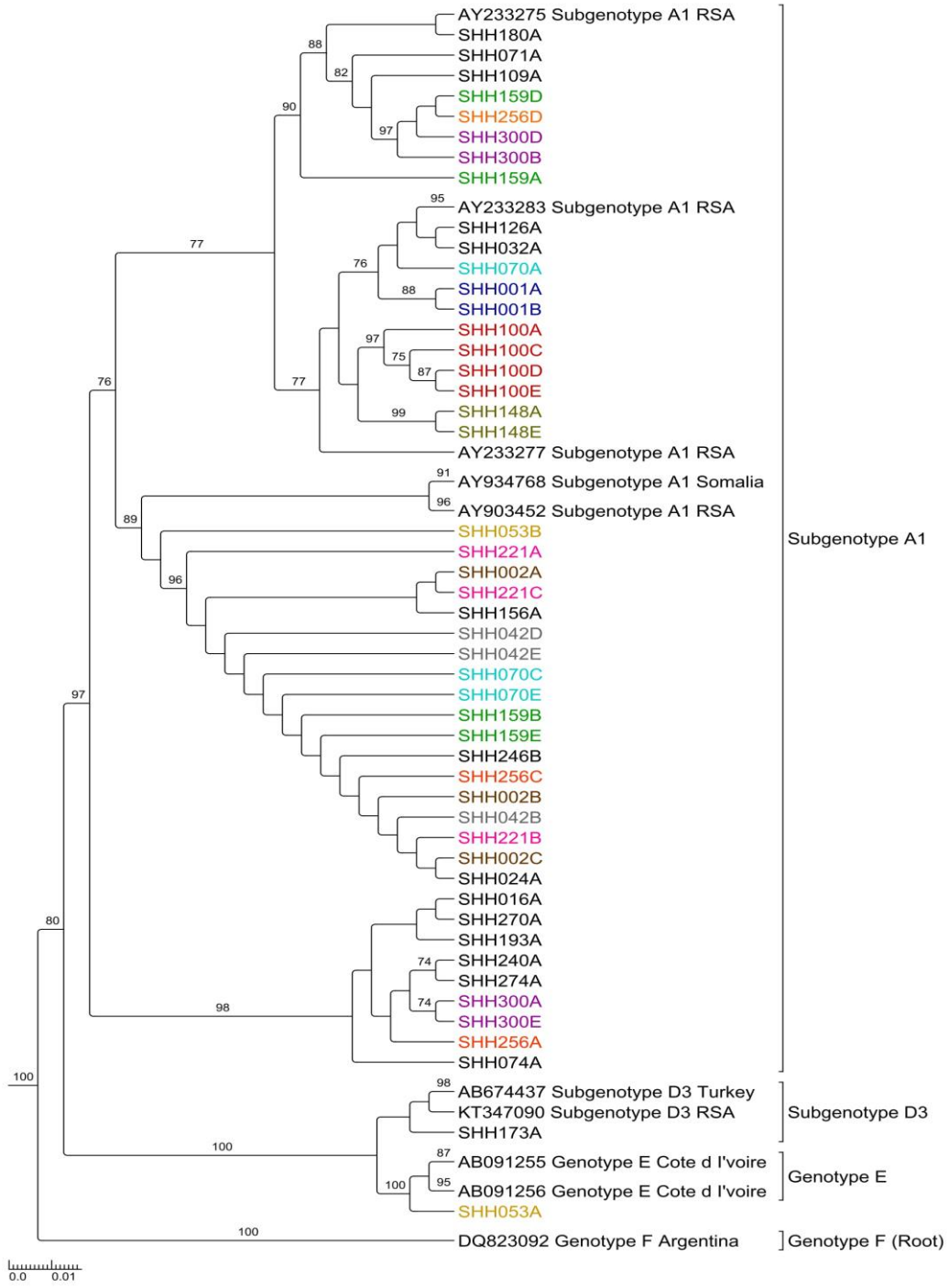

Figure 3.9 (this phylogenetic tree is the same as figure 3.8) This figure shows the changes in genotypes or clades of isolates at different time-points. SHH053A belonged to genotype E but SHH053B belonged to subgenotype A1. SHH300A and E clustered together the same finding was observed for SHH001A and B, SHH042D and E, SHH070C and E, SHH100A,C,D and E, SHH148A and E, SHH159B and E, SHH300A and E as well as SHH300B and D,

3.8 Molecular Analysis

3.8.1 Mutations in BCP/PreC Region

There are numerous mutations found in the BCP/PreC region that prevent secretion of HBeAg. HBeAg known for its immune-tolerogenic properties allows the virus to establish an infection. Ten isolates were HBeAg+ at a minimum of one time-point during the study. Two isolates SHH159 and SHH274 were HBeAg+ positive at baseline, seroconversion occurred at time-points E and C, respectively. Most mutations were detected at baseline, decreasing at each follow-up but this could be as a result of the loss to follow-up, as not every subject returned at each follow-up time point. Various loci (nt 1762, 1764, 1809-1812, 1814-1816, 1862 and 1888) of interest in the BCP/PreC region from nt 1750-1900 (from the *EcoR1* site) were investigated. Mutations at these loci lead to down-regulation or prevention of HBeAg synthesis or secretion.

The A1762T/G1764A double mutation associated with a reduction of transcription was found in HBV isolates from five strains (SHH148A+E, SHH180A, SHH221A, SHH264A and SHH300E). Four A1762T/G1764A mutations occurred at baseline and twice at 18 months. A G1764A mutation occurred once in the absence of the A1762T mutation, in isolate SHH240A (Table 3.10, Appendix B).

Subgenotype A has a unique Kozak sequence ^{1809}TCAT^{1812}, whereas in other genotypes ^{1809}GCAC^{1812} is found in this region.The ^{1809}GCAC^{1812} mutation on Kozak region occurred in seven HBV subgenotype A1 isolates from theHBeAg-group, of which five mutations were found at baseline. The genotype E isolate SHH053A had the ^{1809}GCAC^{1812}Kozak sequence. One HBeAg+ isolate (SHH159A) had the ^{1809}GCAC^{1812} mutation in this region (Table 3.9, Appendix B).^{1809}TCAC^{1812} was found at baseline and at the 18 -month follow-up in HBV

from SHH148. Mutations ^{1809}TCCT^{1812}, and ^{1809}ACAT^{1812}) were found in isolates SHH240A and SHH300A, respectively.

Three isolates (SHH001A, SHH070A and SHH221A) had HBV with^{1814}CTG^{1816}precore start codon mutations at baseline only. In isolate SHH148 an ^{1814}AAG^{1816} start codon mutation was found at baseline and the last follow-up visit, whereas in SHH300 the ^{1814}ACG^{1816} was found also at baseline and the last follow-up visit.Of interest, the ^{1814}ACG^{1816} start codon mutation was present in HBV isolateSHH274, at baseline when the individual was HBeAg+.

This PreC start codon mutation was found in one HBeAg+ isolate, SHH173A. The G1862T mutation was observed in nine HBeAg- isolates and three HBeAg+ isolates. The mutation occurred in five isolates from five baseline, thereafter was found in eight isolates from follow-up. This mutation found to be significantly higher in the HBsAg- individuals in the initial study (Makondo, Bell and Kramvis, 2012), but could not be determined for the follow-up study as the number of sequences per group were insufficient, to conduct statistical significance calculations.

A1888T occurred once during this study, at baseline isolate SHH070A belonging to the HBeAg- group. A1888G of HBV was found in seven baseline isolates, two of which were HBeAg+. Of the five follow-up A1888G mutations, one isolate belonged to HBeAg+ group. Twelve isolates with BCP/PreC mutations were wild-type. The BCP/PreC region had numerous mutations in this study which can be correlated to the high prevalence of HBeAg- infections found in the Southern African HBV subgenotype A1 strain, however the significance of these findings could not be determined as the number of isolates in the follow-up groups were insufficient for statistical analysis. Isolate SHH053A belonging to genotype E had HBV with^{1809}GCAC^{1812} Kozak sequence, as well as C1858T, G1862T and

A1888T. SHH173A belonging to subgenotype D3 had only the G1862T mutation in the BCP/PreC region in HBV isolated at this time-point.

3.8.2 Complete S Region Analysis

Amplification of the complete S-region (nt 2854 – 835 from the *EcoR*1 site) yielded 47 sequences. Twenty-three of these sequences were from baseline samples, and the remaining from follow-up time-points. Fifteen baseline sequences and 24 follow-up sequences were from the HBsAg+ group Table 3.3. These sequences were used to construct a neighbour-joining phylogenetic tree (Figure 3.8 and 3.9). Lower viral loads in the follow-up samples of the HBsAg- group can account for the fewer sequences in this group.

3.8.2.1 PreS1 Mutations

The I48V mutation was found in ten HBsAg+ isolates and one HBsAg- isolate this was associated with T90V/L/A/K in 17 HBsAg+ isolates and four HBsAg- (Table 3.10). In addition, ten V88A/L/K mutations were found in HBV from HBsAg+ isolates and HBV from two HBsAg-. Nine P94T mutations were found in HBV isolates in the HBsAg+ group and one isolate in the HBsAg- group. Other PreS1 mutations found during this study were S5T/N/L, F25L and L85V/M. In SHH053A infected with genotype E, the following PreS1 mutations were found I48N, V88L and T90A. HBV isolate from SHH173A, infected with subgenotype D3 had S5G, I48N V88L and T90A, this participant was HBsAg+ at this time-point.

Table 3.10 Complete S and Polymerase mutations at baseline and follow-up											
	Mutation	HBsAg + Group					HBsAg- Group				
		Percentage Frequency of Mutation (%)					Percentage Frequency of Mutation (%)				
		A	B	C	D	E	A	B	C	D	E
		n=15	n=8	n=7	n=5	n=6	n=8	n=0	n=0	n=0	n=0
PreS1	S5T/N/L	6.7	0	0	0	16.7	25.0				
	F25L	26.7				16.7	25.0				
	I48V/T	26.7	75.0	80.0	20.0	50.0	62.5				
	L85V/M	6.7			40.0	16.7					
	V88A/L	46.7	12.5		60.0	16.7	37.5				
	T90V/L/A/K	80.0	100	80.0	80.0	50.0	87.5				
	P94T	26.7	12.5	20.0	20.0	33.3	12.5				
PreS2	M1I/L	20.0	12.5	20.0	20.0	3.3	12.5				
	A7T	40.0	75.0	80.0	20.0	83.3	37.5				
	Q10K/R	26.7				16.7	25.0				
	A11T	26.7	12.5			16.7	37.5				
	F22L	46.7	25.0	20.0	20.0	33.3	12.5				
	L32H/R	33.3	12.5		60.0		25.0				
	R48K/T	46.7	25.0		60.0	16.7	25.0				
	A53V	20.0			80.0	16.7	25.0				
	L54P	46.7	75.0	80.0	20.0	66.7	37.5				
HBsAg	V14G/A	20.0				16.7					
	I68T	6.7			60.0	16.7	12.5				
	Y100C						12.5				
	S117N						12.5				
	T118K						12.5				
	Q129R						12.5				
	N131K	6.7					12.5				
	V168A	20.0	12.5				25.0				
	S174N						25.0				
	I195M						12.5				
	Y206C					16.7					
Polymerase	E1D	33.3					12.5				
	S105T	26.7					12.5				
	H122I/L/N	26.7					37.5				
	Q125E	53.3									
	T128N	6.7									
	L129M/V	6.7					25.0				
	L180M						12.5				
	M204I/V	6.7					12.5				

3.8.2.2 PreS2 Mutations

The M1I/L/T stop codon translation mutation in the PreS2 region, resulting in vaccine-escape, was observed in HBV from eight HBsAg+ isolates and one HBsAg- isolate (Table 3.11). This amino-acid was lost in one isolate (SHH180A) due to a deletion in this region. Sixteen isolates belonging to the HBsAg+ group had HBV isolates with the A7T mutation whilst two isolates from the HBsAg- group had this mutation. Four isolates of the HBsAg+ had a deletion of 7 aa of the PreS2 region. All, but one isolate/s with the A7T mutation were associated with the L54P mutation. Other PreS2 mutations found were Q10K/R, A11Tand A53V. Five HBsAg+ HBV isolates and one HBsAg- isolate had deletions at aa 22 of the PreS2 region. The F22L mutation was observed in nine HBV isolates belonging to the HBsAg+ group. PreS2mutation L32H occurred in eight isolates from the HBsAg+ group, however, a different mutation L32R was observed in one HBsAg- isolate. The L32H mutations in the HBsAg+ group were associated with R48K/T in all isolates. The R48K/T mutation 'alone' was found in an additional five HBsAg+ and two HBsAg- isolates. The genotype E isolate, SHH053A, had the following PreS2 mutations, A7T,L32V and T54P while the genotype D isolate , SHH173A, had A7T, L32V and A11T in the PreS2 region of the isolated HBV.

3.8.2.3 HBsAg Mutations

The S-region from nt 155 – 835 (using the *EcoR1* site) was the most well-conserved region of the three S -regions, with the least number of mutations found in this portion of the complete S (Table 3.11). The following mutations, V14A/G, I68T and Y206C were observed in HBV isolates in the HBsAg+ group. Isolates belonging to the HBsAg- group had mutations Y100C, S117N, T118K, Q129R, K131N, S174N and I195M. The A168V mutation was observed in six HBsAg+ and two HBsAg- HBV isolates. The significance of mutational frequencies between the HBsAg+ and HBsAg- groups could not be determined.

3.8.2.4 PreS1/PreS2 Deletion Mutants

Deletions of up to 33 can be found in the PreS1 and PreS2 regions, in this study six HBV isolates had PreS1 and/or PreS2 nucleotide deletions. SHH053 had a 3 nucleotide deletion at baseline, from nt 2853 – 2855 (using the *EcoR1* site) Table.). This deletion is found in Genotype E sequences, which was the genotype this isolate belonged to at this time-point which was confirmed by phylogenetic analysis. At the first follow-up time-point (B) isolate SHH053 had a 33 nt in the PreS2 region from nt 13 – 45 (From the *EcoR1* site) however at this time-point the isolate belonged to HBV subgenotype A1 (Figure 3.). SHH053 was HBsAg- at both these time-points but no further complete S sequences were available for this isolate. A 119 nucleotide deletion from nt 3135 – 30 (from the *EcoR1* site) was found in HBV from SHH180 at baseline. This lead to a 21 aa deletion in the PreS1 region not causing any deletions at important loci, however in the PreS2 region deletions of approximately 33nt affecting aa positions 1, 7, 10 and 11. At baseline,an HBsAg- isolate (SHH240) had a 32 nucleotide deletion in the PreS2 region from nt 10 – 42 (from the *EcoR1* site). Another PreS2 deletion of 34 nucleotides from nt 1 – 34 (from the *EcoR1* site), was observed in isolate SHH274 at baseline, at which time the participant was HBsAg+. SHH300, an isolate from the HBsAg+ group, had a 53 nucleotide deletion in the PreS2 region from nt 1 – 53 (from the *EcoR1* site) at baseline and a 52 nucleotide from nt 1 - 52 (from the *EcoR1* site) at the 18-month follow-up time-point. SHH300 was HBsAg+ at all time-points, despite these deletions.

Table 3.11 Analysis of PreS deletion mutants

Isolate number	Nucleotide position	Number of nucleotides deleted	Amino-acid position	Number of Amino-acids deleted	Functional Domain affected by deletions
SHH053A	2853 - 2855	3	**PreS1** G2	1	Myristolated N-Terminal Glycine
SHH053B	13 - 45	33	**PreS2** 12L - 23P	11	B and T cell epitopes
SHH180A	3135 - 30	119	**PreS1** 97T - 119A	21	B and T cell epitopes
			PreS2 1M - 18R	18	
SHH240A	10 - 42	33	**PreS2** 11A - 22F	11	B and T cell epitopes
SHH274A	1 - 34	34	**PreS2** 4N - 19G	12	B and T cell epitopes, pHSA binding site
SHH300A	1 - 53	53	**PreS2** 4N - 22F	18	B and T cell epitopes, pHSA binding site
SHH300E	1 - 52	52	**PreS2** 5S - 23P	18	B and T cell epitopes, pHSA binding site

Nucleotides were numbered using the *EcoR1* site

3.9 Polymerase Mutations

The overlapping ORFs of the HBV genome allow for analysis of a portion of the Pol gene that overlaps with the complete S-region. The Pol region spanning from nt 2307 – 1623 (from the *EcoR1* site) overlaps with the complete S-region which spans from nt 2848 – 835 (from the *EcoR1* site) (Lin and Kao, 2010), it is within this region that the Spacer and the first 236 aa's of reverse transcriptase overlap with the complete S-region . A two nucleotide frame-shift change in the alignment of the complete S-region allows for analysis of the Pol region. Mutations within the spacer region, whose function is not clearly defined, are well-tolerated and donot affect polymerase activity. Ten aa lociof interest in the reverse transcriptase region were investigated for mutations.

The rtE1D start codon mutation together with S105T and H122N occurred in five sequences (SHH173A, SHH240A, SHH274A and SHH300A and SHH300E). SHH173A was the only isolate of the HBsAg- group found to have a mutation in the Pol region. Isolates SHH300A+E showed almost identical sequences at baseline and the final time-point, this was also found when analysing the BCP and complete S regions. In isolate SHH180A the rtE1D was found in conjunction with Q125E and L129V (Table 3.10). The Q125E mutation was found in 5 isolates (SHH071A, SHH109A, SHH159A, SHH159D, SHH180A, SHH256D, SHH300B and SHH300D), all of these were HBsAg+ isolates however this was not a significant finding ($p = 0.29$) in comparison to the HBsAg- group. Only one of these participants experienced a VBT.

The rtL180M drug resistance mutation was found only once during the study, occurring in SHH074, this was found together with H122N, L129M, and another drug resistance mutation rtM204V. Notably SHH074 was HBsAg- throughout the study and experienced full suppression of HBV by the end of the study. The rtM204I mutation was found in isolate SHH148, at the 18 month time-point. Isolate SHH053A belonging to Genotype E had only one mutation in the Pol region, H122I. The isolate belonging to subgenotype D3, SHH173 had two Pol mutations namely, H122L and L129N. However these mutations are poorly described and their effects remain unknown.

CHAPTER 4: DISCUSSION

This was an 18 month long follow-up study in HIV-infected Lam-treatment naïve participants at a rural hospital in the Mpumalanga province of South Africa (RSA), participants qualified for ART at initiation. Blood samples were collected at baseline (treatment-naïve) , for this serological profiling of HBsAg, HBeAg, HBcAg, anti-HBs, anti-HBe, anti-HBc, ALT levels, weight, BMI, CD4+ counts and HIV-viral-loads were tested and recorded. Serological results of the HBs, HBe and anti-HBc antigens and antibodies were available for all follow-up time points. ALT scores and HIV indicators of immune reconstitution were not available at follow-up. Viral-load quantification and PCR amplification were carried out on blood samples from follow-up and used to determine viral-suppression, and mutations in the BCP/PreC, complete S and Pol regions, which were also used in genotypic profiling. These regions were analysed to determine the molecular evolution of HBV isolates from HIV-infected Lam-treated study participants over an 18 month period. HBV is largely transmitted during childhood in sub-Saharan Africa, therefore, HBV and HIV are not contracted simultaneously. Studies of this nature are important, not only because HBV is endemic to sub-Saharan Africa, but also because of the high prevalence of HBsAg-negative and HBeAg-negative HBV infections in the region.

4.1 Follow-up visit participation

A minimum of 12% loss to follow-up was found at all consecutive follow-up time-points affecting the amount of molecular data generated. Auodjane *et al.*, 2014 also document low retention rates of participants in their 1-year study in sub-Saharan Africa, their study had a 72% return to follow-up at 6 months and a 41% return at 12 months.

4.2 Demographic, Clinical, Serological and Virological Characteristics

In the baseline study by Bell et al. 2012 it was found that the HBsAg+ group had a higher prevalence of liver fibrosis based on APRI scores conducted, these could not be determined for follow-up, samples as no liver biochemistry results were recorded at follow-up. A significant clinical characteristic was a higher baseline weight in the HBsAg+ positive group compared to the HBsAg- group (Table 3.2). When comparing baseline ALT levels of the FS and NS groups it was found that ALT levels were significantly higher in the NS group (Table 3.4 and Figure 3.4).

4.3 HBsAg-positive versus HBsAg-negative

All participants who were HBsAg+ were expected to lose the HBsAg within 18 months of Lam treatment, as HBsAg sero-clearance is a goal of treatment (WHO, 2015). All HBsAg+ group participants had detectable HBV DNA at the end of the study, therefore HBsAg positivity at any time is an indication that the HBV-infection has not cleared. Of these HBsAg+ participants 28.2% remained HBsAg+ at the last follow-up (Table 3.3). Although seven participants lost the HBsAg during the study, their viral-loads remained comparable to when they were HBsAg+. Similar findings were observed in a study carried out on HBV subgenotype A1 in Malawi, which found that participants that were HBsAg at any point during the study were unable to clear HBV DNA, moreover HBV viral-loads were similar during HBsAg positivity and HBsAg negativity in these participants (Aoudjane *et al.*, 2014).

A high frequency, (48.72%) of HBsAg negativity was found in this study cohort of HBV-HIV co-infected participants which has been previously reported in this region (Mphahlele *et al.*, 2006). When an immune-compromised individual has an active HBV-infection in the absence of HBsAg, this could be due to the formation of immune-complexes between anti-HBs and HBsAg which explains the high

prevalence of HBsAg- in HIV-infected Southern Africans (Mphahlele *et al.*, 2006). If more is understood regarding the natural course of HBsAg-negative HBV infection in HIV+ participants, this will lead to improved care and management of HBV-HIV co-infected participants. The absence of HBsAg is commonly used as an indicator of no active HBV infection (Terrault *et al.*, 2015; WHO, 2015), yet this study has shown that high HBV-DNA levels persist in HBsAg- participants. Thus, HBsAg sero-clearance may be a poor indicator of resolution of the HBV infection, in a large percentage of individuals, who are otherwise considered uninfected or cured. Undetected HBV infection, may progress to HCC, cirrhosis or ESLD. HBV DNA has been found in HBsAg- individuals suffering from HCC (Pollicino *et al.*, 2004). Following the development of HCC patients may be exposed to immune-suppressive chemotherapeutic agents that could further exacerbate the HBV, as well as HIV infection.

4.4 Full Suppression and No Suppression Group Comparison

Baseline HBsAg negativity was a predictor of full suppression, only participants in the HBsAg- group experienced a full suppression (Table 3.4 and Figure 3.6). The viral suppression observed in this group occurred after 12 months of initiating Lam, which is in accordance with the findings of a similar study by Hafkin et al. 2014. Therefore it may be beneficial to use 12 months as a better indication response to treatment. Incomplete viral suppression was associated with higher baseline HBV viral-loads (Hafkin, 2015) however in the present study baseline HBV viral-loads between the FS and NS were similar. An important outcome in anti-HBV therapy in CHB infected individuals is to reduce HBV viral-loads, this leads to a decreased risk of CHB disease sequelae and liver damage. A median of 56.06% (IQR: [-100%] – [+23.46%]) decrease in HBV viral-loads was experienced by 34 participants in the present study.

4.5 Virologic Breakthrough Positive and Negative Groups

Of the 20 participants in the NS group, ten experienced a VBT. Although other studies have found that the baseline HBV-DNA level is the most important factor associated with VBT in Lam treated participants (Chae and Hann, 2007), the HBV viral-loads between the NS VBT+ and NS VBT- groups were similar at baseline. This study found that 70% of NS VBT+ participants were HBsAg- throughout, all these participants fully suppressed HBV before experiencing a VBT. This study was limited to an 18-month period post-initiation of ART, the HBsAg- group that maintained full-suppression of HBV by the end of the study may experience a VBT at a later stage, since this study found that full suppression of HBV may be followed by a VBT in HBsAg- participants. Therefore VBT is possible in HBsAg-negative participants that have cleared infection which was shown in the present study, therefore, continuous monitoring of HBsAg- HBV-infection in HIV-positive participants is required at regular intervals.

4.6 Genotyping

4.6.1 Genotyping using BCP/PreC sequences

Bartholomeusz & Schaefer 2004 found nucleotides or nucleotide polymorphisms specific to certain genotypes using the BCP/PreC region, using positions 1762, 1764, 1809-1812, 1814-1816, 1858, 1862, 1888 and 1896. Using these loci SHH053A was found to have a non-A HBV infection, and was later confirmed to have a Genotype E infection by complete S-region phylogenetic analysis, this genotype prevails in Western and Central Africa (Kramvis, Kew and François, 2005). However further investigation of this genotype is required to improving understanding of it. BCP/PreC loci analysis indicated SHH173A had a subgenotype A1 HBV infection, however, this contradicted the findings of the complete S region that indicated that this was a subgenotype D3 isolate. The remaining 90.91% of sequences in the study belonged to subgenotype A1, this is in accordance with previous findings from the region which demonstrated that

subgenotype A1 is the predominant strain found in this region in both monoinfected (Kimbi et al, 2004) and HBV/HIV co-infected individuals (Makondo, Bell and Kramvis, 2012).

Although genotyping by BCP/PreC sequencing is a simple and inexpensive method of genotyping this method cannot distinguish between genotypes D and E, since these regions share great homogeneity in the X and and PreC/Core ORFs However, this region can be used for differentiating genotype A from other genotypes and subgenotype A1 from A2. The advantage of using this region is that the BCP/PreC region is a shorter region that easily amplifies during PCR yielding more amplicon for genotyping in comparison to the complete S or full genome because these regions are quite large 1200 – 3200 nucleotides long.

4.6.2 Complete S Phylogenetic Analysis

Fewer amplicon were generated by PCR amplification of the complete S-region (47 sequences), compared to the BCP/PreC region which yielded 77 sequences. These 47 complete S region sequences were used to construct a neighbour-joining phylogenetic tree including genotypes and subgenotypes A-F, which was used to determine the genotype or subgenotype of isolates in this study.

Phylogenetic analysis revealed that two isolates (SHH053A and SHH173A) in this study did not belong to subgenotype A1 at baseline rather, these isolates belonged to genotype E and subgenotype D3, respectively. SHH053A had the characteristic genotype E 3 nucleotide deletion (Norder, Couroucé and Magnius, 1994) in the PreS1 region from nt 2853 – 2855 (Table 3.11). However the 33 nt deletion characteristic of genotype D was not found in isolate SHH173A (Kramvis, 2014) and a bootstrap value <65% was found for this isolate in relation to the subgenotype D3 reference sequences used in the phylogenetic tree (Figure 3.8). This could be explained by the presence of a mixed infection or a

recombinant genotype A/D strain however full length amplification or cloning and sequencing is required to determine this . Previous studies from sub-Saharan Africa have documented the existence of these two genotypes in this region, however, subgenotype A1 is the predominant strain found in this region (Kimbi *et al.,* 2004), whereas genotype D is found in northern Africa and genotype E is found predominantly in western Africa (Kramvis and Kew, 2007; Kimbi *et al,.* 2004). Other than isolates SHH053A and SHH173A all HBV other isolates belonged to subgenotype A1.

Previous studies documented that infection with HBV genotype-A is associated with an increased risk of developing HCC in black Africans than those infected with non-A genotypes (Kew *et al.*, 2005). There is also lower frequency of HBeAg-positivity in individuals infected with subgenotype and this is highly significant in carriers younger than 30 years of age (Kramvis and Kew 2007; Tanaka *et al.*, 2004). In comparison to genotype D, sera from both the HBeAg and anti-HBeAg positive phases have lower HBV viral-loads in subgenotype A1 infections (Tanaka *et al.,* 2004). Both genotype D and E found in Africa have different mutations resulting in the anti-HBe seroconversion in comparison to subgenotype A1 (Kramvis and Kew 2007).

Phylogenetic trees, such as the one constructed during this study, use specific algorithms to determine the evolutionary relatedness of sequences to each other and reference sequences (McCormack and Clewley, 2002). The construction and analysis of phylogenetic trees are more labour intensive and require specialised skills in comparison to the method employed using BCP/PreC analysis. However a major advantage of using this type of genotyping method analyses a larger region of the HBV genome than the limited amount loci analysed in the BCP/PreC region, this lends more statistical support to the complete S-region phylogenetic analysis method. Therefore the complete S region is more

informative but if a mixed infection or recombinant infection is suspected, cloning and sequencing of the complete HBV genome is required.

4.7 Molecular Characteristics

Sequence data was used to molecularly characterise the BCP/PreC and complete S genes of the HBV genome in 39 HBV-HIV co-infected adults at various times before and after initiation of a Lam-containing ARV regimen. The BCP/PreC and complete S regions were characterised at each time point, followed by the construction of a phylogenetic tree using complete S sequences to determine the HBV genotypes and subgenotypes present in this study. Each region was subsequently analysed for various mutations that affect disease progression, response to treatment, in addition detection- or immune-escape variants were determined.

4.7.1 BCP/PreC Mutations

There were a total of 78 sequences yielded from PCR amplification of the BCP/PreC region. Of these 24 sequences were from baseline, 15 from time-point "B", 14 from "C", 13 from "D", lastly 12 from time-point E. Mutations in this region affect the production of HBcAg and HBeAg. The A1762T/G1764A double mutation appeared in six HBeAg- isolates and one HBeAg+ (Table 3.10). This down regulates the production of PreC/C mRNA transcripts, therefore decreasing the amount of HBeAg produced, however, viral replication is increased as a result of the increased pgRNA packaging (Takahashi et al. 1995; Buckwold et al. 1996). An increased risk associated with this mutation is it contribution to the development of HCC by an unknown mechanism (Baptista, Kramvis and Kew, 1999). This mutation has also been implicated in an increased progression to liver disease (Buckwold *et al.*, 1996; Baptista, Kramvis and Kew, 1999), this can be attributed to the increase in viral replication which leads to an increase in viral-load. Four isolates had the double mutation in the BCP/PreC region this was

found together with the T1753C was found in three of these isolates isolates, this is sometimes referred to as the triple mutation. T1753C has been described as an HCC marker and is often found in HBeAg- individuals or those with active liver disease (Pujol *et al.*, 2009).

Subgenotype A1 has a distinct Kozak sequence namely, ^{1809}TCAT^{1812}(Baptista, Kramvis and Kew, 1999; Kramvis *et al.*, 2008)' which is the region located immediately upstream of the PreC initiation codon (Tong *et al.*, 1992). Most 76.62% isolates in this study had the ^{1809}TCAT^{1812} Kozak sequence (Table 3.10). Mutations in the Kozak region, nt 1809 -1812 (numbering from the *EcoR1* site) negatively affect HBeAg production at a translational level (Kozak, 1986). HBeAg may be abolished when three mutations are found within this sequence (Ahn *et al.*, 2003), this is postulated to result in earlier seroconversion to HBeAg-. A leaky scanning mechanism arises as a result of a mutation in the Kozak region, causing a reduction in HBeAg production (Ahn et al 2003). The majority, of HBV strains circulating in sub-Saharan Africa contain double or triple mutations within the Kozak region, this contributes to the high prevalence of HBeAg negativity in the strains found in this region of Africa (Kramvis and Kew, 2007). Other mutations such as TCCT, TCTT, TTCT, and TCAC have also been implicated in HBeAg translational impairment. When mutations in the Kozak region are coupled with A1762T/G1764A double mutation HBeAg production is abolished (Ahn *et al.*, 2003).

Mutations at PreC start codon nt 1814-1816 (from the *EcoR1* site) were found in seven isolates in this study (Table 3.10). Mutations in this region prevent initiation of HBeAg translation (Laras *et al.*, 1998), therefore reducing the production of HBeAg. Isolate SHH274A had an ^{1814}ACG^{1816} mutation despite being HBeAg+ at the time (Table 3.10), this could be attributed to a mixed infection with HBV, whereby PreC mutant strains and wild-type strains are present in the same host, with the wild-type being the non-dominant strain thus was not amplified during

PCR but provided sufficient production of HBeAg which was detected upon serology testing for this antigen.

Another BCP/PreC mutation affecting expression of HbeAg expression at the post-translational level is the G1862T transversion mutation, which was found in 22% BCP/PreC sequences. Previous studies (Kramvis *et al.*,1997; Kramvis *et al.*, 1998) found a higher frequency of this mutation in subgenotype A1, from sub-Saharan Africa. It is the HBeAg precursor that is affected by this mutation, whereby this missense mutation inhibits signal peptide cleavage (Chen *et al.*, 2008). This results in the inhibition of HBeAg secretion thus causing an accumulation of HBeAg and its precursors in the endoplasmic reticulum and Golgi (Chen *et al.*, 2008). HBeAg may be reduced to undetectable levels when G1862T is found together with the A1762T/G1764A double mutation, this was found in isolate SHH221A.

The G1888A mutation, found in nine HBeAg- isolates and two HBeAg+ isolates (Table 3.10), which prematurely introduces a start codon upstream from the core start codon and thus impairs translation of HBcAg (Kimbi, Kew and Kramvis, 2012). This mutation is unique to subgenotype A1. The G1896A mutation, like loci 1862 is found in the encapsidation signal (ε) of the PreC/C region. This mutation introduces a premature stop codon truncating the HBeAg, results in the abolishment of HBeAg production at the translational level (Carman *et al.*, 1989). This mutation was found together with C1858T in two isolates. This mutation is precluded in the presence of 1858C because this would interfere with the secondary structure of the encapsidation signal (ε) and prevent viral replication (Li *et al.*, 1993; Lok, Akarca and Greene, 1994). In genotype D the development of the G1896A stop codon and the A1762T/G1764A double mutation in the BCP/PreC region, result in the anti-HBeAg sero-conversion (Kramvis and Kew, 2007).

4.7.2 Complete S mutations

The complete S-region encodes the three HBV viral surface proteins, LHBs, MHBs and SHBs using the PreS1, PreS2 and S ORFs. These surface proteins are integral to HBV viral attachment to hepatocytes and are pivotal to virus assembly(Prange and Streeck, 1995; Glebe and Urban, 2007). The HBsAg contains the crucial 'a' determinant, this is the region of HBsAg targeted by antibodies as it contains major B and T-cell epitopes. Mutations in this region may result in vaccine-escape, detection-escape, immune-escape or clinical manifestations, such as reactivation, VBT or HCC and liver disease progression (Fan *et al.*, 2001; Sugauchi *et al.*, 2003). In the present study 47 complete S sequences were generated, of these 25 sequences were from baseline isolates.

4.7.2.1 PreS1 Mutations

Numerous PreS1 mutations, S5T/N/L, F25L, I48V/T, L85V/M, V88A/L, T90V/L/A/K and P94T within the neutralising epitope and those involved in hepatocyte attachment were observed during this study.

4.7.2.2 PreS2 Mutations

The M1I/L PreS2 stop codon mutation found during this study (Table 3.10) prevents the initiation of translation of MHBs, thereby preventing its production. A mutation unique to subgenotype A1, L32H was observed intermittently during the study. The Q10R mutation located in the major antigenic region of PreS2, was observed at baseline and the last follow-up. This mutation affects B-cell, T-cell and cytotoxic T lymphocyte epitopes reducing the ability of antibody binding thereby avoiding the neutralising effect of antibodies raised against this antigen (Paulij *et al.*, 1999).

4.7.2.3 HBsAg Mutations

The T118K mutation located in the major hydrophilic loop of HBsAg, was found in 12.5% of HBsAg- individuals which has been previously reported in Northern Europe. (Salpini *et al.*, 2015; Caligiuri *et al.*, 2016) The vaccine induced immune-escape mutation Q129R, located in the crucial 'a' determinant region(Lazarevic, 2014; Caligiuri *et al.*, 2016), was found in 12.5% of HBsAg- isolates, resulting in diagnostic failure by currently available HBsAg detection assays(Raimondo *et al.*, 2008). The HBsAg region had fewer mutations than the PreS1 and PreS2 regions, and was found to be a highly conserved region. Two reactivation markers, V168A and S174N were found in two HBsAg- isolates however both were from the FS group therefore did not experience a re-activation of HBV infection. However HIV immune suppression is the associated cause of reactivation in the presence of these mutations (Gerlich *et al.*, 2010).

4.7.2.4 PreS1/PreS2 Deletion Mutants

Seven isolates in this study were found to have PreS1/PreS2 deletions (Table 3.11), including the characteristic genotype E 3 nt deletion found in the PreS1 region of SHH053A. Most PreS2 deletions found in this study occurred in the major B and T cell epitopes and/or the polymerized serum albumin binding sites. PreS deletions in the S-region are possibly involved in the development of occult infection as these deletions can affect HBsAg expression synthesis and secretion (Chen *et al.*, 2012; Huang *et al.*, 2014). PreS deletions are more frequently found in HBV-HIV co-infection (Audsley *et al.*, 2010; Makondo, Bell and Kramvis, 2012) and HCC patients infected with HBV subgenotypeA1 (Kew *et al.*, 2005). The prevention of HBsAg secretion results in the accumulation of this protein in ER of hepatocytes. This leads to oxidative stress resulting in DNA damage, which contributes to the development of HCC (Chen *et al.*, 2006; Caligiuri *et al.*, 2016). Transcriptional activators of HBV promoters have also been implicated in the development of HCC, as a result of PreS deletion mutants(Hildt *et al.*, 1996).

4.7.3 Polymerase Mutations

The HBV polymerase enzyme is integral to the replication cycle moreover this region is the target of many drugs. The lack of proof-reading of the polymerase results in an error rate of 1 x 10^{-5} - 1 x 10^{-7} nucleotide change per site per year (Girones and Miller, 1989). Despite the introduction of TDF in South Africa, Lam remains widely used in the treatment of HBV- and/or HIV-infection, as it is more affordable than TDF. All strains in this study were receiving the nucleoside analogue Lam, this drug is widely used despite the development of HBV resistance mutations. The YMDD catalytic site is vital to reverse transcription function, this is the specific site targeted by Lam. Anti-viral agents may cause selection pressure leading to drug mutations, caused by dominant quasi-species strains (Caligiuri *et al.*, 2016). Moreover, mutations in the Pol region may affect the overlapping S-region, causing vaccine-, immune- or detection-escape mutations (Sheldon *et al.*, 2005).

Mutations rtS105T, rtH122I/L/N, rtQ125E and rtL129V/N are poorly described and their effects are yet to be elucidated. The rtE1D mutation was found in 13.33% of HBV isolates from this study, however the resultant amino-acid change does not lead to any functional changes of the polymerase, given the structural and chemical similarities between glutamic acid (E) and aspartic acid (D) (Makondo, Bell and Kramvis, 2012). The Q125E mutation was the only mutation found in HBV isolates from the NS VBT+ group, occurring once at baseline.

Two drug resistance mutations, rtL180M and rtM204V/I, were found in this study(Table 3.10). These mutations may cause vaccine escape mutations in the overlapping S-region and prevent secretion of HBsAg (Sheldon *et al.*, 2005). The rtM204I/V mutation is found within the YMDD (tyrosine-methionine-aspartate-aspartate) region, located in the C-domain of HBV polymerase. Spontaneous mutations in the YMDD region, have been associated with liver cirrhosis and HCC (Yang *et al.*, 2013; Caligiuri *et al.*, 2016). In addition a lower rate of VBT has been reported in HBV isolates from treatment-naïve individuals without

YMDD mutations in comparison to isolates with naturally occurring YMDD mutations (Yang *et al.*, 2013). The rtM204V mutation reduces replication *in vitro*, however compensation mutations such as rtL180M develop, to restore replication competence (Delaney *et al.*, 2003).

In this study the rtM204I mutation, was the only Pol mutation found in isolate SHH148E whilst the rtM204V + rtL180M were found together in HBV isolated from SHH074A. Previous studies from sub-Saharan Africa have found the rtM204I mutation in the HBV genome of therapy-naïve HBV-HIV co-infected individuals (Selabe *et al.*, 2009) whereas in the present study this mutation was found after 18 months of Lam treatment in the isolate SHH074 from the NS VBT-group.

4.8 Limitations to this Study

Inconsistent return for follow-up was a major limitation to the study. Due to financial constraints and a shortage of personnel, it was not possible to obtain liver biochemistry data and CD4+ counts for the time-points post-initiation of ART. This data would have assisted us in understanding at what time-point if at all, there was the development of liver fibrosis, cirrhosis or HCC and if the CD4+ count could be related to disease progression. Changes in CD4+ count could affect the suppression and virologic breakthrough. Availability of information such as HIV viral-loads and CD4+ counts would have assisted us in understanding why some participants were able to successfully clear the virus. One of the other limitations of the study is that participants did not return to follow-up, this could possibly reflect a poor adherence as they may not have had access to medication. The low statistical power of the follow-up molecular analysis was also influenced by low viral-loads, this also decreases the amplicon generated when amplifying larger regions such as the complete S region or complete genome.

CHAPTER 5 CONCLUSION

There are a limited number of studies from this high endemicity area on the changes that occur during anti-viral treatment and the effect it has on HBV over time. Therefore the present study was undertaken to investigate the effect of Lam on HBV in HIV-infected individuals from a sub-Saharan African perspective. The BCP/PreC and complete S-regions were amplified and sequenced to characterise molecular evolution of HBV during ART in comparison to pretherapy.

Sequence heterogeneity is expected since HBV Pol lacks proof-reading activity, various studies have described the effect of mutations and their relevance in HBV infection(Bell, 2013; Kramvis, 2014; Matthews *et al.*, 2014; Yousif *et al.*, 2014; Colson *et al.*, 2015). The present study objectives to identify the genotypes or subgenotypes were achieved as well as monitoring the effect of Lam in terms of viral suppression.BCP/PreC, complete S and Pol mutations were identified and correlated to their clinical relevance.

The current study found that serum HBV DNA persisted regardless of HBsAg clearance (HBsAg+ group). Baseline HBV DNA levels in the HBsAg-negative group were comparable to those in the HBsAg-positive group, although higher levels of VBT's occurred in the HBsAg-negative group. This study re-enforces the need for NAT to detect HBV infection, which has already been proposed by others (Raimondo *et al.*, 2008; Bell *et al.*, 2012; Yousif *et al.*, 2014; González *et al.*, 2015), especially in high-risk populations, such as HIV-positive individuals prior to and post initiation of Lam-containing ART. There are severe clinical consequences to undetected HBV-infection, such as an increased risk of HCC, more common in subgenotype A1, which was the predominant strain isolated in this study, (Kew *et al.*, 2005).

The need for treating HBV-infection in this HIV burdened population using Tenofovir is preferred considering that mutants identified in this study are also

sensitive to this drug(Mphahlele *et al.*, 2006; Matthews *et al.*, 2010; Bell *et al.*, 2012; Makondo, Bell and Kramvis, 2012). Unfortunately, both NAT testing and TDF treatment are costly measures that may not be conducive to developing or third world countries. Furthermore, molecular analysis may be required to detect resistance mutations or response to treatment, which adds to the cost of HBV management. Sub-Saharan Africa is an area of HBV and HIV endemicity, with a significantly higher prevalence of HBV in HIV-infected individuals (Thio *et al.*, 2002). Subgenotype A1 is the most prevalent strain found in sub-Saharan Africa (Bowyer and Sim, 2000; Kimbi, Kramvis and Kew, 2004). Disease progression, response to treatment and the development of mutations differ among the various genotypes or subgenotypes, thus studies specific to genotypes or subgenotypes are necessary.

This study showed that ALT levels are an important predicator of HBV suppression, because lower ALT levels were found in the FS group. ALT levels are also indicative of the severity of disease, which may correlate to the abilty of the drug to suppress the virus, patients with persistently elevated ALT levels are an indication the treatment might be failing, allowing for lengthy periods of HBV replication leading to high viral-loads, which is known to increase progression of liver disease (Pollicino *et al.*, 2004; Coffin *et al.*, 2014; Kwak and Kim, 2014).

The most significant finding of the study was that participants that achieved control of HBV infection by fully suppressing the virus following treatment was associated with HBsAg loss. However VBT was still possible. This study can be repeated in a larger study population thereby increasing the amount of sequence data generated. Longer studies within such a population will also allow more indepth insight into drug resistence emergence patterns and the long-term effects of Lam and HIV on HBV infection.

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APPENDIX A

Ethics Documents

University
of the Witwatersrand,
Johannesburg

Human Research Ethics Committee (Medical)
(formerly Committee for Research on Human Subjects (Medical)

Secretariat: Research Office, Room SH10005, 10th floor, Senate House • Telephone: +27 11 717-1234 • Fax: +27 11 339-5708
Private Bag 3, Wits 2050, South Africa

6 July 2009

Professor Anna Kramvis
Hepatitis Virus Diversity Research Programme
Department of Internal Medicine
Medical School
University

Dear Dr Kramvis

RE: Protocol M080450: 'The Molecular and Functional Characteristics of Hepatitis B Virus (HBV) Genotypes isolates from HIV Infected South Africans'

This letter serves to confirm that the Chairman of the Human research Ethics Committee (Medical) has reviewed and approved your request to include a negative control cohort as detailed in your letter dated 1 July 2009.

Thank you for keeping us informed and updated.

Yours sincerely,

Anisa Keshav (Ms)
Secretary
Human Research Ethics Committee (Medical)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Kramvis

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080450**
PROJECT	The molecular and functional characteristics of Hepatitis B virus (HBV) genotypes isolated from HIV infected South Africans
INVESTIGATORS	Prof A Kramvis
DEPARTMENT	Department of Medicine
DATE CONSIDERED	08.04.25
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 08.05.20 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor :

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

health
Department:
Health
MPUMALANGA PROVINCE

No. 7 Government Boulevard
Riverside Park
Extention 2
NELSPRUIT
1200

Private Bag X 11285
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1200
Tel.: +27 13 766 3429
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Litiko LeteMphilo UmNyango WezaMaphilo Departement van Gesondheid

Enquiries: Molefe Machaba (013) 766 3009/3235

02 February 2009

Prof Anna Kramvis
Wits
No 7, York Road
Parktown
JHB
2193

Dear Prof Anna Kramvis

APPLICATION FOR RESEARCH & ETHICS APPROVAL: MOLECULAR AND FUNCTIONAL CHARACTERISATION OF HEPATITIS B VIRUS (HBV) GENOTYPES ISOLATED FROM HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTED SOUTH AFRICANS.

The Provincial Research and Ethics Committee has approved your research proposal in the latest format that you sent. No Issues of ethical consideration were identified.

Kindly ensure that you provide us with the report once your research has been completed.

Kind regards

Molefe Machaba
Research and Epidemiology

17-02-2009
Date

Mpumalanga PHREC
Chairperson: Mosa Moshabela

17-02-2009
Date

MPUMALANGA PROVINCE
DEPARTMENT OF HEALTH
PLANNING & INFORMATION
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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ms Euphodia Makondo

CLEARANCE CERTIFICATE	**M090414**
PROJECT	Genotyping and Molecular Characterisation of Hepatitis B Virus (HBV) from Human Immuno-deficiency Virus (HIV) Infected Individuals in Southern Africa
INVESTIGATORS	Ms Euphodia Makondo.
DEPARTMENT	Department of Internal Medicine
DATE CONSIDERED	09.04.29
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 09.05.29 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof A Kramvis

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

.....

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Trevor Bell

CLEARANCE CERTIFICATE	**Protocol M090107**
PROJECT	Internal medicine Molecular Evolution of Hepatitis B Virus in Antiretrovial-Treated Human Immunodeficiency Virus Infected Southern Africans
INVESTIGATORS	Mr Trevor Bell.
DEPARTMENT	Internal Medicine
DATE CONSIDERED	09.01.30
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 09-02-16 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr A kramvis

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

.....

APPENDIX B

Table 3.10 Molecular Characteristics of the BCP/PreC Region of HBV isolated from HBeAg+ and HBeAg-							
Group	**Mutation**	**Participant Number**	**Time-point**				
			A	**B**	**C**	**D**	**E**
HBeAg Negative	**A1762T/G1764A**	SHH148	+	-	-	-	+
		SHH221	+	NS	-	-	-
		SHH264	+	NS	NS	NS	NS
		SHH274	+	-	-	-	-
		SHH300	-	-	NS	-	+
	^{1809}GCAC^{1812}	SHH001	-	-	+	NS	NS
		SHH016	+	-	NS	-	-
		SHH032	+	NS	NS	NS	NS
		SHH042	+	+	-	+	-
		SHH053	+	-	-	-	-
		SHH070	-	-	-	+	-
		SHH221	+	-	-	-	-
		SHH274	+	-	-	-	-
	^{1809}TCAC^{1812}	SHH148	+	-	-	-	+
	^{1809}TCCT^{1812}	SHH240	+	-	-	-	-
	^{1809}ACAT^{1812}	SHH300	+	-	NS	-	-
	^{1814}CTG^{1816}	SHH001	+	-	-	NS	NS
		SHH070	+	-	-	-	-
		SHH221	+	NS	-	-	-
	^{1814}AAG^{1816}	SHH148	+	-	-	-	+
	^{1814}ACG^{1816}	SHH300	+	-	NS	-	+
	C1858T	SHH016	+	-	NS	-	-
		SHH042	+	-	-	-	-
		SHH053	+	+	-	-	-
		SHH070	-	+	-	+	-
	G1862T	SHH029	-	+	NS	NS	NS
		SHH042	-	-	+	-	-
		SHH053	+	-	+	-	-
		SHH070	-	-	+	-	-
		SHH071	NS	NS	+	-	NS
		SHH074	NS	+	NS	NS	NS
		SHH156	+	+	+	NS	-
HBeAg Negative	**G1862T**	SHH173	+	NS	-	NS	NS
		SHH219	+	-	NS	NS	NS
		SHH221	+	NS	-	-	-
		SHH246	+	NS	NS	NS	NS
	A1888G	SHH001	+	-	-	NS	NS
		SHH016	+	-	NS	-	-
		SHH032	+	NS	NS	NS	NS
		SHH042	+	-	-	+	-

Table 3.10 Molecular Characteristics of the BCP/PreC Region of HBV isolated from HBeAg+ and HBeAg-							
Group	**Mutation**	**Participant Number**	**Time-point**				
			A	**B**	**C**	**D**	**E**
HBeAg Negative	**A1888G**	SHH053	+	-	+	-	-
		SHH148	+	-	-	-	+
		SHH221	-	NS	+	-	-
		SHH264	+	NS	NS	NS	NS
		SHH300	+	-	NS	-	+
	A1888T	SHH070	-	-	+	-	-
	G1896A	SHH016	+	-	NS	-	-
		SHH032	+	NS	NS	NS	NS
		SHH042	+	-	-	-	-
HBeAg Positive	**A1762T/G1764A**	SHH180	+	-	-	-	-
	G1764A	SHH274	+	-	-	-	-
	^{1809}GCAC^{1812}	SHH159	+	-	-	-	-
	^{1809}TTCT^{1812}	SHH180	+	-	-	-	-
	^{1809}TCTT^{1812}	SHH193	+	-	NS	NS	NS
	^{1814}ACG^{1816}	SHH274	+	-	-	-	-
	G1862T	SHH002	-	+	-	-	-
		SHH159	-	+	-	-	-
		SHH193	NS	+	NS	NS	NS
	A1888G	SHH180	+	-	-	-	-
		SHH274	+	-	-	+	-