UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA

THE IMPACT OF LAMIVUDINE-BASED ANTIRETROVIRAL THERAPY ON HEPATITIS B VIRUS (HBV) GENETIC EVOLUTION AMONG HIV-HBV CO-INFECTED PATIENTS

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Antonio Adrián González del Castillo

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Vicente Soriano
Anna Maria Geretti
José Prieto

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TESIS DOCTORAL

ANTONIO ADRIÁN GONZÁLEZ DEL CASTILLO

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DIRECTORES:

Doctor Vicente Soriano
Hospital Carlos III de Madrid

Profesora Anna Maria Geretti
University of Liverpool

Profesor José Prieto
Universidad Complutense de Madrid

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PREFACE

The Hepatitis B Virus (HBV) affects mainly to the liver, infecting the hepatocytes, and it can be either acute or chronic. Globally, it is estimated that 2 billion of people are infected with HBV and 350-400 million people suffer chronic infection. Furthermore, more than 600.000 HBV carriers die every year from end-stage liver diseases including fulminant hepatitis, cirrhosis or hepatocellular carcinoma (HCC) caused by HBV infection and hence it represents a major global health problem.

HBV prevalence is higher in low-income countries including Africa, the middle-east and Asia than in western world, where horizontal transmission of HBV is the most common route. Conversely, in western countries, sexual and parenteral (intravenous drug used) are the main routes of transmission. Furthermore, as HBV shares the routes of infection with Human Immunodeficiency Virus (HIV), co-infection is relatively frequent. Among 36 million people living with HIV worldwide and approximately 4 million people (~10%) are chronically co-infected with HBV. Similarly, the prevalence of this co-infection is higher in Africa and Asia (up to 20% in some regions) than in western countries (<10%) [1].

HBV is characterized by an extraordinary genetic variability mainly due to its reverse transcriptase, which lacks of proof-reading activity producing a high mutation rate. The high genetic variability allows HBV the ability to develop drug resistance mutations to evade pharmacological pressure. Additionally to Interferon α, there are 5 nucleoside/nucleotide analogues approved for HBV treatment (lamivudine, telvibudine, entecavir, adefovir, and tenofovir). Nowadays, tenofovir (TDF) is one of the most widely

nucleotide analogue used for the treatment of HBV infection, which is also active against HIV and is commonly used in western countries for the treatment of HIV/HBV co-infected patients due to its high antiviral efficacy and high genetic barrier for resistance. However, in low-income countries, the use of this drug is still restricted because its high cost and lamivudine (3TC) is still the most nucleoside analogue used against HBV infection included within the HIV antiretroviral therapy because is cheaper and also active against HIV. As consequence of this regimen, HBV drug resistance is expected among these patients since the long-term exposure to 3TC monotherapies is associated with high rates of HBV drug resistance in both HBV monoinfected and HIV/HBV co-infected patients.

HIV/HBV co-infected patients show higher HBV DNA levels and lower serum alanine aminotransferase (ALT) levels than those infected with HBV alone. Moreover, liver fibrosis tends to be more advance and the risk of end-stage liver disease is increased [2–4]. Nevertheless, there is scarce information regarding how HIV could drive the HBV genetic evolution. In this thesis, two HIV/HBV co-infected cohorts under 3TC-based treatment have been studied from two different endemic countries, Ghana and Malawi. The genetic variability within the genes that encodes the polymerase and the Hepatitis B Surface Antigen (HBsAg) was assessed. The Ghana cohort was under long-term treatment and three clinically different cohorts were evaluated at genetic level to evaluate how HIV drives HBV evolution (HIV/HBsAg positive, HIV/HBsAg negative and HBV monoinfected). In Malawi, the rates and predictors of virological responses in HIV/HBV co-infected patients under 3TC-based regimens were evaluated after 48 weeks of 3TC-based therapy. Additionally, the genetic evolution of both polymerase and HBsAg were also assessed.

CHAPTER 1

Hepatitis B Virus

1. Genomic Organization

HBV is a member of the *Hepadnaviridae* family which has a strong preference for infecting liver cells, although it is also possible to find reservoirs in the kidney, pancreas and mononuclear cells [5,6]. HBV virions are envelope particles, 40 to 42 nm in diameter, surrounded by lipoproteins and three different glycoproteins [7,8]. Within the envelope is the viral nucleocapside or core, which contains the viral genome and the viral polymerase responsible for the synthesis of viral DNA inside the infected liver cells [9–11].

The viral genome is a relaxed-circular, partially duplex DNA of 3.2 kb. It is a compact and small structure, organized into four open reading frames (ORF). These encode the core/precore (*C* ORF), polymerase (*pol* ORF), envelope (*S* ORF), and *X* ORF. The biggest is the *pol* ORF which encodes the viral polymerase and its accessory functions. The *S* ORF is totally overlapped within the *pol* ORF and encodes the different envelope proteins. *C* ORF encodes the e antigen and the core (HBeAg and HBcAg) and partially overlapping the *pol* ORF is the *X* ORF which encodes the HBxAg. (Figure 1.1) [12].

Within the *preC-C* ORF the internal AUG codon produce the HBcAg, which is a protein formed by 183-185 amino acids. It is essential for the RNA packaging and second DNA strand synthesis, playing an important role in the viral cycle. The HBeAg is 19 amino acids larger and is modified in the endoplasmic reticulum (ER). Although its function is not completely clear, it is related to high level of viral replication. Nevertheless, due to particular genomic organization, lacking of this protein is not necessarily associated with low level of viremia [13].

The *pol* ORF encodes a 90 kDa protein which is divided into four domains, the N-Terminal or primase, a linker region, the reverse transcriptase (RT) domain and the C-Terminal RNAse H. The RT domain contains the most conserved region which can be divided into 7 domains A to G. The C domain contains the catalytic pocket which has the conserved sequence Tyrosine, Methionine and two Aspartic acids (YMDD).

The *S* ORF is divided into pre-S1, pre-S2 and S regions by two internal AUG start codons, but shares the same termination codon. Translation results in three different proteins in size: Large (L-HBsAg); Medium (M-HBsAg); and Small (HBsAg). These proteins are the main component of the virus envelope and they play an important role in interactions with the host, virion assembly and infection of new hepatocytes.

Finally, the X ORF encodes a small 154 amino acids protein known as HBxAg. It is a soluble protein whose function is related with several factors, being crucial in starting or maintaining viral replication [14]. It is involved in the epigenetic regulation of the covalent closed circular DNA (cccDNA) and is suggested to be important for promoter activation in the development of HCC [15–18].

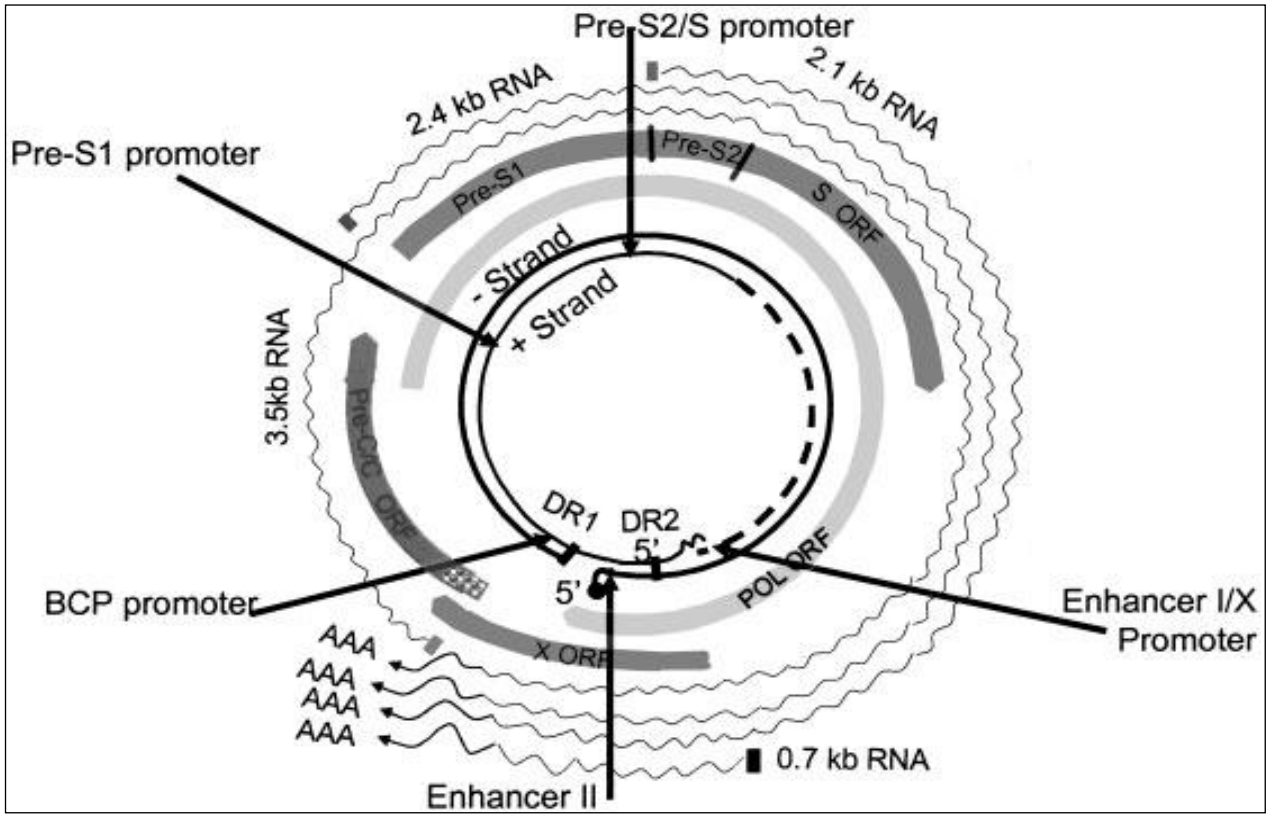

Figure 1.1 The HBV genome is a relaxed-circular, partially duplex DNA of 3.2 kb. It is organized in four open reading frames (ORF); the core/precore (*C* ORF), polymerase (*pol* ORF), envelope (*Surface* ORF), and *X* ORF.

2. Viral Cycle.

The viral cycle can be divided in several steps; (i) attachment to the hepatocyte, (ii) penetration into the cell, (iii) transport into the nucleus, where viral replication and transcription occur, (iv) translation, assembly and reverse transcription in the cytosol, (v) virion maturation or amplification of the cccDNA pool and (vi) release of viruses (Figure 1.2) [19,20]. The mechanism of viral entry into the hepatocytes is still unknown; however, it is known that binding of L-HBsAg to its receptor is required. Once inside the hepatocyte, viral core is released and a nuclear localization signal located in the capsid leads the core to the nucleus. The relaxed circular HBV DNA (rcDNA) is repaired in the nucleus by the host enzymes producing the cccDNA, which serves as a template for transcription [15].

Transcription is directed by four different promoters (enhancer II/BCP, pre-S1, pre-S2/S, enhancer I/X) producing four different sized species of mRNA (3.5, 2.4, 2.1 and 0.7 kb). The largest one is the pre-genomic RNA (pgRNA) which encodes the HBcAg, HBeAg and the HBV polymerase. The smallest mRNA encodes the HBxAg and the remaining two encode the surface proteins. The pgRNA also serves as a template for reverse transcription which occurs after the genome is packaged into the core particle. The new nucleocapsid has two potential fates; it can either be re-imported to the nucleus to generate more cccDNA molecules (typical in the initial stage of infection) or it can be transfered to the endoplasmic reticulum (ER) where envelope proteins are incorporated before release from the cell. Mature virions containing HBsAg proteins are secreted along with spherical and filamentous enveloped subviral particles (SVPs), which lack a nucleocapsid and are non-infectious [20].

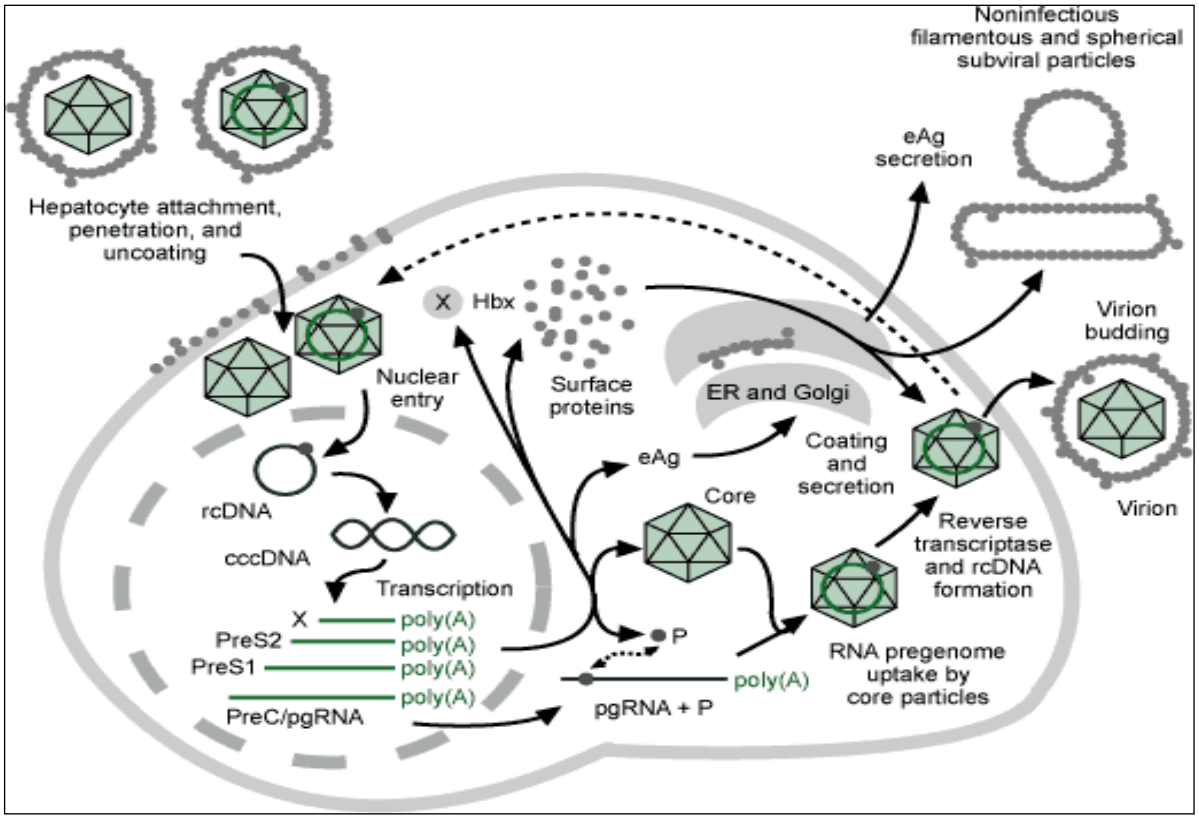

Figure 1.2 HBV cycle. (Adapted from www.clinicalcareoptions.com).

3. Genomic Evolution.

The HBV evolution is characterised by particular features of its DNA genome as well as by the viral polymerase required for replication. Genomic diversity results from two mechanisms; genotypic variability that is produced in the absence of selective pressure and phenotypic variability that results from adaptation to selective pressures produce by the host or drugs.

Two forms of the viral genome exist in infected hepatocytes; the rcDNA and the cccDNA. HBV RT lacks a proof-reading activity increasing the number of errors incorporated into a genome during replication; this increases the genetic diversity of the viral population. The rate of substitutions during replication falls in the range of 10^{-4} to 10^{-5} per site per year. Despite being a DNA virus, this rate is similar to many RNA viruses. This results in 0.1 to 1.0 substitutions per genome during each replication cycle producing many variants in the same patient. Furthermore, HBV has a high viral turnover with up to 10^{11} to 10^{13} virions produced daily [21–23]. These factors result in a high genotypic variability and have led to the classification of up to 10 different genotypes (A-J) based on >8% intergenotype and <4% of intragenotype divergences along the full length genome. The genotypes are different not only molecularly but also in their localization. Genotype A is prevalent in Africa (A1 and A3), Europe (A2) and USA; Genotypes B and C are prevalent in Asia or among Asian immigrants; Genotype D is prevalent in Mediterranean countries, Middle East, Central Asia and India; Genotype E is prevalent in Western Africa; Genotype F is prevalent in South and Central America; Genotype G is prevalent in USA and France; Genotype H is prevalent in Mexico and

Latin America; Finally, Genotypes I and J were recently found in Vietnam/Laos and Japan, respectively [24–26].

Phenotypic variability results from adaption to selective pressure. It can be produced by several factors including the host immune system, antiviral drugs, passive immunisation with HB immunoglobulin (HBIg) or active immunisation using the HBV vaccine. Moreover, demographic factors (race, age, gender) and host genetic factors also play an important role. The factors associated with development of resistance to antiviral therapy are determined by the characteristics of the drug, like adherence or potency, which establish the concept of the drug genetic barrier [19,27,28]. Viral infections will modify the host environment and the stage in the natural history of the infection will also have an effect (i.e., the immune response differs depending on whether the infection is acute or chronic). Furthermore, the viral replicative space, which is the number of cells available for viral infection, determines the number of fresh infections that can occur. During clonal expansion in the liver, the number of hepatocytes available for infection is limited due to the expansion of cells that are resistant to HBV infection or by cells which have been previously infected and are resistant to re-infection.

Mutational epistasis is defined as the dependence of phenotypic effects of one mutation on a mutation at another site. Therefore, mutations at one site can change the direction and intensity of selection at another genomic site. For example, the development of secondary mutations or co-evolution of mutations which help the virus restores fitness. Moreover, a large population containing a mixture of potentially beneficial mutations is maintained in competition by selective pressures such as antiviral drug or immunological pressure. This competition for survival between these independent members of the quasispecies is known as “clonal interference”. Thus, both

epistasis and clonal interference among advantageous mutations coordinate genotypic evolution not just among individual strains but at a level of population or quasispecies. Therefore, there is cooperation among the different strains helping the population evolve. For example, the rtA181T substitution in the HBV polymerase, which is associated with drug resistance, generates a stop codon in the overlapping *S* gene, resulting in a defective envelope protein. The deleterious effect of this mutation cannot be compensated by another mutation in the same HBV genome (epistasis) but the mutation in the *pol* gene produce an advantage since it results in drug resistance. Then, this strain will require the help of the *wild-type (wt)* strains to provide a functional envelope protein establishing a cooperation between the strains that allow the HBV to both survive and evolve [29,30].

However, the rate of variability is restricted by two HBV features particular to HBV. Firstly, the overlapping nature of the HBV genome limits the viability of mutant strains. Secondly, the long half-life of the hepatocytes infected by HBV reduces the replicative space reducing the opportunity for mutant viruses to replace the *wt* strain [28].

4. Natural history: Monoinfected vs. Co-infected.

The HBV infection is not cytopathogenic and liver injuries are a consequence of host´s immune responses. The natural history of chronic HBV infection can be divided into different phases. Firstly, the “immune tolerant phase” is characterized by HBeAg seropositive, high serum HBV DNA level, normal serum alanine aminotransferase (ALT) and near-normal liver histology. Secondly, the “immune clearance phase” is characterized by continuing hepatitis activity or episodic acute flares with ALT over five times the upper limit of normal. This could lead to fibrosis progression or cirrhosis in HBeAg positive patients. The HBeAg serconversion depends on factors like age, ALT or genotypes (A, B > C, D). Thirdly, the “residual inactive phase” is characterized by normal ALT, low serum HBV DNA level and no or minimal necro-inflammatory histological changes. Finally, the “reactive immune clearance phase” is produce when HBV is reactivated with either HBeAg seroreversion or with pre-core (PC) or basal core promoter (BCP) mutations [31].

The influence of HIV into the natural history is a consequence of the immune deficiency produced. For that reason, the HBV chronicity rate in these patients is higher as well as the level of HBV replication. Furthermore, the spontaneous loss of HBeAg and/or HBsAg is lower with a high rate of seroreversion after CD4+ cell are depleted. These facts can be prevented with Highly Active Antiretroviral Treatment (HAART) using dual action antivirals [32].

5. Treatment: Monoinfected vs. Co-infected.

There are three major international guidelines of treatment: (i) the Asian Pacific Association for the Study of the Liver (APASL), (ii) the American Association for the Study of the Liver Diseases (AASLD) and (iii) the European Association for the Study of the Liver (EASL) [33–35]. In general, these guides are based on the same parameters: ALT level, HBV DNA and histology data. However, they are slightly different when it is necessary decide when start the treatment.

The major endpoint in the treatment of HBV is achieving the replication levels of the virus as lowest as possible and maintains it. The guidelines consider that treatment is not indicated for inactive carriers (HBeAg negative, ALT normal, <2000 IU/ml) and immune tolerant patients (HBeAg positive, ALT normal or less than 2xN, high HBV DNA). Regard to which patients need to be treated is controversial. Although the APASL and the AASLD are similar and consider the HBeAg serostatus, the EASL do not consider it. Furthermore, there is no consensus in minimum level of HBV DNA to consider treatment being 2.000 IU/mL in the EASL and 20.000 IU/mL in the APASL and AASLD.

There are seven drugs approved for the treatment of chronic Hepatitis B (CHB). Interferon-α (IFN) and pegylated interferon-α (peg-IFN) together with five nucleoside/nucleotide analogues (NAs) which target the viral polymerase. These are cytidine or thymidine L-Nucleoside analogues, lamivudine (3TC) and telvibudine (LdT), a D-Cyclopentane entecavir (ETV), and two alkyl phosphanates, adefovir (ADV) and tenofovir (TDF) (Figure 1.3). The advantages and the inconvenient between using

interferons or NAs are summarized in the table 1.1. Both American and European guidelines recommend avoiding 3TC, LdT and ADV in naïve patients and using TDF or ETV are the suitable drugs. The Asian guidelines were done a year before and recommends ETV and LdT.

Regarding the HIV/HBV co-infected populations, the European AIDS Clinical Society (EACS) Guidelines recommend starting treatment of these patients when the ALT are elevated and HBV DNA is higher than 2.000 IU/mL [36,37]. However, in patients with significant liver fibrosis, anti-HBV treatment might be considered even when serum HBV-DNA is below 2 000 IU/mL and ALT are not elevated. The drugs used for the treatment will depend on two factors: i) indication for HIV treatment and ii) 3TC-experience or naïve. Taking into account the last factor, TDF is indicated in both cases adding 3TC or emtricitabine in the case of 3TC-naïve patients.

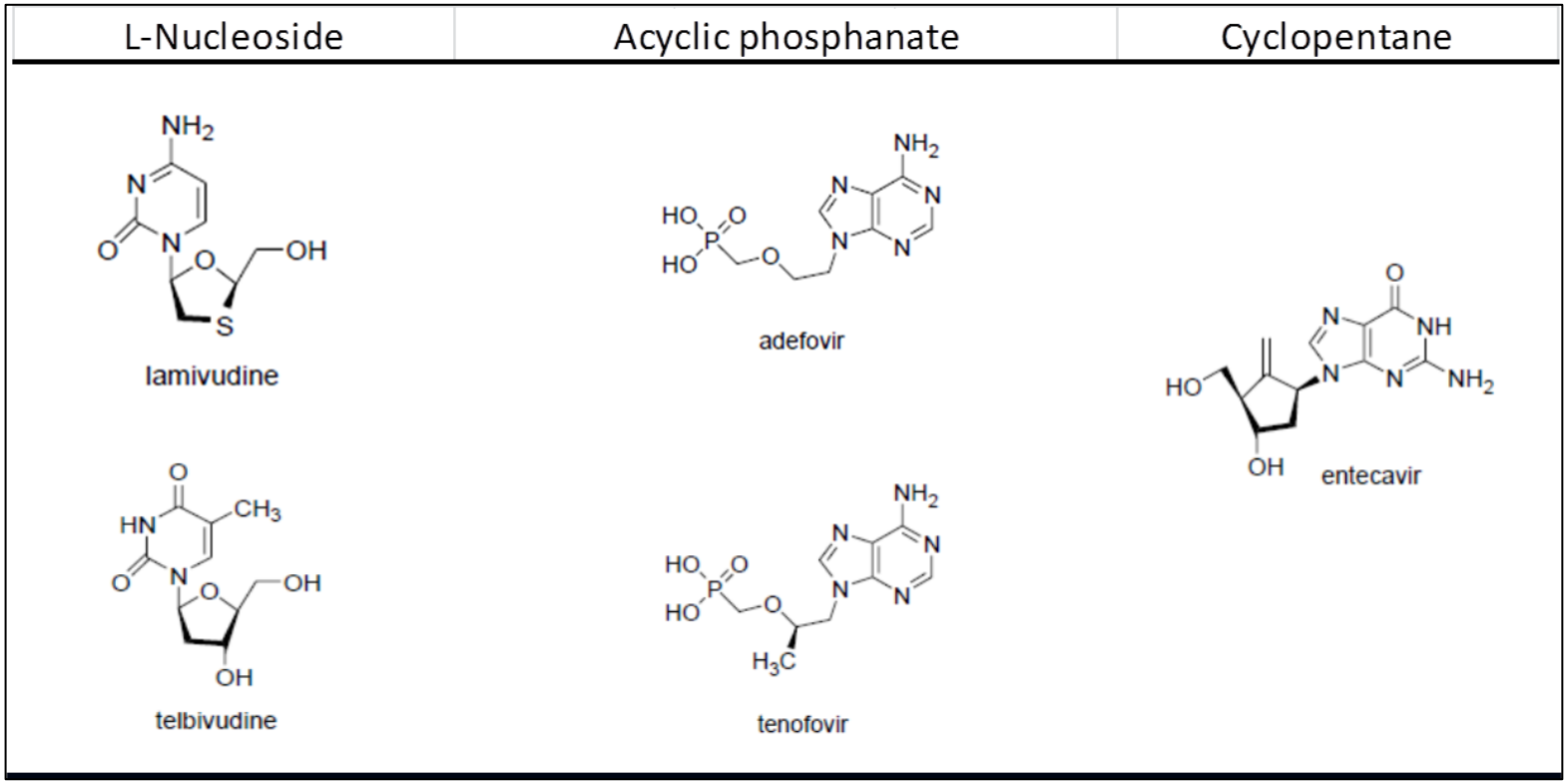

Figure 1.3 Nucleoside/nucleotide analogues structures approved for HBV treatment; L-Nucleosides family includes Lamivudine (3TC) and Telvibudine (LdT); Acyclic or Alkyl phosphanates family includes Adefovir (ADV) and Tenofovir (TDF); Cyclopentane family includes Entecavir (ETV).

ADVANTAGES	INCONVENIENTS
INTERFERON	
Short-term treatment HBsAg negatively more frequent No drug resistance	Adverse effects Poor response in high viral load patients or immune tolerant
NUCLEOS(T)IDES ANALOGUES	
Oral administration Better tolerability Good inhibition of replication	Long-term treatment Low HBsAg negatively Drug resistance

Table 1.1 Difference in availability treatments (Interferon vs. Nucleos(t)ide analogues).

6. Antiviral therapy and drug resistance

6.1 Drug resistance

The desired endpoint of HBV treatment is achieving complete suppression of HBV DNA levels. Failure to control the DNA levels is a major factor associated with the development of drug resistance. Other factors include adherence of the patient and the ability of the drug to suppress viral replication. Hence, a drug with low antiviral activity does not exert substantial selection pressure on the virus and the likelihood of drug resistance developing is increased. No drugs can target the cccDNA archive so complete eradication of the infection is not possible. Therefore, long term treatment regimens are necessary which increase the risk of drug resistance.

The antiviral efficacy of each drug has been assessed in different studies. The pathway in which mutations accumulate in order for the virus to develop drug resistance varies for each drug and determines the genetic barrier of each compound. The drug with the lowest genetic barrier is 3TC, reaching drug resistance in up to 80% of patients after 4 years of treatment [38]. It is followed by LdT which can reach 29% or 11% drug resistance in the second year of treatment depending on the patients HBeAg positive or negative status [39,40]. In HBeAg negative ADV-treated patients, 29% develop drug resistance over 5 years. If the patient already has 3TC resistance, then up to 20% develop drug resistance within the first year [41,42]. ETV and TDF are the antivirals with the highest genetic barrier. In the case of ETV, the rate of drug resistance development is up to 1.2% after 6 years of treatment. However, ETV can reach up to 57% after 6 years in patients that harbours 3TC resistance [43]. Recent clinical trials about TDF reported no resistance after 96 weeks of treatment [44].

Furthermore, HIV co-infection is another problem in terms of HBV drug resistance. HIV is able to modify the course of HBV which shows higher HBV DNA levels and lower serum alanine aminotransferase (ALT) levels than those infected with HBV alone. These levels of HBV DNA increase the probability of developing HBV drug resistance. In fact, it has been already shown this effect [3,45,46].

6.2 Pathways and drug genetic barrier

Antiviral drug resistance is associated with selection of primary drug resistance mutations which facilitate viral synthesis in the presence of the drug. Viral fitness is often reduced by the primary mutations and secondary mutations are needed to restore viral fitness. There are four major different pathways according to the drug structure to generate drug resistance explaining the drug genetic barrier (Figure 1.4) [47]:

(i) the *L-nucleoside pathway*: the primary mutation to the L-nucleoside drugs is rtM204V/I which is associated to resistance to 3TC and LdT. Using the crystal structure of the HIV polymerase, the mechanism of resistance is based on the reduce accessible surface area between the polymerase pocket and the drug. In other words, it produces a steric hindrance and impairs in the catalytic activity of the RT. The secondary mutations related to this pathway are rtL180M, rtV173L and rtL80IV [48–52].

(ii) the *alkyl phosphonate sugar pathway*: it is associated with ADV resistance. The ADV structure is similar to its natural substrate dATP, which allows ADV a great accessibility to the polymerase and a low rate of resistance. The primary mutations associated with ADV treatment are rtN236T and rtI233V in the D domain and rtA181V in the B domain. However, mutations in 3 different regions are necessary for development of drug resistance: (1) mutations involving the D and A domains, rtI233V, rtN236T, rtP237H, rtN236T/D, rtV84M, and rtS85A. A Hydrogen bond between residues rtN236 and rtS85 can be disrupted by any of these mutations; (2) mutations involving the B domain, rtA181V/T, result in an allosteric change in the catalytic site; (3) mutations involving inter- C-D domain, rtV214A and rtQ215S, cause an allosteric change despite not interacting with the catalytic pocket or the DNA template [53,54].

(i) the *D-cyclopentane or treatment-naïve ETV resistance pathway;* ETV is a potent and selective drug against HBV. It can affect multiple functions on the polymerase including priming, reverse transcription and DNA elongation. Mutations at rtL180M and rtM204V/I are required for the development of ETV resistance and at least one of following: rtT184G, rtS202I and rtM250V. A secondary mutation, rtI169T, is also common in this pathway. Interestingly, two mutational patterns have been observed: (1) rtM250V + rtI169T + M204V + L180M and (2) rt184G + rtS202I + rtM204V + rtL180M. Two molecular mechanisms have been proposed to explain these mutational patterns. Mutations at positions rtM250V and rtI169T impact on the primer binding while mutations at rt184G + rtS202I are responsible for the interaction with the hydrophobic core region. In both cases, the resistance level to ETV is enhanced by the 3TC-associated mutations [55,56].

(ii) the "*shared" pathway* between the L-nucleosides and alkyl phospanates compounds; The mutation rtA181T/V conferring resistance to 3TC and ADV and reduced sensitivity to TDF.

The role of rtA194T and its association with TDF resistance remains controversial. The rtA194T mutation was identified in addition to rtL180M and rtM204V in two HIV/HBV co-infected patients who failed treatment. A role for the rtA194T mutation in the development of TDF resistance was confirmed from *in vitro* studies. However, in 2006, this experiment could not be reproduced by *Delaney et al.* and this finding has subsequently been supported by two further studies in 2009 by *Amini-Bavil-Olyaee et al*. and 2011 by *Zhu et al.* [57–60].

The genetic barrier has also been described in terms of the probability of each nucleotide suffer either a transition (A ↔ G, C ↔ T) or transversion (A ↔ T, A ↔ C, G ↔ C, G ↔ T) by *Svicher et al.* [61]. Depending on whether a transition or transversion occurred, a score of 1 or 2.5 was given respectively. In this way, primary drug resistance were associated with a low genetic barrier (score = 1). In this group appeared mutations such as rtM204V, rtA181T/V/S and, interestingly, rtA194T. This fact could explain why these mutations appeared in naïve patients when minority species are studied by ultra-deep sequencing [62,63]. They also showed the case of rtM204I. This mutation results in sW196Stop in the *S* gene that produce a truncated HBs protein. As these changes affect both replication and HBsAg production, the virus would need a co-infection with a *wt* strain to survive. Therefore, in contrast with the other primary mutations, it showed a higher genetic barrier to be selected.

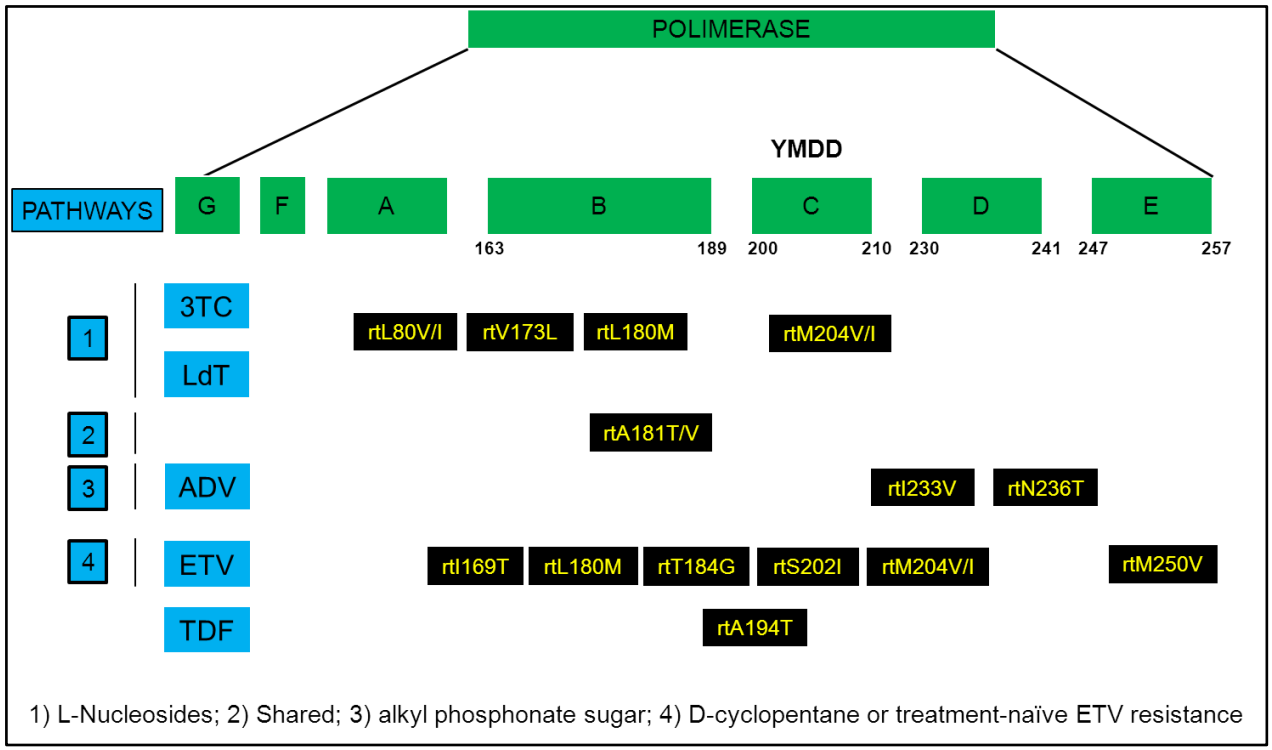

Figure 1.4 Position and combinations of drug resistance mutations in polymerase. Mutations are depicted alongside the domain of RT in which they are located. [1] 3TC and LdT belong to the L-Nulceosides group; [2] The shared pathway is related to L-Nucleosides and alkyl phosphonate sugar pathways; [3] ADV is an alkyl phosphonate sugar; [4] ETV is a D-cyclopentane; rtA194T is related to TDF resistance but data about this mutation is controversial.

Drug resistance can be classified into low, intermediate or high level as determined by *in vitro* experiments. Sometimes, one mutation can confer resistance to a drug that the virus has not been exposed, called cross-resistance, i.e. rtA181V (Table 1.2) [64,65]. This is more likely when different drugs share the same drug resistance pathway. Multidrug-resistant (MDR) strains of HBV have been observed in patients exposed to more than two antivirals. Both sequential monotherapy, especially in patients treated with similar drugs, and/or “add on” regimens due to incomplete viral suppression increase the likelihood of developing MDR viruses [66]. The generation of secondary mutations plays an important role in these strains because viral fitness has to be maintained even though the virus has accumulated multiple resistant mutations which are usually detrimental to fitness.

Table 1.2 Cross-resistance for the main drug-resistance mutants and the approved antivirals

Pol gene mutations	3TC	LdT	ETV	ADV	TDF
rtM204I	R	R	I	S	S
rtL180M+rtM204V	R	R	I	S	S
rtN236T	S	S	S	R	I
rtA181TV	I/R	R	S	R	I
rtN236T+rtA181T/V	R	R	S	R	I
rtL180M+rtM204VI ± rtI169T ± rtV173L ± rtM250V	R	R	R	S	S
rtL180M+rtM204VI ± rtT184G ± rtS202IG	R	R	R	S	S

I, intermediate; R, resistant; S, sensitive

Table 1.2 Mutations associated to cross-resistance according to the approved antivirals [67].

7. Variability of the HBsAg, vaccine escape mutants (VEM) and clinical implications and antiviral drug-associated potential vaccine escape mutants (ADAPVEM).

The HBsAg is the major envelope protein and a key biomarker of HBV infection and contains the major immunological epitope used for developing the vaccine [68–71]. The HBsAg protein is a typical membrane protein formed by 226 amino acids. The C-terminal hydrophobic 57 amino acid crosses the membrane twice to position to both the ER lumen and cytosol dividing the protein into different domains [72,73] (Figure 1.5): (i) the first trans-membrane domain (TMD-I) residues 8 to 24; (ii) the first cytosolic domain (CYL-I), contains the T-cell epitope (residues 28 to 51); (iii) the second trans-membrane domain (TMD-II) between residue 80 - 98; (iv) the double loop, called the antigenic loop (AGL) or "a" determinant (101 – 164) is orientated into the lumen. It contains the major immunological epitope of the S protein, including the B-cell epitope (124-148); (v) finally, a small cytosolic region (CYL-II) is located between two additional trans-membrane domains (TMD-III and TMD-IV). It is stabilized by 14 Cysteines (Cys), which are cross-link to each other forming disulphide bonds (Figure 1.5, green residues) [74,75]. Cys 48, 65 and 69 are strictly conserved among all *hepadnaviruses* and are essential for HBsAg secretion. In contrast Cys 76, 90 and 221 are not conserved and are dispensable for viral replication. Cys 90 and 221 are inside the TMD-II and TMD-IV regions so their impact on antigenicity and infectivity is limited [74–76]. All Cys located inside the AGL are indispensable for HBsAg secretion with the exception of Cys 149 [75].

Biochemical studies have been conducted to characterize the different domains, summarized in the table 1.3. Most of the amino acids inside the CYL-I (25-79) were able to tolerate deletions without impaired folding or secretion. In general, there were no essential residues between amino acid 39 to 58, with the exception of the cysteine sC48 and between amino acids 49 to 53. Deletion between amino acids 59 to 80 was not tolerated since sHis60 and a cluster of positively charged residues containing amino acid 73, 78 and 79 were essential [74,77,78]. *Blanchet and Sureau* used Alanine scanning of the cytosolic domains of HBs to determine the important positions for subviral particle (SVP) secretion. By quantifying the amount of HBsAg detected on the cell surface or in the cytoplasm by western blotting, they identified amino acids sD33, sC48, sH60, sC65, sP66, sY72, sR73 and sC76 as being essential for secretion (Figure 1.5, red or black residues) [77]. Three areas inside the putative TMD-III and IV (amino acid 184 to 188, 209 to 213 and 219 to 223) cause the complete failure in HBsAg secretion but were still detected in the cell lysates. This indicates correct folding of the AGL and supports the putative trans-membrane position. The deletion between 194 to 198 were not found either in the cells lysate or supernatant, indicating that its production was completely blocked [79]. Similar studies have shown that amino acids 104-108 and 139-163 inside AGL are also essential for the secretion, whereas 109 to 133 had no affect (not shown in the figure 1.5).

The infection capacity depends on the N-terminal preS1 domain and the AGL, where the Cys network plays an essential role [80]. The region between 119 and 128 is critical for the infectivity of the virus (Figure 1.5, yellow residues). *Salisse and Sureau* published a detailed study of the non-cysteine residues in the AGL involved in infectivity. Apart from the cysteines from 121 to 149 which had previously been implicated, they

showed a role for; prolines 105, 108, 120, 142 and 153; positively charged residues sR122 and sK141; hydrophobic residues at sV106, sI110, sM133, sI150 and sW156; and finally uncharged polar residues sN146, sT118, sT123 and sT148; and sG119 [81,82].

Van Hemert et al. proposed a 3D-structural model of HBsAg protein [83]. This study was focused on the influence of overlapping both *pol* and *S* genes. The 1st nucleotide position of the *pol* codon corresponds to the 3rd nucleotide position of the *S* codon (p1s3), the 2nd nucleotide of the *pol* codon is the 1st in the *S* codon (p2s1) and the 3rd nucleotide of the *pol* codon is the 2nd of the *S* codon (p3s2). The model showed that variation in one frame of the gene is accompanied by conservation in the other gene. Recently, *Svicher et al.* showed that *pol gene* is more conserved than the *S* gene, which was different according to the genotype and could lead a different immune host response, vaccine efficiency and/or even different diagnostics results. In detail, the probability of nucleotide transitions or transversions was restricted by the sequence of the overlapping ORF. Then, the likelihood of producing a VEM was determined by the sequence of the *S* gene, which is genotype specific. For example, they showed that the codon used for encoding residue sG130, which was different in two genotypes, A and G, and had a lower genetic barrier for the development of immune escape sG130N. Furthermore, the immune escape sT131N was constitutive in these genotypes. Similar situation amongst different genotypes occurred for residues s114, s127 and s161. Therefore, the genotype of the virus can also determine the ease of developing specific escape mutations [61]

Amino acids	Domain
1 → 3	N-terminal
4 → 24	TMD - I
28 → 51	T-cell epitope
25 → 79	CYL - I
80 → 100	TMD - II
101 → 172	AGL
124 → 148	B-Cell epitope
173 → 193	TMD - III
194 → 201	CYL - II
202 → 222	TMD - IV
223 → 226	C-terminal

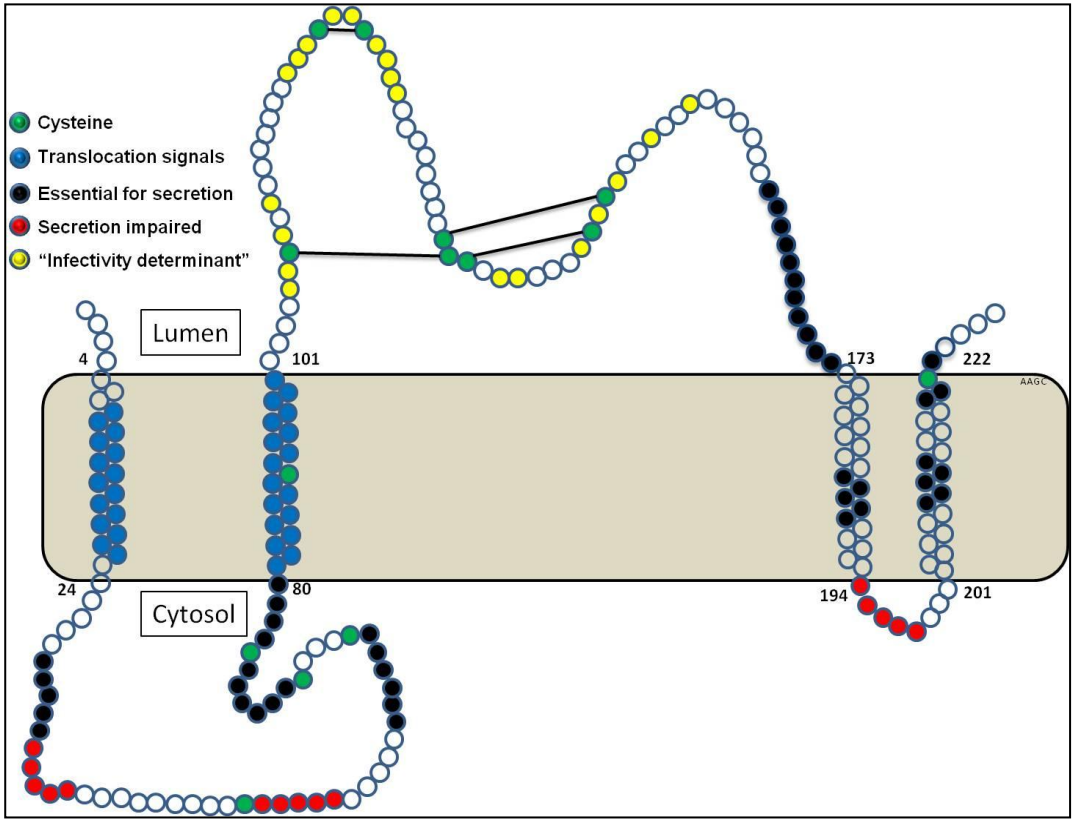

Figure 1.5 The HBsAg structure is initiated by two translocation signals (blue circles). There are four trans-membrane domains (TMD), two Cytosolic loops (CYL) and two lops toward the lumen which harbour the Antigenic loop (AGL). The structure is stabilized by the Cysteine network (green circles). Inside the AGL, there are some residues that give raise the infectivity determinant (yellow circles). Furthermore, there are plenty residues along the structure that are conserved and are essential for maintain it (red and black circles).

Amino Acids and Secretion	
Dispensable	Reduced or crucial
Δ24-28KL	Δ29-33KL
Δ39-43KL	Δ34-38KL
Δ44-47KL	Δ49-53KL
Δ54-58KL	Δ59-64KL
Δ174-178KL	Δ70-75KL
Δ179-183KL	Δ76-80KL
Δ184-188GA	Δ164-168KL
Δ189-193KL	Δ164-168GA
Δ194-198GA	Δ169-173KL
Δ195-197AAA	Δ169-173GA
Δ199-203KL	Δ184-188KL
Δ204-208KL	Δ194-198KL
Δ209-213GA	Δ209-213KL
Δ214-218KL	Δ219-223GA
	Δ219-223KL
	Δ223-226

Table 1.3) Functional *in vitro* studies on the HBsAg were carried out to study the crucial amino acids in the secretion of the protein by deleting/substitution different regions [79,84,85]. The left column shows the regions which were dispensable to HBsAg production. The right column depicts those regions which were essential for HBsAg secretion.

The AGL of the HBsAg was taken for developing the HBV vaccine in the early 80´s. The HBV vaccine has been able to induce protection in up to 95% of immune-competent recipients. Since vaccination programs were introduced, the prevalence of chronic hepatitis B has drastically decreased in the western world. However, evidence of breakthrough infection quickly became apparent giving raise the concept of vaccine escape mutant (VEM). The first description was made in a child vertically infected with HBV despite vaccination and passive immunization with HBV immunoglobulin (HBIg) [86]. A substitution at position 145 of HBsAg (G145R) was found which led to alterations in the "a determinant" loop (amino acid139-147) which resulting in neutralizing antibody escape [87]. Since then, different studies have been carried out in populations where HBV is endemic and primary infection occurs during infancy or early childhood. After the launch of the universal vaccination programme in Taiwan the proportion of children carrying virus with "a determinant" mutations rose to 19.6%, 28.1% and 23.1% among children chronically infected for 5, 10 and 15 years respectively [88,89]. Therefore, despite HBV vaccine, horizontal or vertical transmission has been observed in vaccinated individuals due to immune escape [90–103].

One of the main characteristics of HBV, the overlapping reading frames, can lead to affect the virus in different situations. In this sense, variations in the S gene can modify the antigenicity, the infectivity and the secretion of the protein, reducing the number of virions but can also affect the *pol gene* decreasing viral fitness. Additionally, VEM´s not only affect vaccine response but also potentially affect diagnostic detection. Therefore, it is usually correlated clinical situations to variations in the HBsAg, i.e., the occult HBV infection (OBI). In this sense, mutations in the *S* gene could affect to

diagnostic, either by defect in HBsAg production or by the high variability in the AGL that leads to the disruption in the conformation preventing the immune detection. However, the last hypothesis has been called as a "false" OBI [104]. The defect of HBsAg can be also explained due to the low viral fitness or by generation of stop codon mutations, then producing fewer HBsAg proteins.

Furthermore, VEM´s were found in different strains from patients receiving a transplantation which are, previously or not, passive and/or active immunized [5,105–111]. These mutations could become more aggressive producing a worse progression; it has been observed in patients who presented a fulminant hepatitis where it was found sG145R, alone or in combination, with an insertion between amino acids s122 and s123 or sT126A. The HBsAg retention together with the enhanced viral replication could be the cause of this clinical course of infection [94,112,113]. Moreover, there are also mutations that have been associated with the HCC development [114,115]. This is related with mutations that are able to induce a truncated protein and include sL21Stop, sW156Stop, sW172Stop, sW182Stop [116–118].

Unfortunately, there is no a crystallographic structure either of HBV polymerase or HBsAg proteins and *in vitro* studies are the main tool for studying the effect of mutations on the viral replication, the secretion, the infectivity and/or the antigenicity capacity. *In vitro* assays showed that sG145R modifies the loop conformation causing a negative effect in the antigenicity and 30% reduction in HBsAg secretion [113,119–121]. Although sG145R results in rtW153Q in the *pol gene*, HBV replication is not affected [122]. It was also analysed in combination with 3TC-associated resistance mutations (rtL180M/M204V and rtM204I). sG145R showed its capacity to restore viral fitness in the presence of this 3TC-pattern resistance by *in vitro* assays [113,123–125]. The mutation

sP120T produces the change rtT128N in the *pol* gene, which affects viral replication in the presence of 3TC-resistant mutations. However, contradictory effects of this mutation in combination with rtL180M/M204V have been described [124,125]. *Torresi et al.* showed that sP120T restored replication fitness in 3TC resistant viruses whereas *Amini-Bavil-Olyaee et al.* did not find the same effect [123,125].

Other potential VEM´s have been assessed by *in vitro* studies (table 1.4). These mutations were sT123N (rtQ130P), sM133L (rtY141S), sK141E (rtK149R), sK160N, sW172Stop (rtA181T), sW196L/S/Stop (rtM204I) and sW199Stop (rtM204I) [65,122,124,126–128]. The mutation sT123N was able to restore the viral fitness in 3TC-resistant strains. A new N-Glycosilation site is introduced and the mutant shows reduced levels of secretion and antigenicity. Similar effects were observed with sK160N, but were less pronounced. The mutations sM133L and sK141E impaired viral replication and were less antigenic. The variant sW199Stop impairs the virus replication capacity since it is overlapped with the 3TC-resitant mutation rtM204I. This mutation also results in a truncated HBsAg which affects the level of secretion. Then, it is a good example of cooperation among different strains as this strain will need the help of another *wt* strain to survive. Similar situation appears with the mutations sW196L/S/Stop (rtM204I) and sW172Stop (rtA181T). Furthermore, sW172Stop can cause viral rebound later than expected and inhibition of *wt* virion expression, potentially causing a late diagnosis of drug resistance [65].

Other mutations that have been analysed for secretion by *in vitro* assays are sY100C, sI110M, sG119E, sK122I, sM133T, sA159G and sR169P [128,129]. The mutation sY100C, which has been related with occult HBV, was was found that neither secretion nor production was affected by this mutation. In contrast, the mutation sI110M and

sR169P blocked secretion completely whereas, sG119E, sK122I and sG159G reduced secretion of HBsAg. When sG159G appears in combination with sK160N, secretion of HBsAg was partially restored. It is of interest to note that sM133T also produced a new N-Glycosylation that restored HBsAg secretion. Furthermore, this mutation was able to rescue the secretion of strains harbouring either sG145R or sI110M without altering its immune escape phenotype.

Finally, there are certain mutations that have been assessed solely for their antigenicity. These are sW74Stop, sY100S, sT116N, sK122P, sT125N, sT131I, sE164D, sI195M and sM198I [122,127,130–132]. The mutations sW74Stop and sT125N have been identified in two patients experiencing seroconversion after peg-IFN therapy [132]. Antigenicity of these mutants was tested against monoclonal and polyclonal antibodies. The mutant harbouring sT125N was not recognized by one monoclonal and one polyclonal antibodies whereas the sW74Stop was not recognized at all. The mutation sY100S was not detected in cell-culture using the ABBOTT Architect HBsAg assay. When sS143L was introduce in combination with sY100C, the strain remained undetected. Similar effect was shown sT116N and the combination sT116N plus sS143L as well as in sK122P, SK122P plus sQ101R and in sK122P plus s167L strains (table 1.5). sT131I altered the AGL folding, impairing the antigenicity. The immune detection also failed when the mutations sE164D, sI195M and sM198I were tested.

Similar tests have been performed using combinations of more than one mutation in the same strain (table 1.5). The double mutation sD144E plus sG145R was able to restore the replication capacity in 3TC-resistant strains. This strain will be potentially transmissible because it will produce a higher level of replication that is not only conferring antiviral resistance but is also able to avoid both the immune response and

vaccine induced immunity. Similarly, the double mutation sE164D and sI195M, which is generated by the triple mutation rtV173L, rtL180M, rtM204 is able to avoid the immunologic response to a similar degree as sG145R [127,133].

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There are variants containing multiple mutations do not normally appear *de novo* but are usually produced by interactions among several strains. *Villet et al.* studied a patient firstly treated with 3TC after suffering viral breakthrough ADV was added to the regimen [134,135]. This patient did not respond to combination 3TC and ADV and subsequently underwent an orthotropic liver transplantation (OLT) receiving intramuscular HBIg in combination with 3TC and ADV. Post-transplant viral breakthrough forced the change from ADV to TDF while maintaining 3TC. The viral quasispecies contained many variants, which were studied for their viral fitness, antigenicity and infectivity. One of the variants harboured a deletion between the amino acids 102 to 111 (sF20S, ∆102-111, sP120S, sE164D, sL173F) and was not detected by polyclonal antibodies. Another variant showed a mutation in sR79H (sR79H, sP120S, sE164D, sL173F, sI195M, sY206F) causing a defect in secretion and reduced viral fitness. However, the strain remained able to replicate. The dominant variant (sF20S, sP120S, sE164D, sL173F) presented a reduced viral fitness in comparison with the *wt* strain: however it presented a strong antiviral resistance profile becoming the dominant variant. Furthermore, it showed fewer defects in secretion and higher infectivity capacity than the others variants. Another example of dominant virus population was shown in a patient who developed fulminant Hepatitis B after an OLT. The analysis showed a higher replication capacity of the virus but a defect in the HBsAg secretion [113]. They found the mutation sG145R, which impairs secretion, together with sT45K, sL49I, sS204R and sL205V which had the additive effect of restoring folding and secretion.

Surface gene	*pol gene*	Effect	Reference
sY100C		Secretion is not altered	130
sY100S		Not detected	131
sI110M		Blocked secretion	129
sT116N		Strong decrease in detection	131
sG119E		Impaired secretion	129
sP120T	rtT128N	Contradictory effects in terms of viral fitness	123,127,136
sK122P		Strong decrease in detection	131
sK122I		Impaired secretion/Altered antigenicity	128
sT123N	rtQ130P	Restore viral fitness. Antigenicity impaired.	124,128
sT125N		N-Glycosylation site, secretion reduced	137
sT131I		Altered antigenicity	122
sM133L	rtY141S	Altered antigenicity	124
sM133T		Viral fitness impaired. Altered antigenicity	129
sK141E	rtK149R	It restores secretion via introduces a new N-Glycosylation site.	122
sG145R	rtG153E	Viral fitness impaired. Altered antigenicity	113,136
sA159G		Antigenicity and secretion impaired	
sK160N		Decreased secretion/reduced antigenicity	128
sE164D		Viral fitness normal. Antigenicity reduced.	
sR169P		N-Glycosylation site, secretion slightly reduced	127
sW172*	rtA181T	Altered antigenicity	129
sI195M		Blocked secretion	65
sW196/L/S/*	rtM204I	It produces a truncated protein	
sM198I		Altered antigenicity	127
sW199*	rtM204I	It produces a truncated protein	126

Table 1.4 Effects produce by alone major "single" mutations in both Surface and polymerase genes in vitro. These are mutations that have been shown in different clinical situations, including vaccine failure and drug-related mutations among others.

Diagnostic tests have been improved over the last few years and are able to detect the most common HBsAg variations found in the literature. They require both high levels of sensitivity for detection of different variants and high specificity to avoid the risk false positive results. Detection levels of recombinant HBsAg proteins differ from patient samples, which contains a quasispecies of variants [138]. For this reason, most kits include some polyclonal antibodies in combination with monoclonal ones to allow detection of most of the variants [139–143]. The most typical variants, which pose a problem for detection, are usually located between amino acid 120 to 150, which correlates with the region mutated due to immunological pressure. At this moment, it is a matter of discussion as to which factor has the higher influence in the detection of HBsAg variants including: (i) the position of the amino acid variant, the nature of this variant, (ii) the number of the variants inside the sample, (iii) the variants affect on secretion and (v) folding or even the effect that the HBV genotype can produce [61,130,144–146].

The tight relationship between the genes that encode the polymerase and the HBsAg mean they have to be considered together when a genotypic study is conducted in a patient. The long-term regimens commonly used for HBV treatment produce a high risk of developing drug resistance. The clinical effect observed including resistance to the different drugs used, even to drugs that have never been exposed, and variations that are able to avoid the immune response, both the host and the active/passive immunization (VEM´s and ADAPVEM´s). Additionally, different studies have shown that drug naïve viruses are able to spontaneously develop minority species associated with drug resistance or vaccine escape or both effects [62,63]. *Clements et al.* characterised the criteria required to be an ADAPVEM, to be a

stable mutant, to have undergone sufficient changes in antigenicity for not being neutralized by the anti-HBs generated by the vaccine, to be transmissible and cause infection in immunized individuals, and finally to cause disease in infected individuals [133]. These ADAPVEM´s could jeopardize the vaccine programmes because the transmission risk of these strains. It has been shown HBV strains harbouring the triple 3TC-resistant pattern (rtV173L, rtL180M, rtM204V) produce a double mutation in the envelope (sE164D, sI195M) that were able to infect and replicate in vaccinated chimpanzees [147]. This pattern has been found in newly diagnosed patients showing that the risk of transmission is not a lucubration but it is fact [148,149].

Surface gene	*polymerase* gene	Effect	Reference
sD144E, sG145R	rtG153E	Restore viral fitness, altered antigenicity	127
sE164D, sI195M	rtV173L, rtL180M, rtM204V	Altered antigenicity	
sF20S, sP120S, sE164D, sL173F	rtT128I, rtV173L, rtL180M, rtA181V, rtN236T	Dominant variant. Secretion impaired but high infectivity capacity	
sF20S, Δ102-111, sP120S, sE164D, sL173F	Δ111-120, rtT128I, rtV173L, rtL180M, rtA181V	Deletion blocked secretion	135
sR79H, sP120S, sE164D, sL173F, sI195M, sY206F	rtT128I, rtV173L, rtL180M, rtA181V, rtM204V	Secretion and fitness impaired but infectious	
sT45K, sL49I, sG145R, sS204R, sL205V		Defect in secretion but high viral fitness	113
sY100S, sS143L		Not detected	
sT116N, sS143L		Strong decrease in detection	131
sR122P, sQ101R		Strong decrease in detection	
sR122P, sS167L		Strong decrease in detection	
sA159G, sK160N		Restored secretion. Altered antigenicity	128

Table 1.5 Virological and diagnostic impact produce by mutations in combination in both *Surface* and *polymerase* genes *in vitro*. These are mutations that have been shown in different clinical situations, including vaccine failure and drug-related mutations among others.

ENDPOINTS

1. Assess the prevalence of HBsAg into a HIV positive population from Ghana and the impact of long-term exposure to a lamivudine-based Highly Active Antiretroviral Treatment (HAART) on the *polymerase* and *Surface* genes of the HBV.

2. Study the effect of HIV co-infection on the development of HBV drug resistance and the genetic evolution in both *polymerase* and *Surface* genes.

3. Assess the efficacy of lamivudine-based HAART during 48 weeks within a HIV/HBV co-infected population from Malawi.

4. Study the genomic evolution within the *polymerase* and *Surface* genes after 48 weeks of lamivudine-based HAART among the Malawian population.

CHAPTER 2

Genetic variability of HBV in three different cohort from Kumasi, Ghana

1. Introduction

Ghana is a country located in West Africa which had an HIV prevalence of 3.6% in 2003 [150]. The HAART became available in 2004, consisting of a first-line therapy combination of 3TC with either zidovudine or stavudine and nevirapine or efavirenz. Although TDF is also available, it is usually reserved for using after failure of the initial regimen. HBV is also highly endemic, with 15% of population being chronically infected and usually occurs vertically/horizontally before the age of 10 years old [151]. The HIV/HBV co-infection is common in Africa, reaching a prevalence of up to 20% in some areas; however, these data have not been established in Ghana because HBV is not routinely screening. As a consequence, it is predicted that up to 50% of HIV/HBV co-infected patients have started antiviral treatment, including 3TC as a single anti-HBV agent. Using 3TC as a sole anti-HBV agent increases both the development of HBV drug resistance and progression of liver disease. Drug resistance development can reach up to 70% of population after 4 years of treatment of chronic HBV patients, however the percentage of resistance development can increase up to 90% after 4 years of treatment in HIV co-infected patients [3,38,45,46,152,153].

In general, in low-income countries, HBV transmission usually occurs during the childhood, by vertical or horizontal via. In Ghana, systematic HBV vaccination of new-borns started in 2003, and only a minority of adults has been immunized. Similarly to the drug resistance mutations into the *pol* gene, due to the overlapped genes, mutations can also be produced into the *S* gene, which could lead to a vaccine failure as it has already seen in Taiwan or China [88,89,154]. These mutations not only can affect to the vaccine since it could have more consequences as failure detection by commercial assays, change of

its infectivity capacity or modify the morphogenesis of the protein and, hence, its secretion [81,82,155]. One of these consequences could be associated to the OBI concept, which is defined as detectable HBV DNA in serum with undetectable HBsAg. The prevalence of OBI ranges from 0% to 89% in different HIV infected cohorts [104].

In this study we have established the prevalence of HBV from an HIV cohort in Ghana where most of the patients were under 3TC-based HAART. Furthermore, samples tested negative for HBsAg were also studied to establish the prevalence of OBI in this region. All samples with detectable HBV DNA were characterized in order to evaluate the development of drug resistance and the potential vaccine escape mutants. Finally, using a CHB cohort from the same region, the genetic evolution of HBV was studied to evaluate the potential effect of HIV on HBV.

2. Material and methods

2.1 *Study population*

Two different cohorts were evaluated from Ghana: HIV/HBV co-infected and chronic HBV monoinfected cohorts. The study was approved by the ethics committee of the Kwame Nkrumah University of Science & Technology, Kumasi, Ghana.

2.1.1 HIV/HBV co-infection

The study population comprised a cohort of 2138 HIV infected patients attending routine HIV care at the Komfo Anokye Teaching Hospital (KATH) in Kumasi, Ghana during 2007. Approximately, 50% of the patients were receiving 3TC-based HAART. Paired serum and plasma samples were collected from 838/2138 (39%) unselected HIV-infected patients and they were stored at -20ºC prior to shipping on dry ice to the Royal Free Hospital in London where they were kept at -70ºC before testing. Stored samples from a further 1300/2138 (61%) HIV-infected patients were tested for HBsAg using the rapid Determine assay. Of these, 178 samples tested HBsAg positive and were retrieved for repeat testing in London.

2.1.2 HBV monoinfection

Pregnant women and blood donors attending the KATH hospital in Kumasi, Ghana were screened for HBV by rapid test (Determine HBsAg/AgHBs; Abbott Laboratories). HBV positive samples were amplified and sequenced by Jean-Pierre Allain´s group from the Department of Haematology, University of Cambridge, Cambridge, UK. These cohorts had been fully studied previously [151,156,157].

2.2 *Serology*

Serum samples were tested for the presence of HBsAg using five different assays including three gold-standard assays and two rapid immunocromatography (lateral flow) assays. The gold-standard comprised the automated chemilumiscent immunoassays Architect HBsAg (Abbott Diagnostics, Maidenhead, UK) and Liason HBsAg Ultra (Diasorin, Bracknell, UK), and the manual Murex version 3 plate enzyme-immunoassay (Abbott Diagnostics). The rapid immunocromatography assays included Determine HBsAg assay (Inverness Medical, Storckport, UK) and the Vikia HBsAg assay (bioMerieux, Basingstoke, UK. All assays were performed according to the manufacturers' instructions. The HBV e antigen and anti-e antibody (HBeAg, anti-HBeAb), anti-HBcAb, and anti-HBsAb were measured by the Architect assay (Abbott Diagnostics) [158].

2.3 *HBV DNA Quantification*

The HBV DNA was quantified for both HIV/HBsAg+ (co-infected) and HIV/HBsAg-/anti-HBcAb+ (OBI) cohorts. Samples underwent nucleic acid extraction by the m2000sp automated system (Abbott Laboratories), followed by quantitative real time PCR with an in-house assay with a lower limit of quantification of 14 IU/mL. The assay was calibrated against the 2nd World Health Organization International standard for HBV DNA [159].

2.4 *HBV Amplification*

Samples with a detectable HBV DNA underwent population sequencing of the *pol gene* reverse transcriptase domain (amino acids 1-344) and the *S* gene (amino acids 1-226). Plasma DNA was extracted using the NucliSens EasyMag platmform (NucliSens, Biomereux, Netherlands). Both *pol* and *S* gene were amplified by two consecutive PCR using the primers HEPB1, HEPB2 and HEPBN (table 2.1). The first round amplification was performed in a volume of 50 μl, containing 1x AmpliTaq Gold buffer, 3 mM Mgcl2, 0.6 mM dNTPs, 0.2 μM each primer, 2.5 U AmpliTaq Gold and 5 μl DNA. The nested PCR was carried out with the suitable primers and 5 μl of the first round PCR product. The thermocycling conditions were set as follows: 94ºC for 3 minutes, 40 cycles of 94ºC 1 minute, 55ºC 1 minute and 72ºC 1 minute and a half, followed by a final extension of 72ºC for 7 minutes. A nested PCR was carried out in the same conditions as before but with 25 cycles.

2.5 *Purification*

Samples were purified prior to sequencing on the Amicon 2ml ultra centrifugal filters (millipore). 300 μl of sterile nuclease-free water were placed into each filter unit. The 50 μl PCR product was placed onto the top of the water in the spin column and centrifuged at 500xg for 15 minutes at room temperature. Thereafter, 35 μl of sterile nuclease-free water was added to each filter unit. The filter unit was inverted into a clean labelled 1.5 ml centrifuge tube and centrifuged at 500xg for 5 minutes at room temperature. Finally, the spin filter was removed and discarded.

2.6 *Sequencing*

The amplified products were separated by electrophoresis on 1.0% agarose gel and detected by SYBR® Safe DNA Gel Stain (Invitrogen) using an UV transluminator. Those samples that gave the expected size band were sequenced with four overlapping primers (HEPB2, HEPBSQ, HEPBN and HEPB1731 (table 2.1). The round amplification was performed in 4 different tubes in a volume of 20 μl, containing 9 μl water RNAase free, 2 μl Big Dye, 1 μl primer and 8 μl PCR product. The thermocycling conditions were set as follows: 25 cycles, first of all 96ºC for 10 minutes followed by 5 minutes at 50ºC and 4 minutes at 60ºC. The sequencing was carrying out according to the manufacturer's instructions and using an ABI Prism 3730 sequencer (Applied Biosystems). Sequences obtained were aligned with the distinct HBV genotypes obtained from GeneBank and using the SeqScape v2.6 software (Applied Biosystems).

Primers	Sequence (5'-3')
HEPB1	GCC TCA TTT TGT GGG TCA CCA TA
HEPB2	TCT CTG ACA TAC TTT CCA AT
HEPBN	TTG GGG AGC CCT CAG GCT
HEPBSQ	TTG GCC AAA ATT CGC AGT C
HEPB1731	CTC CTG CCT CCA CCA ATC

Table 2.1 Primers used to amplify and sequence the HBV *pol* and *S* genes. HepB1, HepB2 and HepBN were used to amplify the DNA and all of them, except HepB1, were used for sequencing the samples.

2.7 Analysis

2.7.1 Genetic characterization of pol/S genes

HBV drug resistance to NAs is defined as selection of HBV strains with amino acid variations known to confer reduced susceptibility to the administered NAs. Cross-resistance was considered when variants selected affect the susceptibility of drugs to which patients have never been exposed. Mutations scored included both *pol* and *S* genes and were taking into consideration according with geno2pheno database and bibliography [47,67,160,161]. Mutations in the *pol gene* were associated to: (i) LdT and 3TC-resistance: rtL80I/V, rtV173L, rtL180M/C, rtA181T, rtM204I/V/S [48–51]; (ii) ETV-resistance: combination of rtI169T or rtT184A/G/I/S or rtS202G/I or rtM250V plus rtL180M/C and rtM204I/V. It was also associated with rtI169T, rtT184A/G/I/S, rtS202G/I, rtM250V [55,56]; ADV-resistance: rtA181T/V, rtQ215H, rtI233V, rtN238T and rtN236T [53,54]; (iv) TDF-resistance: rtA194T but it is not completely clear [57–60]. Mutations in the *S* gene associated with immunologic or vaccine escape were associated with sY100C, sQ101KH, sI110L, sT118MRK, sP120AST, sT126NS, sA128V, sQ129HR, sG130DNS, sN131IKT, sM133I, sF134L, sP142LS, sT143M, sD144EA, sG145R [61,87,122–125,127,130,162]. The combination sE164D + sI195M is associated to triple 3TC-resistance mutation and has been shown that produce an immunologic escape similar to sG145R [124]. In general, any variation inside the AGL of the HBsAg was considered as potential immune escape although it has not been shown its effect *in vitro* yet.

2.7.2 Statistical Analysis

The Fisher's exact test was used to compare the number of samples that developed drug resistance and the immune escape mutants among co-infected, OBI and monoinfected populations.

2.7.3 Shannon Entropy

Total Shannon entropy was assessed using BioEdit version 7.0.9.0 (URL: http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and comparative analyses between alignments were conducted using the Los Alamos website (URL: http://www.hiv.lanl.gov/content/sequence/ENTROPY/entropy.html).

The population selected for this study comprised 138 co-infected patients including 40/138 (29%) naïve patients; 39 OBI patients, including 2/39 (5%) naïve patients and 30 HBV monoinfected patients. T-Student test was used for determining the cumulative entropy among each codon at each population. Site by site comparisons of codon selective pressure were performed following removal of duplicate sequences using FEL in CompareSelectivePressure.bf in the HyPhy package.

3. Results

3.1 Study population

1016 out of 2138 HIV patients from routine HIV care at the KATH hospital in Kumasi (Ghana) were selected for HBsAg screening. 318 (31%) patients were positive for HBsAg whereas 84 (26%) of them were also HBeAg positive (table 2.2 & 2.3). HBV DNA was detected in 212 (67%) among co-infected cohort with a median [range] 19073 [23 – $10.0x10^{13}$ IU/mL]. 202 (97%) were successfully sequenced being genotype E predominant (98%) over the genotype A (2%). Among the remaining 698 (85%) HIV/HBsAg negative patients, 555 (80%) were anti-HBcAb positive and 111 samples had detectable DNA which gave a prevalence of OBI of 20%, similar to previous studies in Africa [163–166]. The HBV DNA median [range] was 68 [15 – $56.0x10^{3}$] IU/mL in this group, which was lower than in the co-infected cohort. As the HBV DNA was low, only 39 of the 111 (35%) could be sequenced. All 39 of the OBI samples were genotype E. The lowest HBV DNA level that yielded a sequence was 34 IU/mL.

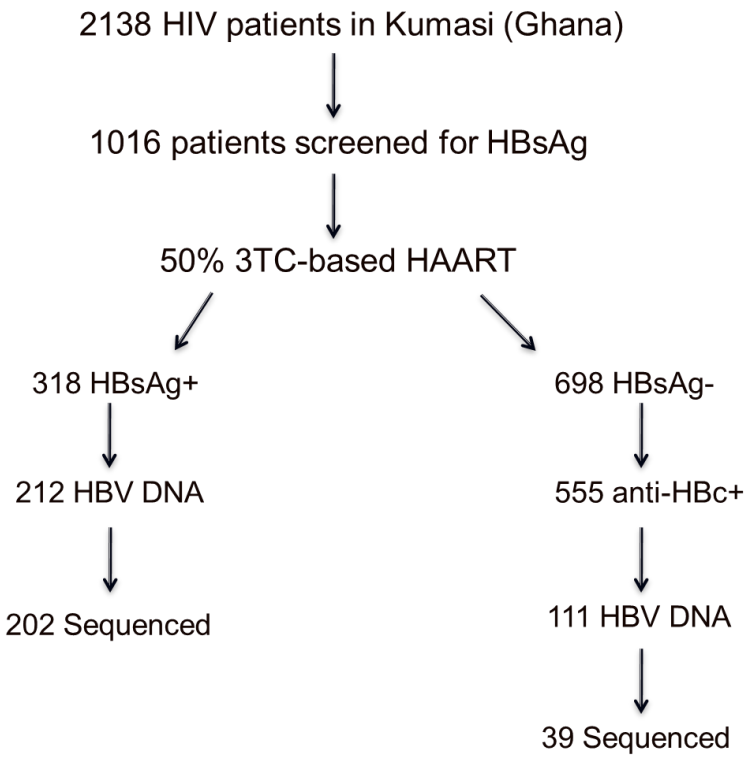

Table 2.2 Study population from Kumasi, Ghana.

	HIV/HBsAg+ (co-infected)	HIV/HBsAg-/anti-HBcAb+ (OBI)
Samples	318	555
HBeAg positive	84 (26%)	NA
HBV DNA >14 IU/mL	212 (67%)	111 (20%)
HBV DNA load median	19073 IU/mL	68 IU/mL
(range)	(23 - 10.0E+13) IU/mL	(15 - 56.0E+3) IU/mL
Samples sequenced	206/212 (97%)	39/111 (35%)
HBV genotype	E (98%), A (2%)	E (100%)

Table 2.3. Characteristics of the study cohorts. Serological and molecular characteristics according to HBsAg status in HIV patients.

3.2 Genetic characterization of pol/S genes from HIV/HBsAg positive (co-infected) patients

206 out of 212 HBsAg positive samples with detectable DNA were sequenced to study the genetic evolution after long-term treatment based on 3TC. *Pol* drug-resistance mutations were detected in 18/206 (9%) patients, 13 (72%) receiving 3TC based HAART and 5 (28%) naïve patients (table 2.4). The mutational pattern reflected predominantly 3TC exposure, and comprised rtM204V/I either alone (n = 1, 8%) or with rtL180M (n = 4, 30%), rtV173L+ rtL180M (n = 7, 54%) or rtL80I (n = 1, 8%). One naïve patient showed 3TC resistance (20%), rtV173L+ rtL180M + rtM204V. Moreover, there was a drug-naïve patient with the associated resistance to TDF, rtA194T plus rtV173L. Two patients harboured rtQ215H and another one presented rtN238T previously implicated in ADV failure but with uncertain effects on drug susceptibility. With regard to the variability of the *S* gene in these patients, major immune escape mutations that have previously been described were found, included well-recognized mutants such as sD144AE (n = 1, 8%), sI195M (n = 1, 8%), and sE164DG + sI195M (n = 2, 16%) and other less recognized as sG159A + sW196L (n = 1, 8%), sL127V + sW196L (n = 1, 8%), sW196L + sI195M (n = 1, 8%) and sD144E + sE164DG + sI195M (n = 1, 8%) in the 3TC based HAART patients (table 2.4).

Immune escape mutations were also detected in the subset of 188 HBsAg positive patients who were not developed drug resistance mutations in *pol* (table 2.5). These include sI110L (n = 4, 2%), sP120AT (n = 3, 2%), sT123A (n = 2, 1%), sQ129H (n = 1, 1%), sM133IT (n = 2, 1%), sS143L (n = 3, 2%), sD144AE (n = 5, 3%), sG145R (n = 3, 2%). It also appeared combinations as sP120T + sT126I (n = 2, 1%) or sE164D + sI195M (n = 4, 2%).

Patient	HAART	HBV DNA (IU/mL)	*polymerase* Gene	*Surface* Gene
1	3TC	6.60E+04	rtM204I	sG159A sW196L
2	3TC	3.90E+02	rtL180M rtM204V	sD144E
3	3TC	1.3E+04	rtL180M rtM204V	-
4	3TC	1.8E+06	rtL180M rtM204V	-
5	3TC	4.40E+07	rtL180M rtM204V	sI195M
6	3TC	1.40E+02	rtL80I rtM204I	sL127V sW196L
7	3TC	2.6E+04	rtV173L rtL180M rtM204V	sW196L sI195M
8	3TC	2.1E+07	rtV173L rtL180M rtM204V	
9	3TC	1.2E+03	rtV173L rtL180M rtM204V	sE164G sI195M
10	3TC	2.0E+04	rtV173L rtL180M rtM204V	sE164G sI195M
11	3TC	9.50E+03	rtV173L rtL180M rtM204V	sD144E sE164V sI195M
12	3TC	4.80E+02	rtV173L rtL180M rtM204V	-
13	3TC	3.30E+06	rtV173L rtL180M rtM204V	sS132F
14	Naïve	4.70E+05	rtV173L rtL180M rtM204V	sE164D sI195M
15	Naïve	4.50E+03	rtV173L rtA194T	-
16	Naïve	1.10E+07	rtQ215H	sT126I
17	Naïve	1.80E+06	rtQ215H	sT126I
18	Naïve	2.60E+05	rtN238T	sT125M

Table 2.4 Mutations within the *pol* and *S* genes related to antiviral resistance and immune escape in the co-infected cohort. The treatment and HBV-DNA levels from each patient are also indicated.

Mutation	HBsAg+		Mutation	HBsAg+	
	n	%		n	%
sQ101H	1	1	sN146D	1	1
sM103I/K	2	1	sE164G	2	1
sP105R	1	1	sI195M	2	1
sV106F	1	1	sM103K sL104M	2	1
sL109V/P/Q	5	2	sL109P sT115N	1	1
sI110L	4	2	sL110I sE164G	1	1
sP120A/T	3	2	sT116N sS117I	1	1
sK122R	1	1	sP120T sT126I	2	1
sT123A	2	1	sL127R sS143L	1	1
sT125M	2	1	sL127V sW196L	1	1
sT126I	5	2	sT131S sG145R	1	1
sT126N	1	1	sG159A sW196L	1	1
sQ129H	1	1	sK160N sE164V	1	1
sT131N	2	1	sE164G sI195M	2	1
sS132F	1	1	sE164D sI195M	2	1
sM133I/T	2	1	sI195M sW196L	1	1
sT140S	2	1	sQ101H sT126N sI128V	1	1
sS143L	3	2	sQ129P sP142L sG145K	2	1
sD144A	3	2	sD144E sE164V sI195M	1	1
sD144E	2	1	sI150T sK160R sF161Y sE164G	3	2
sG145R	3	2			

Table 2.5 Prevalence of mutations associated to immune escape within the *S* gene in the co-infected cohort. Include major immune-escape (bold) and other MHR mutations.

3.3 Genetic characterization of pol/S genes from HIV/HBsAg-/anti-HBcAb+ (OBI) patients

Despite the high number of OBI samples (111), it was possible to sequence just 39 samples due to the low viral load of the samples. 31% of this population contained mutations in the *pol gene*, 17% of which were primary drug resistance mutations and the remaining were drug-associated resistance mutations. Most of the patients harboured more than 2 mutations inside the AGL of *S* gene which could explain the seronegativity of the HBsAg (n = 19, 49%) (Appendix 2.1). Two patients showed primary 3TC-resistance mutations, yielding a prevalence of 5.1% (table 2.6). Seven patients (18%) showed polymorphisms associated to ADV-resistance (Q215H in 5 and N236I and N238H in the others 2), while two other patients (5%) showed I169L. The *S* gene showed some different patterns or combinations of mutations, i.e., sI110L + sT125M + sT126I + sD144E, sE164G + sI195M, sT126I + sE164G, sS143L + sE164D + sI195M or sS143L + sD144E.

In the other 30 samples sequenced, no drug resistance mutations were found but other variations/polymorphisms were detected. Overall, there was more than one variation in both *pol* and *S* genes that could also help explain the failure to detect HBsAg (Appendix 2.1). Mutations were accumulated along the whole length of the S gene, even outside the AGL. These samples included strains harbouring the following mutations: sI110L (n = 2, 4%), sC139S (n = 1, 2%), sG145A (n = 1, 2%), sS143L/sD144E (n = 1, 2%) or sD144E/sG145R (n = 1, 2%) among others. Other samples contained a greater number of mutations within the AGL, including strains harbouring sQ101H + sS143L + sD144E (n = 1, 2%), sQ129H + sM133I + sF134L + sE164A (n = 1, 2%) or I110L + sP120S + sM133L + sF134I + sD144A (n = 1, 2%).

Patient	HBV DNA (IU/mL)	*polymerase* Gene	*Surface* Gene
1	593	rtV173L rtQ215H	sQ101K, sT125M, sL127P, sS140L, sD144E, sE164V,
2	393	rtN236I	sQ101H, sT125M, sL127P, sE164A
3	325	rtV173M	sI110L
4	263	rtQ215H	sF134L
5	230	rtI169L	sY100C, sQ101H, sI110L, sT116N, sT125M, sT126I,s L127P, sT131A, sD144E
6	170	rtL80I rtL180M rtM204V	sE164G, sI195M
7	166	rtQ215H	sQ101H, sT126I, sE164G
8	155	rtV173L rtL180M rtM204V	sL127P, S143L, E164D, I195M
9	152	rtQ215H	s L127P, sS143L, sD144E
10	139	rtQ215H	sE164V
11	99	rtN238H	Y200Stop
12	77	rtI169L	sQ101H, sI110L, sS117I

Table 2.6 Mutations in both *pol* and *S* genes corresponding to antiviral resistance and immune escape in the OBI cohort. HBV-DNA level from each patient is also indicated.

Mutation	HBsAg⁻		Mutation	HBsAg⁻	
	n	%		n	%
sI110L	2	4	sR122P sT126I sQ129H	1	2
sC139S	1	2	sS143L sE164G sS167L	1	2
sG145A	1	2	sS143L sE164D sI195M	1	2
sS154A/V	1	2	sS154L sE164G sI195M	1	2
sM103I sE164V	1	2	sQ101H sT126I sI150T sE164G	1	2
sL109R sS114A	1	2	sQ101R sT125M sK160R sE164G	1	2
sS113T sE164V	1	2	sK122R sE164V sS167L sV168A	1	2
sT126I sQ129H	1	2	sQ129Hs M133I sF134L sE164A	1	2
sS143L sD144E	1	2	sQ101H sI110L sS117I sK160N sW165L	1	2
sD144E sG145R	1	2	sM103I sF134L sS136L sS154P sE164G	1	2
sQ101R sR122K sT123A	1	2	sI110L sP120S sM133L sF134I sD144A	1	2
sQ101R sT125M sE164A	1	2	sQ101H sQ129R sF134S sS154L sK160R sE164G	1	2
sQ101R sS136L sS154L	1	2	sL109I sQ129H sD144E sS154L sG159V sS167L	1	2
sQ101H sS143L sD144E	1	2	sQ101H sI110L sT116N sT125M sT126I sT131A sT148I sD144E sN146S sK160N/S sE164G sW165L sS167L sR169H	1	2
sG112R sT126I sS154P	1	2			

Table 2.7 Prevalence of main mutations associated to immune escape in the AGL in the OBI cohort. It includes the major immune-escape (bold) and other mutations inside AGL.

3.4 Genetic Variability (Shannon entropy)

The entropy method is based on the nucleotide diversity along the genome. As entropy can be used to study variation at individual nucleotide positions, the entropy was divided into the p1s3, p2s1 or p3s2 positions to give an indication as to whether variation is being directed at *pol*, *S* or both genes. Comparison of sequence entropy between populations identified sites with significantly different entropies. The proportion of p3s2 sites with differential entropies was greater when HBV monoinfected and co-infected cohorts were compared suggesting positive selection on the *S* reading frame, then a substitution at p3s2 is more likely to be non-synonymous. Similarly, substitutions at p1s3 are more likely to cause a non-synonymous change in *pol* (Figure 2.1 and Appendix 2.4).

The median viral load in each group was: $4.5x10^6$ [14 – $3.6x10^8$] IU/mL for (HIV-/HBsAg+) HBV monoinfected cohort; $1.5x10^4$ [74 – $1.0x10^4$] IU/mL for (HIV+/HBsAg+) co-infected cohort and 148 [29 – $7.2x10^4$] IU/mL for (HIV+/HBsAg-/anti-HBc+) OBI cohort (Table 2.7). The number of samples with drug resistance-associated mutations was: 1/30 (3.3%) for HBV monoinfected; 13/138 (9.4%) for co-infected cohort; 6/39 (15.4%) for OBI cohort. The immune escape-associated mutations were: 3/30 (10%) for HBV monoinfected cohort; 31/138 (22.4%) for co-infected cohort; 17/39 for OBI cohort (43.6%). Interestingly, the rate of mutations associated to VEM form the OBI cohort was significantly higher than the other two cohorts ($p = 0.0029$ compared to HBV monoinfected cohort and $p < 0.001$ compared to co-infected cohort) (Table 2.8).

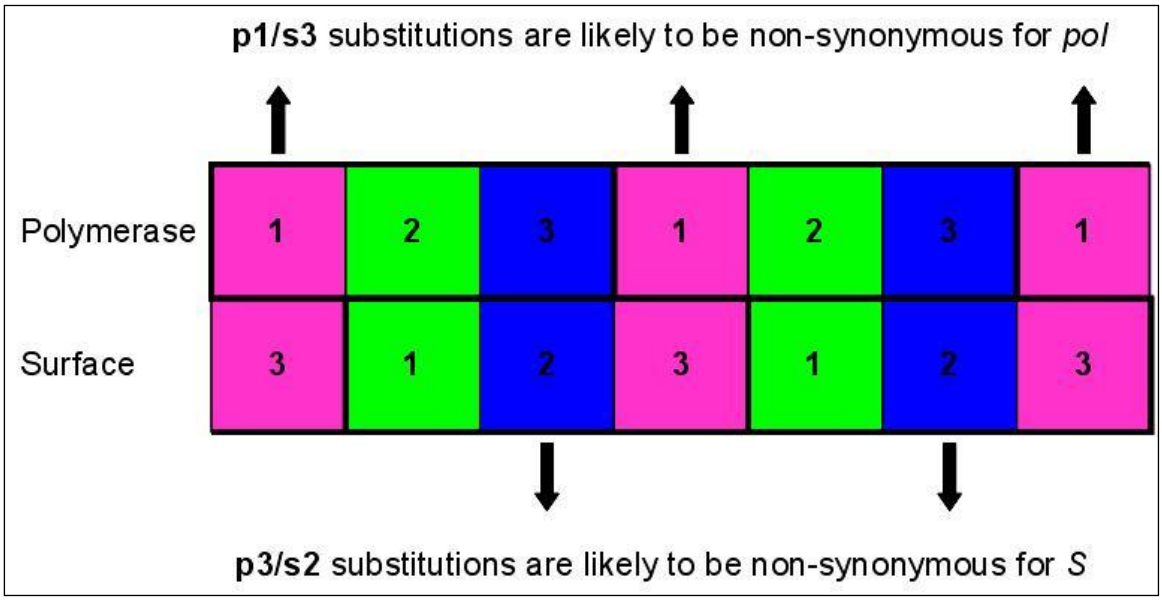

Figure 2.1 Distribution of codon positions within *pol* and *S* genes according with their ORFs. The first nucleotide in *pol gene* coincide with the 3 nucleotide in the *S* gene (p1s3, pink), the second one with the first of the *S* gene (p2s1, green) and the last one coincide with the second in the *S* gene (p3s2, blue).

Cohort	Median (IU/mL)	Range (IU/mL)	Drug-resistance mutation in *pol gene*	Immune escape mutations in *S* gene
HBV monoinfected	4.5×10^6	$14 – 3.6 \times 10^8$	1/30 (3.3%)	3/30 (10%)
Co-infected	1.5×10^4	$74 – 1.0 \times 10^{14}$	13/138 (9.4%)	31/138 (22.4%)
OBI	148	$29 – 7.2 \times 10^4$	6/39 (15.4%)	17/39 (43.6%)

Table 2.7 HBV DNA level and range, drug resistance and immune escape mutations observed among the three different cohorts; HBV monoinfected, co-infected and OBI cohorts.

	HBV monoinfected	co-infected	p-value
Drug resistance	1/30, (3.3%)	13/138 (9.4%)	0,4686
VEM´s	3/30 (10%)	31/138 (22.4%)	0,1410

	HBV monoinfected	OBI	p-value
Drug resistance	1/30, (3.3%)	6/39 (15.4%)	0,1283
VEM´s	3/30 (10%)	17/39 (43.6%)	**0,0029**

	co-infected	OBI	p-value
Drug resistance	13/138 (9.4%)	6/39 (15.4%)	0,3773
VEM´s	31/138 (22.4%)	17/39 (43.6%)	**<0,001**

Table 2.8 Development of drug resistance and inmune escape mutations in each group; HBV monoinfected, co-infected and OBI cohorts. There is statistic difference in the variability of the S gene when OBI cohort was compared with the other two ($p < 0.05$).

The total entropy was measured for each nucleotide (p1s3, p3s2, p2s1) respect to the *S* gene, in total 226 codons or amino acids (Appendix 2.2). The HBV monoinfected cohort showed less variability than the other two populations finding the highest entropy in this group located around codons 25 to 80, which corresponds to the first cytosolic loop (CYL-I), including the T-cell domain. In the co-infected cohort, the total entropy showed higher entropy in the codons 100 to 150 which indicates a higher variability in the AGL, including the B-cell epitope. The OBI cohort showed more variability than the co-infected patients inside the AGL, with the affected area stretching to codon 190, affecting the whole region of the HBsAg that is exposed to the cytoplasm. In all cases, the variability stems mainly from the codon p3s2.

By comparing the total mean entropy at each codon position in the different cohort we found the greatest entropy in the p3s2 codon (blue bar in the Figure 2.2 and Appendix 2.3). Comparison of the different entropies (p2s1, p3s2, p1s3 and total) showed significant differences between HBV monoinfected cohort versus both co-infected and OBI groups ($p < 0.0001$). However, when the co-infected and OBI cohorts were compared, it was significance just the total entropy ($p < 0.001$). Furthermore, when HBV monoinfected and co-infected naïve cohorts were evaluated, it was found a difference in all entropies expect the codon entropy p1s3.

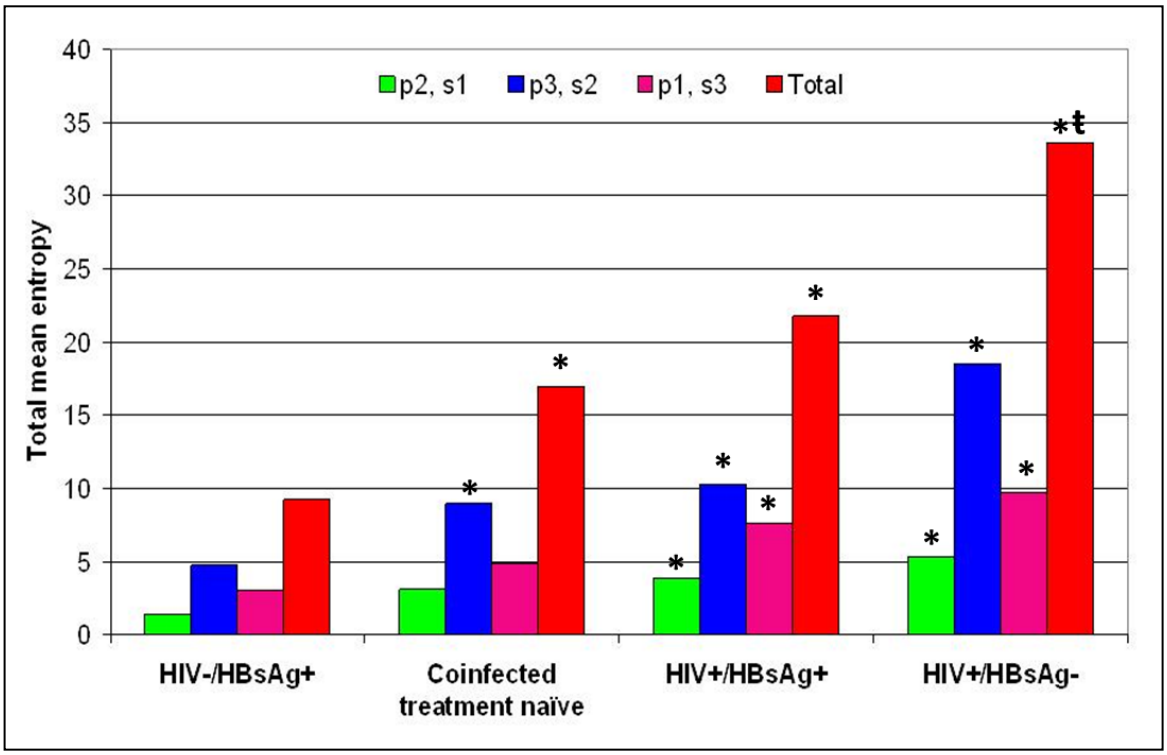

Figure 2.2 Representation of the total mean entropy at each codon position (green, blue and pink bars) and total entropy (red bars). Total entropy significantly increased when comparing the different populations. * Higher entropy respect to the monoinfected group; ŧ Higher entropy respect to the co-infected treated group (*ŧ p value < 0.05).

Next, we conducted a pairwise comparison of the three populations at specific nucleotide positions to determine sites where a significant difference in entropy occurred. The most variable nucleotide position was p3s2 (n = 14) followed by p1s3 (n = 7) and p2s1 (n = 3) (Appendix 2.5). The number of nucleotides with significantly different entropies in the p3s2 position, compared to p1s3 and p2s1, was greater when the OBI cohort was compared to the co-infected one. As mutations in p3s2 were more likely to cause non-synonymous substitutions in the *S* gene, it suggested that was under diversifying selective pressure or was more tolerant of non-synonymous substitutions in the OBI cohort. Figure 2.3 depicts some of the nucleotide sites with significantly different entropies with the corresponding codon in *pol* and *S*. When HBV monoinfected cohort was compared either to co-infection or OBI cohorts, there were three positions in common, all p3s2 positions within *S* codons: 64, 127 and 164. An additional p3s2 position in *S* codon 56 has differential entropy when HBV monoinfected and co-infected cohorts were compared. Comparison of the OBI cohort against HBV monoinfected ones showed four additional p3s2 sites in the *S* codon position 10, 59, 71 and 181. On the other hand, the number of affected nucleotide positions was higher when the co-infected and OBI cohorts were compared. Nucleotides with significantly different entropies were located in codon 10, 56, 101, 127, 134, 143, 154, 164, 174, 177, 203, 207 and 220 of S. It was also noticeable that positions 10, 101, and 154 had differential entropies at 2 out of the 3 possible positions within its codon.

HIV+/HBsAg+ vs HIV+/HBsAg-

nt Position		*pol* codon	Surface codon
53	p2, s1	18	10
54	p3, s2	18	10
192	p3, s2	64	56
327	p3, s2	109	101
328	p1, s3	110	101
405	p3, s2	135	127
427	p1, s3	143	134
453	p3, s2	151	143
485	p2, s1	162	154
486	p3, s2	162	154
516	p3, s2	172	164
546	p3, s2	182	174
555	p3, s2	185	177
633	p3, s2	211	203 *
645	p3, s2	215	207
684	p3, s2	228	220

* Increased entropy in HBsAg positive coinfections

HIV-/HBsAg+ vs HIV+/HBsAg+

nt Position		*pol* codon	Surface codon
192	p3, s2	64	56 *
216	p3, s2	72	64 *
405	p3, s2	135	127
516	p3, s2	172	164

* Increased entropy in HBV monoinfection

HIV-/HBsAg+ vs HIV+/HBsAg-

nt Position		*pol* codon	Surface codon
53	p2, s1	18	10
201	p3, s2	67	59
216	p3, s2	72	64 *
238	p1, s3	80	71
405	p3, s2	135	127
516	p3, s2	172	164
567	p3, s2	189	181

* Increased entropy in HBV monoinfection

Figure 2.3 Sites with significant differential entropy. A selection of all the sites with differential entropies, appendix 2.2 has the full list. Green, blue and pink represent p2s1, p3s2 and p1s3 respectively; i) co-infected vs. OBI, ii) HBV monoinfected vs. co-infected and iii) HBV monoinfected vs. OBI.

We also analysed the site by site selection of codons in both *pol* and *S* genes between the different cohorts (Table 2.10). In the HBV monoinfected cohort we found that amino acids rtE1, rtR110 and sS64 were positively selected (non-synonymous substitutions) with respect to the co-infected cohort and rtR110 was positively selected when compared to the OBI cohort. In contrast, rtQ215 and sQ101 were non-synonymous substitutions in the co-infected and OBI cohorts, whereas sG185 was only a non-synonymous substitution in the co-infected cohort, in comparison to HBV monoinfected one. When co-infected and OBI cohorts were compared, the amino acids rtL66, rtL80, rtS117 and sS154 were statistically more variable in the last group. Finally, when the non-synonymous were compared in the co-infected naïve cohort with the co-infected cohort on treatment and the HBV monoinfected ones, in the co-infected cohort treated, the amino acids rtV112, sI4, sS117 appeared as non-synonymous whereas the sQ101 appeared as non-synonymous substitution in the HBV monoinfected cohort.

	Polymerase Gene		Surface Gene	
	HIV+/HBsAg+	HIV+/HBsAg-	HIV+/HBsAg+	HIV+/HBsAg-
HIV-/HBsAg+	rtE1* rtR110* rtQ215	rtR110* rtQ215	sS64* sQ101 sG185	sQ101
	HIV+/HBsAg-		HIV+/HBsAg-	
HIV+/HBsAg+	rtL66 rtL80 rtS117		sS154	
	Co infected on treatment	HIV-/HBsAg+	Co infected on treatment	HIV-/HBsAg+
Co infected naive	rtV112		sI4 sS117	sQ101

* Non Synonymous substitutions in HBV monoinfected samples

Table 2.10 Mutations selected at the *pol* and *S* genes when the selective pressure at the codon level was compared for the HBV monoinfeced, co-infected and OBI cohorts.

4. Discussion

This study evaluates HBV prevalence within a HIV population from Kumasi (Ghana) and the impact of long-term exposure to a lamivudine-based therapy on the HBV polymerase and surfaces genes. We established an overall prevalence of HBV infection over a population of 6000 HIV-infected patients of 15% (n=318). The phenomenon of occult HBV infection (OBI cohort), which is characterized by HBsAg serum negative and low HBV DNA was observed in 20% (n=111) of HIV infected patients. In both cases, the prevalence is higher than previous data from Sub-Saharan Africa [163,164]. The HBV genotype E was the most prevalent in both cohorts, as previous reported and only 2% of co-infected patients were genotype A [157,167]. A high prevalence of HBV drug resistance to NAs was observed at the HBV polymerase in both co-infected and OBI populations (9% vs. 31%, respectively). Similarly, high rate of mutations at the HBsAg was observed in both cohorts of patients (20% vs. 43.5%, respectively). Finally, the analyses of the genetic variability within *pol and S* genes demonstrated a higher variability within HIV/HBV co-infected compared with HBV monoinfected patients. Interestingly, the genetic variability was even higher in the OBI population which might explain the lack of HBsAg detection by diagnostic assays.

The prevalence of chronic Hepatitis B in Ghana has been previously estimated in 15% and it usually occurs vertically/horizontally during the childhood, similarly to other countries in Africa [151]. Furthermore, HIV infection is also common in Ghana (3.6% in 2003) [150]. However, no data has been shown about prevalence of HIV/HBV co-infection due to the lack of routine test for HBV in this area and, in general, in Africa. Nevertheless, due to the high prevalence of HIV/HBV co-infection found in other African countries (up to 20%), a high prevalence could be also expected for the Ghana

population. From a total of 6000 HIV positive patients under clinical follow-up in Kumasi, we have established for first time an overall prevalence of HIV/HBV co-infection of 15%. Additionally, we found a prevalence of HBV occult infection of 20%, which pose the risk of severe HBV reactivation as it has been previously documented [32,104,165].

The lack of routine HBV testing among the HIV population is the main cause of the large number of patients receiving long-term treatment including 3TC as the only active drug against HBV infection. The use of 3TC monotherapies against HBV has been shown to be inadvisable in HIV/HBV co-infection because favoured the faster development of HBV drug resistance [153]. Indeed, several studies have reported that HBV drug resistance emerged faster in HIV/HBV co-infected (90%) compared with HBV monoinfected (67%) after 4 years of 3TC-monotherapy treatment [3,38,45,152,153]. The selection of mutations at both *pol* and *S* genes is generally associated with poor clinical outcomes due to flares on liver enzyme, cross-resistance, transmission of drug-resistant strains, development of occult forms of chronic hepatitis B and evasion of vaccine protection [168]. Therefore, it is important the early detection of virological failure and the detection of resistance mutations in HIV/HBV co-infected patients under 3TC-based therapies. This issue is of especial interest in countries such as Ghana with limited treatment options. Overall, the prevalence of resistance mutations within the *pol* gene was high for both co-infected and OBI cohorts (9% vs. 31%). In the co-infected cohort, all mutations were related to 3TC (rtL80I, rtV173L, rtL180M or rtm204IV) and in the OBI cohort was also found mutations associated with resistance to other NAs (rtI169L, rtQ215H, rtN236I, rtN238H). It was also found the mutation rtA194V in combination with rtV173L. This mutation has been previously identified in two HIV/HBV co-infected patients failing TDF in combination with rtL180M and rtM204V. However, subsequent *in*

vitro assays could not confirm its association with TDF resistance. Currently, this is still matter of discussion among scientists [57–60].

Regarding the *S* gene, a high prevalence of major immune escape mutants was observed in the OBI than in the co-infected cohort (20 vs. 43.5%, respectively). These included the most common vaccine escape mutants, sD144E/A or sG145R/A and other as sQ101HR, sQ129H and sF134Y. While mutations sQ101H and sQ129H were associated with vaccine breakthrough, mutations sF134Y in combination with sI110L and sT126I was identified previously in OBI patients [169,170]. Overall, we found multiple amino acids substitutions inside the AGL region. These results are agree with those obtained in previous studies that found an average of 4.29 residues substitutions within the AGL that was also associated with OBI [170,171]. However, in another study performed in Ghana population among HBV genotype E patients, the AGL was mainly wild type [157]. Therefore, the high number of mutations within the *S* gene found might explain the lack of HBsAg detection among the OBI cohort either due to impair viral fitness or by an impaired HBsAg secretion/antigenicity.

The HBV overlapped genome produce restriction in the HBV variability since the selection of specific mutations within HBV polymerase or S genes must generate infectious HBV particles. In this context, mutations selected at the polymerase could enhance the HBV viral fitness and produce at the same time a different folding in the HBsAg leading to escape from the immune response. These mutations would provide an advantage over the *wt* and would be dominant after a while. Interestingly, we found a naive patient harbouring the triple mutant rtV173L, rtL180M, rtM204V which leads to a double mutant sE164D and sI195M. This mutation pattern is associated with resistance to 3TC and FTC and the mutations within the *S* gene confer an immunological evasion

similar to sG145R, which is one of the most common vaccine escape mutant described so far [127]. This finding suggest the transmission of HBV primary resistance as previously has been described in newly diagnosed HIV patients [148,149]. Therefore, there is a potential risk of transmission of 3TC-resistance strains mainly in areas where HBV and HIV are highly prevalent. Furthermore, the long-term exposure to a 3TC-based might enhance this problem taking into account the high rate of resistance to 3TC found in this population.

The HBV genetic evolution varied depending on whether infection is acute or chronic due to the different immune pressure. This should be more critical in HIV/HBV co-infected patients since HIV infection does alter the course of HBV infection and HIV/HBV co-infection is associated with higher rates of HIV replication. However, little is known about the role of HIV on the HBV genetic evolution. We evaluated the HBV genetic variability into *pol and S* genes in both HIV/HBV co-infected and a HBV monoinfected cohort in Ghana. The Shannon entropy method was used for this purpose, which is a measure of the ability to guess what nucleotide or amino acid would be in the next sequence you took from the population. The entropy was found significantly different when HIV was present; this influence of HIV in the HBV variability was mainly in the codon p3s2, within both *pol* and *S* genes. The variability observed in p3s2 suggests that *S* gene is able to produce more non-synonymous substitutions, which could explain the high rate of OBI in our cohort. Furthermore, HBV genetic variability is also influenced by several factors including the lack of a proofreading function in the RT, overlapping ORF aforementioned and external factors which can vary from the race of the patient to the drug's characteristics (potency, adherence, long-term treatments, etc.) [19,30,67].

In summary, we have shown the problem of using 3TC as monotherapy against HBV infection in Ghana, due to the high level of HIV/HBV co-infection (15%) and occult HBV infection (20%). The lack of HBV screening in the routine diagnostic tests leads the widely use of a 3TC-based HAART among HIV/HBV co-infected patients favouring the selection of HBV drug resistance. Moreover, we found a high HBV genetic variability in HIV/HBV co-infected population compared with HBV monoinfected subjects. Thus, it would be recommend the implementation of HBV testing in the routine diagnostic tests and promote the use of alternatives therapies to 3TC to favor the virological success and to avoid the selection of resistance mutations.

CHAPTER 3

Clinical response & genetic evolution of HBV in a HIV/HBV co-infected population from Malawi receiving a 3TC-based HAART

1. Introduction

The Republic of Malawi is a small country located in southeast Africa bordered by Zambia, Tanzania and Mozambique. It is an endemic region for HIV where, in some areas, can reach up to 76% prevalence. Viral hepatitis is common in HIV individuals in Malawi and prevalence rates of HIV/HBV co-infection in Malawi have reached up to 16.9% in certain areas [172–174]. The natural history of HBV infection in HIV infected patients is characterized by high levels of HBV viremia, increased rates of chronic active infection, reactivation episodes and fibrosis progression. In addition, side effects caused by antiretroviral drugs such as hepatotoxicity are also increased [175,176]. Since HAART was established, hepatitis-associated liver disease has become one of the leading non-AIDS causes of mortality in HIV patients in both Europe and North America [177].

A HIV/HBV co-infected population was studied from the largest city in the country, Blantyre, located in the south of Malawi. The national Antiretroviral Therapy program for the treatment of HIV/HBV co-infection includes nevirapine, stavudine and 3TC as the standard first-line regimen. Nevirapine is known to cause hepatotoxicity, which is increased by viral hepatitis co-infection [178,179]. In the same way, Stavudine is related with hepatic steatosis that may compound liver toxicities [180]. 3TC is an antiviral drug active to both HIV and HBV; however, when it is used as sole agent against HBV, in both mono and co-infection, is able to produce a high rate drug resistance [3,38,45,153]. Previously, HAART toxicity, morbidity and mortality was studied in patients accessing this program for a year [181]. They showed that HIV virological failure was similar in HIV mono-infected and co-infected patients and reported hepatotoxicity as uncommon event using this regimen.

We have investigated the effect of 3TC used as sole antiviral drug against HBV infection under HIV infection. We quantified both HBV DNA and HIV RNA in the serum of patients at three different time points of the following up (week 0/untreated, 24 weeks and 48 weeks during treatment). We also evaluated the efficacy of treatment according to the HBeAg serostatus and finally we carried out the amplification and sequencing of *pol* gene of patients with detectable viral load in order to detect drug resistance mutations. Furthermore, we also analysed the *Surface* gene to characterize potential VEM´s.

2. Material and methods

2.1 Study

A longitudinal study was performed in a cohort recruited at the outpatient ART clinic of Queen Elizabeth Central Hospital (QECH) in Blantyre, in the southern region of Malawi, who started the standard first-line antiretroviral regimen (stavudine/lamivudine/nevirapine) in 2007-8. Three different time points (week 0, week 24 and week 48) were studied. Parameters analysed at each time point included HBsAg, and HBeAg serostatus, HBV virological failure, HBV suppression, HIV virological outcome and emergence of HBV drug resistance. In addition, Hepatitis Delta Virus (HDV) infection was tested within this cohort. The HIV/HBV co-infected patients were subsequently stratified as HBeAg positive or negative in order to evaluate the difference in treatment efficacy as well as rate of development of drug resistance in both subgroups.

2.2 *Serology.*

1117 serum samples from HIV patients from Blantyre were tested in the Royal Liverpool University Hospital in Liverpool, UK, for the presence of HBsAg using a sensitive EIA (Biokit, Barcelona, Spain). HBsAg positive patients (n = 133, 12%) underwent further testing at the Department of Virology at Royal Free Hospital in London. At this hospital, samples were tested for HBeAg and antibodies (anti-HBe) by the Architect assay (Abbott Diagnostics).and Hepatitis Delta Virus (HDV) by ETI-AB-DELTAK-2 (Diasorin).

2.3 HBV DNA & HIV RNA Quantification

HBV DNA was extracted from plasma samples by QIAsymphony Virus/Bacteria Midi Kit Kits (QIAGEN, Crawley, UK) and quantified by an in-house TaqMan real-time PCR assay. The in-house assay has been previously calibrated against the 2nd World Health Organisation (WHO) International Standard for HBV DNA and showed a lower limit of detection of 14 IU/ml [159].

HIV RNA was extracted from plasma by High Pure Viral RNA Kit (Roche Diagnostics Systems, Basel, Switzerland) and quantified by the Abbott Real Time HIV-1 assay with a limit of quantification of 40 copies/mL. However, some samples were diluted 1:5 previous HIV viral load testing as volume was insufficient otherwise. Therefore, the limit of quantification for such samples was increased to 200 copies/mL.

2.4 Amplification & Sequencing

Samples with detectable HBV DNA underwent population sequencing of the polymerase gene reverse transcriptase (RT) domain (amino acids 1 to 344) and the *S* gene (amino acids 1 to 226) as previously described (Chapter 2, page 46-48).

2.5 Analysis

2.5.1 Clinical response

Outcome associated parameters were defined as current guidelines, these are the EASL the AASLD [34,182]. HBV Virological response was defined as undetectable HBV DNA by real-time PCR assay within 48 weeks of therapy. HBV partial virological response was defined as a decrease in HBV DNA of more than 1 $\log_{10}$ IU/ml but detectable HBV DNA by real-time PCR assay at week 24 or 48. HBV virological failure was defined as a confirmed increase in HBV DNA level of more than 1 $\log_{10}$ IU/ml compared to the nadir HBV DNA level on therapy. Finally, HIV virological failure was defined as HIV RNA > 50 copies/mL at week 24 or 48.

2.5.2 Genetic characterization of *pol*/*S* genes

Analysis of drug resistance and VEM´s was done following the literature and databases as shown in the previous chapter (page 50).

2.5.3 Statistical

The HBV DNA and HIV RNA, measured as a continue variables, were compared using the Mann-Whitney U test. The Fisher's exact test was used to compare the other parameters of the study according to HIV or HBV data, including the number of patients who had viral load undetectable, had a partial response to the treatment, failed to the treatment or were HBeAg positive or negative. Multivariate analysis was carried out to establish the predictor of HBV treatment response. Differences were considered significant when P values were < 0.05. Statistical Analysis was performed using SAS Institute Inc., SAS 9.1 Cary, NC: SAS Institute Inc., 2008 (v 9.1, SAS Inst. Inc, PROC REG and AUTOREG).

3. Results

3.1 *Population and serology*

133 HIV patients were HBsAg positive out of 1117 from Blantyre, Malawi, establishing the prevalence of 12% in our cohort. Three time points were available for 33/133 (25%) patients, 53/133 (40%) patients had at least two time points and 47/133 (35%) patient had just one of the time points. Overall, we had 111/133 (83%) samples at week 0, 91/133 (68%) at week 24 and 50/133 (38%) samples at week 48 (Figure 3.1). The serology for HBeAg markers were carried out successfully in 125 patients (94%). The HBeAg distribution was as follow: 62/125 (49.6%) patients were HBeAg+/anti-HBe- and 39/125 (31.2%) were HBeAg-/anti-HBe+. Additionally, 2/125 (1.6%) patients were positive for both markers and 22/125 (17.6%) were negative for both. The serology for presence of HDV antibodies resulted in one positive patient out of 128 (0,8%). The genotypes distribution showed that most of the patients were found to be infected by genotype A1 96/97 (99%) and only 1/97 (1%) by genotype E (Table 3.1).

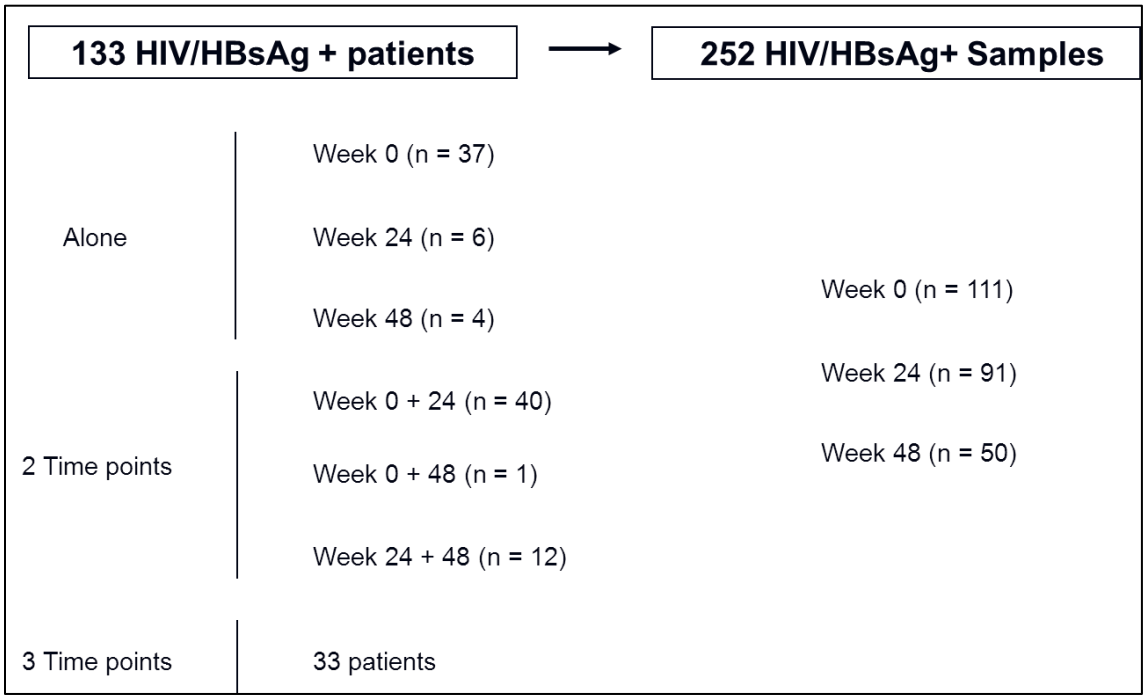

Figure 3.1 Patients and samples received from Blantyre, Malawi, via University of Liverpool, UK.

	Sample size		%
HBsAg positive patients	133		
HBeAg+/anti-HBe-	125	62	49,6
HBeAg+/anti-HBe+	125	2	1,6
HBeAg-/anti-HBe+	125	39	31,2
HBeAg-/anti-HBe-	125	22	17,6
HDV Ab	128	1	0,8
Genotype A1	97	96	99
Genotype E	97	1	1

Table 3.1 Serological characteristics for the entire HIV/HBsAg positive population. HBeAg serostatus, HDV co-infection and genotype characterization are shown.

3.2 *HBV and HIV viral load results*

HBV DNA was detected in 104/111 (94%) patients at week 0, with a median [range] of $21.3x10^6$ [$22.7x10^1$ - $63.5x10^7$] IU/mL; in 53/91 (58%) patients at week 24, with a median [range] of 43.4[14.0 - $67.4x10^4$] IU/mL; and in 20/50 (40%) patients at week 48, with a median [range] of 14.0 [14.0 - $38.5x10^7$] IU/mL. HIV RNA was detected in all patients tested (76/111, 68%) at week 0, 10/91 patients (11%) at week 24 and 4/48 (8%) patients at week 48. Evaluation of HBV DNA after 48 weeks showed that 60% of patients were responders to 3TC-based HAART (Figure 3.2). Moreover, the median HBV DNA quantified showed a statistically significant decrease from week 24 to week 48 ($p = 0.0001$). Despite these results, 32% of patients showed HBV virological failure at week 48.

The median [range] HIV viral load at each week was; 4.02 [2.75 - 2.07] $\log_{10}$ copies/mL at week 0, 2.3 [2.3 - 2.3] $\log_{10}$ copies/mL at week 24 and 2.3 [2.3 - 3.58] $\log_{10}$ copies/mL at week 48, respectively (Table 3.2). The evaluation of HIV RNA showed treatment response close to 90% at week 24 and up to 96% at week 48. Therefore, there was no difference between values of HIV viral load from week 24 to week 48 ($p = 0.3821$).

	n	Week 0	n	Week 24	n	Week 48	p-value
HBV DNA , *IU/ml, median (range)*	104	$21.3x10^{6}$ [$22.7x10^{1}$ - $63.5x10^{7}$]	53	43.4 [14.0 - $67.4x10^{5}$]	20	14.0 [14.0 - $38.5x10^{7}$]	**0,0001**
Patients with HBV DNA < 14 IU/ml, *number (%)*	111	7 (6.3)	91	38 (42)	50	30 (60)	0,0523
HBV partial virological response, *number (%)*	-	-	91	44 (48)	50	4 (8)	-
HBV virological failure, *number (%)*	-	-	91	-	50	16 (32)	-
HIV RNA , *Log copies/mL, median (range)*	76	4.02 [2.75 - 2.07]	91	2.3 [2.3 - 2.3]	48	2.3 [2.3 - 3.58]	0,3821
Patients with HIV RNA < 2.3 Log cp/mL, *number (%)*	-	-	91	81 (89)	48	44 (91.7)	0,7709
HIV virological failure, *number (%)*	-	-	91	-	48	4 (6.3)	-

Table 3.2 Clinical response to both HBV and HIV on the overall study population during 48 weeks.

3.3 HBeAg serostatus

We stratified the data according to the HBeAg serostatus in order to study in detail the response to HAART and the potential development of drug resistance (Table 3.3).

At week 0, patients who were HBeAg positive had higher median HBV DNA viral load ($p<0.0001$) than those that were HBeAg negative. In accordance to this result, the number of patient who had not HBV DNA detectable was higher in the HBeAg negative group (2% vs. 14%, $p = 0.0185$) (Figure 3.2). Regarding HIV status, all samples tested in both group had HIV RNA detectable with a similar viral load.

At week 24, the median HBV DNA viral load was still significantly lower in the HBeAg negative group ($p < 0.0001$) as well as the number of patients that had HBV DNA under the limit of detection, reaching 70% (9% vs. 70%, $p < 0.0001$). In this sense, the number of patients with partial virological response was higher in the HBeAg positive group because the higher viral load than HBeAg negative group (84% vs. 15%, $p < 0.0001$). Furthermore, due to the higher level of viral load, this group also showed a higher proportion of patients who developed drug resistance (16% vs. 2%, $p = 0.0270$). The HIV data showed a similar result in both groups with most of the patients responding to the treatment (84% vs. 94%).

HBV response to treatment and drug resistance development were finally evaluated at week 48. Following to previous weeks, the median HBV DNA was higher in the HBeAg positive group ($p = 0.0009$) and the number of patients with undetectable

viral load was lower (23% vs. 96%, p< 0.0001) (Figure 3.2). In this group, both the HBV virological failure (59% vs. 4%, p < 0.0001) and the HBV drug resistance development (68% vs. 4%, p < 0.0001) were significantly than in HBeAg negative group. Therefore, HBeAg and HBV DNA viral load are the main predictors in failure treatment and drug resistance development. Moreover, HIV virological response rate was quite homogeneous in both groups (9% vs. 4%, p = 0,6078).

In the multivariate analysis the only independent predictor to HBV response at week 48 was the HBeAg negative sero-status in comparison with the HBV DNA [OR: 0.028 (0.003-0.278), p < 0.002].

Week		n	**HBeAg+/anti-HBe-**	n	**HBeAg-/anti-HBe+**	p-value
0	HBV DNA , *IU/ml, median (range)*	62	$12.4x10^{7}$ [$16.5x10^{4}$ - $82.5x10^{7}$]	38	$42.5x10^{2}$ [$10.9x10^{1}$ - $10.3x10^{7}$]	**<.0001**
	Patients with HBV DNA < 14 IU/ml, *number (%)*	63	1 (2)	44	6 (14)	**0,0185**
	HIV RNA , *Log copies/mL, median (range)*	38	4.22 [3.02 - 4.88]	36	3.92 [2.73 - 5.08]	0,1515
	HIV RNA detectable, *number (%)*	38	38 (100)	36	36 (100)	1,0
24	HBV DNA , *IU/ml, median (range)*	44	$37.3x10^{2}$ [14.0 - $21.9x10^{5}$]	47	14.0 [14.0 - $48.9x10^{1}$]	**<.0001**
	Patients with HBV DNA < 14 IU/ml, *number (%)*	44	4 (9)	47	33 (70)	**<.0001**
	HBV partial virological response, *number (%)*	44	37 (84)	47	7 (15)	**<.0001**
	HBV drug resistance	44	7 (16)	47	1 (2)	**0,0270**
	HIV RNA , *Log copies/mL, median (range)*	44	2.30 [2.30 - 2.30]	47	2.30 [2.30 - 2.30]	1,0
	HIV RNA detectable, *number (%)*	44	7 (16)	47	3 (6)	0,1885
48	HBV DNA , *IU/ml, median (range)*	22	$73.2x10^{5}$ [14.0 - $47.4x10^{7}$]	23	14.0 [14.0 - 14.0]	**0,0009**
	Patients with HBV DNA < 14 IU/ml, *number (%)*	22	5 (23)	23	22 (96)	**<.0001**
	HBV partial virological response, *number (%)*	22	4 (18)	23	0	**0,0491**
	HBV virological failure, *number (%)*	22	13 (59)	23	1 (4)	**<.0001**
	HBV drug resistance	22	15 (68)	23	1 (4)	**<.0001**
	HIV RNA , *Log copies/mL, median (range)*	22	2.30 [2.30 - 4.45]	23	2.30 [2.30 - 2.30]	1,0
	HIV virological failure, *number (%)*	22	2 (9)	23	1 (4)	0,6078

Table 3.3 HIV and HBV status according to HBeAg serostatus.

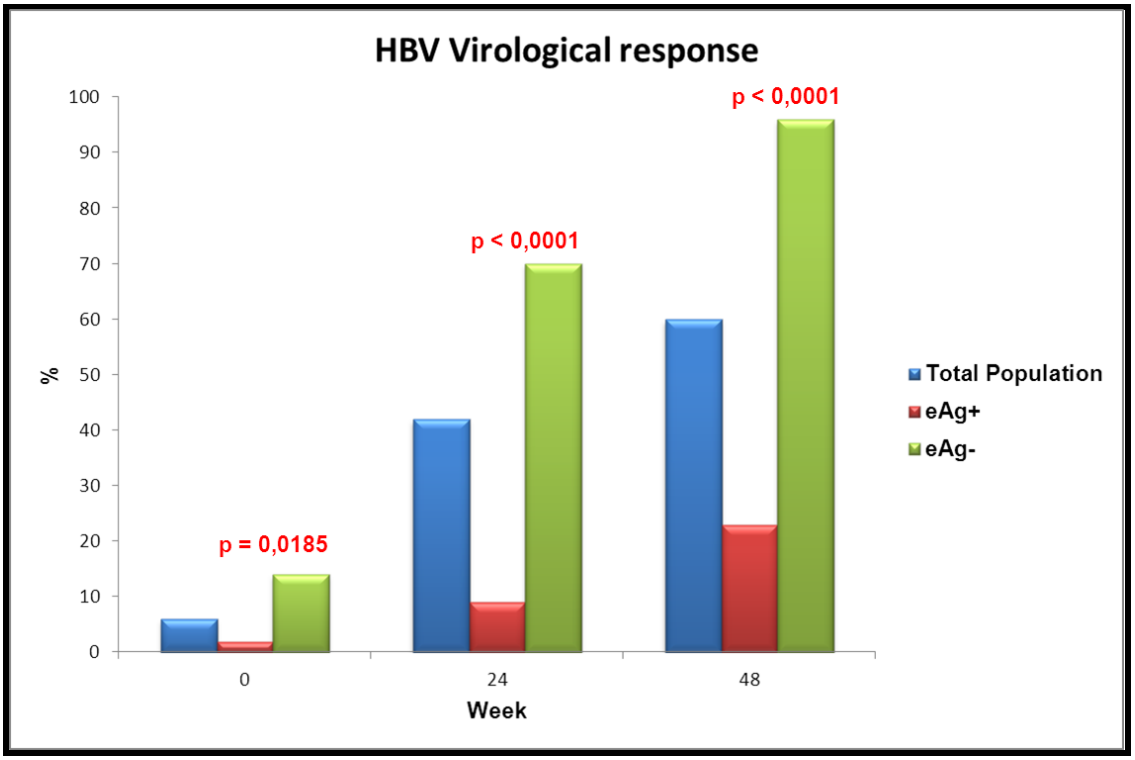

Figure 3.2 HBV virological response at week 24 and week 48 for the entire population and according to its HBeAg serological status.

3.4 *Genetic characterization of pol/S genes*

Sequencing of the *pol* gene was attempted in all samples with detectable viral load. Overall, 93/104 (89 %) samples at week 0, 33/53 (62%) samples at week 24 and 18/20 (90%) at week 48 were successfully sequenced and analysed. The alignment of the *wt* sequences showed a consensus sequences corresponding to genotype A1.

3.4.1 ***Polymerase*** **gene**

In total, 27 different patients showed at least one drug-associated resistance mutation at some time point. Prevalence of HBV drug-associated resistance mutations was as follow: 6/104 (6%) at week 0, 8/53 (15%) at week 24 and 17/20 (85%) at week 48. The evolution of mutations in each patient is shown in the table 3.4.

At week 0, naïve patients showed HBV drug resistance mutations associated to the ETV pathway; rtM250L in 1/104 (2%) and rtI169L in 2/104 (4%) [55,56]. At week 24, secondary mutation rtI169L appeared in one patient (2%) in combination with rtM204I, which confers primary drug resistance. Another patient showed rtI169L alone (2%). Furthermore, the primary drug resistance rtM204VI emerged in 6 more patients (11%). At week 48, different drug resistance combinations were found showing the low genetic barrier of the 3TC, which is enhanced in HIV co infected patients [153]. The combination rtI169L, rtV173L, rtL180M, rtM204V was found in 1/20 (5%) and rtL80I, rtL180M, rtM204IV in 2/20 (10%). In the other 14 patients, we found five different patterns of mutations as rtL180M, rtA181S, rtM204IV in 2/20 (10%), rtL80I, rtM204I in 1/20 (5%), rtL180M, rtM204V in 8/20 (40%), rtV173L, rtL180M, rtM204V in 2/20 (10%), rtI169L,

rtV173L, rtL180M, rtT184I, rtM204IV in 1/20 (5%). Most of them harboured secondary mutations that restore the viral fitness pointing the high viral load out observed in these samples.

3.4.2 *Surface* gene

The genetic evolution on the Surface gene was also studied since both genes are overlapped finding mutations associated to immune escape (Table 3.5). It is necessary highlight that, in most cases, these mutations did not appeared alone since they were in combination with others along the gene.

Few mutations increased its prevalence at the end of the follow up. These included sQ101K (up to 10%), sT118MRK (up to 20%), sA128V (up to 10%) and sG145R (up to 10%). It is noticeable that the most common immune escape mutant, sG145R, appeared in two naïve patients. In another patient appeared at week 24 but was reverted at week 48 and another patient selected it at week 48. The double mutant sE164D + sI195M appeared at week 48 with a prevalence of 20% due to the development of triple 3TC-resitance rtV173L + rtL180M + rtM204V 121.

Other mutations were also found with possible relation to immune escape. However, similar prevalence during the three different time points was observed for these mutations, which included sL109IP, sP120TS, sT126SN, sQ129R, sG130DNS, sN131KS, sM133IT, sF134L, sP142LS, sT143M, sD144EA.

	Week 0	Week 24	Week 48
Sequences (n)	93	33	18
Samples detected	104	53	20
% Drug resistance	**6**	**15**	**85**
Patient (no.)			
1	rtM250L	NA	NA
2	rtM250L	*wt*	NA
3	rtI169L	rtI169L	NA
4	rtI169L	rtI169L, rtM204I	rtI169L, rtV173L, rtL180M, rtM204V
5	rtI169L	*wt*	*wt*
6	rtI169L	NA	NA
7 to 10	*wt*	rtM204I	NA
11	*wt*	rtM204V	NA
12	*wt*	rtM204V	rtL80I, rtL180M, rtM204IV
13, 14	NA/*wt*	*wt*	rtL180M, rtA181S, rtM204IV
15	*wt*	NA	rtL80I, rtM204I
16 to 23	*wt*	*wt*	rtL180M, rtM204V
24, 25	*wt*	*wt*	rtV173L, rtL180M, rtM204V
26	NA	*wt*	rtI169L, rtV173L, rtL180M, rtT184I, rtM204IV
27	*wt*	*wt*	rtL80I, rtL180M, rtM204IV

Table 3.4 Evolution of mutations associated to drug resistance in the *polymerase* gene at each time point. Overall, 27 different patient showed some mutation associated to drug resistance at least in one time point. (NA: Not Available, *wt*: wild type)

	Week 0	Week 24	Week 48
Sequences (n)	93	33	18
Samples detected	104	53	20
Mutation			
sQ101K	3	6	10
sL109IP	3	2	5
sI110L	1	-	0
sT118MRK	7	4	20
sP120TS	6	2	5
sT126NS	2	2	-
sA128V	1	2	10
sQ129R	3	4	-
sG130DNS	3	2	-
sN131KS	2	2	5
sM133IT	2	-	-
sF134L	3	4	5
sP142LS	3	2	-
sT143M	2	4	-
sD144EA	2	2	-
sG145R	2	6	10
sE164D, sI195M	-	-	20

Table 3.5 Prevalence (%) of immune escape associated mutations in the Surface gene at each week

4. Discussion

In this study we have evaluated the efficacy of 3TC-based HAART during 48 weeks of follow-up within a HIV/HBV co-infected population from Blantyre, the largest city of Malawi. We assessed the HBV virological outcomes at three different points: before HAART initiation, at week 24, and at week 48. Moreover, the HBV genetic variability in both *pol* and *S* genes was evaluated. We also established the prevalence of HIV/HBV co-infection in this region (12%), which was similar to previous reports [172–174]. The overall virological response (HBV-DNA < 10 IU/mL) was at week 48 was 60%. However, when patients were stratified by HBeAg sero-status, HBV virological reponse was significantly lower in HBeAg positive patients compared with HBeAg negative (23% vs. 96%, respectively). The multivariate analysis identified HBeAg as the only independent predictor to HBV response at week 48 after one year of 3TC-based HAART in HIV/HBV co-infected patients. Regarding HBV variability, we observed a very high incidence of 3TC resistance after one year of treatment (85%) which is quite high in comparison with previous reports.

Similarly to other countries in South Africa, Malawi is a highly endemic country for both HIV (up to 76%) and HBV chronic infections (17.5%). HIV/HBV co-infection ranged from 10 to 20% [172–174]. We had established a prevalence of HIV/HBV co-infection in Blantyre of 12% among the HIV population randomly selected for HBV testing. The national antiretroviral regimen in Malawi included 3TC as the unique drug active against HBV.

The virological success of treatment against HBV infection using 3TC as monotherapy might be highly compromise for the high risk and incidence for the development HBV resistance. In this study population, we observed that 60% of patients reached HBV virological response at week 48. Interestingly, the proportion of patients who achieved virological response was significantly higher in HBeAg negative patients compared with HBeAg positive (96% vs. 23%, respectively, $p < 0.0001$). Most part of patients showed undetectable HIV viral load at week 48 which demonstrated the compliance to the regimen. In the multivariate analysis we identified HBeAg negative as the only independent predictor to HBV response at week 48 ($p < 0.002$). This fact, likely lead to higher HBV DNA levels in HBeAg positive patients ($p = 0.0009$) and consequently, more probability for the development of HBV drug resistance at week 48 in HBeAg positive compared with HBeAg negative patients (68% vs. 4%, respectively $p < 0.0001$). The association between the HBeAg positive sero-status and a higher risk for the development of HBV drug resistance has been previously described by *Ramos et al.* which found that nearly 90% of patients who had developed 3TC resistance were HBeAg positive [183,184].

We also found a high level of HBV drug resistance development after less than one year of 3TC-based HAART (85%). This rate is worrying in comparison with previous reports that established a similar prevalence of 3TC-resistance (>80%) but after 4 years of treatment in HIV/HBV co-infected patients [46,152,185,186]. At baseline (before HAART initiation), the overall prevalence of HBV drug resistance mutations to NAs in the study population was low (6%). Mutation rtM250L (2%) and rtI169L (4%) related with resistance to entecavir were the only found at baseline. However, these mutations alone were not able to confer HBV drug resistance to NAs. The presence of HBV drug resistance mutations in naïve patients shows the risk of transmission of these strains.

There are several articles that previously have shown HBV drug resistance mutations in newly diagnosed patients. *Thibault et al.* showed in 2002 the first report of 3TC-resistance transmission in a HIV infected patient who developed acute hepatitis [187]. Additionally, within a Spanish cohort, two patients recently diagnosed were found infected with strains which harboured rtM204V and later were also found four more patients (rtL180Mx2, rtL80V, rtV173L) [148,149]. Recently, one naïve patient harbouring he triple mutant (rtV173L, rtL180M, rtM204V) was identified in Japan [188] and 13 HBV monoinfected patients who were also naïve showed HBV drug resistance mutations (rtM204V+rtL180Mx3, rtL80Vx2, rtV173L, rtI233Vx4, M250LVx3) [189].

During the follow-up, the pattern of HBV drug resistance mutations recognized was mainly associated with 3TC resistance. At week 24, the main drug resistance mutation selected was rtM204IV in 87.5% of patients (7/8). At week 48, 8 different patterns of mutations were detected which included the triple mutant (rtV173L, rtL180M, rtM204V) in 20% of patients (4/20). Additionally, we found mutations that potentially confer cross-resistance to ETV such as rtT184I (1/20, 5%) or rtI169L (2/20, 10%). Interestingly, one patient showed an interchange between Isoleucine and Valine at position 204 from week 24 to 48. This has been recently explained by *Svicher et al.* who pointed out that this interchange could be an easy phenomenon since rtM204I would have a higher genetic barrier because it produces a stop codon within the *S* gene at the same time [61,190,191]. Furthermore, as expected, all patients who developed the triple 3TC-associated resistance (n = 4, 20%), produced the double mutation (sE164D, sI195M) in the *S* gene. These mutations are capable to avoid the immunologic response at the same level of sG145R [124,134]. The mutation rtV173L more likely restores the replication capacity in strains that previously harbours mutations rtL180M and rtM204V [192]. A previous report by *Matthews et al*. found a higher prevalence (17%) of the triple 3TC

resistance mutation (rtV173L, rtL180M, rtM204V) in HBV patients infected with non-genotype A. We could not evaluate this association due to the homogenous nature of our population which was also similar to previous studies in the area (genotype A1, 99%) [186,193,194]. Another study by *Ramos et al.* showed a high prevalence of 3TC-associated resistance mutations among patients after 34 months (47%) which was more frequently in HBeAg positive patient with higher HBV DNA levels [183]. The genetic analysis of the *Surface* gene showed a high variability after 48 weeks because the drug pressure; these mutations included sP120TS, N131KS, sM133IT, sF134L, sD144EA, sG145R and the double mutant sE164D, sI195M among others [61,87,122–125,127,130,162]. The fact whether a single mutation is able to produce a conformational change in the AGL or it is necessary more than one mutation for being clinically relevant is not totally clear. Interestingly, we found in a naïve patient the most common mutation sG145R; this mutation is able to avoid the immune response and has been widely identified among vaccinated patients that are infected by HBV. Unfortunately, we could not confirm if the patient was previously vaccinated.

The data of HDV prevalence in Africa are scarce and seems to differ among regions [195,196]. Transmission of Hepatitis Delta has been related to Mediterranean areas and intravenous drug addicts with HBV infection. After HBV vaccination programmes were implemented, the HDV infection significantly decreased in Europe. However, after the 90s when immigration came from areas where HBV is endemic such as Central Africa, Eastern Turkey, Central Asia, some Eastern European countries and some regions in Brazil have increased the prevalence of HDV [196–199]. We have estimated the first data of HDV infection (0,8%) in the region. Since sexual is the main route of transmission for HBV in our population might explain the very low prevalence of HDV infection observed in our population compared with that reported in other countries.

In summary, we have shown a high rate of HBV virological response to a 3TC-based HAART at week 48 in a HIV/HBV co-infected population from Malawi especially in those HBeAg negative patients. Indeed, HBeAg negative was identified as the unique independent predictor of HBV virological response. These data suggest that the HBeAg test might be considered at the beginning of HIV/HBV co-infection therapy to avoid the rapid selection of 3TC resistance mutations. In case of HBeAg positive patients the use of more potent drugs such as TDF instead of 3TC it would be desirable.

CONCLUSIONS

1. There is a high prevalence of HIV/HBV co-infection in Kumasi, Ghana (15%) and Blantyre, Malawi (12%). In addition, a high prevalence of occult HBV infection in HIV positive patients was observed in Ghana (20%).

2. In Ghana, the overall prevalence of resistance to nucleos(t)ide analogues in the *polymerase* gene was higher in the occult HBV than in the HIV/HBV co-infected cohort (9% vs. 31%, respectively). Similarly, the rate of mutations in the *Surface* gene was also higher in the occult HBV cohort (20% vs. 43,5%).

3. The genetic variability was higher in the S than in the *polymerase* gene among HBV monoinfected, HIV/HBV infected and occult HBV cohort. A higher variability was generally observed in both HIV co-infected and occult HBV cohorts. This result in the latter cohort might explain the HBV diagnostic failure either by a defect in the secretion of HBsAg and/or by an impaired of viral fitness.

4. In Malawi, higher rates of HBV virological response (HBV DNA < 14IU/mL) after 48 weeks of lamivudine-based HAART was observed in HBeAg negative compared with HBeAg positive patients (96% vs. 23%, respectively, $p < 0.001$). Indeed, HBeAg was recognized as the unique independent predictor of HBV virological response at week 48.

5. A high level of HBV resistance (85%) to nucleos(t)ide analogues was observed at week 48 in patients with detectable HBV-DNA levels. These results highlight the high risk of development drug resistance using lamivudine-monotherapies in HIV/HBV co-infected patients.

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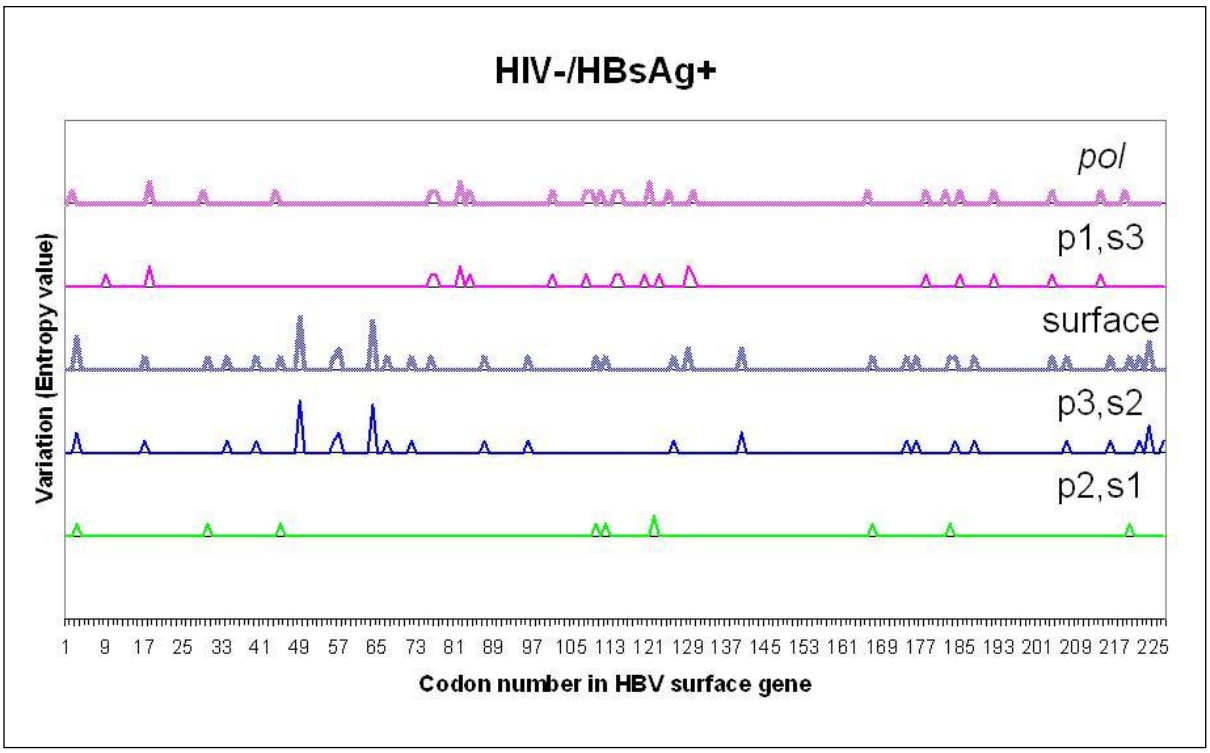
APPENDIX

APPENDIX 1: ADDITIONAL FIGURES AND TABLES OF INTEREST.

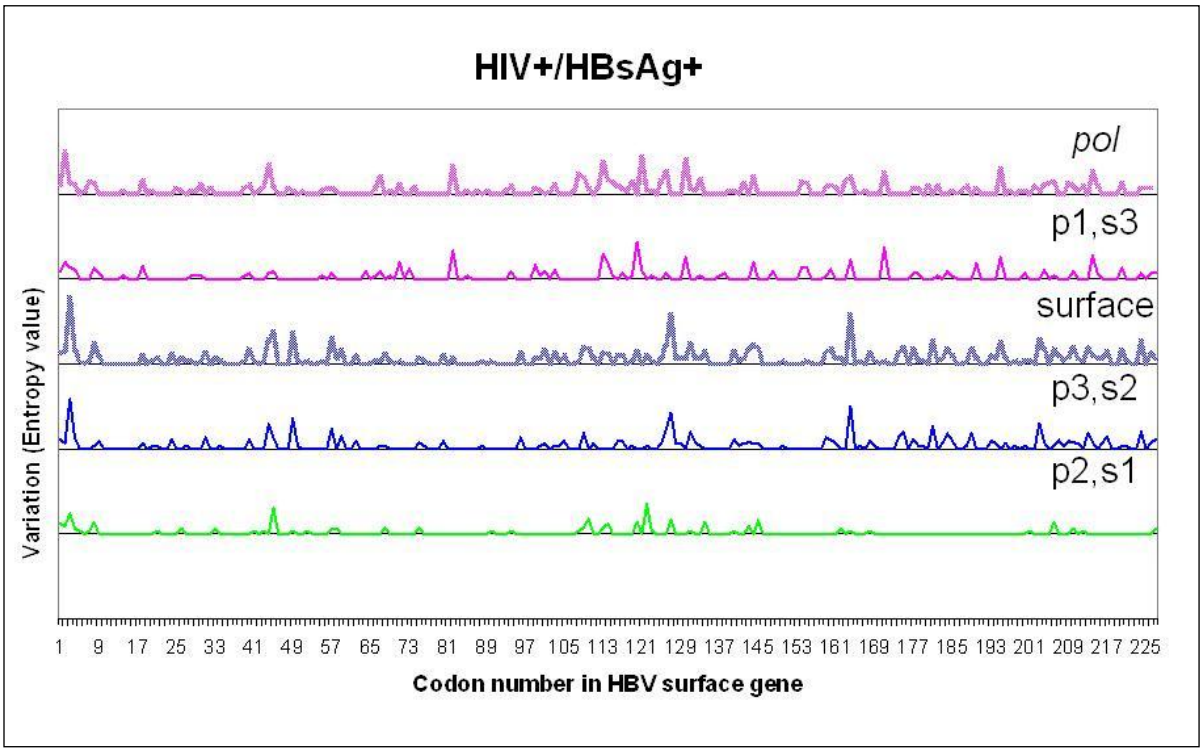
HBV VL (IU/mL)	Gen pol variations	Gen S variations
56,108	V27I	-
3,769	R120K, I162IT, R266T, M267L	G112R, **T126I**, L127P, S154P, Q181R, P214H
1,877	V142I, S143T, S223A, S256R, R266T, M267L	**Q129H, M133I, F134L, E164A**
784	S53AP, S54APT, Y221FY, I233IL	T57N, Y206F, L209W, F212L, L216S, P217L, V224A
772	H234Q, R266T, M267Q	V14A, S31NS, **Q101H, S143L, D144E**
593	K11EQ, R18K, A38E, S53N, L77V, S109Q, N123D, I162S, **V173L**, A211P, **Q215H**, L217R	E2D, T5I, G10KR, Q30K, A45T, **Q101K**, **T125M**, L127P, **S140L, D144E, E164V**, S154AV, S174N, L175S, V177A, N207T, L209V, F220C
510	S53I, P130Q, E263D	A45S
393	N236I, N279H	N59S, **Q101R, T125M**, L127P, **E164A**, V177A, P178L
367		L127P, G145A, I213T
352	V27I, S53N	A45AT
325	L91I, N118T, P130Q, **V173M**, S246C, R266K	S3N, L49P, **I110L**, P217L
272	R153KQ, R266T, M267L	**D144E, G145R**, P178L
263	V112I, H124Y, S143T, I162T, I187L, Q215H, S219A, S256C	G10E, V96G, M103I, F134L, S136L, S154P, E164G, Q181R, N207T, S210K, P217L, V224A
230	K11Q, R18K, N118T, H124Q, N139S, **I169L**, L175I	E2D, G10R, S34L, N40S, P56L, **Y100C, Q101H, I110L, T116N, T125M, T126I**, L127P, **T131A, D144E**, N146S, T148I, K160NS, E164G, W165L, S167L, R169H, S174N, Q181R, P217L, Y225S
220	L29P, V30A, S53I, S54C, M267L, V278I	A45S, P46A
199	K11N, G210A, R266T, M267L	S3I, G202R
188	S40A, N118T, R266T, M267L, D271N	I110L, T27I, S31R
172	N118T, T128I, Y141S, V142D, R266T, M267L	G44E, **I110L, P120S**, L127P, **M133L, F134I**, **D144A**, S204N, P211R, F220C
170	R18K, **L80I, L180M, M204V**, R266T	G10R, L127P, S154L, **E164G**, S174N, P178Q, **I195M**
166	E8A, I162V, Q215H, N248H	T27I, L84H, **Q101H, T126I**, I150T, **E164G**, L175S, N207T
155	**V173L, L180M, M204V**, R266T, M267L	L127P, **S143L**, **E164D, I195M**
152	K11R, **Q215H**	S3G, P62L, L127P, **S143L, D144E**, N207T, P217L
146	P130Q, N131S, L229V, R266K	S3N, Q30R, N40S, S64F, **Q101R**, R122K, **T123A**, L127P, F220L

141	N248H, G258X, R266T, M267L	S31N, N40S, G44E, L49R, **Q101H**, L127P, **Q129R, F134S**, S154L, K160R, E164G, S174N, P214L, V224G
139	D7T, E8D, R18K, Q48X, I53V, N122H, M129L, W153R, V163I, L164M, **Q215H**, T259S	G10R, V14G, N40X, K122R, **E164V**, S167L, V168A, L175S, A194V, S207T, V209L, P217L
138	V23I, L91I, H234Q	**Q101R, T125M**, L127P, K160R, **E164G**,168AV
125	V112I, I254X, S256R, R266T, M267L, P281S, W284*	M103I, L127P, **E164V**, P217L
100	P130Q, N248H	L84HP, L127P, A184V, L175S
99	K11R, L209M, N238H, S256N, G258*	S3G, G44E, S174N, Y200*
77	P17R, T19A, L42V, V44E, D45E, G52E, S53C, S54*, R55G, W58C, P59Q, K60N, F61LR, N118T, Q125H, **I169L**, S185R	L9V, V14A, D33E, W36R, T37N, G44S, P46DE, V47A, G50A, Q51K, N52I, S53A, **Q101H, I110L**, S117I, K160N, W165L, L175S, V177AG, A184V
76	V27I	-
65	A38E	Q30K, **S143L, E164G**, S167L, V177A
63	H13Y, I163V, R266T, M267L	**E164G**, W182S
59	D45X, Q48H, S50X, R51W, S53I, I121T, I122L, V214A	T37L, N40S, L42S, A45S, **S113T**, L127P, **E164V**, Y206H
55	S53I, L66DN, R110G, S117Y	E2G, S3N, S34L, A45S, P56L, S58T, **Y100S**, L109I, L127P, **Q129H, D144E**, S154L, G159V, S167L, L175S, Q181R, L209S, P217L
53	R266T, M267L	**T126I, Q129H**, L209W
53	S53I, I122L, V214A	A45S, L127P, **E164V**, Y206H
35	A113T, I122S, P130Q	S3N, L109R, S114A, L127P, S174N
34	R55S, A87E, F148I, H197Q, L229V	S34L, P56Q, T57I, W74LW, R79S, **C139***, T189N, F220L

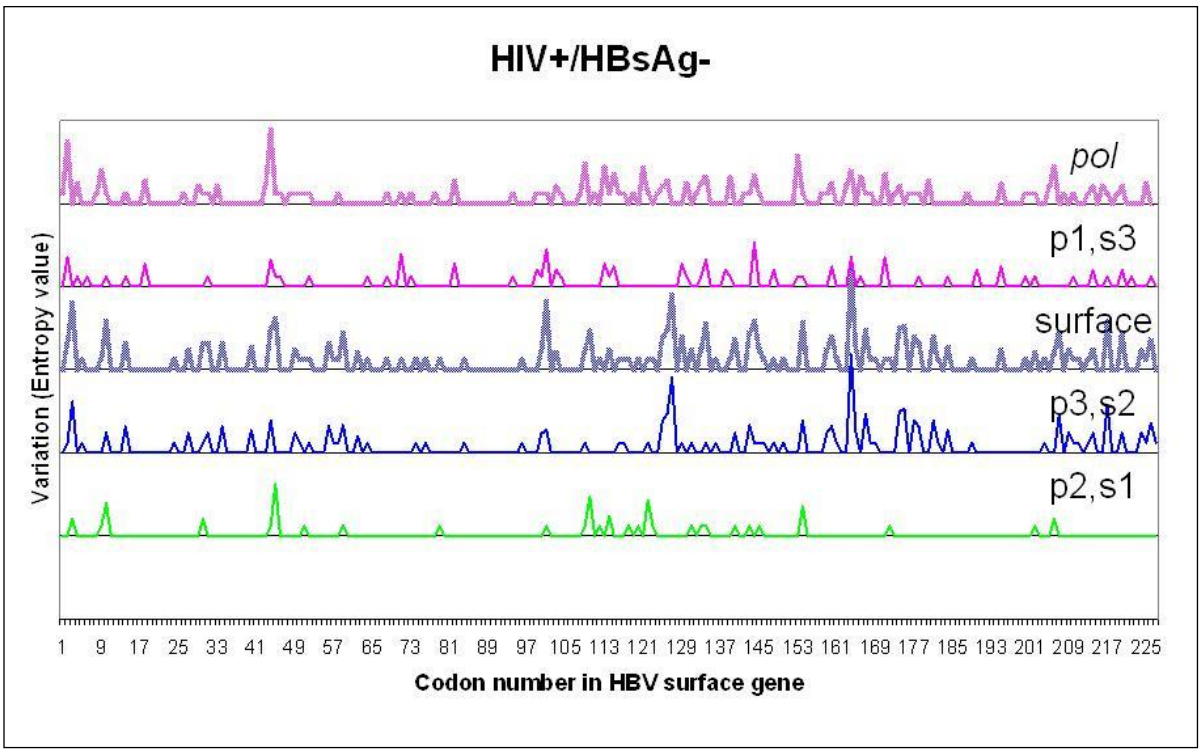
Appendix 2.1) Mutations in the *pol* and *S* genes corresponding to antiviral resistance and immune escape in HIV/HBsAg negative patients. The treatment and the viral load from each patient are also indicated.



Appendix 2.2a) Mean nucleotide entropy values at each codon position along the *S* gene were determined for HIV-/HBsAg+ population. The entropy was measured for each group according to the codon number in the *S* gene. Variability was observed in both *pol* and *S* genes and at each codon p1s3, p3s2, p2s1. Each peak represents the entropy value or variation at one codon position.



Appendix 2.2b) Mean nucleotide entropy values at each codon position along the *S* gene were determined for HIV+/HBsAg+ population. The entropy was measured for each group according to the codon number in the *S* gene. Variability was observed in both *pol* and *S* genes and at each codon p1s3, p3s2, p2s1. Each peak represents the entropy value or variation at one codon position.



Appendix 2.2c) Mean nucleotide entropy values at each codon position along the *S* gene were determined for c) HIV+/HBsAg- population. The entropy was measured for each group according to the codon number in the *S* gene. Variability was observed in both *pol* and *S* genes and at each codon p1s3, p3s2, p2s1. Each peak represents the entropy value or variation at one codon position.

Appendix 2.3a Total Entropy between HIV-/HBsAg+ vs. HIV+/HBsAg-

	HIV-/HBsAg+	HIV+/HBsAg-	p-value
Codon			
p2s1	1,41405	5,37578	**<0,001**
p3s2	4,75188	18,53071	**<0,001**
p1s3	3,07303	9,74248	**<0,001**
Total	9,23896	33,64897	**<0,001**

Appendix 2.3b Total Entropy between HIV-/HBsAg+ vs. HIV+/HBsAg+

	HIV-/HBsAg+	HIV+/HBsAg+	p-value
Codon			
p2s1	1,41405	3,91174	**<0,001**
p3s2	4,75188	10,27766	**<0,001**
p1s3	3,07303	7,59155	**<0,001**
Total	9,23896	21,78095	**<0,001**

Appendix 2.3) Shannon Entropy. Each table show the total entropy of each gene or by surface codon; a) HIV-/HBsAg+ vs. HIV+/HBsAg-; b) HIV-/HBsAg+ vs. HIV+/HBsAg+. * P-value < 0.05 was significant.

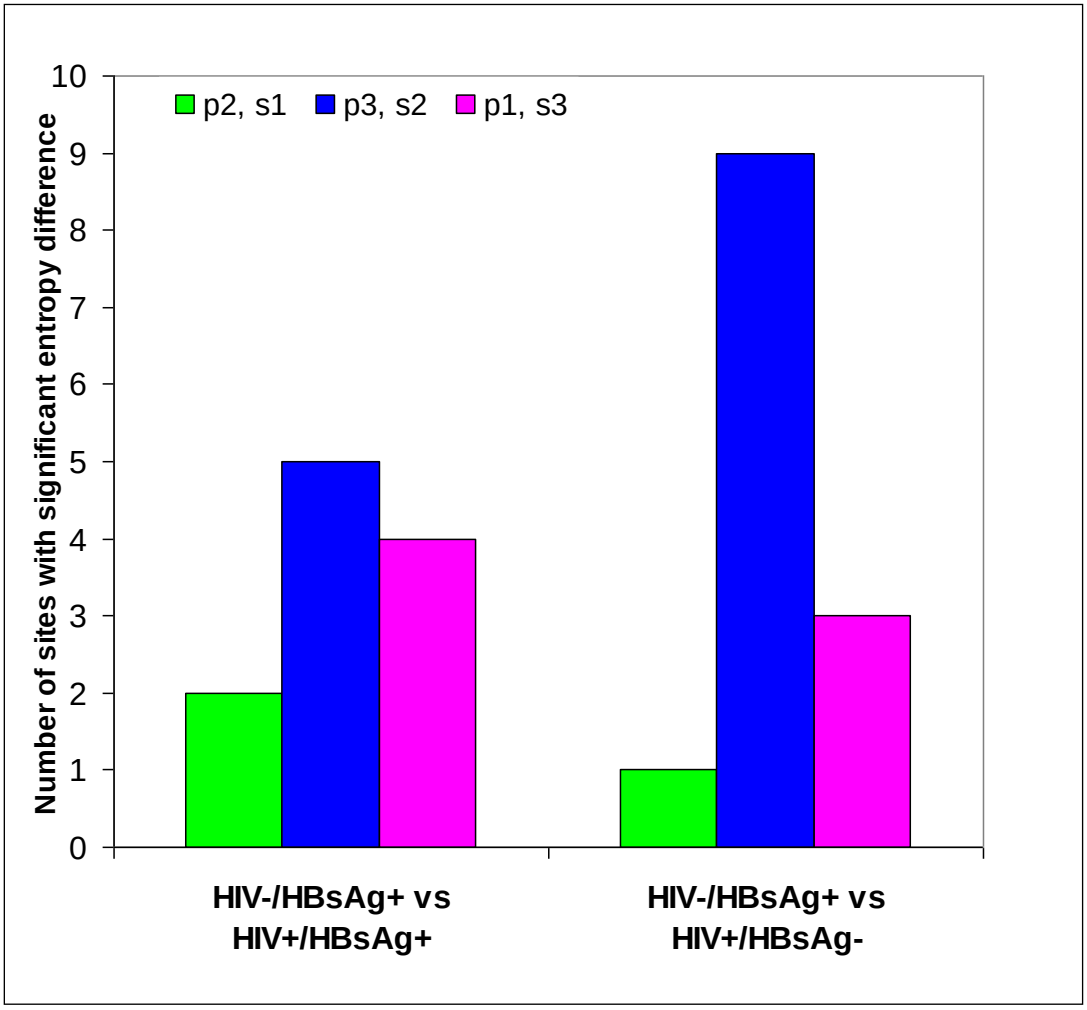
Appendix 2.3c Total Entropy between HIV+/HBsAg+ vs. HIV+/HBsAg-

	HIV+/HBsAg+	HIV+/HBsAg-	p-value
Codon			
p2s1	3,91174	5,37578	1.0
p3s2	10,27766	18,53071	1.0
p1s3	7,59155	9,74248	1.0
Total	21,78095	33,64897	**<0,001**

Appendix 2.3d Total Entropy between HIV-/HBsAg+ naïve vs. HIV+/HBsAg+ naïve

	HIV-/HBsAg+ naïve	HIV+/HBsAg+ naïve	p-value
Codon			
p2s1	1,413	3,09	**0,016**
p3s2	4,75	8,982	**<0,001**
p1s3	3,071	4,887	0,068
Total	9,234	16,959	**<0,001**

Appendix 2.3) Shannon Entropy. Each table show the total entropy of each gene or by surface codon; c) HIV+/HBsAg+ vs. HIV+/HBsAg-; d) HBsAg+ naïve vs. HIV+/HBsAg+ naïve. * P-value < 0.05 was significant.



Appendix 2.5. Number of sites with significant entropy difference between the monoinfected patients and the co-infected HBsAg positive or negative, respectively. It was found that the most variable nucleotide positions were located in the p3, s2 codons.

Sequences (n)	93		33		18
Samples detected	104		53		20
Week 0	Prevalence	Week 24	Prevalence	Week 48	Prevalence
Q101K	3%	Q101K	6%	NA	10%
Q101K		Q101K		Q101K	
Q101K		NA		NA	
NA		Q101H		ND	
ND		ND		Q101K	
L109P	3%	ND	2%	NA	5%
L109I		L109I		L109I	
L109P		NA		NA	
I110L	1%	NA	-	NA	-
T118MRK	7%	NA	4%	NA	20%
T118MRK		NA		NA	
T118MRK		N		T118M	
T118MRK		T118R		T118R	
T118MRK		T118K		T118K	
T118MRK		ND		NA	
T118MRK		NA		ND	
-		NA		T118K	
P120TS	6%	NA	2%	NA	5%
P120TS		NA		NA	
P120TS		NA		NA	
P120TS		NA		NA	
P120TS		NA		NA	
P120T		P120T		NA	
wt		*wt*		P120S	
T126S	2%	NA	2%	NA	-
T126N		T126N		NA	
NA	1%	NA	2%	A128V	10%
A128V		A128V		A128V	

Appendix 3.5a Evolution of mutations associated to immune escape in the AGL of the *Surface* gene at each week. The prevalence is also shown at each week. (NA: Not Available, ND: Not detected *wt*: wild type)

Sequences (n)	93		33		18
Samples detected	104		53		20

Week 0	Prevalence	Week 24	Prevalence	Week 48	Prevalence
Q129R	3%	Q129R	4%	NA	-
Q129R		Q129R		NA	
Q129R		NA		NA	
G130DNS	3%	ND	2%	ND	-
G130DNS		G130NS		NA	
G130S		NA		NA	
N131K	2%	N131K	2%	N131K	5%
N131S		ND		ND	
M133I	2%	NA	-	NA	-
M133T		NA		NA	
F134L	3%	ND	4%	NA	5%
F134L		F134L		F134L	
F134L		F134L		ND	
P142L	3%	NA	2%	NA	-
P142S		NA		ND	
P142S		P142S		NA	
T143M	2%	NA	4%	NA	-
T143M		NA		NA	
ND		T143M		ND	
ND		T143M		ND	
D144E	2%	D144E	2%	NA	-
D144A		NA		ND	
G145R	2%	G145R	6%	G145R	10%
G145R		G145R		NA	
ND		G145R		ND	
ND		ND		G145R	
ND	-	ND	-	E164D, I195M	20%

Appendix 3.5b Evolution of mutations associated to immune escape in the AGL of the *Surface* gene at each week. The prevalence is also shown at each week. (NA: Not Available, ND: Not Detected, *wt*: wild type)

Appendix 2: Publications generated in this thesis

- Ian Harrison, R Odame Phillips, A Gonzalez, F Sarfo, A Garcia, D Candotti, J-P Allain, D Chadwick, and AM Geretti. *Impact of HIV Infection on HBV Polymerase and Surface Variability among Patients Accessing 3TC-containing ART: Ghana*. 18th Conference on Retroviruses and Opportunistic Infections, 2010.

- Antonio Gonzalez del Castillo, Mas Chaponda, Ana Garcia-Diaz, Vicente Soriano, Mark Hopkins, Miriam Taegtmeyer, Joep J Oosterhout, Robert S Heyderman, Saye Khoo, and Anna Maria Geretti on behalf of the HepB Study Group. *Outcomes of Lamivudine-based First-line ART in HIV/HBV Co-infected Patients in Malawi*. 19th Conference on Retroviruses and Opportunistic Infections, 2011.

ABBREVIATIONS

- **3TC**: Lamivudine
- **AASLD**: American Association for the Study of the Liver Diseases
- **ADAPVEM´s**: Antiviral drug-associated potential vaccine escape mutants
- **ADV:** Adefovir
- **AGL:** antigenic loop or "a" determinant
- **ALT**: serum alanine aminotransferase
- **APASL**: Asian Pacific Association for the Study of the Liver
- **BCP**: basal core promoter
- **cccDNA**: close covalent circular DNA
- **CHB**: chronic Hepatitis B
- **CYL-I**: First cytosolic domain
- **CYL-II**: second cytosolic region
- **Cys**: Cysteines
- **EACS**: European AIDS Clinical Society
- **EASL**: European Association for the Study of the Liver
- **ER**: endoplasmic reticulum
- **ETV**: Entecavir
- **HAART**: Highly Active Antiretroviral Treatment
- **HBcAg**: Hepatitis B core Antigen
- **HBeAg**: Hepatitis B e Antigen
- **HBsAg**: Small Hepatitis B Surface Antigen
- **HBxAg**: Hepatitis B x Antigen
- **HCC**: Hepatocellular carcinoma

- **HDV**: Hepatitis Delta
- **IFN**: Interferon-α
- **KATH**: Komfo Anokye Teaching Hospital
- **LdT**: Telvibudine
- **L- HBsAg**: Large Hepatitis B Surface Antigen
- **MDR**: Multidrug-resistant
- **M-HBsAg**: Medium Hepatitis B Surface Antigen
- **NAs**: nucleoside/nucleotide analogues
- **OBI**: occult HBV infection
- **OLT**: orthotropic liver transplantation
- **ORF**: Open Reading Frame
- **PC**: pre-core
- **PEG-IFN**: pegylated interferon-α
- **pgRNA**: pre-genomic RNA
- **rcDNA**: relaxed circular HBV DNA
- **RT**: reverse transcriptase
- **SVP**: subviral particle
- **TDF**: Tenofovir
- **TMD-I**: First trans-membrane domain
- **TMD-II**: second trans-membrane domain
- **TMD-III**: third trans-membrane domain
- **TMD-IV**: four trans-membrane domain
- **VEM´s**: Vaccine Escape Mutants
- ***wt*:** wild type

Impacto del tratamiento antirretroviral basado en Lamivudina sobre la evolución genética del virus de la Hepatitis B (HBV) entre pacientes HIV/HBV co-infectados

Cerca de 400 millones de personas están infectadas crónicamente por el virus de la Hepatitis B (HBV), de las cuales se estima una mortalidad de 600.000 personas al año. Es por ello que constituye un grave problema de salud pública que, además, está estrechamente relacionado con hepatitis fulminante, cirrosis y Carcinoma Hepatocelular (HCC). Las vías de transmisión van, desde la vertical/horizontal (principalmente en África a edades tempranas) a la sexual o por uso de drogas (en el mundo occidental). Al compartir vías de transmisión con el virus de la inmunodeficiencia humana (HIV), la co-infección de estos dos virus es frecuente. Se estima que alrededor de 4 millones de personas están coinfectadas en el mundo, siendo la prevalencia en África mayor que en el mundo occidental (>15% vs. <10%) [1].

El HBV es un virus DNA parcialmente de doble cadena de 3.2 kb cuya principal característica es el solapamiento de sus genes. Este solapamiento provoca que el gen que codifica la polimerasa (gen *pol*) del virus este parcialmente solapado con el gen que codifica las proteínas de la envuelta (gen *S*) (Figura 1.1) [12]. La segunda característica a destacar es su polimerasa, con actividad transcriptasa inversa y similar a la del HIV, su tasa de variabilidad es alta para tratarse de un virus DNA [21–23]. Por otro lado, su ciclo viral se caracteriza, principalmente, por la generación de un DNA circular covalentemente cerrado (cccDNA), con una estructura y regulación parecida a la cromatina, es el principal culpable de la cronicidad de la enfermedad así como el molde usado en la transcripción. La retro-transcripción del RNA pre-genómico (pgRNA) se produce al mismo tiempo que se forma la cápside y, una vez formada la cápside y el

DNA circular relajado (rcDNA), puede o bien ser envuelto por las proteínas superficie y salir a infectar nuevas células o bien regresar al núcleo para producir mayor cantidad de cccDNA, lo que ocurre en los primeros estadios de la infección (Figura 1.2) [19,20].

Para tratamiento de la Hepatitis B existen a día de hoy 7 fármacos; IFN, peg-IFN y 5 análogos de nucleos(t)ido (NAs), 3TC, LdT, ETV, ADV y TDF (tabla 1.1/figura 1.3). El principal objetivo de la terapia antiviral es la supresión de la carga viral del virus algo que, sin embargo, no es posible hacerlo por completo debido a que estos fármacos no actúan contra cccDNA y al dejar el tratamiento los niveles de carga viral repuntan de nuevo. Por ello son frecuentes los tratamientos de larga duración lo que lleva a un aumento de posibilidades de generación de resistencias. En el caso de co-infección con HIV, las guías de la EACS recomiendan empezar el tratamiento a partir de las 2000 IU/mL y usar TDF en combinación con 3TC o emtricitabina [33,34,36,182]. En África el diagnóstico de HBV es limitado y depende del país por lo que no es raro que pacientes HIV cuya co-infección por HBV sea desconocida sean tratados durante un gran periodo de tiempo con regímenes que incluyen 3TC como único fármaco activo frente a HBV. El tratamiento con 3TC en el mundo occidental está contraindicado debido al gran número de resistencias que produce, el cual se incrementa en co-infección con HIV [3,38,45,153].

La resistencia a fármacos se define como la selección de cepas de HBV con variaciones en la secuencia que confieren una susceptibilidad reducida a los fármacos administrados. La resistencia cruzada ocurre cuando las variantes producidas afectan a la susceptibilidad de otros fármacos para los cuales el virus nunca ha sido expuesto. Las mutaciones de resistencia afectan directamente al gen *pol* pero, debido al solapamiento de los genes, el gen *S* también se puede ver afectado [47,67,160,161]. Mutaciones en el gen *pol* se asociaron; Resistencia a LdT y 3TC se asocia con rtL80I/V,

rtV173L, rtL180M/C, rtA181T, rtM204I/V/S [48–51]; La resistencia a ETV se produce por una combinación de rtI169T o rtT184A/G/I/S o rtS202G/I o rtM250V mas rtL180M/C y rtM204I/V. También se asocia con rtI169T, rtT184A/G/I/S, rtS202G/I, rtM250V [55,56]; Resistencia a ADV se relaciona con rtA181T/V, rtQ215H, rtI233V, rtN238T and rtN236T [53,54]; Resistencia a TDF está asociada con rtA194T aunque aún existe controversia sobre esta resistencia [57–60]. Mutaciones en el gen *S* se asocian con escape inmunológico o a la vacuna, la cual utiliza el bucle antigénico (AGL) como molde, y pueden ser sY100C, sQ101KH, sI110L, sT118MRK, sP120AST, sT126NS, sA128V, sQ129HR, sG130DNS, sN131IKT, sM133I, sF134L, sP142LS, sT143M, sD144EA, sG145R [61,87,122–125,127,130,162]. La doble mutación sE164D + sI195M se asocia con la triple mutación asociada a 3TC (rtV173L, rtL180M, rtM204V) y se ha demostrado que produce un escape immune similar a sG145R [124]. En general, cualquier variante dentro del AGL del HBsAg se podría considerar potencial mutante de escape (VEM´s) aunque no se haya demostrado *in vitro*.

Objetivos de la tesis

1. Estudiar la prevalencia de HBsAg en una población HIV positiva procedente de Ghana y el impacto de la exposición al HAART basado en lamivudina durante largo tiempo sobre los genes *pol* y *S* del HBV.

2. Estudiar el efecto de la co-infección con HIV sobre el desarrollo de resistencias a fármacos en el HBV y la evolución genética de los genes *pol* y *S*.

3. Estudiar la eficacia de HAART basado en lamivudina durante 48 semanas en una población coinfectada con HIV/HBV procedente de Malawi.

4. Estudiar la evolución genética en los genes *pol* y *S* durante las 48 semanas de HAART basado en Lamivudina en la población de Malawi.

***Ghana*.** Se estudió una cohorte de 2138 pacientes HIV que, de manera rutinaria, acudían la consulta de HIV en el Hospital Komfo Anokye Teaching (KATH) en Kumasi durante 2007 y en los que, aproximadamente, el 50% recibía tratamiento antirretroviral basado en 3TC. Se recogieron muestras de sangre y plasma; 838 (39%) fueron enviadas al Royal Free Hospital (RFH) de Londres y en 1300 (61%) fueron analizadas para HBsAg usando el ensayo Determine. De estas últimas, 178 HBsAg positivas fueron también enviadas a Londres para repetir los test y caracterizarlas. Además, se recibieron las secuencias de muestras mono-infectadas por HBV de mujeres embarazadas y donantes de sangre que atendían el mismo hospital, KATH y que habían sido estudiadas previamente por el grupo de Jean-Pierre Allain en el Departamento de Hematología de la Universidad de Cambridge, UK [151,156,157]. En total, se seleccionaron en Londres 1016 muestras para el estudio del HBsAg. 212/318 (67%) tenían HBsAg positivo y DNA detectable, constituyendo la cohorte HIV/HBV coinfectada, con una mediana de carga viral (rango) de 19073 (IQR 23 – $10.0x10^{13}$) IU/mL. Por otro lado, 111/555 (20%) muestras con HBsAg negativo y anti-HBc positivo tenían carga viral detectable, 68 (15 – $56.0x10^{3}$) IU/mL, y constituían la cohorte de infección oculta por HBV (OBI) (tabla 2.2). En el grupo de pacientes co-infectados, 206 muestras pudieron ser secuenciadas encontrándose mutaciones asociadas a resistencias en 18 (9%) pacientes, 13 (72%) de ellos recibiendo HAART basado en 3TC y 5 (28%) naïve (tabla 2.3). En estas muestras, además, encontraron también VEM´s en el gen *S*. En el resto de muestras de pacientes co-infectados secuenciadas donde no se encontraron mutaciones asociadas a resistencias (188), si se encontraron VEM´s (tabla 2.4). Dentro de la cohorte de OBI, 39 (35%) muestras pudieron ser secuenciadas de las cuales el 31% contenía mutaciones de resistencia (tabla 2.5). En 30 de las muestras no se encontraron mutaciones dentro del gen *pol* (tabla 2.6). Por otra parte, la mayoría de los

pacientes mostraban más de dos mutaciones dentro del gen S, no solo dentro del AGL sino a lo largo de todo el gen. Por otro lado, la variabilidad de estas dos cohortes se estudió junto con una cohorte monoinfectada por HBV de la misma región para estudiar el efecto del HIV sobre la evolución de estos dos genes. Para ello, basado en el solapamiento de los genes (figura 2.1), se estudió la entropía de las secuencias genéticas de cada grupo. La cohorte monoinfectada por HBV mostró una menor variabilidad que las otras dos, estando su mayor variabilidad localizada entre los codones 25 a 80 (Apéndice 2.4). En la cohorte coinfectada, la mayor variabilidad se encontraba entre los codones 100 a 150 y en la cohorte OBI esta zona se aumentaba hasta el codón 190. Comparando las diferentes entropías de los codones (p2s1, p3s2, p1s3 y total), la mayor diferencia se encontraba en el codón p3s2 y todos estos eran significativamente diferentes cuando la cohorte de muestras monoinfectadas se comparaba con la coinfectada o la OBI (figura 2.2).

Concluyendo, en una cohorte HIV procedente de Ghana establecimos la prevalencia de co-infección HIV/HBsAg positivo (15%) y de la infección oculta por HBV en pacientes HIV (20%). En ambos casos, los datos eran más elevados que los datos previos en África sub-sahariana [163,164]. Además, encontramos una alta prevalencia de mutaciones asociadas a resistencia a fármacos tanto en la cohorte coinfectada como en la OBI, 9% vs. 31%, y de mutaciones asociadas a escape inmunológico, 20% vs. 43.5%. Finalmente, mediante el estudio de la entropía, que mide la variabilidad de las secuencias en función de la composición de nucleótidos, demostramos que el HIV influye en la variabilidad de estos dos genes, posiblemente debido a la diferente presión inmunológica a la que está sometido el paciente que, entre otras cosas, se caracteriza por una mayor carga viral de HBV. Esta variabilidad es incluso mayor en la cohorte OBI, lo que podría explicar el fallo en diagnóstico clínico del HBsAg.

***Malawi*.** Se llevó a cabo un estudio longitudinal de una cohorte de pacientes HIV positivos seleccionada en el Hospital Central Queen Elizabeth (QECH) en Blantyre, al sudeste de Malawi, donde comenzaron un régimen basado en Stavudina, 3TC y Nevirapina en 2007/8. Se recogieron muestras de 133 pacientes en tres momentos diferentes, antes del tratamiento así como 24 y 48 semanas después de iniciarse el mismo (Figura 3.1). La serología de HBeAg mostró que el 50% de los pacientes eran positivos y el estudio de anticuerpos de Hepatitis Delta (HDV) mostró una baja prevalencia en la zona (1%). Se cuantificó la carga viral de todas las muestras posibles para estudiar la respuesta al tratamiento tras esas 48 semanas. Estudiando la población en general, se encontró un alto índice de respuesta tanto para HIV como para HBV, 91.7% vs. 60% (tabla 3.2/figura 3.2). Sin embargo, cuando los pacientes se estratificaban en función del HBeAg se observó un fracaso en la respuesta virológica del HBV (DNA < 14 IU/mL) en pacientes HBeAg positivo frente a HBeAg negativo, 23% vs. 96% ($p < 0.0001$, tabla 3.3). Esto no ocurría para HIV donde más del 90% de los pacientes en ambos grupos presentaban respuesta virológica efectiva (tabla 3.3/figura 3.2). El análisis multivariante para determinar el factor responsable de la respuesta virológica de HBV mostró que el HBeAg positivo era el único predictor independiente [OR: 0.028 (0.003-0.278), $p < 0.002$]. Se llevó a cabo caracterización de los genes *pol* y *S* en todas aquellas muestras con carga viral detectable para un estudio más exhaustivo; 93/104 (89%) en semana 0; 33/53 (62%) en semana 24; y 18/20 (90%) en semana 48. El 99% de los pacientes mostró un genotipo A1 y tan solo uno de ellos mostró genotipo E, lo que se correspondía con la zona geográfica [193,194]. Se observaron mutaciones asociadas a resistencia en cada una de las tres semanas de seguimiento (tabla 3.4). A semana 0 se encontró en un 6% de los pacientes, si bien no conferían resistencia primaria sino que han sido asociadas con resistencia secundaria, rtM250L y

rtI169L. A semana 24, un 15% de los pacientes mostraba resistencia donde ya se incluía rtM204IV en seis de ellos (75%). Finalmente, el 85% de los pacientes con DNA detectable al final del seguimiento presentaban mutaciones asociadas a resistencia, todos ellos conteniendo la principal mutación rtM204IV y el 23,5% de ellos la triple mutación asociada a 3TC (rtV173L, rtL180M, rtM204V). Además, la generación de resistencias en esta última semana era significativamente mayor en el grupo HBeAg positivo [68% vs. 4%, $p < 0.0001$, tabla 3.3]. El estudio del gen *S* mostró, además, una mayor variabilidad en donde la mayoría de las mutaciones aparecían en combinación con otras. En este sentido, algunas de las mutaciones aumentaron su prevalencia según avanzaba el tratamiento, lo que podía ser causa de la presión inmunológica (tabla 3.5). Estas mutaciones incluían sQ101K (hasta un 10%), sT118MRK (hasta un 20%), sA128V (hasta un 10%) and sG145R (hasta un 10%). Además se observó el mutante doble sE164D + sI195M, producto de la generación del triple mutante asociado a 3TC.

Concluyendo, hemos establecido la prevalencia de co-infección HIV/HBV en la mayor ciudad de Malawi, Blantyre (12%). Durante un año, 133 pacientes fueron seguidos al iniciar HAART con 3TC como único agente activo frente a HBV. La respuesta al tratamiento en estos pacientes se veía afectada de manera significativa por el HBeAg, siendo más frecuente el fallo y la generación de resistencias cuando este marcador era positivo. De este modo, el alto porcentaje de resistencias tras 48 semanas (85%) es bastante alto y alarmante en comparación con estudios previos, lo que sugiere la necesidad de evitar este tipo de regímenes, al menos, entre la población con HBeAg positivo [186].

Conclusiones generales

1. Se encontró una gran prevalencia de co-infección HIV/HBV en Kumasi, Ghana (15%) y en Blantyre, Malawi (12%). Además, se observó una alta prevalencia de infección oculta por HBV en pacientes HIV positivo de Ghana (20%).

2. En Ghana, el global de resistencias a análogos e nucleós(t)idos en el gen de la polimerasa era mayor en la cohorte de infección oculta por HBV que en la de HIV/HBV co-infectados (9% vs. 31%, respectivamente). De modo similar, el porcentaje de mutaciones en el gen de la envuelta también era mayor en la cohorte de infección oculta que en la de HIV/HBV co-infectados (43,5% vs.20%, respectivamente).

3. La variabilidad genética era mayor en el gen de la envuelta que en el de la polimerasa entre las cohortes de pacientes HBV monoinfectados, HIV/HBV co-infectados y con infección oculta. Se observó una mayor variabilidad tanto en la cohorte HIV/HBV coinfectado como con infección oculta. El resultado de esta última podría explicar el fallo diagnóstico de HBV ya sea, bien por defecto en la secreción de HBsAg y/o por una reducción de la replicación viral.

4. En Malawi, se observaron mayores porcentajes de respuesta virológica (carga viral < 14IU/mL) tras 48 semanas de HAART basado en Lamivudina en pacientes HBeAg negativo frente a aquellos HBeAg positivos (96% vs. 23%, respectivamente, $p < 0.001$). De este modo, se demostró que el HBeAg el único predictor independiente de la respuesta virológica a la semana 48.

5. Por último, en pacientes con carga viral detectable a semana 48 se observó una alta prevalencia de mutaciones de resistencias asociadas a lamivudina (85%). Estos resultados subrayan el alto riesgo de desarrollo de resistencias en el HBV tras la exposición a monoterapias con lamivudina en pacientes co-infectados por HIV.