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RESEARCH DOCTORATE IN TRANSPLANT SCIENCES

Cycle *XXIV*- MED 04

BIOMOLECULAR TECHNIQUES AND BIOINFORMATICS TOOLS FOR THE IDENTIFICATION AND STUDY OF HBV AND HCV DRUG-RESISTANT VARIANTS

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Abbreviations

AASLD American Association for the Study of Liver Diseases

ADV Adefovir

ALT Alanine aminotransferase

APASL The Asian Pacific Association for the Study of the Liver

ARF Alternate Reading Frame

ASRVPCR Allele Specific PCR for Resistant Vriants

ATP Adenosine Triphosphate

BCP Basic Core Promoter

BLAST Local Alignment Search Tool

CHB Chronic Hepatitis B

Cp/ml Copies per milliliter

CTL Cytotoxic T Lymphocytes

CURS Core Upstream Regulatory Sequence

CVR Complete viral response

DAA Direct-acting antivirals

DHBV duck hepatitis B virus

DNA Deoxyribonucleic acid

dNTP Deoxyribonucleotide triphosphate

DS Direct Sequencing

EASL European Association for the Study of the Liver

EIA Enzyme linked immuno assay

ER Endoplasmic Reticulum

ETOH Alcoholic Cirrhosis

HCC Hepatocellular Carcinomas

HMMs Hidden Markov models

ETV Entecavir

FCH fibrosing cholestatic hepatitis

GGH Ground Glass Hepatocyte

GSHV ground squirrel hepatitis virus

HAV Hepatitis A Virus

HBcAg Hepatitis B core antigen

HBeAg Hepatitis B e antigen

HBIg Hepatitis B Immunoglobulin

HBsAg Hepatitis B surface antigen

HBV Hepatitis B virus

HCV Hepatitis C virus

HCVcoreAg Hepatitis C virus core antigen

HDV Hepatitis D virus

HHBV heron hepatitis B virus

HIV Human immunodeficiency virus

HLA Human Leucocyte Antigen

IFN Interferon

IL28B Interleukin 28 B

ISG Interferon stimulated genes

IU/mL International units per milliliter

IVDU Intravenous drug use

IVR Incomplete viral response

JFH Japan Fulminant Hepatitis

LAM Lamivudine

LDL low-density lipoproteins

LDLR Low Density Lipoprotein Receptor

LdT Telbivudine

LHBsAg Hepatitis B Large Surface Antigen

LT Liver Transplant

MC mixed cryoglobulinemia

MHBsAg Hepatitis B Medium Surface Antigen

NA Nucleos(t)ide Analogue

NAT Nucleic acid amplification technique

NGS Next Generation Sequencing

NS Non-structural

ORF Open Reading Frame

PBC Primary Biliary Cirrhosis

PCR Polymerase chain reaction

peg-IFN Polyethylene glycol Interferon

pg pregenome

PK Kinase Protein

Pol/Rt Polymerase/Retrotranscriptase

PreC precore

PSC Primary Sclerosing Cholangitis

Pt Patient

RACVR Resistance after Complete Viral Response

RdRp RNAdependent RNA polymerase

RIBA Recombinant immunblot assay

RIG-I retinoic acid-inducible gene-I

RNA Ribonucleic acid

RT Reverse Transcriptase

RV Resistant Variant

SHBsAg Hepatitis B Small Surface Antigen

SNP Single nucleotide polymorphism

SOC Standard of Care

SPP Signal Peptide Peptidase

SR-BI Scavenger receptor class B type I

TFV Tenofovir

TM Transmembrane

TNF Tumor Necrosis Factor

TP Terminal Protein

ULN upper limit of normal

UTR Untranslated region

VAP vesicle-associated membrane proteins

VLDL very-low density lipoproteins

WHO World Health Organization

WHV woodchuck hepatitis virus

WMHV woolly monkey hepatitis virus

1. INTRODUCTION

About three thousand of patients had organ transplantation during the 2011 in Italy, with more than one thousand of cases, liver is the second transplanted organ, mostly due to cirrhosis as a consequence of hepatitis [Ministero della salute, report 2011].

The hepatitis is an inflammatory disorder of the liver that may be caused by viruses, drugs, toxins, autoimmune illnesses and metabolic disorders of copper or iron (Wilson's disease and hemochromatosis) (figure 1). Symptoms include jaundice and fever-like symptoms. In particular, five unrelated hepatotropic viruses (hepatitis A to E) are the primary causes of viral hepatitis. Hepatitis A and E are typically caused by ingestion of contaminated food or water. Hepatitis B, C and D usually occur as a result of parenteral contact with infected body fluids. Common modes of transmission for these viruses include receipt of contaminated blood or blood products, invasive medical procedures using contaminated equipment and for hepatitis B transmission from mother to baby at birth, from family member to child, and also by sexual contact. Among the hepatitis viruses, only hepatitis B virus (HBV) and hepatitis C virus (HCV) are capable of establishing chronic infections, while hepatitis D virus (HDV) is considered a subviral satellite (virusoid) that can establish chronic infection only in the presence of an ongoing HBV infection. The World Health Organisation (WHO) has estimated that there are more than 500 million people worldwide living with chronic HBV and/or HCV infections [Te HS *et al*., 2010], all with an increased risk of developing progressive destruction and

regeneration of the liver parenchyma leading to fibrosis, cirrhosis and possibly hepatocellular carcinoma (HCC).

Recognizing the tremendous burden caused by viral hepatitis, the WHO established the Global Hepatitis Programme in the attempt to reduce: i) the transmission of agents that cause viral hepatitis; ii) the morbidity and mortality due to viral hepatitis through improving the care of patients with viral hepatitis; iii) the socio-economic impact of viral hepatitis at individual, community and population levels.

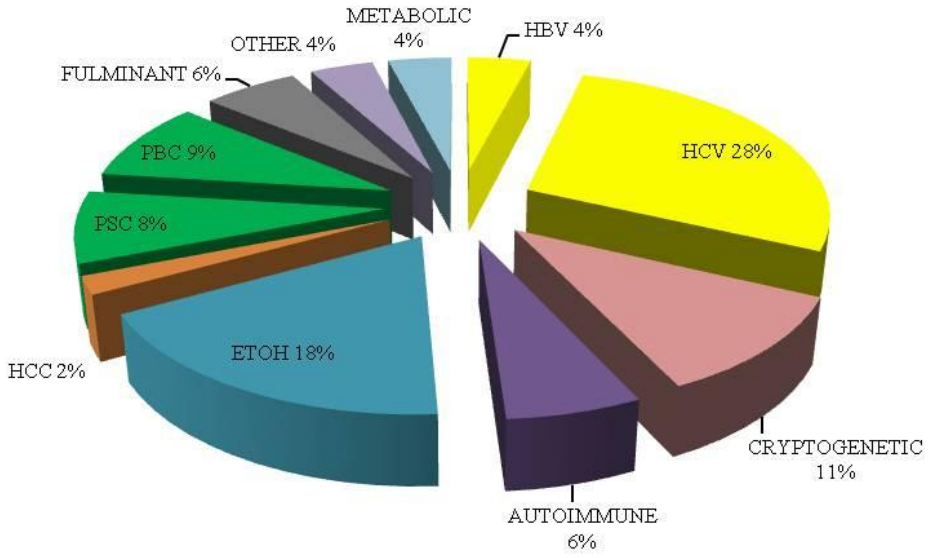


Figure 1. Frequency of liver transplants by diagnosis. With a total of 32% viral hepatitis, represented by HBV and HCV, are the most present (yellow colored). Alcoholic Cirrhosis (ETOH) is the second cause for liver transplant followed by the group of cholestatic diseases, Primary Biliary Cirrhosis (PBC) and Primary Sclerosing Cholangitis (PSC), green portion. Hepatocellular Carcinomas (HCC) are also present (orange colored, 2%). Figureure adapted from *Manzarbeitia C et al., 2012 (source http://emedicine.medscape.com).*

Vaccines and antiviral treatment play a topic role in the Global Hepatitis Programme, particularly in the case of Hepatitis A Virus (HAV),

HBV and HCV prevention / infection. To date several vaccines against hepatitis A are available and highly efficacious providing long-lasting protection in adults and in children above one to two years of age. In countries where clinical hepatitis A is an important health problem, immunization is likely to be a cost-effective public health tool to control the disease. No drugs against HAV are currently available, and antiviral medication is unlikely to become a realistic alternative to appropriate vaccines. Immune globulin may be used for pre- and post-exposure prophylaxis, for example, shortly before entering a disease-endemic area or just after likely HAV exposure. However, passive immunization with immune globulin gives only short-term protection (three to five months) and is relatively costly compared to the long-term immunity from vaccination. Hepatitis B vaccine is 95% effective in preventing HBV infection and its chronic consequences, and is the first vaccine against a major human cancer. The vaccine has an outstanding record of safety and effectiveness. Since 1982, over one billion doses of hepatitis B vaccine have been used worldwide. In many countries where 8% to 15% of children used to become chronically infected with HBV, vaccination has reduced the rate of chronic infection to less than 1% among immunized children. In 2010, 179 countries reported that they had included the hepatitis B vaccine into their national infant immunization programmes (two of these countries reported introducing in part of the country only). This is a major increase compared with 31 countries in 1992, the year that the World Health Assembly passed a resolution to recommend global vaccination against hepatitis B. Furthermore, during the last 15 years, several drugs has been introduced for

the treatment of HBV: immune-modulatory agents like interferon α, and nucleos(t)ide analogues (NAs) direct acting on the virus replication mechanism. The latter, in particular, can avoid the viral replication reducing the liver damage due to the inflammation but unfortunately is not able to eradicate the Hepatitis B virus from infected hepatocytes determining a lifelong treatment for patients affected by chronic hepatitis B (CHB) to prevent the recurrence of the virus. Despite advances in treatment, the chronic HBV infection can still make progress in the disease in case of selection and emergence of drug-resistant viral variants, immunosuppression or poor adherence to therapy. For this reason, liver transplant (LT) may be the only hope for many CHB infected patients, particularly for those with end-stage liver disease.

Because of the impossibility to completely clear HBV from infected cells, a main issue after liver transplant is the HBV graft reinfection, probably due to enhanced virus replication resulting from immunosuppression and direct stimulatory effects of steroid therapy on the glucocorticoid-responsive enhancer region of the HBV genome [McMillan JS *et al*., 1995; Tur-Kaspa R *et al*., 1988]. Results of LT for HBV have improved significantly during the past two decades [Kim WR *et al*., 2004]. In the 1980s, HBV recurrence was highly prevalent post LT, leading to poor patient and graft survival [Starzl TE *et al*., 1984]. Mechanisms of HBV recurrence in the graft included: i) immediate reinfection of the graft due to the circulating HBV particles, or ii) reinfection of the graft due to HBV particles coming from the extra-hepatic sites. Large, single-center data in the early 1990s demonstrated increased mortality and graft dysfunction in

hepatitis B surface antigen (HBsAg)-positive patients as compared to the HBsAg-negative patients undergoing LT [Todo S *et al*., 1991]. It was seen that HBV recurrence in the graft was of a very severe nature, including an aggressive variant of HBV-associated liver injury known as fibrosing cholestatic hepatitis (FCH) [Davies SE *et al*., 1991]. However, with the introduction of hepatitis B immunoglobulin (HBIg) in the peri transplant period, there was a significant reduction in the rate of HBV recurrence post LT, with improved patient and graft survival rates, and HBV-induced fulminant or end-stage hepatic failure became an accepted indication for LT. Refinements made in the antiviral prophylaxis and treatment of HBV infection, in particular, the recent introduction of nucleoside analogs, alone or in combination, for the treatment of established graft infection have remarkably improved the outcome of LT for HBV with the result that few if any grafts should succumb to HBV-inflicted damage.

To date there are no effective vaccines to prevent HCV infection and therapy has been limited to a combination of ribavirin and interferon α or pegasys interferon α (standard of care, SOC) until 2011, when a new class of drugs started to be available for the treatment of chronic hepatitis C: the Direct Acting Anti-viral (DAA). The latter has been developed to be used, in a triple combination therapy (SOC + DAA), firstly in patients with low or no response to the SOC and promising results has been obtained from the first clinical trials. Unfortunately the DAA can select HCV variants inducing resistance and the risk of progression in liver disease is high like in HBV during NAs therapy if not greater. Therefore, management of chronic HCV treatments will be one of the main challenge for hepatologist clinician

in the next years, particularly considering that end-stage liver disease due to chronic HCV infection is the leading indication for LT with a frequency of 28% of the all diagnosed causes (Figure 1). Moreover, from the data available to date, infection is expected to recur, after the LT, in almost all patients with active HCV infection before transplant and approximately 20–30% of these HCV-reinfected patients will develop cirrhosis within 5 years. Unfortunately, many transplant recipients who develop cirrhosis are not candidates for retransplantation, and outcomes in patients who do undergo retransplantation are usually not good. The most reliable way to prevent post-transplantation HCV infection is to cure the infection before transplantation. However, this approach is not feasible in patients who present with decompensated cirrhosis, as use of interferon is contraindicated in these patients. Moreover, the achieving of undetectable levels of HCV RNA while on treatment may not be sufficient to prevent recurrence of HCV infection post-transplantation. A new way to prevent the graft from becoming reinfected post-transplantation is to use monoclonal antibodies targeting the HCV proteins that help the virus gain entry into hepatocytes. A phase II study of these antibodies showed some promise, but an effective regimen will require a DAA drug in addition to the antibody [Gonzalez SA. 2011].

2. HEPATITIS B VIRUS

2.1 HBV biology

Human Hepatitis B virus (HBV) is classified as a member of the Hepadnaviridae family [Gust *et al*, 1986] (hepatotropic DNA viruses), that can be divided into two genera: the Orthohepadnaviruses, infecting only mammals, and the Avihepadnaviruses infecting birds. To date, two major species have been assigned to the Avihepadnaviruses, the duck hepatitis B virus (DHBV) and the heron hepatitis B virus (HHBV). The Orthohepadnavirus genus includes the four best-known distinct species human hepatitis B virus (HBV), woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV) and woolly monkey hepatitis B virus (WMHV). The host range of Orthohepadnavirus varies in the different species allowing in some case the possibility to have more infecting animal models. WHV, is a well studied orthohepadnavirus that occurs naturally in marmots and cannot be transferred to other rodents like its relative GSHV [Summers *et al*., 1978; Mason *et al*., 1980]. GSHV can infect woodchucks, thus its host range is not as narrow as the WHV host range. Finally, the WMHV, despite having a non-human primate as its natural host, in contrast to HBV, is not infectious for chimpanzees [Lanford *et al*. 1998].

2.1.1 Morphology of HBV

The infectious HBV virion (Dane particle) is spherical shaped and has a diameter of 42-47 nm. External envelope consists of a cell derived

phospholipidic bilayer with three different surface protein embedded in it. These proteins, according to their size are named HB small surface antigen (SHBsAg), middle (MHBsAg), or large (LHBsAg). The nucleocapsid, which forms the inner part of the Dane particle, is around 28 nm in size and contains a single copy of the circular partially double-strand genomic DNA which is covalently linked to the viral reverse-transcriptase (figure 2).

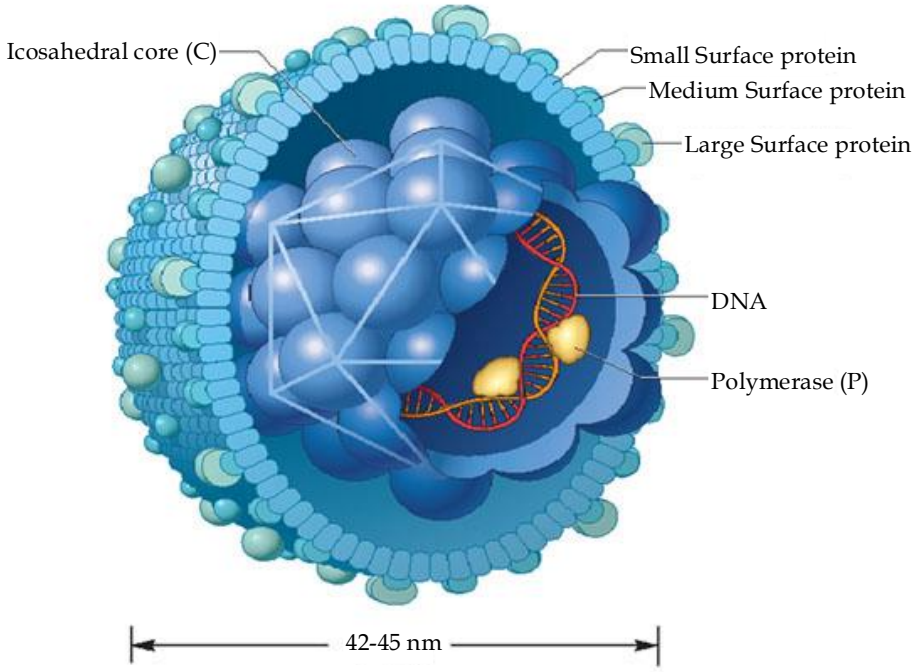


Figure 2. HBV structure

The average size of the viral genome is around 3.2 kbp, varying slightly from genotype to genotype and from isolate to isolate. The viral genome encodes for the core protein (HBcAg), the pre-core protein also known as the e-antigen (HBeAg), the polymerase, the three surface proteins, and the X protein.

2.1.2 HBV Genome and Proteome

The HBV genome is a relaxed circular, partially double stranded DNA of approximately 3,200 base pairs. There are four partially overlapping open reading frames encoding the envelope (Pre-S/S), core (Precore/Core), Polymerase, and X proteins (figure 3) [Tiollais *et al*. 1985; Seeger *et al*. 2000]. All open reading frames are in an identical orientation and overlap at least partially. Within the Dane particle the negative strand of the viral genome is present in full-length, thus carrying the whole genome. In contrast, the positive strand spans only ~ 2/3 of the genome in length, being its 3'-end is variable in size [Lutwick *et al*. 1977; Summers *et al*. 1975). The viral polymerase is covalently bound to the negative strand by a phosphotyrosine bond. At the 5'-end of the positive strand a short RNA oligomer originating from the pre-genomic (pg) RNA remains residually bound covalently after the viral DNA synthesis. The negative strand, in contrast to the positive strand, contains on both the 5'-end and the 3'- end a small redundancy of 8-9 nucleotides in length, named the R-region. These redundant structures are essential for viral replication [Seeger *et al*. 1986; Will *et al* 1987; Lien *et al*. 1986]. The viral genome covers four open reading frames, all of them encoded by the negative strand, with 6 start codons, four promoters, two transcription enhancing elements, a poly-adenylation signal motif, and a number of signals for DNA replication.

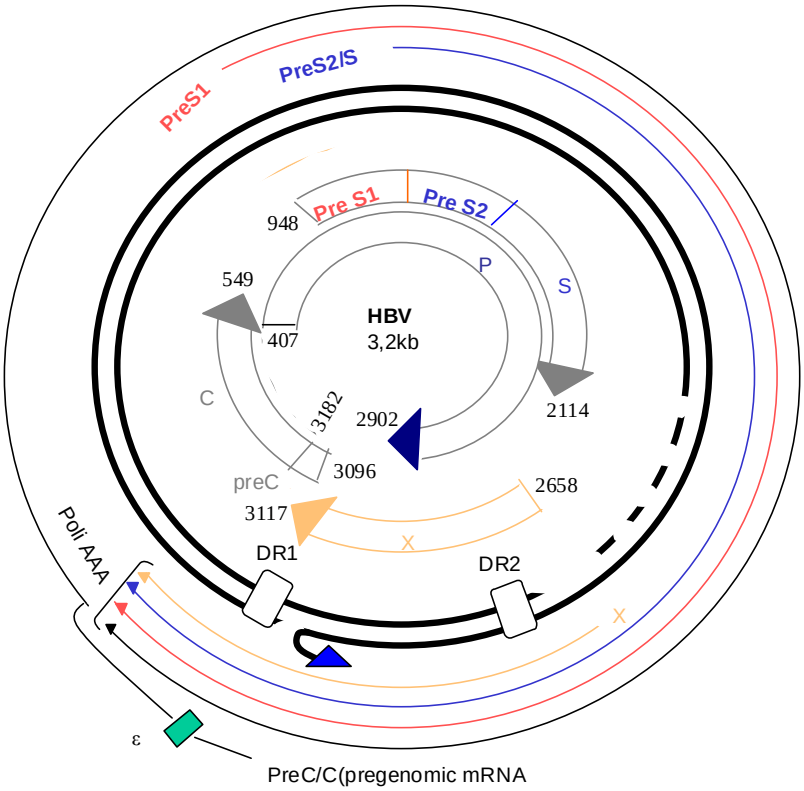


Figure 3. HBV genome organisation

The major RNA transcripts are polyadenylated, capped and named pre-C/C, preS, and S mRNAs [Enders *et al*., 1985; Cattaneo *et al*., 1984]. Moreover, a 0.7 kb long mRNA termed X mRNA occurs occasionally. The 3'-end of all HBV transcripts is common for all of them and created by the polyadenylation signal in the core (C) gene.

2.1.3.a S gene/HBsAg

The S gene encodes for the three distinct but related surface proteins termed S, M and L by a single open reading frame referred as ORF-E. This ORF is 389 or 400 codons in length depending on viral subtype and consists of three 5′ in-phase ATG codons for the initiation of translation and one 3′ TAA termination codon. Thus, the three HBV surface proteins only differ by the length of their N-terminal domains and are essential for envelopment of nucleocapsids. All three envelope components are glycosylated, type II transmembrane proteins, that can form multimers stabilized by disulfide bridges formed by cysteine residues present in the S domain. The L protein is characterized by the addition of a 108 to 119 residue sequence named "PreS1" on the N-terminus of M. Thus, this large protein basically incorporates the preS domain (preS1 and preS2) to the N-terminus of S [Heermann *et al*. 1984]. Currently, it is clearly recognized that L plays a specific role in the viral cycle: recruitment of a mature viral nucleocapsid for virion budding [Bruss, 2007] and recognition of the cellular (co-)receptor(s) for virus entry [Glebe and Urban, 2007], respectively. The 226 aa S protein traverses the membrane at least twice with transmembrane region 1 (TM1) and TM2 and is produced at the rough endoplasmic reticulum (ER) [Bruss, 2007]. The biosynthesis of the 281 aa M protein is quite similar to S and has a similar topology. This protein differs from S by the N-terminal addition of a 55 residue sequence named "PreS2". However, the exact role of M protein in the viral cycle remains unclear. Indeed, its in vivo absence in infected cells does not disrupt viral morphogenesis or particle functionality [Fernholz *et al*., 1991]. Thereby,

this protein seems not to be essential for virus spread and its complete absence from the Avihepadnavirus genomes reinforces this hypothesis.

The S, L, and M proteins are all found as components of the Dane particles. However, the surface proteins are not only incorporated into virion envelopes but also form subviral spherical particles without nucleocapsids. These particles assemble at a pre-Golgi membrane [Huovila *et al*., 1992] together with lipid, have a diameter of 20 nm, and are spherical or filamentous in shape (figure 4). The Large surface protein is an essential component of both virions and filaments and represents 10% of their envelope proteins. In contrast, it represents only 1% of the 20 nm spherical particles. The M protein is present in equal amounts in Dane particles and filaments and constitutes 10% of the 20 nm spherical particles. The S protein by itself is an essential component of virions, filaments and spheres (70-89%). Subviral HBsAg particles exceed virions by a variable factor of 10^2–10^5 and can accumulate up to concentrations of several hundred micrograms per milliliter of serum in the blood of HBV-infected patients [Seeger *et al*., 2000].

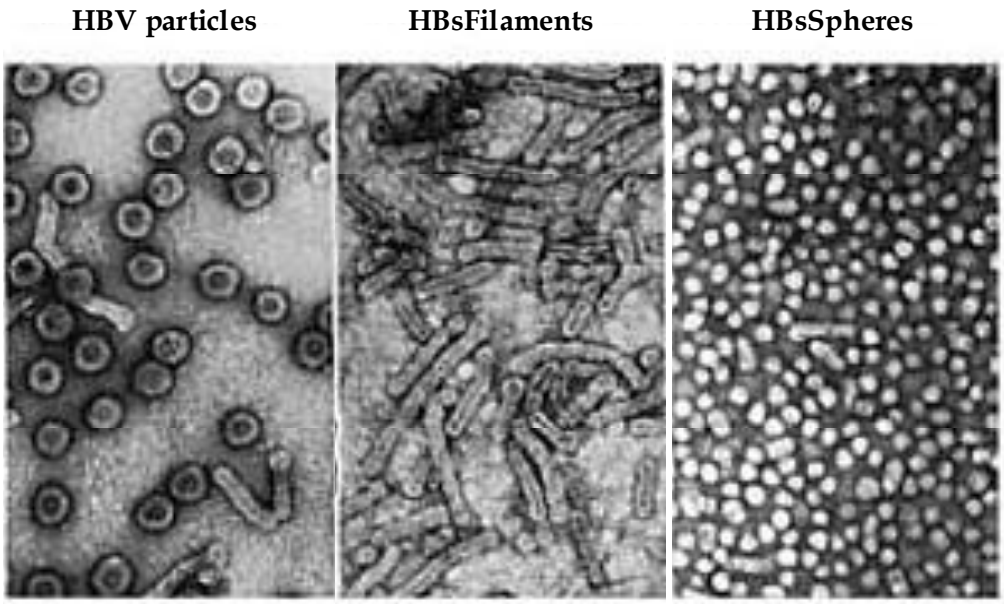


Figure 4. Morphology of HBV particles, HBs filaments and HBs spheres - [adapted from: Molecular Biology of Hepatitis B virus- Heermann KH and Gerlich WH- CRC press (1991)]

In addition to the serum, HBsAg can be detected in the infected hepatocytes with different expression patterns according to the different phases of infection. It has been shown that there is membranous staining of HBsAg associated with variable degrees of cytoplasmic staining of HBsAg during the phase of active hepatitis B virus replication and, by the contrast, solely cytoplasmic staining of HBsAg during the non-replicative phase [Gudat *et al*., 1975; Ray *et al*., 1976; Montano *et al*., 1982]. Membranous staining of HBsAg on the hepatocyte correlated excellently with serum HBV DNA and could be recognized as a marker of active hepatitis B virus replication [Chu *et al*., 1995].

2.1.3.b PreC/C gene/ HBeAg, HBcAg

The PreC/C ORF contains two in-frame translation initiation signals separated by 28 codons, thus encoding both precore protein, precursor for the viral e antigen (HBeAg), and Hepatitis B virus core antigen (HBcAg) [Uy *et al*., 1985; Seeger *et al*., 1986]. Synthesis of precore protein is initiated from the first ATG and core protein from the second at positions 1814 and 1901 respectively. The precore protein therefore initially contains all of the core protein sequence plus 29 amino acids at its N-terminus. These constitute a signal peptide that directs the nascent chain to the endoplasmic reticulum and the secretion pathway.

The first 19 amino acids are cleaved off and during transport to the cell surface the protein is further matured by removal of the highly basic C-terminal tail. Production and secretion are modulated at both transcriptional [Okamoto *et al*., 1994; Laskus *et al*., 1994] and translational [Brunetto *et al*, 1989; Carman *et al*., 1989; Okamoto *et al*., 1990] levels and the protein is released as a soluble antigen, HBeAg [Standring *et al*., 1988]. HBeAg is not a structural component of the virion, it is not required for viral replication, however its secretion is conserved in both Orthohepadnaviruses and Avihepadnaviruses and its synthesis is tightly regulate at transcriptional and translational levels. This it is supposed to be due to an immune-regulatory function promoting virus persistence by inducing neonatal tolerance and modulating the immune-response in adults [Chang *et al*., 1987; Chen *et al*., 1992, Milich *et al*., 1997]. The core protein which itself can be phosphorylated by several kinases, is essential for the formation of nucleocapsids. It plays an active role in binding and packaging of the

pregenomic RNA, recruitment of the viral polymerase, and thus enables the RT-polymerase/RNA complex to initiate reverse transcription within the newly forming nucleocapsids [Lan *et al*., 1999; Gerlich *et al*., 1982 1982; Watts *et al*., 2002]. Although HBcAg and HBeAg show substantial amino acid sequence homology, they are serologically distinct, and the immune responses to these antigens appear to be regulated independently. HBcAg is highly immunogenic (0,025 mg of HBcAg elicit antibodies production) and functions as both T-cell independent and T-cell dependent antigen. Immunization with HBcAg preferentially primes Th1 cells; and HBcAg-specific Th cells mediate anti-envelope as well as anti-HBc antibodies production.

The HBcAg is a major target of CTL response. High levels of soluble anti-HBc, as observed in most of HBV infected individuals, may compete with B cells Ig receptor mediated uptake of HBcAg with inhibition of TH cell activation. The immune response to HBeAg is strictly T cell-dependent [Milich *et al*., 1986] and HBeAg preferentially, but not exclusively, elicits Th0 or Th2-like cells which stimulate the humoral immune reaction [Milich *et al*., 1997]. In particular, HBeAg-specific Th2 cells may cross-regulate HBcAg-specific Th1 cells or the secreted HBeAg may preferentially behave as a tolerogen and inactivate HBcAg-specific T cells through deletion or clonal anergy in the periphery [Chen *et al*., 2005; Milich, 1997; Milich *et al*., 1998]. T-cell anergy is a tolerance mechanism in which the T cell is functionally inactivated following an initial antigen encounter but remains alive in a hypoactivated state.

2.1.3.c P gene/Polymerase

The P ORF, covering nearly 80% of the hepadnaviral genome (1685 nt), encodes a multifunctional protein including the terminal protein (TP) that acts as a primer for HBV DNA synthesis and the viral polymerase that possesses DNA polymerase, reverse transcriptase (RT) and RNaseH activities. During replication, a pre-genomic RNA is produced that encodes for both HBcAg and polymerase [Nassal *et al*. 1996; Nassal 1999]. Polymerase is the second ORF on this messenger RNA (mRNA) and partially overlaps with the 3' end of the HBcAg cistron. The HBV polymerase is not proteolytically cleaved to mature enzymatically active proteins, but consists of 4 domains [Lanford *et al*. 1999]: a TP domain, which becomes covalently linked to negative-strand DNA during initiation of reverse transcription [Weber e al. 1994; Zoulim *et al*. 1994] a spacer domain, which is very tolerant to mutations and can be partially deleted without affecting polymerase activity [Li *et al*. 1991; Radziwill *et al*. 1990; Kim *et al*. 1999]; the reverse transcriptase/polymerase (RT domain), which contains the conserved regions A through F, characteristic of RNA-dependent RNA polymerase (RdRP) and RNA-dependent DNA polymerase (RdDP) [Poch *et al*. 1989; Lesburg *et al*. 1999]; and the ribonuclease H domain (RH domain)[Wei *et al*. 1996].

2.1.3.d X gene/X protein

The X ORF is located downstream to enhancer 1 (Enh I) and is partly overlapped by the P ORF at its N terminus and by the PreC/C ORF at its C terminus [Tiollais *et al*. 1985]. Furthermore, the X ORF is overlapped by several cis-elements including enhancer II (Enh II) [Yee, 1989], several cis-elements for transcriptional regulation, and the PreC-C promoter. The X gene is transcribed independently of the other viral transcripts and a separate X mRNA of 0.8 kb has been detected in liver tissues of both human and woodchuck [Kaneko *et al*.,1988; Guo *et al*., 1991] as well as in transgenic mice [Kim *et al*., 1991]. So far, the role of the X protein is not fully understood, indeed, although HBx does not bind DNA, the first activity identified for this viral protein was the ability to activate transcription of viral and cellular genes [Twu *et al*., 1987]. HBx is a weak transactivator, but it is capable to activate a variety of cellular and viral promoters. This broad spectrum of activities relies on two different mechanisms: either direct interaction with nuclear transcriptional regulators, or activation of signal transduction pathways [Wei *et al*., 2010]. HBx has also been implicated in pleiotropic activities such as cell cycle regulation, activation of signaling pathways, modulation of apoptotic pathways, and inhibition of DNA repair [Andrisani *et al*. 1999; Bouchard *et al*., 2004; Tang *et al*. 2006].

2.1.4 HBV life cycle

Hepatocytes are the primary site of viral DNA replication. It is assumed that viral entry and the host range of hepadnavirus is dependent on the N-terminus of the large surface antigen [Ishikawa *et al*., 1995; Chouteau *et al*., 2001; Lambert *et al*., 1990; Gripon *et al*., 2005; Urban *et al*., 2002]. So far, the intrinsic HBV receptor has not been discovered, but from studies on DHBV in primary duck hepatocytes it is assumed that around 104 receptor molecules per cell mediate the rapid binding, followed by a slow uptake of the virus to the cell which can take up to 16 hours [Pugh *et al*.,1989; Klingmuller *et al*.,1993; Pugh *et al*., 1995]. Following entry the nucleocapsid is transported into the cell nucleus, where the viral nucleic acid is released. Release of the viral DNA and disintegration of the nucleocapsid is assumed to take place at the nuclear core complex [Kann *et al*., 1997; Rabe *et al*., 2003] (figure 5).

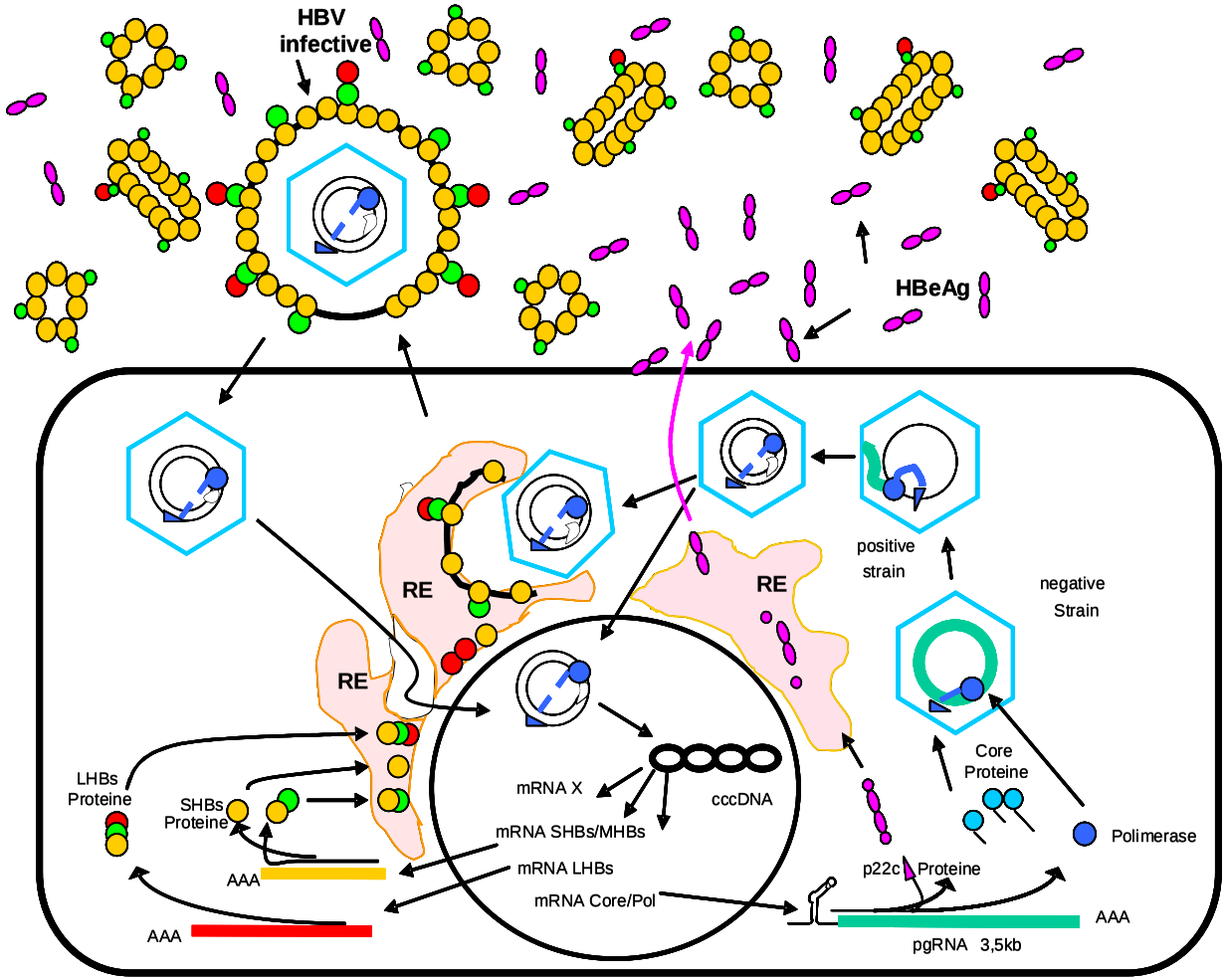


Figure 5. Schematic representation of the HBV life cycle

The life cycle of hepadnaviruses is characterized by the synthesis of a 3kb partially double-stranded, relaxed-circular DNA (rcDNA) genome by reverse transcription of an RNA intermediate, the pregenome. The mechanism of RNA-directed DNA synthesis has been well characterized [Ganem *et al*., 1994; Seeger *et al*., 1996]. After virus penetration the DNA reaches the nucleus and is immediately converted by host enzymes to complete open circular double-stranded DNA and then to supercoiled DNA (cccDNA) (figure 5). Since cccDNA, which accumulates only in the nucleus, is the template for the transcription of all viral mRNAs, its formation indicates a successful initiation of infection. The viral RNAs include pregenomic RNA (pgRNA), which serves as the template for reverse transcription, as well as three subgenomic mRNAs necessary for the translation of the envelope proteins and the mRNA for the X protein. The pgRNA is both the template for core and polymerase protein translation and is the matrix for the progeny genomes. The pgRNA bears a secondary structure - named ε-structure - that is present at both the 5'- and the 3'-ends. The ε-hairpin loops at the 5'-end are first recognized by the viral polymerase and act as the initial packaging signal [Bartenschlager *et al*., 1992; Hirsch *et al*., 1990; Huang *et al*., 1991] (figures 5). In the cytoplasm, the core protein forms the basis for the nucleocapsid. It plays an active role in binding and packaging of the pregenomic RNA, recruitment of the viral polymerase, and thus enables the RT-polymerase/RNA complex to initiate reverse transcription within the newly forming nucleocapsids. Finally, in the endoplasmic reticulum, the nucleocapsid acquires the external coat. In

the early stages of infection the nucleocapsid is also transported to the nucleus in the hepatocyte to increase cccDNA copies [Nassal *et al*., 1993; Nassal *et al*., 2000] (figure 5).

The HBV DNA is able to integrate into the host genome. The integration occurs in different sites and usually affects only one part of the viral genome, which retains the ability to be transcribed. The integration of HBV DNA is an early event in the natural history of infection and is implicated in the pathogenesis of hepatocellular carcinoma: the integration within or near cellular genes involved in regulating cell cycle, could result in an alteration of their function promoting in the hepatocytes affected a neoplastic transformation.

2.1.5 Viral Heterogeneity

Changes in the viral genomes occur randomly as errors in the replication. These can be nucleotide substitutions (point mutations), deletion or insertions. Usually DNA viruses have a more stable genome than those RNA, because of the proofreading capacity of the host enzyme that they exploit for replication. The Hepadnaviridae genome represent an exception. Although their genome is a double-strand DNA, these viruses replicate through a RNA template which is reverse transcribed by a enzymatic complex that lacks the proofreading capacity. On the other hand, the HBV genome in particular, has a very compact organization and even a single nucleotide substitution may result in pleiotropic effect. For this reason, the number of mutations that can be tolerated without losing infectivity and

replication capacity is rather limited [Miller *et al*., 1989]. Indeed, any mutant selection is related to its biological efficiency and ability to escape immune pressure, both humoral and cellular, and/or selective pressure exerted by antiviral drugs that directly interfere with viral enzymes. Moreover, in chronic hepadnavirus infections, net virus expansion cannot occur indefinitely. The maximum amount of virus, or viral cccDNA, in the liver is limited by the number of hepatocytes that can be infected and the maximum number of cccDNA copies per hepatocyte. In a fully infected liver, new cccDNA synthesis is prevented unless uninfected cells are generated by liver growth or cell turnover or unless existing cccDNA molecules are lost and replaced within the cell. Turnover of cells or cccDNA thus provides an opportunity for enrichment of one virus strain over another through competitive growth [Zhang *et al*., 2000]. This explains why, despite the relatively high mutation rate of HBV (approximately 2 x 10^4 nucleotide substitutions/site/year), only a small proportion of mutants can survive [Girones *et al*., 1989].

2.1.5.a S gene mutations

The envelope proteins of HBV are targets of both humoral and cellular immune response that are involved in viral clearance. Anti-HBs antibodies show neutralizing activity and are essential to limit the spread of the infection. As a consequence, envelope proteins have been used in the prophylaxis of HBV infection for preparation of the vaccines and production of antibodies for therapeutic purposes [Neurath *et al*., 1986;

Vento *et al*. 1987; Nayersina *et al*.,1993; Jin *et al*., 1995]. Some of the envelope mutants determine the different subtypes of HBV and may have been selected over centuries perhaps under HLA pressure.

In the recent years, many variants have been identified in the S gene and some of these mutations produce changes in the epitopes recognized by the current diagnostic assays determining the undetectability or a lower sensitivity to the HBsAg quali / quantitative assays [Carman *et al*., 1995; Carman *et al*., 1997; Mühlbacher A *et al*., 2007]. Most frequently, point mutations occur in the area of the "a" determinant (124-147 aa), and also the immune response induced by recombinant vaccines that are current available seems to focus on this region as target. The first "vaccine escape mutant" described [Carman *et al*., 1990] was substitution of a Glycine residue at position 145 by an Arginine residue (G145R), which has been identified also in chronic carriers [Carman *et al*., 1990]. Many other substitutions in the "a" determinant (I/T126A/N, A128V, Q129H/R, G130N, M133L/T, K141E, D144A/H) have since been associated with immune escape [Cooreman *et al*., 2001], but G145R is by far the most common variant. Immune escape mutants with substitutions outside of the "a" determinant have also been described [Oon *et al*., 1999], the most important of which is P120S/T and the loss of cysteine 124 abolishing HBsAg secretion in a HBsAg negative/HBV DNA positive patient. The other medical condition associated with the emergence of envelope variants is treatment with monoclonal antibodies or hyperimmune human immunoglobulin (HBIg) which is used to prevent HBV recurrence in patients transplanted for HBsAg-positive cirrhosis. Similar to vaccine

recipients, a glycine/arginine substitution at position 145 and aspartic acid/alanine at aa 144, are the most common variants found in these cases [Carman et a., 1996]. Another mutant described in a HBsAg negative/HBV DNA positive patient was the loss of cysteine 124, abolishing HBsAg secretion.

Many mutations affect the PreS domains of the envelope proteins, most of which are deletions [Kay *et al*., 2007]. PreS mutants emerge in chronic infections, often in patients treated with interferon [Gerner *et al*., 1998; Santantonio *et al*., 1992], and probably represent attempts by the virus to evade host immune responses. The emergence of pre-S mutants could play a role in viral persistence; however, their implication in the pathogenesis of liver damage cannot be excluded [Brunetto *et al*. 1999]. The region of the S gene coding the PreS1 and PreS2 domains is overlapped by the region of the P gene coding the spacer domain of the viral polymerase. This domain gives flexibility to the viral polymerase, but its sequence is not absolute and can suffer in-phase deletions and insertions without affecting the enzymatic activities of the protein. However, there are constraints on PreS1 mutations due to the fact that its N-terminus (residues 21–47 of the genotype D PreS1) is important in viral attachment to hepatocytes [Neurath *et al*., 1986] and the S promoter is located in the 3' extremity of the PreS1 coding region. In addition, some mutations can result in intracellular retention of the PreS1 protein [Bock *et al*., 1997; Melegari *et al*., 1994] which inhibits virion secretion and is cytotoxic. As a result, although PreS1 deletion mutants are replication competent, they usually need a helper virus and are found as minor viral populations. On the other hand, there appear to

be few constraints on PreS2 mutations since its product is not essential for viral replication, particle morphogenesis and secretion and infectivity [Fernholz *et al*., 1991]. Mutations include deletion or missense mutation of the PreS2 ATG, thereby abrogating synthesis of the protein, and deletions or alterations of B- and T-cell epitopes. HLA class I-restricted, cytotoxic T cells recognize short viral peptides that are generated by the intracellular processing of endogenously synthesized viral antigens within infected cells, and are expressed at the cell surface in the binding groove of selected HLA class I molecules. Single amino acid substitutions of MHC anchor residues or TCR contact sites have been shown to abrogate CTL responses *in vitro* by inhibiting either HLA binding or TCR recognition of the peptide. The most common mutation affecting the PreS2 region involves the first part in which is MHC class I-restricted T-cell epitope [Bertoletti *et al,* 1994; Chisari *et al*., 1995; Sobotta *et al*., 2000; Fan *et al*., 2001;]. An association between pre-S2 mutant (start codon mutants preventing pre-S2 protein synthesis) infection and fulminant hepatitis has been described, and a direct role of these variants in the induction (via an abnormal immune response or a direct cytopatic effect) of severe liver damage has been hypothesized [Pollicino *et al*., 1997]. Mutations in the S gene may lead to different histological liver features. Glassy or ground glass hepatocytes (GGH) ultrastructurally are characterized by an abundance of smooth endoplasmic reticulum (ER), among which HBsAg is accumulated and are different in morphology and distribution at different replicative stages of chronic HBV infection [Hadziyannis *et al*., 1973]. Recently, intracellular study have revealed that ground glass hepatocyte may contain specific mutants and

exhibit differential biological activities [Wang *et al*., 2003; Su et a., 2008]. Type I GGHs expressed an inclusion-like pattern of hepatitis B surface antigens and harbored mutants with deletions over pre-S1 region, whereas type II GGHs, distributed in clusters and emerged at late replicative phase, contained mutants with deletions over pre-S2 region that defines a cytotoxic T lymphocyte (CTL) immune epitope, and may represent an immune escape mutant.

2.1.5.b PreC/C region mutations: HBeAg defective Mutants

During chronic HBV infection, two major types of HBV core gene variants frequently occur that affect the expression of HBeAg: the PreCore (PreC) mutants and the basic core promoter (BCP) mutants.

As described above, the secretion of the antigen "e" depends on the expression of a specific leader peptide which is encoded by HBV PreC region. In 1989 two independent studies performed in anti-HBe positive patients of the Mediterranean area showed that the most frequent cause of the discrepancy between the presence of HBV DNA and the absence of HBeAg was the infection with HBV variants unable to secrete the soluble form of the HBV nucleocapsidic protein (HBeAg minus mutant) [Brunetto *et al,* 1989; Carman *et al*., 1989]. The HBeAg production and secretion are modulated both at the transcriptional [Okamoto *et al*. 1994; Laskus *et al*., 1994, Jaw *et al*., 1991] and translational levels [Brunetto *et al*., 1989, A; Carman *et al*., 1989, Okamoto *et al*., 1990, Raimondo *et al*., 1990; Brunetto *et al*., 1999]. The most prevalent PreC mutation that affect the HBeAg

expression at the translational level, is a guanine to adenine transition at nucleotide position 1896 (G1896A), which creates a TAG stop codon at codon 28 of the PreC protein [Brunetto *et al*., 1989; Carman *et al*, 1989; Raimondo *et al*., 1990; Ganem *et al*., 2001] (figure 6).

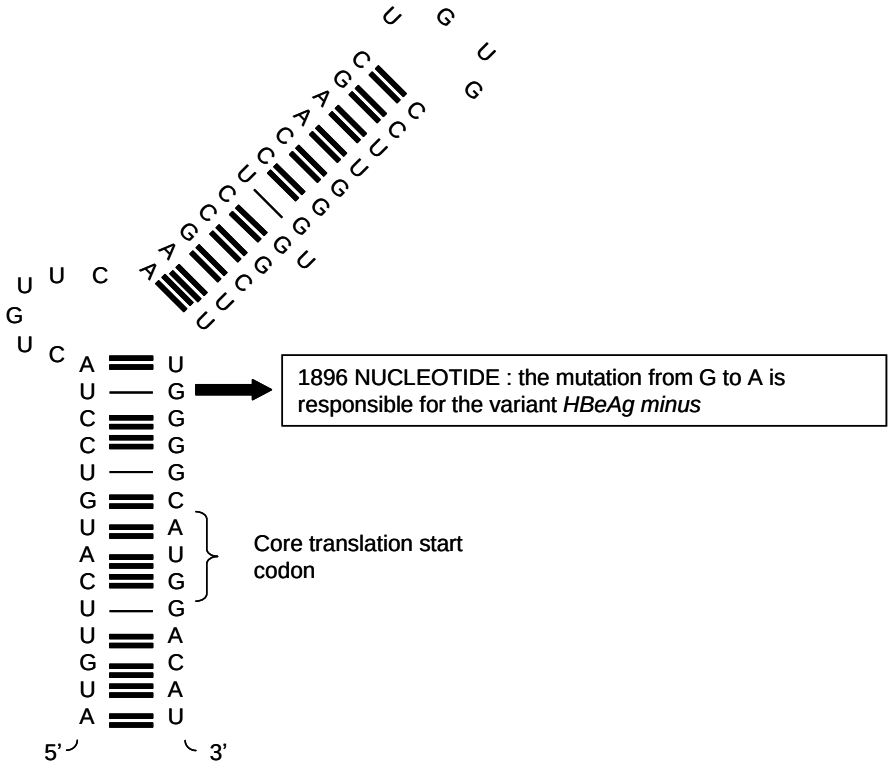


Figure. 6 Nucleotide composition of epsilon signal.

However, this mutation is located within the epsilon (ε) structure, a highly conserved stem-loop essential for initiation of encapsidation within the viral replication cycle. In order to stabilize this ε structure, the nucleotide at position 1896 is paired with the nucleotide at position 1858, which naturally is a thymidine in genotypes B, C, D, E, and G and a cytidine

in genotype A. Therefore, in HBV genotype A, the G1896A mutation usually arises together with a C1858T nucleotide exchange [Lok *et al*., 1994; Rodriguez-Frias *et al*., 1995]. These HBV mutant, termed HBeAg minus or defective is predominant, in the mediterranean area, in 90% of HBV chronic carriers anti-HBe positive [Brunetto *et al*, 1989; Raimondo *et al*., 1990; Kojima *et al*., 1991; Santantonio *et al*., 1992; Tong *et al*, 1992]. As previously shown, wild-type and HBeAg minus HBV may coexist in a HBV carrier and their relative ratio vary over time [Brunetto *et al*., 1991; Brunetto *et al*., 1993; Brunetto *et al*., 1994; Brunetto *et al*., 1994b; Brunetto *et al*., 1997]. Furthermore, as discussed later, follow-up studies suggested the important association between different ratios of circulating wild type and HBeAg minus HBV and pathogenetic events during the course of chronic hepatitis B [Brunetto *et al*., 1994; Brunetto *et al*., 1997]. *In vitro* experiment showed that the precore region and pgRNA transcription are under control of regulatory elements such as the Basic Core Promoter (BCP) and the Core Upstream Regulatory Sequence (CURS) [Yuh *et al*., 1992]. Mutations in these domains appear to affect mRNA transcription, notably by decreasing the synthesis of HBeAg [Okamoto *et al*., 1994]. Cytokines, such as tumor necrosis factor alpha (TNF-α) and interferons alpha and gamma (IFN-α and γ), held the transcription inhibitory activity by acting directly on BCP [Romero *et al*., 1996].

Other mutations that act at translational level are a thymine (T) instead of a cytosine (C) at nucleotide 1817 and an A instead of a G to 1897, both of which give rise to translational stop codons. Two mutations were also observed: G⇒T and T⇒C in position 1816 and 1815 respectively,

responsible for the elimination of translation start codon of the pre-core region. All these mutations leads to the same result: a defective virus of "e" antigen [Brunetto *et al*, 1989; Carman *et al*., 1989 Santantonio *et al*., 1992; Tong *et al*, 1992].

The most common BCP mutation is the double A1762T and G1764A nucleotide exchange, which results in a decrease of HBeAg expression of up to 70% but enhanced viral genome replication [Buckwold *et al*., 1996; Hunt *et al*., 2000; Locarnini *et al*., 2003]. Moreover, considering the regulatory activity of these region, carriers infected with HBV with the BCP mutations resulting in gene deregulation, may be at increased risk for hepatocellular carcinoma [Lee, 1997].

2.1.5.c P gene mutations

Mutations in the P gene are not very frequent during the HBV natural infection and limited to those that result from the host immune pressure on the S gene region overlapping the polymerase/retrotranscriptase. Mutations in the P gene especially occur during NAs prolonged treatment and are selected because allow the Pol/Rt to recover the replication efficiency avoiding and resisting to the chain terminator nucleos(t)ide analogue. Because of their strong association with NAs treatment the HBV resistant variants will be described later, along with the HBV treatments.

2.1.5.d X gene mutations

Mutations in the X region may involve replication regulatory elements such as basic core promoter (BCP) and enhancer II: three AT-rich regions are present in BCP, each of them would represent an independent binding site for liver-enriched factors for initiating the transcription of the different 3.5 mRNAs [Lopez-Cabrera *et al*., 1990]. The BCP mutations are located most frequently in the second AT-rich region (1762 A to T switch and 1764 G to A switch); occasionally, point mutations have been described in the first AT-rich region, whereas no mutations have been observed in the third region, which could induce a decrease in the transcription of genomic mRNA [Okamoto *et al*., 1994; Sato *et al*. , 1995]. In fact, it has been shown that point mutations in the TATA box of eukaryotic promoters drastically decrease *in vitro* transcription, and mutations in the AT region of viral promoters should have similar effects [Corden *et al*., 1980].

2.1.5.e Genotype and Geographical distribution

Genotypes or genetic subtypes are genetically related strains and have been described for viruses belonging to several different families. Four genotypes, A–D, of HBV, originally designated as genomic groups, were based on > 8% inter genotype and <4% intra genotype divergences, when 18 complete genomes were compared [Okamoto *et al*. , 1988]. Later research has identified other four HBV genotypes, E–H [Norder *et al*., 1992; Norder *et al*. 1994; Naumann *et al* 1993; Stuyver at al. 2000; Arauz-Ruiz *et al*. 2002]. Two genotypes, A and F, have been further subdivided into

subgenotypes identified by arabic numerals [Kramvis *et al*., 2002; Kimbi *et al*. 2004, Norder *et al*. 2003]. The eight genotypes identified to date, are distributed in different geographical areas. Genotype A is present in North Europe and North America, genotypes B and C are typical of the East Asian countries, genotype D is spread all over in the world but mainly in the mediterranean area, genotype E is most common in Africa, F and H are endemic of South and Central America respectively, finally genotype G was firstly isolated in France but is rare and always found in coinfection with other genotypes (mainly A). HBV genotypes may influence the course of disease and among these relevant biological differences has been recognized. The classical precore mutation, located at HBV nucleotide 1896 consisting of a G-A substitution that creates a stop codon, is not found in genotype A. Genotype A (and F2) contains a cytosine at position 1858 instead of a uracil, that stabilizes the PreC loop, not allowing the 1896 G-A precore mutation to occur [Li *et al*., 1993]. Indeed, the HBV genotypes B, C, D and E are predisposed to develop the mutation at 1896 precore, which will result in an earlier seroconversion to anti-HBe. Genotype A (presumably A2) infection was associated with a significantly higher cumulative rate of sustained biochemical remission, HBV DNA clearance, and HBsAg clearance in patients with chronic HBV infection than genotype D infection [Sanchez-Tapias *et al*., 2002]. Multiple studies have shown that patients with genotype C experience HBeAg seroconversion at an older age and are more likely to be HBeAg positive at any given age than HBV genotype B [Chen *et al*., 2004; Kao *et al*., 2002; Kao *et al*., 2004]. HBV genotype C is associated with an increase risk of liver inflammation, flares

of hepatitis, liver fibrosis, and cirrhosis. Persons infected with genotype D usually convert from HBeAg to anti-HBe in adolescence or early adulthood. The precore mutant is frequently associated with HBV seroconversion in this genotype. While it appears that many persons go into and remain in the inactive carrier phase, some persons develop HBeAg-negative/anti-HBepositive chronic hepatitis B. This can lead to cirrhosis and HCC [Naoumov *et al*., 1992; Grandjacques *et al*., 2000; Hadziyannis *et al*., 2001; Brunetto *et al*., 2002; McMahon, 2005; Zacharakis *et al*.,2005]. Genotype F is divided into four subtypes: F1–F4 [Devesa *et al*., 2008]. Genotype F2 codes for C at position 1858 and therefore PreC mutation does not occur, whereas F1 does not and thus PreC mutation can occur [Schaefer, 2005].

2.2 HBV infection and disease

HBV can cause a transient or chronic liver disease. The transient infection may be mild and asymptomatic or cause an acute hepatitis with varying degrees of severity to result in 0.5% of patients a fulminant hepatitis. The HBV is not directly cytopathic for the infected cell [Guidotti *et al*., 1995; Chisari *et al*., 2000; Guidotti *et al*., 2006] and damage is induced by the host immune reaction. Correlative clinical studies show that in acute, self-limited hepatitis B, strong T-cell responses to many HBV antigens are readily demonstrable in the peripheral blood [Chisari *et al*., 1995]. These responses involve both major-histocompatibility-complex (MHC) class II–restricted, CD4+ helper T cells and MHC class I–restricted, CD8+ cytotoxic T lymphocytes. By contrast, in chronic carriers of HBV,

such virus-specific T-cell responses are greatly attenuated, at least as assayed in cells from the peripheral blood. It has been show that the resolution of infection occurs through the coordinated activity of cytokines (IFN α and TNF α released by lymphocytes and the cytolitic response that leads to infected hepatocytes elimination [Ando *et al*., 1994; Guidotti *et al*., 1994a; Guidotti *et al*., 1994b; Guidotti *et al*., 1996; Guidotti *et al*., 1999a; Guidotti *et al*., 1999b; Guidotti *et al*., 2000; Kakimi *et al*., 2001; Tsui *et al*., 1995]. The natural history of chronic HBV infection develops over decades, the subject is often infected at birth or early in life and keeps for several years a state of tolerance or weak activation during which the level of liver damage is minimal compared to high replication. Indeed, it is assumed that an "immune-tolerant" conditions it is established in those cases in which the host immune system is compromised or does not recognize the viral antigens (i.e., if the exposition to the virus occurs during the intrauterine life) [Milich *et al*., 1990]. This condition is common in children born from HBeAg-positive mothers and infected with HBV in the perinatal period [Lee *et al*., 1990; Bortolotti *et al*., 1990; Moyes *et al*., 1993]. In this case, the exposure during pregnancy to circulating HBeAg promotes a tolerance state towards viral nucleocapsid antigens (HBcAg and HBeAg), which is the main target of cell-mediated response. The immune-tolerant phase can last for a few years to more than 30 years [Hui *et al*., 2007]. During this phase, the markers of infection (HBsAg and anti-HBc) and replication (HBeAg and HBV DNA), are present in the sera of infected individuals whereas are absent those of virus-induced damage (IgM anti-HBc). In the liver there is either no or minimal liver inflammation or fibrosis, a high percentage of

hepatocytes express hepatitis B core antigen, predominantly in the nuclei, while HBsAg show an extensive membranous staining.

Once the viral antigens are recognized by the immune system, the attempt to control the infection through the block of viral replication (cytokine-mediated) and infected hepatocytes elimination (cell-mediated) begins. Liver infiltration by lymphocytes, observed during viral hepatitis, is the main pathogenetic moment. These phase, termed "immune-activation", can persist for years with alternating phases of exacerbation and remission. In the serum are present markers of viral replication, with typical fluctuations in viremia levels, of liver damage and of virus-induced liver damage [Brunetto *et al*., 2001]. In the liver the expression of HBcAg appears to be focal in the nuclei or cytoplasmic whereas HBsAg stains mainly in the cytoplasm either in perinuclear blush to a dense signal corresponding to ground-glass cells. Most frequently, the immune activation leads to an inactive hepatitis B phase, characterized by the absence of HBeAg and the presence of anti-HBe, normal ALT levels, low HBV DNA levels (<2000 IU/ml), and absence or minimal liver fibrosis and inflammation [De Franchis *et al*., 1993; Martinot-Peignoux *et al*., 2002; Zacharakis *et al*., 2005]. However, a significant proportion of patients, after seroconversion, retain evidence of viral replication and virus-induced liver damage. This condition is typically associated with the prevalence of a viral population unable to secrete the HBeAg [Bonino *et al*., 1981; Bonino *et al*., 1986; Brunetto *et al*., 1989b; Ulrich *et al*., 1990; Bonino *et al*., 1991; Brunetto *et al*., 1991; Bortolotti *et al*., 1993; Brunetto *et al*., 1994; Hadzyannis, 1995; Brunetto *et al*., 1997; Brunetto *et al*., 2001; Brunetto *et al*., 2002]. This

HBeAg defective mutant has been demonstrated during the exacerbation of the immune elimination phenomena preceding HBeAg seroconversion to anti-HBe, becoming the majority viral population in the HBe negative/anti-HBe positive chronic hepatitis B, which is prevalent in patients infected with genotype D of HBV in the Mediterranean area. The disease caused by HBeAg minus HBV runs usually asymptomatic for three to four decades and reaches the stage of cirrhosis at a median age of about 45 years [Brunetto *et al*., 2002]. Thereafter cirrhosis progresses to end stage complications in about 25% of patients in about 10 years; recurrent hepatitis B exacerbations accelerate disease progression [Brunetto *et al*., 2002]. The virological and biochemical patterns of chronic anti-HBe positive hepatitis B vary from intermittently to persistently detectable *viraemia* and elevated ALT levels. In these patients, viraemic levels tend to be lower fluctuanting within the range 10^4-10^7 genomes/ml than those HBeAg positive (range 10^6 - 10^9 gen/ml). In spite of an intermitting disease profile associated with frequent and sometimes long lasting remissions spontaneous recovery from anti-HBe positive chronic hepatitis B is very rare [Lok *et al*., 2001; Hadziyannis *et al*., 2005; Brunetto *et al*., 1989; Brunetto et aòl., 2002]. Persistent viral replication is a major cause of chronic liver damage and development of cirrhosis: in a cohort study after a mean follow-up of 10 years about 50% of the patients with chronic hepatitis at baseline developed cirrhosis and persistently detectable HBV DNA was a factor independently associated with disease progression [Brunetto *et al*., 2002]. Further, cirrhosis development as an end point complication was associated with recurrent hepatitis exacerbations [Brunetto *et al*., 2002].

Active carriers have a significantly increased risk of life-threatening liver complications such as hepatic decompensation, liver cirrhosis and hepatocellular carcinoma [Beasley, 1988]. HBV infection appears to play both an indirect (via liver cirrhosis) and direct role in hepatocellular carcinoma development: the mechanisms of direct HBV oncogenesis are not completely understood, nevertheless either integration of HBV genomic DNA into cellular chromosomes, with disregulation of cellular gene function and transactivating activity of some HBV proteins (such as HBx and HBs truncated forms) had been shown to alter cellular homeostasis.

2.2.1 Diagnosis of HBV infection

Virological diagnosis and monitoring of hepatitis B virus infection are based on serologic assays detecting specific anti-HBV antibodies, and assays that can detect, quantify or characterize the components of HBV viral particles, such as HBV DNA and various viral antigens. Polymerase chain reaction (PCR) based assays allow to detect, by specific molecular hybridisation of nucleic acid probes to the viral sequence target, less than 10 copies/ml of virus starting from a serum or plasma sample of 0.2 ml [Raimondo *et al*., 2003]. The presence of HBV DNA in peripheral blood is a reliable marker of active HBV replication and reflect the viral activity within the liver. However, the diagnosis of the infection does not necessary imply that the liver is damaged by the virus. As described above, florid virus replication can persist for years without liver damage if the host's immune system does not react against viral antigens [Bonino *et al*., 1991b; Brunetto

et al., 1991b]. Liver disease begins as soon as immunotolerance is lost and the virus infected cells start to be eliminated, therefore hepatitis B represents an injurious way of recovering. Serum IgM anti-HBc are detected in any form of liver disease caused by HBV, independent on the duration of virus infection. Serum anti-HBc IgM are usually detected with high titers during an acute hepatitis B, and with lower titers after the typical flare-ups of alanine aminotransferase (ALT) which occur in chronic hepatitis. These episodes of ALT flare up are preceded by an increase or reappearance of *viraemia* [Colloredo *et al*., 1992] followed by rapid decline, whereas the elevation of IgM anti-HBc persist for a longer period due to their extended half life.

HBsAg has been recently shown to represent a new diagnostic tool in the management of HBV infection. HBsAg serum levels vary during chronic hepatitis B infection, becoming lower during the transition from the active to the inactive phase of HBV infection [Nguyen *et al* 2010; Jaroszewicz *et al*., 2010; Brunetto *et al*., 2010]. Recent findings confirm that serum HBV DNA and HBsAg levels provide complementary information on the status of HBV infection and showed that the single point combined quantification of HBV DNA (<2000 IU/mL) and HBsAg (<1000 IU/mL) allows the identification of inactive carrier with a very high diagnostic accuracy (94.3%) that is comparable with that of 1 year monthly monitoring [Brunetto *et al*., 2010]. HBsAg serum levels were shown to correlate with intrahepatic covalently closed circular DNA (cccDNA) levels [Volz *et al*., 2007] that vary in different patient populations but persist through all phases [Werle-Lapostolle *et al*, 2004].

2.3 Treatment anti-HBV: Indications and Goals

The guidelines from the American Association for the Study of Liver Diseases (AASLD) [Lok *et al*., 2009] and the Asian-Pacific Association for the Study of the Liver (APASL) [Liaw *et al*., 2008] advocate treatment for patients with hepatitis B e antigen (HBeAg)–positive chronic hepatitis B virus infection with serum HBV DNA levels > 20,000 IU/mL and persistently elevated ALT levels (> 2 times the upper limit of normal (ULN) over a 3-6 month period). The updated normal ALT levels have been established at 30 U/L for men and 19 U/L for women [Prati *et al*., 2002].

According to the AASLD guidelines, the same HBV DNA and ALT criteria apply to HBeAg-negative patients, but treatment should also be considered in patients with normal ALT if the HBV DNA concentration is > 2000 IU/mL and if they have a liver biopsy showing moderate to severe necroinflammation with or without fibrosis [Dienstag *et al*., 2008]. The APASL guidelines recommend to consider antiviral treatment for HBeAg-negative patients with HBV DNA ≥ 2000 IU/mL and ALT levels persistently > 2 x ULN [Liaw *et al*., 2008].

According to the 2012 HBV guidelines of the European Association for the Study of the Liver (EASL), patients should be considered for antiviral therapy if they have HBV DNA levels > 2000 IU/mL, ALT levels > 1 x ULN, and a liver biopsy that shows moderate to severe necroinflammation or fibrosis, regardless of their HBeAg status. In addition, treatment may be initiated in patients with normal ALT levels if they have HBV DNA levels >

2000 IU/mL and a liver biopsy that shows moderate to severe necroinflammation or fibrosis. Immediate liver biopsy or therapy is not necessary in HBeAg-negative patients with persistently normal ALT levels and HBV DNA levels > 2000 IU/mL but < 20,000 IU/mL and no evidence of liver disease. Conversely, both HBeAg-negative and HBeAg-positive patients with ALT levels > 2 x ULN and serum HBV DNA > 20,000 IU/mL may initiate therapy without the results of a liver biopsy.

Patients who are not candidates for immediate initiation of therapy should be closely monitored. Hepatitis B e antigen positive patients with HBV DNA > 20,000 IU/mL but normal ALT levels or an absence of necroinflammation on the liver biopsy (ie, so-called immunotolerant patients) should have their ALT levels measured every 3-6 months and should be regularly tested for the presence of HBeAg (approximately every 6-12 months [Lok *et al*., 2009]. In HBeAg-negative patients with HBV DNA < 2000 IU/mL and normal ALT (ie, so-called inactive hepatitis B surface antigen carriers), ALT and HBV DNA levels should be reassessed every 3 months during the first year, with subsequent biannual assessments [Lok *et al*., 2009]. Patients with low levels of serum HBV DNA (< 2000 IU/mL), as well as low levels of HBsAg (< 1000 IU/mL), may perhaps require less frequent monitoring due to a very low probability of disease reactivation [Brunetto *et al*., 2010].

Complete eradication of HBV from host hepatocytes cannot be achieved with currently available agents because of the persistence of HBV covalently closed circular DNA. The main goal of treatment for chronic HBV infection, therefore, is to halt the progression of liver inflammation to

fibrosis, cirrhosis, or hepatocellular carcinoma [Feld *et al*., 2009]. Because these outcomes typically do not occur until decades after active infection, surrogate outcomes are used as measures of therapeutic efficacy and success. Several independent studies have shown that lower levels of HBV DNA, as well as clearance of HBeAg and HBsAg, are associated with a lower risk of cirrhosis, hepatocellular carcinoma, and death [Iloeje *et al*., 2006; Chen *et al*., 2006; Yang *et al*., 2002]. Therefore, the major endpoints of treatment are a reduction of HBV DNA to undetectable levels (virologic response), a loss of HBeAg with or without the appearance of antibodies to HBeAg (serologic response), a reduction of alanine aminotransferase to levels considered normal (biochemical response), and a reduction in liver necroinflammation with or without an improvement of liver histology [Lok *et al*., 2009]. Loss of HBsAg from serum, accompanied by the appearance of antibodies to HBsAg, is currently considered the optimal surrogate endpoint, although it is rarely achieved [Perrillo *et al*., 2006].

2.3.1 Interferons

Interferons belong to the family of cytokines, products of nature polypeptide processed by different types of cells after appropriate stimuli that, by exerting a flow modular nature of other functional cells (target cells), evoke various and complex metabolic and biological responses. Interferons are a group of glycoproteins of molecular weight between 15,000 and 20,000 Dalton (D); Interferons are processed by virus-infected cells and induce a state of antiviral resistance in other cells in the body.

To date 3 different classes of interferons, derived from distinct cell populations, are known: interferon alpha (or leukocyte) interferon beta (or fibroblast) and interferon gamma (or immune). The mechanism of the antiviral effect of interferons, still somewhat indefinite, resides in the induction of an antiviral state in the target cells. This depends on the synthesis of at least two cellular enzymes: the 2'-5'-oligoadenilatesintetase (2'-5'-A-polymerase) and the kinase protein (PK). The first promotes the activation of a ribonuclease that degrades the viral messenger RNA and the second providing the phosphorylation, with consequent inactivation, of the peptide that initiates the viral protein synthesis. Genetic engineering, with the recombinant DNA technology, has allowed to produce large quantities of highly purified interferons. Particularly, interferon alpha-2a (IFN-α-2a) and interferon alpha-2b (IFN-α-2b) are highly purified proteins with a molecular weight of about 19,000 D; each contain 165 amino acids, are produced by a recombinant bacterium (*E. coli*) and differ by a single amino acid.

The distribution and metabolism of the interferons in the body are not completely known. Their elimination is primarily by renal excretion, although a small proportion of the drug is eliminated via the bile. The plasma half-life of recombinant interferon alfa is about five hours.

The use of IFN-α has some limitations, as it is often burdened by side effects and poor tolerability. This treatment has been shown to induce seroconversion from HBeAg to antiHBe in approximately 30-40% of patients with HBeAg positive chronic hepatitis B and stop the disease in about 20% of patients with antiHBe.

A significant proportion (30-60%) of patients who respond to IFN can also have HBsAg loss, months or years after the treatment cycle.

The pegylated interferon (PEG-Interferon, produced by a covalent bond with a branched molecule of methoxy-polyethylene glycol) is the ultimate formulation developed among the interferon alpha recombinants, characterized by a different pharmacokinetic that allows a slow elimination and a single weekly administration.

Although treatment with PEG-interferon is effective in patients with advanced fibrosis and compensated cirrhosis, it is contraindicated in patients with decompensated liver disease because of the risk of worsening liver disease and infectious complications [Lok *et al*., 2009; Buster *et al*., 2007] Nucleos(t)ide analogue treatment is, therefore, the best option in this patient population, and use of agents with high potency and low risk of resistance is recommended by the American Association for the Study of Liver Diseases [Lok *et al*., 2009] and the European Association for the Study of the Liver.

2.3.2 Lamivudine

Lamivudine (2', 3' dideoxy tiacitidina) is an analogue L-nucleoside that phosphorylated to adenosine triphosphate (3TC-TP) competes with dCTP for incorporation into the nascent chain of HBV DNA. If embedded by the viral polymerase acts as a chain terminator [Conjeevaram *et al*. 2003]. Lamivudine (LAM) is a high efficacy and tolerability drug but it may lead to the selection of HBV resistant variants (as previously described) during therapy. Several data has been published during the last decade

showing the yearly incremental probability of developing resistance in patients under LAM mono-therapy (figure 7).

2.3.3 Adefovir dipivoxil

Adefovir dipivoxil (ADV) is a second generation nucleotide analogue, an adenosine monophosphate that, once in the hepatocyte, become diphosphate after two phosphorylation reactions and in this form it can interact with the viral polymerase inhibiting the replication cycle by ending the nascent nucleic acid chain [Marcellin *et al*. 2005]. The ADV has proven to be an efficient drug with a good antiviral effect but lower than that of the lamivudine. It is effective against viral strains resistant to lamivudine and before the advent of the NAs third generation was the rescue drug in case of LAM therapy failure. The probability of developing HBV resistant mutants during the treatment is lower than LAM but still present with 29% of cumulative probability after 5 years of therapy (figure 7). Moreover, because of its nephrotoxicity, adefovir must be administered in dose (10 mg/day) lower than all the other NAs and lower than that needed to achieve the maximum inhibitory effect of viral replication [Jannsen H 2012].

2.3.4 Telbivudine

Telbivudine (LdT) was approved for chronic hepatitis B viral infection in the United States in October 2006 and is indicated for patients

with compensated chronic HBV infection and with evidence of viral replication and either evidence of persistent elevations in aminotransferases or histologically active disease. Telbivudine has greater antiviral efficacy compared with lamivudine in both hepatitis B e antigen (HBeAg)–positive and HBeAg-negative chronic HBV infection [Lai *et al*., 2007]

Despite the high potency of telbivudine, virologic response rates deteriorated over time due to the emergence of telbivudine resistance; indeed, telbivudine resistance mutations were found in up to 17% of patients after 2 years of therapy (Figure 7) [Zoulim *et al*., 2009]. Notably, telbivudine resistance mutations confer cross-resistance to lamivudine. Telbivudine, therefore, has limited application in the treatment of patients with chronic HBV infection.

2.3.5 Entecavir

Cyclopentyl guanosine analogue, entecavir (ETV) is a potent inhibitor of HBV reverse transcriptase. Entecavir functionally inhibits all the activities of the HBV Pol/Rt: (1) base priming, (2) reverse transcription of the negative strand from the pregenomic messenger RNA, and (3) synthesis of the positive strand of HBV DNA. Upon activation by kinases, the drug can be incorporated into the nascent viral DNA inhibiting the HBV Pol/Rt activity [Marcellin *et al*., 2005]. There are no significant adverse effects reported to date. Its antiviral efficacy is superior to that of LAM and ADV and in *naive* subjects shows a very low rate (1.2%) of resistance after 5 years of treatment (figure 7). Different is the situation in LAM resistant

patients as they require higher doses of ETV because of the serious risk of resistance, that in this case, is 25% after 3 years of treatment [Balfour 1999, Yamanaka *et al*. 1999, De Man *et al*., 2001].

2.3.6 Tenofovir

Such as adefovir, tenofovir (TFV) is an acyclic nucleoside phosphonate and belong to the third generation of NAs. The TFV is a nucleotide analogue of adenosine monophosphate and act as a chain terminator when embedded in the nascent viral DNA. Approved for the treatment of HBV infection in the United States in 2008, tenofovir is indicated for the treatment of compensated chronic HBV infection in adults. It has demonstrated superior antiviral efficacy compared with adefovir in both HBeAg–positive and HBeAg-negative patients [Marcellin *et al*., 2008]. Recently, a large investigator-initiated study showed that 79% of patients who failed previous nucleos(t)ide analogue treatment achieved HBV DNA levels < 400 copies/mL after a median follow-up of almost 2 years of tenofovir therapy [Van Bommel *et al*., 2010]. This study also suggests a reduced antiviral effect of tenofovir in the adefovir-resistant population. To date, tenofovir resistance has not been detected in treatment-*naive* patients treated for up to 4 years (Figure 7) [Snow-Lampart *et al*., 2010] although viraemic patients were allowed to switch to a combination of tenofovir and emtricitabine at Week 72 [Heathcote *et al*., 2011; Heathcote *et al*., 2010; Marcellin *et al*., 2010].

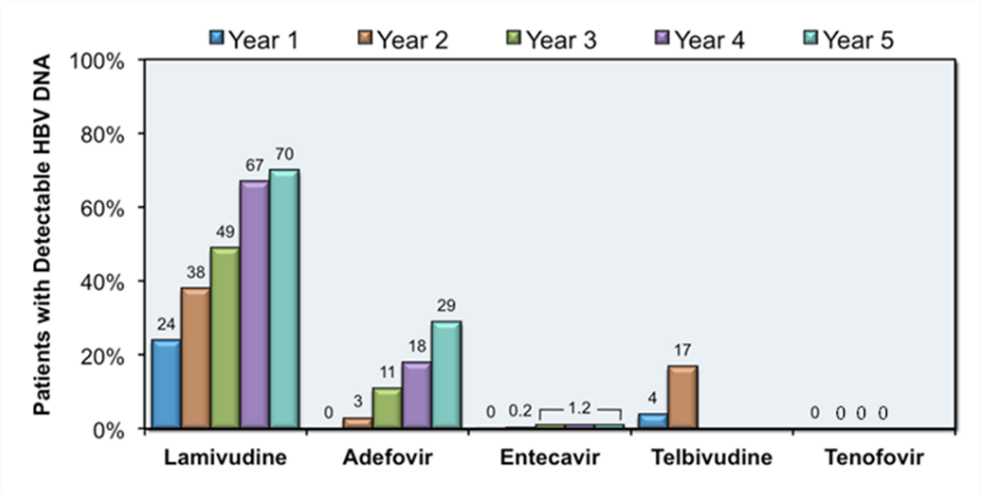


Figure 7. Cumulative incidence of HBV resistance against the five approved NAs for antiviral therapy (Adapted from: EASL Clinical Practice Guidelines: management of chronic hepatitis B - 2009).

2.4 Nucleos(t)ide Analogues treatment monitoring and definition of antiviral response and resistance.

The currently approved nucleos(t)ide analogues can adequately suppress HBV DNA levels, normalize alanine aminotransferase levels, and improve liver histology [Dienstag *et al*., 2009], but their antiviral efficacy is markedly reduced in patients who develop drug-resistant hepatitis B virus variants, resulting in virologic breakthrough, hepatitis flares, and a reduction in clinical benefits [Liaw *et al*., 2004]. To avoid emergence of viral resistance is therefore essential to successful antiviral therapy with nucleos(t)ide analogues.

Persistent viral replication during antiviral therapy (measured by HBV DNA levels) is a major risk factor for the subsequent development of antiviral resistance [Zeuzem *et al*., 2009; Hadziyannis *et al*., 2006; Liaw *et al*., 2009b; Yuen *et al*., 2001]. Therefore, HBV DNA levels should be

monitored at least every 3-6 months with a sensitive assay (lower limit of detection of newest assays is < 20 IU/mL) in patients treated with nucleos(t)ide analogues according to the AASLD and the EASL guidelines.

Current guidelines recommend treatment modification in patients with detectable HBV DNA levels after a predefined duration of treatment [Zeuzem *et al*., 2009; Janssen *et al*., 2009; Lok *et al*., 2009]. Particularly, the European Association for the Study of the Liver guidelines have attempted to classify response patterns to nucleos(t)ide analogues and to advise treatment modification in suboptimal responders to prevent the development of resistance (Figure 8):

- Complete virological responders are those patients who accomplish undetectable levels of HBV DNA within 48 weeks of therapy. These patients have a low probability of emergence of viral resistance.
- Primary nonresponders are those patients with a suboptimal response, failing to achieve ≥ 1 log10 IU/mL drop in HBV DNA levels by 3 months after commencing therapy.
- Partial response is defined as an on-treatment decline in HBV DNA of ≥ 1 log10 IU/mL but failure to achieve undetectable HBV DNA levels at Week 24 (for lamivudine and telbivudine) or Week 48 (for adefovir, entecavir, tenofovir). Different requirements have been set for lamivudine and telbivudine *vs* adefovir, entecavir, and tenofovir because of the lower barrier to resistance of lamivudine and telbivudine.

- Virologic breakthrough is defined as an increase in serum HBV DNA > 1 log10 above nadir after achieving a virologic response during continued treatment [Lok *et al*., 2009].

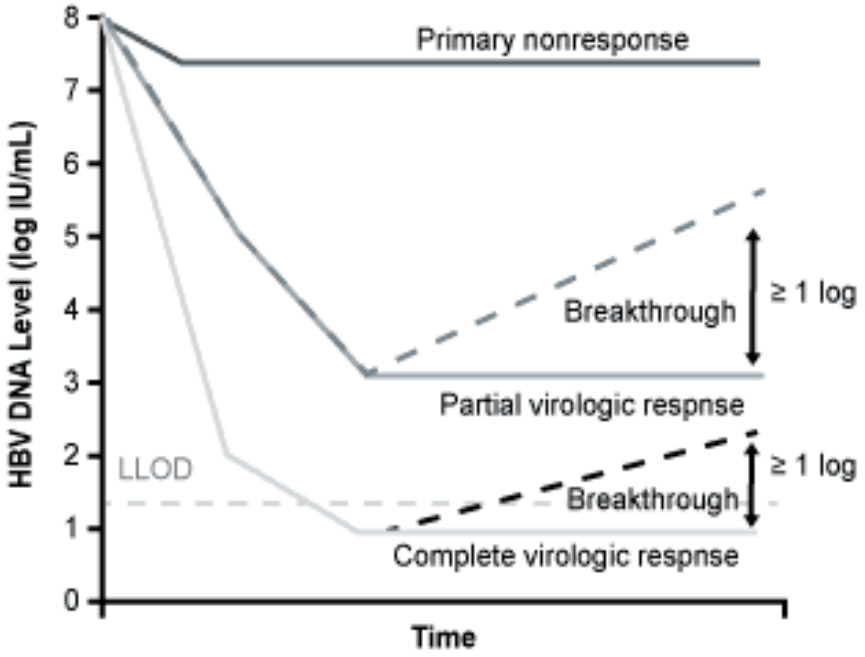


Figure 8. The different type of antiviral responses during the NAs antiviral therapy. Sequence analysis should be performed before the start of treatment and repeated, in case of detectable *viraemia*, every 6 months (LLOD = Lower Limit of Detection).

2.5 Nucleos(t)ide Analogues Resistant Variants

The resistant mutations locate on the HBV Polymerase/Retrotranscriptase region with codons rt204 and/or rt181 involved in all the different class of drugs. Particularly rtM204I or V quasi-species can be selected and emerge during Lamivudine treatment as primary resistance mutations [Ling R *et al*., 1996; Bartholomew M *et al*., 1997; Yeh C *et al*., 2000; Marcellin P *et al*., 2003]. Moreover, secondary/compensatory

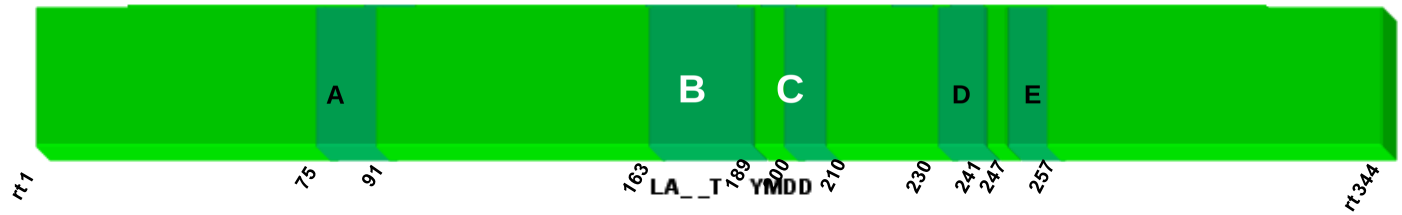
mutations are often co-selected with the primary ones to generate multi-mutational patterns affecting rescue therapies. The rtL180M + rtM204I or V is the most common resistant pattern selected during LAM treatment and in particular is the background for ETV resistance since the addition of only one more mutation among rtI169T, rtT184G, rtS202T, rtM250I or V is required. As entecavir resistance mutations has never been detected in *naive* patients, LAM treatment exposure and/or failure play a critical role in the clinical outcome [Tenney DJ *et al*., 2004; Colonno RJ *et al*., 2006; Zoulim *et al*., 2006; Villet S *et al*., 2007].

The rtA181S or T or V mutations can be selected and emerge during Nas therapy and like the rtM204I or V are classified as primary resistant mutations. Interestingly, the rtA181 variants are also classified as cross resistant mutations since their ability to replicate in presence of both LAM and ADV [Fung SK *et al*., 2005; Yeon JE *et al*., 2006; Lacombe K *et al*., 2006; Moriconi F *et al* 2007; Karatayli E *et al*., 2007].

The rtN236T is also a primary resistant mutations although to date it has been found, in vivo, during ADV monotherapy failure only and rescue therapy worked fine with ETV or TFV [Angus P *et al*., 2003; Bartholomeusz A *et al*., 2006; Ratziu *et al*., 2006; Shim JH *et al*., 2009] (Figure 9).

Recently, resistant variants has been detected, as minor viral populations, in *naive* patients sera also. Such a data has been obtained with high sensitive laboratory method. Indeed, considering the HBV high rate of mutation ($0.9x10^{-3}$ base substitutions/site/year) and the estimated HBV production rate of at least 10^{13} virions/day [Whalley S *et al*., 2001], it was

probable to find several viral variants, the drugs resistance included, when a deep viral population study would be performed in HBV chronic infected patients.



ANTIVIRAL		MUTATIONS
Lamivudine	LMV	L80V/I I169T V173L L180M **A181T/V/S** T184G S202I **M204V/I**
Adefovir	ADV	**A181T/V/S** **N236T**
Telbivudine	LdT	**M204I**
Entecavir	ETV	I169T or T184S/G or S202I or M250V + L180M + **M204V/I**
Tenofovir	TFV	L180M + A194T + **M204V**

Primary resistance mutations are writ large.

Figure 9. Resistant Variants mutations locate on the HBV Polymerase gene with codons 181 and/or 204 involved in all the different class of drugs.

2.6 Laboratory Method for the Identification of HBV Resistant Variants.

Several laboratory methods have been developed over the years to help clinicians to optimize the treatment of CHB patients avoiding resistance:

- Polymerase chain reaction (PCR) is a biochemical technology in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The PCR is the method on wich many of the actual diagnostic assay are based on and relies, in particular, on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

  Most PCR methods typically amplify DNA fragments of up to ~10 kilo base pairs (kb) requiring the following reagents: (i) DNA template that contains the DNA region (target) to be amplified; (ii) Two primers that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target; (iii) Taq polymerase or another DNA polymerase with a optimal working temperature at around 70 °C; (iv) Deoxynucleoside triphosphates (dNTPs; nucleotides containing triphosphate groups), the building-

blocks from which the DNA polymerase synthesizes a new DNA strand; (v) Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase; (vi) Magnesium (Mg2+), a divalent cation whose final concentration affect the yield of the reaction (DNA synthesis).

The PCR method is a multistep process easily carried out today by automated thermal cycler. The different steps include: (i) denaturation, heating the reaction to 94–98 °C for 20–30 seconds to melt DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules; (ii) annealing, the reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the Tm of the primers used. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation; (iii) extension/elongation, a temperature of 72 °C is commonly used at this stage where the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends on the length of the DNA fragment to be amplified.

- After PCR discovery, several variants of this method has been developed. Here we are going to briefly describe two of the most used variants: Nested PCR and Allele Specific PCR. The former is a double PCR increasing the specificity of DNA amplification but above all increasing the yield of the reaction. A small (2ul) volume of the first PCR is amplified in a second roud using two internal primers encompassing nucleotide regions present on the DNA target.

  The Allele Specific is based on single-nucleotide polymorphisms (SNPs) (single-base differences in DNA). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3' ends encompass the SNP. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

- Direct PCR-based DNA sequencing (DS) is to date the gold standard for HBV mutations analysis. The method involves DNA denaturation, annealing, and elongation. During elongation, DNA polymerase randomly incorporates deoxynucleotides or dideoxynucleotides that have been labeled with different fluorochromes, resulting in chain elongation or termination, respectively. Chains of different lengths are therefore generated, each ending with a labeled nucleotide. The amplicons are then migrated in a capillary system that separates them according to their lengths. Bioinformatic software measures the fluorescence peak at

each position of the analyzed sequence and identifies the corresponding dideoxynucleotides to determine a consensus sequence. The presence of multiple peaks at the same position indicates a mixed sequence.

Critical issues concerning the DS are based on the fact that HBV, like other viruses able to establish chronic infections (ie HIV, HCV) behave as quasispecies, complex mixtures of genetically related but distinct viral populations in equilibrium in a given replicative environment. Therefore, at any given time, patients carry a large number of different viral genome sequences. The classic sequencing techniques can only identify the consensus sequence of a viral quasispecies at a given time point, whereas intermediate and minor viral populations go undetected if not present in at 20% of the total *viraemia*. In addition, this approach cannot link different substitutions present in the same viral variant or distinguish substitutions present in different variants.

For all the reasons above described, Direct PCR-based DNA sequencing is not the recommended method to characterize minor viral populations within patient quasispecies [Shaw T *et al*., 2006]. Direct sequencing after cloning can overcome this problem, but analysis of large numbers of clones is required and makes this method too cumbersome and time consuming for a systematic application to a diagnostic routine [Pallier *et al*., 2006].

- An alternative method, based on reverse hybridization, is the Line Probe Assay (INNO-LiPA) where different mutant probes, spotted

on nitrocellulose strips, are incubated with HBV polymerase fragment amplified from serum sample. INNO-LiPA is the most commonly used technique for the analysis of HBV RVs quasispecies in clinical practice and require a total viremia >10E+03 cp/ml to detect 5-10% of mutant prevalence. The advantage of this technique is the early time in resistant variants detection compare to DS. The major limit is that only the known RVs can be detected and several important secondary / compensatory mutations are missing, particularly those associated to entecavir resistance [Hussain *et al*., 2006; Jardi *et al*., 2009].

- New promising methods has been developed during the last years to increase sequencing capacity while generating clonal sequences. Classified as Next Generation Sequencing (NGS), these methods are actually the most powerful to analyze and study viral quasispecies. The NGS methods use a 3 step sequencing process: library preparation, DNA capture and enrichment, and sequencing. In library preparation, DNAs of appropriate lengths are prepared and labeled with the primers required for subsequent isolation and sequencing. DNA is then captured, and clonal DNA is enriched. The DNA is then sequenced, via polymerization combined with detection based on pyrophosphate (pyrosequencing), H^+ ions (pH variation) or fluorescence, or ligation combined with fluorescence detection. The detected signals are transformed into readable sequences by an integrated browser and specific software to produce biologically relevant information.

The NGS methods are susceptible to generating sequencing errors and to date there is still some controversy about the actual error rates of these methods, so prospective assessments are required by independent investigators. Moreover, NGS methods generate unreadable sequences (called junk data) at variable rates. This is associated with an over cost but not with lower quality final results because these sequences can be easily identified and removed. False-positive or false-negative results for substitutions have been also reported and are easily corrected when the sequencing depth is high, as in Genome Wide Association Study or gene expression profiling studies, but are problematic for studies of rare variants [Harismendy O *et al*., 2009; Suzuki S *et al*., 2011]. The relatively short read-length of the sequences obtained with the NGS methods is another deal that can affect analysis and studies on variants. The maximum length of individual sequencing runs are in fact shorter (maximum 400bp, primers included) than those obtainable with DS method (≈700bp).

However, to date, the main need in the field of NGS is for bioinformatic tools to analyze NGS sequences in the context of viral infections. Complex analyzing software might be suitable for research purposes, but clinicians will require bioinformatic tools able to translate millions of generated sequences into simple and clinically relevant information on which therapeutic decisions can be made.

- Real-Time PCR is a recently developed methods following the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. A common method for detection of products in real-time PCR consist of a sequence-specific DNA probe labeled with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end of the probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence; after specific hybridization of the probe with its complementary DNA target, breakdown of the probe itself by the 5' to 3' exonuclease activity of the Taq polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected after excitation with a laser. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter.

  Fluorescence is detected and measured in a real-time PCR machine, and its geometric increase corresponding to exponential increase of the product used to determine the threshold cycle (CT) in each reaction. Real-Time PCR account for high sensitivity and specificity and its application to the identification of HBV resistant variants showed interesting results detecting quasispecies down to 0.1% or lesser. The main disadvantages of Real-Time PCR are the set up of a specific assay including designing of the primers and

probe, the fluorophores labeling and the development of a sophisticated software and algorithms for the calculation of the target quantity. All these phases are also cost affecting the available commercial kit. In addition, studies performed up to date on HBV resistant variants developed and used methods requiring high viremia levels >10E+04 cp/ml to reach high sensitivity and to avoid aspecific amplifications [Lupo *et al*., 2009; Zhao *et al*., 2010; Liu *et al*., 2011].

2.7 Aim of the study

Antiviral therapy for HBV has reached today a high safety level with the use of third generation NAs (Entecavir, Tenofovir). Nevertheless, none of the drugs available today against HBV is able to eradicate the virus from the epatocytes and long-term therapy continue to be the treatment of choice. For this reason, the risk of selection and emergence of resistant variants is not averted, particularly in patients previously treated with NAs first generation and treatment failure experienced.

High sensitive laboratory methods have shown the presence of resistant variants, at low concentration level, in *naive* patients but very few or nothing is known about their influence on the response to antiviral treatment. Thus, a better understanding of the dynamics of drug resistants variants could contribute to the improvement in the management of CHB pts.

The aim of our study was to develop a sensitive and specific assay to detect rt204 and rt181 HBV resistant variants in low viraemic sera (<10E+04 cp/ml) and to investigate the RVs early dynamics during lamivudine treatment. Thus, considering the

2.8 Patients and Methods

2.8.1 Sera Panel and Criteria for patients inclusion

A retrospective panel of 73 serum samples from 15 CHB patients (median age 56 years, range 27 – 73 years; 11 males, 4 female), treated with LAM from the year 2000 at the Hepatology Unit of Pisa University Hospital, was tested (Table 1). Patients (pts) were selected from a large LAM treated cohort monitored in our clinic and were followed-up for a median period of 24 months (range 4 – 143). The selection was done according to three main different viral response profiles observed during treatment: five pts had Complete Viral Response (CVR), defined as HBV DNA < 200 copies/mL at month 6 or earlier, that was maintained throughout the follow-up; six pts had Resistance After Complete Viral Response (RACVR), defined as a rebound of HBV DNA > 200 cp/ml in patients who showed CVR; four pts had Incomplete Viral Response (IVR), defined as HBV DNA > 200 cp/ml during the entire follow-up. Pre-treatment serum and on treatment serum samples were tested in every patient during the first year of treatment. One or more samples were analysed during the further of follow up in 2 IVR and 5 RACVR pts.

Eight out of 15 pts had a histological diagnosis of chronic hepatitis, 3 pts had chronic hepatitis with cirrhosis, 4 pts had hepatocellular carcinoma. Antibodies against HBeAg were present in all the pts but one, positive for the HBe antigen.

Patients were monitored for liver function tests and HBV DNA levels monthly during the first year of therapy and every 3 months thereafter. HBV DNA levels were measured by Roche's COBAS® AMPLICOR HBV MONITOR Test, linear dynamic range 200 – 200000 cp/ml, and by Roche's COBAS TaqMan 48, linear dynamic range 20 – 1,7E+08 UI/ml (1UI/ml equivalent to 5,82 cp/ml). Clinical evaluations were performed every 3 months, ultrasound every 6 months in patients with cirrhosis, yearly in pts with chronic hepatitis. All the patients were negative for antibodies against hepatitis C virus (HCV), hepatitis D virus (HDV) and human immunodeficiency virus (HIV).

Table 1. Demographic and clinical data

Median Age (range)	56 years (27-73 years)
Male/female	10/4
Chronic hepatitis/cirrhosis/liver transplant	8/3/3
Median follow-up (range)	41 months (4-143 months)
Median ALT baseline (range)	138 U/L (45-558 U/L)
Genotype A/D	3/11
HBeAg-positive/HBeAg-negative	1/13
Median HBV-DNA baseline levels (range)	3,2E+08 cp/ml (4,7E+04-6,0E+08 cp/ml)

2.8.2 HBV DNA extraction and amplification

Viral DNA was extracted from 200 µl of serum samples with QIAamp MinElute Virus Spin Kit (QIAGEN, GmbH) according to the

manufacturer protocol. The HBV DNAs were finally resuspended in 50 µl of distilled water and stored at -20°C. Five microliters of extracted DNA were used to amplify the HBV RT region in a 50 µl reaction volume containing Buffer 1x [(67 mM TrisHCl (pH 8.7), 16 mM (NH4)2SO4)], 2.0 mM MgCl2, 0.1 mM dNTPs, 2 U Dream Taq polymerase (Fermentas) and 0.25 µM of each HBV universal primers, Pol1s nt 412-432 5'-CCTGCTGCTATGCCTCATCTT-3' and Pol4as nt 1058-1033 5'-GGCATTAACGCAGGATAWCCACATTG-3'. The first round PCR cycle profiles was: pre-heating 3 min at 94ºC, 40 cycles including denaturation 30 sec at 94ºC, annealing 30 sec at 55ºC and extension 30 sec at 72ºC, followed by 7 min at 72ºC. Negative samples after first round PCR were amplified in nested PCR using internal HBV universal primers Pol2s nt 449-469 5'-GACTATCAAGGTATGTTGCCC-3' and Pol181as and a thermal profile like the first round but for 20 cycles. Five microliters of PCR products were analyzed by ultraviolet fluorescence after ethidium bromide staining in a 2% agarose gel.

2.8.3 Direct Sequencing

PCR products were purified by Ethanol precipitation protocol (Beckman Coulter) and directly sequenced by chain terminator method using CEQ 8000 Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter); briefly 2µl of purified template were added to 8.0 µl of DTCS Quick Start Master Mix, 4.0 µl of 1.6 µM primer (POL2s and POL181as) and 6.0 µl of H_2O. Thermal cycling profile was 30 cycles of 20 sec at 96ºC, 20 sec at 50ºC, 4 min at 60ºC followed by holding at 4ºC.

Sequencing was carried out using a CEQ 8000 XL analysis System (Beckman). Alignment of nucleotide and amino acid sequences were performed with CLUSTAL W program at npsa-pbil.ibcp.fr/, NPS@: Network Protein Sequence Analysis (Combet C *et al*., 2000).

2.9 Results

2.9.1 Development and standardization of Allele Specific PCR for drug resistant mutants: Standards

A total of 9 constructs containing the HBV wild-type and the 8 different mutations causing resistance associated amino acid substitutions at codon rt181 and rt204 were generated. Particularly the 8 nucleotide mutations were responsible for rtA181S, rtA181T, rtA181T* (sW172stop), rtA181V, rtM204I (codon ata), rtM204I (codon atc), rtM204I (codon att), rtM204V (Table 2). We cloned a 647 bp fragment encompassing the B-E dominions of HBV RT region (412 to 1058 bp) from sera samples of 7 CHB NAs therapy failure patients. They were used as reference standards for the standardization and optimization of the assay. Briefly, serum HBV DNA was amplified using HBV universal primers POL1s and POL4as in PCR mixture and cycle profile as previously described. When HBV DNA level was below 10E+04 IU/ml, a nested PCR technique with HBV universal primers Pol2s and Pol181as was used to amplify a 540 bp region including the HBV RT B - E catalytic domains. The PCR products were ligated to pGEM vector (Promega) and cloned into JM109 competent cells. Transformants were grown on ampicillin plates and clone screening for RVs

was performed by sequencing after DNA extraction and reamplification by PCR. Approximately 20 clones per sample were sequenced with primers Pol2s and Pol181as.

The DNA plasmids containing the reference standards were quantified spectrophotometrically and stocked at final concentration of 10E+10 cp/ml. Ten fold serial dilutions of the stocks were prepared to generate standard curves with mixtures composed by every single mutant and the HBV wild-type. Mixture stocks were prepared in ratios of 1/10, 1/100, 1/1000, 1/10000 at final concentration of 10E+04 cp/ml.

Table 2. The 8 different nucleotide mutations causing resistance by amino acid substitutions at codon rt181 and rt204.

codon Wt	aa Wt	aa position	aa RV	codon RV	codon Wt	aa Wt	aa position	aa RV	codon RV
gct	A	181	S	tct	atg	M	204	I*	ata
gct	A	181	T	act	atg	M	204	I	atc
gct	A	181	T*	act	atg	M	204	I	att
gct	A	181	V	gtt	atg	M	204	V	gtg

* Premature stop codon on HBsAg coding frame

2.9.2 Allele Specific Resistant Variant PCRs: strategy and primers design

The Allele Specific PCR followed by emiNested PCR was the method we decided to develop combining specificity and sensitivity. As the 8 standards representing the different RVs at codon rt181 and rt204 were obtained, eight specific primers for each RV were designed (Table 3) and tested separately in the standard mixtures prepared as described in the Standard session. In every RV specific primer, a mismatch was introduced at position 3 or 4 from the 3'-end to increase the specificity of the amplification [Flichman *et al.*, 2005]. The 4 rt181 RVs specific primers

were coupled with HBV universal primers Pol4as and Pol181as in first round and eminested PCR respectively. The 4 rt204 RVs specific primers were coupled with HBV universal primers Pol1s and Pol2s in first round and eminested PCR respectively (figure 10). Five microliters of standard or extracted DNAs were amplified by Allele Specific Resistant Variant PCRs (ASRVPCR) in a 50 µl reaction volume containing Buffer 1x, 1.0 mM MgCl2, 0.6 mM dNTPs, 5 U Hot start Taq polymerase (QIAGEN) and 0.25 µM of each primer. Different ramped ('touchdown') cycle profiles for the different RVs specific primers were used to achieve the best sensitivity and specificity for the Allele Specific PCR method [Grace S *et al*., 1994]. In particular, the first round amplifcation conditions were: pre-heating 15 min at 94ºC, 30 - 40 cycles including denaturation 30 sec at 94ºC, annealing 30 sec at 53 - 61ºC (-1°C per cycle in the first 5 cycles) and extension 30 sec at 72ºC, followed by 5 min at 72ºC. Two microliters from the first round were amplified in a 50 µl reaction volume containing Buffer 1x, 2.4 mM MgCl2, 0.58 mM dNTPs, 5 U Hot start Taq polymerase (QIAGEN) and 0.25 µM of each primer. The cycle profiles for eminested PCR had similar conditions but were not a touchdown and lasted for 20 cycles (Table 4). Finally, 7 microliters of PCR products were analyzed by ultraviolet fluorescence after ethidium bromide staining in a 2% agarose gel.

Table.3 Primers used in the study for classic PCR, Allele Specific PCR and Direct Sequencing

HBV Primers	nt sequence	Position (EcoRI ref)
Universal		
Pol4as	5′-GGCATTAACGCAGGATAWCCACATTG-3′	1058-1033
Pol181as	5′-GACCCACAATTCKTTGACATACTTTCC-3′	1006-980
Pol1s	5′-CCTGCTGCTATGCCTCATCTT-3′	412-432
Pol2s	5′-GACTATCAAGGTATGTTGCCC-3′	449-469
rt181 RVs specific		
rt181S	5′-CTCCAGCCCGTTTCTGHAGT-3′	651-670
rt181T*	5′-CTCCAGCCCGTTTCTGHAGA-3′	651-670
rt181T	5′-CTCCAGCCCGTTTCTGYACA-3′	651-670
rt181V	5′-TCAGCCCGTTTCTCHAGGT-3′	653-671
rt204 RVs specific		
Rt204I*(ata)	5′-CCCAATACCACATCAGCT-3′	758-747
Rt204I (atc)	5′-CCCAATACCACATCACCG-3′	758-747
Rt204I (att)	5′-CCCAATACCACATCAACA-3′	758-747
Rt204V	5′-CCCAATACCACATCATCAAC-3′	758-745

* Premature stop codon on HBsAg coding frame

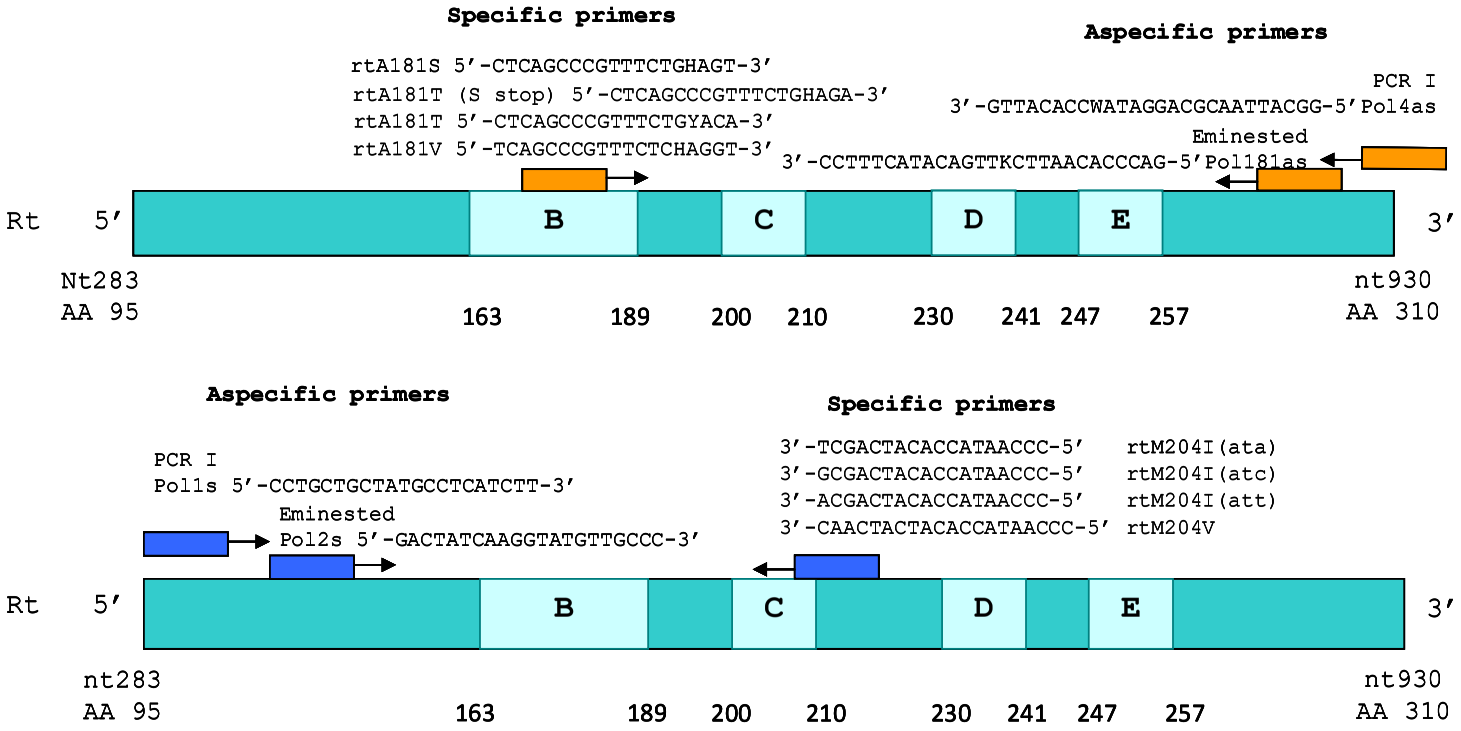


Figure 10. The 8 specific probes, able to identify the resistant variants in position rt181 or rt204, were used in ASRVPCR round 1 and eminested with 4 HBV universal primers. Particularly, rt181 RVs forward specific primers were designed to be coupled with Pol4as and Pol181as reverse primers for PCR round 1 and eminested respectively. The rt204 RVs reverse specific primers coupled with Pol4as and Pol181as forward primers for PCR round 1 and eminested respectively. Nt = nucleotide, AA = amino acid, * = premature S gene stop codon.

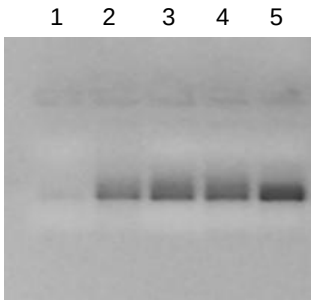
2.9.3 Specificity and Sensitivity of the method

To set up amplification profile of Allele Specific Resistant Variant PCR, every single standard (n = 8) representing a RV at codon rt181 or rt204 was mixed with HBV wild type and selectively amplified with the 8 different specific primers.

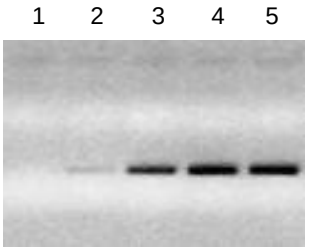
To avoid aspecific amplification different ramped ("touchdown") cycle profiles were used during first round of ASRVPCR for every RVs specific primers. Particularly, a common backbone profile for denaturation and elongation phase was maintained: 95°C for 30 seconds and 72°C for 45 seconds respectively. A similar strategy was used in the eminested PCRs excepting for the touchdown option. The best results according to specificity and sensitivity could be obtained modifying the annealing temperature and the number of cycles of the thermal profiles. All the final annealing temperature and number of cycles are resumed in Table 4. Using those profiles the specific primers for mutant rtA181S and rtA181T could detect 10 copies of the resistant variant construct on a background of 10E+04 copies of wild-type (Figure 11A). Similar results were obtained for mutant primers rtA181T* (stop codon in S gene) and rtA181V (data not shown). The specific primers for mutant rtM204I (ata) and rtM204I (att) could detect 100 copies of the resistant variant construct on a background of 10E+04 copies of wild-type (Figure 11B). Similar results were obtained for mutant primers rtM204I (atc) and rtM204V (data not shown).

A)

1-Wild type 1E+04 cp/ml
2-A181S 1E+01cp / Wild type 1E+04cp (fold 3)
3-A181S 1E+02cp / Wild type 1E+04cp (fold 2)
4-A181S 1E+03cp / Wild type 1E+04cp (fold 1)
5-A181S 1E+04 cp/ml

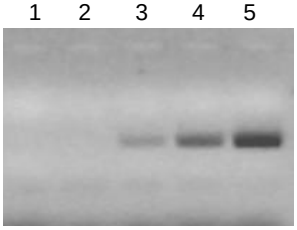


1-Wild type 1E+04 cp/ml
2-A181T 1E+01cp / Wild type 1E+04cp (fold 3)
3-A181T 1E+02cp / Wild type 1E+04cp (fold 2)
4-A181T 1E+03cp / Wild type 1E+04cp (fold 1)
5-A181T 1E+04 cp/ml



B)

1-Wild type 1E+04 cp/ml
2-M204I (att) 1E+01cp / Wild type 1E+04cp (fold 3)
3-M204I (att) 1E+02cp / Wild type 1E+04cp (fold 2)
4-M204I (att) 1E+03cp / Wild type 1E+04cp (fold 1)
5-M204I (att) 1E+04 cp/ml



1-Wild type 1E+04 cp/ml
2-M204V 1E+01cp / Wild type 1E+04cp (fold 3)
3-M204V 1E+02cp / Wild type 1E+04cp (fold 2)
4-M204V 1E+03cp / Wild type 1E+04cp (fold 1)
5-M204V 1E+04 cp/ml

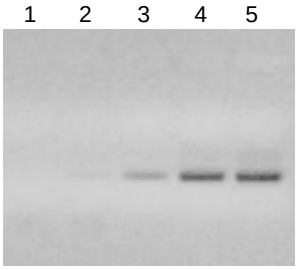


Figure 11. Selective amplification of RVs end point diluted and mixed with HBV wild-type. A) Sensitivity and specificity for primers rtA181S and rtA181T are shown. B) Sensitivity and specificity for primers rtM204I and rtM204V are shown.

Table 4.

RVs specific primer	First Round Ta °C	First Round Touch Down program	Eminested Ta °C	Sensitivity (cp/ml)	Specificity (fold)
204V	61	-1°C per cycle during the first 8 cycles. Ta = 53°C during the following 25 cycles.	55	100	100
204I_att	51	-1°C per cycle during the first 5 cycles. Ta = 46°C during the following 30 cycles.	49	100	100
204I_ata	49	-1°C per cycle during the first 5 cycles. Ta = 44°C during the following 30 cycles.	49	100	100
204I_atc	54	-1°C per cycle during the first 5 cycles. Ta = 49°C during the following 30 cycles.	49	100	100
181S	53	-0,5°C per cycle during the first 6 cycles. Ta = 50°C during the following 34	55	10	1000
181T*	61	-1°C per cycle during the first 8 cycles. Ta = 53°C during the following 27 cycles.	55	10	1000
181T	59	-1°C per cycle during the first 8 cycles. Ta = 51°C during the following 25 cycles.	55	10	1000
181V	61	-1°C per cycle during the first 8 cycles. Ta = 53°C during the following 25 cycles.	55	10	1000

* Premature stop codon on HBsAg coding frame

2.9.4 Clinical validation of the assay and early dynamic study on a sera panel from patients treated with LAM.

A total of 73 serum samples from 15 CHB LAM treated patients were tested with Allele Specific for Resistance Variants technique and the Direct Sequencing was used as a comparator.

Analysis on CVR group

In each of 5 Complete Viral Response patients (pts n. 2, 3, 12, 13, 14) 3 or 4 sequential samples collected at pre-treatment time and during the first 6 months of therapy were tested and no RVs were detected with ASRVPCR nor with DS (table 5).

Analysis on RACVR group

In 6 Resistance After Complete Viral Response patients (pts n. 1, 4, 5, 6, 9, 11) a total of 33 sera, collected at pre-treatment time and during the follow-up were analyzed. Direct sequencing was firstly performed at the time of virological rebound and RVs were identified in all the cases: the rtA181S, rtA181T, rtM204I (codon att) and rtM204I (codon atc) were present in patient n. 11, 6, 1 and 5 respectively; the rtM204V was detected in patient n. 4 and 9. Moreover, the secondary/compensatory mutation rtL180M could be identified in all the sequences where a rtM204 resistant variant could be found.

The pre-treatment serum and sequential samples of the RACVR pts were tested with ASRVPCR using the primer specific for the resistant variant previously identified by DS. Particularly, the mutant rtA181S was

present in all the samples tested of pt n. 11; in the pre-treatment serum, when total *viraemia* was 4.7E+04 cp/ml, during the LAM responding time (HBV DNA < 200 cp/ml) and at the virological rebound when viral load was 1.0E+04 cp/ml. The rtA181T and rtM204I (att) were only detected at the time of virological rebound in pt n. 6 and n. 1 respectively (table 5). In pt n. 5 the rtM204I (atc) could be detected for the first time at month 6 of therapy when viral load was 1.9E+04 cp/ml and its presence was confirmed in the subsequent samples at month 13 and 16 when *viraemia* was 6.0E+03 and 3.3E+03 cp/ml respectively. Unexpectedly the rtM204I was not detected in the next available sample, month 25 when HBV DNA was low but still detectable with 300 cp/ml. That resistant variant was amplified again at month 41, when viral load was < 200 cp/ml and at the viral rebound time, month 51 with HBV DNA = 1.4E+03 cp/ml (table 5). The rtM204V was present in pt n. 4 in the pre-treatment sample when viral load was 6.0E+05 cp/ml and in all the other samples tested, with a *viraemia* ranging from < 200 to 2.4E+03 cp/ml, but one at month 1 with HBV DNA = 1.0E+03 cp/ml. The rtM204V was also detected at pre-treatment and at virological rebound time of pt n. 9 (table 5). The Direct Sequencing performed on samples of RACVR patients n. 1, 4, 5, 6 and 9 didn't identify any RVs at pre-treatment time nor in the follow up sera preceding virological rebound. Differently, in pt n. 11 the DS detected a mixed rtA181A/S population after 1 month of treatment and thereafter.

Analysis on IVR group

In patients n. 7, 8, 10 and 15, IVR grouped, the Direct Sequencing was performed at first in the last follow-up sera available to investigate the

cause of the incomplete viral response and, resistant variants could be found in all the cases. In particular, pt n. 7 showed rtM204V and that mutation was also present in patient n. 8 and n. 15 along with (mixed population) rtM204I (att) and rtA181V respectively. In patient n. 10 the DS detected rtM204I (att), mixed with wild-type population, since the pre-treatment serum. The rtL180M mutation could be found as well, associated to the rtM204 mutations. A total of 23 samples including the pre-treatment and follow up sera of 4 IVR pts were tested by ASRVPCR and DS as for the RACVR group. The rtM204V and rtM204I (att) variants were amplified by ASRVPCR in all the samples of pts n. 7 and 10 respectively (table 5). In patient n. 8, the rtM204V was detectable in all the samples while the rtM204I (att) could be amplified in the pre-treatment serum, HBV DNA = 4.0E+06 cp/ml, but not later, at month 1 and 3 having viral load of 2.8E+05 and 5.6E+05 cp/ml respectively. The rtM204I (att) could be detected again at months 5, 8 and 10 (end of follow-up, EOF) with increasing *viraemia* levels from 6.0E+04 to 2.0E+06 cp/ml. The rtA181V was detected in pt n. 15 in the pre-treatment serum when viral load was 9.6E+05 cp/ml and, intermittently, in other 4 out of 8 follow-up samples tested, where *viraemia* levels ranged between 3.3E+02 and 1.5E+03 cp/ml (table 5). The rtM204V could also be amplified in pt 15 but only at month 10 of therapy (EOF). The comparator DS performed on IVR patients sequential sera revealed a mixed viral population, wild-type/rtM204I (att), in the pre-treatment sample of pt n. 10 and the RV was detected as the prevalent one in the following samples of the same patient. The DS found no resistant variants in pre-treatment

samples of the remaining IVR patients nor during the follow-up samples but in the EOF as previously described (Table 5).

Table 5

Pt n	LAM Res	RV firstly detected by DS at EOF or breakthrough	Group	PreT: RV positive test+tot viraemia (cp/ml)	LAM Treatment 1M	2M	3M	4M	5M	6M	8M	9M	10M	11M	13M	15M	16M	20M	24M	25M	27M	38M	39M	41M	51M	Sera tested	Follow up (months)
1	Yes	M204I/att	RACVR	nd 1,0E+06			nd 4,0E+02								nd 7,0E+05			nd 7,8E+03				nd 5,0E+02	nd <200	DS+AS 1,3E+04		7	41
2	No	no	CVR	nd 2,7E+05	nd <200		nd <200																			3	117
3	No	no	CVR	nd 1,0E+07	nd 5,0E+04		nd 1,0E+03			nd <200																4	12
4	Yes	M204M/V	RACVR	AS 6,0E+05	nd 1,0E+03		AS <200												AS 6,4E+02	DS+AS 2,4E+03						5	25
5	Yes	M204I/atc	RACVR	nd 1,5E+08						AS 1,9E+04					AS 6,0E+03		AS 3,3E+03			nd 3,0E+02				AS <200	DS+AS 1,4E+03	7	51
6	Yes	A181A/T	RACVR	nd 5,3E+06	nd 5,0E+02		nd <200													nd <200	DS+AS 3,6E+02					5	27
7	Yes	M204V	IVR	AS 2,0E+06	AS 1,5E+05		AS 4,6E+03			DS+AS 5,4E+03																4	6
8	Yes	M204I/att M204V	IVR IVR	AS AS 4,0E+06	nd AS 2,8E+05		nd AS 5,6E+05		AS AS 6,0E+04		AS AS 6,3E+04		DS+AS DS+AS 2,0E+06													6	10
9	Yes	M204V	RACVR	AS 3,8E+07		nd 1,0E+03		nd <200														nd <200		DS+AS 1,2E+03		5	41
10	Yes	M204I/att	IVR	DS+AS 4,7E+07							DS+AS 2,9E+06			DS+AS 3,6E+06	DS+AS 6,9E+06											4	15
11	Yes	A181A/S	RACVR	AS 4,7E+04	DS+AS <200	DS+AS <200		DS+AS 1,0E+04																		4	4
12	No	no	CVR	nd 6,0E+06		nd <200		nd <200																		3	143
13	No	no	CVR	nd 3,0E+02			nd <200			nd <200																3	117
14	No	no	CVR	nd 7,6E+04	nd 5,0E+03		nd 4,0E+02			nd <200																4	82
15	Yes	A181V M204M/V	IVR IVR	AS nd 9,6E+05		AS nd 6,6E+02	nd nd 1,5E+03	AS nd 7,4E+02	nd nd 9,3E+02	nd nd 3,3E+02	nd nd 6,6E+02	AS nd 5,7E+02		DS+AS DS+AS 1,2E+03												9	11

M = month
DS = RV detected by Direct Sequencing
AS = RV detected by Allele Specific RV PCR
DS + AS = RV detected by Direct Sequencing and Allele Specific RV PCR
nd = RV not detected

2.9.5 ASRVPCRs product extensive analysis by sequencing method: some preliminary results

Pre-treatment and EOF sera of 2 RACVR (n. 4, 9) and 3 IVR patients (n. 7, 8, 10), were sequenced, after ASRVPCR, with Pol2s primer to extensively characterize the Pol/Rt region of the amplicon obtained (figure 10). In all the patients, analyzed in this session, but number 10, the Pre-treatment sequences showed no additional mutation associated to the rtM204I or V already identified with ASRVPCR. Patient n. 10 showed a mixed viral population rtL180L/M detected, as previously described, with the Direct Sequencing method also.

In all the patients, sequences obtained by the EOF amplicons showed the rtL180M mutation with no other RV or secondary / compensatory mutation.

3. Discussion.

Antiviral therapy for HBV has been improved in the last years with the use of powerful and high genetic barrier nucleos(t)ide analogs (Entecavir, Tenofovir). The rates of treatment failure associated to the selection and emergence of resistant variants (RVs) showed to be very low after 5 years of ETV administration (1.2%) or 4 years of TFV (0.0%) treatment in *naive* patients [Tenney *et al*., 2009; Snow-Lampart *et al*., 2010]. Despite these improvement, the HBV eradication from the epatocytes remain to date an unreached goal and long-term therapy with NAs is required to avoid recurrence of viral replication and disease reactivation. Therefore the risk of emerging drug resistant mutant and treatment failure is not completely abolished, even using ETV or TFV, particularly in patients pre-exposed to the first generation of NAs and in particular groups of patients like the immunocompromised in whom undetectability of *viraemia* may be difficult to achieve (Zoulim *et al*., 2009; Moriconi *et al*., 2007) increasing the risk of RVs selection.

The aim of our study was to develop a high sensitive and specific assay detecting Resistant Variants at the most important HBV codons involved in drug resistant mutations: rtA181 and rtM204. We aimed to set up the method in selective amplification of the RVs in a range of low total *viraemia* (1.0E+01 - 10E+04 cp/ml), where none of the actual available method are working properly because of scarce sensitivity (Direct Sequencing, INNO-LiPA) or lack of specificity (Real Time PCR, Next Generation Sequencing).

We decided to develop a combination of two different PCR methods, Allele Specific PCR and Nested PCR, to achieve the specificity and sensitivity required.

We succeeded in our purpose designing and using 8 specific primers in eminested single tube PCRs and dedicated ramped ('touchdown') cycle profiles. Our methods, high sensitive in-house Allele Specific Resistant Variant PCRs (ASRVPCR), were firstly developed testing standard curves made by mixtures of RVs and Wild-Type HBV clones.

Optimal results were obtained in therm of specificity and sensitivity with the detection of at least 1% of mutant clones in 10E+04 cp/ml of total *viraemia* for every RVs. Sensitivity was even lower (0.1%) for primers encompassing the rtA181 mutations (Table 4). Such divergence could be due to the different specific primers that had to be forcedly designed in the position where the 3'-end locate on the resistant nucleotide mutation. That is the peculiarity of the Allele specific assay to have specificity but at the same time may be a disadvantage for sensitivity allowing very few chances of primer modification to improve the yield of the reaction.

The high specificity of our assay combined with optimal specificity prompted us to proceed to the next step of our project: the study of the early dynamics of drug resistant quasispecies on a sera panel of CHB patients treated with LAM.

The Allele Specific PCRs for HBV rtA181 and rtM204 codons showed no RVs in the Complete Viral Response group. On the contrary, resistance variants were amplified before the treatment start in sera of 7 out of 10 patients who experienced therapy failure (RACVR + IVR).

Particularly, resistant quasispecies were found in 3 out of 6 RACVR pts and in all the 4 IVR pts, showing a different distribution among the different groups of patients. Moreover, the application, in 4 pts (2 RACVR + 2 IVR), of the sequencing method to the ASRVPCR products showed the addition of the rtL180M secondary / compensatory mutation to the rtM204 variants during the follow-up. Another IVR patient (n. 10) showed the rtL180M in both the pre-treatment and the EOF samples. These data suggest that, in our cohort, the RVs as primary resistant mutations can circulate in CHB *naive* patients and their presence can affect the LAM treatment, particularly when the rtL180M is acquired.

The presence, as minor population, of drug resistant quasispecies in CHB *naive* patients has been already demonstrated in previous studies using several highly specific methods but all of them requiring levels of HBV DNA > 10E+04 cp/ml (Lupo *et al*., 2009; Fung *et al*., 2009; Zhao *et al*., 2010) or an intense work screening hundreds of HBV clones (Pallier *et al*., 2006). Our method proved to be specific and high sensitive (10-100 cp/ml) in a *viraemia* range of 10E+01 - 10E+04 cp/ml. In addition, the sequencing of the ASRVPCR product can reveal information about other important mutations locating on the HBV Pol/Rt region including the B dominion and the surrounding nucleotides like the A determinant (overlapping S gene) for a deep and extensive analysis of the RV strain.

Today, in clinical practice of CHB patients, those drugs have been replaced, at least in Western Countries, by the more efficacy NAs third generation but their use is still wide in Asiatic CHB patients and for prophylaxis in particular group of patients like those undergoing organ

transplantation. In fact in treatment-naive patients receiving a donor liver HBcAb positive, the adoption of long-therm combined prophylaxis with lamivudine and anti-HBs immunoglobulin is recommended by the Italian Guidelines [Marzano et al., 2007].

Lamivudine is also administered in HBV inactive carrier patients undergoing immunosuppressive therapy or anti-tumoral chemotherapy since reactivation of HBV replication is frequently associated to immunocompromised conditions.

Another group of patients in wich ASRVPCRs could be used is the LAM treatment failure expericed patients going to be affected by patterns of resistance based on rtM204 and rtA181 variants. and help clinicians in the tailoring and optimization of the treatment for CHB patients, HBV infected OLT and immunocompromized patient, from the start and the on-going phases where HBV DNA could persist at low level.

In conclusion, whether confirmed in a larger cohort of patients, such a highly sensitive assay for the detection of Resistant Variants could warrant a safer use of NAs first/second generation like lamivudine, adefovir and telbivudine, prolonging their life in the HBV treatment in favour of a cost effective therapy regimen.

3 HEPATITIS C VIRUS

4.1 HCV biology

HCV is an enveloped virus with a positive sense RNA genome in the Hepacivirus genus of the Flaviviridae family. Each virus particle is approximately 55-65 nm in size [Kaito *et al*., 1994; Shimizu *et al*., 1996]. Electron microscopy of the virus has been hampered by the lack of a cell culture system that produces a sufficient amount of virus for visualization. However, recent work using the JFH-1 cell culture system has allowed for further characterization of the virus [Wakita *et al*., 2005]. By examining other viruses of the Flaviviridae family, it is believed that HCV has an icosahedral arrangement in which the structural glycoproteins E1 and E2 are embedded into a bi-layer lipid envelope derived from the host cells (figure 12). The core protein forms the nucleocapsid of the virus that encloses the RNA genome [Ishida *et al*., 2001]. There are three forms of the virus present in the serum of infected individuals including free virions, immunoglobulin associated virions and virions associated with very-low-density and low-density lipoproteins [Bradley *et al*., 1991; Thomssen *et al*., 1993].

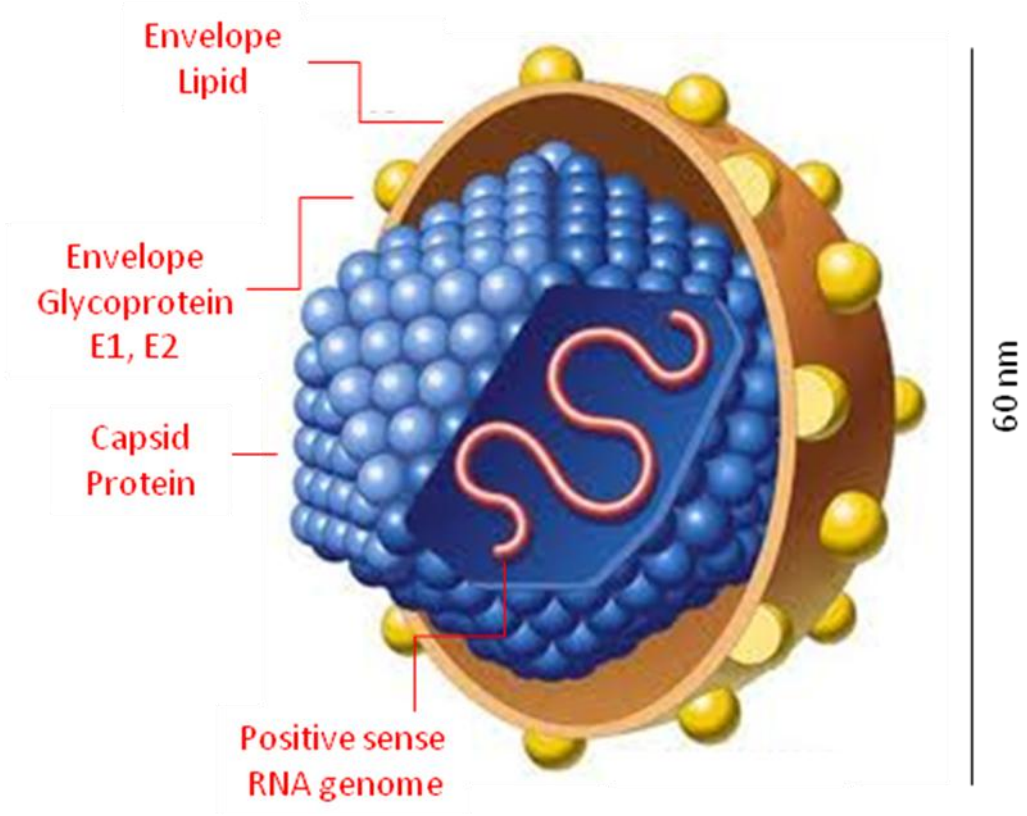


Figure 12. HCV structure (adapted from Perkins JA, 2001)

4.2 Genome Structure

Hepatitis C virus contains a positive sense, single stranded RNA genome of approximately 9.5 kb. The genome consists of a single open reading frame flanked by 5' and 3' untranslated regions (UTR) which are important for replication of the genome (figure 13).

The highly conserved 5' UTR is 341 nucleotides long and contains the internal ribosome entry site consisting of four major RNA domains with extensive secondary structure. The entire 5' UTR is believed to be important

for IRES activity and HCV translation [Beales *et al*., 2001; Jubin *et al*., 2000; Lukavsky *et al*., 2000]. The 3' UTR is potentially important in initiating viral replication. It consists of a poly (U)/polypyrimidine tract, a variable 40 nucleotide sequence, and a highly conserved 98 nucleotide sequence with stable secondary structure [Tanaka *et al*., 1996; Kolykhalov *et al*., 1996].

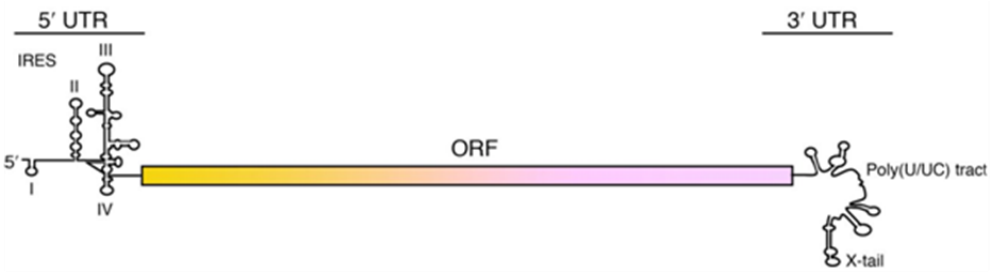


Figure 13. HCV genome structure

4.3 HCV Genotypes and Subtypes

HCV strains are to date categorized into at least six major genotypes based on nucleic acid sequence. Genotypes are approximately 65% identical across the whole HCV genome. Within each genotype, subtypes are also evident with approximately 80% nucleic acid similarity [Simmonds *et al*., 1994]. The different genotypes and subtypes have different geographical distributions and prevalence, with genotype 1 being the most prevalent genotype in North America and Europe while genotype 4 is most common in Egypt and North Africa and genotypes 5 and 6 are most common in South Africa and Hong Kong. Genotypes 2 and 3 are common in North America, Europe and Japan, but to a lesser extent than genotype 1. A high

number of subtypes are evident in Africa and Southeast Asia, suggesting that this region may be the original source of HCV as fewer subtypes are evident in Europe and North America [Smith and Simmonds, 1997] (figure 14). Currently, genotyping of the virus in an infected individual is accomplished by DNA hybridization, restriction length fragment polymorphism, direct nucleotide sequencing using polymerase chain reaction, and serologic genotyping. Many of the current methods, excluding direct nucleotide sequencing, cannot discriminate between viral subtypes.

It has become very clear that the HCV genotype is clinically relevant. In acute infection, the rate of progression to chronic infection has been related to genotype, with an increased number of genotype 1 infected individuals progressing to chronicity in comparison with other genotypes [Amoroso *et al*., 1998]. Perhaps more importantly, treatment response rates to combination therapy are directly correlated with HCV genotype. Genotype 1 or 4 infected individuals will only respond to treatment approximately 50-60% of the time, and only with a longer course of therapy than genotype 2 and 3 infected individuals who are able to clear the virus in approximately 70 to 80% of cases with a shorter treatment duration. The reason for this dramatic difference between genotypes is largely unknown, but may be related to an interferon sensitivitydetermining region in the NS5a protein [Enomoto *et al*., 1995; El-Shamy *et al*., 2008; Torres-Puente *et al*., 2008]. Interestingly, HCV genotype has also been correlated with the development of steatosis in HCV infected individuals. Several studies have shown that a substantially increased number of HCV genotype 3a infected patients have steatosis, even in the absence of contributing factors such as

obesity, diabetes and alcoholism. Furthermore, steatosis is abolished upon successful treatment and clearance of the virus, suggesting that HCV genotype 3a may play a direct role in altering hepatic lipid metabolism that is not as evident with other genotypes. Within each HCV infected individual, many different sequences of the virus exist which are 90-99% identical at the nucleotide level and are members of the same infecting genotype. These populations of virus, called quasispecies, are generated by the errorprone nature of the HCV RNA-dependent RNA polymerase during genomic replication.

These quasispecies populations exists in high numbers during acute infection; as infection progresses, the number of dominant quasispecies populations decreases with the major quasispecies strain changing intermittently. The role of these quasispecies populations is two fold. Firstly, high mutation rates can result in the development of new viral strains that have increased viral fitness over the parent strain. Secondly, viral quasispecies likely serve as a mechanism for immune evasion and viral persistence. As the immune system mounts an antibody response against a particular viral epitope, the virus quickly mutates and the dominant viral population changes to one that the immune system no longer recognizes. In a similar fashion, the virus can also quickly generate mutants that are resistant to antiviral drugs. The high mutability of the HCV virus poses several challenges to the development of an effective vaccine or antiviral drug.

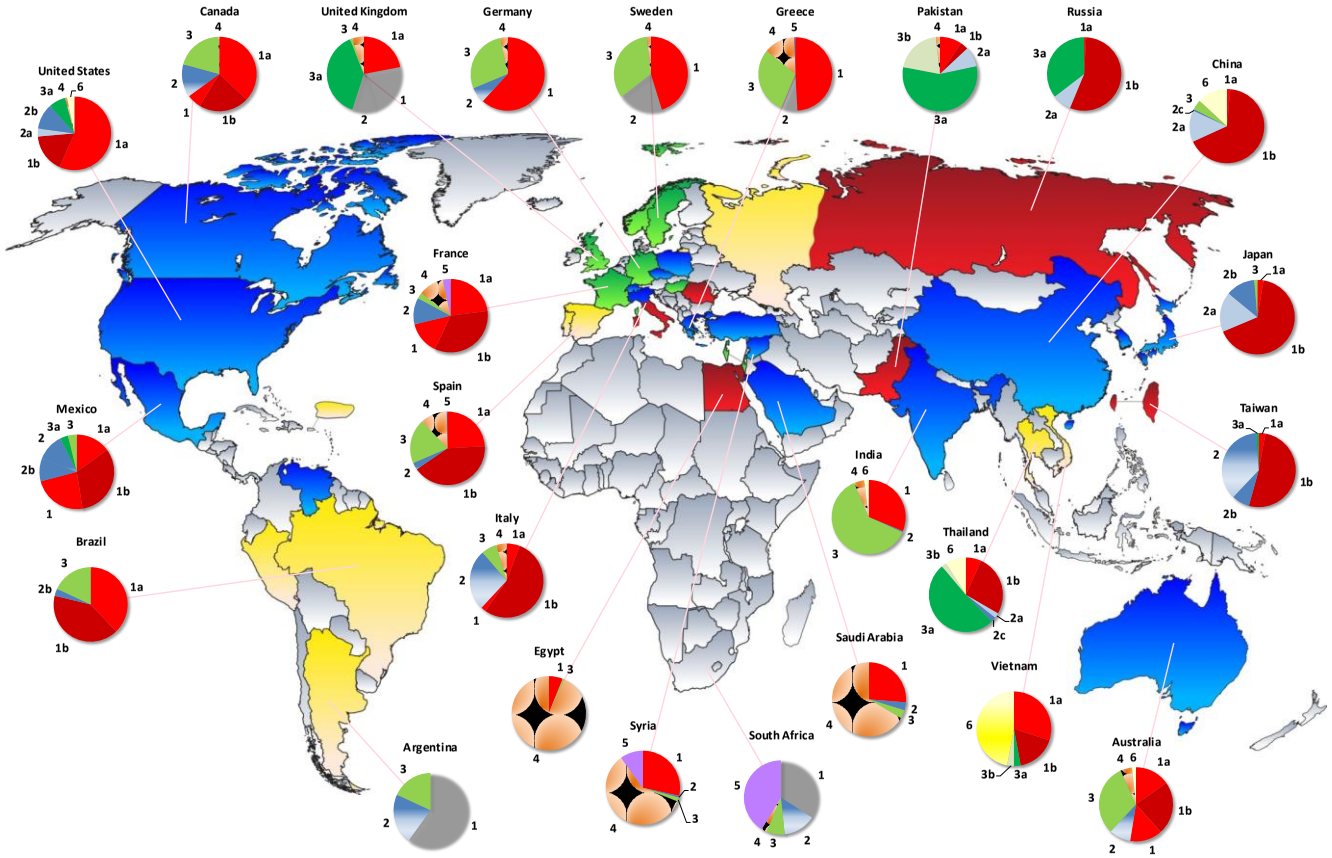


Figure 14. HCV Prevalence and Subtypes distribution around the world. Source: Kromite – Center for Disease Analysis, International Conquer C Coalition (I-C3)

4.4 HCV proteins

The HCV genome is translated into a polyprotein that is subsequently cleaved, by host peptidase and viral protease, into approximately 10 proteins in the sequence Core-E1-E2-p7-NS2-NS3-NS4a-NS4b-NS5a-NS5B. The Core, E1, E2 and p7 proteins are considered structural proteins of the virus while NS2, NS3, NS4a, NS4b, NS5a and NS5B are non-structural proteins (figure 15).

4.4.1 Structural proteins

The core protein is cleaved from the polyprotein via host signal peptidase to form a 191 amino acid immature form of the protein. The core protein is then cleaved between amino acids 173 to 179 by host Signal Peptide Peptidase (SPP) to form the mature protein [Okamoto *et al*., 2004]. The core protein is a dimeric alpha-helical protein that can bind RNA and consists of two major domains. The D1 domain is primarily hydrophilic and is located in the N-terminus while the D2 domain is primarily hydrophobic and located in the C-terminus. D2 assists in the folding of the protein as well as membrane and lipid droplet association [Boulant *et al*., 2005; Santolini *et al*., 1994]. The core protein is localized on the endoplasmic reticulum and on the surface of lipid droplets in infected cells. Some studies have also shown a small amount of core protein in the nucleus and on mitochondria, but this localization has not been observed in infected cells [McLauchlan, 2000; Hope *et al*., 2002; Suzuki *et al*., 2005; Rouille *et al*., 2006]. Localization of core with lipid droplets is also mediated by proteolytic

cleavage by SPP [McLauchlan *et al*., 2002]. Mature core proteins form the nucleocapsid of the virus. However, many studies have shown alternative roles for the core protein in the pathogenesis of HCV including apoptosis, insulin resistance, cell cycle, and lipid metabolism. Interestingly, the core protein-encoding region also contains a +1 alternative reading frame that results in translation of the ARF (alternative reading frame) protein. The F protein does not contain an AUG start codon, so translation of this protein is a random event. No known function of the F protein is currently known. Although antibodies against it are present in HCV infected individuals, it is not required for viral replication [Branch *et al*., 2005].

The E1 and E2 proteins of HCV are envelope glycoproteins that are essential for virus entry and form heterodimers on the viral envelope. The E2 protein contains a hypervariable region that mutates frequently to allow for the development of immune escape variants [Forns *et al*., 2000; Callens *et al*., 2005].

The p7 protein has recently been found to have ion-channel activity and it is essential for virus infectivity. As such, new research has been directed toward developing antiviral compounds to block the function of this protein [Saint *et al*., 2009].

4.4.2 Non-structural proteins

The NS2 protein is a hydrophobic protease that cleaves the HCV polyprotein between NS2 and NS3 and it may function as a novel cysteine protease. Efficient cleavage of NS2/3 is required for HCV replication, but the presence of NS2 is not required for genome replication. Following

cleavage, NS2 is localized in the ER membrane. Besides its protease activity, NS2 appears to be involved in apoptosis, cell proliferation, innate immunity and lipid metabolism [Dumoulin *et al*., 2003; Erdtmann *et al*., 2003; Yang *et al*., 2006; Oem *et al*., 2007].

The NS3 protein is a protease and RNA helicase. The Protease activity of NS3 is mediated by the NS4a protein and both proteins are localized on the ER. The NS3/4a protease complex cleaves the HCV polyprotein downstream of NS3 and this action is essential for formation of the viral replication complex [Lindenbach *et al*., 2005] (figure 15). The NS3/4a is able to inhibit the host innate immune response via disrupting the RNA helicase retinoic acid-inducible gene-I (RIG-I) pathway by cleaving Cardif, an adaptor protein of interferon regulatory factor-3 [Foy *et al*., 2003]. The function of the NS3/4a helicase activity is still unknown.

The NS4b protein is present on the ER membrane and induces intracellular membrane changes that may be involved in the formation of the replication complex membranous webs [Hugle *et al*., 2001; Egger *et al*., 2002]. It also contains a nucleotide binding motif that hydrolyzes GTP [Einav *et al*., 2004]. NS4b is thought to play a major role in viral replication, assembly and release as well as lipid metabolism [Jones *et al*., 2009; Park *et al*., 2009].

The NS5a is also an ER-associated protein that is particularly multi-functional. It can bind to HCV RNA and it is essential for viral replication [Huang *et al*., 2005]. It can be hyperphosphorylated in order to inhibit viral replication [Evans *et al*., 2004]. NS5a is also able to decrease the immune response via inhibition of interferon-induced double stranded RNA

activated protein kinase PKR [Gale *et al*., 1998]. NS5a may also play a role in mediating lipid metabolism as it interacts with core proteins on the surface of lipid droplets and it also interacts with apolipoproteins [Shi *et al*., 2002].

The NS5B is an RNA dependent RNA polymerase that synthesizes RNA using an RNA template. It is also localized on the ER membrane and it is essential for viral replication. As such, it is a prime target for the development of antiviral compounds [Lohmann *et al*., 2000].

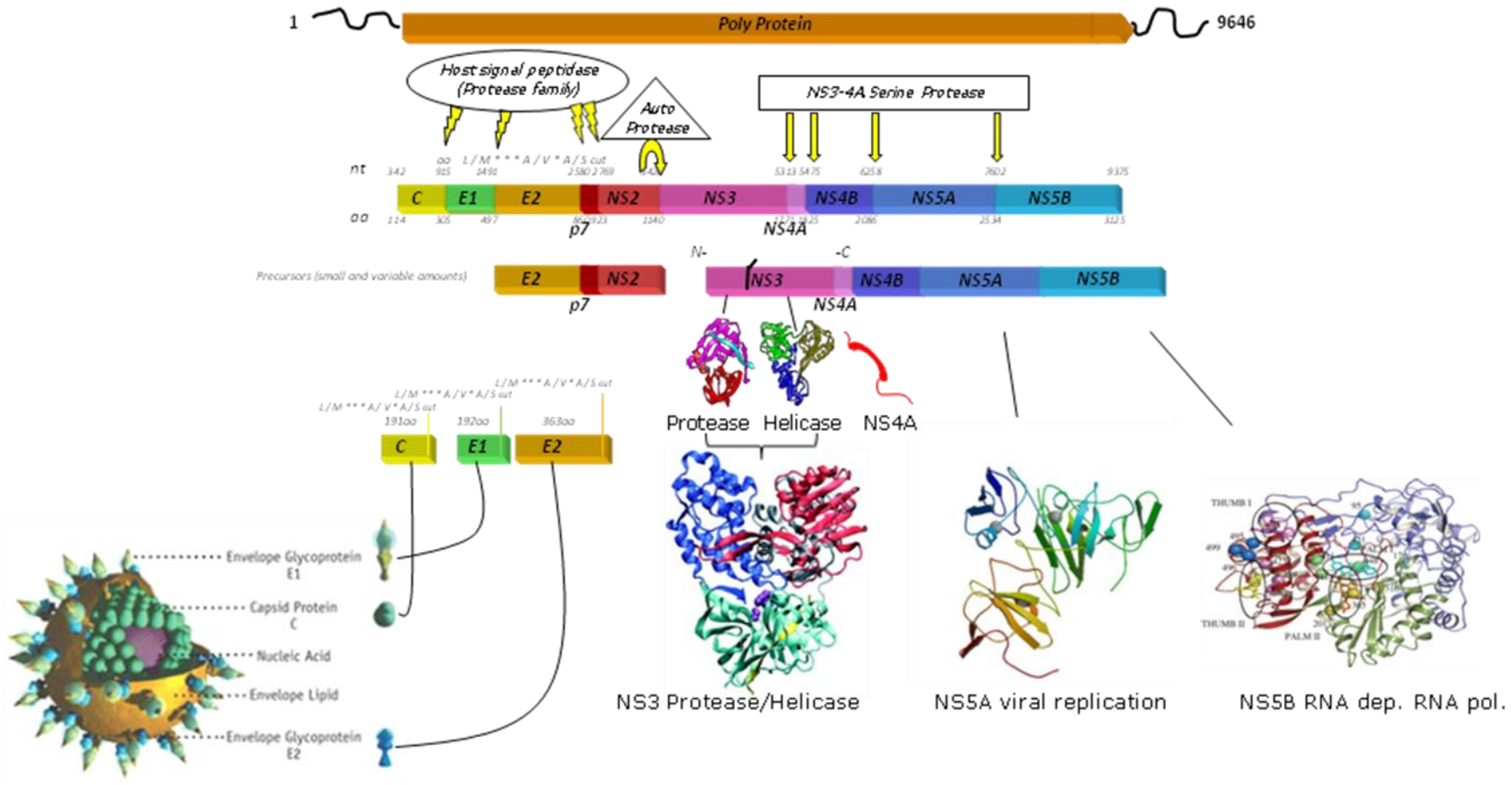


Figure 15. Schematic representation on HCV translation and proteins functions. Polyprotein is cleaved by host peptidase and viral serine protease in Structural and Non-Structural Proteins. The former contribute to the Core and Envelope (E1, E2) protein, the latter include multiple function enzymes involved in the cleavage of the polyprotein or in the replication of the HCV genome.

4.5 HCV life cycle

4.5.1 Receptors and entry

The entry of HCV into a naïve host cell appears to be a complicated, multi-step process involving several different host receptors. The initial entry steps have only been recently characterized thanks to the newly developed infectious cell culture system, and many details are still to be discovered. Heparin, a glycosaminoglycan, has been long suspected to play a role in viral entry with several studies showing inhibition of viral attachment in the presence of heparinase, an enzyme that disrupts heparin [Koutsoudakis *et al*., 2006; Barth *et al*., 2003; Barth *et al*., 2006]. Low-density lipoprotein receptor (LDLR) is very likely a critical component of viral attachment. HCV in the serum of infected patients is bound to low density and very low-density lipoproteins. Adsorption of HCV from the serum of infected patients can be inhibited through the use of antibodies against LDLR suggesting that the association of HCV with lipoproteins is an important step in attachment of the virus [Thomssen *et al*., 1992; Andre *et al*., 2002; Huang *et al*., 2007a; Monazahian *et al*., 1999; Molina *et al*., 2007]. CD81, a cell surface protein in the tetraspanin family, is considered one of the major players in HCV attachment. Antibodies against CD81 effectively inhibit HCV infectivity in different models. In addition, HepG2 and HH29 hepatoma cells, which are not normally permissive to HCV infection, can be infected following ectopic expression of CD81 [Cormier *et al*., 2004; Wakita *et al*., 2005]. However, expression of CD81 in other non-permissive cell lines does not allow HCV infection to occur, suggesting that other host cell factors may be involved in the attachment and entry process

[Bartosch *et al*., 2003]. Scavenger receptor class B type I (SR-BI) is another cell surface protein that may be involved in viral attachment and entry. SR-BI is a receptor for lipoproteins and it can also change the lipid composition of membranes. Blocking of SR-BI with antibodies can prevent virus entry. It is still unclear whether SR-BI is interacting directly with the virus proteins or if it is interacting with virus associated lipoproteins to mediate virus entry as both mechanisms have been observed [Bartosch *et al*., 2003; Catanese *et al*., 2007; Acton *et al*., 1996; Maillard *et al*., 2006; Huang *et al*., 2003]. Recently, two tight junction cell proteins Claudin-1 and Occludin have been shown to be involved in HCV entry. Claudin-1 can be ectopically expressed in non-permissive, non-hepatic 293T cells and allow HCV infection to occur. Knock-down of Claudin-1 in permissive Huh7 cells can reduce HCV infectivity. It has also been found that Claudin-1 and Occludin are involved in late entry of the virus into the cell, which likely occurs through interaction with the tight junctions [Evans *et al*., 2007]. The use of the tight junctions by HCV may also allow for cell to cell spread of the virus [Timpe *et al*., 2008]. It has been shown that HCV entry is mediated by endocytosis in a pH-dependant manner. In addition, knock-down of clathrin, a critical component of the endocytotic vesicles, prevents HCV infection [Meertens *et al*., 2006; Blanchard *et al*., 2006]. Fusion of the virus envelope with the endosome membrane is not well characterized and both E1 and E2 are likely involved in the process [Lavillette *et al*., 2007].

4.5.2 Replication, assembly and release

HCV replication occurs in a membrane associated replication complex that contains several viral and host proteins and replicating RNA. The positive strand of the virus RNA genome is made into a negative strand that is then used to make more positive strand copies for packaging. The NS5B protein is the major player in the synthesis of both the positive and negative strands. The HCV replication complex appears to contain a membrane alteration called a membranous web that can be observed by electron microscopy in infected cells *in vitro* and in vivo, as well as in cells expressing the NS4b protein alone. The source of the membranous web is likely the ER as most of the HCV proteins are associated with the ER upon translation [Shi *et al*., 2003; Gosert *et al*., 2003]. It is also possible that viral replication is occurring on lipid rafts that contain large amounts of cholesterol and sphingolipids. Inhibitors of sphingolipid synthesis can prevent HCV replication and both non-structural proteins and replicating RNA are found on lipid rafts [Sakamoto *et al*., 2005; Matto *et al*., 2004; Aizaki *et al*., 2004; Shi *et al*., 2003]. Recently, it has been determined that lipid droplets play an important role in virus assembly. Core protein localizes around lipid droplets where it recruits non-structural proteins and replication complexes. Inhibiting lipid droplet localization of core protein decreased the production of infectious virus particles [Miyanari *et al*., 2007]. There are also many different host cell proteins with putative roles in HCV replication. For example, vesicle-associated membrane proteins A and B (VAP-A and VAP-B) localize in the ER and bind both NS5a and NS5B and are essential for viral replication [Hamamoto *et al*., 2005]. Cyclophilin

B, a peptidyl-prolyl cis-trans isomerase, binds to NS5B and enhances its RNA binding activity [Watashi *et al*., 2005]. Similarly, p68, and RNA helicase, binds NS5B and moves into the cytoplasm from the nucleus to assist in viral replication [Goh *et al*., 2004]. Very little is known about HCV assembly and release. There are several different forms of the virus found in infected serum, including free mature virions, virions bound to low-density and very low-density lipoproteins, virions bound to immunoglobulins and non-enveloped nucleocapsids. It is believed that viral RNA can interact with the core protein, which then oligomerizes to form the nucleocapsid around the viral RNA [Tanaka *et al*., 2000]. The envelope of the virus is then obtained through core interaction with E1 glycoprotein on the ER membrane, from which budding occurs [Ezelle *et al*., 2002; Murakami *et al*., 2006]. From this point it is thought that the virus is released from the cell after transit through the Golgi apparatus and the secretory pathway [Serafino *et al*., 2003].

4.5.3 Virus-like particles

In the serum of HCV infected individuals, several different forms of HCV can be found that differ in both size and density. Currently very little is known about HCV assembly and how these different types of HCV particles are formed and secreted. HCV RNA can be found in infected serum at a density between 1.03 and 1.25 g/ml [Thomssen *et al*., 1992; Thomssen *et al*., 1993]. Interestingly, the lower density particles appear to be more infectious than the higher density particles when tested in

chimpanzees [Bradley *et al*., 1991; Hijikata *et al*., 1993]. The low-density particles contain high amounts of triglycerides, as well as HCV RNA, core protein and apolipoproteins B and E (ApoB and ApoE). Triglycerides along with ApoB and ApoE are the major components of very-low density lipoproteins (VLDL) and low-density lipoproteins (LDL) which are particles packaged in the ER of hepatocytes to be secreted into the bloodstream and transport their triglyceride load to adipocytes for storage [Rustaeus *et al*., 1999]. During HCV infection, assembled virus particles are hypothesized to use this pathway to acquire ApoB, ApoE and triglycerides prior to secretion from the cells. Virus particles that do not obtain the necessary triglycerides and apolipoproteins are not secreted from the cell and undergo degradation. Exploitation of the VLDL assembly pathway by HCV ultimately decreases lipid export from the cells and could be an important factor in the development of steatosis in infected patients. The addition of apolipoproteins and triglyceride to the virus particle could also be advantageous to the virus as it could be providing a mechanism of immune escape by hiding viral epitopes and also by increasing the number of potential receptors (ie. CD81, LDLR and SR-BI) for attachment [Gastaminza *et al*., 2008; Huang *et al*., 2007a; Nielsen *et al*., 2006; Andre *et al*., 2002; Perlemuter *et al*., 2002].

Another fraction of the HCV population in infected serum appears to be nonenveloped. These particles have a higher density and vary in size. The core protein is displayed on the surface of these particles and antibodies against core protein are a hallmark of HCV infection. Currently it is unknown whether these nonenveloped particles are infectious or what role

they play in the course of HCV infection and pathogenesis [Maillard *et al*., 2001].

4.6 HCV Clinical Characteristics

4.6.1 Acute viral infection

Upon infection with HCV, most patients enter an acute phase that is largely asymptomatic. Most of the infected individuals (~80%) will progress to chronic HCV infection while the other 20% will clear the virus within the first 3 months after infection.

The asymptomatic nature of the acute stage of infection results in difficulty in diagnosing and reporting HCV infection, as well as difficulty in studying the aspects of the immune response that allow for viral clearance during this time. The greatest risk of infection with HCV is through intravenous drug use (IDU), unprotected sex, exposure during medical procedures, vertical transmission from mother to child, or needle injury in health care professionals. Blood transfusion prior to 1992 is also an important risk factor [Centers for Disease Control and Prevention, 2007].

The HCV RNA can be detected in the serum approximately 1-3 weeks after exposure. Symptoms include fatigue, jaundice, dyspepsia, and abdominal pain but are generally not specific or severe enough to warrant consultation of a doctor. Elevated alanine aminotransferase (ALT) levels can be detected 4-12 weeks after exposure and are the first indication of liver injury [Santantonio *et al*., 2003]. Serum HCV RNA and anti-HCV

antibody seroconversion is generally used to detect HCV infection [Pawlotsky, 2002].

Host and viral factors appear to contribute to viral clearance in acute infection including genotype of the virus, HIV co-infection, gender, race, age and HLA [Lehmann *et al*., 2004]. A strong cellular immune response is also thought to increase viral clearance, and symptomatic infection appears to correlate with viral clearance [Gruner *et al*., 2000; Thimme *et al*., 2001]. Patients who spontaneously clear the virus need to be monitored as disease relapse may occur up to 6 months after clearance [Gerlach *et al*., 1999].

4.6.2 Chronic viral infection

Infection with HCV is one of the leading causes of liver disease worldwide. As previously discussed, a small proportion of people are able to clear the virus following an acute infection but the majority progress to a chronically infected state. Chronic infection can persist for many years without causing the death of the patient. However, most cases eventually lead to the development of hepatitis, fibrosis, steatosis and cirrhosis with the eventual need for a liver transplant if left untreated. A small number of HCV infected individuals will also develop hepatocellular carcinoma. Host factors such as age, obesity, alcohol use, gender, race and co-infection with HIV or HBV can increase the rate of progression of liver damage [Soto *et al*., 1997; Pontisso *et al*., 1998; Corrao and Arico, 1998]. Genotype does not appear to play a role in disease progression [Silini *et al*., 1996]. However, the presence of non-alcoholic steatohepatitis can increase progression of disease, and infection with genotype 3a is associated with a higher

prevalence of steatosis [Mihm *et al*., 1997; Adinolfi *et al*., 2001]. In general, the rate of progression to cirrhosis is unpredictable in chronic HCV infection [Ascione *et al*., 2007].

4.6.3 Associated clinical pathology

4.6.3a Hepatitis, steatosis, fibrosis and cirrhosis

The HCV is not generally considered to be a cytopathic virus. Damage to the liver is thought to be primarily the result of immunopathogenic mechanisms. However, HCV has been associated with direct cytopathic effects on the liver such as apoptosis, stellate cell activation, the development of steatosis and insulin resistance. Damage to the liver is clinically observed by increased ALT levels. In the case of HCV, liver biopsies are often taken to assess the stage of liver disease. Hepatitis of the liver occurs following an infiltration of inflammatory cells into the liver and damage of the hepatocytes leads to steatosis and fibrosis. Steatosis of the liver is the accumulation of lipid within hepatocytes that can eventually lead to the development of fibrosis. Fibrosis of the liver is characterized by the accumulation of extracellular matrix proteins, like collagen, that are produced by different liver cell types following damage.

Liver fibrosis can be reversed [Bataller and Brenner, 2005]. Cirrhosis is the final stage of liver disease in which liver tissue is replaced by scar tissue and nodules that decrease liver function. Cirrhosis is irreversible and patients in the advanced stages of liver cirrhosis will need a liver transplant [Mengshol *et al*., 2007].

4.6.3b Lymphoproliferative disorders

One of the best characterized extra-hepatic disorders associated with HCV infection is mixed cryoglobulinemia (MC). MC is caused by the development of cryoglobulins consisting of polyclonal IgG and monoclonal IgM or polyclonal IgM with rheumatoid factor that precipitate in the blood at less than 37°C [Zignego 1997]. Symptoms include weakness, arthralgias, and palpable purpura in the lower extremities and complications can include kidney damage and possibly liver damage [Ferri 1992; Kayali 2002; Saadoun 2006]. Approximately 5-10% of HCV patients exhibit MC [Lunel 1994]. Successful treatment of the HCV infection results in eradication of MC in most patients [Zignego 2007b]. HCV is also associated with malignant lymphomas that may be related to MC. B-cell derived Non-Hodgkin's Lymphoma is the most common lymphoma associated with HCV infection [De Vita 1997].

4.6.3c Hepatocellular carcinoma

Infection with HCV is a major risk factor for the development of hepatocellular carcinoma (HCC). The mechanism by which HCV causes HCC is unknown but several studies have indicated a role for the core protein, including the induction of oxidative stress and steatosis. Cellular signaling pathways are also changed by the core protein, as previously discussed. The development of liver fibrosis and cirrhosis also increases the risk for HCC. Approximately 1-3% of HCV patients will develop HCC after 30 years of chronic infection [Hassan *et al.*, 2002]. Age, alcohol

consumption, obesity, diabetes, genotype and co-infection with HBV or HIV can all increase the risk of developing HCC in HCV infected patients [El-Serag, 2002].

4.7 HCV test for diagnosis and monitoring

Diagnosis of chronic HCV infection is based on the presence of both anti-HCV antibodies, detected by enzyme immunoassays (EIA), and HCV RNA, detected by molecular assays. HCV RNA testing is essential for the management of HCV therapy [Chevaliez S *et al*., 2008]. As previously described for HBV (session 2.2.1) the most recent assays are based on the use of real-time polymerase chain reaction (PCR). This method can detect very little amounts of HCV RNA (down to 10 international units IU/ml) and accurately quantify HCV RNA levels up to approximately 10^7 IU/ml. Their dynamic range of quantification adequately covers the clinical needs for diagnosis and monitoring [Vermehren J *et al*., 2008, Chevaliez S *et al*., 2009, Fytili P *et al*., 2007].

HCV genotype and subtype can be determined via various methods, including direct sequence analysis, reverse hybridization (INNO-LiPA), and genotype-specific real-time PCR [Chevaliez S *et al*., 2008] with the first two more commonly used in laboratory for routinary use. However, these assays targeting the 5' noncoding region (5' UTR) of the HCV genome, fail to differentiate HCV subtypes 1a and 1b. In particular, Trugene HCV Genotyping Kit (Direct sequencing based method) and INNO-LiPA HCV 1.0, failed to correctly identify HCV subtype 1a in 22.8% and 29.5% of cases, and HCV subtype 1b in 9.5% and 8.7% of cases, respectively. The

INNO-LiPA HCV has been recently improved adding, in the version 2.0, the core-encoded oligonucleotide probes to better discriminate between HCV subtypes 1a and 1b. With INNO-LiPA HCV 2.0, subtype identification was corrected in 97.5% and 96.2% of subtype 1a and 1b respectively [Chevaliez S *et al*., 2009].

Although genotype information is enough for SOC management, the correct subtype identification is becoming more and more important with the introduction of the DAAs since subtype, specific, single nucleotide polymorphisms can affect the development of drug resistant variant as in the case of subtype 1a and 1b [Kieffer TL, *et al*. 2007]. Therefore, sequencing or reverse hybridization-based methods targeting segments other than the 5' UTR region are going to be required in the very next future [Chevaliez S *et al*., 2009].

4.8 HCV Treatment

Until recently, standard of care (SOC) for chronic HCV infections has been limited to combination of pegylated interferon-α 2a/2b plus ribavirin. Whilst SOC is generally successful in eradicating the virus in 80% of genotype 2 and 3 patients, the efficacy of this regimen is lower in case of infection with HCV genotype 1 and 4 (eradicating the 42% and 69% respectively). Moreover, SOC is associated with significant Flu-like symptoms following each injection (fever, chills, headache, muscle aches and pains, malaise). These symptoms vary from mild to severe and occur in up to half of all patients. For this reasons, Direct Acting Antiviral (DAAs), new classes of anti-HCV agents, have been developed in the last years.

Many of them are currently going through clinical trials while two, Telaprevir and Boceprevir, have been approved by the Food and Drug Administration (FDA) in April and May 2011 respectively, to be used in combination with SOC.

4.8.1 Ribavirin plus interferon combination therapy: mechanism of action.

As previously described in the HBV session 2.3.1, interferons are cellular proteins able to induce an antiviral state in their target cells, as well as cytokine secretion, recruitment of immune cells, and cell differentiation. Concerning the HCV infection, their metabolism and mechanisms were recently reviewed [Chevaliez S *et al*., 2009].

Immediately after injection, IFN-α binds to receptors present on various cells including hepatocytes, triggering a sequence of intracellular reactions that activate IFN-inducible genes (ISGs). The products of these genes are responsible for the IFN-α-mediated antiviral effects, achieved via two mechanisms. One, indicated as the first phase, is the induction of a nonvirus-specific replication inhibition in infected cells. The IFN-α was found to directly inhibit HCV replication *in vitro* in the subgenomic replicon (a synthetic replication system using HCV nonstructural proteins in various cell lines) [Kim CS *et al*., 2007]. The second mechanism, second phase of response, involves immunomodulatory effects that enhance the host's specific antiviral immune response, thus clearing the infected cells [Chevaliez S *et al*., 2009] (figure 16). The induction of the antiviral state could potentially extend to noninfected cells, thus reducing the chance that

they will become infected. Upon the interaction of IFN-α with its receptor, many complex effects are generated, including the induction of major histocompatibility complex class I (MHC I) antigen expression, activation of macrophages, natural killer cells and T lymphocytes, production of primarily T-helper 1 (Th1) cells, and decreased production of T helper-2 (Th2) cells. PEG-IFN-α also interacts with cytokines such as CCL chemokines and tumor necrosis factor (TNF)-α. Soluble TNF-α receptors (sTNF-R), which are released by activated neutrophils, mononuclear blood cells, and fibroblasts [Porteu F *et al* 1991, Lien E *et al*., 1995] in response to mediators, such as interferon and TNF- α itself [Lien E *et al*., 1995, Lantz M *et al*., 1990, Joyce DA *et al*., 1994, Tilg H *et al*., 1995], retain their ability to bind circulating TNF- α and are important in regulating its activity. These sTNF-R may contribute to the anti-inflammatory action of IFN-α. All of these effects suggest that IFN-α simply accelerates the host immune response, though no studies have clearly proven it. HCV genotypes 1 and 4 are intrinsically more resistant to IFN-α than genotypes 2 and 3. Consequently, the clearance of infected cells in patient who are interferon responders occurs much slower in genotypes 1 and 4, as compared to 2 and 3 [Neumann AU *et al*., 2002, Pawlotsky JM *et al*., 2002]. The mechanisms underlying these differences are yet to be defined. The combination of IFN induced proteins and pathways responsible for inducing an antiviral state has not been completely mapped out, though various effectors have been proposed including the 2'–5' oligoadenylate synthetase (2'–5' OAS) system, Mx proteins, double-strand-RNA-dependent protein kinase (PKR),

as well as other, less well-characterized/unknown IFN-induced intracellular pathways [Chevaliez S *et al*., 2009].

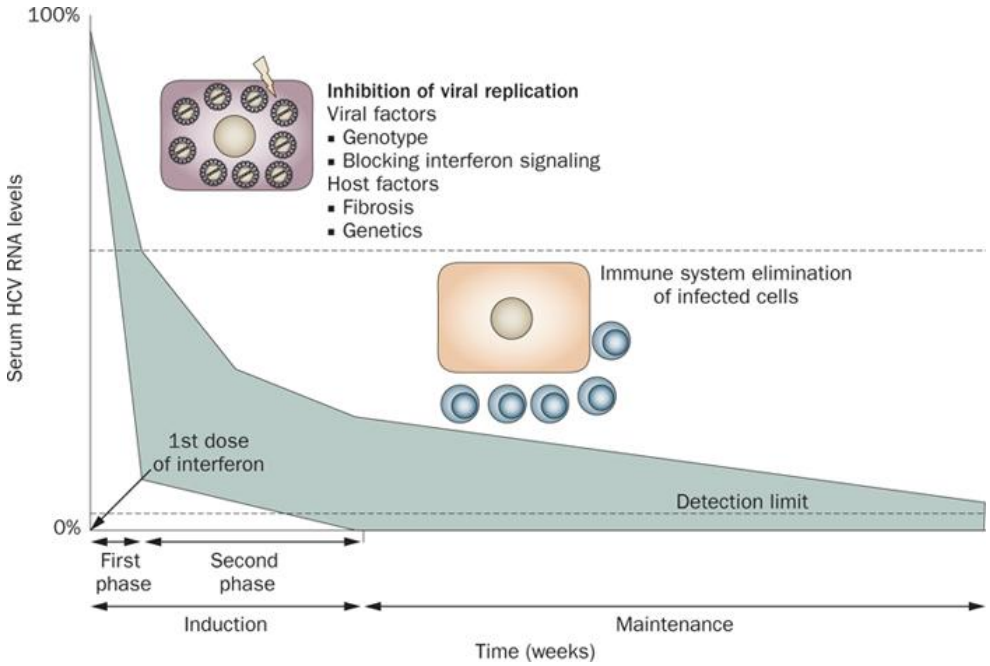


Figure 16. Phases of antiviral treatment of chronic hepatitis C. Source: Ferenci P. *Nature Reviews Gastroenterology & Hepatology 7, 191-193 (April 2010).*

Ribavirin is a synthetic guanosine analog that undergoes intracellular phosphorylation, with the ultimate product, ribavirin triphosphate, being responsible for ribavirin's effects. In vivo, ribavirin has a moderate (<0.5 log HCV RNA reduction) and transient inhibitory effect of only 2-3 days duration on HCV replication, and this is only seen in about 50% of patients [Pawlotsky JM *et al*., 2004]. These effects are consistent with those seen in *in vitro* studies, and the modesty of the effect could be related to the drug's weak inhibitory action on the RNAdependent RNA polymerase (RdRp) [Lau JYN *et al*., 2002]. Fortunately, this effect is too weak and transient to account for selecting viral resistance to ribavirin. Despite its apparently

weak antiviral effects, ribavirin remains essential to HCV treatment, as it appears to accelerate the clearance of infected cells through unknown mechanisms and to prevent viral breakthrough during treatment, and relapses following treatment, in patients on IFN-α [Bronowicky JP *et al*., 200633]. Studies have suggested that ribavirin is an RNA mutagen, causing loss of viral "fitness" via lethal nucleoside accumulation during replication [Contreras AM *et al*., 2002], though no excessive mutagenesis was noted during ribavirin therapy in HCV infection [Chevaliez S *et al*., 2007, Lutchman G *et al*., 2007]. Ribavirin, much like IFN-α, has immunomodulatory effects, including preferentially driving the immune system to produce more Th1 cells relative to Th2 [Fanf SH *et al*., 2000]. Some reports have suggested that ribavirin can also amplify the intracellular IFN-α responses, via unknown mechanisms [Feld JJ *et al*., 2007]. According to Tang *et al*., IFN-α attachment to interferon alpha receptors (IFNAR) [Tang KH *et al*., 2005] and the very rapid activation of Interferon Stimulated Genes (ISG) after interferon alpha administration [Feld JJ *et al*., 2005] could explain the effect of IFN-α as a potent immunostimulant of an innate response in the first few hours and an adaptive response after the first four weeks. [Ji X *et al*., 2003]. The degree of the ISG-induced innate response may result in a rapid decline of HCV replication (as measured by a decrease of HCV-RNA levels), which, if significant enough, causes very distinct CD4 and CD8 responses (shifting the immune response from innate to adaptive). Clinically, this phenomenon defines a group of patients known as rapid responders (HCV RNA serologic negativity achieved by week 4 of treatment) and differentiates them from patients with a less vigorous early

reduction in viral load, known as slow responders. Sustained virologic response (SVR) is achieved when no virus is detected in the blood for six months after finishing treatment and prognosticates a 99% chance of indefinitely eradicating HCV, which for many experts is equivalent to curing HCV. Several events need to work in concert in order to achieve SVR, including (i) successfully achieve a rapid phase 1 effect by turning off viral replication, (ii) effectively suppress the viral load throughout treatment, and (iii) induce a solid and persistent Phase 2 effect [Araujo ES *et al*., 2007] (figure 16). It has been hypothesized that phase 2 is driven by the host's adaptive immune response in the context of sustained inhibition of virus production, while restoration of the innate immune response by viral inhibition leads to clearance of residual HCV-infected cells [Neumann AU *et al*., 1998].

4.8.2 Direct Acting Anti-viral drugs

The five steps in HCV replication that are potential targets for direct-acting antivirals include initial binding of HCV to hepatocyte surface receptors (via LDLR and CD81), translation and polyprotein processing (via the HCV protease complex), RNA replication (via the HCV RNA-dependent polymerase complex), virion assembly and maturation, followed by release from the hepatocyte. To date, the most successful approaches have been targeting the HCV protease (via inhibition of NS3A4 protease) and the HCV polymerase complex (via inhibition of NS5A, NSAb and indirectly through NS3A4). The *in vitro* replicon and transgenic models for HCV replication and the application of rapid screening techniques for small

molecules have triggered an explosion in drug development. During the last years, about a hundred of protease and polymerase inhibitors have entered clinical development, of which several have halted because of toxicity (BILN2061, NM283, HCV796, R1626) and many more have been abandoned because of preclinical toxicity signals or lack of clinical efficacy (table 6).

Table 6. Direct Acting Antiviral for HCV therapy.

Drug name	**Company Target**	**Target**	**Active site**	**Phase**
NS3-4A protease Inhibitors				
Ciluprevir (BILN 2061)	Boehringer Ingelheim	Active site	macrocyclic	Stopped
Telaprevir (VX-950)	Vertex Janssen	Active site	linear	Approved
Boceprevir (SCH503034)	Merck (S-P)	Active site	linear	Approved
Simeprevir (TMC435350)	Janssen Medivir	Active site	macrocyclic	III
Danoprevir (R7227)	Roche InterMune	Active site	macrocyclic	II
Vaniprevir (MK-7009)	Merck	Active site	macrocyclic	Halted II
MK-5172	Merck	Active site	macrocyclic	II
BI201335	Boehringer Ingelheim	Active site	linear	III
Narlaprevir (SCH900518)	Schering-Plough	Active site	linear	Halted
Asunaprevir (BMS-650032)	Bristol-Myers Squibb	Active site		II
PHX1766	Phenomix	Active site		I
GS-9256	Gilead	Active site		II
GS-9451	Gilead	Active site		I
ABT450	Abbott	Active site		II
IDX320	Idenix	Active site		II
ACH-1625	Achillion	Active site	macrocyclic?	II
Nucleoside analog NS5B *polymerase Inhibitors*				
Valopicitabine (NM283)	Idenix Novartis	Active site		Stopped
Mericitabine (R7128)	Roche Pharmasset	Active site		II
R1626	Roche Active site			Stopped
PSI-7977	Pharmasset	Active site		II
PSI-938	Pharmasset	Active site		II
IDX184	Idenix	Active site		II
Non-nucleoside NS5B *polymerase Inhibitors (NNI)*				
BILB 1941	Boehringer Ingelheim	NNI site 1	thumb 1	Stopped
BI207127	Boehringer Ingelheim	NNI site 2	thumb 1	II

Drug name	Company Target	Target	Active site	Phase
MK-3281	Merck	NNI site 1	thumb 1	II
TMC647055	Janssen	NNI site 1	thumb 1	
Filibuvir (PF-00868554)	Pfizer	NNI site 2	thumb 2	II
VCH759	ViroChem Pharma	NNI site 2	thumb 2	II
VCH916	ViroChem Pharma	NNI site 2	thumb 2	II
VCH222	ViroChem Pharma	NNI site 2	thumb 2	II
ANA598	Anadys	NNI site 3	palm 1	II
ABT-072	Abbott	NNI site 3	palm 1	II
ABT-333	Abbott	NNI site 3	palm 1	II
HCV-796	ViroPharma Wyeth	NNI site 4	palm 2	Stopped
GS-9190	Gilead	NNI site 4	palm 2	II
IDX375	Idenix	NNI site 4	palm 2	II
NS5A Inhibitor				
Daclatasvir (BMS-790052)	Bristol-Myers Squibb	NS5A domain 1	Inhibitor	II
BMS-824393	Bristol-Myers Squibb	NS5A protein		I
PPI-461	Presidio Pharmaceuticals	NS5A protein		I
GS-5885	Gilead	NS5A protein		I
Indirect Inhibitors unknown mechanism of action				
Alisporivir (Debio-025)	Debiopharm	Cyclophilin	Inhibitor	III
NIM811	Novartis	Cyclophilin	Inhibitor	I
SCY-635	Scynexis	Cyclophilin	Inhibitor	II
Miravirsen	Santaris	miRNA122	antisense RNA	II

4.8.2a NS3-4A protease inhibitors and main resistant patterns

HCV NS3-4A protease inhibitors (PI) can be divided into two classes, the macrocyclic inhibitors and linear tetrapeptide α-ketoamide derivatives. In general, NS3-4A protease inhibitors have been shown to strongly inhibit HCV replication during monotherapy, but also may cause the selection of resistant mutants, which is followed by viral breakthrough. The additional administration of pegylated interferon and ribavirin, however, was shown to reduce the frequency of development of resistance. Future strategies aim for combination therapies with different antiviral drugs

to prevent the development of resistance. To date the most advanced compounds are telaprevir and boceprevir, both approved in 2011 for the treatment of chronic hepatitis C virus genotype 1 infection by the FDA, European Medicin Agency (EMA) and in several other countries.

Telaprevir and boceprevir are protease inhibitors belonging to the class of α-ketoamid derivatives. Like other NS3-4A inhibitors, telaprevir and boceprevir are characterized by a remarkable antiviral activity against HCV genotype 1. Phase II and III clinical studies have shown that the addition of pegylated interferon α plus ribavirin leads to significantly higher SVR rates in both treatment-*naïve* and treatment-experienced HCV genotype 1 patients compared to treatment with pegylated interferon α and ribavirin alone [Bacon *et al*., 2011, Jacobson *et al*., 2011, Poordad *et al*., 2011, Sherman *et al*., 2011, Zeuzem *et al*., 2011]. Other NS3 protease inhibitors are currently in various phases of development (danoprevir (R7227/ITMN191), vaniprevir (MK-7009), BI201335, simeprevir (TMC435350), narlaprevir (SCH900518), asunaprevir (BMS-650032), PHX1766, ACH-1625, IDX320, ABT-450, MK-5172, GS-9256, GS-9451) and will significantly increase treatment options for chronic hepatitis C in the near future, possibly with improved tolerability, broader genotypic activity and different resistance profiles.

Because of the high replication rate of HCV and the poor fidelity of its RNAdependent RNA polymerase, numerous variants (quasispecies) are continuously produced during HCV replication. Among them, variants carrying mutations altering the conformation of the binding sites of DAA compounds can develop.

During treatment with specific antivirals, these preexisting drug-resistant variants have a fitness advantage and can be selected to become the dominant viral quasispecies. Many of these resistant mutants exhibit an attenuated replication with the consequence that, after termination of exposure to specific antivirals, the wild type may displace the resistant variants [Sarrazin *et al*., 2007]. Nevertheless, HCV quasispecies resistant to NS3-4A protease inhibitors or non-nucleoside polymerase inhibitors can be detected at low levels in some patients (approx. 1%) who have never been treated with these specific antivirals before [Gaudieri *et al*., 2009]. The clinical relevance of these pre-existing mutants is not completely understood, although there is evidence that they may reduce the chance of achieving an SVR with DAA-based triple therapies if the patient's individual sensitivity to pegylated interferon α + ribavirin is low. The NS3-4A inhibitors resistance profile are summarize in Table 7. Although the resistance profiles differ significantly, R155 is an overlapping position for resistance development and different mutations at this position confer resistance to nearly all protease inhibitors currently in advanced clinical development [Sarrazin *et al*., 2010]. Importantly, many resistance mutations could be detected in vivo only by clonal sequencing. For example, mutations at four positions conferring telaprevir resistance have been characterized so far (V36A/M/L, T54A, R155K/M/S/T and A156S/T), but only A156 could be identified initially *in vitro* in the replicon system [Lin *et al*., 2005]. These mutations, alone or as double mutations, conferred low (V36A/M, T54A, R155K/T, A156S) to high (A156T/V, V36M + R155K, V36M + 156T) levels of resistance to telaprevir [Sarrazin *et al*., 2007]. It is

thought that the resulting amino acid changes of these mutations alter the confirmation of the catalytic pocket of the protease, which impedes binding of the protease inhibitor [Welsch *et al*., 2008].

4.8.2b NS5B Polymerase Inhibitors

NS5B RNA polymerase inhibitors can be divided into two distinct categories: Nucleoside analog inhibitors (NIs) and Non-nucleoside analog Inhibitors (NNI). Among the former, valopicitabine (NM283), mericitabine (R7128), R1626, PSI-7977, PSI-938 or IDX184 mimic the natural substrates of the polymerase and are incorporated into the growing RNA chain, thus causing direct chain termination by tackling the active site of NS5B [Koch *et al*., 2006]. Because the active centre of NS5B is a highly conserved region of the HCV genome, NIs are potentially effective against different genotypes. Single amino acid substitutions in every position of the active centre may result in loss of function or in extremely impaired replicative fitness. Thus, there is a relatively high barrier in the development of resistances to NIs.

In contrast to NIs, the heterogeneous class of non-nucleoside inhibitors (NNIs) achieves NS5B inhibition by binding to different allosteric enzyme sites, which results in conformational protein change before the elongation complex is formed [Beaulieu *et al*., 2007]. For allosteric NS5B inhibition high chemical affinity is required. The NS5B is structurally organized in a characteristic “right hand motif”, containing finger, palm and thumb domains, and offers at least four NNI-binding sites, a benzimidazole-(thumb 1)-, thiophene-(thumb 2)-, benzothiadiazine-(palm 1)- and

benzofuran-(palm 2)-binding site [Lesburg *et al*., 1999] (Figure 17). Because of their distinct binding sites, different polymerase inhibitors can theoretically be used in combination or in sequence to manage the development of resistance that might be already there, before any treatment, as a consequence of the poor fidelity leading to a high rate of errors in the HCV RNA progenies. Indeed, numerous different HCV quasispecies are generated during HCV replication in a given patient. It is reasoned that due to the lack of proofreading of the NS5B polymerase together with the high replication of HCV, every possible mutation is generated each day.

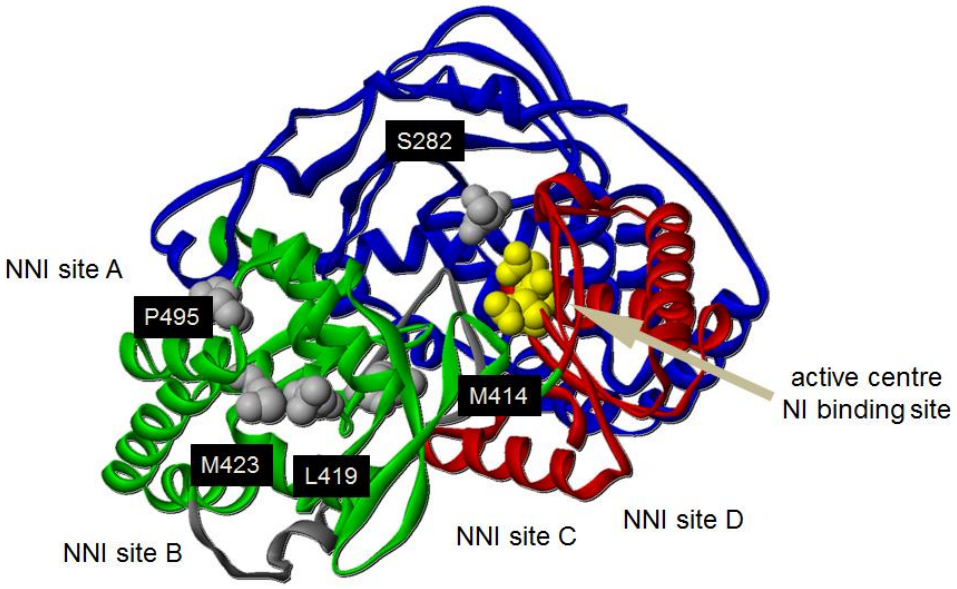


Figure 17. Structure of the HCV NS5B RNA polymerase and binding sites.

4.8.2c NS5A Inhibitors and resistant mutants associated

Daclatasvir BMS-790052 was the first NS5A inhibitor to be clinically evaluated. Even low doses of this drug display high antiviral efficacy against all HCV genotypes *in vitro*. Monotherapy with BMS-

790052 led to a sharp initial decline of HCV RNA concentrations, though its genetic barrier to resistance is relatively low [Gao *et al*., 2010].

According to an interim analysis of a Phase IIb clinical trial in treatment-*naïve* HCV genotype 1 and 4 patients, treatment with 20 or 60 mg BMS-790052 once daily in combination with PEG-IFN α and ribavirin for 24 or 28 weeks, 54% of all patients achieved an extended RVR, compared to 13% in the control group [Hezode *et al*., 2011]. SVR rates of this study are awaited. During monotherapy, rapid selection of variants resistant to BMS-790052 occurred [Nettles *et al*., 2011]. The most common resistance mutations in HCV genotype 1a patients were observed at residues M28, Q30, L31, and Y93 of NS5A. In HCV genotype 1b patients, resistance mutations were observed less frequently, predominantly at positions L31 and Y93 (table 7). These resistance mutations increased the EC50 to BMS-790052 moderately to strongly [Fridell *et al*., 2011]. However, no crossresistance between BMS-790052 and other DAA agents has been reported.

Collectively, BMS-790052 is a highly promising agent for both triple therapy as well as all-DAA combination therapy approaches.

Other NS5A inhibitors (e.g., BMS-824393, PPI-461, GS-5885) are in early clinical development.

Table 7. HCV drug resistance pathways associated to DAAs resistance

Pathway *	Resistance Level	Amino Acid Substitutions
NS3 PI Class Specific	High	A156S/T/V; R155K/Q
NS3 PI Macrocyclic	High	D168A/V; R155K+D168V/A
NS3 PI Linear Ketoamide	Low	V36M; T54A; V36M+T54A
	High	A156T+V36M; R155K+V36M; V170A
NS5A Imino-thiazolidinone	Low	M28; Q30H/R; Q54L; A92V; R157W; Y93C+Q30H
	High	L31V/M; L31V/M+P58L; Y93C
NS5B NA	High	S282T/R
NS5B NNA	Moderate	H96Q/R; C315Y/F/S; N411S
	High	S365A/T/L; M414I/L/T/V

* PI = protease inhibitor; NA = nucleos(t)ide analogue inhibitor; NNA = non-nucleos(t)ide analogue inhibitor

4.8.3 Management of HCV chronic infection: Standard of Care and Direct Acting Anti-viral

The goal of therapy is to eradicate HCV infection in order to prevent the complications of HCV-related liver disease, including necroinflammation, fibrosis, cirrhosis, HCC, and death. The endpoint of therapy is sustained viral response (SVR), intermediate endpoints are used during Standard of Care (SOC) treatment to assess the likelihood of an SVR and tailor treatment duration. They include HCV RNA level measurements at 4, 12, and 24 weeks of therapy, which are interpreted in comparison to the baseline HCV RNA level. When HCV is eradicated, necroinflammation ceases and fibrosis progression is halted in non-cirrhotic patients.

As already mentioned the SOC treatment has different efficacy levels among the different HCV genotypes. For this reason guidelines

recommend 48 weeks of SOC administration in patients infected with HCV genotype 1 and 4 or 24 weeks in case of infection with genotypes 2, 3, 5, 6. Several predictors, other than HCV genotypes, for SVR after therapy have been identified in the past. Some of them are viral factors like baseline HCV RNA levels, some other are related to the host characteristic like body mass index, age, insulin resistance, gender, or related to the liver disease, including levels of ALT, GGT, the stage of fibrosis or co-infection with another hepatotropic virus or with HIV. However, one of the strongest predictors in case of HCV genotype 1 infection, have been only recently identified as a genetic polymorphisms located in chromosome 19, close to the region coding for IL28B (or IFN k3). In a study involving HCV genotype 1 infected patients, Ge *et al*., reported a twofold greater rate of SVR for the allele rs12979860 CC genotype in comparison with the TT genotype. Such a strong association was not found in HCV genotypes other than 1.

Boceprevir (BOC) and Telaprevir (TVR) are the Direct Acting Anti-viral to date available for HCV chronic infection. Results from previous studies have shown rapid selection and emergence of resistance mutations during boceprevir or Telaprevir monotherapy [Susser *et al*., 2009; Hiraga N *et al*., 2011]. For this reason triple therapy combination including DAA plus SOC was tested in clinical trials showing an increment of 24% and 33% in the response rates of HCV genotype 1 infected patients to BOC + SOC (4wks lead in of SOC + 48wks of combination) and TVR (12wks) + SOC (48wks) treatments respectively, comparing to the use of SOC alone [Manns *et al*., 2001; Nelson DR 2010].

Therefore, telaprevir- and boceprevir-based triple therapy can be considered the novel standard of care for HCV genotype 1 patients.

4.8.4 Treatment failure

The loss of therapy efficacy or the lack of a response *ab initio* are events known as treatment failure. During HCV chronic infection treatment various patterns of HCV treatment failure have been reported, including viral nonresponse, viral breakthrough on treatment, and viral relapse after treatment completion.

Non-responders are patients who failed to achieve a decline of 2 log HCV RNA IU/ml after 12 weeks of treatment or who never achieved undetectable HCV RNA during treatment of a minimum duration of 24 weeks.

Relapsers are defined as patients who achieved an end-of-treatment response (undetectable HCV RNA at the end of treatment) but subsequently relapsed and did not achieve an SVR. The relapse rate after treatment with pegylated IFN-a and ribavirin is on the order of 15–25%, but varies according to when HCV RNA becomes undetectable during therapy.

To date patients SOC failure experienced can have the possibility to reach an SVR with the new DAAs in a triple therapy. Despite the encouraging results of telaprevir- and boceprevir-based triple therapy, several cases of drug resistance were observed during treatment, and they were confirmed by *in vitro* phenotype testing. In particular, the prevalence of resistance among HCV genotype 1 infected patients after combination therapy SOC + BOC (44wks) was 14% and 15% for treatment-naïve and

SOC pretreated patients respectively [Poordad *et al*., 2011; Bacon *et al*., 2011].

Resistant mutation a part, the total failure rates in treatment-*naïve* patients are 20%–30% on triple therapy. In previously treated patients, the failure rates range as high as 50% – 60%. Development of persistence of viral resistance depends in part on several host and treatment-related variables. Failure rates are higher in patients with less favorable genetic background (IL- 28 phenotypes CT or TT), including the African-American population, prior treatment nonresponders, HIV- or HBVcoinfected patients, post-liver-transplant patients, noncompliant patients, and patients with advanced fibrosis/cirrhosis.

Telaprevir is taken, with PEG-IFN-α and ribavirin, at a dose of 750 mg every eight hours for the first 12 weeks of the treatment. If HCV RNA is equal to or greater than 1000 IU/mL at weeks 4 or 12, virologic failure has occurred and triple therapy should be discontinued immediately. If the virus is detected to any degree at week 24, dual therapy should also be discontinued at that point, as the likelihood of achieving SVR is very low.
When using boceprevir, the treatment algorithm is different and involves a 4-week period of PEG-INF-α and ribavirin dual therapy, also known as the "lead in" period, followed by triple therapy with addition of 800 mg of boceprevir every eight hours for a variable period of time; PEG-IFN- α and ribavirin will continue until treatment completion. A viral load equal to or greater than 100 IU/mL at week 12 is equivalent to virologic failure, and all three drugs should be stopped to avoid resistance. Alternatively, any

detectable viral load at week 24 again implies again lack of response, and treatment should be terminated.

Failing triple therapy raises concerns regarding possible adverse consequences for the liver disease and for this reason these patients must continue to be clinically monitored. To evaluate is also the role of resistant quasispecies selected and emerged during the treatment for the influence on the response to future DAA-based therapy.

5 Bioinformatics: definitions and application to virology

Bioinformatics is the application of computer science, statistics and information theory to the field of biology and medicine, and has become indispensible for virology research in recent years. Numerous bioinformatics tools are available for general analyses of viral genomes such as multiple nucleotide/amino acid sequence alignment, motif identification, recombination, genome annotation, and phylogenetic relationships. Most of these tools are available as freeware, and many can be found at the National Center for Biotechnology Information (NCBI) at http://www.ncbi.nlm.nih.gov or the European Molecular Biology Laboratory (EMBL) at http://www.embl.org websites [Yan Q, 2008].

The complex genome structure and arrangement of some viruses such as overlapping open reading frames (ORF) and translational frameshifts, is often challenging for general bioinformatics tools; thus software packages specifically designed or optimized for virology research have been developed in recent years. Moreover, dedicated public databases that function as data repository for treatment history, viral and clinical data

have been established for specific viruses such as influenza virus (Influenza Virus Resource at http://www.ncbi.nih.gov/genomes/FLU/FLU.html), human immunodeficiency virus (The HIV databases at http://www.hiv.lanl.gov/content/index) and coronavirus (SARS Bioinformatics Suite http://athena.bioc.uvic.ca/database.php?db=Coronaviridae).

5.1 Bioinformatics methods and public databases for HCV

The natural histories for chronic HCV infections have been well characterised, yet the mechanism of pathogenicity of this virus remain largely unknown; these include the receptors responsible for viral entry into hepatocytes, the viral and host genetic factors required to establish chronic infections, and the viral and host genetic factors required for progression to HCC. Recent advances made in sequence genomics of a large number of organisms will enable further studies and aid in understanding the structure-function correlation between the virus and its respective host providing a unique insight into the process of deciphering the pathogenic mechanisms of HCV.

Within the last ten years, several bioinformatics tools dedicated to HCV research have become available. They are mainly nucleotide/amino acid sequence analysis tools that can be accessed in public databases established for hepatitis viruses (table 8).

Table 8: HCV Databases described in this thesis

Database	Internet Address	Reference
Hepatitis C		
Los Alamos HCV Sequence Database	http://hcv.lanl.gov/content/index	[Kuiken C *et al*., 2005]
European HCV Database (euHCVdb)	http://euhcvdb.ibcp.fr/edHCVdb/	[Combet C *et al*., 2007]
Japan Hepatitis Virus Database	http://s2as02.genes.nig.ac.jp	[Shin IT *et al*., 2008]
SeqHepC	(under development)	

Databases dedicated for HCV were initially developed up for research and epidemiological purposes. Excluding the alternate reading frame protein (ARFP/F), whose function remains unknown, the genome structure of HCV is simpler than HBV. The HCV is a positive-sense, single-stranded RNA virus with a genome that is approximately 9.6kb in size and encodes for a unique polyprotein with no overlapping regions. Whilst this structure may make it easier to identify clinically important mutations and to study their effects compared to the HBV genome, there is less constraint for mutations to occur and the rate of mutation is substantially higher (0.9x10-3 base substitutions/site/year). This is the primary cause of the high genetic heterogeneity observed in HCV, accounting for at least 6 HCV genotypes and 18 subtypes with genomes that differ by 24-30% and 15-23% respectively [Simmonds P *et al*., 2005].

The main public databases established for HCV research and epidemiological studies include the Los Alamos HCV Sequence Database (http://hcv.lanl.gov/content/index) [Kuiken C, 2010], the European HCV Database (http://euhcvdb.ibcp.fr/euHCVdb/ - euHCVdb) [Combet C *et al*.,

2007] and the Japan Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp) [Shin IT *et al*., 2007]. Both Los Alamos HCV Sequence Database and euHCVdb contain >1,000 HCV genome sequences, and have dedicated tools that enable sequence retrieval using selection criteria such as HCV full genome/specific region, genotype/subtype, and treatment history. In recent years, new HCV treatment databases and dedicated tools have been developed in provision for studying and analyzing the effects of new anti-HCV drugs, the directly acting antiviral (DAAs).

The high genetic heterogeneity of HCV will affect the future use of DAAs, and drug resistance associated mutations are expected to emerge and progress to complex profiles as more DAAs are approved for the management of chronic HCV infections. *In vitro* drug susceptibility phenotype studies have already showed different patterns of amino acid substitutions can confer different levels of drug resistance. A summary of amino acid substitutions that are currently known to confer resistance to the main classes of DAA is shown in Table 7.

5.2 Aim of the study

In 2010, a joint collaboration was established between the Victorian Infectious Diseases Reference Laboratory (VIDRL) of Melbourne Health, Australia and the Hepatitis C Virus Research Laboratory of the Institute of Medical and Veterinary Science (IMVS), Australia to develop a web assessable sequence analysis tool (SeqHepC) for the management of patients with resistance to the DAAs for chronic hepatitis C.

My personal aims inside this main project were to:

- List all major HCV genotypes (references if possible)
- List as many HCV subtypes as possible (references if possible)
- Calculate proportion of sequence divergence between all HCV subtypes (pairwise analyses)
- Focus on HCV NS3 protease and NS5B polymerase to start off with how useful would these genes be for subtyping and If useful, what is the minimum region for subtyping
- Collect representative full length HCV sequences as references for SeqHepC
- Determine all before any DAA drugs became available.

5.3 Materials and Methods

A total of 1,387 full-length HCV sequences downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/) were analysed for genotyping, subtyping and divergence analysis. Los Alamos HCV database tools were used to select and align sequences. Alignments in particular, have been carried out with a new implementing method that use probabilistic models called profile hidden Markov models (profile HMMs, http://hmmer.janelia.org/). Compared to the Basic Local Alignment Search Tool (BLAST), HMMs is more accurate and more able to detect remote homologs because of the strength of its underlying mathematical models.

Sequence editing and manual correction were performed with Bioedit (www.mbio.ncsu.edu/bioedit/bioedit.html) while MEGA 4.0 program (www.megasoftware.net/mega4/mega.html) was used for

phylogenetic analysis. Particularly bootstrap consensus trees were obtained with the Neighbor-Joining method (1000 replicates).

5.4 Results

Development of SeqHepC began with a comprehensive evaluation of all available full-length HCV genome sequences from the GenBank database. A total of 1,387 full-length HCV sequences were downloaded from GenBank. Phylogenetic analysis showed that 248 sequences were duplicate submissions (identical sequences with different submission number), and thus were removed from subsequent analysis (figure 18).

The HCV genotype and subtype of the remaining 1,139 unique sequences were as follow: 933 HCV genotype 1 (1a = 550, 1b = 380, and 1c = 3) and 206 non-1 HCV genotypes. Based on the HCV genotype nomenclature system proposed by Simmonds *et al* [Simmonds *et al*., 2005], the genotype, subtype and drug-*naïve* status could be confirmed for 228 of the HCV full length genome sequences analysed using the data available in the Los Alamos HCV Sequence Database and the euHCVdb database (Figure 18-19). Of these, 185 sequences were HCV genotype 1 (126 = 1a; 59 = 1b) and 43 were non-1 HCV genotypes.

Thirty-two sequences representing all the genotypes and most of the subtypes were then selected from different intragenotype clades as the reference set to use for genotyping and drug resistance analysis in SeqHepC (Figure 18, 20). The percentages of divergence between these 32 HCV sequences are shown as a matrix in Figure 21.

As the amino acid substitutions, known to be associated with PI resistance, are located within the first one third of the NS3 gene, a region where the catalytic site of the enzyme is encoded, we identified and tested a minimal region of first 543 nucleotides including PI resistant mutation. Phylogenetic analysis and pairwise comparisons performed on this minimal NS3 region showed it is sufficient to determine HCV genotypes/subtypes (Figures 22 - 23) [data submitted to Antiviral Therapy].

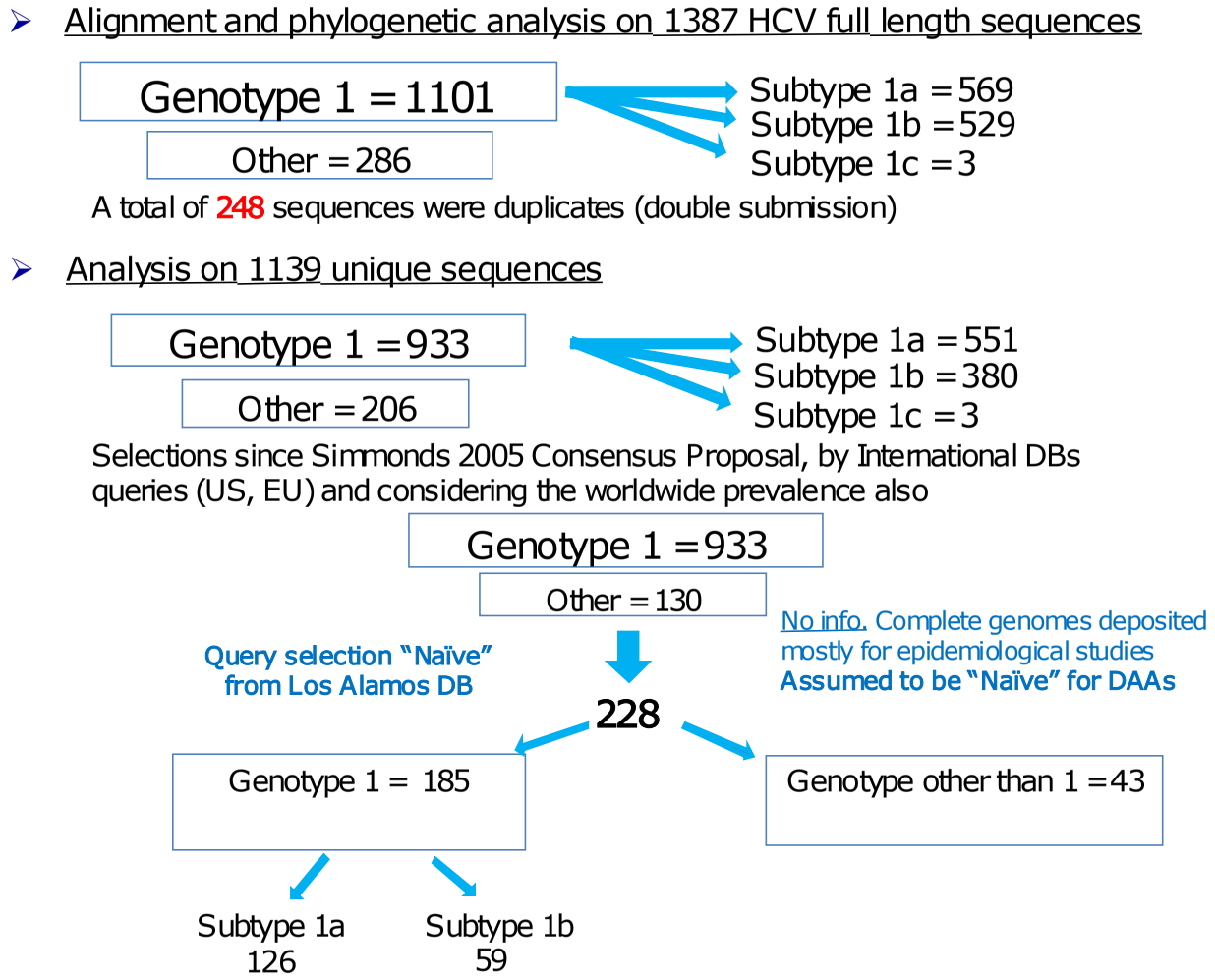


Figure 18. Flowchart on the analysis for the collection of representative full length HCV sequences as references for SeqHepC project.

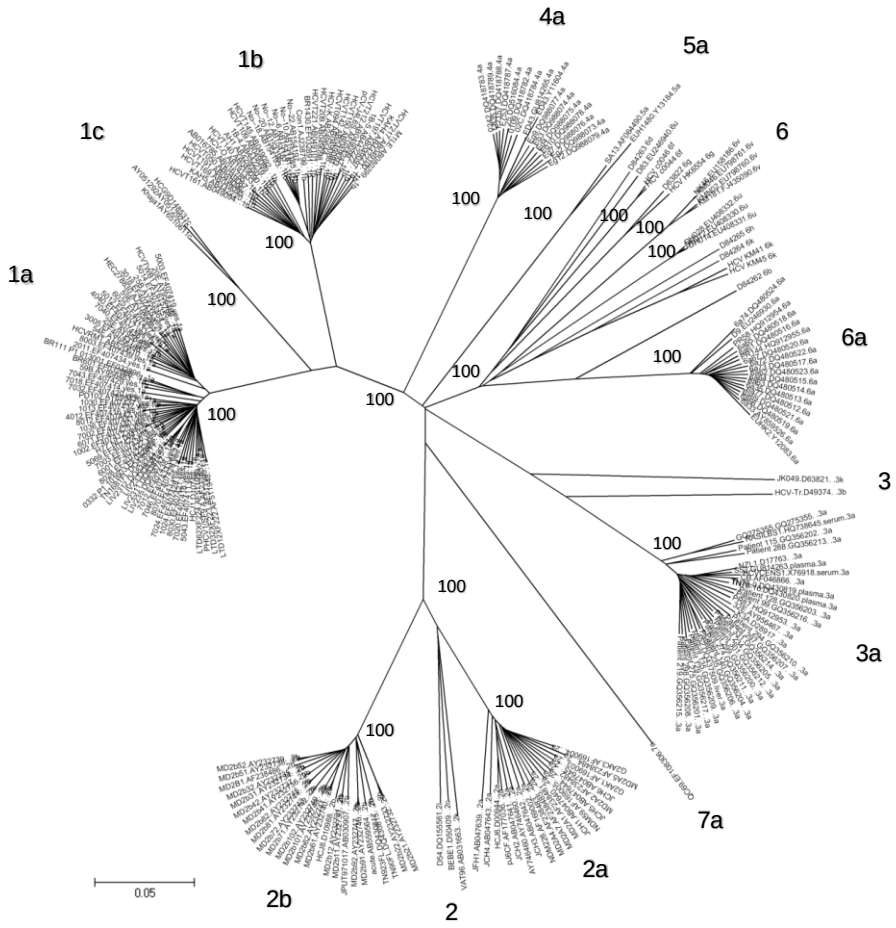


Figure 19. An unrooted neighbor-joining tree of 228 full-length HCV genome sequences determined from drug-naïve individuals, using the Maximum Composite Likelihood substitution model. The tree is a consensus of 1,000 datasets generated using the bootstrap re-sampling method. Similar results were obtained from unrooted trees of the HCV regions coding for the DAAs targets (NS3, NS5A, NS5B).

A B

Input Sequence Name	Length
H77.NC_004102._.1a	9646
7043.EF407429.yes.1a	9086
4040.EF407417.yes.1a	9383
Con1.AJ238799.1b	9605
HCVK1S1.D50483.yes.1b	9410
TN28.EU781828.yes.1b	9359
AY051292.1c	9441
pJ6CF.AF177036._.2a	9711
JFH1.AB047639._.2a	9678
MD2b71.AY232742._.2b	9406
AB559564._.2b	9511
BEBE1.D50409._.2c	9513
D54.DQ155561.2i	9370
VAT96.AB031663._.2k	9488
K3A.D28917._.3a	9454
GQ275355._.3a	9442
HCVTr.D49374._.3b	9444
JK049.D63821._.3k	9450
25.DQ516084.4a	9309
Eg4.DQ988075.4a	9057
EUH1480.Y13184.5a	9343
6a72.DQ480522.6a	9358
D9.EU246930.6a	9376
D84262_6b	9628
D84263_6d	9615
HCV_c0046_6f	9454
HCV_HK6554_6g	9462
D84265_6h	9621
HCV_KM41_6k	9430
DH012.EU408330.6u	9451
KM046.EU798761.6v	9429
QC69.EF108306.7a	9357

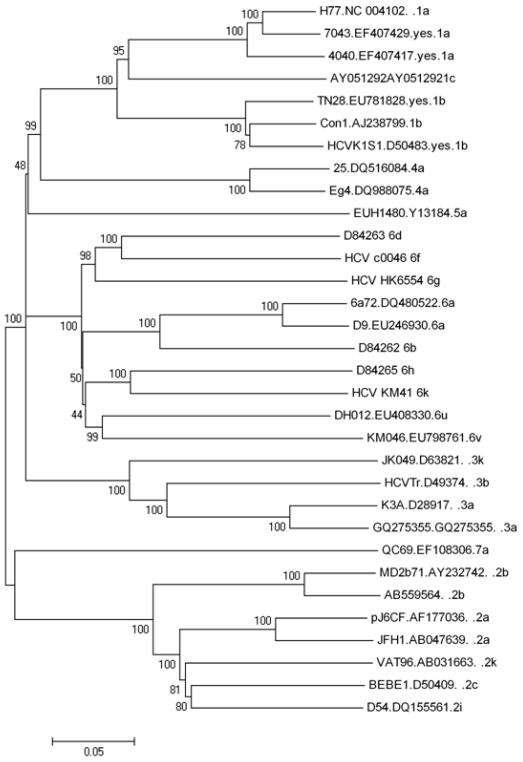


Figure 20. The 32 HCV full-length sequences from GenBank used as reference sequences in SeqHepC. (A) List of the 32 HBV sequences showing the corresponding GenBank accession number, HCV genotype and subtype in parenthesis, and length of sequence. (B) A consensus neighbor-joining tree of 1,000 datasets (by the bootstrap re-sampling method) generated using Maximum Composite Likelihood substitution model. The tree shows the clustering profiles of the 32 reference HCV full-length sequences.

Sub	1a	1b	1c	2a	2b	2c	2i	2k	3a	3b	3k	4a	5a	6a	6b	6d	6f	6g	6h	6k	6u	6v	7a
1a		20.80	20.12	31.47	31.99	30.60	31.11	31.66	30.80	30.97	31.05	27.70	29.28	29.25	29.86	29.42	29.28	29.05	29.65	29.85	28.60	30.50	31.47
1b	1799		21.12	31.42	32.18	31.36	31.08	31.46	30.17	30.46	30.67	27.36	29.45	29.16	29.91	29.23	28.86	29.19	29.56	29.78	28.57	30.42	31.83
1c	1740	1827		31.77	31.85	31.75	31.39	32.23	30.43	30.89	30.69	27.86	29.35	29.84	29.88	29.65	29.73	29.55	29.92	29.76	29.18	30.28	31.92
2a	2722	2718	2748		22.31	19.51	19.05	19.93	33.87	34.15	33.46	31.78	32.91	32.83	33.09	32.00	32.57	32.62	32.92	32.86	32.12	33.42	33.02
2b	2767	2784	2755	1930		21.98	22.09	22.76	33.86	34.24	33.62	32.13	32.92	32.95	32.84	32.69	33.25	33.03	33.25	32.95	31.94	33.05	32.86
2c	2647	2713	2746	1688	1901		18.07	19.01	33.62	33.98	33.60	32.37	32.71	32.72	32.84	31.65	32.51	32.29	32.79	32.00	31.73	33.06	33.47
2i	2691	2688	2715	1648	1911	1563		18.99	33.62	33.84	33.31	32.09	32.35	32.64	32.94	32.45	32.36	32.61	32.36	32.13	31.29	32.68	33.42
2k	2739	2721	2788	1724	1969	1644	1643		33.56	33.83	33.64	32.00	32.92	32.91	33.98	32.54	32.46	32.90	32.71	32.09	32.14	33.10	33.25
3a	2664	2610	2632	2930	2929	2908	2908	2903		21.38	24.57	30.98	31.49	31.16	31.40	30.92	30.38	31.25	31.24	31.17	30.55	31.46	33.05
3b	2679	2635	2672	2954	2962	2939	2927	2926	1849		24.60	30.64	31.14	31.62	31.32	30.92	31.90	31.51	31.82	31.18	31.12	31.86	33.11
3k	2686	2653	2655	2894	2908	2906	2881	2910	2125	2128		30.37	31.16	31.31	31.84	30.90	31.19	31.33	32.16	31.27	31.57	31.63	33.46
4a	2396	2367	2410	2749	2779	2800	2776	2768	2680	2650	2627		29.60	29.61	29.91	29.38	29.29	29.97	29.78	29.42	28.95	29.91	32.59
5a	2533	2547	2539	2847	2848	2829	2798	2848	2724	2694	2695	2560		30.44	30.68	29.87	29.98	30.30	30.54	30.37	30.24	31.28	33.56
6a	2530	2522	2581	2840	2850	2830	2823	2847	2695	2735	2708	2561	2633		19.71	26.15	26.08	26.37	26.43	26.34	25.68	26.43	32.73
6b	2583	2587	2585	2862	2841	2841	2849	2853	2716	2709	2754	2587	2654	1705		26.32	26.29	26.13	26.23	26.46	25.77	26.61	33.20
6d	2545	2528	2565	2768	2828	2738	2807	2815	2675	2675	2673	2541	2584	2262	2277		22.25	24.86	25.79	25.80	24.51	26.67	33.16
6f	2533	2496	2572	2817	2876	2812	2799	2808	2628	2759	2698	2534	2593	2256	2274	1925		24.66	25.83	25.09	25.01	26.58	33.32
6g	2513	2525	2556	2822	2857	2793	2821	2846	2703	2726	2710	2592	2621	2281	2260	2150	2133		25.90	26.49	26.09	27.51	33.02
6h	2565	2557	2588	2848	2876	2836	2799	2829	2702	2752	2782	2576	2642	2286	2269	2231	2234	2240		22.32	25.77	26.28	33.87
6k	2582	2576	2574	2842	2850	2768	2779	2776	2696	2697	2705	2545	2627	2278	2289	2232	2170	2291	1931		25.47	26.23	33.31
6u	2474	2471	2524	2778	2763	2745	2707	2780	2643	2692	2731	2504	2616	2221	2229	2120	2163	2257	2229	2203		24.42	33.28
6v	2638	2631	2619	2891	2859	2860	2827	2863	2721	2756	2736	2587	2706	2286	2302	2307	2299	2380	2273	2269	2112		33.45
7a	2722	2753	2761	2856	2842	2895	2891	2876	2859	2864	2894	2819	2903	2831	2872	2868	2882	2856	2930	2881	2879	2893	

Num. of nt. differences on a total of 8650 sites

Divergence (%)

Figure 21. Analysis on the set of 32 reference sequences. Between Subtype analysis on full length genomes: strains belonging to a same subtype have been grouped using the Taxa and Group definition option in Mega 4.0; the phylogenetic distances are expressed as means of nucleotide differences between groups (below the diagonal, computed in Mega 4.0) or as percentage divergence calculated on 8650 valid sites for the analysis (above the diagonal). The highlighted areas show the low levels of divergence between strains of the same genotype (blue for genotype 1; red for genotype 2; green for genotype 3; light blue for genotype 6).

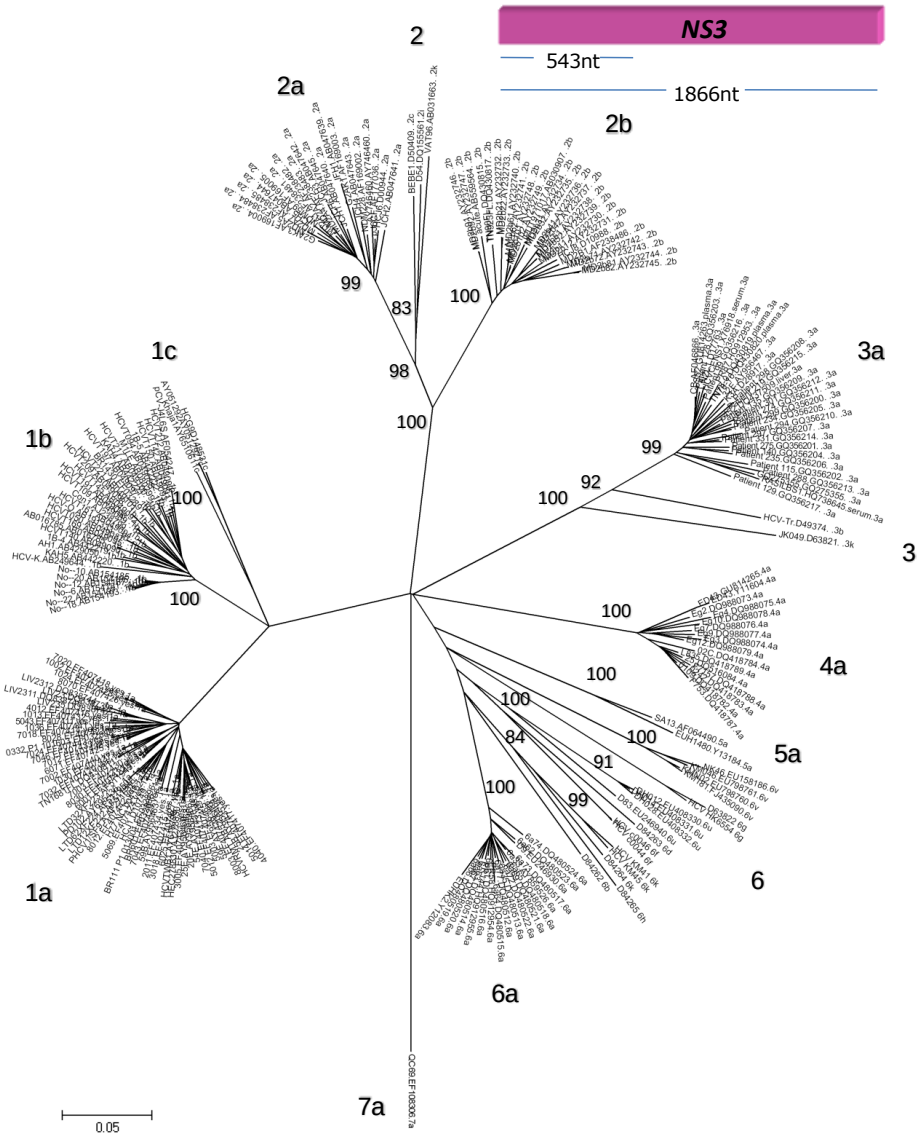


Figure 22. An unrooted neighbor-joining tree of 228 HCV sequences. The genomic region analysed was the NS3 minimal region (543nt) that correspond to the protease domain where the PI resistance mutations would be found. The tree is a consensus of 1,000 datasets generated using the bootstrap re-sampling method and analysed with Maximum Composite Likelihood substitution model.

Complete NS3 region

Genotype	1	2	3	4	5	6	7
1		29.26	29.90	26.21	27.22	27.71	30.98
2	546		31.51	29.42	29.42	30.33	30.98
3	558	588		30.17	29.47	30.55	31.14
4	489	549	563		27.38	28.19	31.30
5	508	549	550	511		28.35	31.89
6	517	566	570	526	529		31.67
7	578	578	581	584	595	591	

Divergence (%)

Num of nt. differences on a total of 1866 sites

NS3 minimal region (543nt)

Genotype	1	2	3	4	5	6	7
1		34.27	33.90	30.71	30.52	29.78	36.70
2	183		36.52	34.08	34.83	34.46	36.52
3	181	195		34.46	33.71	32.58	35.21
4	164	182	184		33.33	32.02	37.08
5	163	186	180	178		29.03	37.83
6	159	184	174	171	155		36.89
7	196	195	188	198	202	197	

Divergence (%)

Num of nt. differences on a total of 543 sites

Figure 23. Analysis on the set of 32 reference sequences for SeqHepC system. Between Genotype analysis in the NS3 complete region (upper matrix) and in the NS3 minimal region (lower matrix). Strains belonging to a same genotype have been grouped using the Taxa and Group definition option in Mega 4.0; the phylogenetic distances are expressed as means of nucleotide differences between groups (below the diagonal, computed in Mega 4.0) or as percentage divergence calculated on 1866 and 543 valid sites for the analysis in NS3 complete region and NS3 minimal region respectively [Submitted to Antiviral Therapy].

5.5 Discussion

Bioinformatics for hepatitis viruses started some years ago with mainly general tools and few dedicated allowing purely research analysis. Until 2 years ago three main web acess databases were available for HCV allowing phylogenetic analysis including sequence alignment, genotyping, subtyping.

That was considered enough since the only viral predicting factor for HCV treatment outcome was the genotype.

The arising DAAs era in the HCV treatment is now the driving force for the development of new databases and bioinformatic tools HCV dedicated including not only analisys for genotype/subtype prediction but also resistance variants identification.

Despite the new interest in creating HCV web access databases, few of them are actually including the necessary informations and tools for a correct identification of resistance. None of the available Databases is intended to be a real updated diagnosis instrument helping the clinician in taking therapy decision.

In 2010, a joint collaboration was established between the Victorian Infectious Diseases Reference Laboratory (VIDRL) of Melbourne Health, Australia and the Hepatitis C Virus Research Laboratory of the Institute of Medical and Veterinary Science (IMVS), Australia to develop a web assessable sequence analysis tool (SeqHepC) for the management of patients with resistance to the DAAs for chronic hepatitis C.

The project needed at first to collect a limited and manageable number of full length reference sequences from all the major HCV genotypes and subtypes.

We used the HCV Los Alamos Database and the tools included for query selection of a large number of full length HCV sequences. A total of 228 sequences were selected by unique submition, confirmed genotypes/subtypes by Simmonds consensus proposal and by naïve status.

An unrooted tree was generated to confirm genotype/subtype of the 228 sequences and among them, 32 strains were chosen from the main branches of the tree.

The big skimming prompet us to proceed to the next phase of our project: to focus on HCV NS3 protease and NS5B polymerase to start off with how useful would these genes be for subtyping and If useful, what is the minimum region for subtyping.

The NS3 and NS5B regions were both found to be useful for genotyping and subtyping. We succeeded in identifying a minimal region of 543 nucleotides located in the NS3 sequence including all the identified protease inhibitor resistant mutations. Such a minimal region was also useful for genotyping and even subtyping as showed by the phylogenetic tree and divergence data (table 23).

A working version of SeqHepC is currently undergoing alpha testing. The program is designed to enable genotyping and mutational analysis of HCV sequences, which can be submitted either as a nucleotide sequence or deduced amino acid sequence. The input sequence would be compared to the 32 full length HCV reference sequences to determine the genotype, followed by comparison with the reference sequence of an equivalent genotype for the assessment of drug-resistance associated amino acid substitutions. Reports generated are date and time stamped, and will display data including the HCV genome region covered by the submitted sequence, a list of clinically significant amino acid substitutions, and predicted PI susceptibility. All drug resistance associated amino acid substitutions reported are currently based on the peer-reviewed literatures

for the HCV NS3 protein. The data generated can be also used to further develop HCV drug resistance interpretation algorithms, especially when it can be integrated in a local database with the clinical data (therapeutic histories, HCV viral load levels, and liver function test results) of patients from whom the viral sequences were determined. A future goal is to enable the database to be mined using artificial intelligence algorithms to aid in the rapid identification of novel HCV drug resistance mutation(s) (Figure 24).

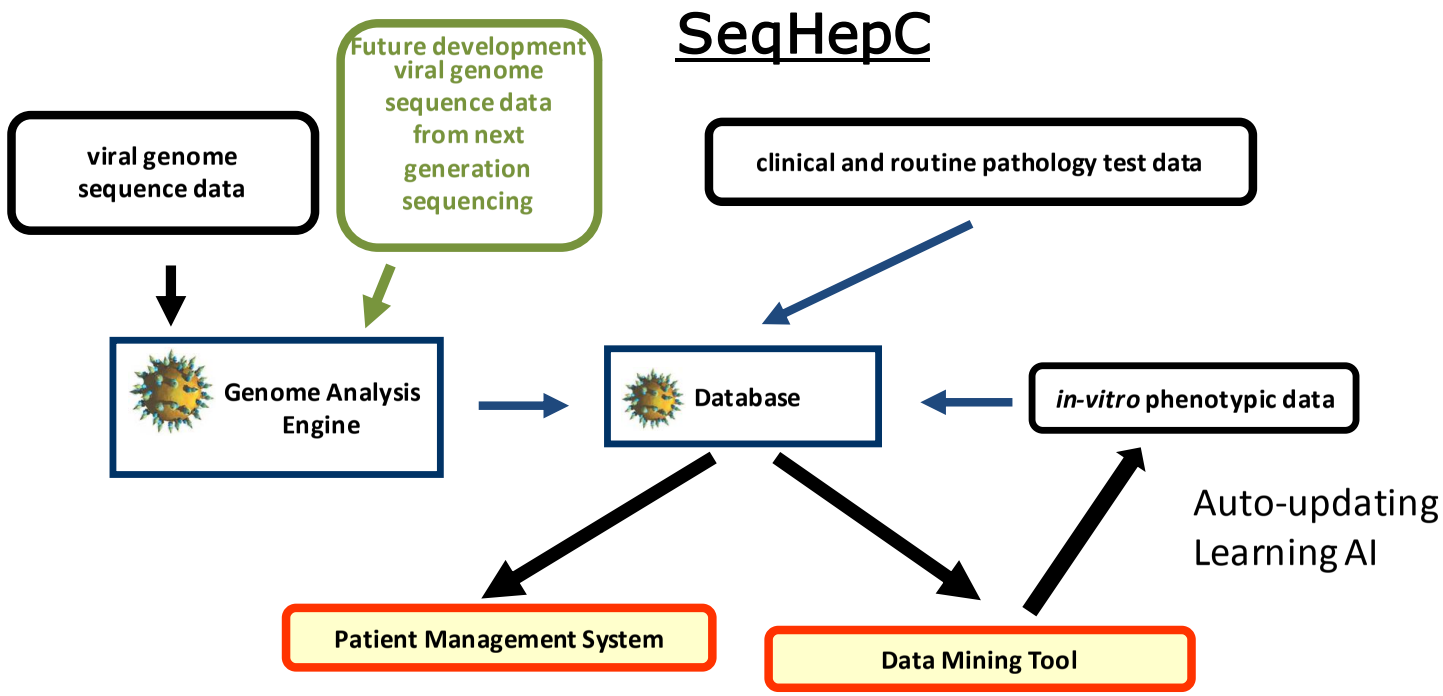


Figure 24. SeqHepC project and workflow. Merging the information from the clinical, virological and *in vitro* phenotypic data will generate an auto-updating learning artificial intelligence tool for the managing of DAAs therapies.

6. Conclusion

Chronic hepatitis viruses are a huge burden affecting health of hundreds million people worldwide and new infection number is costantly incrementing because of the high infectivity level of these viruses and their ability to persist and replicate in the host for many years or lifelong.

The Hepatitis B and C viruses (HBV, HCV) are causing the great majority of the viral chronic hepatitis and if not treated they lead to progressive liver diseases including fibrosis, chirrosis and hepatocellular carcinoma.

From the time of their first identification several drugs against HBV and HCV have been developed in the attempt to cure or slow down the liver disease and possibly to eradicate the infection.

The high viral heterogeneity is one of the main factors affecting the success of the therapy as treatment failure is mostly associated to viral resistant variants.

In this thesis we aimed to give a contribution to the study of the hepatitis viruses heterogeneity considering its pivotal role in case of anti-viral therapy failure.

We succeeded developing a sensitive and specific assay for the study of early dynamics HBV resistant variants and demonstrating their presence, as minor population, in *naïve* patient that later showed a no response therapy associated to resistant variants. Further analysis and a larger cohort of patient are needed to better understand the role of the HBV resistant variants but given the results obtained in this thesis, our assay will be useful in the future as both a research instrument for deep and extensive analysis of

resistant quasispecies and a predictive test for sustained responders in HBV treatment.

Concerning HCV, our contribution was in an accurate selection and analysis of a large number of HCV sequences to reach a limited but well represented number of references needed by the SeqHepC program. The SeqHepC will be a web access database working as a sequence repository but also including tools for extensive and rapid bioinformatic analysis comparing an imput sequence to the references collected during our study. Clinical informations from patients and *in vitro* phenotypic data for resistance confirmation will be also included and managed by the system as an auto-updating learning artificial intelligence. Finally, SeqHepC will be a very useful tool for research and helping clinician in the complex management of the new therapies available in the next years.

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Refernces

Ando K, Guidotti LG, Wirth S, Ishikawa T, Missale G, Moriyama T *et al*.
Class I-restricted cytotoxic T lymphocytes are directly cytopathic for their target cells in vivo
J Immunol (1994); 152(7):3245-3253.

Andrisani OM and Barnabas S
The transcriptional function of the hepatitis B virus X protein and its role in hepatocarcinogenesis
Int J Oncol (1999); 15:373–9.

Angus P, Vaughan R, Xiong S, Yang H, Delaney W, Gibbs C, Brosgart C, Colledge D, Edwards R, Ayres A, Bartholomeusz A and Locarnini S
Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase
Gastroenterology. (2003); 125(2) : 292 - 297.

Arauz-Ruiz P, Norder H, Robertson BH and Magnius LO
Genotype H: A new Amerindian genotype of hepatitis B virus revealed in Central America
J Gen Virol (2002); 83: 2059–2073.

Bartenschlager R and Schaller H
Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome
EMBO J (1992); 11(9):3413-3420.

Bartholomeusz A., Groenen LC and Locarnini SA
Clinical experience with famciclovir against hepatitis B virus
Intervirology (1997) ; 40 : 337 – 342

Beasley RP
Hepatitis B virus. The major etiology of hepatocellular carcinoma
Cancer (1988), 61:1942-1956.

Belloni L, Pollicino T, De Nicola F, Guerrieri F, Raffa G, Fanciulli M, *et al*.
Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function
Proc Natl Acad Sci U S A (2009); 106:19975-19979.

Bertoletti A, Costanzo A, Chisari FV, Levrero M, Artini M, Sette A, Penna A, Giuberti T, Fiaccadori F, Ferrari C..
Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope
J Exp Med. (1994) Sep 1;180(3):933-43

Bock, C.T., Tillmann, H.L., Maschek, H.J., Manns, M.P. and Trautwein, C.
A preS mutation isolated from a patient with chronic hepatitis B infection leads to virus retention and misassembly
Gastroenterology (1997); 113 (6), 1976–1982.

Bock CT, Tillmann HL, Manns MP and Trautwein C
The pre-S region determines the intracellular localization and appearance of hepatitis B virus
Hepatology (1999); 30:517–525.

Bonino F, Hoyer B, Nelson J, Engle R, Verme G and Gerin J
Hepatitis B virus DNA in the sera of HBsAg carriers – a marker of active hepatitis B virus replication in the liver
Hepatology (1981); 1:386–391.

Bonino F, Brunetto MR, Purcell RH and Zuckerman AJ.(b)
Genetic heterogeneity of hepatitis viruses: Clinical implications
J Hepatol 1991; 13 (suppl): S 1-S 174.

Bonino F, Rosina F, Rizzetto M, Rizzi S, Chiaberge E, Tardanico R, *et al*.
Chronic hepatitis in HBsAg carriers with serum HBV-DNA and anti-HBe
Gastroenterology (1986); 90:1268–1273.

Bonino F. (b)
The importance of hepatitis B viral DNA in serum and liver
Hepatol (1986); 3: 136-41.

Bonino F, Brunetto MR, Rizzetto M and Will H.
Hepatitis B virus unable to secrete e antigen
Gastroenterology. (1991) Apr;100(4):1138-41

Borgniet O, Parvaz P, Bouix C, Chevallier P, Trépo C, André P and Zoulim F.
Clearance of serum HBsAg and anti-HBs seroconversion following antiviral therapy for chronic hepatitis B
J Med Virol (2009); 81:1336-1342.

Bortolotti F, Cadrobbi P, Crivellaro C, Guido M, Rugge M, Noventa F, Calzia R and Realdi G.
Long term outcome of chronic type B hepatitis in patients who acquire hepatitis B virus infection in childhood
Gastroenterology (1990); 99:805 – 810

Bortolotti F, Crivellaro C, Brunetto MR, Cadrobbi P, Bertolini A, Alberti A.J
Selection of a precore mutant of hepatitis B virus and reactivation of chronic hepatitis B acquired in childhood
Pediatr. (1993) Oct;123(4):583-5.

Bouchard MJ and Schneider RJ
The enigmatic X gene of hepatitis B virus
J Virol (2004); 78:12725–34.

Brunetto MR, Stemler M, Schdel F *et al*.
Identification of HBV variants which cannot produce precore derived HBeAg and may be responsible for severe hepatitis
Ital. J. Gastroenterol. (1989); 21: 151-154

Brunetto MR, Oliveri F, Rocca G, Criscuolo D, Chiaberge E, Capalbo M, *et al*. Natural course and response to interferon of chronic hepatitis B accompanied by antibody to hepatitis B e antigen
Hepatology (1989);10:198–202.

Brunetto MR, Stemler M, Bonino F, Schodel F, Oliveri F, Rizzetto M, Verme G and Will H J
A new hepatitis B virus strain in patients with severe anti-HBe positive chronic hepatitis B
Hepatol. (1990) Mar;10(2):258-61.
.
Brunetto MR, Giarin MM, Oliveri F, Chiaberge E, Baldi M, Alfarano A, Serra A, Saracco G, Verme G, Will H and Bonino F
Wild-type and e antigen-minus hepatitis B viruses and course of chronic hepatitis
Proc Natl Acad Sci U S A. (1991); 88(10):4186-90.

Brunetto MR, Giarin M, Oliveri F, *et al*. (b)
e antigen defective hepatitis B virus and course of chronic infection
J Hepatol (1991); 13: S82-6.

Brunetto MR, Giarin M, Saracco G, Oliveri F, Calvo PL, Capra G, *et al*.
Hepatitis B virus unable to secrete e antigen and response to interferon in chronic hepatitis B
Gastroenterology (1993); 105:845–850.

Brunetto MR, Capra G, Randone A, Calvo PL and Bonino F
Wild-type and HBeAg minus HBV fluctuations: cause or effect of chronic hepatitis B pathogenetic mechanisms?

Viral hepatitis and liver disease. Tokyo: Springer; (1994) p. 261–264.

Brunetto MR, Randone A, Ranki M, Jalanko A, Piantino P, Giarin M, Capra G, Calvo PL, Oliveri F and Bonino F.
Quantitative analysis of wild-type and HBeAg minus hepatitis B viruses by a sequence-dependent primer extension assay
J Med Virol. (1994) Jul;43(3):310-5.

Brunetto MR, Monti Gorin J, Citico G, Oliveri F, Colombatto P, Capalbo M, *et al*. Pre-core/core gene mutants of hepatitis B virus: pathogenetic implications in viral hepatitis and liver disease
In: Rizzetto M, Purcell R, Gerin J, Verme G, *et al*., editors. Minerva Medica; (1997). p. 127–137.

Brunetto MR, Rodriguez UA and Bonino F
Hepatitis B Virus Mutants
Intervirology (1999); 42:69–80

Brunetto M. R., Oliveri F.and Bonino F
Diagnosi eziologica delle epatite in corso di infezione virale
Ligandassay (2001) ; 6 : 75 – 85

Brunetto MR, Oliveri F, Coco B, Leandro G, Colombatto P, Gorin JM, *et al*. The outcome of anti-HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study
J Hepatol (2002); 36:263-270.

Brunetto MR, Moriconi F, Bonino F, Lau GK, Farci P, Yurdayin C, *et al*. Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B
Hepatology (2009) ;49:1141–1150.

Brunetto MR, Oliveri F, Colombatto P, Moriconi F, Ciccorossi P, Coco B, Romagnoli V, Cherubini B, Moscato G, Maina AM, Cavallone D, Bonino F. Hepatitis B surface antigen serum levels help to distinguish active from inactive hepatitis B virus genotype D carriers
Gastroenterology. 2010 Aug;139(2):483-90.

Brunetto MR
A new role for an old marker, HBsAg
J Hepatol. (2010); 52(4):475-7.

Bruss V and Ganem D
The role of envelope proteins in hepatitis B virus assembly
Proc Natl Acad Sci U S A. (1991) Feb 1;88(3):1059-63

Bruss V
Hepatitis B virus Morphogenesis
World J. Gastroenter. (2007); 13 (1):65-73

Buckwold, V. E., Z. Xu, M. Chen, T. S. Yen, and J. H. Ou
Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication
J. Virol.(1996); 70:5845–5851.

Budkowska A, Dubreuil P, Gerlich WH, *et al*.
Occurrence of pre-S1 antigen in viremic and nonviremic carriers of hepatitis B surface antigen
J Med Virol (1988); 26(2):217–25.

Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, Zuckerman AJ and Thomas HJ
Vaccine-induced escape mutant of hepatitis B virus
Lancet (1990); 336:325–329.

Carman WF, Korula J, Wallace L, MacPhee R, Mimms L and Decker R
Fulminant reactivation of hepatitis B due to envelope protein mutant that escaped detection by monoclonal HBsAg ELISA
Lancet (1995); 345:1406–1407.

Carman WF, Trautwein C, van Deursen FJ, Colman K, Dornan E, McIntyre G, Waters J, Kliem V, Müller R, Thomas HC and Manns MP
Hepatitis B virus envelope variation after transplantation with and without hepatitis B immune globulin prophylaxis
Hepatology (1996); 24(3):489-93.

Carman WF, Van Deursen FJ, Mimms LT, Hardie D, Coppola R, Decker R and Sanders R
The prevalence of surface antigen variants of hepatitis B virus in Papua New Guinea, South Africa, and Sardinia
Hepatology (1997);26:1658–1666.

Cattaneo R, Will H, and Schaller H
Hepatitis B virus transcription in the infected liver.
EMBO J (1984); 3(9):2191-2196.

Chan HL, Wong VW, Tse AM, Tse CH, Chim AM, Chan HY, *et al*.
Serum hepatitis B surface antigen quantitation can reflect hepatitis B virus in the liver and predict treatment response
Clin Gastroenterol Hepatol (2007);5:1462-1468.

Chang C, Enders G, Sprengel R, Peters N, Varmus HE and Ganem D
Expression of the precore region of an avian hepatitis B virus is not required for viral replication
J Virol. (1987); 61(10):3322-5.

Chen JY, Hsu HC, Lee CS, Chen DS, Zuckerman AJ and Harrison TJ
Detection of hepatitis B virus DNA in hepatocellular carcinoma: methylation of integrated viral DNA
J Virol Methods (1988); 19:257-263.

Chen HS, Kew MC, Hornbuckle WE, Tennant BC, Cote PJ, Gerin JL, Purcell RH and Miller RH.
The precore gene of the woodchuck hepatitis virus genome is not essential for viral replication in the natural host
J Virol. (1992); 66(9):5682-4.

Chen HC, Eng HL, Lee CM, Fang YK, Kuo FY, Lu SN, *et al*.
Correlations between hepatitis B virus genotype and cirrhotic or non-cirrhotic hepatoma
Hepatogastroenterology (2004); 51: 552–555.

Chen B, Liu C, Jow G, Chen P, Kao J and Chen D
High Prevalence and Mapping of Pre-S Deletion in Hepatitis B Virus Carriers With Progressive Liver Diseases
Gastroenterology (2006);130:1153–1168

Chen CH, Hung CH, Lee CM, Hu TH, Wang JH, Wang JC, Lu SN and Changchien CS.
Pre-S deletion and complex mutations of hepatitis B virus related to advanced liver disease in HBeAg-negative patients
Gastroenterology. (2007) Nov;133(5):1466-74. Epub 2007 Sep 6.

Chisari FV
Hepatitis B Virus immunopathogenesis
Annu. Rev. Immunol. (1995); 13: 29-60

Chisari FV and Ferrari C
Hepatitis B virus immunopathogenesis
Annu Rev Immunol (1995); 13:29-60.

Chisari FV.
Rous-Whipple Award Lecture. Viruses, immunity, and cancer: lessons from hepatitis B.
Am J Pathol (2000); 156:1117–32.

F.V. Chisari, M. Isogawa and S.F. Wieland
Pathogenesis of hepatitis B virus infection
Pathologie Biologie 58 (2010) 258–266

Chouteau P, Le Seyec J, Cannie I, Nassal M, Guguen-Guillouzo C and Gripon P
A short N proximal region in the large envelope protein harbors a determinant that contributes to the species specificity of human hepatitis B virus
J Virol (2001); 75(23):11565-11572.

Chu CM and Liaw YF
Membrane staining for hepatitis B surface antigen on hepatocytes: a sensitive and specific marker of active viral replication in hepatitis B

Clin Pathol (1995);48:470-473

Colloredo G, Bellati G, Leandro G, Colombatto P, Rho A, Bissoli F, Brunetto MR, Angeli G, Ideo G, Bonino F.
Quantitative analysis of IgM anti-HBc in chronic hepatitis B patients using a new gray-zone for the evaluation of borderline values
J Hepatol. 1996 Nov;25(5):644-8

Cooreman MP, Leroux-Roels and Paulij WP
Vaccine- and hepatitis B immune globulin-induced escape mutations of hepatitis B virus surface antigen
J. Biomed. Sci. (2001); 8 (3), 237–247.

Corden J, Wasylyk B, Buchwalder A, Sassone CP, Kedinger C and Chambon P
Promoter sequences of eukaryotic protein-coding genes
Science (1980) ;209:1406–1414.

De Franchis R, Meucci G, Vecchi M, Tatarella M, Colombo M, Del Ninno E, *et al*.
The natural history of asymptomatic hepatitis B surface antigen carriers
Ann Intern Med (1993); 118:191-194.

Delaney WE 4th, Yang H, Westland CE, Das K, Arnold E, Gibbs CS, Miller MD and Xiong S
The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication *in vitro*
J Virol (2003); 77(21) : 11833 - 11841.

Devesa M, Loureiro CL, Rivas Y, Monsalve F, Cardona N, Duarte MC, *et al*. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia
J Med Virol (2008); 80(1):20–26.

Dienes HP, Gerlich WH, Worsdorfer M, Gerken G, Bianchi L, Hess G, *et al*.
Hepatic expression patterns of the large and middle hepatitis B surface proteins in viremic and non viremic chronic hepatitis B
Gastroenterology (1990) ;98:1017–1023.

Dienstag JL.
Hepatitis B virus infection
N Engl J Med (2008); 359:1486–1500.

Enders GH, Ganem D and Varmus H
Mapping the major transcripts of ground squirrel hepatitis virus: the presumptive template for reverse transcriptase is terminally redundant
Cell (1985); 42(1):297-308.

Fan YF, Lu CC, Chang YC, Chang TT, Lin PW, Lei HY and Su IJ

Identification of a pre-S2 mutant in hepatocytes expressing a novel marginal pattern of surface antigen in advanced diseases of chronic hepatitis B virus infection
J Gastroenterol Hepatol (2000), 15:519–528

Fan YF, Lu CC, Chen WC, Yao WJ, Wang HC, Chang TT, Lei HY, Shiau AL, Su IJ.
Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at different replicative stages of chronic HBV infection
Hepatology. (2001) Jan;33(1):277-86.

Fattovich G, Giustina G, Schalm SW, Hadziyannis S, Sanchez-Tapias J, Almasio P, *et al*.
Occurrence of hepatocellular carcinoma and decompensation in western European patients with cirrhosis type B
The EUROHEP Study Group on Hepatitis B Virus and Cirrhosis.
HEPATOLOGY (1995);21:77-82.

Fernholz D, Stemler M, Brunetto M, Bonino F, Will H.
Replicating and virion secreting hepatitis B mutant virus unable to produce preS2 protein
J Hepatol. (1991) ;13 Suppl 4:S102-4

Fernholz D, Galle PR, Stemler M, Brunetto M, Bonino F, Will H
Infectious hepatitis B virus variant defective in pre-S2 protein expression in a chronic carrier
Virology. (1993) May;194(1):137-48

Flichman D, Maina A, Colombatto P, Bonino F, Brunetto MR.
Validation and comparison of different PCR-based methods for detection of hepatitis B virus precore region mutants.
J Virol Methods. 2005 Oct;129(1):64-74.

Funk, M. L., D. M. Rosenberg, and A. S. Lok.
World-wide epidemiology of HBeAg-negative chronic hepatitis B and associated precore and core promoter variants
J. Viral Hepat. (2002); 9:52–61.

Ganem D
Assembly of hepadnaviral virions and subparticles
Curr Top Microbiol Immunol (1991);168:61–83.

Ganem, D., J. R. Pollack, and J. Tavis.
Hepatitis B virus reverse transcriptase and its many roles in hepadnaviral genomic replication
Infect. Agents Dis. (1994) 3:85–93.

Ganem D and Schneider RJ
Hepadnaviridae and their replication
In: Knipe DM, Howley C, editors. Fields: in virology, 4th ed.

Philadelphia: Lippincott-Raven; (2001). p. 2703–2737.

Ganem D and Prince AM.
Hepatitis B virus infection--natural history and clinical consequences
N Engl J Med (2004); 350(11):1118-1129.

Gerlich WH, Goldmann U, Müller R, Stibbe W, Wolff W.
Specificity and localization of the hepatitis B virus-associated protein kinase.
J Virol. (1982); 42(3):761-6.

Gerken G, Kremsdorf D, Capel F, Petit MA, Dauguet C, Manns MP, Meyer zum Buschenfelde KH ad Brechot C
Hepatitis B defective virus with rearrangements in the preS gene during chronic HBV infection
Virology (1991); 183:555–565.

Gerner PR, Friedt M, Oettinger R, Lausch E and Wirth S
The hepatitis B virus seroconversion to anti-HBe is frequently associated with HBV genotype changes and selection of preS2-defective particles in chronically infected children
Virology (1998); 245 (1): 163–172.

Girones R and Miller RH
Mutation of the Hepadnavirus genome
Virology (1989); 170: 595-597

Glebe D and Urban S
Viral and cellular determinants involved in hepadnaviral entry
World J Gastroenterol. (2007); 13(1):22-38.

Grandjacques C, Pradat P, Stuyver L, *et al*.
Rapid detection of genotypes and mutations in the pre-core promoter and the precore region of hepatitis B virus genome: correlation with viral persistence and disease severity
J Hepatol (2000); 33(3):430–439.

Gripon P, Cannie I and Urban S
Efficient inhibition of hepatitis B virus infection by acylated peptides derived from the large viral surface protein
J Virol (2005); 79(3):1613-1622.

Gudat F, Bianchi L, Sonnabend W, Thiel G, Aenishaenslin W and Stalder GA
Pattem of core and surface expression in liver tissue reflects state of specific immune response in hepatitis B
Lab Invest (1975) ;32:1-9.

Guidotti LG, Matzke B, Schaller H and Chisari FV
High-level hepatitis replication in transgenic mice

J Virol (1995); 69:6158–69.

Guidotti LG, Ando K, Hobbs MV, Ishikawa T, Runkel L, Schreiber RD *et al*.
Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice
Proc Natl Acad Sci U S A (1994); 91(9):3764-3768.

Guidotti LG, Borrow P, Brown A, McClary H, Koch R and Chisari FV
Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte
J Exp Med (1999); 189(10):1555-1564.

Guidotti LG, Guilhot S and Chisari FV
Interleukin-2 and alpha/beta interferon down-regulate hepatitis B virus gene expression in vivo by tumor necrosis factor-dependent and - independent pathways
J Virol (1994); 68(3):1265-1270.

Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R and Chisari FV
Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes
Immunity (1996); 4(1):25-36.

Guidotti LG, McClary H, Loudis JM and Chisari FV
Nitric oxide inhibits hepatitis B virus replication in the livers of transgenic mice
J Exp Med (2000); 191(7):1247-1252.

Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R and Chisari FV
Viral clearance without destruction of infected cells during acute HBV infection
Science (1999); 284(5415):825-829.

Guidotti LG, Chisari FV
Immunobiology and pathogenesis of viral hepatitis
Annu Rev Pathol (2006); 1:23–61.

Guo WT, Wang J, Tam G, Yen TS and Ou JS
Leaky transcription termination produces larger and smaller than genome size hepatitis B virus X gene transcripts
Virology (1991); 181:630–636.

Guo H, Mason WS, Aldrich CE, Saputelli JR, Miller DS, Jilber AR and Newbold JE
Identification and characterization of avihepadnaviruses isolated from exotic anseriformes maintained in captivity
J. Virol. (2005), 79(5): 2729-2742

Guo Y, Li Y, Mu S, Zhang J, Yan Z

Evidence that methylation of hepatitis B virus covalently closed circular DNA in liver tissues of patients with chronic hepatitis B modulates HBV replication
J Med Virol (2009); 81:1177-1183.

Hadziyannis S, Gerber MA, Vissoulis C and Popper H.
Cytoplasmic hepatitis B antigen in ground-glass hepatocytes of carriers
Arch Pathol. (1973) Nov;96(5):327-30.

Hadziyannis SJ, Vassilopoulos D.
Hepatitis B e antigen-negative chronic hepatitis B
HEPATOLOGY (2001); 34:617-624.

Heermann KH, Goldmann U, Schwartz W, Seyffarth T, Baumgarten H, Gerlich WH.
Large surface proteins of hepatitis B virus containing the pre-s sequence
J Virol. (1984); 52 (2):396-402.

Hildt E, Saher G, Bruss V, Hofschneider PH
„The hepatitis B virus large surface protein (LHBs) is a transcriptional activator Virology (1996), 225:235–239

Hildt E, Hofschneider PH
The preS2 activators of the hepatitis B virus: activators of tumour promoter pathways
Recent Results Cancer Res (1998), 154:315–329

Hildt E, Munz B, Saher G, Reifenberg K, Hofschneider PH
„The preS2 activator MHBs(t) of hepatitis B virus activates c-raf-1/Erk2 signaling
in transgenic mice
EMBO J (2002), 21:525–535

Hirsch RC, Lavine JE, Chang LJ, Varmus HE and Ganem D
Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as wel as for reverse transcription
Nature (1990); 344(6266):552-555

Hoofnagle JH, Dusheiko GM, Seeff LB, Jones EA, Waggoner JG and Bales ZB
Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis
Ann Intern Med (1981); 94:744-748.

Hoofnagle JH, Doo E, Liang TJ, Fleischer R and Lok ASK
Management of hepatitis B: Summary of a clinical research workshop
Hepatology (2007); 45:1056-1075.

Hsieh YH, Su IJ, Wang HC, *et al*.

Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and DNA damage
Carcinogenesis (2004);25:2023–2032.

Hsu HC, Lai MY, Su IJ, Chen DS, Chang MH, Yang PM, Wu CY and Hsieh HC
Correlation of hepatocyte HBsAg expression with virus replication and liver pathology
Hepatology (1988), 8:749–754

Hsu YS, Chien RN, Yeh CT, Sheen IS, Chiou HY, Chu CM, *et al*.
Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B HEPATOLOGY (2002); 35:1522-1527.

Huang MJ and Summers J
Infection initiated by the RNA pregenome of a DNA virus
J Virol (1991); 65(10):5435-5439.

Hui DK, Leung N, Yuen ST, Yuen ST, Zhang HY, Leung KW, *et al*.
Natural history and disease progression in Chinese chronic hepatitis B patients in immune tolerant phase
Hepatology (2007); 46:395-401.

Hunt, C. M., J. M. McGill, M. I. Allen, and L. D. Condreay
Clinical relevance of hepatitis B viral mutations
Hepatology (2000); 31:1037–1044.

Huovila AP, Eder AM and Fuller SD
Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment
J Cell Biol. (1992); 118(6):1305-20

Ishikawa T and Ganem D
The pre-S domain of the large viral envelope protein determines host range in avian hepatitis B viruses
Proc Natl Acad Sci U S A (1995); 92(14):6259-6263.

Jaroszewicz J, Serrano BC, Wursthorn K, Deterding K, Schlue J, Raupach R, *et al*. Hepatitis B surface antigen (HBsAg) levels in the natural history of hepatitis B virus (HBV)-infection: a European perspective
J Hepatol (2010) ;52:514–522.

Jaw Ching Wu, Chen P. J., Kuo M. Y. P., Lee S. D., Chen O. S and Ting L. P
Production of hepatitis delta virus and suppression of helper hepatitis B virus in a human hepatoma cell line
J. Virol. (1991) ; 1099-1104

Jin Y, Shih JW-K and Berkower I
Human T-cell response to the surface antigen of heptitis B virus (HBsAg)
J Exp Med (1995); 168:293–299.

Jung MC, Diepolder HM, Spengler U, Wierenga EA, Zachoval R, Hoffmann RM *et al*
Activation of a heterogeneous hepatitis B (HB) core and e antigen-specific CD4+ T-cell population during seroconversion to anti-HBe and anti-HBs in hepatitis B virus infection
J Virol. (1995); 69(6):3358-68.

Kakimi K, Lane TE, Chisari FV and Guidotti LG
Cutting edge: Inhibition of hepatitis B virus replication by activated NK T cells does not require inflammatory cell recruitment to the liver
J Immunol (2001); 167(12):6701-6705.

Kaito M, Watanabe S, Tsukiyama-Kohara K, Yamaguchi K, Kobayashi Y, Konishi M *et al*
Hepatitis C virus particle detected by immunoelectron microscopic study
J Gen Virol. 1994 Jul;75 (Pt 7):1755-60.

Kaneko S and Miller RH
X-region-specific transcript in mammalian hepatitis B virus-infected liver
Virol. (1988); 62:3979–3984.

Kann M, Bischof A, Gerlich WH.
In vitro model for the nuclear transport of the hepadnavirus genome
J Virol (1997); 71(2):1310-1316.

Kao JH, Chen PJ, Lai MY and Chen DS
Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection
J Clin Microbiol (2002);40:1207–1209

Kao JH, Chen PJ, Lai MY and Chen DS
Hepatitis B virus genotypes and spontaneous hepatitis B e antigen seroconversion in Taiwanese hepatitis B carriers
J Med Virol (2004);72:363–369.

Kay A and Zoulim F
Hepatitis B virus genetic variability and evolution
Virus Research (2007); 127: 164–176

Kuhns M and Busch M
New Strategies for Blood Donor Screening for Hepatitis B Virus: Nucleic Acid Testing versus Immunoassay Methods.
Molecular Diagnosis & Therapy (2006);10(2):77–91.

Kidd-Ljunggren K, Ekdahl K, ObergM, Kurathong S and Lolekha S.
Hepatitis B virus strains in Thailand: genomic variants in chronic carriers
J Med Virol (1995); 47:454–461.

Kim CM, Koike K, Saito I, Miyamura T and Jay G

HBx gene of hepatitis B virus induces liver cancer in transgenic mice
Nature (1991); 351: 317–320.

Kim Y, Hong YB and Jung G
Hepatitis B virus: DNA polymerase activity of deletion mutants
Biochem Mol Biol Int (1999); 47:301-308.

Kimbi GC, Kramvis A and Kew MC
Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa
J Gen Virol (2004); 85:1211–1220.

Klingmuller U and Schaller H
Hepadnavirus infection requires interaction between the viral pre-S domain and a specific hepatocellular receptor
J Virol (1993); 67(12):7414-7422.

Kojima M. Shimizu M., Tsuchimochi T., Koyasu M., Tanaka S., Lizuka H., Tanaka T., Okamoto H., Tsuda F., Miyakawa Y and Mayumi M.
Posttransfusion fulminant hepatitis B associated with precore defective HBV mutant
Vox Sang (1991) ; 60 : 34-39

Kramvis A, Weitzmann L, Owiredu WK and Kew MC
Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa
J Gen Virol (2002), 83:835–839.

Lambert V, Fernholz D, Sprengel R, Fourel I, Deleage G, Wildner G *et al*.
Virus-neutralizing monoclonal antibody to a conserved epitope on the duck hepatitis B virus pre-S protein
J Virol 1990; 64(3):1290-1297.

Lan YT, Li J, Liao W, Ou J.
Roles of the three major phosphorylation sites of hepatitis B virus core protein in viral replication.
Virology (1999);259(2):342-8

Lanford RE, Chavez D, Brasky KM, Burns RB 3rd and Rico-Hesse R
Isolation of a hepadnavirus from the woolly monkey, a New World primate
Proc Natl Acad Sci U S A (1998), 95(10): 5757-61.

Lanford RE, Kim YH, Lee H, Notvall L and Beames B
Mapping of the hepatitis B virus reverse transcriptase TP and RT domains by transcomplementation for nucleotide priming and by protein-protein interaction
J Virol (1999); 73:1885-1893.

Laskus T., Rakela J., Tong M. J and Persing D. H.

Nucleotide sequence analysis of the precore region in patients with spontaneus reactivation of chronic hepatitis B
Dig Dis Sci (1994); 39: 2000-2006

Lau JYN, Bain VG, Davies SE, Alexander GJM and Williams R
Export of intracellular HBsAg in chronic hepatitis B virus infection is related to viral replication
Hepatology (1991);14:416–421.

Lee P. I., Chang M. H., Lee C. Y., *et al*.
Changes of serum hepatitis B virus DNA and aminotransferases levels during the cours of chronic hepatitis B virus infection in children
Hepatology (1990); 12, 4: 657 – 660

Lee, W. M
Hepatitis B virus infection
N. Engl. J. Med.(1997); 337:1733–1745.

Lesburg CA, Cable MB, Ferrari E, Hong Z, Mannarino AF and Weber PC
Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site
Nat Struct Biol (1999); 6:937-943.

Levrero M, Pollicino T, Petersen J, Belloni L, Raimondo G and Dandri M
Control of cccDNA function in hepatitis B virus infection
J Hepatol (2009); 51:581-592.

Li JS, Fourel I, Jaquet C and Trepo C
Decreased replication capacity of a duck hepatitis B virus mutant with altered distal pre-S region
Virus Res (1991); 20:11-21.

Li JS, Tong SP, Wen YM, Vitvitski L, Zhang Q and Trepo C
Hepatitis B virus genotype A rarely circulates as a HBe-minus mutant: possible contribution of a single nucleotide in the precore region
J Virol (1993); 67:5402–5410.

Li, J., V. E. Buckwold, M. W. Hon, and J. H. Ou
Mechanism of suppression of hepatitis B virus precore RNA transcription by a frequent double mutation
J. Virol.(1999); 73:1239–1244.

Liaw YF, Chu CM, Su IJ, Huang MJ, Lin DY and Chang-Chien CS
Clinical and histological events preceding hepatitis B e antigen seroconversion in chronic type B hepatitis
Gastroenterology (1983) ; 84:216-219.

Liaw YF, Brunetto MR and Hadziyannis S.
The natural history of chronic HBV infection and geographical differences.
Antivir Ther. (2010); 15 Suppl 3:25-33.

Lien JM, Aldrich CE and Mason WS
Evidence that a capped oligoribonucleotide is the primer for duck hepatitis B virus plus-strand DNA synthesis
J Virol (1986), 57(1):229-236.

Lin LY, Wong VW, Zhou HJ, Chan HY, Gui HL, Guo SM, *et al*.
Relationship between serum hepatitis B virus DNA and surface antigen with covalently closed circular DNA in HBeAg-negative patients
J Med Virol (2010);82:1494-1500.

Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, Harrison TJ.
Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine
Hepatology. (1996) Sep; 24(3):711-3.

Livingston SE, Simonetti JP, Bulkow LR, Homan CE, Snowball MM, Cagle HH, *et al*
Clearance of hepatitis B e antigen in patients with chronic hepatitis B and genotypes A, B, C, D, and F Gastroenterology (2007); 133: 1452-1457.

Locarnini SA
Hepatitis B virus surface antigen and polimerase gene variants: potential virological and clinical significance
Hepatology (1998); 27: 294 – 297

Locarnini, S., J. McMillan, and A. Bartholomeusz
The hepatitis B virus and common mutants
Semin. Liver Dis. (2003); 23:5–20.

Lok AS, Lai CL, Wu PC, Leung EK and Lam TS
Spontaneous hepatitis B e antigen to antibody seroconversion and reversion in Chinese patients with chronic hepatitis B virus infection
Gastroenterology (1987); 92:1839-1843.

Lok, A. S., U. Akarca, and S. Greene
Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal
Proc. Natl. Acad. Sci. USA(1994); 91:4077–4081.

Lok AS, Heathcote EJ and Hoofnagle JH
Management of hepatitis B: 2000– summary of a workshop
Gastroenterology (2001); 120:1828-1853.

Lopez-Cabrera M, Letovsky J, Hu KQ, Siddiqui A
Multiple liver specific factors bind to hepatitis B virus core/pregenomic promoter: Transactivation and repression by CCAAT/enhancer binding protein
Proc Natl Acad Sci USA (1990); 87:5069–1073.

Lutwick LI and Robinson WS
DNA synthesized in the hepatitis B Dane particle DNA polymerase reaction
J. Virol. (1977), 21(1): 96-104

Manesis EK, Hadziyannis ES, Angelopoulou OP and Hadziyannis SJ
Prediction of treatment-related HBsAg loss in HBeAg-negative chronic hepatitis B: a clue from serum HBsAg levels
Antivir Ther (2007); 12:73-82.

Manesis EK, Papatheodoridis GV, Tiniakos DG, Hadziyannis ES, Agelopoulou OP, Syminelaki T, Papaioannou C, Nastos T and Karayiannis P.
Hepatitis B Surface Antigen:Relation to Hepatitis B Replication Parameters in HBeAg-Negative Chronic Hepatitis B
J Hepatol. 2010 Dec 5. [Epub ahead of print]

McMahon BJ, Holck P, Bulkow L and Snowball MM
Serologic and clinical outcomes 1536 Alaska Natives chronically infected with hepatitis B virus
Ann Intern Med (2001): 759-768.

McMahon BJ
Epidemiology and natural history of hepatitis B
Semin Liver Dis (2005); 25(Suppl 1):3-8.

Marion P, Oshiro LS, Regnery DC, Scullard GH and Robinson WS
A virus in Beechey ground squirrels that is related to Hepatitis B virus of humans
Proc Natl Acad Sci U S A (1980), 77: 2941-2945

Martinot-Peignoux M, Boyer N, Colombat M, Akremi R, Pham BN, Ollivier S, *et al*
Serum hepatitis B virus DNA levels and liver histology in inactive HBsAg carriers
J Hepatol (2002); 36:543-546.

Marzano A, Angelucci E, Andreone P, Brunetto M, Bruno R, Burra P, *et al*
Prophylaxis and treatment of hepatitis B in immunocompromised patients.
Dig Liver Dis 2007; 39: 397-408

Mason WS, Seal G and Summers J
Virus of Pekin ducks with structural and biological relatedness to human Hepatitis B virus
J. Virol. (1980), 36: 829-836

McMahon BJ
Epidemiology and natural history of hepatitis B
Semin Liver Dis (2005) ;25(Suppl 1):3–8.

Melegari, M., Bruno, S.,Wands, J.R.
Properties of hepatitisBvirus pre-S1 deletion mutants
Virology (1994); 199 (2), 292–300.

Milich DR, McLachlan A.
The nucleocapsid of hepatitis B virus is both a T-cell-independent and a T-cell-dependent antigen
Science. (1986) Dec 12;234(4782):1398-401.

Milich DR, Chen M, Schödel F, Peterson DL, Jones JE and Hughes JL
Role of B cells in antigen presentation of the hepatitis B core
Proc Natl Acad Sci U S A. (1997) Dec 23;94(26):14648-53.

Milich, DR
Influence of T-helper cell subsets and crossregulation in hepatitis B virus infection
J. Viral. Hep (1997); 4:48–59.

Milich, D. R., MK Chen, JL Hughes and JE Jones.
The secreted hepatitis B precore antigen can modulate the immune response to the nucleocapsid:a mechanism for persistence
J. Immunol. (1998); 160:2013–2021.

Miller RH, Kaneto S, Chung CT *et al*
Compact organization of hepatitis B virus genome
Hepatology (1989); 9: 322 – 327

Minami M, Okanoue T, Nakajima E, Yasui K, Kagawa K and Kashima K
Significance of pre-S region-defective hepatitis B virus that emerged during exacerbation of chronic type B hepatitis
Hepatology (1993); 17:558–563.

Missale G, Redeker A, Person J, Fowler P, Guilhot S, Schlicht HJ, Ferrari C and Chisari FV
HLA-A31- and HLA-Aw68-restricted cytotoxic T cell responses to a single hepatitis B virus nucleocapsid epitope during acute viral hepatitis
J Exp Med (1993), 177:751–762

Montano L, Miescher GC, Goodall AH, Wiedmann KH, Janossy G and Thomas HC
Hepatitis B virus and HLA antigen display in the liver during chronic hepatitis B virus infection. Hepatology (1982) ;2:557-61.

Moriconi F, Colombatto P, Coco B, Ciccorossi P, Oliveri F, Flichman D, Maina AM, Sacco R, Bonino F, Brunetto MR.
Emergence of hepatitis B virus quasispecies with lower susceptibility to nucleos(t)ide analogues during lamivudine treatment.
J Antimicrob Chemother. (2007) Aug;60(2):341-9. Epub 2007 Jun 13.

Moucari R, Mackiewicz V, Lada O, Ripault MP, Castelnau C, Martinot-Peignoux M, *et al*
Early serum HBsAg drop: a strong predictor of sustained virological response to pegylated interferon alfa-2a in HBeAg-negative patients
Hepatology (2009); 49:1151-1157.

Moyes D., Milne A., Waldon F., *et al*.
Liver function of hepatitis B carriers in children
Pedriatr Infect Dis J (1993); 12:120 – 125

Nakajima E,MinamiM, Ochiya T, KagawaK, Okanoue T
PreS1 deleted variants of hepatitis B virus in patients with chronic hepatitis
J Hepatol (1994); 20:329–335.

Nakanishi H, Kurosaki M, Asahina Y, Onuki Y, Ueda K, Nishimura Y, Tsuchiya K, Kitamura T, Uchihara M, Miyake S, Enomoto N and Izumi N
Polymerase domain B mutation is associated with hepatitis relapse during long-term lamivudine therapy for chronic hepatitis B
Intervirology (2005); 48 (6) : 381-8

Nassal M and Schaller H
Hepatitis B virus replication—an update
J Viral Hepat (1996); 3:217-226.

Nassal M
Hepatitis B virus replication: novel roles for virus-host interactions
Intervirology (1999); 42:100-116.

Naumann H, Schaefer S, Yoshida CF, Gaspar AM, Repp R and Gerlich WH
Identification of a new hepatitis B virus (HBV) genotype from Brazil that expresses HBV surface antigen subtype adw4
J Gen Virol (1993), 74:1627–1632.

Naoumov NV, Schneider R, Grotzinger T, *et al*.
Precore mutant hepatitis B virus infection and liver disease
Gastroenterology (1992);102:538.

Nayersina R, Folwer P and Guilhot S
HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection
J Immunol (1993); 150:4659–4671.

Nguyen T, Thompson AJV, Bowden S, Croagh C, Bell S, Desmond PV, *et al*.
Hepatitis B surface antigen levels during the natural history of chronic hepatitis B: perspective on Asia
J Hepatol (2010) ;52:508–513.

Netter, H. J., S. Chassot, S. F. Chang, L. Cova, and H. Will

Sequence heterogeneity of heron hepatitis B virus genomes determined by full-length DNA amplification and direct sequencing reveals novel and unique features
J. Gen. Virol. (1997), 78:1707–1718.

Neurath AR, Kent SBH and Parker K
Antibodies to a synthetic peptide from the pre-S(2) 120145 region of the hepatitis B virus envelope are
virus-neutralizing
Vaccine (1986); 4:35–41.

Norder H, Couroucé AM and Magnius LO
Molecular basis of hepatitis B virus serotype variations within the four major subtypes
J Gen Virol 1992;73:3141–3145.

Norder H, Hammas B, Löfdahl S, Couroucé AM and Magnius LO
Comparison of the aminoacid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains
J Gen Virol (1992), 73:1201–1208.

Norder H, Couroucé AM and Magnius LO
Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes
Virology (1994), 198: 489–503.

Norder H, Arauz-Ruiz P, Blitz L, Pujol FH, Echevarria JM and Magnius LO
The T(1858) variant predisposing to the precore stop mutation correlates with one of two major genotype F hepatitis B virus clades
J Gen Virol (2003), 84: 2083–2087.

Okamoto H, Tsuda , Akahane Y, Sugai Y, Yoshiba M, Moriyama K, Tanaka T, Miyakawa Y and Mayumi M Hepatitis B virus with mutations in the core promoter for an e antigen- negative phenotype in carriers with antibody to e antigen
J Virol (1994); 68:8102–8110.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y and Mayumi M
Typing Hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigens subtypes
J. Gen. Virol (1998), 69: 2575-2583

Oon CJ, Chen WN, Koh S and Lim GK
Identification of hepatitis B surface antigen variants with alterations outside the a determinant in immunized Singapore infants
J. Infect. Dis. (1999); 179(1): 259–263.

Ou JH and Rutter WJ

Regulation of secretion of the hepatitis B virus major surface antigen by the preS-1 protein
J Virol (1987), 61:782–786

Pai SB, Bozdayi AM, Pai RB, Beker T, Sarioglu M, Turkyilmaz AR, Grier J, Yurdaydin C, Schinazi RF Emergence of a novel mutation in the FLLA region of hepatitis B virus during lamivudine therapy
Antimicrob Agents Chemother. (2005); 49(7) : 2618-2624.

Parekh, S., F. Zoulim, S. H. Ahn, A. Tsai, J. Li, S. Kawai, N. Khan, C. Trepo, J. Wands and S. Tong
Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants
J. Virol. (2003); 77:6601–6612.

Perillo R., Rakela J., Martin P., Levy G., Shiff E., Wright T., Diestang J., Gish R., Villeneuve J., Caldwell S., Brown N. and Self P
Lamivudine Transplant Group: Lamivudine for hepatitis B after liver transplantation
Hepatology (1996) ; 24 : 182

Persing DH, Varmus HE and Ganem D
Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide
Science (1986), 234:1388–1391

Poch O, Sauvaget I, Delarue M and Tordo N.
Identification of four conserved motifs among the RNA-dependent polymerase encoding elements
EMBO J (1989); 8:3867-3874.

Pollicino T, Campo S and Raimondo G
PreS and core gene heterogeneity in hepatitis B virus (HBV) genomes isolated from patients with long-lasting HBV chronic infection
Virology (1995); 208: 672–677.

Pollicino T, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G and Levrero M.
Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones
Gastroenterology (2006);130:823- 837

Popper H
The ground glass hepatocyte as a diagnostic hint
Hum Pathol (1975), 6:517–520

Pugh JC and Summers JW
Infection and uptake of duck hepatitis B virus by duck hepatocytes maintained in the presence of dimethyl sulfoxide
Virology (1989); 172(2):564-572.

Pugh JC, DI Q, Mason WS and Simmons H
Susceptibility to duck hepatitis B virus infection is associated with the presence of cell surface receptor sites that efficiently bind viral particles
J Virol (1995); 69(8):4814-4822.

Rabe B, Vlachou A, Pante N, Helenius A, Kann M.
Nuclear import of hepatitis B virus capsids and release of the viral genome
Proc Natl Acad Sci U S A (2003); 100(17):9849-9854.

Radziwill G, Tucker W and Schaller H
Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity
J Virol (1990); 64:613-620.

Raimondo G., Schneider R., Stemler M. and Will H.
A new hepatitis B virus variant in a chronic carrier with multiple episodes of viral reactivation and acute hepatitis
Virology (1990); 179 : 64-68

Raimondo G, Pollicino T and Squadrito G
Clinical virology of hepatitis B virus infection
J Hepat. (2003); 39: S26-30

Ray MB, Desmet VJ, Bradbume AF, Desmyter J, Fevery J and DeGroote J
Differential distribution of hepatitis B surface antigen and hepatitis B core antigen in liver of hepatitis B patients
Gastroenterology (1976);71:462-7.

Rodriguez-Frias, F., M. Buti, R. Jardi, M. Cotrina, L. Viladomiu, R. Esteban, and J. Guardia
Hepatitis B virus infection: precore mutants and its relation to viral genotypes and core mutations
Hepatology (1995); 22:1641–1647.

Romero R and Lavine J. E.
Cytokine inhibition of the hepatitis B virus core promoter
Hepatology (1996); 23 : 17-23

Rothbard JB.
Major histocompatibility complex-peptide interactions
Curr Opin Immunol. 1989 Oct;2(1):99-105.

Sanchez-Tapias JM, Costa J, Mas A, Bruguera M and Rodes J
Influence of hepatitis B viral genotype on the long-term outcome of chronic hepatitis B in western patients
Gastroenterology (2002); 123:1848–1856.

Santantonio T., Jung M. C., Miska S., Pastore G., Pape G. R and Will H

Prevalence and type of pre-C HBV mutants in anti-HBe positive carriers with chronic liver disease in a highly endemic area
Virology (1991) ; 183 : 840-844

Santantonio T, Jung MC, Schneider R, Fernholz D, Milella M, Monno L, Pastore G, Pape GR and Will H
Hepatitis B virus genomes that cannot synthesize pre-S2 proteins occur frequently and as dominant virus populations in chronic carriers in Italy
Virology (1992); 188 (2):948–952.

Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, Tsuda T, Tanaka Y, Okamoto H, Miyakawa Y and Mayumi M
Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis
Ann Inter Med (1995); 122:241–248.
Schaefer S.
Hepatitis B virus: significance of genotypes
J Viral Hepat (2005) ;12:111–124.

Seeger C, Ganem D and Varmus HE.
Biochemical and genetic evidence for the hepatitis B virus replication strategy
Science (1986), 232(4749):477-484.

Seeger C and Mason WS
Replication of the hepatitis virus genome
In M. L. DePamphilis (ed.), DNA replication in eukaryotic cells (1996) pp. 815–831
Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Seeger C and Mason W
Hepatitis B Virus Biology
Microbiol. Mol. Biol. Rev. (2000), 64: 51-68

Seeger C, Zoulim F and Mason WS
Hepadnaviruses
In: Knipe DM *et al*. Editors. Field Virology. Lippincott Williams & Wilkins (2007): 2977-3029

Sobotta D, Sominskaya I, Jansons J, Meisel H, Schmitt S, Heermann KH, Kaluza G, Pumpens P, Gerlich WH.
Mapping of immunodominant B-cell epitopes and the human serum albumin-binding site in natural hepatitis B virus surface antigen of defined genosubtype.
J Gen Virol. (2000) Feb;81(Pt 2):369-78.

Sprengel R, Kaleta EF and Will H
Isolation and characterization of a Hepatitis B virus endemic in herons
J. Virol. (1988) 62: 932-937

Stibbe W and Gerlich WH
Variable protein composition of hepatitis B surface antigen from different donors
Virology 1982;123(2):436–442.

Stibbe W and Gerlich WH
Characterization of pre-s gene products in hepatitis B surface antigen
Dev Biol Stand (1983); 54:33–43.

Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF and Rossau R
A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness
J Gen Virol (2000), 81: 67–74.

Su IJ, Kuo TT and Liaw YF
Hepatocyte hepatitis B surface antigen. Diagnostic evaluation of patients with clinically acute hepatitis B surface antigen-positive hepatitis
Arch Pathol Lab Med (1985), 109: 400–402

Su IJ, Lai MY, Hsu HC, Chen DS, Yang PM, Chuang SM and Sung JL
Diverse virological, histopathological and prognostic implications of seroconversion from hepatitis B e antigen to anti-HBe in chronic hepatitis B virus infection
J Hepatol (1986), 3:182–189

Sugauchi F, Ohno T, Orito E, *et al*.
Influence of hepatitis B virus genotypes on the development of preS deletions and advanced liver disease
J Med Virol (2003); 70:537–544.

Summers J, O'Connell A and Millman I
Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles
Proc. Natl. Acad. Sci. USA (1975), 72(11), 4597-4601

Summers J, Smolec J and Snyder R
A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks
Proc Natl Acad Sci U S A (1978), 75: 4533-4537

Tai PC, Banik D, Lin GI, Pai S, Pai K, Lin MH, Yuoh G, Che S, Hsu SH, Chen TC, Kuo TT, Lee CS, Yang CS and Shih C
Novel and frequent mutations of hepatitis B virus coincide with a major histocompatibility complex class I-restricted T-cell epitope of the surface antigen
J Virol (1997), 71:4852–4856

Tai PC, Suk FM, Gerlich WH, Neurath AR and Shih C

Hypermodification and immune escape of an internally deleted middleenvelope (M) protein of frequent and predominant hepatitis B virus variants
Virology (2002); 292:44–58.

Tang H, Oishi N, Kaneko S and Murakami S
Molecular functions and biological roles of hepatitis B virus X protein
Cancer Sci (2006); 97:977–83.

Tenney DJ, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, Plym M, Pokornowski K, Yu CF, Angus P, Ayres A, Bartholomeusz A, Sievert W, Thompson G, Warner N, Locarnini S, Colonno R J
Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine
Antimicrob Agents Chemother. (2004); 48(9) : 3498-3507.

Thompson AJ, Nguyen T, Iser D, Ayres A, Jackson K, Littlejohn M, *et al*.
« Serum hepatitis B surface antigen and hepatitis B e antigen titers: disease phase influences correlation with viral load and intrahepatic hepatitis B virus markers
Hepatology (2010); 51:1933-1944.

Thung SN, Gerber MA, Kasambalides EJ, Gilja BK, Keh W and Gerlich WH
„Demonstration of pre-S polypeptides of hepatitis B virus in infected livers
Hepatology (1986), 6:1315–1318

Tiollais P, Pourcel C and Dejean A
The hepatitis B virus
Nature (1985), 317: 489-495

Tisdale M., Kemp S. D., Parry N. R. and Larder BA
Rapid *in vitro* selection of human immuno-deficiency virus type 1 resistant to 3-thiacyttidine inhibitors due to a mutation in the YMDD region of reverse transcriptase
Procl Natl Acad Sci USA (1993); 90 : 5653 – 5656

Tong S. P., Li J. S., Vitvitski L. and Trepò C
Replication capacities of natural and artificial precore stop codon mutants of hepatitis B virus: relevance of pregenome encapsidation signal
Virology (1992) ; 191 : 237-245

Tsui LV, Guidotti LG, Ishikawa T and Chisari FV
Posttranscriptional clearance of hepatitis B virus RNA by cytotoxic T lymphocyte-activated hepatocytes Proc Natl Acad Sci U S A (1995); 92(26):12398-12402.

Twu JS and Schloemer RH
Transcriptional trans-activating function of hepatitis B virus
J Virol (1987); 61:20–30.

Urban S and Gripon P
Inhibition of duck hepatitis B virus infection by a myristoylated pre-S peptide of the large viral surface protein
J Virol (2002); 76(4):1986-1990.

Ulrich PP, Bhat RA, Kelly I, Brunetto MR, Bonino F and Vyas GN.
A precore-defective mutant of hepatitis B virus associated with e antigen-negative chronic liver disease
J Med Virol. 1990 Oct;32(2):109-18.

Vento S, Chen S-H, Giuliani-Piccari G
Cellular immunity to nucleocapsid and pre-S determinants in asymptomatic carriers of Hepatitis B Virus
Immunology (1987); 62: (593-598)

Volz T, Lutgehetmann M, Wachtler P, Jacob A, Quaas A, Murray JM, Dandri M, and Petersen J.
Impaired intrahepatic hepatitis B virus productivity contributes to low viremia in most HBeAg-negative patients.
Gastroenterology. 2007 Sep;133(3):843-52.

Wang HC, Wu HC, Chen CF, Fausto N, Lei HY and Su IJ
Different types of ground glass hepatocytes in chronic hepatitis B virus infection contain specific pre-S mutants that may induce endoplasmic reticulum stress
Am J Pathol. (2003) Dec;163(6):2441-9.

Warner N, Locarnini S, Kuiper M, Bartholomeusz A, Ayres A, Yuen L and Shaw T
The L80I substitution in the reverse transcriptase domain of the hepatitis B virus polymerase is associated with lamivudine resistance and enhanced viral replication *in vitro*
Antimicrob Agents Chemother. (2007) ; 51(7): 2285-2292

Watts NR, Conway JF, Cheng N, Stahl SJ, Belnap DM, Steven AC, Wingfield PT
The morphogenic linker peptide of HBV capsid protein forms a mobile array on the interior surface.
EMBO J. (2002); 21(5):876-84.

Weber M, Bronsema V, Bartos H, Bosserhoff A, Bartenschlager R and Schaller H
Hepadnavirus P protein utilizes a tyrosine residue in the TP domain to prime reverse transcription
J Virol (1994); 68:2994-2999.

Wei X, and Peterson DL
Expression, purification, and characterization of an active RNase H domain of the hepatitis B viral polymerase

J Biol Chem (1996); 271:32617-32622.

Y. Wei, C. Neuveut, P. Tiollais and Buendia MA
Molecular biology of the hepatitis B virus and role of the X gene
Pathologie Biologie (2010); 58: 267–272

Werle-Lapostolle B, Bowden S, Locarnini S, Wursthorn K, Petersen J, Lau G, Trepo C, Marcellin P, Goodman Z, Delaney WE 4th, Xiong S, Brosgart CL, Chen SS, Gibbs CS and Zoulim F
Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy.
Gastroenterology. (2004) Jun;126(7):1750-8.

Will H, Reiser W, Weimer T, Pfaff E, Buscher M, Sprengel R *et al*.
Replication strategy of human hepatitis B virus
J Virol (1987), 61(3):904-911.

Xu Z, and Yen TS
Intracellular retention of surface protein by a hepatitis B virus mutant that releases virion particles
J Virol (1996), 70:133–140

Xu Z, Jensen G and Yen TS
Activation of hepatitis B virus S promoter by the viral large surface protein via induction of stress in the endoplasmic reticulum
J Virol (1997), 71:7387–7392

Yee JK
A liver-specific enhancer in the core promoter region of human hepatitis B virus
Science (1989); 246:658–661.

Yu MW, Hsu FC, Sheen IS, Chu CM, Lin DY, Chen CJ, *et al*.
Prospective study of hepatocellular carcinoma and liver cirrhosis in asymptomatic chronic hepatitis B virus carriers
Am J Epidemiol (1997); 145:1039-1047.

Yuh C-H., Chang Y-L., Ting L-P.
Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus
J Virol (1992); 66, 7 : 4073 - 4084

Zacharakis GH, Koskinas J, Kotsiou S, Papoutselis M, Tzara F, Vafeiadis N, *et al*.
Natural history of chronic HBV infection: A cohort with with up to 12 years folllow-up in North Greece (Part of the Interreg I-II/ECProject)
J Med Virol (2005); 77:173-179.

Zhangh Y and Summers J

Low Dynamic State of Viral Competition in a Chronic Avian Hepadnavirus Infection
J Virology (2000); 74(11): 5257-5265

Zoulim F and Seeger C
Reverse transcription in hepatitis B viruses is primed by a tyrosine residue of the polymerase
J Virol (1994); 68:6-13.

Zoulim F and Locarnini S
Hepatitis B Virus Resistance to Nucleos(t)ide Analogues
Gastroenterology (2009);137:1593–1608

1- CNT: Report 2011 sui trapianti d'organo in Italia e in Europa.

2- Te HS, Jensen DM. Epidemiology of hepatitis B and C viruses: A global overview. Clin Liver Dis 2010, Feb; 14(1):1-21, vii.

3- Hepatitis B Fact sheet N°204, July 2012; World Health Organization.

4- McMillan JS, Shaw T, Angus PW, Locarnini SA. Effect of immunosuppressive and antiviral agents on hepatitis B virus replication *in vitro*. Hepatology 1995; 22:36.

5- Tur-Kaspa R, Shaul Y, Moore DD, *et al*. The glucocorticoid receptor recognizes a specific nucleotide sequence in hepatitis B virus DNA causing increased activity of the HBV enhancer. Virology 1988; 167:630.

6- Kim WR, Poterucha JJ, Kremers WK, *et al*. Outcome of liver transplantation for hepatitis B in the United States. Liver Transpl 2004; 10:968.

7- Starzl TE, Iwatsuki S, Shaw BW Jr, Van Thiel DH, Gartner JC, Zitelli BJ, *et al*. Analysis of liver transplantation. Hepatology 1984;4:47S-9S.

8- Todo S, Demetris AJ, Van Thiel D, Teperman L, Fung JJ, Starzl TE. Orthotopic liver transplantation for patients with hepatitis B virus-related liver disease. Hepatology 1991;13:619-26.

9- Davies SE, Portmann BC, O'Grady JG, Aldis PM, Chaggar K, Alexander GJ, *et al*. Hepatic histological findings after transplantation for chronic hepatitis B virus infection, including a

unique pattern of fibrosing cholestatic hepatitis. Hepatology 1991;13:150-7.

10- Gonzalez SA. Management of recurrent hepatitis C following liver transplantation. Gastroenterol Hepatol (N Y). 2010;6:637-645.

11- Rubín A, Aguilera V, Berenguer M. Liver transplantation and hepatitis C. Clin Res Hepatol Gastroenterol. 2011;35:805-812.

12- Yan Q. Bioinformatics databases and tools in virology research: An overview. In Silico Biol 2008; 8(2):71-85.

13- Te HS, Jensen DM. Epidemiology of hepatitis B and C viruses: A global overview. Clin Liver Dis 2010, Feb; 14(1):1-21, vii.

14- Locarnini SA. Hepatitis B virus surface antigen and polymerase gene variants: Potential virological and clinical significance. Hepatology 1998, Jan; 27(1):294-297.

15- Panjaworayan N, Roessner SK, Firth AE, Brown CM. HBVRegDB: Annotation, comparison, detection and visualization of regulatory elements in hepatitis B virus sequences. Virol J 2007; 4:136.

16- Kramvis A, Kew M, François G. Hepatitis B virus genotypes. Vaccine 2005, Mar 3; 23(19):2409-2423.

17- Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000, Mar; 118(3):554-559.

18- Gnaneshan S, Ijaz S, Moran J, Ramsay M, Green J. HepSEQ: International public health repository for hepatitis B. Nucleic Acids Res 2007, Jan; 35(Database issue):D367-D370.

19- Warner N, Locarnini S. The antiviral drug selected hepatitis B virus rta181t/sw172* mutant has a dominant negative secretion defect and alters the typical profile of viral rebound. Hepatology 2008, Jul; 48(1):88-98.

20- Lai M-W, Yeh C-T. The oncogenic potential of hepatitis B virus rta181t/ surface truncation mutant. Antivir Ther 2008; 13(7):875-879.

21- Lai M-W, Huang S-F, Hsu C-W, Chang M-H, Liaw Y-F, Yeh C-T. Identification of nonsense mutations in hepatitis B virus S gene in patients with hepatocellular carcinoma developed after lamivudine therapy. Antivir Ther 2009; 14(2):249-261.

22- Rhee S-Y, Margeridon-Thermet S, Nguyen MH, Liu TF, Kagan RM, Beggel B, *et al*. Hepatitis B virus reverse transcriptase sequence variant database for sequence analysis and mutation discovery. Antiviral Res 2010, Dec; 88(3):269-275.

23- Beggel B, Neumann-Fraune M, Döring M, Lawyer G, Kaiser R, Verheyen J, Lengauer T. Genotyping hepatitis B virus dual infections using population-based sequence data. J Gen Virol 2012, Jun 6.

24- Yuen LKW, Ayres A, Littlejohn M, Colledge D, Edgely A, Maskill WJ, *et al*. SeqHepB: A sequence analysis program and relational database system for chronic hepatitis B. Antiviral Res 2007, Jul; 75(1):64-74.

25- Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, *et al*. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 2005, Oct; 42(4):962-973.

26- Kuiken C. Designing a low-cost drug resistance database for viral hepatitis. Antivir Ther 2010; 15(3 Pt B):517-520.

27- Combet C, Garnier N, Charavay C, Grando D, Crisan D, Lopez J, *et al*. EuHCVdb: The european hepatitis C virus database. Nucleic Acids Res 2007, Jan; 35(Database issue):D363-D366.

28- Shin-I T, Tanaka Y, Tateno Y, Mizokami M. Development and public release of a comprehensive hepatitis virus database. Hepatol Res 2008, Mar; 38(3):234-243.

29- Manns MP, Cornberg M, Wedemeyer H. Current and future treatment of hepatitis C. Indian Journal of Gastroenterology: Official Journal of the Indian Society of Gastroenterology 2001; 20:C47.

30- Nelson DR, Benhamou Y, Chuang W-L, Lawitz EJ, Rodriguez-Torres M, Flisiak R, *et al*. Albinterferon alfa-2b was not inferior to pegylated interferon-α in a randomized trial of patients with chronic hepatitis C virus genotype 2 or 3. Gastroenterology 2010, Oct; 139(4):1267-1276.

31- Bacon BR, Gordon SC, Lawitz E, Marcellin P, Vierling JM, Zeuzem S, *et al*. Boceprevir for previously treated chronic HCV genotype 1 infection. N Engl J Med 2011, Mar 3; 364(13):1207-1217.

32- Poordad F, McCone J, Bacon BR, Bruno S, Manns MP, Sulkowski MS, *et al*. Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med 2011, Mar 3; 364(13):1195-1206.

33- Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, *et al*. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009, Sep 9; 461(7262):399-401.

34- Kuiken C, Yusim K, Boykin L, Richardson R. The los alamos hepatitis C sequence database. Bioinformatics 2005, Feb 1; 21(3):379-384.

35- Gaudieri S, Rauch A, Pfafferott K, Barnes E, Cheng W, McCaughan G, *et al*. Hepatitis C virus drug resistance and immune-driven adaptations: Relevance to new antiviral therapy. Hepatology 2009, Apr; 49(4):1069-1082.

36- López-Labrador FX, Moya A, Gonzàlez-Candelas F. Mapping natural polymorphisms of hepatitis C virus NS3/4A protease and antiviral resistance to inhibitors in worldwide isolates. Antivir Ther 2008; 13(4):481-494.

Samuel D, Muller R, Alexander G, Fassati L, Ducot B, Benhamou JP, *et al*. Liver transplantation in European patients with the hepatitis B surface antigen. N Engl J Med 1993;329:1842-7.