Hepatitis B Virus RNA in serum and liver tissue – quantification using digital PCR

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Cover illustration : *Genesis* by Kasthuri Prakash

To my dearest Nanapa and Chemmi

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ABSTRACT

Hepatitis B virus (HBV) infection is a global health issue that is responsible for approximately 900,000 deaths each year, by inducing liver cirrhosis and hepatocellular carcinoma (HCC). A few markers are used to classify HBV infection and monitor treatment efficacy, including HBV DNA, surface antigen (HBsAg) and e antigen (HBeAg) in serum as well as HBV DNA and RNA in liver tissue. The recent discovery of the receptor NTCP facilitates *in vitro* studies of HBV.

The aims of this thesis were (I) to characterize a new marker of HBV infection, HBV RNA in serum (II) to investigate *in vitro* the neutralizing effect of HBV encoded subviral (HBsAg) particles (III) to develop and apply a new method to discriminate viral and integrated DNA in liver tissue (IV) to analyze focal differences within the liver of HBV and hepatitis D virus (HDV) and (V) to explore HBV RNA profile in liver biopsies by digital PCR.

High levels of serum HBV RNA was found in the majority of 95 patient samples utilized in this study. This RNA was of full genome length, appeared in fractionation together with HBV DNA. Sequencing data supported that HBV RNA in serum represents virus-like particles with failing reverse transcription of the pregenomic RNA (pgRNA).

The role of subviral particles (SVP) during HBV infection was explored in HepG2-NTCP cell line. The results support that SVP functions as a decoy to neutralize antibodies synthesized by the host.

A novel droplet digital PCR (ddPCR) method was developed and applied on 70 liver biopsies to quantify circular and linear HBV DNA, in order to estimate the amount of integrated HBV DNA in the human genome. A complimentary study on the same material was performed to obtain an RNA profile using ddPCR to amplify six target regions. Together, these results indicate that integrated DNA represents the majority of intrahepatic HBV DNA in late stages of infection and is responsible for maintaining high HBsAg levels in serum. The results also suggest that reduced transcription of pgRNA via a novel mechanism may contribute to low HBV replication in HBeAg-negative phase.

ddPCR analysis of a range of HBV markers was used to study focal differences in infection in 15-30 pieces of liver explant tissue from six patients with HBV or HDV induced cirrhosis. Large differences in focality was observed especially in patients with low degree of viral replication or with HDV coinfection and the results also support expression of S RNA from integrated HBV DNA. HDV infection was less focal with presence of high HDV RNA levels in the absence of HBV.

In summary, this thesis compilation contributes to better understanding of HBV serum and tissue markers and their relationship to replication and integration.

SAMMANFATTNING PÅ SVENSKA

Infektion med hepatit B virus (HBV) är ett globalt hälsoproblem som står för ungefär 900 000 dödsfall årligen genom att orsaka skrumplever och levercancer. De biomarkörer som idag används för att klassificera HBV-infektionen och övervaka behandlingseffekt inkluderar HBV-DNA, ytantigenet HBsAg, e-antigenet HBeAg i serum samt HBV-DNA och HBV-RNA i levervävnad. Nyligen upptäcktes HBV-receptorn, NTCP, vilket underlättar *in vitro*-studier av HBV.

Syftet med denna avhandling var att: (I) karakterisera en ny biomarkör för HBV-infektion (HBV-RNA i serum); (II) undersöka den neutraliserande effekten av HBV-kodade subvirala partiklar (HBsAg) *in vitro*; (III) utveckla och applicera en ny metod för att skilja på viralt och integrerat HBV-DNA i levervävnad; (IV) analysera fokala skillnader i förekomst av HBV och hepatit D-virus (HDV) i levervävnad; (V) utforska HBV-RNA-profiler i leverbiopsier med digital PCR.

I en majoritet av serumprover från 95 patienter uppmättes höga nivåer av HBV-RNA. Detta RNA motsvarade hela HBV-genomets längd och förekom vid fraktionering i partiklar med samma densitet som de med HBV-DNA. Sekvensering indikerade att HBV-RNA i serum motsvarar viruslika partiklar där omvänd transkription av pregenomiskt RNA (pgRNA) ej fungerat.

Funktionen av HBsAg-bärande subvirala partiklar (SVP) i HBV-infektion undersöktes i cellinjen HepG2-NTCP. Resultaten stöder hypotesen att HBsAg/SVP minskar den virusneutraliserade effekten av antikroppar riktade mot virusets ytprotein.

En ny metod, baserad på droplet digital PCR (ddPCR), utvecklades och användes för analys av 70 leverbiopsier. Metoden kvantifierade cirkulärt och linjärt HBV-DNA för att kunna uppskatta mängden integrerat HBV-DNA i det humana genomet. En kompletterande studie på samma material utfördes för att ta fram virus-RNA-profiler genom att med ddPCR amplifiera sex olika målregioner. Sammantaget pekar dessa resultat på att integrerat HBV-DNA utgör den största andelen av intrahepatiskt HBV-DNA

i senare stadier av infektionen, och att detta integrerade DNA uttrycks så att höga nivåer av HBsAg i serum bibehålls. Resultaten tyder också på att nedreglering av pgRNA via en ny mekanism skulle kunna bidra till lägre replikation av HBV i HBeAg-negativa patienter.

ddPCR-analys av flera olika HBV-markörer användes för att studera fokala skillnader i HBV-infektionen i 15-30 bitar av leverexplantat från sex patienter med HBV- eller HDV-orsakad skrumplever. Stora skillnader i fokalitet observerades särskilt i patienter med låggradig virusreplikation och resultaten stöder hypotesen att S-RNA uttrycks från integrerat HBV-DNA. HDV-infektion (hos två av patienerna) var jämntare utspridd i levervävnaden med höga nivåer av HDV-RNA oberoende av HBV.

Sammanfattningsvis bidrar denna avhandling till bättre förståelse av serum- och levermarkörer vid HBV-infektion och deras koppling till replikation och integration.

LIST OF PAPERS

The thesis is based on the following studies, referred to in the text by their roman numerals.

I   **Kasthuri Prakash**, Gustaf E. Rydell, Simon B. Larsson, Maria Andersson, Gunnar Norkrans, Heléne Norder, Magnus Lindh. High serum levels of pregenomic RNA reflect frequently failing reverse transcription in hepatitis B virus particles. Virology Journal 2018; 15 : 86.

II   Gustaf E. Rydell, **Kasthuri Prakash**, Heléne Norder, Magnus Lindh. Hepatitis B surface antigen on subviral particles reduces the neutralizing effect of anti-HBs antibodies on hepatitis B viral particles in vitro. Virology 2017; 509: 67-70.

III   Gustaf E. Rydell, Simon B. Larsson, **Kasthuri Prakash**, Maria Andersson, Heléne Norder, Kristoffer Hellstrand, Gunnar Norkrans, Magnus Lindh. Abundance of non-circular HBV DNA suggests integrations to be the predominant form of viral DNA in chronic hepatitis B. Manuscript.

IV   **Kasthuri Prakash**, Simon B. Larsson, Catarina Skoglund, Gustaf E. Rydell, Johan Ringlander, Maria Andersson, Maria Castedal, Heléne Norder, Magnus Lindh. Intrahepatic focality of hepatitis B infection analysed by quantification of viral RNA in multiple tissue pieces from explanted livers. Manuscript.

V   **Kasthuri Prakash**, Simon B. Larsson, Gustaf E. Rydell, Maria Andersson, Johan Ringlander, Gunnar Norkrans, Heléne Norder, Magnus Lindh. Analysis of HBV RNA in liver biopsies by digital PCR reveals differences in transcript levels, length and origin between e antigen positive and negative individuals. Manuscript.

ABBREVIATIONS

AuAg	Australian Antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
NA	Nucleoside / nucleotide analogues
rcDNA	Relaxed circular DNA
HBcAg	Hepatitis B core antigen
HBsAg	Hepatitis B surface antigen
HBeAg	Hepatitis B "e" antigen
VP	Viral Particles
SVP	Subviral particles
NTCP	Sodium-taurocholate cotransporting polypeptide
cccDNA	Covalently closed circular DNA
PreC RNA / PC	Precore RNA / precore
PgRNA	Pregenomic RNA
dslDNA	Double stranded linear DNA
ssDNA	Single stranded DNA
ALT	Alanine aminotransferase
ORF	Open reading frame
BCP	Basal core promoter
RT	Reverse transcriptase / reverse transcription
HDV	Hepatitis delta virus
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RT-PCR	Reverse transcriptase PCR
ddPCR	Droplet digital PCR
cDNA	Complimentary DNA

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1   INTRODUCTION TO HEPATITIS B VIRUS

In 1967, Dr Blumberg and his associate discovered a new protein in serum of patients that underwent blood transfusions, specifically in Australian aboriginals. This protein named "Australian Antigen" (AuAg) became the first of many discoveries confirming the presence of viral hepatitis [1]. In 1970, Dr Davis S Dane described 42nm virus-like particles in patients with AuAg and named them "Dane Particles" [2] which is now known as Hepatitis B Virus (HBV).

The viral infection caused by HBV still remains a global public health issue in-spite of decades of research and availability of a preventive vaccine since 1982. HBV belongs to the family of hepatotropic DNA viruses that can cause both acute (infection cleared within 6 months of exposure) and chronic (infection that persists >6 months) infection of the liver. The World Health Organization as of 2017 estimates that 2 billion individuals are or have been infected with HBV of which 257 million have a chronic infection [3]. Approximately 900,000 deaths each year is caused by HBV related complications such as liver cirrhosis and hepatocellular carcinoma (HCC) [4].

HBV can be transmitted percutaneously via sharing of blood products, through sexual transmission or by means of vertical transmission, i.e., to a newborn from an infected mother. According to the World Health Organization's (WHO) statistics, about 80-90% of infants and 30-40% of children under the age of 6 who are infected with HBV will develop a chronic disease, but when acquired as adults 95% will clear the infection. The pathogenesis of complications involves immune mediated killing of hepatocytes causing liver injury with subsequent regeneration by clonal expansion of hepatocytes eventually leading to scarification of the liver (fibrosis) and later on cirrhosis and HCC. Other contributing factors for pathogenesis are integration of viral DNA into the host genome and oncogenic effects of viral protein [5]. Patients showing signs of progressive liver damage are given long-term treatment with nucleoside / nucleotide analogues (NA) in order to prevent these complications, but with it arises the possibility for resistant strains to emerge via mutation [6-8].

During the course of this PhD, various aspects of HBV infection was investigated in order to answer some pressing questions. Interesting associations were explored, and intriguing hypotheses are presented as a compilation with the hope that these studies will aid better understanding of disease progression and assist the clinical community in development of new treatments for patients with HBV.

1.1 Molecular structure

HBV is classified as one of the smallest known DNA viruses measuring approximately 42 nm in diameter belonging to the family of Hepadnaviridae viruses. It has a 3.2 kb sized relaxed circular (rcDNA) genome that is partially double stranded (ds) with a complete minus (-) strand and an incomplete plus (+) strand that is covalently bound to a viral polymerase at its 5' end. This genome is enclosed within an icosahedral nucleocapsid core protein (core antigen, HBcAg) that is surrounded by viral surface protein (hepatitis B surface antigen, HBsAg).
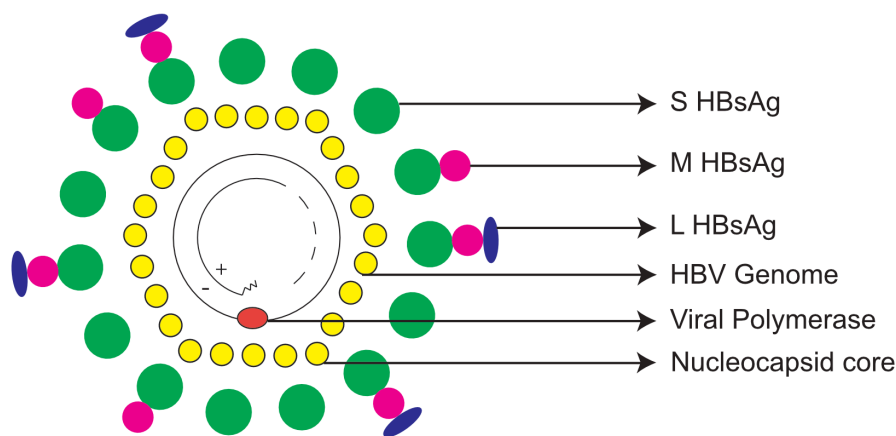
Figure 1: HBV particle containing the partially double stranded genome with complete – strand and an incomplete + strand covalently bound to the viral polymerase

HBV genome has four overlapping reading frames (ORF) that codes for viral proteins (i) precore / core gene for nucleocapsid core and secretory "e" protein (HBeAg) (ii) PreS / S gene for small (S HBsAg), middle (M HBsAg) and large (L HBsAg) envelope proteins (iii) X gene for regulatory X protein and (iv) polymerase gene for viral polymerase.
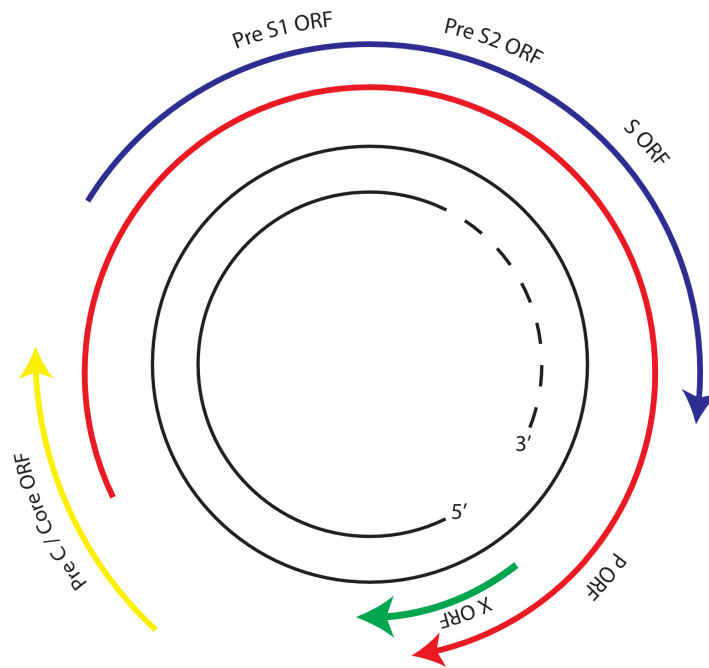
Figure 2: HBV genome that is partially double stranded. Represented here are 4 overlapping reading frames responsible for translation of viral proteins.

Along with HBV DNA containing viral particles (VP), an excess production of 22 nm sized rod and circular empty subviral particles (SVP) comprised of only HBsAg can be detected in the patient's serum.

Figure 3: Sphere and rod forms of HBV subviral particles with large, middle and small surface proteins that lack a viral genome.

1.2 Viral entry and replication

Although presence of hepadnaviruses in extrahepatic tissues such as lymph nodes, kidneys, skin and colon have been detected, the only target for these viruses are hepatocytes [9] because of the presence of cell specific receptors expressed by the hepatocytes.
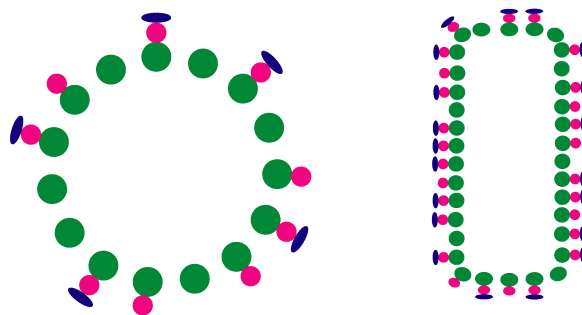
The first step in viral entry involves interaction of the pre S1 domain of viral surface antigen with hepatocyte specific heparin sulphate proteoglycan [10] followed by attachment to the sodium taurocholate co-transporting polypeptide (NTCP) receptor [11]. The uptake of the virus into the hepatocyte is hypothesized to take place via clathrin or caveolin-1 mediated endocytosis through which the encapsidated viral genome is transported into the host cell [12, 13]. The microtubules in the cytoplasm then transport the nucleocapsid containing the viral genome and the polymerase into the cell's nucleus where the capsid disassociates and the rcDNA is released [14]. A sequence of highly precise steps to convert rcDNA into the highly stable mini chromosome cccDNA takes place. The incomplete + strand is elongated to the 5' end of the – strand. After this step, the polymerase is removed followed by the elimination of eight terminally redundant nucleotides from the – strand. At this juncture the 5' and the 3' ends of the + and the – strand can be ligated and supercoiled to form cccDNA [15].

It has been estimated that around 1-50 copies of the cccDNA remain within each infected hepatocyte as mini chromosomes that serves as a transcriptional template for viral replication. Transcription of cccDNA results in the formation of four mRNAs of sizes 3.5kb, 2.4kb, 2.1kb and 0.7kb that are translated into seven viral proteins (Figure 4). The 3.5kb genomic mRNA includes precore (preC) mRNA that is translated into secretory "e antigen" HBeAg protein and pregenomic RNA (pgRNA) that is translated into core and polymerase proteins needed for viral replication. They are larger that genome length because of the presence of terminal redundancies on both 3' and 5' ends. The other transcripts termed subgenomic RNAs are represented by PreS1 mRNA (2.4 kb) that translates into L HBsAg, the PreS2/S mRNA (2.1 kb) for M and S HBsAg, and X mRNA (0.7 kb) for regulatory HBx protein.
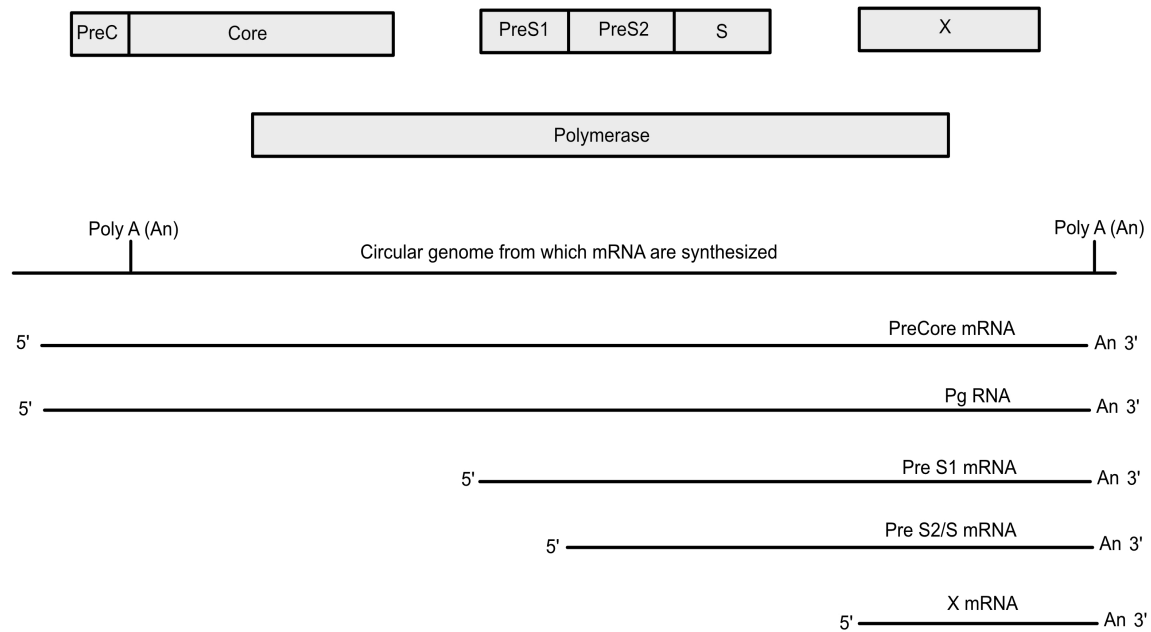
Figure 4 : Genomic placements of different ORFs and genome with polyadenylation at 3' and 5' regions are both represented in a linear form. All RNAs transcripts of different length with a 5' cap and a 3' polyadenylation (An) is represented.

The pgRNA and precore RNA encompasses two highly conserved sequence regions termed as epsilon (ε) that forms a secondary stem loop structure at the 5' and 3' regions. The 5' ε structure directs encapsidation of RNA along with viral polymerase that undergoes reverse transcription to form functionally infectious DNA containing virions [16-18]. However, only pgRNA is encapsidated to form infectious DNA [19] while precore RNA is cleaved in the endoplasmic reticulum and is secreted as HBeAg. Figure 5 describes highly precise steps required to convert pgRNA into infectious viral genome. These replication steps generate encapsidated relaxed circular HBV DNA (rcDNA), that can either (i) acquire HBsAg and be secreted as viral particles or (ii) be recycled within the hepatocyte to increase or maintain cccDNA pool [20].

Along with the mature virion, secretion of empty nucleocapsids, RNA containing viral particles and to some extent double stranded linear forms (dslDNA) has also been observed [21, 22]. During plus strand synthesis, primer translocation failure may cause viral genome to remain linear instead of circularizing to form rcDNA. This double

stranded linear (dslDNA) can recirculate into the hepatocyte nucleus and be integrated into the host chromosome [23].
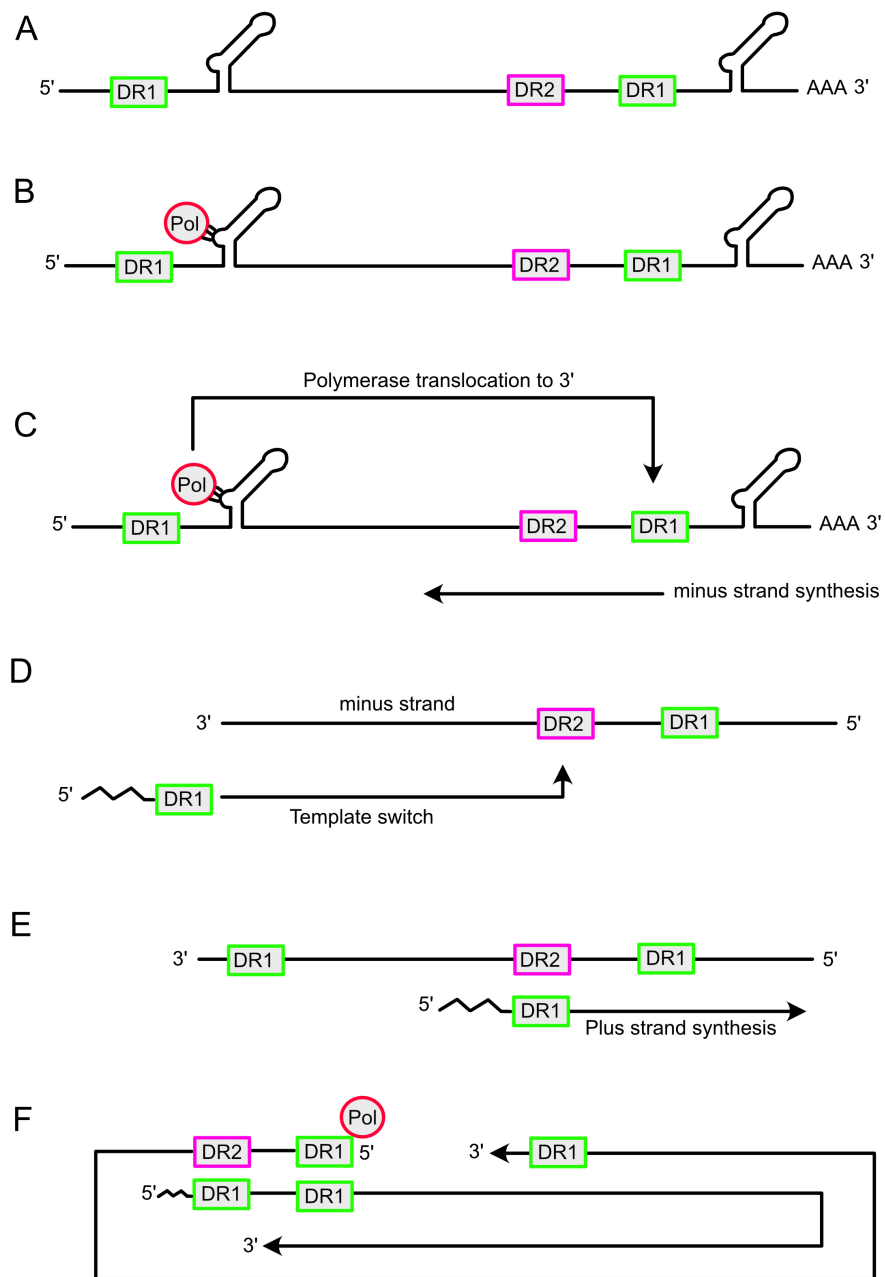
Figure 5 : Sequence of steps following pgRNA encapsidation is represented. (A) shows linear pgRNA within capsid. (B) Binding of polymerase to the ε loop at the 5' end. (C) Synthesis of short oligo followed by translocation of the polymerase and the oligo to the direct repeat 1 (DR1) region where minus strand synthesis is initiated. (D) Minus strand synthesis is accompanied by degradation of the pgRNA template where only DR1 sequence and the short oligo from the 5' end remains. This acts as precursor for the plus strand synthesis and after a template switch (represented in E) plus strand synthesis begins and subsequently circularized (represented in F).
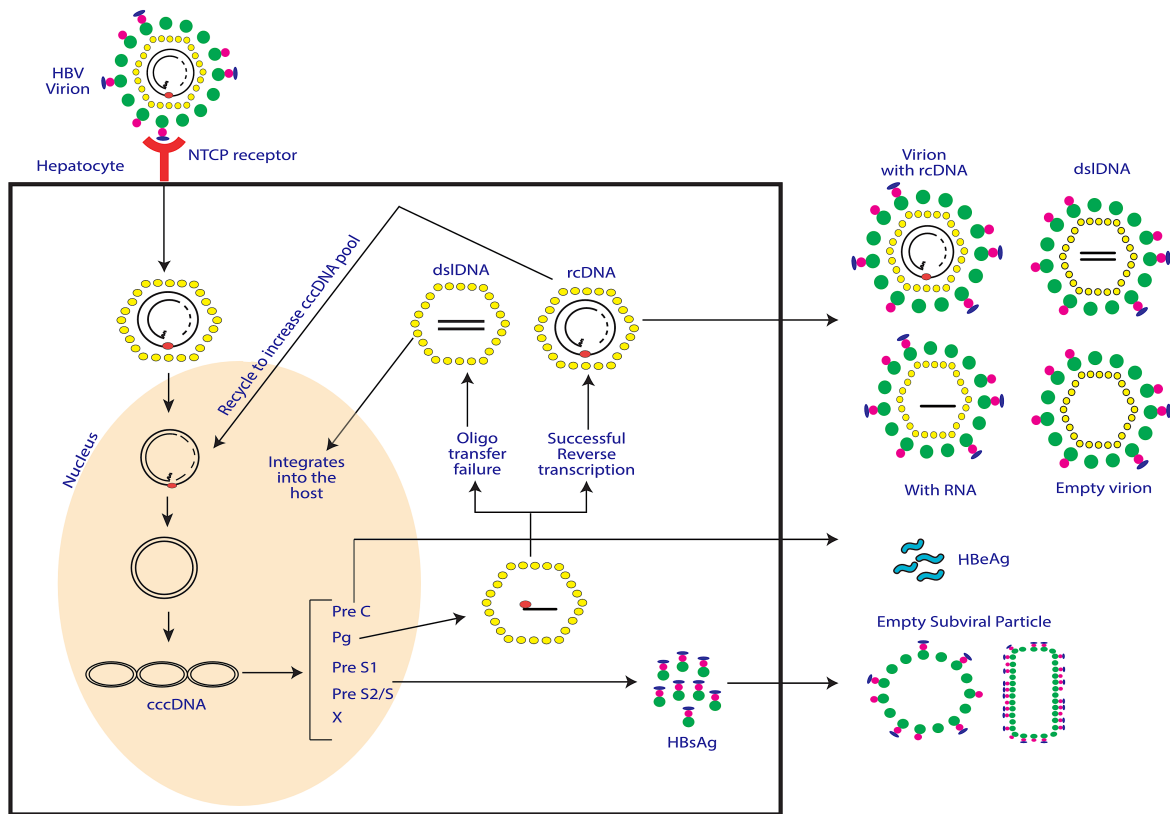
Figure 6: HBV replication cycle

1.3 Serological and intrahepatic markers

Clinical staging of HBV infection is primarily done using quantifiable serological and intrahepatic markers. HBV is what is known as a stealth virus, that depending on the age at which infection is acquired, can replicate for decades before immune flare is initiated. Replication of HBV starts immediately post infection and mature viral DNA along with HBsAg becomes detectable in the blood. Presence of HBsAg for longer than 6 months is defined clinically as "chronic infection" and its clearance is usually accompanied by the appearance of anti-HBs antibodies. Therefore, anti-HBs is an accepted marker to determine end-point for treatment [24]. HBeAg secreted into the blood from infected hepatocytes is a marker to determine viral replication [25, 26]. High HBV DNA levels may however be present also in patients seronegative for HBeAg, because mutations especially in the precore region, may emerge and preclude synthesis of HBeAg, particularly in HBV genotypes B, D or E [27-29]. Presence of HBV RNA in serum first reported in 2001 has been suggested as a diagnostic marker since then, in particular for monitoring the effect

on cccDNA during antiviral treatment [30-33]. During treatment of HBV infection using NA, HBV DNA is reduced to levels below the quantification limit, after which the effect of treatment cannot be assessed. Quantification of HBsAg in serum was proposed to represent cccDNA, but it was later found that this was found to not be true, since the decline of HBsAg levels was minimal. Since HBV RNA is not directly reduced by drugs that inhibit reverse transcription, it should be useful as serum marker representing cccDNA levels in the liver [34].

Intrahepatic cccDNA a highly stable mini-chromosome is the template needed to synthesize HBV RNA transcripts necessary for virus production. Reduction of viral load by two $\log_{10}$ units during HBeAg negative stage is caused by the loss of intrahepatic cccDNA copies [35, 36]. In duck model, it has been found that the number of cccDNA molecules may vary over time, with mean number of cccDNA per infected hepatocyte ranging between 3 and 9 [36, 37]. Because of its role in maintaining chronic infection and ability to avoid drug induced clearance [38, 39], quantifying cccDNA and understanding its relationship to other viral markers are important. Many approaches have been designed to quantify cccDNA in the liver [36, 40, 41] but because of its presence in low concentrations, conventional approaches such as southern blot are not adequately sensitive [42]. A challenge for these assays is to be able to differentiate between cccDNA from rcDNA, therefore a polymerase chain based assay was designed to target the gap in plus strand region that would be present only in cccDNA [36, 43]. In addition to intrahepatic molecular markers such as cccDNA and HBV DNA immunohistochemistry can be performed to visualize HBsAg, HBcAg, HBxAg and pre-S peptides on liver tissue.

Although liver biopsies provide valuable data, this invasive sample collection method has to a large extent been replaced by non-invasive techniques such as elastography assessment of liver fibrosis, a trend that will hamper future research. A drawback to using biopsy tissues is the risk of sampling error, where the material analyzed is not a true representation of the disease or infection state.

1.4 Natural history of infection

Diverse nomenclatures have been defined to describe the clinical phases of HBV infection. Discussed here are phases according to European Association for the Study of Liver (EASL) guidelines on HBV infection management (i) HBeAg positive chronic HBV infection (ii) HBeAg positive chronic hepatitis B (iii) HBeAg negative chronic HBV infection (iv) HBeAg negative chronic hepatitis B (v) HBsAg negative phase [24].

1.4.1 HBeAg positive chronic HBV infection / Immune tolerance

HBV infection that is acquired during early childhood (<2 years), usually through vertical transmission at birth or later horizontal transmission, usually induces weak immune responses for several decades. During this phase of HBV infection (often termed the immune tolerance phase), HBV infects almost all liver cells and replicates actively with typically > 10^7 IU/mL HBV DNA detected in patient serum. The individual remains asymptomatic at this stage and shows normal to mild elevation of alanine aminotransferase (ALT) levels with normal liver histology and detectable HBeAg detected in serum. So far, treatment interventions during immune tolerant phase has not been recommended [44-48].

1.4.2 HBeAg positive chronic hepatitis B

Typically seen to last from the 3rd to 4th decade post infection. This phase is characterized by active immune mediated clearance of HBV infection, during which, infected liver cells are eradicated, causing elevation in ALT levels. The duration and level of cellular damage to the liver determines if the patient gets HBV mediated fibrosis or cirrhosis. Eventually most patients seroconvert to anti-HBe with suppression of HBV DNA. However, some patients fail to control viral HBV replication thereby progressing to HBeAg negative hepatitis.

1.4.3 HBeAg negative CHB / inactive carrier

The detection of anti-HBe along with undetectable or significant reduction of viral DNA and substantial reduction or normalization of ALT levels in serum of CHB patient indicates

clinical remission. HBV DNA levels vary between < 2000 – 20,000 IU/mL at this point and in some patients this can lead to HBsAg seroconversion [49].

1.4.4 HBeAg negative chronic hepatitis

Progression from being an inactive carrier to an individual with chronic infection is highly variable and is associated with factors like age at which infection was acquired, age of HBeAg seroconversion, duration of infection and genotype. Increase in serum HBV DNA and ALT levels have been observed in long term follow up studies in the absence of HBeAg [49-53]. This is mainly due to the presence of mutations in precore region of the viral genome that survive as an escape mutant and causes relapse of chronic infection (discussed in detail under the topic genomic variations)[54, 55].

1.4.5 HBsAg negative phase

During this phase patients undergo loss of HBsAg with or without development of anti-HBs along with anti-HBc antibodies and show normal ALT levels along with undetectable HBV DNA and presence of cccDNA in liver tissue. In some cases development of "Occult HBV" takes place, where patient remains negative for HBsAg in the presence of low or undetectable circulating viral load. Loss HBsAg that occurs either spontaneously or by treatment interference although considered as "functional cure" does not eliminate the probability of HCC. Development of cirrhosis before HBsAg seroclearance has been associated with HCC incidence thereby increasing the need for surveillance after reaching treatment endpoint [56-58].
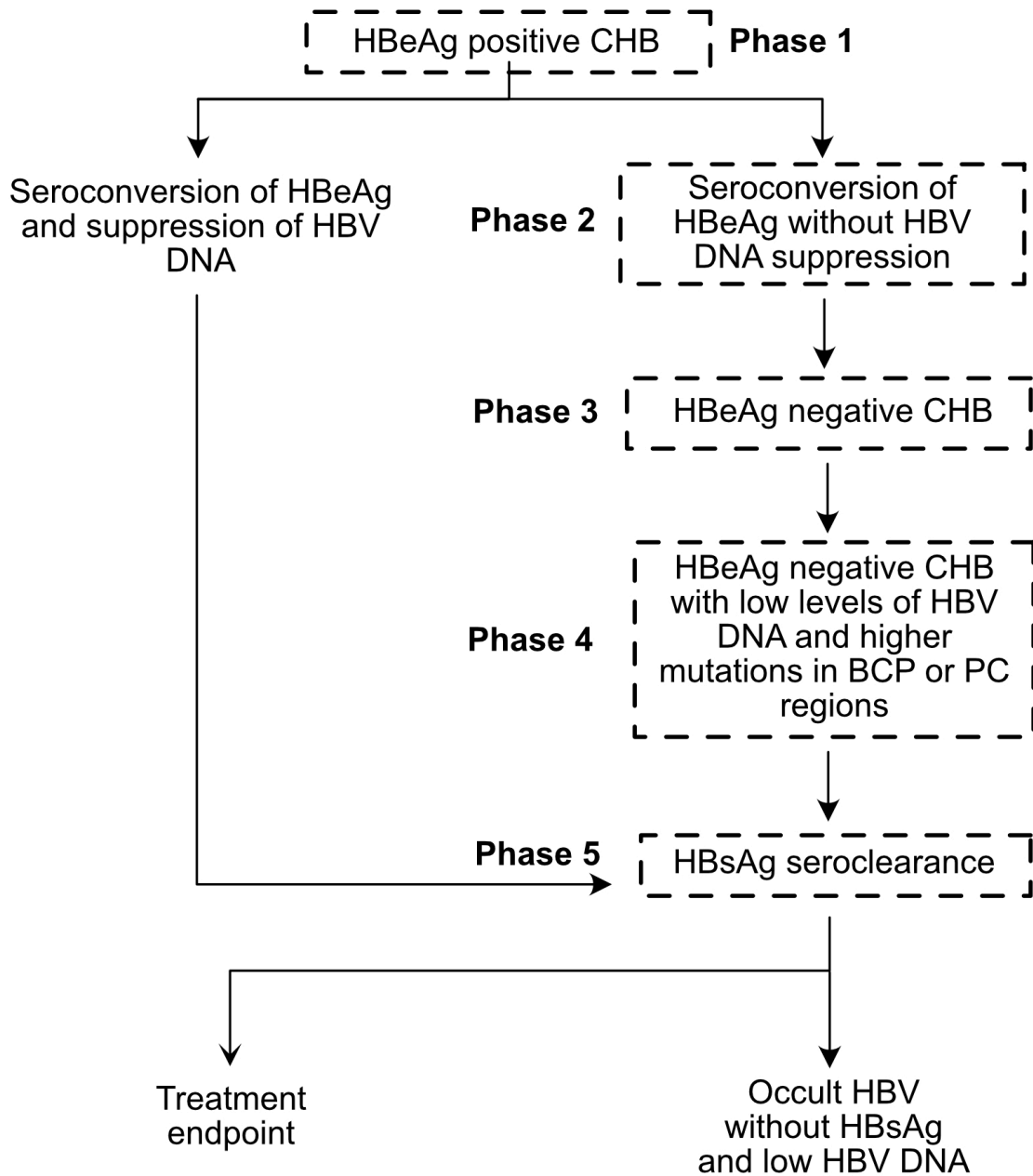
Figure 7 : History of HBV infection as defined by EASL guidelines. There is no clear distinction between these phases and only a rough estimate is defined here.

1.5 Integrations

Similarities between HBV and retroviruses have been discussed including the potential for successfully integrate into the host chromosomes [59]. But unlike retroviruses that need integration for replication, HBV integrations are defined as replicative dead ends

with the potential to produce viral proteins even in the absence of active replication [60, 61]. Primer translocation failure during plus strand synthesis results in the formation of double stranded liner DNA (dslDNA) which separates the precore/core gene from its promoter. Therefore, this form can neither synthesize pregenomic RNA nor generate capsids. Since dslDNA is the precursor for integration [62], only S gene that remains intact is suspected to be active and be capable of producing HBsAg even in the absence of HBV replication as indicated by low HBV DNA. This phenomenon was first observed in hepatocellular carcinoma cell line PLC/PRF/5 [63] and has been described in a recent study conducted on chimpanzees [61]. Integrations have been observed at early stages of infection and has been associated with incidence of HCC and affect survival of infected individuals [64]. Double stranded breaks in host chromosomes have been established as the preferential site for integrations [65], where the host's cellular mechanisms integrates viral into chromosomal DNA damage response (DDR). It has been accepted that DDR pathways lead to HBV integrations, however the exact methodology by which this is achieved is debated. Some studies suggest non-homologous end joining (NHEJ) [65] for double stranded break repair are responsible for viral integrations whereas others argue that it occurs via microhomology-mediated end joining (MMEJ) method [66].

1.6 Genomic variations

1.6.1 Genotypes and Sub-genotypes

The polymerase present in mature HBV lacks proof reading ability causing the virus within the capsid to contain genomic sequences with variations and has resulted in an array of viral strains. When the genomic sequences have a variation >8% they are classified as genotypes and with variation within a genotype between 4-8% they are defined as sub-genotypes. Ten different genotypes and 30 sub-genotypes have been identified so far with a distinct geographical distribution pattern. Variations in these genotypes also extend to their clinical and virological features. Largely discussed are the observations with regards to disease progression [67], treatment response, development of cirrhosis and capability to advance toward HCC [68, 69]. Two studies comparing genotypes B and C showed that the latter has the potential in association with HBeAg to cause severe liver disease [27, 70]. Results from a Taiwanese study observed higher

frequencies of cirrhosis and HCC in genotype C when compared to genotype D [69]. The same study also discussed larger incidents of HCC in non-cirrhotic patients compared to genotype B. In a European study comparing genotype A and D, the former was seen to cause more chronic infections [71] and showed larger incidents of remission post HBeAg seroconversion than the latter [72]. Frequencies of HBV related deaths are reported to be higher in patients with genotype F when compared to genotype A [72].

These studies further press on the importance of understanding differences caused by genotypes on viral load, clinical manifestations and response to treatment; support better comprehension and aid in advancement of clinical practices.

Figure 8: Geographical distribution of HBV genotypes

In addition to the lack of polymerase's proof-reading ability, genetic variability because of mutations are a product of immune selective pressure and drug resistance. A study conducted on woodchucks in 1989 approximated 1 error occurring every 10^7 bases [73, 74]. Selection pressure from immune responses that target wild type viruses may lead to emergence of genomes with mutations. Mutations have been observed in all 4 ORFs with

their contribution to clinical outcomes and attribution to disease progression extensively researched [75].

1.6.2   S ORF mutation

A highly conserved amino acid (aa) region termed as "a" determinant in HBsAg is selected as a target for vaccine against HBV as well as for clinical diagnosis of disease status. Mutations in this region was first documented in a patient that had vertically transmitted HBV even after vaccination with both HBV DNA and anti-HBs presence in serum [76]. Lack of epitope recognition can thus lead to vaccine failure [76] and unreliable results from diagnostic assays [77].

1.6.3   Precore / core ORF mutation

The precore and core regions are responsible for the production of two proteins, the structural core and the secretory 'e' protein. HBeAg is not required for viral production but has been suspected to serve as a decoy to curb immune response against core [78], but immune tolerance is eventually lost and anti-HBe is produced along with reduction and in many patients, clearance of HBV DNA. Failure to clear HBV DNA is seen with emergence of several mutations in the basal core promoter (BCP) and precore (PC) regions suggesting selection due to immune pressure [79]. The most common PC mutation occurs at position 1896 where guanine (G) is replaced by adenine (A) (G1896A) which induces a stop codon in HBeAg sequence and abrogates HBeAg synthesis [80]. Another common mutation is seen at 1899 where guanine (G) is replaced by adenine G1899A and this mutation was found both by itself and along with A1896T mutation [80]. Mutations in the BCP region, 1762 (A1762T) and 1764 (G1764A) have also been associated with contributing to the reduction in HBeAg expression and is correlated with higher HCC incidents [81]. Low HBV DNA quantifications are observed in patients with PC mutations along with lower ALT levels that suggest lesser liver damage compared to infection with wild type virus. However BCP mutations such as thymine replacement with cytosine (C) at 1753 (T1753C) and also at C1766T reduce HBe secretion via transcriptional mechanisms and have shown increase in HBV DNA and ALT levels therefore suggesting higher prevalence of progression towards cirrhosis [82, 83].

1.6.4   X ORF mutation

The X gene is only found in mammalian hepadnavirus and has important functions including ability to establish an infection [84], promote viral replication [85], and support development of HCC [86]. The mutations in the X ORF have been correlated to enhanced progression towards liver cirrhosis (LC) and HCC [83, 87-89].

1.6.5   Polymerase ORF mutation

Polymerase ORF encodes for the polymerase protein that is encapsidated along with pgRNA and is responsible for many steps that are necessary for successful completion of HBV replication cycle [90-92]. A mutation in the RT region of the polymerase ORF can dictate and alter replication events. Investigations regarding naturally occurring mutations have been undertaken in the past few years because it could give information about possible drug resistance [93, 94]. This may alter a clinician's decision about treatment of a patient with these mutations. Associations between different mutations in the RT region and viral load, degree of liver disease have been published [94-96]. RT inhibitors are commonly used for the treatment of HBV but can induce a selection pressure that leads to proliferation of drug-resistant HBV strains. One of the most common mutations induced by the antiviral drug Lamivudine is seen in the tyrosine-methionine-aspartate (YMDD) motif of HBV polymerase around nucleotide 552 [97]. This mutation however is associated with low serum titers of the virus and shows low probability to cause adverse liver disease [8].

1.7   **Treatment**

Two classes of drugs have been approved for the treatment of CHB, immunomodulating agents and nucleoside / nucleotide analogues in order to reduce viral load during infection to achieve seroconversion of HBeAg and within short duration to minimize immune mediated liver damage and prevention of adverse events such as LC and HCC. The drugs used for management of HBV that exists now only manage to keep viral replication under control, and does not eliminate the highly stable mini chromosome, cccDNA [38, 98-100].

1.7.1 Immunomodulators

Interferons are naturally produced proteins that are synthesized by cells in response to viral infections to induce immune response in retaliation. Immunomodulators such as interferon alpha (IFN-α) and Pegylated interferon (PEG-IFN) are used in CHB treatment because of its potential to bind to cellular receptors and activate protein synthesis needed for cellular defense against invading virus [101]. These drugs cannot however be prescribed to patients who are affected by decompensation of the liver such as cirrhosis [102].

1.7.2 Nucleoside / Nucleotide analogues

Drugs under these classifications were initially developed for treatment against herpesvirus and human immunodeficiency virus (HIV) because of their potential to impair polymerase function and can be adapted to HBV treatment. There are currently six drugs that are approved for the treatment of CHB, (i) lamivudine (ii) entecavir (iii) adefovir (iv) tenofovir (v) tenofovir alafenamide (vi) telbivudine. These medications are taken orally and are safe to use however, since they only block RT, long term treatment is required. The formation of drug induced resistant mutations was a main problem earlier, since lamivudine resistance developed in 15-32% of patients within one year of treatment [8]. The currently used drugs tenofovir and entecavir however, have high barriers against development of resistance mutations [103-105].

1.8 Cell culture & animal models

Cell culture and animal models facilitate better understanding of a disease and aids with treatment development. Due to specific host requirements for HBV, there are limitations on animal models that can be used for research [106]. Some animals such as chimpanzees, tupaias, ducks and human chimeric mice have been used to study HBV [107]. Of these, the immune response of chimpanzees are the closest to humans and it was precisely this reason that led to the use of this model to test efficacy of preventive HBV vaccine [108].

Cell culture systems such as hepatoma cell lines HepG2 and Huh7 have been available for a long time now and have supported research regarding transcription and synthesis of

new virion particles [109-111]. Even though they are hepatoma cell lines and are available in plenty, they do not support infection due to the lack of the receptor NTCP. HepRG cells can support infection, however they require complicated differentiation steps in a fashion similar to the induced hepatocytes (iHep) [112]. Comparing these two cell lines, HepRG has lower efficiency of infection than iHep and can support the entire life cycle of the virus. Primary human hepatocytes (PHH) is another system that is used to study HBV infection and although this is the closest condition possible to mimicking natural state of infection, it is less efficient, more expensive and is hugely dependent on donor availability [113]. NTCP was recently discovered as the cellular receptor for HBV / HDV viral entry [11, 114]. The receptor was then expressed in the HepG2 hepatoma cell line to make it susceptible to HBV / HDV infection. This discovery opened up new avenues to explore different facets of infection and replication. Although these cells can be infected there are certain limitations that needs to be addressed. This system requires large quantities of input and does not promote long term infection; since this is cancerous cell line, it lacks many of the pathways needed to study host-virus interactions; and post infection only a small amount of cccDNA is expressed; although the cell system can be infected, it needs in addition polyethylene glycol (PEG) to enhance infection by promoting glycosaminoglycan binding and dimethyl sulphoxide (DMSO) to aid infection.

2   INTRODUCTION TO HEPATITIS DELTA VIRUS

In 1977 a new antigen associated with HBV was discovered in patients who were HBsAg positive [115]. It was later in 1980 using chimpanzees as a model system that delta antigen was determined to be a defective infectious agent that interferes with HBV replication [116]. Since then many studies have shown hepatitis delta virus (HDV) to be a satellite virus to HBV that requires surface antigen production from HBV to envelope its genome to gain access into hepatocytes for successful infection [116].

HDV is the smallest known RNA virus that infects mammals [117] and is the only member of the deltavirus genus. Transmission of HDV happens in the same routes as HBV, percutaneously via sharing of blood products or through sexual transmission or in rare cases via vertical transmission. A study in 2003 estimated that 5% of the world's population with HBV also has HDV [118], but according to WHO many countries do not test for HDV co-infection in HBV positive patients and in countries such as Mongolia where 60% of the population tested positive for HBV may also be infected with HDV [3].

HDV may be acquired as a simultaneous infection with HBV causing mild to severe acute hepatitis, where development into chronic HDV infection is rare occurring in less that 5% of the cases [119]. If an individual with chronic HBV is superinfected with HDV, development of chronic HDV infection is almost inevitable, leading to LC more often and faster than in a patient with HBV monoinfection [116, 120].

2.1   HDV genome and replication

HDV is a small single stranded RNA virus with a circular genome containing 1679 nucleotides. With 74% intramolecular base pairing [121], the genome forms a rod like structure that can be found along with delta antigen (small, S-δAg and large, L-δAg).

For viral replication, HDV engages in a mechanism termed as "the double rolling-circle amplification" where it uses the host's cellular polymerase to synthesize a complimentary RNA strand called the antigenomic δ RNA and an mRNA with 5' cap and a polyadenylated

3' end that codes for the delta antigen protein [122]. With the production of antigenome, viral replication continues and the virus is secreted with an envelope containing HBsAg .
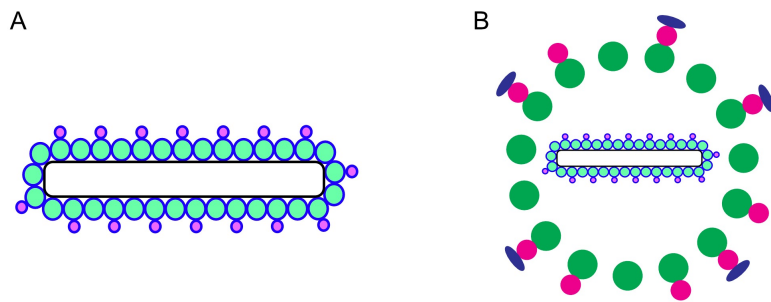

Figure 9: (A) Single stranded HDV with large and small forms of δ-antigen. (B) HDV genome surrounded by S, M and L surface proteins from HBV.
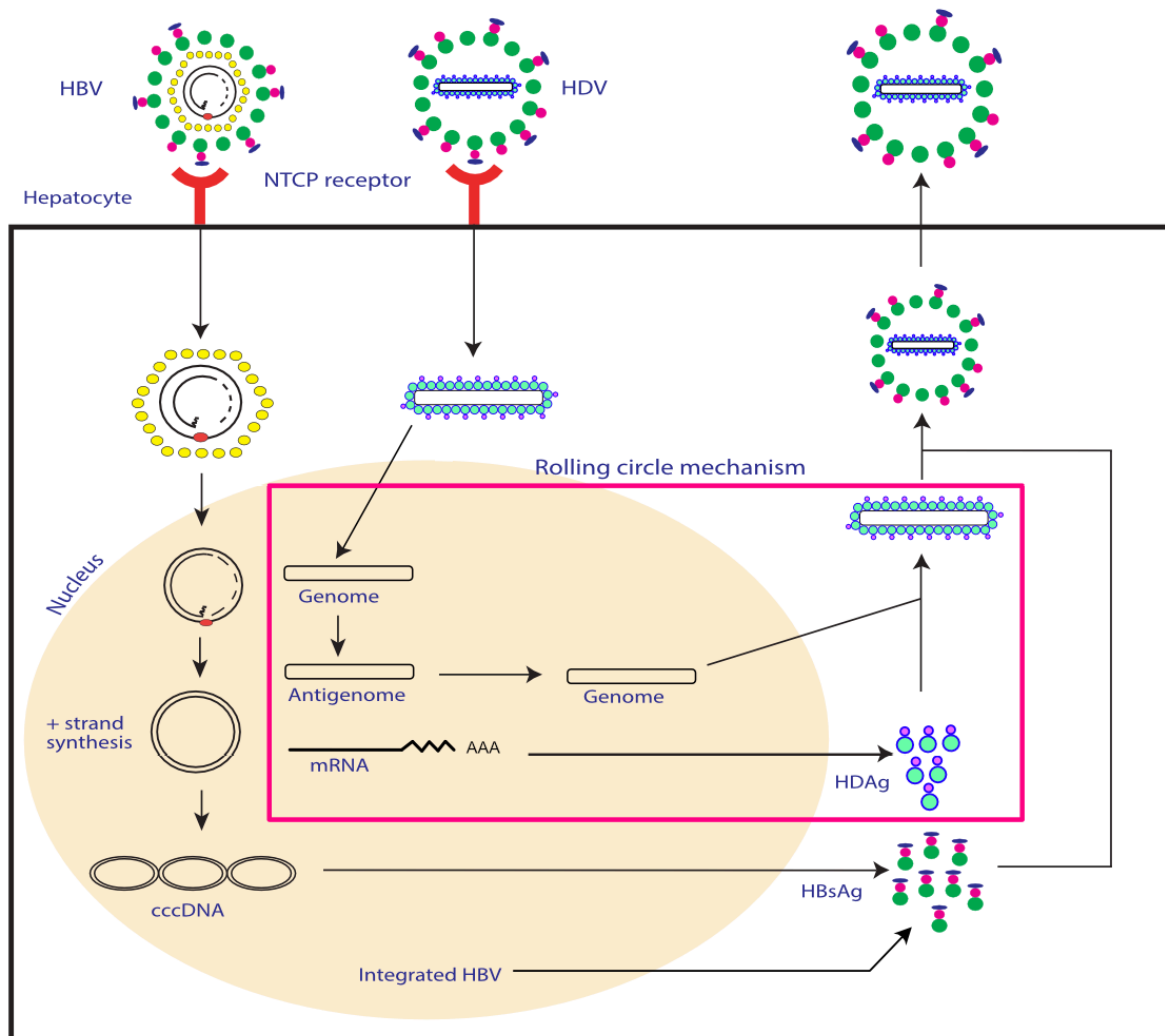

Figure 10 : HDV replication cycle aided by HBsAg synthesis by HBV. Highlighted in pink is the "Rolling circle mechanism" of HDV replication.

Two variants of delta antigen are synthesized from the mRNA and they have two distinct functions. The S-δAg is responsible for viral replication where-as the L-δAg inhibits replication and promotes virion assembly. Therefore, the balance in the production of these proteins is very important to establish a successful infection.

2.2   HBV suppression by HDV

It has been recorded in cell culture and animal model systems that the presence of HDV reduces the expression of HBV [123]. The mechanism through which this is achieved remains unknown, but in cell culture model systems the L-δAg and S-δAg have shown the ability to activate MxA gene to increase IFN-α response towards HBV and activate cellular immune protein synthesis to control infection [124-126].

2.3   Treatment

Since HBsAg is necessary for successful HDV infection and subsequent replication, the current preventive vaccine for HBV that targets and neutralizes the surface antigen works very effectively against HDV [127]. But once infection is actived the treatment options are quite bleak. Since HDV only requires surface antigen from HBV, blocking HBV replication is not effective [128], and the only treatment for HDV currently available is INF-α [129]. Treatment with INF-α has shown significant improvements in histological response and loss of HDV RNA and HBsAg in some patients [130], but long term studies have also shown relapse in most patients with failure to clear HDV [131]. A few new treatment options for chronic HDV infections are underway, including Myrcludex B that blocks HBV/HDV viral entry into hepatocytes [132], but in-vivo it has been shown that HDV can propagate via cell division even in the presence of this drug [133].

3 AIMS

The overall aim of this project was to explore and understand different facets of chronic hepatitis B virus infection by means of studying patient serum and liver tissue from biopsies and explanted liver. Specific aims were as follows:

Paper I

To quantify and characterize HBV RNA in serum in terms of sequence length, particle association, concentration and correlations with HBV RNA in liver tissue and HBV DNA in serum

Paper II

To investigate *in vitro* the influence of subviral particle on infection and explore its effects on anti-HBs.

Paper III

To develop a new method to analyze HBV DNA in liver tissue in order to discriminate viral and integrated DNA.

Paper IV

To analyze intrahepatic focal differences in the patients undergoing transplantation due to HBV or HBV/HDV related liver disease.

Paper IV

To explore HBV RNA profiles in liver biopsies of patients representing different stages of chronic infection by using droplet digital PCR method.

4   MATERIALS AND METHODS

4.1   **Materials**

Paper I : 95 patient serum from a previous study that included 160 samples was stored and available for further analysis [134]. These samples represent both HBeAg positive and negative groups belonging to genotypes A, B, C and D. One anonymous patient sample with HBV DNA concentration of 10^8 IU/mL that also tested positive for HBeAg and HBsAg was included in this study.

Paper II : Two anonymous serum samples one with positive HBeAg and HBsAg with HBV DNA concentration with 10^8 IU/mL and the other negative for HBsAg and anti-HBc from a vaccinated individual were selected. Both these samples were also negative for anti-HCV, anti-HIV, anti-HAV IgM and positive for anti-HAV IgG.

Paper III : 76 stored liver biopsies out of 160 that were included in a previous cross sectional study were available for further analysis [134] of which 70 were positive with digital PCR method. The samples chosen represented genotypes A, B, C and D, with liver damage ranging from mild to severe, either with or without HBeAg. They were also negative for HIV, hepatitis C or D and were untreated for HBV when biopsy was performed.

Paper IV : This study included tissue material obtained from 6 patients undergoing liver transplant because of HBV related chronic liver disease where all patients presented liver cirrhosis. Two patients had HCC; one patient with cirrhosis developed acute liver decompensation brought on by acute-on-chronic hepatitis and presented with very high serum HBV DNA; two patients were also co-infected with HDV. Samples were collected during surgery and subsequently stored at -80°C until it was prepared for analysis.

Paper V : 76 of the 77 liver biopsies that were used in paper III were also used for analysis in this work.
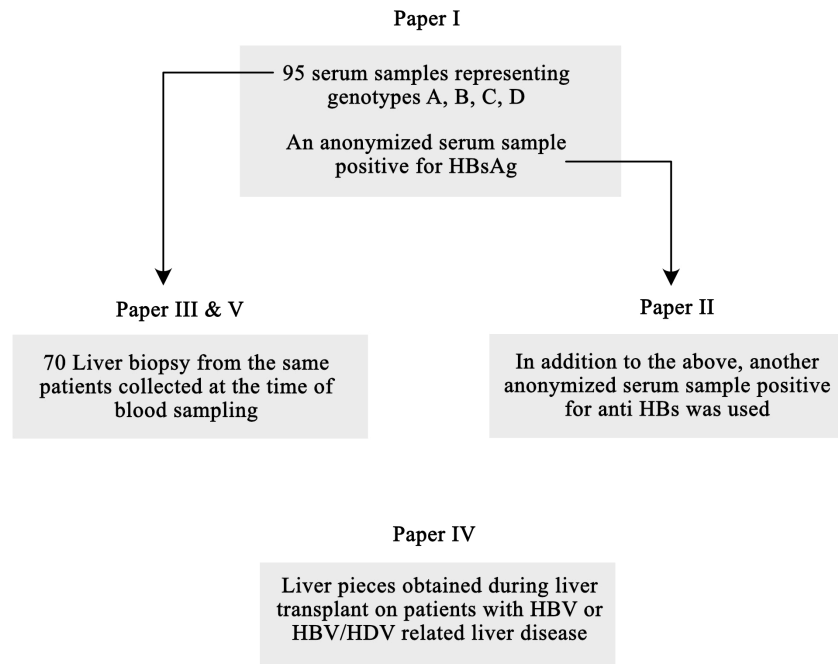
Paper I

95 serum samples representing
genotypes A, B, C, D

An anonymized serum sample
positive for HBsAg

Paper III & V

70 Liver biopsy from the same
patients collected at the time of
blood sampling

Paper II

In addition to the above, another
anonymized serum sample positive
for anti HBs was used

Paper IV

Liver pieces obtained during liver
transplant on patients with HBV or
HBV/HDV related liver disease

Figure 11 : Schematic representation of patient samples used in different papers compile din this thesis.

4.2 Methods

4.2.1 Nucleic acid extraction from serum & tissues

Total nucleic acid extraction was carried out on both tissue and serum samples in an automated MagNA Pure LC system (Roche Applied Science) according to manufacturer's protocol. For serum extraction MagNA Pure LC total nucleic acid isolation kit (Roche Applied Science) was used and for tissue material MagNA Pure LC DNA isolation kit II (Roche) was used.

4.2.2 DNase and RNAse treatment

A portion of the total nucleic acid extracted was treated with TURBO DNA-free kit (Thermo Fisher Scientific) in a rigorous two-step protocol according to manufacturer's instruction to remove contaminating DNA, for RNA analysis.

A Serum sample was treated with RNAse (Thermo Fisher Scientific) prior to and post MagNA Pure LC (Roche Applied Science) extraction to quantify free HBV RNA content in serum.

4.2.3 Polymerase chain reaction

Molecular biology techniques have come a long way since establishing the Watson-Crick model in 1953. The polymerase chain reaction (PCR) method was invented in 1983 and allows amplification of specific DNA segment of interest. It was based on the identification of the species *Thermus aquaticus* in 1969 that requires a temperature of $70 - 79°C$ for its growth [135], and the isolation of its polymerase termed as the "Taq Polymerase" in 1976 [136], an enzyme that could withstand high temperatures. This enzyme is used to copy a specific DNA sequence in a PCR reaction that is supplemented with (i) a target to be amplified (ii) oligonucleotide sequences called primer that are specific to the target (iii) nucleotides needed for creating new target sequences (iv) Taq polymerase to place each nucleotide in the right order for sequence extension.

4.2.3.1 Reverse Transcriptase PCR

The only difference between a standard PCR and a reverse transcriptase PCR (RT-PCR) is the template used for amplification. The former uses double stranded DNA (dsDNA) whereas the latter uses RNA as starting material. During an RT-PCR, an enzyme termed as reverse transcriptase is used to create a complimentary DNA (cDNA) strand to the RNA. This enzyme also contains the RNAse H activity where the RNA template is degraded after copying. Now, using the new cDNA as template a second strand is synthesized to have a complete dsDNA. Amplification of this newly synthesized DNA is carried out using the principles of standard PCR.

4.2.3.2 Quantitative PCR and quantitative RT PCR

A quantitative PCR (qPCR) is a method that is widely used in clinical diagnostics and research alike. There are typically two methods available to quantify the sequence of interest, (i) dye-based method and (ii) probe-based method. In the first method, a green fluorescent dye that intercalates with all double stranded products is used and the increase in fluorescence is measured at each cycle [137]. The technique that is extensively used in this study is probe based PCR method that just like the dye-based method can

measure in "real time" the amplification of target sequence. This procedure makes use of a strand specific probe that is tagged with a fluorophore on one end and a quencher on the other. When kept in close proximity, the quencher quenches that fluorescence emitted by the fluorophore. The polymerase in addition to adding specific nucleotides for strand extension has a second responsibility to remove any double stranded sequences in its path and replace it with available nucleotides this is termed as "exonuclease activity". Therefore, when it encounters the probe, it cleaves the probe thereby separating the fluorophore from the quencher and the fluorescence now emitted can be captured and measured. An advantage to using this technique is the ability to measure multiple targets in the same reaction which was pursued on paper IV and V using the digital PCR method. For an RNA target, reverse transcriptase (RT) enzyme was included to create the first cDNA strand and the standard PCR protocol was followed.

4.2.3.3 Digital PCR

The term Digital PCR (ddPCR) was coined in 1999 when two researchers Bert Vogelstein and Kenneth Kinzler studying rare mutations in colorectal cancer developed a PCR system where multiple reactions were used to study a single target from the same patient sample [138]. The disadvantage with a qPCR system is that it requires a standard curve to determine the initial quantity that was present in the reaction which means small variations in the samples go unnoticed. To avoid this, Vogelstein and Kinzler hybridized two fluorescent probes to the amplified product one that binds to the specific target and the second that binds to all sequences as a control and measured the fluorescence. By doing this they obtained an absolute quantification of the mutation. This came with certain disadvantages like time required for the analysis and the need for large quantity of starting material. The simplified commercialized procedure now enables the separation of each reaction into 20,000 nano droplets using water-emulsion technology. Following a standard PCR, targets amplified is assessed by an automated system where the fluorescence is measured within each droplet. Using Poisson statistics an absolute quantification of the target can be obtained.

4.2.4 Nycodenz fractionation

Fractionation of serum sample in paper I to show the presence of viral RNA within capsids and in paper II to separate viral and subviral particles was carried out using Nycodenz –

a substance used commonly for isolation of many cell types. This substance is widely used because of its non-cytotoxic nature, ability to be autoclaved without compromise and non-interference with downstream analysis [139-141]. This method was utilized in paper I and II where different products synthesized by HBV infection present in serum was separated in different Nycodenz fractions depending on their density. The separated fractions were then subjected to analysis such as TaqMan PCR, RT PCR and *in-vitro* infection of Hep G2 NTCP cells.

4.2.5 Sanger sequencing

Extracted HBV RNA sequence from patient serum was in the presence of RT enzyme and a reverse primer that targets the poly-A tail at the 3' end, used to create a complimentary cDNA strand. The cDNA was then subjected to two PCRs that target the 5' and 3' regions of the cDNA and the amplicons obtained were sequenced using Sanger method to identify mutations that may contribute to the presence of RNA in serum where reverse transcription has not occurred.

4.2.6 In-vitro infection

In vitro cell culture techniques are being developed rigorously to efficiently replicate an infection outside the host in order to improve understanding of the causative agent and develop appropriate treatment. HepG2 NTCP cell line was recently established after the discovery of the receptor NTCP needed for HBV to gain entry into the cells [114]. Hep G2 cells stably transfected with HBV specific receptor NTCP was maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) containing high glucose (Invitrogen). In accordance with the protocol defined by Ni et al., in 2014, the media used for culture maintenance and subsequent infection was substituted with 10% FBS, 100U/mL penicillin, 0.1 mg/mL streptomycin and 2mM L-glutamine [11]. Using a hemocytometer cells were counted and seeded to give approximately $1.25*10^5$ cells per well. 24 hours later when cells reached optimum confluency, HBV serum sample with or without prior incubation with anti-HBs antibodies was added to the cells along with 2.5% dimethyl sulfoxide (DMSO) (Sigma) and 4% polyethylene glycol 800 (PEG800) (Sigma) and incubated to start infection experiment. The cells were then washed three times with DMEM after 24 hours and replaced with fresh culture media (CM). Every second day the

CM from each well was collected and stored for downstream analysis and replaced with fresh CM spiked with 2.5% DMSO until the end of the experiment.

4.2.7   Architect Assay

This technique was used to quantify HBeAg secreted by infected HepG2 NTCP cells. In this two-step immunoassay, the sample is first flooded with anti-HBe coated microparticles followed by introduction to acridinium labeled conjugated anti-HBe. A washing step is included after both stages to remove any unbound products. The chemiluminescence that is produced by the bound, labelled target is captured and compared to signal cut off ratio measured during calibration of the instrument to obtain a quantification of HBeAg in CM from infected cells.

5 RESULTS AND DISCUSSION

5.1 Paper I

NAs used for CHB treatment, block polymerase activity that leads to rapid reduction of HBV DNA, a serological marker quantified to monitor disease progression [24] as well as a predictor for liver cirrhosis and HCC development [142]. HBV RNA however, is not affected by the treatment and therefore can be used as a marker of the infection in the liver [31-33] in addition to serum HBsAg [143-145].

In order to characterize HBV RNA detected in serum, density-based fractionation was performed using six concentrations of Nycodenz gradients (8, 16, 25, 33, 42, and 50 wt%) followed by quantification using real time PCR. HBV RNA and DNA particles were detected in the same fractions suggesting similar densities (figure 12 A). An earlier study using sucrose gradient to fractionate HBV DNA and RNA post entecavir treatment has shown similar results [146]. Since mature HBV virions are present within nucleocapsids, treatment with detergent Tween-80 disrupted these capsids causing the peak fraction containing HBV DNA to move toward higher Nycodenz concentration (figure 12 B). A similar pattern in RNA suggested presence of RNA within enveloped capsids (figure 12 B).

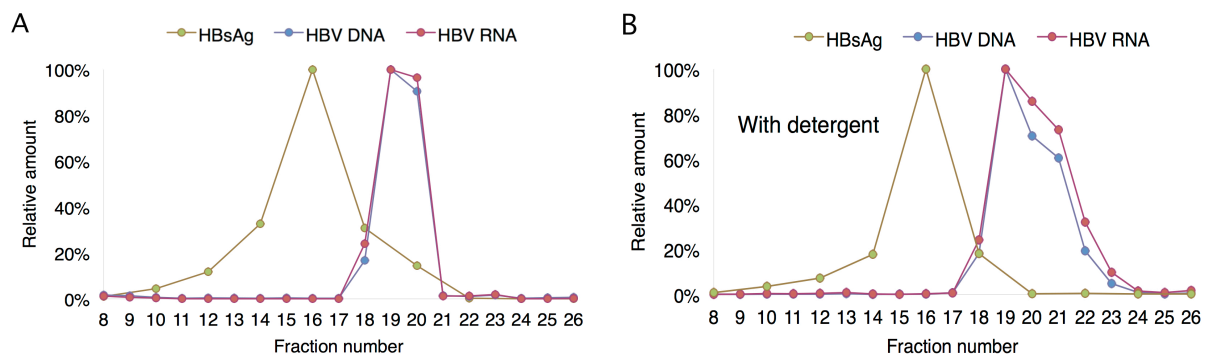*Figure 12 : (A) Nycodenz gradient centrifugation of serum sample showing separation of HBV DNA and RNA (B) Nycodenz fractionation of HBV positive serum after Tween-80 treatment.*

HBV DNA and RNA were quantified in serum collect from 95 patients chronically infected with HBV. The levels of HBV RNA present were similar to HBV DNA in both HBeAg positive and negative patient groups (figure 13 A).
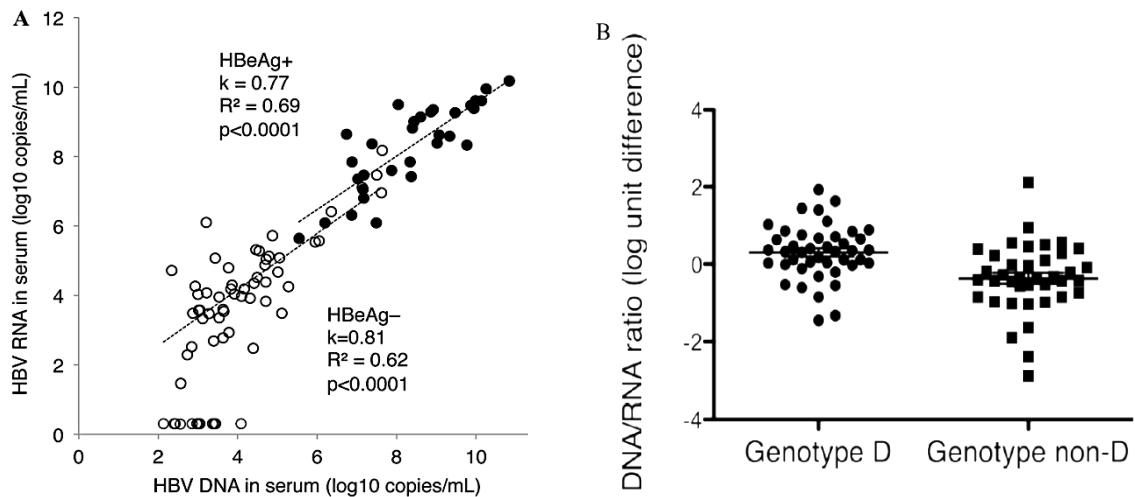
Figure 13 : (A) correlation between HBV DNA and RNA present in serum (B) Genotype D showing significantly higher ratio between HBV DNA and RNA in comparison with non-genotype D samples (p = 0.0001).

Sequencing was used to characterize the 3' part of HBV RNA from serum. First cDNA from was synthesized by using a primer with tag sequence targeting the polyadenylated 3' end. This was followed up with a forward primer at nt 1550 and a reverse primer targeting tag sequence inserted during cDNA synthesis. Agarose gel electrophoresis of the PCR products (figure 14 A) indicated that only a small proportion of RNA was prematurely polyadenylated. In order to assess if the HBV RNA was of full length, real time PCR targeting core at 5' region and X at 3' region was performed on the cDNA generated by the poly-T reverse primer (figure 14 B). The results showing similar quantities suggest that the HBV RNA present in serum was of full (genome) length.

From these results we concluded that HBV RNA in serum was pgRNA inside capsids that probably were enveloped, likely because the reverse transcription step had failed. To study if this might be due to mutations in the ε sequence, Sanger sequencing was performed on 3 samples after synthesizing cDNA using a reverse primer targeting the 3' polyadenylation region. A correct ε sequence is necessary for encapsidation [17, 147] and to initiate reverse transcription [18]. No mutations that would influence the function of the ε sequence was detected. Yet, sequence variation might be of importance for the failure of reverse transcription, because the ratio between HBV DNA and HBV RNA was higher in genotype D in comparison to genotype A, B or C (figure 13 B).
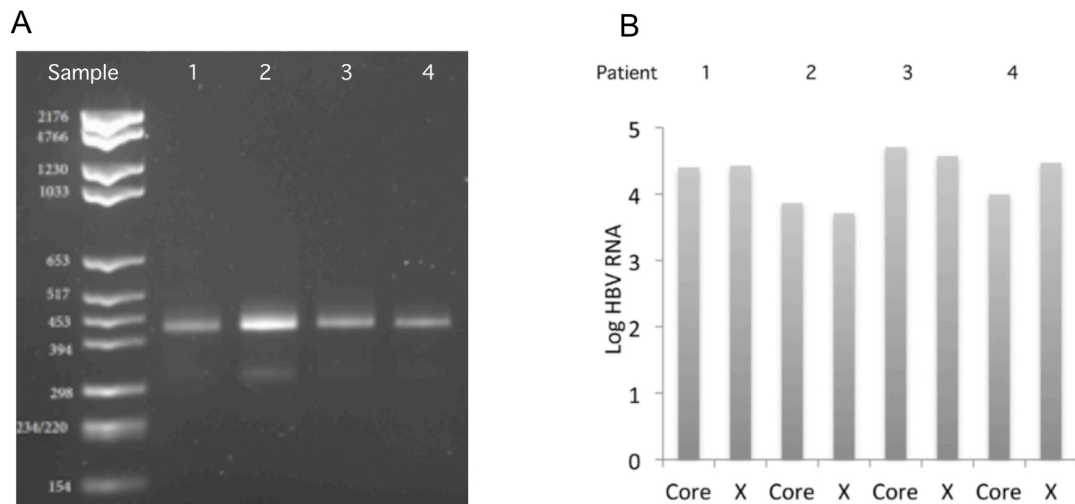
Figure 14 : (A) Agarose gel electrophoresis showing PCR products obtained from serum sample (B) Real time PCR targeting 5' and 3' genomic sequences (core and X) after cDNA targeting polyadenylated tail at 3' region.
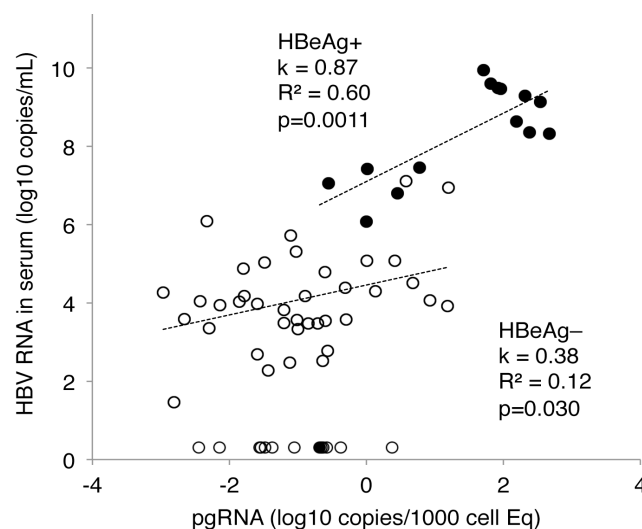
Figure 15 : Low correlation between pgRNA in liver to HBV RNA present in serum for HBeAg positive and negative patients.

The levels of HBV RNA in liver tissue and serum correlated significantly, although less in the HBeAg-negative group (Figure 15). Comparing results in figure 13 A to data in figure 15, shows that the correlation between levels of HBV DNA and HBV RNA in serum was stronger than between pgRNA in liver and HBV RNA in serum. This shows that secretion of virus-like particles with HBV RNA is linked to secretion of mature viral particles (containing HBV DNA), rather than representing intrahepatic RNA levels.

In conclusion, the results presented in this article represent high concentrations of encapsidated, full genomic length RNA in serum and encourages further research to explain failure of reverse transcription.

5.2 **Paper II**

Excess production of SVP during HBV infection in comparison to genome containing VP has been observed previously at the rate of 2000 SVP for every VP [141, 148]. Both VP and SVP contain the envelope protein HBsAg, however the ratio of preS1 domain that recognizes the cell specific receptor NTCP is higher in VP in comparison to SVP. Excess SVP production has been proposed to act as a decoy to reduce effect of anti-HBs antibody, but no experimental proof to support this hypothesis is available.

In order to study the effects of SVP, Nycodenz fractionation of patient serum showing signs of high viral replication was carried out to separate SVP from VP, to be used for subsequent infection on HepG2-NTCP cells. Serum from an individual with high anti-HBs levels after being vaccinated for HBV was also used in this study to assess effects of SVP on host antibodies. HBeAg produced and secreted by infected HepG2-NTCP cells were measured to quantify infectivity.

Anti-HBs was serially diluted and preincubated with (i) unfractionated serum (containing both VP and SVP), (ii) VP and (iii) mixture of VP + SVP in 1:1 ratio. Incubation of VP with anti-HBs showed a marked reduction in the infection of HepG2-NTCP cells (lower HBeAg levels for the straight as compared with the dotted lines in figure 16). In the presence of subviral particles (VP+SVP), this neutralizing effect of anti-HBs was reduced, ie higher concentration of anti-HBs was required to reduce infection. This effect was more pronounced in unfractionated serum (which likely contained SVP in much higher concentrations than VP (figure 16 A and B).

To study if SVP can also block VP entry, different concentrations of SVP were mixed with fixed VP concentrations in the presence or absence of anti-HBs antibodies (table 1 A and B). SVP neutralized activity of anti-HBs by 55% at a concentration of 200 IU/mL, whereas SVP alone, in the absence of anti-HBs, showed minimum reduction in infectivity, suggesting that competition between SVP and VP for binding and cellular entry is not of significant importance. This result is comparable to previously published data where artificially synthesized SVP did not block VP entry [149], but a few studies conducted on

Duck HBV have exhibited SVPs potential to both neutralize anti-HBs and compete for viral entry [150, 151].
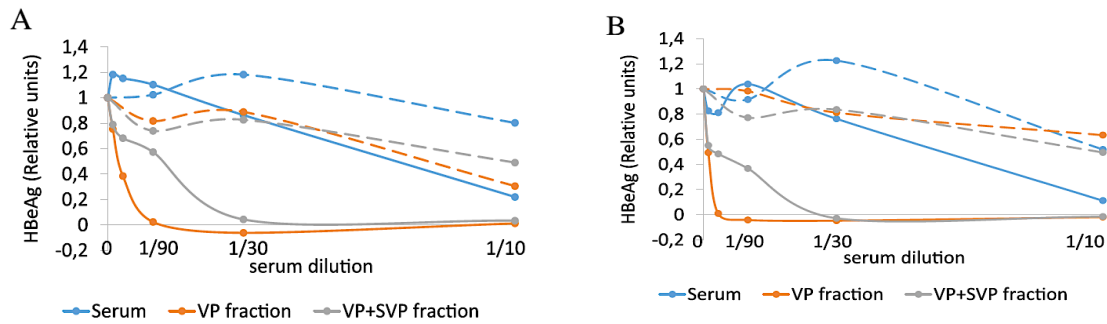
Figure 16 : Serial dilution of anti-HBs concentration starting from (A) 10,000 IU/L or (B) 30,000 IU/L. Straight and dotted lines represent presence or absence of anti-HBs antibodies. Infection potential was quantified using HBeAg secreted by cells.

A

SVP concentration (IU/mL)	0	50	100	200
VP concentration (GE/cell)	3.2	3.2	3.2	3.2
Anti-HBs (IU/L)	250	250	250	250
Reduction in antibody mediated inhibition (%)	0	5	25	55

B

SVP concentration (IU/mL)	0	50	100	200
VP concentration (3.2 GE/cell)	3.5	3.5	3.5	3.5
Anti-HBs (IU/mL)	0	0	0	0
Decrease in infection	-	-	-	Minimal

Table 1 : Experimental design and tabulated result showing higher capacity of SVP to adsorb anti-HBs (A) than block entry of VP (B).

In conclusion, observations from this study suggest that the main role of SVP during infection is to act as a decoy and neutralize antibodies produced by the host.

5.3 Paper III

In this article a method was first developed to quantify the circular and linear forms of intrahepatic DNA from CHB patient biopsies. Nucleotide (nt) position 1830 has been recognized as a preferential site for HBV integrations and therefore a PCR spanning this region would differentiate between integrated and non-integrated transcripts. A discriminating PCR assay was set up using two sets of primers, one spanning nt 1830 (for circular DNA) and another placed in the X region that would amplify all forms of HBV DNA (circular and linear).
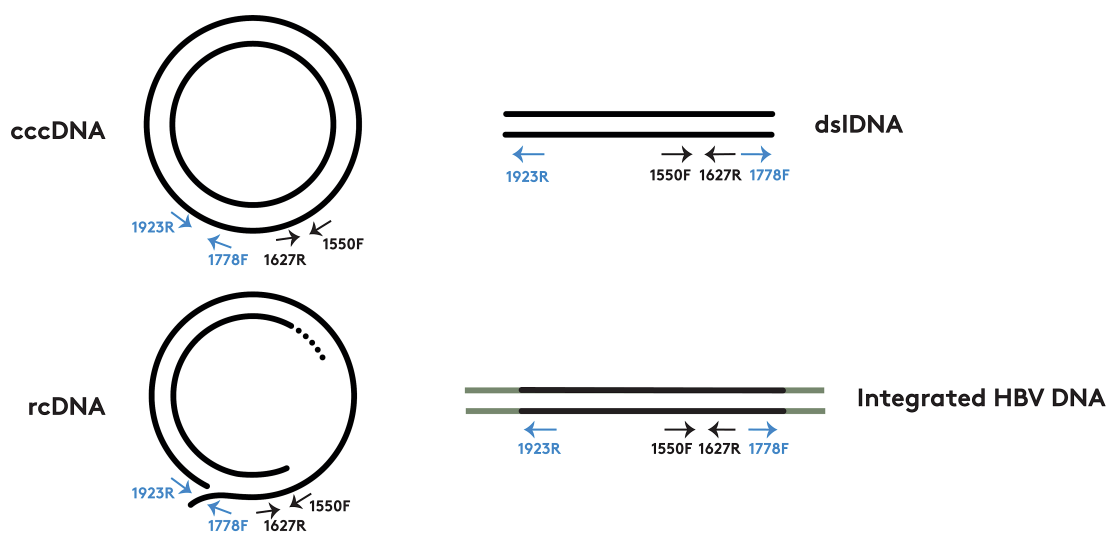
Figure 17 : Discriminating PCR using forward primer at nt 1550 in combination with a reverse primer at nt 1627 to amplify all HBV DNA forms, and forward primer at nt 1776 in combination with reverse primer at nt 1924 to amplify only circular forms of HBV DNA.

This discriminating PCR was applied on 70 liver biopsies from patients in different stages of chronic infection. The results revealed that linear DNA constituted a large fraction of the total intrahepatic HBV DNA in both HBeAg positive (54%) and negative group (89%). The variation between these two groups were statistically significant (P<0.0001) indicating higher quantity of linear DNA in e-negative patients (figure 18).

Control experiments on serum and liver samples showed that linear HBV DNA in liver tissue (but much less in serum) was sensitive to degradation by endonuclease

(benzonase), indicating that it was not encapsidated but rather represents HBV DNA integrated in the chromosomal DNA.
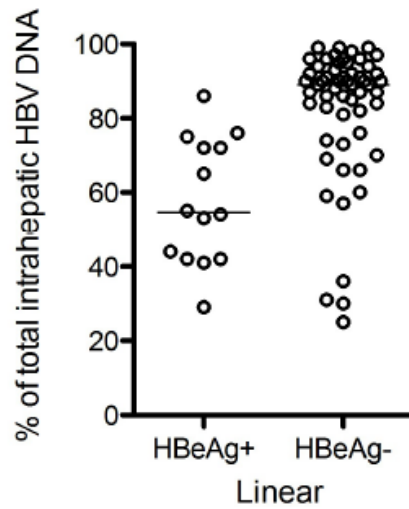
Figure 18 : Percentage of linear DNA in HBeAg positive and negative HBV patients
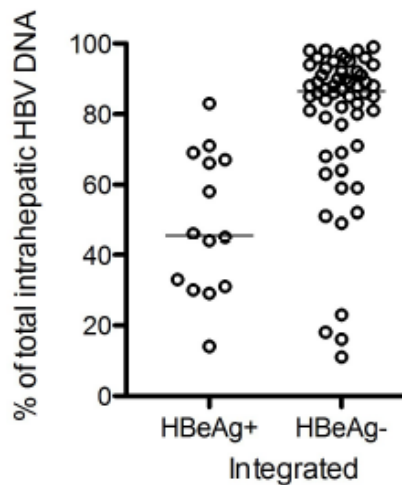
Figure 19 : Percentage of integrated HBV DNA out of total intrahepatic DNA.

To calculate the fraction of integrated HBV DNA out of total intrahepatic DNA, two assumption were made: (i) dslDNA (not integrated) is approximately 20% of rcDNA and (ii) all circular DNA is rcDNA (cccDNA is much lower than rcDNA). These assumptions are supported by published data, including studies estimating cccDNA to be <10% of total circular DNA in liver [36, 39, 142]. By using the formula Integrated DNA = total linear DNA – dslDNA (assumed to constitute 20% of total rcDNA), integration in HBeAg positive and negative patients were calculated to constitute 46% and 87% of total intrahepatic linear

DNA respectively (figure 19). Together, these experiments indicate presence of integrations in large proportions occurring very early during HBV infection.

Previously published large scale study using inverse PCR technique has estimated presence of integrations in approximately about 1% of total liver cells [152]. However due to technical challenges, inverse PCR is said to detect only about 10% of total integration events [153] which suggests approximately 10% of liver cells carry integration. This is lower than our estimates, but almost at a similar level.

The high degree of integration, in particular in HBeAg-negative patients, supports that expression of integrated HBV DNA might contribute to maintaining high levels of serum HBsAg in patients with low rate of replication. Such high HBsAg levels have been noticed in clinical diagnostics for a long time [60], and illustrates the striking difference in immune mediated reduction of HBV DNA and HBsAg. In our samples this was observed as a large (thousand-fold) difference in the HBsAg/HBV DNA ratio in HBeAg-positive as compared with HBeAg-negative patients. As shown in Figure 20 this HBsAg to HBV DNA ratio in serum significantly correlated with the fraction of total HBV DNA in liver tissue that was linear HBV DNA, likely integrated in chromosomal DNA. This finding supports that integrated DNA significantly contributes to HBsAg production in HBeAg-negative patients.
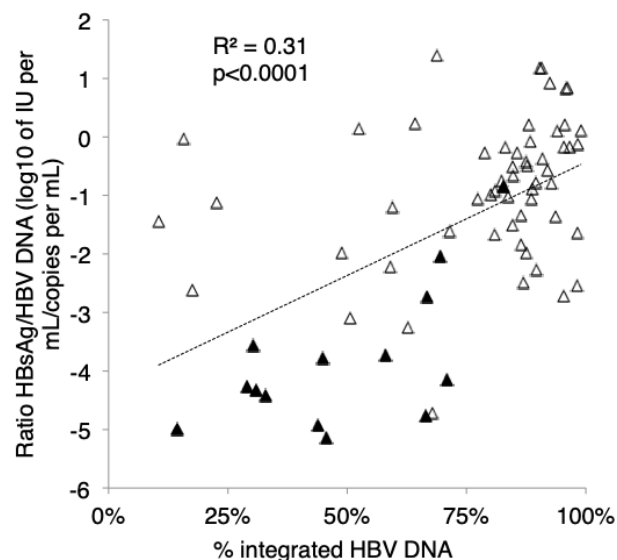
Figure 20 : Significant correlation (p<0.0001) between ratio of HBsAg and HBV DNA in HBeAg positive and negative patient serum and proportion of integration indicative of linear DNA in biopsy.

In conclusion, this study describes a novel and reliable technique to quantify integrations. In addition, this study also contributes experimental proof using natural HBV infection in patients, to support the hypothesis that integrations contribute to maintenance of HBsAg even during low replication rates.

5.4 Paper IV

Liver biopsies were widely used in the past to estimate intrahepatic injury and analyze HBV markers by immunohistochemistry staining or molecular methods. In this study, 15-30 liver tissue from 6 patients undergoing transplantation for HBV related liver disease was analyzed. The aim of the study was to investigate the relation between different HBV markers, in particular those representing integration in the host genome, that was previously hypothesized to be variable by Lindh et al. [154]. This strategy was also employed to estimate the risk of sampling error, that is, the risk that an analyzed piece of liver tissue is not representative of the infection status in the whole liver

All patients were on antiviral therapy of short or long duration. Patients 1-4 had HBV-induced liver cirrhosis, but the infection activity differed greatly. Patients 1 and 3 had recently experienced reactivation of hepatitis with serum levels of HBV DNA at ≈ 8 log IU/mL prior to initiation of entecavir or tenofovir treatment three weeks and four months, respectively, before transplantation. Patient 2 had an infection that had been highly active for a long time and had relatively high levels of HBV DNA in serum as a result of antiviral resistance. Patient 4 had low-active infection and had been on tenofovir for > two years. Patients 5 and 6 had HBV/HDV co-infection with low HBV DNA and relatively high HDV-RNA levels in serum.

As shown in Figure 21, the degree of infection focality differed markedly between patients; between viruses; and between different HBV viral markers. For HBV infection, the most abundant marker was S RNA, followed by core RNA, total HBV DNA and cccDNA. Patient 1 expressed the highest levels of all HBV markers, and had relatively small variation in the distribution of infection (all markers detected in all pieces, with CV ranging between 4.4% and 8.6%). Patients 2 and 3 (P3) had lower levels and higher CV values for both HBV DNA and RNA, but these markers were still detected in all pieces. Patient 4 (P4) (on tenofovir for >2 years before transplantation) had low HBV DNA in most pieces and much higher CV values (Table 2).
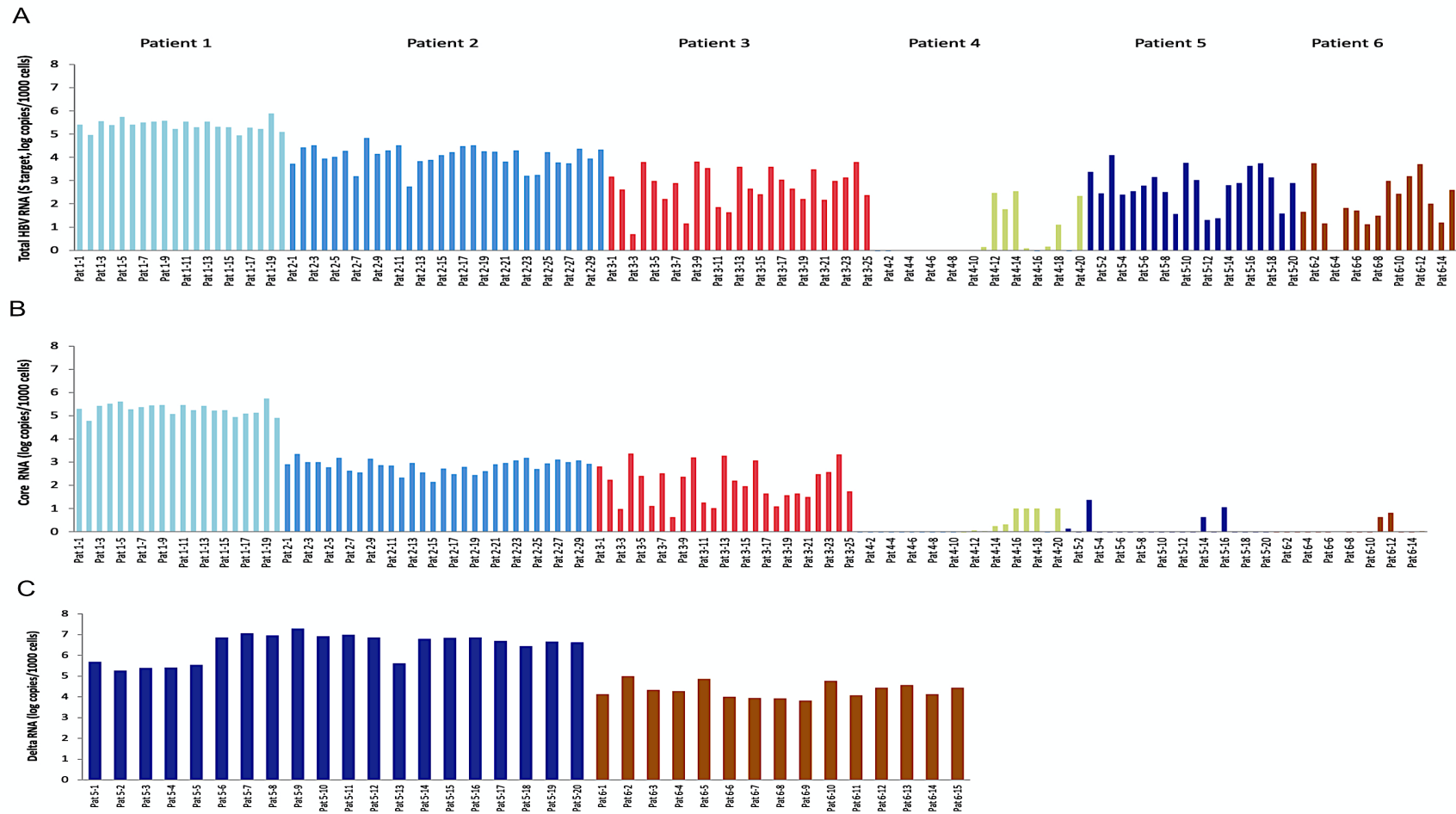
Figure 21 : Quantification of (A) total HBV RNA measured by S transcript PCR and (B) core RNA (C) Delta RNA in patient 5 (in blue) and patient 6 (in brown).

	Patient 1	Patient 2	Patient 3	Patient 4
Indication for transplantation	Acute liver failure, cirrhosis	Cirrhosis	HCC and cirrhosis	Cirrhosis
Age at transplantation	43.6	50.5	54.8	34
Geographic origin	East Africa	Balkan	Middle East	East Africa
Antiviral treatment	Entecavir, 3 weeks	Lamivudine, resistance[a]	Tenofovir 4 months	Tenofovir >2 years
HBsAg serum (log IU/mL)	4.16	3.79	3.61	1.00
HBV DNA serum (log IU/mL)	6.64[b]	5.55	2.09[c]	Neg
Liver tissue data				
Pieces analyzed	20	30	25	20
Cells per piece, median (IQR)	2735	1962	2900	5940
HBV DNA, log copies/1000 cells				
Total (S target)	3.69 (3.07-4.24)	2.41 (1.66-2.83)	1.32 (0.59-1.72)	0.14 (-0.32-1.72)
Coefficient of variation	8.6%	11%	22%	135%
Pieces PCR positive	20 (100%)	30 (100%)	25 (100%)	16 (80%)
cccDNA, log copies/1000 cells	3.27 (2.78-3.66)	1.04 (0.31-1.91)	0.84 (-0.02-1.45)	< -1
Coefficient of variation	7.3 %	33%	48%	
Pieces PCR positive	20 (100%)	30 (100%)	24 (96%)	0
HBV RNA, log copies/1000 cells				
S target	5.40 (4.96-5.89)	4.24 (2.75-4.83)	2.65 (0.69-3.82)	0.15 (<-1-2.54)
Coefficient of variation	4.4%	12%	30%	187%
Pieces PCR positive	20 (100%)	30 (100%)	25 (100%)	12 (60%)
Core target	5.29 (5.11-5.45)	2.78 (2.55-2.97)	2.24 (1.12-2.81)	< -1
Coefficient of variation	4.7%	10%	40%	110%
Pieces PCR positive	20 (100%)	30 (100%)	25 (100%)	9 (45%)

[a] M204V mutation in the reverse transcriptase region of HBV. [b] The HBV DNA level was 8.19 log IU/mL when entecavir treatment was initiated 3 weeks before transplantation. [c] The HBV DNA level was 7.90 log IU/mL when tenofovir treatment was initiated 4 months before transplantation.
Median and (range) values. CV, Coefficient of variation=standard deviation/mean.

Table 2 : Characteristics of four liver transplanted patients with HBV infection and results from ddPCR analyses of liver explant tissue piec

	Patient 5	Patient 6
Indication for transplantation	Cirrhosis	HCC and cirrhosis
Age at transplantation	53.3	47.2
Sex	Male	Male
Geographic origin	Middle East	Middle East
Antiviral treatment	Tenofovir 2 years	Entecavir 2 months
HBsAg serum (log IU/mL)	3.27	2.86
HBV DNA serum (log IU/mL)	Neg	Neg
HDV RNA serum (log copies/mL)	4.99	3.94
Liver tissue data		
Pieces analyzed	20	15
Cells per piece, median (IQR)	2130	4721
HBV DNA, log copies/1000 cells		
Total (S target)	1.03 (0.43-2.29)	<1 (-0.08-1.87)
Coefficient of variation	45%	86%
Pieces PCR positive	19 (95%)	7 (47%)
cccDNA, log copies/1000 cells	< -1	<- 1
Coefficient of variation		
Pieces PCR positive	0	0
HBV RNA, log copies/1000 cells		
S target	2.85 (1.31-4.10)	1.91 (1.12-3.74)
Coefficient of variation	29%	42%
Pieces PCR positive	20 (100%)	14 (93%)
Core target	0.75 (0.13-1.38)	< -1
Coefficient of variation	68%	18%
Pieces PCR positive	4 (20%)	2 (13%)
HDV RNA, log copies/1000 cells	6.70 (5.26-7.28)	4.28 (3.82-4.99)
Coefficient of variation	11%	8%
Pieces PCR positive	19 (95%)	15 (100%)

Median and (range) values. Coefficient of variation, CV=standard deviation/mean, calculated for levels in samples with detected target.

Table 3: Characteristics of two liver transplanted patients with HBV/HDV infection and and results from ddPCR analyses of liver explant tissue pieces

Patients 5 and 6 (P5 and P6) had co-infection with HDV low HBV DNA and relatively high HDV RNA in serum. In liver tissue, cccDNA was undetected in all pieces, HBV DNA and core RNA was low or undetected. Yet, S RNA ranged from moderate to high levels in all pieces. HDV RNA was detected at high levels in all pieces (figure 21 and table 3).

The findings in the HDV cases agrees relatively well with those in a previous study comparing intrahepatic markers in 21 HBV/HDV co-infected and 22 HBV monoinfected patients [155]. In that study, liver tissue from HDV-infected patients had lower levels of cccDNA, lower pgRNA/cccDNA and higher S RNA/cccDNA than patients with HBV monoinfection.

The results show significant focal differences in the distribution and activity of both HBV and HDV and demonstrates the risk involved when analyzing a single tissue piece to quantitatively represent intrahepatic levels especially in patients with low viral load. The risk of obtaining false negative results was relatively low except for HBV markers in tissue with low HBV load (such as often seen when there is HDV co-infection)

In the following figures, markers are presented pairwise in order to show correlations and to display differences in more detail by showing values for individual pieces. Figure 22 shows that HBV core RNA and S RNA values correlated strongly in pieces from Patients 1 and 3. This probably reflects that in these patients, core and S RNA were mainly transcribed from cccDNA, and at similar rates in different pieces. By contrast, the levels did not correlate in pieces from Patients 2, 4, 5 and 6. In Patient 2, core and S RNA were both present in relatively high amounts, but without correlation. In Patient 4 the lack of correlation might to some extent be due to the poorer analytical precision for low target levels. In Patients 5 and 6 (with HDV co-infection) core RNA was undetectable or much lower than S RNA.

Figure 23 shows reflection of cccDNA on HBV RNA levels (in the three patients where cccDNA was detected, P1-3). Overall, core RNA correlated strongly with cccDNA, whereas for S RNA a correlation was only observed in P1 and P3 but not in P2.

In figure 24 the same data is presented as RNA copies per cccDNA. In Patient 1 who had a highly active infection the core and S RNA levels per cccDNA were similar and at levels around 100 RNA copies per cccDNA with little variation between the pieces. In Patient 2 the core RNA levels were slightly lower with relatively little variation, whereas S RNA levels were much higher and with large variation, from 60 to 8000 S RNA copies per cccDNA.
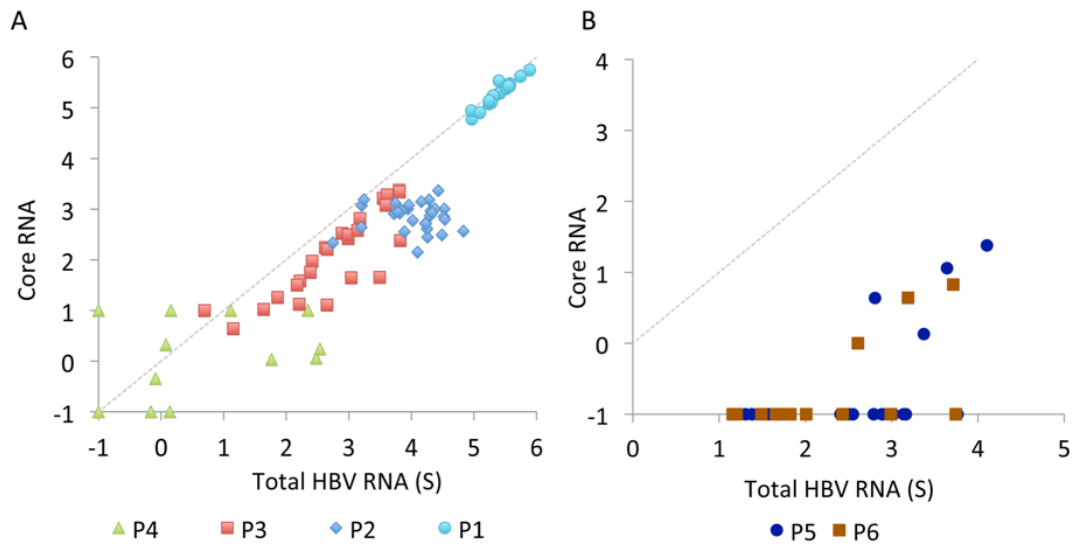
Figure 22. HBV core RNA and S RNA in 15-30 pieces of from four patients (P1-P4) with HBV (A), and two patients (P5-P6) with HDV/HBV coinfection (B). The values are in log$_{10}$ copies/1000 cells.
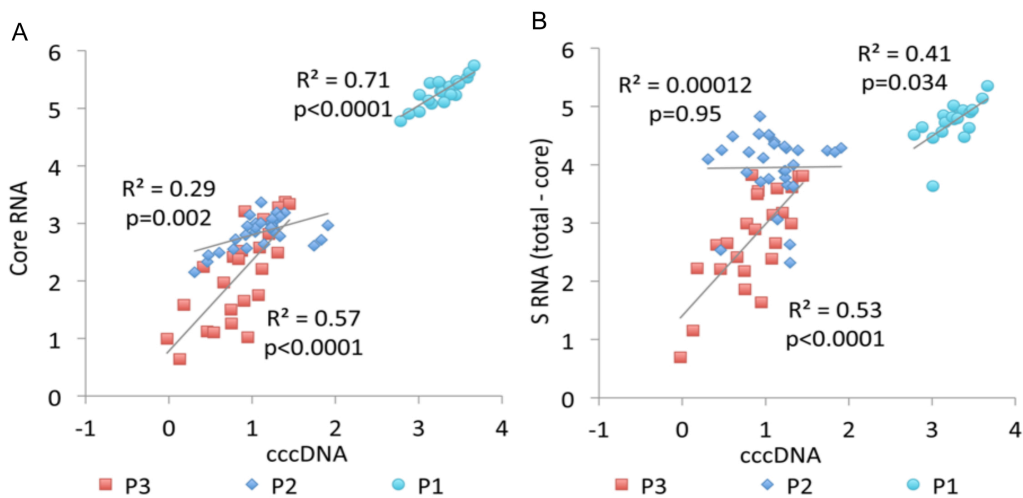

Figure 23 : Correlations between (A) cccDNA and core RNA and (B) cccDNA and S RNA in pieces of explanted liver from P1-3. The values are in log$_{10}$ copies/1000 cells.

Since cccDNA is the only template for core RNA whereas S RNA can be transcribed from cccDNA or from integrated sequences [63, 156], the results suggest that a large proportion of the S RNA in Patient 2 (and to some extent in Patient 3) may be due to transcription from integrated HBV DNA. This observation fits both with our findings in Paper 3 and with data from a recent publication on experimental HBV infection in chimpanzees [61].
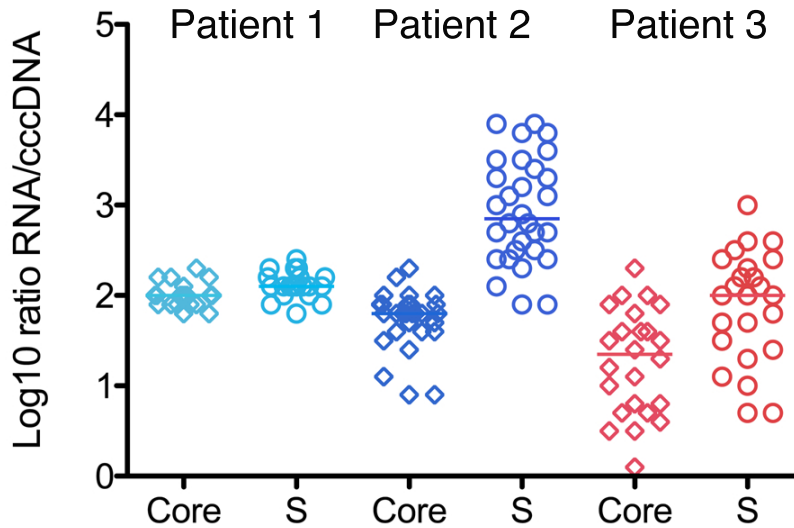
Figure 24 : Levels represented in P1 -3 as copies/cccDNA.

In the two patients with HDV co-infection, there was no correlation observed between HDV RNA and HBV S RNA. Presence of high levels of HDV RNA was observed even when S RNA levels were low, as shown in figure 25A. Accordingly, as shown in figure 25B, the HDV RNA:S RNA ratio was highly different, spanning from 10:1 to >100,000:1. This finding suggests that production of HDV RNA is independent of HBsAg and that HDV RNA might be produced also in cells that do not produce HBsAg. Thus, it seems as if HDV infection may be present in hepatocytes in different forms: (i) a true co-infection together with replicating HBV which provides HBsAg from cccDNA; (ii) in cells without replicating HBV but with integrated HBV DNA that provides HBsAg so that HDV virus particles can be formed and secreted; (iii) HDV RNA in cells without HBsAg production, replicating and translated to delta antigen but not secreted since HBsAg is not provided. This model is supported by data from previous publications. In one study, HBsAg from integrations was shown to support HDV replication *in vitro* [157], and a recent study reported persistence of HDV during hepatocyte replication and its presence in daughter cells that did not contain HBV [133].
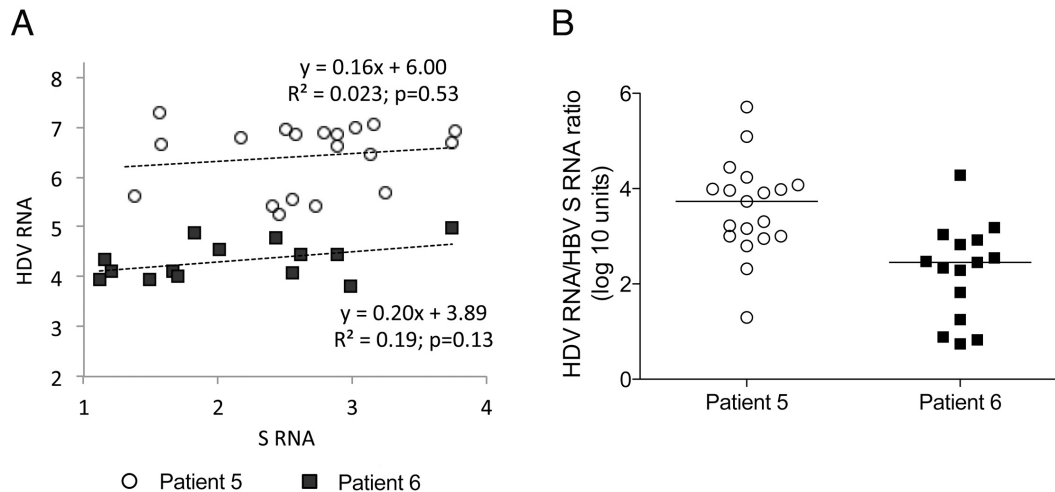
Figure 25 : (A) Lack of correlation between HDV RNA and HBV S RNA in P5 and P6 (B) Varied ratio between HDV RNA and HBV S RNA inP5 and P6.

The model proposes that only a minority of cells capable of producing HBsAg (from cccDNA or transcripts integration) are required for production of high quantities of intrahepatic HDV RNA (as seen by the lack of correlation between HBV S RNA and HDV RNA). This may have clinical implications. It would mean that production of HDV virus particles may occur in a small proportion of hepatocytes that produces HBsAg (from cccDNA or integrations), but that HDV RNA from these particles might enter a much larger number of hepatocytes and drastically increase the production of HDV antigens and expression of their epitopes on the cell surface to provoke harmful immune responses. This model would not contradict the association between HDV RNA in serum and liver damage, but rather propose a mechanism by which the pathogenic effects of secreted HDV could be amplified. If true, this model may help to understand mechanism involved in the development of severe necroinflammation that is often seen during HDV infection. Further research is required to clarify these hypotheses.

In conclusion, the analysis of multiple pieces of liver tissue shows wide range of focality within the same infected liver especially in individuals with low viremia; shows integrations to be a source of HBV S RNA especially in HDV coinfected patients with low HBV viremia; and also proposes novel mechanisms to pave way for future research to understand aggravated liver injury during HBV / HDV co-infection.

5.5 Paper V

In this article, biopsy material representing both HBeAg positive and HBeAg negative infection in 76 patients were used to describe RNA profile of the different transcripts seen during HBV infection. Levels of the transcripts were quantified to also distinguish its source (cccDNA or integrated HV DNA).

The PCR targets are shown in figure 26. These PCRs involve amplification of overlapping targets, and droplet digital PCR technique gives a methodological advantage over real time PCR since absolute quantification can be obtained without amplification efficiency bias.
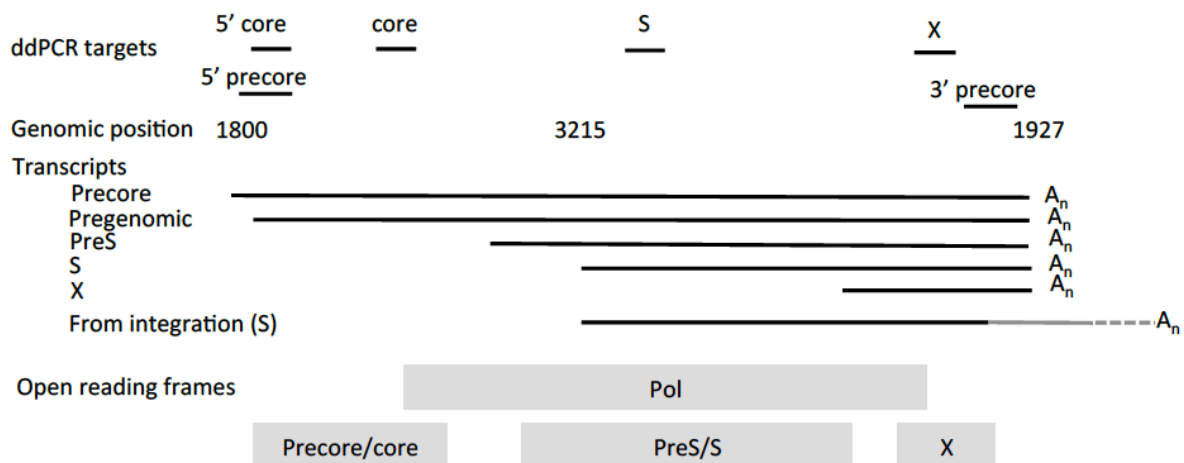

Figure 26 : PCR strategy to quantify different RNA species.

Figure 27 shows levels of all RNA species measured in the 76 biopsies, demonstrating that the RNA profile differed significantly between HBeAg positive and negative patients. In particular, HBeAg-negative patients had lower levels of pgRNA, core RNA and 3' precore regions, a finding that agrees well with recent transcriptome data from liver biopsies taken from experimentally infected chimpanzees [61].
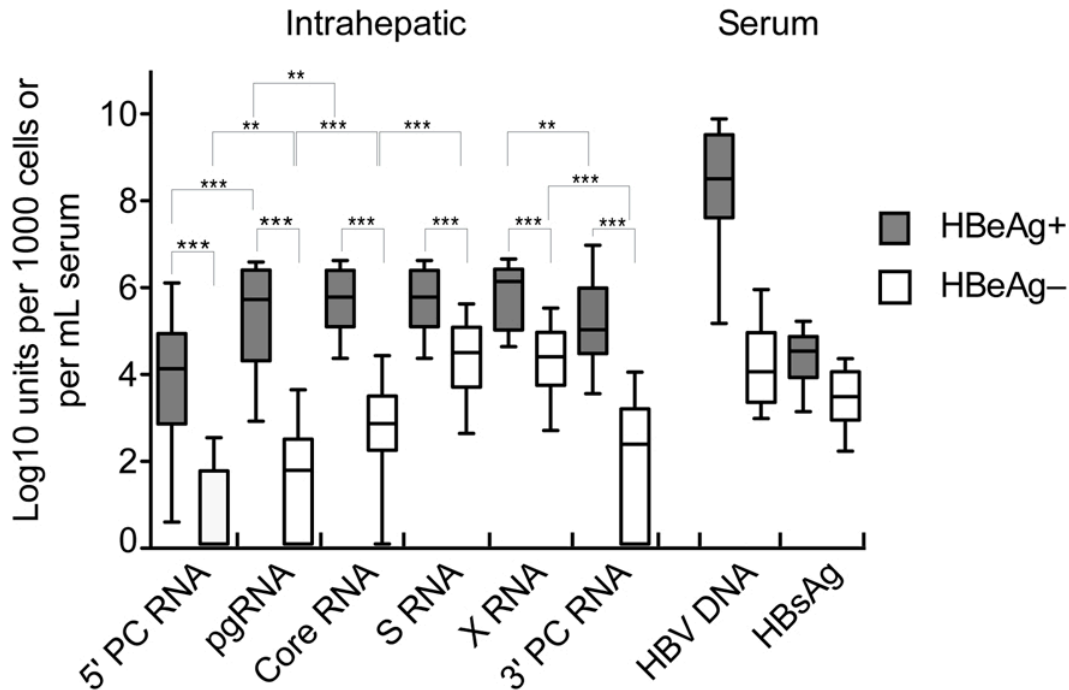
Figure 26 : Intrahepatic HBV RNA profiles in patients positive or negative for HBeAg. The box plots show median levels and interquartile ranges by digital PCR assays targeting different segments of the genome. The levels of HBsAg and HBV DNA in serum are shown for comparison. The whiskers show 10th and 90th percentile. PC, precore; pg, pregenomic. . ***, p<0.0001; **, p<0.001.

Figure 27 shows levels of core and S RNA from 76 patients. The HBeAg-positive patients had similar levels of core and S RNA. By contrast, HBeAg negative patients had 10-100 times lower core than S RNA, illustrated in the figure by the distance from the dotted line. The difference could be due to specific transcriptional down- regulation of core RNA synthesis. Evidence of epigenetic regulation in HBV has been presented [158], but so far no data suggests specific control of core RNA.

Quantification of core RNA was performed using two additional primers, targeting its 5' part ("pgRNA") or a target region downstream of the core gene. Figure 28 A shows lower levels of pgRNA (5' core RNA) than core RNA, in particular in HBeAg negative patients. Figure 29 presents the levels from 28A as box plots, and with the core RNA values obtained after subtraction of pgRNA (performed because the values by the core PCR detects also the pgRNA molecules). This plot clearly demonstrates lower pgRNA levels in HBeAg-negative patients.
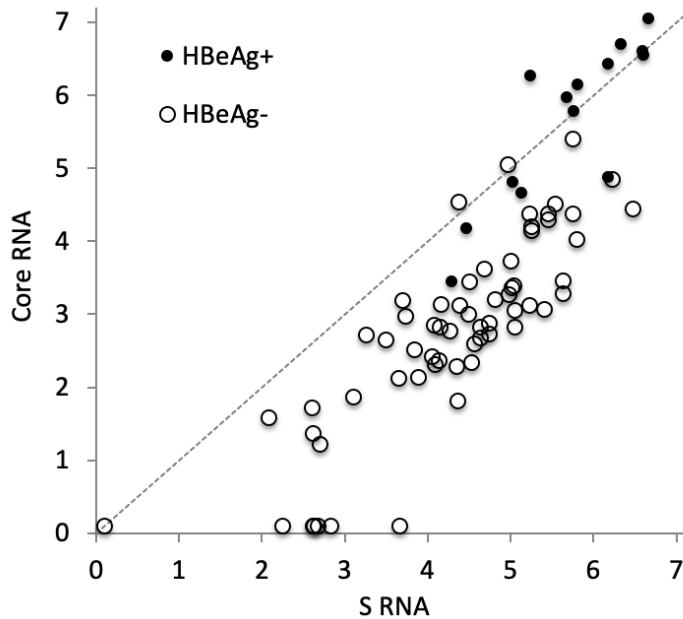
Figure 27 : Correlation between core and S RNA in HBeAg positive and negative patients.

Since the transcripts lack the 5' part containing the ε region essential for encapsidation and initiation of reverse transcription, these cannot serve as pregenomic RNA and may contribute to explain lower level of HBV replication observed in HBeAg negative in comparison with HBeAg positive patients [24].

 Primers targeting the 3' precore region (the 3' redundancy) should amplifies the 3' terminal part of core RNA. The finding that the levels by 3' precore PCR were lower as compared to core (figure 28 B) suggest that part of the core RNA is shorter because an upstream polyadenylation signal at nt 1930 was used [159, 160]. Degradation of the 3´ part might be an alternative explanation to this observed reduction.

All RNA that are transcribed from cccDNA should contain the terminal 3' redundancy representing nt 1830-1927. Therefore, if cccDNA was the source of S RNA, amplification by the S RNA and 3' PC assays should give similar results. As shown in Figure 30 there was a striking discrepancy, with much lower 3' RNA than and S RNA levels in HBeAg negative than in HBeAg positive patients. The lower 3' levels might be due to RNA degradation, but the finding that 3' RNA levels were almost as high as S RNA in HBeAg-positive patients argue against this possibility. The discrepancy between S and 3' RNA was greatest in HBeAg-negative patients with lower levels of replication. In these cases,

3' RNA was 3 $\log_{10}$ units lower than S, indicating that>99% of the S RNA was derived from integrated HBV DNA.
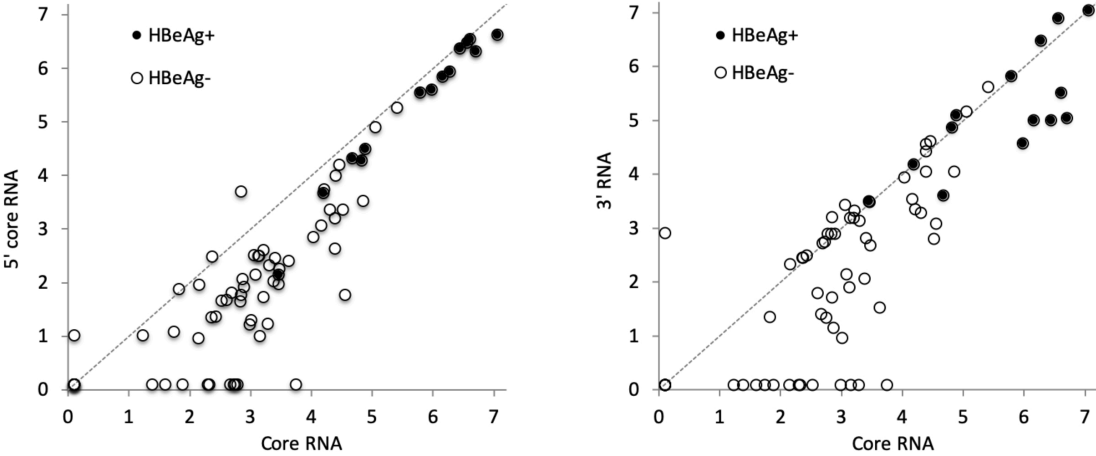

Figure 28 : RNA levels detected by (A) core and 5' core (pgRNA) assays and (B)core and 3' PC RNA.
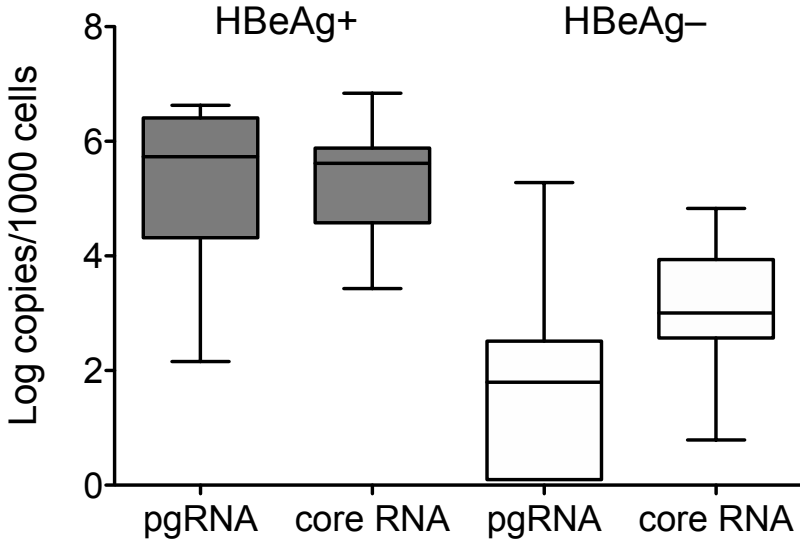

Figure 29 : Box plot showing the same markers as in figure 27A, but with core RNA values obtained after subtraction of pgRNA values.
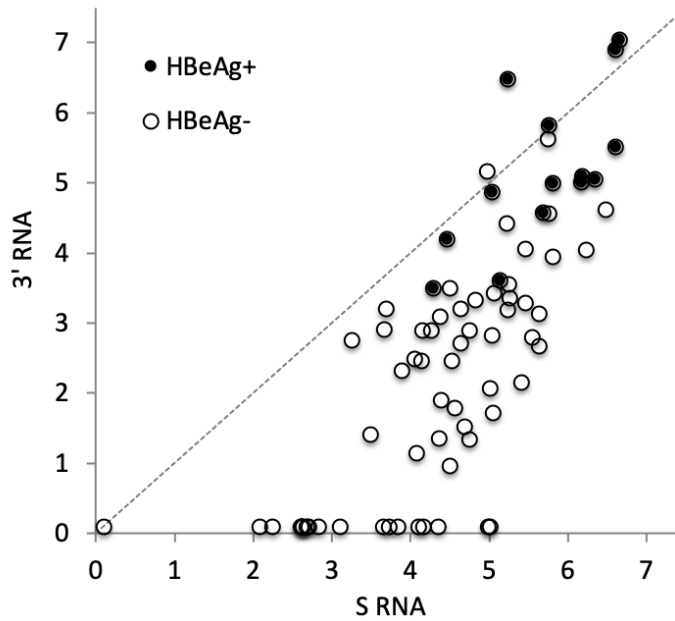
Figure 30 : Correlation between 3' and S RNA transcripts in HBeAg positive and negative patients.

To explore the putative importance of cccDNA and integrated HBV DNA as sources of HBV DNA and HBsAg in blood we compared the ratios of these markers in liver tissue and serum. Figure 31 A shows ratio between cccDNA and total intrahepatic DNA correlating with ratio between pgRNA (that comes from cccDNA) and X RNA (which can come from both cccDNA and integrated DNA). The significant correlation fits with the explanation of cccDNA being the source for pgRNA synthesis. Figure 30 B shows that in the next step, the ratio between serum HBV DNA and HBsAg correlate with intrahepatic proportions of between pgRNA and X RNA. These results agree well with the idea that a large proportion of HBsAg synthesized in HBeAg negative patients are a product of integrations, in accordance with a recent publication where transcriptome analysis in chimpanzee model system was used to study integrations [61].
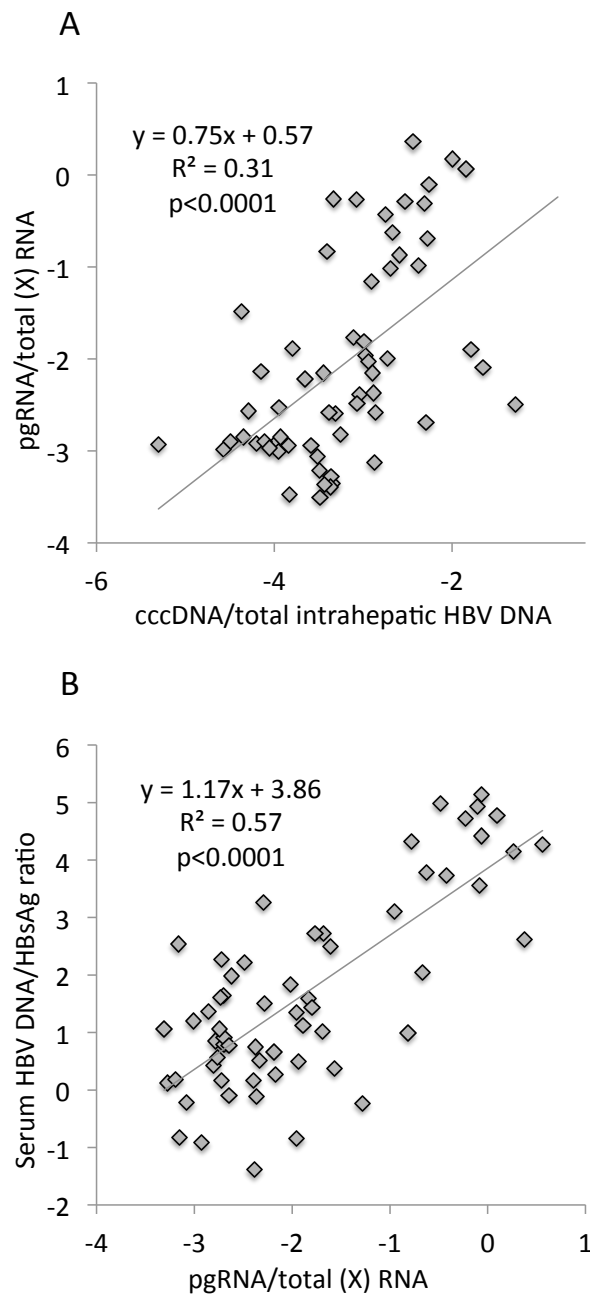
Figure 30. Correlations between (A) the core RNA/total HBV RNA ratio and the ratio between cccDNA and total intrahepatic HBV DNA, and (B) the core RNA/total HBV RNA ratio and the serum HBV DNA/serum HBsAg ratio.

Analyses of multiple biopsies and exploration of RNA transcript ratio by means of digital PCR has provided valuable support to the theory that most of the HBsAg in HBeAg negative patients have integrated transcripts as their source. The results also show presence of intrahepatic HBV core RNA that lacks the 5' or both 5' and 3' end, eliminating the possibility to function as pgRNA. This is hypothesized to be contribute to the reduction of HBV replication in patients with HBeAg negative infection.

6   CONCLUSIONS

Hepatitis B virus (HBV) infection has the potential to cause severe liver damage including cirrhosis and hepatocellular carcinoma (HCC) which is predicted using diagnostic markers such as HBV DNA, HBsAg and HBeAg in serum and HBV DNA and RNA in liver tissue. Nucleoside / nucleotide analogue treatments currently available effectively blocks viral replication but fails to clear reminiscent cccDNA in liver which can reactivate replication when treatment is terminated.

Due to rapid reduction in viral replication, HBV DNA in serum cannot be used to evaluate the long-term effect of treatment on intrahepatic cccDNA. HBV RNA in serum has been proposed as a potential marker for this purpose since it is not directly influenced by treatment. In paper 1, this marker was characterized. Analysis of serum from 95 patients indicate that HBV RNA in serum is encapsidated and of full genomic length RNA and possibly less abundant in genotype D.

Subviral particles (SVP) in the ratio of >10,000:1 to viral particles (VP) during active replication has been proposed to act as a decoy to host antibodies (anti-HBs) against HBsAg. The first experimental support for this hypothesis was described in paper 2, in results obtained using a recently developed cell line HepG2-NTCP. It was shown that SVP significantly reduced the neutralizing effect of anti-HBs in *in vitro* infection, but had a rather small competing impact on binding to the viral specific receptor.

During HBV replication, a double stranded linear form of the genome (dslDNA) rather than the functional circular form, is produced in minority, and may become integrated into the host genome. A novel strategy to estimate integration by means of measuring circular and linear forms of HBV DNA in liver using digital PCR was developed and applied on liver biopsies. A panel of digital PCR assays were also developed to obtain a quantitative profile of HBV RNA in these biopsies. The results from these analyses suggest that a majority of HBV DNA found in liver during late infection stage is in integrated forms that contributes to maintaining high levels of HBsAg in the serum even during low HBV replication. In addition, results suggesting a novel mechanism associating low pgRNA transcription to reduced replication during HBeAg negative state were obtained.

In a separate study, focal differences in the distribution of HBV infection in the liver were studied using liver explant material from patients undergoing transplantation due to HBV or HDV induced liver disease. By analyzing multiple tissue pieces extensive differences in HBV viral load and transcription (a hundred-fold or more between some pieces), especially in patients with low viremia or with HDV coinfection were found. High levels of S RNA in the absence of cccDNA or core RNA support the expression of HBsAg from integrated HBV DNA. The levels of HDV RNA were generally high with lower degree of focal differences, and without correlation to S RNA levels. We propose that partial HDV replication and expression of delta antigens may occur also in hepatocytes that are not co-infected by HBV or even express HBsAg from integrations, and that this might contribute to the severe necroinflammation that is observed in many HDV infected patients.

The compiled works presented in this thesis address important questions and proposes novel mechanisms that hopefully will aid further research and contribute to the understanding of infections caused by HBV and HDV.

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