Genomic and transcriptomic profiles in chronic hepatitis B infection

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Cover illustration: Colors of hepatitis B by Johan Ringlander, depicting an alignment of hepatitis B virus DNA sequences.

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To Laura

I'm just a chap who messed about in the laboratory

– Frederick Sanger

ABSTRACT

Chronic infection with hepatitis B virus (HBV) affects >250 million people globally and is the most common cause of hepatocellular carcinoma and liver cirrhosis worldwide. Approximately one million deaths each year are attributed to HBV infection. The virus is adapted to the human host and has developed mechanisms to evade immunity. For example, integration of HBV DNA into the chromosomal DNA of hepatocytes yields the formation of HBV surface antigen (HBsAg). HBsAg derived from integrated HBV DNA is not required for virus replication but may facilitate viral persistence by dampening antiviral immunity. In paper I we utilized deep sequencing to show that HBV integrations, with ensuing production of HBsAg, are much more common than previously appreciated. We also found that integrated HBV DNA may facilitate co-infection with the hepatitis delta virus (HDV), which propagates only in the presence of HBsAg. In paper II we show that samples from patients lacking HBV replication still carried HBV DNA integrations along with HDV RNA, implying that HDV replication may utilize integration-derived HBsAg. Accordingly, we observed that HBsAg levels in blood correlated with the number of HBV integrations in liver tissue samples from HDV-infected patients. By combining sequencing methods and digital PCR we provide evidence to support that the HBV particle often contains both degraded RNA as well as incomplete HBV DNA genomes (paper III). The thesis additionally includes deep sequencing-based methods for HBV whole genome sequencing, which generated highly accurate consensus sequences much faster and less costly compared to Sanger sequencing (paper IV and V). In conclusion, we show that HBV integrations are highly expressed and contribute to HBsAg levels in blood, may support HDV replication and that novel deep sequencing methods may be valuable in routine diagnostics.

Keywords: hepatitis B virus, hepatitis delta virus, integration, deep sequencing, cirrhosis, HCC

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SAMMANFATTNING PÅ SVENSKA

Hepatit B-virus (HBV) är ett DNA-virus som endast infekterar leverceller. HBV orsakar varje år nära en miljon dödsfall i världen och cirka 250 miljoner personer är kroniskt infekterade. HBV utnyttjar unika mekanismer för att undkomma immunförsvaret och upprätthålla kronisk infektion. En sådan strategi är att viralt DNA integreras i infekterade levercellers DNA, vilket leder till produktion av ett ytprotein (HBsAg) som kan dämpa immunförsvar mot HBV. En huvuddel av denna avhandling beskriver förekomst och betydelse av HBV-genom som integrerats i levercellers DNA. Avhandlingen presenterar därtill nya metoder för att bättre kartlägga virusets arvsmassa.

Vuxna som smittas med hepatit B får oftast en akut infektion som läker ut spontant Vid smitta i barndomen blir infektionen nästan alltid kronisk. En anledning till den låga utläkningsgraden vid kronisk hepatit B tros vara att HBsAg försvagar immunsvaret mot HBV. HBsAg är också nödvändigt för att HBV skall kunna infektera leverceller, eftersom det binder till en särskild receptor på levercellers yta.

HBV använder en komplicerad replikationscykel där en linjär RNA-kopia av genomet, pregenomiskt RNA, omvandlas till ett cirkulärt virus-DNA. Det har tidigare visats att en del HBV-partiklar ändå innehåller RNA, vilket tolkats som att replikationen ibland inte fungerar alls. I ett av delarbetena visas att dessa partiklar inte endast består av RNA utan av RNA och DNA i olika proportioner.

I vissa HBV-partiklar (≈ 10%) blir virusets DNA inte cirkulärt utan dubbelsträngat linjärt. Sådant linjärt HBV DNA kan integreras i det humana genomet i leverceller. Avhandlingen visar genom djupsekvensering av HBV-RNA i levervävnad att dessa integrationer är mycket vanliga och bidrar till produktion av HBsAg. Vi visar också att ett annat virus, hepatit D-virus (HDV), kan utnyttja ytantigen från HBV-integrationer. HDV förekommer alltid ihop med HBV eftersom HDV behöver HBsAg för att bygga egna viruspartiklar.

Det är sedan tidigare känt att mutationer i HBV-genomet kan ge upphov till resistens mot antivirala läkemedel. Vi har utvecklat två djupsekvenseringsmetoder som kan användas för att avgöra vilken genetisk grupp som virus tillhör och för att identifiera resistensmutationer. Metoderna kan tillämpas i rutindiagnostik för att skapa förutsättningar för individanpassad behandling liksom för ökad kunskap om HBV-mutationers kliniska betydelse.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. **Ringlander J**, Skoglund C, Prakash K, Andersson ME, Larsson SB, Tang KW, Rydell GE, Abrahamsson S, Castedal M, Norder H, Hellstrand K, Lindh M. Deep sequencing of liver explant transcriptomes reveals extensive expression from integrated hepatitis B virus DNA. *J Viral Hepat* 2020;27(11): 1162-1170.

II. **Ringlander J**, Strömberg LG, Stenbäck JB, Andersson ME, Abrahamsson S, Larsson SB, Rydell GE, Lindh M. Enrichment reveals extensive integration of hepatitis B virus in hepatitis delta infected patients. In manuscript.

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CONTENT

ABBREVIATIONS

ALT alanine transaminase

cccDNA covalently closed circular DNA

dslDNA double-stranded linear DNA

HBcAg hepatitis B core antigen

HBeAg hepatitis B e antigen

HBsAg hepatitis B surface antigen

HBV hepatitis B virus

HCC hepatocellular carcinoma

HDV hepatitis delta virus

HIV human immunodeficiency virus

MMEJ microhomology-mediated end joining

NHEJ non-homologous end joining

nt nuclecotide

NTCP sodium/bile acid cotransporter

PCR polymerase chain reaction

pgRNA pregenomic RNA

rcDNA relaxed circular DNA

RT reverse transcriptase

TAF tenofovir alafenamide

TDF tenofovir disproxil

1 INTRODUCTION

The hepatitis B viral particle was first described by Dane and coworkers in 1970 (1), but the discovery of the hepatitis B virus (HBV) is attributed to Baruch S. Blumberg who identified the hepatitis B surface antigen (HBsAg). In the late 1960s Blumberg and colleagues reported HBsAg seroprevalence in various ethnic groups and patient cohorts and its role in hepatitis (2-11). HBsAg was initially called the ”Australia antigen” as it was first found in sera from Australian aboriginals (3). With the discovery of HBV, the majority of viral hepatitis infections globally, both chronic and acute, could be explained, as hepatitis A also was discovered during this period. A link between HBV, chronic hepatitis and severe liver complications such as cirrhosis and hepatocellular cancer (HCC) was later observed (10) and in 1976 Blumberg was awarded the Nobel prize. Vaccine development was initiated shortly after the discovery of HBV and resulted in a peptide vaccine with almost 100% protection against infection. Given the strong association between HBV and liver cancer, HBV vaccination is regarded as the single intervention that has saved the largest number of patients from cancer (12-14). Despite the vaccine, 1.5 million people are infected every year and transmission from mother to child still occurs. With no curative treatment, eradication of the virus is not yet in sight.

The lack of curative treatment may partly be explained by the ability of HBV to evade immunity. The virus replication is associated with varying degree of liver inflammation, from very mild to severe, depending on the phase of infection and the intensity of the host immune response. There is an acute and a chronic form of HBV infection, the first named being more common in adults. Children typically develop a chronic infection that probably persists due to a partial immune tolerance against viral antigens. The disease severity varies greatly and a prolonged inflammation, representing an active immune response without the capacity of clearing the virus, is considered the most important risk factor for liver complications.

One of the strategies utilized by HBV is to integrate its genome into the chromosomes of host hepatocytes (15). HBV integration is considered to serve as a significant source of HBsAg and thereby promotes viral persistence, immune escape and chronic infection. Integrations of HBV DNA in liver cells may also contribute to development of HCC (16).

Recent findings support that HBsAg derived from HBV integrations facilitate hepatitis delta virus (HDV) co-infection (17, 18). HDV is a small RNA virus

that only infects individuals carrying HBV. The basic elements of the HDV were first described in 1977 by Rizetto *et al,* and was fully characterized during the 1970s and 80s (19-22). HDV is the smallest virus known to infect humans and unlike other human pathogens resembles viroids found in plants. HDV uses HBsAg to envelope its own RNA genome and delta antigen and uses the same cellular receptor as HBV for cell entry. Infection with HDV is associated with a more severe inflammation and poorer outcome than infection with HBV alone. HDV is difficult to treat and suppression of HBV replication with antivirals typically does not decrease HDV replication or HDV-induced inflammation. In recent years, however, an entry inhibitor mimicking the HBsAg, bulevirtide, has been introduced. This compound reduces HDV replication and partially diminishes liver inflammation (23-26).

Many aspects of co-infection with HBV and HDV have remained unknown. In this thesis, we have utilized techniques such as digital PCR and deep sequencing aiming to extend knowledge about details of HBV/HDV interactions.

1.1 HEPATITIS B VIRUS

1.1.1 Structure

HBV is one of the smallest viruses known and is an enveloped DNA virus 42 nm in diameter. The HBV particle carries a relaxed circular partially double-stranded DNA (rcDNA) genome of 3.2 kB that is encapsidated by core proteins and attached to a polymerase. The structure of HBV is shown in figure 1.

The viral proteins are translated from mRNAs with overlapping open reading frames (ORFs) on the HBV genome. There are five HBV transcripts: precore, pregenomic (pgRNA), preS1, preS2 and X.

The precore transcript is translated to e antigen (HBeAg). A slightly shorter transcript pgRNA (also known as core RNA) is translated to either the core protein or polymerase. PreS1 translates to large S, preS2 to middle and small S antigens and X mRNA to the X protein. All mRNAs share a polyadenylation signal at 1919, resulting in a poly(A)-tail at approximately nt 1940. The transcripts in relation to the HBV genomic positions are shown in figure 1.

Core proteins are essential for the formation of the capsid. Most commonly, the icosahedral capsid is assembled of 240 HBV core proteins but there is also a smaller form with 180 core proteins (27, 28).

The polymerase is also translated from the core/pgRNA and acts both as a reverse transcriptase and DNA polymerase, enabling the unusual HBV replication that includes an RNA intermediate that is template for a circular, partly double-stranded DNA or a linear double-stranded DNA (29), as seen in figure 2.

The virus envelope contains the small, middle and large surface antigen proteins (HBsAg) (Figure 1). The large S protein includes a preS1 part that binds to the cellular receptor sodium/bile acid co-transporter (NTCP) (30). HBsAg also forms non-infectious particles without capsids and genomes, known as subviral particles (31). These can be either spherical or rod-like (32). These particles are very abundant and are believed to act as decoys attaching antibodies that would otherwise inhibit infectious particles (33).

A non-structural protein, the HBeAg, is also expressed in amounts that reflect viral replication in acute infection and the early phases of chronic infection.

HBeAg was first described by Magnius *et al.* and is still an important biomarker in chronic HBV infection and is used to separate the different phases of the disease (34, 35). In later stage of chronic infection, transcription of the precore RNA encoding the protein is often reduced or its translation terminated, probably due to immune response induced selection of HBeAg defective variants (36-38). The pgRNA is transcribed throughout all phases of infection enabling continued replication.

Another non-structural protein, the X protein, is assumed to maintain viral replication and protect the cccDNA from being silenced by host factors. It was long unknown how the X protein interferes with infected cells but recent studies show that X binds to host SMC5/6 complexes and thereby enables HBV transcription and replication. In the absence of X, SMC5/6 bind to HBV cccDNA and decrease HBV expression (39-41).

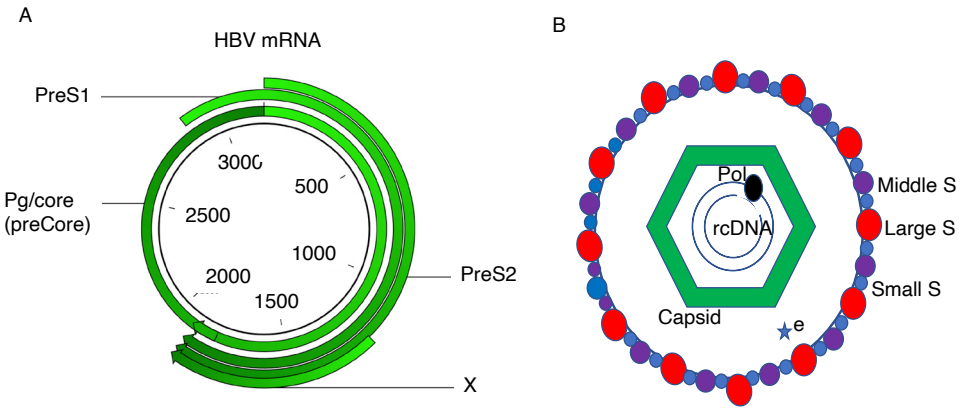

Figure 1. A) Hepatitis B virus (HBV) mRNAs of the circular HBV genome, shown as broad, green arrows. All transcripts share the same polyadenylation site but have different promoters. B) Structure of the HBV particle. The envelope consists of the surface antigens, core proteins constitute the capsid that harbors the relaxed circular DNA (rcDNA) and the polymerase (Pol). The e antigen has no structural role.

1.1.2 Replication and integration

Circulating HBV particles reach the sinusoids of the liver and enter the space of Disse, located between the sinusoids and hepatocytes. The virus binds to NTCP receptor on the surface of hepatocytes with the HBsAg large domain (30). Cellular import is mediated through clahtrin-dependent endocytosis (42). After cell entry, the particle is de-enveloped and the capsid is transported to the nucleus where the capsid is dissolved. The rcDNA is subsequently released into the nucleus and host factors start the completion of rcDNA into a covalently closed circular DNA (cccDNA) that is fully double-stranded (43).

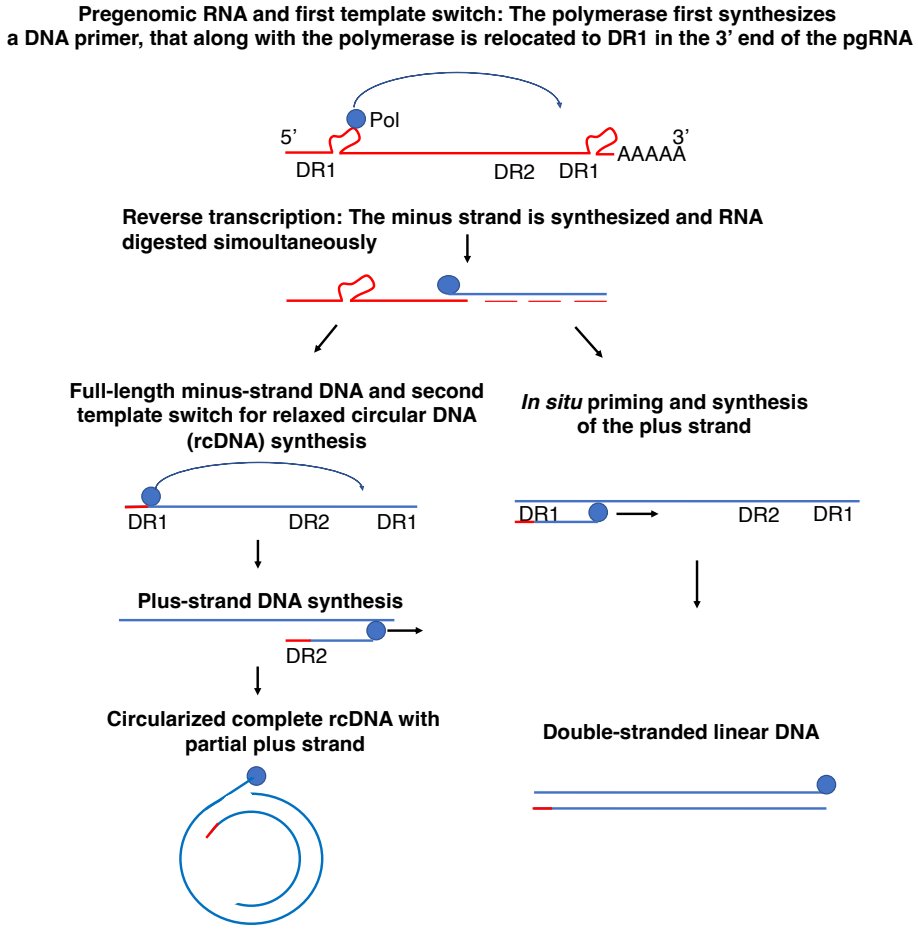

Figure 2. Hepatitis B virus replication. The figure depicts the reverse transcription of pregenomic RNA (pgRNA), resulting in either relaxed circular (rcDNA) or double-stranded linear DNA (dslDNA).

The pgRNA is transcribed from cccDNA and exported to the cytosol. In the cytosol the pgRNA is either encapsidated by core proteins or translated to core proteins or polymerase in the ribosomes. If encapsidated, the pgRNA is reversely transcribed to a negative sense DNA from which the HBV polymerase synthesizes a shorter, complementary plus strand (29). During reverse transcription the pgRNA is digested by the Rnase H activity of the polymerase. Only the end portion 5' part of pgRNA is spared and act as a primer for polymerase template switch to another position (DR2) at the minus strand DNA (44). This switch is required for creating rcDNA. If it fails, the RNA 'primer' remains at DR1 and double-stranded linear DNA (dslDNA) is formed (45). The HBV genome replication is schematically depicted in figure 2.

HBV integrations were first detected in hepatoma cell lines in 1980 by Edman *et al.* (15) and in liver tumor tissue by Bréchot *et al.* (46). However, only more recently the importance of HBV integration has been widely accepted. The integrations are transcriptionally active and contribute to HBsAg levels in blood (17, 47).

HBV dslDNA may integrate into the host chromosomal DNA in the nucleus of hepatocytes if double-strand breaks are present. Two principal mechanisms are proposed to mediate HBV integration. Non-homologous end joining (NHEJ) is a pathway for double-strand break repair that directly ligates the dsDNA ends without need of any homologous template of the ligated strands. For HBV dslDNA integration this means that DNA may be integrated anywhere in the genome, independently of similarities between human and viral sequences. A second integration mechanism is microhomology-mediated end joining (MMEJ). MMEJ is similar to NHEJ but requires a few homologous nucleotides between the two DNAs being ligated (48). If integrations are caused by MMEJ, certain human genomic regions homologous to the flanking HBV sequence would have a predisposition for integration. Accordingly, some studies show that integration occurs in a non-randomly; however, these results may be biased by the sequencing methods used. Also, clonal selection of specific integrations may skew results as integrations with low expression and/or not clonally expanded may not be detected (49, 50). Others have proposed that HBV integrations occur randomly in the human genome (51).

In dslDNA, the core/pgRNA promoter is separated from the core open reading frame. Thus, core/pgRNA is not transcribed from integrated HBV DNA (48). As a result, viral particles cannot be produced solely from integration-derived HBV proteins. In theory, presence of a human promoter

located just upstream of an integration could induce transcription of a human/HBV chimeric core/pgRNA that might be translated to a truncated core protein and be reverse transcribed, but this is unlikely to impact on the viral replication (16, 17, 47, 52). The promoters of preS1, preS2 and X transcripts are intact in dslDNA and these RNA species can be transcribed from integrations resulting in translation to corresponding proteins.

While the clinical implications of HBV DNA integrations are only partly understood, integrations have been suggested to increase the risk for HCC and recurrence of HCC after surgery (53). Recent studies have presented sequencing-based methods for detection of circulating HBV/human DNA fragments in blood that thus might be clinically valuable (16, 45, 53).

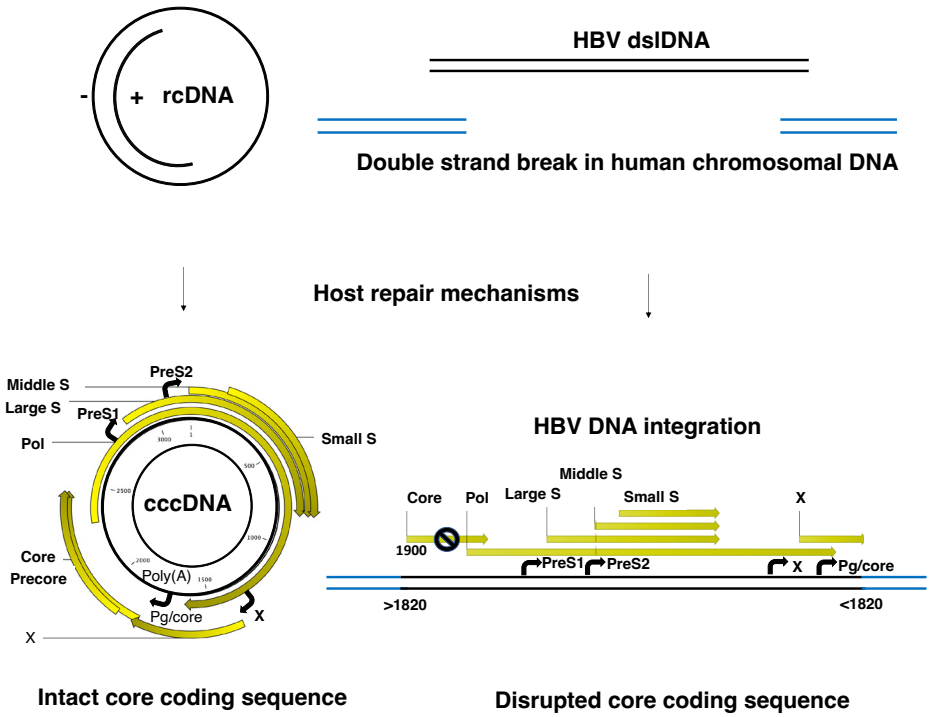

Figure 3. The completion of HBV relaxed circular DNA (rcDNA) to cccDNA (covalently closed circular DNA) and integration of HBV double-stranded linear DNA (dslDNA). Black arrows represent transcript promoters. Yellow arrows indicate the coding regions for the viral proteins. All HBV transcripts can be transcribed from the cccDNA and share the poly(A)-site. DslDNA can integrate with the human genome and express preS1, preS2 and X mRNA but not pg/core RNA, as the promoter is separated from the open reading frame.

1.1.3 Evolution and genotypes

HBV is an ancient virus that belongs to the *Blubevirales* order, the *Hepadnaviridae* family and the *Orthohepadnavirus* genera, the other genus of *Hepadnaviridae* being *Avihepadnavirus* (54). All *Hepadnaviridae* carry a polymerase with reverse transcriptase and DNA polymerase activity. The virus order probably evolved from escape RNA retroelements in insects, translated to proteins with reverse transcriptase and RNase activity, and later gained a gene encoding the capsid (55). *Nackednaviridae*, found in fish, have a similar structure as the *Hepadnaviridae* but without an envelope and is considered to be the evolutionary precursor (56). After the advent of an envelope gene the *Hepadnaviridae* separated into two genera: the *Orthohepadnavirus* that gained the X gene and the *Avihepadnavirus* lacking X (55).

Other *Hepadnaviridae* comprise duck hepatitis virus and woodchuck hepatitis virus causing chronic infection of the liver in the respective species. These viruses are structurally very similar to HBV and have been extensively studied and used as models for HBV infection (57).

The HBV that infects higher primates is closely related to the human HBV, suggesting that HBV has co-evolved with its human host and that the human HBV genotypes developed prior to human migration waves out of Africa (55).

HBV subtypes were originally based on differences in the surface antigen and by detection of certain epitopes. Determinant a and q are present in almost all HBV genotypes but the combination of determinants d/y and w/r differs (58, 59). W is divided into W1-4. The antigen-dependent method dividing HBV into different serotypes is now replaced by genotypes that are based on nucleotide differences in the genomic sequence. Genotypes A-D were first established and later E-H were found. More recently genotypes I and J have also been described (60, 61). Genotypes A-H and other non-human hepadnaviruses are shown in the phylogenetic tree in figure 4.

Several studies have presented evidence for differences in severity of chronic infection between genotypes. The genotype C has been proposed to cause liver cirrhosis and cancer faster compared to other variants (62-65). However, there are several host and lifestyle dependent factors that may influence the risk for complications to chronic HBV infection, such as aflatoxin exposure and alcohol abuse. In papers II and IV we describe new methods for deep sequencing-based genotyping.

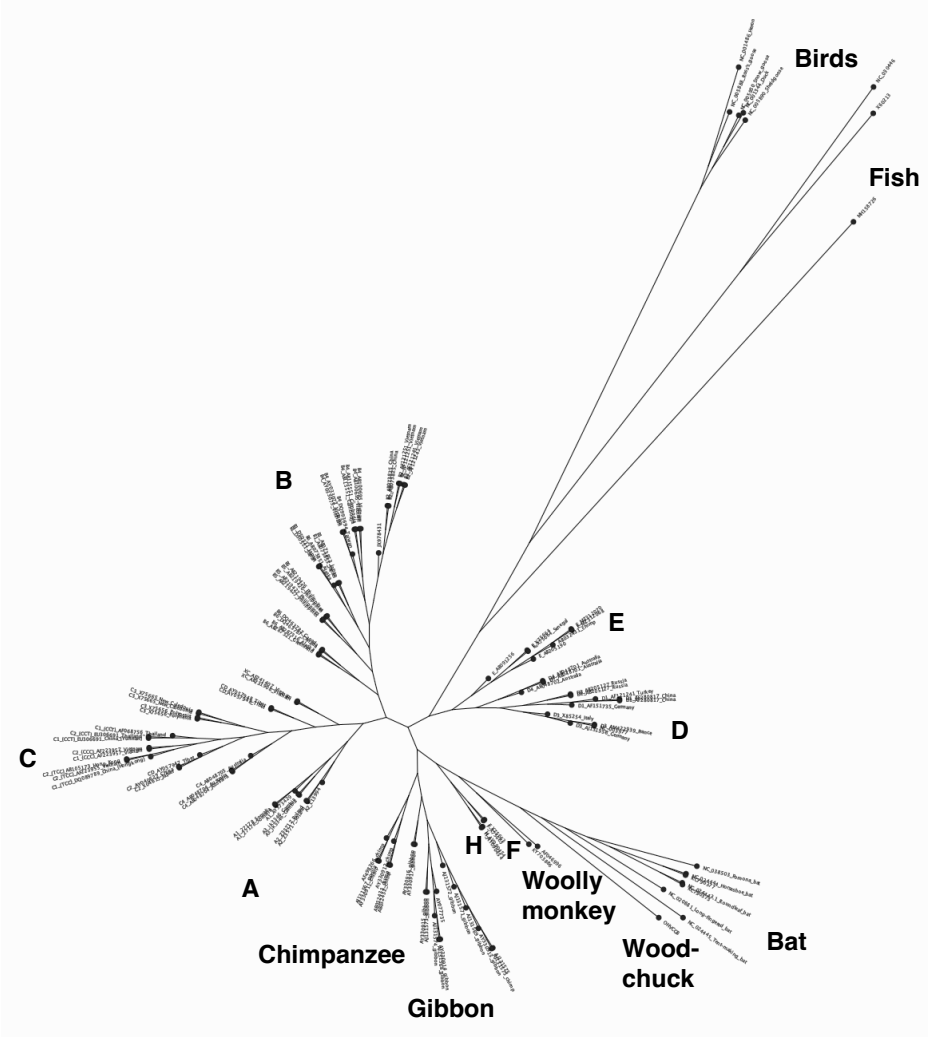

Figure 4. Radial phylogenetic tree (compressed and branches shortened) with human hepatitis B genotypes A-H and related hepadnaviruses in different hosts.

1.1.4 Mutations

Several HBV mutations are associated with the course of infection and patient outcome, the most well-known being the precore, core promoter mutations and antiviral resistance-associated mutations.

The precore encoding region carries mutations that have been associated with HBeAg loss. At position 1896 a G to A change creates a stop codon, which abolishes translation of precore RNA to HBeAg. In genotypes A and C1 presence of a C nucleotide at nt 1858 is common and prevents the G to A mutation at nt 1896 because it would lead to unfavorable basepairing in the stem-loop of precore RNA (66).

Mutations in the pg/core promoter, in particular A1762T and G1764A, are reportedly associated with decreased expression of HBeAg and usually develops before the advent of the 1896 mutation as described above (67, 68).

Deletions in the core region result in a defective core protein. These mutations are more frequent in HBeAg-positive patients, and co-exist with a wild-type virus that supplies a functional core protein (69).

Table 1. Mutations causing antiviral resistance

Amino acid	Adefovir	Entecavir	Lamivudine	Telbivudine	Tenofovir
L80V/V[1]			x	x	
I169T[1]		x			
V173L[1]			x		
L180C/M		x	x		
A181T/V	x			x	
M184A/G/I/S[1]		x			
S202G/I[1]		x			
M204I/V		x	x		
M204S		x	x	x	
N236T	x				x
M250V[1]		x			

[1]Compensatory mutations not directly causing resistance, typically occur with one or more of the other mutations listed.

Mutations in the S region have been linked to vaccine escape, that is, antibodies formed after vaccination do not bind to the mutated HBsAg and do not protect against infection (70, 71). The amino acid substitution Gly145Arg

is reported as a vaccine-escape variant (72). Also, deletions occur in the S region, which may cause false negative results in HBsAg assays and may herald poorer outcome in HBeAg-negative patients, including the development of HCC development (73).

Clinically relevant mutations in the reverse transcriptase region of the polymerase typically occur after treatment with antivirals. Table 1 shows the currently established resistance mutations and corresponding antivirals (74, 75).

There is limited knowledge about the impact of mutations in the X region of HBV (76). One study suggested that certain mutations in X contribute to earlier development of liver cirrhosis in patients with genotype C (77).

1.2 HEPATITIS B VIRUS INFECTION

1.2.1 Transmission

HBV is typically transmitted sexually or through contact with blood, the latter being represented by mother-to-child transmission during pregnancy or birth ('vertical'), transmission by blood products in unsafe healthcare procedures or injection drugs abuse (78). Blood donor screening and improved routines in healthcare have markedly reduced iatrogenic HBV infections. Transmission leading to chronic HBV is thought to be most common from mother to child over the placenta during delivery, or later in childhood from parents or siblings. HBV has been found in multiple body fluids from children; however, the contribution by transmission between children is not completely known (79).

1.2.2 Acute hepatitis B virus infection

The focus of this thesis is chronic HBV infection. However, the infection is often acute and self-resolving. In subjects exposed to HBV after childhood or adolescence the most common outcome is acute hepatitis. The infection may cause a moderate to severe hepatitis but an efficient immune response typically clears the infection. Symptoms in the acute phase include jaundice, abdominal pain, pronounced fatigue and nausea. The incubation time is two to three months and the symptomatic phase mostly lasts for some weeks and by definition no longer than six months. HBV infections that persist for more than six months are considered chronic. A Swedish study showed that out of 100 patients with a history of acute HBV, none developed chronic infection (80) but a Chinese study showed that 8.5% of patients progressed to chronic infection and that genotype C was a risk factor for chronicity (81). While such results favor the view that genotype C is more commonly associated with chronicity, differences in patient age or ethnicity may confound these findings.

Although patients do not harbor replicating virus after the acute infection the virus may reactivate, in particular among immunosuppressed patients (82). Reactivation is explained by the persistence of HBV cccDNA in hepatocytes, which is present in a minute fraction of hepatocytes several decades after infection (83).

1.2.3 Chronic hepatitis B virus infection

Chronic hepatitis B is divided into different phases depending on the viral expression and host immune response (84). In the modern classification, viral antigens and the degree of inflammation defines the phases of chronic infection, as depicted in figure 5.

Typically, HBeAg-positive chronic infection/immune tolerant phase is seen in children and young adults. During this phase there is little or no inflammation or liver damage, and patients are usually not considered candidates for antiviral treatment but monitored regularly. As this phase is associated with high viral replication there is a considerable risk for transmission and household members and other close contacts should be vaccinated. Transition into the immune reactive or HBeAg-positive hepatitis phase usually occurs during adulthood at around age 20-40. A moderate to severe inflammation is seen, probably provoked by the matured T cell response against the virus.

The HBeAg-positive chronic hepatitis phase is most often followed by HBeAg seroconversion a (referring to the formation of antibodies against HBeAg) and transition into the HBeAg-negative chronic infection/inactive carrier phase, but the time for this transition varies greatly between patients, from months to decades. HBV DNA level and inflammation, reflected by alanine transaminase (ALT) levels, are typically dramatically reduced and occasionally HBsAg loss is also achieved relatively short after HBeAg seroconversion. However, if the HBeAg-positive chronic hepatitis phase is prolonged there is increased risk for cirrhosis and cancer (85). This risk is also increased if HBeAg loss is not accompanied by a significant reduction of viral replication and liver inflammation. High HBV DNA and ALT levels are seen in HBeAg-negative chronic hepatitis, which may occur directly after HBeAg-positive chronic hepatitis or following years of the HBeAg-negative chronic infection/inactive carrier phase. Differences in age at HBV infection, time until HBeAg negativity and comorbidities may impact the risk of having HBeAg-negative chronic hepatitis. Also, certain viral genotypes and/or precore mutations may increase the risk for HBeAg-negative chronic hepatitis (68).

HBsAg loss is considered as a functional cure of the HBV infection. As mentioned, it does not mean that cccDNA or HBV integrations are completely removed in the HBsAg-negative phase but usually HBV DNA is undetectable and anti-HBsAg IgG becomes detectable. The risk for HCC is

still slightly elevated and reactivation of the infection may occur if the patient is immunosuppressed, *e.g.* in transplanted patients (86).

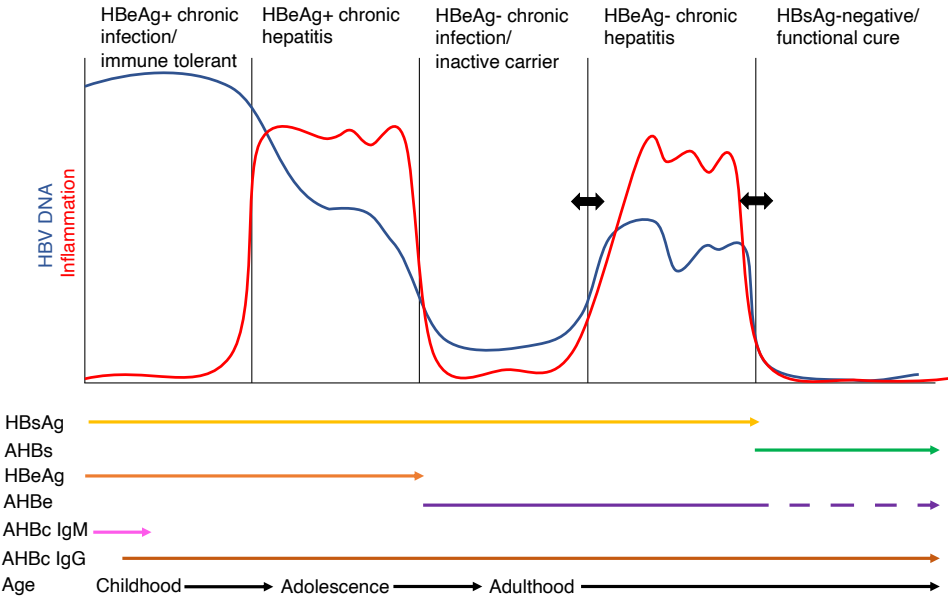

Figure 5. The phases of chronic hepatitis B virus infection are depicted as a graph with HBV DNA and inflammation seen as blue and red curves, respectively. Usually, inflammation is quantified by alanine transaminase (ALT). The intervals for antigen and antibody detection in patient blood are found beneath the graph, and as seen, there are several seroconversions during a typical chronic infection. The time for each phase varies greatly between patients, but typically follows the general timeline in the figure.

1.2.4 Monitoring of chronic hepatitis B virus infection

Most commonly, patients with chronic hepatitis B infection in high or middle-income countries are monitored by gastroenterologists or specialists in infectious diseases. Blood samples are drawn regularly for analysis of ALT and prothrombin complex concentration (PCC) and platelet count. The interval of sampling depends on the disease phase and the extent of liver damage. The most important viral marker is HBV DNA as the viral load in blood correlates with disease severity, including risk for cirrhosis and liver cancer (87, 88). Quantification of HBsAg is also used to monitor the infection but is of limited use in the early phases of chronic infection. In later

phases HBsAg levels may predict viral clearance and may potentially serve as a surrogate for intrahepatic HBV DNA integration (89). HBeAg and anti-HBeAg are also relevant serological markers, as seroconversion often heralds improved outcome and decreased viral replication. Serological test to exclude co-infection with hepatitis C virus (HCV), human immunodeficiency virus (HIV) and HDV are performed at initial diagnosis and occasionally later during monitoring. Also, HBV genotyping and antiviral resistance testing should be considered.

1.2.5 Vaccine

There is an effective vaccine against HBV consisting of the small S protein. This vaccine neutralizes all HBV genotypes and offers almost 100% protection against HBV (90, 91). The recombinant antigen presently used is produced from yeast cells but in the early 1980s it was obtained from HBV patient blood and harvested through a cumbersome filtering method, including an inactivation step (92-96). It was debated whether the antigen from HBV patient blood could be contaminated by other bloodborne viruses, such as HIV, but the vaccine was proved to be safe and lacked any infectious agents (97). The recombinant protein synthesis offered a much more efficient production for the billions of doses needed. Immunity can be quantified by measuring anti-HBs IgG, where 10 IU/L or more in blood is considered protective against infection (98).

1.2.6 Treatment

Nucleos(t)ide analogs are commonly used in HBV treatment and were first introduced in the 1990s. Previously, interferon, an antiviral and immune-modulating cytokine, was the only treatment option but its effectiveness is low with considerable toxicity (99). In recent years a viral entry inhibitor, bulevirtide, has been approved; however, this compound is not yet widely used in chronic hepatitis B and its main indication is severe HDV infection.

Interferon (or pegylated variants) has antiviral properties and induces a host immune response towards viruses by stimulating expression of several factors important for innate immunity; the precise mechanism of interferon therapy in chronic hepatitis B is however not known. Side-effects are common and include symptoms characteristic of acute viral infection, such as

fever, headache, nausea but also extreme fatigue and depression. Approximately 40% of interferon-treated patients achieve reduced viral load and decreased liver inflammation (100).

During the 1990s nucleoside analogs were introduced as antiretroviral treatment for HIV (101, 102). Similar to HIV, HBV replication includes reverse transcription and certain nucleoside analogs were found to be effective also in HBV infection.

Lamivudine was one of the first nucleoside analogs to show efficacy against HBV and is still used (103). Evolvement of resistance during therapy is common and lamivudine use has therefore decreased (104). Telbivudine is a thymidine analog shown to better reduce HBV DNA in blood compared with lamivudine but this drug shares the relatively high rate of antiviral resistance and has been discontinued (105).

Entecavir was developed in the early 1990s with the intention of creating a herpesvirus antiviral. Entecavir shows antiviral efficacy against HBV and was approved for treatment of HBV infection in 2005 (106). Entecavir has a high barrier for resistance, and it is uncommon with *de novo* resistance mutations during treatment, however, previous treatment with lamivudine increase the risk of entecavir resistance (106, 107). Entecavir is widely used and side-effects are typically mild.

Nucleotide analogs differ from nucleoside inhibitors by carrying a phosphonate bound to the nucleotide. The nucleotide analog adefovir was among the first direct antivirals used against HBV. It shares the tendency of lamivudine for resistance development but the time for such mutations to occur is longer. Another nucleotide analog, tenofovir, reduces HBV DNA levels in patients and shows a higher resistance barrier. The introduction of tenofovir disproxil (TDF) (108) was followed by tenofovir alafenamide (TAF), which is a prodrug of TDF (109). The prevalence of resistance to TDF or TAF is apparently low and only a few case reports and *in vitro* data imply resistance or reduced susceptibility. Recent studies show that tenofovir treatment is associated with less long-term complications than entecavir (110, 111). Side-effects to TDF vary but nausea, rash and weakness may occur. In patients with severe side-effects from TDF or kidney failure, TDF can be replaced by TAF or entecavir (109).

Current treatment recommendations advocate nucleos(t)ide treatment for patients with high viral replication and signs of liver inflammation as reflected by ALT elevation. HBeAg-positive patients with high replication

but low-grade inflammation do not qualify for treatment according to most guidelines (112-114). This aspect of guidelines has been questioned as antiviral treatment may lower the number of integrations, as nucleos(t)ide analogs reduce the substrate for integration, *i.e* HBV dslDNA. On the other hand, it has been proposed that antiviral treatment might lower the likelihood of achieving HBeAg seroconversion, thus arguing against treating HBeAg-positive patients in the immune tolerant phase (115).

The novel entry inhibitor bulevirtide was recently incorporated into the treatment arsenal. It binds to NTCP with higher affinity than HBsAg and thus prevents HBV and HDV to enter hepatocytes. The efficacy of bulevirtide in HBV mono-infection is not fully known, however, recent studies of HBV/HDV infection have shown promising results (25)

Cessation of antiviral treatment and viral clearance

In the past decade, studies have shown that cessation of nucleoside analog treatment after several years of treatment can trigger a host antiviral immunity that is able to eradicate or HBV or markedly decrease HBV DNA levels, without need for further antiviral treatment. A study by Hadziyannis *et al.* showed HBsAg loss in 39% of patients who stopped treatment with adefovir (116). Later studies have reported variable rates of functional cure and that Caucasian descent and genotype D infection predict a favorable outcome (117, 118). The mechanism for viral clearance after stopping antiviral treatment is not yet known, but the regained viral replication was hypothesized to stimulate a more efficient T cell response if viral replication has been shut down for a longer period (119).

1.2.7 Liver fibrosis and cirrhosis

Liver fibrosis is a common complication of chronic hepatitis B and may eventually lead to cirrhosis. In cirrhosis, the fibrosis is extensive and there is also an altered architecture of liver tissue. Lobules with central veins are replaced by noduli, and there is often portal hypertension. Milder forms of fibrosis may regenerate completely if the virus is removed and inflammation ceases (120).

In HBV, male sex, high viral load and longer periods of elevated ALT, a proxy for inflammation, or co-infection with HDV are risk factors for developing fibrosis and cirrhosis (121). The pathogenesis of cirrhosis

includes oxidative stress that accompanies inflammation. Reactive oxygen species and danger-associated molecular patterns activate Kupffer cells. Signaling from Kupffer cells and other macrophages, which presumably are recruited from the bone marrow through chemokine signaling, leads to the transformation of quiescent stellate cells into activated stellate cells. Increased expression of TGF-β1 is the most important factor but also TNF-alpha, PDGF and IL-1 are assumed to participate in stellate cell activation. The cytokines may also attract peripheral fibroblasts and transdifferentiate stellate cells into myofibroblasts that are mediators of scaring due to extensive production of extracellular matrix (ECM). The ECM is deposited throughout the liver, completely transforming the normal tissue organization and cause reduced blood flow that eventually leads to portal vein hypertension. TGF-β1 is also assumed to induce stellate cells to express factors TIMP (tissue inhibitor of metalloproteinase) 1 and 2 that inhibit matrix metalloproteinases from degrading ECM/fibrotic tissue, leading to irreversible liver damage (122).

Cirrhosis is the dominant risk factor for HCC in any liver disease. The role of HBV integrations for the development of cirrhosis is not completely understood but several studies show that integrations in the *FN1* gene, (encoding fibronectin-1, a soluble protein involved in coagulation and tissue repair) are more common in cirrhotic livers than in HCC tissue (123, 124). It is it not known if *FN1* causes cirrhosis or if these integrations are passengers during cirrhosis development.

1.2.8 Hepatocellular carcinoma

HCC is developed from premalignant dysplastic noduli and usually occurs in a cirrhotic liver. The noduli are divided into low-grade and high-grade variants, the latter having higher risk of malignant transformation. Early HCC lesions are typically <2 cm in diameter and restricted to one focus with stromal, non-vascular invasion. As HCC progresses the size of the tumor increases, vascular invasion occurs followed by metastasis (125).

Chronic HBV infection is the leading cause of HCC worldwide (126). In contrast to HCC induced by hepatitis C virus or autoimmune diseases, HBV may cause HCC in a non-cirrhotic liver, in part because of its inherent oncogenic mechanisms such as X antigen expression and chromosomal integration (16). However, most HBV-induced cases of HCC are seen in patients with severe liver fibrosis or cirrhosis caused by inflammation (127).

This implies that both HBV-driven inflammation and viral factors jointly promote cancer development. The risk for HCC may differ between viral genotypes and is also dependent on host factors. A diet with high levels of aflatoxin, alcohol abuse, African or Asian descent and male gender are suggested as dominant risk factors (85). Viral risk factors include genotype C, late HBeAg seroconversion and superinfection with HDV (128). HBV integrations are known to occur in HCC and are suggested to participate in oncogenesis. Integration in several human protooncogenes is known to occur in HBV-induced HCC, among them *CCNE1*, *MLL4* and *TERT*. In HCC, HBV integrations in *TERT* are typically associated with increased expression of this gene. *TERT* transcription leads to conservation of telomer length and thereby increases cell survival to promote oncogenesis. Notably, the *TERT* gene is also frequently mutated and upregulated in non-HBV-induced HCC (49). HBV integrations may also reduce the expression and transcription of tumor suppressor genes. Currently it is debated whether integrations cause the initial malignant transformation of hepatocytes or if inflammation-associated mutations and mechanisms are the main drivers of HCC (129-131).

1.2.9 Liver resection and transplantation

Cirrhosis cause irreversible liver damage and liver failure. HBV-induced liver failure and HCC are among the most common reasons for liver transplantation in the western world (132). In end-stage cirrhosis and in advanced HCC, transplantation is often inevitable. Liver resection (removal of a part of the liver) is an option for smaller tumors with limited vascular invasion in patients with preserved liver function. Starzl *et al*. performed the first liver transplantations in the 1960s (133) but it was rarely practiced as the survival rate was low. When more efficacious immune-suppressive treatments were introduced in the 1980-90s and the transplantation-related mortality declined the use of liver transplantations increased (134-136).

Prior to transplantation, HBV-infected patients usually receive antivirals to suppress viral replication. Immunoglobulins are administered during and after transplantation to reduce the risk of infection of the transplanted liver. Liver transplantation can result in clearance of HBV infection; however, life-long immunosuppressive treatment is needed. The 5-year survival rate after the procedure is 70-75% (137, 138).

1.3 HEPATITIS DELTA VIRUS

HDV is a satellite virus to HBV that only infects already HBV-infected cells. It consists of a circular, rod-shaped, minus-stranded RNA genome, delta antigens (small and large) and an envelope consisting of HBsAg from HBV (22, 139).

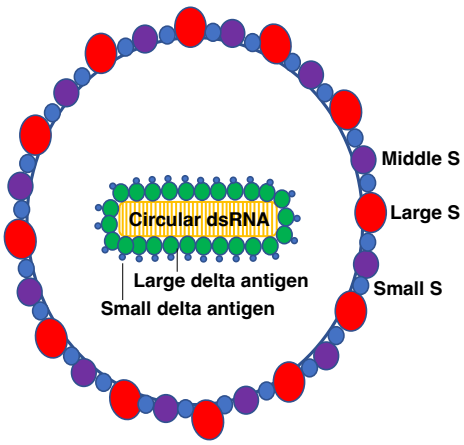

Figure 6. Hepatitis delta virus structure. The virus uses the hepatitis B virus surface antigen. The large and small delta antigens surround the circular RNA genome that base pairs to itself and therefore has a rod-like shape.

The replication cycle resembles that of viroids, including a rolling circle replication and ribozyme activity that cleaves the long RNA molecules to HDV genome length, which thereafter are circularized (140). HDV differs from true viroids by expressing an mRNA encoding two proteins. HDV mRNA are translated to the small and large delta antigens. For translation of the large delta antigen host RNA-editing by the enzyme ADAR is needed. ADAR deaminates the nucleotide adenosine to an inosine (which is interpreted as a guanosine in replication) at the stop codon for the small delta antigen. The novel codon encodes an amino acid instead of a stop and the translation of deaminated mRNA therefore results in the large delta antigen (141).

Infection with HDV is most common in central Asia where Mongolia has the highest prevalence. Superinfection with HDV in previously acquired HBV infection is the most common cause of infection. The virus often causes

severe liver inflammation and is considered to entail cirrhosis and cancer more rapidly than mono-infection with HBV (142).

HDV genome replication in hepatocytes is probably not dependent on active HBV replication and perhaps not even on presence of HBsAg. Recent findings suggest that HDV may spread also through cell division, thus bypassing the need of HBsAg (143). It has been suggested that non-HBV viral antigens encapsidate HDV, although only shown in *in vitro* (144). In order to produce infective particles that are distributed into the blood, the virus must probably infect a cell with HBsAg production from either cccDNA or integrations (18).

Usually, the monitoring of HDV-infected patients includes the regular follow-up regime for chronic HBV infection with the addition of HDV RNA quantification in blood (145). Interferon was previously the only treatment for HDV but the discovery of a viral entry inhibitor, as mentioned, may provide a treatment option with reduced toxicity and better outcome (24, 25).

2 AIMS

This thesis work aimed to contribute to the knowledge of HBV DNA and RNA, including the possibility of developing diagnostics, in patients with chronic HBV infection. We utilized serum samples and liver tissue to determine the role of integrated HBV DNA for the formation of HBsAg. In serum samples, we aimed to define HBV RNA to DNA content in viral particles. Additionally, we applied sequencing methods aiming at providing diagnostic tools. In detail, the aims were to:

I. quantify HBV integrations by Illumina deep sequencing of the entire transcriptome in liver tissue,
II. determine the extent and expression of HBV integrations with enhanced sensitivity by Ion Torrent deep sequencing after PCR enrichment of RNA fragments carrying an HBV/human fusion point,
III. utilize multitarget digital PCR to characterize the DNA and RNA content in HBV particles in serum samples,
IV. determine the feasibility of a short reads deep sequencing method for the whole genome analysis of HBV, and
V. develop a long reads deep sequencing method for whole genome analysis of HBV.

3 PATIENTS AND METHODS

3.1 PATIENTS AND MATERIAL

Paper I investigated explant liver tissue and serum samples from five patients with HBV induced liver cirrhosis including two patients with HCC. The detailed patient characteristics are presented in paper I.

In paper II, five liver-explanted patients with chronic hepatitis B and HDV co-infection were included, two of whom were also included in paper I. Patients contributed with explant liver tissue and serum samples. All had cirrhosis and one patient had HCC. Detailed patient characteristics are presented in paper II.

I paper III, 75 serum samples from 46 chronic hepatitis B patients monitored at the Sahlgrenska University Hospital were analyzed. A majority of these patients were HBeAg-positive. Three patients were sampled also after initiation of antiviral treatment.

I paper IV, we analyzed 23 serum samples from patients with chronic hepatitis B who were monitored at the Infectious diseases clinic, Sahlgrenska University Hospital.

In paper V, seventeen serum samples from chronic hepatitis B patients were analyzed.

Liver explant samples were collected in the operation theatre at the time for transplantation. All transplantations were performed at the Department of Transplantation Surgery, Sahlgrenska University Hospital, Gothenburg, Sweden. Immediately after explantation, small pieces from all liver lobes of were cut out. In patients with HCC, separate samples were taken from the tumor. Samples were immediately put in either RNAlater, AllProtect or no medium for storage at -80°C. Serum samples were collected as part of routine care and stored at -20°C at the Department of Clinical Microbiology at Sahlgrenska University Hospital, Gothenburg.

Ethics

Ethical approval for paper I, II and IV was granted by the Ethical Review Board in Gothenburg (registration numbers 835-17, 165-09 and 828-15) and for paper III and V by the Swedish Ethical Review Authority (registration number 2022-01628-01).

3.2 METHODS

3.2.1 Tissue preparation and nucleic acid extraction

Tissue homogenization

MagNA Lyser (Roche, Basel, Switzerland) was used for the homogenization of liver tissue. Liver samples were cut into pieces of approximately 5x5 mm in paper I, and for paper II the pieces were 1x1 mm. The tissue pieces were transferred to a MagNA Lyser Green Beads tube (Roche) containing buffer solution for either of the extraction kits.

Nucleic acid extraction

For optimal RNA quality we used a spin column extraction for RNA for liver tissue deep sequencing experiments. For papers I and II, the RNeasy kit (Qiagen) was used for RNA extraction of liver tissue, according to the manufacturer's instructions. Briefly, the homogenate was placed in a spin column, after which denaturation and nucleic acid purification was performed according to manufacturer instructions. The nucleic acid was diluted in superQ H_2O and a TurboDNAse treatment was performed twice (Thermo-Fisher, Waltham, MA, USA) was performed.

Serum samples in papers III, IV and V were extracted with the MagNA Pure LC Total Nucleic Acid Isolation Kit with a MagNA Pure 96 instrument (Roche).

3.2.2 Polymerase chain reaction

Polymerase chain reaction (PCR) is a process in which a known DNA sequence is amplified as first described in 1983 by Mullis and Faloona (146). PCR can be applied also on RNA if an initial reverse transcription step to generate cDNA is included. This RT step requires presence of an enzyme, reverse transcriptase, as well as a primer that anneals to the RNA target. This primer may target the poly(A)-tail if all mRNA is wished to be converted unbiasedly, alternatively, random primers or target-specific primers may be used.

Realtime PCR (qPCR)

In a real-time PCR a fluorescent probe is included in the PCR. The probe is designed to be complementary to a target in the region between the primers, and carries a fluorophore that emits light of a specific spectrum that is detected by a sensor (147). The key parameter in real-time PCR is the Ct value that refers to the cycle when the fluorescence from the probe reaches a certain level. The Ct value is inversely related to the concentration of the target and can be translated to the concentration of the nucleic acid target by defined algorithms.

Digital PCR

In a digital PCR, the amplification of a target is performed as parallel reactions in several thousand nanodroplets (148). Based on the relationship between positive and negative, along with mathematics based on the Poisson distribution, a highly accurate quantification of DNA is achieved.

3.2.3 Sequencing methods

Sanger sequencing

The Sanger sequencing method, developed by Frederick Sanger and first published in 1977 (149), was the first method capable of determining a DNA sequence and is still widely used. Before the Sanger method, successful sequencing attempts of the tRNA of *Saccharomyces cerevisiae* with selective ribonucleases had been made by Holley and colleagues (150). Briefly, an enrichment of the DNA that is to be sequenced is usually made prior to sequencing to avoid background noise. A complementary oligo, known as a sequencing primer, enables the polymerase to bind to the single stranded DNA to start synthesis of the complementary strand. Deoxynucleotides and dideoxynucleotides are used as substrate; the latter are not compatible with chain elongation because of the lack of a 3' OH-group needed for phosphodiester binding, which means that the primer elongation halts randomly and that the chain terminates at different positions. This produces fragments of all possible lengths of the target that are separated by gel electrophoresis and by using separate reaction wells for A, C, G and T stop nucleotides, and the sequence can be read directly from the gel. Sanger sequencing was further refined by Hood and colleagues to involve detection of fluorophores and capillary gel electrophoreses (151). Fragments of

different length reach a light spectrum-detecting laser at different time points. The fluorophore of the shortest fragment, *i.e.*, the first nucleotide, is detected first and the last nucleotide, present on the longest fragment, is detected last. The maximum read length for Sanger sequencing is approximately 700 nt. Sanger sequencing was used in studies IV and V for comparison with the deep sequencing-based methods we have developed.

Deep sequencing (next generation sequencing, massive parallel sequencing)

Deep sequencing refers to techniques that determine the sequence nucleic acids in parallel reactions, *i.e.*, not only generate one consensus sequence but typically thousands to million nucleic acid reads of fixed of various lengths. A read is the sequence of one (whole or part of) nucleic acid molecule. We have used the three most common deep sequencing platforms: Illumina, Ion Torrent and Nanopore. Figure 7 and 8 depict the mechanism for calling nucleotides of these sequencing techniques.

Illumina sequencing

The Illumina technique (Illumina inc., San Diego, CA, USA) offers sequencing platforms based on the detection of incorporated fluorescent nucleotides from several million DNA molecules in the same reaction. The method was first developed by Balasubramanian and was previously called Solexa sequencing (152). The luminescent A, C, G and T nucleotides emit different colors, and the sequence is determined by detecting the different light spectra produced. The method is commonly referred to as Bridge Amplification Sequencing, as the DNA molecules folds to a bridge-like shape during the initial steps of the sequencing reaction. Briefly, oligo adapters containing a known sequence complementary to those on the surface of the sequencing chips are attached on the DNA. Thereafter, the DNA folds and the other end of the DNA also binds to a complementary oligo on the chip. After folding, a complementary strand is synthesized and clonal expansion of the strands is achieved; one strand is denatured where after fluorescent nucleotides are introduced that bind to the strands left on the chip. The clonally expanded molecules are positioned very close to each other on the chip and therefore the light signal produced by each DNA clone is accumulated to one light signal, increasing sensitivity and specificity (153, 154).

The main advantages of Illumina sequencing are accuracy and high throughput. Several hundred million reads can be obtained from one sample and the quality of each read is typically very high, which makes this approach

suitable for metagenomic sequencing, transcriptomic analysis and minor variant analyses (154). Disadvantages include a relatively short read length, 100-250 nt from each end of the DNA sequenced depending on kit used. The paired-end sequencing sometimes results in reads that have an unknown middle part, but if the number of reads is high, this part is almost always covered by overlapping reads. However, structural variants such as detecting splice sites may still be problematic.

Illumina was employed in paper I for sequencing of RNA from liver explants and a metagenomic shotgun approach was used, that is, no HBV enrichment was performed and all RNA in the sample was sequenced. The metagenomic method may have reduced sensitivity for HBV but enabled simultaneous analysis of human gene expression.

Ion Torrent

The Ion Torrent (Thermo Fisher) sequencing method is based on the release of hydrogen ions, *i.e.* protons, which occurs when complementary nucleotides are incorporated. This release entails a pH change that can be detected. The DNA that is to be sequenced binds to beads and an emulsion PCR is performed. The beads carry oligos on their surface, complementary to a sequence on the DNA, as the DNA molecules have been equipped with an adapter sequence during preparation. The emulsion PCR amplifies DNA with adapters and for each cycle, more DNA bind to the non-occupied oligos of the beads. After the emulsion PCR, the DNA is denatured and only single strands are left. The beads are loaded onto a chip with wells, in which the sequencing reaction occur. The chip is flushed with different nucleotide solutions in a cyclical manner and the exact timepoint for binding of a nucleotide, *i.e* a pH change, is noted and the sequences can therefore be determined (155, 156). Ion Torrent sequencing was employed in paper II, IV and V.

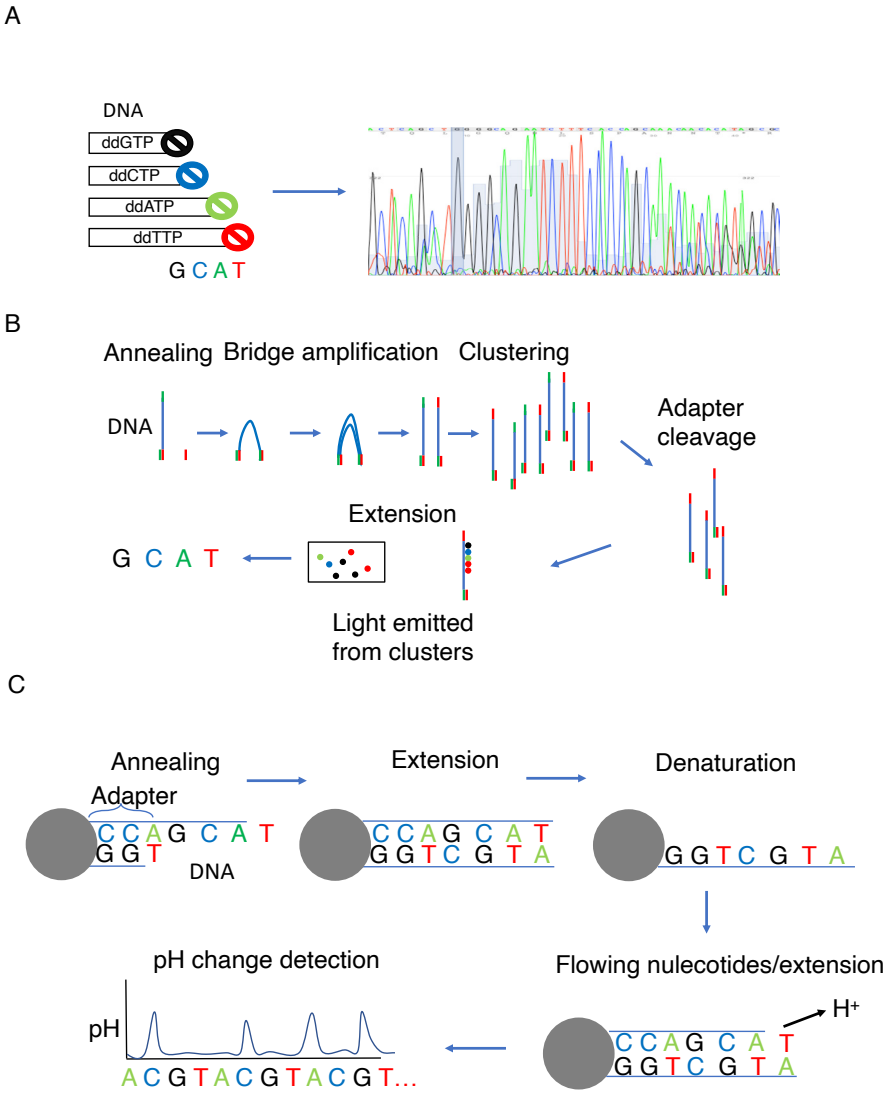

Figure 7. A) Sanger sequencing. Dideoxynucletotides terminate chain elongation and the last complementary nucleotide fluorophore of each fragment can be detected. B) Illumina sequencing is based on bridge amplification, clustering of DNA and detection of complementary fluorescent nucleotides. C) Ion Torrent reads are generated through emulsion PCR and pH detection after the addition of a matching nucleotide.

For paper II we developed a method that amplifies all HBV RNA with a 3' end surpassing position 1690 in the HBV genome, enabling enrichment of HBV integrations. Briefly, RNA 3' ends were ligated with a dsDNA oligo with a known Ion Torrent sequencing adapter sequence. RT was performed with primers targeting this sequence, followed by a fusion PCR targeting HBV region 1690-1710 simultaneously incorporating the other Ion Torrent adapter. The protocol enables direct Ion Torrent sequencing of the amplicons after bead clean-up.

For paper IV, we developed a method for whole genome sequencing of HBV using Ion Torrent. This method allowed for overlapping amplicons covering the entire HBV genome. Primers were equipped with adapter and barcodes sequences, making the library preparation significantly faster and less expensive than standard ligation-based protocols.

Nanopore

The Nanopore sequencing technique was originally developed by David Deamer in 1989 when he realized that small protein channels could be fitted into a membrane with an ionic current and when a nucleotide passes through the pore, a change in the ionic current would occur (157). The sequencing platforms from Oxford Nanopore (Oxford Nanopore technologies, Oxford, UK) is an adaptation of protein pore-based sequencing. Briefly, nucleic acid is prepared and loaded onto so called flow cells that comprise several thousand nanopores, membranes and sensors that allow for the detection of the small and unique current changes of individual nucleotides. As the nucleic acid passes the pore, the whole sequence of the DNA is determined and the reading is continuous rather than requiring cycles of the reaction, thus enabling sequencing of very long DNA. When the nucleic acid chain has passed the pore, a new molecule may enter and the process starts again. The pores are eventually degraded as they consist of proteins, which eventually limits the sequencing reaction. Nanopore sequencing of double-stranded DNA is performed from the 5' end. A motor protein with helicase activity feeds the strand through the pore and the opposite strand is fixed on the membrane in singleplex protocols (158). For sequencing of both strands duplex protocols have recently been made available; however, such kits were not used in our studies.

The direct detection of nucleotides makes Nanopore sequencing faster than previous sequencing techniques that rely on cycles of complementary nucleotide addition and detection of a signal produced when a complementary nucleotide binds. Protocols for library preparation are also

typically less time-consuming and the reagent cost, if samples are barcoded and pooled prior to sequencing, is lower.

We have used the ligation sequencing kit and rapid barcoding kits (Oxford Nanopore Technologies), both adapted for dsDNA sequencing. For RNA sequencing, reverse transcription was first performed and cDNA converted to double-stranded DNA with PCR. We also evaluated the direct RNA sequencing kit from Nanopore but the quality of the reads was insufficient for our purposes.

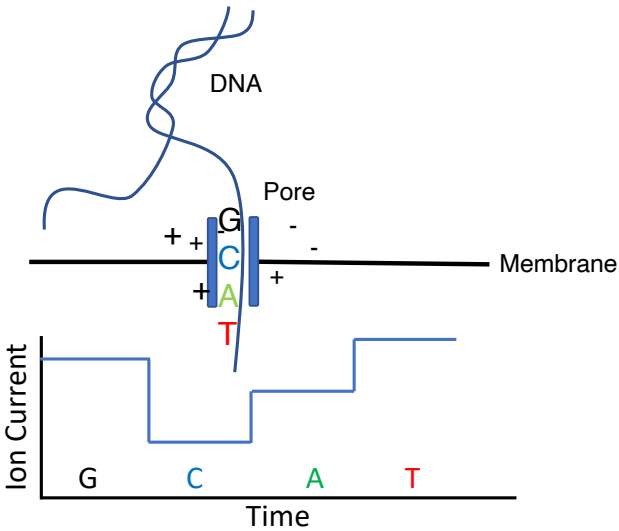

Figure 8. Nanopore sequencing. DNA molecules flow through pores, inducing a change of the ion current (represented by + and -) in the membrane that can be translated to the corresponding nucleotide, as seen in the graph.

In paper II and IV we used a reverse transcription method targeting the poly(A) tail, either native or attached prior to RT with *E.coli* poly(A) 3' polymerase. In paper II we used probes to capture HBV sequences prior to Nanopore sequencing. In paper IV, PCRs were performed with HBV-specific forward primers and reverse primers targeting the tag sequence introduced on the 5' end of the cDNA during reverse transcription. In paper V the material used was HBV DNA from serum samples and two conventional PCRs were carried out prior to library preparation. Library preparation was performed with either the ligation sequencing kit or the rapid barcoding kit.

The nanopore method has a higher error rate than the methods described above, and the errors therefore must be corrected or removed by advanced algorithms as described in detail below.

3.2.4 Bioinformatics and statistics

Bioinformatics

Deep sequencing yields immense amounts of data that must be analyzed with mathematical algorithms, often called bioinformatic tools. The bioinformatics are adjusted to the sequencing platform used as they each have different biases.

Briefly, bioinformatic analysis often includes multiple programs that are connected and perform different tasks, often referred to as pipelines. Most bioinformatical pipelines start with filtering of low-quality reads that are discarded from further analyses. The next step is usually mapping of reads to a reference sequence, after which only treads that map to the organism studied are kept; the rest are discarded. Alternatively, a *de novo* assembly can be made. *De novo* assemblers align all reads to each other rather than to one reference and can thereby create mappings and consensus sequences that are unaffected by the structure of the reference genome. *De novo* assembly is often needed for large variable genomes, typically found in bacteria, but for small viral genomes with few genomic aberrations the most common method is mapping to a reference.

Illumina and Ion Torrent reads may be mapped to the reference genome using BWA, BowTie2, STAR and TopHat, all performing short reads alignment. These tools accept reads stored as sequences in fastq files for mapping. The fastq files always contain essential information about the reads; the name of the read, nucleotide content, quality of the nucleotide calling, which instrument that was used and at which time the read was produced.

Most mapping tools are based on dynamic programming, which is a mathematical optimization method developed by Richard Bellman in the 1950s (159). The method allows matching of similar but not identical sequences based on a scoring system for each read and its potential match in the reference genome. Dynamic programming requires a high computing capacity as the number of calculations performed in analysis high-throughput data can sum up to billions. That is why the most common mapping tools

BWA and BowTie2 use a Burrow-Wheeler transformation that rearranges the nucleotide sequences into strings that are better suited for computations. This compressing of data increase of memory-efficiency and makes mapping of reads possible to perform on a standard personal computer (160).

Minimap2 is a relatively new method for mapping of reads and is designed for long reads, such as those from Nanopore. Longer sequences require other mapping tool features, such as allowing splicing and handling insertions/deletions differently to properly map reads to a reference. Minimap2 is also memory-efficient and can be used for short read data (161).

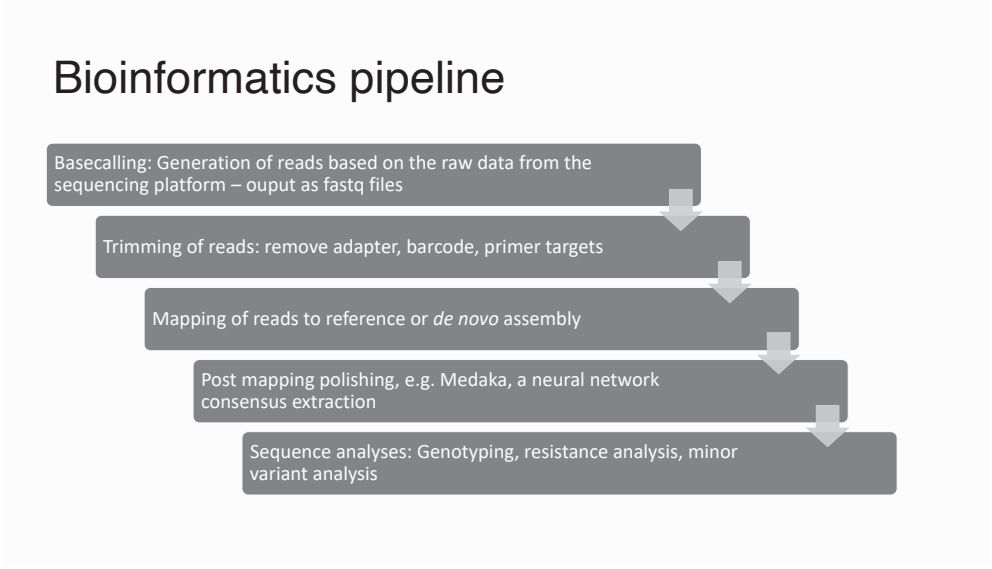

Figure 9. Schematic picture of a typical bioinformatics pipeline.

We developed in-house bioinformatics pipelines that not only map reads to the HBV reference but also extract reads with an HBV/human breakpoint. These reads are analyzed in detail aiming to detect HBV genomic breakpoint and human breakpoint, thus identifying human gene involved. In RNA sequencing, the number of reads covering each gene give an estimation of the gene expression. Our pipelines are publicly available on GitHub (https://github.com/SannaAb/Viral_Integration_Pipe). Figure 9 depicts a typical bioinformatics pipeline.

The CLC Genomic Workbench (Qiagen, Hilden, Germany) bioinformatical program provides classical tools such as alignment and phylogenetic analyses as well as methods for analyzing deep sequencing data, including those mentioned above. CLC provides a user-friendly graphical interface. There is

no need for advanced programming skills. The program provide visualization of bioinformatical data and was utilized to create graphs of mapping coverage, extract consensus sequences and for minor variant analyses. The consensus sequences from the samples were aligned with the classic alignment tool in CLC Genomics Workbench. Phylogenetic trees, such as those in paper IV and V were also made in CLC with the Jukes-Cantor neighbor-joining method.

Statistics

In paper III, linear mixed effects model was used for all comparisons except for RNA ratios in duplicate samples taken at different timepoints, for which Pearson correlation coefficient was used, statistical analyses were performed in R (version 4 or later). In paper II, simple linear regression analysis was used, performed in Prism (version 9 or later) (GraphPad, San Diego, CA, USA).

4 RESULTS AND DISCUSSION

Paper I.

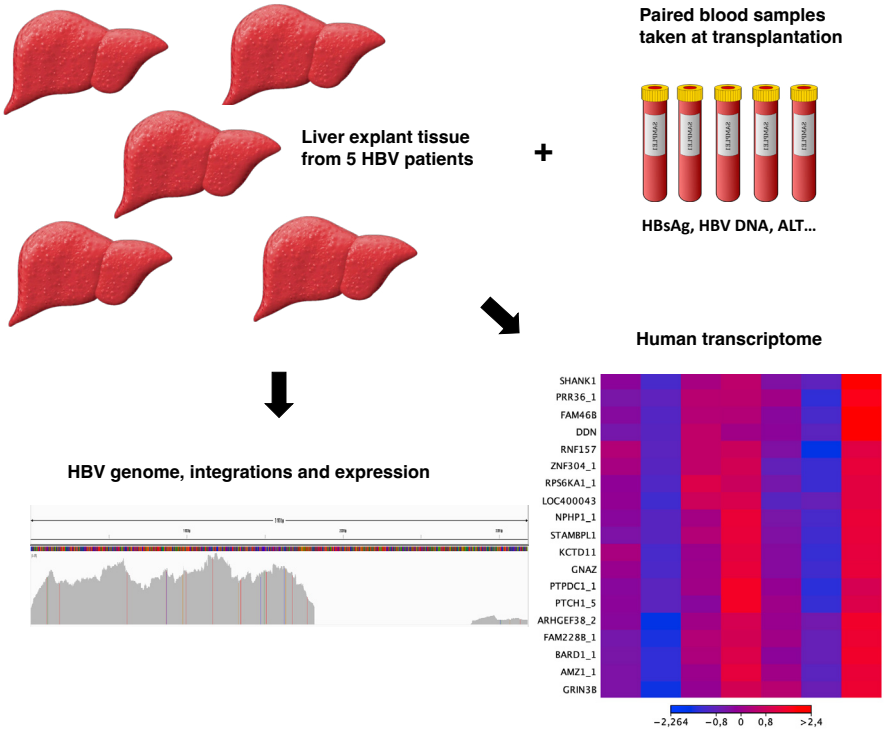

Figure 10. Graphical abstract for paper I. The five liver explants were transcriptome sequenced, HBV and human gene expressions were analyzed. Paired blood samples were available for analysis of HBV-related markers. HBV integrations were seen to impact both the expression of HBV mRNAs and also human gene expression.

This study used Illumina sequencing of all RNA in liver explant tissue from five patients (tumor and non-tumor tissue) with liver cirrhosis caused by HBV or HDV/HBV co-infection. The results showed that HBV integrations are extensively expressed, with an RNA profile (figure 11) that supports that integrations contribute to HBsAg levels in blood. More than 99% of fusion reads had an HBV 5' part and a human 3' end, indicating transcription start at the HBV S promoters and probably an intact S open reading frame.

The HBV expression profile showed a lack of reads in the core region and of reads with a 3' end reaching the polyadenylation site at approximately nt

1930-40, which would be present if the RNA were transcribed from cccDNA. Therefore, the observed RNA profile supports that most of the HBV RNA originated from integrations rather than from cccDNA. Accordingly, the relatively high HBsAg levels in serum of these patients likely were due to abundant presence and expression of integrated HBV DNA. Based on the lack of reads in the 3' part (up to the polyadenylation site) we estimated that on average 95% of the HBV RNA from all samples were derived from integrations (range 24-100%).

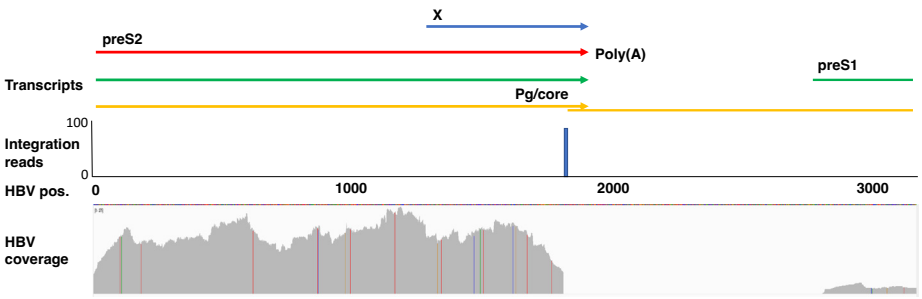

Figure 11. Graph showing a typical HBV expression profile in explant liver tissue. Pg/core RNA is not expressed, preS1 is scarcely expressed and preS2 and X dominate the expression. Integration derived transcripts constitute most of the HBV RNA which is shown by the high number of reads carrying a human 3' end and the lack of reads covering the conventional poly(A)-site used in cccDNA.

The ratio of fusion reads to all HBV reads was 3-7%, but the HBV/human integration breakpoint would most often be missing because of the technical limitations. Integrations mostly occur in the 1700-1820 region of the HBV genome and the short reads (250 nt long) generated by Illumina sequencing are evenly distributed over the entire length of the HBV transcripts they were derived from. Thus, this ratio does not give a representative ratio of transcription of cccDNA vs. integrated HBV DNA. An alternative estimation, which considers that the fusion reads found do not represent more than 10% of the reads actually derived from fusion RNA, instead give a span of 10-70% integration derived HBV RNAs, more similar to the calculations presented in the paragraph above. At the time of the study, long reads sequencing, such as PacBio and Nanopore, was less available, however, those methods can presumably offer a better estimation of this ratio, as whole integration and cccDNA derived HBV transcripts can be captured and characterized.

We also analyzed data from a Japanese collection of RNA-sequencing data from HBV-infected patients that had undergone liver transplantation or

resection, stored in the International Cancer Genome Consortium database (162). The results were similar to those from our samples and the majority of the samples, both tumor and non-tumor, showed a high expression of HBV/human chimeric RNA.

Several integrations changed the expression of the host gene adjacent to the HBV integration. An overexpression of the human gene was typically found, which hypothetically may contribute to oncogenesis. Among the genes with integrations we found *TERT* which is a driver mutation for HCC and other neoplasms (163). Notably, the *TERT* expression of the sample was 1000-fold higher than that of normal liver tissue. *TERT* has been reported to carry integration in previous studies and potentially HBV integrations in *TERT* are selected during tumor development as this mutation entails longer cell survival (16). In the ICGC dataset we also found integrations in the *MLL4* gene, previously described as a common site for HBV integrations and possibly contributing to HCC development. Most HBV integrations were found in intronic regions of the human genome that usually lack RNA expression. Twelve integrations were found in genes that normally are expressed in hepatocytes. Eight of these genes had a higher expression than normal liver tissue (164), three had a decreased expression and one gene was unchanged.

More recent articles have shown similar results (165, 166), including a study of liver biopsies from non-cirrhotic/non-tumor patients that reported similar expression profiles and a similar number of HBV integrations (47). These findings further support our conclusion that the majority of HBsAg in HBeAg-negative patients originates from integrations.

The finding that integrated HBV DNA may maintain high levels of HBsAg in serum in the absence of detectable HBV replication challenges the use of HBsAg as marker for HBV infection, and possibly also the dogma that HBsAg loss is required for cure. Some patients with undetectable HBsAg in serum would probably be considered “cured” if the lack of viral replication (undetected HBV DNA) were the main criterion. On the other hand, integrations could be the reason why HBsAg-positive patients without inflammation are at higher risk of developing HCC than those never infected. Therefore, HBsAg loss may still be the best way of defining HBV cure, and treatments also targeting integrations should be the developed, not least since integrations are potentially associated with oncogenesis (16, 49). In the absence of such treatment a way to reduce integrations might be to initiate antiviral treatment earlier and on broader indication, which should decrease accumulation of integrations over a lifetime.

Paper II.

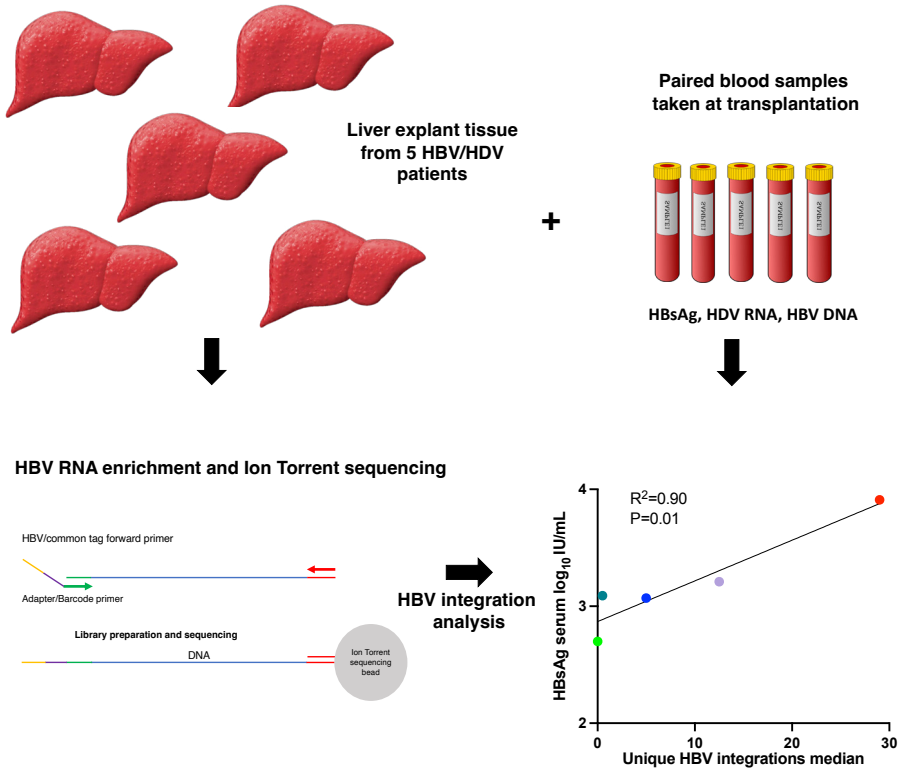

Figure 12. Graphical abstract of paper II. Liver explant tissue from five HBV/HDV positive patients were used. Ten samples from each patient were enriched for HBV RNA and sequenced. Paired blood samples were analyzed for HBV DNA, HBsAg and HDV RNA. Results show a correlation between the mean number of HBV integrations and HBsAg levels in blood.

The aim of this study was to further explore HBV integrations by deep sequencing of RNA with enhanced sensitivity for integrations. The strategy was to enrich RNA reads that contain the fusion between HBV and human sequences prior to Ion Torrent deep sequencing. This was achieved by ligating one of the Ion Torrent 'adapters' to the 3' end of all RNA fragments and then run a PCR with an HBV-specific forward primer upstream of the typical integration position in the HBV genome and a reverse primer targeting the ligated adapter. By this strategy, 90-95% of all reads were derived from HBV, which was >1,000-fold higher than with the Illumina sequencing approach used in paper I. We applied the technique on liver

explant tissue from five patients with HDV-induced liver cirrhosis. We chose HDV/HBV co-infections because of the low HBV replication typically seen in HDV infection which indicates that integrations may be an important source of HBsAg.

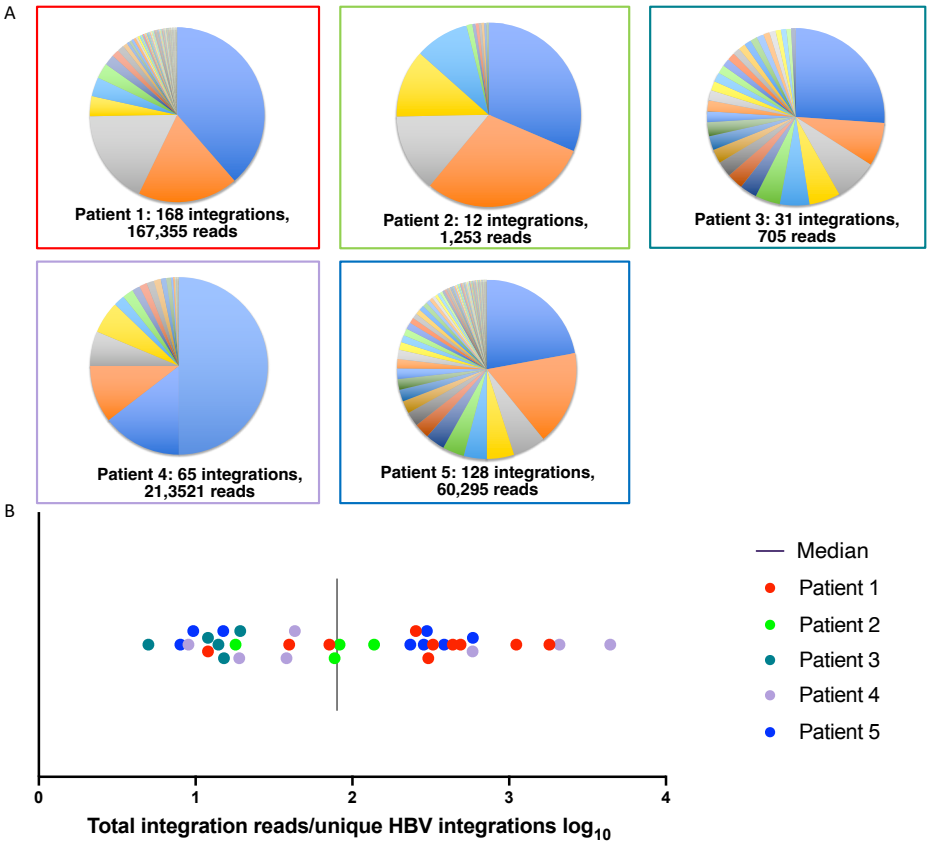

Figure 13. A) Total number of integration-derived RNA reads and number of unique integrations in five patients with HBV/HDV co-infection. B) Ratio of number of total integrations reads and unique integrations found in the samples.

Previous studies have used more expensive and arguably less sensitive methods for detecting HBV integrations (17, 50, 52, 53, 130). Typically, only one sample from each patient is analyzed, which yields a sampling bias as HBV integrations are unevenly and focally distributed (167). To obtain a more representative result we analyzed 10 pieces from each of the five liver explants.

The results showed that HBV integrations were abundant and highly expressed albeit with large variation between the tissue pieces and between

patients. A high number of unique integrations were identified but only a few of these integrations also showed high amounts of reads, indicating high expression. Figure 13 illustrates the number of integrations and the variation of expression between and within patients. In the 50 samples from the five patients, integrations were found in 420 different regions or genes of the human genome. On average there were 20 different HBV integrations in each patient.

Each unique integration was supported by a median of 80 reads (mean 419), which was similar to the ratio between HBV S RNA and S DNA by real-time PCR (median 36, mean 290). The HBV DNA copies per 100 cells (number of HBV S DNA copies/100 betaglobin DNA copies) varied from less <1 to >1000. The latter finding implies that some cells contain more than one HBV DNA copy per cell. The number of fusion reads (*i.e.,* derived from integrations) was compared with the serum level of HBsAg, showing a significant correlation (Figure 14). This finding, in combination with low HBV DNA levels in serum, supports that both HBsAg present in subviral particles and HBsAg in HDV particles are assembled by integration derived HBsAg. Interestingly, the number of integration-derived reads and HDV RNA in liver tissue did not correlate, as did not HBV S RNA and HDV RNA, suggesting that synthesis of HDV RNA, but not infectious HDV particles, might take place in hepatocytes that lack HBsAg production.

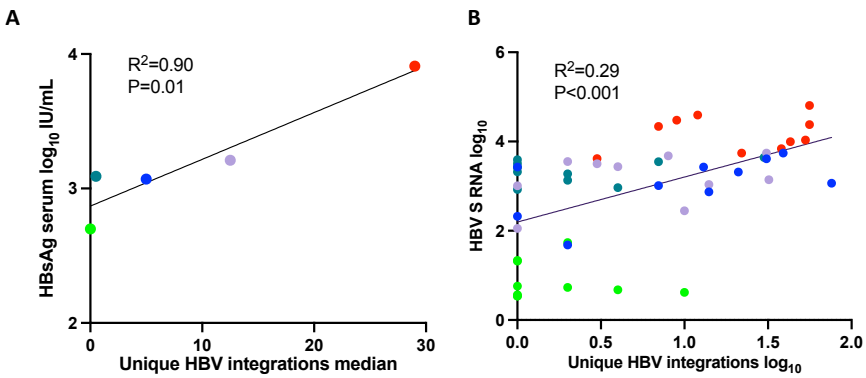

Figure 14. Correlation between (A) the median number of HBV integrations in 10 liver samples from each of the five patients and their HBsAg levels in serum and (B) number of integrations and S RNA levels of the total 50 tissue samples. Each color represents a specific patient.

As mentioned, there was high variation in number of reads representing each unique integration. A striking finding was that a few integrations contributed to a major part of the integration expression. When we applied the analysis

on publicly available sequences (obtained by Illumina) from a study by Furuta *et al*. (130) we confirmed that a few unique HBV integrations were responsible for the majority of the fusion reads.

Nanopore sequencing, following probe-based enrichment of samples, was performed in two of the patients. Both samples showed an almost complete lack of pgRNA and a high ratio of integration-derived reads, further supporting the notion that most HBV RNA in these samples was derived from integrations, HBV coverage profiles were very similar to those presented in paper I (Figure 11).

Taken together these results imply that the expression of integrated HBV DNA in liver tissue varies between patients and between tissue pieces from individual patients, and that some integrations are represented by very high number of reads. Such high expression of a few integrations is likely due to both clonal expansion of hepatocytes carrying the integrations along with a high rate of transcription of these integrations. Our interpretation is that most integrations are lowly expressed while a small proportion of integrations contribute to the majority of the HBsAg. Thus, the high HBsAg levels that are typically maintained also when HBV replication has ceased (such as in HDV infection) might be explained by a random integration strategy that, by unknown mechanisms results in high HBsAg production in a small fraction of hepatocytes.

In conclusion, these results suggest that many unique integration events increase the likelihood that a few integrations become highly expressed. These few integrations seem to produce a large proportion of the HBsAg and might contribute to HDV particle assembly.

Paper III.

This study aimed to explore the nucleic acid content in HBV particles in blood by digital PCR targeting different regions of the genome. The results suggested that HBV particles may contain both RNA and DNA in inverse amounts because of incomplete reverse transcription.

The high accuracy of digital droplet PCR (ddPCR), allowed us to compare levels of HBV RNA and HBV DNA in blood when measured in different genomic regions: core, S and X. The results showed higher levels of core RNA than in S or X, as depicted in figure 15. The mean levels of core RNA

were 0.4 $\log_{10}$ units higher than S RNA, and S RNA levels were 0.2 $\log_{10}$ units higher than X. The DNA levels were higher than RNA and constituted 96% of total nucleic acid in X, 93% in S and 80% in core. Possibly the relatively lower levels in S could be due to splicing, which should influence the ddPCR targeting S but in most cases not core or X.

The gradient in RNA levels with the lowest levels in X most fits with the direction (3'→5') of degradation from RNase H during reverse transcription. By using nanopore sequencing on three samples we observed that the reverse transcription and RNase H halt during DNA-synthesis, as the HBV RNA reads were distributed very similar to the ddPCR profile, with the highest coverage at the pgRNA start. We also noted that splicing (most commonly joining of nt 2467-489, 2087-489 or 2067-2350:2447-489) was present in up to 50% of the reads spanning the S region in the studied samples, although the method did not allow for absolute quantification. Figure 16A shows the RNase H activity from the 5' to the 3' end of HBV pgRNA, resulting in a higher reduction of RNA in the X and S regions than in core (figure 15A).

HBV particles containing RNA have been extensively investigated (168-173). Although gradients of RNA levels similar to those observed in our study have been reported (173, 174), the view has remained that particles with DNA or RNA are different entities. Our results indicate that this dichotomous view may have to be modified in that particles with RNA rather represent a normal HBV particle with incomplete reverse transcription. The reverse transcription seems to halt before completion of the minus strand in approximately 20% of particles. It is possible that there are also other types of HBV RNA-containing particles, for example RNA in capsids without an envelope (naked capsids) (173) or RNA without an active polymerase (with a maintained poly(A) terminal). However, the gradient observed suggests that these particles are much less common than particles carrying partially degraded RNA.

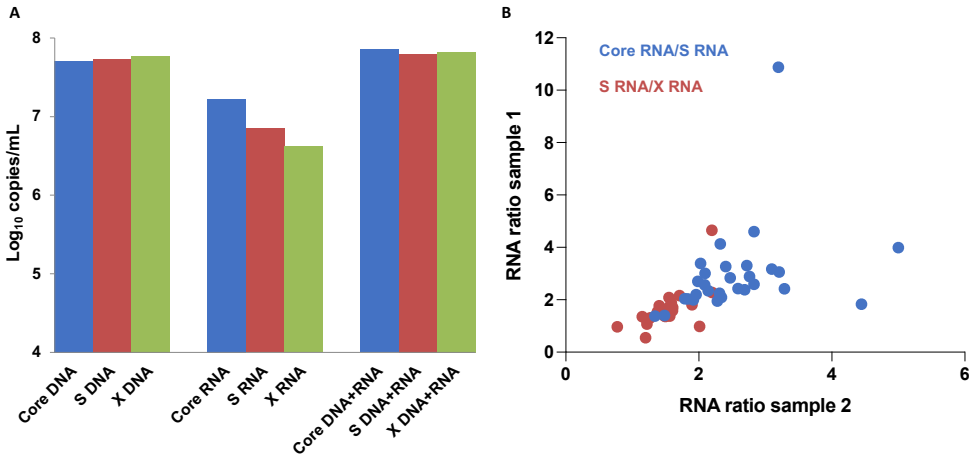

Figure 15. RNA and DNA content in HBV particles. A) Median values of HBV DNA and RNA in the core, S and X regions. B) Correlation between ratios for core/S RNA and S/X RNA in duplicate samples from 29 patients.

The variation in termination of reverse transcription was remarkably large, spanning the entire genome. The previously reported finding that the plus strand has a variable length likely also reflects that the polymerase might stop at different positions. The explanation to the putative halt in reverse transcription is unknown. A lack of substrate (nucleotides) within the capsid might explain the halted reverse transcription. Previous studies have shown that influx of nucleotides is not possible through the envelope and only through a naked capsid (175, 176). If so, reverse transcription would stop at different positions depending on the amount of substrate available before the particle becomes enveloped. This could be affected by factors such as the concentration of nucleotides in the cells or how quickly the virus is enveloped. Thus, both host and viral factors might influence this gradient. This is supported by the unexpected finding that the gradient profile, represented by the quantitative relation between core, S and X RNA (the ratio between the values), was maintained over time in patients that were sampled at two time points (Figure 15B). It is not known which host or viral factors that might contribute to influence the rate of reverse transcription. This rate would determine the ratio between core DNA and core RNA. Interestingly, the HBV DNA levels in blood ($p=0.001$) and the HBV genotype were associated with this ratio (D vs. non-D, $p=0.006$). These associations remained significant in multivariate analysis implying that viral factors, such

as mutations or pgRNA structure, might influence on the rate of reverse transcription.

A

B

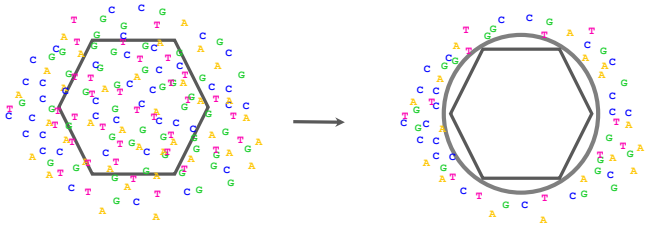

Figure 16. Panel (A) shows the direction of reverse transcription and Rnase H that is responsible for the RNA- and DNA-containing particles. Panel (B) illustrates that in a naked capsid (left), nucleotides can diffuse freely. However, the enveloped particle (right) does not allow influx of nucleotides and if the capsid is enveloped rapidly, this may reduce the availability of substrate for reverse transcription.

Paper IV. We show that deep sequencing with Ion Torrent after library preparation with fusion primers is a sensitive and efficient method for whole genome characterization of HBV (177). This method produced reproducible HBV sequences at viral concentrations exceeding HBV DNA levels of 3 $\log_{10}$ IU/mL. The resulting genotypes agreed with Sanger sequencing with 99.50 % to 99.97 % sequence agreement. The same antiviral resistance mutations were detected using both methods. Approximately 75% of discrepancies were due to ambiguous nucleotides in the Sanger sequence, 18% from gaps in the Sanger sequence and 6% from indels caused by Ion Torrent. Indels (referring to false insertions and deletions) by Ion Torrent have previously been reported (178) and could automatically be removed by using appropriate bioinformatic filters.

An advantage of a fusion primer-based library preparation is the reduction of hands-on time. The cost is also reduced as the ligation protocols are more expensive. Pooling of several samples is also made possible thanks to the barcoding, which likely provides an advantage over Sanger sequencing.

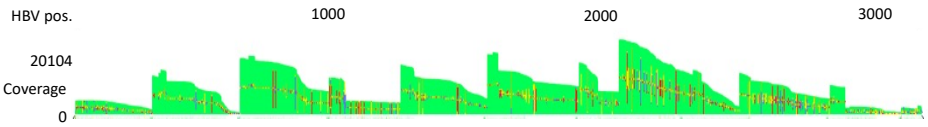

Figure 17. Typical coverage profile of reads over the HBV genome. As seen, the regions where amplicons overlap have higher coverage and there are differences between amplicons, depending on PCR efficiency.

The high number of reads obtained from each sample (mean coverage 30,000x) along with the relatively low error rate of Ion Torrent enabled accurate minor species analysis. This resulted in fewer ambiguous nucleotides than with Sanger sequencing and allowed identification of minor variants or (intra-host single nucleotide variants, iSNVs) which may emerge from polymerase errors, immune response selection and host enzyme editing.

We also performed reproducibility analysis by sequencing serial dilutions of a sample with an original HBV DNA level of 6.5 $\log_{10}$ IU/mL. At 2.5 $\log_{10}$ IU/mL we noted that the reproducibility was lower and several nucleotide differences compared to the sequences obtained from concentrations 6.5 and 4.5 $\log_{10}$ IU/mL were seen. This could partly be due to sequencing errors, but as the samples still had a relatively high number of reads, the differences cannot solely be explained by technical issues. Probably, the lower number of genome copies makes any sequencing analysis less reliable, as the genomes amplified are less likely to be represent the entire viral population. However, we did not perform a similar reproducibility analysis for the Sanger sequencing method, which is a limitation of this study.

The large depth in Ion Torrent sequencing allows the study of quasispecies with A to G mutations that may be induced by ADAR, a human enzyme converting adenosine in double-stranded RNA to inosine (interpreted as a guanosine in replication and sequencing). We have recently reported ADAR-derived mutations in SARS-CoV-2 (179), and in future studies we aim to clarify the role of ADAR and other host mechanisms that edit viral RNA/DNA for infection with HBV or HDV. Previous studies of ADAR on HBV show partly diverging results. Yuan *et al*. suggested that ADAR may promote HBV replication (180), opposite of our findings in SARS-CoV-2, while Liu *et al* found that ADAR activity inhibited HBV (181).

The deep sequencing data from Ion Torrent also provide higher resolution in the analysis of HBV transmission. In a not yet published case report we

found that minor variant analysis could support transmission of HBV despite a relatively large number of sequence differences in consensus sequences obtained by Sanger sequencing. Previously, minor variant analysis has been used to distinguish acute from chronic infection, suggesting that the viral sequence may revert to an "original" version upon infection of a new host (182, 183).

Paper V. A method for whole genome sequencing of HBV using Nanopore was developed. During the COVID-19 pandemic, Nanopore sequencing has been increasingly used in routine laboratories. The method offers simple library preparation protocols and provides less expensive deep sequencing than other platforms. We found that Nanopore analysis of HBV provided sequences with a 99.9% agreement with Sanger and 99.7% with Ion Torrent sequencing. The method comprised amplification of the whole genome in two PCR reactions, and showed high sensitivity. All seventeen samples had satisfactory coverage over the entire genome and consensus sequences of high quality. Nested PCR of the amplicon covering the RT region was applied in four of the samples that lacked obvious bands in gel electrophoresis after the first PCR. The RT sequence could be determined in those samples, additionally, the non-nested amplicon also showed a sufficient number of reads (approximately >100x). However, these samples lacked whole genome sequences from Sanger or Ion Torrent methods for validation.

The main advantages of Nanopore are simplicity and speed. We applied two kits, the ligation sequencing kit and the rapid barcoding kit, where the latter reduced hands-on time to less than one hour. We did not observe any differences in accuracy or sensitivity between these kits, based on seven samples that were sequenced using both methods (HBV DNA levels 1.8 to 9 log10 IU/mL). The rapid barcoding kit allows for pooling of 96 samples simultaneously which reduces costs as the reagent cost per flow cell run is approximately 900 euro. If all barcodes are used and 96 samples sequenced on one flow cell, the cost per sample is approximately 10 euro, including PCR reagents. If less samples are to be sequenced, the smaller and less expensive flongle flow cell can be used. We pooled up to 12 samples on one flongle and the cost per sample was 20 euro.

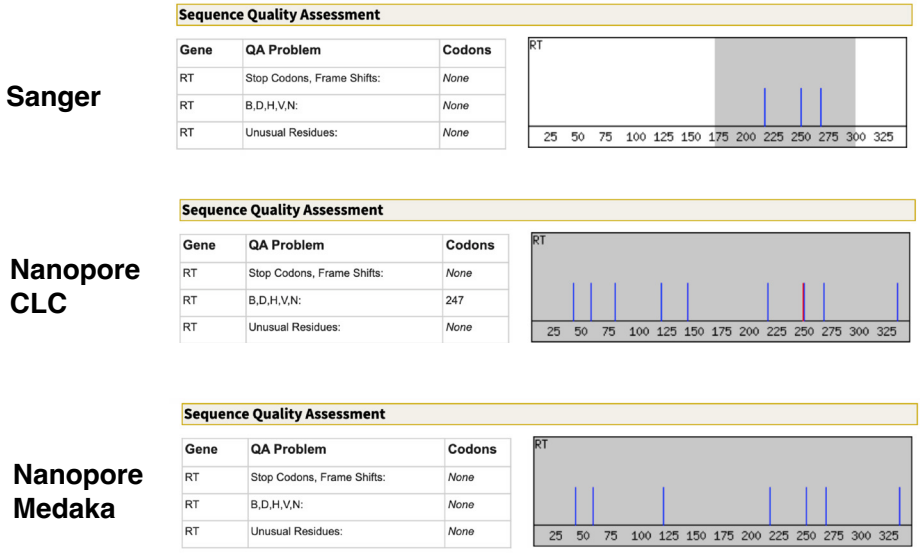

Figure 18. Example of the quality improvement gained by the Medaka Nanopore sequence polishing. Sanger- and Nanopore-derived sequences from one sample are shown, red lines indicate quality problems and blue lines represent differences compared to the HBV reference that are not considered quality issues in the Stanford HBV database. As shown, Medaka removes Nanopore sequencing errors, as compared to the CLC sequence extraction that does not have this capacity. The shadowing shows that the Nanopore sequences span the entire reverse transcriptase region, as compared to the shorter Sanger sequence.

A robust bioinformatic pipeline is key for creating an accurate consensus sequence from Nanopore reads, as this method yields higher error rate than other deep sequencing platforms. We used the basecalling program Guppy on a graphics processing unit (GPU) using the super-accurate mode, enabling fast production of reads and high accuracy. GPU-based basecalling has recently been developed and is compatible with Linux systems, reducing the time to complete all reads from a run from days to real-time completion (184). The reads were mapped using minimap2, which is an aligner developed for the mapping of long reads (161). Consensus sequences, representing the major viral population of the sample, were generated with Medaka, a neural network-based tool from Oxford Nanopore Technologies that has proven to provide highly accurate SARS-CoV-2 consensus sequences (185). In our study, Medaka created consensus sequences of high quality also for HBV. This was shown by comparing sequences from samples analyzed using Nanopore and Sanger sequencing. Figure 18 shows the

quality improvement of a Nanopore-derived sequence after polishing with Medaka, as compared to the consensus sequence from the CLC consensus extraction tool.

Although the Nanopore method produced high quality consensus sequences, minor variant analysis was inferior to Ion Torrent sequencing. This is probably due to the higher error rate and might be possible to overcome. McNaughton *et al.* reported that a method utilizing rolling circle amplification reduced false mutations and thus offers an interesting alternative for sequencing HBV (186). Our method was developed for clinical use; thus sensitivity and protocol simplicity had to be considered and therefore a standard PCR prior to sequencing was used.

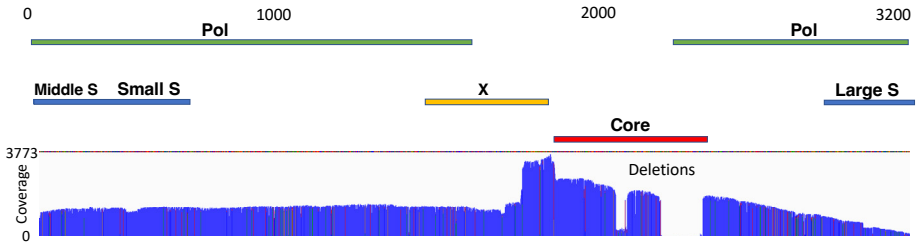

Figure 19. Example of core deletion detected. Blue graph depicts coverage over the HBV genome and above, lines represent open reading frames for the HBV proteins. As seen, there are obvious drops in coverage in the core region, very similar to core deletions previously described. This sample also carried mutations coding for antiviral resistance.

The minor variant analysis used in this study might be suboptimal for Nanopore data, as the CLC basic variant detection tool is developed for short reads data. For extraction of the consensus sequence a neural network-based tool (Medaka) was used, which produced consensus sequence of high quality by removing sequencing errors. A similar method for minor variant analysis is warranted.

With Nanopore, whole genome sequencing of HBV can be carried out rapidly and at low cost. This opens for sequencing of the virus of all HBV patients, which may translate into personalized treatment for a larger proportion of patients. Interestingly we found core deletions in 2/17 samples, of which one also carried resistance mutations. Core deletions have been proposed to herald cirrhosis development and are associated with high viral load (69). Normally, HBV sequencing for clinical diagnostics only includes analysis of the RT region, and such anomalies would be missed. The

proposed method allows for the application of WGS on all samples, and includes sequence mutations or aberrations outside of the RT region. We plan to conduct additional studies of HBV patient samples referred to our laboratory and combine results with clinical data to better understand which mutations are clinically important.

5 CONCLUSION AND FUTURE PERSPECTIVES

Our studies show that HBV integrations are abundant and transcriptionally active. We also found that integrations are a significant source of HBsAg levels in blood. Integration of HBV DNA is likely a strategy for the virus to produce more HBsAg, which is believed to maintain an insufficient immune response against HBV. Also, integrations probably support HDV infection in the absence of active HBV infection by supplying the HBsAg that builds the envelope of HDV.

Loss of HBsAg is considered the hallmark of HBV cure. However, relatively high levels of HBsAg may be maintained despite lack of HBV replication. There are currently no drugs that remove HBV integrations, and immunological clearance of integrations is usually slow, also when viral replication is suppressed with antivirals. One might argue that HBsAg loss is an inadequate marker for cure since it may be present in the absence of active infection, when detectable HBsAg rather reflects integration. Clearance of hepatocytes with integrations may on the other hand be required for a true HBV cure with complete reduction of the risk of HCC.

Two deep sequencing methods for analysis of the whole HBV genome were presented, which both appeared to provide advantages over Sanger sequencing in terms of cost efficiency and performance. The Ion Torrent-based method described in paper IV allows intra-host viral variant detection and antiviral resistance identification with high sensitivity, which holds promise for future studies of minor variants and their interplay with host mechanism altering the viral genome, such as ADAR. The Nanopore-based protocol appears to be well suited for clinical diagnostics, being fast, inexpensive and easy to perform. Also, analysis of structural aberrations of the HBV genome can be performed, which may be useful in deciphering the impact of aberrant HBV DNA on clinical outcome of infection.

A future application of deep sequencing might be the search for specific HBV integrations in blood samples aiming to detect recurrent HCC after liver resection. This notion is supported by recently published results (53). Future studies of the usefulness of detecting circulating chimeric HBV/human DNA for earlier detection of HCC would be interesting. Currently, HBV patients at high risk of developing HCC are screened with regular ultrasound examination with low sensitivity for small tumors. Results in this thesis

imply that integrations of HBV DNA likely reflect clonal expansion of hepatocytes, which further supports their use as a biomarker for HCC.

The finding that HBV DNA and HBV RNA in serum do not represent different entities of viral particles but are present in different proportions in the same type of particle suggests that HBV in blood represents a wide spectrum of DNA-RNA mixtures, albeit with predominance of DNA. We hypothesize that this is explained by a variable progress of the reverse transcription. This finding, and its interpretation, agrees with previous studies showing that the plus DNA strand of HBV show variable length. In future studies we will investigate variation in the DNA plus strand using digital PCR techniques. We will also expand the analysis of the impact of HBV genotype on the DNA:RNA proportions. While the clinical importance of the finding of these RNA/DNA HBV particles is uncertain, it should mean that the recorded levels of HBV DNA or HBV RNA in diagnostics may be influenced by the target region for PCR, and points to the importance of using the same target region.

The eradication of HBV is not in sight despite an efficient and globally available vaccine. A global campaign to eliminate HBV has been initiated by the WHO comprising expanded identification of infection by screening, improved vaccination programs and the development of additional therapeutics. Antiviral treatment for pregnant women and modified immunization programs that include general HBV vaccination directly after birth should be available in low-income countries. Such programs have shown to be effective on a large scale, for example in Taiwan (187). To achieve the task of eradication of HBV, molecular diagnostics, vaccine, and antivirals must be made less expensive, more available and implemented in robust healthcare systems.

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