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Evaluation of hepatitis B virus surface protein variations in patients under therapy with nucleic acid polymer REP 2139-Ca

Hrvoje Mijočević

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C. Abbreviations

aa	amino acid
anti-HBc	antibodies against HBV core antigen
anti-HBe	antibodies against HBV “e” antigen
anti-HBs	antibodies against HBV surface antigen
cccDNA	covalently closed circular DNA
HBeAg	hepatitis B virus “e” antigen
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HDV	hepatitis D (delta) virus
MHR	major hydrophilic region
mRNA	messenger RNA
MSM	men who have sex with men
NAP	nucleic acid polymer
NGI	National Genetic Institute, Los Angeles, California, USA
NGS	next generation sequencing
NTCP	sodium taurocholate cotransporting polypeptide
NUC	nucleos(t)ide analogues
pegINF	pegylated interferon alfa
pgRNA	pregenomic RNA
rcDNA	relaxed circular DNA
QS	quasispecies
SVP	subviral particles
TUM	Technische Universität München
UDE	University of Duisburg-Essen

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1. Introduction

1.1. Hepatitis B virus

Hepatitis B virus (HBV) is an enveloped DNA virus with an icosahedral nucleocapsid, has a small (approx. 3200 bp), partially double-stranded circular DNA and is a member of the *Hepadnaviridae* virus family. Based on a divergence of more than 8% in the complete nucleotide sequence, ten genotypes (A-J) with distinct geographical distribution have been identified to date. Genotype A is prevalent in north-western Europe, sub-Saharan and Western Africa, and the USA; genotypes B and C in Asia; genotype D in the Mediterranean countries, Middle East and India; genotype E in West Africa; genotype F in South and Central America; genotype G in the USA, France and Germany; genotype H in Central and South America; genotype I has recently been reported in Vietnam and Laos, while genotype J has been identified in the Ryukyu Islands in Japan (Sunbul, 2014). HBV genotypes are related to the clinical progression of hepatitis B, response to antiviral treatment, and prognosis.

1.1.1. HBV life cycle

After unspecific attachment of HBV to heparin sulfate proteoglycans on the surface of hepatocyte, HBV binds with its large envelope protein to the sodium taurocholate cotransporting polypeptide (NTCP), a transmembrane protein that mediates the transport of bile acids (figure 1). After endocytosis of the viral particle in the hepatocyte and uncoating, the nucleocapsid migrates from the cytoplasm to the nucleus and delivers the viral genome in form of relaxed circular DNA (rcDNA). rcDNA is converted to covalently closed circular DNA (cccDNA), also known as episomal DNA or minichromosome, which is crucial for the persistence of HBV infection. cccDNA serves as a template for the transcription of four messenger RNAs (mRNA), which are then transported to the cytoplasm and translated into viral proteins. The longest (3,5 kb) mRNA, pregenomic RNA (pgRNA), serves also as a template for reverse transcription into (-) strand DNA, that takes place within the nucleocapsid after its assemble in the cytoplasm. Synthesis of (-) strand DNA is followed with the synthesis of (+) strand DNA. The mature nucleocapsids migrate either to the nucleus and begin the new replication cycle, or reach endoplasmatic reticulum and assemble with the lipid membrane containing surface proteins. Such complete viral particle, also called Dane particle, is now ready to be secreted through the Golgi apparatus (figure 1). Besides infectious Dane particles, relatively smaller non-infectious subviral particles (SVP) in the form of spheres and filaments are secreted. SVP are composed only of HBV

envelope proteins, without capsid and genome, and represent more than 99.99% of circulating HBsAg (Ganem and Prince, 2004).

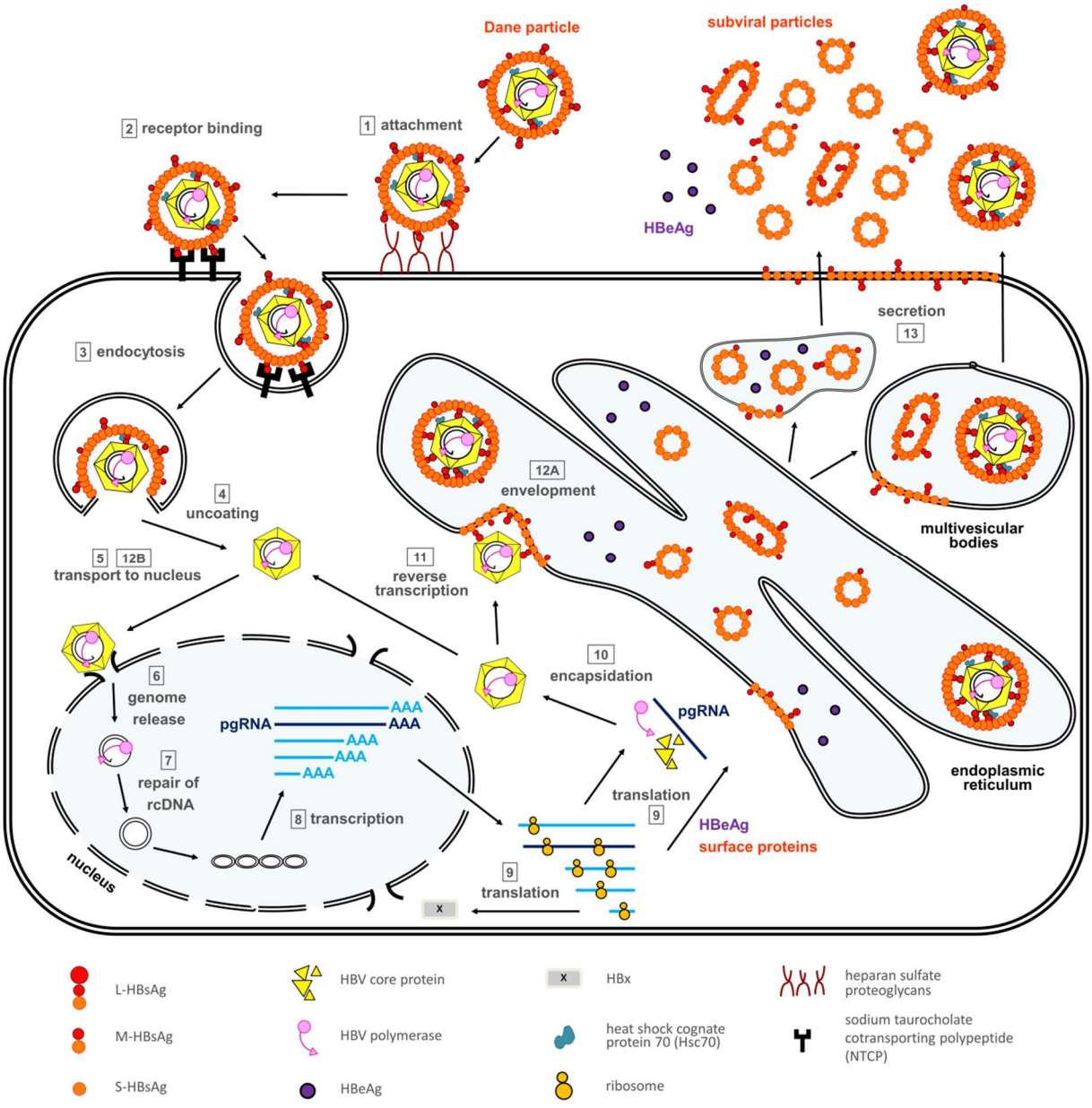


Figure 1. HBV life cycle. [1] unspecific attachment of HBV to heparin sulfate proteoglycans, [2] specific binding to NTCP, [3] endocytosis, [4] uncoating and [5] intracellular trafficking of the viral capsid, [6] release of the rcDNA genome into the nucleus, [7] conversion of rcDNA to the cccDNA persistence form, [8] transcription, [9] translation, [10] encapsidation and [11] reverse transcription of a pgRNA, [12A] envelopment and [12B] re-import of mature capsid into the nucleus, [13] secretion of virions and subviral particles (SVP). Adapted from Ko et al, 2017.

1.1.2. Organization of the viral genome

The viral genome encodes four overlapping open reading frames (ORF): P, pre-C/C, pre-S/S, and X (figure 2). The P ORF encodes viral polymerase which consists of four domains. The first domain, the terminal protein, is covalently bound to the 5' end of the (-) strand DNA and is essential for the encapsidation of the viral pgRNA. The second domain is a spacer which so far seems to have no specific function. The third domain has polymerase and reverse transcriptase activity, while the fourth domain has ribonuclease H activity, and degrades the pgRNA during (-) strand DNA synthesis. The pre-C/C ORF encodes the pre-core/core (HBeAg) and the core (HBcAg) proteins. The pre-S/S ORF encodes the viral surface (envelope) proteins, whereas the smallest X ORF encodes the small non-structural HBx protein. The pre-S/S ORF can be structurally and functionally divided into pre-S1, pre-S2 and S regions and corresponding proteins: large (L-HBsAg), medium (M-HBsAg) and small (S-HBsAg) surface protein (figure 2 and 3), which are synthesized from alternative initiation codons. S and M proteins are translated from a 2.1-kb RNA transcript, while the L protein is translated from a longer (2.4-kb) RNA transcript.

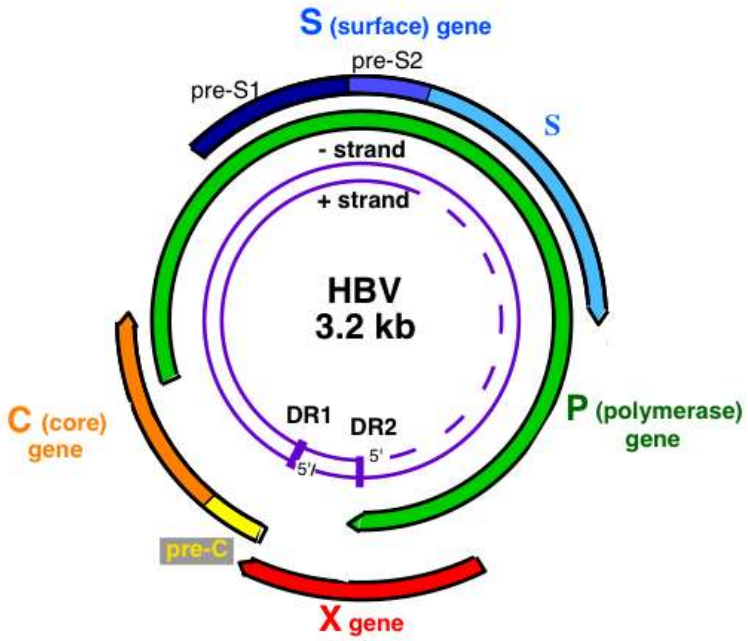


Figure 2. Organisation of the HBV viral genome. HBV genome encodes four overlapping open reading frames (ORF): P, pre-C/C, pre-S/S, and X. Adapted from Roggendorf, 2018.

Because of this compact genomic organization, mutations within one ORF may affect another ORF. The pre-S/S ORF overlaps the initial two thirds of RT-polymerase region of P ORF (702 of 1032 nucleotides). Nucleotides coding amino acids (aa) 1-7 of RT-polymerase overlap the 3' end of the pre-S2 region, whereas nucleotides coding aa 8-234 overlap the whole S region of pre-S/S ORF (Cento et al, 2013). Mutations in the S region of pre-S/S ORF may simultaneously result in mutations in RT-polymerase region of P ORF and vice versa, and may alter viral fitness (Sheldon et al, 2006). Mutations in the HBV genome occur more than 10-fold more frequently compared to other DNA viruses due to reverse transcriptase which is necessary for viral replication and has no proofreading activity.

1.1.3. HBV surface proteins

All three HBV surface proteins, L-, M- and S-HBsAg, have different N-terminus and identical c-terminus, and contain the major hydrophilic region (MHR) which encompasses aa 99-169 of S region (figure 3). The MHR includes the "a" determinant (aa 124-147) which contains the major B cell epitopes involved in recognition of HBsAg by antibodies (Wu et al, 2010).

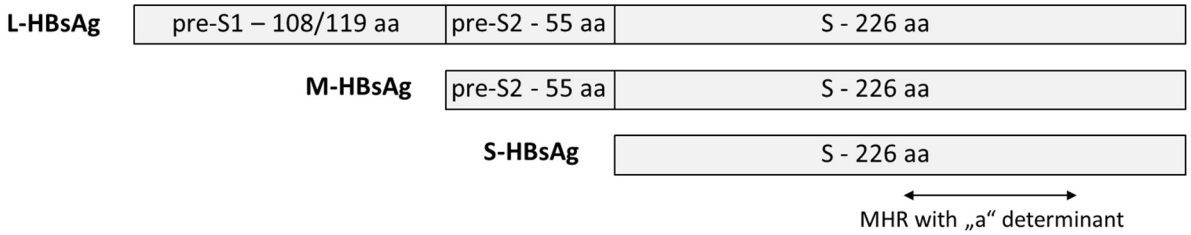


Figure 3. Organization of hepatitis B virus surface proteins. Pre-S1 protein sequence is in genotype A and C 119 amino acids (aa) long, whereas genotype D has 11 aa shorter pre-S1 sequence at the N-terminus. Pre-S2 and S consist of 55 aa and 226 aa, respectively. Large (L-) HBsAg consists of all 3 regions, medium (M-) HBsAg is shorter for pre-S1 region, whereas small (S-) HBsAg has just S region. MHR is common for all three forms of HBsAg. It is located in S region, consists of aa 99-169 and involves "a" determinant (aa 124-147).

Mutations in the S region of pre-S/S ORF, especially in the "a" determinant, can result in HBsAg variants due to conformational changes impairing the binding to anti-HBs (Chotiyaputta and Lok, 2009). Antibodies induced by vaccination or passive administrated anti-HBs immunoglobulin as post exposure prophylaxis may not be protective in case of infection with the virus containing these mutations in the

"a" determinant (Chotiyaputta and Lok, 2009; Lazarevic, 2014). In addition, these mutations can affect the reactivity of HBsAg in standard diagnostic assays causing false negative or false low HBsAg values (Carman, 1997; Lazarevic, 2014; Zhang et al, 2016). The most important and most frequently detected vaccine escape mutation is G145R (Cao, 2009; Kay and Zoulim, 2007), first described in infants of carrier mothers, who have been infected despite a properly administered passive and active immunisation (Carman et al, 1990).

L-HBsAg and S-HBsAg, but not M-HBsAg, are essential for viral assembly and infectivity of HBV (Bruss and Ganem, 1991b; Le Seyec et al, 1998; Ni et al, 2010). Nevertheless, the presence of M protein enhances the efficiency of virion secretion (Ito et al, 2010). L-HBsAg interacts with its pre-S domains with core particles and is critical for viral assembly. Additionally, the N-terminus of L-HBsAg contains binding domains for the HBV specific receptor, sodium taurocholate cotransporting polypeptide (NTCP) (Yan et al, 2012). Amino acid residues 20-29 are essential, and residues 40-59 are accessory for virion binding. Deletions in this domain may impair or inhibit HBV attachment (Churin et al, 2015; Glebe et al, 2005).

All three surface proteins have a glycosylated form. N-linked glycosylation is an attachment of the oligosaccharide glycan to an asparagine (Asn) residue of a protein, which affects the structure and function of the proteins, and is important for protein folding, stability, sorting, degradation, and secretion. The L and S proteins occur in both, nonglycosylated and monoglycosylated forms, due to an optional N-linked glycosylation site at N146 of the S region, whereas the M protein contains an additional, mandatory N-linked glycosylation site at position 4 in the pre-S2 region and occurs in monoglycosylated and diglycosylated forms (Ito et al, 2010). Lack of glycosylation due to mutations at N-linked glycosylation sites may impair virion secretion, although this may be compensated by an extra N-linked glycosylation site (Ito et al, 2010; Julithe et al, 2014). In addition, a modification of the glycosylation affects not only virion secretion, but also virion assembly, infectivity and immune escape (Julithe et al, 2014).

1.2. Hepatitis B virus infection

Hepatitis B virus infection is one of the major global health problems, with a wide spectrum of clinical manifestations ranging from acute and fulminant hepatitis to chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC). Approximately 292 million people have an acute or chronic hepatitis B infection worldwide, corresponding to a global prevalence of 3.9% (Razavi-Shearer et al, 2018). Of these, approximately 887000 die each year mostly from complications of chronic hepatitis B infection as cirrhosis and hepatocellular carcinoma (WHO, 2018a). The prevalence of HBsAg in Europe is relatively low and has been estimated as 0.6-1.7, contrary to high endemic regions as sub-Saharan Africa and Asia with the prevalence of 5.1-10.1 and 1.1-6.8, respectively (Razavi-Shearer et al, 2018).

1.2.1. Transmission of HBV

The HBV is transmitted through percutaneous or permucosal contact with the infected blood or other body fluids, including saliva, semen and vaginal fluid. Percutaneous transmission occurs through sharing and reuse of needles and syringes among intravenous drug users, through tattooing, or through use of razors and similar objects that are contaminated with infected blood, but may also occur in health-care settings, during medical, surgical and dental procedures. A sexual transmission of the virus is especially of importance in men who have sex with men (MSM) and persons with multiple sex partners or contact with sex workers. Sexual transmission and intravenous drug use are the most common routes of transmission in low endemic regions, such as Europe (Fattovich, 2003). In highly endemic areas, hepatitis B is most commonly spread perinatally from mother to child, or horizontal by close person-to-person contact (presumably by non-intact skin), especially among children. Approx. 90% of HBeAg positive mothers transmit hepatitis B virus to their infants, compared with 10–20% of HBeAg negative mothers after seroconversion to anti-HBe (Okada et al, 1976).

1.2.2. Prevention of hepatitis B

HBV infection can be prevented by a safe and effective vaccine available since 1981. The vaccine induces protective antibody levels in more than 90% of vaccinated individuals (Mendy et al, 2013). A complete immunization consists of three doses, where the second and the third dose are scheduled one and six months after the first dose, respectively. The protection lasts at least 20 years but is probably lifelong (Bruce et al, 2016; Wang et al, 2015). The HBV vaccine is currently in standard vaccination schedules for infants, children and adolescents in most countries. In addition, it is highly recommended for non-immune individuals belonging to high-risk groups including people who

frequently receive blood or blood products, dialysis patients, recipients of transplants, intravenous drug users, household members and sexual contacts of people with chronic HBV infection, persons with chronic liver disease, healthcare workers, MSM, people with multiple sexual partners, and travellers before leaving for endemic areas.

1.2.3. Clinical presentation of HBV infection

The incubation of the HBV infection is 75 days on average but can vary from 30 to 180 days. HBV has a strict tropism for human hepatocytes but it is not directly cytotoxic. The symptoms of infection are mainly caused by the immune response, not by the virus itself (Chisari and Ferrari, 1995). In the case of weak immune response, the virus proliferates very strong, but there are hardly any clinical symptoms of hepatitis.

HBV infection in adults causes acute icteric hepatitis in about one third of the infected individuals, while one third of the infected individuals have just unspecific general symptoms like tiredness, loss of appetite, abdominal discomfort, nausea, vomiting and fever, but without icterus. Approximately one third of the infections is completely asymptomatic and can only be detected serologically. About 1% of all infections can be fulminant with the development of acute liver failure, which can lead to death (Tillmann et al, 2006). Acute hepatitis B cures completely in more than 90% of adults and lead to lifelong immunity. However, the genome of HBV remains in form of cccDNA preserved mostly lifelong in a small proportion of hepatocytes and can reactivate, particularly in immunosuppressed patients.

Less than 5% of infected otherwise healthy adults develop chronic infection. As opposed to that, 90% of newborns, and 25–30% of children infected before the age of 5 years develop chronic infection (Terrault et al, 2018). In addition to the age at infection, the infants of HBeAg positive mothers have increased risk of chronic infection. On the contrary, less than 10% of newborns of HBeAg negative and anti-HBe positive mothers develop chronic infection, but they have increased risk for develop of fulminant hepatitis B (Chang, 2000; Fattovich, 2003).

1.2.4. HBV/HDV coinfection

Hepatitis D (delta) virus (HDV) is a small satellite virus, has a small (approx. 1700 bp), single-stranded, circular RNA, and is the smallest and the only circular animal RNA virus. The HDV can propagate only in the presence of the HBV, since HDV needs the HBV envelope proteins, which are necessary for viral entry in to hepatocytes. Therefore, a hepatitis B vaccine is also efficient prevention of HBV/HDV coinfection.

It has been estimated that at least 5% of HBV chronic infected individuals are infected with HDV, resulting in a total of 15 to 20 million people worldwide (WHO, 2018b). HDV infection is endemic in Eastern Europe, Turkey, Middle East, Central and Northern Asia (e.g. Mongolia), Central and West Africa, Pacific Islands, South America, and Greenland (WHO, 2018b). In western countries, HDV is mostly detected in immigrants from endemic areas and in intravenous drug users (Rizzetto, 2015).

Although the transmission routes of HDV are the same as for HBV, direct parenteral inoculation seems to be the most efficient way of transmission (Rizzetto, 2015). Vertical transmission from mother to child is rare. Transmission of HDV can occur either simultaneously with HBV (coinfection) or as a superinfection of chronic HBV carriers. Coinfection has been associated with severe acute hepatitis and may progress to acute liver failure. Though, the vast majority of coinfected patients recovered from both infections. Superinfection results in the vast majority of cases with a chronic course of the infection. Both coinfection and superinfection with HDV results in more severe disease compared to monoinfection with HBV, with rapid progression of liver disease to cirrhosis and an increased risk of developing HCC in chronic infected patients (Rizzetto and Alavian, 2013).

1.2.5. Diagnostic of HBV infection

Laboratory diagnostic of an acute or chronic HBV infection is based on the detection of HBsAg in serum or plasma. For determination of an acute, chronic or resolved HBV infection additional serological parameters are used (table 1). In addition, HBV DNA detection is used to quantify viral load (e.g. under therapy).

Anti-HBc antibodies are the first that occur in HBV infected individuals. Anti-HBc IgM is a marker of an acute HBV infection and disappears after approx. 6 months, while anti-HBc IgG remains positive lifelong as a marker of a past HBV infection. Anti-HBc IgM may also occur during an acute exacerbation of a chronic hepatitis B. Chronic infection is defined by the persistence of HBsAg for at least 6 months, with or without HBeAg, which is a marker of a high level of replication of the virus and indicates increased infectiousness of the infected individual. Anti-HBs is detectable in persons who recovered

from HBV infection, indicates immunity and protect from re-infection with HBV. Anti-HBs also develops as single positive HBV marker in persons who have been successfully vaccinated against HBV and represents immunity, if > 10 mIU/ml.

Table 1. Interpretation of HBV serology.

Serological parameter						Interpretation
HBsAg[‡]	anti-HBs[‡,†]	anti-HBc[‡,†]	anti-HBc IgM	HBeAg	anti-HBe[†]	
+	-	+	+	+	-	Acute HBV infection
+	-	+	-	+/-	+/-	Chronic HBV infection
-	+	+	-	-	+/-	Resolved HBV infection
-	+	-	-	-	-	Successfully vaccinated
-	-	-	-	-	-	No contact with HBV

[‡]part of a standard HBV screening program; [†]total antibodies (IgG+IgM)

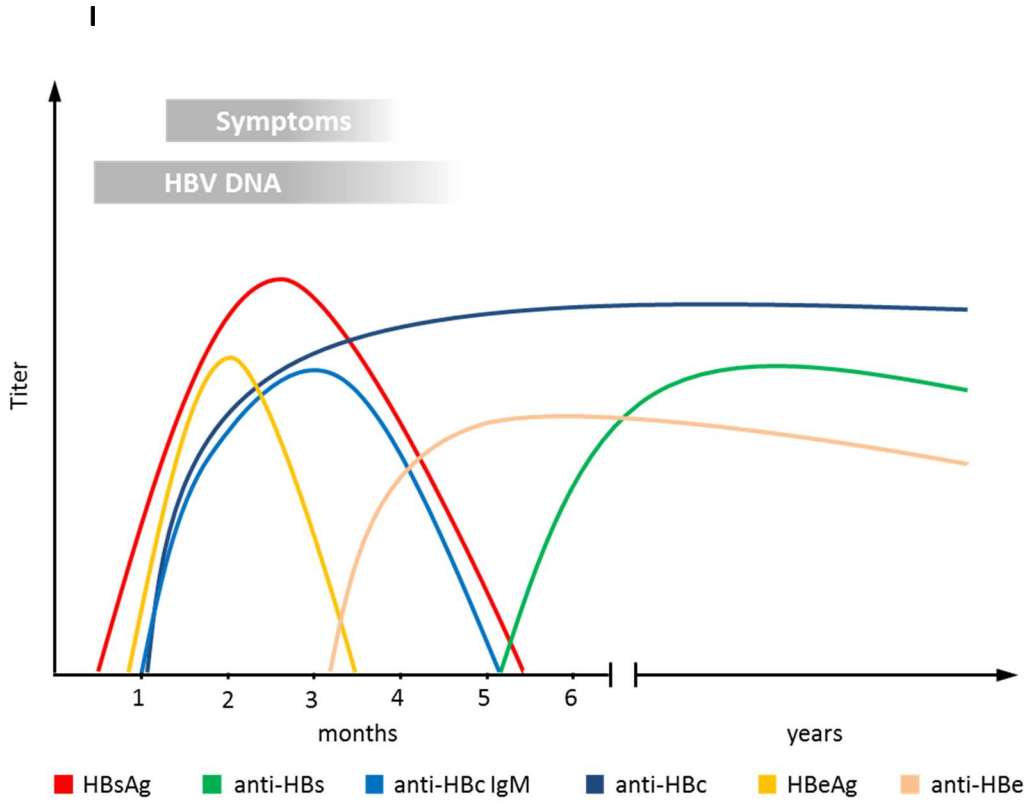


Figure 4. Acute (I) and chronic (II) HBV infection with typical serologic course.

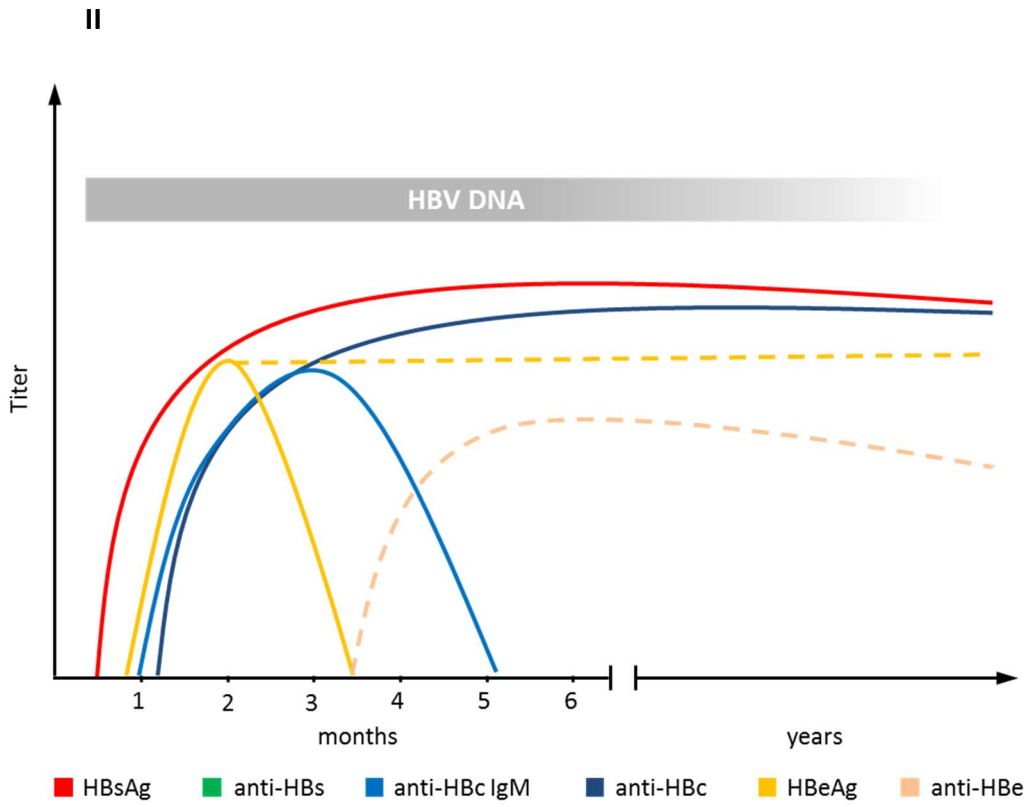


Figure 4 (continued). Acute (I) and chronic (II) HBV infection with typical serologic course.

1.2.6. Therapy of hepatitis B and D

Acute hepatitis B in adults is selflimiting disease in more than 95% of cases and requires only symptomatic therapy. Nevertheless, antiviral therapy is indicated for patients with fulminant or protracted severe acute hepatitis (Lok and McMahon, 2007). Therapy of chronic hepatitis B is according to current guidelines indicated for "patients with cirrhosis or decompensated liver disease and, for patients without cirrhosis, evidence of modest to high viremia and biochemical or histological evidence of hepatic necroinflammation" (Lok et al, 2017).

1.2.6.1. Approved therapies

There are currently two groups of drugs approved for therapy of chronic hepatitis B: direct antiviral drugs (nucleos(t)ide analogues, NUCs) and immunomodulators (pegylated interferon alfa, pegINF).

Immunomodulating pegINF therapy has few advantages for therapy of chronic hepatitis B as compared to NUCs such finite treatment duration (48-52 weeks) and higher rates of HBeAg seroconversion (24%

- 27% compared to 12% - 22%) and HBsAg seroconversion (3% - 7% compared to 0% - 3%) after the same duration of NUC therapy (Yapali et al, 2014). Despite these advantages, pegINF requires parenteral administration, has greater adverse effects and is less effective at suppressing viral replication. NUCs are administrated oral, have relatively few adverse effects, and have a potent direct antiviral effect. Therefore, nucleoside analogue entecavir, and nucleotide analogues tenofovir disoproxil fumarate and tenofovir alafenamide are today the first-line drugs for therapy of chronic hepatitis B. NUCs act as inhibitors of reverse transcriptase and are efficient in reducing of HBV DNA to undetectable levels, as monotherapy or combination therapy with pegINF, but they have marginal effect on cccDNA, which serves, together with integrated DNA, as a template for transcription of HBV mRNAs and synthesis of HBsAg. The addition of pegINF in patients who already have viral suppression with NUCs has been reported to enable significantly higher HBsAg loss than with either monotherapy alone (Lok et al, 2017). However, rates of HBsAg loss are still very low (Marcellin et al, 2016; Hagiwara et al, 2018) and long-term or lifelong treatment with NUCs is needed. Additionally, prolonged use of NUCs results in the evolution of drug-resistant mutants (Liang et al, 2015; Zhang et al, 2015; Lok et al, 2017). Although the reduction of HBV DNA improves liver function and decelerates disease progression, a risk to develop HCC, especially in patients with liver cirrhosis, is still increased (Papatheodoridis et al, 2015). Therefore, new drugs are needed.

PegINF therapy is to date the only one used for chronic hepatitis D.

1.2.6.2. New therapies

A number of new therapeutically approaches are being investigated with the aim to achieve functional cure of hepatitis B, characterized with clinical resolution of infection, undetectable HBV DNA, loss of HBsAg, and potential seroconversion to anti-HBs, or even a complete cure, with complete eradication of HBV including loss of cccDNA and integrated HBV DNA in the liver.

Approaches with direct antiviral action include inhibitors of capsid formation, RNA translation inhibitors, agents that block cccDNA, inhibitors of HBsAg release, or entry inhibitors (table 2).

Entry inhibitor myrcludex B is a myristolated peptide containing 47 aa of the pre-S1 domain of the L-HBsAg (Ni et al, 2014). It competes for the binding with the natural HBsAg to the NTCP receptor which results in the restriction of virion entry in the hepatocyte. This should protect uninfected hepatocytes from new infection, leading to the eradication of the virus. The concentration of myrcludex B that blocks HBV and HDV entry is 100 times lower than that which inhibits bile acid transport. Therefore, viral entry inhibition should be achieved without significantly interfering with bile acid transport (Blank

et al, 2016). A recent clinical study showed that the patients on myrcludex B monotherapy or combination therapy with pegINF, achieved also decline of serum HDV RNA, with normalization of ALT in some patients, but there was no effect on serum HBsAg, which remained unchanged (Bogomolov et al, 2016).

Among newly investigated therapeutically approaches, nucleic acid polymers (NAPs) are a group of the first drugs to be able to achieve rapid reduction or complete loss of HBsAg during treatment of chronic HBV infection and chronic HBV/HDV coinfection (Vaillant, 2018).

Immunological approaches to treat chronic hepatitis B include stimulation of antiviral defence, limitation of T cell exhaustion/depletion, stimulation of innate immunity, and activation of B and T cell responses with diverse therapeutic vaccines (Dembek et al, 2018; Bartenschlager et al, 2019).

Table 2. New approaches for therapy of chronic HBV infection.

mode of action	target	drug	status of development
direct antiviral action			
inhibition of RNAseH	viral polymerase	only experimental compounds	preclinical
inhibition of capsid formation	capsid	diverse	phase I/II trials
RNA translation inhibitors	viral mRNA	siRNAs, antisense oligonucleotides	phase I/II trials
transcriptional silencing	cccDNA	only experimental compounds	preclinical
inhibition of assembly and/or secretion of subviral particles	HBsAg? Unknown cellular factor(s)?	nucleic acid polymers (e.g. REP 2139-Ca)	phase II trials
entry inhibition	NTCP	Myrcludex B	phase II trials
immune approaches			
limiting T cell exhaustion/depletion	immune checkpoints	anti-PD1, anti-PDL1, anti-CTLA4	approved for cancer therapy
innate immune activation	pattern recognition receptors	agonist of TLR-7, TLR-8, STING; Rig-I activation	phase II trials (TLR-7), remaining drugs preclinical of phase I
activation of B and T cell responses	adaptive immunity	diverse therapeutic vaccines	phase I – III trials

[adapted from Bartenschlager et al, 2019]

1.3. Nucleic acid polymers

Nucleic acid polymers (NAPs) are phosphorothioate oligonucleotides with a broad spectrum of sequence independent but length and phosphorothioate dependent antiviral activity (Vaillant, 2018). This antiviral activity is based on a high affinity interaction of phosphorothioate linkage with hydrophobic surface of alpha helix in proteins. This interaction was first demonstrated in a study investigating antiviral activity of early variants of NAPs against human immunodeficiency virus (Vaillant et al, 2006).

In HBV infected patients NAPs interfere with the assembly and/or secretion of HBV SVP by a still unknown mechanism (Noordeen et al, 2015; Quinet et al, 2018; Vaillant, 2018). HBsAg is abundantly expressed, and its majority is excreted in form of non-infectious SVP (Ito et al, 2010). The clearance of this abundantly produced HBsAg from the blood of infected patients dramatically potentiates the ability of immunotherapies to restore host immune response with subsequent elimination of the virus and functional cure of chronic hepatitis B (Cornberg et al, 2017; Dusheiko et al, 2016; Frenette and Gish, 2009).

Recent clinical studies in patients with chronic HBV infection and chronic HBV/HDV coinfection report a multilog reduction or loss of HBsAg in the blood after treatment with the lead NAP compound, REP 2139. This HBsAg clearance is associated with seroconversion to anti-HBs, loss of HBeAg and seroconversion to anti-HBe, reduction of HBV DNA, and additionally elimination of HDV RNA in HBV/HDV coinfected patients (Al-Mahtab et al, 2016; Bazinet et al, 2017). REP 2139-mediated HBsAg clearance appears to potentiate the host response to pegIFN, resulting in seroconversion to anti-HBs, therapeutic liver flares and the functional cure of HBV and HDV infections. A fraction of patients showed only minor (i.e. < 1 log) or no reduction of HBsAg during REP 2139 therapy. The reasons for this non-responsiveness have not yet been elucidated.

2. Aims

Therapy of chronic hepatitis B with currently approved first line drugs (i.e. NUCs) is efficient in reducing of HBV DNA to undetectable levels. However, NUCs cannot eliminate cccDNA. Furthermore, due to HBsAg transcription from cccDNA and HBV DNA integrated in the cell genome, levels of HBsAg remain high and rates of HBsAg loss are very low. Although the reduction of HBV DNA improves liver function and decelerates disease progression, risk to develop HCC is still increased, especially in patients with liver cirrhosis, and long-term or lifelong treatment is needed. Therefore, new drugs are required.

Among a number of newly investigated therapeutically approaches, NAPs (e. g. REP 2139-Ca) are the first drugs to be able to achieve rapid reduction or complete loss of HBsAg during treatment of chronic HBV infection and chronic HBV/HDV coinfection by a still unknown mechanism. Nevertheless, a fraction of patients showed only minor or no reduction of HBsAg during REP 2139 therapy. The reasons for non-responsiveness have not yet been elucidated.

The following questions were examined in this project:

1. Is the failure of response to REP 2139-Ca therapy observed in 3 of 12 patients in the REP 102 study (Al-Mahtab et al, 2016) related to differences in the amino acid sequences of pre-S/S ORF present in non-responders versus responders? To explore this issue, we analysed the sequences of the whole pre-S/S ORF of the HBV genome obtained by direct sequencing in patient's samples prior, during and after treatment with NAPs.

2. Do quasi-species populations of HBV in non-responders to REP 2139-Ca differ to patients having an early reduction of viremia and HBsAg? Possible sequence differences between responders and non-responders could help to obtain a better understanding of the mechanism of drug action. To address this question, we performed the next generation sequencing of the MHR of the pre-S/S ORF and analysed the sequences.

3. Are reductions of HBsAg concentration in patients' sera observed in the REP 102 study caused by a failure in detection by standard diagnostic assays due to the presence of mutations in the "a" determinant? To clarify this issue, we analysed the sequences of the whole S region of the pre-S/S ORF obtained by direct sequencing and compared to the data obtained by next generation sequencing of the MHR.

4. Were the reduction of HBsAg, HBV DNA and HDV RNA observed in diagnostic assays in the REP 102 (Al-Mahtab et al, 2016) and REP 301 (Bazinet et al, 2017) studies based on a failure in diagnostic assays due to a direct interference of REP 2139-Ca with corresponding routine diagnostic assays? To answer this question, we performed the testing of direct interference of REP 2139-Ca with these diagnostic assays.

3. Materials and methods

3.1. Sequencing of the pre-S/S ORF

3.1.1. Patients and blood-samples for sequencing of the pre-S/S ORF

Sera form all 12 patients (9 responders and 3 non-responders) in the REP 102 study under therapy with REP 2139-Ca (the calcium chelate complex formulation of REP 2139) were collected at different time points during therapy with REP 2139-Ca as described (Al-Mahtab et al, 2016). All patients in the REP 102 study had confirmed chronic HBV infection and were serum HBsAg and HBeAg positive with HBV DNA >10^6 copies/ml and treatment naïve. The samples were transported on dry ice and stored at -80°C until analysis. Analysis was performed on samples harvested prior or at the beginning of treatment, during the treatment and as close as possible to the end of the treatment or early in treatment-free follow-up with a viral load sufficient for sequencing analysis. Baseline (pre-treatment) samples were analysed in 6/9 responders and 2/3 non-responders. Baseline samples of the remaining 4 patients were unfortunately not available. Sample characteristics are shown in table 3. The same samples were used for direct sequencing and next generation sequencing (NGS) with exception of the third time point in patient 3. A responder was defined as a patient with >1 log declines in HBV DNA and HBsAg. All procedures followed in the REP 102 study were in accordance with the ethical standards of the responsible institutional ethics committee on human experimentation in Dhaka, Bangladesh (Viral Hepatitis Foundation of Bangladesh) and with the Helsinki Declaration of 1975, as revised in 2008.

3.1.2. Extraction and amplification of HBV DNA

HBV DNA was extracted using the mSample Preparation System DNA kit (Abbott, Wiesbaden, Germany) on an m24sp extraction device (Abbott, Wiesbaden, Germany). Single PCR was performed for samples with high viral load using specific primers targeting pre-S, S or MHR, respectively. Semi-nested (for small HBsAg and pre-S region) and nested PCR (for MHR) was performed for samples with lower viral load using specific primers targeting the entire large HBsAg region in the first round, and the small HBsAg, pre-S and MHR in the second round, respectively. The primers were designed and adapted according to reference sequences (figure 5). The first round of semi-nested and nested PCR was the same for all amplificated regions – pre-S, S and MHR. For the first round PCR we used F1 and R1 primers. For the single and second rounds of S region PCR we used F2 and R1, for the single and second rounds of MHR PCR we used DF and DR, whereas we used F1 and R2 for the single and second

rounds of pre-S region PCR. The PCR was carried out in a final volume of 50 µl containing 10 µl of 5× Q5 Reaction Buffer, 2 µl of 5 mM dNTP, 2.5 µl of each primer (10 µM), 0.3 µl of Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Frankfurt am Main, Germany), and 5 µl of extracted DNA for the single PCR and first round of semi-nested and nested PCR, or 3 µl of the first round PCR product for the second round of semi-nested and nested PCR.

The DNA amplification for all PCRs was performed on GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). Cycling conditions for the first round of semi-nested and nested PCR, as well as for single and the second rounds of S region and MHR PCR were initiated with denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 56 °C for 20 s, and 72 °C for 20 s. Cycling conditions for single or the second rounds of pre-S region PCR were initiated with denaturation at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 61 °C for 20 s, and 72 °C for 20 s. A final extension step for all PCRs was performed for 2 min at 72 °C. The amplification products (7 µl of each) were analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide and observed under ultraviolet light.

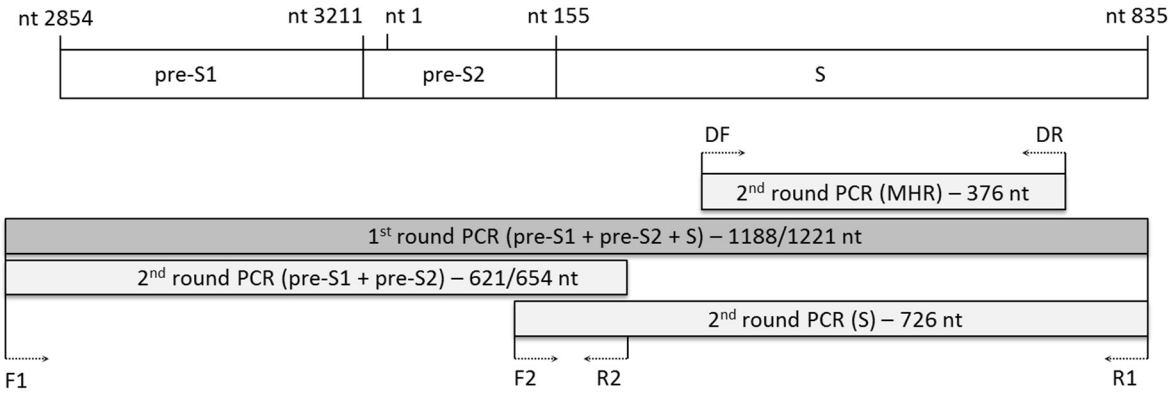


Figure 5. PCR primers used for HBV DNA amplification.

F1: 5'-GAACAAGAGCTACAGCATG-3'; F2: 5'-CATATCGTCAATCTTCTCG-3';
R1: 5'-GTTTAAATGTATACCCA(A/G)AGA-3'; R2: 5'-AGAGAAGTCCACCACGAG -3';
DF: 5'-GTCTGCGGCGTTTTATC-3'; DR: 5'-CAATACCACATCATCCATAT-3'.

The first round of semi-nested and nested PCR was the same for all amplificated regions – pre-S, S and MHR. For the first round PCR we used F1 and R1 primer. For the single and second round of S region PCR we used F2 and R1, for the single and second rounds of MHR PCR we used DF and DR, whereas we used F1 and R2 for the single and second round of pre-S regions PCR. The primers were designed according to the reference sequences. Primer F1 and F2 were adapted from Naito et al, 2001.

Table 3. Samples used for sequencing of hepatitis B surface proteins and their characteristics.

Patient	Sampling time point‡		Therapy completed at sampling time point	HBV DNA (copies/ml)	HBsAg (IU/ml)
Patient 1[NR]	1.	week 1	baseline	>9.98E+08	168950
	2.	week 27	26 weeks of REP 2139-Ca	7.18+08	23951
	3.	week 40	39 weeks of REP 2139-Ca (including 1 week of thymosin alfa 1)	5.90E+08	11900
Patient 2	1.	week -2	baseline	6.23E+08	52296
	2.	week 5	4 weeks of REP 2139-Ca	4.48E+07	30268
	3.	week 11	10 weeks of REP 2139-Ca	4.04E+04	1121
Patient 3	1.	week -3	baseline	1.01E+08	13008
	2.	week 6	5 weeks of REP 2139-Ca	1.69E+07	1665
	3.	week 13[DS]	12 weeks of REP 2139-Ca	5.69E+04	0.08
	3.	week 17[NGS]	16 weeks of REP 2139-Ca	2.18E+04	0.09
Patient 4	1.	week -3	baseline	1.18E+08	3654
	2.	week 13	12 weeks of REP 2139-Ca	8.15E+04	2.65
	3.	week 27	26 weeks of REP 2139-Ca	8.71E+03	2.59
Patient 5[NR]	1.	week 2	1 week of REP 2139-Ca	3.15E+07	5258
	2.	week 23	22 weeks of REP 2139-Ca	5.47E+06	8264
	3.	week 34	33 weeks of REP 2139-Ca (including 2 weeks of thymosin alfa 1)	1.11E+07	8070
Patient 6	1.	week -4	baseline	1.29E+08	47690
	2.	week 12	13 weeks of REP 2139-Ca	6.96E+05	3127
	3.	week 30	29 weeks of REP 2139-Ca	1.19E+04	69
Patient 7	1.	week 7	6 weeks of REP 2139-Ca	>9.89E+08	16832
	2.	week 37	36 weeks of REP 2139-Ca (including 5 weeks of thymosin alfa 1)	5.74E+06	250
	3.	week 76[§]	58 weeks of REP 2139-Ca (including 13 weeks of thymosin alpha 1 followed by 13 weeks of pegylated interferon alfa 2a) 17 weeks of follow-up	4.57E+03	5.76
Patient 8	1.	week 2	1 week of REP 2139-Ca	>9.89E+08	99954
	2.	week 11	10 week of REP 2139-Ca	8.38E+04	62194
	3.	week 20	19 week of REP 2139-Ca	2.75E+03	2012
Patient 9	1.	week 2	1 week of REP 2139-Ca	7.25E+05	22855
	2.	week 25	24 weeks of REP 2139-Ca	1.28E+04	9.60
	3.	week 80[§]	48 weeks of REP 2139-Ca (including 24 weeks of pegylated interferon alfa 2a) 22 weeks of pegylated interferon alfa 2a 10 weeks of follow-up	8.90E+03	3269
Patient 10[NR]	1.	week -2	baseline	>9.89E+08	>1.25E+05
	2.	week 11	10 weeks of REP 2139-Ca	>9.89E+08	>1.25E+05
	3.	week 25	24 weeks of REP 2139-Ca (including 4 weeks pegylated interferon alfa 2a)	>9.89E+08	>1.25E+05
Patient 11	1.	week -2	baseline	>9.89E+08	87504
	2.	week 8	7 weeks of REP 2139-Ca	3.55E+07	159
	3.	week 13	12 weeks of REP 2139-Ca	~1.00E+04	0.08
Patient 12	1.	week -1	baseline	9.09E+06	1289
	2.	week 12	11 weeks of REP 2139-Ca	8.77E+03	0.01
	3.	week 78[§]	33 weeks of REP 2139-Ca (including 13 weeks of pegylated interferon alfa 2a) 43 weeks of follow-up	2.15E+03	7.24

[NR] non-responder; ‡ weeks in relation to initiation of REP 2139-Ca therapy (treatment was given after blood collection for serum samples); [§] taken during follow-up after removal of all therapy; [DS] sample used for direct sequencing; [NGS] sample used for next generation sequencing [adapted from Mijočević et al, 2019]

3.1.3. Direct sequencing of HBV DNA

PCR products were purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) according to manufacturer's instructions and eluted in 30 µl final volume. Direct sequencing was performed in two directions by GATC Biotech (Konstanz, Germany) with Supremerun tube Sanger sequencing. These sequence data have been submitted to the GenBank database under accession numbers MH507517 to MH507552 (www.ncbi.nlm.nih.gov/genbank).

3.1.4. Next generation sequencing of HBV DNA

Next generation sequencing (NGS) of the whole S region of pre-S/S ORF was performed particularly for the patient 5 to confirm the premature stop codon mutation observed in direct sequencing. NGS of the extended MHR corresponding to aa 76-200 was performed for all 12 patients. NGS was performed in collaboration with Institute of immunology and genetics, Kaiserlautern, Germany, and was performed under the direction of Mr. Martin Däumer.

All amplicons were purified using the Agencourt AMPure®XP system on a BioMek NX workstation (Beckman Coulter, Brea CA, USA), quantified fluometrically on a FluoStar Optima (BMG Labtech, Ortenberg, Germany) using Quant-iT Picogreen dsDNA reagent (Life technologies, Darmstadt, Germany). Library preparation for Illumina deep sequencing of the whole S region and MHR was done using Nextera® XT DNA Sample Preparation and Index kit (Illumina, San Diego, CA, USA), and TruSeq Nano DNA Library kit (Illumina, San Diego, CA, USA), respectively, according to the manufacturer's instructions. Resulting libraries were normalized and pooled for subsequent sequencing on an Illumina MiSeq platform using the 2 × 250 cycle paired-end sequencing protocol.

3.1.5. Data analysis

3.1.5.1. Direct sequencing data analysis

Direct sequences were edited with CLC Sequence Viewer, Version 7.7 (QIAGEN, Aarhus, Denmark) and analysed with Geneious, Version 9.0.5 (Biomatters Ltd., Auckland, New Zealand). Amino acid residues of envelope proteins were numbered according genotype A reference sequence for all genotypes (figure 3 and 5). We used following genotype specific reference sequences downloaded from the Hepatitis B Virus database (hbvdb.ibcp.fr; September 2017): EU054331 (Makuwa et al, 2008) and X02763 (Valenzuela et al, 1980) for genotype A, GQ924620 (Meldal et al, 2011) and GQ358158 (Thedja et al, 2011) for genotype C, AF121240 (Hannoun et al, 2000) and FJ904433 (Meldal et al, 2009) for

genotype D. The Hepatitis B Virus database has two representative sequences per genotype. If an amino acid in the analysed sequence was present in one of two reference sequences, it was not considered as aa difference to reference sequences.

In order to confirm the source of the virus which infected each of patients, sequence similarity search was performed using BLAST (Basic local alignment search tool) against the NCBI non-redundant sequence database (Sayers et al, 2012).

Analysis of the overlapping RT-polymerase region was performed with the Geno2pheno [hbv] online service (version 2.0, October 2018) from Max Planck Institute for Informatics.

A t-test was used to determine a difference between the groups (SPSS Statistics, version 24). A p-value of <0.05 was considered as statistically significant.

All samples from patient 12 and the third time point of patient 3 and 8 were excluded from pre-S regions analyses because of low viral load or poor sequence quality. The forward pre-S regions sequence of third time point of patient 7 and the end of the forward S region sequence (45 nucleotides) of the third time point of patient 8, as well as revers S region sequences of the third time point of patient 8 and second time point of patient 9 were not included in the analyses because of poor sequence quality. The end of all S region sequences (approx. 10 nucleotides) could not be analysed because of close reverse primer localisation.

3.1.5.2. NGS data analysis

NGS analysis of the S region and MHR was performed in collaboration with Department of Bioinformatics, Wissenschaftszentrum Weihenstephan, Technische Universität München, Freising, Germany, and was performed by Ms. Zainab Usman. Illumina deep sequencing reads of the S region and MHR were trimmed of adapters and quality clipping from either the 5’ or 3’ end of the reads with a PHRED threshold of 20 was performed using CutAdapt 1.1 (Martin, 2011). The reads were then mapped against the reference sequence (GenBank accession no. NC_003977.2) of genotype D strain ayw using the BWA-mem (Burrows-Wheeler Aligner) algorithm (Li, 2013/05/26). The alignment file was used to determine each nucleotide for each position in every read using Samtools “mpileup” (Li et al, 2009). The sequences were than compared with the same reference sequences used for direct sequencing analysis (see above). If an amino acid in the analysed sequence was present in one of two reference sequences, it was not considered as aa difference to reference sequences.

3.2. Investigation of direct interference of REP 2139-Ca with diagnostic assays

3.2.1. Blood samples

Serum samples used for the testing of direct interference of REP 2139-Ca with diagnostic assays were collected between 2011 and 2016 in cooperation between Institute of Virology, Technische Universität München (TUM), Munich, Germany, and the 2nd Medical Clinic and Polyclinic and Surgical Clinic and Polyclinic of Klinikum rechts der Isar, Munich, Germany. Informed consent was obtained from all patients whose blood samples were included in the study. The samples were stored at -20 °C until analysis. The selected samples were pooled and anonymized. The study is approved by the Ethics Committee of School of Medicine, TUM, Munich, Germany.

3.2.2. REP 2139-Ca and its plasma concentration

The NAP we used in this study was REP 2139-Ca (the calcium chelate complex formulation of REP 2139) as described (Al-Mahtab et al, 2016). The plasma concentration of REP 2139-Ca was determined in previous studies in cynomolgus monkeys model (Roehl et al, 2017). The plasma concentration of REP 2139-Ca at the end of 1st infusion was 155.6 ± 12.1 µg/ml and after 24 hours 0.02 ± 0.007 µg/ml. At the end of 26th infusion the concentration was 131.8 ± 36 µg/ml and after 24 hours 1.0 ± 1.2 µg/ml. These data demonstrate the rapid clearance of REP 2139-Ca from the blood within 24 hours after administration, which is comparable to other phosphorothioated oligonucleotides with 2' ribose modification in monkeys and humans. The REP 2139-Ca application takes place once a week and its plasma concentration decreases continually over the remaining week before the next dose, although this decline becomes slower with repeated dosing.

3.2.3. Sample preparation

We have prepared one serum pool for each qualitative assay, and two serum pools with different concentration (low and high) of analysed parameter for each quantitative assay. The final sample concentrations of REP 2139-Ca were 150 µg/ml, 10 µg/ml, 1 µg/ml and 0.1 µg/ml which cover concentration of REP 2139-Ca at the end of infusion and substantially in excess of serum concentration predicted to be present in human patients one week after receiving a 500 mg (7.69 mg/kg) dose of REP 2139-Ca after 26 weeks of dosing. The interference study protocol is designed to minimize serum dilution by normal saline to avoid an impact on assay performance and to ensure that the dilution of

serum sample is identical for the all REP 2139-Ca concentrations to be tested and to allow volumes which will not introduce a pipetting error.

We used the 50 mg/ml drug vial to prepare 3 mg/ml, 0.2 mg/ml, 0.02 mg/ml and 0.002 mg/ml solution of REP 2139-Ca (dilutions in normal saline). We prepared five aliquots of an equal volume of the each pooled serum sample and did a 1:20 dilutions of 4 prepared solutions of REP 2139-Ca in pooled serum to get the final concentration of REP 2139-Ca (see above). We used one aliquot to prepare a control sample by a 1:20 dilution of normal saline. We gently vortexed prepared samples to ensure complete mixing and incubated at room temperature for 30 minutes before we ran test samples through diagnostic assays.

The samples tested at the University of Duisburg-Essen (UDE), Essen, Germany were pooled and sent anonymously at 4 °C together with prepared dilutions of REP 2139-Ca.

Serum pools for additional HDV RNA PCR interference testing (National Genetics Institute and Eurobioplex) were prepared and sent on dry ice. The samples were finally prepared shortly before running the assays as described above.

3.2.4. Diagnostic assays

The majority of diagnostic assays were performed at TUM. Anti-HBs quantitatively, HBsAg qualitatively, HBsAg quantitatively, anti-HBc total antibodies, anti-HBc IgM, anti-HBe and HBeAg were performed using chemiluminescent microparticle immunoassay (CMIA) (Abbott, Wiesbaden, Germany) on Architect i1000 SR^{TM} device (Abbott, Wiesbaden, Germany). Anti-HDV was performed using enzyme-linked immunosorbent assay (ELISA) (DiaSorin, Dietzenbach, Germany) on BEP III device (Siemens, Erlangen, Germany). HBV DNA was extracted using mSample Preparation System DNA kit (Abbott, Wiesbaden, Germany) on m24sp extraction device (Abbott, Wiesbaden, Germany). HBV PCR was performed using real-time PCR kit (Abbott, Wiesbaden, Germany) on a TaqMan device (Thermo Fisher, Waltham, Massachusetts, USA). HDV RNA was extracted using mSample Preparation System DNA kit with standard protocol for simultaneous extraction of DNA and RNA on the m2000sp device (Abbott, Wiesbaden, Germany). HDV PCR was performed using real time “in-house” PCR (Institut of Virology, TUM) on the TaqMan device.

For HDV PCR performed at UDE, HDV RNA was extracted using Magnapure Extracter system (Roche, Pleasanton, California). HDV PCR was performed using RoboGene® HDV RNA quantification kit (AJ Roboscreen, Leipzig, Germany).

Additional HDV PCR interference tests were performed at National Genetic Institute (NGI), Los Angeles, California, USA, using phenol-chloroform based RNA extraction and "in-house" HDV PCR, and at Centre national de référence des hépatites B, C et Delta, Hôpitaux universitaires de Paris-Seine-Saint-Denis, Bobigny, France, using Abbott m2000sp RNA extraction and Eurobioplex HDV kit (Eurobio, Les Ulis, France).

All assays were tested in triplicate for each REP 2139-Ca serum concentration and performed according to the manufacturer's instructions.

3.2.5. Statistical analysis

Statistical analyses were performed using SPSS Statistics (version 24). Mean and standard deviation was used for graphic presentation of data. Kolmogorov-Smirnov Test was used to test normality. A t-test was used to determine a difference between the control sample and tested sera. A p-value of <0.05 was considered as statistically significant.

Two values were excluded from statistical evaluation due to excessive deviation from the mean value: one value of anti-HDV control sample and the other one of HDV RNA 1 - TUM.

4. Results

4.1. Analysis of the entire pre-S/S ORF

From a total of 12 patients, 9 responders and 3 non-responders, direct sequencing of the entire pre-S/S ORF of HBV DNA was performed and the amino acid (aa) sequences of pre-S1, pre-S2 and S regions were analysed. Additionally, NGS of the S region from the patient 5 was performed. Three time points per patient were analysed to account for potential changes during REP 2139-Ca therapy. The presence of three HBV genotypes were confirmed: genotype A in one patient, genotype C in seven and genotype D in four patients, in agreement with the genotype identified by Al-Mahtab et al, 2016. In addition to comparison with reference sequences, we compared the sequences from these patients against the available sequences in NCBI database using BLAST.

We found that some aa differences to the reference sequences could be attributed to specific subgenotypes. Using the closest sequence relatives resulting from BLAST search, we found that all 7 genotype C sequences showed 99-100% identity with subgenotype C1 isolates from the South Asian region (i.e. Bangladesh, Myanmar). In addition, we found that 4 genotype D sequences have similarity with the genotype D ayw3 strain found in South Asia (i.e. India), while genotype A sequences from one infected patient has similarity to A1 genotype isolates from South Africa.

4.1.1. Comparison of amino acid sequences of the pre-S1 region

The pre-S1 region is N-terminal domain of the L-HBsAg and consists of 119 aa in genotype A and C, whereas pre-S1 sequences in genotype D is 11 aa shorter. This region contains binding domains for the HBV specific receptor (NTCP).

In the genotype A patient (patient 4), three aa were different from the genotype A reference sequences at time point 3 (I/V74L, T86V and Q100K). Additionally, T86A was observed at time point 1 and 2.

Four aa differences to the genotype C reference sequences were observed at all time points in all 6 analysed genotype C patients: K10Q, D27G, E54A and A62S (figure 6, table 4). Additionally, H/P51Q was observed in patients 1, 3, 7, 10 and 11, whereas G73S was observed in patient 1, 3, 6, 10 and 11. D27G coexisted with the wild type in patient 10. Performing the NCBI BLAST search, it was found that these 6 differences to genotype C reference sequences were present in isolates from the South Asian region and are subgenotype C1 specific variations (table 6). G73N was observed at all time points in patient 7. Two additional aa differences were observed at all time points in patient 3 (A90T and A91S).

Amongst genotype D patients, two aa differences relative to the reference sequences (A/T39S and G102R) were observed at all time points in patient 2. In-frame deletions of 183 nucleotides (nt 3025-3207) corresponding to aa 58-118 was observed in patients 5 and 9 and was coexistent with the wild type (figure 7).

There were no significant difference between amino acid differences to reference sequences between responders and non-responders.

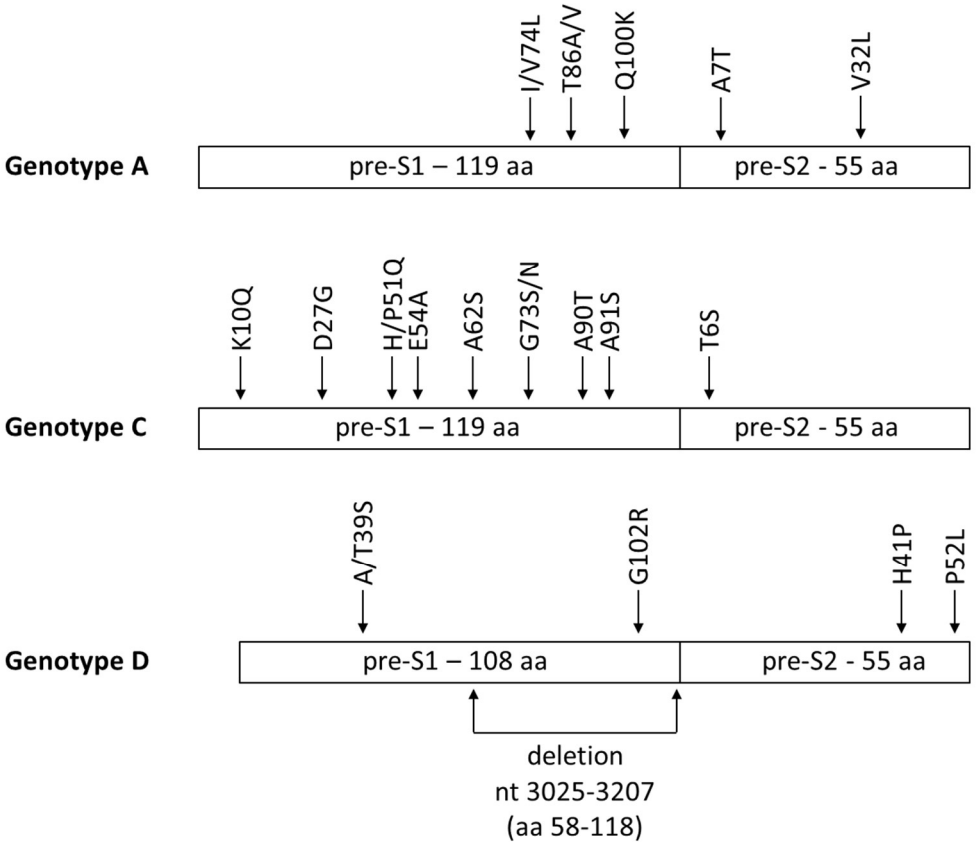


Figure 6. Pre-S1 and pre-S2 amino acid differences to reference sequences observed in the REP 102 study. This figure shows the location of all amino acid differences to genotype specific reference sequences we observed in pre-S1 and pre-S2 regions in the REP 102 study regardless of whether the mutation occurs in one or more patients. Adapted from Mijočević et al, 2019.

Table 4. Pre-S1 and pre-S2 amino acid differences to reference sequences observed in the REP 102 study for each patient (sequences obtained by direct sequencing).

Patient	Genotype	aa variations/mutations									
		pre-S1								pre-S2	
4	A	I/V74L[3]	T86A[1,2‡]/V[3]		Q100K[3]					A7T	V32L
1[NR]	C	K10Q	D27G	H/P51Q	E54A	A62S	G73S			T6S	
3	C	K10Q	D27G	H/P51Q	E54A	A62S	G73S	A90T	A91S	T6S	
6	C	K10Q	D27G		E54A	A62S	G73S			T6S	
7	C	K10Q	D27G	H/P51Q	E54A	A62S	G73N			T6S	
10[NR]	C	K10Q	D27G[‡]	H/P51Q	E54A	A62S	G73S			T6S	
11	C	K10Q	D27G	H/P51Q	E54A	A62S	G73S			T6S	
12	C	excluded from evaluation									
2	D	A/T39S		G102R						H41P	P52L
5[NR]	D	nt 3025-3207 del (aa 58-118)								H41P	P52L[‡]
8	D									H41P	
9	D	nt 3025-3207 del (aa 58-118)								H41P	

[NR] non-responder; [‡]amino acid variant coexists with the wild type; [1,2,3] indicate amino acid difference observed just in the 1[st], 2[nd] or 3[rd] time point [adapted from Mijočević et al, 2019]

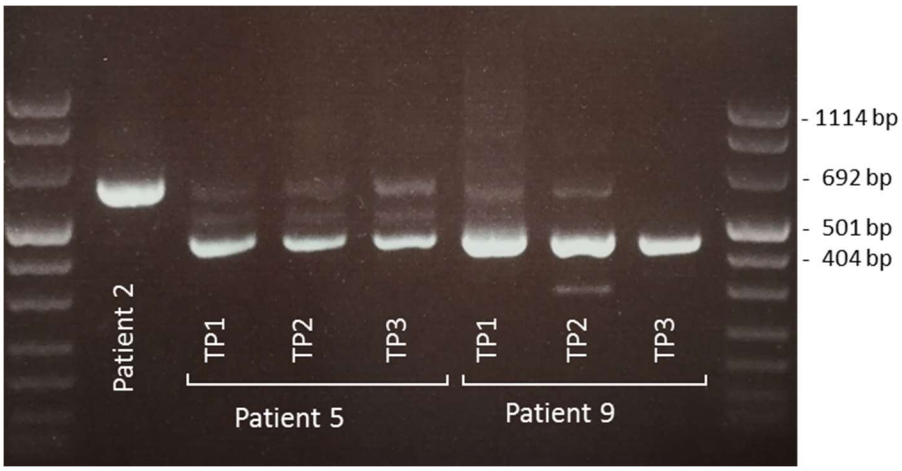


Figure 7. In-frame deletion of 183 nucleotides in the pre-S1 region. The in-frame deletion (nt 3025-3207) corresponding to aa 58-118 was observed in patients 5 and 9. This figure shows electrophoresis of pre-S PCR products of samples from all three time points (TP) from patient 5 and 9. Compared to the patient 2, where no deletion was found, all samples from patient 5 and 9 show shorter PCR products. Though, these samples have low signals at the level of control patient 2, which indicate coexistence of this mutation with the wild type. Adapted from Mijočević et al, 2019.

4.1.2. Comparison of amino acid sequences of the pre-S2 region

Pre-S2 region is encodes N-terminal domain of M-HBsAg and the middle domain of the L-HBsAg, and consists of 55 aa.

In patient 4 (genotype A), two aa differences to reference sequences were observed at all time points: A7T and V32L. Subgenotype C1 characteristic variation T6S was observed in all analysed genotype C patients. Subgenotype D ayw3 characteristic variation H41P was observed in all genotype D patients (table 6). Additionally, P52L was observed in patient 2, which also coexisted with wild type in patient 5 (figure 6, table 4).

There was no significant difference between amino acid differences to reference sequences between responders and non-responders.

4.1.3. Comparison of amino acid sequences of the S region

S region can be found in all three forms of HBsAg and consists of 226 aa in all genotypes. It involves MHR with “a” determinant - the major B cell epitope.

In the single genotype A patient, patient 4, Y161F was the only aa difference observed and was coexistent with the wild type at all 3 time points (table 5).

In genotype C patients, three aa differences to reference sequences, N3S, S53L and S210N, were observed at all time points in all patients (except S210N at time point 2 in patient 12). The nucleotide sequence comparison of these sequences against NCBI’s BLAST results showed that these 3 variations were present in isolates from the South Asian region and are subgenotype C1 specific (table 6). In patient 3, L98V, Q101K, L109P, T115N, K122R and I/T126S were coexistent with the wild type at all time points and P70H was observed coexistent with the wild type at time point 3. In patient 6, G10V was observed at time point 3 and G119R was coexistent with the wild type at time point 2. In patient 7, P120T was observed at time point 3. In patient 12, A159V and W196L were observed at time point 2.

Table 5. S HBsAg amino acid differences to reference sequences observed in the REP 102 study for each patient (sequences obtained by direct sequencing).

Patient	Genotype	aa variants/mutations																		
		aa 1-98					aa 99-169 (MHR)										aa 170-226			
4	A						Y161F‡													
1[NR]	C	N3S		S53L														S210N		
3	C	N3S		S53L	P70H[3‡]	L98V‡	Q101K‡	L109P‡	T115N‡				K122R‡	**I/T126S‡**				S210N		
6	C	N3S	G10V[3]	S53L						G119R[2‡]								S210N		
7	C	N3S		S53L							P120T[3]							S210N		
10[NR]	C	N3S		S53L														S210N		
11	C	N3S		S53L														S210N		
12	C	N3S		S53L											A159V[2]	W196L[2]	S210N[1,3]			
2	D	Q30K						T118V	**P127T**	**A128V**						V184A	T189I		L213I[1‡,2‡]	
5[NR]	D		C69stop‡		L88P‡			T118V	**P127T**	**A128V**										
8	D						G112R[2]	T118V	**P127T**	**A128V**							T189I[1‡,3‡]	S207R[3]		
9	D		C69stop[1‡]	R78Q[2]				T118V	**P127T**	**A128V**	P153T[2]					V184A[3‡]				

[NR] non-responder; ‡ amino acid variant coexist with the wild type; [1,2,3] indicate amino acid difference observed just in the 1st, 2nd or 3rd time point; amino acid differences within "a" determinant are bolded and underlined [adapted from Mijočević et al, 2019]

In all genotype D patients, T118V, P127T and A128V aa differences to the genotype D reference sequences were observed at all time points. NCBI BLAST search showed that these three differences were present in isolates found in South Asia and are subgenotype D ayw3 specific (table 6). Q30K and L213I (time point 1 and 2, coexistent with the wild type) were observed in patient 2. V184A was observed in patient 2 and was coexistent with the wild type at time point 3 in patient 9. T189I was observed in patient 2 at all time points and was coexistent with the wild type in patient 8 at time points 1 and 3. Mutation L88P coexisted with the wild type and was present at all time points in patient 5. G112R (time point 2) and S207R (time point 3) were observed in patient 8. R78Q was observed only at time point 2 in patient 9.

Table 6. Comparison of genotype C and genotype D sequences isolated in the REP 102 study with sequences in NCBI database. Using the NCBI's BLAST search, it was found that the aa differences to reference sequences observed in pre-S1, pre-S2 and S region of HBV surface protein were also present in other isolates from the South Asian region (i.e. Bangladesh, Myanmar) belonging to subgenotype C1 and in isolates found in South Asia (i.e. India) belonging to subgenotype D ayw3, respectively.

Patient	Subgenotype	subgenotype specific amino acid variations		
		pre-S1	pre-S2	S-HBsAg
1[NR]	C1	K10Q, D27G, H/P51Q, E54A, A62S, G73S	T6S	N3S, S53L, S210N
3	C1	K10Q, D27G, H/P51Q, E54A, A62S, G73S	T6S	N3S, S53L, S210N
6	C1	K10Q, D27G, E54A, A62S, G73S	T6S	N3S, S53L, S210N
7	C1	K10Q, D27G, H/P51Q, E54A, A62S	T6S	N3S, S53L, S210N
10[NR]	C1	K10Q, D27G, H/P51Q, E54A, A62S, G73S	T6S	N3S, S53L, S210N
11	C1	K10Q, D27G, H/P51Q, E54A, A62S, G73S	T6S	N3S, S53L, S210N
12	C1	excluded from evaluation		N3S, S53L, S210N
2	D ayw3	-	H41P	T118V, P127T, A128V
5[NR]	D ayw3	-	H41P	T118V, P127T, A128V
8	D ayw3	-	H41P	T118V, P127T, A128V
9	D ayw3	-	H41P	T118V, P127T, A128V

[NR] non-responder [adapted from Mijočević et al, 2019]

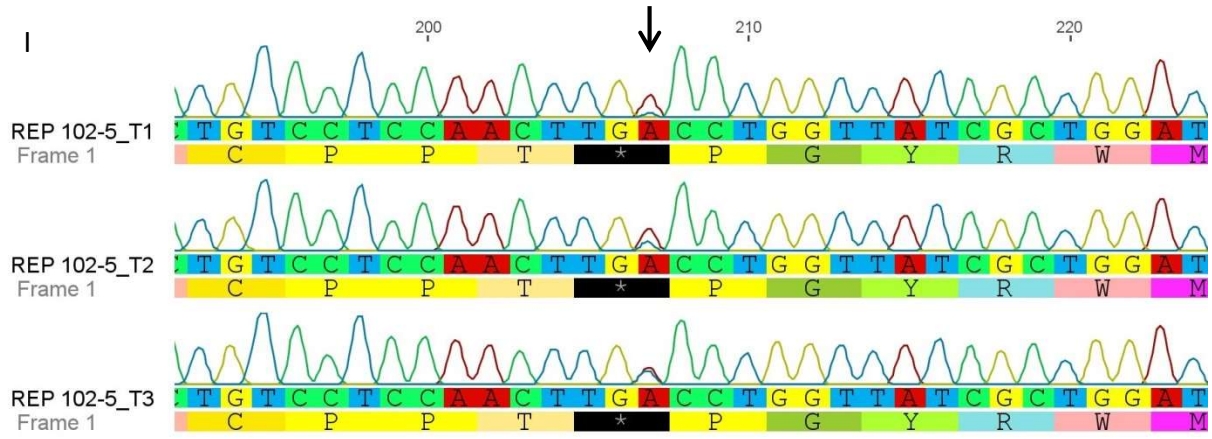
4.1.3.1. An early stop codon mutation

An early stop codon (C69stop) was present at all time points in patient 5 and coexisted with the wild type (figure 8). NGS was performed to confirm this mutation. A nucleotide change thymidine to adenosine at position 207 was observed which led to a change of aa 69 (cysteine) to a stop codon. Interestingly, this nucleotide change at position 207 was observed in higher frequency in all three time points. At time point 1, 80.74% of the reads showed a stop codon at aa position 69 whereas 19.23% of the reads had a cysteine codon. Similarly, at time point 2 and 3, 75.16% and 64.73% of the reads had

adenosine at position 207 (stop codon) while 24.82% and 35.22% of the reads had thymidine (cysteine codon) at position 69.

Similarly, the C69stop was also observed in patient 9. It was coexistent with the wild type and was observed only at time point 1.

I



II

Time point	Thymidine (wild type)	Adenosine (mutation)
T1	19.23 %	80.74%
T2	24.82%	75.16%
T3	35.22%	64.73%

Figure 8. Premature stop codon at amino acid position 69 in patient 5. This mutation coexists in all three time points (T1-T3) with the wild type. This mutation was first observed in Sanger sequences. As the chromatograms show (I), nucleotide substitution of thymidine (T, blue chromatogram) to adenosine (A, red chromatogram) at position 207 (arrow) of S-HBsAg region is coexistent with the wild type (both nucleotides present). This mutation results in an amino acid change at position 69 from cysteine (C) to a premature stop codon (*). This observation was confirmed with next generation sequencing (NGS) which revealed that the nucleotide change thymidine to adenosine at position 207 was observed in higher frequency at all three sampling time points (II). Adapted from Mijočević et al, 2019.

4.1.3.2. Analysis of the "a" determinant

Within the "a" determinant no mutations were observed in the genotype A patient (patient 4). In patient 3 (genotype C) I/T126S coexisted with the wild type and was present at all three time points. Both P127T and A128V, occurred in all time points within the all genotype D patients and are subgenotype D ayw3 specific. No mutations emerged within the "a" determinant during REP 2139-Ca treatment (table 5, figure 9).

Overall, more aa differences to reference sequences were observed in responders compared to non-responders in whole S region, 5.3 versus 3.7, however this difference was not statistically significant (p=0.318) and its clinical impact remains unclear. None of observed amino acid differences to reference sequences were correlated with response or non-response to therapy with REP 2139-Ca.

Genotype A

Patient	Time point	"a" determinant reference amino acid sequence																									
		124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147
		C	T	T	P	A	Q	G	N	S	M	F	P	S	C	C	C	T	K	P	T	D	G	N	C
4	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Genotype C

Patient	Time point	"a" determinant reference amino acid sequence																									
		124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147
		C	T	I/T	P	A	Q	G	T	S	M	F	P	S	C	C	C	T	K	P	S	D	G	N	C
1^{NR}	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	1	.	.	S‡	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	S‡	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	S‡	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
6	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
7	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10^{NR}	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
11	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
12	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Genotype D

Patient	Time point	"a" determinant reference amino acid sequence																									
		124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147
		C	T	T	P	A	Q	G	T	S	M	Y	P	S	C	C	C	T	K	P	S	D	G	N	C
2	1	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
5^{NR}	1	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8	1	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9	1	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	2	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	T	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

^{NR} non-responder; ‡ amino acid variant coexists with the wild type

Figure 9. "a" determinant amino acid sequence (aa 124-147) in genotype A (upper panel), genotype C (middle panel) and genotype D (lower panel). Sequences were obtained by direct sequencing. Amino acids positions without a difference to the reference sequence are marked with dots. Adapted from Mijočević et al, 2019.

4.1.4. Analysis of the overlapping RT-polymerase region

The initial two thirds of RT-polymerase region of the P ORF overlap pre-S/S ORF. Nucleotides coding aa 1-7 overlap the pre-S2 region, whereas nucleotides coding aa 8-234 overlap the whole S region of pre-S/S ORF. Amino acid variations observed in this region are shown in table 7.

Four variations (D7T, N122H, M129L and V163I) have been observed at all three time points in only genotype A patient (patient 4).

H9Y have been observed in genotype C patients 1, 3, 6, 7 and 10 at all time points except time point 3 in patient 3. N13H and I91L were observed in all genotype C patients at all time points except I91L at time point 2 in patient 12. In patient 3, S106C, P109Q, N123K and D134E coexisted with the wild type at all time points and G127E coexisted with the wild type at time point 2 in patient 6. R18S and T128N were observed at time point 3 in patients 6 and 7, respectively. D31N in patient 7, N123D in patient 12, R153W in patient 11 and S223A in patient 1 have been observed at all time points, whereas M204I was observed just at time point 2 in patient 12.

N53D, Y54H and H126R were observed in all genotype D patients at all time points. A38E and F221Y (coexisted with the wild type) in patient 2, R120K in patient 8 and L132M in patient 5 were observed at all time points. S78T coexisted with the wild type in patient 5 at all-time points and in patient 9 just in the first time point. P161H was observed at time point 2 in patient 9. Q215P was observed at time point 3 in patient 8.

Table 7. Amino acid differences to reference sequences in the overlapping RT-polymerase region observed in the REP 102 study for each patient (sequences obtained by direct sequencing).

Patient	Genotype	aa variations/mutations										
4	A	D7T	N122H	M129L	V163I							
1[NR]	C	H9Y	N13H		I91L							S223A
3	C	H9Y[1,2]	N13H		I91L	S106C‡	P109Q‡	N123K‡	D134E‡			
6	C	H9Y	N13H	R18S[3]	I91L				G127E[2‡]			
7	C	H9Y	N13H	D31N	I91L					T128N[3]		
10[NR]	C	H9Y	N13H		I91L							
11	C		N13H		I91L						R153W	
12	C		N13H		I91L[1,3]			N123D				**<u>M204I[2]</u>**
2	D	A38E	N53D	Y54H		H126R			F221Y‡			
5[NR]	D		N53D	Y54H	S78T‡	H126R	L132M					
8	D		N53D	Y54H	R120K	H126R			Q215P[3]			
9	D		N53D	Y54H	S78T[1‡]	H126R		P161H[2]				

[NR] non-responder; ‡amino acid variant coexists with the wild type; [1,2,3] indicate amino acid difference observed just in the 1st, 2nd or 3rd time point; amino acid differences which indicate resistance are bolded and underlined

4.2. Analysis of quasispecies within the major hydrophilic region (MHR)

Because of the importance of mutations that may occur in the MHR, we performed NGS of this region for all 12 patients included in the REP 102 study and compared the results with those obtained by direct sequencing. Three time points were analysed for each patient. All samples used for NGS were the same as for the direct sequencing expect of the third time point in patient 3.

A mean coverage depth ± SD per nucleotide after passing quality control was 201022 ± 114613 for responders and 278307 ± 69252 for non-responders. To eliminate sequencing errors and to characterize the HBV population as accurately as possible, only quasispecies (QS) with prevalence of ≥ 1% were used for analysis.

In four genotype C patients, 1, 7, 10 and 11, we observed just one QS through all three time points. In five patients, 2, 3, 6, 9 and 12, we observed more QS at the first time point with the reduction of QS in the second and third time point. In patient 4 and 8 we observed the increase of QS in the second time point, but decrease of QS in the third time point, whereas in patient 5 the number of QS increased between the second and the third time point (table 8).

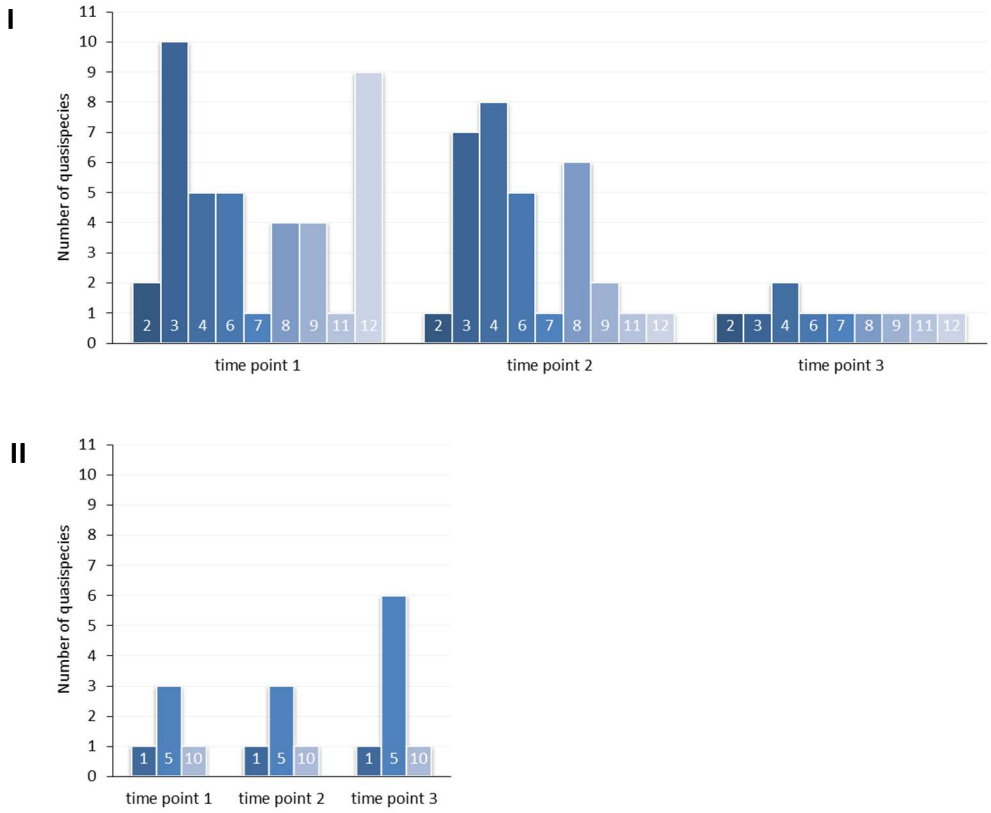


Figure 10. Number of quasispecies in responders (I) and non-responders (II) observed in the REP 102 study. QS with prevalence of ≥1% are presented. Numbers in columns indicate patients.

Table 8. Number of quasispecies observed in the REP 102 study.

Patient	Genotype	Time point	Quasispecies with frequency ≥ 1%	Quasispecies with frequency < 1%
4	A	1	5	184
		2	8	82
		3	2	2
1[NR]	C	1	1	1
		2	1	1
		3	1	1
3	C	1	10	166
		2	7	83
		3	1	115
6	C	1	5	8
		2	5	6
		3	1	1
7	C	1	1	1
		2	1	1
		3	1	1
10[NR]	C	1	1	1
		2	1	1
		3	1	1
11	C	1	1	1
		2	1	1
		3	1	1
12	C	1	9	49
		2	1	1
		3	1	1
2	D	1	2	7
		2	1	1
		3	1	1
5[NR]	D	1	3	66
		2	3	98
		3	6	85
8	D	1	4	4
		2	6	10
		3	1	1
9	D	1	4	17
		2	2	2
		3	1	1

[NR] non-responder [Adapted from Usman et al, 2019]

Table 9. Amino acid differences of the extended MHR corresponding to aa 76-200 to reference sequences observed in the REP 102 study (sequences obtained by NGS).

Variant/Mutation	Patient	Time point 1	Time point 2	Time point 3
		Percentage of reads with specific variant/mutation‡		
F85C	4	-	7.65§	-
L88P	5NR	59.16	42.09	35.74
L88R	12	5.08§	-	-
L98V	3	55.38	56.23	-
D99G	9	4.48§	-	-
Q101K	3	29.75	32.81	-
Q101R	3	14.52§	12.48§	-
M103I	6	11.54§	33.08§	-
V106F	8	-	2.30§	-
P108H	12	6.08§	-	-
L109P	3	41.95	45.03	-
L109Q	4	11.69§	15.21§	-
	8	-	7.57§	-
G112R	8	19.31§	73.05	-
T115N	3	40.47	45.03	-
	8	-	7.57§	-
T118V	2, 5NR, 8, 9	100	100	100
G119R	6	12.42§	37.40	-
P120T	5NR	-	-	2.51§
	7	-	-	100
K122R	3	42.43	45.03	-
I/T126S	3	42.7	44.01	-
T126I	5 NR	1.43§	-	-
P127T	2, 5NR, 8, 9	100	100	100
A128V	2, 5NR, 8, 9	100	100	100
D144E	9	3.46§	-	-
G145R	6	6.71§	3.75§	-
P153T	9	-	61.61	-
A159V	12	-	100	-
Y161F	4	69.66	36.37	88.29
V168A	3	2.37§	3.28§	-
F170S	5NR	1.43§	-	-
	3	23.11§	21.65§	-
L173P	5NR	-	-	2.96§
V184A	2	100	100	100
G185E	12	1.08§	-	-
P188L	9	3.46§	-	-
P188H	12	4.46§	-	-
T189I	2	100	100	100
	5NR	-	-	1.03§
	8	61.43	13.21§	100

‡quasispecies with frequency < 1% were not included in the analysis; NRnon-responder; amino acid differences to reference sequences within "a" determinant are bolded and underlined; §aa difference to reference sequences not observed in direct sequencing

All aa differences to referent sequences observed in the direct sequencing analysis were confirmed in NGS except R78Q and V184A in patient 9, and W196L in patient 12. Furthermore, the are some aa differences to referent sequences that were observed just in NGS analysis (table 9). The vast majority of them were present in less than 10% of reads per sample: V168A in patient 3, F85C in patient 4, P120T, T126I, F170S, L173P and T189I in patient 5, G145R in patient 6, V106F, L109Q and T115N in patient 8, D99G, D144E and P188L in patient 9, and L88R, P108H, G185E and P188H in patient 12. Furthermore, Q101R in patient 3, L109Q in patient 4, M103I and G119R in patient 6, and G112R and T189I in patient 8 were present in 10 - 20% of reads per sample. Two aa differences to referent sequences that were not found in direct sequencing analysis were present in > 20% of reads in NGS: M103I at the second time point in patient 6, and F170S at the first and second time point in patient 3.

4.3. Investigation of direct interference of REP 2139-Ca with diagnostic assays

A strong reduction of HBsAg during REP 2139-Ca treatment was observed in the REP 102 study (Al-Mahtab et al, 2016). In the analysis of the pre-S/S ORF of HBV DNA in patient's sera that we performed, no mutations that could cause false low or false negative HBsAg values have been found. To exclude that this strong reduction of HBsAg is caused by direct interference of REP 2139-Ca with routine diagnostic assays, the interference study was performed. Direct interference of REP 2139-Ca with 13 different assays for 9 serological and molecular diagnostic parameters for diagnosis and follow up of HBV and HDV infection was investigated. For all quantitative assays two concentration ranges were investigated. Descriptive statistic of measured values in the examined samples with four different concentrations of REP 2139-Ca and one control sample is shown in the table 10.

Mean concentrations of anti-HBs in all samples examined were 16.98 IU/l (SD=0.47) and 536.18 IU/l (SD=15.10). Mean concentrations of HBsAg in all tested samples were 47.04 IU/ml (SD=1.93) and 38138.78 IU/ml (SD=1370.96). Mean signals of qualitative serological assays were as follows: 3954.13 S/CO (SD=48.48) for HBsAg, 11.02 S/CO (SD=0.12) for anti-HBc total, 31.67 S/CO (SD=0.48) for anti-HBc IgM, 0.01 S/CO (SD=0.00) for anti-HBe, 815.19 S/CO (SD=11.92) for HBeAg and 0.025 extinction (SD=0.003) for anti-HDV assay.

Mean concentrations of HBV DNA in all tested samples were 1928.80 IU/ml (SD=133.46) and $5.12x10^7$ IU/ml (SD=$2.57x10^6$). Mean concentrations of HDV RNA in all tested samples were 1827.57 copies/ml (SD=417.23) and 43529.67 copies/ml (SD=9716.79) measured with TUM PCR, 4194.67 copies/ml (SD=528.66) and 69313.33 copies/ml (SD=10076.50) measured with RoboGene® PCR, 22702 copies/ml (SD=20253) and 29200 copies/ml (SD=24784) measured with NGI PCR and 2.96 log IU/ml (SD=0.47) and 5.45 log IU/ml (SD=0.39) measured with Eurobioplex PCR.

Table 10. Mean and standard deviation of observed results in tested assays.

REP 2139-Ca	control		0.1 µg/ml		1 µg/ml		10 µg/ml		150 µg/ml	
Assay	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
anti-HBs quant. 1[a]	17.23	0.45	16.67	0.61	16.84	0.63	17.18	0.12	17.17	0.26
anti-HBs quant. 2[a]	529.81	6.15	549.90	25.01	540.34	7.11	524.66	9.29	525.90	15.16
HBsAg qual.[b]	3968.68	65.43	3991.27	26.55	3955.65	19.77	3900.94	45.75	3865.98	53.55
HBsAg quant. 1[c]	47.06	1.77	47.27	1.29	46.02	3.56	47.82	1.55	46.11	3.43
HBsAg quant. 2[c]	3.71×10^4	1827.92	3.87×10^4	1176.50	3.82×10^4	1329.71	3.85×10^4	1548.01	3.88×10^4	547.83
anti-HBc total[b]	10.99	0.11	11.01	0.08	11.13	0.12	10.95	0.14	11.00	0.15
anti-HBc IgM[b]	31.65	0.57	31.89	0.39	31.38	0.61	31.78	0.56	31.64	0.64
anti-HBe[b]	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00
HBeAg[b]	813.05	7.33	822.68	12.50	815.07	13.50	808.48	21.76	816.64	6.12
anti-HDV[d]	0.029	0.001	0.024	0.001	0.024	0.003	0.025	0.002	0.026	0.004
HBV DNA 1[c]	1858.33	60.12	1929.00	43.86	2069.33	162.14	2010.33	132.14	1777.00	51.88
HBV DNA 2[c]	4.89×10^7	8.54×10^5	5.45×10^7	2.31×10^5	5.08×10^7	9.07×10^5	5.18×10^7	2.14×10^6	5.03×10^7	4.16×10^6
HDV RNA 1 (TUM)[e]	2273.67	140.43	2273.33	117.12	1356.67	210.93	1618.00	246.07	1546.33	136.40
HDV RNA 2 (TUM)[e]	5.09×10^4	3747.50	5.55×10^4	1551.57	4.13×10^4	4173.90	4.18×10^4	2297.17	2.82×10^4	3130.06
HDV RNA 1 (RoboGene®)[f]	3836.67	863.10	4220.00	170.59	4313.33	265.77	3810.00	482.80	4793.33	210.79
HDV RNA 2 (RoboGene®)[f]	6.64×10^4	1.25×10^4	6.34×10^4	9657.29	6.39×10^4	8866.23	7.21×10^4	8418.43	8.08×10^4	6518.44
HDV RNA 1 (NGI)[e]	2.67×10^4	1.15×10^4	3.93×10^4	3511.88	4.50×10^4	1.87×10^4	2510.00	3715.66	0.00	0.00
HDV RNA 2 (NGI)[e]	5.67×10^4	7637.63	5.33×10^4	2.02×10^4	1.58×10^4	1.40×10^4	2.02×10^4	1.64×10^4	0.00	0.00
HDV RNA 1 (Eurobioplex)[g]	3.26	0.08	3.23	0.16	3.25	0.03	3.00	0.13	2.05	0.08
HDV RNA 2 (Eurobioplex)[g]	5.74	0.04	5.65	0.02	5.67	0.02	5.49	0.04	4.69	0.03

[a]mIU/ml; [b]S/CO; [c]IU/ml; [d]extinction; [e]copies/ml; [f]U/ml; [g]logU/ml

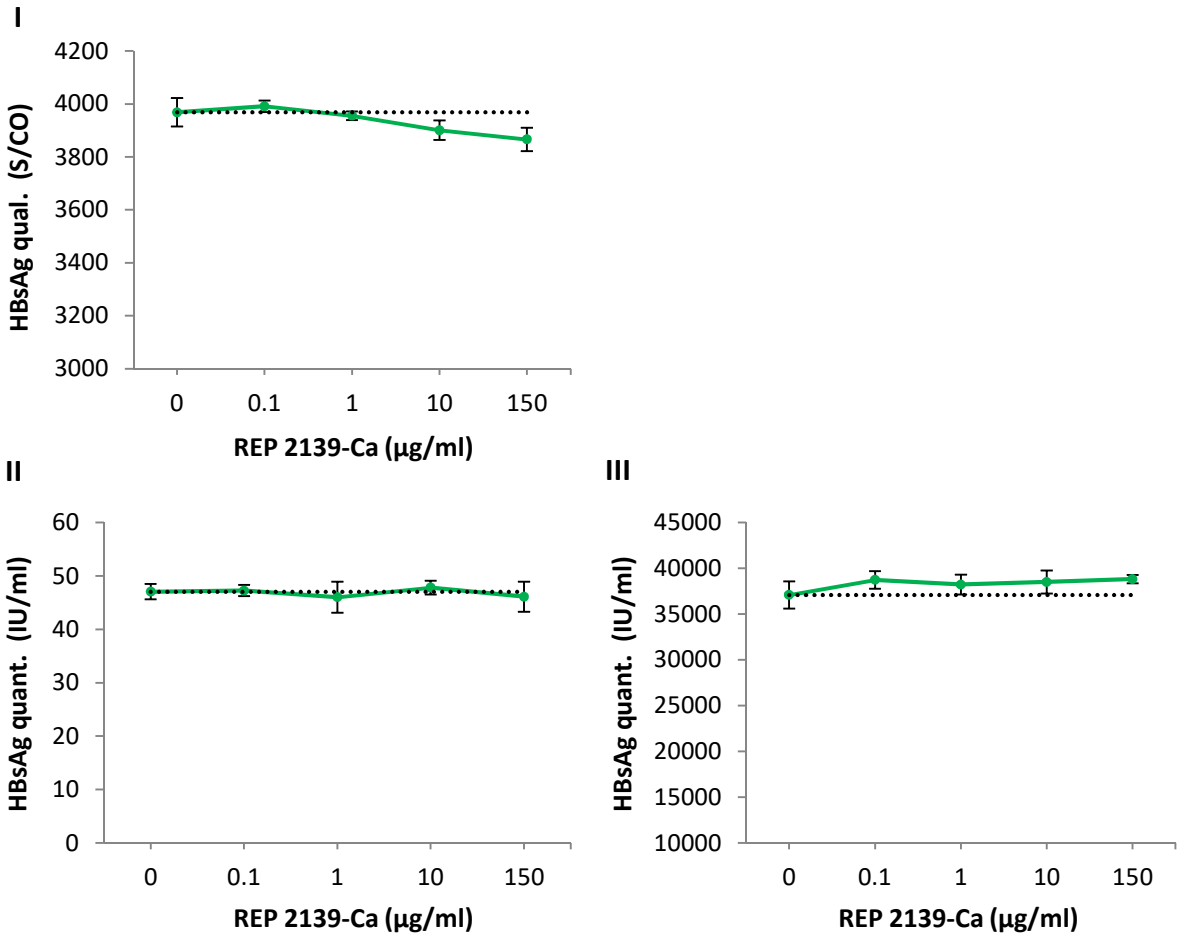


Figure 11. Interference of REP 2139-Ca with HBsAg assay qualitative (I) and quantitative (in samples with low (II) and high (III) concentration of HBsAg). Plotted values represent mean and standard deviations from three independent tests run for each REP 2139-Ca concentration. Dotted lines indicate mean of signals observed in control sample. Adapted from Bazinet et al, 2017.

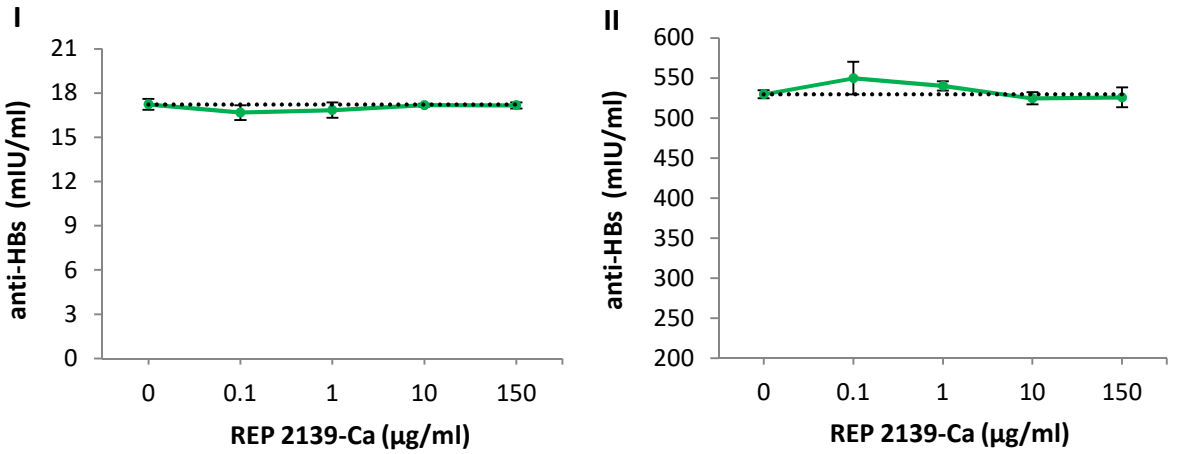


Figure 12. Interference of REP 2139-Ca with anti-HBs assay in samples with low (I) and high (II) concentration of anti-HBs. Plotted values represent mean and standard deviations from three independent tests run for each REP 2139-Ca concentration. Dotted lines indicate mean of signals observed in control sample. Adapted from Bazinet et al, 2017.

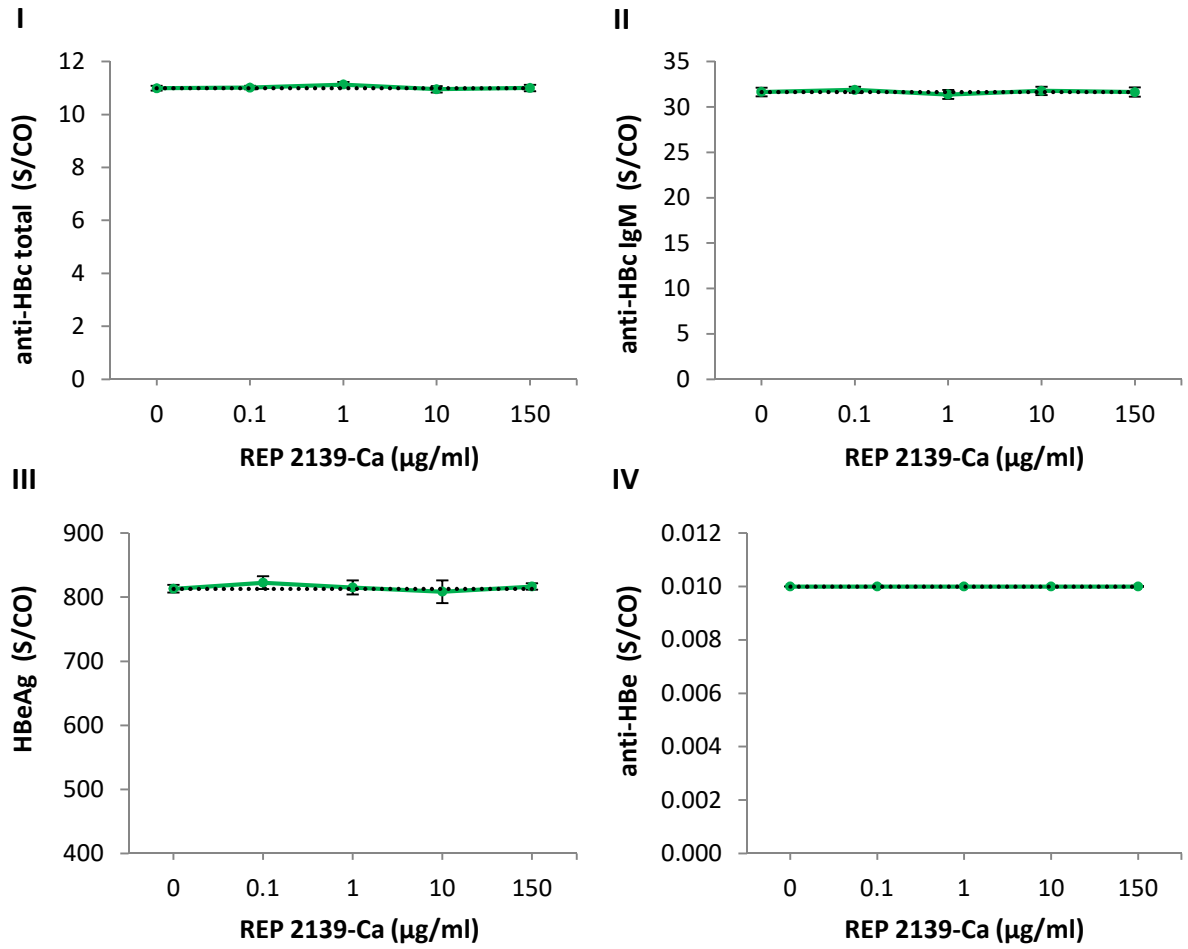


Figure 13. Interference of REP 2139-Ca with anti-HBc total (I), anti-HBc IgM (II), HBeAg (III) and anti-HBe assay (IV). Plotted values represent mean and standard deviations from three independent tests run for each REP 2139-Ca concentration. Dotted lines indicate mean of signals observed in control sample. Adapted from Bazinet et al, 2017.

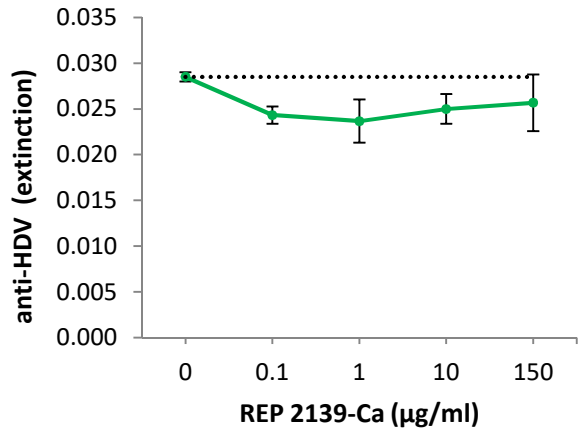


Figure 14. Interference of REP 2139-Ca with anti-HDV assay. Plotted values represent mean and standard deviations from three independent tests run for each REP 2139-Ca concentration. Dotted lines indicate mean of signals observed in control sample. Adapted from Bazinet et al, 2017.

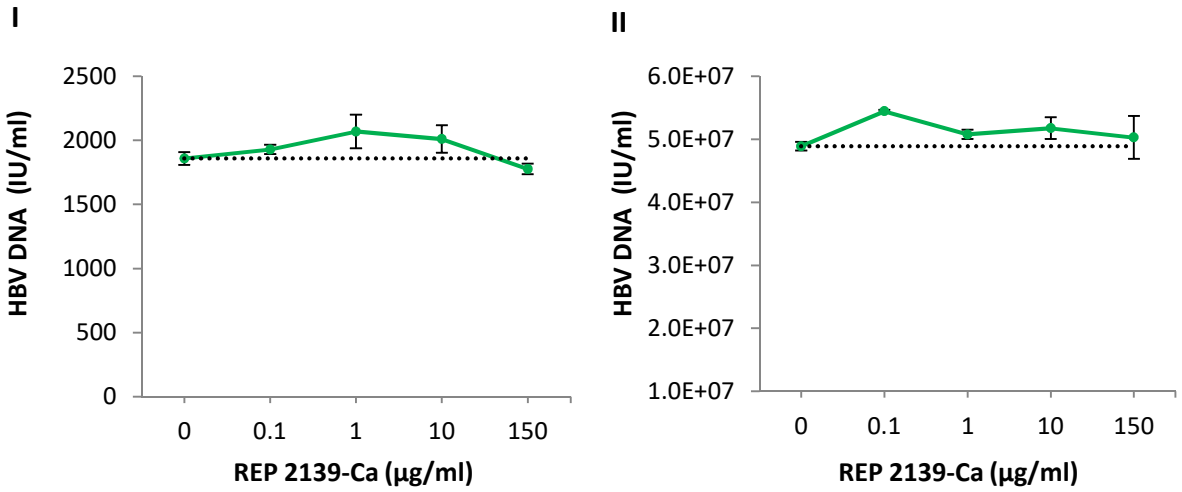


Figure 15. Interference of REP 2139-Ca with HBV DNA PCR in samples with low (I) and high (II) viral load. Plotted values represent mean and standard deviations from three independent tests run for each REP 2139-Ca concentration. Dotted lines indicate mean of signals observed in control sample. Adapted from Bazinet et al, 2017.

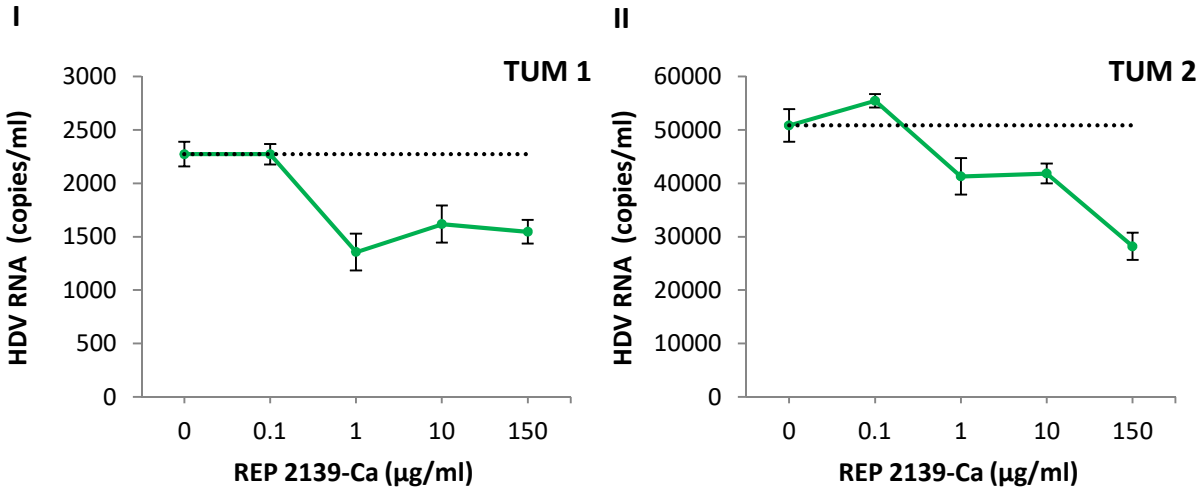


Figure 16. Interference of REP 2139-Ca with HDV RNA PCR in samples with low (I) and high (II) viral load – Technische Universität München (TUM). Plotted values represent mean and standard deviations from three independent tests run for each REP 2139-Ca concentration. Dotted lines indicate mean of signals observed in control sample. Adapted from Bazinet et al, 2017.

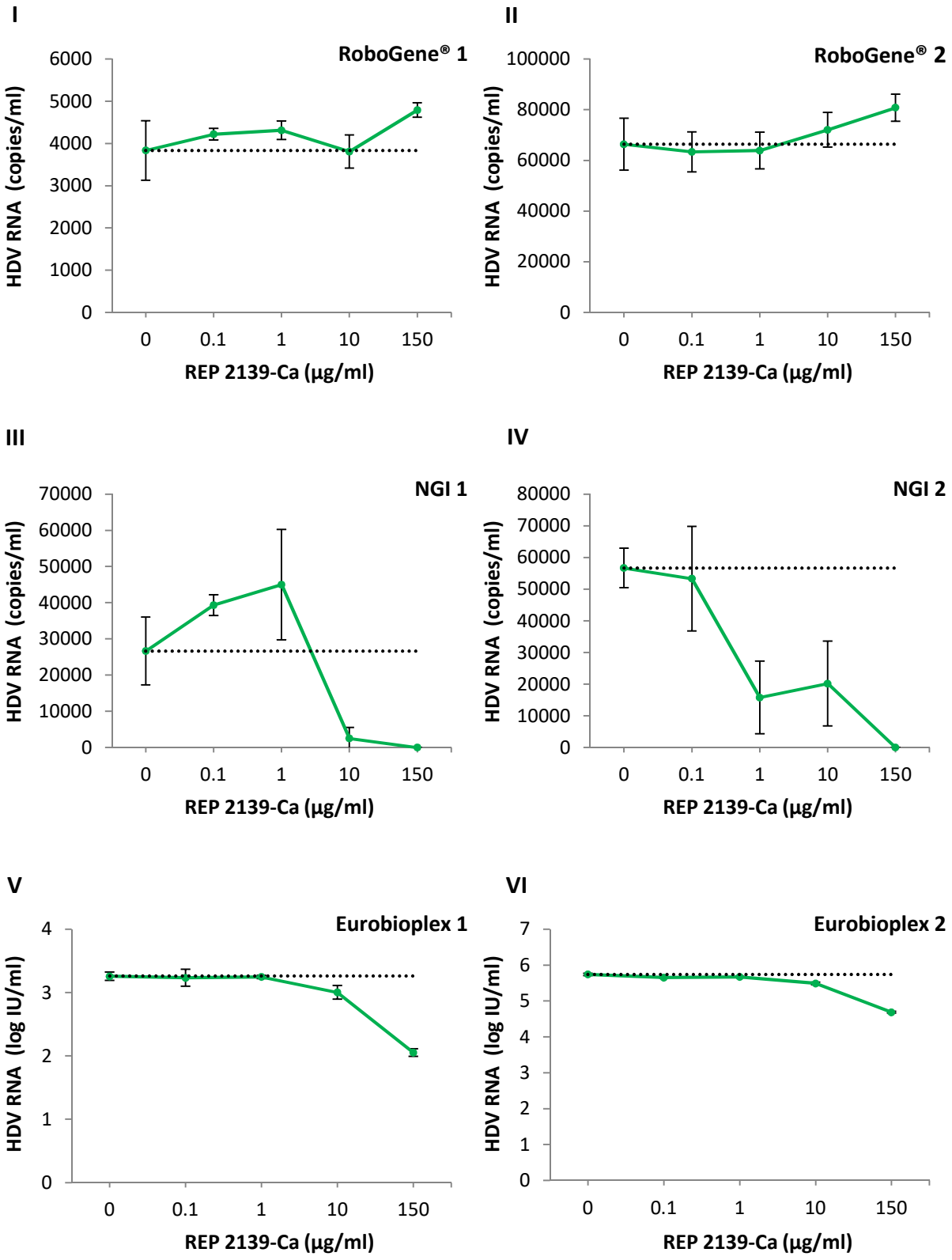


Figure 17. Interference of REP 2139-Ca with HDV RNA PCR: RoboGene® (I and II), National Genetic Institute - NGI (III and IV) and Eurobioplex (V and VI) in samples with low and high viral load, respectively. Plotted values represent mean and standard deviations from three independent tests run for each REP 2139-Ca concentration. Dotted lines indicate mean of signals observed in control sample. Adapted from Bazinet et al, 2017.

Kolmogorov-Smirnov test has shown normally distribution for all variables except anti-HBe and Eurobioplex HDV RNA PCR 1 and 2 (data not shown). A t-test was used to determine a difference between the control sample and samples with four different concentrations of REP 2139-Ca regardless of data distribution. The t-test is a robust method and assumption of normality can be violated without damaging the validity of the statistic (Leech et al, 2005).

Table 11. Determination of difference between control samples and tested sera (t-test).
Statistically significant differences are bolded and underlined. For anti-HBe assay was not possible to perform t-test because of exactly the same values of all measurements.

REP 2139-Ca concentration	**0.1 µg/ml**		**1 µg/ml**		**10 µg/ml**		**150 µg/ml**	
Assay	**t-value**	**p-value**	**t-value**	**p-value**	**t-value**	**p-value**	**t-value**	**p-value**
anti-HBs quant. 1	1.268	0.274	0.872	0.433	0.186	0.861	0.212	0.842
anti-HBs quant. 2	-1.351	0.297	-1.941	0.124	0.801	0.468	0.414	0.700
HBsAg qual.	-0.554	0.609	0.330	0.758	1.470	0.216	2.104	0.103
HBsAg quant. 1	-0.167	0.876	0.453	0.674	-0.563	0.604	0.427	0.692
HBsAg quant. 2	-1.307	0.261	-0.868	0.434	-1.024	0.364	-1.572	0.191
anti-HBc total	-0.252	0.814	-1.459	0.218	0.381	0.723	-0.094	0.929
anti-HBc IgM	-0.590	0.587	0.564	0.603	-0.274	0.798	0.020	0.985
anti-HBe	-	-	-	-	-	-	-	-
HBeAg	-1.151	0.314	-0.228	0.831	0.345	0.748	-0.651	0.550
anti-HDV	**4.443**	**0.021**	2.213	0.114	2.278	0.107	0.995	0.393
HBV DNA 1	-1.645	0.175	-2.113	0.102	-1.814	0.144	1.774	0.151
HBV DNA 2	**-10.894**	**<0.001**	-2.594	0.060	-2.158	0.097	-0.571	0.598
HDV RNA 1 (TUM)	0.003	0.998	**6.268**	**0.003**	**3.934**	**0.029**	**6.435**	**0.003**
HDV RNA 2 (TUM)	-1.974	0.120	**2.948**	**0.042**	**3.552**	**0.024**	**8.039**	**0.001**
HDV RNA 1 (RoboGene®)	-0.755	0.492	-0.914	0.412	0.047	0.965	-1.865	0.136
HDV RNA 2 (RoboGene®)	0.332	0.756	0.282	0.792	-0.654	0.549	-1.766	0.152
HDV RNA 1 (NGI)	-1.824	0.142	-1.447	0.221	**3.461**	**0.026**	**4.015**	**0.016**
HDV RNA 2 (NGI)	0.267	0.802	**4.430**	**0.011**	**3.495**	**0.025**	**12.851**	**<0.001**
HDV RNA 1 (Eurobioplex)	0.222	0.835	0.199	0.852	**2.811**	**0.048**	**18.818**	**<0.001**
HDV RNA 2 (Eurobioplex)	**3.506**	**0.025**	2.711	0.053	**7.319**	**0.002**	**36.248**	**<0.001**

There is no significant difference between control samples and samples with different concentrations of REP 2139-Ca (0.1, 1, 10 and 150 µg/ml) for all tested serological assays with exception of anti-HDV assay, where the sample with the lowest concentration of REP 2139-Ca had statistically significant higher signal compared to the control sample (figure 11-14, table 11).

Among molecular diagnostic assays, no significant difference between control samples and samples with REP 2139-Ca (0.1, 1, 10 and 150 µg/ml) were found for HBV DNA PCR with exception of a higher concentration of HBV DNA in the sample with the lowest concentration of REP 2319-Ca (0.1 µg/ml) in HBV DNA PCR 2 (figure 15, table 11). A statistically significant lower signal of HDV RNA was found in samples with 1, 10 and 150 µg/ml REP 2139-Ca in TUM HDV RNA 1 and 2 and NGI HDV RNA 2. A significant lower signal of HDV RNA was observed in samples with 10 and 150 µg/ml REP 2139-Ca for all HDV RNA assays except RoboGene® HDV RNA 1 and 2 (figure 16 and 17, table 11). However, except for Eurobioplex HDV RNA 2 there was no significant difference observed between control samples and samples with the lowest concentration of REP 2139-Ca (0.1 µg/ml).

5. Discussion

Currently approved direct antiviral therapy for chronic hepatitis B with NUCs can suppress HBV replication and improve liver function. Moreover, immunotherapy with pegINF has higher rates of HBsAg seroconversion compared to NUCs after the same duration of therapy (Yapali et al, 2014). However, rates of functional cure under both therapies are still low, and long-term or lifelong treatment with NUCs is needed.

Phase II clinical study in patients suffering from chronic hepatitis B with nucleic acid polymer REP 2139-Ca combined with immunotherapy has shown promising results (Al-Mahtab et al, 2016). A significant reduction or complete loss of HBsAg, reduction of HBV DNA, and subsequent seroconversion to anti-HBs was observed. Furthermore, absence of HBV DNA and HBsAg persisted after 40 weeks of treatment.

Blocking the assembly and/or release of SVP originating from cccDNA or integrated HBV DNA by NAPs result in seroconversion to anti-HBs and functional cure of chronic HBV infection. This functional cure seems to be immune mediated and provided by reduction of HBsAg, since REP 2139 therapy has no direct immunostimulatory properties (Real et al, 2017). It has been shown that large amounts of HBsAg have immunosuppressive effects in HBV infection (Aillot et al, 2018; Dembek et al, 2018; Kondo et al, 2013; Lebossé et al, 2017). Clearance of HBsAg in the circulation establishes a more permissive immunological environment where functional cure of the HBV infection can be achieved.

Similar results have been seen in the patients suffering from chronic HBV/HDV coinfection. The therapy with REP 2139-Ca combined with pegINF therapy showed a significant reduction or complete loss of HBsAg, reduction of HBV DNA and HDV RNA, and subsequent seroconversion to anti-HBs which persisted after end of therapy of 63 weeks (Bazinet et al, 2017).

5.1. Analysis of the entire pre-S/S ORF

The goal of the analysis of the pre-S/S ORF of HBV DNA was to examine whether differences in amino acid sequences of HBV envelope proteins correlate to response or non-response of patients receiving REP 2139-Ca therapy. The entire protein sequences of pre-S1, pre-S2 and S regions were analysed from all 12 patients included in the REP 102 study (Al-Mahtab et al, 2016).

Non-responders compared with responders showed a trend of a more conserved S region within the pre-S/S ORF with respect to reference sequences through all three analysed time points. However, this

difference was not statistically significant. Some mutations of HBsAg, especially pre-S1 and pre-S2 deletions, have been shown to accumulate with the progression of HBV infection (Cao, 2009), which may reflect attempts of the virus to escape from the host immune response (Kay and Zoulim, 2007). The greater number of mutations in responders might reflect a more active host specific immune response. However, no discernible differences between HBsAg sequences in responders versus non-responders in all three surface proteins have been observed during REP 2139-Ca therapy which correlated with therapy outcome. The addition of pegIFN or thymosin a1 in the REP 102 study might increase mutation rates in HBsAg due to higher immune pressure. However, no additionally mutations were observed at time points following the addition of pegIFN or thymosin a1 therapy except P120T in patient 7 and V184A (coexistent with the wild type) in patient 9.

Analysis of binding domains for the HBV specific receptor (NTCP) in the pre-S1 region of L-HBsAg has been performed to exclude mutations that may affect viral entry. No deletions or mutations in essential NTCP-binding site have been found in analysed sequences. The deletion of two aa seen in one non-responder (patient 5) and one responder (patient 9) at the end of the accessory NTCP-binding site has presumably no functional significance. The differences to reference sequences (D27G, H/P51Q, E54A) that have been found in both essential and accessory NTCP-binding sites in genotype C isolates were observed in all patients, responders and non-responders, respectively. These differences were shown to be subgenotype C1 specific (table 6) and most likely have no influence on NTCP-binding site affinity or response to REP 2139-Ca therapy.

No mutations have been observed at mandatory N-linked glycosylation site at position 4 in the pre-S2 region, or at optional N-linked glycosylation site at position 146 in the S region, which could impair virion secretion.

The in-frame deletion of 183 nucleotides (nt 3025-3207) that have been found in pre-S1 region in two genotype D patients was coexistent with the presence of wild type in both these patients. This type of in-frame deletion within pre-S1 region has already been described (Chen et al, 2006; Fan et al, 2001; Melegari et al, 1994). Deletions can be often found in the pre-S region of HBV envelope proteins, especially at the 3’ terminus of pre-S1 and 5’ terminus of pre-S2, with increasing of pre-S variants prevalence during the natural course of chronic HBV infection (Fan et al, 2001). Pre-S mutations and especially pre-S deletions may cause endoplasmic reticulum stress through intracellular retention of HBV envelope proteins (Chen, 2018) and are associated with progressive liver disease and a higher risk of HCC in chronically infected patients (Chen et al, 2006; Choi et al, 2007). Pre-S regions mutations/deletions and S region mutations are often observed in patients with occult HBV infection

(Kim et al, 2013). In the context of the REP 102 study, this deletion has no impact on response to REP 2139-Ca therapy in these two patients as patient 5 was a non-responder and patient 9 was a responder.

The second objective of the analysis of the pre-S/S ORF was to determine mutations, especially in the "a" determinant, which may occur during therapy and impair the detection of HBsAg by diagnostic assays. Mutations within the "a" determinant that emerge during REP 2139-Ca therapy have been found in none of 12 patients. Three variants observed within "a" determinant (I/T126S, P127T and A128V), were present in initial samples and persisted throughout REP 2139-Ca treatment. Furthermore, the I/T126S mutation seen in patient 3 coexisted with the wild type at all three time points. While several studies suggest that the I/T126S mutation can lead to false negative results (Caligiuri et al, 2016; Coppola et al, 2015), HBsAg harbouring this mutation can be recognized by a spectrum of commercial used HBsAg assays, including the quantitative HBsAg assay (Architect, Abbott) used in the REP 102 study (Huang et al, 2012; Servant-Delmas et al, 2012). P127T and A128V occur within the "a" determinant in all genotype D patients and are subgenotype D ayw3 specific (table 6).

Mutations in the MHR but outside of "a" determinant may also affect the detection of HBsAg. P120T mutation that emerges at the third time point in patient 7 may be the most relevant mutation we observed in this region. The aa residues at positions 120 to 123 were described as important for the antigenicity of HBsAg (Tian et al, 2007) and may impair diagnostic detection of HBsAg. Nevertheless, studies have shown that HBsAg with the P120T mutation was reactive in the majority of commercial diagnostics assays, including the assay used in REP 102 study, however, under-quantified (Huang et al, 2012; Servant-Delmas et al, 2012; Tian et al, 2007).

A premature stop codon found at aa position 69 of S region of pre-S/S ORF in two genotype D infected patients (one responder and one non-responder) lead to the production of truncated, functionally defective HBsAg, which cannot be detected by standard diagnostic assays because of the absence of an "a" determinant. HBV isolates with such altered HBsAg were previously described (Veazjalali et al, 2009), and are able to form morphologically and functionally correct virions and SVP in the presence of coexisting wild type virus (Bruss and Ganem, 1991a; Yang, 2003) which was the case in these two patients.

HBsAg produced from coexisting wild type virus can be detected by diagnostic assays, as observed in one non-responder (patient 5) with positive HBsAg during all three time points, and in one responder (patient 9), where this deletion was detected just at the first time point, where the high concentration of HBsAg was reported. This confirms earlier findings.

The coexistence of this mutation with the wild type was confirmed in patient 5 with NGS. Interestingly, the proportion of quasispecies with premature stop codon at aa position 69 continuously decreased during REP 2139-Ca treatment (figure 8). These data confirm coexistence of the variant with the premature stop codon with the wild type virus in these patients and explain positive HBsAg results despite C69stop mutation that leads to production of truncated HBsAg.

Based on the analysis of the complete pre-S/S ORF of HBV DNA in the patients received REP 2139-Ca there is no indication of mutations causing structural or functional changes in HBV envelope proteins in non-responders compared to responders that may explain lack of response. This suggest that there is no direct interaction of REP 2139-Ca with HBsAg which is in accordance with previous studies (Beilstein et al, 2018; Blanchet et al, 2019).

Mutations that may significantly affect the sensitivity of diagnostic assays used for the detection of HBsAg were found neither in responders nor in non-responders. The observed mutations can be recognized by various commercial used HBsAg assays, including the HBsAg assay we used for testing of patients' samples in the REP 102 study. In addition, the majority of observed variants are present throughout all three time points, which covers both high and low concentrations of HBsAg, and additionally speaks against the possible negative effects of observed mutations on HBsAg detection by standard diagnostics assays.

Importantly, the same lack of inhibition of the release of HBV SVP observed in three patients in the REP 102 study was observed in the recent REP 301 study in HBV/HDV coinfected patients (Bazinet et al, 2017). However, HDV RNA was rapidly cleared from the blood in all treated patients. This indicates that REP 2139 is being efficiently delivered to hepatocytes in all patients but in a small number of patients can not sufficient supress the release of SVP. This discrepancy between the reduction of HDV RNA and HBsAg in serum is likely a result of a direct antiviral activity of REP 2139 against HDV, potentially due to direct interaction with HDV antigen (Shamur et al, 2017), which occurs in the nucleus and/or cytoplasm. The delivery of NAPs to these subcellular compartments is known to be efficient. SVP assembly and release begins in the intermediate compartment located between the endoplasmic reticulum and Golgi apparatus (Patient et al, 2009). The delivery of REP 2139 to this compartment may be suboptimal in some patients.

The analysis of pre-S/S ORF of HBV DNA performed in this study indicate that at least HBV genotypes A, C and D are not affecting response to REP 2139-Ca and suggest a one or more host factors may be potentially mediating the ability of REP 2139 to block assembly and/or release of SVP. The analysis of HBsAg variants and host factors that could be potentially involved in the mechanism of action of REP

2139 need to be explored in future clinical studies in order to identify the patients who will respond to REP 2139 therapy.

5.2. Analysis of quasispecies within the major hydrophilic region (MHR)

Quasispecies (QS) are heterogeneous, simultaneously present variants of a virus within the same host. The HBV QS are caused by mutations of the viral genome during replication, since the required reverse transcriptase, in contrast to most DNA polymerases, has no proofreading activity. Besides, the immune system response and antiviral therapy may contribute to the selection of QS. Therefore, these minor viral subpopulations may have virological and clinical relevance, especially by emerging of immune escape variants, or in the context of resistance testing, if they carry important drug mutations.

In contrary to the direct (Sanger) sequencing where just one predominant viral population can be sequenced, by next generation sequencing (NGS) different QS, even minor subpopulations, that are present in one sample can be sequenced. Using NGS in this study we could confirm all differences to reference sequences we obtained by direct sequencing, with exception of three variations (R78Q and V184A in patient 9, and W196L in patient 12), which may be contributed to the analytical error. In accordance with previous findings (Lindström et al, 2004; Solmone et al, 2009), all aa differences to reference sequences observed in NGS at the frequency > 20% could be confirmed by direct sequencing, with the exception of two variants. F170S that was observed at the frequency of 23% and 22%, at the first and the second time point in patient 3, respectively. M103I was observed at the frequency of 33% at the second time point in patient 6.

Among variants observed just in NGS (table 9), three of them were observed within “a” determinant: T126I in patient 5, D144E in patient 9 and G145R in patient 6, at the frequency of 1.43%, 3.46% and 6.71%, respectively. Even though these mutations may have a significant impact on HBsAg detection by routine diagnostic assays, these minor QS populations in the coexistence with the wild type have no practical influence on detection of HBsAg by routine diagnostic assays. However, these mutations may affect quantification of HBsAg. In addition, all three mutations were present only at the first or the second time point, where the levels of HBsAg were high. These mutations were not found at the third time point or during the follow up, where HBsAg was low.

We observed fewer QS in non-responders compared to responders, with mostly unchanged QS complexity during REP 2139-Ca therapy. The reduction of QS in responders during REP 2139-Ca therapy may indicate selective targeting of certain viral QS during therapy, causing profound declines in HBsAg and HBV DNA. However, we have observed no specific mutation pattern among the non-responders

versus responders to the REP 2139-Ca therapy that may explain the lack of response. Nevertheless, we observed fewer aa differences to reference sequences in non-responders compared to responders, which is in accordance with the results obtained by direct sequencing.

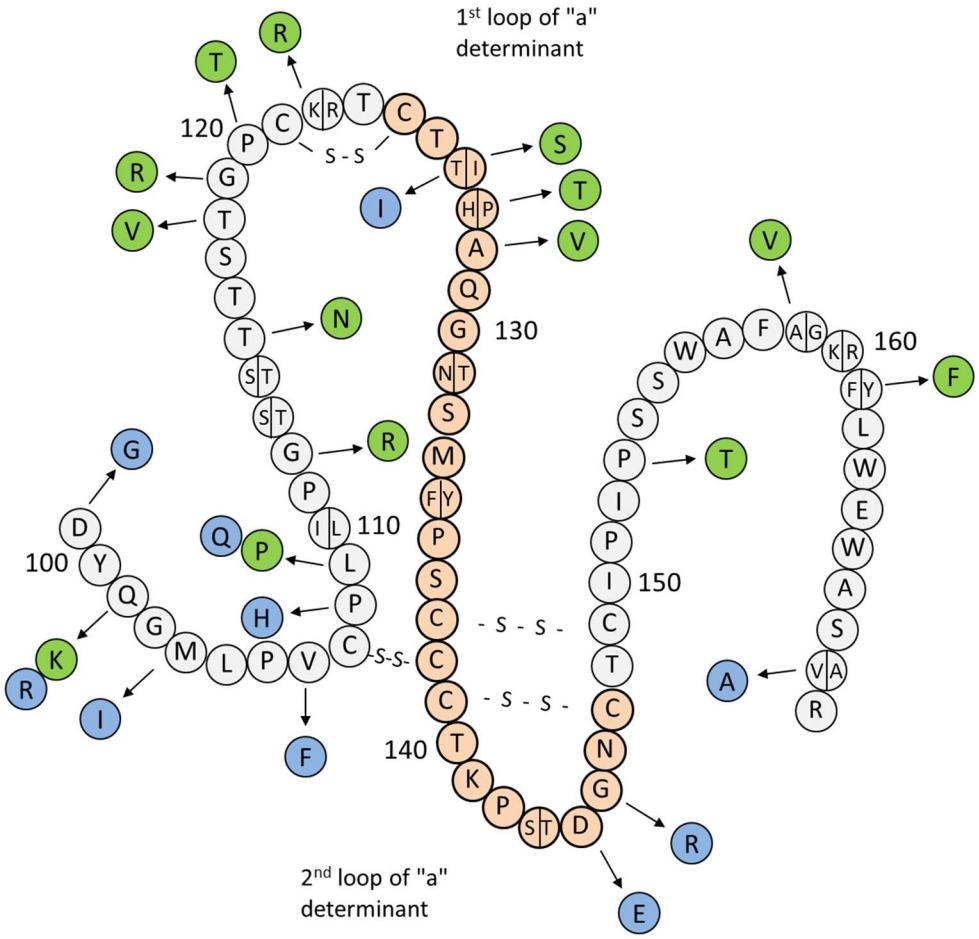


Figure 18. Major hydrophilic region (MHR) of HBsAg and amino acid differences to reference sequences observed in REP 102 study. This figure shows the location of all amino acid differences to genotype specific reference sequences observed within the MHR in the REP 102 study regardless of whether the mutation occurs in one or more patients. The "a" determinant (124-147 aa) is marked with red circles. Divided circles represent genotype-related variants. Green circles represent amino acid differences to reference sequences observed by direct sequencing, while blue circles represent amino acid differences to reference sequences observed just by NGS. Adapted from Locarnini and Yuen, 2010; Zhu et al, 2016 and Mijočević et al, 2019.

5.3. Analysis of the overlapping RT-polymerase region

The goal of the analysis of the overlapping RT-polymerase region of HBV DNA was to investigate whether mutations in the pre-S/S ORF result in mutations in the RT-polymerase region of P ORF that cause resistance to NUCs, which are currently the only approved direct antiviral therapy for chronic hepatitis B. The analysis was limited on N-terminal two thirds of RT-polymerase region of P ORF (aa 1-234) that overlaps pre-S/S ORF.

There was no discernible difference in aa sequences between responders and non-responders. With exception of three variants, R18S in patient 6, T128N in patient 7 and Q215P in patient 8, there were no mutations that occurred during REP 2139-Ca therapy. A single mutation relevant for resistance of HBV to NUCs, M204I, was observed only at the second time point in patient 12. This mutation is according to literature the most frequently resistance mutation found in treatment naïve patients (Choi et al, 2018) and is associated with Lamivudine, Entecavir and Telbivudine resistance (Tenney et al, 2009; Yano et al, 2015).

5.4. Investigation of direct interference of REP 2139-Ca with diagnostic assays

An additional goal of this study was to investigate whether the REP 2139-Ca used in this clinical study interferes with any of standard assays used in the diagnostic of HBV. Additionally, we investigated interference of REP 2139-Ca with diagnostic assays used in diagnostic of HDV infection, in order to validate the results obtained in additional studies with HBV/HDV coinfected patients. Analytical interference can be defined as “the effect of a substance present in the sample that alters the correct value of the result, usually expressed as concentration or activity, for an analyte” (Kroll and Elin, 1994). Testing of direct interference of REP 2139-Ca with 13 diagnostic assays for diagnosis and follow up of HBV and HDV infection was investigated. Because of the consequences of incorrect results, interference testing of REP 2139-Ca with HBsAg assays, HBV DNA PCR and HDV RNA PCR was of great importance.

No significant interference of any concentration of REP 2139-Ca (0.1 – 150 µg/ml) was observed in any tested serological assay for diagnosis and follow up of HBV infection. A statistically significant lower extinction in anti-HDV assay in samples with the lowest concentration of REP 2319-Ca (0.1 µg/ml) was observed. In this competitively assay this result means more positive result and likely appeared due to an analytic variability and have no diagnostic relevance.

A significantly higher values compared to control sample in HBV DNA PCR 1 (low viral load) in samples with the lowest concentration of REP 2139-Ca (0.1 µg/ml) was observed. These interference has not been seen in samples with the higher concentrations of REP 2139-Ca (1 – 150 µg/ml) and most likely appeared due to an analytic variability and should not be considered as relevant interference. In addition, no significant interference of any concentration of REP 2139-Ca (0.1 – 150 µg/ml) was observed in HBV DNA PCR 2 (high viral load).

Significant interference has been observed in samples with higher concentrations of REP 2139-Ca in all HDV RNA PCRs except RoboGene® PCR. A strong interference has been observed in NGI HDV RNA PCR (low and high viral load) which is the only assay that used phenol-chloroform based RNA extraction method. All other PCR assays used standard silica column or magnetic bead based RNA or parallel RNA/DNA extraction. However, except Eurobioplex HDV RNA PCR 2 (high viral load) there was no significant interference of the lowest concentration of REP 2139-Ca (0.1 µg/ml) with any tested HDV RNA PCR. This concentration is substantially above of serum concentration which can be expected in patient sera one week after receiving a 500 mg dose of REP 2139-Ca after 26 weeks of treatment (Roehl et al, 2017). Significantly lower signal in Eurobioplex HDV RNA PCR 2 (high viral load) was not observed in Eurobioplex HDV RNA PCR 1 (low viral load) which should be more sensitive to possible interference. In addition, this interference has not been seen in both samples with 1 µg/ml REP 2319-Ca (low and high viral load) and should not be considered as diagnostic relevant.

The interference study has shown no clinically relevant interference of REP 2139-Ca with any tested diagnostics assay. Nevertheless, because of the strong interference that was seen in NGI HDV RNA PCR, it is not recommended using the phenol-chloroform RNA extraction method for patients on therapy with REP 2139-Ca and other NAPs. Phenol-chloroform based RNA extraction may have advantages comparing to standard silica column based extraction, as it has a higher yield of RNA, however, significant contaminants including phenol, guanidine, chloroform, and salt can remain in samples and may have an inhibitory effect on the RT PCR (Deng et al, 2005; Toni et al, 2018). In RNA extraction methods with phenol-chloroform also small oligonucleotides (including NAPs) may be not efficiently removed, and retained oligonucleotides may interfere with the RT PCR. Furthermore, the interference of higher concentration of REP 2139-Ca with HDV RNA PCRs seen in this study may also be present in other RNA PCRs. Therefore, the blood sampling for diagnostic procedures in patients who are treated with NAPs should be done before drug administration or postpone at least 24 hours after administration of a drug.

Moreover, as in the REP 102 study observed, the patients on REP 2139-Ca therapy become first HBsAg negative, followed by reduction of HBV DNA. This has to be considered in monitoring of patients in the

routine virological diagnostic of chronic hepatitis B. Patients on efficient NUCs therapy have commonly undetectable HBV DNA, but HBsAg remains for a long time or lifelong positive due to transcription and translation from cccDNA and integrated HBV sequences.

6. Summary

Hepatitis B virus infection is one of the major global health problems. About 292 million people have an active hepatitis B virus infection worldwide, of which approximately 887000 die each year from complications as cirrhosis and hepatocellular carcinoma. Nucleos(t)ide analogues (NUCs), as first-line drugs for therapy of chronic hepatitis B, act as inhibitors of reverse transcriptase and are efficient in reducing of HBV DNA to undetectable levels, but rates of reduction of HBsAg concentration are very low and long-term or lifelong treatment is needed. Although the reduction of HBV DNA improves liver function and decelerates disease progression, a risk to develop hepatocellular carcinoma is still present.

Recent phase II clinical study with the lead nucleic acid polymer compound REP 2139 in patients with chronic HBV infection showed a multilog reduction or loss of HBsAg after 40 weeks of treatment. This HBsAg clearance is associated with seroconversion to anti-HBs, loss of HBeAg and seroconversion to anti-HBe, and reduction of HBV DNA, indicating a functional cure of chronic HBV infection. Nevertheless, a fraction of patients showed only minor or no reduction of HBsAg and HBV DNA during REP 2139 therapy. The reasons for this non-responsiveness have not yet been elucidated.

We investigated whether the failure of response to REP 2139-Ca therapy observed in 3 of 12 patients in the REP 102 study was related to differences in the amino acid sequences of pre-S/S ORF present in non-responders versus responders. We analysed sequences of the whole pre-S/S ORF of the HBV genome obtained by direct sequencing and sequences of the extended major hydrophilic region obtained by next generation sequencing (NGS). We also investigated whether the reductions of HBsAg concentration in patients' sera observed in the REP 102 study were false, caused by a failure in detection by diagnostic assays due to the presence of mutations within the "a" determinant. In addition, we investigated whether the REP 2139-Ca used in this clinical study interferes with any of standard assays used in the diagnostic of HBV and HDV infection. This is of importance to validate the results obtained in the REP 102 study and subsequent studies (REP 301, REP 401).

Based on the analysis of pre-S/S ORF of HBV DNA in the patients who received REP 2139-Ca therapy, there is no indication of mutations leading to structural or functional changes in HBV envelope proteins in non-responders compared to responders that may explain lack of response. This finding was also confirmed by NGS. Furthermore, analysing the overlapping RT-polymerase region of HBV DNA, no discernible difference in amino acid sequences between responders and non-responders was found.

Only single mutation relevant for resistance of HBV to NUCs (M204I) occurred in one single patient which however disappeared during the treatment.

In summary, the analysis of pre-S/S ORF of HBV DNA indicate that at least HBV genotypes A, C and D are not affecting response to REP 2139-Ca and also suggest a host factor may be potentially mediating the ability of REP 2139 to block assembly and/or release of subviral particles. The analysis of HBsAg variants and host factors that could be potentially involved in the mechanism of action of REP 2139 need to be explored in future clinical studies in order to predict the patients who will respond to REP 2139 therapy.

Mutations which may have significant influence on the sensitivity of diagnostic assays used for the detection of HBsAg were found neither in responders nor in non-responders. The observed mutations have been described in previous studies using various commercial used HBsAg assays, including the HBsAg assay used for testing of patients' samples in the REP 102 study. Some mutations were observed just by NGS at the very low frequency and have no practical importance for detection of HBsAg by routine diagnostic assays.

In summary, this investigation has shown no clinically relevant direct interference of REP 2139-Ca with any tested serological or molecular diagnostics assay. The interference of some HDV RNA PCRs with higher concentrations of REP 2139-Ca was not observed in samples with the lowest concentration corresponding to the serum concentration of REP 2139-Ca in patients. Nevertheless, the blood sampling for diagnostic procedures in patients who are treated with NAPs should be done before drug administration or postpone at least 24 hours after administration of a drug. Furthermore, because of the strong interference that was seen in HDV RNA PCR, where the phenol-chloroform RNA extraction was used, it is not recommended using this RNA extraction method for patients on therapy with REP 2139-Ca or other NAPs.

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