Sequencing of serum hepatitis B virus (HBV) RNA as a novel method for the genome analysis of HBV

Sequenzierung der Hepatitis-B-Virus (HBV) RNA im Serum als neue Methode der Genomanalyse des HBV

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eingereicht von: Laura Schmalbrock, geboren am 29.04.1983 in München

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Betreuer:
Prof. Dr. med. Th. Berg
Dr. med. F. van Bömmel
Dr. med. S. Böhm

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Meinen Eltern

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II Abbreviations

%	per cent
®	‘registered trademark’
*	stop codon
x g	relative centrifugal force
A	alanine
aa	amino acid
ADV	adefovir
ag	antigen
ALT	alanine aminotransferase
anti-HBe	anti-hepatitis B envelope
anti-HBs	anti-hepatitis B surface
aqua dest	aqua destillata (distilled water)
BCP	basal core promoter
BHQ	black hole quencher
bp	base pairs
C	cysteine
°C	degree Celsius
cccDNA	covalently closed circular DNA
CP	crossing point
cp	copies
D	aspartic acid
DNA	deoxyribonucleic acid
ddNTP	dideoxynucleotide triphosphate
dNTP	deoxynucleotide triphosphate
DR	direct repeat sequence
DTT	dithiothreitol
E	glutamic acid
E. coli	*Escherichia coli*
EDTA	ethylendiaminetetraacetic acid
et al.	lat. ‘et alii’ (and others)

Enh	enhancer
ER	endoplasmatic reticulum
ETV	entecavir
F	forward
FAM	6-carboxyfluorescein
fl	full-length
FRET	fluorescence resonance energy transfer
g	grams
G	glycine
GT	genotype
h	hour(s)
H_2O	water
HBV	hepatitis B virus
HBcAg	hepatitis B virus core antigen
HBeAg	hepatitis B virus envelope antigen
HBsAg	hepatitis B virus surface antigen
HCC	hepatocellular carcinoma
I	isoleucine
INF	interferon
IU	international units
k	kilo
kb	kilo base
LAM	lamivudine
LdT	telbivudine
L	leucine
L	liter
LB	lysogeny broth
LOD	lower limit of detection
log_{10}	decadal logarithm
µ	micro (10^{-6})
m	milli (10^{-3})

M	methionine
M	molar (mol/L)
min	minute(s)
mg	milligrams
mRNA	messenger ribonucleic acid
n	nano (10^{-9})
N	asparagine
NA	nucleos(t)ide analogue
NaCL	sodium chloride
NaOH	sodium hydroxide
NGS	next generation sequencing
nm	nanometer
nt	nucleotide
ORF	open reading frame
p	*p* - value
P	polymerase protein
pat ID	patient identification
PCR	polymerase chain reaction
peg-INF-a	pegylated interferon alpha
pgRNA	pre-genomic RNA
pol	polymerase gene
poly(A)	polyadenylation
R	reverse
RACE	rapid amplification of cDNA ends
rc	relaxed circular
RNA	ribonucleic acid
rpm	rounds per minute
rt	reverse transciptase
RT buffer	Reverse Transcriptase buffer
qPCR	real-time polymerase chain reaction
s	surface

S	serine
sec	second(s)
SDS	sodium dodecyl sulfate
S.O.C.	super optimal broth with catabolite repression
T	threonine
TAMRA	tetramethylrhodamine
TBE	TRIS-borate-EDTA
TDF	tenofovir disoproxil fumarate
tr	truncated
TRIS	Tris(hydroxymethyl)aminomethane
U	units
UDPS	ultra-deep pyrosequencing
UV	ultraviolet
V	valine
Vox	*Volvox aureus*
Y	tyrosine
YMDD	tyrosine - methionine - aspartic acid - aspartic acid
WT	wild type

1 Introduction

Chronic infections with the hepatitis B virus (HBV) rank among the most frequent infectious diseases with an estimated number of 240 million infected people worldwide (Lozano 2012, WHO 2015). Every year, more than 650,000 people die of HBV related complications including liver cirrhosis and hepatocellular carcinoma (HCC). Nucleoside and nucleotide analog polymerase inhibitors, which are used for antiviral treatment in a majority of chronically HBV infected patients, reduce the risk of liver disease progression but do not represent a curative treatment approach. Treatment with nucleos(t)ide analogues (NAs) might thus be life-long and a safe monitoring of the treatment response and a probable disease progression is mandatory.

Sequencing of serum HBV DNA is a standard technique for the genotyping of the HBV genome in patients with chronic HBV infection at diagnosis and for the detection of potential resistant HBV variants during antiviral treatment. In most patients treated with potent NAs, the HBV DNA declines to undetectable levels within 12 to 24 months and sequence analysis becomes impossible. Interestingly, several studies reported that even after the decline of HBV DNA to undetectable levels during antiviral treatment, serum HBV RNA remains measurable in some patients. Based on this observation, we aimed at studying whether the sequencing of serum HBV RNA might represent a novel method to analyze the HBV genome when the HBV DNA had become unavailable under NA treatment. We further wanted to assess if this method might be applicable for the monitoring of the evolution of resistance associated HBV variants during long-term antiviral treatment, which occur in the reverse transcriptase (rt) region of the HBV polymerase (pol) gene, and which may also be associated with changes in the overlapping gene of the HBV surface (s) protein.

1.1 HBV

1.1.1 Classification

HBV belongs to the *Hepadnaviridae*, a family of small enveloped, double-stranded DNA viruses, which are hepatotropic and exhibit a species specificity. *Hepadnaviridae* are transmitted by bodily fluids and can cause acute and chronic infections in mammals (*Orthohepadnaviridae*) and birds (*Avihepadnaviridae*) (Lee 1997, Glebe 2013). A crucial step in the replication cycle of *Hepadnaviridae* is the reverse transcription of the viral RNA into DNA (Summers 1982). The viral RNA-dependent DNA polymerase lacks a proofreading activity. Therefore, nucleotide (nt) exchanges often occur during replication. As a result, the HBV pool in the infected hosts is composed of different HBV variants, the quasispecies (Burda 2001, Locarnini 2003, Kaya 2007). Based on a genomic homology of minimum 8% of these variants (Okamoto 1988), for HBV, 10 genotypes (A-J) have been distinguished so far. For the most common genotypes, the geographic distribution shows predominance for genotype A in Northern Europe and the USA, Genotype D in Middle and Southern Europe, genotypes B and C in Asia and genotype E in Africa (Kaya 2007, Zhang 2016).

1.1.2 HBV virion structure and genomic organization

HBV virions, the ‘Dane particles’ (Dane 1970), have a diameter of 42-44 nanometers (nm). They contain an outer envelope, which consists of lipoproteins and HBV surface proteins (HBsAg). The envelope encloses the nucleocapsid, which is composed of HBV core proteins (HBcAg) and covers the HBV DNA (Dryden 2006, Glebe 2013). The HBV DNA has a size of 3 to 3.3 kilo base pairs (kbp) and is partially double-stranded. The complete minus and the incomplete plus strand overlap at the 5’ end of the HBV genome (Figure 1). In HBV virions, the HBV DNA exists in a relaxed circular (rc) form. After the incorporation of the HBV DNA into the nucleus of the hosts’

hepatocytes, the plus strand is completed and the covalently closed circular DNA (cccDNA) is being formed. The cccDNA represents the matrices for the transcription of HBV messenger (m) RNAs and the pre-genomic (pg) RNA. The HBV genome organization is complex as it includes four overlapping open reading frames (ORFs) and encodes for seven proteins. The transcription of the HBV RNAs initiates at the core, preS1, preS2 and X promoters producing four mRNAs of 3.5, 2.4, 2.1 and 0.7 kb sizes, respectively (Seeger 2000, Ganem 2004, Glebe 2013, Tong 2016). All ORFs use one common polyadenylation (A) signal at the 3' end of the HBV genome (Figure 1) for the polyadenylation of the different RNA transcripts (Nassal 2015).

1.1.3 HBV proteins

The core promoter regulates the transcription of the 3.5 kb long pre-core mRNA and the pgRNA. From the pgRNA, the viral RNA-dependent DNA polymerase, the polymerase (P) protein, and the structural core proteins are translated. The core proteins form the nucleocapsid. Detected in serum samples, the presence of anti-HBc antibodies proves a past infection with HBV.

The P protein catalyzes the reverse transcription of the pgRNA into HBV DNA during the viral replication cycle (Glebe 2013, Tong 2016). It is composed of three catalytic domains - the terminal protein, the rt and the RNaseH - and a highly variable spacer region (Radziwill 1990, Tong 2016). As described in the following chapters, resistance mutations to antiviral treatment are located in the functional important domain of the rt region. The polymerase ORF spans almost the entire HBV genome and completely overlaps with the preS/S ORF (Figure 1). Therefore, mutations in both ORFs can cause mutual nucleotide exchanges (Torresi 2002, Locarnini 2010).

The preCore/Core ORF encodes the pre-core protein, which is translated from the pre-core mRNA. The pre-core is the only post-translational processed HBV protein. After the translation, the envelope (e) antigen (HBeAg) is cleaved from the

pre-core protein and secreted from the hepatocytes. The function of the HBeAg for the viral replication is still elusive, but it presumably exhibits immunomodulatory effects and contributes to the chronification of HBV infection (Milich 2003, Chen 2004). HBeAg is considered to be a surrogate serum marker for the activity of the viral replication. The discrimination of patients with positive or negative serum HBeAg has important clinical implications for the treatment and management of HBV infections (EASL 2012).

Three HBsAgs of different sizes are translated from the 2.4 and 2.1 kb long HBV mRNAs. The 2.4 kb mRNA encodes the large HBs protein and the 2.1 kb mRNA the middle and small HBs proteins. All surface proteins share a common C-terminal domain (Figure 1). The surface proteins are in a distinct proportion part of the viral envelope. Their function is the modulation of the virus entry to the hepatocytes as well as the virion assembly and release (Bruss 1991, Glebe 2013). HBsAg is a serological marker for the presence of HBV infection and its persistence for at least 6 months is defining for a chronic HBV infection (Nguyen 2009, EASL 2012). HBsAg triggers the anti-HBsAg production and is a component of the HBV vaccine. Several immune escape mutations and further s gene variants have been identified and were previously shown to reduce anti-HBs binding properties (Tong 2016).

The X ORF encodes the HBx protein, which is translated from the 0.7 kb long HBx mRNA. The HBx protein supposedly has a regulatory function within the viral replication (Bouchard 2004, Belloni 2009) and maintenance of the infection (Lucifora 2011). It further might play a role in HCC development by interacting with cell proliferation enhancing promoters (Kekulé 1993, Bouchard 2004, Geng 2015).

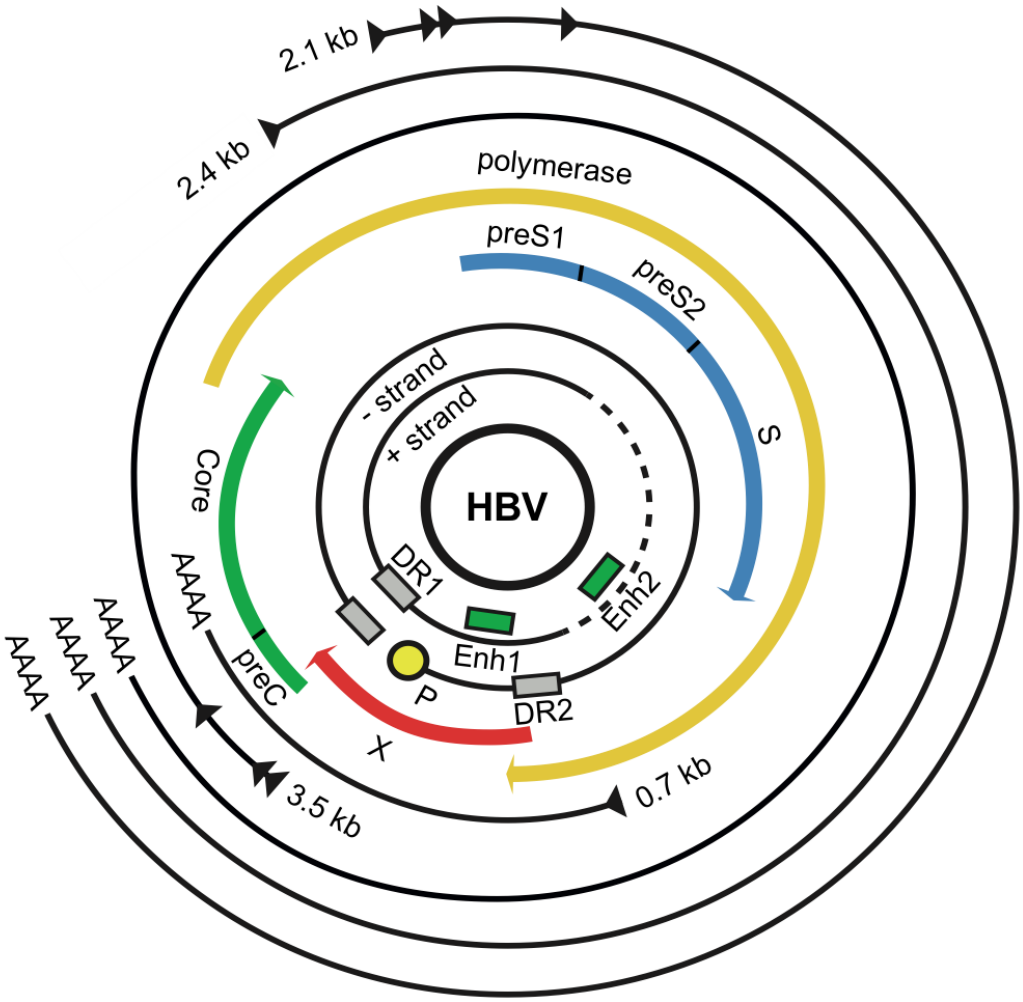


Figure 1: Organization of the HBV genome

The HBV DNA is a circular, partially double-stranded DNA molecule with 3 to 3.3 kbp in length. The complete minus (-) and incomplete plus (+) strands (black inner circles) overlap at the 3' end. The HBV genome is organized in four ORFs: polymerase (yellow), preS1/preS2/S (blue), preC/Core (green) and X (red). From the cccDNA, four sub-genomic mRNAs of 3.5, 2.4, 2.1, and 0.7 kb sizes and the 3.5 kb long pgRNA are transcribed (outer black circles). All mRNAs share a common poly(A) (AAAA) signal at the 3' end. Enh = Enhancer; DR = direct repeat sequence; P = polymerase protein. (Adopted from Gish et al. 2015)

1.1.4 HBV replication cycle

After the endosomal incorporation of the HBV virions into the hepatocytes, the HBV nucleocapsid is released into the cytoplasma and translocated to the nucleus (Figure 2). Here, the cccDNA is formed (Beck 2007, Nassal 2015) and persists in the form of mini-chromosomes during chronic infection (Newbold 1995, Bock 2001). The cccDNA serves as template for the transcription of the sub-genomic and genomic HBV RNAs (Beck 2007), which were described in chapters 1.1.2 and 1.1.3. For the generation of new virions during the viral replication, the pgRNA is packaged with the P protein into an immature core complex in the cytosol and there reverse transcribed into HBV DNA (Summers 1982). The reverse transcription of the pgRNA starts at the 5' end of the minus strand that is covalently linked to the viral polymerase at a stem-loop formation, the *epsilon* signal (Beck 2007, Glebe 2013, Nassal 2015). During the synthesis of the minus and subsequent plus stand, the RNA template is degraded by the RNAse H and the encapsidation is completed (Beck 2007, Glebe 2013). As illustrated in Figure 2, the newly generated HBV nucleocapsids are either enveloped and secreted from the hepatocytes, representing the infective HBV virions, or re-translocated into the nucleus, where they provide templates for the sustainment of the cccDNA pool (Urban 2010, Glebe 2013, Tong 2016).

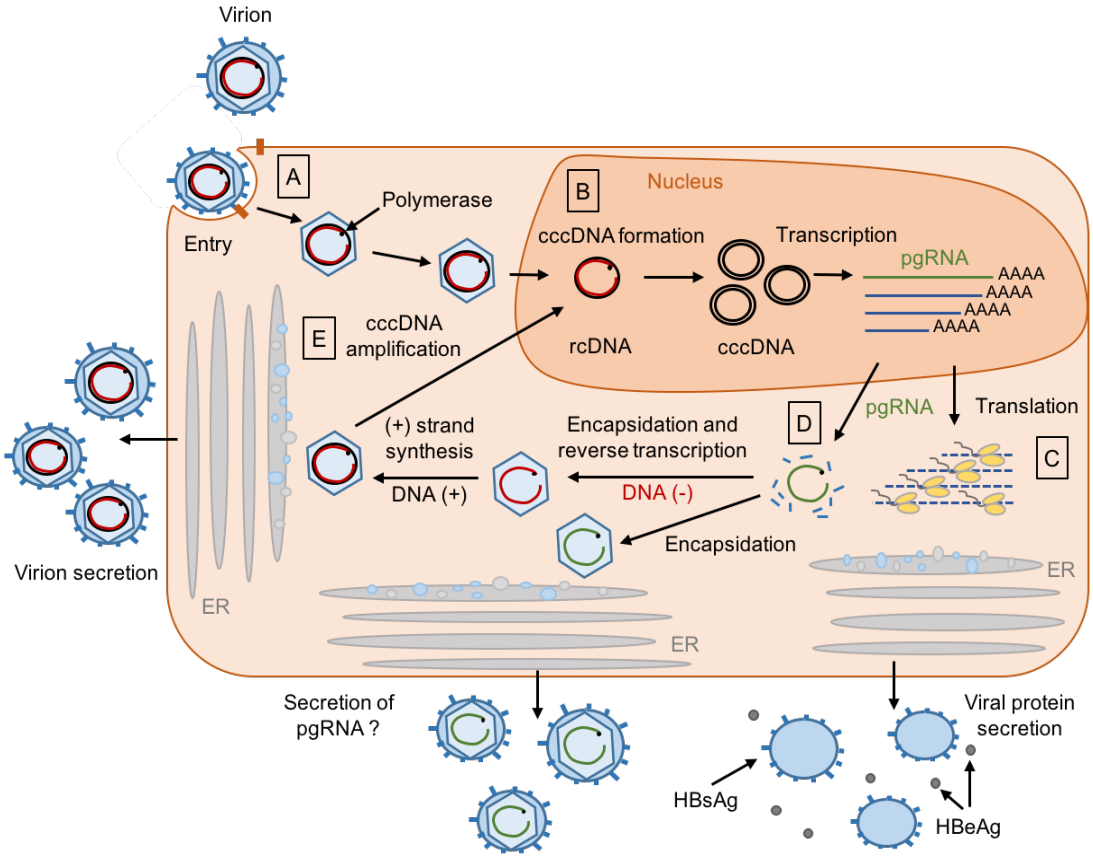


Figure 2: HBV replication cycle in the hepatocytes

A) After the entry of the HBV virions (upper left), the nucleocapsids (pentagons) are translocated to the nucleus and the HBV rcDNA is released; B) In the nucleus, the cccDNA is formed and serves as template for the transcription of the sub-genomic mRNAs (blue) and the pgRNA (green); C) From the mRNAs, the viral surface and envelope proteins are translated and secreted (HBsAg and HBeAg); D) The pgRNA serves as template for the HBV DNA synthesis via reverse transcription. In the cytosol, the minus (-) and plus (+) strands are synthesized during the encapsidation. It has been proposed that pgRNA might further be encapsidated and secreted in virion like particles without prior reverse transcription (Wang 2015); E) The HBV DNA containing nucleocapsids either are enveloped in the endoplasmatic reticulum (ER) and secreted or re-translocated to the nucleus to sustain the cccDNA pool. (Modified after Zoulim et al. 2009)

1.2 Chronic HBV infection

1.2.1 Epidemiology

Worldwide, around 240 million people are carriers of HBsAg with the highest prevalence in the endemic regions of the sub-Saharan Africa and Asia (WHO 2015). In the Western countries, the implementation of the HBV vaccination significantly reduced HBV infection rates. In the German population, the prevalence of HBsAg carriers was estimated to be below 1% in the year 2014 (RKI 2015). About 5% of the infected adults and up to 90% of the infected children develop a chronic HBV infection. HBV promotes the development of liver fibrosis, cirrhosis and HCC, which are the major complications of chronic HBV infection and occur in 20 - 30% of all adult cases (WHO 2015). Compared to HBsAg negative individuals, the lifetime risk of HBsAg carriers to develop HCC is rated with a 15- to 20- fold increase (El-Serag 2012). It is estimated, that about 650,000 deaths per year worldwide relate to the long-term consequences of chronic HBV infection (RKI 2015, WHO 2015).

1.2.2 Natural course of chronic HBV infection

The natural history of chronic HBV infection is divided into five phases, which do not necessarily occur in sequential order (McMahon 2009, EASL 2012, Gish 2015). The ‘immune tolerant phase’ is characterized by a high viral replication activity with measurable HBeAg and high serum HBV DNA levels. The aminotransferases are commonly not elevated in this state of infection, because HBV itself is not considered cytopathic (Chisari 1995) and liver damage caused by the immunological response is low (EASL 2012, Gish 2015). The phase of ‘immune clearance’ is characterized by ineffective viral clearance and persistent necroinflammation that is indicated by a decrease of the serum HBV DNA levels, increased aminotransferases and a progress of liver fibrosis (Ganem 2004, Gish 2015). Most chronic HBV infected patients remain HBeAg positive. However, in some patients HBeAg negativity caused by the loss of

HBeAg and preCore/Core mutations is observed (Chu 2003), resulting in the 'HBeAg negative chronic HBV infection'. In the 'non-replicative' phase, the HBV DNA levels decrease to low levels (<2,000 IU/mL). The normalization of the aminotransferases further reflects the immunological control of the infection in this state (EASL 2012). Only in a minority of patients, HBsAg loss and seroconversion to anti-HBsAg, the 'HBsAg negative/occult chronic HBV infection', occurs (EASL 2012). Serum HBV DNA is low or undetectable in this state of infection but remains measurable in liver cells and reactivation of the HBV infection is possible (EASL 2012, Gish 2015).

1.3 Treatment of chronic HBV infection with nucleos(t)ide analogues

1.3.1 Nucleos(t)ide analogues

Besides the immunomodulatory drugs interferon (INF) and pegylated INF alpha (peg-INF-a), five NAs are approved for the antiviral treatment of chronic HBV infection. NAs are commonly well-tolerated and applied for treatment in the majority of patients. Pharmacologically, polymerase inhibitors are divided into two structural classes, the nucleoside analogues lamivudine (LAM), telbivudine (LdT) and entecavir (ETV) and the nucleotide analogues adefovir (ADV) and tenofovir (TDF) (EASL 2012). NAs compete with the natural nucleotide substrates and lead to the termination of the HBV DNA chain elongation when incorporated into the nascent strand. By blocking the HBV RNA- dependent DNA polymerase in the cytosol, NAs inhibit the reverse transcription of the HBV RNA into HBV DNA and thus the viral replication (Zoulim 2009, DeClercq 2010, Menéndez-Arias 2014).

1.3.2 Treatment goals

The major goal in the treatment of chronic HBV infection is to prevent the progression of the disease to liver fibrosis, cirrhosis and HCC with its related complications and risk of death (EASL 2012, Terrault 2016). HBV DNA levels > 10^4

copies (cp)/mL were identified as independent risk factor for the progression of liver disease and associated with a 3- fold higher risk of HCC development in a large Taiwanese study cohort (Chen 2006). This points out the importance to achieve a virological response to antiviral treatment, which is defined by undetectable HBV DNA measured with sensitive polymerase chain reaction (PCR) assays (for definition of terms see table 1). The measurement of serum HBV DNA levels with PCR is recommended at first diagnosis and every 3 to 6 months during follow-up, because serum HBV DNA level elevation normally precedes an increase of the transaminases, which indicate the damage of liver cells. A re-increase of serum HBV DNA (virological breakthrough) during antiviral treatment with NAs is further an indicator for a possible resistance development of HBV. The normalization of the alanine aminotransferases (ALT) during treatment (biochemical response) signals the regression of liver damage. HBeAg and HBsAg seroconversion (serological response) occur commonly late during NA treatment and indicate a sustained response to treatment (EASL 2012).

Table 1: Definition of terms for treatment response during antiviral treatment according to the EASL practical guidelines 2012

Term	Definition
Primary non-response	< 1 $\log_{10}$ IU/mL decrease of serum HBV DNA levels from baseline at 3 months of therapy
Virological response	Undetectable serum HBV DNA (sensitive PCR assay)
Partial virological response	Decrease of > $\log_{10}$ IU/mL but detectable serum HBV DNA after at least 6 months of treatment
Virological breakthrough	Increase of serum HBV DNA > $\log_{10}$ IU/mL compared to the lowest value of serum HBV DNA during treatment
Biochemical response	Normalization of ALT levels
Serological response	HBeAg loss and seroconversion to anti-HBe in HBeAg positive patients; HBsAg loss and development of anti-HBs in all patients

IU/mL = International Unit/milliliter; PCR = polymerase chain reaction; ALT = alanine aminotransferase. (Adopted from EASL 2012)

1.3.3 Response to nucleos(t)ide analogue treatment

Treatment with NAs reduces the risks of HBV related complications, but cannot eradicate the chronic HBV infection due to the persistence of HBV cccDNA in the nucleus of infected cells (Figure 2; Werle-Lapostolle 2004, EASL 2012). Therefore, and because of the low rates of serologic response, which allows a cessation of NA treatment, life-long treatment is necessary for most patients. The currently licensed NAs show strong differences in the antiviral potency and the risk of resistance development. After 12 months of antiviral treatment, response rates in HBeAg positive (Figure 3A) and HBeAg negative patients (Figure 3B) are high, especially for the recommended first-line NAs ETV and TDF. In HBeAg positive patients, anti-HBeAg seroconversion was found in 12 to 22% of patients after 1 year of treatment (Figure 3C). However, serologic responses are rare, and especially HBsAg loss or seroconversion occur only in few HBeAg positive (Figure 3D) and almost never in HBeAg negative patients (data not shown) (EASL 2012).

Because of the good response rates and the low rates of resistance to antiviral therapy (see chapter 1.4), ETV and TDF are recommended first-line for the treatment of chronic HBV infection (EASL 2012, Terrault 2016). Long-term follow-up studies reported that serum HBV DNA levels were undetectable in 94% of HBeAg positive patients after 5 years of ETV treatment (Chang 2010). For TDF treatment, long-term response over 8 years was demonstrated in >99% of patients (Marcellin 2014), also in special populations like patients with high viral loads at baseline (Gordon 2013) and patients with failure to prior antiviral therapy with NAs (Fung 2014, van Bömmel 2010). HBeAg seroconversion and HBsAg loss were observed in 31% and 5% of patients after 2 years of ETV treatment, respectively (Chang 2010). After 7 years of TDF treatment, HBeAg and HBsAg loss occurred in 54.5 % and 11.8% of patients, respectively (Buti 2014).

Future cornerstones for the treatment of chronic HBV infection are the establishment of predictive markers for a sustained treatment response and the implementation of monitoring regimens, which might allow a safe discontinuation of NA treatment.

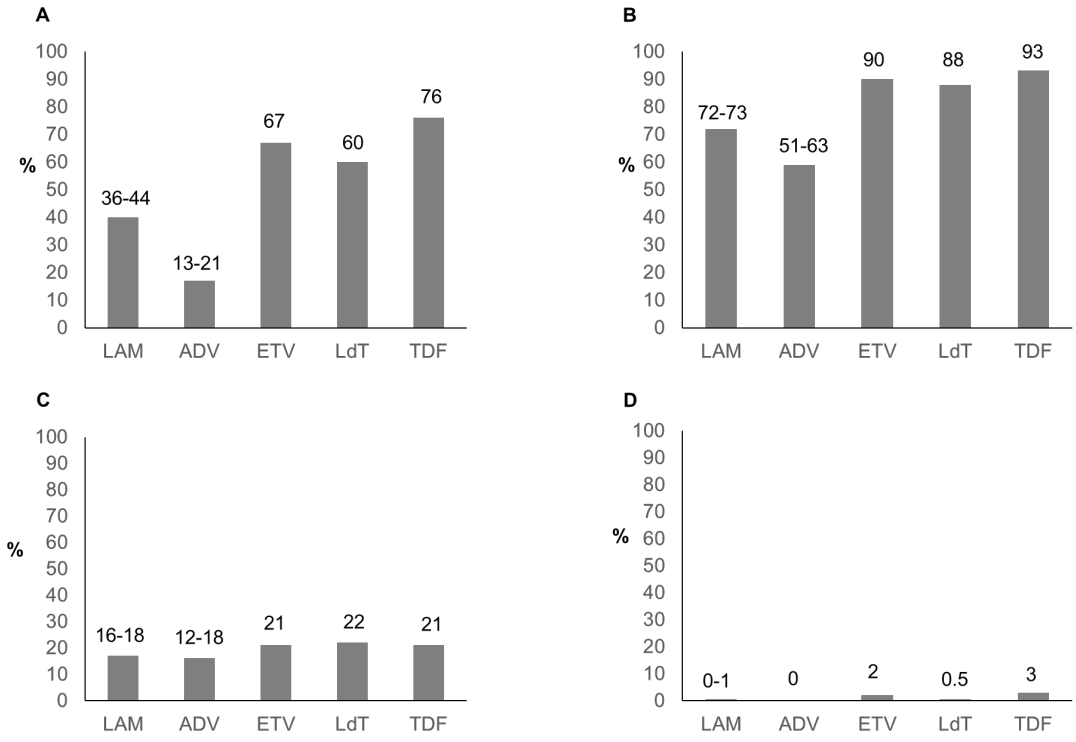


Figure 3: Response to 12 months treatment with nucleos(t)ide analogues

Percentages (%) of patients (numbers above bars) with a suppression of serum HBV DNA to levels < 60 – 80 IU/mL in HBeAg positive (A) and HBeAg negative (B) patients; C) Rates of HBeAg seroconversion in HBeAg positive patients during antiviral treatment; D) Rates of HBsAg loss in HBeAg positive patients after 12 months of treatment with NAs. Of note, the data shown in this figure were not derived from head-to-head studies. (Adopted from EASL guidelines 2012)

1.3.4 Treatment with nucleos(t)ide analogues and liver disease

A sustained virological response during NA treatment was shown to prevent from liver disease progression (Vlachogiannakos 2013, Wu 2014, Papatheodoridis 2015). In cirrhotic and non-cirrhotic patients, the risk of HCC development was

reduced by about 30% and 80%, respectively (Papatheodoridis 2015). Progressed liver disease in the state of fibrosis and cirrhosis, which is considered as a pre-malignant condition, was in part reversed by NA treatment (Marcellin 2013). After achievement of a sustained virological response during NA treatment, the risk for HCC development however remains increased and the monitoring for HCC development is mandatory in all patients. A study by Cho *et al.* compared the cumulative incidence rates between patients with inactive chronic HBV infection - defined by HBeAg negativity and undetectable serum HBV DNA levels - and patients with active HBV infection treated with NAs (Cho 2014). In non-cirrhotic patients, the patient group with complete virological response during antiviral treatment had a significantly higher risk for HCC development compared to the group of inactive carriers, with cumulative incidence rates of 2.3% versus 0.3% at year 1 and 7.2% versus 0.8% after 5 years (Cho 2014). The authors assumed that a more effective and intact immune response with lower necroinflammation in the patient group with inactive HBV infection might explain this observation. It also seems conceivable that genomic alterations and chromosomal instabilities, which occur during early stages of the infection before the beginning of antiviral treatment, might predispose to malignant transformation. Furthermore, the oncogenic potential of several HBV variants in the s gene, which were found to emerge during NA treatment, was discussed in the context of HCC development (Lai 2008, Lai 2009, Lee 2012, Huang 2014, Li 2016) and will be further described in chapter 1.4.3.

1.4 Evolution of HBV variants during antiviral treatment

1.4.1 HBV resistance mutations in the pol gene

The HBV RNA- dependent DNA polymerase is error prone due to a leak of proofreading activity. Therefore, all HBV variants that are replication competent can arise in an infected individual, and these HBV variants represent the HBV

quasispecies (Burda 2001, Locarnini 2003, Kaya 2007). Antiviral treatment with NAs puts selection pressure on the HBV, which promotes the selection of different HBV variants with resistance to the applied drug, a major complication during NA treatment. For the first generation polymerase inhibitor LAM, the resistant variants rtM204V/I, which are located in the highly conserved YMDD motif in the rt region of the pol gene, were identified and extensively studied. The mutated HBV variants replicate less efficiently. Therefore, they commonly co-occur with compensatory mutations at positions rtL80V/I, rtL180M and rtV173L, which are increasing the replication fitness of the virus (Pallier 2006, Zoulim 2009, Warner 2014). Resistance to ADV treatment was associated with the rtN236T mutation and resistance to LAM and ADV with the rtA181T/V substitutions. For EVT resistance, multiple mutations on the base of pre-existing resistance against LAM (rtL180M + rtM204V/I) are required, namely mutations at the positions rtI169T, rtV173L and rtM250V or at codons rtT184G and rtS202I/G (Zoulim 2009, Warner 2014). For TDF, resistance to a mutation at position 194 (rtA194T) was described in a HIV-HBV co-infected patient (Sheldon 2005) but could not be confirmed *in vitro* (Delaney 2006) and did not occur *in vivo* after 8 years of follow-up (Marcellin 2014). The susceptibility to TDF thus might be reduced in the presence of the rtN236T mutation (van Bömmel 2010), which is discussed in the following chapter. Table 2 gives an overview of known resistance mutations to the five approved polymerase inhibitors according to the EASL practical guidelines of 2012 (EASL 2012). Figure 4 shows the location of these mutations in the rt region of the pol gene.

Table 2: Primary resistance mutations to treatment with nucleos(t)ide analogues located in the rt region of the HBV pol gene

Resistance mutations, rt region	LAM	LdT	ETV	ADV	TDF
M204I	R	R	I	I	S
M204V	R	S	I	I	S
L180M + M204V	R	R	I	I	S
N236T	S	S	S	R	I
A181T/V	I	S	S	R	S
L180M + M204V/I ± I169T ± V173L ± M250V	R	R	R	S	S
L180M + M204V/I ± T184G ± S202I/G	R	R	R	S	S

S = sensitive; I = intermediate; R = resistant; LAM = lamivudine, LdT = telbivudine, ETV = entecavir, ADV = adefovir, TDF = tenpfovir. (Modified after EASL 2012)

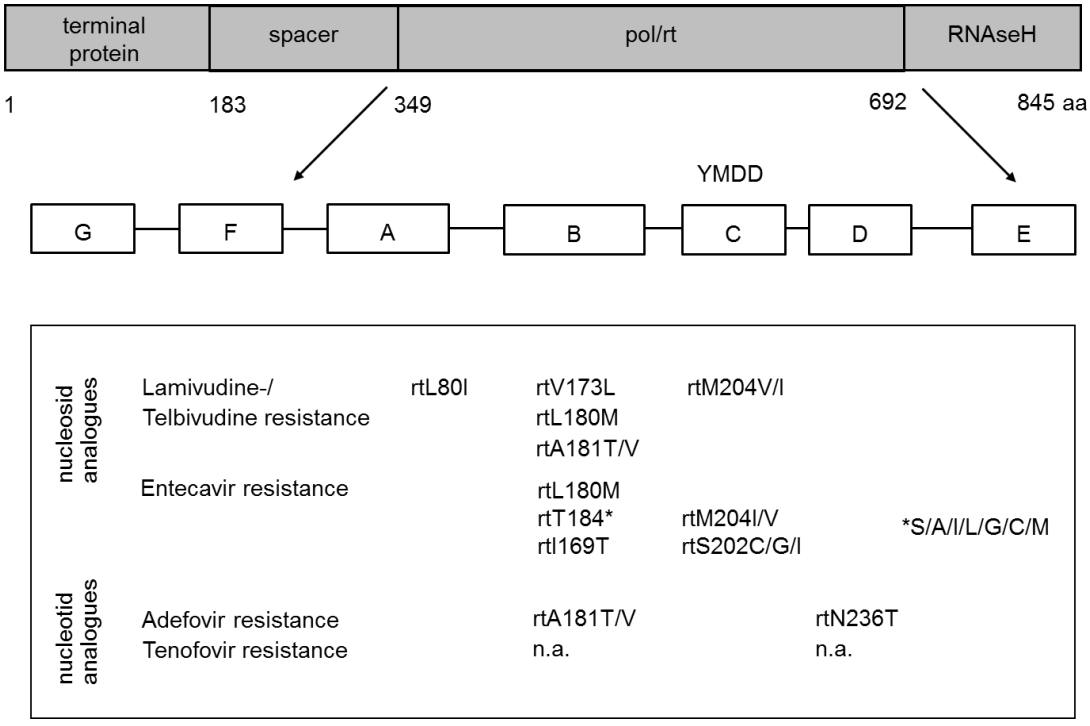


Figure 4: Location of resistance mutations to nucleos(t)ides analogues in the HBV pol gene

The grey bar is a scheme of the P protein consisting of the terminal protein, the spacer, the pol gene with the included rt region and the RNAseH. The numbers indicate the starting and end points of the amino acids (aa). The pol is composed of 7 subdomains (A to G). In the subdomain C, the highly conserved YMDD motif is located. Below, all confirmed resistance mutations to NA treatment are listed. (Adopted from Zoulim et al. 2009)

1.4.2 Resistance rates to treatment with nucleos(t)ide analogues

Because resistance to antiviral treatment with the first generation NAs LAM, LdT and ADV occurs frequently, the polymerase inhibitors ETV and TDF with higher resistance barriers are recommended for first-line treatment (Zoulim 2009, WHO 2015, EASL 2012). After 5 years of LAM and ADV treatment, HBV resistance was observed in around 70% and 29% of patients, respectively (EASL 2012, Zoulim 2009). For LdT, 17% of patients developed resistance to treatment after 2 years (EASL 2012, Zoulim 2009). In contrast, ETV resistance was found only in around 1.2% after 5 years of follow-up in treatment naïve patients (Tenney 2009). For TDF treatment, no resistance mutations have been reported after 8 years in a long-term follow-up study (Marcellin 2014). An overview of the cumulative resistance rates to NA treatment is given in table 3.

In NA pre-treated patients, cross-resistance can occur, which is defined as resistance of HBV to a NA without prior exposure (Zoulim 2009). The presence of HBV mutations at positions rtM204V/I ± rtL180M, which confer resistance to LAM and LdT, led to increased resistance rates up to 51% after 5 years of ETV treatment (Zoulim 2009, EASL 2012). During ADV treatment, resistance mutations at positions rtN236T and rtA181V/T occurred more frequently in patients harboring LAM resistant HBV variants and were estimated with up to 20% after 1 year (Lee 2006, EASL 2012). No cross-resistance of TDF to LAM resistant HBV variants was found so far (van Bömmel 2006, Zoulim 2009), which makes the treatment with TDF the first choice in patients who had developed resistance to LAM treatment (EASL 2012, Terrault 2016). In the presence of the ADV resistant HBV variant rtN236T, a 3- to 4- fold decrease of the susceptibility to TDF treatment was found *in vitro* (Delaney 2006). Also *in vivo*, van Bömmel *et al.* reported that the probability to achieve undetectable serum HBV DNA levels was around 50% lower in patients with ADV resistant HBV variants during

TDF treatment (median duration 23 months). A virologic breakthrough that indicated resistance to TDF treatment however was not observed (van Bömmel 2010). In concordance, Kitrinos *et al.* found no resistance to TDF treatment after 288 weeks of observation irrespective of the presence of HBV resistance variants (Kitrinos 2014). The authors further reported that during long-term treatment with TDF, a suppression of serum HBV DNA to undetectable levels was achieved in all patients after week 240 (Kitrinos 2014).

Table 3: Cumulative rates of HBV resistance to antiviral treatment with nucleos(t)ide analogues

Nucleos(t)ide analogue	1st year (%)	2nd year (%)	3rd year (%)	4th year (%)	5th year (%)
LAM	24	38	49	67	70
LdT (HBeAg positive)	4	17	-	-	-
LdT (HBeAg negative)	2.7	8.6	-	-	-
ADV (naïve)	0	3	11	18	29
ADV (LAM resistant)	Up to 20%				
ETV (naïve)	0.2	0.5	1.2	1.2	1.2
ETV (LAM resistant)	6	15	36	46	51
TDF	0	0	0	0	0

LAM = lamivudine, LdT = telbivudine, ADV = adefovir, ETV = entecavir, TDF = tenofovir. (Adopted from EASL 2012 and Zoulim 2009)

1.4.3 HBV variants in the s gene

HBsAg variants naturally occur during the HBV replication as part of the genetic HBV diversity. Because the HBV genome is organized in overlapping ORFs, mutations in the pol and preS/S gene can cause mutual nucleotide exchanges, which also can become selected during NA treatment (Table 4) (Torresi 2002). In the context of acquired LAM resistance, the HBsAg variants sE164D/rtV173L, sI195M/rt204V and sW196S/rtM204I were associated with a reduced binding to anti-HBs antibodies as a result of changes in the protein structure (Torresi 2002) and a lower infectivity *in vitro*

(Billioud 2012). The relevance of these HBV variants in the context of a possible immune-escape to HBV vaccination - as assumed for exchanges in the 'a' determinant in the HBsAg (Pollicino 2014) - is still under discussion. Besides point mutations, several HBsAg stop (*) mutations were described to arise in the natural course of the infection (Kazim 2006) and also during NA treatment. Some of them were suspected to promote the progression of liver disease and HCC pathogenesis (Lai 2008, Lai 2009, Lee 2012, Huang 2014, Li 2016), which will be discussed below.

The LAM and ADV resistant variant rtA181T causes a stop codon at position s172 (sW172*) that results in a truncation of 55 amino acids at the C terminus of the HBsAg (Warner 2008). Warner *et al.* demonstrated that the secretion of the truncated protein was defective, led to the retention of the concurrent expressed wild type (WT) and reduced quantitative HBsAg and HBV DNA levels (Warner 2008). The authors assumed that a virological breakthrough and possible disease progression could thus remain undetected (Warner 2008). Indeed, Lai *et al.* found the rtA181T/sW172* mutation in a treatment naïve HBsAg carrier who had developed HCC and was HBsAg negative at the time point of HCC diagnosis (Lai 2008). The authors related the mutation to the transactivation of cell growth enhancing promoters and thus assumed a possible contribution to the pathogenesis of HCC (Lai 2008). Further preS/S stop mutations with and without mutual nucleotide exchange in the pol gene (s15*, sL21*, sW163*, sL216*, rtG165S/sW156*, rtV191I/sW182* and rtM204I/W196*) were described in serum and tissue samples of chronic HBV patients who had developed HCC, which suggests a potential role in the pathogenesis (Lai 2009, Lee 2012, Huang 2014). Lee *et al.* found the rtV191I/sW182* variant with a frequency of 26.5% in serum samples of chronic HBV infected patients in a Korean cohort (n = 292). The occurrence was significantly higher in patients with HCC or liver cirrhosis compared to those with chronic HBV infection or carrier status only (31.8%

versus 17.2%, *p* = .01; Lee 2012). Interestingly, Lai *et al.* reported that the detected HBsAg stop mutations s15*, sL21*, sW163*, L216*, rtG165S/sW156*, rtA181T/sW172* and rtM204I/sW196* in their study were exclusively found in 7 out of 8 HCC patients who had received LAM treatment but not in the treatment naïve control group with HCC (Lai 2009). Also in a large meta-analysis, the rates of HCC development were significantly higher in patients with LAM resistance (Papatheodoridis 2010), which - taken together - suggests the possibility of the selection of potential oncogenic variants during antiviral treatment with NAs. Experimentally, the sL21*, rtG165S/sW156* and rtA181T/sW172* mutations were related to the transactivation of oncogene promoters and an increased tumorigenicity could be demonstrated in nude mice (Lai 2008, Lai 2009). For the rtV191I/sW182* mutation, *in vitro* data showed that the translated truncated protein affected the G1/S checkpoint through down-regulation of p53 and p21, thus enhancing cell growth in NIH-3T3 cells (Lee 2012). A better understanding of the mechanisms of HCC development that occurs during antiviral treatment and of the evolution of HBV variants, which might contribute to liver disease progression, is an important issue to improve surveillance strategies for patients at increased risk.

Table 4: HBV resistance mutations in the rt region and corresponding exchanges in the s gene and HBsAg stop mutations

drug	HBV variant, rt region	HBV variant, s gene
LAM	M204V	I195M
LAM	M204I	W196S/L/*
LAM	L180M	no change
LAM	V173L	E164D
LAM	L80V/I	no change
LAM + ADV	A181T	W172L/F/*
LAM + ADV	A181V	L173F
ADV	N236T	no change
	no change	216*
	V191I	W182*
	S78T	C69*
	V208	W199L/*

LAM = lamivudine, ADV = adefovir. (Adopted from Zoulim 2009)

1.5 HBV RNA in serum of chronically infected patients

1.5.1 HBV RNA molecules

As described in chapter 1.1.2, the polyadenylation of all transcribed HBV mRNAs matures at a common poly(A) signal at the 3' ends. In liver biopsies of patients with HBV related HCC, Hilger *et al.* located the poly(A) signal for the full-length (fl) transcripts to position nt1789 (TATAAA) upstream of the HBx gene (Hilger 1991). Furthermore, an internal poly(A) signal at position nt1661 (CATAAA) was identified, which led to a truncation at the 3' end and the production of truncated (tr) RNAs (Hilger 1991, Schutz 1996). In serum samples of patients with chronic HBV infection, both HBV mRNAs were detectable with different methods that used primers binding at the poly(A) tail at the 3' end of the HBV genome (Su 2001, van Bömmel 2015). Only recently, two studies further discriminated the serum HBV RNA molecules with specific primers that targeted the 5' end of the HBV genome and showed that the detected HBV RNA was mostly pre-genomic (Jansen 2015, Wang 2016).

1.5.2 HBV RNA packaging and release

With the observation that HBV RNA was measurable in serum samples of chronically HBV infected patients, the question arised, in which condition the HBV RNA is packaged and released from the hepatocytes. Former studies of the HBV replication cycle did not include HBV RNA packaging in their replication model because the formation of the HBV envelope during the virion generation was linked to the HBV DNA synthesis and the subsequent degradation of the pgRNA (Wai 1996, Gerelsaikhan 1996). Studies that are more recent however demonstrated that also HBV DNA free nucleocapsids were enveloped and secreted, suggesting that alternative ways of virion generation might be present during the HBV replication (Ning 2011, Luckenbaugh 2015). For the HBV RNA, Jansen *et al.* showed that HBV RNA remained stably detectable in serum samples after RNAse treatment, which

suggested its persistence in an enveloped form (Jansen 2015). The authors further showed that HBV RNA was not detectable after anti-HBs immunoprecipitation of patient plasma but increased 100- fold after removal of the envelope and subsequent anti-HBc precipitation. This demonstrated that the HBV RNA, similar to HVB DNA, might be packaged into nucleocapsids and enveloped (Jansen 2015, Wang 2016). In line, Rokuhara *et al.* and Wang *et al.* found serum HBV RNA in the same sucrose density gradient fraction as HBV DNA and HBcAg (Rokuhara 2006, Wang 2016). Because HBV RNA transcription from the cccDNA is not affected by antiviral treatment with polymerase inhibitors (Doong 1991), it was suggested that high loads of serum HBV RNA was a result of the disrupted DNA elongation within NA treatment (Huang 2010). However, serum HBV RNA has also been detectable before the initiation of NA treatment and corresponded well to HBV DNA loads (Rokuhara 2006, Jansen 2015). Assuming that serum HBV RNA is composed of encapsidated, enveloped and pgRNA containing virions, Wang *et al.* proposed a model in which the formation of pgRNA virions represented an additional process in the HBV replication cycle (Wang 2016). It seems conceivable that pgRNA virions become the dominant fraction in serum when the synthesis of HBV DNA virions is inhibited during antiviral treatment with NAs, which was demonstrated in transgenic mice treated with ETV (Wang 2016). This also suits the observation, that serum HBV RNA remained persistently measurable at high levels after the decline of HBV DNA to undetectable levels (Rokuhara 2006, Hatakeyama 2007, Huang 2010, van Bömmel 2015, Jansen 2015, Wang 2016). In concordance, treatment with INF, which also inhibits the transcription of the HBV RNA from the HBV cccDNA (Belloni 2012), induced a more pronounced decrease of serum HBV RNA compared to NA treatment (Huang 2010, Jansen 2015).

1.5.3 HBV RNA as serum marker

Independently of other established serum markers, lower serum HBV RNA levels at the beginning of NA treatment associated with shorter intervals to virological response, which underlines that serum HBV RNA levels might directly reflect the viral replication activity (Huang 2015, Jansen 2015). Correspondently, one study reported that high serum HBV RNA levels at the beginning of LAM treatment increased the risk for the emergence of HBV resistant variants (Hatakeyama 2007), which might be due to higher mutational rates within a more active viral replication. A more rapid decline of serum HBV RNA was observed in HBeAg negative patients compared to HBeAg positive patients (van Bömmel 2015, Jansen 2015) and predicted HBeAg seroconversion during NA treatment (van Bömmel 2015). Wang *et al.* further showed, that among 33 patients with undetectable serum HBV DNA during NA treatment, a viral rebound at week 24 after the cessation of NA treatment occurred significantly more often in patients with detectable serum HBV RNA at the time point of discontinuation (Wang 2016).

1.6 Aim of the study

The genome of HBV can be assessed by sequence analysis of HBV DNA in serum. However, this approach is limited by the fast decrease of HBV DNA during treatment with potent NAs. HBV RNA, in contrast, was shown to persist in serum of some HBV infected individuals receiving NA treatment. There is further evidence that HBV RNA in serum is mainly composed of HBV pgRNA.

The aim of this work was to investigate whether sequencing of serum HBV RNA might allow assessing the HBV genome at time points, at which serum HBV DNA became undetectable during NA treatment. First, we aimed at establishing a method for the sequencing of serum HBV RNA. We analyzed serum HBV DNA derived sequences at the beginning of TDF treatment in patients who had a high

probability to harbor known HBV resistance variants and s gene variants due to prior antiviral treatment. We considered those HBV variants as an individual genetic ‘footprint’ of the underlying cccDNA quasispecies in every patient. We analyzed, for each patient, if these individual quasispecies patterns were present on serum HBV RNA basis, which would be a proof for the common origin of serum HBV DNA and serum HBV RNA.

We then applied this method to observe the evolution of HBV variants during treatment with TDF, a potent inhibitor of the HBV replication. We wanted to find out, if changes in the rt region of HBV, which were previously associated with resistance development to NA treatment, might be lost or acquired during long-term treatment with TDF. We further analyzed the occurrence and courses of HBV s gene variants during TDF treatment, which were discussed in the context of HCC development.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

Agar-agar	SERVA, Heidelberg, Germany
Agarose	SERVA, Heidelberg, Germany
Ampicillin	Roth, Karlsruhe, Germany
Aqua ad injectabilia	Braun, Melsungen, Germany
Boric acid	Sigma-Aldrich, Steinheim, Germany
Bromophenol blue	Sigma-Aldrich, Steinheim, Germany
EDTA	SERVA, Heidelberg, Germany
Ethanol	J.T. Baker, Deventer, NL
Ethidium bromide	Roth, Karlsruhe, Germany
Isopropanol	J.T. Baker, Deventer, NL
Kanamycin	Roth, Karlsruhe, Germany
NaCl	Roth, Karlsruhe, Germany
NaOH	Roth, Karlsruhe, Germany
RNase-free water	Qiagen, Venlo, NL
SDS	SERVA, Heidelberg, Germany
Sodium acetate buffer solution 3M	Sigma-Aldrich, Steinheim, Germany
Sucrose	Roth, Karlsruhe, Germany
TRIS base	Roth, Karlsruhe, Germany
Tryptone	Roth, Karlsruhe, Germany
X-gal	Roth, Karlsruhe, Germany
Yeast extract	Roth, Karlsruhe, Germany

2.1.2 Devices

Alpha Imager Hp	Alpha Innotech, Kasendorf, Germany
Biofuge pico (Rotor: HERAEUS #3325)	Heraeus Instruments, Hanau, Germany
Biometra TI 1	Biometra, Göttingen, Germany
Plate centrifuge 5430R (Rotor: A-2-MTP)	Eppendorf, Hamburg, Germany
Centrifuge 5417R (Rotor: F45-30-11)	Eppendorf, Hamburg, Germany

CO_2 incubator	Heraeus Instruments, Hanau, Germany
C25KC incubator shaker	New Brunswick Scientific, Edison, NJ, USA
Electrophoresis power supply EPS 3501XL	Amersham Pharmacia Biotech, Uppsala, Sweden
Pipettes	Eppendorf, Hamburg, Germany
Pipettes	Gilson, Middleton, WI, USA
Gel chambers	PeqLab Biotechnology, Erlangen, Germany
Herasafe KS18	Thermo Scientific, Schwerte, Germany
Herasafe KS12	Thermo Scientific, Schwerte, Germany
Microwave Pro II 1400	Panasonic, Osaka, Japan
Mono - mixer	Sarstedt, Nümbrecht, Germany
MS 1 minishaker	IKA, Staufen im Breisgau, Germany
Pipetus - akku	Hirschmann, Eberstadt, Germany
Thermomixer compact	Eppendorf, Hamburg, Germany

2.1.3 Laboratory materials

Adhesive clear PCR seal	Biozym Scientific, Oldendorf, Germany
Biosphere filter tips	Sarstedt, Nümbrecht, Germany
Eppendorf tubes	Eppendorf, Hamburg, Germany
Falcon tubes (10 mL, 50 mL)	Greiner, Frickenhausen, Germany
Gloves Peha soft nitril	Hartmann, Heidenheim, Germany
Light Cycler 480 Multiwell plates, white	Roche Diagnostics, Mannheim, Germany
PCR 96 multiwell plates	Sarstedt, Nümbrecht, Germany
Serum pipette tips	Sarstedt, Nümbrecht, Germany
Single tubes (200 µL)	Greiner bio-one, Kremsmünster, Austria

2.1.4 Cycler

Flex Cycler	Analytik Jena, Jena, Germany
Gradient Cycler PTC-200	MJ Research, Reno, NV, USA

LightCycler® 480 II	Roche Diagnostics, Mannheim, Germany
Thermal Cycler 2720	Applied Biosystems, Darmstadt, Germany

2.1.5 Kits

Kit	Manufacturer	Catalogue number
Big Dye v3.1 Terminator Cycle Sequencing Kit	Applied Biosystems, Darmstadt, Germany	4336921
Big Dye v3.1 Terminator Cycle Sequencing Kit	Applied Biosystems, Darmstadt, Germany	4336921
DNA ladder 100 bp	Promega, Madison, WI, USA	G8291
DNeasy Blood and Tissue Kit	Qiagen, Hilden, Germany	69506
dNTP Mix, PCR grade	Invitrogen, Carlsbad, CA, USA	18427-013
LightCycler® 480 Probes Master	Roche Diagnostics, Mannheim, Germany	04887301001
QIAfilter Plasmid Midi and Maxi Kit	Qiagen, Venlo, NL	12262L
RNase OUT Ribonuclease Inhibitor	Invitrogen, Carlsbad, CA, USA	10777-019
SuperScript® III Reverse Transcriptase	Invitrogen, Carlsbad, CA, USA	18080044
TOPO® TA Cloning® Kit TOP10 (pCR®II-TOPO®)	Invitrogen, Carlsbad, CA, USA	K4600-01
Taq PCR Master Mix Kit (1,000 U)	Qiagen, Hilden, Germany	201445
Zymo Clean Gel DNA Recovery Kit	Zymo Research, Freiburg, Germany	D4002

2.1.6 Buffers and solutions

10 x TBE buffer
108 g TRIS base 55 g boric acid 40 mL EDTA solution (0.5 M, pH 8.0) ad 1 L aqua dest
EDTA solution (0.5 M, pH 8.0)
7.3 g EDTA 2.40 g NaOH ad 200 mL aqua dest
DNA loading dye
4.0 g sucrose 400 µL EDTA solution (0.5 M, pH 8.0) 500 µL 10% SDS 5 mg bromophenol blue ad 10 mL aqua dest
LB medium
5.0 g tryptone 2.5 g yeast extract 5.0 g NaCl ad 500 mL aqua dest
LB agar
5.0 g tryptone 2.5 g yeast extract 5.0 g NaCl 7.5 g agar-agar ad 500 mL aqua dest

2.1.7 Primers

TIB Molbiol (Berlin, Germany) provided all primers. The starting position is numbered from the Eco RI restriction site within the HBV genome.

Table 5: Sequencing primers

Primers	Sequences 5'→ 3'	Methods
B2808a-F	GCC TCA TTT TGT GGG TCA CCA TA	1st PCR
B2808b-F	GCC TCA TTT TGC GGG TCA CCA TA	1st PCR
B1417a-R	ACG TCC CGC GAA GGA TCC AGT TG	1st PCR
B1417b-R	ACG TCC CGC GCA GGA TCC AGT TG	1st PCR
B1056a-R	CAT TAA AGC AGG ATA ACC ACA TTG	1st PCR
B1056b-R	CAT TAA AGC AGG ATA TCC ACA TTG	1st PCR
B1056c-R	CAT TAA GGC AGG ATA ACC ACA TTG	1st PCR
B1056d-R	CAT TAA GGC AGG GTA ACC ACA TTG	1st PCR
B0840a-R	AGG GTT TAA ATG TAT ACC CAA AGA CA	2nd PCR; Sequencing
B0840b-R	AGG GTT TAA ATG TAT ACC CAG AGA CA	2nd PCR; Sequencing
B0840c-R	AGG GTT CAA ATG TAT ACC CAA AGA CA	2nd PCR; Sequencing
B660a-F	GTT TCT CCT GGC TCA GTT TAC TAG	1st / 2nd PCR; Sequencing
B660b-F	GTT TCT CTT GGC TCA GTT TAC TAG	1st / 2nd PCR; Sequencing
B1281a-R	GAG TTC CGC AGT ATG GAT CGG	2nd PCR; Sequencing
B3077a-F	TGG GGT GGA GCC CTC AGG CTC A	2nd PCR; Sequencing
B3077b-F	TGG GGT GGA GCC CTC AGG CAC A	2nd PCR; Sequencing
B0401a-R	ATA TGA TAA AAC GCC GCA GAC AC	1st / 2nd PCR; Sequencing
B0401b-R	AGA TGA TAA AAC GCC GCA GAC AC	1st / 2nd PCR; Sequencing
B0321a-F	CAA CCT CCA ATC ACT CAC CAA C	1st / 2nd PCR; Sequencing
B0321b-F	AAA TCT CCA GTC ACT CAC CAA C	1st / 2nd PCR; Sequencing
B0321c-F	CAA CCT CCA ATC ACT CAC CAA T	1st / 2nd PCR; Sequencing

Table 6: Primers for the quantification of serum HBV DNA

Primers	Sequences 5'→ 3'	Methods
s gene		
HBV-Taq1	CAA CCT CCA ATC ACT CAC CAA C	qPCR
HBV-Taq2	ATA TGA TAA AAC GCC GCA GAC AC	qPCR
B0349-Probe	FAM-TCC TCC AAY TTG TCC TGG YTA TCG CT-BHQ1	qPCR; probe
core		
HB-2256F	TGG TYT CTT TYG GAG TGT GGA T	qPCR
HB-2337R	GTY TCC GGA AGT GTT GAT ARG ATA GG	qPCR

HB-2279F-Probe	FAM-CGC ACT CCT CCH GCH TAT AGA CCA CCA A–BHQ1	qPCR; probe
Vox standard		
Vox-F	ACA ACA TGC TGT TTC CAC TGG A	qPCR
Vox-R	AAG GTC CCC GGC CTG AA	qPCR
Vox-Probe	FAM–TCC GGC GAC GGC AGC AGC–TAMRA	qPCR; probe

Table 7: Primers for the reverse transcription and quantification of serum HBV RNA

Primers	**Sequences 5'→ 3'**	**Methods**
HBV trRNA		
x-RACE short a	GAG ACT CGA CTC CAC AAC CA $(dT)_{17}$ GC TGG TGA ACA GAC	Reverse transcription
x-RACE short b	GAG ACT CGA CTC CAC AAC CA $(dT)_{17}$ GC TGG TGC GCA GAC	Reverse transcription
B1577a-F	GTG TGC ACT TCG CTT CAC CTC	qPCR
Anchored short	GAG ACT CGA CTC CAC AAC CA	qPCR
Probe B 1599a-FAM	FAM-CAC GTC GCA TGG AGA CCA CCG TGA ACG C–BHQ1	qPCR; probe
Probe B 1599b-FAM	FAM-CAC GTT GCA TGG AGA CCA CCG TGA ACG C–BHQ1	qPCR; probe
HBV flRNA		
3'RACE-long	ACC ACG CTA TCG CTA CTC AC $(dT)_{17}$ GW AGC TC	Reverse transcription
HBV-x-long	CAA CTT TTT CAC CTC TGC CTA	qPCR
Anchored long	ACC ACG CTA TCG CTA CTC AC	qPCR
HBV-x-long FAM	FAM-CAT GTC CYA CTG TTC AAG CCT CCA AG-BHQ1	qPCR; probe

Table 8: Standard plasmids used for the absolute quantification with qPCR

Plasmids	**Constructs**
s gene	pCRII-HBV-2808-1623
core	pCRII-HBV-1381-2951
HBV trRNA	pMA–T-HBV-1577-x-RACE-short-991 (nt1577 - nt1810); ID construct: 12ABEHLP, Life Technologies, Carlsbad, CA, USA
HBV flRNA	pCRII-HBV-1577-x-RACE-long-991 (nt1577-nt1935)
Vox	pCRII-volvox-F-R

2.1.8 Data analysis

- Analysis of HBV sequences: Chromas Lite 2.01, Technelysium Pty Ltd
- Alignment and analysis of HBV sequences: CLC Main Workbench Version 6.8.3, CLC bio
- Analysis of HBV sequences (Translation to aa sequences, PileUps): HUSAR (http://genome.inet.dkfz-heidelberg.de/husar/hs_home.html)
- Genotyping of HBV sequences: HepSEQ-Research Database (http://www.hepseq.org)
- Source for HBV reference genomes: National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nuccore)
- Analysis of HBV variants: HBV Seq tool of the HIV Drug Resistance Database, Stanford University (http//hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html)
- Analysis of the HBV deletion: BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi)
- Data analysis: Microsoft Excel (versions 2010 and 2013)
- Figures: Microsoft Power Point (versions 2010 and 2013)

2.2 Methods

2.2.1 Patient set and sample selection

We aimed at assessing the value of the sequencing of HBV DNA and HBV RNA as a method for the monitoring of the development of HBV variants during antiviral treatment. Therefore, we composed our study cohort of patients likely of having acquired HBV resistance during previous treatment with NAs. For this reason, a prerequisite for inclusion in this study was a previously confirmed viral breakthrough defined as re-increase of serum HBV DNA > 1 $\log_{10}$ as suggested by the current

treatment guideline (EASL 2012), which occurred during NA treatment that preceded TDF treatment. Furthermore, at least four consecutive serum samples stored at −20°C representing the beginning and time points around months 6, 12 and 24 of TDF treatment had to be available. To observe the evolution of HBV variants during long-term treatment with TDF also serum samples at later time points were included.

2.2.2 Extraction of nucleic acids from serum samples

Total nucleic acids were extracted from 200 µL serum using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and obtained in 100 µL elution buffer. The nucleic acid isolation was performed for each serum sample, a positive and a negative control. In addition, the Vox standard plasmid (Table 8), which served as internal control for the extraction efficacy, was measured for every sample. The isolated nucleic acids were either used immediately for further analysis or stored at -80°C.

2.2.3 Reverse transcription of HBV RNA

The reverse transcription of HBV RNA was performed using the SuperScript® III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). In a first step, 10 µL of the isolated nucleic acids were incubated at 65°C for 5 minutes in the presence of 2.5 µM rapid amplification of cDNA ends (RACE) primers for trRNA and flRNA each (Table 7) and 0.5 mM dNTPs and immediately transferred on ice. In a second step, RNase Inhibitor (2 U/µL), RT buffer, 0,1 M DTT (5 mM) and the reverse transcriptase (10 U/µL) were added to the reaction mix and incubated at 50°C for 60 minutes, followed by an inhibition step of 15 minutes at 70°C, and finally cooled down. The reverse transcription for each sample was carried out in single tubes on the Gradient Cycler PTC-200 (MJ Research, Reno, NV, USA). The reaction mix and cycling conditions are given in Tables 9 and 10, respectively. The cDNA was immediately used for further analysis or stored at -20°C.

Table 9: Reaction mix of the reverse transcription

Reagents	Volume (µL)	Final concentration
dNTP (10 mM)	1.0	0.5 mM
RACE primer short/long (25 µM)	2.0	2.5 µM
HBV nucleic acids	10.0	
5 x RT buffer	4.0	
DTT 0.1 M	1.0	5.0 mM
RNase inhibitor (40 U/µL)	1.0	2 U/µL
RT Super Script III (200 U/µL)	1.0	10 U/µL
Total volume	20.0	

Table 10: Conditions of the reverse transcription

Condition	Cycles	Temperature (°C)	Time (h:min:sec)
Preincubation	1	65	00:05:00
Cooling	forever	4	forever
Reverse transcription	1	50	60:00:00
Enzyme inactivation	1	70	00:15:00
Cooling	forever	4	forever

2.2.4 Quantification of serum HBV DNA and HBV RNA by real-time PCR

2.2.4.1 Real-time PCR

For the absolute quantification of the HBV DNA and reverse transcribed HBV RNA, in-house quantitative real-time PCRs (qPCRs) as described in the following chapters were performed. qPCR is a method to determine quantitatively the concentration of nucleic acids based on PCR. During the amplification, a fluorescent signal emitted by either unspecific fluorescent dyes or specific probes is detected at the respective wavelength (Arya 2005). In our study, we used TaqMan hydrolysis probes designed to detect the region of interest for the qPCR reaction. Hydrolysis probes consist of a reporter fluorophore at the 5’ end and a quencher at the 3’ end. The quencher molecule quenches the fluorescence signal, which is emitted by the

excited 5' end fluorophore through fluorescence resonance energy transfer (FRET). After annealing to a region located between the forward and reverse PCR amplification primers, the probes are cleaved by the 5'-3'exonuclease activity of the polymerase during the elongation (Holland 1991), and the reporter fluorophore is released. Thus, the fluorescence signal is no longer quenched by the quencher fluorophore and becomes detectable at a defined wavelength. Our probes were labelled with 6-carboxyfluorescein (FAM) at the 5' end and with tetramethylrhodamine (TAMRA) or black hole quencher (BHQ) at the 3' end (Table 7).

The qPCRs were all carried out on a Light Cycler® 480 II (Roche Diagnostics, Mannheim, Germany) system. The wavelengths of excitation and detection were 465 and 510 nm, respectively. For the absolute quantification of the HBV DNA and cDNA we used the second derivative maximum method and calculated the copy value for each reaction. Within this method, for each sample, the crossing point (CP) that represents the number of PCR cycles during the amplification, at which the fluorescence signal becomes detectable, is determined (Pfaffl 2001). The CP is then compared to a standard curve, which was calculated by serial dilutions of known nucleic acid concentrations of cloned standard plasmids (Table 8) ranging from 10^6 to 10^1 cp per reaction (Figure 5).

The determined HBV DNA and cDNA quantities represented the serum levels per reaction and were multiplied with the respective dilution factors of the transformation in the unit cp/mL serum as described below. Furthermore, for each assay the lower limit of detection (LOD) was previously determined. The LOD defines the lowest copy level, which is distinguishable from blank samples in 95% of all cases and is experimentally determined by measuring the quantities of serial dilution samples of known DNA and cDNA concentrations.

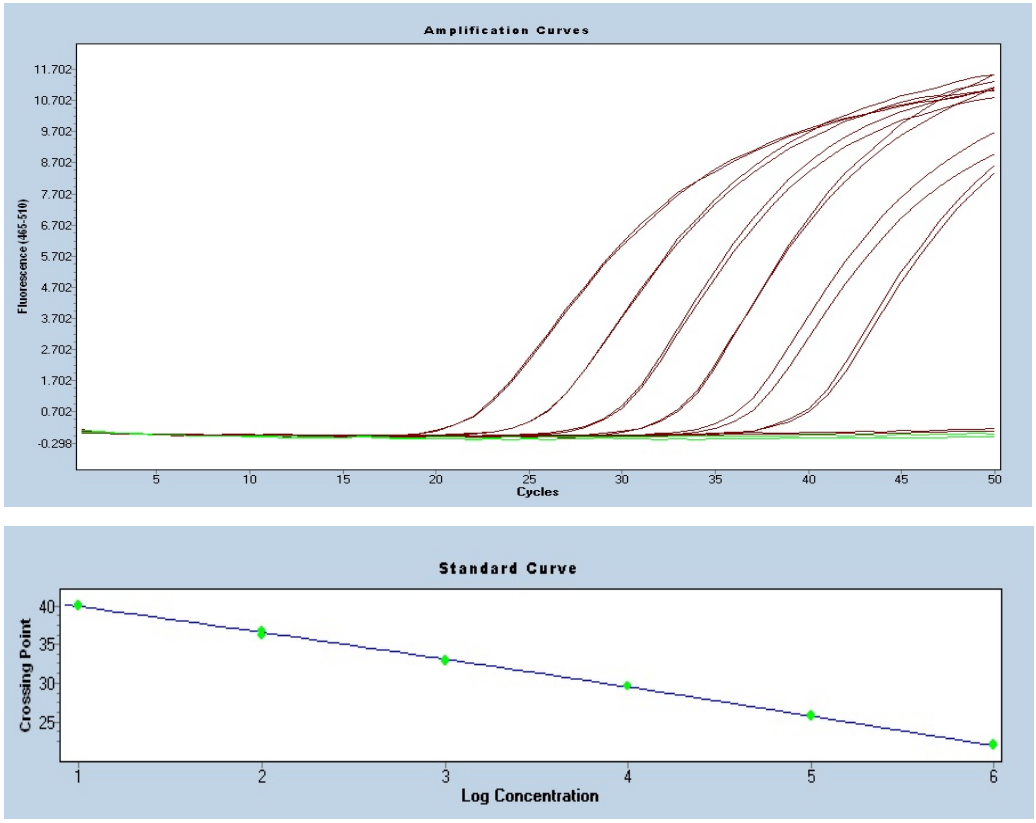


Figure 5: Standard curves of the HBV trRNA assay for the absolute quantification of HBV serum levels on a Light Cycler® 480 II

The amplification curves represent known concentrations of a cloned plasmid representing quantities between 10^6 and 10^1 cp per reaction. The standard curve is calculated based on the determined crossing points (CPs), which each represent the required PCR cycles for the detection of a fluorescence signal. It indicates the lineal correlation between the CP values and the decadal logarithm of the standard concentrations.

2.2.4.2 Quantification of serum HBV DNA

For the HBV DNA quantification, two regions of the HBV genome, the HBV s gene and the core region, were separately targeted by qPCR. In a third reaction, we determined the efficiency of the nucleic acids isolation by the quantification of the Vox plasmid. The reaction mixes consisted of 10 µL LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany), 300 nM forward and reverse primers and 150 nM TaqMan probes. 5 µL of the previous isolated HBV DNA were added to a total reaction volume of 20 µl. The reaction mix and cycling conditions for the quantitative

PCR on the LightCycler® 480 II (Roche Diagnostics, Mannheim, Germany) are given in tables 11 and 12, respectively.

Table 11: Quantification of the serum HBV DNA

Reagents	Volume (µL)	Final concentration
H_2O PCR-grade	3.5	
LightCycler® 480 Probes Master	10.0	
Forward primer 10 µM	0.6	300 nM
Reverse primer 10 µM	0.6	300 nM
Probe 10 µM	0.3	150 nM
Template	5.0	
Total volume	20.0	

Table 12: Cycling conditions of the qPCR on the Light Cycler® 480 II

Cycling step	Cycles	Temperature (°C)	Time (h:min:sec)
Activation	1	95	00:05:00
Amplification	50		
Denaturation		95	00:00:05
Annealing and elongation		60	00:00:20
Cooling	1	40	00:00:10

To obtain the unit cp/mL serum, the determined quantities of each reaction were multiplied by the factor 100. The multiplication factor was calculated considering the dilution factor of the HBV DNA extraction, which was 1:5 because 200 µL instead of 1 mL serum was used for the nucleic acid isolation, and the dilution factor of the qPCR reaction, which was 1:20 because of 100 µL eluted nucleic acids 5 µL were added to the reaction. For the HBV DNA assays, the determined LOD was 400 cp/mL serum. Values below 100 cp/mL serum were defined as negative. To include values between 100 and 400 cp/mL serum for further analysis, we regarded these values as positive and assigned them to a quantity of 250 cp/mL serum. We performed all

reactions in duplicates and applied the negative control from the extraction step for each system. A HBV DNA positive patient sample from the extraction step served as positive control.

2.2.4.3 Quantification of serum HBV trRNA and HBV flRNA

The quantification of the HBV fl and trRNAs was based on a recently described specific qPCR technique (van Bömmel 2015). As described in chapter 2.2.3, the HBV RNA was reversely transcribed into cDNA using RACE primers. RACE primers contain an oligo(dT) sequence that binds to poly(A) tails of RNAs (Frohmann 1988). To detect only the HBV specific RNA, the primers in our study further contained a short sequence complementary to the viral sequence that targeted the standard poly(A) (flRNA) and the internal poly(A) signal (trRNA) as described by Hilger *et al.* (Hilger 1991). Furthermore, the primers consisted of artificial anchored sequences, which were the target sequences of the primers used for the qPCR, which allowed a selective quantification of the reverse transcribed HBV RNA without the need of DNase digestion (Figure 6) (van Bömmel 2015).

The reaction mix of the qPCR for the HBV trRNA consisted of 10 µL LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany), 900 nM of the complementary forward and reverse primers and 75 nM TaqMan probe. To a final volume of 20 µL, 2 µL of the reverse transcribed RNA was added (Table 13). The reaction mix of the qPCR for the HBV flRNA consisted of 10 µL LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany), 500 nM of complementary forward and reverse primers and 150 nM TaqMan probe. 2 µL of the reverse transcribed RNA was added to a final volume of 20 µL (Table 14). The qPCRs for HBV trRNA and flRNA were carried out on the LightCycler® 480 II (Roche Diagnostics, Mannheim, Germany) system as described in Table 12.

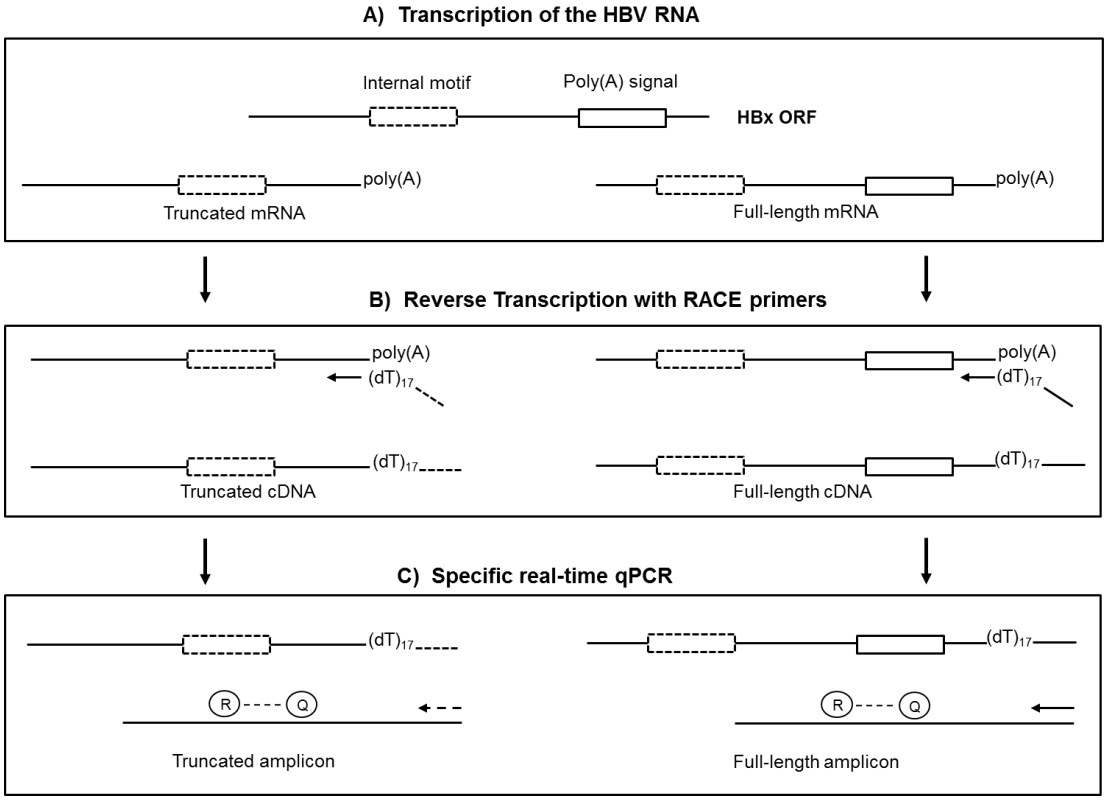


Figure 6: Principle of the specific qPCR for the quantification of serum HBV trRNA and HBV flRNA

The HBV full-length (fl) and truncated (tr) RNAs mature at the standard poly(A) signal and the internal signal upstream the HBx (A). During reverse transcription, oligo-(dT) RACE primers bind to the poly(A) tails at the 3' end with a specific complementary viral sequence for the detection of the HBV flRNA and trRNA (B). The reverse transcribed HBV RNAs contain an artificial anchored sequence (dashed line HBV trRNA, solid line HBV flRNA), which is targeted during the qPCR (C). (Adopted from Hilger et al 1991)

Table 13: Quantification of the serum HBV trRNA

Reagents	Volume (µL)	Final concentration
H_2O PCR-grade	4.25	
LightCycler® 480 Probes Master	10.0	
Primer anchored-short 10 µM	1.8	900 nM
Primer 1577 a-F 10 µM	1.8	900 nM
Probe 1599 ab-FAM 10 µM	0.15	75 nM
Template cDNA	2.0	
Total volume	20.0	

Table 14: Quantification of the serum HBV flRNA

Reagents	Volume (µL)	Final concentration
H_2O PCR-grade	5.7	
LightCycler® 480 Probes Master	10.0	
Primer anchored-long 10 µM	1.0	500 nM
Primer HBV-x-long 10 µM	1.0	500 nM
Probe HBV-x-long-FAM 10 µM	0.3	150 nM
Template cDNA	2.0	
Total volume	20.0	

The determined quantities for HBV trRNA and HBV flRNA per reaction were multiplied with the factor 500. This factor was calculated considering the dilution factors of the HBV DNA extraction (1:5) as described above, the reverse transcription, for which 10 µL of the nucleic acids eluted in 100 µL were applied (1:10) and the qPCR reaction, for which 2 µL cDNA was added to a volume of 20 µL reaction mix (1:10). The previously determined LODs were 5,500 and 3,000 cp/mL serum for the HBV trRNA and flRNA, respectively. We considered values below 500 cp/mL serum as negative in both assays. To include values below the LODs, for HBV flRNA quantities between 3,000 and 500 cp/mL serum were assigned to a level of 1,750 cp/mL serum and for HBV trRNA quantities between 5,500 and 500 cp/mL serum to a level of 3,000 cp/mL serum. All reactions were performed in duplicates and the respective negative controls applied. A HBV RNA positive patient sample served as positive control.

2.2.5 Sequencing of serum HBV DNA and HBV RNA

2.2.5.1 Primer design

Using semi-nested PCRs of three overlapping fragments, we amplified the entire rt region of 1032 nt size (position nt130 - nt1161) that is located within the HBV pol gene followed by Sanger sequencing of the purified PCR products. With this

sequencing strategy, we included the small s gene of 681 nt size (position nt155 – nt835) that overlaps with the rt region (Figure 7). We designed the primers to target conserved regions in the HBV genome and included the most common HBV genotypes. For variable positions, respective primer sequences were designed (Table 5) and added to the PCR reaction in an equimolar relation.

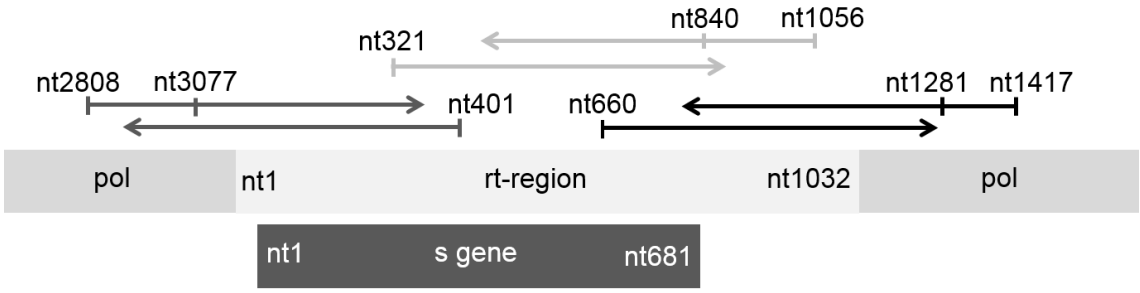


Figure 7: Location of the sequencing primers in the rt region of the HBV genome
The PCR was performed in 3 overlapping fragments of around 500 bp spanning the entire rt region (light grey) of the pol gene (grey) and the s gene (dark grey). Arrows indicate the direction of the elongation and numbers the first nt of the amplified region after the ECO RI restriction site of the HBV genome.

2.2.5.2 Amplification by PCR

The amplification of HBV DNA and cDNA was carried out by optimized PCR reactions with a semi-nested PCR to achieve higher yields of the PCR product. According to the determined quantitative copy levels by qPCR, HBV DNA was used as template for sequence analysis with serum levels between 500 to 1,000 cp/mL. In serum samples with HBV DNA < 500 cp/mL and detectable HBV RNA, the reversely transcribed HBV RNA was used as template for the amplification and subsequent sequencing. The semi-nested PCR consisted of two PCR rounds. In the 1st PCR, a reaction mix with a final volume of 25 µL containing 12,5 µL LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany) and 200 nM of forward and reverse

primers (Table 5) was prepared for the three overlapping fragments, each in single tubes or 96 well plates. 5 µL of isolated HBV DNA or 2 µL of the reverse transcribed HBV RNA were added to each reaction (Table 15). The amplification was carried out on a Gradient Cycler PTC-200 (MJ Research, Reno, NV, USA) or Thermal Cycler 2720 (Applied Biosystems, Darmstadt, Germany) for single tubes or plates, respectively. The cycling conditions are given in Table 16.

Table 15: Reaction mix of the 1st PCR for HBV DNA and cDNA amplification

Reagents	Volume (µL)	Final concentration
H_2O PCR-grade	6.5/9.5	
LightCycler® 480 Probes Master	12.5	
Forward primer 10 µM	0.5	200 nM
Reverse primer 10 µM	0.5	200 nM
Template DNA/cDNA	5.0/2.0	
Total volume	25.0	

Table 16: Cycling conditions for the 1st PCR for HBV DNA and cDNA amplification

Cycling step	Cycles	Temperature (°C)	Time (h:min:sec)
Preincubation	1	95	00:05:00
Amplification	40		
Denaturation		95	00:00:30
Annealing		60 - 55	00:00:30
Elongation		72	00:01:00
Final elongation	1	72	00:07:00
Cooling	forever	10	forever

In the 2nd PCR, 2 µL of the obtained amplification products for each fragment were added to 12.5 µL LightCycler® 480 Probes Master (Roche Diagnostics, Mannheim, Germany) and 200 nM of the correspondent forward and reverse primers

(Table 17). The amplification temperatures were equal to the first PCR with an annealing temperature of 55°C for all fragments (Table 16).

Table 17: Reaction mix 2nd PCR for HBV DNA and cDNA amplification

Reagents	Volume (µL)	Final concentration
H_2O PCR-grade	9.5	
LightCycler® 480 Probes Master	12.5	
Forward primer 10 µM	0.5	200 nM
Reverse primer 10 µM	0.5	200 nM
Product of 1st PCR	2.0	
Total volume	25.0	

2.2.5.3 Purification of amplification products

The amplified HBV DNA and cDNA fragments were analyzed by gel electrophoresis on a 1% agarose gel, which separates DNA fragments based on their size. The gel was prepared by dissolving agarose (SERVA, Heidelberg, Germany) in 1xTBE buffer. The obtained amplification products were mixed with DNA loading buffer in a relation of 5:1 and applied to the gel. For the visualization of the PCR amplification product, 0.05 µL/mL ethidium bromide, which intercalates into DNA, was applied to the gel. A 100 bp DNA marker (Promega, Madison, WI, USA) was used to estimate the sizes of the obtained fragments. The electrophoresis run was performed at a constant voltage of 110 Volt for 45 minutes. The amplification fragments were exposed to ultraviolet (UV) light on an Alpha Imager (Alpha Innotech, Kasendorf, Germany) for detection, sliced out from the gel with a scalpel and extracted using the Zymo Clean Gel DNA Recovery Kit (Zymo Research, Freiburg, Germany) following the manufacturer's instructions. The purified products were eluted in 10 µL H_2O and stored at -20° C.

2.2.5.4 Sanger sequencing of PCR fragments

Sanger sequencing is a method to determine the bp sequence of DNA molecules based on the termination of the chain elongation during the PCR amplification. Sanger and colleagues first described the method in 1977 (Sanger 1977). During the amplification in either the forward or reverse direction of single strand DNA molecules, the incorporation of 2'-3'-dideoxynucleotide triphosphates (ddNTPs) instead of deoxynucleotide triphosphates (dNTPs) leads to the termination of the chain elongation of the nascent strand. In ddNTPs the 3'-OH group, which is necessary for the formation of phosphodiester bonds between the nucleotides is absent and the chain elongation is thus interrupted. A Sanger sequencing reaction commonly contains DNA primer, DNA polymerase, dNTPs and ddNTPs, which are labeled with radioisotopes, chemiluminescence or fluorescent dyes for the later detection of the nucleotides (Franc 2002). In our study, we used a fluorescence dye, the Big Dye v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany). We performed Sanger sequencing for each fragment in the forward and reverse direction. In a final volume of 10 µL, 1 to 2 µL of the purified PCR template was added to a reaction mix consisting of 1,000 nM forward or reverse primer, 1 µL Big Dye v3.1 Terminator Kit and 1 µL of the provided sequencing buffer (Applied Biosystems, Darmstadt, Germany) (Table 18). The reaction was carried out on the Flex Cycler (Analytik Jena, Jena, Germany) for single tubes or the 2720 Thermal Cycle (Applied Biosystems, Darmstadt, Germany) for the 96 well plates with 24 cycles of denaturation at 96°C for 30 seconds, annealing for 15 seconds at 59°C and elongation at 60°C for 4 minutes (Table 19).

Table 18: Reaction mix for the sequencing reaction

Reagents	Volume (µL)	Final concentration
H_2O PCR-grade	6.0	
Buffer 5x	1.0	
BigDye® Terminator v3.1 Cycle Sequencing Kit	1.0	
Primer 10µM	1.0	1,000 nM
Purified PCR product	1.0	
Total	10.0	

Table 19: Cycling conditions of the sequencing reaction

Cycling step	Cycles	Temperature (°C)	Time (h:min:sec)
Amplification	24		
Denaturation		96	00:00:30
Annealing		59	00:00:15
Elongation		60	00:04:00
Cooling	forever	4	forever

The amplification products were precipitated with 96% ethanol and 75 mM sodium acetate and centrifuged at 13,000 rpm (16,060 x g) for 15 minutes (single tubes) or 4,680 rpm (2,204 x g) for 30 minutes (plates) after an incubation step of 15 minutes. The supernatant was discharged and the remaining pellet washed with 70% ethanol. After a short centrifuge step of 5 to 10 minutes (16,060 x g), the supernatant was cautiously removed. The amplification product was further processed at the IZKF (Interdisziplinäres Zentrum für Klinische Forschung) core unit at the University of Leipzig. The remaining pellet was dissolved in 0.1 mM EDTA solution and read out using the Sequencing Analysis v5.4.

2.2.6 Quantification of serum HBsAg and HBeAg

Quantitative HBsAg levels and the presence of HBeAg at baseline were measured with immunosorbent assays on the Abbott Architect platform (ARCHITECT, Abbott Architect, Abbott Diagnostics GmbH, Wiesbaden, Germany). The HBsAg qualitative detection limit was 0.5 - 250.0 IU/mL. For HBeAg, the signal to cut - off values of 1,000 IU/mL were considered reactive.

2.2.7 Cloning of HBV variants

To confirm single nucleotide exchanges, which were only detectable in either the forward or reverse direction of the sequences, the respective region of interest was cloned into a pCR™®II-TOPO® vector using the TOPO® TA Cloning® Kit TOP10 (Invitrogen, Carlsbad, CA, USA) and products analyzed by direct sequencing. The PCR was carried out using the Taq PCR Master Mix Kit (Qiagen, Hilden, Germany) to construct a single ‘a’ overlap at the 3’ end for the subsequent ligation of the insert into the vector. The reaction mix for the PCR and cycling conditions are given in Tables 20 and 21, respectively.

Table 20: Reaction mix for the cloning PCR

Reagents	Volume (µL)	Final concentration
Taq PCR Master mix	12.5	
Primer Forward 10 µM	2.0	800 nM
Primer Reverse 10 µM	2.0	800 nM
H_2O PCR-grade	3.5	
Template	5.0	
Total	25.0	

Table 21: Cycling conditions of the cloning PCR

Cycling step	Cycles	Temperature (°C)	Time (h:min:sec)
Preincubation	1	95	00:05:00
Amplification	40		
Denaturation		95	00:00:30
Annealing		55	00:01:00
Elongation		72	00:02:00
Final elongation	1	72	00:07:00
Cooling	forever	10	forever

The PCR product was visualized on an agarose gel (chapter 2.2.5.3) and purified using the Zymo Clean Gel DNA Recovery Kit (Zymo Research, Freiburg, Germany). Following the manufacturer's instructions, the PCR product was cloned into the TOPO TA Cloning® vector as described in Table 22. The reaction was gently mixed and incubated at room temperature for 5 minutes. On ice, 2 µL of the reaction were added to TOP10 chemically competent *E. coli* cells and incubated for 5 minutes. The cells were heat-shocked for 45 seconds at 42°C and immediately transferred on ice. 250 µL of room temperature S.O.C. medium provided by the TOPO TA Cloning® Kit was added and the mix horizontally shaken with 200 rpm at 37°C for 1 hour. 50 µL of each transformation was spread on a pre-warmed LB plate. The LB plates contained 25 µg/mL Kanamycin and 50 µg/mL ampicillin and 40 mg/mL x-gal for the color selection. The plates were incubated at 37°C for 8 to 12 hours.

Table 22: TOPO® TA cloning® reaction

Reagents	Volume (µL)
H_2O	3.0
Salt solution	1.0
TOPO TA Cloning® vector	1.0
PCR product	1.0
Total volume	6.0

Around 10 colonies were picked and each added to 50 µL LB medium mixed with 1 µL/mL ampicillin and 2.5 µL/mL kanamycin and gently shaken at 37°C for 1 hour. For the inoculation of each colony, 10 µL of the mix was added to a single falcon tube (15 mL) containing 3 mL LB medium mixed with ampicillin and kanamycin and incubated at 200 rpm at 37°C over night. The preparation of the bacterial suspension was performed using the QIAfilter Plasmid Midi and Maxi Kit (Qiagen, Venlo, NL) following the manufacturer's instructions and eluted in 50 µL H_2O. The sequencing of the cloning products was carried out as described in chapter 2.2.5.

2.2.8 Data analysis

2.2.8.1 Serum HBV DNA and HBV RNA quantities

All values of the HBV DNA and cDNA determined by qPCR were measured in duplicates. For further analysis, the mean values of the duplicates were used and transferred to $\log_{10}$. HBV s gene and HBV core derived values did not significantly differ, therefore, only the core values were used for further analysis. The mean copy levels and standard variations of the HBV DNA and HBV RNA were calculated at baseline and during follow-up at months 6, 12, 18 and 24 with Microsoft Excel (Versions 2010 and 2013) and depicted in box plots. Missing values for those time points were linear interpolated. The linear correlation between the HBV DNA, HBV trRNA and HBV flRNA was calculated using Excel regression analysis tool for the Pearson's correlation coefficient (r) and the *p* value. We chose a significance level < .05 for the *p* value.

2.2.8.2 Analysis of HBV DNA and HBV RNA sequences

Sequences were analyzed using the bioinformatics programs Chromas Lite (Version 2.01) and CLC Main workbench (Version 6.8.3). With CLC Main workbench, the obtained forward and reverse sequences of the three fragments were aligned

(Figure 8). Double peak variants were considered as true if they were present in one sequence and covered the entire peak or if present in the forward and reverse sequence and covered at least 50% of the peak's height. Peaks with lower height had to be present also in follow-up sequences. Single peaked nucleotide exchanges were considered true if present in the forward and reverse sequences. Single nucleotide exchanges only present in the forward or reverse sequence were cloned as described in chapter 2.2.7 and considered as true if present in 50% of the analyzed clones.

The entire rt region was analyzed using the HepSEQ-Research Database System (http://www.hepseq.org) for genotyping and a reference genome determined for each sequence. The sequences of the reference genomes were obtained with the respective GenBank annotation (Table 23) using the National Center for Biotechnology Information (NCBI) Nucleotide platform (http://www.ncbi.nlm.nih.gov/nuccore).

Table 23: HBV reference genomes

HBV genotype	GenBank annotation
A	V00866.1
A	X70185.1
A	X51970.1
B	AB073827.1
D	AB073827
D	AF151735.1
D	X65257.1
D	X51970.1
D	X65257.1
D	X85254.1
E	x75657

Using the bioinformatics tool HUSAR (http://genome.dkfz-heidelberg.de/husar/), we translated the nucleotide sequences into the amino acid sequences and compared them to the respective reference genomes using the PileUp tool that allows the alignment of nucleotide and amino acid sequences (Figure 9).

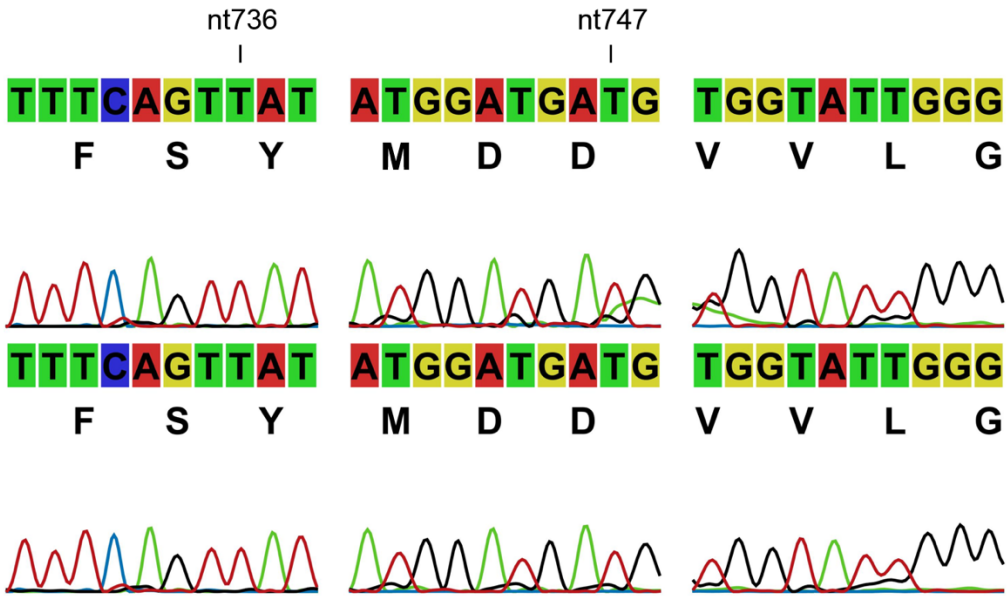


Figure 8: Alignment of HBV sequences using CLC Main Workbench

Alignment of the forward and reverse sequence of a PCR fragment spanning from nt321 to nt840 using CLC Main Workbench. The detail focuses on the highly conserved YMDD (TATATGGATGAT; nt736 to nt747) motif in the rt region of the pol gene, which here represents a wild type sequence.

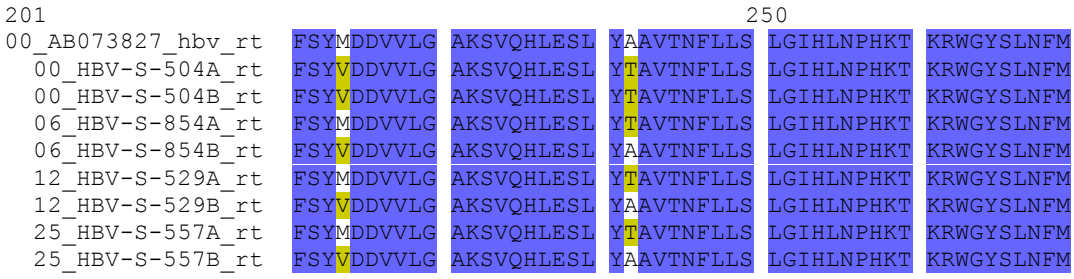


Figure 9: Comparison of sequences during follow-up with PileUp

PileUp of one patient using the HUSAR platform of the amino acid (aa) sequences (aa201 to aa 250) in follow-up samples from month 0 to 25 of TDF treatment. The detail includes the YMDD motif in the rt region (aa203 to aa206). The upper line indicates the reference HBV genome, which was previously determined. Different variants were called 'A' and 'B'. The mutation rtM204V is present in all samples.

The sequences were checked for known primary resistance mutations in the rt region and for s gene variants and stop codons before and during antiviral treatment. We assessed HBV variants described to be associated with primary resistance against LAM (rtM204V/I), ADV (rtN236T) and both NAs (rtA181V/T) as well as the compensatory mutations rtL80V, rtV173L and rtL180M (Zoulim 2009). Within the s gene, we determined the presence of the variants sE164D and sI195M, which occur combined with the overlapping rt gene variants rtV173L and rtM204V, respectively, and the variants sW196S/L, which are caused by the overlapping variant rtM204I. We also assessed variants between positions s90 – s170, defined as 'a' determinant, the major target of anti-HBs (Pollicino 2014), and the occurrence of stop codons (*).

3 Results

3.1 Composition of the patient set

A total of 68 patients receiving mono therapy with TDF 300 mg/day, and 3 patients receiving a combination therapy of TDF and LAM 100 mg/day as second or third line treatment after failure to LAM and ADV were identified. The treatment was conducted at the outpatient clinics of the University Hospitals Charité, Berlin, and Leipzig, and in a large Gastroenterological Center in Herne, Germany, between 2002 and 2013. All participants had given written informed consent for the participation in the study. Of these patients, we included 25 patients with follow-up serum samples available in our study, of which 24 received TDF as monotherapy and 1 patient a combination with LAM. For 4 patients, serum samples at the beginning of TDF treatment were not available but represented months 1 (n = 2), 2 (n = 1) and 3 (n = 1). We excluded these patients from the quantitative analysis of serum HBV DNA and serum HBV RNA at baseline, but included them for the assessment of HBV variants during antiviral treatment. Of 25 patients, 21 were HBeAg positive at baseline (Table 24).

3.2 Quantification of HBV DNA and HBV RNA in serum samples

In total, 156 samples representing time points around months 0, 6, 12, 24 and further available serum samples representing later time points during TDF treatment were retrospectively analyzed. The mean duration of follow-up was 45.2 ± 16.8 (21 - 82) months. We screened all samples for the presence of serum HBV DNA, HBV trRNA and HBV flRNA. The HBV DNA and HBV flRNA serum levels could be determined in all samples. In 1 patient (pat ID 1-15), the quantification of serum HBV trRNA failed and the sequencing of the primer-binding site in the HBx region revealed a high variability of nucleotides in this patient.

Table 24: Patients characteristics at the beginning of treatment with TDF

Parameters	Values
Male/female, n	19/6
Age, years*	44.6 ± 12.6 (25 - 70)
HBeAg positive, n	21
HBV DNA, log_{10} cp/mL*	6.3 ± 1.6 (9.5 – 3.3)
HBV trRNA, log_{10} cp/mL*	5.8 ± 1.4 (8.7 – 3.9)
HBV flRNA, log_{10} cp/mL*	5.6 ± 1.4 (7.9 -3.4)
HBsAg, IU/mL*	3.9 ± 0.6 (6.6 - 4.9)
ALT, IU/mL*	136 ± 324 (23 - 1,523)
Duration of follow-up, months*	45.2 ± 16.8 (21 - 82)
HBV genotype, n	
A	4
B	2[1)]
D	19[1)]
E	1
Preceding antiviral treatment	
LAM, n	4
ADV, n	1
LAM and ADV, n	20

* mean ± standard deviation (range); [1)] one patient had mixed genotypes B and D. ALT = alanine aminotransferase; cp/mL = copies per milliliters; IU/mL = international units per milliliters; n = number of patients; flRNA = full-length RNA; trRNA = truncated RNA;

3.2.1 Quantitative courses of serum HBV DNA

At baseline, the mean serum level for the HBV DNA was 6.2 ± 1.5 (9.5 – 3.1) log_{10} cp/mL. During antiviral treatment with TDF, the HBV DNA serum levels persisted detectable above the LOD (400 cp/mL) for a mean duration of 9.7 ± 10.6 (0 - 38) months in all patients. In 1 patient, the serum HBV DNA level decreased > 1 log_{10}

during the first months of TDF treatment but persisted > 400cp/mL until the end of follow-up at month 37 (pat ID 1-66; Figure 17L). In another patient, the HBV DNA became detectable at high levels at month 17, after a previous decline to undetectable levels, but re-decreased during further follow-up (pat ID 1-37; Figure 16J).

3.2.2 Quantitative courses of serum HBV flRNA and HBV trRNA

The quantitative levels of serum HBV flRNA and trRNA at baseline were 5.6 ± 1.4 (3.4 – 7.9) log_{10} cp/mL and 5.8 ± 1.4 (3.9 – 8.7) log_{10} cp/mL, respectively. The linear correlation between the HBV DNA and HBV RNA serum levels at baseline was calculated with the Pearson's correlation coefficient (Figure 10) and showed a significant correlation for the serum HBV DNA with HBV trRNA (r = 0.83; p < .001) and HBV flRNA (r = 0.65; p < .05).

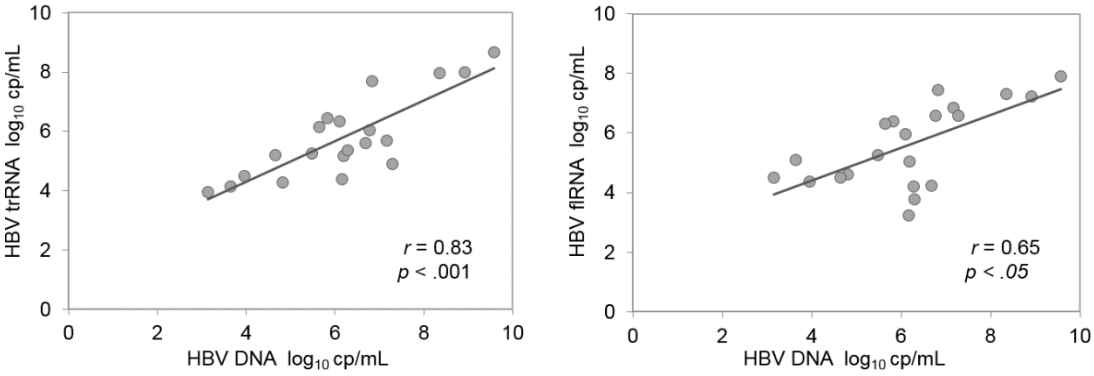


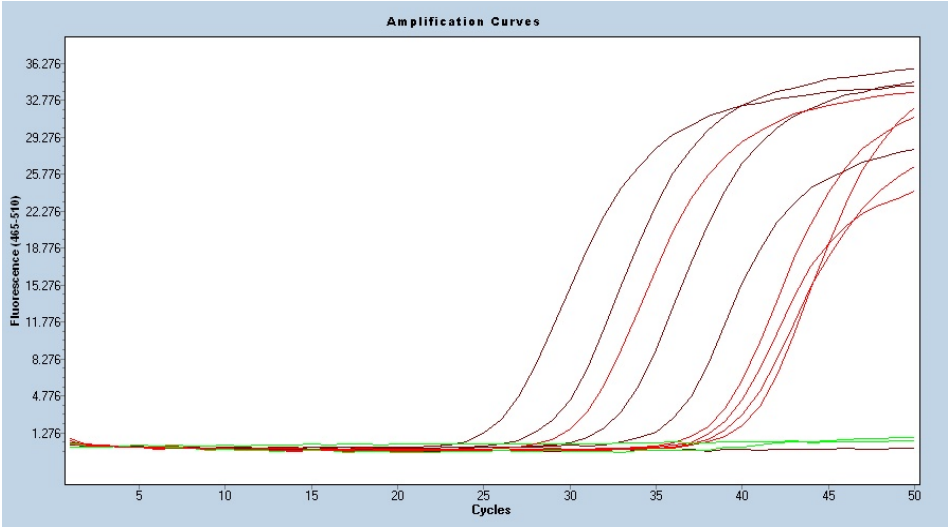
Figure 10: Correlation of serum levels of HBV DNA with HBV trRNA and HBV flRNA at baseline

The Scatter plots depict the linear correlation between HBV DNA serum levels and HBV trRNA (left) and HBV flRNA (right) at baseline. r = Pearson's correlation coefficient; p = p - value.

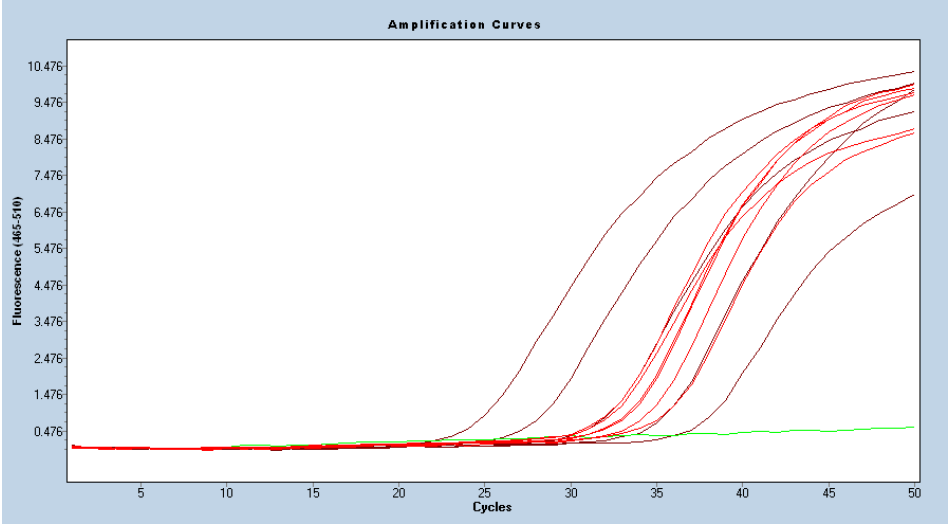
Serum HBV flRNA and trRNA levels persisted above the LODs of 3,000 and 5,500 cp/mL for mean durations of 33.4 ± 18.1 (0 - 76) and 25.1 ± 20.7 (0 - 76) months, respectively. In 9 patients (pat IDs 1-34, 1-60, 1-25, 1-37, 1-12, 1-15, 1-02, 7-02, 7-09), serum HBV flRNA and HBV trRNA decreased to levels < 500 cp/mL during follow-up . In 4 of those patients (pat IDs 1-15, 1-25, 1-37, 7-09), the HBV flRNA and trRNA

was undetectable in follow-up serum samples after the decline of the HBV DNA. 3 of these patients (pat IDs 1-25, 1-37, 7-09) were HBeAg negative. In patients with persistent HBV RNA, the mean durations of HBV flRNA and trRNA levels > 500 cp/mL were 34.1 ± 21.0 (0 - 76) and 31.4 ± 19.7 (0 - 76) months, respectively. Figure 11 shows exemplary the quantification curves of serum HBV DNA, HBV flRNA and HBV trRNA of 1 patient (pat ID 1-76) with persistence of the HBV RNA after the decline of the HBV DNA.

A) HBV DNA



B) HBV trRNA



C) HBV flRNA

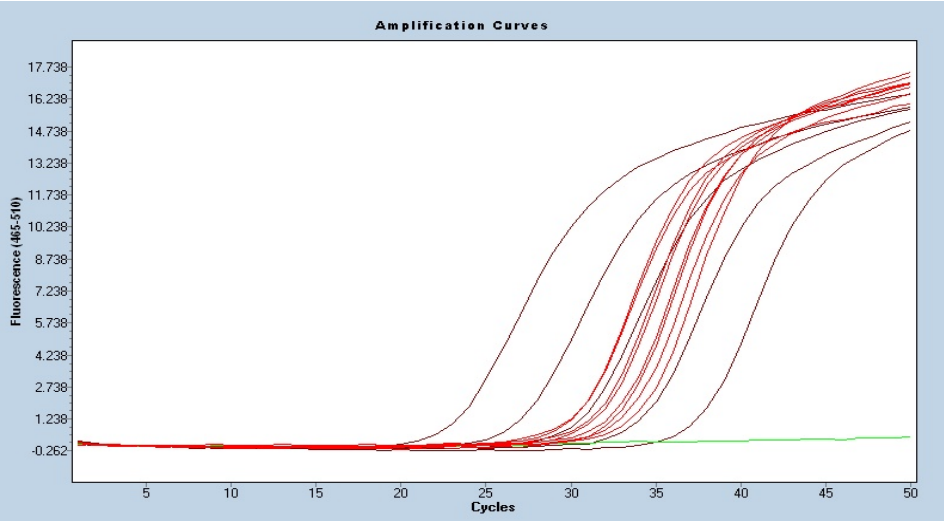


Figure 11: Quantification of HBV DNA and HBV RNA by qPCR – Example of 1 patient (pat ID 1-76)

Amplification curves of the HBV DNA (A), HBV trRNA(B) and HBV flRNA (C) in follow-up samples of 1 patient. The brown curves represent the standard curves. The red curves indicate the samples and the green lines the negative samples and negative controls. While the serum HBV DNA decreased during TDF treatment to undetectable levels (A), HBV trRNA (B) and HBV flRNA (C) remained measurable until the end of follow-up.

The quantitative courses in the entire cohort of the mean HBV DNA, HBV flRNA and HBV trRNA serum levels at baseline and at months 6, 12, 18 and 24 of antiviral treatment depicted in box plots are shown in figure 12.

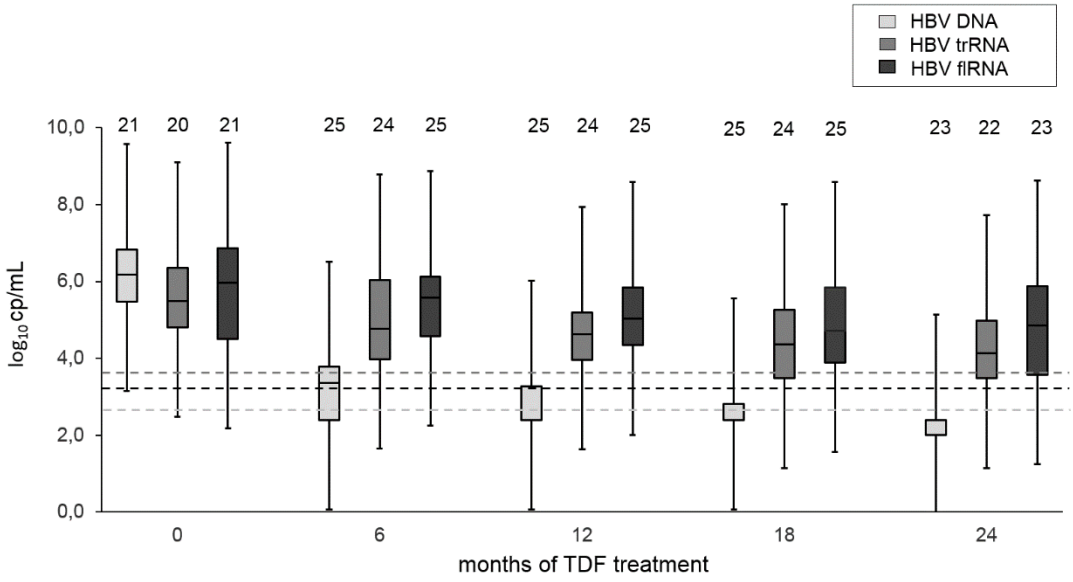


Figure 12: Quantitative courses of serum HBV DNA and HBV RNA during antiviral treatment with TDF in the entire cohort

Median serum levels of HBV DNA (light grey), HBV trRNA (grey) and HBV flRNA (dark grey) at baseline and during follow-up of TDF treatment. The boxes represent the 0.25 and 0.75 quartiles, the solid lines in the boxes the median. The numbers above whiskers indicate the number of results included. The dashed lines represent the respective lower limits of detection for HBV DNA (light grey), HBV flRNA (dark grey) and trRNA (grey).

3.3 Sequencing of HBV DNA and HBV RNA

3.3.1 Method

The main purpose of our study was to establish the sequencing of serum HBV RNA as a method for genotyping of HBV variants after HBV DNA serum levels became undetectable during antiviral treatment. We used a semi-nested PCR for the amplification of the HBV DNA (Figure 13) and HBV RNA (Figure 14) to achieve higher yields of the PCR amplification products and to reach a more sensitive PCR. Thus for a successful PCR amplification, copy levels of around 500 to 1,000 cp/mL were required. An annealing temperature of 55 °C was chosen for the semi-nested PCRs (chapter 2.2.5.2) except for the nt2808 and nt401 primers, for which annealing

temperatures at 60 °C and 58 °C in the first amplification step appeared to detect more specific amplification products determined by a gradient PCR.

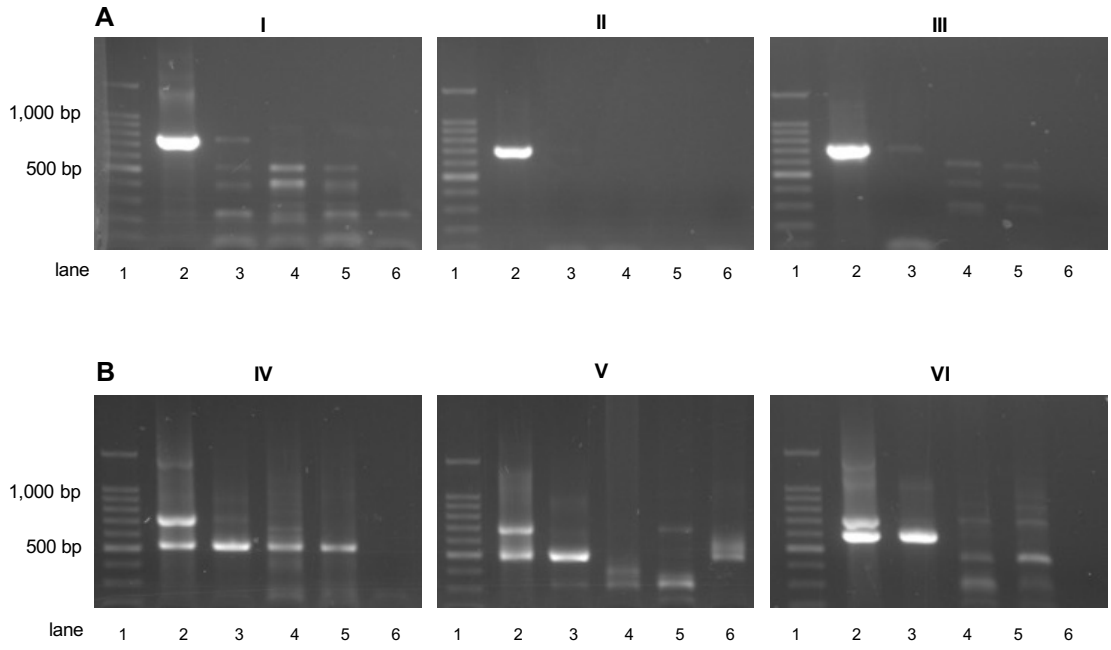


Figure 13: Semi-nested PCRs of the HBV DNA in 1 patient (pat ID 1-21)

Amplification products of around 700 bp sizes from the 1st PCR (A) and around 500 bp sizes of the 2nd PCR (B). In each PCR round, 3 fragments were amplified with primers spanning from nt2808 to nt401 (I), nt321 to nt1056 (II) and nt660 to nt1417 (III) in the 1st PCR, and from nt3077 to nt401 (IV), nt321 to nt840 (V) and nt660 to nt1281 (VI) in the 2nd PCR. Lane 1 in each PCR picture indicates the 100 bp size marker. Lanes 2 to 5 represent follow-up serum samples of the patient at months 0, 6, 12 and 26. The respective quantities are given in Table 25. Lanes 6 show the negative controls. Quantities < 1,000 cp/mL (lanes 4,5) were not sufficient to obtain an amplification product for the sequence analysis after the semi-nested PCR (B). In fragment IV, unspecific amplification products of 500 bp sizes were present before the optimization of the PCR conditions.

Table 25: Serum levels of HBV DNA and HBV flRNA at baseline and during follow-up in 1 patient (pat ID 1-21)

months	HBV DNA cp/mL	PCR - lanes
0	1,49E+07	2
6	3,26E+03	3
12	6,01E+02	4
26	0,00E+00	5

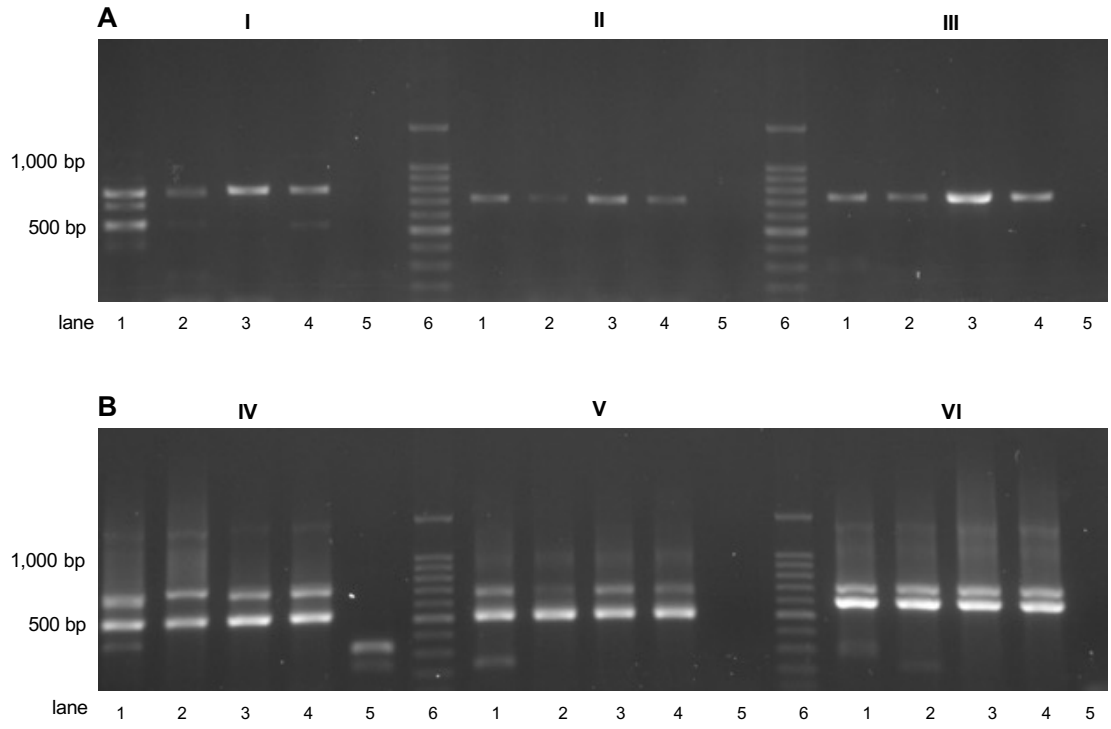


Figure 14: Semi-nested PCRs of the HBV RNA in follow-up samples of 2 patients (pat IDs 1-21, 1-10)

Amplification products of around 700 bp sizes from the 1st PCR (A) and around 500 bp sizes of the 2nd PCR (B). In each PCR round, 3 fragments were amplified using primers as described in figure 13. In each PCR picture, lanes 1 and 2 represent the amplification products at months 12 and 26, respectively, during follow-up in patient 1-21. As shown in figure 13, at these time points, amplification products could not be obtained based on HBV DNA in this patient. For another patient (pat ID 1-10), the PCR amplification products were obtained at months 13 (lanes 3) and 25 (lanes 4) during TDF treatment. The respective serum levels are given in Table 27. Lanes 6 and 12 indicate the 100 bp size markers, lanes 5 the negative controls.

Table 26: Serum levels of HBV flRNA in follow-up samples of 2 patients

pat ID	months	HBV flRNA cp/mL	PCR – lanes
1-21	12	3,60 E+04	1
1-21	26	1,68 E+03	2
1-10	13	6,60E+05	3
1-10	25	7,60E+04	4

3.3.2 Follow-up with sequencing of HBV DNA and HBV RNA

Out of 156 serum samples, the sequencing of the HBV DNA or cDNA could be performed for 61 and 68 probes, respectively. For 27 serum samples, the sequence analysis was not available due to low serum levels of the HBV DNA and HBV RNA.

In the baseline serum samples, sequence analysis based on HBV DNA was possible for all patients (Figure 15). During follow-up, 3 HBeAg negative patients (pat IDs 7-09, 1-37, 1-25) and 1 HBeAg positive patient (pat ID 1-60) had low serum levels of HBV DNA and HBV RNA, therefore, sequence analysis was only available at baseline but not in the sequential samples (Figure 15). In 17 out of the remaining 21 patients with available genotyping during follow-up, sequence analysis based on HBV DNA became unavailable after a mean duration of 6.0 ± 4.5 (0 - 13) months. The HBV DNA derived sequences thus mainly represented early time points during antiviral treatment with TDF. After the decline of the HBV DNA, sequence analysis of the reverse transcribed HBV RNA was possible for an additional mean duration of 33.9 ± 12.7 (16 - 65) months. In 4 patients (pat IDs 7-02, 1-66, 1-02, 1-15), sequence analysis based on HBV DNA remained available for a longer mean duration of 35.3 ± 4.6 (30 - 38) months. In these patients, sequencing of HBV DNA became transiently unavailable at single time points during follow-up whilst sequencing of HBV RNA was still possible (Figure 15).

3.3.3 Genotyping of baseline samples

For HBV genotyping of all baseline serum samples, the HepSEQ-Research Database was used. The distribution of the genotypes was A in 4 patients, B in 1 patient, D in 18 patients and E in 1 patient. In 1 patient, a combination of the genotypes B and D were found (Table 24, pat ID 1-15; Figure 15). This was also the

patient, in which the quantification of the trRNA failed due to the high variability of the primer-binding site in the HBx gene region.

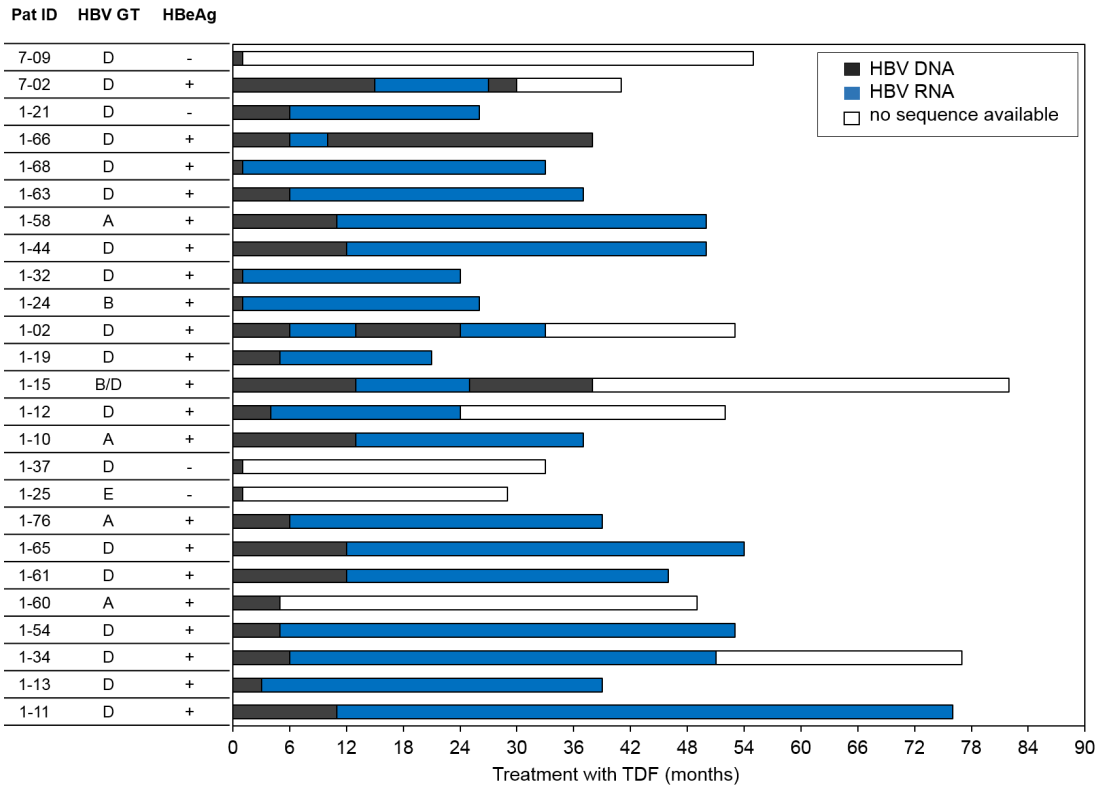


Figure 15: Durations of follow-up of HBV variants by sequencing HBV DNA and HBV RNA

Durations of follow-up during TDF treatment in months (x-axis) by sequence analysis based on HBV DNA (black) and HBV RNA (blue) for each patient (ID; on the left). The white bar indicates that no sequence was available. In 4 patients (pat IDs 7-09, 1-37, 1-25, 1-60), sequences were only available at baseline and not during follow-up. The HBeAg status at baseline (+ positive; - negative) and the HBV genotypes (GT) are given on the left.

3.4 Evolution of HBV variants in the rt region

3.4.1 HBV resistance mutations in the rt region at baseline

We analyzed the occurrence of HBV mutations in the rt region of the pol gene, which confer to primary resistance to treatment with LAM (codon rt204), ADV (codon rt236) or both (codon rt181) as well as compensatory mutations at codons rt80, rt173 and rt180 at the beginning of treatment with TDF or the first sample available in all

patients. In total, 14 out of 25 patients harbored one or a combination of HBV mutations associated with primary resistance to LAM (n = 8) or ADV (n = 6). In 4 patients (pat IDs 1-66, 1-02, 7-02, 7-09), the ADV resistant HBV variant N236T was combined with a A181V/T mutation that confers to ADV and LAM resistance. 1 patient (pat ID 1-12) harbored a sole compensatory mutation at codon rt80 (rtL80V) at the beginning of TDF treatment. In 10 patients, no HBV mutations associated with resistance to antiviral treatment with NAs were detected at baseline (Table 27).

Table 27: Resistance mutations in the rt region at baseline

pat ID	pre-treatment	aa rt80	aa rt173	aa rt180	aa rt181	aa rt204	aa rt236
resistance mutation							
1-10	LAM	L	V>L	L>M	A	M>I/V	N
1-19	LAM	L>V	V	L	A	M>I	N
1-21	LAM	L	V>L	L>M	A	M>V	N
1-44	LAM	L>V	V	L>M	A	M>I	N
1-24	LAM + ADV	L	V	L>M	A	M>V	N
1-58	LAM + ADV	L	V>L	L>M	A	M>I/V	N
1-63	LAM + ADV	L>V	V	L	A	M>I	N
1-68	LAM + ADV	L>I	V	L	A	M>I	N
1-12	LAM + ADV	L>V	V	L	A	M	N
1-15	LAM + ADV	L	V	L	A	M	N>T
1-32	LAM + ADV	L	V	L	A	M	N>T
1-66	LAM + ADV	L	V	L	A>V	M	N>T
1-02	LAM + ADV	L	V	L	A>V	M	N>T
7-02	LAM + ADV	L	V	L	A>T	M	N>T
7-09	LAM + ADV	L	V	L	A>V	M	N>T
no resistance mutation							
1-25	LAM + ADV	L	V	L	A	M	N
1-34	LAM + ADV	L	V	L	A	M	N
1-37	LAM + ADV	L	V	L	A	M	N
1-54	LAM + ADV	L	V	L	A	M	N
1-65	LAM + ADV	L	V	L	A	M	N
1-76	LAM + ADV	L	V	L	A	M	N
1-60	LAM + ADV	L	V	L	A	M	N
1-61	ADV	L	V	L	A	M	N
1-11	LAM + ADV	L	V	L	A	M	N
1-13	LAM + ADV	L	V	L	A	M	N

aa = amino acid; LAM = lamivudine, ADV = adefovir.

Of 24 patients who had received pre-treatment with LAM as monotherapy (n = 4) or in addition with ADV (n = 20), 8 patients had a HBV mutation at codon rt204 (Table 27). 4 patients (pat IDs 1-19, 1-44, 1-63, 1-68) showed a rtM204I mutation, of which 3 patients (pat IDs 1-19, 1-63, 1-68) had a compensatory mutation at codon rt80 (2 patients rtL80V and 1 patient rtL80I), and 1 patient (1-44) a combination of additional rtL80V and rtL180M mutations. 2 patients (pat IDs 1-21, 1-24) showed a rtM204V mutation, both combined with a rtL180M mutation, of which 1 patient harbored an additional rtL173V substitution. In 2 patients (pat IDs 1-10, 1-58), a combination of the rtM204V, rtM204I, rtL180M and rtL173V mutations were detectable at baseline (Table 27). 4 patients (pat IDs 1-66, 1-02, 7-02, 7-09) who were pre-treated with LAM and ADV had a rtA181V/T mutation, which confers to LAM and ADV resistance. In all these patients, a rtN236T substitution that is associated with ADV resistance, was also detectable (Table 27). This mutation was found in 2 additional patients (pat ID 1-15, 1-32) but not combined with other mutations in these cases. 1 patient (pat ID 1-61) pre-treated with ADV mono therapy had no known primary resistance mutation at baseline (Table 27).

3.4.2 HBV resistance mutations in the rt region during antiviral treatment

We assessed the evolution of HBV resistance variants in the rt region during antiviral treatment in all patients. The courses of 10 patients with no resistance mutations at baseline are illustrated in figure 16 (Figure 16A - 16J). The courses of the evolution of HBV variants in patients with resistance to prior antiviral treatment are given in figure 17 (Figure 17A - 17O).

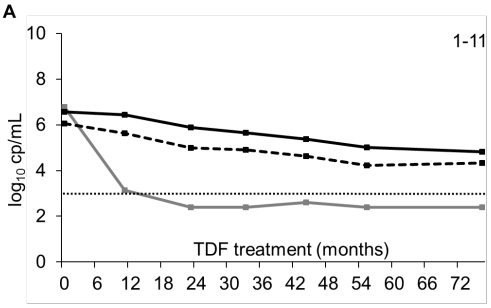
Of 10 patients with no HBV resistance mutations detectable at baseline (Figures 16), sequential sequence analysis based on HBV DNA or HBV RNA was available for 7 patients (Figures 16A - 16D, 16F, 16G, 16H). Only 1 of these patients (pat ID 1-61; Figure 16F) had a rtA181T mutation during follow-up, which was

transiently detectable on HBV DNA basis at month 5. In 1 HBeAg positive patient (pat ID 1-60; Figure 16E) and 2 HBeAg negative patients (pat ID 1-25; Figure 16I, pat ID 1-37; Figure 16J), no follow-up based on sequencing of the HBV RNA was available due to the rapid decline of the serum HBV RNA copy levels.

In 14 out of 15 patients with resistance mutations at baseline (Figure 17), follow-up serum samples could be used for observing the evolution of these variants during antiviral treatment with TDF. In 1 HBeAg negative patient (pat ID 7-09; Figure 17O) harboring a rtA181V and rtN236T mutation, no sequences based on HBV RNA sequencing were available during follow-up. In 13 patients with follow-up sequences available, the resistance mutations detected at baseline based on HBV DNA were present on HBV RNA during follow-up and persisted over long durations in most patients. Only in 1 patient (pat ID 1-19; Figure 17 D), a rtM204I + rtL80V mutation occurred at baseline but was not detectable in sequential HBV DNA and HBV RNA samples. In 3 patients (pat ID 1-12; Figure 17B, pat ID 1-15; Figure 17C, pat ID 1-63; Figure 17J) additional resistance mutations, which were not detected on HBV DNA basis, were found by sequence analysis of HBV RNA at positions rtM204V and rtL180M and rtV173L.

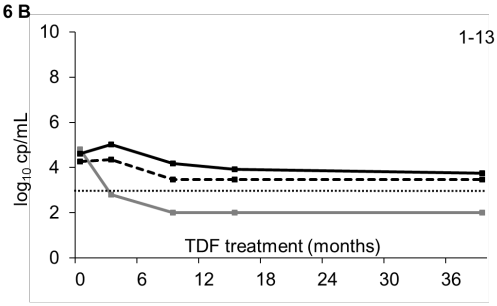
HBV DNA HBV flRNA HBV trRNA

16 A



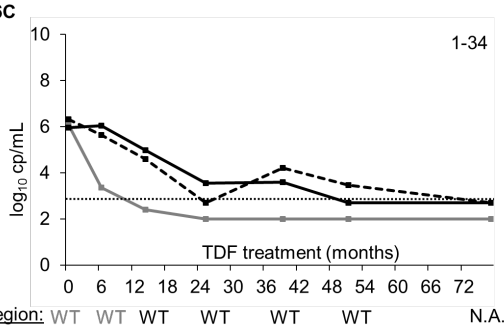
rt region:	WT	WT	WT	WT	WT	WT	WT	WT
s gene:								
sC69	*	*	WT	*	*	*	*	*

16 B



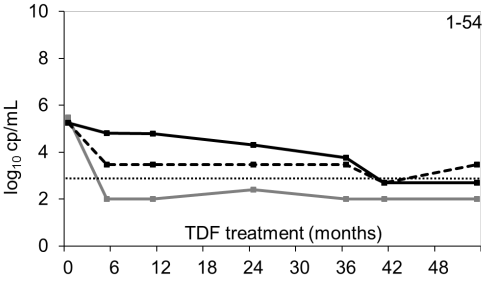
rt region:	WT	WT	WT	WT	WT
s gene:					
sL216	*	*	WT	*	WT
sW181	WT	WT	WT	WT	*

16C



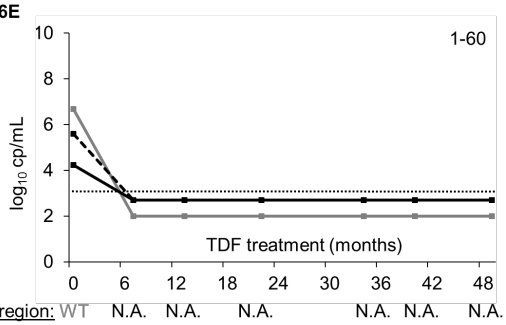
rt region:	WT	WT	WT	WT	WT	WT	N.A.

16D



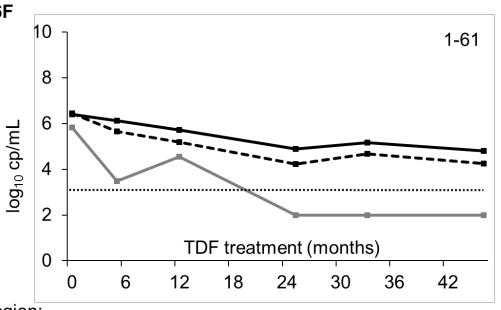
rt region:	WT	WT	WT	WT	WT	WT	WT
s gene:							
sC69	WT	*	WT	WT	WT	*	WT
sL122	*	*	*	*	*	*	WT

16E



rt region:	WT	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

16F



rt region:							
rtA181	WT	T	WT	WT	WT	WT	
s gene:							
sW172	WT	*	WT	WT	WT	WT	
sW182	WT	*	WT	WT	WT	WT	

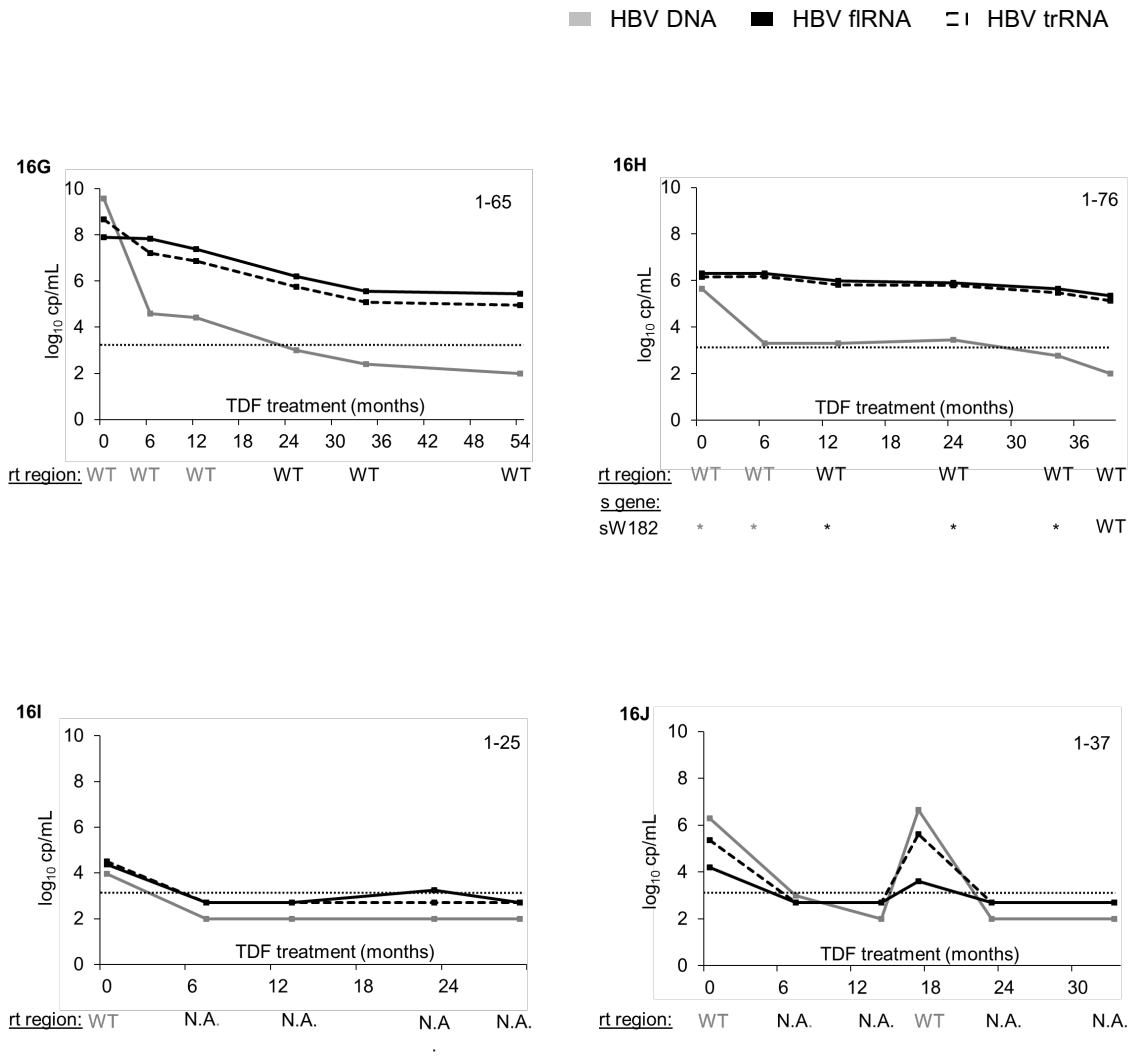
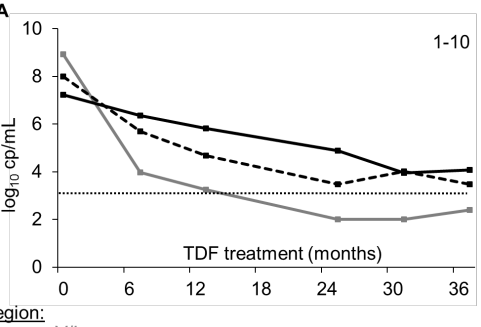


Figure 16: Evolution of HBV variants in patients with no HBV resistance mutations at baseline during TDF treatment

*Figures 16A to 16J show the quantitative courses of serum HBV DNA (solid line, light grey), serum HBV trRNA (dashed line, black) and serum HBV flRNA (solid line, black) during antiviral treatment in 8 HBeAg positive (16A - 16H) and 2 HBeAg negative patients (16I, 16J) with no HBV resistance mutations at baseline. The respective patient IDs are given in the upper right corner of each figure. The y-axis represents the log_{10} of the serum levels in cp/mL, the x-axis the duration of TDF treatment in months. The dashed black line in each figure indicates the limit between 500 and 1,000 cp/mL for a successful sequencing. Below the quantitative curves, the HBV variants in the rt region and the s gene are given. Grey indicates HBV DNA derived sequences and black HBV RNA derived sequences. WT = wild type; N.A. = no sequence available. * = stop mutation.*

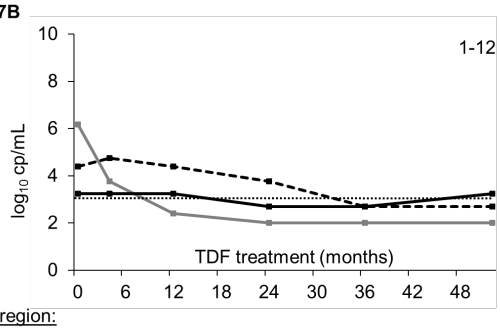
HBV DNA HBV flRNA HBV trRNA

17A



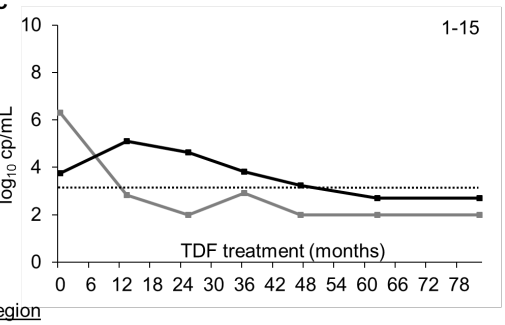
rt region:						
rtM204	V/I	V/I	I	V/I	V	WT
rtV173	L	L	L	WT	WT	WT
rtL180	M	M	M	WT	M	WT
s gene:						
sE164	D	D	D	WT	WT	WT
sI195	M	M	M	WT	M	WT
sW196	L	L	L	L	WT	WT

17B



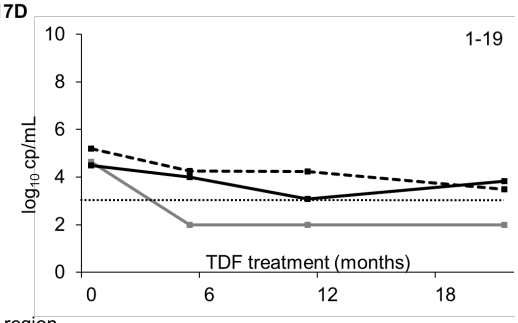
rt region:						
rtL80	V	WT	V	WT	N.A.	N.A.
rtM204	WT	WT	V	V	N.A.	N.A.
rtL180	WT	WT	WT	M	N.A.	N.A.
s gene:						
sI195	WT	WT	M	M	N.A.	N.A.

17C



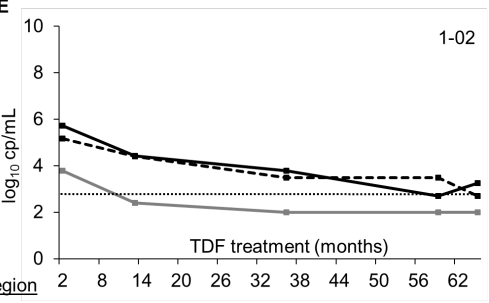
rt region							
rtN236	T	T	T	WT	N.A.	N.A.	N.A.
rtM204	WT	WT	V	WT	N.A.	N.A.	N.A.
rtL180	WT	WT	M	WT	N.A.	N.A.	N.A.
s gene							
sI195	WT	WT	M	WT	N.A.	N.A.	N.A.

17D



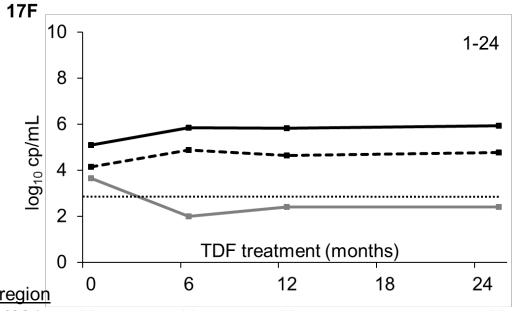
rt region				
rtM204	V	WT	WT	WT
rtL80	I	WT	WT	WT
s gene				
sC69	WT	WT	*	WT
sW196	S	WT	WT	WT

17E



rt region							
rtN236	T	WT	T	WT	WT	N.A.	N.A.
rtA181	V	V	V	V	V	N.A.	N.A.

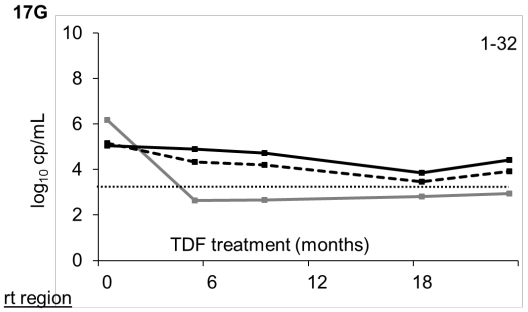
17F



rt region				
rtM204	V	V	V	V
rtL180	M	M	M	M
s gene				
sI195	M	M	M	M

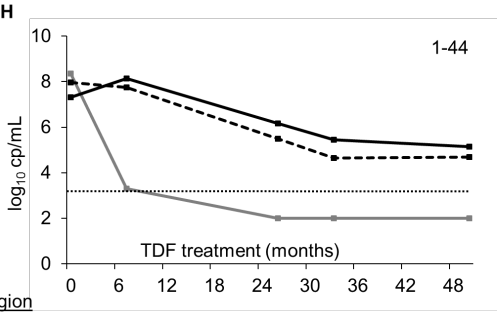
HBV DNA HBV flRNA HBV trRNA

17G



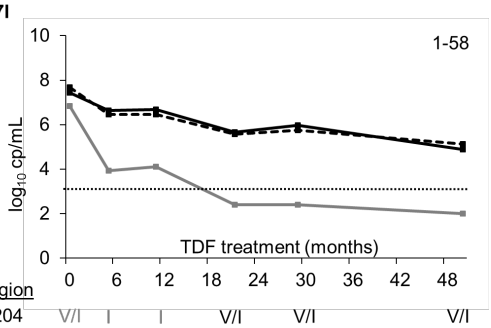
rt region					
rtN236	T	WT	T	WT	WT

17H



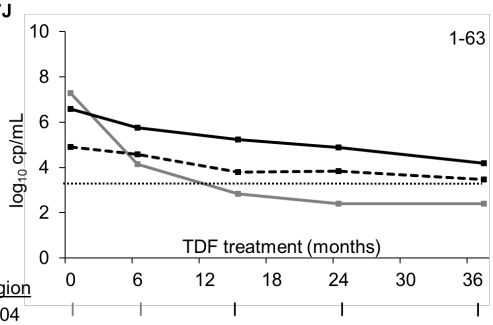
rt region					
rtL80	V	WT	V	V	V
rtM204	I	I	I	I	I
rtL180	M	M	M	M	M
s gene					
sW196	L	L	L	L	L

17I



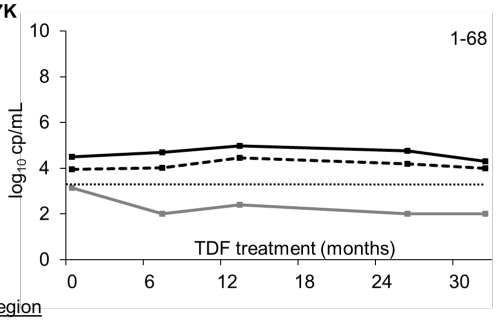
rt region						
rtM204	V/I	I	I	V/I	V/I	V/I
rtV173	L	WT	WT	L	L	L
rtL180	M	M	WT	M	M	M
s gene						
sE164	D	WT	WT	WT	D	WT
sI195	M	WT	WT	M	M	M
sW196	*	*	*	*	*	*
sW199	*	*	WT	*	*	*

17J



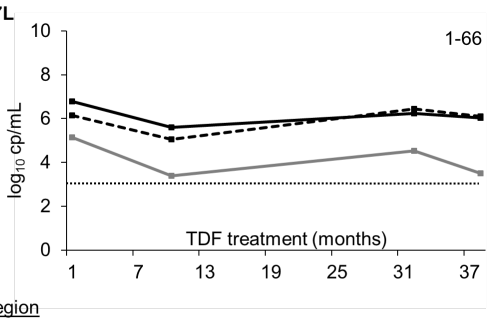
rt region					
rtM204	I	I	I	I	I
rtV173	WT	WT	WT	WT	L
rtL80	V	V	V	V	V
s gene					
sE164	WT	WT	WT	WT	D
sW196	L	L	L	L	L

17K



rt region					
rtM204	I	I	I	WT	WT
rtL80	I	I	I	I	WT
s gene					
sW196	L	L	L	WT	WT

17L



rt region				
rtN236	T	T	WT	WT
rtA181	V	V	V	V

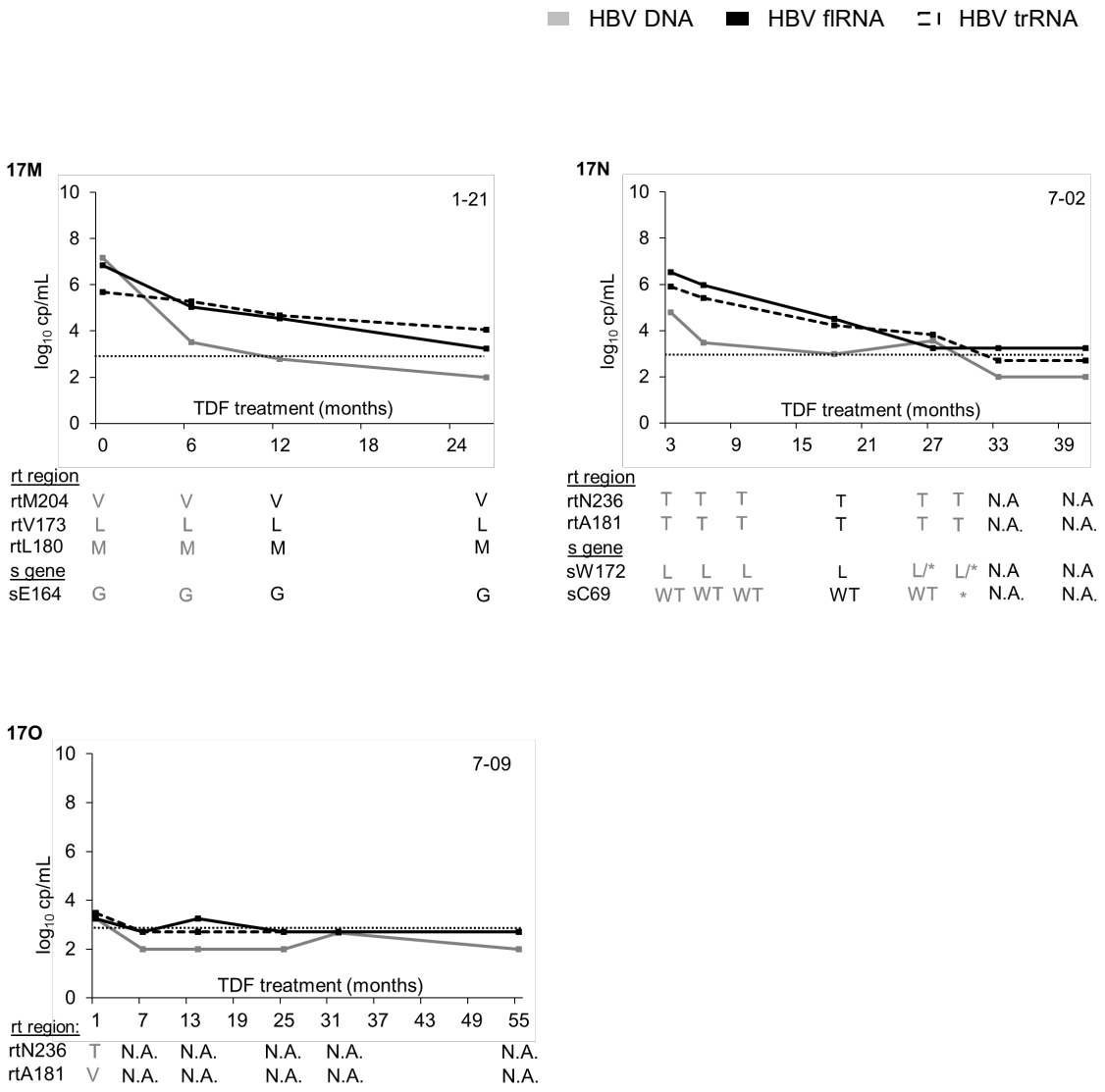


Figure 17: Evolution of HBV variants in patients with HBV resistance mutations at baseline during treatment with TDF

*Figures 17A to 17O show the quantitative courses of serum HBV DNA (solid line, light grey), serum HBV trRNA (dashed line, black) and serum HBV flRNA (solid line, black) during antiviral treatment in 13 HBeAg positive (17A -17M) and 2 HBeAg negative patients (17N, 17O) with HBV resistance mutations at baseline. The respective patient IDs are given in the upper right corner of each figure. The y-axis represents the serum levels of HBV DNA and RNA (cp/mL), the x-axis the duration of TDF treatment in months. The dashed black line indicates the limit between 500 and 1,000 cp/mL for a successful sequencing. Below the quantitative curves, the HBV variants in the rt region and the s gene are given. Grey indicates HBV DNA derived and black HBV RNA derived sequences. WT = wild type; N.A. = no sequence available. * = stop mutation.*

3.5 HBV variants in the s gene

3.5.1 HBV s gene variants at baseline

We screened all sequences for the presence of s gene variants associated with changes in the rt region of the pol gene, which were sE164D/rtV173L, sI195M/rtM204V, sW196S/L/rtV204I and sW172L/rtA181T and for stop mutations. In 13 patients (Table 28) none of the assessed amino acid exchanges within the s gene were found at the beginning of TDF treatment. In 8 LAM resistant patients, s gene variants at positions s196 and s195 that associate with the rtM204I/V mutations were present at the initiation of TDF treatment (Table 28). The sE164D/rtV173L mutation was present in 2 patients (pat IDs 1-10, 1-58) and 1 rtV173L mutated patient (pat ID 1-21) had a sE164G substitution at this position. The s172L/rtA181T mutation was detected in 1 patient (pat ID 7-02). At baseline, stop codons in the s gene were present in 4 patients (pat IDs 1-11, 1-13, 1-58, 1-76), of whom 1 patient (pat ID 1-58) had a LAM resistance associated variant within the rt gene (Table 28).

In 1 patient with no HBV resistance mutation at baseline (pat ID 1-12), a stop codon at position s122* was found, which was produced by a deletion of 27 nucleotides between nt336 to nt364 in the s gene (Figure 18). The deletion resulted in a deletion of 9 amino acids from s113 to s121 and was located in the ‘a’ determinant of the s gene (Figure 18).

Table 28: HBV variants in the s gene at baseline

pat ID	aa s196	aa s195	aa s164	aa s172	aa s199	aa s182	aa s69	aa s216
1-10	W > L	I>M	E>D	W	W	W	W	L
1-11	W	I	E	W	W	W	W>*	L
1-12	W	I	E	W	W	W	W	L
1-13	W	I	E	W	W	W	W	L>*
1-15	W	I	E	W	W	W	W	L
1-19	W>S	I	E	W	W	W	W	L
1-21	W	I>M	E>G	W	W	W>L	W	L
1-24	W	I>M	E	W	W	W	W	L
1-25	W	I	E	W	W	W	W	L
1-32	W	I	E	W	W	W	W	L
1-34	W	I	E	W	W	W	W	L
1-37	W	I	E	W	W	W	W	L
1-44	W>L	I	E	W	W	W	W	L
1-54	W	I	E	W	W	W	W	L
1-58	W>*	I>M	E>D	W	W>*	W	W	L
1-61	W	I	E	W	W	W	W	L
1-63	W > L	I	E	W	W	W	W	L
1-65	W	I	E	W	W	W	W	L
1-68	W > L	I	E	W	W	W	W	L
1-76	W	I	E	W	W	W>*	W	L
1-60	W	I	E	W	W	W	W	L
1-66	W	I	E	W	W	W	W	L
1-02	W	I	E	W	W	W	W	L
7-02	W	I	E	W>L	W	W	W	L
7-09	W	I	E	W	W	W	W	L

aa = amino acid

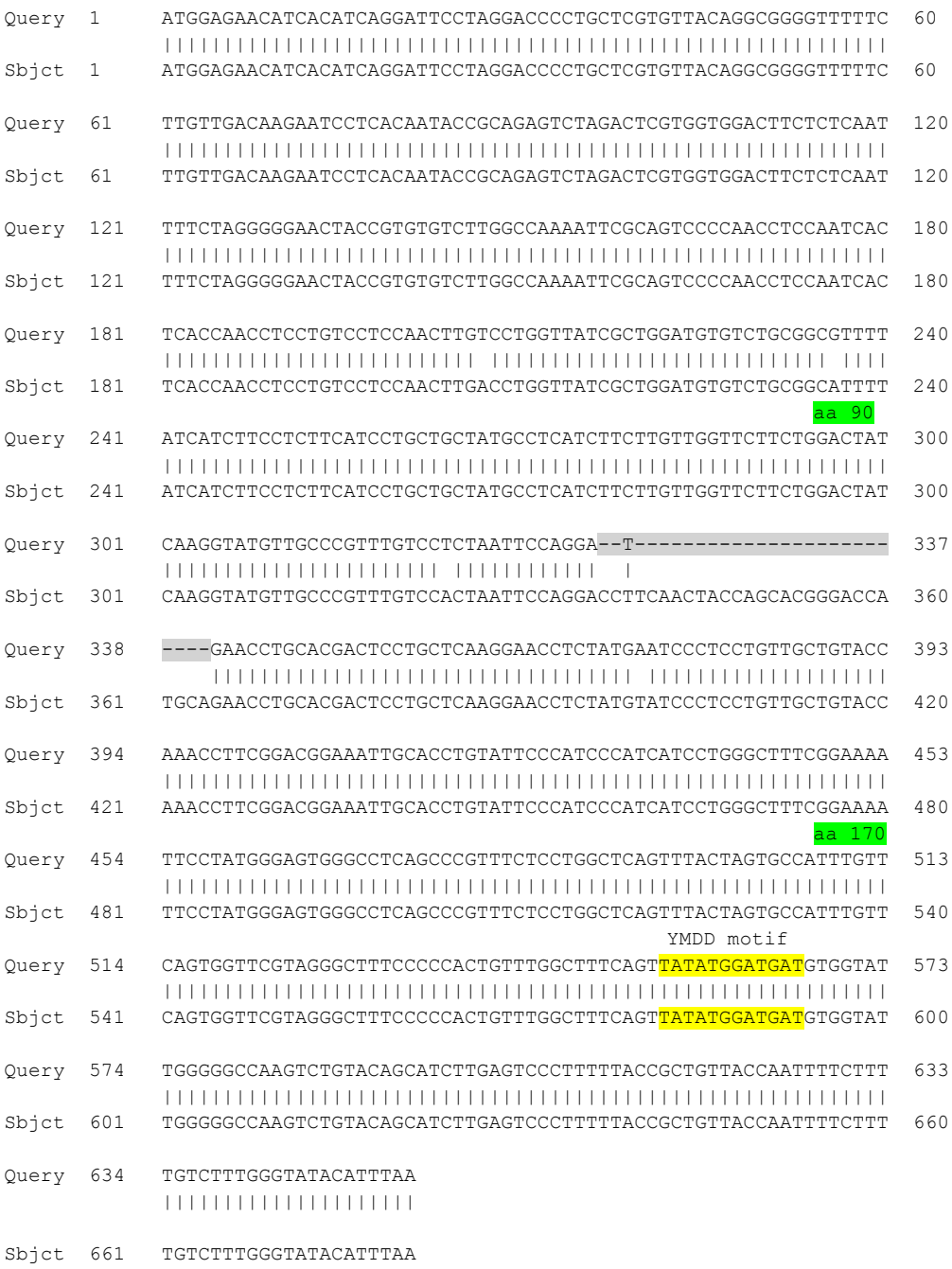


Figure 18: Deletion in the 'a' determinant of the HBV s gene in patient 1-12

Alignment of the baseline HBV DNA s gene sequence that harbors a deletion in the 'a' determinant between nt336 and nt364 (grey) compared to a WT sequence. The 'a' determinant is located between amino acid (aa) 90 and aa170 (green). The YMDD motif in the rt region is indicated in yellow.

3.5.2 HBV s gene variants during antiviral treatment

S gene variants with associated primary resistance mutations at positions sI195M/rtM204V, sI196S/L/rtM204I, and sW172L/rtA181T were found in 9 patients at baseline. Concordantly with the HBV resistance mutations, these variants remained detectable in HBV RNA derived sequences in 8 patients during follow-up (pat ID 1-10; Figure 17A, pat ID 1-15; Figure 17C, pat ID 1-24; Figure 17F, pat ID 1-44; Figure 17H, pat ID 1-58; Figure 17I, pat ID 1-63; Figure 17J, pat ID 1-68; Figure 17K, pat ID 1-21; Figure 17M) and disappeared early at month 6 only in 1 patient (pat ID 1-19; Figure 17D). Stop codons in the s gene, which were present at baseline, could be followed for a mean duration of 32.3 ± 17.8 (10 - 50) months on HBV RNA basis after HBV DNA had become undetectable (pat ID 1-11; Figure 16A, pat ID 1-13; Figure 16B, pat ID 1-76; Figure 16H, pat ID 1-58; Figure 17I). The deletion in the 'a' determinant (pat ID 1-12), that produced a sL122*, was present during follow-up in HBV RNA derived sequences until month 41 (Figure 16D). At month 53, Sanger sequencing detected only the non-deleted HBV variant (Figure 19). However, cloning of this region at this specific time point revealed the presence of the deleted HBV in all clones analyzed (n = 11). At months 6 and 42, sequencing of the HBV RNA further revealed additional s gene stop mutations at codon sC69* in this patient (pat ID 1-12; Figure 16D). This mutation was also found based on HBV RNA sequencing at month 12 in 1 patient (pat ID 1-19; Figure 17D). Stop mutations that occurred during follow-up were also detected based on HBV DNA. In 1 patient (pat ID 1-61; Figure 16F) with a transient rtA181T mutation at month 5, associated stop mutations at positions sW172 and sW182 were found during follow-up. Another patient (pat ID 7-02; Figure 17N) showed s gene stop mutations at positions W172 and C69 at month 27 based on HBV DNA sequencing.

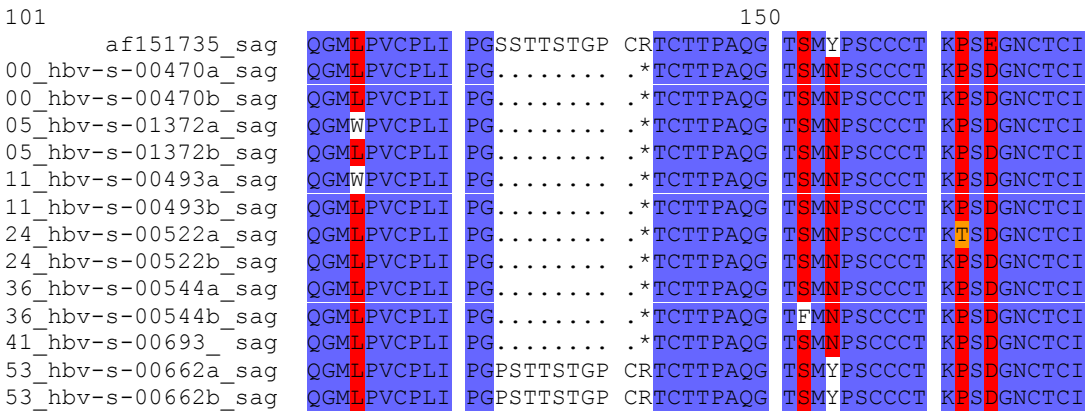


Figure 19: PileUp of the amino acid sequences of patient 1-12 with a deletion in the HBV s gene

PileUp of the amino acid sequences generated with HUSAR of patient 1-12 with a deletion in the 'a' determinant of the s gene. The deletion caused a stop codon at position sL122. The deletion was detectable at baseline in the HBV DNA derived sequence and during follow-up based on HBV RNA until month 41. The upper sequence (af151735) represents the reference HBV genome.

4 Discussion

4.1 Patient cohort

We composed our set of chronic HBV infected patients with failure to prior antiviral treatment with LAM (n = 4), ADV (n = 1) or both drugs (n = 20), which were assumed to be at increased risk for the development of rt and s gene variants under the selection pressure of highly potent polymerase inhibitors. We further focused on HBeAg positive patients (n = 21) because, based on previous reports, we concluded that the persistence of serum HBV RNA might be prolonged compared to HBeAg negative patients (van Bömmel 2015). HBsAg quantities at baseline and the quantitative HBsAg courses during follow-up were no selection criteria for our patient set because no association with serum HBV RNA levels was found in earlier reports (van Bömmel 2015). Consistentently, HBsAg levels in our set persisted in most patients (n = 22) during follow-up also after the decline of the HBV DNA and irrespecitive of HBV RNA levels. Only 1 patient showed loss of the HBsAg (pat ID 1-60) and for 2 patients (pat ID 7-02, 7-09) the information on follow-up was not available. We did not pre-select patients according to HBV genotypes because treatment response to NAs was not associated with the HBV genotype (Wiegand 2008). The included genotypes were A, B, D and E, of which D was most common (n = 19) reflecting the pre-dominance of patients from Central and Southern European countries in our cohort.

4.2 Quantification of serum HBV DNA, HBV flRNA and HBV trRNA

Multiple groups - using different methods for the detection- had previously described the presence of serum HBV RNA (Köck 1996, Rokuhara 2006, Jansen 2015, Wang 2016, Hatakeyama 2007, Huang 2010, Su 2001, Zhang 2004, van Bömmel 2015). Some groups used DNAse digestion before the reverse transcription of the HBV RNA to amplify only the reverse transcribed HBV cDNA (Rokuhara 2006,

Jansen 2015, Wang 2016). A limitation of this approach is a possible insufficient DNAse digestion and the RNA degradation during the DNA digestion. Another approach was the calculation of serum HBV RNA levels by subtracting serum HBV DNA levels in samples without previous reverse transcription from HBV DNA and HBV RNA serum levels after reverse transcription (Hatakeyama 2007, Huang 2010). Su *et al.* used anchored oligo(dT) primers for the selective RNA amplification and performed Southern blots to visualize the HBV RNA quantities, which however does not allow the absolute quantification of the serum HBV RNA (Su 2001, Zhang 2004).

The method we applied for the quantification of serum HBV RNA in our study targeted two poly(A) sites in the HBx region using specific RACE primers. A major advantage of this method is that DNAse digestion is not needed, because the applied RACE primers were designed to specifically detect the viral mRNAs. Within this method it however was not completely excluded, that the primer sequence complementary to the viral genome also annealed to HBV DNA strands. This was especially of concern for the 'x-RACE short' primer, because the complementary primer sequence for the binding to the viral genome was composed of 20 nucleotides, thus exceeding the oligo$(dT)_{17}$ tail in length (Table 5). Because in this study the qPCR based quantification of the HBV RNA served primarily as a screening method to determine HBV RNA serum levels sufficient for sequencing, we considered this not as a major restriction.

4.3 Quantitative courses of serum HBV DNA, HBV flRNA and HBV trRNA

All patients in our set had detectable serum HBV DNA, serum HBV trRNA and serum HBV flRNA at baseline. As demonstrated previously (van Bömmel 2015), the correlation of these serum markers at baseline was significant (Figure 10).

During TDF treatment, a decline of serum HBV DNA levels was observed in all patients and a virological response achieved in the majority. Interestingly, in 6

patients with ADV resistance at baseline conferring to positions rtN236T and rtA181T/V, the duration to suppressed HBV DNA serum levels < 400 cp/mL was prolonged (mean 24.5 ± 21.2 (1 – 56) months) and was not achieved in 2 patients until the end of follow-up at months 23 and 38 (pat IDs 1-32, 1-66). In contrast, the mean duration of serum HBV DNA persistence > 400 cp/mL in patients with LAM resistant HBV variants or no resistance variants was 7.6 ± 9.1 (0 – 34) months. This observation stands in line with studies that described a reduced susceptibility of TDF in the presence of ADV resistant HBV variants (Delaney 2006, van Bömmel 2010). In 2 patients with HBV DNA persistence it is however thinkable that a suppression of the HBV DNA < 400 cp/mL occured after the end of follow-up, because a complete virological response at later time points was observed in most ADV resistant patients (Kitrinos 2014).

During follow-up, serum HBV RNA persisted in 21 out of 25 patients after the decline of serum HBV DNA, which has been reported earlier (Rokuhara 2006, Jansen 2015, Wang 2016, Hatakeyama 2007, Huang 2010, Su 2001, Zhang 2004, van Bömmel 2015). Strikingly, 3 out of 4 patients who showed an early decline of serum HBV RNA during follow-up were HBeAg negative at baseline, which is in concordance with previous reports (van Bömmel 2015, Jansen 2015). Van Bömmel *et al.* reported a more rapid decline of the HBV trRNA and HBV flRNA at months 3 and 6 during antiviral treatment in HBeAg negative patients and demonstrated that the decrease of serum HBV RNA at these time points during antiviral treatment predicted HBeAg seroconversion in HBeAg positive patients (van Bömmel 2015). Concordantly in our cohort, 1 HBeAg positive patient (pat ID 1-60; Figure 17 E) with HBeAg loss during antiviral treatment also showed a rapid decrease of serum HBV RNA. HBeAg negativity and HBeAg seroconversion indicate the immunological control of the HBV infection, and were earlier associated with low HBV DNA loads and a good prognosis.

In some HBeAg negative cases however, high viral loads and progressive liver disease was observed. This led to the identification of specific mutations in the pre-core region (G1896A) and the basal core promoter (BCP; A1762T/G1764A), which suppressed HBeAg expression but actually enhanced the genome replication (Baumert 1996, Jammeh 2008, Kao 2003, Tong 2016). Also in HBeAg negative patients with effective viral suppression during antiviral treatment, the rates of HCC development were estimated with around 2.8% after 5 years (Papatheodoridis 2015). BCP mutations associated with a higher risk of HCC development (Kao 2003) and, independently of the HBeAg status, with HBV related liver cirrhosis (Tseng 2015). Assuming that pgRNA transcription might be enhanced in patients harboring pre-core and BCP mutations (Baumert 1996, Jammeh 2008), it would be interesting to investigate whether serum HBV RNA is persistent in those patients. In HBeAg negative patients and HBeAg seroconverters with persistent serum HBV RNA during NA treatment, serum HBV RNA sequencing might be a promising tool to study the presence of pre-core and BCP mutations in these patients. In our cohort, only in 1 HBeAg negative patient (pat ID 1-21; Figure 17M) serum HBV RNA persisted measurable after the decline of serum HBV DNA at month 12, but the information on pre-core and BCP mutations was not available

4.4 Sequencing of serum HBV RNA as novel method for HBV genome analysis

In this study, we were the first to demonstrate the sequence analysis of HBV RNA from serum of chronically HBV infected individuals as method to observe the HBV genome during NA treatment when serum HBV DNA sequencing became impossible. For this, the extracted HBV RNA was reversely transcribed using specific RACE primers for two HBV RNA species, the HBV flRNA and trRNA, and then

analyzed by Sanger sequencing for mutations in the rt region and the overlapping s gene.

Although we could not completely exclude the possibility of HBV DNA and HBV RNA co-amplification with the applied primers in the reverse transcription step (chapter 4.2), we assumed a high specificity of the detected HBV RNA because also in samples with undetectable HBV DNA, sequencing was possible after the reverse transcription of the HBV RNA (Figures 13 and 14). We further mostly used samples with low or undetectable HBV DNA (< 500 – 1,000 cp/mL) determined by qPCR for the sequencing of the HBV RNA, which made the co-amplification of HBV DNA and HBV RNA molecules during the PCR amplification unlikely. Only in 3 out of the 68 cDNA derived sequences, HBV DNA levels were > 1,000cp/mL determined by sensitive qPCR but not applicable for sequence analysis based on HBV DNA at this time point. In these samples with marginal HBV DNA, there is a probability of HBV DNA and HBV cDNA co-amplification during the PCR based amplification, which should be taken to account if a strict comparative analysis of HBV DNA and HBV RNA derived sequences at a single time point is pursued.

With the here performed reverse transcription of the HBV RNA using RACE primers, the poly(A) signals for the flRNA and trRNA transcripts located upstream the HBx region (Hilger 1991) were targeted (van Bömmel 2015). Because all HBV mRNAs mature at these poly(A) sites, this primer system allowed the reverse transcription of all HBV RNA transcripts (Figure 1). Our sequencing system spanned around two thirds of the HBV genome including the entire rt and preS1/preS2 region. Although the applied sequencing system did not embrace the entire pgRNA, it is likely that the detected HBV RNA sequences in our study were pre-genomic, considering the data presented by Jansen *et al.* and Wang *et al.* who showed that the pgRNA

represented the dominant molecule in HBV RNA positive samples (Jansen 2015, Wang 2016).

Sequencing of the HBV DNA is widely used for the genotyping of the HBV genome at diagnosis and for the detection of HBV resistance variants during antiviral treatment when a virological breakthrough had occurred. For a successful analysis, HBV DNA levels of 500 – 1,000 cp/mL are commonly required (Valsamakis 2007), a sensitivity we could reach with our semi-nested PCR system for the HBV DNA. Below this limit, the analysis of the HBV genome in serum samples is commonly not possible with Sanger sequencing. The here described method of reverse transcribed serum HBV RNA sequencing might overcome this limitation. We demonstrated that sequence analysis based on reverse transcribed HBV RNA was possible for a mean duration of 33.9 ± 12.7 (16 - 65) months during follow-up, thus exceeding the duration of HBV DNA sequencing, which was possible only for a mean of 6.0 ± 4.5 (0-13) months (Figure 15). Resistance variants found on HBV DNA basis were also present in most of the HBV cDNA derived sequences, which underlines the homology between HBV DNA and HBV RNA derived sequences. The homologies between serum HBV DNA and serum HBV RNA derived sequences were further investigated in cooperation with the Max-Planck-Institute for Informatics (Saarbrücken, Germany) by calculating the variability of nucleotide exchanges in the rt region (Beggel 2014). The preliminary data for 20 patients shows similar median values of nucleotide exchanges between serum HBV DNA and serum HBV RNA derived sequences (Supplementary Figure 1). This suggests that the diversity of the quasispecies represented by serum HBV RNA sequencing might well reflect the serum HBV DNA derived sequences, which however should be researched more comprehensively.

It was not possible to reveal whether HBV resistance variants become detectable by serum HBV RNA sequencing before a virological breakthrough occurs.

This was, because no patient in our set developed resistance to TDF after long-term treatment up to 76 months, which is in line with the literature (Marcellin 2014). 1 patient had a re-increase of the HBV DNA at month 17 (pat ID 1-37), which we assumed was due to non-adherence to the antiviral treatment at this time point. Sequencing of serum HBV DNA and serum HBV RNA was not possible in this HBeAg negative patient before and after. To answer the question at what time points HBV resistance mutations are detectable by serum HBV RNA sequencing, the retrospective analysis of patient samples with known resistance during treatment with LAM, ADV or ETV would be a suitable approach. Especially for patients receiving the first-line NA ETV, for which resistance was found in around 1.2% of treatment naïve patients after 5 years (Tenney 2009), the sequencing of serum HBV RNA might be a helpful tool to early detect HBV resistant variants.

A general limitation of the chosen Sanger sequencing approach was that only dominant HBV variants, representing around 20% of all variants, were detectable. Thus, minor HBV resistance variants, which might arise during antiviral treatment, were probably missed and a detailed characterization of the quasispecies diversity was limited. On the other hand, it is questionable whether such minor species play a role in the course of the disease or not. It was further not possible to discriminate, whether mutations detected at different positions were present in the same or in different sequences (Chevalier 2012, Rodriguez-Frias 2013, Lowe 2016). For the detection of minor HBV subclones and a more detailed observation of the HBV quasispecies, sequencing techniques like next generation sequencing (NGS) might provide a more sensitive approach. The main challenges of these techniques are higher error rates, the identification of true insertions and deletions as well as data processing and analysis (Chevalier 2012, Rodriguez-Frias 2013). Besides, NGS is more cost intensive and the clinical significance of the detected minor resistance HBV

variants is elusive (Margeridon-Thermet 2009, Lowe 2016). However, sequencing of reverse transcribed serum HBV RNA with more sensitive techniques might provide deeper insights into the evolution of the HBV quasispecies during antiviral treatment, and should be further investigated.

4.5 Evolution of HBV variants in the rt region

HBV DNA derived sequences could be obtained at baseline for all patients and mainly at early time points during follow-up, whereas HBV RNA based sequences represented mostly later time points. At baseline, 15 out of 25 patients harbored at least one of the known resistance mutations conferring to the prior antiviral treatment with LAM and/or ADV. In concordance with the literature, HBV variants associated with TDF resistance were not observed at baseline or during follow-up (Marcellin 2014), based on HBV DNA and HBV RNA sequencing. 10 patients showed no primary resistance HBV mutations at baseline although a virological breakthrough had occurred during prior antiviral treatment (Table 27). Besides the confirmed and extensively studied primary and compensatory resistance mutations, many other HBV variants with suspected resistance to polymerase inhibitors have been described, among them the substitutions rtS78T, rtV207M/I, rtL229M and rtM309K (Cento 2013). In our cohort, in 5 patients with no known resistance mutations at baseline the substitutions rtS78T (n = 1), V207M/I (n = 1) and rtM309K (n = 3) could be detected, but the impact of these variants on treatment failure - also in our cohort - is elusive. As described in chapter 4.3, it is also possible that minor HBV resistant clones were not detected because of the limited sensitivity of the Sanger sequencing approach. In a study by Margeridon-Thermet *et al.*, ultra-deep pyrosequencing (UDPS) revealed the presence of LAM resistant HBV variants in serum samples, which were not detected by Sanger sequencing, but the clinical relevance of these minor variants remained unclear (Margeridon-Thermet 2013).

In 13 patients with resistance mutations at baseline, the same variants persisted on HBV RNA basis for a median duration of 20.5 (3 - 39) months after the HBV DNA had become undetectable. The persistence of LAM resistance variants after the discontinuation of LAM treatment was previously reported and assigned to the slow emergence of resistance in HBV compared to other chronic viruses (Margeridon-Thermet 2013). In our cohort, excluding the 1 patient with TDF + LAM co-treatment (pat ID 1-66), LAM and ADV resistant variants could be followed for a mean duration of 26.5 ± 15.8 (0 - 50) months during TDF treatment, mostly in HBV RNA derived sequences. Because the information on the exact time point to prior NA treatment discontinuation was not available, the persistence of these HBV variants might have been even longer. In 1 patient treated with TDF + LAM the rtN236T + rtA181V mutations were detectable at baseline (pat ID 1-66; Figure 17L). The LAM resistance mutation rtA181V remained detectable until the end of follow-up at month 38 in serum HBV RNA derived sequences. The rtN236T mutation instead was present until month 10 after the discontinuation of ADV treatment but disappeared during follow-up.

In 3 patients (pat ID 1-12; Figure 17B; pat ID 1-15; 17C, pat ID 1-63; 17J), additional resistance mutations at positions rtM204V, rtL180M and rtV173L were found during follow-up. It is likely that this reflects variations in the compositions of dominant HBV clones within the quasispecies rather than newly acquired mutations. The comparative cloning analysis of 1 patient (pat ID 1-15), whose course of mutations is shown in Figure 17C, indeed revealed pre-dominance of HBV clones harboring the rtN236T at baseline (based on HVB DNA) and the rtM204V + rtL180M mutations at months 25 (based on HBV RNA). Because we demonstrated that sequences derived from serum HBV RNA show a high homology to those sequences

derived from serum HBV DNA we assumed that serum HBV DNA and serum HBV RNA have the same origin fom the HBV cccDNA.

4.6 Evolution of HBV stop mutations in the s gene

Stop codons in the s gene, including the stop mutation caused by the deletion in the ‘a’ determinant, were present in 5 patients at baseline. Interestingly, 4 of those patients (pat ID 1-11; Figure 16A, pat ID 1-13; Figure 16B; pat ID 1-54; Figure 16 D; pat ID 1-76; Figure 16 H) did not harbor any of the assessed s gene variants associated with resistance to NA treatment. The stop codons remained stably detectable during TDF treatment for a mean duration of 37 ± 19.4 (12 - 65) months. The sC69*/rtS78T mutation, which was found in 4 patients (pat IDs 1-11, 1-54, 1-19, 7-02) in our cohort, was described to arise in the natural course of HBV infection (Saha 2014). The variant was further associated with failure to ADV treatment even in the absence of known ADV resistance mutations (Cento 2013). In our set, the sC69*/rtS78T mutation was found in 1 patient (pat ID 1-11¸ Figure 16A) at baseline and during follow-up based on HBV RNA sequencing. This patient was pre-treated with LAM and ADV and harbored none of the assessed resistance mutations. The mutation was additionally found only during TDF treatment based on serum HBV DNA sequencing in 1 patient (pat ID 7-02; Figure 17N) at month 30. The variant further appeared in 2 patients at month 11 (pat ID 1-19; Figure 17D) and at months 6 and 41 (pat ID 1-54; Figure 16D) based on serum HBV RNA sequencing during TDF treatment. Of note, 1 patient (pat ID 1-19; Figure 17D) with emergence of the rtS78T/sC69* mutation in our set was treatment naïve for ADV, thus the mutation was either a natural HBV variant or became selected during TDF treatment. In 1 patient, the sC69* mutation was combined with the sL122* stop mutation (pat ID 1-54; Figure 16D), which was a result of a deletion in the ‘a’ determinant, a region assumed to be the dominant immunological target of polyclonal antibodies (Pollicino 2014). Both

mutations might affect the formation of the ‘a’ determinant by causing N-terminal truncations, thus impairing HBsAg antibody binding properties. Both variants were further located to a region, in which 3’ truncations caused by deletions and stop mutations were suspected to generate proteins that act as transcriptional activators (Lauer 1992). In 3 additional patients, the HBsAg stop mutations sL216* (pat ID 1-13; Figure 17B), rtV191I/ sW182* (pat ID 1-76; Figure 16H) and a combination of the rtM204V/ sW196* and rtV208I/ sW199* substitutions (pat ID 1-58; Figure 17I) were found at baseline and persisted on HBV RNA basis after the decline of serum HBV DNA. In 1 patient (pat ID 1-13; Figure 16B), serum HBV RNA based sequencing revealed the appearance of the stop mutations rtV191I/sW182* at month 39. In none of the patients harboring s gene stop codons, a drop of HBsAg quantities as indicator for a secretory defect, was observed. Because of the small number of patients with stop codons in this study, we could not determine whether the presence of those influenced serum HBV DNA levels (Pollicino 2012). Because all patients were pre-treated, we further could not determine whether the detected HBsAg stop mutations were already present before or acquired during NA treatment, which was a limitation for the observation of the natural course of HBV s gene variants during TDF treatment. We also could not associate the presence of these variants, which all were discussed in the pathogenesis of HCC development, with the clinical outcome of our cohort because the clinical data of the follow-up was not available. For a better understanding of the evolution of these variants during antiviral treatment, and their possible influence on disease progression, future studies should prospectively determine the status of s gene variants before and during NA treatment.

4.7 Conclusion

In summary, this study demonstrates for the first time that the sequencing of reverse transcribed HBV RNA from patient serum is possible and that it is a suitable

method to assess the evolution of HBV variants during antiviral treatment, especially in the group of HBeAg positive patients. In this study, we provided insights into the evolution of HBV variants that circulated in the serum of chronically HBV infected patients during treatment with TDF. In patients achieving a strong suppression of HBV DNA during treatment with TDF, resistance associated HBV variants acquired during previous NA treatments seem to persist for long periods. This was the case for variants with resistance against ADV, which have a possible cross-resistance to TDF, but most interestingly also for those associated with resistance against LAM, which should be no longer under selection pressure during TDF treatment. This persistence without selection pressure indicates a high conservation of those variants in the cccDNA of the infected individuals. Moreover, the long period of persistence of those variants also sheds light on the half-life time of HBV cccDNA, which is probably long. Further studies are needed to investigate this observation more in detail. For a more precise study of the quasispecies, sequencing approaches with higher sensitivity using reverse transcribed HBV RNA (for example NGS) might overcome the currently limited sensitivity of the Sanger sequencing approach. Despite this methodological restriction, sequencing of serum HBV RNA represents an interesting tool to study the composition of HBV variants during antiviral treatment and should be further investigated for clinical applications. Besides the detection of resistance associated HBV variants, the detection of s gene HBV variants, that associated with an increased risk for HCC development, might be one of the most interesting applications of the here presented method of serum HBV RNA sequencing and should be researched in future projects.

III Summary

Dissertation zur Erlangung des akademischen Grades

Dr. med.

Sequencing of serum hepatitis B virus (HBV) RNA as a novel method for the genome analysis of HBV

Sequenzierung der Hepatitis-B-Virus (HBV) RNA im Serum als neue Methode der Genomanalyse des HBV

eingereicht von: Laura Schmalbrock

angefertigt an der Klinik und Poliklinik für Gastroenterologie und Rheumatologie, Sektion Hepatologie, des Universitätsklinikums Leipzig AöR

betreut von:

Prof. Dr. med. Th. Berg

OA Dr. med. F. van Bömmel

OA Dr. med. S. Böhm

Monat und Jahr der Einreichung: November 2016

The genome of hepatitis B virus (HBV) can be assessed by sequence analysis of HBV DNA in serum. In most chronic HBV infected patients treated with potent nucleos(t)ide analogues (NAs) this approach however is limited by the fast decrease of serum HBV DNA during NA treatment. In contrast, HBV RNA was shown to persist in serum of some HBV infected individuals receiving NA treatment.

In this study, we established the sequencing of serum HBV RNA as a method for the monitoring of HBV variants during NA treatment after the decrease of serum HBV DNA to undetectable levels. Using this approach, we studied the evolution of

HBV variants in follow-up serum samples (n=156) of 25 patients treated with the potent polymerase inhibitor tenofovir (TDF) as second or third-line treatment.

In our cohort, specific reverse transcribed full-length and truncated HBV RNA remained detectable with real-time PCR for long periods in most serum samples, also after the decline of HBV DNA during treatment with TDF. The HBV genome could be analyzed based on serum HBV DNA sequencing in 61 serum samples for a mean duration of 6.0 ± 4.5 (0 – 13) months. After this, sequencing of reverse transcribed serum HBV RNA allowed the analysis of the HBV genome for an additional mean duration of 33.9 ± 12.7 (16 - 65) months in 68 serum samples.

The comparison of serum HBV DNA and serum HBV RNA derived sequences showed a high homology. In most patients, acquired HBV resistance variants in the reverse transcriptase (rt) region of the polymerase gene were detectable on HBV DNA and HBV RNA basis. Serum HBV RNA sequencing further revealed a long persistence of these variants during TDF treatment (mean duration of 26.5 ± 15.8 (0 – 50) months), which indicates a high conservation in the cccDNA of the infected individuals. Also HBV stop mutations in the small surface (s) gene, which were discussed in the pathogenesis of hepatocellular carcinoma (HCC), were present at baseline in 5 patients and remained detectable on HBV RNA basis during follow-up.

In this study, we demonstrated that sequencing of reverse transcribed HBV RNA from patient serum is a suitable method to assess HBV variants during NA treatment. We further provided insights into the evolution of HBV variants during strong suppression of the viral replication with the polymerase inhibitor TDF. Future studies should investigate more comprehensively the clinical application of the here presented method of serum HBV RNA sequencing for the early detection of resistant HBV variants during NA treatment and the observation of HBV s gene variants related to HCC development.

IV. References

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V. List of Figures

VI. List of Tables

VII. Supplement

Figure S1: Comparison of nucleotide exchanges in HBV DNA and HBV RNA derived sequences

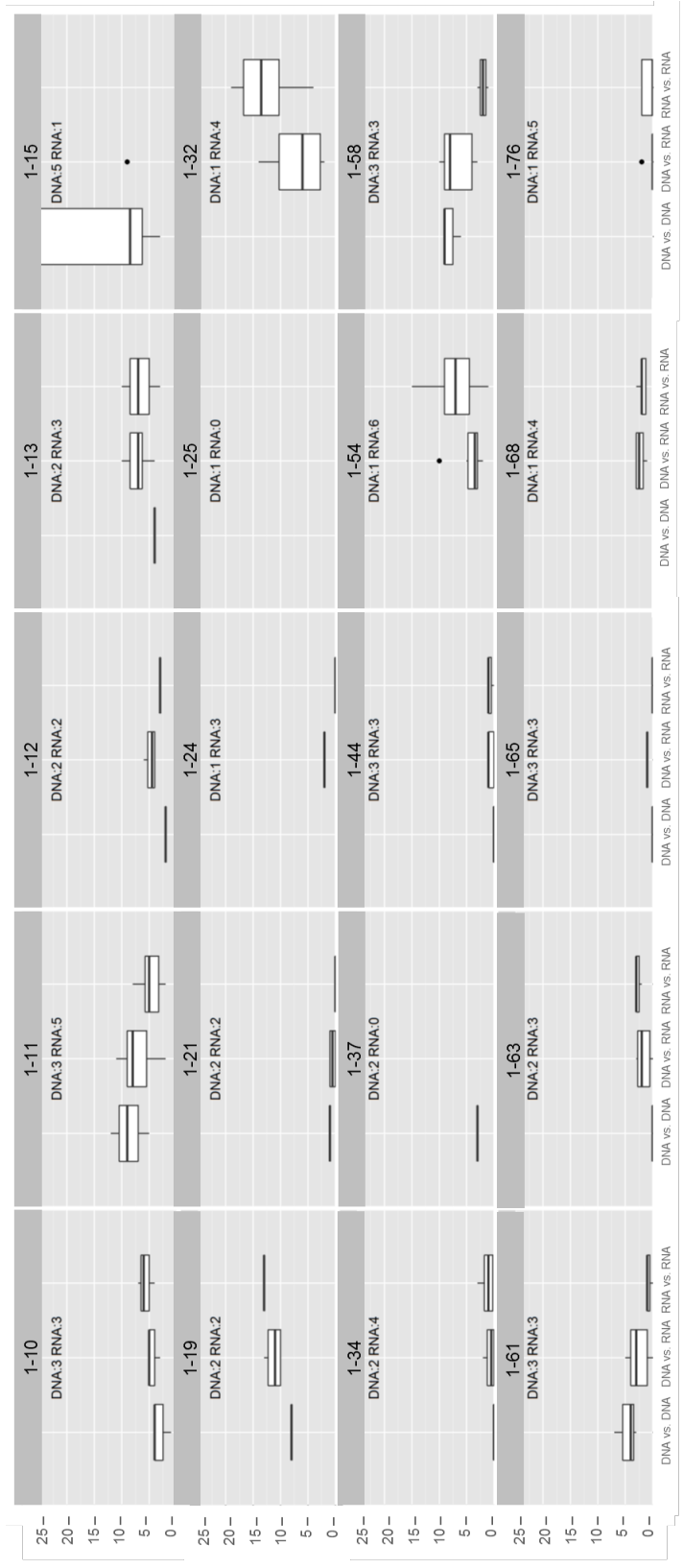

Figure S1

The box-plots show the medians of the nucleotide exchanges in the rt region of 20 patients (numbers in grey bars indicate pat ID) receiving antiviral treatment with TDF. The medians were calculated between HBV DNA derived sequences (DNA vs DNA; left side in each figure), between HBV DNA and HBV RNA derived sequences (DNA vs. RNA; middle in each figure) and between HBV RNA derived sequences (RNA vs. RNA; right side in each figure). The y-axis on the left gives the numbers of nucleotide exchanges. The numbers above the plots give the numbers of the included HBV DNA and HBV RNA derived sequences.

VIII Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar eine Vergütung oder geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren. Die aktuellen gesetzlichen Vorgaben in Bezug auf die Zulassung der klinischen Studien, die Bestimmungen des Tierschutzgesetzes, die Bestimmungen des Gentechnikgesetzes und die allgemeinen Datenschutzbestimmungen wurden eingehalten. Ich versichere, dass ich die Regelungen der Satzung der Universität Leipzig zur Sicherung guter wissenschaftlicher Praxis kenne und eingehalten habe.

.....	.....
Datum	Unterschrift

IX Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

X. Publications

Publications

F. van Bömmel*, **L. Schmalbrock***, B. Beggel, D. Deichsel, D. Hüppe, Th. Berg, S. Böhm. *Sequence analysis of hepatitis B virus (HBV) RNA in serum as a novel method for monitoring the evolution of the HBV genome during antiviral treatment*. (in preparation)

* equal authorship contribution

Conference contributions

2016

Bill M., Jentzsch M., Schuhmann L., Grimm J., Knyrim M., **Schmalbrock L**., Schubert K., Cross M., Vucinic V., Franke G.-N., Pönisch W., Behre G., Lange T., Niederwieser D., Schwind S. *The prognostic impact of differential GLI1 expression in patients with acute myeloid leukemia after non-myeloablative allogeneneic hematopoietic stem cell transplantation.* (DGHO annual meeting 2016, V1012)

Gaber T., Bill M., Jentzsch M., Schubert K., Weidner H., Grimm J., Schulz J., Kloss L., **Schmalbrock L**., Bonifacio L., Wildenberger K., Pönisch W., Vucinic V., Franke G.-N., Lange T., Cross M., Behre G., Niederwieser D., Schwind S. *Differential expression of Pri-miR-320a impacts on outcome in acute myeloid leukemia patients undergoing non-myeloablative allogenic stem cell transplantation.* (DGHO annual meeting 2016, P190)

Grimm J., Dick T., Bill M., Jentzsch M., Schulz J., **Schmalbrock L**., Bonifacio L., Knyrim M., Schubert K., Cross M., Pönisch W., Vucinic V., Behre G., Franke G.-N., Niederwieser D., Schwind S. *The Allelic Ratio (AR) of DNMT3A R882 mutations (Mut) determined by digital droplet PCR (ddPCR) is a potential prognostic factor in Acute Myeloid Leukemia (AML),* (DGHO annual meeting 2016, V384)

Jentzsch M., Bill M., Schumann L., Leiblein S., Schubert K., Grimm J., Bergmann U., Pleß M., **Schmalbrock L.**, Schulz J., Knyrim M., Franke G.-N., Behre G., Pönisch W., Vucinic V., Niederwieser D., Schwind S. *The surface antigen profile in patients (pts) with Acute Myeloid Leukemia (AML) identifies subgroups characterized by distinct biological and clinical features.* (DGHO annual meeting 2016, V783)

J. Grimm, T. Dick, M. Bill, M. Jentzsch, J. Schulz, **L. Schmalbrock**, L. Bonifacio, M. Knyrim, K. Schubert, M. Cross, W. Pönisch, V. Vucinic, G. Behre, G.-N. Franke, D. Niederwieser, S. Schwind. *Assessment oft the allelic ratio of DNMT3A R882 mutations in Acute Myeloid Leukemia by digital droplet PCR.* 20th annual meeting EHA 2016, Abstract E904)

2015

M. Bill, M. Jentzsch, L. Schuhmann, J. Grimm, M. Knyrim, **L. K. Schmalbrock**, K. Schubert, M. Cross, V. Vucinic, G.-N. Franke, W. Poenisch, G.Behre, T. Lange, D. Niederwieser, S. Schwind. *High Expression of the Hedgehog Transcription Factor GLI1 Is Associated with Improved Outcomes in Patients with Acute Myeloid Leukemia Undergoing Hematopoietic Stem Cell Transplantation after Non-Myeloablative Conditioning.* (ASH 57th annual meeting 2015, Blood 2015, 126:2032.)

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M. Jentzsch, M. Bill, S. Leiblein, K. Schubert, M. Pleß, U. Bergmann, K. Wildenberger, **L. Schmalbrock**, M. Cross, W. Pönisch, G.-N. Franke, V. Vucinic, G. Behre, D. Niederwieser, S. Schwind. *The prognostic impact of CD34+/CD38- cell burden at diagnosis and during disease course in acute myeloid leukemia patients undergoing allogeneic stem cell transplantation* (EBMT 41th annual meeting 2015, Abstract P035)

J. Grimm, M. Bill, H. Weidner, M. Knyrim, **L. Schmalbrock**, M. Jentzsch, K. Schubert, A. Wurm, D. Gerloff, M. Cross, T. Lange, G. Behre, D. Niederwieser and S. Schwind. *microRNA (miR)-9 directly downregulates the oncogenic transcription factor ets related gene (ERG) & high expression associates with improved outcomes in acute myeloid leukemia (AML)* (Annual Meeting DGHO 2015)

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H. Weidner, M. Bill, **L. Schmalbrock**, M. Jentzsch, L. Kloss, T. Gaber, L. Bonifacio, K. Schubert, K. Wildenberger, D. Gerloff, V. Vucinic, G.-N. Franke, T. Lange, M. Cross, S. Fricke, G. Behre, D. Niederwieser and S. Schwind. *High Expression of Mir-9 down-Regulates the Poor Outcome Prognosticator ERG and Associates with Reduced Relapse-Rates in Acute Myeloid Leukemia* (ASH 56th annual meeting 2014, Abstract 1575, Blood 2014, 124:1575)

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M. Jentzsch, M. Bill, S. Leiblein, H. Weidner, **L. Schmalbrock**, K. Wildenberger, M. Cross, M. Pleß, U. Bergmann, N. Nehring-Vucinic, N. Jäkel, R. Krahl, W. Pönisch, G.-

N. Franke, V. Vucinic, G. Behre, D. Niederwieser and S. Schwind. *A high CD34+/CD38-cell burden at diagnosis of acute myeloid leukemia predicts worse outcome in patients undergoing reduced intensity conditioning allogeneic stem cell transplantation.* (Annual meeting DGHO 2014, Abstract 252)

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F. van Boemmel, **L. Schmalbrock**, D. Deichsel, E. Schott, Th. Berg, S. Böhm. *Sequence analysis of serum hepatitis B virus (HBV) RNA represents a novel method for HBV genome analysis of HBV variants in patients achieving undetectable HBV DNA during antiviral treatment.* (64th AASLD annual meeting 2013; Hepatology 2013 Vol 58, Abstract 1037)

L. Schmalbrock, S. Böhm, D. Deichsel, E. Schott, Th. Berg, F. van Bömmel. *Monitoring the evolution of Hepatitis B Virus (HBV) variants by sequence analysis of HBV RNA in patients achieving undetectable HBV DNA during antiviral treatment.* (EASL Monothematic Conference: Translational Research in Chronic Viral Hepatitis 2013, Abstract P30)

Oral presentations

Isocitrat dehydrogenase mutations in patients with Acute Myeloid Leukemia undergoing Hematopoietic Stemcell Transplantation after Reduced Intensity Conditioning (Annual meeting of the DGHO 2014, V110)

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