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Identification of host and virologic factors for HBV- and HCV-associated pathogenesis

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Lisa Kuhnhenn
aus Hanau

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Dekan:	Prof. Dr. Clemens Glaubitz
Erstgutachter:	Prof. Dr. Rolf Marschalek
Zweitgutachter:	Prof. Dr. Christoph Sarrazin
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Für meine Lieben

Wenn eine Idee am Anfang nicht absurd klingt, dann gibt es keine Hoffnung für sie.
Albert Einstein

Abstract

Infections with the hepatitis B virus (HBV) or the hepatitis C virus (HCV) lead to complications like the development of cirrhosis or hepatocellular carcinoma. These complications end up in 887,000 and 500,000 deaths per year, respectively. Since the development of new direct acting antiviral agents for HCV in the past years a complete cure of an HCV infection can be achieved in the majority of the patients. In contrast, a complete cure of a chronic HBV infection still remains a challenging problem as current treatment regimens mainly suppress the viral replication and cccDNA as well as integrated DNA still persist in these patients. Several viral and host factors were described to impair the efficacy of treatment regimens or influence the course of the infection. Therefore, in this work viral factors as well as host factors were investigated in HBeAg negative chronic HBV infected patients and in chronic HCV infected patients.

In the present study, it was demonstrated that mutations and/or deletions in the HBV basal core promoter (BCP), the precore and the preS domain occur in a genotype-specific pattern in HBeAg negative HBV infected patients. While the BCP double mutation A1762T/G1764A was found with the highest prevalence in genotype E infected patients, the precore mutation G1896A occurred mostly in genotype B infected patients. Variants in the preS domain could be detected with the highest frequency in patients infected with genotype C. In patients, who had to start an antiviral therapy during the course of the disease, mutations in the precore region could be detected with a higher frequency in the samples right before treatment start in comparison to the baseline sample.

While different HBV genotypes and preS mutations were not associated with HBV-DNA serum levels, precore mutations as well as BCP mutations were significantly associated with HBV-DNA levels. Furthermore, precore mutations showed lower and preS mutations higher HBsAg levels. The HBsAg serum levels varied significantly among the different genotypes. Since HBsAg levels < 1000 IU/ml have been described as a prognostic marker in several studies, the prevalence of patients with HBsAg < 1000 IU/ml was analyzed among the genotypes A - E. While most of the patients infected with HBV genotype B had HBsAg < 1000 IU/ml, only a few patients infected HBV genotype E and A had HBsAg < 1000 IU/ml.

Furthermore, HBV genotype A genomes derived from patients harboring a) A1762T/ G1764A (BCP), b) G1896A/G1899A (precore), c) 15 aa deletion in preS1, d) no mutation (reference genome) were cloned and analyzed *in vitro*. An enhanced expression but reduced

secretion of viral genomes was found in the preS-deletion- and the precore-variant. No differences in the HBsAg production and secretion were observed in the cloned precore- or BCP-variant, while the preS-deletion-variant was characterized with an elevated HBsAg release.

Regarding the secretion of viral and subviral particles, a genotype-specific pattern of the L/M/SHBs ratio was detected in the serum of patients infected with genotypes A - E. This pattern did not change in the serum of patients, who started antiviral treatment. Secreted HBsAg containing particles displayed a higher density as well as a higher filaments/spheres ratio in genotypes B and D compared to genotypes A, C and E.

Population-based and deep sequencing revealed large deletions in the preS domain or preS2 start codon mutations in a certain number of the viral genomes. Theoretically, these mutations/deletions should influence the molecular weight of the expressed protein or abolish the expression of the protein at all. In contrast, LHBs/MHBs were detectable and appeared at the same molecular weight in these patient samples in comparison to patient samples without these mutations. Furthermore, in the *in vitro* analyses comparing the reference genome and the preS1-deletion genome, it was shown that the deletion indeed influenced the molecular weight of LHBs. Therefore, HBsAg might be expressed from a genetically different source than the released viral genomes, meaning the integrated DNA.

Additionally, in the present study the prevalence of resistance associated substitutions (RASs) in the viral genes NS3, NS5A and NS5B of chronic HCV infected patients was analyzed in correlation to single nucleotide polymorphisms (SNPs) in the interferon-λ4 (IFNL4) gene of the infected patients. No significant correlation was found between IFNL4 SNPs and RASs within NS3/NS5B in the present cohort. In contrast, the frequently detected NS5A RAS Y93H could be significantly associated with beneficial IFNL4 SNPs and a high baseline viral load in HCV genotype 1-infected patients.

Taken together, the present study demonstrated that viral genome mutations as well as the morphology of secreted particles occur in a genotype-dependent pattern in HBeAg negative HBV infected patients with no need of antiviral therapy. As the amount of serum qHBsAg levels varied among the different genotypes, the HBsAg cut-off < 1000 IU/ml should be adapted individually among the various genotypes. Because the composition of the secreted subviral particles varied between the different genotypes, a genoytpe-specific immune-response might be induced in these patients. Additionally, the results of the present study indicate that in HBeAg negative HBV infected patients with mutations or deletions in the preS domain MHBs and LHBs might be expressed from the integrated DNA and therefore from a genetically different source than the released viral genomes.

Aside from that, the finding of a significant association of the NS5A RAS Y93H with beneficial IFNL4 SNPs in chronic HCV infected patients may explain a lack of a correlation or an inverse correlation of treatment response with the IFNL4 genotype in some NS5A inhibitor-containing IFN-free regimens.

Zusammenfassung

Eine chronische Infektion mit dem Hepatitis B Virus (HBV) oder dem Hepatitis C Virus (HCV) kann Komplikationen wie die Entwicklung einer Fibrose, Cirrhose oder eines hepatozellulären Karzinoms zur Folge haben. Jährlich sterben an diesen Komplikationen weltweit 887.000 Menschen ausgelöst durch eine HBV-Infektion beziehungsweise bis zu 500.000 Menschen durch eine HCV-Infektion. Sowohl HBV als auch HCV können beispielsweise durch Blut und Blutprodukte, Transplantation infizierter Organe, intravenösen Drogenkonsum, unzureichende Sterilisation medizinischen Materials, sexuellen Kontakt, sowie von infizierten Müttern auf ihre ungeborenen Babys übertragen werden. Daher ist es von besonderer Wichtigkeit, sich gegen eine Infektion zu schützen. In jedem Fall sollten Maßnahmen wie Händedesinfektion, Sterilisation und Desinfektion von Injektions- oder OP-Besteck sowie die Benutzung von Kondomen eingehalten werden. Des Weiteren empfiehlt die Weltgesundheitsorganisation (WHO) seit 1992 eine Impfung aller neugeborenen Babys gegen HBV. Ein funktionelle Vakzinierung gegen HCV konnte bisher noch nicht entwickelt werden.

Allerdings wurden in den letzten Jahren zahlreiche neue direkt wirkende antivirale Substanzen (direct acting antivirals; DAAs) zur Therapie der HCV-Infektion entwickelt. Diese führen in der Mehrheit der Patienten zu einer anhaltenden virologischen Ansprechrate und somit zu einer kompletten Eradikation des Virus (Heilungsraten von mehr als 95 % in praktisch allen Patientengruppen). Im Gegensatz dazu kann mit den aktuell verfügbaren Medikamenten zur Therapie einer chronischen HBV-Infektion keine komplette Eradikation des Virus erreicht werden. Die Medikamente sind bisher nur in der Lage, die Replikation des Virus zu unterdrücken, wobei die virale DNA weiterhin vorliegt und sich außerdem Teile der HBV-DNA in das Wirtsgenom integrieren können.

Des Weiteren sind zusätzliche Virus- und Wirtsfaktoren in der Lage, den Verlauf der Infektion sowie auch die Wirksamkeit verschiedener Therapien stark zu beeinflussen. Beispielsweise konnte in zahlreichen asiatischen Studien gezeigt werden, dass Mutationen im basalen Core Promotor und der preCore Region des HBV mit dem Verlauf der Infektion assoziiert sind. Die Doppelmutation A1762T/G1764A im basalen Core Promotor reduziert die Synthese von HBeAg und erhöht die Menge an prä-genomischer RNA *in vitro*. Des Weiteren konnte die Doppelmutation A1762T/G1764A als unabhängiger Risikofaktor für die Entwicklung einer Fibrose oder eines hepatozellulären Karzinoms identifiziert werden. Durch eine Punktmutation im preCore Gen an der Stelle G1896A wird ein Stopcodon

gebildet, das zu einem Abbruch der Produktion des HBeAg führt. Einige Studien konnten zwar einen Zusammenhang zwischen der G1896A-Mutation und der Entwicklung einer „fulminanten Hepatitis“ herstellen, allerdings widerlegten andere Studien diesen Zusammenhang. Durch bestimmte Mutationen oder Deletionen im preS Gen kann es zu einer intrazellulären Akkumulierung des HBsAg im endoplasmatischen Retikulum kommen und somit zu einer verminderten Sekretion des HBsAg. Dies führt zur Bildung reaktiver Sauerstoffspezies und konnte in zahlreichen Studien mit der Bildung eines hepatozellulären Karzinoms assoziiert werden. Diese Studien wurden im asiatischen Raum durchgeführt, in dem die Übertragung des Virus hauptsächlich vertikal erfolgt und die HBV Genotypen B und C dominieren. Im Gegensatz dazu werden in Deutschland/Europa die Genotypen A und D mit der höchsten Prävalenz gefunden und die Übertragung erfolgt zum größten Teil horizontal. Daher ist es weitestgehend unklar, ob die Erkenntnisse asiatischer HBV-Infektionen auf die Situation in der westlichen Welt übertragen werden können.

Aufgrund dessen war es das Ziel der vorliegenden Studie, den Einfluss genetisch unterschiedlicher HBV-Varianten, wie beispielsweise unterschiedliche Genotypen und Mutationen, auf die Serum-Marker „HBV-DNA“ und „quantitatives HBsAg“ in HBeAg-negativen chronisch HBV-infizierten Patienten zu analysieren. Die HBV-Genotypen wurden entweder während der klinischen Routine oder während der vorliegenden Arbeit durch Amplifikation und populations-basierter Sequenzierung eines Teilstücks des Polymerase-Gens bestimmt. Die Mutationen im viralen Genom wurden durch Amplifikation und populations-basierter Sequenzierung des basalen Core Promotors, des preCore- und des preS-Bereichs detektiert. Die vorliegenden Genotypen sowie Mutationen wurden mittels statistischer Analysen mit den Serum-Markern „HBV-DNA“ und „quantitatives HBsAg“, die zuvor während der klinischen Routine bestimmt wurden, korreliert.

Außerdem wurden unterschiedliche HBV-Genotyp A2 Genome kloniert und die Expression der viralen Genome und Proteine in einer humanen Hepatokarzinom-Zelllinie mittels real-time PCR, HBeAg/HBsAg-spezifischer ELISAs und Western Blot untersucht.

Des Weiteren wurde die Sekretion viraler und subviraler HBV-Partikel im Serum von Patienten, die mit den Genotypen A - E (GTA - GTE) infiziert waren, via real-time PCR, HBsAg-spezifischem ELISA, Western Blot und Elektronenmikroskopie analysiert.

Dabei konnte in der vorliegenden Studie gezeigt werden, dass Mutationen/Deletionen im basalen Core Promotor, preCore- und preS-Bereich des HBV in einer Genotypen-spezifischen Verteilung auftreten. Beispielsweise wurde die basale Core Promotor Doppelmutation A1762T/G1764A bei 93 % der GTE-infizierten Patienten gefunden, wohingegen bei nur 44 % der GTB-infizierten Patienten diese Doppelmutation nachgewiesen werden konnte. Im Gegensatz dazu konnte die preCore Mutation G1896A bei 56 % der GTB-infizierten Patienten und bei nur 6 % der GTA-infizierten Patienten detektiert werden. Mutationen oder Deletionen im preS-Bereich waren hauptsächlich bei GTC-infizierten Patienten (21 %) zu finden. Die in dieser Arbeit gefundene Genotypen-spezifische Prävalenz

der verschiedenen Mutationen bestätigt auch die Ergebnisse vorheriger Studien.

Die verschiedenen HBV-Genotypen sowie preS Mutationen konnten in der vorliegenden Arbeit nicht mit einer erhöhten oder erniedrigten HBV-DNA-Menge assoziiert werden. Im Gegensatz dazu war die Menge an HBV-DNA in Patienten mit der BCP Doppelmutation A1762T/G1764A im Vergleich zu Patienten ohne diese Mutation signifikant erniedrigt. In Patienten mit Mutationen im preCore-Bereich war die virale DNA-Menge signifikant erhöht im Vergleich zu Patienten ohne Mutationen im preCore-Bereich. Auch bezüglich der ins Blut abgegebenen HBsAg-Menge gab es Unterschiede zwischen den Genotypen und Patienten mit bestimmten Mutationen. Während die Mehrheit der GTB-infizierten Patienten HBsAg-Werte < 1000 IU/ml hatten, konnte dieser cut-off, der mit einem vorteilhaften Verlauf der Infektion assoziiert ist, nur bei wenigen GTE- und GTA-infizierten Patienten gefunden werden. Dies würde bedeuten, dass GTE- und GTA-Infektionen im Allgemeinen mit einem schlechteren Krankheitsverlauf assoziiert sind als GTB-Infektionen. Dies konnte in vorherigen Studien bisher allerdings nicht gezeigt werden. Aufgrund dessen sollte der HBsAg cut-off für jeden HBV-Genotypen individuell angepasst werden. Des Weiteren konnten in der vorliegenden Arbeit bei Patienten mit preCore Mutationen signifikant niedrigere HBsAg-Spiegel und bei Patienten mit preS-Deletionen/-Mutationen signifikant höhere HBsAg-Spiegel im Vergleich zu Patienten ohne diese Mutationen verzeichnet werden.

Im Verlauf der Infektion mussten 14 Patienten eine antivirale Therapie beginnen, da sie an mehreren Wiedervorstellungsterminen entweder eine erhöhte Viruslast und/oder erhöhte Transaminasen hatten und/oder sich der Beginn einer Fibrose zeigte. Bei diesen Patienten konnte kein Unterschied in der HBsAg-Menge zu Beginn der Studie (Baseline) im Vergleich zum Therapiestart festgestellt werden. Allerdings war die HBV-DNA zum Zeitpunkt, bevor die Therapie begonnen wurde, leicht erhöht im Vergleich zu Baseline. Außerdem wurden Mutationen im preCore-Bereich bei Therapiestart mit einer höheren Frequenz detektiert als zu Baseline. Aufgrund dessen könnte ein Zusammenhang zwischen preCore Mutationen und der Notwendigkeit des Beginns einer antiviralen Therapie aufgrund einer erhöhten Viruslast bestehen. Allerdings muss dies zukünftig in weiteren Patienten, die eine antivirale Therapie beginnen, evaluiert werden.

Die *in vitro* Analyse verschiedener klonierter 1.5-facher HBV-Genome (diese enthielten die folgenden Variationen: 1. A1762T/G1764A, BCP; 2. G1896A/G1899A, preCore; 3. Deletion von 15 Aminosäuren im preS1 Bereich oder 4. keine der genannten Mutationen/Deletionen - kurz Referenzgenom) in einer humanen Hepatokarzinom-Zelllinie ergab eine erhöhte Expression mit gleichzeitig verminderter Sekretion viraler Genome in Zellen, die mit dem preS-Deletions-Genom oder dem preCore-Genom transfiziert wurden, im Vergleich zu Zellen, die mit dem BCP-Genom oder dem Referenzgenom transfiziert wurden. Des Weiteren war die HBsAg-Produktion und -Sekretion in Zellen, die mit dem preS-Deletions-Genom transfiziert wurden, signifkant höher als in Zellen, die mit dem BCP-, preCore- oder Referenzgenom transfiziert wurden.

In weiteren Versuchen konnten Genotypen-spezifische Unterschiede in der Zusammensetzung der Oberflächenproteine (Large/Middle/Small Hepatitis surface proteins; L/M/SHBs) sowie der sekretierten, nicht infektiösen subviralen Partikel (Sphären und Filamente) in der vorliegenden Arbeit gefunden werden. Bei den Genotypen C, D und E war eine deutlich größere Menge an MHBs im Vergleich zu LHBs vorhanden, wohingegen beim Genotyp B deutlich mehr LHBs als MHBs vorhanden war. Beim Genotyp A war die Menge an MHBs im Vergleich zu LHBs leicht erhöht. Da Filamente einen größeren Anteil an LHBs aufweisen als Sphären, würde dies daraufhin deuten, dass der Genotyp B präferiert mehr Filamente als Sphären sezerniert im Vergleich zu den Genotypen A, C, D und E. Dies konnte in der vorliegenden Arbeit elektronenmikroskopisch bestätigt werden. Die Genotypen A, C und E produzierten vermehrt Spähren und nur wenige Filamente, wohingegen beim Genotyp B hauptsächlich Filamente zu finden waren, die sich zu großen Bündeln zusammenlagerten. Da in zuvor beschriebenen Studien gezeigt werden konnte, dass die subviralen Partikel mit einer erhöhten Infektiösität und der Neutralisation antiviraler Antikörper assoziiert sind, könnte es Genotypen-spezifische Unterschiede auch bezüglich der Pathogenese und Immugenität geben. Diese Genotypen-spezifischen Unterschiede sollen in zukünftigen Studien genauer analysiert werden.

Außerdem konnten in einigen viralen Genomen, die aus dem Serum infizierter Patienten isoliert wurden, sowohl mittels populations-basierter Sequenzierung als auch mittels Tiefensequenzierung große Deletionen in der preS-Region sowie preS2-Startcodon Mutationen detektiert werden. Diese Mutationen/Deletionen sollten theoretisch das Molekulargewicht der exprimierten Proteine verringern oder sogar die gesamte Expression des entsprechenden Proteins unterbinden. Allerdings konnte hier gezeigt werden, dass sich die exprimierten Proteine in ihrer Größe nicht voneinander unterschieden und dass auch MHBs exprimiert wurde, obwohl dies die preS2-Startcodon Mutationen verhindern sollten. Dies deutet darauf hin, dass das sezernierte HBsAg nicht den gleichen genetischen Ursprung hat, sprich von der cccDNA abstammt, wie die virale DNA im Serum der Patienten. Vielmehr stammt es von der in das Wirtsgenom integrierten viralen DNA ab. Diese Annahme wäre auch in Übereinstimmung mit vorangegangenen Studien anderer Arbeitsgruppen, die zum Einen zeigen konnten, dass die cccDNA-Mengen nicht mit der Menge an HBsAg im Serum HBeAg-negativer chronisch HBV-infizierter Patienten assoziiert sind. Zum Anderen konnten die Studien beweisen, dass die HBsAg-Menge in HBeAg-negativen Patienten, die mit einer cccDNA angreifenden siRNA behandelt wurden, nicht reduziert werden konnte im Gegensatz zu behandelten HBeAg-positiven Patienten. Daher scheint auch die integrierte DNA ein wichtiger Punkt zu sein, an dem neue antivirale Strategien ansetzen müssen, wenn eine komplette Eradikation des Virus erzielt werden soll.

Zusätzlich konnte in früheren Studien bewiesen werden, dass nicht nur virologische Faktoren den Verlauf einer Infektion oder die Wirksamkeit einer Therapie beeinflussen, sondern auch wirtsspezifische Faktoren wie Einzelnukleotid-Polymorphismen im Wirtsgenom.

Beispielsweise konnten Einzelnukleotid-Polymorphismen im Interferon-λ3 und Interferon-λ4-Gen mit der spontanen Clearance einer HCV-Infektion assoziiert werden. Aufgrund dessen wurde in der vorliegenden Studie der Zusammenhang zwischen Resistenz-assoziierten Substitutionen in den viralen HCV-Genen NS3/NS5A/NS5B und Einzelnukleotid-Polymorphismen im Interferon-λ4-Gen in chronisch HCV-infizierten Patienten untersucht. Dazu wurde die Prävalenz Resistenz-assoziierter Substitutionen in den HCV-Genen NS3/NS5A/NS5B ermittelt, sowie der Interferon-λ4-Genotyp an der Position rs12979860 mittels real-time PCR bestimmt.

Dabei konnte zunächst gezeigt werden, dass die Resistenz-assoziierte Substitution Y93H im NS5A-Gen sowie die Substitutionen C316N und S556G/N/R im NS5B-Gen hauptsächlich im Genotyp 1b im Vergleich zum Genotyp 1a und Genotyp 3 vorkommen. Dahingegen wurde die Resistenz-assoziierte Substitution Q80K des NS3-Gens häufiger im Genotyp 1a im Vergleich zu den Genotypen 1b und 3 detektiert. Diese Erkenntnisse decken sich mit den Ergebnissen weiterer Studien.

Bezüglich der Resistenz-assoziierten Substitutionen in NS3 und NS5B bestand kein signifikanter Zusammenhang mit dem Interferon-λ4-Genotyp des Wirts. Hingegen dessen konnte in Genotyp 1b-infizierten Patienten ein höchst signifikanter Zusammenhang zwischen dem Vorliegen der Y93H Substitution und dem vorteilhafteren Interferon-λ4-Genotyp rs12979860 C/C gefunden werden. Patienten mit dem vorteilhaften C/C-Genotyp hatten eine höhere Viruslast im Vergleich zu Patienten mit den weniger vorteilhaften non-C/C-Genotypen. Zwischen den gefundenen Resistenz-assoziierten Substitutionen Q80K, L31F/M/V, C316N, S556G/N/R und der Höhe der viralen RNA-Menge gab es keinen Zusammenhang, wohingegen Patienten mit einer Y93H Substitution eine signifikant erhöhte HCV-RNA-Menge aufwiesen. Durch univariate und multivariate Analysen konnten HCV-RNA-Mengen $>$ 5.000.000 IU/ml sowie das Vorliegen des vorteilhafteren Interferon-λ4-Genotyps C/C als unabhängige Faktoren für das Vorliegen einer Y93H Substitution identifiziert werden. Bisher ist es unklar, warum einige der Patienten mit einem vorteilhafteren C/C-Genotyp ein unerwartet schlechteres Therapieansprechen bei bestimmten NS5A-Inhibitor-basierten Therapien haben. Dies könnte an den Resistenz-assoziierten Substitutionen liegen, die schon zu Beginn der Infektion vorliegen und sich im Laufe der Zeit zur Hauptspezies des Virus entwickeln. Da beispielsweise die Substitution Y93H mit einer höheren Viruslast und einem vermehrten Auftreten im eigentlich favorisierten C/C-Genotyp einhergeht, könnte das Vorliegen Resistenz-assoziierter Substitutionen eine fehlende Korrelation zwischen favorisiertem C/C-Genotyp und einer besseren Ansprechrate bei bestimmten Therapien erklären.

Abbreviations

aa	amino acid
ALT	alanine aminotransferase
anti-HBc	antibodies against HBcAg
anti-HBe	antibodies against HBeAg
anti-HBs	antibodies against HBeAg
BCP	basal core promoter
BL	baseline
bp	basepairs
cccDNA	covalently closed circular DNA
cDNA	complementary DNA
CLDN1	claudin-1
CO_2	carbon dioxide
DAA	direct acting antiviral
DNA	desoxyribonucleic acid
dslDNA	dobule-stranded linear DNA
E. coli	*Escherichia coli*
EASL	European Association for the Study of the Liver
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport
GAG	glycosylaminoglycan

GT	genotype
HBcAg	hepatitis B virus core antigen
HBeAg	hepatitis B virus e antigen
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCl	hydrochloric acid
HCV	hepatitis C virus
HRP	horseradish peroxidase
HSPG	heparan sulfate proteoglycan
IFNL3	interferon-λ3
IFNL4	interferon-λ4
IL28B	interleukin-28B
IRES	internal ribosomal entry site
IU	international units
kb	kilobases
kDa	kilodalton
LDL	low-density lipoprotein
LDL-R	low-density lipoprotein receptor
LHBs	large hepatitis B virus surface protein
LVPs	lipo-viro-particles
MHBs	middle hepatitis B virus surface protein
mRNA	messenger ribonucleic acid
nt	nucleotides
NTR	non-translated region
NUC	nucleos(t)ide analogues
OCLN	occludin

ORF	open reading frame
PBS	phophate buffered saline
PCR	polymerase chain reaction
PEG-IFNα	pegylated interferon alpha
pgRNA	pregenomic RNA
qHBsAg	quantitative hepatitis B virus surface protein
RAS	resistance associated substitution
RC	replication complex
rcDNA	relaxed circular DNA
ref. genome	reference genome
RNA	ribonucleic acid
rpm	revolutions per minute
rt-PCR	real time-PCR
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamid-gelelectrophoresis
SEM	standard error of the mean
SHBs	small hepatitis B virus surface protein
SNP	single nucleotide polymorphism
SR-B1	scavenger receptor class B type I
STAT	signal transducer and activator of transcription
SVPs	subvrial particles
SVR	sustained virologic response
Tris	tris(hydroxymethyl)-aminomethane
TS	treatment start
VLDL	very-low-density lipoprotein
w/v	weight per volume

WB	western blot
WHO	World Health Organization

Contents

1 Introduction

1.1 Hepatitis B

1.1.1 Epidemiology, transmission and prevention

Hepatitis B is an infection of the liver caused by the hepatitis B virus (HBV). According to the World Health Organization (WHO) approximately 257 million people are living with an HBV infection and in 2015 about 887,000 people died because of the development of cirrhosis or hepatocellular carcinoma (HCC) [172]. Therefore, HBV is still a major global health problem. As shown in Figure 1.1 the prevalence of chronically infected people varies geographically around the world with the highest prevalence in the Western Pacific Region, Greenland, parts of South America and Africa.

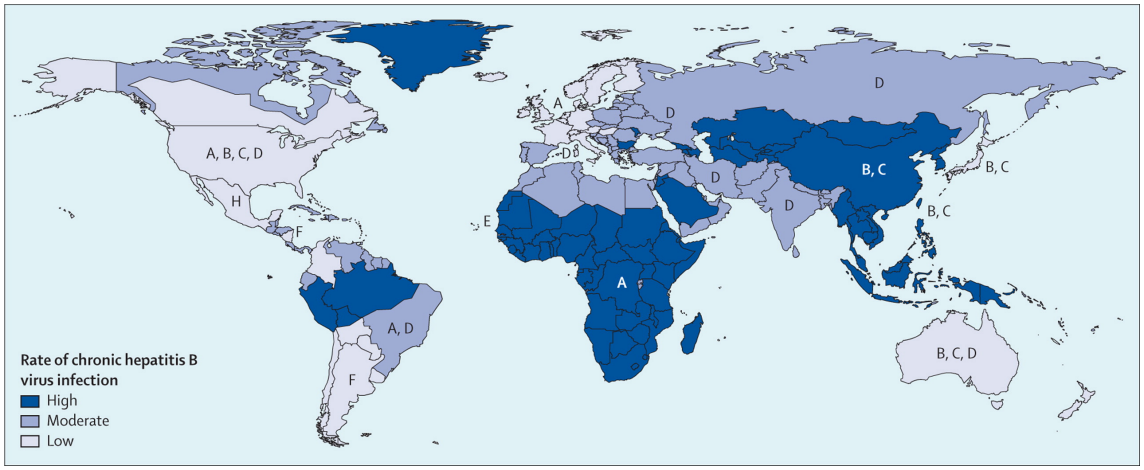

Figure 1.1: Worldwide prevalence of adults chronically infected with HBV. Highest prevalence of chronically infected patients is found in Africa, Western Pacific regions, Greenland and parts of South America. Lowest prevalence of chronic HBV infection occurs in most parts of Europe. Taken from [165].

HBV can be transmitted easily via blood and blood products, body fluids, syringes, razor blades, transplantation of infected organs or perinatal via infected mothers to their child [82]. Since 1992 a vaccination of all newborn babies is recommended by the WHO [130].

1.1.2 Course of disease

An exposure to HBV can lead to an acute infection, which is normally resolved by responses of the innate and adaptive immune system. However, 3 - 5% of the adolescent patients are not able to resolve the acute infection and HBsAg can be detected in the serum of these patients for more than 6 months [95]. Hence, the infection becomes chronic. The natural history of a chronic HBV infection can be divided into five phases, whereas the phases are not necessarily sequential and the management of some patients needs to be individualized [83].

Phase 1: HBeAg positive chronic HBV infection (formerly known as "immune tolerant" phase)
This phase is defined by the detection of HBeAg (hepatitis B virus e antigen) in the serum of the patient, very high HBV-DNA levels, normal values of alanine aminotransferase (ALT), minimal or no liver necroinflammation, but frequent integration of viral DNA into the host's liver DNA. Due to very high levels of HBV-DNA patients are highly contagious, but usually there is no need for treatment [83].

Phase 2: HBeAg positive chronic hepatitis B
Characteristics of this phase are HBeAg positivity, high HBV-DNA levels and elevated ALT. The liver shows a moderate to severe necroinflammation and enhanced progression of fibrosis. Hence, a therapy is indicated in the majority of cases [83].

Phase 3: HBeAg negative chronic HBV infection (formerly known as "inactive carrier" status)
In this stage patients are HBeAg negative but have serum antibodies to HBeAg (anti-HBe), low levels of HBV-DNA (< 2,000 IU/ml) and normal ALT values. Liver biopsies are characterized by minimal necroinflammation and low fibrosis. Loss and seroconversion of HBsAg (hepatitis B virus surface antigen) occurs spontaneously in up to 3% of the patients per year. Only in a few patients the start of an antiviral therapy is indicated [83].

Phase 4: HBeAg negative chronic hepatitis B
Patients are HBeAg negative and anti-HBe positive, have persistent or fluctuating moderate to high levels of HBV-DNA and ALT. Hepatic necroinflammation as well as fibrosis may develop and start of antiviral therapy might be necessary [83].

Phase 5: HBsAg-negative phase (also known as "occult infection")
Characteristics are HBsAg negativity with or without detectable antibodies to HBsAg (anti-HBs), normal ALT and commonly undetectable serum HBV-DNA [83].

1.1.3 Treatment

In the German guideline for prophylaxis, diagnostics and therapy of HBV infections [29] the treatment of patients with chronic hepatitis B with HBeAg-positivity or -negativity is recommended, if HBV-DNA levels are $\geq$ 2000 IU/ml, ALT values $>$ 2 times the upper limit of normal or significant necroinflammation of the liver [29].

The initial agents for adults recommended by the German guideline are a finite treatment with (pegylated) interferon alpha ((PEG)-IFNα), which is immunomodulatory and antiviral, and/or a long-term treatment with nucleos(t)ide analogues (NUC), such as entecavir, tenofovir disoproxil fumarate, tenofovir alafenamide (or lamivudine, telbivudine, adefovir) [161]. The major treatment endpoint in HBeAg-negative as well as in HBeAg-positive patients is sustained off-treatment HBsAg loss, with or without seroconversion to anti-HBs. But unfortunately, only up to 11% of the patients treated with (PEG)-IFNα achieve HBsAg loss and the chance of HBsAg clearance is even lower with NUC therapy [22, 48, 76, 98]. However, long-term NUC administration therapies effectively suppress the viral replication and therefore lower the risk of morbidity and mortality associated with chronic hepatitis B infection. New strategies are currently in development to achieve an eradication of the virus and therefore a complete cure of the disease [83, 161].

1.1.4 The hepatitis B virus

1.1.4.1 General information and genome organization

The hepatitis B virus belongs to the family of the Hepadnaviridae, which can be found in mammals (orthohepadnaviruses) and in birds (avihepadnaviruses) [137]. Until now HBV can be classified into 9 genotypes (A to I; GTA to GTI) and one candidate GT (J) according to phylogenetic analyses with at least 8% of divergence in the nucleotide sequence [159, 181].

Infectious viral particles are spherical and have a diameter of 42 nm. They contain a circular, partially double-stranded 3.2 kb genome, which is covalently linked to the viral DNA polymerase and surrounded by a viral capsid made of the core protein (HBcAg). The viral capsid itself is encapsulated by the surface proteins, which are associated with lipids of the host cells (see Figure 1.2) [47]. Not only infectious viral particles are produced in infected hepatocytes but also so called subviral particles (SVPs). These subviral particles are only made out of the surface proteins and because they are lacking the viral genome, they are not infectious. SVPs can be divided by their shape into spheres with a diameter of 22 nm and filaments with different length between 50 - 1200 nm but also with a diameter of 22 nm [67, 102, 114].

The circular 3.2 kb genome contains an incomplete strand, which is of positive-sense and non-coding, and a complete strand, which is of negative-sense and coding. The genome comprises at least four overlapping open reading frames (ORF) encoding the HBc and HBeAg, the viral polymerase, the regulatory HBx protein and the preS/S gene encoding

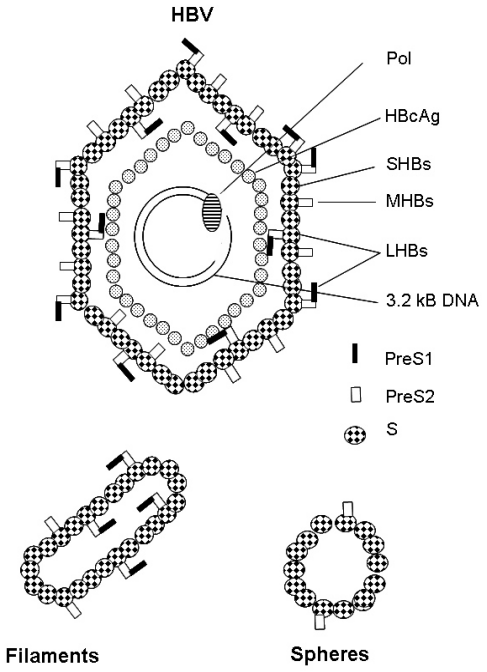

Figure 1.2: Viral and subviral particles produced by the hepatitis B virus. Viral particles contain the viral genome encapsulated by the core proteins and surrounded by surface proteins. Subviral particles are divided into spheres and filaments and are built by surface proteins. Taken from [136].

the surface proteins (see Figure 1.3) [136].

1.1.4.2 Precore and core protein

The HBV genome is surrounded by a nucleocapsid made of the core protein HBcAg. The core protein consists, depending on the viral genotype, of 183 to 185 amino acids (aa) and is divided into an N-terminal and a C-terminal domain. The aa 1 to 149/151 are needed for the spontaneous assembly of the icosahedral capsids, whereas the C-terminal end is required for packaging of a pre-genome/reverse transcriptase complex [21, 185]. Self-assembly of the capsids is initiated by an attachment of two HBcAg monomers to built a dimer. Assembly of more dimers leads to the formation of capsids. Either 90 dimers are attached to built capsids with a diameter of 30 nm (called T3-symmetry) or 120 dimers form capsids with a diameter of 34 nm (T4-symmetry) [30]. Furthermore, a so called precore protein is existing, which contains additionally 29 aa upstream of the core protein. After being transported to the lumen of the endoplasmic reticulum (ER), it is posttranslationally processed and 34 aa at the C-terminal end are cleaved to form a secreted version of the core protein named HBeAg [112].

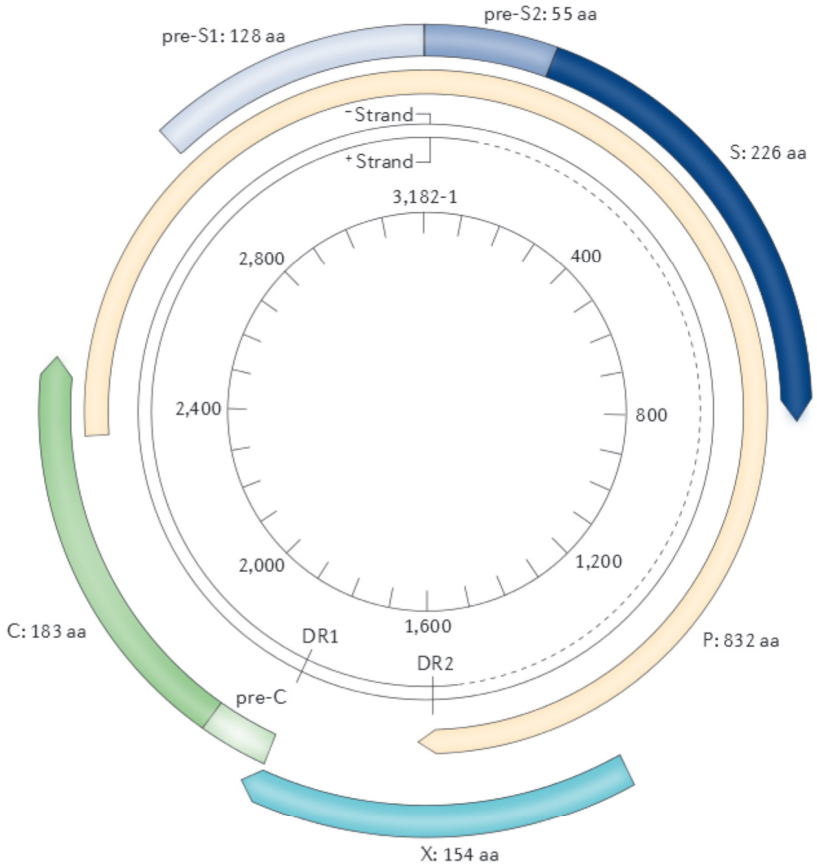

Figure 1.3: Genome of the hepatitis B virus. The genome encompasses a 3.2 kb partially double-stranded DNA containing a complete (−)-strand and an incomplete (+)-strand (compare line and dotted line) as well as four overlapping ORFs encoding the viral polymerase (P), precore and core protein (preC/C), HBx protein (X) and the surface proteins built of pres1, preS2 and S. Taken from [8].

1.1.4.3 Surface proteins

The preS/S gene contains three in-frame AUG initiation codons and depending where the translation is started the three surface proteins LHBs, MHBs and SHBs (large/middle/ small hepatitis B surface proteins) are encoded. LHBs consists of the preS1 domain (108 to 128 aa depending on HBV GT), preS2 domain (55 aa) and S domain (226 aa), whereas MHBs lacks the preS1 domain and contains the preS2 and S domain. SHBs comprises only the S domain.

While SHBs contains an N-glycosylation site at Asparagine-146, it exists in an unglycosylated and a glycosylated form with a molecular weight of 24 kDa or 27 kDa (also named pS/gpS or P24/GP27), respectively. MHBs harbours an N-glycosylation site at Asparagine-4 and an O-glycosylation site at Tyrosine-37. Hence, it can occur in an unglycosylated, monoglycosylated and biglycosylated form with molecular weights of 30, 33 and 36 kDa (also named pM/gpM/ggpM or P30/GP33/GP36), respectively. LHBs contains a glycolysation site in the S domain resulting in an unglycosylated and a glycosylated form of 39 and 42 kDa (also named pL/gpL or P39/GP42) [99].

HBsAg includes a group-specific region between the aa 124 to 147 of the S domain, the so called *"a"* determinant, as well as two subtypic determinants designated as *d* or *y* and *w* or *r* resulting in the four major subtypes *adw*, *adr*, *ayw* and *ayr* [110]. It has been shown that the variations *d* or *y* and *w* or *r* were dependent on a Lysine to Arginine substitution at residues 122 and 160 of the S gene [110]. As indicated in Figure 1.4, subtype-specific size differences of LHBs and differences in the migration pattern of LHBs and MHBs have been observed in previous studies [59, 68]. It could be demonstrated that a smaller distance between GP33/GP36 and P39/GP42 occurs in the *ay* subtype as well as a faster migration of P39/GP42 (Figure 1.4B).

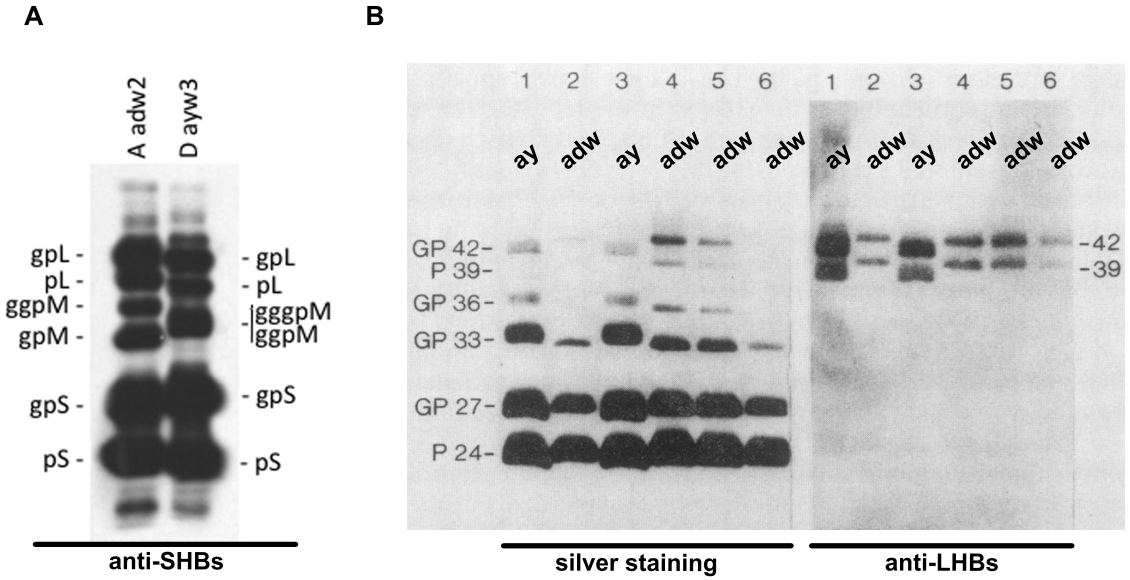

Figure 1.4: Subtype-specific differences of HBsAg. Subtype-specific size differences of LHBs and differences in the migration pattern of LHBs and MHBs in subtype *ay* and *adw*. Adapted from [59] and [68].

1.1.4.4 HBx protein

The smallest ORF of the HBV genome encodes a 17 kDa regulatory protein called HBx protein. Several studies (reviewed by [81]) report post-translational modifications like acetylation, disulfide bond formation and phophorylation by HBx as well as inhibition of p53 tumor suppressor gene, inactivation of negative growth regulator and regulation of cell cycle progression [72]. Furthermore, HBx has been described to trigger the activation of the c-Raf-1/MEK kinase cascade, which is essential for HBV gene expression [147].

1.1.4.5 Viral polymerase

The largest ORF of the HBV genome encodes the viral polymerase (the only enzyme that is encoded by the HBV genome) consisting of three different functional domains. The so called terminal protein is linked to the viral polymerase via a spacer-region and is necessary

for priming during reverse transcription. An RNaseH domain is located N-terminally decomposing the RNA template during reverse transcription [13].

1.1.4.6 Viral life cycle

The viral life cycle (see Figure 1.5) starts with a weak binding of the virion's LHBs to cell surface heparan sulfate proteoglycans of the host's hepatocytes [139]. The preS1 domain of LHBs interacts with the sodium taurocholate cotransporting polypeptide, a liver-specific bile acid transporter, and the specific uptake of the virus via endocytosis is triggered [177]. Processing of the viral particles through endosomal proteases leads to a conformational change of the viral surface proteins and therefore to presentation of a so called translocation motif, which mediates the transport of the viral nucleocapsids across the endosomal membrane [148].

The viral DNA containing nucleocapsids are transported to the nuclear pore complex, disassemble and release the viral DNA into the nucleus. The viral DNA is a partially double-stranded relaxed circular DNA (rcDNA), which consists of a complete (−)-strand covalently bound to the viral polymerase at the 5' end and an incomplete (+)-strand containing a RNA oligonucleotide at the 5' end serving as a primer for (+)-strand synthesis. The rcDNA is converted into covalently closed circular DNA (cccDNA) by host cellular

factors and associated with nucleosomes to form a minichromosome [17]. The cccDNA serves as a template for the three subgenomic mRNA transcripts (2.4 kb, 2.1 kb and 0.7 kb RNA) as well as the pregenomic RNA (pgRNA), which are transcribed by the cellular RNA polymerase II. The subgenomic RNAs are translated into LHBs, MHBs, SHBs and the X protein, whereas the pgRNA is translated into the precore/core protein and into the viral polymerase [23].

Furthermore, the pgRNA serves as a template for reverse transcription. Therefore, the pgRNA and the viral reverse transcriptase are encapsidated by core proteins initiated by the packaging signal ε, which is located at the 5' end of the pgRNA. The pgRNA is reverse transcribed into (−)-single-stranded DNA and the viral polymerase synthesizes two-thirds of the (+)-strand before it terminates [13]. Mature nucleocapsids containing newly synthesized rcDNA are either recycled back to the nuclear pore complex, disassemble and release the rcDNA into the nucleus to maintain a continuous reservoir of cccDNA, or they can be enveloped by the surface proteins and released from the cell [168]. SVPs, namely spheres and filaments, are only made of the surface proteins LHBs, MHBs and SHBs, which are formed in the ER and do not contain a nucleocapsid or viral DNA. While viral particles as well as filaments are released via multivesicular bodies due to an endosomal sorting complex required for transport (ESCRT) system, spheres are released by the ER-Golgi intermediate compartment by the secretory pathway [67, 123, 170].

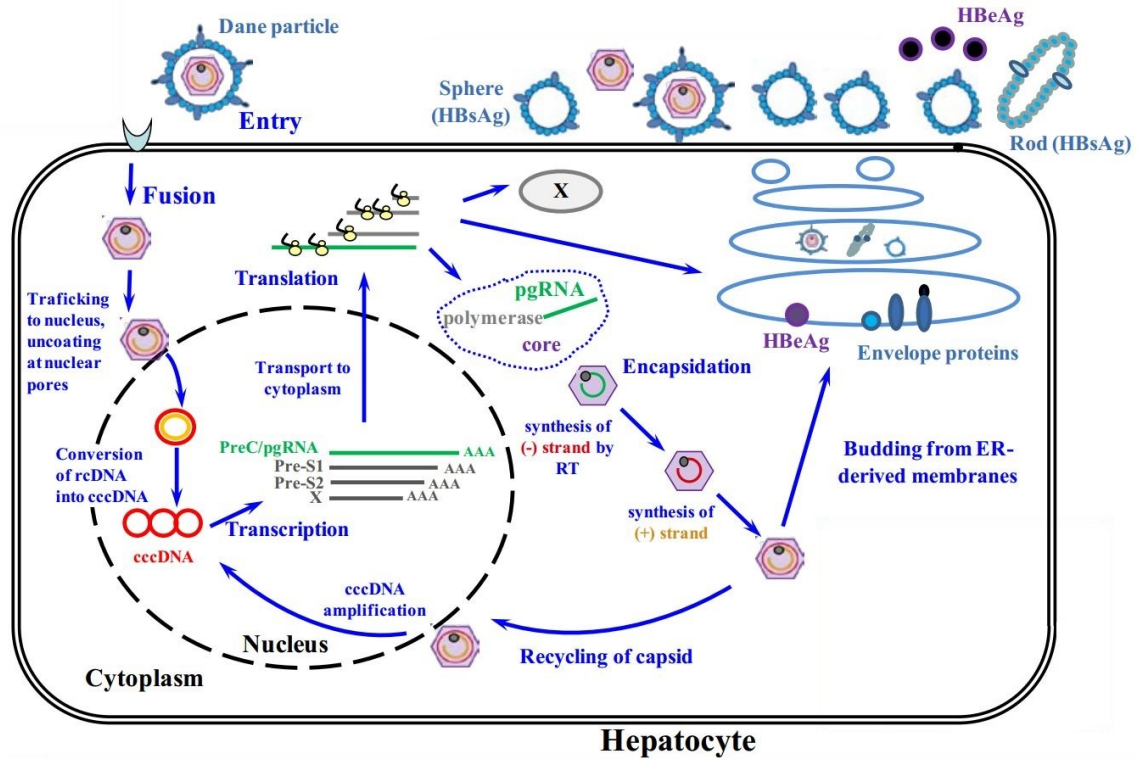

Figure 1.5: Life cycle of the hepatitis B virus. Virions attach to and enter hepatocytes. The nucleocapsid is released into the cytoplasm, transported to the nuclear core complex and rcDNA is released. rcDNA is converted to cccDNA, transcribed into the subgenomic mRNAs and pgRNA and translated into different viral proteins. pgRNA is encapsidated and serves as a template for first-strand DNA synthesis by reverse transcription. After second strand DNA synthesis leading to mature rcDNA, nucleocapsids are either recycled back to the nucleus to form cccDNA or they are enveloped by surface proteins and new virions are secreted. Furthermore, HBeAg and SVPs are secreted. Modified from [1].

1.1.4.7 Viral DNA integrates into the host genome

On the one hand, reverse transcription of the pgRNA occurs within the nucleocapsid resulting in rcDNA. On the other hand, in a minority of nucleocapsids translocation of the RNA primer does not occur and reverse transcription is primed from the direct repeat DR1 producing double-stranded linear DNA (dslDNA). Because primer translocation does not occur, the dslDNA contains a 16 -18 nt insertion. Further, it can either be transported to the nucleus to form cccDNA or it can be released as virions containing dslDNA [179]. Intranuclear dslDNA genomes are the presumed form that integrates into the host genome occurring in one of 10^5 to 10^6 infected cells [150]. Integration occurs at DNA double-strand breaks with no specific chromosomal hot-spots or common recurring sites in the host genome. Terminal deletions of up to 200 bp from the integrated HBV-DNA are observed very frequently [15, 180].

The integrated DNA forms are structurally rearranged and therefore the expression of all viral open reading frames is affected. One exception is the HBsAg ORF, which maintains its position under its native promoter and hence stays intact. Furthermore,

enhancer 1 is active in the integrated form and therefore it can produce transcripts of the HBx ORF [138].

The integrated viral DNA is not capable of producing pgRNA because it lacks the core promoter/enhancer for pgRNA translation upstream of the pgRNA start site and by this means integration of viral DNA into the host DNA represents a "replicative dead-end" for the virus [167]. Multiple rearrangements of the HBV genome and deletions within were found by characterizing integrated HBV sequences in primary tissues [26, 138, 155] not knowing whether those rearrangements occur by pre-integration (integration of defective HBV genomes) or by post-integration via chromosomal instability or a combination of both [167]. Because in its integrated form dslDNA is replication-incompetent, clinical implications of HBV-DNA integration still remain a challenging issue. So far, HBV integration has been connected with chromosomal and genomic instability [176]. Furthermore, the development of HCC through expression of mutated or truncated HBV surface proteins leading to ER stress responses was described [169]. In experiments with animal models integrated forms of HBV were shown to persist after the resolution of the infection [149, 150, 180] and also in patients with an occult HBV infection persistent integrated forms have been detected [125].

1.1.4.8 Mutations in the basal core promoter, precore and preS region

In several Asian studies it was demonstrated that mutations in the basal core promoter (BCP) and the precore region are associated with the course of chronic HBV infection.

The BCP and its corresponding enhancer trigger the transcription of both the precore and the pgRNA [56]. An A to T change at position 1762 in combination with a G to A change at position 1764 (A1762T/G1764A) is the most frequently observed mutation in the BCP. It reduces the synthesis of HBeAg due to a reduced transcription of precore RNA and simultaneously increases the amount of pgRNA *in vitro* [65]. Due to the overlapping ORFs in the HBV genome the BCP double mutation A1762T/G1764A leads to substitutions of codons 130/131 of the HBx protein, which are supposed to cause a weaker T cell response to the immunodominant HBx-derived epitope [96]. Furthermore, the BCP double mutation A1762T/G1764A was identified as an independent risk factor for developing liver fibrosis and HCC [71, 91, 178].

The G to A point mutation at position 1896 of the precore region converts codon 28 from tryptophan (UGG) to a translational stop codon (UAG). Therefore, it prevents the translation of the precore protein and abrogates HBeAg production while the production of HBcAg is not affected. G1896 is located within the ϵ signal, which is a highly conserved stem-loop among all genotypes essential for pregenomic RNA packaging. It has been recognized, that the development of the G1896A mutation is constrained by the nucleotide T or C at position 1858 depending on the viral genotype [93]. For genotypes with T1858, a T-A basepair is created by the G1896A mutation enhancing the stability of the stem-loop. In contrast, the G1896A mutation is unlikely found in variants with C1858 due to

destroying the stable C-G base pair. Since C1858 is mainly found in genotype A and in some genotype C variants the G1896A substitution is only found with low frequencies in these genotypes and is more likely found in genotypes B and D [88]. The contributions of mutations in the precore domain to the progression of the disease are discussed controversially. Whereas in some studies an association of the presence of G1896A with the development of fulminant and severe hepatitis [111, 113] was found, other studies failed to prove this correlation [75, 91, 178].

Recent studies indicate that mutations in BCP or precore are existing as minor variants of the quasispecies besides the predominating wildtype variant in infected patients from the early beginning of the HBeAg positive stage [43, 53, 156]. During the seroconversion of HBeAg to anti-HBe the immune tolerance to HBV is lost and the wildtype strain is suppressed. BCP or precore variants are selected and become the predominating variant of the viral quasispecies in patients with chronic HBV infection [43, 53, 156]. While BCP and precore mutations were described to enhance viral escape and concurrently decrease HBV recognition by cytotoxic T cells, their precise function during the pathogenesis of chronic hepatitis still remains obscure [65].

Variants in the preS/S-gene mostly occur as either point mutations in the preS2 start codon leading to a complete abolishment of the M protein synthesis or as in-frame deletions of different length at the C-terminus of the preS1 region or in the middle of the preS2 domain. Several specific mutations in the preS- and the S-gene have been described with an intracellular accumulation of HBsAg and therefore to a diminished secretion of HBsAg (reviewed by [122]). This has been associated with a severe course of the disease and the development of HCC [37, 63, 91]. The *"a"* determinant between the aa 124 to 147 of the preS domain is the main target of neutralizing B cell responses [85, 119]. Mutations which lead to conformational changes within the *"a"* determinant have been described to affect the antigenicity of HBsAg and therefore escaping vaccine induced immunity and anti-HBV immunoglobulin therapy. Also, false negative results in diagnostic assay detection can be provided depending on the epitope, which is recognized by the assay [35, 47]. The Glycine to Arginine substitution at position 145 of the S-gene is the most common variant found in the *"a"* determinant. Since this substitution has been described to neutralize antibodies induced by vaccination and the mutated epitope is no longer recognized, those variants are called *vaccine-escape mutants* (reviewed by [122]).

1.1.4.9 Longitudinal HBsAg carrier study (Albatros study)

Prognostic markers like mutations in the BCP, precore and preS region as well as cut-offs which might predict the progression of the disease or indicate a necessity of treatment of chronically infected patients were mainly established in large Asian studies [24, 37, 66, 71, 90, 166]. In Asia HBV is mainly transmitted vertically from infected mothers to their unborn babies while in Europe mostly horizontal transmissions occur. Furthermore, in Asian countries HBV genotypes B and C can be found with the highest prevalence,

whereas in European countries HBV genotypes A and D are detected with the highest frequencies. Hence, it is questionable if these cut-offs and prognostic markers can be applied from Asian to European populations. Furthermore, the different phases have to be taken into account, since not every phase of an HBV infection has to be treated. Until now, studies evaluating the natural course of an HBeAg negative chronic HBV infection in European or German patients have not been conducted.

Therefore, the large German prospective, multicenter study Albatros was established in 2009 to investigate the longitudinal course of an HBeAg negative chronic HBV infection in patients who do not need an antiviral therapy. Patients included in the study are HBeAg negative, HBsAg positive, have low HBV-DNA levels ($< 100{,}000$ IU/ml) and low to slightly elevated transaminases. During an individual study duration of 10 years, patients should show up for follow-up monitoring every 12 month. In the course of this, liver values, HBV-DNA levels, HBsAg levels, stage of fibrosis, development of HCC and possible occurring changes in treatment indication will be analyzed. New patients have been included into the Albatros study until the end of 2017.

1.2 Hepatitis C

1.2.1 Epidemiology, transmission and prevention

Hepatitis C is a liver disease caused by the hepatitis C virus (HCV) and according to the World Health Organization (WHO) about 71 million people are infected chronically [173]. Estimated 399,000 people die from hepatitis C each year because of the progression of cirrhosis or hepatocellular carcinoma (HCC). Globally, the highest prevalence (2.3 and 1.5%, respectively) occurs in Eastern Mediterranean and European regions as well as in Eastern and Central Asia as shown in Figure 1.6 [173].

HCV is mainly transmitted via transfusion of blood from infected donors and via intravenous or nasal drug use and to a lesser extend by hemodialysis, inadequate sterilization of medical equipment, sexual contact, tattooing and from infected mothers to their unborn babies [162, 173]. So far, no vaccination against HCV is available [173].

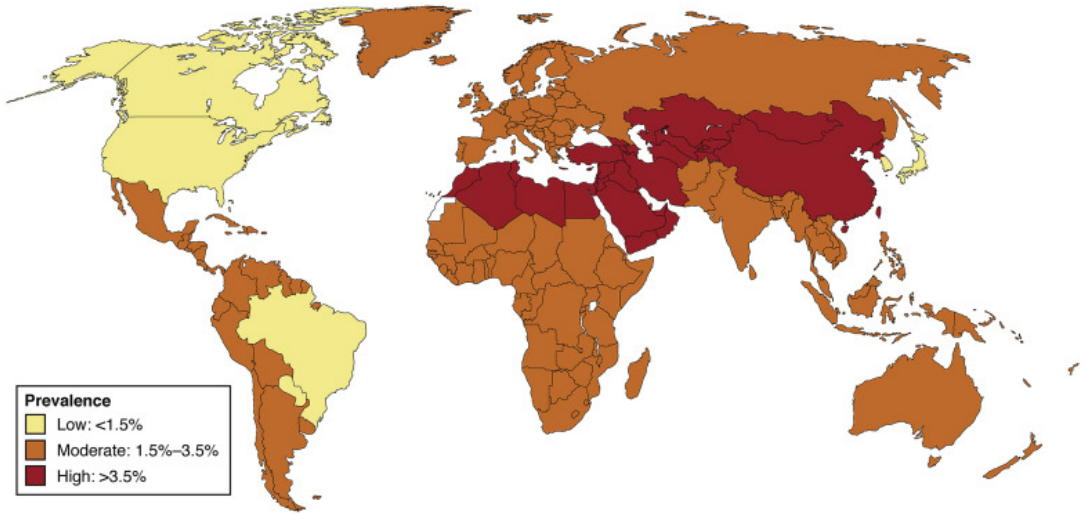

Figure 1.6: Global prevalence of people chronically infected with HCV. Highest prevalence is found in Eastern Mediterranean and European regions and Eastern and Central Asia. Taken from [171].

1.2.2 Course of disease

The hepatitis C virus has an incubation period of 2 weeks to 6 months and often it remains undiscovered due to an asymptomatic or flu-like progression. About 55 to 85% of acutely infected patients fail to clear HCV infection spontaneously and therefore develop chronic hepatitis [173]. A chronic HCV infection is diagnosed based on the detection of both anti-HCV antibodies and HCV RNA for more than 6 months. Since HCV is not cytopathogenic, hepatocytes are harmed due to natural killer cells of the host and cytotoxic T lymphocytes causing metabolic changes, which lead to fibrosis, cirrhosis and HCC [62].

1.2.3 Treatment

Main goals of treating HCV are the elimination of the virus (sustained virologic response; SVR), reduction of the transmission of the infection to other people and to stop development of histological changes and therefore development of HCC. SVR is defined as the durable absence of detectable HCV RNA for at least 12 weeks after end of treatment [27].

The German guidelines on the management of HCV infection [132] recommend the treatment of all patients who are chronically infected with HCV. Until 2010 HCV standard treatment was PEG-IFNα plus ribavirin, which led to an SVR in about 50% of the patients [101]. In the past years several direct acting antivirals (DAAs) have been developed for DAA combination regimens, which lead to SVR rates $> 95\%$ in almost all patient groups [38–40, 184]. The efficacy of the approved treatment regimens is depending on viral geno- and subtype, stage of pre-treatment, fibrosis and occurrence of resistance associated substitutions [183]. Therefore, a cure of HCV still may remain a challenging issue e.g. in patients with cirrhosis, which have been treated unsuccessfully with DAA-based regimens, in people with decompensated cirrhosis and in patients infected with genotype 3 (especially with cirrhosis). Further global problems are the missing knowledge about the infection and the access to HCV treatment: From estimated 71 million people living with an HCV infection worldwide only 20% know about their infection and only 1.1 million people of diagnosed patients started an antiviral treatment in 2015.

1.2.4 The hepatitis C virus

1.2.4.1 General information and genome organization

The hepatitis C virus belongs to the family of the Flaviviridae and can be divided into seven genotypes (1 to 7), which differ in up to 30% of their nucleotide sequence [143], and 67 subgenotypes (a, b, c, ...) showing sequence homology of 75 to 80% [142]. Infectious virions display a spherical or pleomorphic form with a diameter varying between 50 and 80 nm [18, 58]. In contrast to other virions of the Flaviviridae family HCV particles are not characterized very well due to their high content of lipids and lipoproteins. HCV particles interact with very-low-density and low-density lipoproteins (VLDL and LDL) and are therefore termed as lipo-viro-particles (LVPs) [5].

The viral genome consists of a 9.6 kb single-stranded RNA with positive polarity, which is surrounded by the viral capsid and enveloped by heterodimerized glycoproteins. The genome contains one single ORF, which is flanked 5' and 3' by non-translated regions (NTR) and encodes a polyprotein of about 3000 aa. This polyprotein is processed co- and post-translationally by viral and host proteases into the viral proteins. As shown in Figure 1.7A the viral proteins can be divided into the non-structural proteins (NS3, NS4A, NS4B, NS5A and NS5B) essential for viral replication and the structural proteins (core, E1 and E2) forming HCV particles. Figure 1.7B shows the membrane topology and function of the viral proteins.

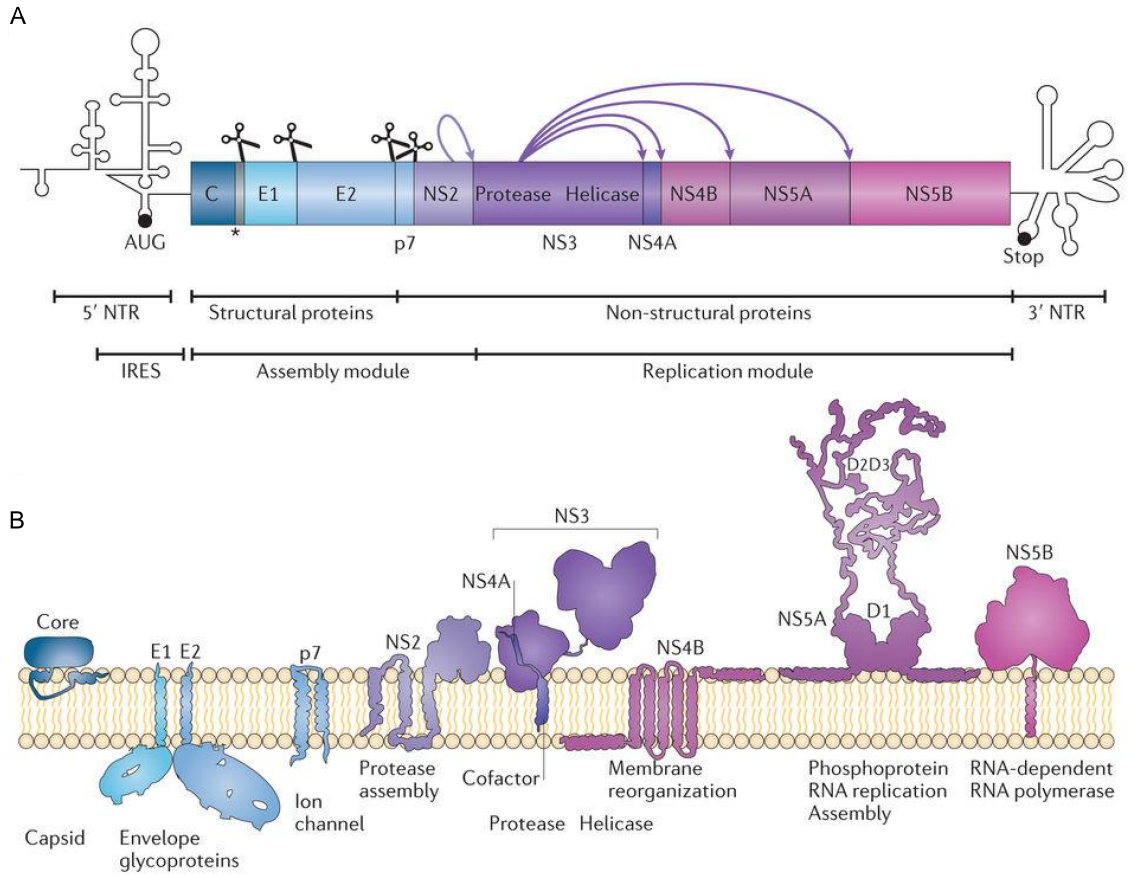

Figure 1.7: Genome of the hepatitis C virus and membrane topology of hepatitis C virus proteins. (A) The viral genome encompasses a 9.6 kb positive single-stranded RNA molecule containing one ORF flanked by non-translated regions (NTR). The ORF encodes a polyprotein of about 3000 aa, which is processed into structural and non-structural proteins. (B) Each protein is associated with intracellular membranes via transmembrane domains or amphipatic α-helices. Adapted from [12].

1.2.4.2 The viral proteins

The HCV genome is surrounded by a nucleocapsid built by the core protein, which is the most highly conserved one within the HCV proteins. Besides its structural function the core protein is involved in the formation of so called lipid droplets, which are necessary for viral assembly [105], and it interacts with several viral and cellular proteins (reviewed by [70]). The viral RNA is encapsulated by the nucleocapsid and surrounded by a host-derived lipid envelope layer, which is embedded with viral glycoproteins E1 and E2 playing a role in receptor binding and fusion of viral and endosomal membranes to release the nucleocapsid into the hepatocytes [87]. p7 is a 63 aa polypeptide with two transmembrane domains located in the ER. There, it models an ion channel, which plays a crucial role in the viral infection process and the assembly of virions [49, 146]. NS2 is a 21 to 23 kDa protein, which forms four transmembrane helices, and it is needed to built mature virions but it is not essential for viral replication [10]. NS3 is a bifunctional 67 kDa protein. The

N-terminal part cleaves the non-structural proteins apart from each other, whereas the C-terminal part harbors NTPase/helicase activity [34, 44, 141]. NS4A is a co-factor and activator of NS3 and embeds the NS3/4A complex to the ER membrane [74]. NS4B is a small protein of 27 kDa with four transmembrane domains [94]. Through restructuring of ER membranes and lipid droplets the formation of a structure called "membranous web" is induced at which viral replication takes place [36]. The RNA-binding phosphoprotein NS5A is attached to the ER membrane and consists of three domains. Domain I builts a dimer with a large RNA binding groove, whereas domain II is essential in viral genome replication and domain III is needed for the assembly of viral particles [19, 160]. NS5A can exist in a basally hypophosphorylated form of 56 kDa and a hyperphosphorylated form of 58 kDa [106]. Replication increases after hyperphosphorylation is inhibited and hence it might be assumed that the phosphorylation status of NS5A serves as a molecular switch from viral replication to viral assembly [7]. NS5B is a 65 kDa RNA-dependent RNA polymerase, which is anchored to the ER membrane via its C-terminal 21 aa and synthesizes new RNA molecules. Since the polymerase lacks proofreading activity 2×10^{-3} mutations per nucleotide position per year are incorporated and lead to an enormous heterogeneity of the viral genome and therefore to a diverse quasispecies [100].

1.2.4.3 Viral life cycle

The viral life cycle (see Figure 1.8) starts with an interaction of HCV particles with several receptors and entry factors, while HCV uptake is triggered via clathrin-mediated endocytosis of the virion [33, 34]. Due to a low endosomal pH, conformational changes in the E1E2 complex are induced, which might lead to a fusion of viral and endosomal membranes whereby the viral capsid is released into the cytoplasm [34].

After disintegration of the viral capsid the released RNA is recognized as mRNA and translation via the internal ribosomal entry site (IRES) located in the 5'-NTR is initiated at the rough ER. The emerged polyprotein is further processed co- and post-translationally by viral and host proteases into the viral structural and non-structural proteins. The non-structural proteins are embedded into restructured membranes of the ER building the replication complex [64]. A (−)-stranded RNA molecule serves as a template for the synthesis of a new (+)-stranded RNA genome, which is used for replication, translation and incorporation into viral particles [12].

The morphogenesis of mature viral particles still remains the step least characterized in the viral life cycle. It is assumed that the viral RNA is recruited to core-coated lipid droplets and that the maturation of viral particles is connected tightly to the maturation of VLDL particles, since viral particles are packaged as LVPs having similar densities as VLDLs. After the assembly the LVPs are budded into the ER and released by the cell via the secretory pathway [28, 103].

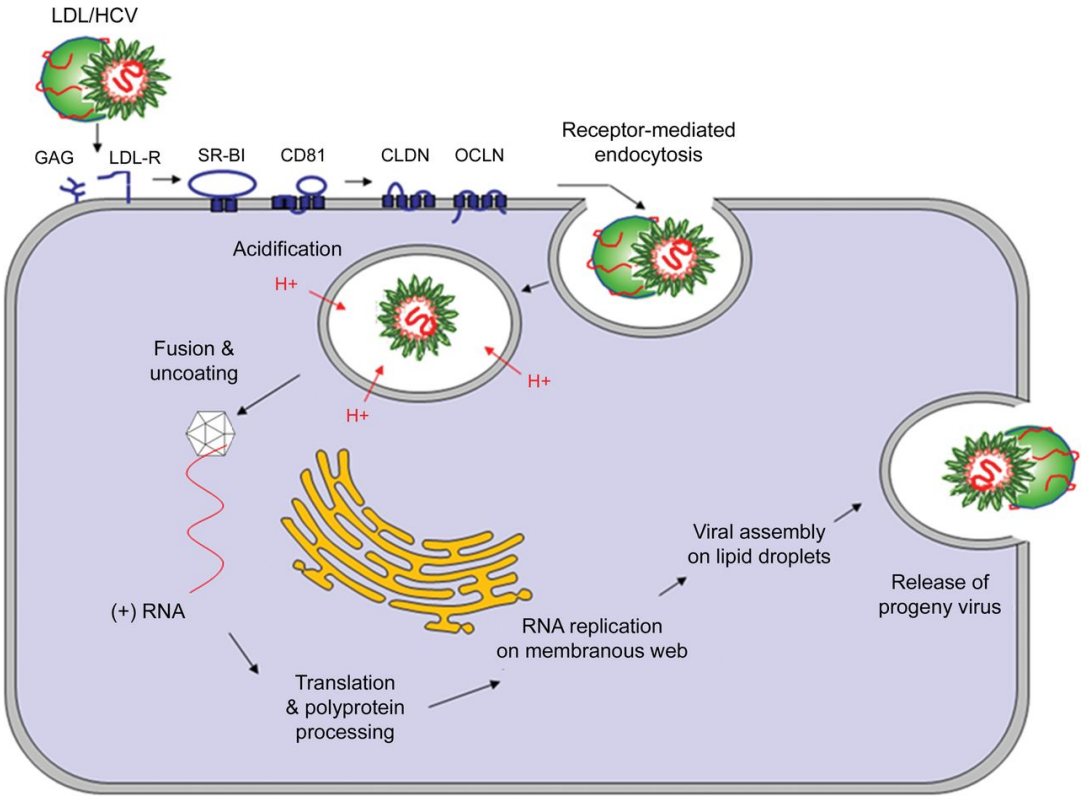

Figure 1.8: Life cycle of the hepatitis C virus. Virions attach to hepatocytes by interactions with glycosylaminoglycans (GAG), LDLR, CD81 and SR-BI with high affinity. The viral receptor complex translocates to the tight junction proteins CLDN and OCLN and receptor-mediated endocytosis is induced. The endosome is acidified in the cytoplasm, leading to fusion of viral and endosomal membranes and release of the nucleocapsid. The viral RNA is released and translated into a polyprotein, which is co- and post-translationally modified to build the viral proteins. Replication takes place at the membranous web. Assembled viral particles bud into the lumen of the ER and are released through the secretory pathway. Taken from [158].

1.2.4.4 Resistance associated substitutions

Although high cure rates can be achieved with the current (IFN)-free, DAA-based combination therapies, in 1 to 15% of the patients (depending on the viral genotype, stage of liver damage, baseline viral load or previously administered treatment regimens) the virus cannot be eradicated [132]. Furthermore, a very high variability of HCV genomic sequences (quasispecies) has been observed in infected patients due to an error prone HCV RNA polymerase. These pre-existing mutations exist also in untreated patients, emerge under the selective pressure of DAAs and confer resistance to several DAAs [129, 134]. Therefore, they are also called resistance associated substitutions (RASs).

The impact of baseline RASs on SVR depend on the susceptibility of the RASs, HCV genotype, treatment regimen and duration as well as the patient population and genetic host factors. Several amino acid changes in the sequence of the NS3-, NS5A- and/or NS5B-gene have been associated with treatment failure. RASs in NS3 have been related to a reduction of the replicative fitness, which explains a more unlikely detection of NS3 RASs as pre-existing RASs. Also, a fast replacement with the wildtype variant has been

observed after NS3 protease inibitor-based therapy was stopped (reviewed by [131]). One exception is the NS3 substitution Q80K, which is not associated with a significant replicative impairment *in vitro*. Furthermore, the various substitutions in the NS3-gene including V36M, T54S, V55A, Q80K, R155K and D168E confer different levels of resistance to the approved NS3 protease inhibitors (e.g. simeprevir, asunaprevir, paritaprevir/grazoprevir) (reviewed by [131]).

In contrast, the replicative fitness of RASs to NS5A inhibitors is not impaired and therefore NS5A RASs are more likely to persist for a long duration. Furthermore, some RASs are found with different frequencies in the various HCV genotypes conferring different levels of resistance. Whereas the majority of NS5A RASs like M28A/G/T/V, L31I/M/V/ and Y93C/F/N found in genotype 1a conferred intermediate to high-level resistance to daclatasvir/ledipasvir/ombitasvir/elbasvir, mainly low to intermediate resistant variants, e.g. L28T, R30H and L31I/F/M/V, are detected in genotype 1b infected patients. One exception is the Y93H substitution, which confers intermediate to high level resistance in genotype 1b to different treatment regimens (reviewed by [131]).

Nucleotide analogue inhibitors of NS5B (sofosbuvir) have a high barrier to resistance and only in a few cases major RASs have been detected (i.e. S282T) [154]. In contrast, non-nucleoside inhibitors of NS5B (dasabuvir) have a low resistance barrier selecting rapidly resistant variants with a higher replicative fitness (reviewed by [115]). RASs conferring low level resistance to dasabuvir were rarely detected in genotype 1a infected patients (C316Y in up to 1.2% and S556G in up to 3.1%), whereas they were detected more frequently in genotype 1b infected patients (C316N in up to 35.6% and S556G in up to 25%) (reviewed by [131]).

Although only limited data are available regarding the global distribution of pre-existing RASs, the frequency of some substitutions, like the Y93H, varies between European HCV genotype 1b infected patients (15%) compared to genotype 1b infected patients from Japan (12.5%) or the US (9.3%) [60, 79].

1.2.4.5 Host factors

Clinical trials identified several host genetic factors that influenced treatment efficacy and the clinical course of the HCV infection. One landmark finding was the association of single nucleotide polymorphisms (SNPs) in or near the interferon-λ3 (IFNL3), formerly known as interleukin-28B (IL28B), and interferon-λ4 (IFNL4) locus [124] with the spontaneous clearance of HCV [126, 162]. Furthermore, those SNPs were related to the response to PEG-IFNα plus ribavirin therapy [45, 151, 157], triple therapy or IFN-free regimens [3, 183]. One of these SNPs is a nucleotide variant within the first intron of the human IFNL4 gene at position rs12979860 (C/C vs. C/T or T/T). The C/C-genotype has been strongly associated with HCV clearance. Recently, another dinucleotide variant 367 bp upstream of rs12979860 in the first exon of IFNL4 at rs368234815 (TT/TT vs. TT/ΔG or ΔG/ΔG) was found. This variant is in strong linkage disequilibrium with rs12979860

and was described in various studies to be a better predictor than rs12979860 for PEG-IFNα/ribavirin treatment response in HCV genotype 1-infected and genotype 3-infected patients [14, 153]. The ΔG deletion (TT/ΔG or ΔG/ΔG) at rs368234815 leads to a frameshift causing the establishment of a novel ORF in the IFNL4 gene encoding the IFN-λ4 protein, whereas the alternative allele at this locus (TT/TT) does not encode IFN-λ4 [124]. By transient over-expression experiments of IFNL4 in hepatoma cells it could be shown that IFN-λ4 proteins induce the phosphorylation of the STAT1 and STAT2 proteins (signal transducer and activator of transcription 1 and 2) and activation of different IFN-stimulated gene, which are essential components of the cellular antiviral response and adaptive immunity. Remarkably, the loss of IFN-λ4 by the TT/TT variant has been described with improved HCV clearance [124].

2 Aim of this study

In several studies HBV-DNA > 2000 IU/ml and qHBsAg > 1000 IU/ml were found to be associated with liver disease progression and development of hepatocellular carcinoma. Hence, these cut-offs are used in the clinical HBV disease assessment of patients with chronic HBV infection. Furthermore, associations between mutations in the hepatitis B virus basal core promoter, the precore and the preS region and the development of liver fibrosis, cirrhosis and/or hepatocellular carcinoma were found. While these markers and cut-offs were evaluated and approved in Asian studies, data regarding European patients remain limited. Since Asian patients are characterized by a high percentage of vertical HBV transmission and a predominant prevalence of HBV genotypes B and C, infection in the Western world is mostly transmitted horizontally and genotypes A and D are prevailing. Hence, it is obscure if these markers can be directly transferred from Asian populations to genetically different populations in the Western world. Therefore, the aim of the present study was to analyze the impact of genetically different HBV variants (different HBV genotypes, basal core promoter-, precore- and preS-mutations) on the established prognostic serum markers HBV-DNA and qHBsAg in a large European study cohort of patients with HBeAg negative chronic HBV infection. Furthermore, the sera of patients infected with HBV genotypes A to E were analyzed regarding their composition of released viral and subviral particles since only very limited data regarding the comparison of genotypes in vivo exist.

But not only virological factors may influence the progression of a disease, but also several host genetic factors that influence treatment efficacy and the clinical course of a infection were identified in several studies. For example, single nucleotide polymorphisms in or near the interferon-λ3 and interferon-λ4 locus were significantly associated with the spontaneous clearance of a hepatitis C virus infection and with the response to different treatment regimens. The present study aimed to analyze the prevalence of substitutions in different HCV genes conferring resistance to direct acting antivirals in a large European population chronically infected with HCV. Additionally, the prevalence of those resistance associated substitutions should be correlated to single nucleotide polymorphisms in the interferon-λ3 and interferon-λ4.

3 Materials

3.1 Patient samples

Hepatitis B virus

In total 504 patients of the Albatros trial (listed at Clinical.Trial.gov: NCT01090531) with HBeAg negative chronic HBV infection were included in the analysis. Main inclusion criteria of the Albatros trial were chronic HBV infection with HBsAg and anti-HBc positivity for at least 6 months, HBeAg negativity, HBV DNA levels $<$ 100,000 IU/ml, ALT levels $\leq$ 2x upper limit of normal, age between 18 and 79 . Main exclusion criteria were current or intended antiviral therapy against HBV, previous antiviral HBV therapy for more than 3 months, chronic "immune tolerant" HBeAg positive HBV infection (HBeAg positive, high levels of HBV DNA, normal liver values), coinfection with HCV and HIV as well as malignant or psychiatric comorbidities, hepatocellular carcinoma or hepatic metastases. HBV genotypes were either determined during clinical routine or by population-based sequencing of the polymerase region within the present work.

Hepatitis C virus

In total 633 patient samples of two German multicenter studies (INDIV-2; Clinical.Trial.gov: NCT00351403 and PIRB/PISA (described in [31]) and from the outpatient department of the University Hospital Frankfurt, Germany, were taken for primary analysis. Samples from treatment-naive as well as treatment-experienced patients chronically infected with HCV genotype 1a, 1b and 3 were analyzed. Additionally, 201 patient samples of the INDIV-2 study and the outpatient department were enrolled as a replication cohort. Patients were infected with genotype 1a or 1b and treatment-naive. HCV genotypes were determined during clinical routine.

Serum or plasma of these patients was prospectively collected and stored at $-80\,^{\circ}\mathrm{C}$. The studies were approved by local ethics committees and written informed consent was obtained from all patients. The studies were performed in accordance with the provisions of the Declaration of Helsinki and good clinical practice guidelines [46].

3.2 Cells

3.2.1 Bacterial cells

Strain	Genotype	Source
E. coli TOP10	F− mcrA Δ(mrr− hsdRMS− mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG	Invitrogen, Karlsruhe
E. coli DH5α	F− Φ80lacZΔM15 Δ(lacZYA− argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 λ− thi−1 gyrA96 relA1	Invitrogen, Karlsruhe

3.2.2 Mammalian cells

Strain	Source
Huh7.5	Human hepatoblastoma cell line derived from Huh7 cells [16]

3.3 Plasmids

Plasmid	Description	Source
pUC18	cloning vector	Invitrogen, Karlsruhe
pUC18_6006	pUC18 + 1.5 fold HBV genome with reference genome sequence	Generated during this work
pUC18_6015	pUC18 + 1.5 fold HBV genome with precore double mutation G1896A/G1899A	Generated during this work
pUC18_011	pUC18 + 1.5 fold HBV genome with 15 aa deletion in preS1-sequence	Generated during this work
pUC18_6006_BCP	pUC18 + 1.5 fold HBV genome with reference genome sequence but with BCP double mutation A1762A/G1764A	Generated during this work

The pUC18 plasmid was kindly provided by Prof. Dr. Eberhard Hildt, Paul-Ehrlich-Institute, Langen.

3.4 Oligonucleotides

3.4.1 Oligonucleotides for amplification and sequencing - HBV

All oligonucleotides used during this work were synthesized by Eurofins Scientific, Luxemburg.

Number	**Sequence 5' → 3'**
1	CAC GTT GCA TGG AGA CCA
2	GGA GTG CGA ATC CAC ACT CC
3	TGT CAA CGA CCG ACC TTG AG
4	GCA ATG CTC AGG AGA CTC TAA GGC
5	ACT CTT GGA CTY TCA GCA ATG
6	GTC AGA AGG CAA AAA AGA GAG
7	TCT CAG CAA TGT CAA CGA CCG
8	AGA GAG TAA CTC CAC AGA WGC TC
9	TGC GGC GTT TTA TCA TCT TCC T
10	GTT TAA ATG TAT ACC CAA AGA C
11	CAG CGG CAT AAA GGG ACT CAA G
12	GCG GGT CAC CAT ATT CTT GG
13	GAG TCT AGA CTC TGC GGT AT
14	TAA CAC GAG CAG GGG TCC TA
15	GCG GGT CAC CAT ATT CTT GG
16	AAC TGG AGC CAC CAG CAG
P1	CCG GAA AGC TTG AGC TCT TCT TTT TCA CCT CTG CCT AAT CA
P2	CCG GAA AGC TTG AGC TCT TCA AAA AGT TGC ATG GTG CTG G
A1	CCC AAG CTT CTA TTG ATT GGA AAG TAT GTC
A2	GAA AAT TGA GAG AAG TCC AC
B1	ACA ARA ATC CTC ACA ATA CC
B2	GAA GAT CTG ATA GGG GCA TTT GGT GGT C
K1	TGC CTC TCA CAT CTC GTC AAT C
K2	GAG CCA AGA GAA ACG GAC TG
K3	TCC AGG AAC AAC AAC AAC
K4	GTT GGC GAG AAA GTG AAA GC
K5	GGC TTT GCT GCT CCA TTT AC
K6	TTG CTG GGA GTC CAA GAG TC
K7	CCG TCT GTG CCT TCT CAT C
K8	CTA GAT CCC TGG ATG CTG
K9	TCA CCA TAC AGC ACT CAG

Number	Sequence 5' → 3'
K10	GTT TCC CTC TTA TAT AGA ATCC
K11	CAA TTT GTG GGC CCT CTC
K12	TTA GAG GTG GAG AGA TGG
K13	CAG GCT CAG GGC ATA TTG
K14	AGC AGC AGG ATG AAG AGG

3.4.2 Oligonucleotides for site-directed mutagenesis - HBV

Number	Sequence 5' → 3'
SDM1	ACT TCT CTC AAT TTT CTA GGG GGA CCA CCC GTG TG
SDM2	CAC ACG GGT GGT CCC CCT AGA AAA TTG AGA GAA GT
SDM3	CCT AAT ACA AAG ACC TTT AAC CTC GTC TCC TCC
SDM4	GGA GGA GAC GAG GTT AAA GGT CTT TGT ATT AGG

3.4.3 Oligonucleotides for amplification and sequencing - HCV

Number	Sequence 5' → 3'
1	CAC GTT GCA TGG AGA CCA
2	GGA GTG CGA ATC CAC ACT CC
3	TGT CAA CGA CCG ACC TTG AG
4	GCA ATG CTC AGG AGA CTC TAA GGC
5	ACT CTT GGA CTY TCA GCA ATG
6	GTC AGA AGG CAA AAA AGA GAG
7	TCT CAG CAA TGT CAA CGA CCG
8	AGA GAG TAA CTC CAC AGA WGC TC
9	TGC GGC GTT TTA TCA TCT TCC T

3.4.4 Oligonucleotides and probes for TaqMan Real Time Assays - HBV

Oligonucleotides and probes for TaqMan Real Time Assays were synthesized by Thermo Fisher Scientific, Darmstadt.

Primer/ Probe	**Sequence 5' → 3'**
HBV-F	GGC TGT AGG CAT AAA TTG GTC
HBV-M	FAM-TGC GTA CCA GCA CCA TGC AAC T-NFQ
HBV-R	AGC TTG GAG GCT TGA ACA GT

3.4.5 Oligonucleotides and probes for TaqMan Real Time Assays - HCV

Primer/ Probe	**Sequence 5' → 3'**
rs368234815IFNL4-F	GCC TGC TGC AGA AGC AGA GAT
rs368234815IFNL4-R	GCT CCA GCG AGC GGT AGT G
rs368234815IFNL4-V	VIC-ATC GCA GAA GGC C-NFQ
rs368234815IFNL4-M	FAM-ATC GCA GCG GCC C-NFQ
rs12979860-F	GCC TGT CGT GTA CTG AAC CA
rs12979860-R	GCG CGG AGT GCA ATT CAA C
rs12979860-V	VIC-TGG TTC GCG CCT TC-NFQ
rs12979860-M	FAM-CTG GTT CAC GCC TTC-NFQ

3.5 Antibodies

Antibody	**Species, Clonality**	**Dilution**	**Manufacturer**
Primary antibodies			
Anti-HBsAg HB01	mouse, monoclonal	1:400	kindly provided by E. Hildt, Paul-Ehrlich-Institute
Anti-LHBs MA18/7	mouse, monoclonal	1:600	kindly provided by E. Hildt, Paul-Ehrlich-Institute
Anti-Transferrin	rabbit, monoclonal	1:200	Santa Cruz Biotechnology, Heidelberg
Anti-β-Actin	mouse, monoclonal	1:10,000	Sigma-Aldrich, Seelze

Antibody	Species, Clonality	Dilution	Manufacturer
Secondary Antibodies			
Anti-mouse IgG-HRP	goat, polyclonal	1:5000	Santa Cruz Biotechnology, Heidelberg
Anti-rabbit IgG-HRP	goat, polyclonal	1:5000	Santa Cruz Biotechnology, Heidelberg

All antibodies were diluted in 10% milk powder in 0.05% Tween/TBST (Carl Roth, Karlsruhe) for western blot analysis.

3.6 Molecular weight markers

Name	Manufacturers
Precision Plus Protein Dual Color Standards	Bio-Rad, München
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific, Darmstadt

3.7 Enzymes

Name	Manufacturers
Restrictionendonucleases	NEB, Frankfurt am Main
T4 DNA-Ligase	NEB, Frankfurt am Main
DNaseI	Promega, Mannheim

3.8 Reagents for cell culture

Name	Manufacturers
DMEM medium (4,5 g/l glucose, L-glutamine)	Thermo Fisher Scientific, Darmstadt
PBS (Phophate Buffered Saline)	Thermo Fisher Scientific, Darmstadt
Fetal Calf Serum (FCS)	Thermo Fisher Scientific, Darmstadt
Trypsin-EDTA (0.05%)	Thermo Fisher Scientific, Darmstadt
Penicillin/ Streptomycin	Thermo Fisher Scientific, Darmstadt

3.9 Proteaseinhibitors

Name	Target	Manufacturers
Aprotitin	serin proteases (1 µM)	Sigma-Aldrich, Seelze
Leupeptin	serin-, cystein-protease (4 µM)	Sigma-Aldrich, Seelze
Peptastin	acid-, aspartatic-proteases (1 µM)	Sigma-Aldrich, Seelze
PMSF	serin proteases (1 mM)	Carl-Roth, Karlsruhe

3.10 Chemicals

All chemicals were purchased from Carl Roth/Karlsruhe, Sigma-Aldrich/Seelze, or Merck/ Darmstadt. Exceptions are listed in the following table.

Name	Manufacturers
peqGOLD TriFast	VWR, Erlangen
Acrylamide 4k solution	AppliChem, Darmstadt
TAE-buffer	AppliChem, Darmstadt
GelPilot DNA Loading Dye, 5x	Qiagen, Hilden
Polyethylenimin (PEI)	Fermentas, St. Leon-Rot
Sephadex G-25 Medium	GE Healthcare, Freiburg
Universal-Agarose, peqGOLD	VWR, Darmstadt

3.11 Commercial kits

Name	Manufacturers
QIAamp Viral RNA Mini Kit	Qiagen, Hilden
SuperScript III First-Strand Synthesis SuperMix	Thermo Fisher Scientific, Darmstadt
QIAamp DNA Blood Mini Kit	Qiagen, Hilden
QIAamp UltraSens Virus Kit	Qiagen, Hilden
QIAquick Gel Extraction Kit	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
QIAprep Spin Miniprep Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden

Name	Manufacturers
High Pure Viral Nucleic Acid Kit	Roche Diagnostics, Mannheim
TA Cloning Kit with pCR 2.1 vector	Thermo Fisher Scientific, Darmstadt
QIAGEN Fast Cycling PCR Kit	Qiagen, Hilden
Expand High Fidelity PCR System	Roche Diagnostics, Mannheim
Titanium Taq PCR Kit	ClonTech, Saint-Germain-en-Laye, France

3.12 Devices

3.12.1 Electrophoresis

Name	Manufacturers
Horizontal elektrophoresis system Horizon 11.14	Analytik Jena, Jena
Consort EV231 power supply	Consort bvba, Turnhout, Belgium
Mini-PROTEAN Tetra Handcast Systems	Bio-Rad, München
Trans-Blot SD Semi-Dry Transfer Cell	Bio-Rad, München
Electrophoresis power supply eps 301	GE Healthcare, Freiburg

3.12.2 Microscopy

Name	Manufacturers
Electron Microscope 109	Zeiss, Jena
Axiovert 135	Zeiss, Jena

3.12.3 Imaging

Name	Manufacturers
Fusion FX	Vilber Lourmat, Eberhardzell
Gel Doc XR System	Bio-Rad, München

3.12.4 PCR cycler

Name	Manufacturers
StepOnePlus Real-Time PCR System	Thermo Fisher Scientific, Darmstadt
LightCycler 480	Roche Diagnostics, Mannheim
LightCycler 1.5	Roche Diagnostics, Mannheim
Applied Biosystems Veriti Thermal Cycler	Thermo Fisher Scientific, Darmstadt
GeneAmp PCR System 9700	Thermo Fisher Scientific, Darmstadt

3.12.5 Centrifuges

Name	Manufacturers
Optima^{TM} L-80 XP Ultracentrifuge	Beckman Coulter, Krefeld
L8-70M Ultracentrifuge	Beckman Coulter, Krefeld
Rotors SW32Ti and SW60 for Ultracentrifuges	Beckman Coulter, Krefeld
Mini centrifuge	Bio-Rad, München
Sigma 4-15C Benchtop Centrifuge	Qiagen, Hilden

3.12.6 Other devices

Name	Manufacturers
Laborwaage Kern Typ 510-63	Kern, Balingen
Präzisionswaage EW-N/EG-N	Kern, Balingen
Nanodrop 2000	Thermo Fisher Scientific, Darmstadt
Tecan Infinite M1000	Tecan Group AG, Männedorf Schweiz
Thermomixer compact	Eppendorf, Hamburg
Vortex Mixer VF2	IKA Werke, Staufen
CAT Roller RM5-30V	CAT, Staufen
New Brunswick Innova 44	Eppendorf, Hamburg
Heraeus Function Line B6	Heraeus, Hanau
Heracell 240i CO_2 Incubator	Thermo Fisher Scientific, Darmstadt
Herasafe KS, Class II Biological Safety Cabinet	Thermo Fisher Scientific, Darmstadt
Ultrasound-Homogenisator Sonopuls HD 2070	Bandelin, Berlin

Name	Manufacturers
Sonotrode MS 73	Bandelin, Berlin
pH-meter	Thermo Fisher Scientific, Darmstadt
Plattenleser Envision 2104 Multilabel Reader	PerkinElmer, Oxon Hill, USA

3.12.7 Other relevant material

Name	Manufacturers
Immobilon Western HRP Substrate	Millipore, Darmstadt
Luminata Forte	Merck, Darmstadt
Rotiload 1	Carl Roth, Karlsruhe
TaqMan Gene Expression Mastermix	Thermo Fisher Scientific, Darmstadt
TaqMan Genotyping Mastermix	Thermo Fisher Scientific, Darmstadt
SYBR Green Real-Time PCR Master Mix	Thermo Fisher Scientific, Darmstadt
LightCycler capillaries (Polycarbonate)	Genaxxon, Biberach
Enzygnost HBsAg 6.0	Siemens Healthineers, Erlangen
Enzygnost HBe monoclonal testkit	Siemens Healthineers, Erlangen
Munktell Filter Paper F1	Munktell & Filtrak GmbH, Bärenstein
Amersham Protran 0.2 Nitrocellulose membrane	GE Healthcare, Freiburg
Neubauer Chamber	Carl Roth, Karlsruhe

3.13 Buffers and solutions

Name	Composition
RIPA buffer	50 mM Tris-HCl pH 7.2 150 mM Natrium chloride 0.1% SDS (w/v) 1% Sodium desoxycholate (w/v) 1% TritonX 100

Name	Composition
2.2% SDS	2.2% SDS (w/v)
0.5% SDS	0.5% SDS (w/v)
SDS running buffer (10x) buffer	0.25 M Tris 2 M Glycine 1% SDS (w/v) pH 8.3
Separation gel buffer	1.88 M Tris pH 8.8
Stacking gel buffer	0.625 M Tris pH 6.8
TBST buffer (10x)	200 mM Tris-HCl pH 7.8 1.5 M Sodium chloride 0.5% Tween
Transfer buffer (10x)	0.25 M Tris 2 M Glycine 1% SDS (w/v) pH 8.3
Transfer buffer (1x)	100 ml Transfer buffer (10x) 20% Methanol (v/v) ad 1000 ml H_2O
Ampicillin stock solution	100 mg/ml

3.14 Software

Name	Composition
LightCycler Software Version 3.5	Roche Diagnostics, Mannheim
iTEM	Olympus, Münster
Fusion. Ink	Vilber Lourmat, Eberhardzell
FinchTV	PerkinElmer, Oxon Hill, USA

Name	**Composition**
BioEdit Sequence Alignment Editor	Ibis Therapeutics, Carlsbad, USA
Graph Pad Prism 5	GraphPad Software, Inc., California, USA
BiAS version 11	Epsilon, Frankfurt
Inkscape vector graphics editor 0.91	Inkscape Community

4 Methods

4.1 Cell biology

4.1.1 Procaryotic cell culture

E. coli TOP10 cells or XL10 Gold Ultracompetent cells were cultivated for 16 h in LB medium with 100 µg/ml ampicillin for the selection of transformed bacteria at 37 °C under agitation in Erlenmeyer flasks or 15 ml Falcons. Stocks were stored in 25% (v/v) glycerol at −80 °C.

4.1.2 Mammalian cell culture

Cells of the human hepatoma cell line Huh7.5 were grown in DMEM (4.5 g/l glucose) containing 10% FCS, 1% penicillin/streptomycin and 2 mM L-glutamine (DMEM complete) at 37 °C, 5% CO_2 and 90% humidity.

Reaching 80 - 90% confluency cells were washed once with PBS and trypsinated using 1x trypsin/EDTA solution for 2 - 5 minutes at 37 °C. The trypsin/EDTA solution was inactivated by adding DMEM complete. The cells were seeded at a dilution of 1:5 into fresh medium in order to obtain optimal growth.

4.1.3 Transfection of Huh7.5 cells with plasmids

Huh7.5 cells were transfected with plasmids by using linear polyethylenimine (PEI) (1 mg/ml). 1 µg plasmid DNA was resuspended in 200 µl PBS, supplemented with 6 µl PEI/µg plasmid DNA and mixed by vortexing for 10 seconds. After incubating the reaction mixture for 10 minutes at room temperature, it was added dropwise to 2.5 x 10^6 cells/well plus 2 ml medium in a six well plate. After 16 h the medium was changed and the transfected cells were incubated for another 48 h at 37 °C, 5% CO_2 and 90% humidity.

4.1.4 Virus purification

17.5 ml of cell culture supernatant containing produced viruses were loaded onto 17.5 ml of 10% sucrose in TN_{150}-buffer and subjected to ultracentrifugation in a SW32Ti rotor for 3 h 10 min at 32,000 rpm at 4 °C. After centrifugation, the supernatant was discarded carefully and the viral pellet was resuspended in 150 µl TN_{150}-buffer.

For further separation of viral and subviral particles the 150 µl of the previous step were layered onto a sucrose gradient, consisting from bottom to top of 40 - 25% sucrose in TN_{150}-buffer in 2.5% steps, and subjected to ultracentrifugation in a SW60 rotor for 17 h at 32,000 rpm at 4 °C. After centrifugation 18 fractions were collected from top to bottom each 210 µl and stored at 4 °C.

For enrichement of viral and subviral particles from patient samples sera were either enriched or separated via ultracentrifugation. For enrichment, 100 - 200 µl patient sera were layered on top of a cushion of 10% sucrose dissolved in TN_{150}-buffer and centrifuged in a SW60 rotor at 42,000 rpm for 2.5 h at 4 °C. For separation, 100 - 200 µl patient sera were layered onto a linear gradient and centrifuged in a SW60 rotor at 32,000 rpm for 17 h at 4 °C. The gradient was composed from bottom to top as follows: 40%, 37.5%, 35%, 32.5%, 30%, 27.5%, 25% sucrose dissolved in TN_{150}-buffer.

4.1.5 Protein lysates of mammalian cells

Transfected Huh7.5 cells were washed once with PBS and RIPA buffer supplemented with a protease-phosphatase-inhibitor cocktail was added to the cells. The lysed cells were transferred to 1.5 ml reaction tubes after 10 minutes, sonificated with a maximum of 20% power for 10 seconds. After a 5 minute centrifugation step at 1,000 g and 4 °C the supernatant was transferred to a new reaction tube and subjected to Western Blot analysis.

4.2 Molecular biology

4.2.1 Agarose gel electrophoresis

1 - 2% (w/v) agarose was mixed with 1x TAE buffer and dissolved by heating. 0.1 µg/ml ethidiumbromide was added into the liquefied agarose and after mixing it was poured into a horizontal gel chamber containing a comb. When the gel had solidified, it was transferred into a gel chamber containing 1x TAE buffer. Samples to be analyzed were supplemented with 5x loading dye and separated at 110 V. DNA was visualized by UV light (254/365 nm).

4.2.2 Purification of DNA

4.2.2.1 Purification via Gelextraction

If multiple bands were separated on an agarose gel, the right band was excised from the gel with a clean and sharp scalpel and the DNA was extracted with the QIAquick Gel Extraction Kit.

4.2.2.2 Purification directly from PCR product

If only one single band DNA was generated by PCR, the DNA was extracted from the PCR approach with the QIAquick PCR Purification Kit.

4.2.3 Determination of nucleic acid concentration

DNA or RNA concentration and purity were determined using a Nanodrop UV-Vis Spectrophotometer. At 260 nm an absorbance/extinction of 1.0 corresponds to a concentration of 40 µg/ml RNA or 50 µg/ml double stranded DNA. Measuring the absorbance at 280 nm contemporaneously will give information on the purity of your sample.

4.2.4 Isolation of plasmid DNA

Plasmid DNA was either isolated of 5 ml bacterial culture with the QIAprep Spin Miniprep Kit or of 200 ml with the QIAGEN Plasmid Maxi Kit according to the manufacturer's instructions.

4.2.5 Restriction endonuclease digestion

Either circular plasmids were linearized or inserts and plasmids were digested prior to ligation. For analytical purposes 0.5 µg plasmid DNA were digested with the corresponding restriction endonucleases according to the manufacturer's protocol. For cloning purposes 10 µg plasmid DNA were digested. The DNA was separated via agarose gelelectrophoresis.

4.2.6 DNA ligation

4.2.6.1 Self-ligation of HBV monomers to dimers

In order to generate HBV full-length genomes in a dimeric form, 400 ng DNA were self-ligated in a total volume of 20 µl with T4 DNA ligase from NEB. The reaction was incubated at 16 °C over night.

4.2.6.2 Ligation of vector and insert

In a total volume of 10 µl 100 ng linearized vector and a three- to five-fold excess of insert DNA with compatible ends were ligated with T4 DNA ligase from NEB. The reaction was incubated at 16 °C over night.

4.2.7 Transformation of competent bacteria

2 µl TOPO cloning reaction or DpnI digested site directed mutagenesis reaction were transformed into One Shot TOP10 *E. coli* or XL10 Gold Ultracompetent cells, respectively, according to the manufacturer's instructions.

2 µl of ligated vector and insert were added to chemically competent *E. coli* TOP10 cells (Invitrogen) and the transformation was performed according to the manufacturer's protocol.

4.2.8 Polymerase Chain Reaction

PCR reactions were either set up in a total volume of 20 µl using the Fast Cycling PCR Kit from QIAGEN (Table 4.1, 4.2) or in 50 µl using the Expand High Fidelity PCR System from Roche (Table 4.3, 4.4) or in 50 µl using the Titanium Taq PCR Kit from ClonTech (Table 4.5, 4.6).

Table 4.1: PCR reaction mixture Fast Cycling PCR Kit

Component	**Volume [µl]**
Template DNA	1 - 3
Fast Cycling Mastermix (10x)	10
Q solution	4
10 µM Primer forward	0.5
10 µM Primer reverse	0.5
H20	ad 20

Table 4.2: PCR amplification program Fast Cycling PCR Kit

Temperature	**Time**	
95 °C	5 min	
96 °C	5 sec	
x	5 sec	35x
68 °C	1 min	
72 °C	1 min	

Annealing temperatures (x) according to the corresponding oligonucleotides were used as follows: 52 °C and 55 °C with oligonucleotides 1/2 and 3/4 (outer and inner PCR), 60 °C and 60 °C with oligonucleotides 5/6 and 7/8 (outer and inner PCR), 50 °C and 55 °C with oligonucleotides 9/10 and 9/11 (outer and inner PCR), 55 °C and 58 °C with oligonucleotides 12/13 and 12/14 (outer and inner PCR).

Table 4.3: PCR reaction mixture Expand High Fidelity PCR System

Component	Volume [µl]
Template DNA	10 - 40
Expand High Fidelity Buffer (10x) with 15 mM $MgCl_2$	5
Deoxynucleotide mix (10 mM of each dNTP)	1
10 µM Primer forward	1
10 µM Primer reverse	1
Expand High Fidelity Enzyme Mix	2
H_2O	ad 20

Table 4.4: PCR amplification program Expand High Fidelity PCR System

Temperature	Time	
94 °C	2 min	
94 °C	40 sec	
x	1.5 min	35x
68 °C	3 min + 2 min every 10 cycles	

Annealing temperatures (x) according to the corresponding oligonucleotides were used as follows: 56 °C with oligonucleotides P1 and P2, A1 and A2 or B1 and B2.

Table 4.5: PCR reaction mixture Titanium Taq PCR Kit

Component	Volume [µl]
Template DNA	1 - 2
Titanium PCR buffer	5
dNTPs	1
10 µM Primer forward	1.5
10 µM Primer reverse	1.5
Taq polymerase	1
H_20	ad 50

Table 4.6: PCR amplification program Titanium Taq PCR Kit

Temperature	Time	
95 °C	5 min	
95 °C	45 sec	
x	45 sec	40x
72 °C	90 sec	
72 °C	1 min	

Annealing temperatures (x) were 55 °C for the outer PCR and 60 °C for the inner PCR.

4.2.9 Cloning of 1.5-fold HBV genotype A genomes

The entire HBV genome was amplified from viral DNA extracted from patients with a chronic HBeAg negative HBV infection with modified primers P1 and P2 from Günther et al [50] as described in Tables 4.3 and 4.4. The amplified full-length HBV genotype A genomes were cloned into the pCR^{TM}2.1 -TOPO vector with the TOPO TA Cloning Kit according to the manufacturer's protocol. Afterwards, the plasmid DNA was extracted with the QIAprep Miniprep Kit and digested with the restriction enzyme SapI to release the inserted full-length HBV DNA. The released DNA was cleaned via gelextraction and subjected to a self-ligation step overnight at 16 °C with T4 DNA ligase. Full-length monomers were ligated with each other to form head-to-tail-dimers. The dimers were cleaned via gelextraction and futher used as template for the generation of the fragments A (positions 961 to 278 of the HBV genotype A genome) and B (positions 221 to 2317 of the HBV genotype A genome) via PCR as described in Tables 4.3 and 4.4. Fragments A and B were cleaned with the QIAprep PCR purification kit. Fragment A was digested with the restriction enzymes HindIII and XbaI and fragment B with XbaI and BglII and cloned into the pUC18 vector digested with HindIII and BamHI.

4.2.10 Real-Time PCR (rt-PCR)

Maxima SYBR Green qPCR-Kit (Fermentas) (Table 4.7, 4.8) or TaqMan Assays (Table 4.9, 4.10) were used.

Table 4.7: rt-PCR reaction mixture Maxima SYBR Green qPCR-Kit

Component	**Volume [µl]**
Template DNA	3
2x Maxima SYBR Green qPCR Master Mix	5
10 µM Primer forward	0.25
10 µM Primer reverse	0.25
Expand High Fidelity Enzyme Mix	2
H_2O	ad 10

Table 4.8: rt-PCR program Maxima SYBR Green qPCR-Kit

Program	**Temperature** (°C)	**Hold time** (sec)	**Slope** (°C/sec)	**Cycles**
denaturating	95	600	20	1
cycling	95	15	20	45
	56	30	20	
	72	30	5	
melting	95	60	20	1
	60	30	20	
	95	0	0.1	
cooling	40	30	20	

Table 4.9: rt-PCR reaction mixture TaqMan Assay

Component	**Volume [µl]**
Template DNA	1 - 2
2x Master Mix	10
20x Assay Mix	1
H_2O	ad 20

Table 4.10: rt-PCR program TaqMan Assay

Temperature	Time	
50 °C	2 min	
95 °C	20 sec	
95 °C	1 sec	
60 °C	20	40x

4.2.11 Population-based sequencing

For sequencing, the DNA which was amplified and purified before was subjected to a PCR using only one primer and the BigDye Terminator 3.1 Ready Reaction Mix (Thermo Fisher) following the protocol described in Tables 4.11 and 4.12.

Table 4.11: Reaction mixture for sequencing PCR

Component	Volume [µl]
Template DNA	1
BigDye Terminator 3.1 Ready Reaction Mix	4
10 µM Primer forward or reverse	0.5
H_2O	ad 10

Table 4.12: Sequencing PCR amplification program

Temperature	Time	
96 °C	2 min	
96 °C	10 sec	
45 °C	15 sec	35x
60 °C	5 min	
72 °C	10 min	

4.2.12 Deep sequencing

PreS amplicons were generated by semi nested PCR as described in section 4.2.8. For each of the samples the second round of PCR was conducted in triplicate. The three approaches were pooled together to reduce PCR bias. Samples were sent to SeqIt Laboratories, Kaiserslautern, and analyzed by Illumina deep sequencing as described [32, 152].

According to previous investigations, a conservative 1% frequency (occurrence rate of the variant in percent of the quasispecies) cut-off was used for calling variants and to exclude potential false variants associated with the amplification and sequencing steps [32].

4.2.13 RNA Isolation

Total RNA was isolated from transfected cells using peqGold TriFast according to the manufacturer's protocol.

4.2.14 cDNA synthesis

1 µg isolated RNA was digested with DNaseI as described by the manufacturer in a total volume of 10 µl. Therefrom 8 µl were reverse transcribed using SuperScript III First-Strand Synthesis SuperMix (Thermo Fisher); compare Table 4.13.

Table 4.13: Reverse transcription protocol

RNA	8 µl
Random Hexamer Primer (50 ng/µl)	1 µl
65 °C	5 min
on ice	1 min
2x FS Reaction Mix	10 µl
SuperScript III SuperMix	2 µl
25 °C	10 min
50 °C	50 min
85 °C	5 min

4.3 Protein biochemistry

4.3.1 Protein quantification by Bradford assay

The protein amount in total cell lysates was quantified using the Bradford reagent, which contains the dye Coomassie Brilliant Blue G250. Its absorbance is shifted in the presence of proteins from 465 to 595 nm. Protein quantification was performed according to the manufacturer's instructions.

4.3.2 SDS-PAGE

The SDS-polyacrylamid-gelelectrophoresis (SDS-PAGE) can be used to separate proteins depending on their molecular weights. A density of 10 - 15% of the separation gel was used in accordance to the molecular weight of the target protein. Proteins were denaturated in 4x Roti Load 1 by heating for 10 min at 95 °C and separated at 25 mA.

4.3.3 Western blot analysis

Proteins which were separated by SDS-PAGE were transferred to a nitrocellulose membrane by semi-dry blotting (see figure 4.1). The membrane was blocked after blotting with 10% skim milk in 1x TBST for 1 h at room temperature. Afterwards, the membrane was incubated with the first antibody diluted in blocking solution at 4 °C over night. To remove unbound antibody, the membrane was washed three times with 1x TBST and incubated with a secondary antibody, which was coupled with a horseradish peroxidase (HRP), for one hour at room temperature. The membrane was washed three times with 1x TBST to remove unbound secondary antibody. Using an enhanced chemiluminescent substrate, proteins conjungated with HRP were detected.

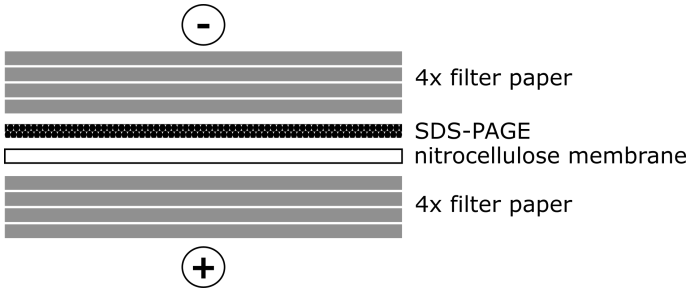

Figure 4.1: Scheme of semi-dry blot stack. Onto four layers of filter paper the nitrocellulose membrane and the SDS-PAGE were layered and covered with four additional layers of filter paper. Each layer was soaked in western blot transfer buffer before.

4.4 Electron microscopy analysis

4.4.1 Negative contrast staining

Freshly glow-discharged carbon-coated nickel grids were incubated with sucrose cushion- or sucrose density gradient-derived samples for 10 min and washed with distilled water. Viral and subviral particles were fixed with 3.7% formaldehyde, washed once with PBS and afterwards two times with distilled water. The grids were negatively stained with 2% uranylacetate for 10 sec and analyzed by electron microscopy (EM 109, Zeiss, Jena).

4.5 Immunological methods

4.5.1 HBsAg/HBeAg ELISA

Enzyme-linked Immunosorbent Assays (ELISA) for the detection of HBsAg and HBeAg were performed in accordance to the manufacturer's instructions.

4.6 Statistics

Significance of results was evaluated by unpaired two-tailed students t-test, Wilcoxon-Mann-Whitney-U test or Kruskal-Wallis test with BiAS 11.06 for Windows. Results are described as mean or median ± standard error of the mean or median (SEM) from at least 3 independent experiments. Error bars in the figures represent SEM. Statistical significance is represented in the figures as follows: ns = not significant; $p < 0.05$ low significance ; $p < 0.01$ medium significance and $p < 0.001$ high significance.

5 Results

5.1 Hepatitis B Virus

The samples which were used in this interim study analysis were derived from patients chronically infected with HBV included into the ALBATROS trial. These patients are characterized by HBeAg negativity, HBsAg positivity, low HBV-DNA levels (< 100,000 IU/ml), low to slightly elevated transaminases and no need of antiviral treatment. Patient characteristics are described in more detail in Table 5.1.

Table 5.1: Epidemiological, serological and virological patient characteristics

Parameter	**Total**	**GTA**	**GTB**	**GTC**	**GTD**	**GTE**
n (%)	504 (100)	125 (24.8)	36 (7.1)	20 (4.0)	295 (58.5)	28 (5.6)
Age (years, mean ± SD)	40.6 ± 11.9	45.0 ± 12.5	37.6 ± 12.3	39.0 ± 12.5	39.4 ± 12.5	37.6 ± 12.4
Male gender n (%)	209 (41.5)	48 (38)	13 (36)	3 (15)	131 (44)	14 (50)
HBV-DNA (mean log IU/ml ± SD)	2.9 ± 0.8	2.8 ± 0.8	3.0 ± 0.7	2.9 ± 0.5	2.9 ± 0.8	3.0 ± 0.8
qHBsAg (mean log IU/ml ± SD)[1]	3.1 ± 1.0	3.6 ± 0.8	2.3 ± 0.9	3.4 ± 0.9	3.0 ± 0.9	3.7 ± 0.6
ALT (mean U/l ± SD)	28.8 ± 14.2	29.8 ± 13.9	24.3 ± 11.9	24.7 ± 10.4	29.2 ± 14.6	27.9 ± 15.0
Ethnicity[2]						
Caucasian	273	102	2	0	167	2
Asian	70	8	34	19	9	0
African-American	42	11	0	0	6	25
Oriental	116	2	0	1	113	0

[1] qHBsAg was available in 469 patients.

[2] Ethnicity was available in 501 patients.

5.1.1 The Prevalence of mutations in the BCP, precore and preS region is genotype-dependent

Genotyping of the viral strains with which the patients were infected was done during clinical routine or during this study by amplification/sequencing of the polymerase gene as described in Table 4.1 and 4.2. Patients were mainly infected with HBV genotype D (GTD: 59%; 295/504) and HBV genotype A (GTA: 25%; 125/504). Genotypes B (GTB: 7%; 36/504), E (GTE: 6%; 28/504) and C (GTC: 4%; 20/504) were found with lower prevalences (compare Figure 5.1A).

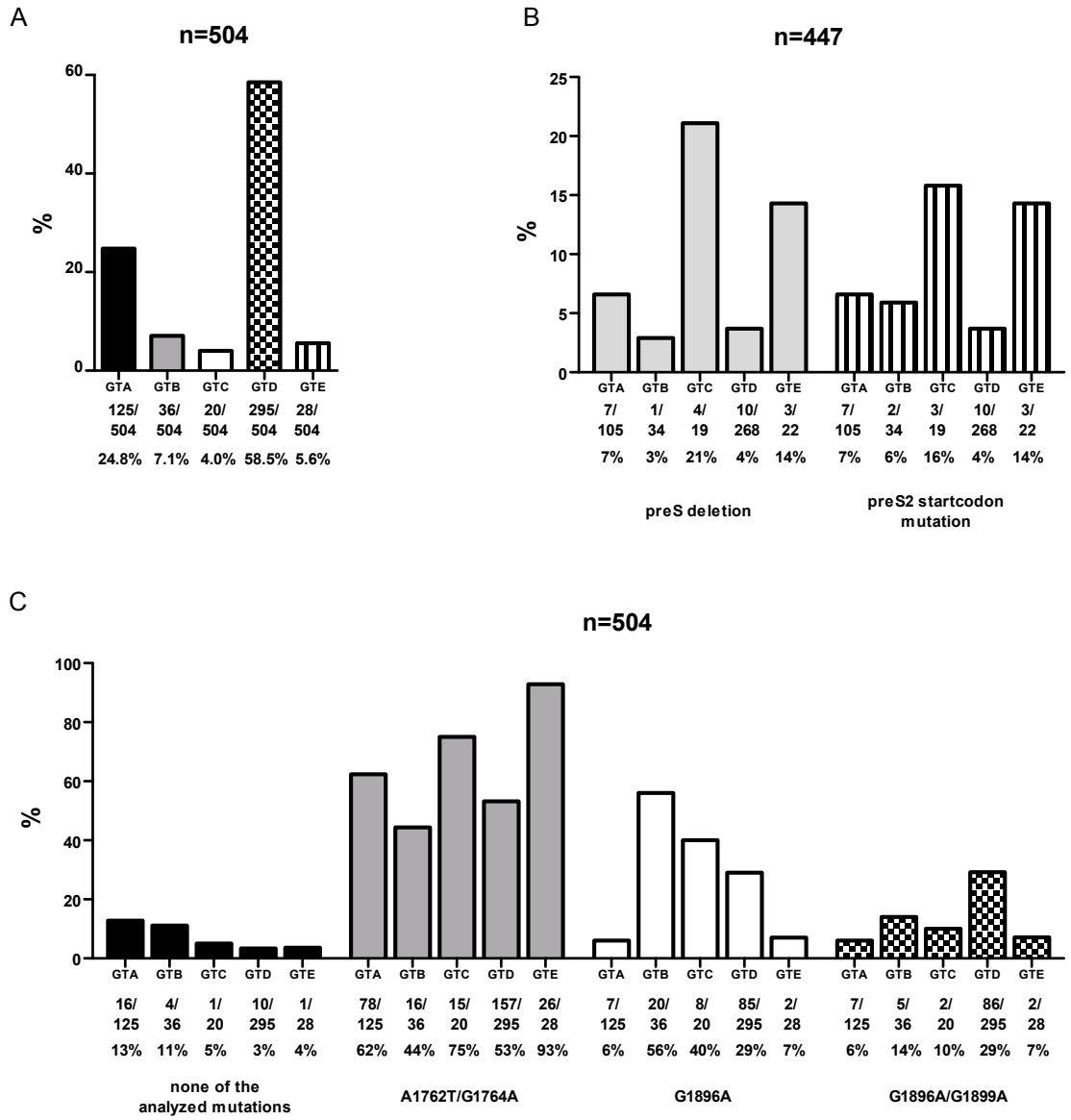

Figure 5.1: Prevalence of BCP, precore and preS mutations is genotype-dependent. Prevalence of (A) HBV genotypes A - E (GTA - GTE); (B) BCP double mutation A1762T/G1764A, precore single G1896A and double mutation G1896A/G1899A and (C) deletions in preS and start codon mutations in the preS2 domain

Furthermore, mutations in the basal core promoter (A17562T/G1764A) and the precore region (G1896A+/-G1899A) as well as mutations and deletions in the preS region were analyzed, since they have been associated with the progression of the disease in several Asian studies. The BCP double mutation A17562T/G1764A could be detected frequently (58%; 292/504) in the study population. The highest frequency of 93% was found in patients infected with GTE (26/28) followed by GTC (75%; 15/20), GTA (62%; 78/125) and GTD (53%; 157/295). In patients infected with GTB the BCP double mutation A17562T/G1764A was found with the lowest frequency (44%; 16/36) (Figure 5.1B). In 24% (122/504) of the study population the precore single mutation G1896A was found. Highest prevalences were observed in patients infected with GTB (56%; 20/36), GTC (40%; 8/20) and GTD (29%; 85/295). Respectively, 7% and 6% of the patients infected with GTE (2/28) and GTA (7/125) had the precore single mutation G1896A (Figure 5.1B). The precore double mutation G1896A/G1899A could be detected in 20% (102/504) of all patients. It was predominantly found in patients infected with GTD (29%; 86/295), GTB (14%; 5/36) and GTC (10%; 2/20) but less often in GTA (6%; 7/125) and GTE infected patients (7%; 2/28) (Figure 5.1B). Due to limited sample volumes, deletions and point mutations within the preS domain were analyzed in 447 patients (GTA: 105/447; GTB: 34/447; GTC: 19/447; GTD: 268/447; GTE: 22/447) (Figure 1C). Deletions (varying in length from one aminoacid (aa) to 49 aa) and point mutations at the preS2 start codon were found in 11% (48/447) of the patients. In patients infected with GTE and GTC these mutations/deletions were found with the highest prevalences (23%, 5/22; 26%, 5/19). In GTA (13%, 14/105), GTB (9%, 3/34) and GTD (8%, 22/268) variants regarding the preS domain were found less prevalent (Figure 5.1C).

In conclusion, the BCP double mutation A17562T/G1764A, precore mutations (G1896A and G1896A/G1899A) and mutations/deletions in the preS domain were detected in a genotype-specific pattern.

5.1.2 Precore mutations are associated with higher and BCP mutations with lower HBV-DNA levels

The correlation between the different genotypes and HBV-DNA serum levels was evaluated as well as interrelationships between analyzed mutations and HBV-DNA serum levels.

When HBV-DNA serum levels were compared, no significant differences were detected among the various HBV genotypes A to E (Figure 5.2A). In patients with the precore G1896A single mutation or the precore G1896A/G1899A double mutation significant higher HBV-DNA levels could be observed compared to patients without these mutations (3.1 log IU/ml versus 2.6 log IU/ml; $p < 0.001$) (Figure 5.2B). However, patients which harbored the BCP A17562T/G1764A mutation had significantly lower HBV-DNA levels in comparison to patients without this double mutation (2.7 log IU/ml versus 3.1 log IU/ml; $p < 0.001$) (Figure 5.2B).

In contrast, similar HBV-DNA levels were observed in patients with or without variants in the preS domain (2.9 log IU/ml versus 2.9 log IU/ml; p = 0.626) (Figure 5.2B).

Taken together, different genotypes and preS mutations were not associated with HBV-DNA serum levels in our study population, whereas precore G1896A+/-G1899A mutations as well as the BCP double mutation A17562T/G1764 were significantly associated with HBV-DNA levels.

5.1.3 qHBsAg levels vary among HBV genotypes and are associated with mutations in precore and preS

To evaluate the impact of qHBsAg serum levels, different HBV genotypes and HBsAg serum levels as well as analyzed mutations and HBsAg serum levels were correlated.

The amount of HBsAg found in the patient sera varied significantly between genotypes A to E. The highest qHBsAg levels could be found in patients infected with GTA (3.6 log IU/ml) and GTE (3.7 log IU/ml), whereas patients infected with GTB (2.3 log IU/ml) had significantly lower levels of qHBsAg ($p < 0.001$) (Figure 5.2C). Also in GTD qHBsAg levels were lower (3.0 log IU/ml; $p < 0.001$) in comparison to GTA, whereas higher qHBsAg levels could be found in patients infected with GTC (3.4 log IU/ml) and GTD (3.0 log IU/ml) as compared to GTB ($p = 0.001$ and $p = 0.004$, respectively).

A HBsAg cut-off of < 1000 IU/ml was evaluated in several Asian studies as a prognostic marker, whereas the impact in European populations has not been analyzed well so far. In this interim study cohort only 11% (3/27) of GTE and 17% (20/115) of GTA infected patients had qHBsAg levels < 1000 IU/ml, whereas significantly more GTD (45%; 122/274) and GTB (69%; 24/35) infected patients had qHBsAg levels < 1000 IU/ml (Figure 5.2E).

Irrespective of the HBV genotype qHBsAg levels varied in patients with different mutations. In patients with the precore single mutation G1896A or double mutation G1896A/G1899A a significant lower amount of qHBsAg was observed in comparison to patients without these mutations (3.1 log IU/ml versus 3.4 log IU/ml; $p = 0.002$) (Figure 5.2D). However, patients with a preS deletion or preS2 start codon mutation showed significantly higher amounts of qHBsAg in comparison to patients without preS variants (3.6 log IU/ml versus 3.1 log IU/ml; $p < 0.001$) (Figure 5.2D). In contrast, no association of qHBsAg levels and the BCP A17562T/G1764A double mutation could be detected in this interim study cohort (3.2 log IU/ml versus 3.1 log IU/ml; $p = 0.662$) (Figure 5.2D).

In summary, qHBsAg serum levels differed up to 1.4 log between different genotypes. While only a few patients infected with GTE and GTA had qHBsAg < 1000 IU/ml, most of the patients infected with GTB and almost half of the GTD infected patients had qHBsAg < 1000 IU/ml. Precore mutations were associated with lower and preS mutations with higher qHBsAg levels.

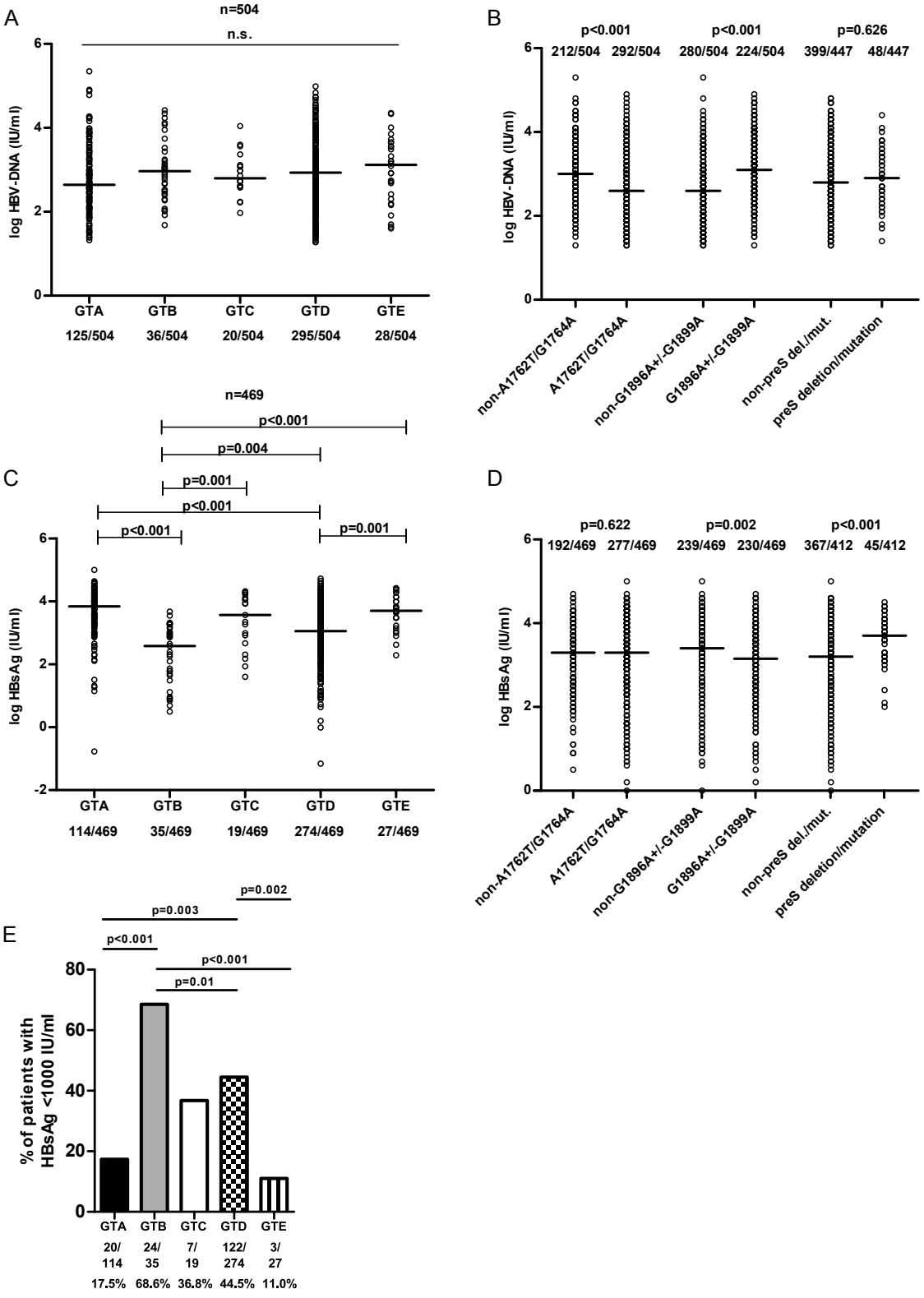

Figure 5.2: Comparison of HBV-DNA levels (IU/ml) and qHBsAg levels (IU/ml). (A, C) Among different genotypes and (B, D) with BCP double mutation A1762T/G1764A, precore single mutation G1896A and/or double mutation G1896A/G1899A, preS deletions and/or preS2 start codon mutations. (E) Prevalence of patients with qHBsAg < 1000 IU/ml in different HBV genotypes. Horizontal lines represent the median.

5.1.4 Mutations, qHBsAg and HBV-DNA in patients who started an antiviral therapy

During the years of follow-up 14 patients had to start an antiviral therapy (as recommended by the German guideline [29]). Mutations in the BCP, precore and preS domain as well as HBsAg levels and HBV-DNA levels were analyzed in these patients at baseline (BL; timepoint when patients were included into the study) and right before they started antiviral treatment (treatment start, TS). At BL only 1/14 patients had none of the analyzed mutations, while at TS 14/14 patients had mutations in the different regions. The BCP double mutation A17562T/G1764A was found with the highest frequency (86%; 12/14) both at BL and at TS in these patients (Figure 5.3A). Mutations in the precore region (single mutation G1896A or double mutation G1896A/G1899A) could be detected

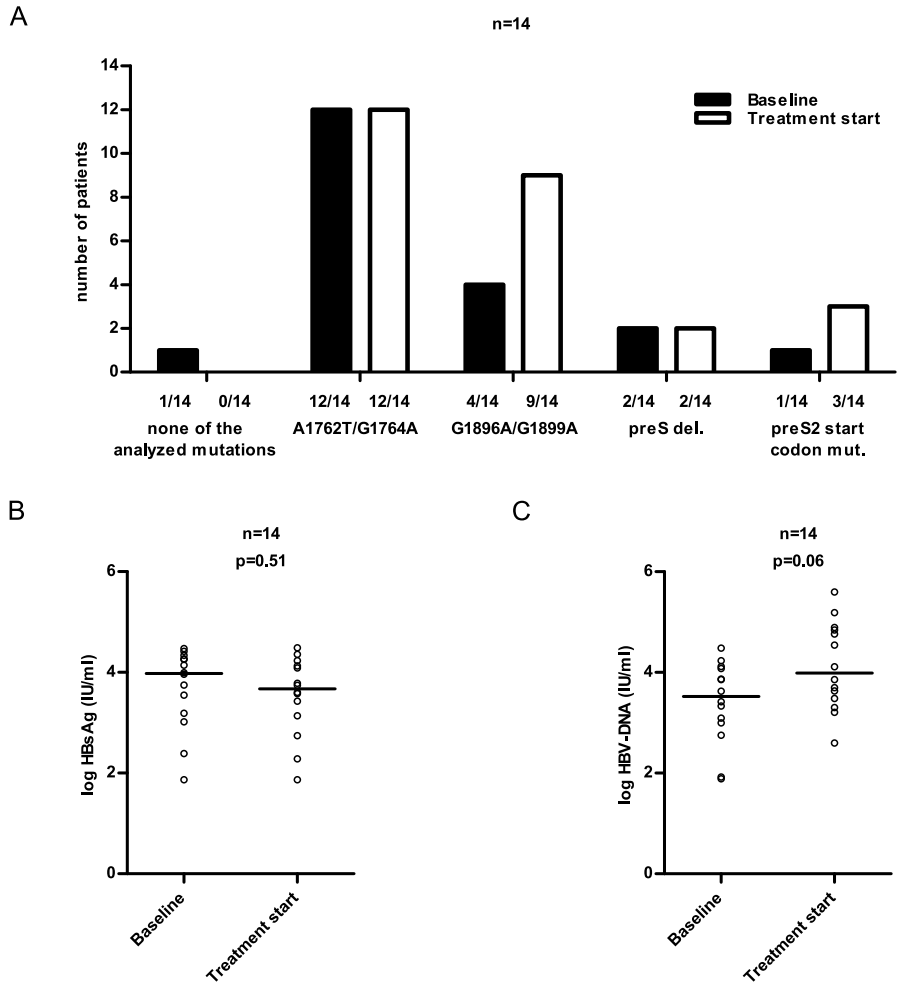

Figure 5.3: Mutations and correlation of HBV-DNA/qHBsAg levels in patients at baseline compared to treatment start. (A) Number of patients with BCP double mutation A1762T/G1764A, precore single G1896A and/or double mutation G1896A/G1899A and deletions in preS and/or start codon mutations in the preS2 domain, (B) Comparison of HBV-DNA levels (IU/ml) at baseline and at treatment start, (C) Comparison of qHBsAg (IU/ml) at baseline and at treatment start.

in 4/14 patients at BL and in 9/14 patients at TS. While preS deletions were found in 2/14 patients at BL as well as at TS, preS2 start codon mutations could be detected in 1/14 patients at BL and in 3/14 patients at TS (Figure 5.3A). As shown in Figure 5.3B, no differences in the qHBsAg levels at BL in comparison to TS could be detected (3.67 log IU/ml versus 3.98 log IU/ml; $p = 0.51$). In contrast, HBV-DNA levels were tending to be higher in the patient samples at TS in comparison to the BL samples (4.0 log IU/ml versus 3.5 log IU/ml), although it was not significant ($p = 0.06$) (Figure 5.3C).

5.1.5 *In vitro* characterization of mutations in BCP, precore and preS

To further characterize the molecular virology of the analyzed BCP-, precore- and preS-variants HBV GTA 1.5-fold genomes from patients harboring the BCP double mutation A17562T/G1764A (BCP-variant), the precore double mutation G1896A/G1899A (precore-variant) or a preS1-deletion (preS-del.-variant; 45 nucleotides deletion at position 2944-2988) were used. An HBV GTA genome lacking these mutations (termed below: reference (ref.) genome) was used as a control.

HBsAg is released at higher amounts in the preS-del.-variant in comparison to BCP-, precore- and ref. genome-variants

On the one hand, cellular RNA from HuH7.5 cells transiently transfected with the different cloned variants was extracted and reverse transcribed into cDNA. On the other hand, viral DNA released into the supernatant was extracted in order to quantify expressed and released viral genomes via rt-PCR.

No difference in the quantity of viral RNA isolated from HBV-expressing cells was observed in the BCP-variant ($p = 0.179$) in comparison to the ref. genome-variant, while significantly higher HBV-RNA levels were found in cells expressing the precore- and the preS-del.-variant ($p = 0.012$ and $p = 0.009$, respectively) in comparison to the ref. genome (Figure 5.4A). The levels of secreted viral genomes differed only very slightly in the supernatants of cells expressing the BCP- and the preS-del.-variant compared to the ref. genome-variant. However, in the supernatant of cells expressing the precore-variant significantly fewer viral genomes were detected in comparison to the ref. genome ($p < 0.001$) (Figure 5.4B).

Furthermore, the supernatants were analyzed by HBsAg- and HBeAg-specific ELISA and the whole cell lysates by HBsAg-specific ELISA. In the lysates of preS-del.-variant expressing cells lower amounts of HBsAg were found, whereas marginally higher amounts were observed in the lysates of precore-variant expressing cells in comparison to the ref. genome (Figure 5.4C). In contrast, the secreted HBsAg amount was significantly higher in supernatants of preS-del.-variant expressing cells in comparison to the other variants ($p < 0.001$) (Figure 5.4D), which is also in accordance to the *in vivo* observations (Figure 5.2D).

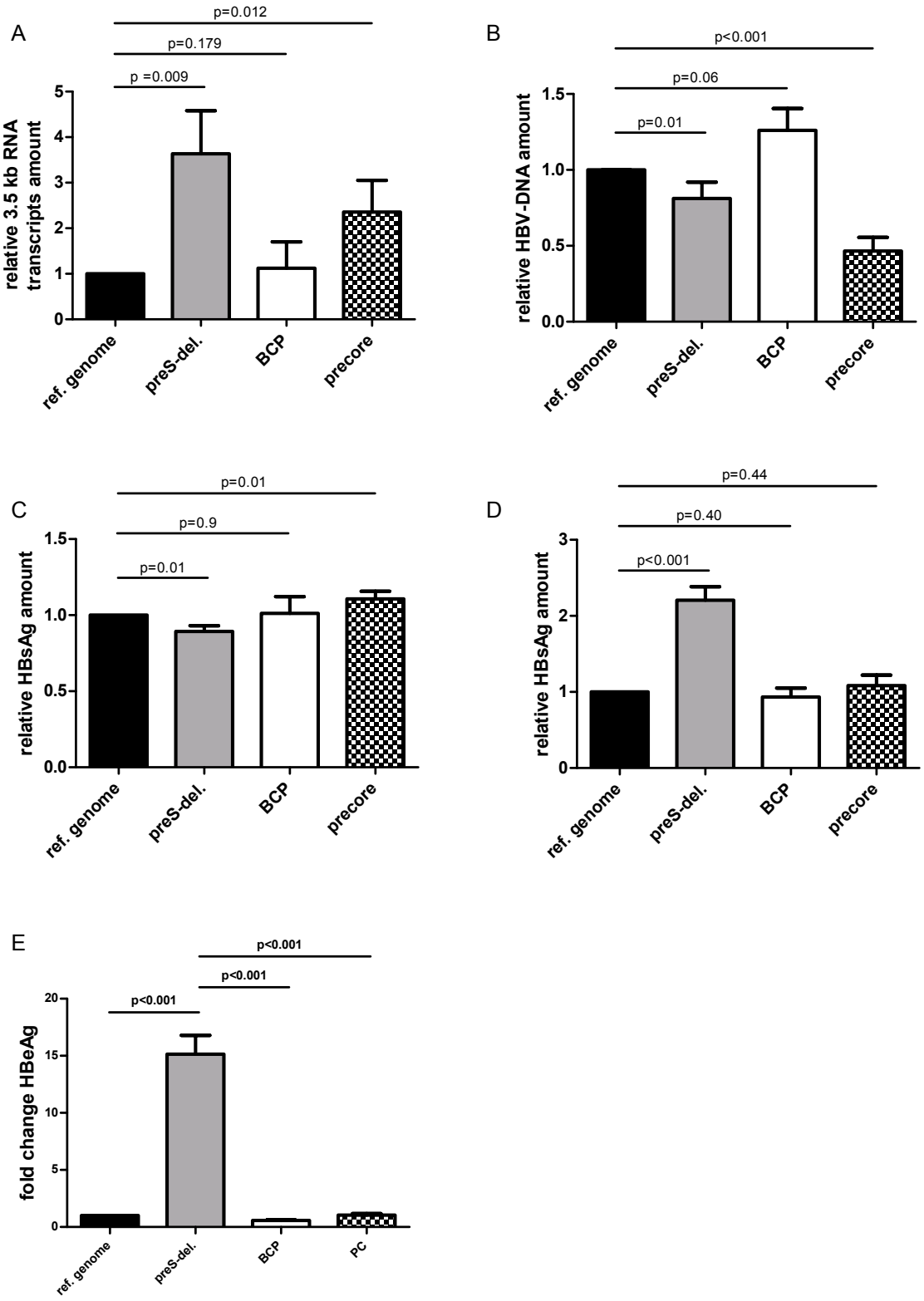

Figure 5.4: The preS-del.-variant is characterized with an enhanced HBsAg release and capability of HBeAg release. Quantification of (A) HBV 3.5 kb RNA transcripts by rtPCR, (B) HBV-DNA in supernatants by rt-PCR, (C) HBsAg by ELISA in cellular lysates, (D) HBsAg and (E) HBeAg by ELISA in supernatants of HuH7.5 cells transfected with ref. genome-, BCP-, PC- and preS-del.-variant.

The precore-, BCP- and ref. genome-variants failed to secret significant amounts of HBeAg. In contrast, in the supernatant of preS-del.-variant expressing cells HBeAg could be detected in high amounts, although the cloned genome was derived from a confirmed HBeAg negative and anti-HBe positive patient (Figure 5.4E).

In conclusion, this indicates an enhanced expression but reduced secretion of viral genomes in the preS-del.- and precore-variant. While no differences in HBsAg production and secretion were observed in precore- and BCP-variants, the preS-del.-variant is characterized with an elevated HBsAg release. Furthermore, the preS-del.-variant is capable of HBeAg release.

The results presented in section 5.1.1 - 5.1.5 are included in [80].

5.1.6 Large, middle and small HBsAg occur in a specific pattern in the different HBV genotypes A - E

To enrich viral and subviral particles, sera of patients infected with HBV genotypes A - E (GTA - GTE) were subjected to ultracentrifugation. The distribution of the different surface proteins (SHBs, MHBs, LHBs) within the enriched particles was investigated by western blot (WB) analysis using the HBs-specific monoclonal antibody HB01.

On average 10 samples of each genotype were analyzed and representative samples are shown in Figure 5.5A. In all genotypes SHBs (unglycosylated: 24 kDa; monoglycosylated: 27 kDa) could be identified as the predominant fraction (Figure 5.5A). The ratio of SHBs to MHBs/LHBs varied between the different genotypes. It was higher in GTA, GTC and GTE and lower in GTB and GTD (Figure 5.5B). More MHBs (monoglycosylated: 33 kDa; biglycosylated: 36 kDA) than LHBs (unglycosylated: 39 kDa; monoglycosylated: 42 kDa) was found in GTC, GTD and GTE, whereas a higher amount of LHBs in comparison to MHBs was found in GTB. In GTA slightly more MHBs than LHBs could be detected (Figure 5.5A and B).

During the progression of the disease 14 patients had to start an antiviral therapy. In order to investigate if there is a change in the ratio of LHBs to MHBs to SHBs during the course of the disease, patient serum samples from BL and TS were analyzed by WB analysis using HBs-specific antibody HB01. As shown in Figure 5.5C, no changes in the ratio of LHBs to MHBs to SHBs could be observed from BL to TS independent from the analyzed genotype (representative examples of GTA to GTD are shown in Figure 5.5C).

In summary, a genotype-specific pattern of the L/M/SHBs ratio was detected in HBV genotypes A to E. This pattern did not change in the serum of patients who experienced a reactivation to active Hepatitis and started antiviral treatment.

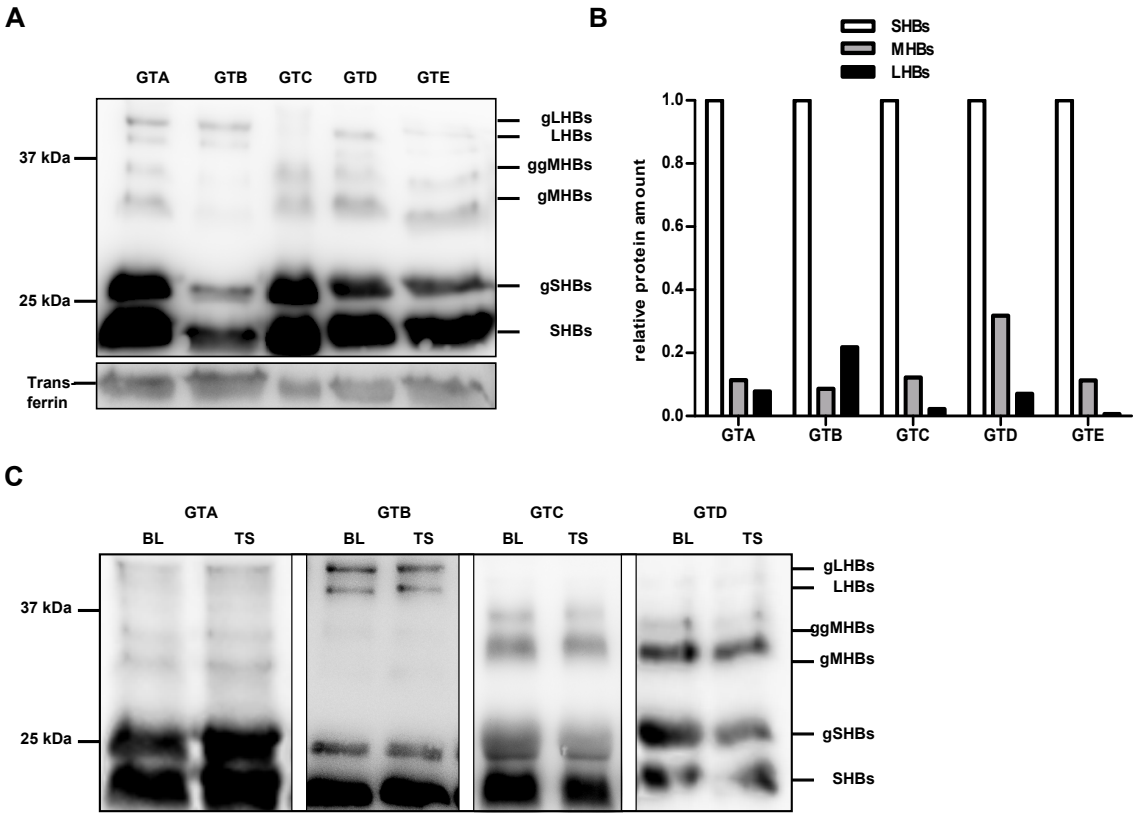

Figure 5.5: Distribution of large, middle and small HBsAg varies in HBV genotypes A to E. (A) WB analysis using HBs-specific antibody (HB01) of serum samples from HBeAg negative patients infected with HBV genotypes A to E (GTA-GTE) after enrichment of viral/subviral particles via ultracentrifugation over a 10% sucrose cushion. (B) Bar chart showing the quantification of the relative amounts of the large, middle and small HBsAg (LHBs, MHBs and SHBs) shown in the WB analysis in (A). (C) WB analysis using HBs-specific antibody (HB01) of serum samples from HBeAg negative HBV infected patients at baseline (BL) and right before treatment start (TS).

5.1.7 Density and morphology of HBsAg containing particles vary in different HBV genotypes

To separate viral particles, spheres and filaments, serum samples of patients infected with HBV genotypes A to E were layered onto a linear sucrose density gradient. The gradient was subjected to ultracentrifugation and 18 fractions were collected. These fractions were analyzed via WB, HBsAg ELISA, rt-PCR and electron microscopy.

Regarding the amounts of HBV-DNA detected via rt-PCR it must be stated that only very low HBV-DNA levels were detected due to the low replicative stage in the HBeAg negative HBV infected patients. Nevertheless, the highest HBV-DNA levels could be detected in the last fraction with the highest amount of sucrose (compare Figure 5.6B) independent of the HBV genotype.

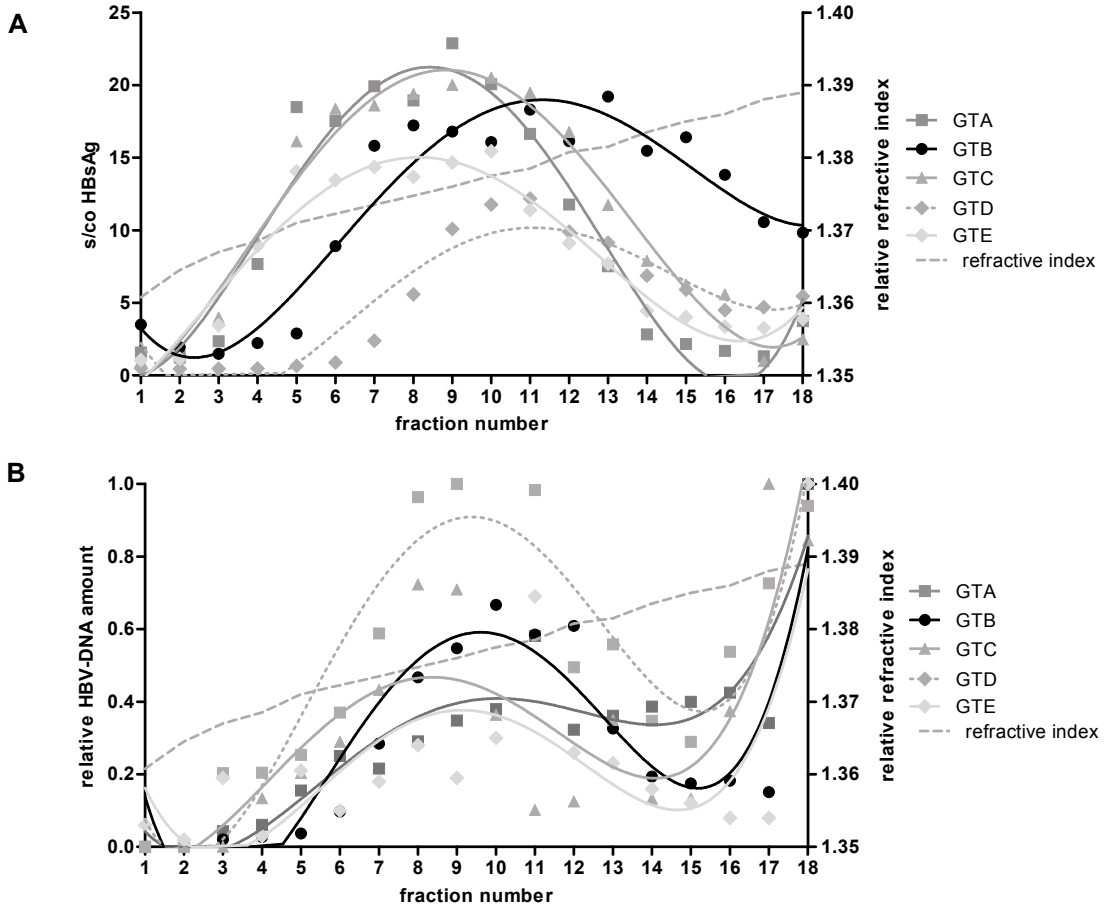

Figure 5.6: The composition of HBsAg-positive particles varies in different HBV genotypes. Analysis of sucrose fractions 1 - 18 of serum samples from HBeAg negative patients infected with HBV genotypes A to E (GTA - GTE) after separation of viral and subviral particles via ultracentrifugation over a continuous sucrose gradient (25 - 40% sucrose from top to bottom) by (A) HBsAg ELISA and (B) rt-PCR. s/co = signal-to-cutoff ratio.

As shown in Figure 5.6A, in GTA, GTC and GTE samples the largest amounts of HBsAg could be detected in fractions 8 and 9 by the HBsAg-specific ELISA, while in GTB and GTD samples (GTA: n = 9, GTB: n = 7; GTC: n = 4; GTD: n = 12; GTE: n = 2) the HBsAg peak was shifted to fractions 11 and 12, which contained a higher density of sucrose. This could also be observed in the WB using HBs-specific antibody (Figure 5.7B). The highest amounts of HBsAg were detected in fractions of a lower density (fractions 8 - 10) in GTA and GTE samples, whereas the HBsAg peak was shifted to fractions with a higher density in GTC (fractions 9 - 12). An even more distinct shift to fractions with a higher density was identified in GTB and GTD (fractions 10 - 14). In contrast to GTA, GTC and GTE, detectable amounts of LHBs were observed in GTB in fractions with the highest density (fractions 17 and 18). Furthermore, the HBsAg pattern was analyzed during the course of the disease in samples of patients who had to start treatment during the follow up of the study. Exemplary, one GTD sample at BL and at TS is depicted in Figure 5.7C. No changes in the density or composition of the secreted particles could be observed in the TS sample in comparison to the BL sample.

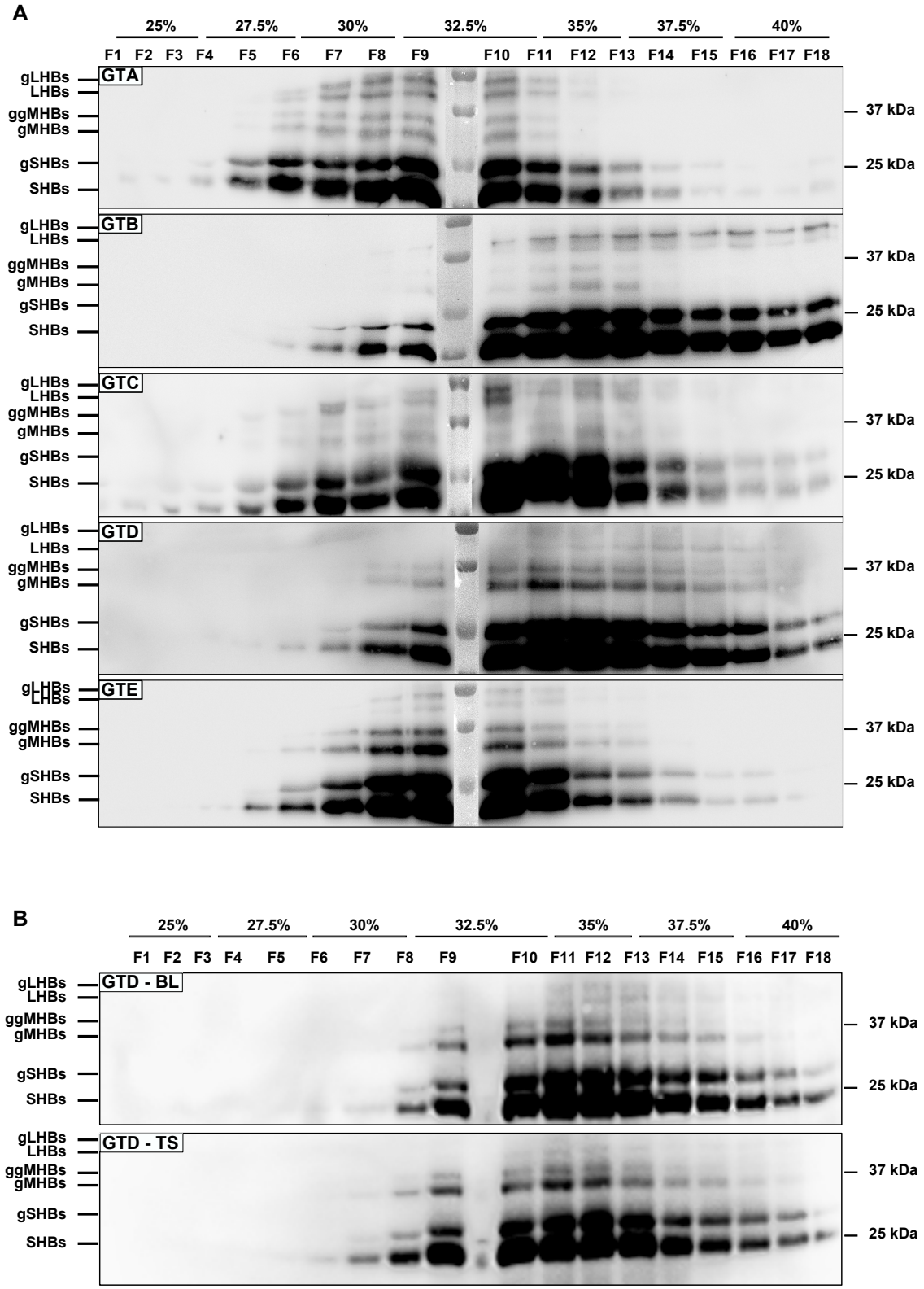

Figure 5.7: Densities of HBsAg-positive particles vary in different HBV genotypes. Analysis of sucrose fractions 1 - 18 of serum samples from HBeAg negative patients infected with (A) HBV genotypes A to E (GTA - GTE) and (B) genotype D at baseline (BL) and before treatment start (TS) after separation of viral and subviral particles via ultracentrifugation over a continuous sucrose gradient (25 - 40% sucrose from top to bottom) by WB analysis using HBs-specific antibody HB01.

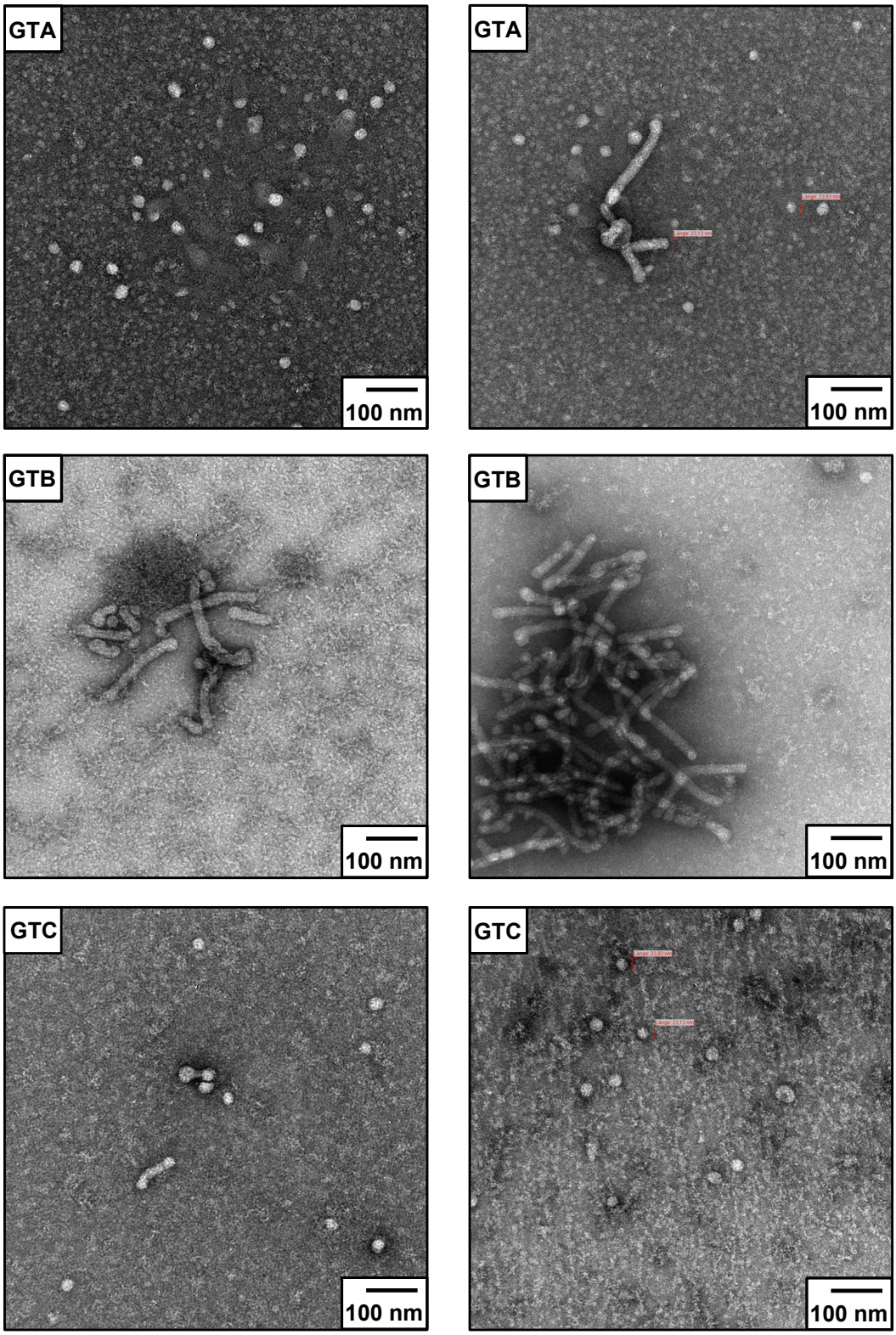

Figure 5.8: Composition of subviral particles varies in different HBV genotypes (GTA - GTC). Electron microscopy analysis of viral and subviral particles of serum samples from HBeAg negative patients infected with HBV genotypes A to C (GTA - GTC) after enrichment via ultracentrifugation over a 10% sucrose cushion.

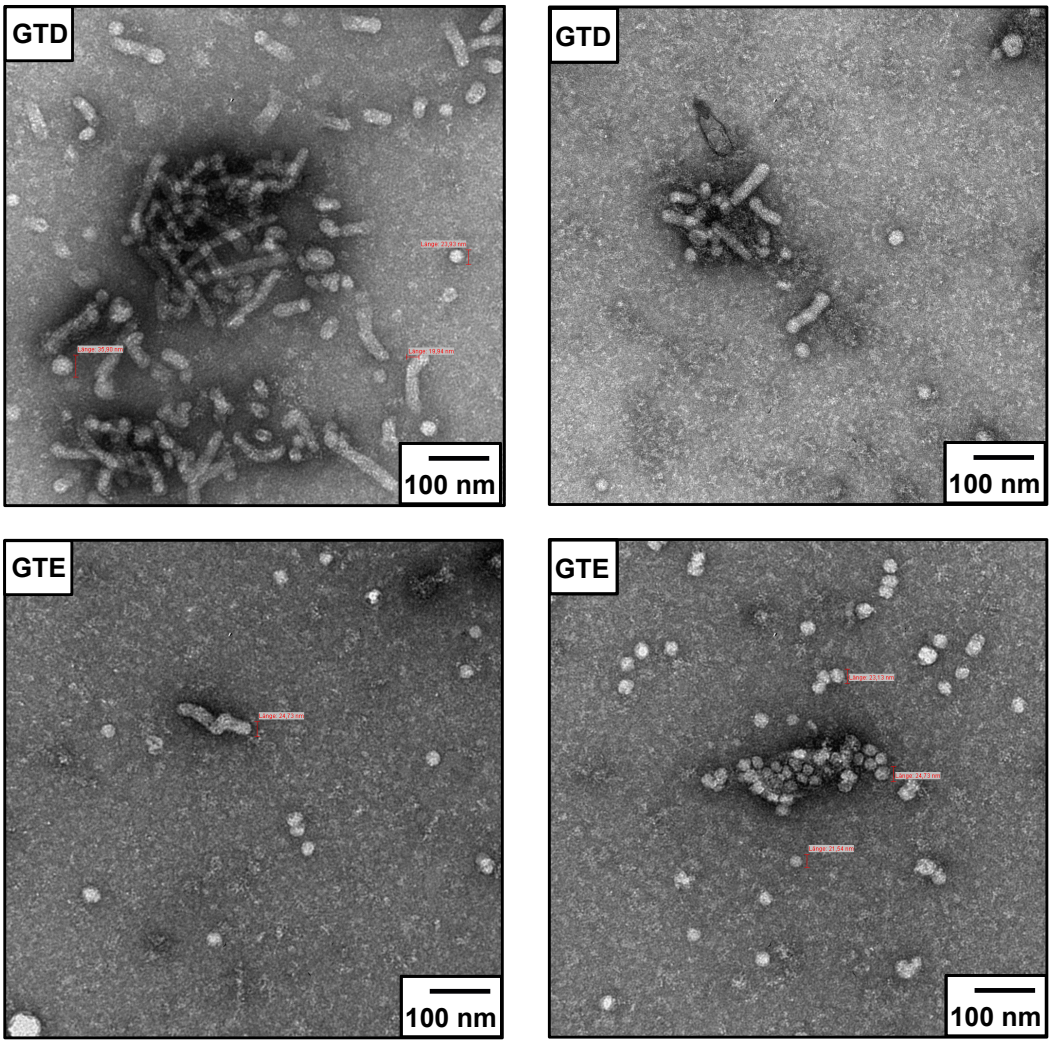

Figure 5.9: Composition of subviral particles varies in different HBV genotypes (GTD - GTE). Electron microscopy analysis of viral and subviral particles of serum samples from HBeAg negative patients infected with HBV genotypes D or E (GTD or GTE) after enrichment via ultracentrifugation over a 10% sucrose cushion.

Electron microscopy analysis of enriched viral and subviral particles of patient sera was performed in order to investigate, if the observed genotype-specific pattern can be reflected by a genotype-specific morphology of the secreted particles.

Mostly spheres with a diameter of 22 nm and a few filaments with a diameter of 22 nm and variable length were observed in GTA, GTC and GTE, whereas in GTB and GTD less spheres were detected (compare Figure 5.8 and 5.9). In contrast, a high amount of filaments with variable length was found in GTB and GTD. Furthermore, in GTB long filaments accumulated to form large bundles (see Figure 5.8 and 5.9). In all of the analyzed genotypes infectious viral particles could only be detected very rarely due to the low viral replicative status in the study cohort.

Taken together, secreted particles containing HBsAg display a higher density as well as a higher filaments/spheres ratio in GTB and GTD compared to GTA, GTC and GTE.

5.1.8 Mutations or deletions in the preS1 and preS2 domain do not affect HBsAg expression *in vivo*

Variants harboring different mutations or deletions in the preS1 and/or preS2 domain were detected by amplification and population-based sequencing of the preS domain. To evaluate the impact of those deletions or mutations on the pattern of the secreted HBsAg, viral and subviral particles of serum samples were enriched via ultracentrifugation and analyzed via WB using HBs-specific antibody.

Deletions and start codon mutations should theoretically affect the molecular weight of the different surface proteins, but as shown in Figure 5.10 no differences in the expression pattern of HBsAg could be detected in variants harboring mutations or deletions in the preS region in comparison to variants without mutations. In Figure 5.10, three examples of each of the genotypes A, D and C are shown. Two of the samples per genotype harbored deletions or mutations in the preS region and were compared to one reference sample without mutations regarding the preS domain.

For example, the LHBs expression signal (unglycosylated: 39 kDa; monoglycosylated: 42 kDa) of a GTA variant with a 15 amino acid (aa) deletion in the preS1 domain appeared at the same molecular weight as LHBs of a GTA variant harboring no mutations. The same could be observed for a GTA variant with a 49 aa deletion and an additional premature stop codon (Figure 5.10 left panel).

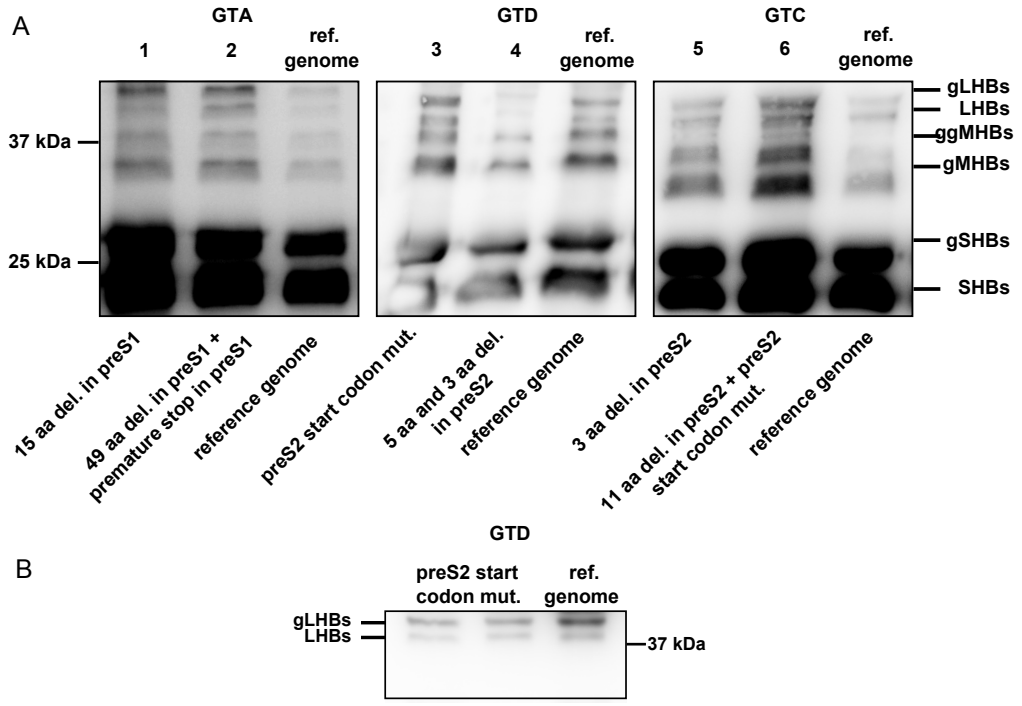

Figure 5.10: Large deletions and mutations in the preS1 and/or preS2 domain do not affect the appearance of MHBs and LHBs isolated from patients. WB analysis of serum samples from HBeAg negative patients infected with HBV after enrichment of viral/subviral particles via ultracentrifugation over a 10% sucrose cushion using HBs-specific antibody (HB01).

As another example, LHBs and MHBs (monoglycosylated: 33 kDa; biglycosylated: 36 kDa) of a GTD variant harboring a preS2 start codon mutation could be detected and appear at the same molecular weight compared to LHBs and MHBs of a GTD variant without mutations. Also in a GTD variant harboring two deletions in the preS2 domain (5 aa and 3 aa deleted), LHBs appeared identically as compared to the reference sample (Figure 5.10 middle panel). Likewise, LHBs and MHBs of a GTC variant with a 11 aa deletion in the preS2 domain in combination with a preS2 start codon mutation were detectable and appear at the same molecular weight as a GTC variant without mutations. The same occurred in a variant with a 3 aa deletion in the preS2 domain (Figure 5.10 right panel).

It might be possible that in the variants with the preS2 start codon mutation the unexpected expression signal at 33 and 36 kDa is not MHBs, but is a truncated form of LHBs. Therefore, three GTD samples (two with preS2 start codon mutation, one reference genome) were analyzed with an LHBs-specific antibody. As it can be seen in Figure 5.10B, only LHBs signals at 39 and 42 kDa could be detected. If truncated variants of LHBs would have caused the unexpected signals at 33 and 36 kDa there should have also been detectable signals at 33 and 36 kDa with this LHBs-specific antibody.

A huge heterogeneity of the viral genome emerges due to the lacking proofreading activity of the viral polymerase. To examine if there is a correlation of the diverse variability of the viral quasispecies and the unexpected HBsAg pattern in the WB analysis, deep sequencing analysis of the preS region of the six patients described above was performed. Therefore, amplicons of the preS domain were subjected to Illumina deep sequencing analysis. The predominant mutant variant found by population-based sequencing was also detected as the major variant in the deep-sequencing analysis in five out of six patients as shown in Table 5.2. The major variant was even found with a percentage of $> 99\%$ in patient number 1 and 6.

Table 5.2: Variants found by population based sequencing and deep sequencing

Sample	Variant found by population-based sequencing	Variant found by deep sequencing	Percentage of the viral quasispecies found by deep sequencing
1	15 aa Δ preS1	15 aa Δ preS1	99.2
2	stop in preS1 + 49 aa Δ preS1	stop in preS1 +/− 49 aa Δ preS1	84.7/ 10.9
3	preS2 start codon mutation	preS2 start codon mutation	88.7
4	5 aa + 3 aa Δ preS2	5 aa + 3 aa Δ preS2	69.9
5	3 aa Δ preS2	3 aa Δ preS2	17.4
6	11 aa Δ preS2 + preS2 start codon mutation	11 aa Δ preS2 + preS2 start codon mutation	99.9

To prove that such mutations should affect the HBsAg expression pattern, a comparative analysis of a GTA 1.5-fold HBV genome with the 15 aa deletion in the preS1 domain (preS-del.-variant, patient number 1 from Figure 5.10 left panel) and a reference genome (ref. genome) without mutations in the preS domain was carried out. Cell lysates of hepatoma cells transiently transfected with the preS-del.- and the ref. genome-variant were analyzed by WB using an HBs-specific antibody.

As shown in Figure 5.11A, LHBs is shifted to a lower molecular weight in the preS-del.-variant in comparison to the ref. genome-variant. Furthermore, the specific binding site for the LHBs-specific antibody MA18/7 is deleted in the preS-del.-variant. Therefore, LHBs cannot be detected in the WB analysis of lysates of transiently transfected HuH7.5 cells by this antibody (Figure 5.11B). But in contrast to that, in the serum of the patient with the 15 aa deletion in preS1 a strong specific LHBs signal could be detected. It appeared at the same molecular weight compared to LHBs of a patient infected with the reference genome (compare Figure 5.11C).

In conclusion, the *in vivo* as well as the *in vitro* data indicate that in HBeAg negative HBV infected patients with mutations or deletions in the preS domain MHBs and LHBs might be expressed from a genetically different source than the released viral genomes.

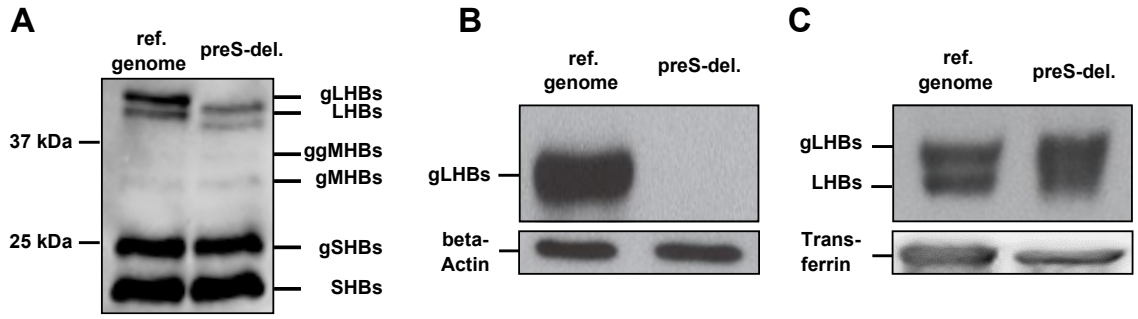

Figure 5.11: A large deletion in the preS1 region affects the appearance of LHBs *in vitro*. WB analysis using (A) HBs-specific antibody (HB01) and (B) LHBs-specific antibody (MA18/7) of (A, B) lysates of HuH7.5 cells transfected with ref. genome- and preS-del.-variant and (C) serum samples from patients with the ref. genome-variant and the preS-del.-variant after enrichment of viral/subviral particles via ultracentrifugation over a 10% sucrose cushion.

The results presented in section 5.1.6 - 5.1.8 are included in [116].

5.2 Hepatitis C Virus

The samples used for the primary analysis were taken from patients chronically infected with HCV from two German multicenter studies (INDIV-2 and PIRB/PISA) as well as from the outpatient department of the University Hospital Frankfurt, Germany. Furthermore, for validation of the results treatment-naive genotype 1a and genotype 1b (GT1a and GT1b) patients from the INDIV-2 study and the outpatient department were enrolled as a replication cohort. For further characteristics of the patients compare Table 5.3 and 5.4.

Table 5.3: Epidemiological, serological and virological characteristics of patients infected with HCV GT1 and GT3 (primary cohort) [117]

Parameter	**Total, n (%)**	**GT1b, n (%)**
n	633 (100)	323 (100)
Age (years, mean ± SD)	48.1 ± 12.9	48.2 ± 12.9
Sex (male)	376 (59)	163 (50)
Alanine aminotransferase (mean ± SD)	94.7 ± 82.2	95.1 ± 82.5
HCV genotype		
1a	259 (41)	
1b	323 (51)	323 (100)
3	51 (8)	
HCV RNA (mean log IU/ml ± SD)[1]	5.8 ± 0.7	5.8 ± 0.7
rs12979860 genotype		
CC	180 (28.4)	78 (24.1)
CT	326 (51.5)	176 (54.5)
TT	127 (20.1)	69 (21.4)
METAVIR fibrosis stage[2]		
F0-F3	313 (82)	256 (89)
F4 (cirrhosis)	67 (18)	33 (11)
Treatment history[3]		
Naive	337 (53)	180 (56)
Experienced	164 (26)	88 (27)
Unknown	132 (21)	55 (17)

[1]HCV RNA concentrations were available in 409 patients.

[2]METAVIR fibrosis stages were available in 380/633 of all and 289/323 of HCV GT1b-infected patients.

[3]Pretreatment history was IFN monotherpay or IFN/ribavirin or pegylated IFN/ribavirin therapy.

Table 5.4: Epidemiological, serological and virological characteristics of patients infected with HCV GT1 (replication cohort) [117]

Parameter	Total, n (%)	GT1b, n (%)
n	201 (100)	131 (100)
Age (years, mean ± SD)	48.7 ± 13.5	49.2 ± 13.9
Sex (male)	112 (56)	70 (53)
Alanine aminotransferase (mean ± SD)	86.5 ± 68.3	82.3 ± 63.0
HCV genotype		
1a	70 (35)	
1b	131 (65)	131 (100)
HCV RNA (mean log IU/ml ± SD)	5.8 ± 0.8	5.8 ± 0.7
rs12979860 (formerly IL28B) genotype		
CC	70 (35)	44 (33.6)
CT	110 (55)	73 (55.7)
TT	21 (10)	14 (10.7)
METAVIR fibrosis stage[1]		
F0-F3	124 (90)	66 (85)
F4 (cirrhosis)	14 (10)	9 (10)
Treatment history		
Naive	201 (100)	131 (100)
Experienced	0 (0)	0 (0)

[1]METAVIR fibrosis stages were available in 138/201 of all and 75/131 of genotype 1b-infected patients.

5.2.1 IFNL4 status

Since single-nucleotide polymorphisms (SNPs) in the IFNL4 gene were found to be predictors for treatment success in patients with HCV infection, the IFNL4 status regarding the SNPs at rs12979860 (formerly known as IL28B) and rs368234815 was evaluated in n = 633 patients of the primary cohort. Patients were mainly infected with GT1b (51%; 323/633) and GT1a (41%; 259/633) and to a lesser extend with GT3 (8%; 51/633).

The beneficial C/C genotype at rs12979860, which is associated with treatment success, could be found in the primary analysis in 28% (180/633) of the patients (GT1a: 31%; GT1b: 24%; GT3: 45%), while 72% (453/633) had the unfavorable non-C/C genotypes (C/T or T/T; GT1a: 69%; GT1b: 76%; GT3: 55%), see Table 5.5. In the replication cohort the favorable rs12979860 C/C genotype was observed in 35% (70/201) of the patients (GT1a: 37%; GT1b: 34%) (Table 5.6).

Table 5.5: rs12979860 and rs368234815 status in HCV GT1 and GT3 (primary cohort) [117]

	GT1 (total) n (%)	GT1a n (%)	GT1b n (%)	GT3 n (%)
rs12979860				
C/C	157 (27)	79 (31)	78 (24)	23 (45)
non-C/C	425 (73)	180 (69)	245 (76)	28 (55)
rs368234815				
TT/TT	116 (29)	58 (34)	58 (25)	17 (46)
non-TT/TT	288 (71)	114 (66)	174 (75)	20 (54)

Due to limited sample volumes, the dinucleotide variant at rs368234815 (TT/TT or non-TT/TT) was analyzed only in 441 patients (GT1a: 172/441, GT1b: 232/441, GT3: 37/441). The favorable TT/TT genotype was found in 30% (133/441) of the patients (GT1a: 34%; GT1b: 25%; GT3: 46%), whereas 70% (308/441) of the patients were non-TT/TT, see Table 5.5. Among the SNPs at rs12979860 and rs368234815 a strong linkage disequilibrium ($r^2 = 0.925$) could be detected. For that reason and because of the amount of patients without data for the rs368234815 variant, only data for rs12979860 are shown in further analyses.

Table 5.6: rs12979860 Status in HCV GT1 (replication cohort) [117]

	GT1 (total) n (%)	GT1a n (%)	GT1b n (%)
rs12979860			
C/C	70 (35)	26 (37)	44 (34)
non-C/C	131 (65)	44 (63)	87 (66)

5.2.2 Prevalence of resistance associated substitutions (RASs) in NS5A, NS3 and NS5B

Since the viral polymerase lacks proofreading activity and because of a high replicative rate of the virus, a heterogeneous viral population - the quasispecies - is established by the virus in infected patients. Mutations that confer resistance (RASs) to DAAs may pre-exist in untreated patients at lower levels and emerge under the selective pressure of the DAAs. Therefore, RASs in the non-structural proteins NS3, NS5A and NS5B were analyzed by amplification and population-based sequencing of the corresponding genes during the clinical routine. The available results were used to determine the prevalence of

different RASs in NS3, NS5A and NS5B retrospectively. Therefore, the frequency of RASs in NS5A could be analyzed in n = 633 patients. Substitutions at Y93 (Y93C/H/N/F) were detected most frequently in these patients (total: 9%, 55/633; GT1a: 3%, 8/259; GT1b: 14%, 46/323; GT3: 2%, 1/51). Interestingly, in patients infected with GT1b only the Y93H variant occurred and in four GT1a samples other variants (Y93C/N/F) could be detected. RASs at positions M28T, M28V, R30H, Q30E/R were identified very rarely; the prevalences are summarized in Table 5.7.

Sequencing data regarding the prevalence of different NS3 RASs were available in 592/633. The substitution Q80K was the most frequently observed NS3 RAS in the analyzed cohort (16%, 93/592) with a major prevalence in GT1a infected patients (38.8%, 89/242). Other substitution like V36M, T54S, V55A, R155K, and D168E were only found with a very low prevalence as shown in Table 5.7.

RASs associated with resistance to NS5B non-nucleoside polymerase inhibitors were determined in a subgroup of genotype 1 patients (38.9%, 246/633), which had available sequencing data. Whereas the RASs at C316N and S556G/N/R could be frequently detected in GT1b samples (33%, 37/112 and 19%, 22/114, respectively), they were found only with a low prevalence or were completely absent in GT1a samples (see Table 5.7).

Table 5.7: Prevalence of known RASs in NS3, NS5A, and NS5B (primary cohort)

Variant	GT1a n (%)	GT1b n (%)	GT3 n (%)	Total n (%)
NS5A (n=633)				
M28T	1 (0.4)	1 (0.4)	0 (0)	1 (0.2)
M28V	8 (3.1)	0 (0)	1 (2)	9 (1.4)
R30H	0 (0)	3 (0.9)	0 (0)	3 (0.5)
L31F/M/V	3 (1.2)	15 (4.6)	0 (0)	18 (2.8)
Y93C/H/N/F	8 (3.1)	46 (14.2)	1 (2)	55 (8.7)
Y93H	4 (1.5)	46 (14.2)	1 (2)	51 (8.1)
NS3 (n=592)				
V36M	2 (0.8)	0 (0)	0 (0)	2 (0.3)
T54S	11 (4.5)	7 (2.3)	0 (0)	18 (3)
V55A	13 (5)	3 (1)	0 (0)	16 (2.7)
Q80K	89 (36.8)	4 (1.3)	0 (0)	93 (16)
R155K	4 (1.7)	0 (0)	0 (0)	4 (0.7)
D168E	1 (0.4)	2 (0.7)	0 (0)	3 (0.5)
NS5B (n=242; 246)				
C316N	0 (0)	37 (33)	n.a.	37 (15.3)
S556G/N/R	4 (3)	22 (19.3)	n.a.	26 (10.6)

5.2.3 Correlation of IFNL4 genotype and RASs in NS3, NS5A and NS5B

Statistical analysis was performed to investigate a possible correlation between detected RASs in NS3, NS5A, NS5B and the IFNL4 SNPs of the host. No significant correlation of IFNL4 SNPs with any rare RASs (a prevalence of $< 10\%$ was designated as rare) within the NS3, NS5A and NS5B genes was found (compare Figure 5.12A-C).

For the common NS5B RASs C316N and S556G/N/R and for the NS3 variant Q80K, which was found with a high prevalence in GT1a, no significant association with IFNL4 could be observed in any HCV genotype, although a slightly higher prevalence of Q80K was detected in the favorable C/C genotype.

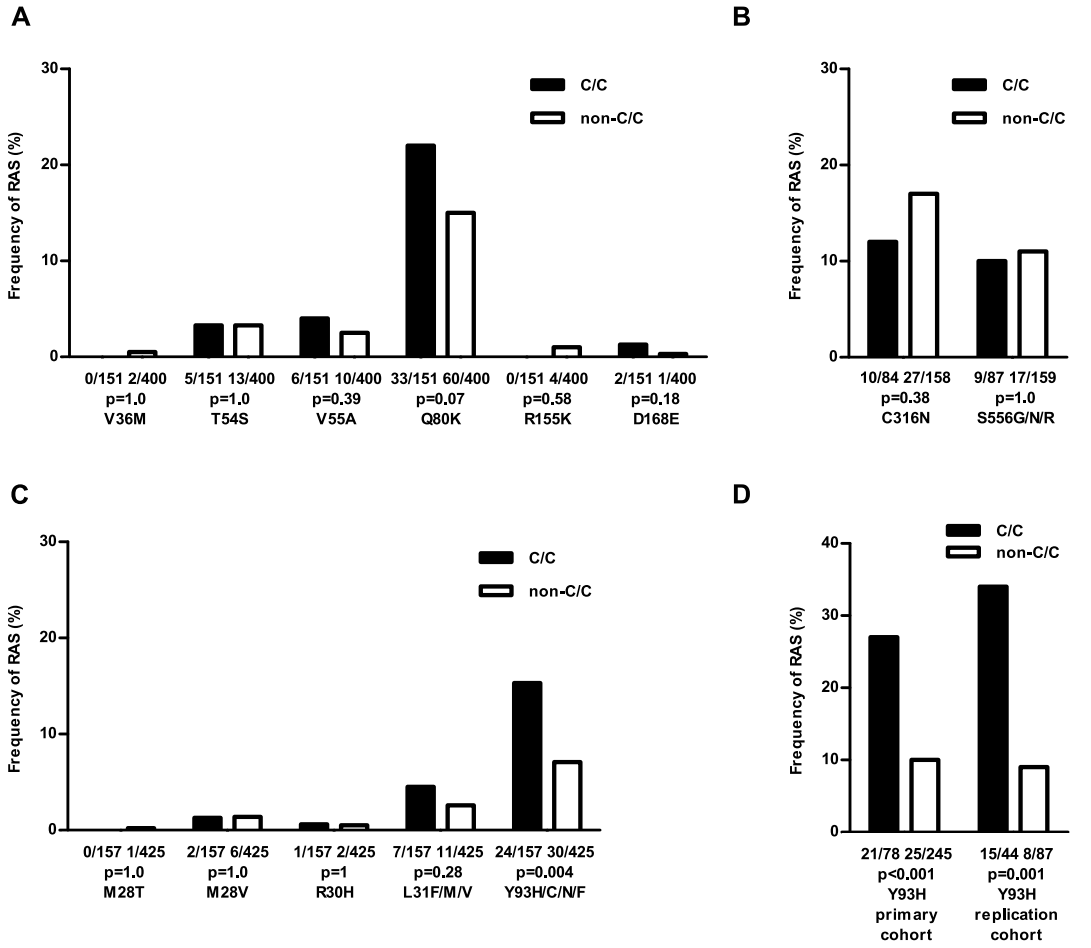

Figure 5.12: Prevalence of RASs in HCV GT1 infected patients in (A) NS3 (n=551), (B) NS5B (n=246), (C) NS5A (n=582) in correlation to rs12979860 (C/C versus non-C/C) in the primary cohort and (D) prevalence of Y93H (NS5A) in HCV GT1b infected patients in correlation to rs12979860 in the primary and replication cohort.

Interestingly, a highly significant association between the Y93H substitution and the beneficial rs12979860 C/C genotype was found in HCV GT1b ($p < 0.001$) as shown in Figure 5.12D. Also in the replication cohort it could be shown that the Y93H variant is significantly associated with the C/C genotype in rs12979860 ($p = 0.001$) (Figure 5.12D).

In summary, no significant correlation between the occurrence of rare and common RASs in NS3, NS5B and the IFNL4 status could be observed in this study population. In contrast, the NS5A substitution Y93H showed a highly significant interrelation with the beneficial rs12979860 C/C genotype.

5.2.4 Prevalence of Y93H detected by population-based and deep sequencing

Deep sequencing analysis of 109 treatment-naive GT1 patients (GT1a: n = 38; GT1b: n = 71) of the replication cohort was performed to examine a possible overlap of Y93H-positive and Y93H-negative populations. The results of deep sequencing and population-based sequencing are comparatively shown in Table 5.8. Y93H was absent in GT1a-infected patients by population-based sequencing as well as by deep sequencing. In each patient, which was infected with GT1b and in whom the Y93H substitution was detected by direct sequencing (n = 16), Y93H was found also with a high frequency (35% - 99% of the quasispecies) by deep sequencing. Furthermore, Y93H could be detected in 10 patients only by deep sequencing since the detection limit of population-based sequencing is assumed to be > 15%. Therefore, no correlation with IFNL4 genotypes could be observed in patients with Y93H at frequencies below the detection limit of population-based sequencing .

Table 5.8: Detection of Y93H in HCV GT1-infected patients by population-based (pop.-bas.) and deep sequencing (replication cohort) [117]

Y93H detected by	Total	GT1a	GT1b
Population-based seq. only	0/109	0/38	0/71
Population-based + deep seq. (freq. 35% - 99%)	16/109	0/38	16/71
Deep seq. only (freq. 35% - 99%)	1/109	0/38	1/71
Deep seq. only (freq. 7% - 34%)	0/109	0/38	0/71
Deep seq. only (freq. 2% - 6%)	7/109	0/38	7/71
Deep seq. only (freq. 1% - 2%)	2/109	0/38	2/71

5.2.5 Association of viral load with IFNL4 and RASs

Since the beneficial C/C genotype at rs12979860 has been described to be correlated with a higher viral load in comparison to the non-C/C genotype in previous studies, the correlation of IFNL4 SNPs with viral load in 409/633 patients of the present study cohort was evaluated.

In patients with the beneficial rs12979860 (C/C) genotype significantly higher viral loads (6.2 log IU/ml versus 5.8 log IU/ml; $p < 0.001$) were observed in comparison to patients with the non-C/C genotypes (Figure 5.13A). No significant association between viral load and the prevalent RASs Q80K (NS3), L31F/M/V (NS5A), C316N and S556G/N/R (both NS5B) was detected.

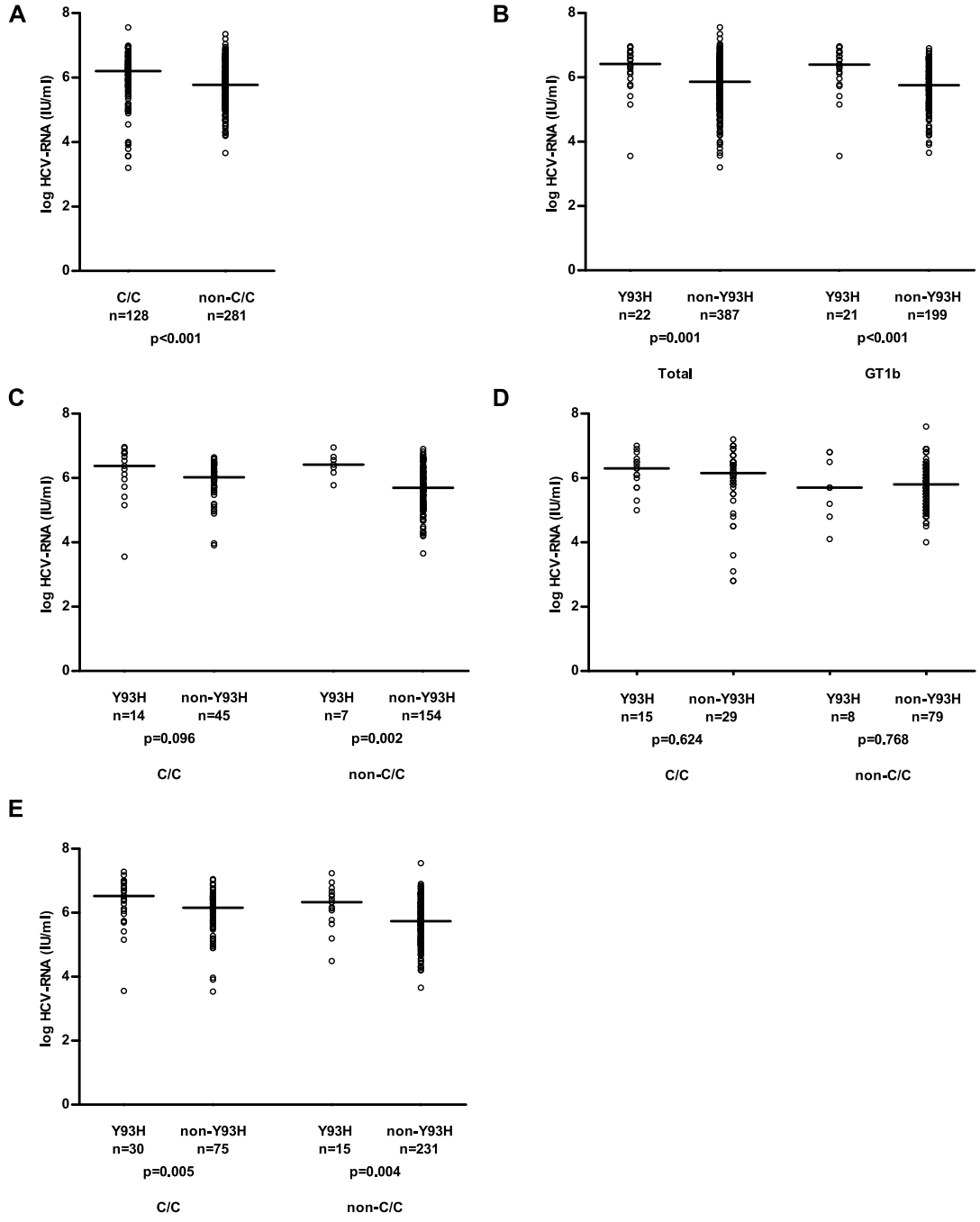

Figure 5.13: Correlation of viral load (IU/mL). (A) With rs12979860 genotypes in HCV GT1 (primary cohort), (B) with Y93H variant in HCV GT1 and GT1b (primary cohort), (C) within rs12979860 genotypes and Y93H variant in HCV GT1b (primary cohort), (D) within rs12979860 genotypes and Y93H variant in HCV GT1b (primary and replication cohort analyzed together).

In contrast, Y93H (NS5A) was significantly associated with a higher viral load (6.4 log IU/ml versus 5.8 log IU/ml; p = 0.001) in all genotypes due to its high prevalence in GT1b (Figure 5.13B). Because Y93H might be a replication enhancing factor leading to increased viral loads, HCV RNA levels were analyzed in Y93H-positive and Y93H-negative populations within the same IFNL genotype. Remarkably, in the primary cohort viral loads were higher in Y93H-positive samples than in Y93 wild-type samples within the same IFNL4 genotype (C/C: 6.2 log IU/ml vs. 6.0 log IU/ml; p = 0.096; non-C/C: 6.4 log IU/ml vs. 5.7 log IU/ml; p = 0.002) as shown in Figure 5.13C. In the replication cohort the viral load was slightly higher in Y93H-positive GT1b samples within the C/C genotype (6.3 log IU/ml vs. 6.2 log IU/ml), but it did not reach significance (p = 0.624; Figure 5.13D). Therefore both cohorts were analyzed together and hence independent from the IFNL4 genotype a significant correlation of Y93H with high viral load was observed in the non-C/C group (6.3 vs. 5.7 log IU/ml; p = 0.004) as well as in the C/C group (6.5 log IU/ml vs. 6.2 log IU/ml; p = 0.005) (Figure 5.13E).

Furthermore, univariate and multivariate logistic regression analysis were performed to explore independent predictors for Y93H occurrence. The following possible predictors were set up: HCV RNA cut-off > 800,000 IU/ml, HCV RNA cut-off > 5,000,000 IU/ml and rs12979860 genotype. Very high viral loads (> 5,000,000 IU/ml) and the rs12979860 genotype could be identified as independent predictors for the occurrence of Y93H (Table 5.9). Interestingly, in the group with very high viral loads (> 5,000,000 IU/ml) Y93H was detected with a high prevalence (60%, 6/10).

Table 5.9: Univariate and multivariate logistic regression analysis of potential predictors for Y93H Occurrence [117]

	P	**Odds Ratio**	**95% Confidence Interval**
Univariate logistic regression analysis			
rs12979860	< 0.001	0.15	0.006 - 0.38
HCV RNA > 800,000 IU/mL	0.005	4.51	1.59 - 12.83
HCV RNA > 5,000,000 IU/mL	< 0.001	19.6	4.95 - 77.6
Multivariate logistic regression analysis			
rs12979860	< 0.001	0.15	0.05 - 0.44
HCV RNA > 5,000,000 IU/mL	< 0.001	19.1	3.85 - 94.6

5.2.6 Association of T83M With Y93H

Different NS5A variants were investigated for a possible correlation with Y93H in order to determine additional factors associated with Y93H.

Interestingly, not known as a RAS another viral polymorphism (T83M) was identi-

fied to be associated with Y93H in GT1b-infected patients. While T83M is the wildtype variant in GT1a-infected patients, it is found to be a polymorphism in GT1b. T83M was detected in 13.2% (29/220) of the GT1b-infected patients and in GT1b-infected patients harboring the Y93H mutation the T83M substitution was detected even with a higher prevalence (32%, 7/22; $p = 0.014$); see Table 5.10. This could also be confirmed in the replication cohort with an overall T83M prevalence of 35.1% (46/131) and in 74% (17/23) in patients with Y93H ($p < 0.001$). Remarkably, this could only be observed for samples with a high frequency of Y93H variants in the quasispecies (in those samples Y93H was detectable by population-based sequencing). Also in the deep sequencing analysis the prevalence of T83M was significantly higher in patients with a high Y93H frequency ($> 35\%$) compared to patients with lower Y93H frequencies (12/17 versus 2/9; $p = 0.046$). When T83M was correlated with IFNL4 genotypes, no independent association between T83M and rs12979860 genotype could was detected in contrast to Y93H (Table 5.10).

Table 5.10: Association of T83M with Y93H and rs12979860 [117]

	Primary Cohort (n)	**Replication Cohort (n)**
T83M		
Y93H	7	17
Non-Y93H	22	29
Non-T83M		
Y93H	15	6
Non-Y93H	176	79
p	0.014	< 0.001
T83M		
rs12979860 C/C	11	20
rs12979860 non-C/C	18	26
Non-T83M		
rs12979860 C/C	50	24
rs12979860 non-C/C	141	61
p	0.274	0.117

The results presented in section 5.2 are included in [117].

6 Discussion

6.1 Hepatitis B Virus

According to the WHO approximately 887,000 people died due to complications of an infection with HBV, like the development of cirrhosis or HCC, in 2015 [172]. Although potent antiviral treatment regimens against a chronic HBV infection are approved, only up to 11% of the patients (depending on the treatment regimen) achieve a functional cure (loss of HBsAg) and the rates of achieving a complete cure (eradication of cccDNA) are even lower [22, 48, 76, 98]. Also, integration of viral DNA into the host genome and therefore a hidden source for the production of viral proteins might occur. These steps have not been investigated well so far and cannot be attacked by the current NUC therapies. Hence, also the integrated DNA might prevent a complete cure and might be responsible for reactivation of an HBV infection in patients who previously achieved a long-term ($>$ 10 - 15 years) sustained virological response (undetectable HBV-DNA) [140].

Furthermore, mutations in the BCP, precore and preS region have been described to be associated with a severe course of the disease in several Asian studies [63, 71, 91, 111, 113]. Also, the cut-offs of the serum markers HBV-DNA and qHBsAg, which might predict the progression of the disease, were established in Asian cohorts [66, 90, 166]. In Asian populations HBV is mostly spread vertically and HBV genotypes B and C dominate, while in European populations HBV is mainly transmitted horizontally and genotypes A and D are found with the highest prevalence. Hence, it is unclear whether these prognostic markers as well as the association of certain mutations with the progression of the infection can be transferred from Asian to European patients.

Therefore, the German prospective, multicenter Albatros study including patients with an HBeAg negative chronic HBV infection was established to investigate the longitudinal course of a low replicative form of a chronic HBV infection in patients who do not need an antiviral therapy. The prevalence of the different HBV genotypes and mutations in the BCP/precore/preS domains was determined. The detected genotypes and mutations were correlated with prognostic serum markers as well as the progression of the disease. Additionally, certain variants containing BCP, precore and preS mutations were cloned and the amount of expressed and secreted viral genomes/proteins was analyzed in a hepatoma cell line. Furthermore, different HBV genotypes were investigated regarding the expression and secretion of viral and subviral particles.

6.1.1 Impact of Genomic variability on HBsAg and HBV-DNA levels

In this interim study analysis 504 patients with HBeAg negative chronic HBV infection were analyzed. As expected, HBV genotype A and D infected patients were found with high frequencies (section 5.1.1) since these genotypes have been described to be mostly prevalent in Europe [77]. As genotypes B and C are mostly prevalent in Asian countries and genotype E is highly prevalent in Western and Central Africa, the majority of patients infected with genotype B/C and E was originally from Asia and Africa, respectively.

As shown in section 5.1.1 mutations in the BCP, precore and preS domain were found in a genotype-specific pattern. This is in accordance to other studies where it has been described for preS mutations in genotypes B and C and for BCP and precore mutations in genotypes A-D, respectively [164]. So far, no data have been published concerning the prevalence of BCP/PC/preS mutations in HBV genotype E. Therefore, in this study it was described for the first time that the predominant majority of patients infected with GTE contained the BCP double mutation. Furthermore, preS mutations and deletions could also be detected with moderate frequencies, while mutations in the precore region were detected only infrequently in HBV genotype E. The BCP double mutation A1762T/G1764A was found with a high prevalence in HBV genotype C. While mutations in the precore region were detected frequently in genotypes B, C and D, they occured only infrequently in HBV genotype A in accordance with other studies [164]. Deletions regarding the preS domain or mutations in the preS2-start codon were found with the highest frequency in GTC as described in line with another study [164].

Although it was described that the HBV genotype influences viral replication *in vitro* [145], in the present study (section 5.1.2) as well as in a previous study from Denmark [78] it could be demonstrated that the HBV genotype is not associated with HBV DNA serum levels in patients with an HBeAg negative HBV infection. In contrast, in the present study it could be shown that mutations in the precore domain are associated with significantly higher HBV-DNA levels compared to patients without these mutations. This is also in line with *in vitro* studies showing a replication enhancing effect of precore mutations [135, 145]. This replication enhancing effect could also be confirmed in the present *in vitro* analysis (section 5.1.5). Interestingly, despite an enhanced intracellular replication the extracellular HBV-DNA levels were lower in this variant, which is in line with another *in vitro* study [89]. As patients with precore mutations had higher HBV-DNA levels in the present study, this *in vivo* observation cannot be explained by the molecular virological characteristics of the precore variant found in different *in vitro* analyses. The BCP double mutation A1762T/G1764A was associated with lower HBV-DNA levels in the present study population. Interestingly, only marginal differences in the intracellular HBV-RNA levels and in the secreted amounts of HBV-DNA in cells expressing the BCP-variant could be observed. This is in contrast to other *in vitro* studies where an up to 2-fold enhancing impact on replication of this mutant was shown [164]. Regarding a large deletion in the preS1 domain the *in vitro* analysis of the present study revealed a significantly enhanced

production of viral genomes but a slightly reduced secretion of viral DNA, which is in line with a previous study investigating different preS/S variants [121].

Regarding the quantitative HBsAg serum levels it could be shown that qHBsAg levels varied significantly among the HBV genotypes A to E in patients with HBeAg negative chronic HBV infection (section 5.1.3). This was also previously found in a Spanish study including 135 patients with HBeAg negative chronic HBV infection [128]. The widely used qHBsAg cut-off < 1000 IU/ml has been associated with a favorable disease course and also has been implemented in the recent guideline of the European Association for the Study of the Liver (EASL) for monitoring HBeAg negative patients [83]. This cut-off was developed to differentiate between inactive carriers and patients who are at risk for reactivation and disease progression [83]. While in the present study only a low percentage of the patients infected with HBV genotype A and E had qHBsAg levels < 1000 IU/ml, the majority of patients infected with GTB showed levels below this cut-off (section 5.1.3). This might imply that genotype A and E infected patients are at a higher risk of disease progression, which has not been published in the literature so far. These striking differences in qHBsAg serum levels might be rather due to a genotype-dependent efficiency of expression and/or release of HBsAg. This was also demonstrated in two other *in vitro* studies [57, 145]. Consequently, the qHBsAg cut-off as a prognostic marker for disease progression should be specified for each HBV genotype.

Furthermore, mutations and deletions in the preS and the precore region were associated with qHBsAg levels in the present study (section 5.1.3). Patients with preS mutations/deletions had significantly higher qHBsAg levels compared to patients without variations in the preS domain, while lower qHBsAg levels could be detected in patients with precore mutations in comparison to patients without these mutations. However, in the present *in vitro* analysis the HBsAg expression and release were not diminished in the precore variant which is in line with a recent Australian study [145]. In contrast, the enhanced HBsAg release which was found in the patient population with preS deletions/mutations could also be found in preS-del.-variant expressing cells in comparison to the BCP-, precore-, and reference genome-variants. This is in contrast to another *in vitro* study, where three preS/S variants showed a significant reduction of HBsAg secretion and therefore an enhanced retention of envelope proteins in the cell, especially in the ER [121].

Since the cloned 1.5-fold HBV genotype A2 genomes were derived from confirmed HBeAg negative, anti-HBe positive patients, they should not be able to produce HBeAg *in vitro*. Interestingly, as shown in section 5.1.5 the preS-del.-variant produced significant amounts of HBeAg *in vitro*. This might indicate that other mechanisms (i.e. immunological) than a diminished protein expression may lead to HBeAg negativity in this patient despite the fact that the variant was still virological capable of HBeAg production and release *in vitro*. However, in further experiments of our working group in collaboration with the working group of Prof. Hildt at the Paul-Ehrlich-Institute it could be excluded that HBeAg causes the observed enhanced HBsAg release. Additionally, lower amounts

of LHBs were found in this variant (described in [80]) and as LHBs is known to inhibit HBsAg secretion, lower amounts of LHBs in the preS-del.-variant might be causative for the observed enhanced secretion of HBsAg [25]. Furthermore, it was demonstrated that the preS-del.-variant produced filaments with a lower density and a shorter length in comparison to cells expressing a reference genome [80]. The filament length of the reference genome-variant showed a similar appearance as the morphologically different filaments of the HBV genotype G [118]. The HBV genotype G was described to produce HBsAg-particles with a higher density and different morphology *in vitro* and that therefore the secretion of subviral particles is impaired in this genotype [118]. This might indicate that also in the present study the reference genome-variant is characterized by an impaired HBsAg release due to a higher amount of long filaments.

In immunofluorescence microscopy analyses it was observed that HBsAg is localized throughout the extracellular region in the preS-del.-variant, while HBsAg is accumulated in the perinuclear region in cells expressing the BCP-, precore- or reference genome-variant [80]. However, in cells expressing an HBV genotype A genome which was derived from a patient with a highly replicative HBeAg positive HBV infection, a similar HBsAg distribution as compared to the preS-del.-variant was observed [118]. Hence, the release of HBsAg seems to be reduced in all the analyzed genomes derived from patients with an HBeAg negative chronic HBV infection except for the preS-del.-variant. Because of that, these lower qHBsAg serum levels which are commonly observed in patients with HBeAg negative chronic HBV infection [83] might be caused partially by a lower HBsAg release.

Although many patients might live with an HBeAg negative chronic HBV infection for several of decades, about 10 to 30% of the patients lose their "inactive carrier" status and develop chronic hepatitis B with high HBV-DNA levels accompanied by necroinflammatory activity (called reactivation) [4, 54]. Individuals with an HBeAg negative chronic HBV infection are the most prevalent patient group seen in the outpatient departments worldwide [4]. The occurrence of reactivation might depend on certain characteristics like the HBV genotype, the age of spontaneous HBeAg seroconversion, mode and age of transmission and/or HBV-DNA levels at entry in the study [51]. So far, no other as the present long-term observational study has been established to evaluate the correlation between mutations in the viral genome and the progression of the disease in European patients with an HBeAg negative chronic HBV infection.

In the present study only a small number of patients had to start an antiviral therapy during the course of the disease. Therefore, no meaningful correlation could be observed between found mutations to the start of an antiviral therapy (Table 5.3). According to the results described in section 5.1.4, one probable indication might be, that the prevalence of precore mutations was higher in the samples at treatment start than in the baseline samples (64% versus 40%), although the results were not significant ($p = 0.128$). Since new patients will be included into the Albatros trial until the end of the year 2017 and patients will be monitored for 10 years, it is of high interest to evaluate the status of

mutations in the BCP, precore and preS domain in the future as more patients might have to start an antiviral therapy.

Taken together, HBV genotypes A to E are not associated with HBV-DNA serum levels but they are significantly correlated to qHBsAg levels in patients infected with HBeAg negative chronic HBV infection. Hence, a genotype-dependent qHBsAg cut-off level as a prognostic marker should be evaluated. Additionally, common mutations in BCP, precore and preS regions are found in a genotype-specific pattern and have an impact on HBV-DNA and qHBsAg serum levels *in vivo* and *in vitro*. In future years of follow-up the prevalence of patients who reactivate and have to start an antiviral therapy has to be further analyzed and the impact of commonly found mutations in these patients has to be evaluated.

6.1.2 Viral and subviral particles of HBV genotypes A to E

It has been described that different genotypes lead to various courses of an HBV infection, e.g. patients infected with HBV genotype C were more likely to develop HCC [178]. Though, it has not been evaluated so far, if there might be an influence of a different composition of viral and subviral particles in the distinct genotypes to the course of the disease. Therefore, the composition of the secreted HBsAg as well as the ratio of the secreted viral and subviral particles were analyzed in HBeAg negative HBV infected patients included in the Albatros study. In the present study it was shown that the morphology of secreted subviral particles and the composition of secreted HBsAg is strongly genotype-dependent in HBeAg negative chronic HBV infected patients (sections 5.1.6 and 5.1.7). More LHBs than MHBs was found in patients infected with genotype B, while a higher amount of MHBs than LHBs was detected in patients infected with genotypes C, D and E. So far, comparable *in vivo* studies are not available. A comparison of the present *in vivo* data with two recently published *in vitro* studies showed conflicting results as they described higher amounts of LHBs than MHBs in genotypes A - D in general [57, 128]. It might be possible that these differences are due to the individual genomes, which were used to be representative for the different genotypes in the *in vitro* studies. Furthermore, HBeAg negative chronic HBV infected patients were included in the present study, while HBeAg positive HBV genomes were analyzed in the *in vitro* studies [57, 128]. This suggests, that genotype-specific variations in the HBsAg composition might depend as well on the stage of the disease (e.g. HBeAg negative versus HBeAg positive). Regarding patients who had to start antiviral therapy, no changes in the composition of HBsAg could be detected during the progression of the disease (section 5.1.7). Hassemer et al. [57] could not detect any differences regarding the density of HBsAg containing particles among genotypes A - D, while in the present study it could be shown that HBsAg containing particles secreted from genotypes B and D display a higher density (section 5.1.7). Furthermore, an increased ratio of filaments to spheres in the sera of genotype B and D infected patients was

observed as well as long filaments, which accumulated to larger bundles, in genotype B infected patients. SVPs have been described to enhance HBV infectivity, neutralize antiviral antibodies (anti-HBs) and thereby allowing viral spread and persistence in the host [20, 67, 108]. Hence, genotype-specific differences regarding the ratio and morphology of different SVPs might result in a genotype-specific pathogenesis and immunogenicity. To validate this hypothesis, immune cells, like monocyte derived dendritic cells, could be incubated with purified viral and subviral particles of the various genotypes. Cell viability, T cell proliferation and expression of e.g. CD80, CD86, IL6, IL12 and TNFα could be analyzed to prove, if the provoked immune responses differ among the various genotypes.

Furthermore, the impact of different deletions/mutations in the preS domain on the composition of HBsAg was analyzed by WB analysis in the HBeAg negative HBV infected patients of the Albatros study (section 5.1.8). Surprisingly, neither deletions in the preS1/preS2 region led to the formation of LHBs or MHBs with a lower molecular weight, nor mutations abolishing the preS2 start codon had an impact on MHBs expression at all. It might be possible that a heterogeneous quasispecies circulating in the serum of the infected patients explains the conflicting results of found mutations in the viral DNA and the non-expected protein pattern in the WB. Therefore, preS amplicons were subjected to deep sequencing analysis. The mutations which were first detected by population-based sequencing were also found to be the major variant by deep sequencing analysis (section 5.1.8). Hence, it could be excluded that the variability in the viral quasispecies explains the unexpected MHBs and LHBs pattern. In contrast, WB analysis of lysates of hepatoma cells transfected with a GTA genome harboring a 15 aa deletion in the preS1 region proved that this deletion results in a different LHBs pattern when expressed *in vitro* in comparison to a reference genome without this deletion. Furthermore, the 15 aa deletion in the preS1 region removes the specific binding domain of a preS1-specific antibody (D31-F34 of the preS-region [144]) and therefore this antibody cannot bind in this sample. *In vitro* it could be observed that the antibody is not able to bind and hence no expression signal could be detected in the preS-del.-variant. In contrast, a strong LHBs-specific signal could be observed in the serum sample of the patient carrying the preS-del.-variant, although the preS1 deletion abolishes the binding site of this antibody (section 5.1.8).

Several studies demonstrated evidence that HBsAg transcripts are not only derived from the cccDNA but also from HBV-DNA integrated into the host genome (reviewed by [175]). Furthermore, it has been shown that the amount of integrated DNA is higher in HBeAg negative than in HBeAg positive patients, whereas the amount of cccDNA is higher in HBeAg-positive patients [41, 174]. Additionally, in HBsAg positive individuals with no detectable serum HBV-DNA, integrated HBV-DNA was found to be the productive source of HBsAg [52]. Therefore, also the results of the present study indicate that in the analyzed patients harboring preS1/preS2 mutations HBsAg must be expressed from a genetically different source than the secreted viral genomes. While the viral genomes analyzed in the present study were derived from cccDNA, MHBs and LHBs must be derived

most probably from the integrated DNA. This is in accordance with two other studies, which demonstrated that cccDNA amounts and HBsAg serum levels were not associated with each other in HBeAg negative patients [86, 163]. In contrast, in HBeAg positive patients a strong correlation could be detected [163]. These studies as well as the present study indicate that HBV surface proteins might be expressed in functionally sufficient quantities from viral DNA integrated into the host genome in HBeAg negative chronic HBV infected patients. This was also assumed by another study in which HBsAg was not sufficiently reduced by treatment with a small interfering RNA targeting cccDNA in HBeAg negative chronic HBV infected patients, while a sufficient reduction was observed in HBeAg positive patients [182]. If HBsAg is produced from the integrated DNA, the production is not dependent on the viral replication. Therefore, the sustained suppression of the immune system might be supported and the formation of new virions is kept up. And since only a few HBV genomes, which can escape the surveillance of the immune system, are necessary to maintain the cccDNA pool in the infected cells, chronic HBV infection persists for several decades in the patients [9]. While HBsAg loss as a functional cure due to PEG-IFNα treatment is a very rarely observed event and because currently available NUC therapies target reverse transcription, therefore only suppressing the virus, a complete cure cannot be achieved so far. Furthermore, not only the elimination of cccDNA seems to be required to achieve a complete cure of a chronic HBV infection, but also the integrated DNA is a potential and most likely highly productive HBsAg source, which has to be considered as a target for novel antiviral strategies.

In summary, the composition and morphology of HBsAg containing subviral particles vary among the different HBV genotypes A to E. While preS mutations and deletions do affect the HBsAg expression *in vitro*, they do not alter the HBsAg expression pattern in HBeAg negative chronic HBV infected patients. Hence, the present study demonstrates that HBsAg is expressed from the integrated viral DNA in HBeAg negative chronic HBV infected patients. Therefore, this should be considered for novel treatment strategies in this certain group of patients.

6.2 Hepatitis C Virus

According to the WHO about 350,000 to 500,000 people die from complications like cirrhosis and HCC related to a hepatitis C virus infection every year[173]. In 2016 several new DAAs were approved leading to SVR rates of $> 95\%$ in certain patient groups [38, 40, 184]. Still a major problem is the access to treatment, since in 2015 only 7% of the 71 million people chronically infected with HCV could access antiviral drugs [173].

Not only the reduced availability in high-endemic areas is a challenging problem, which must be overcome in the future to eliminate HCV, but also viral and host factors influence the efficacy and the outcome of antiviral treatment regimens. For example as a viral factor, RASs which are pre-existing mutations found in untreated patients remain a challenging issue. They emerge under the selective pressure of DAAs and confer resistance to several DAAs [129, 134]. Furthermore, several host genetic factors influencing treatment efficacy have been identified in recent clinical trials. For example, SNPs in or near the interferon-λ3 (IFNL3) and interferon-λ4 (IFNL4) gene were significantly associated with the spontaneous clearance of HCV and with the response to triple therapy or IFN-free regimens [3, 124, 126, 162, 183]. Ansari et al. demonstrated an interplay between the innate immune system and the viral HCV genome [6]. They conducted a comparative analysis of whole-human-genomes to whole-HCV-genomes in 542 patients chronically infected with HCV and found a significant impact of SNPs in the genes for the human leukocyte antigens and for IFNL3/4 on viral polymorphisms. Furthermore, they showed a correlation between the IFNL4 genotype and the viral load depending on a specific aa substitutions in the HCV NS5A region [6].

Therefore, in the present study the prevalence of commonly known RASs in the non-structural HCV genes NS3, NS5A and NS5B was investigated in patients infected with HCV genotypes 1 and 3 and correlated with SNPs in the IFNL4 gene of the host's genome. As shown in section 5.2.2 the prevalence of RASs in NS3, e.g. Q80K, was higher in GT1a than in GT1b, which is in line with the findings of previous studies [11, 133, 134]. In contrast, RASs in NS5B, e.g. C316N and S556G/N/R, were detected with a higher prevalence in GT1b infected patients than in GT1a infected patients as it was also stated by former investigations [73]. Also, RASs against NS5A inhibitors, e.g. L31M and Y93H, were described to be highly prevalent in HCV GT1b infected patients [11], which could also be confirmed in the present study. The Y93H substitution was found in 14% of the patients infected with HCV GT1b, whereas only in 3% of the GT1a samples a RAS at this position (Y93C/H/N/F) could be found. The prevalence of Y93H in GT3 infected patients might vary geographically as a low prevalence was described in the present study as well as in another European study population [120], whereas a significantly higher prevalence of Y93H was observed in a phase 3 trial of GT3-infected patients from the US [107].

It was shown by multiple studies [84, 104], that several immunological mechanisms

can lead to the selection of certain HCV variants and that pre-existing RASs can reduce the overall barrier to resistance and compromise treatment success. Exemplary, in a large phase 3 study, which evaluated the combination of the NS3 protease inhibitor asunaprevir plus the NS5A inhibitor daclatasvir in HCV GT1b-infected patients, pre-existing viral RASs and host IFNL4 genotypes were examined as predictors for treatment success. The NS5A RAS Y93H could be significantly correlated with reduced SVR rates (38% versus 92%) [97]. Although the beneficial rs12979860 C/C genotype was characterized by higher viral loads, it could be associated with slightly higher SVR rates in sofosbuvir/ledipasvir-based all-oral regimens in HCV GT1-infected patients [109, 127] and with higher SVR rates in GT1b-infected patients treated with daclatasvir-based triple therapy [55, 61, 109].

Furthermore, the SNP at rs368234815 was described to be a functional variant of the rs12979860 genotype. The ΔG deletion in IFNL4 induces a frameshift leading to a novel ORF and the expression of the protein IFN-λ4. The induction of IFN-λ4 leads to phosphorylation of STAT1/STAT2 in hepatoma cells and activates IFN-stimulated genes inducing a more efficient antiviral response [124]. Since a strong linkage disequilibrium ($r^2 = 0.925$) between rs12979860 and rs368234815 was found in the present study only the rs12979860 genotype was correlated with RASs in NS3, NS5A, and NS5B. By multivariate regression analysis the beneficial C/C genotype could be identified as a predictor for occurrence of the NS5A RAS Y93H indicating a regulating influence of host factors like IFNL4 on the nature of the viral quasispecies (section 5.2.5). In addition, Y93H was associated with a significantly higher viral load suggesting an replication enhancing influence, which was also in line with previous studies [2, 69]. In contrast to this, in an *in vitro* system the Y93H variant was found to be characterized by an impaired fitness and a decreased replication level [42]. These contrasting finding might be due to the artificial character of the modified replicons with several adaptive mutations in NS5A and a different viral backbone, which are used *in vitro* HCV-replicating systems [92]. Therefore, the beneficial IFNL4 genotypes might come across the infection with a more efficient antiviral response which either could lead to eradication of the virus or to selection of certain variants of the quasispecies enhancing viral replication and a greater resistance against the innate immune response [2].

In conclusion, while naturally occurring RASs against NS3/4A protease inhibitors and non-nucleoside NS5B polymerase inhibitors were not significantly associated with IFNL4 SNPs, the NS5A RAS Y93H could be associated highly significant with the beneficial IFNL4 genotypes in HCV GT1b-infected patients. This might explain a lack of correlation of treatment response in some patients with a favorable IFNL4 genotype and unknown status of RASs. Therefore, the significant association of Y93H with IFNL4 SNPs in HCV GT1b patients might be of clinical interest for consideration of IFN-free treatment options for patients with known IFNL4 status.

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* These authors share first-authorship.

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Erklärung und Versicherung

Erklärung

Ich erkläre hiermit, dass ich mich bisher keiner Doktorprüfung im mathematisch-naturwissenschaftlichen Bereich unterzogen habe.

Ort, Datum	Lisa Kuhnhenn

Versicherung

Ich erkläre hiermit, dass ich die vorgelegte Dissertation über *Identification of host and virologic factors for HBV- and HCV-associated pathogenesis* selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass alle Entlehnungen aus anderen Schriften mit Angabe der betreffenden Schrift gekennzeichnet sind.

Ich versichere, die Grundsätze der guten wissenschaftlichen Praxis beachtet und nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

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