Quantification of the three hepatitis B virus surface proteins (large, middle and small) and the potential as novel biomarkers during treatment of chronic HBV patients

Inauguraldissertation
zur Erlangung des Grades eines Doktors der Medizin
des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

vorgelegt von Kathrin, Schröder
aus Harare/Simbabwe

Gießen 2024

Aus dem Fachbereich Medizin der Justus-Liebig-Universität Gießen

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Tag der Disputation: 09.12.2024

Dedication

In loving memory of my mother Jane Schröder and my aunt Caroline Evans.

For everything there is a season,
a time for every activity under heaven. Yet God has made everything beautiful for its own time. He has planted eternity in the human heart, but even so, people cannot see the whole scope of God's work from beginning to end.
Ecclesiastis3:1and 11 (New Living Translation)

Content

1 Introduction

1.1 A Historical Insight into Hepatitis B

The symptoms that occur during acute liver infections were first mentioned around 3000 B.C. by the Sumerians, who observed jaundice concerning the liver but associated its symptoms with spiritual causes (Gregory A. Michelotti et al. 2013; Trepo 2014). Around 400 B.C., Hippocrates described epidemic jaundice, specific observations about liver infections, and the body's systemic reactions. He documented that certain groups of patients died within two weeks of the fulminant course of the disease (Oon 2012). Throughout many wars of the New Age, millions of soldiers were most likely infected by the hepatitis virus due to poor living and sanitary conditions (Purcell 1993; Lee et al. 1988).

Nevertheless, it was still unknown that the hepatitis virus was the leading cause of liver infections and the growing worldwide epidemics, despite increasing data correlating with possible risk factors and transmission pathways such as faecal-oral or parenteral transmission during the 19th and early 20th centuries. In 1963, Blumberg et al. made a fundamental discovery when they identified the Australia antigen in an indigenous Australian (Gerlich 2013; Ray 1979; Blumberg et al. 1965). Many subsequent studies showed close relationships between this newly discovered protein and different ethnic groups and the symptom constellation of hepatitis B infections (Beecher 1966; Carson and Burns 2002). With investigations into potential antigens, subviral particles (SVPs) attracted attention and led to a significant breakthrough in 1970 when D.S. Dane described viral proteins, later known as Dane particles (Dane et al. 1970). Different viral particles, including the Australian Antigen, could be examined by electron microscopy (Fig. 1) (Vos et al. 1979; ALTER 2003). Soon afterwards, using molecular biological methods to analyse the virus's deoxyribonucleic acid and polymerase, Robinson et al. finally identified these SVPs as the actual hepatitis B virus and the hepatitis B surface antigen (HBsAg; formerly the Australian antigen), respectively.

While many theories have been discussed about the origin of HBV, many open questions remain. In most cases, the emphasis lies on molecular phylogenetic methods to reconstruct the source of the HBV (Simmonds 2001). The geographical distribution of the HBV genotypes challenges many of these theories since they cannot explain current genotypical patterns. The latest viral DNA analysis extracted from prehistoric

bones from a human infected by HBV about 7000 years ago appears to answer some questions (Barbara Mühlemann et al. 2018; Mühlemann et al. 2018). Mühlemann et al. proposed that the virus migrated with the first *Homo sapiens* out of Africa more than 60,000 years ago, which is still controversially discussed. The fact is that the virus itself is far older. Recent estimations suggest that the virus already existed at the time of the dinosaurs, but most likely underwent recombination and adjusted due to cross-species infections (Krause-Kyora et al. 2018). Suh et al. argue that the earliest hepatitis virus appeared approximately 82 million years ago based on DNA found in Mesozoic birds (Suh et al. 2013).

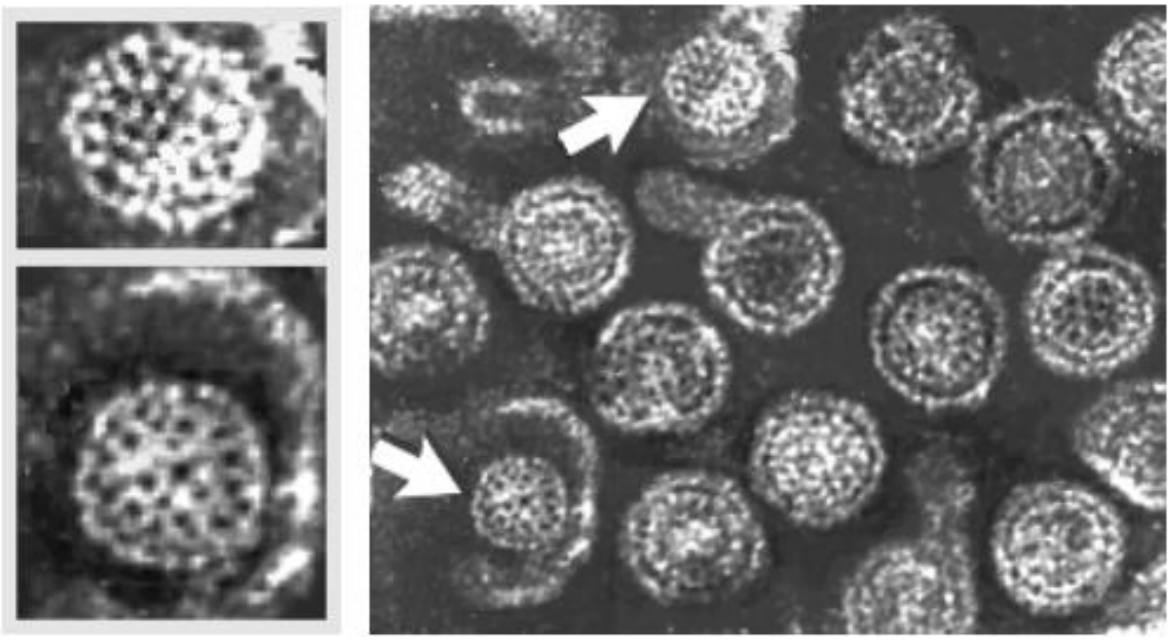


Figure 1 Dane particles. Electron micrographs showing characteristic hepatitis B virions (right) and enlargements of the two exposed cores (left; indicated by arrows). Photograph by Dr Linda M. Stannard (1995) reused with kind permission of Dr Jane Yeats (© Copyright University of Cape Town [2021]) (Hepatitis B Virus | Division of Medical Virology 2021)

1.2 HBV

1.2.1 Classification and Distribution

As one of the smallest mammalian viruses, HBV is an outstanding representative of the Hepadnaviridae family known for its narrow host range, strong hepatotropism, and complex replication mechanisms, particularly its combination of open reading frames (ORFs) and highly efficient management of ribonucleic acid (RNA) transcription. Hepadnaviridae viruses are generally divided into two main genera based on their genomic variations and species specificity. The Avihepadnaviridae are avian viruses,

and the Orthohepadnavirideae cause infections in mammals, including acute and chronic HBV infections in humans (Tiollais et al. 1985; Miller et al. 1984; Miller and Robinson 1986).

The distinctively high error rate (Osiowy et al. 2006) of its reverse transcriptase and its ability to generate high replicant concentrations makes HBV a perfect adaptor to its host, resulting in different genotypes and subgenotypes in humans and apes (Sa-Nguanmoo et al. 2009). Genomic analysis of the entire viral genome has identified ten genotypes (A-J) and their subgenotypes in humans. Eight genotypes are widely recognised based on an intergroup divergence of >7.5% and for the subgenotypes 3.5% (Kramvis et al. 2005; Olinger et al. 2008; Lin and Kao 2015). The latest statistics show that Southeast Asia and Africa have the highest hepatitis B prevalences worldwide (Global Hepatitis Report, 2017; Sunbul 2014). Most HBV genotypes, including their subgenotypes, have distinct geographical distributions and regional specifications (Sunbul 2014). Genotypes A1 and A3-5 predominate in Sub-Saharan Africa. Genotype A2 predominates in Europe. Genotypes C and D are highly prevalent in Central America, South America, Asia, and Australia. Genotype D1-3 is globally distributed in all ethnicities and is the most common genotype. Genotype E is mainly found in Central Africa. Genotype F is found in South America. There are few descriptions of genotypes G and H, and their distributions remain unclear. Recently detected in a few patients, genotypes I and J could be recombinants of different primates (Godoy et al. 2020; Sozzi et al. 2018). Therefore, they are only tentatively recognised (Fig. 2).

Besides differences in their global distribution, they also differ significantly at the molecular-biological level. Several genotypes include genome-specific insertions and deletions, giving them different nucleotide sequence lengths. For example, genotype A had a six-base-pair insertion in the terminal protein region. In contrast, genotype D has a 33-base-pair deletion in the polymerase gene’s spacer region. These genomic variants cause differences in HBV replication (Locarnini 2004).

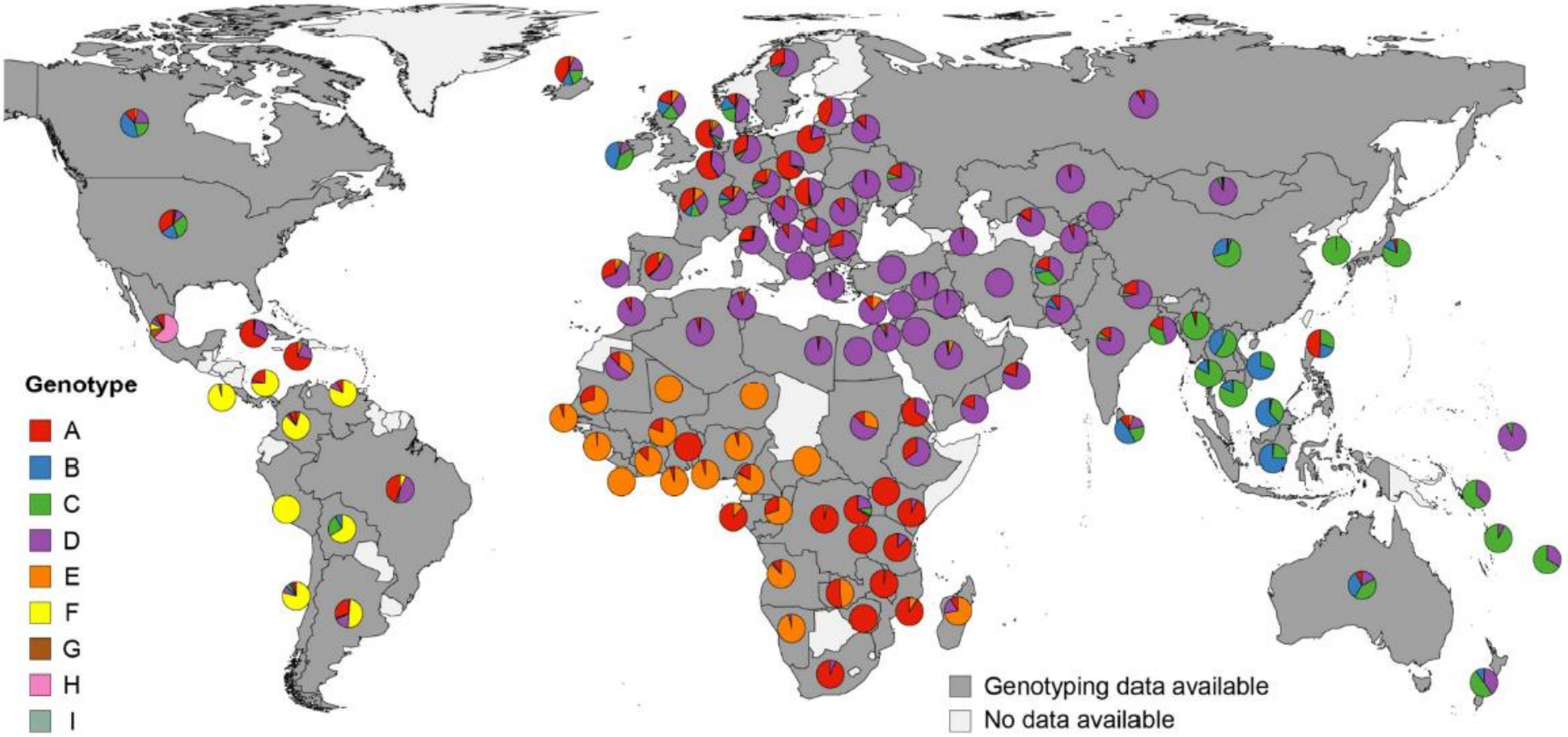
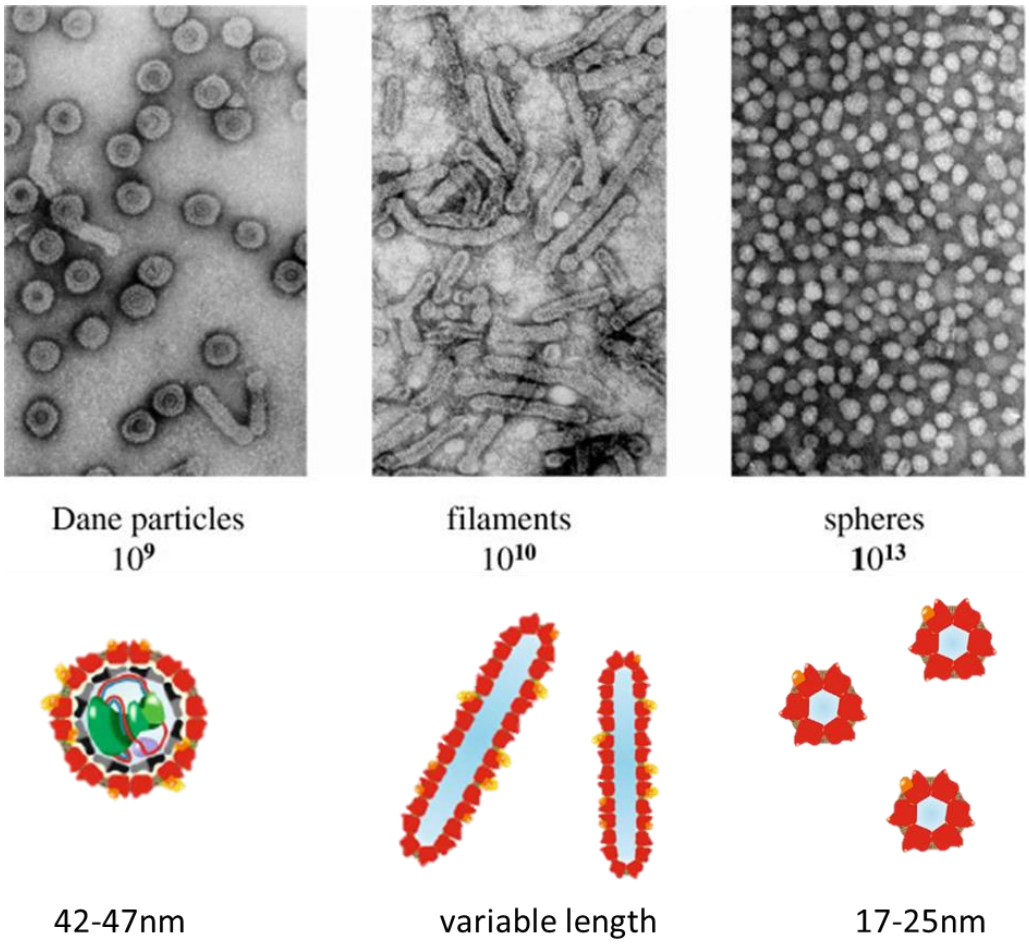


Figure 2 Global Distribution of HBV Genotypes. Pie charts indicate the proportions of HBV genotypes in countries with available data. Kind permission to reuse this image was given by Stoyan Velkov and Thomas Michler (Institute of Virology, Technische Universität München) (Velkov et al. 2018).

1.2.2 Morphology and Structure

HBV is a DNA virus with a partially double-stranded genome. First detected by Dane et al., HBV can be described as enveloped double-shelled particles with a diameter of about 45 nm (Fig. 3) (Dane et al. 1970). The HBV envelope is surrounded by a lipid membrane comprising three major surface proteins—large (LHB), middle (MHB), and small (SHB) hepatitis surface proteins—that are summarised as HBsAgs (Sun et al. 2018; Ben-Porath et al. 1985). HBsAg is proportionally most presented and takes different forms and shapes, such as filaments and spheres (Fig. 3), which are not infectious and cannot replicate since they lack viral DNA. Gerlich et al. found up to 10,000-fold higher concentrations of SVPs than infectious virions in the sera of infected patients (Gerlich 2013; Ganem 1991). The virus has a core nucleocapsid comprising 120 core proteins that form dimers and, for the most part, enclose its double-stranded DNA genome (Zlotnick et al. 1996) and the viral polymerase linked covalently to the primer domain of the genome's minus strand (Fig. 4) (Unchwaniwala et al. 2016; Shih



et al. 2018).

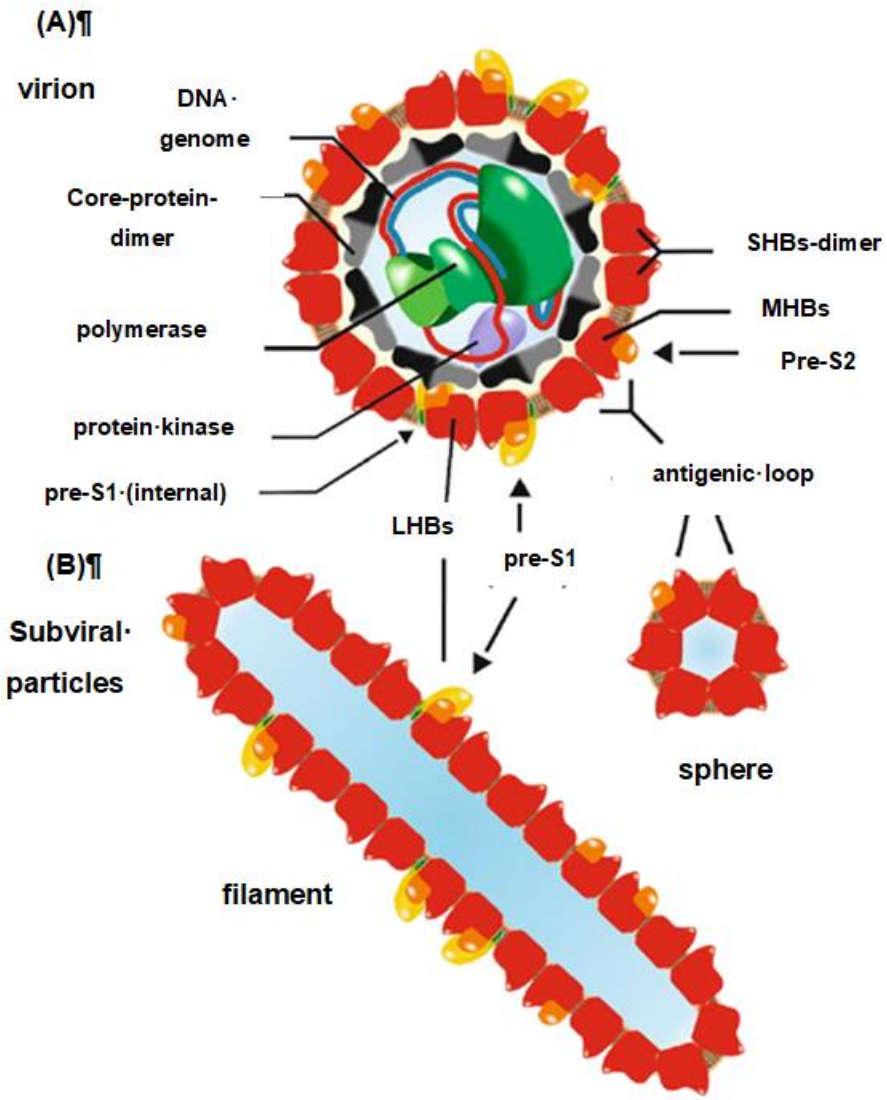


Figure 4 Morphologies and structures of virions and SVPs. The dimeric SHBs (Small Hepatitis B Virus surface protein) **(in red)**, MHBs (Middle Hepatitis B Virus surface protein) with the additional pre-S2 domain **(in orange),** and LHBs (Large Hepatitis B Virus surface protein) with both pre-S2 and pre-S1 **(in yellow)** are integrated into the double lipid membrane either as **(B)** SVPs (spheres and filaments) or **(A)** enclose the virion's nucleocapsid. The nucleocapsid comprises core-protein dimers **(grey and black)**. The partial double-stranded genomic DNA **(red and blue**), protein-kinase **(purple)**, and polymerase are part of the viral core (Glebe and Bremer 2013). The licence for use is provided in the Appendix.

1.2.3 Genomic Structure and Organisation

As mentioned above, HBV has a 3.2 kb small, partially double-stranded, relaxed-circular DNA genome with a complex, four times overlapping ORF (Fig. 5) (Hruska et al. 1977; Landers et al. 1977; Venkatakrishnan and Zlotnick 2016). The largest ORF region encoding the viral polymerase is covalently linked at the 5' end of the minus strand, comprising different subdomains. These include priming and spacer domains, and reverse transcriptase and ribonuclease (RNase) H domains (Fig.5) that can reverse

transcribe an RNA intermediate into the first strand of the DNA genome (Bartenschlager and Schaller 1988; Seeger and Mason 2015).

The longer minus-strand has a complete length of 3182–3248 nucleotides, varying according to the genotypes (Summers et al. 1975; Glebe and Bremer 2013; Gerlich 1980). The shorter incomplete plus strand has a variable 3' end and measures 1700–2800 nucleotides (Karayiannis 2017). The circular formation of the viral DNA results from overlapping regions on the minus strand with 240 base pairings (Orthohepadnaviridae) between the two strands (Fig. 5) (Mizokami et al. 1997; Sattler and Robinson 1979). Therefore, the plus-strand does not undergo transcription or play any significant role in encoding viral proteins (Nguyen et al. 2008)(Landers et al. 1977). In contrast, the minus strand with its four conserved ORFs is greatly important in the viral replication cycle since it encodes all viral proteins (Fig.5). The different messenger RNAs (mRNAs) are produced from different gene sequences and start codons. The second-largest ORF encodes the three major surface proteins (LHBs, MHBs, and SHBs) that together comprise HBsAgs (Schlicht et al. 1993; Schlicht and Schaller 1989). Another ORF is associated with the viral capsid proteins (Qazi et al. 2018), such as the pre-core HBV E antigen (HBeAg) (Carlier et al. 1995) initiating at the first start codon and the dimerizing core protein initiating at the second start codon (Fig.5). The last and smallest genomic region encodes the HBV X protein (HBx), which mainly has regulatory functions (Slagle and Bouchard 2016) and is needed in the replication cycle but is not secreted (Murphy et al. 2016; Kornyeyev et al. 2019).

The latest investigations suggest an epigenetic influence on HBV transcription (Belloni et al. 2009). HBx might be involved in carcinogen processes in infected hepatocytes (Wu et al. 2018). The two viral genome's direct repeats (DR1 and DR2) have proven essential during viral replication (Will et al. 1987; Habig and Loeb 2006). Finally, transcription terminates with the polyadenylation signal at the RNA (Lee et al. 2008; Ganem and Varmus 1987).

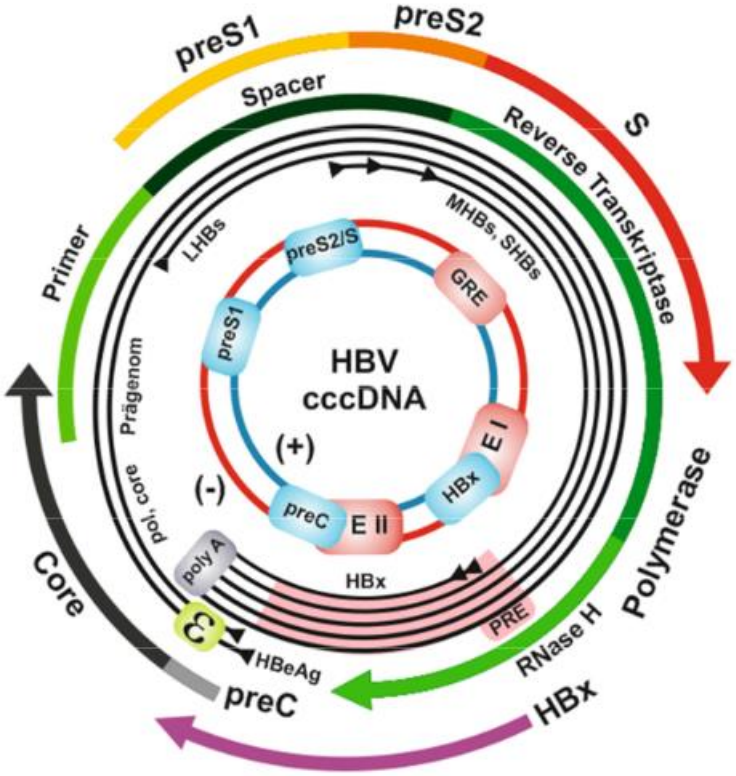


Figure 5 Schematic diagram of the HBV genome. The semicircular organisation of the covalently closed circular DNA (cccDNA) is shown. Its four ORFs are transcribed into the subgenomic mRNAs shown, resulting in the viral proteins (surface-proteins in yellow, orange, and red; core proteins in grey; HBx in purple). According to the four ORF regions' start codons, the enhancer (E I and E II) and glucocorticoid responsive (GRE) elements are highlighted in light red and light blue boxes in the double-stranded cccDNA (in blue and red). The black circles represent the transcripts with one polymerase (grey box) and a posttranslational regulatory element (PRE; in pink) encoding the viral proteins. The pre-genomic mRNA contains an encapsidation signal and encodes the viral polymerase (in green). The longest mRNA encodes the HBeAg (dark grey). Taken from (Glebe and Bremer 2013). The licence for use is provided in the Appendix.

1.2.4 Surface Proteins

The three HBV surface proteins are integrated into the lipid membrane encapsulating all viral particles and virions and enveloping the core capsid (Ueda et al. 1991). The surface proteins are encoded by the S-ORF and transcribed into specific mRNAs (2.1 and 2.4 kb long) from two individual promoters (Almeida 1971; Heermann et al. 1984a). The LHB (39 and 42 kDa) is produced from the longer mRNA (2.4 kb). In contrast, the MHB (31 kDa) and SHB (24 kDa) are produced from the smaller mRNA (2.1 kb) using different start codons. These proteins have different N-termini (J. F. Conway et al. 1998) due to their different start codons but have the same C-termini due to their shared stop codon (Roger H. Miller et al. 1989). The surface proteins combine into the HBsAg. After transcription, the proteins undergo further processing in the endoplasmic reticulum (E.R. (Gavilanes et al. 1982), where they are N-glycosylated

(Lambert and Prange 2007), and Golgi apparatus, when they are O-glycosylated (Patzer et al. 1986; Huovila et al. 1992). They either find their final place in the lipid membrane of infectious virions or leave the cell as spheres and viral particles without nucleocapsids.

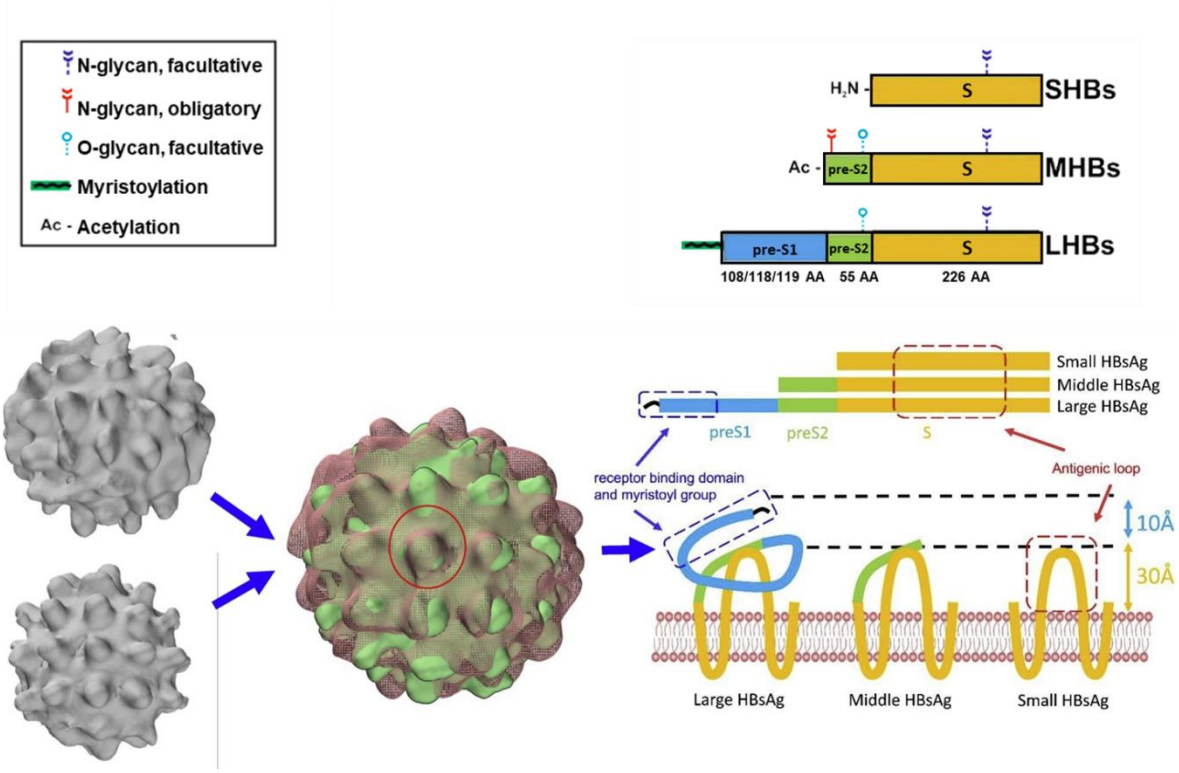


Figure 6 HBV surface proteins. All three surface proteins contain an S domain (226 amino acids [aa], yellow) in their N-terminal region. The MHBs and LHBs have an additional pre-S2 domain at their C-terminus (55 aa, green). LHBs also have a pre-S1 domain, which can vary in length depending on the genotype (108, 118, and 119 aa, blue). The three surface proteins can be altered by post-translational modification. Their S domain can be N-glycosylated (facultative). LHBs have an N-terminal myristylated glycine (obligatory) and an O-glycosylated pre-S2 domain. MHBs have the same modifications in the S domain and pre-S2 domain but always have N-terminal acetylation and an N-glycosylated pre-S2 domain. The essential components for infectivity and coating are in the pre-S1 and pre-S2 domains, which contain the major epitopes of neutralising antibodies and, with their extracytosolic domains, play a vital role in the viral binding mechanism. Another critical region is the receptor binding region in the pre-S1 domain with its necessary myristoyl group. The cytosolic anchorage domain is believed to determine the cytosolic protein portion during maturation. The cryogenic electron microscopy (cryo-EM) image makes it even more apparent that protein organisation and the different protein components define viral functionality and highlight possible antigenic regions. The cryo-EM image was taken from Cao et al. and the modified schematic structure of the surface proteins were taken from Gelbe and Bremer (Glebe and Bremer 2013; Cao et al. 2019). The licence for use is provided in the Appendix.

The Small HBV Surface Antigen

SHBs are the major component of virions and SVPs and only contain the S domain (Bruss and Thomssen 1994). At 226 aa long, it comprises multiple transmembrane domains that form inner (28–79 aa) and outer (99–161 aa) loops (W. P. Paulij et al. 1999; Berting et al. 1995). The outer loop with the glycosylation site (asparagine [N]-

146) presents the antigenic HBs epitope. The HBs epitope is the main target for the immune system and the emerging neutralising antibodies (Helene Norder et al. 2004). Immune cells recognise the different loci of the epitope and initiate either the synthesis of monoclonal antibodies against the SHB's HB-1 (CRTCTT/CKTCTT) region (121–126 aa) (Cerino et al. 2015) or, for example, produce anti-C20/2 against the antigen-loop's conformational epitope (120–160 aa) (Wolfram H. Gerlich 2015). Indeed, during vaccination, this neutralising antibody production effect is the main aim of protecting against HBV (D L Peterson et al. 1984; Emini et al. 1986).

The virus's presented loop also has an essential role in the prime attachment to the host cell's heparan sulfate proteoglycans (HSPGs) (Somiya et al. 2016; Sureau and Salisse 2013). A specific part of the inner loop functions as a binding partner for the completed nucleocapsids and interacts in virion assembly (Xu et al. 1997; Bruss 1997). Some circumstances, such as stress during antiviral therapy, can trigger mutations in the S domain and create escape mutants, emphasising the importance of SHBs in the viral life cycle (Cooreman et al. 2001; Suffner et al. 2018; Melegari et al. 1997). The interlinkage of the loop's cysteines is necessary to stabilise the SHB-envelope complex and hold the tertiary complex together (Mangold and Streeck 1993).

The Middle HBV Surface Antigen

The function of MHBs is not yet fully understood. Like the other surface proteins, MHBs include the S domain but also have an N-terminal pre-S2 domain (55 aa long). The pre-S2 domain is partially N-glycosylated at position N-4 in the E.R. (Sureau et al. 2003). The facultative O-glycosylation at position threonine (T)-37 is performed in the Golgi apparatus (Stibbe and Gerlich 1982; Tolle et al. 1998). O-glycosylation was detected for all genotypes except A (Schmitt et al., .1999). MHBs are suspected to be associated with virion secretion since experiments show that faulty or incomplete MHBs (N-glycosylation site at N-4) are problematic in the secretion process and lead to immune escape (Yu et al. 2014; Liu et al. 2015; Sheu and Lo 1994).

The Large HBV Surface Antigen

The S, pre-S2, and pre-S1 domains comprise the largest HBV surface protein (LHBs) (Heermann et al. 1984b). Their aa length depends on the genotype (108, 118, or 119 aa). SVP and virions comprise SHBs and LHBS and exist in two isoform topologies (Dienes et al. 1990). LHBs can be found either outside the viral membrane or directed inside the

virus (Xu et al. 1997; Bruss and Thomssen 1994; Lambert and Prange 2003). LHBs are essential for the viral replication cycle (Bruss et al. 2004) and determine the success of the HBV infection and coinfection with the hepatitis D virus (HDV) (Gudima et al. 2007). The internal LHBs interact with the core proteins. In contrast, the external surface proteins are involved in binding hepatocytes' sodium taurocholate cotransporting polypeptide (NTCP) and HSPG receptors (Schulze et al. 2007). Receptor attachment initiates the uptake of the virus and enables the final entry and, consequently, infection (Yan et al. 2012).

The internal SHB component acts as a signal transducer and inter-functions with LHBs. Therefore, SHBs play a vital role in the binding of enveloped mature nucleocapsids (Xu et al. 1997; Siegler and Bruss 2013). Moreover, SHB-LHB imbalances favouring LHBs inhibit virion and SVP secretion (Le Seyec et al. 1999, 1999; Blanchet and Sureau 2007). In contrast, an SHB overload enhances infection and secretion (Hildt et al. 1996; Liu et al. 2013).

Conformational changes in the LHBs impact either their internal or external alignment (P. Ostapchuk et al. 1994; Prange and Streeck 1995). The most critical element for infectivity is the N-terminal 77 aa of the pre-S1 domain that signals HBV's entry (Taylor 2013; Glebe and Urban 2007; Gripon et al. 1995). It is assumed that myristoylation (Bruss et al. 1996) interacts with the pre-S1 domain and is necessary for HBV's specific attachment to the host cell. Other aas in the pre-S1 domain interact with the NTCP (L-11 und P-14) to stabilise the attachment (Yan et al. 2012). The aas 49–77 do not affect virus assembly but are associated with HBV infectivity (Blanchet and Sureau 2007; Le Duff et al. 2009)

1.2.5 Replication Cycle

The HBV life cycle is inseparable from the hepatocyte's structural and molecular-biochemical processes. The virus depends on the functionality of its host cell system to survive and replicate. Depending on the infectious viral particles' performance and the host's immune response, the virus is more or less likely to enter and successfully infect the cells and multiply. HBV is a blood borne pathogen that primarily circulates in the blood system after first contact until it reaches the liver via the portal vein. The virions manage to escape through the endothelial barrier. At first, HBV binds with relatively low specificity and affinity to hepatocytes' HSPGs (Schulze et al. 2007; Sureau and Salisse 2013; Somiya et al. 2016). The pre-S1 domain and N-terminal myristic acid are

likely involved in the attachment process (Bremer et al. 2011; Bruss et al. 1996; Glebe et al. 2005).

The hepatocyte plasma membrane's nearby NTCP receptors are essential for the virus to stably bind and enter the cell via receptor-mediated endocytosis via the pre-S1 domain of its LHBs (Yan et al. 2012; Eller et al. 2018; König et al. 2014). Only present in the hepatocyte's basolateral membrane, the NTCP is well-known for its role in the reuptake of conjugated bile acids into hepatocytes. However, it has gained significant importance with growing insight into its function in viral infection. It is suspected that the NTCP is the virus's limiting and restricting factor. Therefore, it has become highly attractive as a target for new antivirals (Kaneko et al. 2018; Tu et al. 2018).

Attachment triggers fusion with the endosomal membrane and uncoats the viral particles into the hepatocyte cytoplasm (Delgado et al. 2015; Rodríguez-Crespo et al. 1995). Then, the bare nucleocapsids are actively transported to the nucleus via microtubule filaments due to the nuclear localisation sequence at the core proteins' C-terminus (Rabe et al. 2006).

The nucleocapsids are released into the karyoplasm at the nuclear pore complex, where their disassembly occurs, exposing the HBV genome (Gallucci and Kann 2017; Paci et al. 2020; Kann et al. 1999). The host DNA repair mechanisms remove the viral polymerase, RNA primer, and positive strand of the covalently linked DNA. Consequently, the relaxed circular DNA is converted into the partially double-stranded cccDNA. Together with a viral chromatin-histone complex, the cccDNA forms a stable minichromosome and acts as the template for HBV and pre-genomic (pg-mRNA) mRNAs (Mohd-Ismail et al. 2019; Liu et al. 2018; Claus-Thomas Bock et al. 1994). The minichromosome is a resource that remains for an extended period in the hepatocyte and is an essential part of the virus's continuous life cycle. HBx is argued to play a role in maintaining cccDNA expression due to its anti-silencing effect on the host's regulation mechanisms (Ramakrishnan et al. 2019; Fang et al. 2019; Qiao et al. 2013). Compared to retroviruses, the HBV does not integrate its genome into the host DNA but uses its cccDNA, which is transcribed by cellular polymerase II into the six known viral RNAs (Rall et al. 1983).

The mRNAs are exported into the cytoplasm. At the E.R., the mRNAs are translated into viral surface proteins, and the pg-mRNAs into the viral polymerase and HBV core

protein (HBcAg). These core proteins dimerise and assemble spontaneously into core capsids (Seifer et al. 1993). Additionally, the polymerase can reverse transcribe the pg-mRNA into partly double-stranded relaxed circular DNA (rcDNA) (Tan et al. 2015; Summers and Mason 1982), which is encapsidated by core proteins (Hu and Seeger 1996). The polymerase is activated in the synthesised nucleocapsids, which is necessary for their maturation. Then, the nucleocapsids are either further processed in the E.R. or transported back to the nucleus via microtubules, where cccDNA production is repeated (Nassal 2015; Inoue and Tanaka 2019).

The nucleocapsids' interactions with membranes containing pre-S1 domains complete their coating with all HBV-surface proteins. Multivesicular bodies connect with mature infectious virions for secretion into the bloodstream (Lambert et al. 2007; Hu and Liu 2017). Surface protein formations without nucleocapsids that remain in the E.R. produce notable amounts of SVPs, which can be secreted via the Golgi apparatus (Huovila et al. 1992), explaining their accumulation in the blood of infected patients (Gavilanes et al. 1982; Patient et al. 2007). The synthesis of HBsAg and HBx occurs in the cytoplasm via the translation of other viral mRNAs. The HBeAg is a co-product of the core protein and translocates to the E.R., where it undergoes proteolysis. As the final step, HBeAg leaves the hepatocyte and remains in the blood. While its functions are not fully understood, it influences the progression of the infection, suggesting it has an immunomodulatory effect (Kramvis et al. 2018; Revill et al. 2010; Liu and Liang 2018).

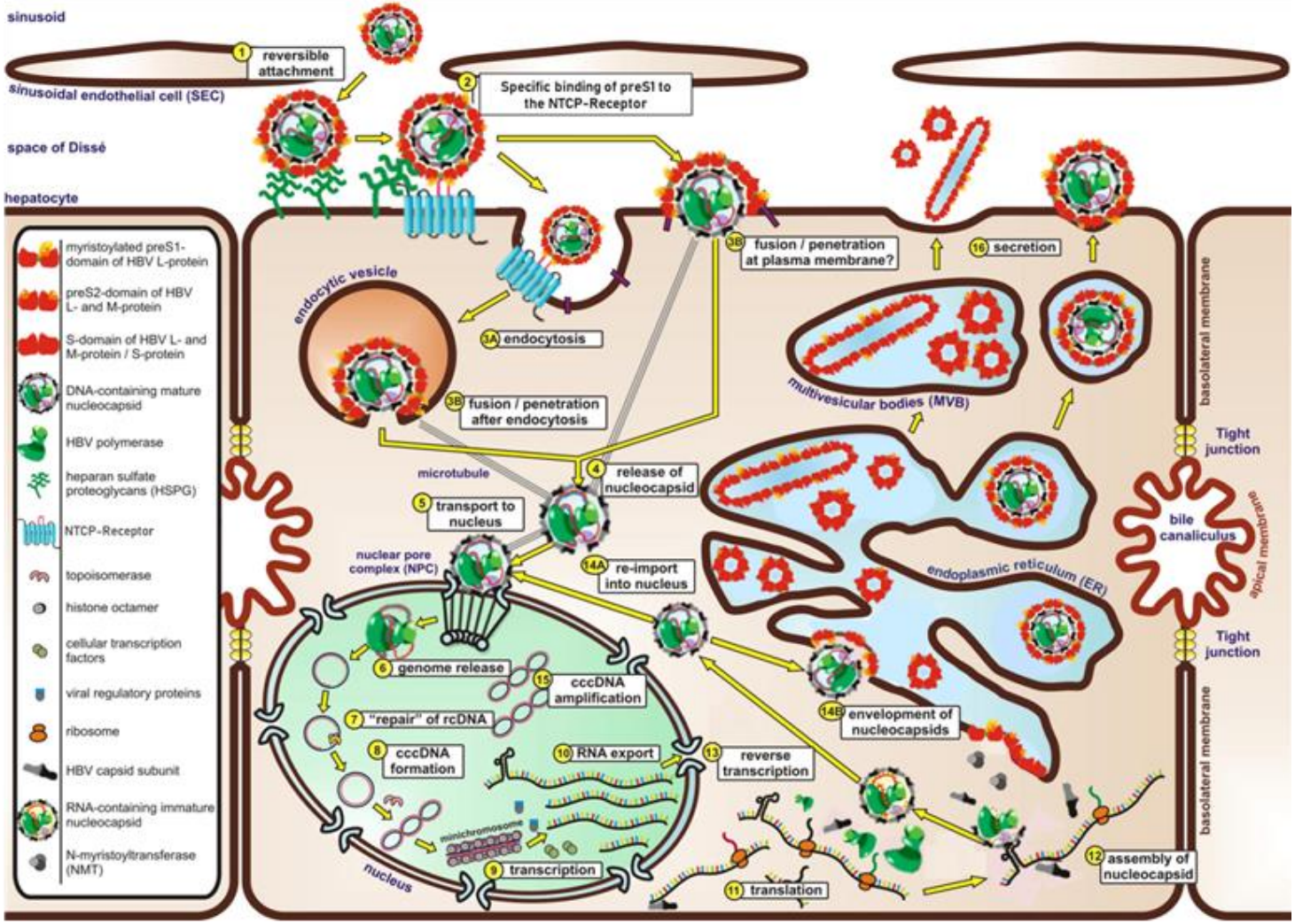


Figure 7 The HBV replication cycle. Modified figure taken from (Urban et al. 2010) with modified and added subviral particles, virions and viral proteins from (Glebe and Bremer 2013) The licence for use is provided in the Appendix.

1.3 Clinical Aspects of HBV Infection

The course of an HBV infection can vary depending on the infected individual's immune response, age, and sex. In addition, the virus's fitness and the concentration of infectious virus particles are decisive in the complex host-virus interaction. Acute phase and chronic infections can be broadly distinguished (European Association for the Study of the Liver [EASL] 2017). First and foremost, the infected individual's immune system predominantly induces symptoms that do not result from the virus. HBV can reproduce quickly if the immune system is weakened or fails to fight the virus. However, the infection does not present with symptoms typical of liver inflammation (Ganem and Prince 2004) and includes hepatocyte destruction by immunoreactions. The first symptoms generally appear 40–200 days after inoculation, depending on the dose of infectious virus particles and the host's immune status (Wright and Lau 1993).

Acute HBV infection can often start with very nonspecific symptoms, such as flu-like symptoms, tiredness, abdominal pain, nausea, and vomiting, with one-third of adult infections leading to jaundice and liver-specific inflammation signs. In 95% of adults with a competent immune response who recover spontaneously, the liver cells regenerate and create immunity against HBV (anti-HBsAg negative and anti-HBV core protein [HBc] and often anti-HBs positive). A full recovery represents a decrease in HBsAg and a substantial decrease in HBV-DNA in patients' blood (Liaw and Chu 2009).

Many patients are asymptomatic and only discovered by serological testing. Only about 1% of infected individuals experience a fulminant course of the disease leading to hepatic-encephalopathy, coagulation disorders, acute liver failure, or even death. Often severe hepatitis is associated with superinfections of HDV or HBV mutants (Kanada et al. 2007).

The infection course in children differs in some aspects. Children can develop Gianotti–Crosti syndrome, characterised by papular acrodermatitis. HBeAg negative carriers can provoke severe inflammation that can cause death (Sokal et al. 2013).

HBV can become chronic and has a risk of complications such as liver fibrosis and, ultimately, hepatocellular carcinoma (HCC) (Bruix and Llovet 2003). Per its definition, chronic infection is accompanied by detectable HBsAg serum levels for >6 months. About 10% of infected adults develop chronic disease, often without noticing an acute

phase. In contrast, in perinatal and infancy, infections become chronic in about 95% of cases. This age-based difference in symptoms and chronification rate can be explained by immune competence and maturity and lack of control over virus replication, meaning individuals who are either immunocompromised or weakened are more likely to suffer under a chronic infection course (Hepatitis B 2020.000Z; Pungpapong et al. 2007).

A closer look at the disease's chronic phase can recognise four phases defined by the dynamics of biomarkers in laboratory diagnostics. These biomarkers aid in monitoring and managing the infection course. The quantification and detection of liver transaminases, HBV-DNA, and several immune markers make it possible to differentiate the different phases and help predict the outcome or treatment response, which this chapter will look at in detail.

As previously mentioned, HBV-related complications are feared most in chronic infection. Limiting long-term liver destruction is only possible by reducing the viral load. About 15% of adults and 40% of children develop liver cirrhosis over 5–20 years due to the constant inflammatory processes. Indeed, many chronic HBV carriers have a 20–100-fold higher risk of developing HCC and dying from the disease. According to the World Health Organization (WHO), about 887,000 are associated with chronic HBV infections, making it essential to identify individuals at risk and determine useful predictors to limit the factors contributing to liver disease progression.

Over an extended period, many HBV carriers show a decrease in viremia and HBsAg, and their HBeAg becomes undetectable. During this phase, their liver enzymes normalise. In singular cases, HBV replication cycle reactivation with an inflammatory relapse can mimic a new acute infection phase. The phases are hard to distinguish serologically from actual acute infection. This effect is frequently found in infections with HBV genotypes D and A2 (Lok et al. 2017).

1.3.1 Transmission

The hepatitis virus spreads via anthropogenic transmission, meaning the virus's main reservoir is infected humans, especially in highly viraemic stages, who pass it onto other humans. The viral load is an important factor for contagiousness, which occurs foremost in the chronic infection stage and the early stage of the acute phase. It can reach up to a concentration of $>10^8$ infectious viral particles/mL blood (Wolfram H. Gerlich 2014; Hsia et al. 2006). Since the virus is highly contagious, ten infectious viral particles are

sufficient for successful transmission (Hoofnagle and Di Bisceglie 1991). The HBV presumably survives outside its host since it takes about a week to infect a new host if it enters a new organism that is insufficiently immune to it. The incubation period is between 30 and 180 days and has a diagnostic window of about four weeks in which the virus is undetectable (Whalley et al. 2001; Krajden et al. 2005; Hepatitis B WHO 2020.000Z).

Asymptomatic carriers have the highest potential of transmitting the virus. In HBeAg-positive patients, HBV reaches high blood concentrations (Sharma et al. 2005; Wolfram H. Gerlich 2014). Other body fluids such as saliva, semen, menstrual blood, vaginal secretion, colostrum, and tear fluid can also be contagious but mostly have lower HBV concentrations (Taha et al. 2011). The transmission pathway varies depending on the endemicity of different regions, the individual's age, and the HBV genotype. Perinatal/vertical HBV transmission and genotype C dominance are common in highly endemic countries. In up to 95%, the HBsAg-positive mother infects the new-born by exposure to blood and infectious fluids. Infected children often develop a chronic infection course, accounting for many globally registered chronic infections. At this point, vaccination (passive and active immunisation) is crucial in preventing further transmissions since the child-to-child transmission rate is very high (Hepatitis B WHO 2020.000Z; Nayagam et al. 2020; Ladhani et al. 2014).

In Western industrialised nations, specific risk-related behaviours are primarily responsible for spreading the virus. Horizontal transmission with a significant number of genotype A-associated HBV infections justifies listing HBV as one of the most critical sexually transmitted diseases. The highest rate of new infections is associated with sexual transmission in young adolescents (Louise Heiberg and Hogh 2012; A.D. Green and S.J. Harbour 1991).

A further risk group is individuals using injection needles due to drug abuse, sharing equipment, and reusing syringes multiple times. Co-transmission of other infections often occurs, such as the human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS), due to high-risk sexual behaviour.

Related to needle injuries and exposure to infectious fluids, tattooing, piercing, and medical staff are possible sources of HBV transmission (Hughes 2000). Therefore, the recommendation is to vaccinate medical staff and employees in healthcare settings. This

proposal has been extended and strongly supported for individuals frequently exposed to potential transmission risks. Occupational HBV infections remain underestimated despite broad vaccination awareness, as shown by the significant percentage of new infections that trace to work-related exposure (Hepatitis B WHO 2020.000Z).

The virus persists in hepatocytes and the bloodstream. Before implementing HBsAg-screenings in the 1970s and introducing anti-HBc titer testing and DNA detection in the 2000s, the incidences of HBV infections related to liver transplantations and blood donation or transfusion were significant. Today, these cases are sporadic thanks to modern standardised inactivation methods of blood/plasma products (Candotti and Allain 2009; Arababadi et al. 2011; (Buddeberg et al. 2008)).

1.3.2 Epidemiology

Hepatitis B is a widespread infectious disease that remains a global health concern. According to the WHO, about 257 million humans live with chronic hepatitis B (CHB; global prevalence = 3.5%). A significant proportion are women of childbearing age who are at particular risk of transmitting the virus to their children. While vaccination provided outstanding achievements in battling the spread of the virus, an estimated 887,000 deaths can be attributed to the disease's consequences, such as HCC and liver cirrhosis (Stasi et al. 2017; European Centre for Disease Prevention and Control 2020) (Willis C. Maddrey 2000).

Prevalences in Africa and the West-Pacific (6%) are higher than in the US (<1%). The transmission patterns can explain regional differences. The main transmission route is perinatal in high-prevalence countries such as Sub-Saharan Africa. In contrast, in regions with low infection rates, sexual transmission and intravenous drug injection are the leading causes of Hepatitis B (Surveillance Atlas of Infectious Diseases 2020.000Z).

1.3.3 Treatment

HBV infections are highly variable in their symptoms, progress, morbidity, and mortality. Many factors influence the course of the disease and require individualised therapeutic approaches. The viral infection's complexity has made it challenging to identify successful treatments. Consequently, until today, the main emphasis of therapy is to prevent complications and co-morbidities such as cirrhosis, liver failure, and HCC (Hou et al. 2017; Ward et al. 2016). Therefore, most therapeutic strategies aim to slow

infection progression, suppress reactivation, reduce transmission, improve life quality, and increase survival. In most cases, lifelong therapy is inevitable (Gerlich 2013).

Due to the high spontaneous recovery rate in the acute infection phase, supportive symptom-orientated treatment is mainly provided. Antiviral medication is avoided if possible. In exceptional cases with severe fulminant hepatitis, current guidelines consider Lamuvidin beneficial to prevent further liver damage. However, chronic HBV infection strongly indicates antiviral therapy (WHO 2015).

The current guidelines for HBV present two treatment options for chronically infected patients (EASL 2017). One suggests treatment with nucleos(t)ide analogues (N.A.s) adefovir, entecavir, telbivudine, and tenofovir. The N.A.s are divided based on their barrier against HBV resistance and classified into nucleotide and nucleoside analogues. They target the viral reverse transcriptase, resulting in ineffective replication and inhibition of virion production (Menéndez-Arias et al. 2014). N.A.s are structurally similar to nucleotides or nucleosides and compete in the DNA synthesis mechanism with the cell's functional equivalents, leading to the terminations of the transcription process (Dienstag 2009). It causes HBV levels to drop below the detection limit, especially with long-term suppression. The WHO recommends oral medications such as tenofovir or entecavir. Their advantages over the other drugs include the most effective reduction in viral replication, fewer side effects based on the reliable safety profile, and lower drug resistance (Xiaoyun Zhang et al.).

The other therapy option is treatment with pegylated-interferon-alpha (PegIFNa), which has immune-modulatory effects on the inflammation processes causing liver destruction and infected patients' systemic reactions (Tseng et al. 2014). Indeed, interferon-alpha (IFNa binds to its specific interferon receptor on the cell surface. The binding of IFNa triggers a signalling pathway that activates antiviral DNA transcription. About 40% of IFNa-treated hepatitis B patients seroconvert to HBeAg negativity, depending on the genotype. In a few cases, treatment leads to successful HBsAg clearance (Korenman et al. 1991; Stephanos J Hadziyannis 2002).

Unfortunately, the treatment has many side effects, limiting the length of the medication period (Sonneveld and Janssen 2010). Many influential variables underlie response to therapy, which is not always predictable or ensured. Therefore, monitoring biomarkers is crucial for evaluating treatment success and indicating infection development.

Hepatitis B treatment and access to resources are limited in developing countries, and globally, there is a greater need for effective new drugs against HBV. Therefore, many novel approaches and investigations target the treatment and cure of HBV infections.

1.3.4 Prevention

While recent major improvements in treatment and prevention remain the most crucial aspects in combating hepatitis B and reducing new infections, the most effective tool besides hygiene measures is the hepatitis B vaccination, which was first implemented in 1981 (Szmuness et al. 1980) in the United States and soon afterwards found its way onto the World Health Organization's List of Essential Medicines (WHO 2019).

The recombinant vaccine is created using yeast (*Saccharomyces cerevisiae*) (Valenzuela et al. 1982; Hilleman 1987) to produce the small HBV surface proteins comprising SVPs belonging to genotype A2 (Peterson et al. 1984) and co-protects against HDV infections due to its similarity to HBV. Lately, vaccines have been refined with pre-S proteins to limit non-response and improve the anti-HBs immune response (Shouval et al. 2015). However, HBV mutants pose a challenge since the vaccine fails to immunise and neutralise these escape mutants, which occur naturally or develop under antiviral therapy (Di Lello et al. 2019; Kamili et al. 2009). Consequently, vaccination does not prevent infection (Carman et al. 1990).

The WHO recommends immunising all infants and children up to age 18 years and documents an estimated global coverage of 84% in 2017, reflecting the efforts to widely spread the vaccine and introduce a routine vaccination schedule (World 2019). The effectiveness of the over one billion vaccinations worldwide since their introduction in the early 1980s is reflected in the significant reduction in chronic infections in children from 8%–15% to <1% (Meireles et al. 2015).

A successful immunisation lasts about 20 years, and it is even suggested that it likely has a lifelong protective effect. It is also recommended that adults in high-risk groups be vaccinated in many countries, such as medical staff exposed to pathogenic bodily fluids, sexually active individuals, or intravenous drug users. Including adults helped decrease the overall disease burden and affected the mortality rates associated with HBV infection. Despite successes, social and economic imbalances still hinder further global distribution and expansion of vaccine implementation. Therefore, to complement projects and initiatives, healthcare programs focus on different strategies, such as blood

product safety, safe injection practices, and safer sex initiatives (Hepatitis B WHO 2020.000Z; (Chang and Chen 2015).

1.4 Current Biomarkers in Hepatitis B Diagnostics

Accurate diagnostic testing is one of the elementary keystones in the battle against the spreading of the disease and progression of the HBV infection. A set of useful biomarkers has been established, which can be categorised as serological and molecular biological markers. Currently, the most important markers are HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc immunoglobulin M (IgM) and IgG, which are considered serological biomarkers, and HBV-DNA, cccDNA, and HBV-RNA, which are considered molecular biological markers (Chakravarty 2012). It is broadly possible to differentiate between acute and chronic phases of infection, pre-screen blood products, monitor the infection, and confirm immunisation. Assessing liver inflammation and progression requires comparing and including transaminases, biochemical inflammation parameters, and immune indicators such as cytokines. The complex interplay of all biomarkers in the different phases of the infection presents specific overall diagnostic patterns (compare Figs. 8 and 9) (Jackson et al. 2018, 2018; Gerlich 2013; Coffin et al. 2019).

HBsAg was one of the first biomarkers and has remained indispensable ever since. HBsAg is detectable about 1–10 weeks after inoculation. It is suggested to correlate with viral replication, especially cccDNA transcription, and is more pronounced in HBeAg-positive carriers than in HBeAg-negative carriers (Chan et al. 2007). The viral surface proteins vary in quantity across the different phases of the infection (Pfefferkorn et al. 2017) and guide and assist in response during PegIFNa therapy (Chuaypen et al. 2017b; Limothai et al. 2019).

In a subclinical or asymptomatic course, the adaptive immune system eliminates all infected hepatocytes before the virus spreads further into the liver. A low concentration of HBsAg in the serum is detectable during this short period, completely disappearing after a few weeks below the detection limit.

Acute HBV infection is indicated by the detection of HBsAg, followed by HBcAg and anti-core immunoglobulin IgM, and high levels of HBV DNA (Whalley et al. 2001; Du et al. 2017). However, normal alanine aminotransferase (ALT) levels are indicators for the initial phase. In the high replication phase, HBeAg is secreted and is strongly

correlated with infectivity (Kramvis et al. 2018). HBeAg indirectly correlates with reduced mortality and morbidity and indicates CHB (Yang et al. 2002; Lok and McMahon 2007). Seroconversion from HBeAg positivity to negativity is often observed due to recovery processes in the liver or mutations in the viral promotor regions (Funk et al. 2002; Liaw 2009; Hsu et al. 2002). HBsAg, HBeAg, or HBV-DNA persisting for >6 months indicates a chronic infection that can maintain or lose HBeAg positivity. High levels of anti-HBc can be found during this period. Therefore, HBeAg loss is accompanied by decreased HBV DNA and indicates fewer risks of hepatitis-induced complications (Cornberg et al. 2017). The decrease in the HBsAg level is mainly accompanied by an increase in anti-HBc level and liver cell destruction, releasing transaminases ("HBeAg-positive immune-active" phase, when ALT levels become elevated). The inflammation caused by the immune response explains symptoms associated with hepatitis (EASL 2017). Immunity is defined by the production of anti-HBs and the complete loss of HBsAg (Robério Amorim de Almeida Pondé 2019). The immune response appears to shift or slow as the virus escapes the immune system for several weeks, spreading into the liver and replicating without disturbance. The only difference to the infection's asymptomatic course is that serum levels of HBsAg and transaminases are far higher. Before the outbreak of symptoms, viral DNA and HBeAg are detectable and steadily increase until they drop again after a few weeks. While HBsAg decreases slower and is undetectable, a small amount of DNA remains detectable for a long time (Ganem and Prince 2004; Gerlich 2013).

Anti-HBs and anti-HBc are specific products of HBV infection and can be measurable even after decades. Anti-HBs titers are used to assess immunity (Pondé 2019). It is thought that the anti-HBs quantified by diagnostics are only those not bound or captured in immunocomplexes with virions or SVPs (Bruss 2007; Glebe et al. 2008) and, therefore, are most likely only an estimation of the actual possible neutralisation power of the antibodies (Ciupe et al. 2014).

Anti-HBc antibodies are essential for distinguishing between exposure to HBV and vaccination (which only includes HBs) (Wang et al. 2017; Tiffany Wu et al. 2017). New test systems can detect HBV core-related antigen (HBcrAg), which may be a useful tool for representing virus replication in the liver (Mak et al. 2019; Inoue and Tanaka 2019), particularly in settings where the infected patient is under N.A. therapy and is HBeAg-seroconverted (Chuaypen et al. 2017a; Mak et al. 2017). It is argued that cccDNA

activity, which is supposed to be an adequate indicator for viral replication rates, possibly is reflected by HBcrAg (Wang et al. 2019). A recent study reported a correlation with cccDNA transcriptional activity in CHB patients (Testoni et al. 2019).

Another marker to estimate the virus's replication activity within the liver is the HBV DNA. It is used in managing N.A. therapy in CHB (Cornberg and Manns 2018; EASL 2017). The viral load is directly proportional to DNA quantities in the serum and appears about one month after infection. After three months, DNA levels can exceed 10^8 copies/mL but decline in chronically infected patients. High levels indicate the need for treatment or therapy failure and are associated with a higher risk for HCC (Liu et al. 2016)(Lin et al. 2010). Looking at molecular biological markers, HBV pre-genomic RNA can be used to investigate the response to therapy under HBV DNA suppression, which, for example, occurs during N.A. therapy (Wu et al. 2019; Fujita et al. 2018; Liu et al. 2019; Limothai et al. 2019).

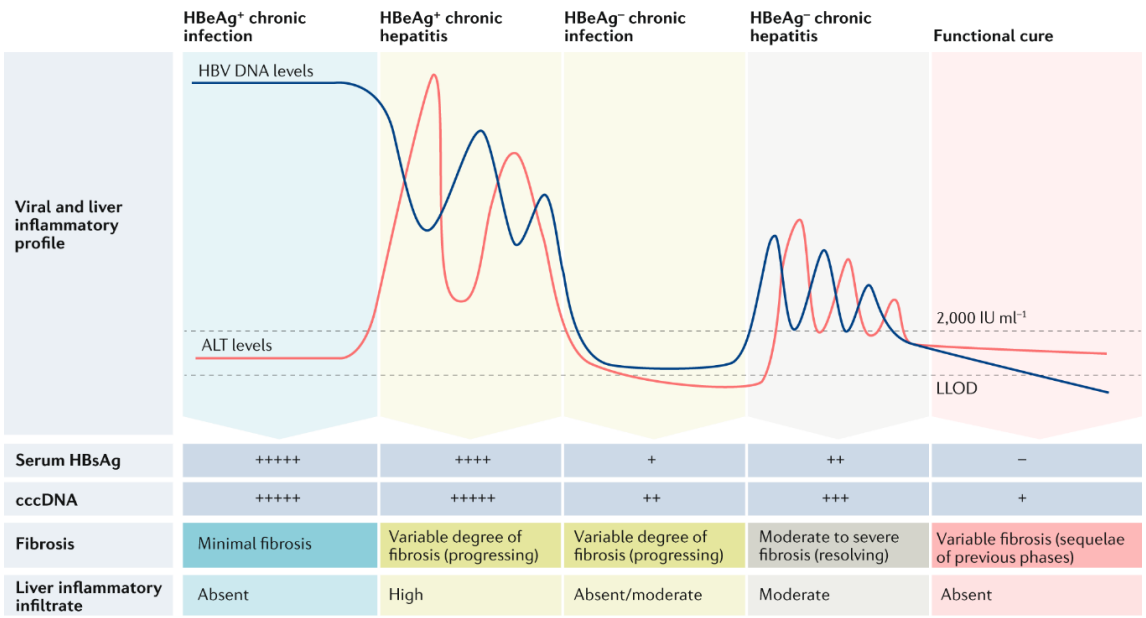


Figure 8 Natural history of HBV infection. All stages of the chronic infection are shown. The highest grades/levels (indicated by: −, not detected; +, rarely detected; ++ to +++++, detected) of HbsAg and cccDNA are seen in HBeAg-positive infections and reach the lowest concentration in functional cure (below the lower limit of detection [LLOD]). The graph highlights the alanine transaminase (ALT) level (red line) and cccDNA detection throughout all phases of viral hepatitis (blue line). Inflammatory signs such as fibrosis and liver infiltrates are additional and complementary in the lower panels. The image is reproduced from (Fanning et al. 2019). The licence to use is provided in the Appendix.

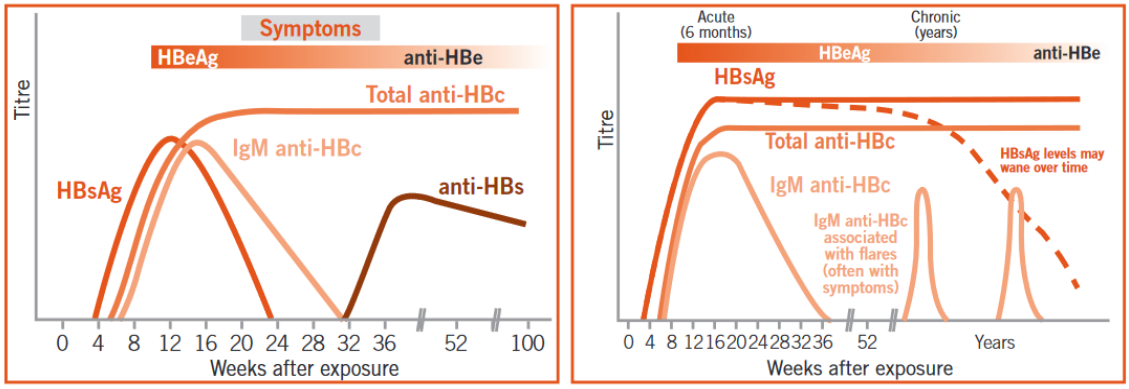


Figure 9 Time course of serological markers of HBV infection. The graphs depict titres of HBsAg, total and IgM anti-HBc, HBeAg, and anti-HBs in acute, chronic infection (left) and CHB infection (right). The licence to use is provided in the Appendix.

2 Aim and Objectives

Infections related to the HBV are challenging and due to >300,000 cases, they can be considered a major global health burden. The various stages and courses make the infection complex and demanding to manage and treat. While diagnostic tools are available to monitor the course of the infection with the help of biomarkers, it is still difficult and does not always lead to detailed and individualised adjusted treatment for the patient. Currently, there is no cure for the disease, and limited predictive biomarkers make it even more important to maximize therapy effectiveness. Closer investigations on biomarkers during the infection course have great potential in detecting predictive outcome tools. These would also allow monitoring of patients' infection courses by signalling treatment failure or recurrence, leading to individualised therapy.

Aim:

This study mainly focuses on quantifying the HBsAg protein components by targeting specific epitopes with modified in-house immunoassays. Its priority is assessing the potential of the three HBV surface proteins LHBs, MHBs and SHBs as novel clinical biomarkers for monitoring immune-based therapy in chronically HBV-infected HBeAg-positive patients.

Objectives:

Method-Validation and Collaborative Study on Exemplary Cohort of Treatment-Naïve Samples

In a collaborative ring trial with Prof. Florian van Boemmel and Dr. Maria Pfefferkorn et al. (University of Leipzig), exemplary samples from patients in different phases of viral infection without antiviral treatment are analysed and compared. In advance, the research team in Leipzig quantifies the three HBs proteins with a commercial ELISA kit and provides the samples for analysis with the in-house immunoassays established in Giessen. The following aspects and questions will be assessed:

- Validation of the modified in-house immunoassays (ELISA and WB).
- Are the results of the different methods comparable regarding the surface proteins' quantification and relative distribution? Are any further optimisations or modifications necessary?

Can this collaborative investigation help to understand the utility of the surface proteins as an instrument for determining the different phases of HBV infections? Are there similar trends and patterns in the relative HBs distribution compared to exemplary samples of different HBV infection phases?

Quantification and Analysis of HBV Surface Proteins in Samples of Chronic Infected Patients with PegIFNa-2a-Treatmet

In cooperation with the research team of Prof. Dr. Markus Cornberg at the Medical University of Hannover (MHH) and the working group of Prof Dieter Glebe at the Institute for Medical Virology at the University in Gießen (JLU), it was possible to include >100 samples of HBV-infected patients previously enrolled in a trial on treatments with PegIFNa-2a (global cohort F. Hoffmann-La Roche Ltd). These samples will be quantified and analysed with modified in-house immunoassays (enzyme-linked immunosorbent assay [ELISA] and western blot [WB]) targeting specific epitopes of all three viral surface proteins. A compressed structural overview of this study's objectives is provided in Fig.10. It will address the following questions:

ELISA Analysis:

- Do the quantification and calculation results for LHBs, MHBs and SHBs contribute information on the relative proportions of HBs surface proteins in the serum of HBeAg-positive CHB patients? Is there a stable distribution pattern?
- Are any determining kinetics of HBs proteins during PegIFNa treatment detectable, and is there a possible predictivity to treatment response?

WB Analysis:

- Can the performance of the in-house ELISA be validated, and are the quantification and relative distribution results replicable in the WB analysis?
- Are there any possible patterns or relationships for the glycosylation of HBV surface proteins?
- Can we fill any additional gaps regarding differences between the methods and distributional factors by visualising the different SVP formation statuses (e.g. SHB dimers) and HBs components?

Statistical Analysis

Essential data and information obtained from the two global, randomised phase III/IV studies published before this investigation (Lau et al. 2005; Liaw et al. 2011) will be retrospectively integrated into the statistical analysis. The statistical analysis will involve the following tasks:

- Are LHBs, MHBs, and SHBs quantities significant biomarkers in predicting response to antiviral treatment?
- Is it possible to determine an ideal sampling time for outcome prediction?
- Do other variables such as genotype, age, and sex significantly influence the outcome or response?

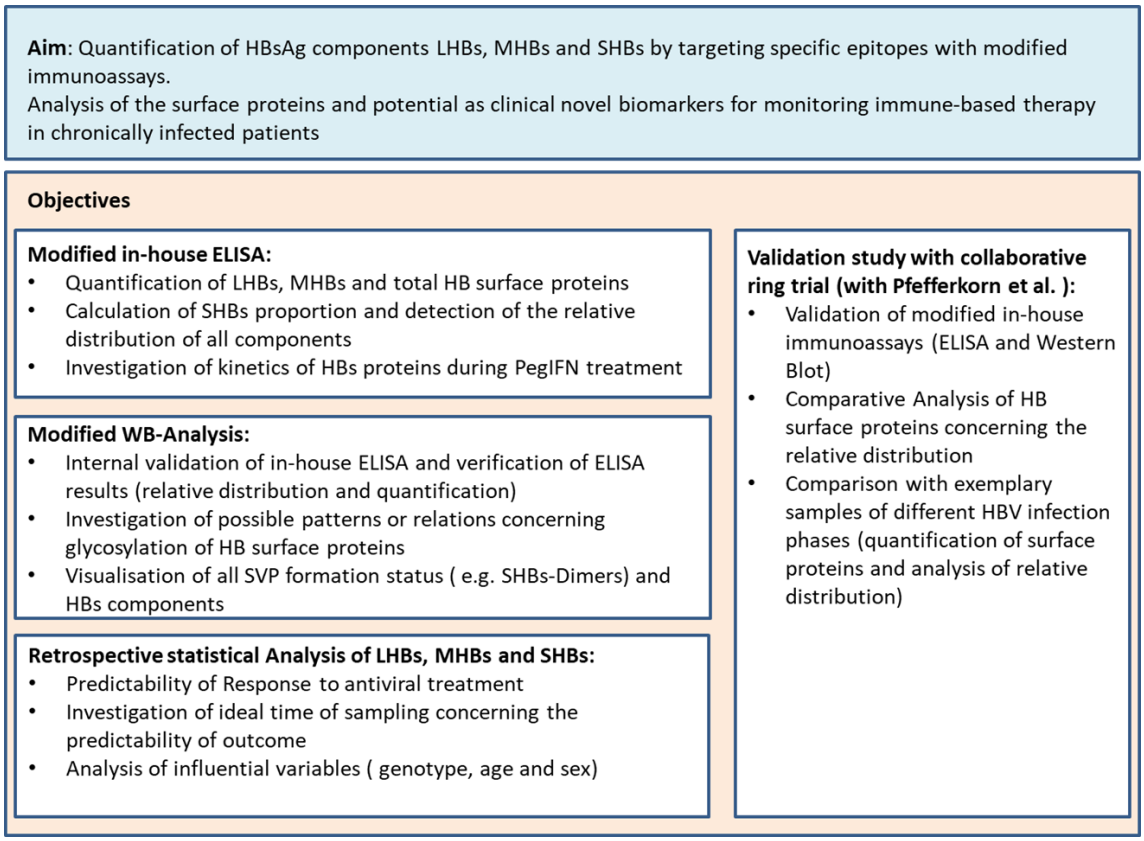


Figure 10 Overview of Aims and Objectives

3 Materials and Methods

3.1 Materials

3.1.1 Sample Cohort

All patients who participated in the studies had been informed about their contents and agreed to all terms in a written approval, including permission for future analysis of stored samples. Therefore, all research is based on the fundamental principles of Good Clinical Practice and the Helsinki Declaration. Every research group and centre was responsible for the ethical approval of their studies by an affiliated committee or board. This study that did only work with plasma samples that were sent from our collaborators was approved by the ethics committee of the Medical Faculty of Justus-Liebig University in Gießen (Register Number 56/17). This study was registered at ClinicalTrials.gov (NCT01705704). The detailed sample characteristics are listed in Table 1.

Characteristic	**PegIFN (*n* = 74)**	**PegIFN+LAM (*n* = 53)**
Sex, male	51 (69%)	39 (74%)
Race, Asian	70 (85%)	52 (98%)
Age, years	31 ± 7.5	30 ± 8.2
HBV genotype A	2 (3%)	1 (2%)
HBV genotype B	21 (28%)	24 (45%)
HBV genotype C	49 (66%)	28 (53%)
HBV genotype D	2 (3)	–
HBV DNA, $\log_{10}$ IU/mL	8.6 ± 1.8	9.2 ± 1.8
ALT, $\log_{10}$ U/L	2.0 ± 0.3	2.0 ± 0.3
HBeAg, $\log_{10}$ PEIU/mL	2.3 ± 0.9	2.3 ± 0.9
HBsAg, $\log_{10}$ IU/mL	4.1 ± 0.8	4.1 ± 0.7
HBeAg seroconversion[a]	38 (51%)	26 (49%)

Table 1 Summary of patient characteristics. Data were taken from Franziska Rinker et al. (2020). Data for age and concentrations: mean ± standard deviation at 24 weeks post-treatment. a) HBeAg seroconversion as measurement to Treatement-Response after 24 weeks

3.1.2 Antibodies

Primary antibodies					
Name	**Specificity**	**Epitope**	**Concentration**		**Source**
			ELISA	**WB**	
HB1	Monoclonal anti-SHBs IgG (1 mg/mL)	S-domain Binding motif: CRTCTT/CKTCTT Conformational independent/linear		1:1000	Dr. Aurelia Zvirbliene, Institute of Biotechnology, Vilnius, Lithuania
C20/02	Monoclonal anti-SHBs	S-domain, AS 120–160	1:500		Prof W. Gerlich, Institute for Med. Virology

	IgG (1 mg/mL)	A-determinant, conformational			JLU-Gießen
Q19/10	Monoclonal anti-MHBs IgG (1 mg/mL)	Pre-S2 domain N-glycosylation Linear	1:80		Prof W. Gerlich, Institute for Med. Virology JLU-Gießen
MA18/7	Monoclonal anti-LHBs IgG (1 mg/mL)	Pre-S1 domain AS 20–23 Binding-motif: LDPAF Linear	1:1000		Prof W. Gerlich, Institute for Med. Virology JLU-Gießen
HBsAg	Monoclonal anti-HBs biotin conjugate Enzygnost HbsAg 6.0	HBsAg	1:40		Siemens Healthcare Diagnostics

Secondary antibodies					
Name	**Specificity**	**Concentration**	**Concentration used**		**Source**
			ELISA	**WB**	
IRDye 680	Goat anti-mouse antibody	1.0 mg/mL		1:10000	LI-COR Biosciences, Homburg, Germany
IRDye 800CW	Goat anti-Mouse	1.0 mg/mL		1:10000	LI-COR Biosciences, Homburg, Germany
Streptavidin-POD	Biotin	1 mg/mL	1:1000		Dianova, Hamburg, Germany

3.1.3 Buffer, Solutions, and Chemicals

Buffers, solutions, and chemicals			
Name	**Ingredients**	**Concentration/ amount**	**Company**
ELISA coating buffer (NaPP) pH 7.4	NaCl $Na_2HPO_4 \times H_2O$ KH2PO4	83 mM 8.6 mM 2.2 mM	
PBS (10×) pH 7.4	NaCl, pH 7.4 KCl $Na_2HPO_4 \times 2\ H_2O$ KH_2PO_4	137 mM 3.4 mM 10 mM 1.8 mM	
ELISA coating buffer (NaPP) pH 7.4	NaCl $Na_2HPO_4 \times H_2O$ KH_2PO_4	83 mM 8.6 mM 2.2 mM	
TNE pH 7.4	NaCl EDTA Tris-HCl	140 mM 1 mM 20 mM	
PBS++	$CaCl_2$ $MgCl_2$ $MgSO_4$ PBS (1×)	0.9 mM 0.52 mM 0.16 mM detached in 400 mL	
PBS++	$CaCl_2$ $MgCl_2$ $MgSO_4$ PBS (1×)	0.9 mM 0.52 mM 0.16 mM detached in 400 mL	
Running buffer			
Loading dye			
Substrate for ELISA	OPD tablets Perhydrol (30) H_2O	 4µl 12 mL	DAKO, Glostrup, Dänemark Merck, Darmstadt

1% casein Blocking solution		1% or 0.1% (in PBS)	Thermo Scientific
Blot washing buffer	PBS Tween 20	 1%	Merck, Darmstadt
Blocking WB	Soy milk (light: 2.1g protein/100 mL)	40 mL	Alpro GmbH, Danone
ELISA wash buffer	PBS Tween 20	1 L 0.1%	Merck, Darmstadt
DTT			
Tris-glycine			
Ammonium persulfate			
Acrylamide/Bis			
Ammonium persulfate (APS)			
Tetramethyl-ethylenediamine			
Tris-HCl (LaemmLi) 1.5M pH 8.8			
Tris-HCl (LaemmLi) 0.5M pH 6.8			
10x Tris/glycine/SDS SDS (%)			
Isopropanol			

3.1.4 Consumable Supplies, Equipment, and Software

Consumable supplies	
Name	Company
Reaction tube (0.5, 1.5, and 2 mL)	Eppendorf
Falcon 15 mL 17×120 mL	BD Biosciences, Heidelberg

Falcon 50 mL 30×115 mL	BD Biosciences, Heidelberg
Pipette tip unplugged (10, 200, and 1000µl)	Sarstedt, Nümbrecht
Single-serving pipette (1, 5, 10, 25, and 50 mL)	BD Biosciences, Heidelberg
Deepwell-Plates 2.2 mL 96-well	Thermo Fisher, Schwerte
Glass containers (e.g., measuring cylinders and beaker glasses)	Schott, Mainz
Pipettes	Eppendorf, Hamburg
Pipettes	Gilson, Limburg-Offheim
Low-bind pipette tips (10 and 200 µl)	Sorenson
Mini-PROTEAN short plates Mini-PROTEAN spacer plates with 0.75 mm integrated spacers Mini-PROTEAN combs, 15-well, 1.5 mm, 40 µl Mini-PROTEAN 3 multi-casting chamber Mini-PROTEAN tetra cell casting stand and clamps	Bio-Rad Laboratories

Equipment	
Name	Company
Centrifuge 5417R Eppendorf	Eppendorf, Hamburg
EIA-Reader BioTek	BioTek, Bad Friedrichshall
EIA-Shaker ZLI 164	Amersham Biosciences, Braunschweig
EIA-Washer COLUMBUS	Tecan Deutschland GmbH
Pipettierhilfen Integra	Biosciences, Fernwald
Reinstwasseranlage	Millipore, Eschborn

Vortexer	IKA, Staufen
Tablecentrifuge 5430	Eppendorf, Hamburg
Rotina 420R	Hettich
SW28.38 rotor	Beckmann
Tablecentrifuge 5430	Eppendorf
Mini-PROTEAN Tetra Vertical Electrophoresis Cell	Bio-Rad Laboratories
Mini Trans-Blot Cell Blottingsystem	Bio-Rad Laboratories
Kit TGX Stain-Free FastCast Acrylamide Solutions	Bio-Rad Laboratories

Software	
Name	Company
SPSS Statistics 24	IBM
Microsoft Office 2010 Excel	Microsoft
Microsoft Office 2010 Word	Microsoft
Microsoft Office 2010 PowerPoint	Microsoft
LI-COR Image Studio Lite Software (Version 5.x)	LI-COR Biosciences
Citavi 6.0	Swiss Academic Software GmbH

3.2 Methods

3.2.1 External measurements and recorded data

It was of vital interest to cooperate with other research centres to perform a complete and comprehensive analysis. The study's sponsor, F. Hoffmann-La Roche Ltd, provided a significant part of the clinical and laboratory data. HBeAg was identified and quantified with an immunoassay using the AxSYM Hbe 2.0 (Abbott, Wiesbaden, Germany). HBeAg levels were detected using suitable standards made available by the Paul Ehrlich Institute in Langen (Germany). The HbsAg levels used in the analysis

derived from the study registered under WV16240, which used the Architect HbsAg (Abbott; LLOQ 0.05 IU/mL) and the data generated in study WV19432 via quantifying HbsAg with the Elecsys HbsAg II Quant (Roche Diagnostics; LLOQ 0.05 IU/mL) assay.

3.2.2 ELISA

ELISAs were used to detect and quantify LHBs, MHBs, and SHBs in HBeAg-positive patients receiving Peg-Interferon. Gerlich and Glebe et al. at the Justus Liebig University in Giessen designed and modified the ELISA system. The test was based on a sandwich ELISA, an immunological method Engvall et al. first investigated in 1971 that leverages specific antigen-antibody-binding visualised through an enzyme-linked antibody reaction. Therefore, it is possible to identify and indirectly determine the actual concentrations of the different surface proteins.

The viral surface LHBs, MHBs and SHBs, which are present on the HBV, are secreted as SVPs, uncoated capsids, or spheres and form a significant proportion of unbound viral proteins in the sera of infected patients. Explicit antibodies against specific epitopes are fundamental for quantifying these surface proteins. The sandwich ELISA used for this study is based on the principle that the analyte is captured between two antibodies, of which one makes the binding visible through enzymatic reactions. The following primary antibodies were used: Ma18/7 targeting the pre-S1 epitope of the LHBs, Q19/10 against the N-glycosylated pre-S2 domain of the MHBs, C20/02 against a conformational structure subtype-independent epitope in the S domain of the HbsAg representing total HBs protein levels (1 µg/mL MA18/7), MHBs (2 µg/mL Q19/10), and SHBs (1 µg/mL C20/02).

Inhouse pre-analysed (known concentration) and processed sera were used to create a standard curve or were used as controls. The standard curve was essential for quantifying the surface proteins and comprised a dilution series of a standard stock solution (1.2 mg/mL) and was run for each plate. The measured absorption was plotted against the standard dilution concentrations. The generated calibration curve made it possible to calculate the concentrations of the proteins with the help of a linear equation.

The immunoassay comprised the following steps:

(1) Coating

The mentioned capture antibodies, expected to specifically bind the surface proteins to the solid phase/plate, were transferred into 0.1 mol/L sodium phosphate coating buffer (pH 7.4) and linked to a highly binding 96-well microtiter plate (Nunc MaxiSorp). Then, the microtiter plates were incubated for 24 h at 4°C.

(2) Washing

Next, the wells were washed twice with 300 µl 0.1% Tween in PBS and twice with 300 µl PBS using the automated ELISA Washer (Tecan) to remove excess antibodies.

(3) Blocking

Then, the wells were blocked with 200 µl of bovine serum albumin-blocking solution diluted to 1% in PBS for 2 h at room temperature. This step is necessary to saturate nonspecific binding spaces and reduce background noise.

(4) Loading

After washing, the prepared microplates were loaded with 100 µl of patient sera, positive control, and negative control in triplicate to minimise the effects of pipetting error or other disturbing factors. The sera were diluted in 1% casein/PBS according to their HbsAg levels (but at least 1:5) to fit into the quantitation range (0.625–20 ng/mL). The absolute quantification of the detected HbsAg in the samples used a 1:2 serial dilution with the purified HbsAg stock solution in 1% casein/PBS with a known concentration (0.167–10 ng/mL; ID1); a 1:2 dilution in 1% casein/PBS (10–0.3 ng/mL HbsAg) was prepared. A negative control of 100 µl of standard diluent buffer was used to confirm that the buffer does not produce a signal. Serum from healthy individuals and negative for HBV and markers were diluted 1:10 and functioned as the negative controls.

In contrast, HbsAg-positive serum from the in-house serum collection was used as a positive control. Here, 100 µl of each standard solution was transferred to the designated well. If necessary, a fixed amount (100 µl) of serum diluted with 900 µl of 1% casein/PBS was placed in the appropriate wells.

The primary antibodies and surface antigens bind and remain as complexes during 24 h of incubation at 4°C.

(5) The plates are washed to remove excess antigens.

(6) Antibody detection

In the next step, 100 µl of biotinylated polyclonal anti-HBs detection antibodies diluted 1:40 in 0.1% casein/PBS (Enzygnost HbsAg 6.0; Siemens Healthcare, Munich, Germany) were pipetted into the wells.

During the 1 h incubation on the shaker at room temperature, the conjugate binds to its epitope of the already bound target structure.

(7) An additional washing step removes all unbound antibodies and other components.

(8) Substrate

The visualisation used o-phenylenediamine dihydrochloride (OPD), a suitable substrate for horseradish peroxidase (HRP), which is converted to a detectable product by the conjugated enzyme. Two OPD substrate tablets (Dako, Glostrup, Denmark) were dissolved in 6 mL water a 2,5 perhydrol per the manufacturer's instructions. Then, 100 µl of the prepared solution was added to the well and incubated on the plate shaker in the dark for 5–15 min.

(9) Stopping the enzyme reaction

The oxidative reaction of HRP into hydrogen peroxide, which causes a blue colour, was stopped with 50 µl of 0.5 M sulphuric acid. The stop solution causes an apparent change in colour to yellow.

(10) Measurement

The intensity of the colour, which is proportional to the antigen concentration, was photometrically measured with an extinction wavelength of 492 nm and a reference wavelength of 620 nm using a plate reader. The measurements were automatically transferred to the reader software, where they were transformed into the standard calibration curve needed to calculate actual surface protein concentrations. SHB protein levels were quantified indirectly by subtracting the LHB and MHB fractions from total HBs. The relative proportions of SHBs, MHBs, and LHBs proteins were calculated relative to total HBs protein.

3.2.3 Western Blot

The Western Blot approach combines two methods: sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) first separates the proteins, which are then electrophoretically transferred onto a membrane for antibody-based detection. SDS-PAGE was developed by Ulrich K. Laemmli in 1970 and is a biochemical analytical method for separating charged macromolecules, such as proteins and nucleic acids. It leverages the different mobility properties of proteins due to their molecular masses, length confirmation, and charge. After successfully fractioning the proteins, they can be differentiated or identified. An essential step is a denaturation with SDS, which linearises the proteins and evenly changes their polypeptide chains' charge to negative, making them migrate towards the positive pole. As mentioned, the fractioned products can be visualised in the following steps using a blotting method and immunological detection system.

3.2.3.1 Preparation of Samples, Standards, Controls, and Markers

Like in the ELISA, the purified standard ID1 (144 ng/mL) with a known concentration was used in each measurement. The standard and patient sera (9 µl) were diluted in 20 µl aqua dest, 11.25 µl sample buffer, and 4.5 µl of 10% dithiothreitol (DTT). Then, 15 µl of the standard and 15 µl of the analytes were loaded into the wells. Each gel was loaded with the same positive and negative controls used in the ELISA.

The Licor chameleon molecular-weight ladder marker was loaded next to the samples and controls on each gel. The molecular-weight ladder comprises known proteins ranging between 8 and 260 kDa. The samples' actual protein sizes could be estimated by comparing their bands with the marker bands.

Pre-trials showed a clear advantage in precipitating the sera before use with an optimal concentration of 12.5%. The optimal method was polyethylene glycol (PEG) precipitation according to the instructions in Marchionni et al.

The viral surface proteins were precipitated from serum by adding 200 µL PEG 6000 in TNE buffer (10 mmol/L Tris, 130 mmol/L sodium chloride, and 1 mmol/L ethylenediaminetetraacetic acid; pH 7.4) to 400 µL serum to achieve the 12.5% concentration. Next, the tubes were incubated overnight at 4°C. Then, the samples were centrifuged at 6800 g for 5 min. Finally, the supernatant was removed, and the pellets

were washed twice in ice-cold 12.5% PEG in TNE buffer and redissolved in 100 µL of PBS.

3.2.3.2 Gel Preparation

The gels installed in the WB System were prepared one day before use. All gels were produced using the Mini-Protean Tetra Cell System. The discontinuous SDS-PAGE gel comprised a collecting gel with integrated loading chambers in which the proteins are concentrated before migrating into the second gel phase. The collecting gel merges into the separating gel (pH), where the surface proteins are separated. The different attributes of the two successive gels are based on their pore size (12% for the collecting gel and 4% for the separating gel), pH values, and ionisation.

The pH gradient between the two gel buffer systems requires a stacking effect. Due to changes in ionic charges and pH increase, the bands of the different proteins (visible after staining) become narrower and sharper.

The Biorad Mini Gel Sealed Glass Plates System and fitting spacers were installed in a clamp stand for gel production.

The SDS–PAGE was performed according to Laemmli (1970). The gel comprised a 4% (w/v) acrylamide stacking gel and a 10%–12% (w/v) acrylamide separating gel, as described below. Methylene-bis-acrylamide, which functions as a crosslinker, is mixed with the gel buffer. Additionally, 50 µl of 10% ammonium persulfate, water, and SDS are added. Tetramethylethylenediamine initiates the radical polymerisation process and must be poured into the cavities of the glass plates. The lower gel (separating gel) is poured first and covered with a few drops of water-soluble alcohol (usually buffer-saturated butanol or isopropanol), eliminating bubbles from the meniscus and protecting the gel solution of the radical scavenger oxygen while solidifying. After the polymerisation of the separating gel, the alcohol solution is discarded, and the residual alcohol is removed with filter paper. The procedure is repeated for the loading gel.

3.2.3.3 Quantification and Fractioning of HBV Surface Proteins

(1) Denaturation

The reaction tubes with the prepared analytes, controls, and standards were heated for 10 min at 70°C to denature the proteins. This process is necessary to transform the proteins into a linear structure by disrupting the hydrogen bonds of their secondary and

tertiary structures. The previously added DTT (10 mM) or dithioerythritol (10 mM) reduces the disulphide bridges. The SDS buffer assures that the surface proteins are equally negatively charged by binding to the protein and further denaturing them. After cooling at room temperature, the samples are ready for pipetting into the gel wells.

(2) Vertical Electrophoresis

Vertical electrophoresis was performed in Biorad chambers in Tris-glycine-SDS (pH 8.8) running buffer at 100 V for about an hour. An electrical field is applied, and the negatively charged proteins move towards the positive pole.

(3) Transfer

The electrophoretic transfer of the proteins from the SDS-PAGE gel onto a nitrocellulose or polyvinylidene difluoride membrane was performed directly after SDS-PAGE according to the Millipore (Immobilon-P Transfer Membrane) user guide using a Mini Trans-Blot Electrophoretic Transfer Cell System (Bio-Rad Laboratories, München, Germany). Instructions for a semi-dry blotting technique (Khysen-Andersen, 1984). For this purpose, a semi-dry blotting chamber with graphite electrodes was used. The nitrocellulose membrane was trimmed to fit precisely on the gel and equilibrated in methanol anode buffer. The membrane is placed onto the gel between pads and filters and should be free from air bubbles construction.

The prepared sandwich system was enclosed in the blotting chamber and inserted into the BioRad chamber before the electrodes were connected. Electrophoresis was performed in a transfer buffer with a voltage supply source for 1.5 h at 30 V at 4°C. Membranes were pre-stained with chameleon ladder dye to confirm a successful transfer.

(4) Blocking

After blotting, the membranes were blocked overnight at 4°C in soy milk in PBS (blocking buffer), which proved the most effective in the pre-testing. Four 5-min washing steps with 75 mL of washing buffer on a shaker were used to prevent non-specific binding.

(5) Primary Antibodies

The HBV surface proteins were detected with monoclonal mouse primary antibodies mAbHB1 (2 µg/mL) against the conserved linear epitope in the S domain (aa 121-

CR[K]TCT-125). The antibodies were diluted to the appropriate concentrations (1:200-1:1000) and incubated overnight at 4°C.

(6) Secondary Antibodies

The membrane was washed three times before being incubated with the HRP-conjugated secondary antibody (Dianova) and six secondary IRDye 680 goat anti-mouse antibodies (LI-COR Biosciences, Homburg, Germany) against the primary mouse antibodies. The secondary antibodies were applied at a final concentration of 0.12 µg/mL, diluted 1:10,000 in soy milk/Tween-PBS, for 1 h at room temperature. Membranes were visualised using an Odyssey CLx Imaging System (LI-COR Biosciences, Homburg, Germany).

(7) Substrate

Finally, after one last washing step, a detection kit with an HRP substrate was added. After repeated washing, the membrane was incubated with the alkaline phosphatase substrate (Immobilon Western Chemiluminescence A.P. Substrate; Merck, Millipore) for 1–2 min.

(8) Detection

The Licor secondary antibodies enabled direct detection of antigen-antibody complexes on the membrane by fluorescence under a charge-coupled device camera that records the fluorescence signals and directly sends the data to a linked odyssey analysis that generates digital images of the proteins on the membrane.

3.2.4 Statistical Analysis

The data were collected, organised, and processed in Microsoft Excel. Then, all statistical analyses were run in IBM SPSS Statistics. In SPSS, the log-transformed data were described and underwent correlation analyses evaluating relationships between variables. A mixed analysis of variance (ANOVA) with repeated measures was performed and complemented by binary logistic regression analysis to show possible causalities. Finally, associations between biomarker levels and responses were assessed using binary logistic analysis.

4 Results

4.1 ELISA-based Quantification and Analysis of the HBsAg (LHBs, MHBs and SHBs)

In clinical diagnostics, quantifying HBsAg with commercially available immunoassays plays a major role in monitoring the infection course. An in-house modified ELISA was designed to detect LHBs, MHBs and SHBs, which together comprise HBsAg. Microtiter wells were coated with specific capture antibodies targeting epitopes of the surface protein components. As shown in Fig. 11, three monoclonal antibodies served as primary antibodies. The monoclonal antibody C20/02 targets the conformational epitope of the S-domain, which exists in all HBs and has a similar significance as the quantification of HBsAg. It is impossible to distinguish between SVPs (filaments, spheres), or actual virions. However, most viral surface proteins in serum are expected to reflect SVPs. The monoclonal antibody Q19/10 targets the N-glycosylated epitope of the MHBs' pre-S2 domain. The monoclonal antibody Ma18/7 targets the pre-S1 domain and is necessary to differentiate LHBs. SHBs is not detected directly, because it is the building block of both MHBs and LHBs and must be determined by subtracting MHBs and LHBs levels from the total HBs serum level (Fig. 11). The captured antigen was detected with a secondary antibody against HBs forming a sandwich structure. The enzyme-substrate reaction produces a colour reaction and emits light, which is measured with a photometer (Fig. 11).

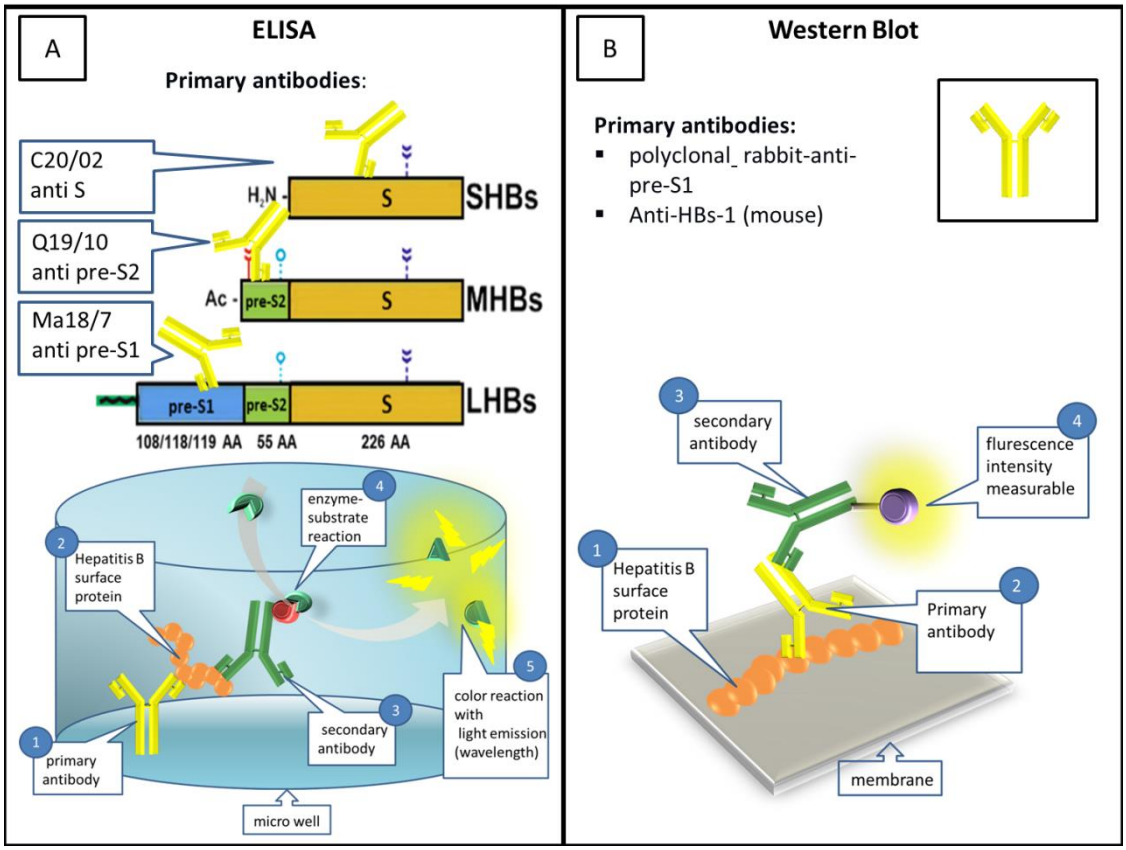


Figure 11 Quantification and Analysis Methods. (A) ELISA method: (1) primary antibodies are bound to microwells (Ma18/7; Q19/10, and C20/2, shown in yellow) and target specific epitopes on LHBs (Large Hepatitis B Virus surface protein), MHBs (Middle Hepatitis B Virus surface protein)and HBs (Hepatitis B Virus surface proteins) (upper graphs); (2) the HB surface proteins bind to their respective primary antibody; (3) the secondary antibodies against HBsAg bind as a sandwich to surface proteins; (4) substrate is added to activate the enzyme conjugated to the detection antibody; (5) the enzyme-substrate-reaction emits light of a specific wavelength that is detectable with a photometer. (B) WB method: (1) HBV surface proteins are blotted onto a membrane (2) and targeted by two primary antibodies (anti-pre-S1 in the LHBs domain and anti-HBs1 for total surface proteins); (3) a secondary antibody bound to a fluorescent dye binds to the primary antibodies; (4) ultraviolet light evokes fluorescence, which is measurable according to the intensity**.** The pictures illustrating the ELISA and Western Blot Method are own created graphics. The ideas for the model is taken from (https://www.immunopaedia.org.za/treatment-diagnostics/diagnostic-tests 2024). The HBV surface protein domain graphs where modified and taken from (Glebe and Bremer 2013)

In each ELISA run, it is essential to pipet a dilution series of standard serum (ID1) from a patient with a known HBs protein concentration (144 ng/mL) (Fig. 12). The diluted standards' measured absorption is transferred to a table with the corresponding diluted concentration and used to generate an Excel-based linear graph, which is important for the subsequent steps (Fig. 12). The absorptions of the surface proteins are converted into ng/mL concentrations using the linear equation generated from the standard linear graph (Fig. 12). When calculating LHBs and MHBs concentrations, their mean relative proportion in total HBs must be considered, which is about 10% for LHBs and 20% for MHBs (implying 14.4 ng/mL and 28.8 mg/mL concentrations in the standard serum; Fig. 13).

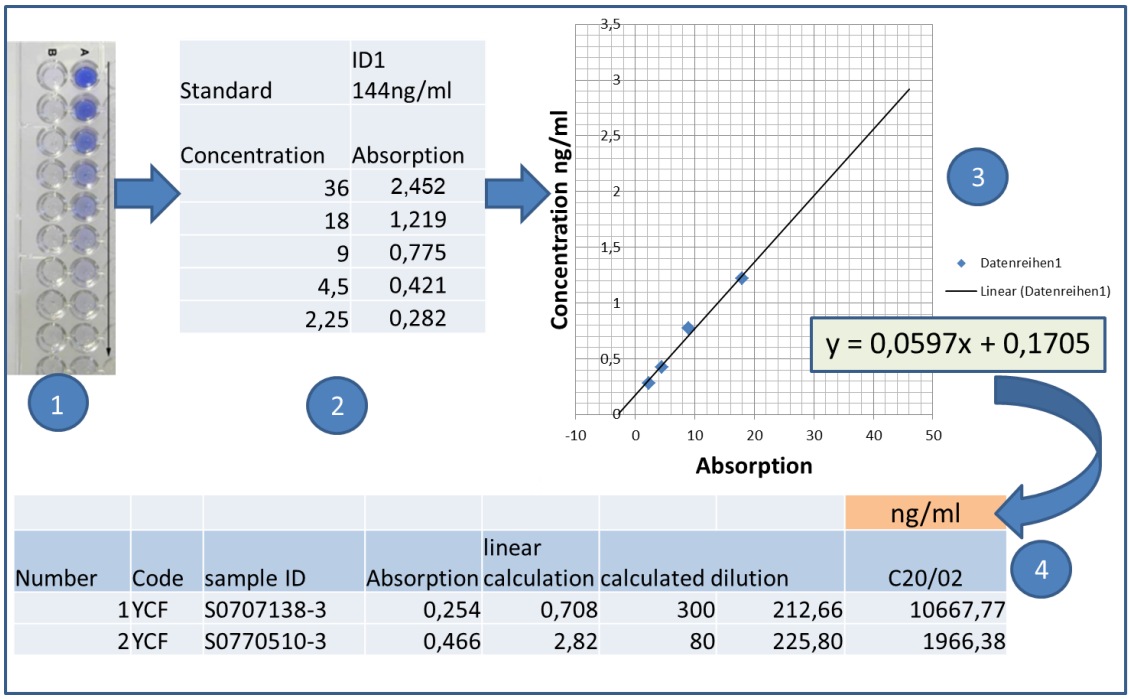


Figure 12 Quantification of LHBs, MHBs, and total HBs and calculation of actual concentrations. (1) A standard of known concentration is diluted, and (2) the measured absorptions are correlated with the corresponding diluted concentrations. (3) A linear graph is created, which can be translated into a linear function. (4) All patient sample ELISA results can be converted into actual concentrations using the linear function.

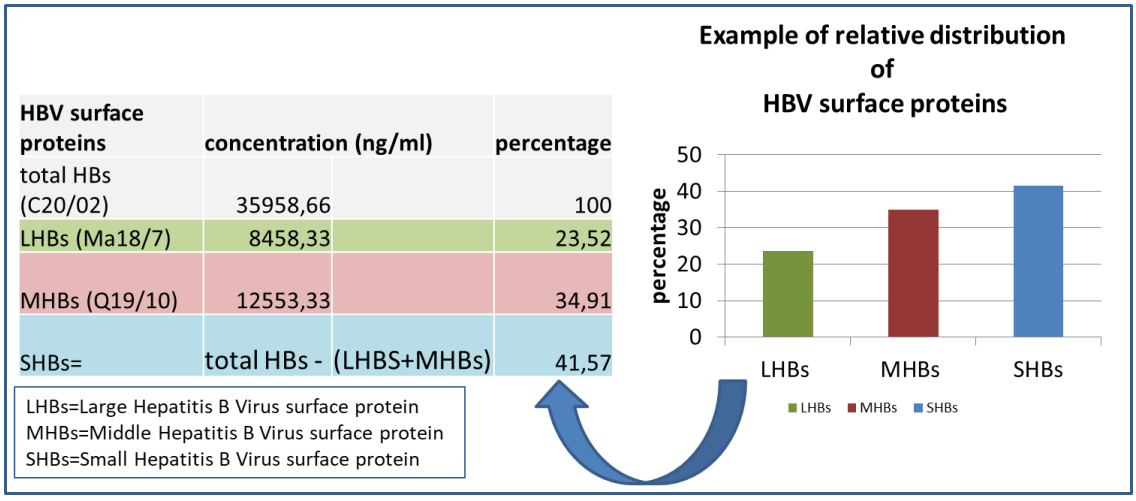


Figure 13 Calculation of the relative distributions of HBV surface proteins. (Antibodies used in ELISA are in brackets). The relative quantity of the total Hepatitis B Virus surface proteins (total HBs) was used to calculate the relative SHBs-quantity by subtracting relative LHBs -and MHBs-quantities (as shown in the table).

4.2 Western Blot Analysis of HBV Surface Proteins

The Western Blot (WB) analysis was important for determining the relative distributions of the HBV surface proteins in the standard serum since they are necessary for calculating the concentrations in the ELISA-examined sera. WB investigations have

the advantage of actually visualising the different HBsAg components. It is possible to distinguish between glycosylated and non-glycosylated proteins; all surface proteins are detectable without further calculations.

The immunoblot was modified and optimised due to pretesting with different blotting solutions and trials of serum preparation, such as ideal PEG (Creighton) precipitations.

The HBV surface proteins were processed according to WB-Method explained in the methods (pgs. 38-42, compare also Fig.11). Initially, the standard serum used in the ELISA was analysed with WB analysis. It was possible to differentiate all three surface proteins and define the mean relative distribution, which was about 10% for LHBs, about 20% for MHBs, and about 70% for SHBs, with small amounts of SHBs-dimers (Fig.13 and Fig.14B). A dilution series confirmed that the measured light intensity correlated highly with the concentrations of the different proteins, making it possible to quantify and calculate the relative distributions of all samples. A significant advantage of the WB analysis was detecting both glycosylated and non-glycosylated forms of the surface proteins (Figs. 14). The different light wavelengths highlight the LHBs fractions and enable explicit allocations, which helps identify actual LHBs and SHBs dimers (Fig. 14B), which are usually difficult to distinguish. In the standard serum (ID1) investigation, the dilution series showed definite limits for detecting the surface proteins, which was important for understanding all subsequent analyses (Figs. 14).

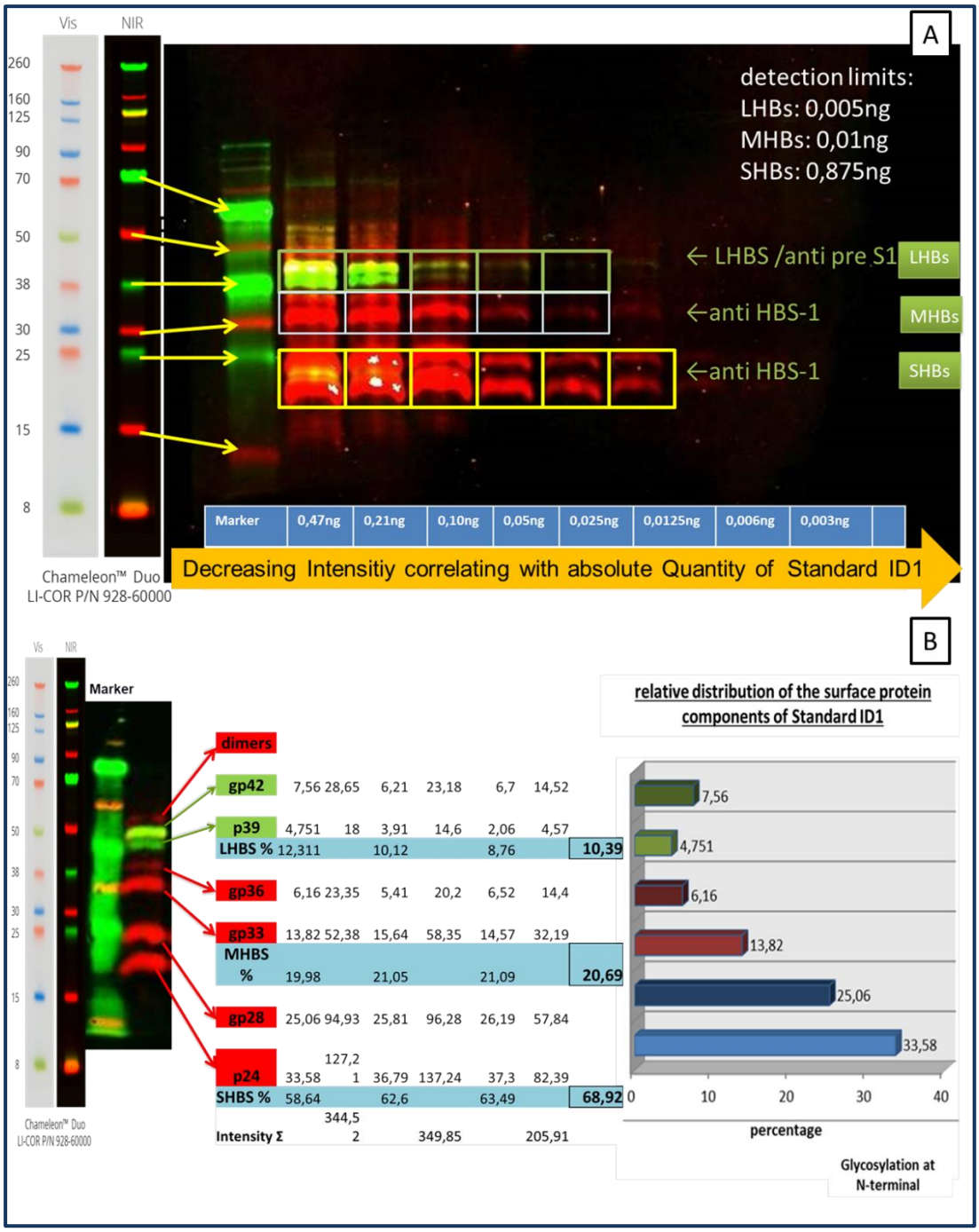


Figure 14 WB analysis: (A) Dilution, calibration, and correlation analysis of the standard HBV-serum ID1. The visible and measured intensities are directly proportional to the absolute protein quantity loaded into the WB. The WB allows detection of the HBV surface proteins as listed above: LHBs (Large Hepatitis B Virus surface protein); MHBs (Middle Hepatitis B Virus surface protein) and SHBs (Small Hepatitis B Virus surface protein). The standard serum ID1 was diluted as shown in the dilution table below the WB (absolute quantities in ng) that where transferred into the gel-pockets. With decreasing absolute viral surface-protein quantity, the immunofluorescence intensity decreased directly proportional until no more visible/detectable. The absolute quantities (in ng) of the different HBV surface proteins which were still detectable with the WB-Analysis are listed in the upper right corner (detection limits) concentrations**. (B) WB analysis of the relative distribution of the surface protein components.** Based on immunological markers targeting specific HBV antigens, the WB shows a specific pattern of the different viral protein fractions (left) of Standard serum ID1. The quantitative measurements of fluorescent light intensity allow the relative distribution to be detected, as shown in the bar chart (right)and showing all HBs components (glycosylated glycoprotein (gp) and protein weight [number]) and the calculation steps from measured intensity to relative distribution (calculation table with all intensity measurements and calculation in relative fractions of all proteins).

4.3 Method Validation and Western-Blot Analysis of Exemplary HBV-Cohort without Antiviral Treatment

The cooperation with Pfefferkorn et al. aimed to validate the modified and optimised serological methods used to detect, quantify, and analyse the HBV surface proteins. The focus was to compare the HBsAg compositions of an exemplary cohort representing different clinical phases of acute/chronic HBV infection. Pfefferkorn et al. investigated the three main surface proteins to differentiate possible biomarkers staging the various phases of the HBV infection.

Pfefferkorn et al. primarily used a commercial ELISA kit to quantify HBsAg. In contrast, this study detected LHBs and MHBs with specific antibodies designed in Gießen. The similar ELISA setups in Leipzig and this study made this an ideal setting for an inter-laboratory performance comparison. A small representative sample cohort of selected patient sera that differ in infection phases (Fig. 15) but had not been treated with antiviral drugs was run on both ELISA setups and then compared. The adjusted and modified WB method was used to analyse further aspects of the surface proteins and to validate the ELISA results. The methods provided ideal intersection points for validation and comparison.

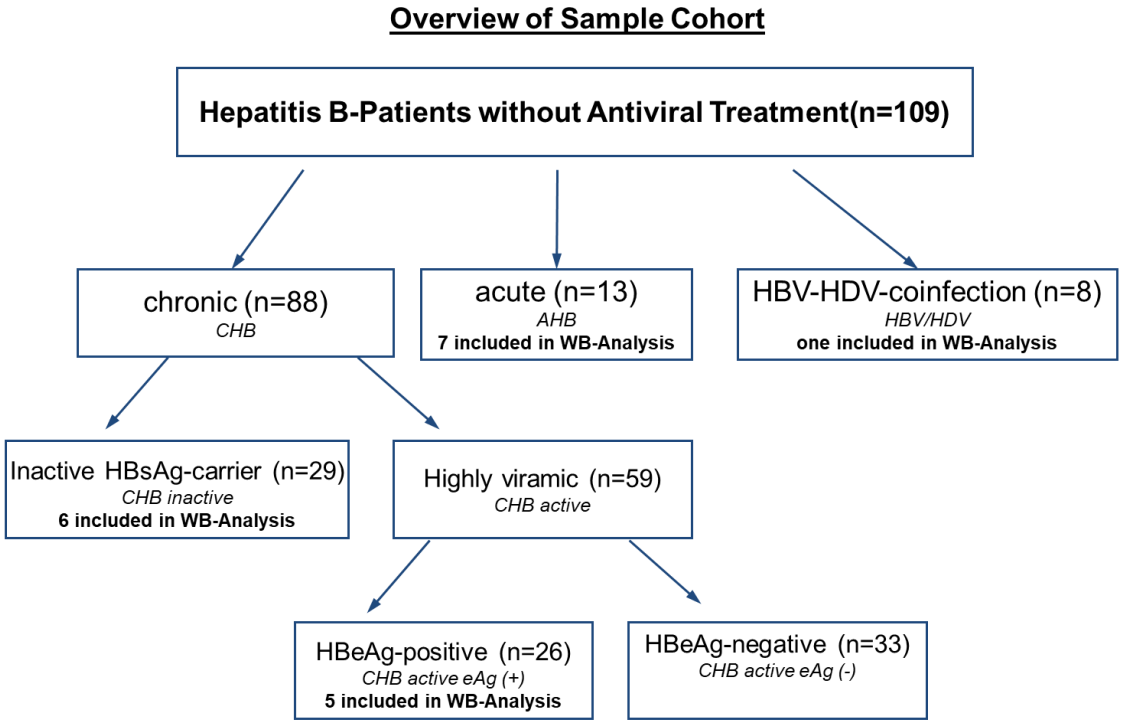


Figure 15 Overview of the sample cohort used for method validation.

WB Analysis of the Surface Proteins in Exemplary Samples of Different Infection Phases with a Focus on the Distribution Patterns

It was possible to confirm a stable relative distribution of the HBV surface proteins in acutely infected compared to chronically infected patients analysed in the main investigation of this thesis (Fig. 16). The HBsAg carriers differed but showed a similar trend. The WB analysis of acute phase samples indicated a relative mean LHB fraction of 7.86%, MHB fraction of 12.70%, and SHB fraction of 79.44% (Fig. 17). The distribution pattern implied probable stability. HBsAg carriers had, on average, a third of the amount of MHBs and half the amount of LHBs. SHBs relatively predominate. The relative distribution of the HBV surface proteins roughly defines three groups: acute infection, chronic (active) infection, and HBsAg carriers.

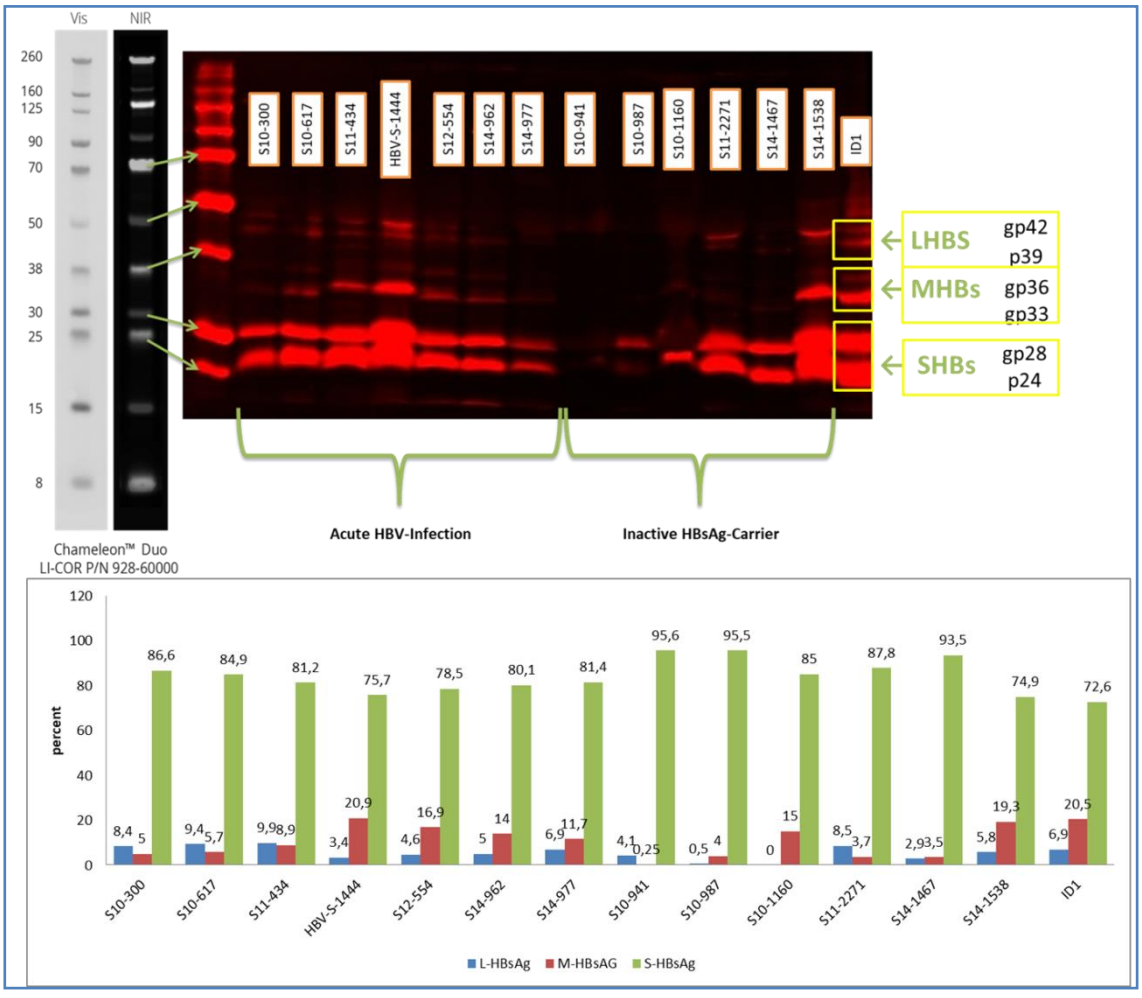


Figure 16 WB analysis and calculation of the relative proportions of the HBV surface proteins. Upper figure: WB of selected samples comparing all surface protein components (Large-; Middle- and Small Hepatitis B Virus proteins) (glycosylated at the N-terminus [glycoprotein] and protein weight [number]) in acute and inactive HBsAg carrier infections. **Lower figure:** Graph presenting calculated relative distributions (numbers above bars) of the HBV surface proteins (Large-; Middle- and Small Hepatitis B Virus proteins).

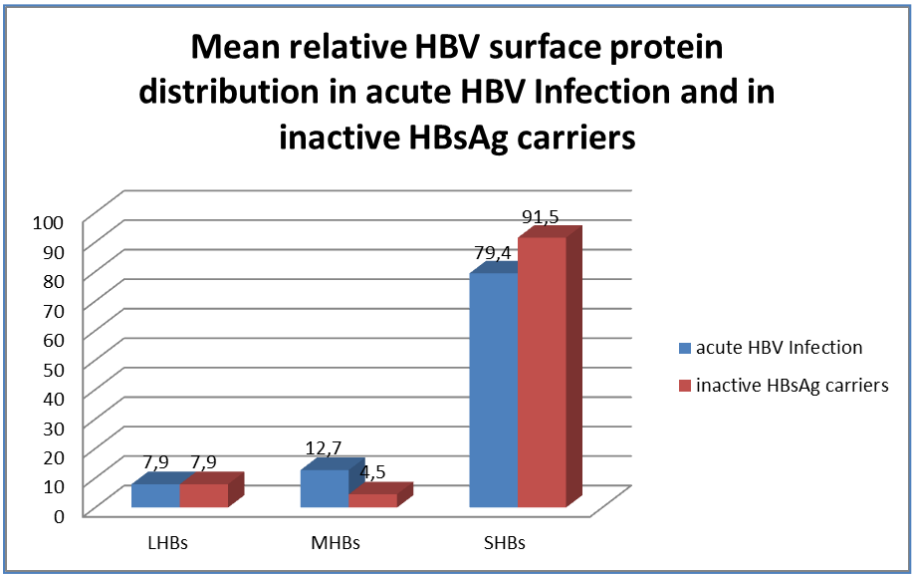


Figure 17 Mean relative surface protein distributions comparing acute HBV infections and inactive HBsAg carriers using analysed with the WB. The bar chart shows the three HBV surface proteins, LHBs, MHBs and SHBs (Large-; Middle- and Small Hepatitis B Virus surface proteins) and their mean relative quantity (number above bar).

HBsAg carriers are included in the chronic infection phases but do not fully represent the different phases. The primary investigation focuses on chronic HBeAg-negative infections. Therefore, representative samples from HBeAg-positive, HBeAg-negative (representing examples at baseline before antiviral treatment from the primary cohort of this thesis), and HBsAg-carrier samples were analysed by WB to understand possible distributional differences and to provide a better comparison with the ELISA results as part of the interlaboratory method validation. Within the group of chronically infected patients, there were different trends in the relative surface protein distributions. Inactive HBsAg carriers and HBeAg-positive samples appeared to have, on average, lower levels of MHBs and LHBs than HBeAg-negative samples. HBeAg-negative infections stand out with relatively high LHBs and MHBs levels (Fig. 18).

A likely stable, consistent pattern is that SHBs relatively predominate in all stages regardless of the infection phase; the variability lies in the distributions of LHBs and MHBs and implies their greater importance in distinguishing the different phases.

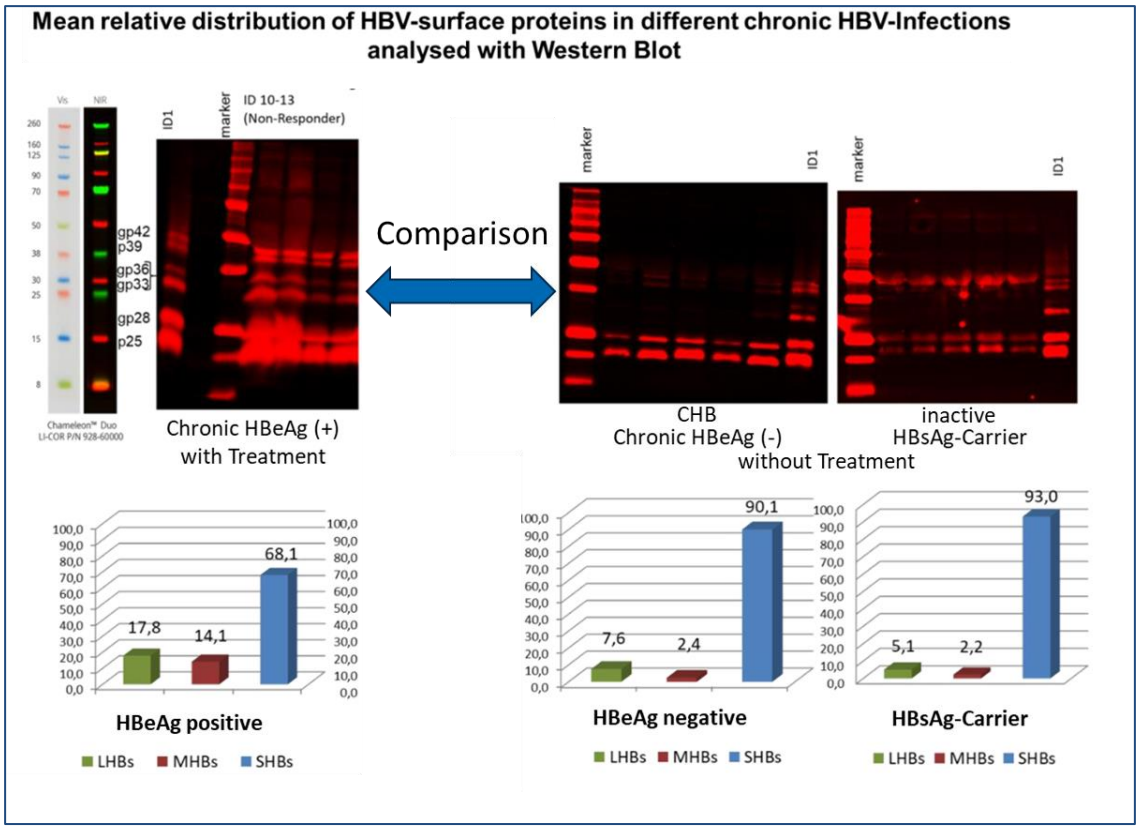


Figure 18 WB analysis of relative surface protein distribution in different HBV infections and comparison. The upper row shows WB-Analysis of three different phases of the HBV infection (left: a Non-Responder to antiviral Treatment from the Cohort with chronic HBeAg negative HBV infections, middle: non-treated chronic HBeAg positive HBV infection and right: non-treated HBsAg-Carrier). **The lower row** presents the equivalent bar charts with mean relative HBV surface protein quantities of the WB-Analysis above. LHBs, MHBs and SHBs (Large-; Middle- and Small Hepatitis B Virus proteins) can be compared directly due to the adjusted scales of the three bar charts)

4.4 Cohort and Samples of chronic Hepatitis B Virus Infected Patients with antiviral Treatment

This study analysed human serum samples derived from multi-centred, global, randomised phase III/IV studies (WV16240 [NCT00048945] and WV19432 [NCT00435825]). Lau et al. and Liaw et al. collected essential data and published their results (Liaw et al. 2011), contributing to the following investigations that analysed them retrospectively. It was confirmed at the time of the trials that their participants were CHB patients who were HBeAg negative and had elevated serum alanine aminotransferase (>1–10× upper limit of normal) and HBV DNA (>500 000 copies/mL or >100,000 IU/mL (van Bömmel et al. 2018, 2018)) levels (Liaw et al. 2011; Lau et al. 2005). The main excluded categories were decompensated liver disease, co-existing severe medical conditions, and coinfection with hepatitis C or D viruses or HIV. The

enrolled patients were randomised into groups treated with PegIFNa 180 µg/week with or without lamivudine for 48 weeks. Essential to the analysis of this thesis was choosing samples that could be assigned with baseline measurements and sequel sampling after 12, 24 and 36 weeks of treatment. Most test subjects included in this study are of Asian origin and young males (average age of 30 years). Their genotypes, treatments, and additional relevant examinations or characteristics are summarised in Table 1 in materials (p.34). An overview of all samples analysed with the in-house ELISA and Western Blot are shown in Figure 19.

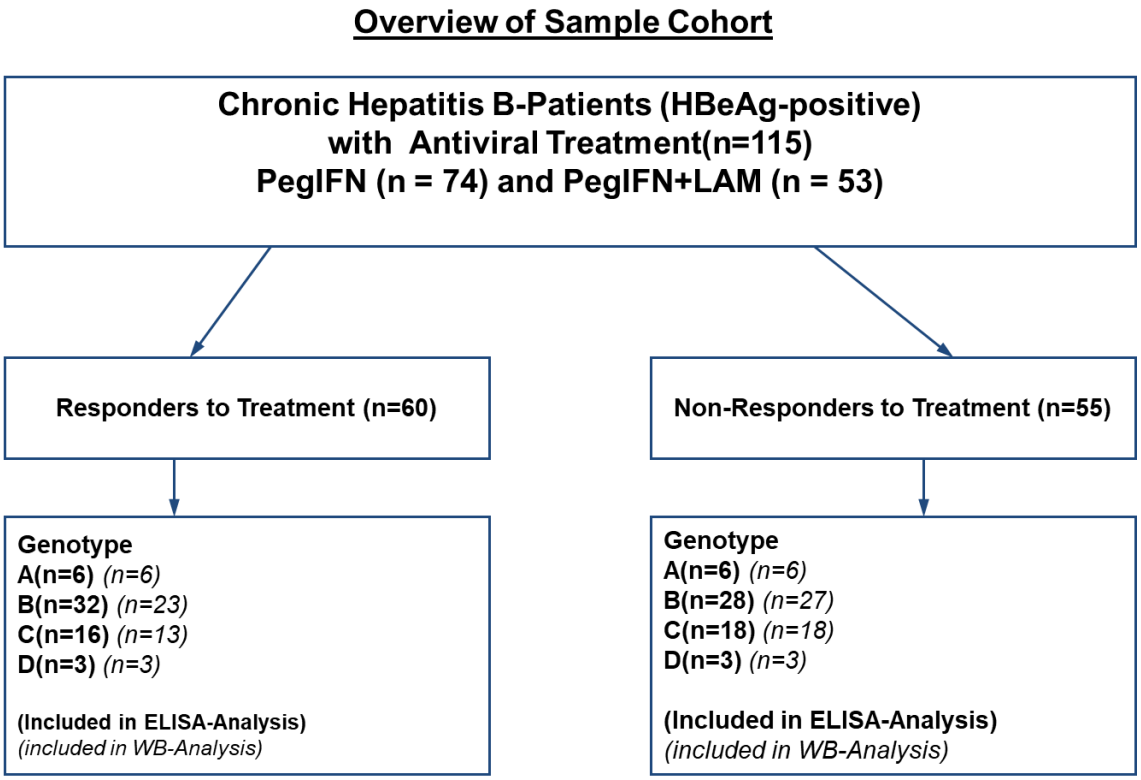


Figure 19 Overview of Sample Cohort of Chronic Hepatitis B-Patients with Antiviral Treatment (Response to treatment was defined as HBeAg seroconversion after 24 weeks of antiviral treatment)

4.5 Preparation of Data for Descriptive and Correlation Analysis

Out of the 115 samples, 112 could be processed to quantify the surface proteins via ELISA detection with the C20/2 (equivalent to total protein), Ma18/7 (LHBs), and Q19/10 (MHBs) antibodies.

Due to limited sample volume, 93 samples were left to be analysed by WB, aiming to investigate distributions; visualise specific patterns of all components of LHBs, MHBs and SHBs; and compare them with the surface proteins quantified by ELISA. The

proteins were calculated by recording the intensity of the fluorescent light emission measured by the Odyssey scanner.

A general overview of the descriptive statistic of the ELISA test is provided in Table 2 (see Appendix), and the results of the WB analysis are summarised in Table 3 (see Appendix). Furthermore, the tables indicate a non-normal distribution of the collected data related to skewness and kurtosis, which means that the raw data showed a non-linear and widly variable distribution. In order to meet the assumption, that the data assumes normality, all metrical variables were log-transformed. The log-transformation was necessary in order to use the date for all further statistical calculation. The log-transformed data is close-to-normal distributions, handling outliers and assuring linearity, which are essential assumptions for the statistical models used in this study. A summary of the descriptive statistics for the transformed data is provided in the Appendix Fig 1

4.6 Descriptive Overview of Distribution of the Quantification of LHBs, MHBs, SHBs, and their Glycosylation

The samples of chronically HBV-infected patients were pre-tested for HBsAg with a commercial ELISA (Architect HBsAg (Abbott; LLOQ 0.05 IU/mL) assay in Study WV16240 and the Elecsys® HBsAg II quant (Roche Diagnostics; LLOQ 0.05 IU/mL) assay in Study WV19432) The quantification results were included in the investigation and compared with the ELISA measurements obtained with the standardised ELISA at the Institute of Medical Virology at the University of Gießen. We first sought to understand how the surface proteins were distributed. As shown in Figure 20 the quantification of the HBV surface proteins measured in the patient sera reveal an expected high quantity of total HBs (mean 3,41ng/ml) in comparison to the LHBs- and MHBs- concentrations. There was in this sample cohort more LHBs (mean 2,48ng/ml) than MHBs (mean 1,79ng/ml).

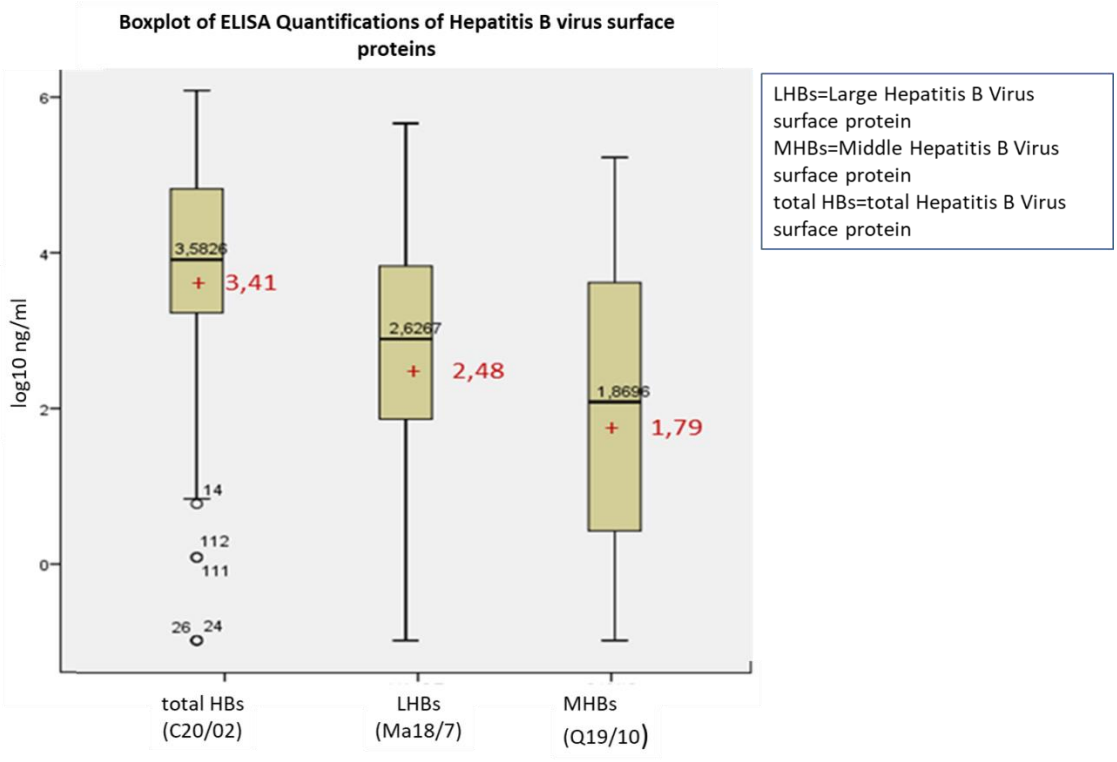


Figure 20 Boxplots of ELISA-based surface protein quantities. The lower (Q1) and upper (Q3) quartiles represent observations outside the 9–91 percentile range. The median (black bar) observation for ELISA-based surface protein quantities (log_{10}) is shown in black. Outlying data are plotted outside the Q1–Q3 range. The means are highlighted in red (+). The antibodies used to detect the HBV surface proteins are in brackets below.

After assessing the information given by the ELISA results, the next step was to examine all components of the HBV surface proteins, assuming that the WB analysis would visualise the separated proteins and reveal the N-glycosylated and non-N-glycosylated forms of LHBs, MHBs and SHBs. The measured fluorescence intensity could be used as an absolute and relative scale to describe the surface proteins, as shown in Figure 21.

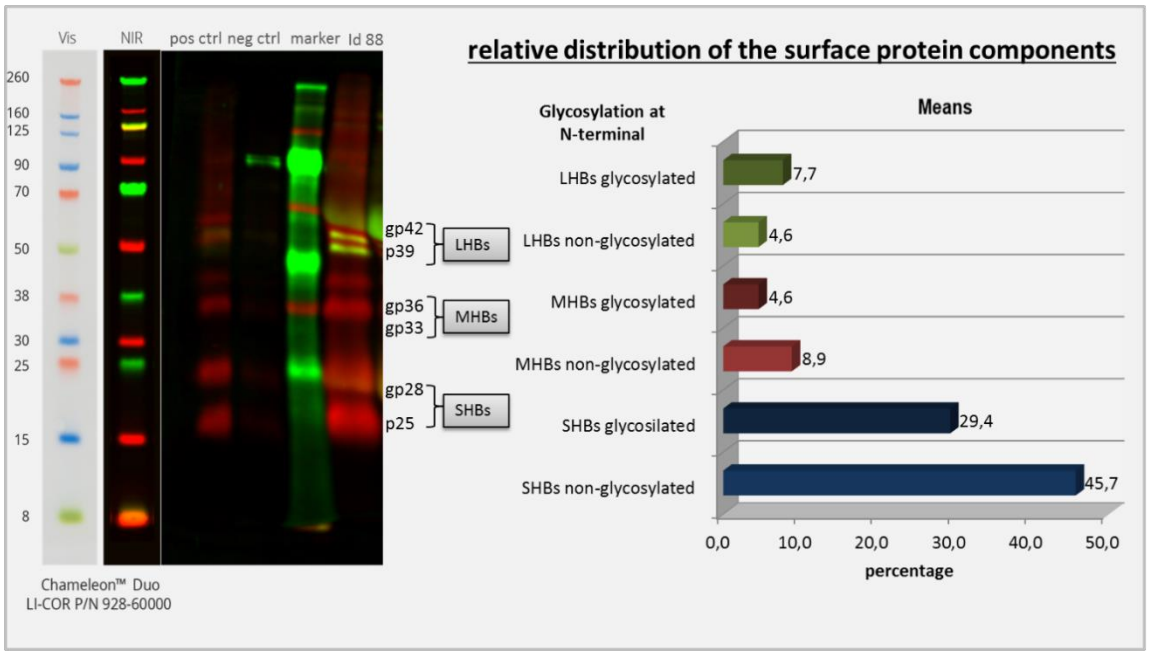


Figure 21 WB analysis of the relative distribution of the surface protein components. Based on immunological markers targeting specific HBV antigens, the WB shows a specific pattern of different viral protein fractions (here of the exemplary patient sample ID88). The three HBV surface proteins, LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein) can be detected with the WB analysis and present glycosylated and non-glycosylated protein fractions (gp(glycoprotein), p(protein) and their molecular weights (number)) (**left**). The quantitative measurement of fluorescent light intensity enables the relative distribution to be determined, as shown in the bar chart (**right**).

The consequential logic step was to compare the WB findings with the ELISA-based relative distribution of the proteins, particularly since the N-glycosylated fractions could not be distinguished entirely by ELISA. Therefore, LHBs and MHBs could be directly differentiated and counted. However, SHBs had to be calculated by subtracting LHBs and MHBs from the total C20/10 protein concentration. The similarities in the relative distribution of the surface proteins are visible in Figure 22, confirming average fractions of about 73% for SHBs, about 10% for MHBs, and about 17% for LHBs.

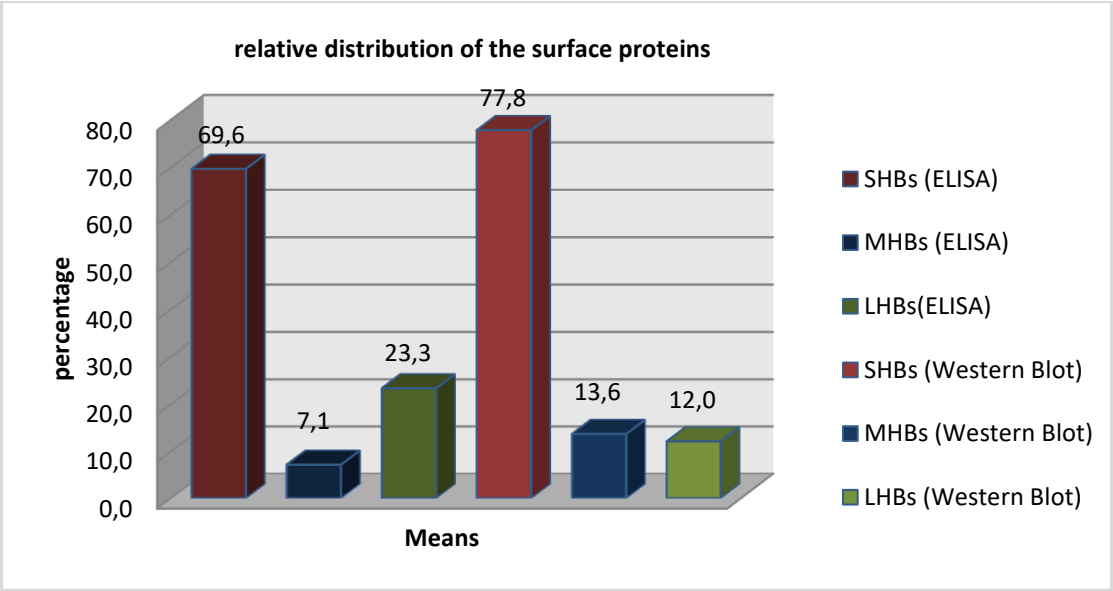


Figure 22 Bar chart comparing the mean relative distributions of the surface proteins measured by ELISA and Western Blot. (HBV surface proteins: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein))

A closer look at the absolute distribution of the surface proteins in Figure 23 provides additional information. Non-N-glycosylated MHBs can reach very high levels, resulting in a higher mean than LHBs and contrasting with the relative distribution. The N-glycosylated MHBs range from very low to high concentrations compared to the other proteins.

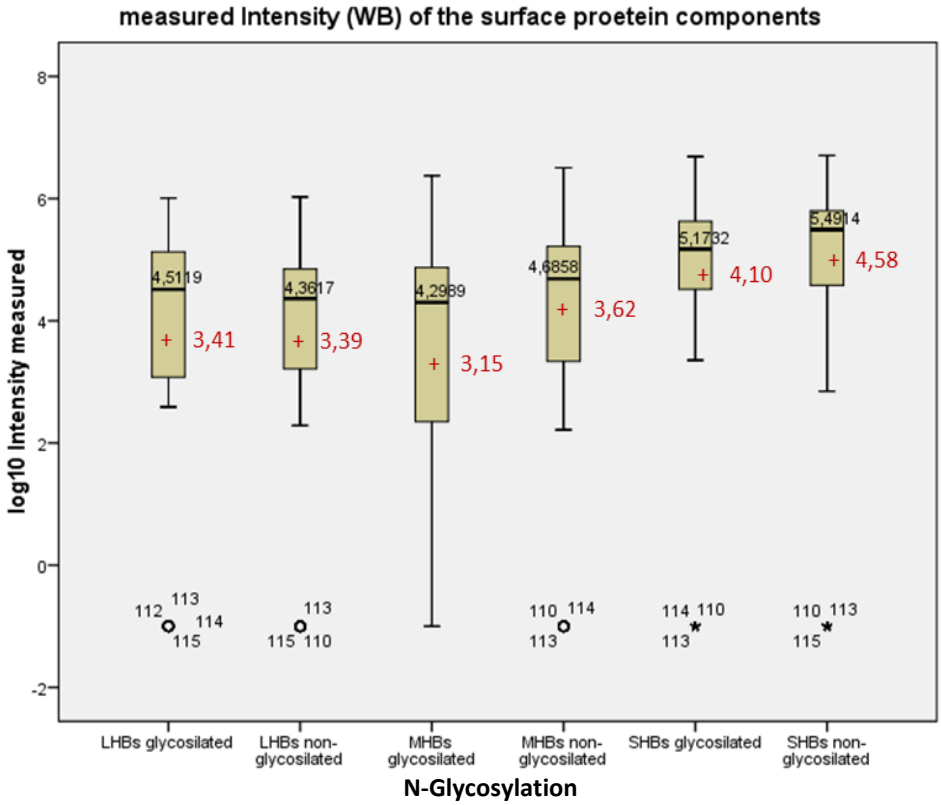


Figure 23 Boxplots of the measured intensity of the surface protein components. The intensities are $\log_{10}$ transformed. The lower (Q1) and upper (Q3) quartiles represent observations outside the 9–91 percentile range. The median observation for Western Blot-based surface protein intensities ($\log_{10}$) are shown in black. Outlying data are plotted outside the Q1–Q3 range. The means are highlighted in red (+). HBV surface proteins: LHBs (Large HBV surface protein) , MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein)

One of the leading questions was whether there were any correlations between the quantifications obtained by the ELISA measurements and WB analysis. Therefore, a Pearson correlation (r) was computed between them in SPSS. All correlations between the measured variables are listed in the Appendix. The strongest correlations (Cohen 1992 classification) were between the surface proteins examined in the WB, notably between glycosylated and non-glycosylated SHBs ($r_{(115)} = +0.989$, $p < 0.01$, two-tailed). Similarly, there was a very strong correlation between non-glycosylated MHBs and LHBs ($r_{(115)} = +0.984$, $p < 0.01$, two-tailed) and glycosylated MHBs and LHBs ($r_{(115)} = +0.951$, $p < 0.01$, two-tailed). Figure 6 shows the strong correlations. Regarding the proteins detected with MA18/7, Q19/10, C20/2 and the pre-quantified HBsAg with the commercial ELISA, the strongest correlation was between Ma18/7 and Q19/10 ($r_{(115)} = +0.857$, $p < 0.01$, two-tailed), closely followed by the correlation between Ma18/7 and C20/2 ($r_{(115)} = +0.853$, $p < 0.01$, two-tailed). Interestingly, there were few significant relationships with HBsAg quantifaction; the most notable was with Ma18/7 ($r_{(115)} = +0.594$, $p < 0.01$, two-tailed). In summary the quantification of HBsAg with the

commercial ELISA and the quantification with the in house ELISA and the results of the WB-Analysis correlated strongest with LHBs as indicated in Figures 24 and 25. The correlation analysis provides a significant linearity between the quantification of the surface proteins and their glycosylation but do not allow any conclusions or causality concerning prediction to response to therapy. Therefore, a binary regression analysis is needed as performed in the following chapter.

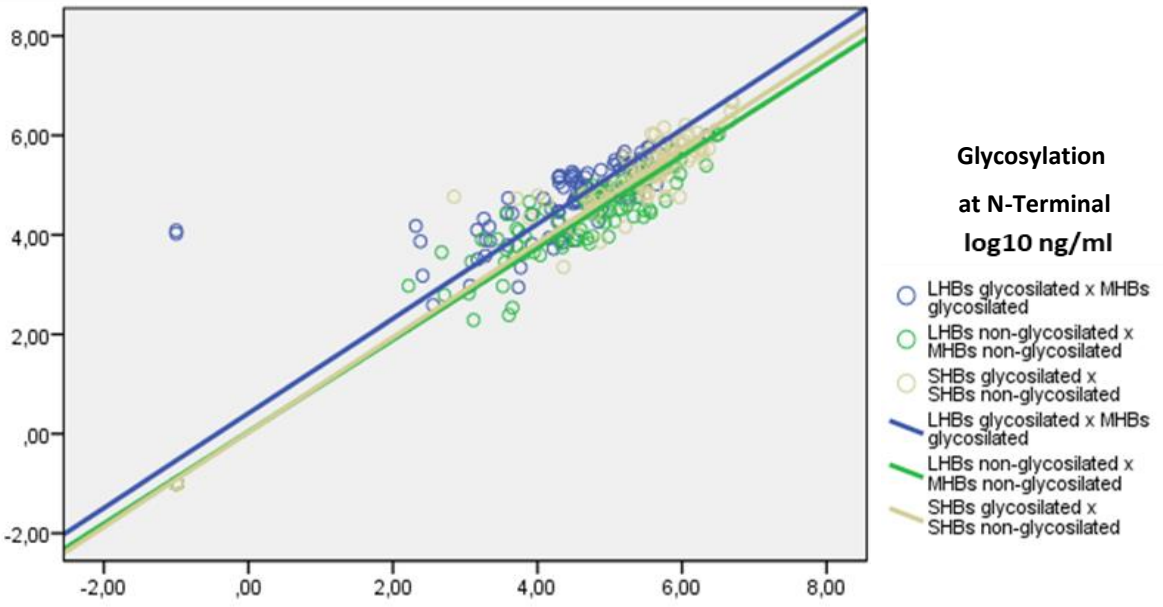


Figure 24 Line graphs of correlations between all HBV surface protein fractions detected by Western Blot. HBV surface proteins: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein)

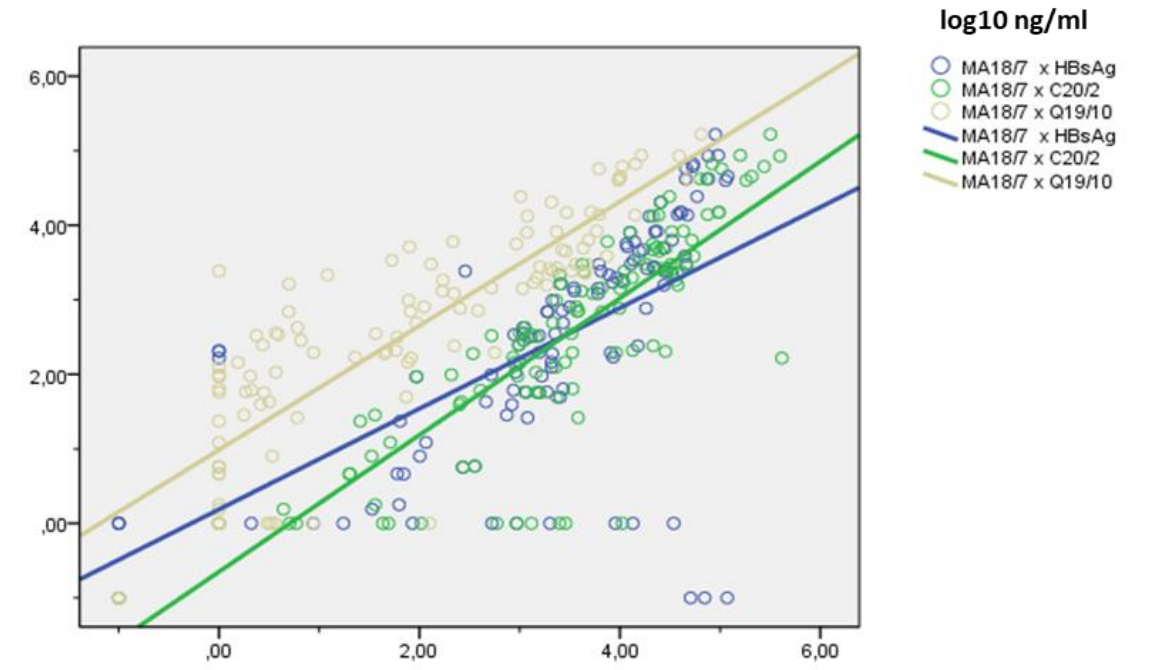


Figure 25 Line graphs of correlations between ELISA-quantified LHBs (Ma18/7), MHBs (Q19/10), total surface proteins (C20/02), and HBsAg. HBV surface proteins: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein)

A key attribute of an efficient biomarker is to mirror biochemical changes in the patient as signs of improvement or worsening in their health condition. In this case, responses to long-term treatment of chronic HBV carriers were categorised in the former study and defined by significant decreases in specific parameters (see the cohort description in Methods for more details). Before answering the core question of whether surface protein quantities can serve as suitable biomarkers, it is necessary to investigate other possible influences on the outcome of responding or failing to respond. Table 4 lists the samples included in this analysis and the frequencies of the two categories (response or non-response to treatment).

		Frequency	Per cent	Valid per cent	Cumulative per cent
Valid	Non-response	55	47.8	47.8	47.8
	Response	60	52.2	52.2	100.0
	Total	115	100.0	100.0	

Table 2 Frequencies of the response variable.

The graphs in Figures 26 and 27 show the variables, genotypes, and treatments grouped according to the response to treatment. The differences between responders and non-responders related to the treatment received and the HBV genotype initially appeared to be very small. A Chi-square test with Cramer's V coefficient was applied to the

categorised data, indicating non-significant associations with the outcome response for genotype ($\chi^2_{(3,N112)}$ = 3.49, p = 0.951, φ = 0.056) and treatment ($\chi^2_{(3,N115)}$ = 2.44, p = 0.786, φ = 0.146).

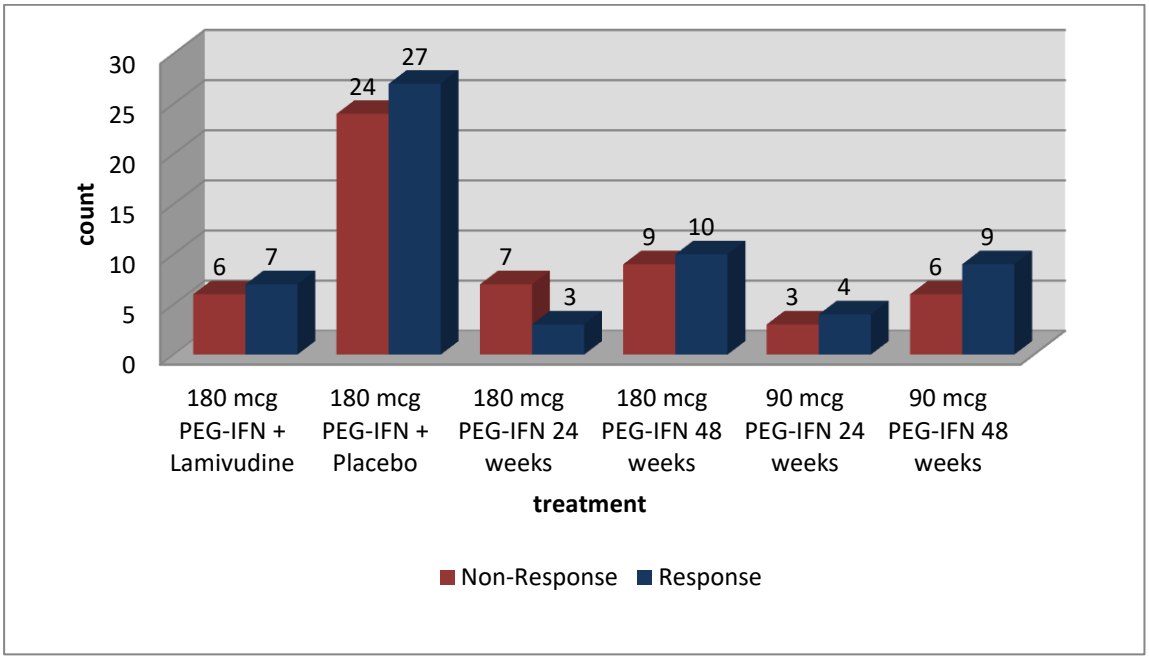


Figure 26 Distribution by the Response to PegIFNa (peginterferon alfa-2a) treatment combinations (PegIFNa combined with Lamividine, Placebo and monotherapy and 24 weeks or 48 weeks treatment)

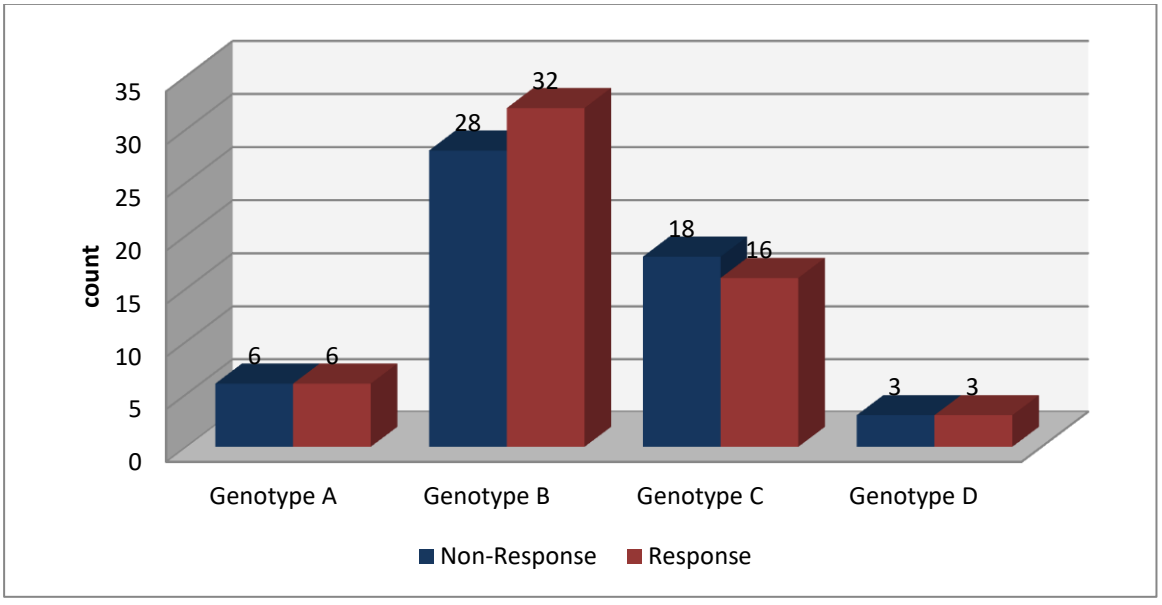


Figure 27 Genotype absolute counts of Response to PegIFNa treatment.

More relevantly, an association was found between the measured surface protein concentrations and response to treatment. All results are shown in Table 5 in descending association strength with the response to treatment. Therefore, HBsAg results in the

highest Eta and the non-N-glycosylated SHBs the weakest Eta which can be interpreted as highest correlation with response to treatment is associated with quantification of HBsAg with the commercial ELISA. The quantification of the surface proteins with in the in house ELISA come close but have a weaker correlation with response to treatment. The WB-analysis of all viral proteins are rather not very strong correlated with response to treatment.

Nominal by interval Response by protein	Dependent Surface protein (log_{10} ng/mL)	Eta (N = 115)
	HBsAg	0.584
	Q19/10	0.452
	Ma18/7	0.401
	C20/02	0.373
N-terminal glycosylation status	MHBs (glycosylated)	0.206
	LHBs (glycosylated)	0.206
	MHBs (non-glycosylated)	0.198
	LHBs (non-glycosylated)	0.193
	SHBs (glycosylated)	0.185
	SHBs (non-glycosylated)	

Table 3 **Cohen Eta analysis on interrelations between response and viral surface protein components.** HBV surface proteins: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein). According to Cohen (1988), the relationship is quite strong for all Eta ≥0.30 and ≥0.16.

In order to simplify and visualise the indicated correlations the results where reproduced by using box-plotts of all surface proteins and categorising them according to their outcome (Figs. 28 and 29). Regarding HBsAg quantification with the commercial ELISA, the most substantial effect was detected with non-N-glycosylated SHBs measured with the WB, with apparent differences in distribution scattering and the main centred weight of the protein concentration. The variance of the response and non-response medians was remarkable for Q19/10, while the difference was not as prominent for non-N-glycosylated SHBs. All together SHBs detected with the WB and LHBs quantified with the in-house ELISA come statistically closest to HBsAg quantified with the commercial ELISA but cannot outperform in effect and significance concerning the correlation with treatment response.

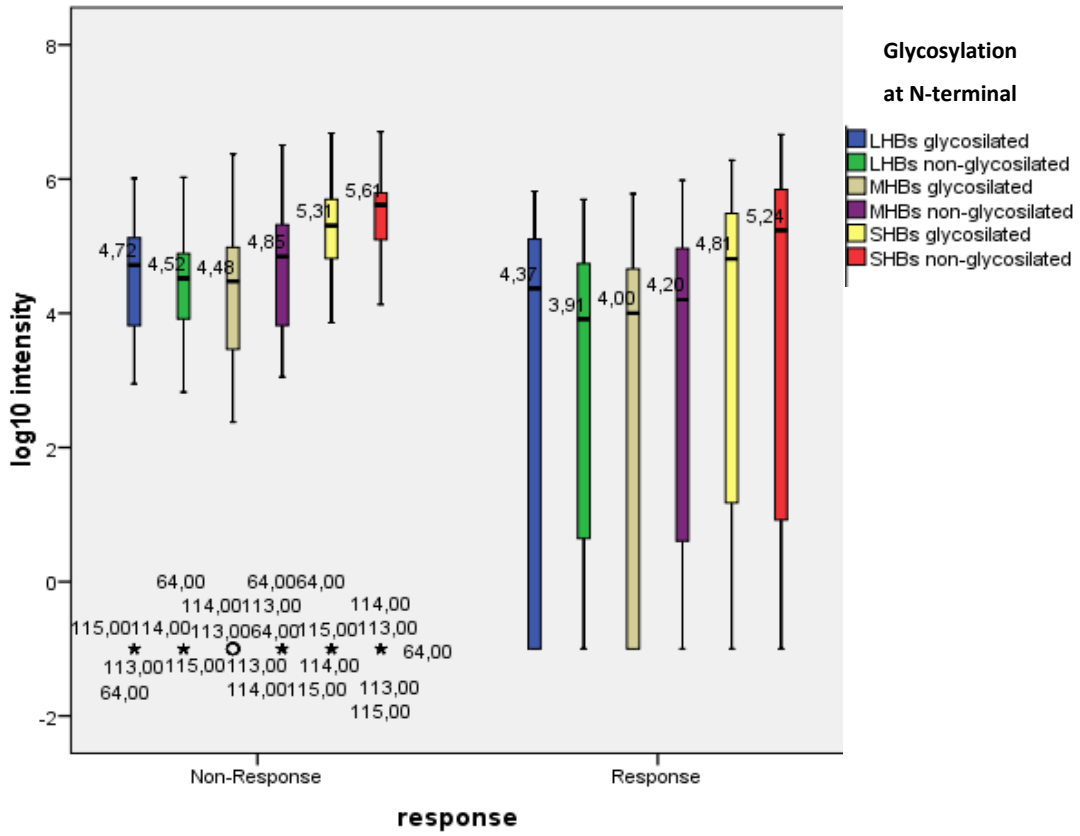


Figure 28 Boxplots of WB Quantities categorised by the Response to Treatment. The intensities are $\log_{10}$ transformed. The lower (Q1) and upper (Q3) quartiles represent observations outside the 9–91 percentile range. The median observation for Western Blot-based surface protein intensities ($\log_{10}$) are shown in black. Outlying data are plotted outside the Q1–Q3 range. The means are highlighted in red (+). HBV surface proteins: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein)

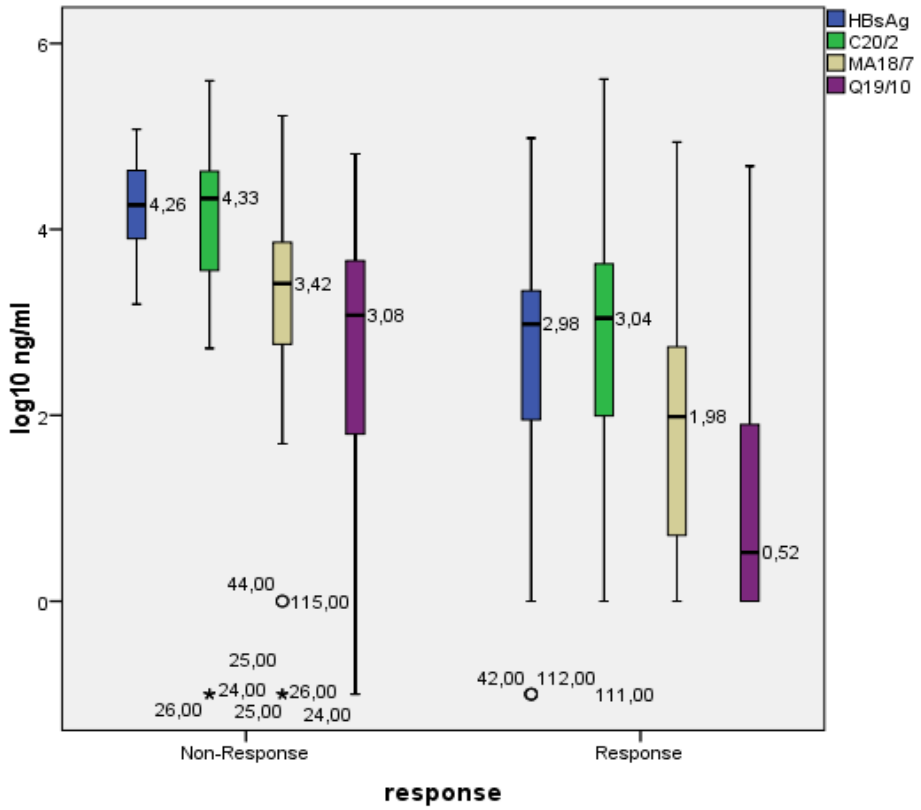


Figure 29 Boxplots of ELISA quantities categorised by the Response to pegINFa treatment. The quantities (ng/ml) are $\log_{10}$ transformed. The lower (Q1) and upper (Q3) quartiles represent observations outside the 9–91 percentile range. The median observation for ELISA-based surface protein intensities ($\log_{10}$) are shown in black. Outlying data are plotted outside the Q1–Q3 range. The means are presented with a black middle line **(-)**. HBV surface proteins: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein), the numbers marked with stars and circles are representing outliers and have no further statistical meaning.

The WB (Fig. 30) shows a similar visual characteristic between response and non-response. In this case, the contrast between Id52 (a responder sample) and Id18 (a non-responder sample) is outstanding, confirmed by the bar chart in Figure 30.

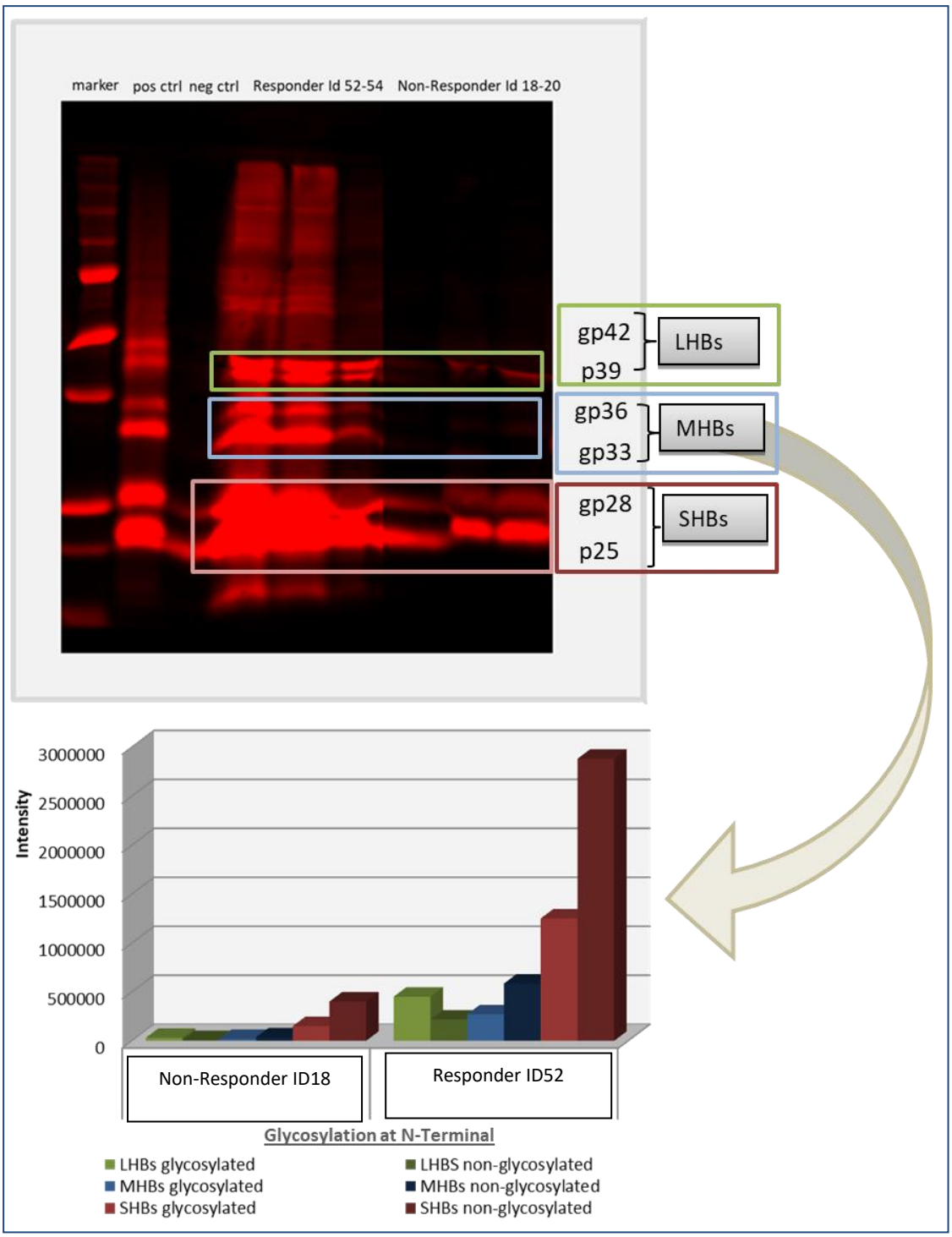


Figure 30 Western Blot quantities (measured Intensities) categorised by the Response to PegIFNa Treatment. Viral surface protein fractions were visualised by WB (upper) and analysed after fluorescence intensity quantification (absolute count dimensionless). The graph (below) shows the difference in counts of N-glycosylated and non-N-glycosylated surface protein components between responders (patient sample ID52) and non-responders (patient sample ID18). HBV surface proteins: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein) and their glycoproteins (gp) (molecular weight [number]).

4.6.1 Analysis of the Sampling Time in Aspect of Predictability of Response to Treatment

Blood was collected from each patient at two-week intervals throughout the 36 to 48 weeks of antiviral treatment and before treatment initiation. The crucial question was whether sampling time potentially statistically impacted the quantification of surface proteins or whether any changes in quantity and distribution could be observed. The blood sampling times were almost equally distributed in all groups, as shown in the bar chart in Figure 31.

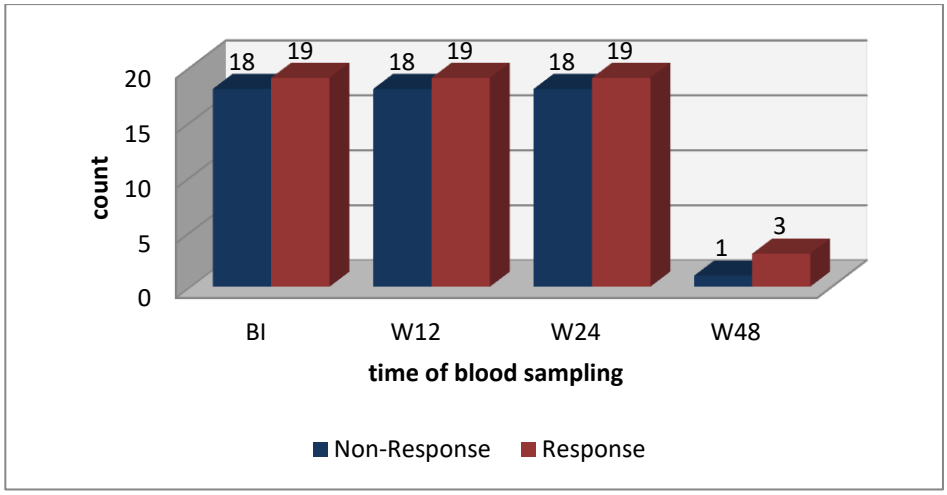


Figure 31 Counts of response to pegINFa treatment with respect to sampling time. All patients were treated with pegINFa and where sampled at baseline (BI), after 12 weeks (W12), 24 weeks (W24) and 48 weeks (W48). The graph shows patients that responded to the treatment (red) are compared to no response to treatment (blue). Response was defined as HBeAg sero-clearance after 24 weeks of pegINFa treatment**.**

A Spearman's rank correlation coefficient (r_s) was calculated to assess any association between quantification of surface proteins and blood sampling time. All analysed surface proteins showed a significant moderate negative (mono-linear) correlation with blood sampling time: HBsAg ($r_{s(115)} = -0.368$, $p < 0.001$), C20/2 ($r_{s(115)} = -0.343$, $p < 0.001$), Ma18/7 ($r_{s(115)} = -0.278$, $p = 0.003$), and Q19/10 ($r_{s(115)} = -0.288$, $p = 0.002$). The boxplots in Figure 32 summarise the results of the descriptive statistics and show the general tendencies for each time point. In summary there were apparent differences in the average concentrations of the surface proteins across the four time points and especially already in week 12 we can observe a significant drop of HBsAg concentration (measured with commercial ELISA) and LHBs (quantified with the in-house ELISA) (Fig. 32). In Fig.33 one can see that sera from patients that responded to antiviral treatment disrespectable of sample timing have significant lower surface protein levels than those of Non-Responder.

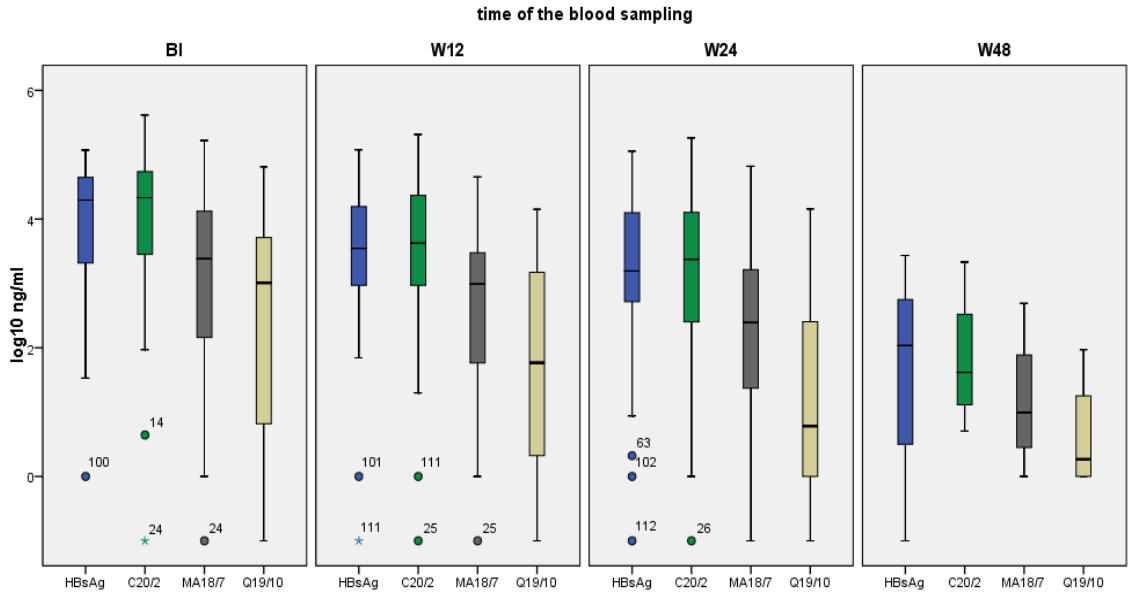


Figure 32 Boxplots of ELISA-based HBV surface protein quantities by sampling time. The quantities (ng/ml) are $\log_{10}$ transformed. The lower (Q1) and upper (Q3) quartiles represent observations outside the 9–91 percentile range. The median observation for ELISA-based surface protein intensities ($\log_{10}$) are shown in black. Outlying data are plotted outside the Q1–Q3 range. The means are presented with a black middle line **(-)**. All patients were treated with pegINFa and where sampled at baseline (BI), after 12 weeks (W12), 24 weeks (W24) and 48 weeks (W48). HBV surface proteins: detection antibodies -> Ma18/7= LHBs (Large HBV surface protein), Q19/10= MHBs (Middle HBV surface protein) and C20/02= total HBV surface protein

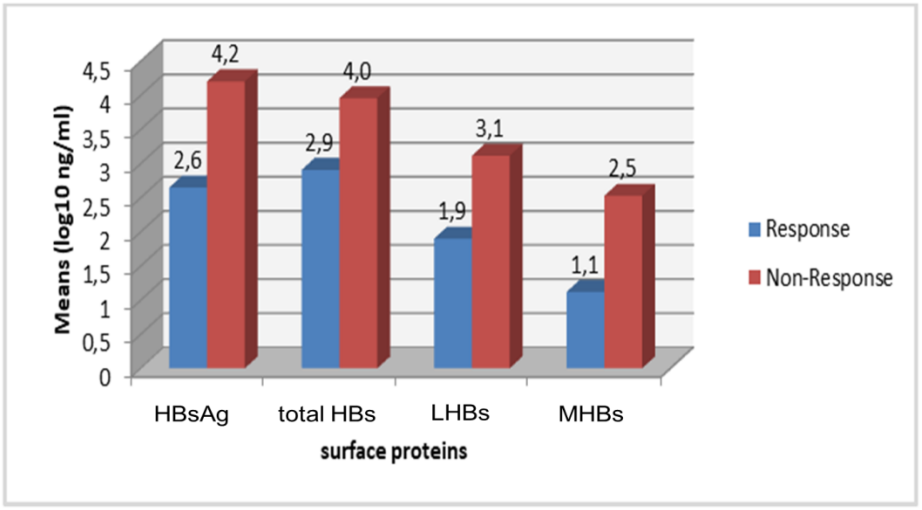


Figure 33 Mean quantities of surface proteins measured by ELISA grouped by response to antiviral Treatment. Response defined by HBeAg sero-clearence after 24 weeks of treatment. HBV surface proteins: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and total HBs (total HBV surface proteins)

4.6.2 The Genotype Parameter as possible important influencial Variable

Due to pre-genotyping, another fundamental variable could be included in this study and serve as an important parameter with a possible effect on the quality of a predictive biomarker. While the previous correlation analysis on response and genotype did not identify any associations, the core question remains about possible variations in the surface antigens and their distributions for individual genotypes. Moreover, it is

interesting to obtain further insight into patterns that may enable further specifications and characterisations and could explain interactions or manifestations of the virus in chronically infected individuals. The frequencies of the included genotypes are listed in Table 6.

Genotype

		Frequency	Per cent	Valid per cent	Cumulative per cent
Valid	Genotype A	12	10.4	10.7	10.7
	Genotype B	60	52.2	53.6	64.3
	Genotype C	34	29.6	30.4	94.6
	Genotype D	6	5.2	5.4	100.0
	Total	112	97.4	100.0	
Missing	−1	3	2.6		
Total		115	100.0		

Table 4 Frequency of Genotype included in the cohort

As expected, SHBs levels were relatively dominant for all genotypes (Fig. 34). Genotype B differs because there were more LHBs than MHBs. In addition, Genotype A stands out with a high percentage of MHBs. Unlike the other genotypes, genotypes C and D show relatively low percentages of LHBs.

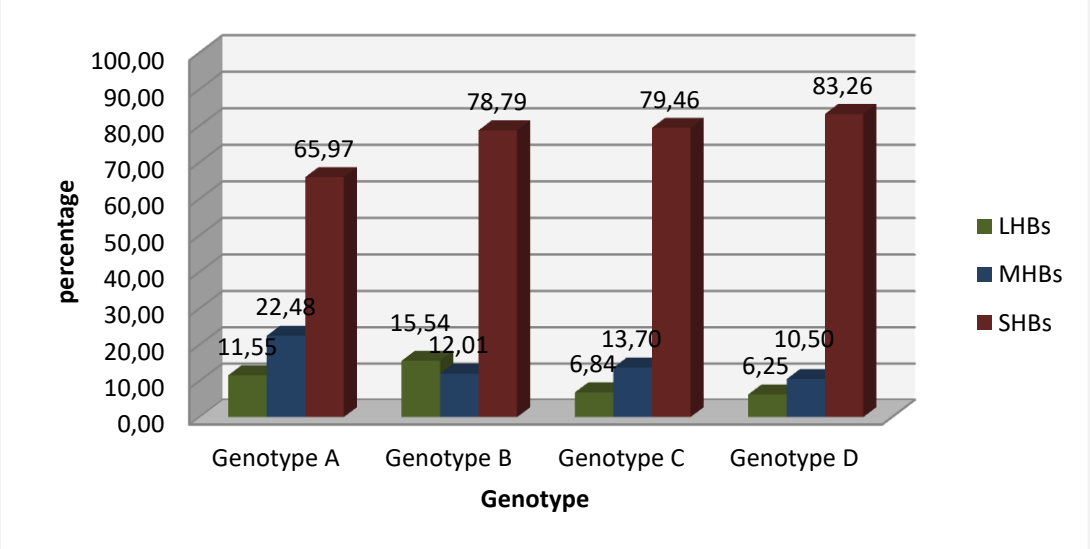


Figure 34 Relative distribution of HBV surface proteins in the different Genotypes under pegInterferon treatment. HBV surface proteins shown: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and total HBs (total HBV surface proteins)

A closer look at the separate components of the virus surface antigens shows that there appears to be a stringent pattern in their relative distribution across all genotypes. Therefore, the bar charts in Figure 35 indicate relatively more N-glycosylated than non-N-glycosylated SHBs and MHBs. However, LHBs showed the opposite pattern. The results are transferable to individual cases, as shown in Figure 36.

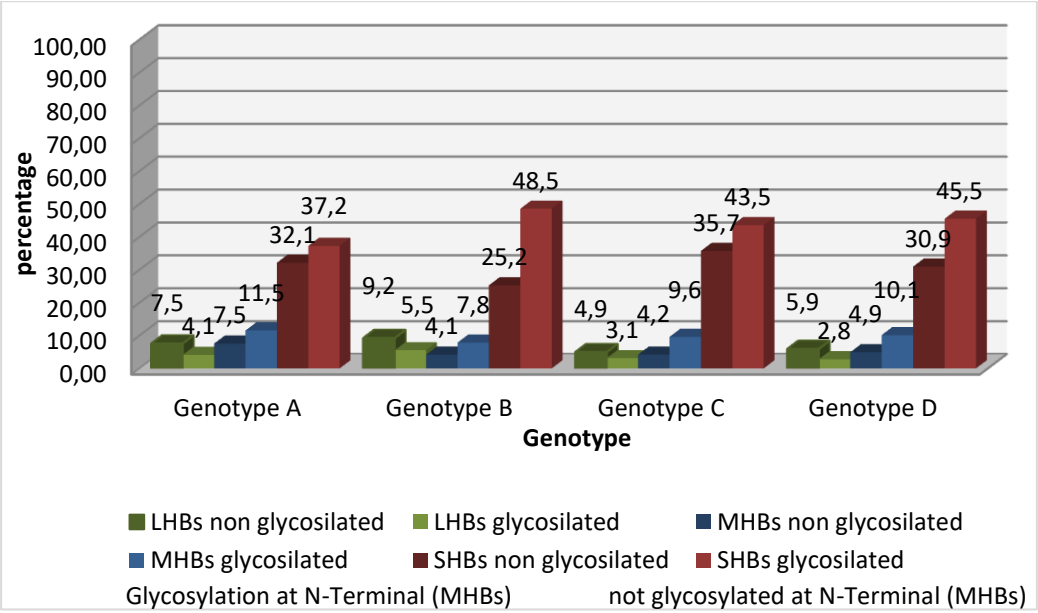


Figure 35 Mean Relative distributions of all WB-based HBV surface protein quantities and their different glycoproteins by genotype. Samples from patient cohort with antiviral treatment. HBV surface proteins shown: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and total HBs (total HBV surface proteins).

An Eta coefficient test was conducted for the association between the log-transformed surface protein concentrations and the genotype variable Table 7 lists all Etas in descending order showing that MHBs and LHBs quantified with the in house ELISA (Q19/10 and Ma18/7) correlates most with the variable genotype. In other words, LHBs levels vary significantly in the different genotypes. The ELISA quantification of LHBs and MHB can vary according genotype and as the graphs in Fig.35 shows, are for example relatively higher in Genotype A compared to Genotype D.

Directional Measures

Nominal by Interval Genotype by Protein	Dependent surface protein (ng/ml)	Eta Value (N=112)	Dependent surface protein (ng/ml)	Eta Value (N=112)
	LHBs glycosylated	.310	Q19/10	.389
	MHBs glycosylated	.291	Ma18/7	.388
	LHBs non-glycosylated	.274	C20/2	.358
	MHBs non-glycosylated	.250		
	HbsAg	.233		
	SHBs glycosylated	.218		
	SHBs non-glycosylated			

Table 5 Eta coefficient test for the association between the log-transformed surface protein variables and the genotype variable. According to Cohen (1988), the relationship is quite strong for all Eta ≥0.30 and ≥0.16.

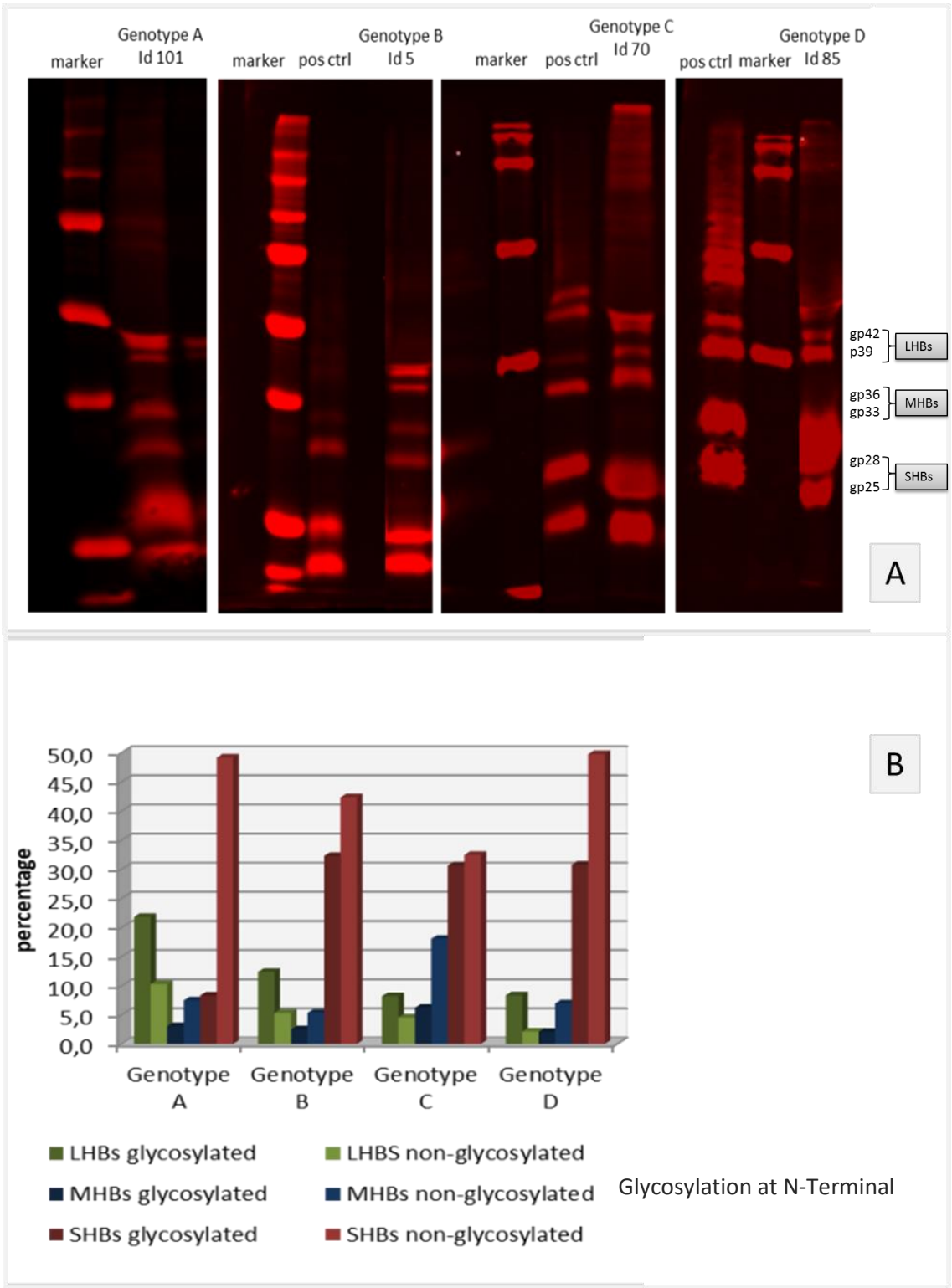


Figure 36 WB analysis of the relative distributions of HBV protein components by genotype. HBV surface proteins: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein) and their glycoproteins (gp) (molecular weight [number]). (A) Representative examples of WB-Analysis HBV surface proteins of pegINFa treated patients for each genotype in the cohort. (B) Bar charts of relative fractions for all viral surface protein components reveal a consistent pattern across all genotypes.

4.7 Mixed ANOVA with Repeated Measures

Descriptive statistics allow for a general assessment of associations and correlations between all variables, simplifying the understanding of more detailed and complex analyses. Therefore, a mixed ANOVA with repeated measures was used to compare total HBs (C20/02), LHBs (Ma18/7), and MHBs (Q19/10) quantities measured with the in-house ELISA (within-subject factors) with blood sampling time and response to treatment (between-subject factors). The main aim was to detect an interaction of effects and examine the main effects, focusing on the directional causality of the influence between the chosen variables mentioned.

Probes were collected respectively during the 12-week interval, categorised according to their response to treatment, and measured with the in-house ELISA. Table 8 lists the frequencies of the included categorised variables. Due to the low sample count in week 48 and the weak universal representability, that data will be excluded from further descriptions and figures.

Between-subject factors

		Label	*N*
Blood sampling time	1	BI	37
	2	W12	37
	3	W24	37
	4	W48	4
Response	1	Non-response	55
	2	Response	60

Table 6. Overview of sample proportions for response and blood sampling time after PegIFNa treatment

In summary, the essential information given by the descriptive analysis suggests higher surface protein concentrations in responders to treatment than in non-responders (Fig. 37).

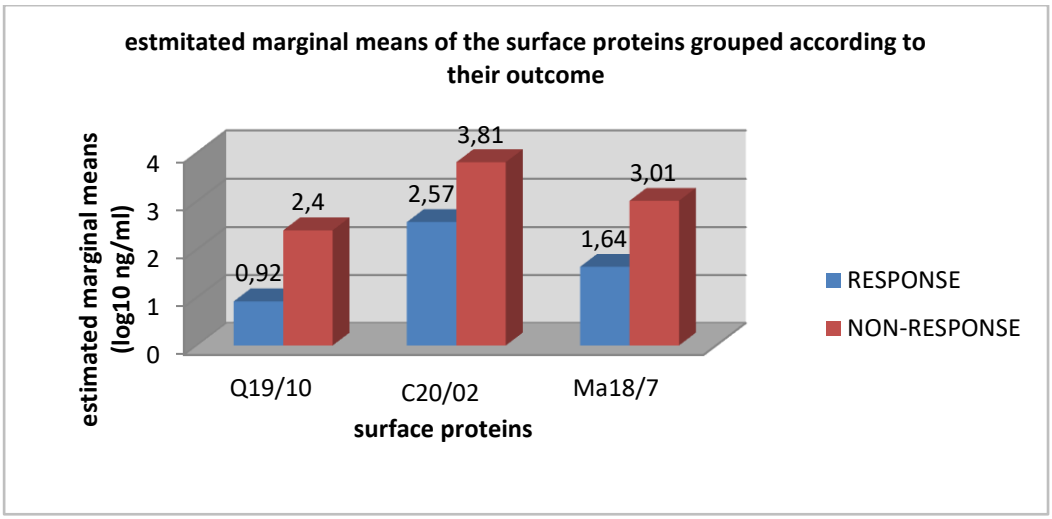


Figure 37 Estimated marginal means of ELISA-based surface proteins quantities categorised by the response to treatment. HBV surface proteins quantified with ELISA: Ma18/7= LHBs (Large HBV surface protein), Q19/10= MHBs (Middle HBV surface protein) and C20/02= total HBs (total HBV surface proteins)

The detailed descriptive statistics of the ANOVA analysis are listed in the Appendix. Its observations are reflected in Figure 38, which shows a WB analysis comparing a responding and a non-responding patient serum series.

All assumptions were met, meaning the residuals were tested on the normal distribution (the comparison test is in the Appendix). Outliers and linearity were addressed through a logarithmic transformation of the interval-scaled data. There was no violation of the homogeneity of the error variances, as assessed by Levene's test ($p > 0.05$). Box's test ($p = 0.341$) confirmed the homogeneity of covariance. Mauchly's test of sphericity ($p = 0.471$) was calculated, with the Greenhouse–Geisser adjustment used to correct for sphericity violations. There was no significant interaction between the surface proteins, time, and response (Greenhouse–Geisser $F_{(5.92,\ 211.025)} = 0.484$, $p = 0.82$, partial $\eta^2 = 0.013$). However, the main effect for the surface proteins was significant (Greenhouse–Geisser $F_{(1.97,\ 76.526)} = 63.71$, $p < 0.001$, partial $\eta^2 = 0.52$). The categorised variables showed significance for their main effects, implying differences in sampling times and response-related causality. The main effect for time (Greenhouse–Geisser $F_{(3,\ 495.269)} = 3.82$, $p = 0.012$, partial $\eta^2 = 0.097$) and response (Greenhouse–Geisser $F_{(1,\ 495.269)} = 11.63$, $p = 0.001$, partial $\eta^2 = 0.098$).

Bonferroni-adjusted posthoc analysis was performed on time, showing a significant ($p = 0.014$) difference between week 24 and the other sampling times (0.90, 95% confidence

interval: 1.34–47.7). The line graphs in Figure 39 show the results and highlight the contrasts.

To sum up the core findings of the statistical analysis, it is possible to state, that the ANOVA analysis (which analysis any causalities between variables) failed to make clear statements (no significance) on the predictability of the response to treatment based on C20/02 (total Hbs), Ma18/7 (LHBs) and Q19/10 (MHBs) quantities measured with the in house ELISA. The ANOVA analysis could provide a significant statistical effect concerning timing of blood sampling at week 12 for possible response to treatment. Figs. 38 and 39 give a graphic overview of significant surface protein level trends and differences in responder and non-responder to antiviral treatment. Responder tend to drop with the surface protein levels earlier (mostly week 12) and decrease in concentration throughout the entire 48 weeks. In comparison the surface protein level dynamic in non-responders is rather late or only insignificant in decrease of surface protein concentrations.

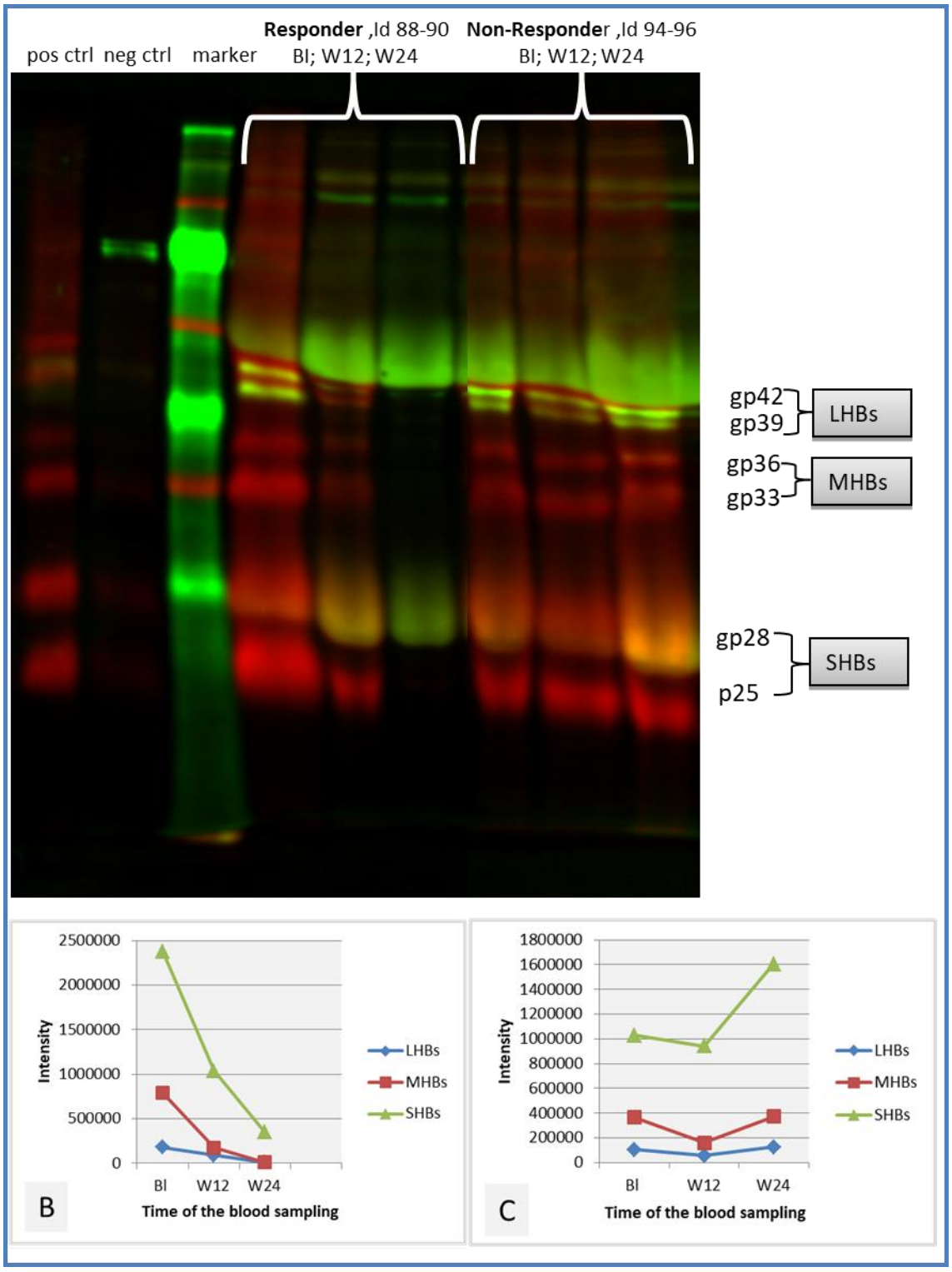


Figure 38 WB analysis of a Responder and a Non-Responder to treatment with PegIFNa grouped by blood sampling time. All patients were treated with pegINFa and where sampled at baseline (BI), after 12 weeks (W12), 24 weeks (W24) and 48 weeks (W48). HBV surface proteins: LHBs (Large HBV surface protein), MHBs (Middle HBV surface protein) and SHBs (Small HBV surface protein). (A) WB quantification and presentation of viral protein components (gp = glycoprotein; p= protein). Graphs of intensity measurements for the presented WB at the three sampling times for the Responder (ID88-90) (B) and Non-responder (ID94-96) (C).

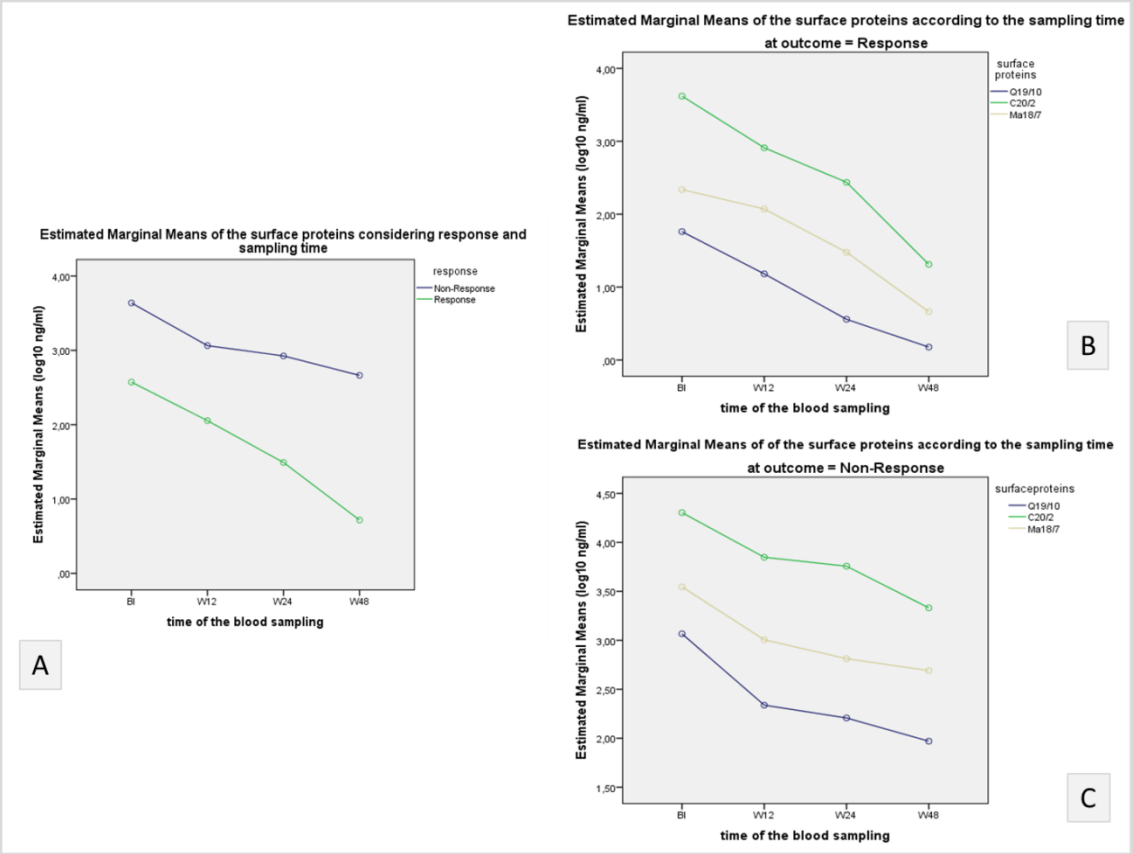


Figure 39 Mixed ANOVA estimated marginal means of the ELISA quantities of LHBs (MA18/7), MHBs (Q19/10), and total HBs (C20/2) by sampling time of pegInterferon treated patients. (A) Estimated marginal means in responders and non-responders show significant main effects by sampling time, implying differences in the sampling times and response-related causality. Time variable analyses in (B) responders and (C) non-responders show a significant difference in week 24 compared to the other sampling times.

4.8 Binary Logistic Regression

Furthermore, it is interesting to validate the performance of the surface proteins in the combination of influential parameters. Therefore, a binary logistic regression was performed to ascertain the effects of in house ELISA-based LHBs (Ma18/7), MHBs (Q19/10), and HBsAg quantities measured with a commercial ELISA, the measured protein components (including glycoproteins), sampling time, and genotype on the likelihood that a patient is a responder to pegINFa-treatment. (Compare in Table 9, all cases are identified and included in the analysis)

Case Processing Summary

Unweighted Cases		*N*	Per cent
Selected cases	Included in the analysis	112	97.4
	Missing cases	3	2.6
	Total	115	100.0
Unselected cases		0	0
Total		115	100.0

Table 7. Overview of the cases (samples) selected for mixed binary logistic regression.

The statistic model was considered significant ($\chi^2_{(15)}$ = 85.373, p < 0.0001) and explained 71.1% (Nagelkerke R^2) of the variance in the response to treatment. Table 10 shows that 88.4% of all cases were correctly classified.

		Predicted		
		Response		
Observed		Non-response	Response	Per cent correct
Response	Non-response	49	6	89,1
	Response	7	50	87,7
Overall per cent				88,4

Table 8. The percentage of correctly classified cases included in the mixed ANOVA analysis.

HBsAg measured with a commercial ELISA (Wald = 14.150, p < 0.001), genotype B (Wald = 4.446, p = 0.035), and sampling time at week 12 (Wald = 8.792, p = 0.003) were significant predictors of response to treatment. Consequently, the following formula was used to calculate the likelihood of the outcome response:

$$P(\text{Response}=1) = \frac{1}{1+e^{-(-23.689+(-5.404)*\log 10\ \text{HBsAg}+(-3.564)*\text{Genotype B}+(-3.432)*\text{week 12})}}$$

In conclusion, if all other parameters are kept constant, an increase of one unit in HBsAg quantity results in a 95% increase in the relative probability that the patient is a responder. Genotype B is almost three times more likely to be related to response than genotype C. The difference between genotype A was nearly negligible. However, the influence on the outcome did not pass the significance test in this setting. Interestingly,

sampling after 12 weeks of treatment was a strong predictor, contrary to the expectation that a later point would contribute more to the response probability. All results are compiled in the graphs in Figures 40 and 41, which highlight the differences and insights gained through logistic regression.

In short summary the binary regression is able to calculate the probability of a variable to predict response to antiviral treatment (Figures 40 and 41). HBsAg quantification with the commercial ELISA remains the strongest biomarker in order to predict response and combined with the sampling time at week 12 improves the strength of prediction to treatment. LHBs levels quantified with the in house ELISA come close in predicting treatment outcome but is not as significant as the already established HBsAg-ELISA.

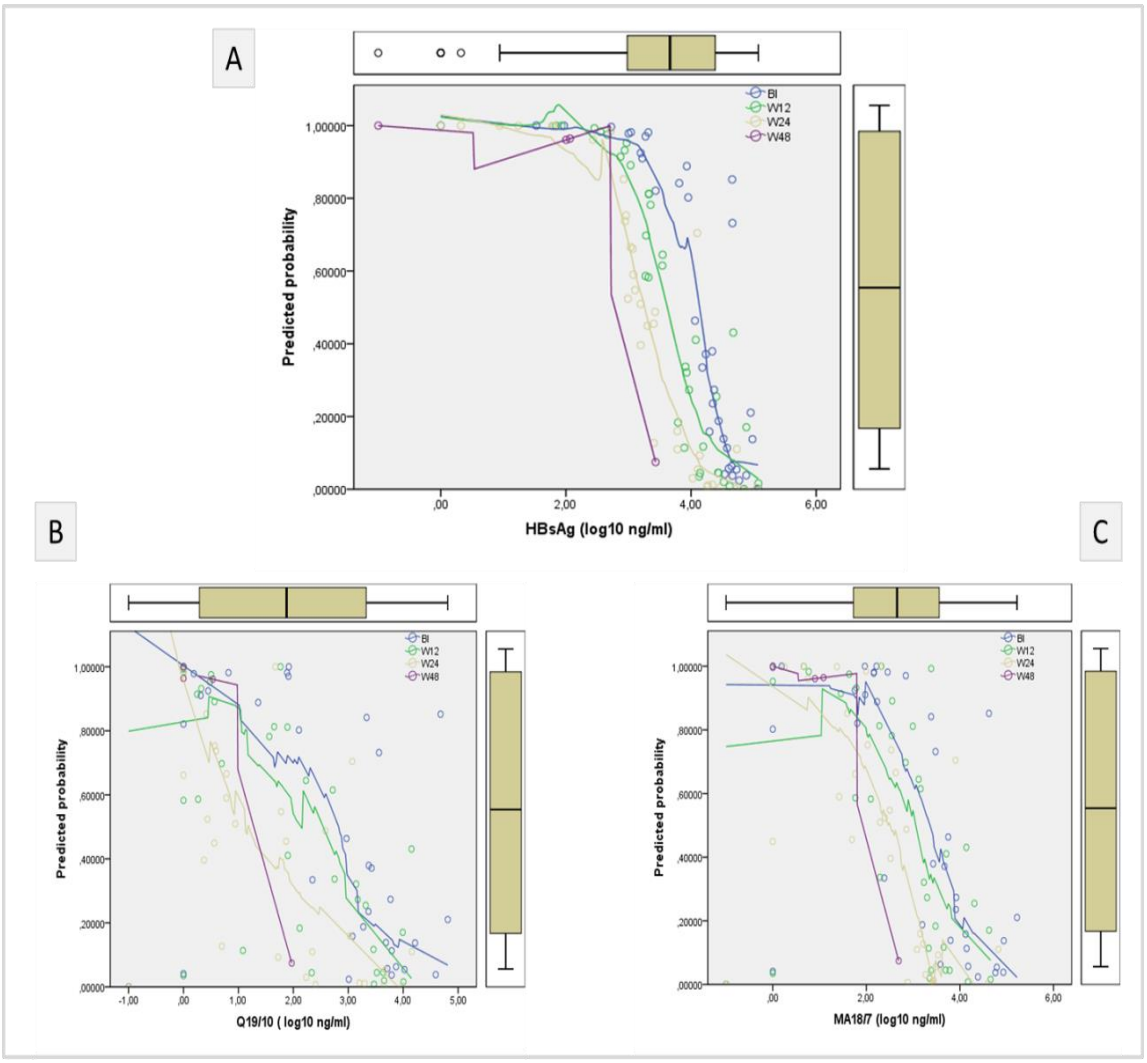


Figure 40 Correlations between predicted probability of response to pegInterferon-Therapy and ELISA based HBV surface protein quantities (mean log_{10}) of (A) HBsAg, (B) Middle Hepatitis B Virus surface proteins (**Q19/10)**, and (C) Large Hepatitis B Virus surface proteins **(MA18/7)** by **sampling time** (at baseline (BI), after 12 weeks (W12), 24 weeks (W24) and 48 weeks (W48)).

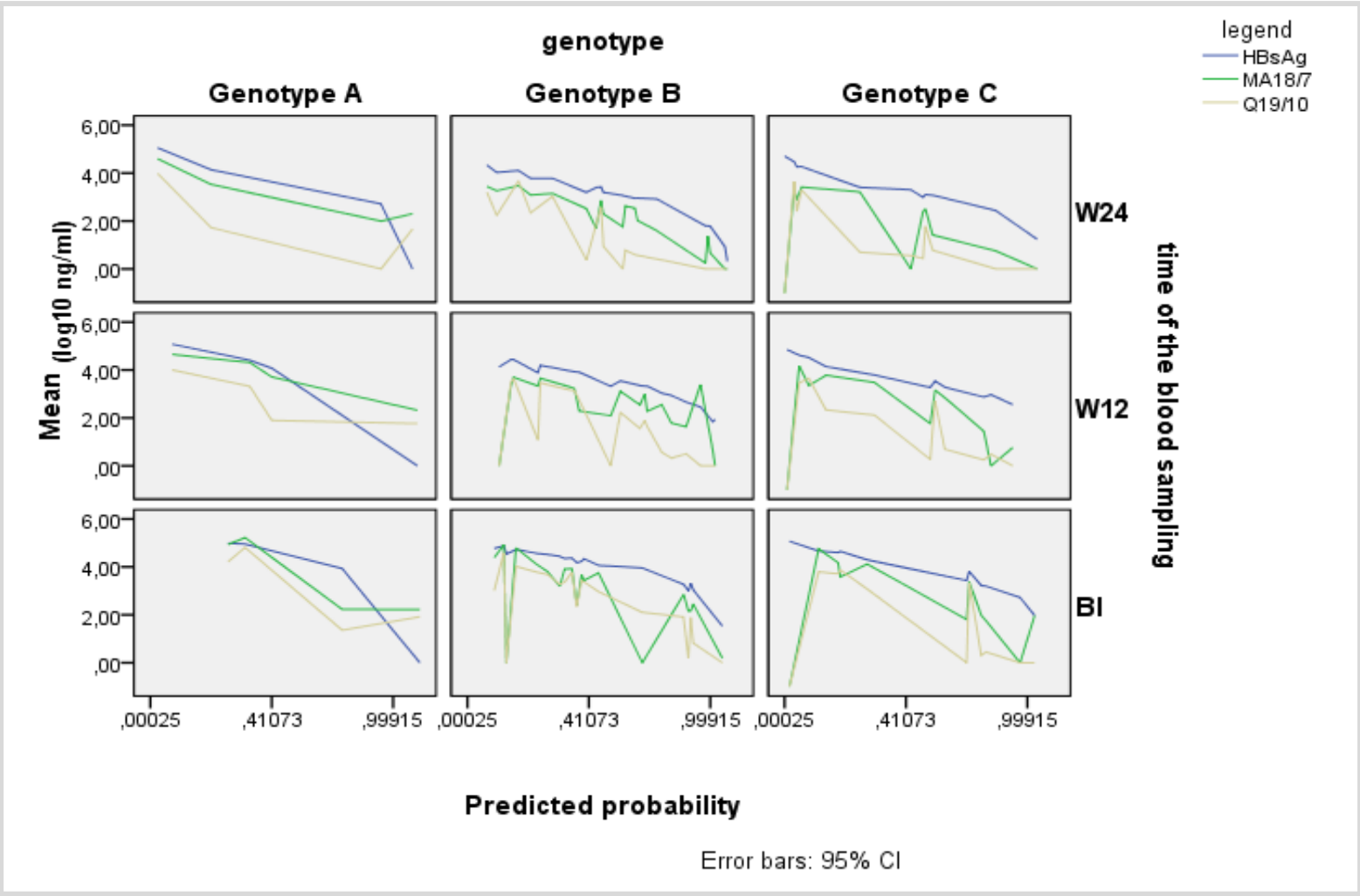


Figure 41 Correlations between predicted response probability and quantities (mean log_{10}) of HBsAg, LHBs (MA18/7), and MHBs (Q19/10) by sampling time and genotype.

5 Discussion

With more than 300,000 individuals infected with HBV, we face the challenges of one of the significant global health burdens (Hepatitis B WHO 2020.000Z). Due to the complexity of its infection and the limitations of diagnostic tools, the demand for detailed investigation is high. Investigations on biomarkers are essential to understand how the virus affects the human bio system and finally discover a cure. At present, finding efficient predictive biomarkers of sustained response is more important and relevant to deliver individually adjusted treatment and patient care. The primary purpose of potent biomarkers is to help monitor the infection and signal recurrence or treatment failure in its early stages. Tackling these issues in a setting with many important variables requires examining several aspects of the infection and influencing factors, necessitating the cooperation of many research teams.

This study's main focus was quantifying the HBsAg components (LHBs, MHBs, and SHBs) by targeting specific epitopes with in-house built immunoassays and analysing their potential as novel clinical biomarkers for monitoring immune-based therapy in chronically infected patients. This study's essential tasks were optimising the ELISA, internal validation with an adjusted WB analysis, and cooperative external validation with M. Pfefferkorn and the working group at the University of Leipzig.

Essential data and information obtained from two global, randomised phase III/IV studies published before this investigation (Lau et al. 2005; Liaw et al. 2011) contributed fundamentally to this investigation. These studies included samples of >100 HBV-infected patients enrolled in a trial on treatments with PegIFNa-2a that were retro-respectively integrated into this analysis. Moreover, the knowledge gained in the different projects was helpful in interpreting the data generated by this study's statistical analyses. Furthermore, in close cooperation with F. Rinker and the research team from the Medical University of Hannover, who independently ran their HBsAg components analysis in parallel, it was possible to compliment and confirm the results obtained by the research group of Prof Dr Dieter Glebe at the Institute for Medical Virology at the University in Gießen. The findings were cooperatively published (Franziska Rinker et al. 2020).

A further critical question arose concerning the fragmentation and kinetics of the surface antigen components in different phases of the infection in different settings. M.

Pfefferkorn and the working group at the University of Leipzig committed themselves to addressing the issue. In a productive partnership with AG Glebe, the WB testing, which visualised and hinted at differences, contributed to the significant results, which were presented together in a publication (Pfefferkorn et al. 2017).

The following sections will highlight and discuss this study's results and evaluate the different steps of the findings and analysis of the HBs surface proteins LHBs, MHBs and SHBs as potential novel biomarkers for predicting viral response to PegIFNa treatment in HBeAg-positive chronically infected patients.

5.1 LHBs, MHBs, and SHBs as Novel Biomarkers for Predicting Response to Therapy with PegIFNa

This study's core focus was on the HBsAg and its subunits LHBs, MHBs, and SHBs. This study highlights the serum levels of different HBs components during the trial. The results of this analysis contribute information on the relative proportions of HBV surface proteins in the serum of HBeAg-positive CHB patients. A closer look at the kinetics of HBs protein levels during PegIFNa treatment showed the grade of predictivity to the response (see the binary logistic analysis for predictivity on pp. 60–62). With the knowledge gained using modified in-house ELISA quantifications and improved WB analysis, more gaps in the interrelationships and characteristics of the viral surface proteins could be closed.

A careful selection of suitable antibodies was essential, which targeted specific conserved epitopes on LHBs, MHBs, and SHBs. The in-house developed and validated ELISA (Gerlich/Glebe, Gießen) using the previously mentioned well-defined monoclonal antibodies against the different HBsAg components proved highly efficient in previous studies and met the ideal requirements of this project. It was possible to achieve meaningful results with a calibration curve generated with a standard serum (see pp. 41–43). The advantage of the WB was the visualisation of the different viral components and the beneficial effect that the proteins did not suffer from masking by other antibodies or serum components. Additionally, it enabled analysis of N-terminal glycosylation, which will be further discussed in the relevant section. Nevertheless, the ELISA had greater sensitivity.

Interestingly, both methods reacted comparatively well (see validation pp. 46–49). The WB results showed a strong significant linear correlation with the ELISA results,

especially for LHBs. The effect of a significant linear correlation between WB- and ELISA- analysis, as shown with the LHBs-analysis, could not be equally shown with MHBs. Rinker et al. quantified all three HBs components in the same samples used in this study and came to similar conclusions like this study. The in-house ELISA and modified WB were externally validated using a small cohort of sera from patients sampled at different stages of HBV infection at the Institute in Leipzig. After solving methodical differences, the measurements were highly comparable between the commercial immune assay in Leipzig and the in-house ELISA in Giessen (see pp. 42–44).

The collaborative ring trial suggested differences in LHBs levels in the different stages of the HBV infection and hinted at quantitative variations in the three viral surface proteins between acute HBV infections and inactive HBsAg carriers. Further detailed and meaningful analyses were conducted by Pfefferkorn et al. The HBV surface protein levels quantified by ELISA showed significant similar trends in the first 24 weeks post-initiation of PegIFNa treatment independent of additional N.A. (LAM). The combinations of antiviral therapies had no significant effect on the response to antiviral treatment and had no significant statistic influence on the HBV protein levels or distribution (see pp. 56–60). The WB analysis confirmed the decrease in HBs proteins, especially in responders. In general, the analysis of LHBs, MHBs, and SHBs generated data showing that LHBs especially come closest to the performance of HBsAg measurements and produce similar results on the predictability of treatment outcome after 12 weeks. MHBs and SHBs show the same trends and cutoff after 12 weeks but with less significant power for identifying non-responders to PegIFNa.

The low baseline HBs protein levels during 24 weeks of treatment were significantly correlated with response. The analyses showed a clear trend towards a more significant decline in HBs levels in responders than in non-responders during the entire trial period. In many cases, MHBs and LHBs dropped below the detection limit in responders. Overall, ELISA-based SHBs quantities closely resembled the HBsAg levels of well-established commercial ELISAs, underlining the similar sensitivity and specificity of the in-house HBs assay to the commercial HBsAg ELISA.

Regarding the subviral fractions, the WB analysis detected high MHBs levels, especially in responders. Based on the ELISA surface protein quantities, the relative proportions of the three HBsAg components follow a stable pattern that does not change

throughout the therapy period. SHBs predominate with an average proportion of 70%, followed by LHBs at about 20% and MHBs at about 10% (see p. 43). The detailed WB analyses show comparable results and suggest that even the glycosylated fractions remain in a stable relative order. However, in this study, quantitative WB showed a relatively high proportion of MHBs in the SVPs from HBeAg-positive CHB patients. Altogether, the core information taken from this study is the significant correlation between LHBs and HBsAg. Therefore, LHBs stand out as the most promising predictive biomarker but do not outperform HBsAg.

The virus HBsAg comprises three transmembrane proteins. As suggested by numerous researchers, they have designated functions and specific antigenicity. Many questions related to the actual purpose and meaning of the viral infection remain to be discovered. The virus uses the host's polymerase and mRNA to produce the viral surface proteins and secrete them as virions, spheres, and viral particles that circulate in the patient's serum. The SVPs were mainly detected by immune assays in this study. Therefore, HBsAg is the significant biomarker currently available and essential in HBV diagnostics. However, there are limited resources or biomarkers to predict response to antiviral therapy and outcome. HBsAg is hardly replaceable in HBV management and is currently recommended in the guidelines for monitoring HBsAg levels during PegIFNa therapy (EASL 2017). With the advent of different therapeutics and the aim of seroclearance and functional cure, biomarkers are even more essential in the adjusted treatment of CHB. HBsAg levels are essential to guide therapy decisions in HBeAg-positive patients treated with PegIFNa (Chuaypen et al. 2017b).

All samples underwent HBsAg quantification before the analysis of the SVPs and, ideally, were integrated into the statistical comparison. This study agreed with other studies that low HBsAg levels at week 12 are more likely found in responders and decrease during immune-modulating therapy. The latest studies suggest that HBsAg levels <1500 IU/mL at week 12 strongly indicate HBeAg seroconversion and confirm treatment continuation. HBsAg levels <20,000 IU/mL or any decline in HBsAg after 24 weeks make treatment response and achieving HBeAg seroconversion very unlikely. Therefore, treatment with PegIFNa should be stopped (Sonneveld et al. 2013).

This study did not compare cccDNA and HBs levels and, therefore, cannot conclude whether there is a strong correlation between them. Nevertheless, research on cccDNA and integrated HBV DNA has revealed them as essential determinants of cure (Li et al.

2017; Li et al. 2016). Interestingly, many studies describe a significant correlation between serum HBsAg levels and cccDNA in the liver and discuss the linkage of the host immune control (Henry Lik–Yuen Chan et al. 2007; Wang et al. 2016a; Liang et al. 2016). The quantitative and qualitative production of HBsAg is modulated by the interrelationship of host and virus and appears to correlate negatively with immune control and HBsAg levels (Cornberg et al. 2017; Nguyen et al. 2010). In this case, changes in HBsAg levels in the different phases of the infection could be interpreted accordingly. They can assist in distinguishing between immune tolerance and clearance in HBeAg-positive patients and predict spontaneous HBsAg seroclearance in HBeAg-negative and inactive-disease patients (Zhu et al. 2013; Wang et al. 2016b).

The remaining question was whether the three components of HBsAg (LHBs MHBs, and SHBs) provide additional information. In a collaborative study with Pfefferkorn et al. (Pfefferkorn et al. 2017), it was possible to show that LHBs were a useful instrument for determining the different phases of HBV infection. The quantification of the LHB and MHB components of HBsAg showed a significant difference between inactive HBV carriers and patients in other phases of HBV infection. In the cooperative ring trial with a small representative cohort from Pfefferkorn et al., we could visualise the suspected differences with the modified WB analysis and show similar ELISA HBs levels. The HBs surface protein levels quantified by ELISA in this study showed significantly similar trends in the first 24 weeks post-initiation of PegIFNa treatment independent of treatment variations. The addition of N.A. did not change the statistical outcomes and, therefore, hints that the main therapeutic impact is due to PegIFNa, which presumably interferes with viral replication in the host cell, resulting in reductions in cccDNA and HBsAg (Chuaypen et al. 2017b). Another possible explanation is the difference in the targets of LAM or, rather, the lack of interference in the actual viral transcription (Chan et al. 2011). Therefore, HBsAg can be produced and secreted uninterrupted from the viral cccDNA and the transcription via HBV RNA and its specific polymerase.

Xiang et al. highlighted another important aspect of low HBsAg levels due to novel SHB mutations in the central antigenic region (Xiang et al. 2017). Depending on the period, the virus expresses such mutations. Other external variables have a further impact and provoke viral mutations. Therefore, a decrease in HBsAg may no longer be

relatable to the actual response to PegIFNa but rather to the result of faulty viral mechanisms for HBsAg secretion and lower binding affinity to test antibodies.

The constant proportionality shows that neither PegIFNa nor the combinational therapy significantly influenced the processing of the three surface proteins, despite two independent liver mRNAs being involved in their transcription. As known, LHBs derive from the ORF starting from the pre-S1 promotor in the liver cells. In contrast, the virus depends on the pre-S2/S promotor to generate the MHBs and SHBs. In all sera, SHBs predominate clearly in quantity. One explanation could be that more SHBs are secreted as part of spheres or SVPs. Alternatively, the difference may reflect the specific functions of LHBs and MHBs, which are only expressed and secreted in lower amounts.

The study's crucial question centres around predictive factors related to response to the treatment with PegIFNa in HBeAg-positive CHB patients. Patients in this study with low baseline HBs protein levels during 24 weeks of treatment were more likely to respond to PegIFNa treatment. Responders showed a stronger decline in HBs levels than non-responders. Indeed, MHBs and LHBs decreased below the detection limit in responders. LHB quantification produced similar results to the HBsAg measurements and was as effective in predicting the treatment outcome at the 12-week cutoff. MHB and SHB quantification performed similarly but not as significantly. Several previous studies investigated probable variables that could function as predictive biomarkers (see pp. 56–62). The results of the quantitive analysis agree on the effect of individual genotypes described in the relevant section and confirm the probability of response with low viral load at baseline or a decline in serum HBsAg levels during the first 12 weeks of treatment (Sonneveld et al. 2010). While the time interval of this study was limited to a maximum of 46 weeks, another study examining a longer period of PegIFNa treatment determined a similar cutoff at week 24 and confirmed significant HBsAg loss after 48 weeks of treatment in a follow-up investigation (Li et al. 2017). Therefore, the continuation of treatment or relatively long-term therapy could promise success. The latest HBV infection guidelines emphasise the importance of predicting response and determining further treatment based on a significant reduction in HBsAg level resulting in a rapid HBsAg loss (EASL 2017).

The strongest correlation was observed between LHBs and HBsAg and showed the most promising predictive features as a possible novel biomarker. Previous studies have shown a strong correlation between serum HBV-LP and HBV DNA and that LHB

expression can reflect HBV replication (Wang et al. 2011; Wu et al. 2008). Furthermore, Zhu et al. examined a similar cohort. They found LHBs to be a strong predictor of virological response to PegIFNa in the early stages of the infection course. They even discussed that a combination of quantifying LHBs, HbsAg, and HBV DNA could discriminate and predict viral response sooner and more effectively (Zhu et al. 2013). While the emphasis lies in close relation to HBV DNA and is not associated with HBsAg kinetics, this study instead showed a definite correspondence to HBsAg levels but agreed on the predictive aspect of LHBs and the divergence of the different antiviral therapies and their effects.

The differences regarding the HBsAg correlation may lie in the various influences on the different assays. The latest studies reported divergence in accuracy (sensitivity), vulnerability to failures, and the need for global assimilation of test standards (Murayama et al. 2019; Höner Zu Siederdissen et al. 2017; Alavian et al. 2013). Interestingly LHBs and HBs play a significant role in distinguishing different phases of HBV infection. Pfefferkorn et al. highlighted that serum levels of LHBs and MHBs were significantly lower in HBeAg-negative HBV carriers than in chronic HBeAg-positive patients (Pfefferkorn et al. 2017). If proven, both surface proteins could serve as indicators of viral activity and HBV DNA integrated into the host chromosomes, read by the specific promotors.

Unfortunately, a direct comparison to untreated patients is yet to be performed to show the actual differences and effects of PegIFNa that lead to seroconversion. It can be presumed that PegIFNa has other immunological mechanisms than those in spontaneous HBeAg seroconversion (Vyas et al. 2018). The WB results, especially for LHBs, showed a strong significant linear correlation with the ELISA results. MHBs were not as outstanding as LHBs, suggesting that to no small degree, the MHBs' pre-S2 domain is masked and undetectable by ELISA (Bernd Krone et al. 1990). To some degree, this likely also applies to LHBs. This phenomenon is known and has been described before (R. Deepen et al. 1990). For example, Deepen et al. found high levels of free MHBs in HBeAg-positive carriers only when the HBsAg concentration was >20,000 ng/mL (R. Deepen et al. 1990). However, in this study, quantitative WB showed a relatively high proportion of MHBs in the SVPs from HBeAg-positive CHB patients, especially in responders and even with low HBsAg levels.

SHB quantification with the in-house ELISA came close to the HBsAg levels of commercial ELISAs, suggesting similar sensitivity and specificity. Therefore, it also became apparent that there is a certain stability to the ratio of the internal and external pre-S1 domains of virions, the same as for filaments. SHBs could correlate significantly with quantitative HBsAg measurements. Surprisingly, the relative proportions of the three HBsAg components follow a stable pattern that does not change during therapy. The WB analysis visualised and affirmed the relative and absolute order of the HB surface proteins. Closer examination even suggests that glycosylation at the N-terminals follows a stable relative pattern.

The right choice of methods is greatly important when examining the efficiency of a potential biomarker. Recently, many quantitative immunoassays for HBsAg have been designed and approved as suitable methods in hepatitis B diagnostics. Most HBsAg ELISAs fulfil the biomarker detection and quantification requirements: reproducibility, automated quantification, and standardisation. Therefore, it was logical to use ELISA to quantify and analyse the HBV surface proteins. Another strong argument was that immune assays have recently been proven capable of detecting all circulating forms generated by the virus, including spherical and filamentous SVPs. While it was expected that the sera might contain immune complexes of anti-HBs and HbsAg, it is unlikely that they were overrepresented or disturbed in any immunoassay. Further investigations with appropriate methods are needed to investigate the amount and effect of possible immune complexes.

While there were many clear benefits, advantages, and meaningful results, both tests also had weaknesses. The WB method has lower sensitivity and is a complex method with multiple steps and, therefore, less than ideal reproducibility. Due to the pre-treatment of the sera, there is potentially a loss of viral particles. While the ELISA is highly sensitive, recent studies have shown that changes in the viral genome cause undetectable escape mutants, resulting in false-negative outcomes (Di Lello et al. 2019; Cui et al. 2016). Additionally, pre-S/S mutants can negatively impact HBsAg production (Melegari et al. 1997; Wang et al. 2006; Bi and Tong 2018). Other influencing factors include immune complexes, albumin binding, and other molecular interactions that often partially mask HBsAg surface proteins (Quiroga et al. 1987). One aspect that needs further investigation but has been addressed before is the different

performances of immune assays due to HBV genotype- or strain-specific polymorphisms (Murayama et al. 2019).

5.1.1 The Role of N-Terminal Glycosylation in the Distribution Pattern of the Surface Proteins and its Possible Effects on Response

With the help of the WB analysis, it was possible to take a closer look at the N-glycosylation of the surface proteins. Surprisingly, the investigations showed that the relative distribution of the different components was stable, showing a particular pattern independent of the outcome or response. The non-glycosylated SHBs predominate in quantity. In addition, the proportion of non-glycosylated MHBs was always higher than that of glycosylated proteins. A reversed proportionality was found with LHBs. While no significant correlations were found between other variables, such as genotypes, some genotype differences were observed. For example, the percentage of glycosylated LHBs was higher in genotype A than in the other genotypes, while the percentage of glycosylated MHBs was much lower in genotype D. There were no significant correlations between glycosylation and response. However, all quantitative measurements of the fractions were, on average, higher in non-responders than responders (compare the results on pp. 45–51).

Ito et al. showed that N-linked glycosylation of HBV envelope proteins was critical for virion secretion and that an additional glycosylation site could rescue secretion defects caused by mutations in the S protein (Ito et al. 2010). Julithe et al. investigated the surface of HBV particles, finding that the immunodominant a-determinant was the main target of neutralising antibodies and an essential determinant of infectivity. It contains an N-glycosylation site at position 146, which is functional on only half of the envelope proteins (Blanchet and Sureau 2007). Therefore, this finding suggests that the coexistence of nonglycosylated and glycosylated N146 on the surface of HBV particles reflects the dual function of this determinant in infectivity and immune escape. Consequently, changes to the HBV glycosylation pattern affect virion assembly, infectivity, and immune escape (Sureau et al. 2003)(Julithe et al. 2014). Altogether, these findings suggest that the nonglycosylated N146 is essential for infectivity. Moreover, in addition to its importance for HBV virion secretion, the glycosylated form is instrumental in shielding the a-determinant from neutralising antibodies.

Not all samples could be analysed by WB, and future studies would benefit from a broader spectrum of samples and methods to confirm these findings. This study did not

perform a detailed examination of the glycosylation sites. Additional or more specific analyses on glycosylation patterns may provide more insight into possible pathways for the virus and its escape mechanisms. These aspects might contribute to the probability of response to PegIFNa therapy. Investigations on mutations in the S protein and the resulting changes in glycosylation could further explain effects on different HBsAg serum levels during CHB.

5.1.2 Genotype as possible influential variable

Despite a prevailing proportional distribution pattern of the surface proteins and their glycosylated components, the analysis indicated differences for the genotypes. The SHB fraction predominates quantitatively over the other fractions regardless of the genotype. Remarkably, patients with genotype B had the highest relative concentrations of LHBs and MHBs. Genotype A stood out with a higher percentage of MHBs than the other genotypes, although samples for genotypes A and D were too few for a representative analysis. Interestingly, genotypes C and D had relatively lower serum LHB levels. In the overview, LHBs were most strongly represented in genotype B. The ELISA and WB analyses of the surface proteins provided corresponding results and produced a consistent overall picture (compare results on pp. 53–56)

Several investigations on the different genotypes imply that different causalities are interlinked. The same observations apply to the serum levels of LHBs, MHBs, and SHBs. Recent studies have produced increasing evidence showing that HBV genotypes influence the outcome of the infection and response to PegIFNa and play a role in HBeAg seroconversion and even specific mutations. Sonnevelt et al. found that patients with genotype A showed a notable decline in HBsAg levels, while those with genotype D showed the smallest decline in HBsAg levels, consistent with the findings of this study (Sonneveld et al. 2012). Erhardt et al. argued that genotypes A and D are important predictors of response to PegIFNa in CHB patients (Erhardt et al. 2005).

Unlike the results of this study, Pfefferkorn et al. report lower MHBs and LHBs levels for genotype A than for genotype D (Pfefferkorn et al. 2017). HBV genotypes are most likely important and independent predictors of IFN responsiveness in CHB. There is a correlation between the decrease in HBsAg during therapy and the HBV genotype (Sonneveld et al. 2012). In HBeAg-positive chronically infected patients, this decline was more pronounced with genotypes A and B than with genotypes C and D. Shen et al. investigated the in vitro response of infected hepatocytes to therapy, showing

hepatocyte sensitivity to IFN. Here, the intrinsic response rather than the genotype of the virus plays a superficial role (Shen et al. 2018).

The investigations of Sunbul et al. connected further aspects with the genotypes. Here, the genotypes differed in their mutation frequency; the mutation rate for genotype C appeared higher, and genotype A showed a higher tendency to chronicity. Genotype B highlights both aspects more strongly. An increased progression towards HCC was observed with genotypes C and D (Sunbul 2014). Essential aspects include the PegIFNa response and the surface antigens' general expression and intracellular and extracellular distribution. Thang et al. could contribute significant findings in this regard. In their in vitro studies, less HBsAg was detected in cells infected with genotype-D virus (Zhang et al. 2017). Genotype D also had less intracellular HBsAg than genotype A. These effects are explained by the SHBs in genotype D having higher secretion efficiency and LHBs most likely having a crucial function in inhibiting HBsAg secretion.

It is assumed that pre-core genes are responsible for the expression of both core proteins and HBeAg and, therefore, correlate with infection activity at high viral loads. These observations agree with the assumptions of this study and very likely explain the reduced expression of the surface antigens in genotype D, possibly related to increased promoter performance. Surprisingly, patients with genotype B infections had significantly higher mean HBsAg, MHB, and LHB protein levels than those with genotype C infections. Furthermore, LHB proportions were significantly higher with genotype B. However, they may result from different relative amounts of virions, spheres, and filaments. Interestingly, Pfefferkorn et al. also observed differences in HBs proteins between genotypes. Indeed, genotype A had lower MHB and LHB levels than genotype D (Pfefferkorn et al. 2017).

The representation of the various genotypes in this study's cohort was unbalanced. Therefore, not all genotypes were investigated and had sufficient statistical power. Consequently, while both independent methods used in this study provided similar results and it was unlikely that any major weaknesses in the detection techniques or substantial interfering factors existed, it was impossible to differentiate between mutation patterns or other influential factors that further impact the response or HBs serum levels. Given the known differences in IFNa treatment responses and outcomes associated with the different genotypes (Zhang et al. 2017; Erhardt et al. 2005; Shen et al. 2018), genotype-based differences in HBs proteins merit further investigation.

Adapted treatment regimens may further improve treatment efficacy in CHB. Whether differences in surface protein expression and secretion account for differences in spontaneous and antiviral therapy-induced HBsAg clearance and recovery rates from acute HBV infection warrants further investigation.

5.1.3 The Meaning of the Treatment with PegIFNa in the Research Cohort

This study showed no significant difference between PegIFNa monotherapy and combinations with LAM over time and no difference in response with dosage modifications. The statistical analysis showed no significant correlation to HBs protein serum levels or any other variables in this investigation (see pp. 45–51)

The patients enrolled in this study underwent PegIFNa therapy for 24–36 weeks. The cohort was primarily grouped by PegIFNa monotherapy and combination therapy with LAM at different dosages. PegIFNa was given according to official guidelines in settings with chronic infections to reduce the viral load. LAM is recommended when PegIFNa is contraindicated or with low inflammatory activity. The actual goal of any therapy is to cure the patient by eliminating the pathogen from them. Since no such cure is yet available, the closest achievement is seroconversion to reduce the viral load by suppressing the viral replication with N.A.s. Response to therapy is aligned with an evident decline in serum HBsAg levels (EASL 2017; Cornberg et al. 2017).

Multiple factors that play a particular role in response to treatment must be considered. An important, influential factor is the immune system of the infected patient and its capability to adequately fight the infection and respond to the therapy (Sonneveld et al. 2013; Ma et al. 2019). In most cases, the patients have to be continuously treated with N.A.s and only rarely achieve HBsAg loss and HBeAg seroconversion resulting in treatment termination. Interestingly, as previously stated, combining LAM with PegIFNa did not show significantly different effects. Other studies showed that different N.A.s or even combinations of N.A.s had no significant effect on HBsAg kinetics (Zoulim et al. 2015; Seto et al. 2013). Sonneveld et al. agreed that predictiveness was not influenced by the inclusion of an additional N.A. (Sonneveld et al. 2010). Extended follow-up studies could give more insight into possible shortening or reduction of the antiviral therapy, which could benefit the patient given the side effects of the substances (Wong et al. 2014). The latest studies on the possible advantages of combinational therapy regimens showed, contrary to expectations, no additional benefit compared to the already established therapy options and resulted in no significant change in response

rate (Lau et al. 2005)(Marcellin et al. 2004). Again, other studies argue specific effects related to HBsAg seroclearance relating to therapy modulations or combinations and discuss that PegIFNa monotherapy causes delayed HBsAg seroclearance by comparison (Chan et al. 2015; Hou and lai 2015).

To understand the general effectiveness of monotherapy with PegIFNa, we must take a closer look at the mechanisms of actions of the different therapeutics. PegIFNa provides superior HBsAg decline but is not as effective as LAM in HBV DNA suppression, which goes back to the different pathways of the N.A. (Brunetto et al. 2009; Reijnders et al. 2011). As mentioned earlier, immunomodulation is one of the crucial aspects of PegIFNa and is presumably responsible for the HBsAg decline. T cell activity is likely deterministic in HBsAg production and secretion. LAM interferes in the reverse transcription of the pg-mRNA and is associated with the cytokine pathways that cause HbsAg decline, whereas PegIFNa targets the cccDNA. It must be considered that, for example, interferon-gamma or tumour necrosis factor-alpha can affect the infected cells and lead to cytolyses. Therefore, it would also explain the relatively small decline in HBsAg with LAM since it generally has an influential indirect potential (Xia et al. 2016; Butler et al. 2018).

Due to the different target points and utterly different pharmacokinetics of PegIFNa and LAM, it is difficult to define the effects of the therapy on the patients in this cohort. In addition, this study did not investigate in detail the immune status of the patients. Many factors, such as age or sex, are not broadly presented in the cohort and, therefore, do not show all possible influencing factors in the treatment setting of this study. In future studies, it would be interesting to explore whether combinations with N.A.s other than LAM show significant differences in response and HbsAg component levels during the infection course.

5.1.4 Possible Influencing Variables: Origin, Age, Sex, and HBeAg Status

Interrelations between age, origin, or sex could not be shown. There is no significant correlation between these variables and response to PegIFNa therapy. Nevertheless, the data suggest possible differences in the kinetics of the infection. In terms of nationality or origin, there are evident genotypical distribution patterns, as discussed before (p. xx). A comparison with HBeAg-negative patients was not possible within this study. All included patient samples were HBeAg-positive (see pp. 40–41).

While origin and sex play a minor role in this study, it is worth investigating age as an influential factor given that PegIFNa functions immunomodulatory and the HBV infection interacts with the host immune system. It is conceivable that age-related changes in the immune system could influence the outcome and response. LeMaoult et al. reported that the induction of the antibody response to new antigens declines significantly with age and correlates with the immune response (LeMaoult et al. 1997). It is also conceivable that older patients deviate in the course of the infection compared to younger patients and, therefore, differ in response rates. This question was addressed by Rosenberg et al. They confirmed the assumption of an immunosenescence effect, meaning that immune system degradation accompanies the ageing processes (LeMaoult et al. 1997; Rosenberg et al. 2013; Sadighi Akha 2018).

Furthermore, Jang et al. showed that HBsAg titres gradually fall due to long-term immune pressure during HBV infection. Age inversely correlated with HBsAg levels that, while barely detectable in some cases, were not indicative of liver disease progression (Jang et al. 2011). Interestingly, age-specific patterns in the distribution of HBsAg and remarkable differences in dynamics between HBeAg-positive and HBeAg-negative patients could be shown. For example, elderly HBeAg-negative patients had fewer fluctuations in HBsAg levels, while HbsAg-positive patients had more significant decreases in HBsAg levels at different stages (Chu et al. 2002; Chen et al. 2009; Iloeje et al. 2006).

Regarding HBeAg status, it is possible to relate some aspects to the course of the infection and HBsAg levels. The decrease in HBsAg is known to be higher in HBeAg-positive than in HBeAg-negative patients (Zoutendijk et al. 2011; Wai-Kay Seto et al. 2015). Differences in responses to specific therapies are also observed. It has been reported that HBeAg-negative patients integrate more HBV DNA, leading to greater HBsAg production (Seto et al. 2013). Some studies interpret a substantial HBsAg decrease in HBeAg-positive patients as a higher probability of seroconversion (Lee et al. 2011), while others cannot confirm this association (Singh et al. 2014). Thompson et al. discussed that these facts add another layer of challenge to achieving a complete cure for CHB but do account for the lack of correlation between serum HBsAg levels and intrahepatic cccDNA levels in patients with HBeAg-negative CHB (Lin et al. 2010; Thompson et al. 2010). Interestingly, most studies of HBeAg-positive patients have

noted a positive correlation between serum HBsAg and HBV DNA titers and liver cccDNA. Similar results were obtained in 197 Chinese patients (Xun et al. 2013).

Therefore, the age, sex, and origin of this cohort do not reflect the global population and cannot be generally transferred. The patients were mainly middle-aged male participants aged about 30 years. The patients were mostly of Asian origin (see p. xx). Better distribution and representation of the different groups are essential to obtain a complete picture and to understand linkages between the mentioned variables. Age dependence could imply adjusting the interpretation of serum surface protein levels, which would require correcting guidelines, cutoffs, and therapies. It would suggest further research into mechanisms that impact viral HBsAg production with age, sex, and HBeAg status. Comparisons in a larger cohort with different HBeAg could provide more information for interpreting HBeAg responses to PegIFNa therapy and the meaning of HBV surface antigen quantification.

6 Summary and Conclusions

HBV infections remain a global health burden, and their diagnostics and treatment management are challenging. Therefore, this study investigated whether the quantities of HBsAg components (LHBs, MHBs, and SHBs) measured with specific immunoassays serve as novel predictive biomarkers for monitoring CHB patients undergoing immunomodulatory treatment with PegIFNa.

The data suggest that LHBs come closest to the performance of HBsAg measurements and produce similar results when predicting treatment outcomes after 12 weeks. LHB quantification had comparable applicability for monitoring during PegIFNa therapy and is promising as a useful tool to determine the different phases of HBV infection. MHBs and SHBs provided similar trends with a cutoff at 12 weeks but were not as significant in their prediction and detection of non-responders. The HBs quantification underlined a significant correlation between low baseline HBs protein levels during 24 weeks of treatment and response and a greater decline in responders than in non-responders during the trial period. Broadly translated, the results indicate an agreement with already published knowledge of a higher probability of response with low viral load at baseline or a serum decline in HBsAg levels during the first 12 weeks of treatment. SHBs levels correlated with HBsAg levels. Therefore, the main conclusion that can be

drawn is that the ELISA quantification of the three HBsAg components did not outperform the already established HBsAg quantification.

The WB analysis confirmed that the relative proportions of the surface proteins and their N-glycosylated fractions have a stable pattern that persists throughout the therapy period. The SHB fraction dominates quantitatively over the other fractions regardless of the genotype, and patients with genotype B had the highest relative concentrations of LHBs and MHBs. Genotype A stood out with a higher percentage of MHBs, whereas genotypes C and D had relatively low serum LHB levels. Overall, LHBs are strongly represented in genotype B.

While the already established HBsAg assay is hard to replace as a meaningful biomarker for predicting response, the different HBsAg fractions are still worth investigating closely, as this research showed, despite their previously discussed limitations. While the genotypes were not equally distributed, future studies on the meaning of genotypes might extend the explanations of treatment failures and different outcomes. A closer exploration of the role of glycosylation patterns may provide more insight into possible pathways of the virus and its escape mechanisms. Future studies should replicate these results in larger cohorts of a global population with broader age, sex, and origin representation to better understand the influencing factors.

Other interesting questions could be the differences in HBsAg kinetics and immune system responses associated with HBeAg and the meaning of HBV surface antigen quantification. With emerging new methods to detect proteins, further attempts could prove beneficial and show potential for detailed investigation of the surface proteins. Altogether, the properties and functions of LHBs, MHBs, and SHBs remain an exciting and promising research field and provide hope for improvements in HBV diagnostics and management and maybe even solutions approaching a cure.

7 Zusammenfassung und Fazit

Hepatitis-B-Virus-Infektionen stellen nach wie vor eine globale Gesundheitsbelastung dar, und die Diagnostik und das Management der Behandlung sind eine Herausforderung. Daher wurde in dieser Studie die zentrale Frage untersucht, ob die Quantifizierung der HBsAg-Komponenten LHBs, MHBs und SHBs mit spezifischen Immunoassays als prädiktive Biomarker eignet um den Verlauf chronisch infizierter Patienten zu monitoren, die sich einer immunmodulatorischen Behandlung mit PegIFN unterziehen.

Die Daten deuten darauf hin, dass LHBs und HBsAg-Messungen ähnliche, bezogen auf die Vorhersagbarkeit des Behandlungsergebnisses nach 12 Wochen, ähnliche Ergebnisse liefern Die LHBs-Quantifizierung ist für die Überwachung während der PegIFN-Therapie vergleichbar gut geeignet und ist ein vielversprechendes Instrument zur Bestimmung der verschiedenen Phasen der HBV-Infektion. MHBS und SHBS liefern ähnliche Trends mit einem Cut-off bei 12 Wochen, sind aber nicht so signifikant in ihrer Vorhersagbarkeit und Erkennung von Non-Respondern. Die HBs-Quantifizierung zeigte eine signifikante Korrelation zwischen niedrigen HBs-Spiegeln zu Beginn der 24-wöchigen Behandlung und dem Ansprechen sowie einen signifikanten Rückgang bei Respondern. Im Großen und Ganzen deuten die Ergebnisse darauf hin, dass eine höhere Wahrscheinlichkeit für ein Ansprechen besteht, wenn zu Beginn der Behandlung die HBsAg-Spiegel im Serum während der ersten 12 Wochen niedrig sind oder unter der Behandlung stark abnehmen. Die SHBs-Werte korrelierten mit den HBsAg-Werten. Daraus lässt sich ableiten, dass die ELISA-Quantifizierung der drei HBsAg-Komponenten, die bereits etablierte Quantifizierung von HBsAg, nicht übertrifft.

Die WB-Analyse bestätigte, dass die relativen Anteile der Oberflächenproteine und ihrer N-glykosylierten Fraktionen einem stabilen Muster folgen und während des gesamten Therapiezeitraums vorherrscht. Die SHBs-Fraktion dominiert quantitativ über die anderen Fraktionen, unabhängig vom Genotyp. Patienten mit Genotyp B haben die höchsten relativen Konzentrationen von LHBs und MHBs. Genotyp A zeichnete sich

durch einen hohen Prozentsatz an MHBs aus, während Genotyp C und D relativ niedrige Serumspiegel an LHBs aufwiesen. Insgesamt sind LHBs im Genotyp B stark vertreten.

Trotz Limitierungen und der Erkenntnis, dass der aussagekräftige HBsAg-Elisa nicht leicht ersetzbar ist, lohnen sich genauere Analysen der HBs-Proteine. Trotzdem, dass die Genotypen nicht gleichmäßig verteilt waren, könnten künftige Forschungen zur Bedeutung der Genotypen im Zusammenhang von Behandlungsversagen beitragen. Eine genauere Untersuchung der Rolle von Glykosylierungsmustern könnte mehr Aufschluss über mögliche Übertragungswege des Virus und Ausweichmechanismen geben. Um ein besseres Verständnis der Einflussfaktoren zu erlangen, sollten künftige Studien darauf abzielen, die Ergebnisse in größeren repräsentativen Kohorten der Weltbevölkerung zu wiederholen, um Korrelationen von Alter, Geschlecht und Herkunft darzustellen. Eine weitere interessante Frage könnten die Unterschiede in der HBsAg-Kinetik und der Reaktion des Immunsystems in Verbindung mit HBeAg und die Bedeutung der HBV-Oberflächenantigen-Quantifizierung sein. Mit den sich abzeichnenden neuen Methoden zum Nachweis von HBs-Proteinen könnten sich weitere Untersuchungen als sehr vorteilhaft erweisen und ihr Potenzial für eine detaillierte Untersuchung der Oberflächenproteine zeigen. Alles in allem bleiben die Eigenschaften und Funktionen von LHBs, MHBs und SHBs ein spannendes und vielversprechendes Forschungsfeld und geben Anlass zur Hoffnung auf eine Verbesserung der Hepatitis-B-Diagnostik und des Managements und vielleicht sogar auf Lösungsansätze zur Heilung.

Abbreviation List

aa	amino acid
ALT	Alanine Aminotransferase
cccDNA	covalently closed circular DNA
CHB	chronic Hepatitis B
DNA	deoxyribonucleic acid
E.R.	Endoplasmatic Reticulum, Endoplasmatic Reticulum
ELISA	Enzyme-linked Immunosorbent Assay
HBc	Hepatitis B core protein
HBcAg	HBV core protein
HBcrAg	Hepatitis B core-related antigen
HBeAg	Hepatitis B envelope antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B Virus
HBx	Hepatitis B Virus X-protein
HCC	hepatocellular carcinoma
HDV	Hepatitis D Virus
HSPG	heparan sulfate proteoglycane
INFa	Pegylated-Interferon-Alpha
kb	kilobase
kDa	kilodalton
LAM	Lamivudin
LHBs	Large Hepatitis Surface Antigen
MHBs	Middle Hepatitis B surafce protein
mRNA	messenger ribonucleic acid
N.A	nucleos(t)ide analogue
NTCP	Sodium taurocholate cotransporting polypeptide
OPD	o-phenylenediamine dihydrochloride
ORFs	overlapping open reading frame
PegIFNa	Pegylated-Interferon-Alpha
pg-mRNA	pregenome RNA
rcDNA	partly double-stranded relaxed circular DNA
RNA	ribonucleic acid

RNase	Ribonuclease
SHBs	Small Hepatits B surface protein, Small Hepatits B surface protein
SVP	subviral particles
WHO	World Health Organisation, World Health Organisation

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Publications

Papers published

Rinker, F, Bremer, CM**, Schröder, K**, et al. **Quantitation of large, middle and small hepatitis B surface proteins in HBeAg-positive patients treated with peginterferon alfa-2a**. *Liver Int*. 2020; 40: 324– 332. https://doi.org/10.1111/liv.14298

Pfefferkorn, Maria; Böhm, Stephan; Schott, Tina; Deichsel, Danilo; Bremer, Corinna M.; **Schröder, Kathrin** et al. (2017): **Quantification of large and middle proteins of hepatitis B virus surface antigen (HBsAg) as a novel tool for the identification of inactive HBV carriers**. In: Gut. DOI: 10.1136/gutjnl-2017-313811.

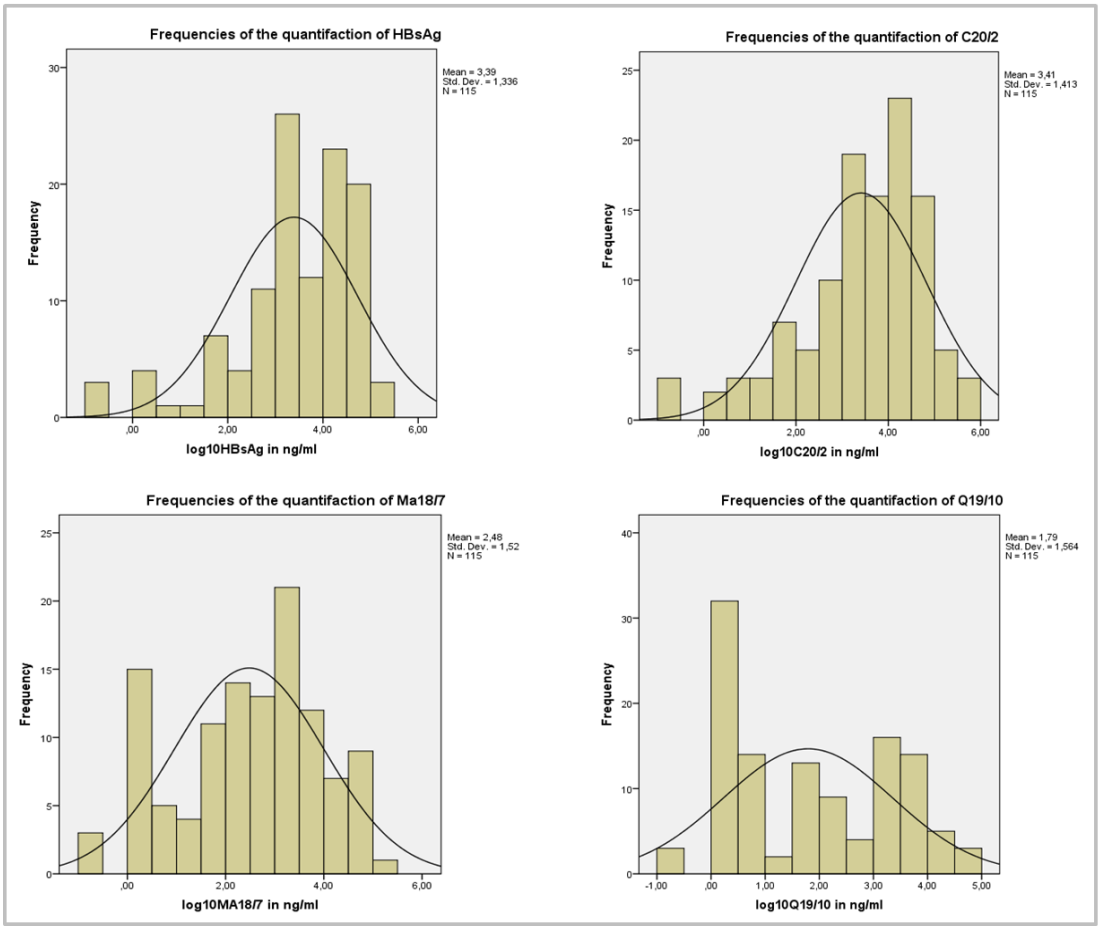
Poster Presentation

Quantification of hepatitis B surface proteins (large, middle and small) and the potential as novel biomarkers in HBeAg-positive patients treated with peginterferon alfa-2a

Schröder, K.;Rinker, F, Bremer, CM**;** Glebe,D

Laboratory Poster Presentation at Institute of Medical Microbiology, Immunology and Hygiene (MIH) of the Technical University of Munich; Laboratory Retreat ; May 2019, Venedig - Isola di San Servolo

Appendix



Appendix Figure 1 Normal Distribution of all data included for the statistical analysis (after log10)

N

Percentiles

Descriptive Statistics

		Time of the blood sampling	Origin of patient	Treatment	Genotype	Response	HBsAg (ng/mL)	C20/02 (ng/mL)	MA18/7 (ng/mL)	Q19/10 (ng/mL)
N	Valid	115.00	115.00	115.00	112.00	115.00	112.00	112.00	112.00	112.00
	Missing	0.00	0.00	0.00	3.00	0.00	3.00	3.00	3.00	3.00
Mean		2.07	1.23	3.01	2.30	1.52	17834.78	31737.10	8320.57	2968.78
Std. Error of Mean		0.08	0.05	0.15	0.07	0.05	2523.15	6785.86	2117.43	826.70
Median		2.00	1.00	2.00	2.00	2.00	4775.00	3989.72	590.13	76.38
Std. Deviation		0.89	0.58	1.58	0.73	0.50	26702.46	71814.79	22408.79	8748.98
Variance		0.78	0.34	2.50	0.54	0.25	713021277.06	5157364603.13	502154069.34	76544714.31
Skewness		0.17	2.36	0.72	0.29	−0.09	2.11	3.76	4.45	5.19
Std. Error of Skewness		0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23
Kurtosis		−1.10	4.22	−0.72	−0.03	−2.03	4.38	15.06	24.26	30.11
Std. Error of Kurtosis		0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Range		3.00	2.00	5.00	3.00	1.00	119141.41	413595.23	166256.78	64775.00
Minimum		1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00
Maximum		4.00	3.00	6.00	4.00	2.00	119141.41	413595.23	166256.78	64775.00
Sum		238.00	142.00	346.00	258.00	175.00	1997495.19	3554554.80	931903.41	332503.55
Percentiles	25%	1.00	1.00	2.00	2.00	1.00	985.13	936.45	55.88	1.43
	50%	2.00	1.00	2.00	2.00	2.00	4775.00	3989.72	590.13	76.38
	75%	3.00	1.00	4.00	3.00	2.00	24886.87	28659.87	3731.61	2136.39

Table 9 SPSS output and summary of the descriptive statistics with the collected data and categorisations by the Roche study and the surface proteins quantified by ELISA.

Descriptive Statistics

		LHBs (N-glycosylated)	LHBs (non-N-glycosylated)	MHBs (N-glycosylated)	MHBs (non-N-glycosylated)	SHBs (N-glycosylated)	SHBs (non-N-glycosylated)	Total intensity (LHBs/MHBs/SHBs)
N	Valid	89.00	93.00	87.00	93.00	93.00	93.00	93.00
	Missing	26.00	22.00	28.00	22.00	22.00	22.00	22.00
Mean		137009.48	84780.61	135146.66	237576.82	477492.26	773128.04	1830521.85
Std. Error of Mean		20484.98	17018.81	37566.08	53921.13	82525.18	110138.98	298100.28
Median		58200.00	31800.00	33000.00	68100.00	203000.00	421000.00	863400.00
Std. Deviation		193254.94	164123.47	350393.02	519996.53	795844.03	1062141.84	2874775.04
Variance		37347472266.50	26936513422.09	122775269863.70	270396395468.30	633367716635.00	1128145283027.00	8264331502991.00
Skewness		2.62	4.54	5.48	4.32	3.89	2.62	3.75
Std. Error of Skewness		0.26	0.25	0.26	0.25	0.25	0.25	0.25
Kurtosis		7.99	23.67	32.05	20.46	17.58	7.11	16.59
Std. Error of Kurtosis		0.51	0.50	0.51	0.50	0.50	0.50	0.50
Range		1019614.00	1059807.00	2369794.00	3209837.00	4847740.00	5079302.00	17076410.00
Minimum		386.00	193.00	206.00	163.00	2260.00	698.00	37590.00
Maximum		1020000.00	1060000.00	2370000.00	3210000.00	4850000.00	5080000.00	17114000.00
Sum		12193844.00	7884597.00	11757759.00	22094644.00	44406780.00	71900908.00	170238532.00
Percentiles	25%	14850.00	8285.00	14400.00	9885.00	66050.00	163500.00	323290.00
	50%	58200.00	31800.00	33000.00	68100.00	203000.00	421000.00	863400.00
	75%	168500.00	87550.00	128000.00	222000.00	545500.00	845000.00	2086600.00

Table 10 Descriptive statistics of all components detected in the WB and their intensity measured by the Odyssey scanner

			Kohorte chronisch Infizierter Patientin mit pegINFa-Therapie					ELISA-Analyse			ELISA-Analyse		
			Blut-Entnahme				Response to Therapy	mit Standard berechnet ng/ml			Berechnung mit Standard prozentueller Anteil		
ID	Code	Proben ID	Zeitpunk	Country	Behandlung	Genotype	1=NR, 2=R	C20/02	MA18/7	Q19/10	SHBs	LHBs	MHBs
1	YCF	S0707138-3	BI	China	90 mcg PEG-IFN 48 weeks	B	2	2827,99	144,28	75,73	92,22	5,10	2,68
2	YCF	S0770510-3	W12	China	90 mcg PEG-IFN 48 weeks	B	2	259,48	41,83	2,19	83,04	16,12	0,84
3	YCF	S0831908-1	W24	China	90 mcg PEG-IFN 48 weeks	B	2	35,09	0,78	0,00	97,78	2,22	0
4	YZJ	S0770517-7	BI	China	90 mcg PEG-IFN 48 weeks	B	1	43042,98	8342,23	5872,15	66,98	19,38	13,64
5	YZJ	S0770522-4	W12	China	90 mcg PEG-IFN 48 weeks	B	1	13236,87	1982,60	1485,32	73,80	14,98	11,22
6	YZJ	S0836237-7	W24	China	90 mcg PEG-IFN 48 weeks	B	1	18279,53	2994,81	4716,24	57,82	16,38	25,80
7	ZZH	S0770520-6	BI	China	180 mcg PEG-IFN 24 weeks	B	1	21408,79	4685,39	2653,29	65,72	21,89	12,39
8	ZZH	S0770525-5	W12	China	180 mcg PEG-IFN 24 weeks	B	1	24807,39	4486,52	2881,60	70,30	18,09	11,62
9	ZZH	S0836239-7	W24	China	180 mcg PEG-IFN 24 weeks	B	1	10050,30	1415,64	1069,80	75,27	14,09	10,64
10	XX	S0810403-4	BI	China	180 mcg PEG-IFN 24 weeks	B	1	395484,24	84922,77	39090,02	68,64	21,47	9,88
11	XX	S0836232-5	W12	China	180 mcg PEG-IFN 24 weeks	B	1	10882,52	1699,20	1389,43	71,62	15,61	12,77
12	XX	S0932080-6	W24	China	180 mcg PEG-IFN 24 weeks	B	1	3846,93	715,63	383,56	71,43	18,60	9,97
13	XX	S0988614-5	W48	China	180 mcg PEG-IFN 24 weeks	B	1	2143,65	489,36	92,37	72,86	22,83	4,31
14	YJ	S0988562-4	BI	China	90 mcg PEG-IFN 24 weeks	B	2	3,40	0,55	0,00	83,88	16,12	0
15	YJ	S0988581-5	W12	China	90 mcg PEG-IFN 24 weeks	B	2	18,90	3,59	0,00	80,99	19,01	0
16	YJ	S0932079-6	W24	China	90 mcg PEG-IFN 24 weeks	B	2	19,32	3,63	0,00	81,22	18,78	0
17	YJ	S1219366-4	W48	China	90 mcg PEG-IFN 24 weeks	B	2	50,23	11,14	0,00	77,81	22,19	0
18	KW	S0932043-6	BI	China	90 mcg PEG-IFN 48 weeks	B	1	274808,0	61474,64	10567,51	73,78	22,37	3,85
19	KW	S0932069-4	W12	China	90 mcg PEG-IFN 48 weeks	B	1	28727,16	4942,22	4315,07	67,78	17,20	15,02
20	KW	S0988587-7	W24	China	90 mcg PEG-IFN 48 weeks	B	1	5357,53	1214,56	219,83	73,23	22,67	4,10
21	ZYH	S0932048-7	BI	China	180 mcg PEG-IFN 48 weeks	B	2	3744,99	695,28	80,33	79,29	18,57	2,15
22	ZYH	S0988584-5	W12	China	180 mcg PEG-IFN 48 weeks	B	2	2312,32	983,65	77,02	54,13	42,54	3,33
23	ZYH	S0988597-4	W24	China	180 mcg PEG-IFN 48 weeks	B	2	251,14	38,25	1,61	84,13	15,23	0,64
24	HZP	S0988555-7	BI	China	180 mcg PEG-IFN 48 weeks	C	1						
25	HZP	S1219342-4	W12	China	180 mcg PEG-IFN 48 weeks	C	1						
26	HZP	S1219347-4	W24	China	180 mcg PEG-IFN 48 weeks	C	1						
27	HBJ	S0828776-3	BI	China	90 mcg PEG-IFN 48 weeks	B	2	885,83	143,24	0,54	83,77	16,17	0,06
28	HBJ	S0920053-4	W12	China	90 mcg PEG-IFN 48 weeks	B	2	1003,20	355,70	2,67	64,28	35,46	0,27
29	HBJ	S0920060-3	W24	China	90 mcg PEG-IFN 48 weeks	B	2	1314,23	339,96	2,91	73,91	25,87	0,22
30	LRH	S0920041-4	BI	China	180 mcg PG-IFN 48 weeks	B	1	75086,91	13916,47	6183,95	73,23	18,53	8,24
31	LRH	S0920047-4	W12	China	180 mcg PG-IFN 48 weeks	B	1	2292,77	123,20	0,00	94,63	5,37	0
32	LRH	S0920063-4	W24	China	180 mcg PG-IFN 48 weeks	B	1	3355,47	195,03	7,77	93,96	5,81	0,23
33	LJ	S0920042-4	BI	China	180 mcg PG-IFN 48 weeks	B	1	52317,10	6309,37	4860,52	78,65	12,06	9,29
34	LJ	S0920057-4	W12	China	180 mcg PG-IFN 48 weeks	B	1	29412,36	2426,63	3300,24	80,53	8,25	11,22
35	LJ	S1010261-2	W24	China	180 mcg PG-IFN 48 weeks	B	1	46611,11	2782,87	1594,33	90,61	5,97	3,42

Appendix Table 1Appendix Table 1 Part1 of Data and Results of entire ELISA- Analysis

ID	Code	Proben ID	Blut-Entnahme Zeitpunkt	Country	Behandlung	Genotype	Response to Therapy 1=NR, 2=R	ELISA-Analyse mit Standard berechnet ng/ml			ELISA-Analyse Berechnung mit Standard prozentueller Anteil		
36	HYL	S0920043-4	Bl	China	90 mcg PEG-IFN 48 weeks	B	2	37782,67	1570,19	1874,70	90,88	4,16	4,96
37	HYL	S0920056-4	W12	China	90 mcg PEG-IFN 48 weeks	B	2	3287,09	349,53	35,56	88,28	10,63	1,08
38	HYL	S1010255-7	W24	China	90 mcg PEG-IFN 48 weeks	B	2	1451,56	105,31	2,70	92,56	7,26	0,19
39	HWZ	S0976439-7	Bl	China	180 mcg PEG-IFN 48 weeks	B	2	1093,47	284,51	5,58	73,47	26,02	0,51
40	HWZ	S0920055-4	W12	China	180 mcg PEG-IFN 48 weeks	B	2	405,98	60,01	1,11	84,95	14,78	0,27
41	HWZ	S1010256-2	W24	China	180 mcg PEG-IFN 48 weeks	B	2	24,63	22,46	0,00	8,80	91,20	0,00
42	HWZ	S1182070-4	W36	China	180 mcg PEG-IFN 48 weeks	B	2	4,06	0,00	0,00	100	0	0
43	LJH	S0976438-7	Bl	China	90 mcg PEG-IFN 24 weeks	B	1	21512,62	240,23	221,84	97,85	1,12	1,03
44	LJH	S0920054-4	W12	China	90 mcg PEG-IFN 24 weeks	B	1	1310,25	0,00	0,00	100	0	0
45	LJH	S1010257-2	W24	China	90 mcg PEG-IFN 24 weeks	B	1	36091,16	1831,70	170,29	94,45	5,08	0,47
46	YJ	S0823607-5	Bl	China	180 mcg PEG-IFN 48 weeks	B	2	10611,31	0,00	125,30	98,82	0	1,18
47	YJ	S0882313-3	W12	China	180 mcg PEG-IFN 48 weeks	B	2	4371,09	2428,97	0,00	44,43	55,57	0
48	YJ	S0954632-5	W24	China	180 mcg PEG-IFN 48 weeks	B	2	1476,88	56,45	0,00	96,18	3,82	0
49	HFL	S0135070-5	Bl	China	180 mcg PEG-IFN + Placebo	C	2	1568,28	55,69	1,81	96,33	3,55	0,12
50	HFL	S0173626-6	W12	China	180 mcg PEG-IFN + Placebo	C	2	355,75	4,85	0,00	98,64	1,36	0
51	HFL	S0173651-10	W24	China	180 mcg PEG-IFN + Placebo	C	2	270,42	4,66	0,00	98,28	1,72	0
52	LJL	S0120029-3	Bl	China	180 mcg PEG-IFN + Placebo	C	2	947,87	93,69	1,06	90,00	9,88	0,11
53	LJL	S0142144-2	W12	China	180 mcg PEG-IFN + Placebo	C	2	1118,62	57,06	0,85	94,82	5,10	0,08
54	LJL	S0131033-3	W24	China	180 mcg PEG-IFN + Placebo	C	2	3823,85	25,16	5,04	99,21	0,66	0,13
55	WTQ	S0120033-2	Bl	China	180 mcg PEG-IFN + Placebo	C	1	97864,44	15022,69	5156,50	79,38	15,35	5,27
56	WTQ	S0142147-2	W12	China	180 mcg PEG-IFN + Placebo	C	1	95974,77	14844,57	2939,01	81,47	15,47	3,06
57	WTG	S0131030-2	W24	China	180 mcg PEG-IFN + Placebo	C	1	25322,18	2615,14	1975,89	81,87	10,33	7,80
58	GZY	S0127533-5	Bl	China	180 mcg PEG-IFN + Placebo	C	1	54593,70	3845,84	7407,09	79,39	7,04	13,57
59	GZY	S0142145-3	W12	China	180 mcg PEG-IFN + Placebo	C	1	36980,63	2248,73	4479,12	81,81	6,08	12,11
60	GZY	S0131037-3	W24	China	180 mcg PEG-IFN + Placebo	C	1	41440,73	2375,95	4435,78	83,56	5,73	10,70
61	ZC	S0118957-3	Bl	China	180 mcg PEG-IFN + Placebo	B	2	2874,41	0,00	0,00	100	0	0
62	ZC	S0142449-2	W12	China	180 mcg PEG-IFN + Placebo	B	2	103,64	0,00	0,00	100	0	0
63	ZC	S0142441-2	W24	China	180 mcg PEG-IFN + Placebo	B	2	48,30	0,00	0,00	100	0	0

Appendix Table 2Appendix Table 1 Part 2 of Data and Results of entire ELISA- Analysis

ID	Code	Proben ID	Blut-Entnahme Zeitpunk	Country	Behandlung	Genotype	Response to Therapy 1=NR, 2=R	ELISA-Analyse mit Standard berechnet ng/ml			ELISA-Analyse Berechnung mit Standard prozentueller Anteil		
36	HYL	S0920043-4	BI	China	90 mcg PEG-IFN 48 weeks	B	2	37782,67	1570,19	1874,70	90,88	4,16	4,96
37	HYL	S0920056-4	W12	China	90 mcg PEG-IFN 48 weeks	B	2	3287,09	349,53	35,56	88,28	10,63	1,08
38	HYL	S1010255-7	W24	China	90 mcg PEG-IFN 48 weeks	B	2	1451,56	105,31	2,70	92,56	7,26	0,19
39	HWZ	S0976439-7	BI	China	180 mcg PEG-IFN 48 weeks	B	2	1093,47	284,51	5,58	73,47	26,02	0,51
40	HWZ	S0920055-4	W12	China	180 mcg PEG-IFN 48 weeks	B	2	405,98	60,01	1,11	84,95	14,78	0,27
41	HWZ	S1010256-2	W24	China	180 mcg PEG-IFN 48 weeks	B	2	24,63	22,46	0,00	8,80	91,20	0,00
42	HWZ	S1182070-4	W36	China	180 mcg PEG-IFN 48 weeks	B	2	4,06	0,00	0,00	100	0	0
43	LJH	S0976438-7	BI	China	90 mcg PEG-IFN 24 weeks	B	1	21512,62	240,23	221,84	97,85	1,12	1,03
44	LJH	S0920054-4	W12	China	90 mcg PEG-IFN 24 weeks	B	1	1310,25	0,00	0,00	100	0	0
45	LJH	S1010257-2	W24	China	90 mcg PEG-IFN 24 weeks	B	1	36091,16	1831,70	170,29	94,45	5,08	0,47
46	YJ	S0823607-5	BI	China	180 mcg PEG-IFN 48 weeks	B	2	10611,31	0,00	125,30	98,82	0	1,18
47	YJ	S0882313-3	W12	China	180 mcg PEG-IFN 48 weeks	B	2	4371,09	2428,97	0,00	44,43	55,57	0
48	YJ	S0954632-5	W24	China	180 mcg PEG-IFN 48 weeks	B	2	1476,88	56,45	0,00	96,18	3,82	0
49	HFL	S0135070-5	BI	China	180 mcg PEG-IFN + Placebo	C	2	1568,28	55,69	1,81	96,33	3,55	0,12
50	HFL	S0173626-6	W12	China	180 mcg PEG-IFN + Placebo	C	2	355,75	4,85	0,00	98,64	1,36	0
51	HFL	S0173651-10	W24	China	180 mcg PEG-IFN + Placebo	C	2	270,42	4,66	0,00	98,28	1,72	0
52	LJL	S0120029-3	BI	China	180 mcg PEG-IFN + Placebo	C	2	947,87	93,69	1,06	90,00	9,88	0,11
53	LJL	S0142144-2	W12	China	180 mcg PEG-IFN + Placebo	C	2	1118,62	57,06	0,85	94,82	5,10	0,08
54	LJL	S0131033-3	W24	China	180 mcg PEG-IFN + Placebo	C	2	3823,85	25,16	5,04	99,21	0,66	0,13
55	WTQ	S0120033-2	BI	China	180 mcg PEG-IFN + Placebo	C	1	97864,44	15022,69	5156,50	79,38	15,35	5,27
56	WTQ	S0142147-2	W12	China	180 mcg PEG-IFN + Placebo	C	1	95974,77	14844,57	2939,01	81,47	15,47	3,06
57	WTG	S0131030-2	W24	China	180 mcg PEG-IFN + Placebo	C	1	25322,18	2615,14	1975,89	81,87	10,33	7,80
58	GZY	S0127533-5	BI	China	180 mcg PEG-IFN + Placebo	C	1	54593,70	3845,84	7407,09	79,39	7,04	13,57
59	GZY	S0142145-3	W12	China	180 mcg PEG-IFN + Placebo	C	1	36980,63	2248,73	4479,12	81,81	6,08	12,11
60	GZY	S0131037-3	W24	China	180 mcg PEG-IFN + Placebo	C	1	41440,73	2375,95	4435,78	83,56	5,73	10,70
61	ZC	S0118957-3	BI	China	180 mcg PEG-IFN + Placebo	B	2	2874,41	0,00	0,00	100	0	0
62	ZC	S0142449-2	W12	China	180 mcg PEG-IFN + Placebo	B	2	103,64	0,00	0,00	100	0	0
63	ZC	S0142441-2	W24	China	180 mcg PEG-IFN + Placebo	B	2	48,30	0,00	0,00	100	0	0

Appendix Table 3 Appendix Table 1 Part 3 of Data and Results of entire ELISA- Analysis

ID	Code	Proben ID	Blut-Entnahme Zeitpunk	Country	Behandlung	Genotype	Response to Therapy 1=NR, 2=R	ELISA-Analyse mit Standard berechnet ng/ml			ELISA-Analyse Berechnung mit Standard prozentueller Anteil		
64	WN	S0119006-3	Bl	China	180 mcg PEG-IFN + Placebo	B	1	30240,90	2679,92	2357,60	83,34	8,86	7,80
65	WN	S0144830-2	W12	China	180 mcg PEG-IFN + Placebo	B	1	9116,49	194,34	562,41	91,70	2,13	6,17
66	WN	S0136202-2	W24	China	180 mcg PEG-IFN + Placebo	B	1	2355,95	48,43	73,07	94,84	2,06	3,10
67	HAY	S0129766-5	Bl	China	180 mcg PEG-IFN + Placebo	C	2	592,90	0,00	0,00	100	0	0
68	HAY	S0144831-4	W12	China	180 mcg PEG-IFN + Placebo	C	2	34,96	27,38	0,77	19,45	78,34	2,21
69	HAY	S0136189-5	W24	China	180 mcg PEG-IFN + Placebo	C	2	4,92	0,00	0,00	100	0	0
70	LL	S0119370-2	Bl	China	180 mcg PEG-IFN + Placebo	C	1	21556,82	13233,27	1193,00	33,08	61,39	5,53
71	LL	S0144286-2	W12	China	180 mcg PEG-IFN + Placebo	C	1	7500,00	6032,25	215,00	16,70	80,43	2,87
72	LL	S0119413-7	W24	China	180 mcg PEG-IFN + Placebo	C	1	9909,09	767,27	253,00	89,70	7,74	2,55
73	ZLP	S0131565-2	Bl	China	180 mcg PEG-IFN + Placebo	B	1	31318,66	24320,07	1019,86	19,09	77,65	3,26
74	ZLP	S0142931-2	W12	China	180 mcg PEG-IFN + Placebo	B	1	2504,46	2150,45	11,10	13,69	85,86	0,44
75	ZLP	S0142934-3	W24	China	180 mcg PEG-IFN + Placebo	B	1	522,74	328,63	1,37	36,87	62,87	0,26
76	CZP	S0130393-9	Bl	China	180 mcg PEG-IFN + Placebo	C	2	92,26	91,98	0,00	0,30	99,70	0
77	CZP	S0118750-7	W12	China	180 mcg PEG-IFN + Placebo	C	2	932,64	0,00	2,04	99,78	0	0,22
78	CZP	S0168672-5	W24	China	180 mcg PEG-IFN + Placebo	C	2	979,84	246,65	1,73	74,65	25,17	0,18
79	CGJ	S0130411-9	Bl	China	180 mcg PEG-IFN + Placebo	C	1	103393,94	57205,55	6168,00	38,71	55,33	5,97
80	CGJ	S0118666-8	W12	China	180 mcg PEG-IFN + Placebo	C	1	4231,06	3000,60	129,33	26,02	70,92	3,06
81	CGJ	S0169369-11	W24	China	180 mcg PEG-IFN + Placebo	C	1	2604,04	1635,44	4,00	37,04	62,80	0,15
82	ZTG	S0175741-2	Bl	China	180 mcg PEG-IFN + Placebo	B	2	32880,87	8215,19	2330,50	67,93	24,98	7,09
83	ZTG	S0194520-2	W12	China	180 mcg PEG-IFN + Placebo	B	2	340,90	188,61	43,69	31,86	55,33	12,82
84	ZTG	S0190184-3	W24	China	180 mcg PEG-IFN + Placebo	B	2	1131,81	422,30	5,07	62,24	37,31	0,45
85	KI	E0832450-12	Bl	German	180 mcg PEG-IFN + Placebo	D	2	73058,08	41742,62	47903,33	-22,71	57,14	65,57
86	KI	E0836853-4	W12	German	180 mcg PEG-IFN + Placebo	D	2	63272,73	41974,68	9812,00	18,15	66,34	15,51
87	KI	E0895088-6	W24	German	180 mcg PEG-IFN + Placebo	D	2	12666,67	8008,44	1178,67	27,47	63,22	9,31
88	NJ	E0832451-8	Bl	German	180 mcg PEG-IFN + Placebo	A	2	158829,23	86735,99	16430,00	35,05	54,61	10,34
89	NJ	E0895096-8	W12	German	180 mcg PEG-IFN + Placebo	A	2	25750,39	20405,06	2077,33	12,69	79,24	8,07
90	NJ	E0895095-4	W24	German	180 mcg PEG-IFN + Placebo	A	2	207,14	97,53	0,00	52,92	47,08	0
91	ISC	E0830810-12	Bl	German	180 mcg PEG-IFN + Placebo	D	1	32410,60	3008,44	3627,50	79,53	9,28	11,19
92	ISC	E0932137-8	W12	German	180 mcg PEG-IFN + Placebo	D	1	24395,93	13709,46	14165,00	-14,26	56,20	58,06
93	ISC	E0932143-12	W24	German	180 mcg PEG-IFN + Placebo	D	1	83149,65	66200,72	14349,00	3,13	79,62	17,26

Appendix Table 4 Appendix Table 1 Part 4 of Data and Results of entire ELISA- Analysis

ID	Code	Proben ID	Blut-Entnahme Zeitpunk	Country	Behandlung	Genotype	Response to Therapy 1=NR, 2=R	ELISA-Analyse mit Standard berechnet ng/ml			ELISA-Analyse Berechnung mit Standard prozentueller Anteil		
94	TL	E0831306-12	Bl	Poland	180 mcg PEG-IFN + Placebo	A	1	317443,18	166256,78	64775,00	27,22	52,37	20,41
95	TL	E0887130-8	W12	Poland	180 mcg PEG-IFN + Placebo	A	1	206486,49	45198,41	10090,46	73,22	21,89	4,89
96	TL	E0984963-12	W24	Poland	180 mcg PEG-IFN + Placebo	A	1	182162,16	39841,27	9658,52	72,83	21,87	5,30
97	WL	S1204223-4	Bl	China	180 mcg PEG-IFN 24 weeks	B	2	21185,05	5611,11	929,18	69,13	26,49	4,39
98	WL	S1209029-4	W12	China	180 mcg PEG-IFN 24 weeks	B	2	4132,51	1306,85	168,73	64,29	31,62	4,08
99	WL	S1268621-7	W24	China	180 mcg PEG-IFN 24 weeks	B	2	42,04	0,00	0,00	n	n	n
100	MK	E0665625-4	Bl	Poland	180 mcg PEG-IFN + Placebo	A	2	413595,23	163,69	81,56	99,94	0,04	0,02
101	MK	E0722991-4	W12	Poland	180 mcg PEG-IFN + Placebo	A	2	13413,10	208,22	57,38	98,02	1,55	0,43
102	MK	E0734369-4	W24	Poland	180 mcg PEG-IFN + Placebo	A	2	28458,00	201,00	45,94	99,13	0,71	0,16
103	JC	E0668908-8	Bl	Poland	180 mcg PEG-IFN + Lamivudin	A	1	858,11	168,15	21,89	77,85	19,60	2,55
104	JC	E0734392-4	W12	Poland	180 mcg PEG-IFN + Lamivudin	A	1	23351,35	5115,08	78,25	77,76	21,90	0,34
105	JC	E0734433-4	W24	Poland	180 mcg PEG-IFN + Lamivudin	A	1	15513,51	3388,89	52,00	77,82	21,84	0,34
106	XXF	S0066868-4	Bl	China	180 mcg PEG-IFN + Lamivudin	C	2	11067,57	2423,61	2156,07	58,62	21,90	19,48
107	XXF	S0131085-6	W12	China	180 mcg PEG-IFN + Lamivudin	C	2	6600,00	1445,24	521,03	70,21	21,90	7,89
108	XXF	S0152871-1	W24	China	180 mcg PEG-IFN + Lamivudin	C	2	1447,75	316,07	58,76	74,11	21,83	4,06
109	XXF	S0180326-2	W48	China	180 mcg PEG-IFN + Lamivudin	C	2	32,30	6,97	2,41	70,95	21,59	7,46
110	CGQ	S0067428-2	Bl	China	180 mcg PEG-IFN + Lamivudine		2	3675,68	802,58	110,23	75,17	21,83	3,00
111	CGQ	S0079627-2	W12	China	180 mcg PEG-IFN + Lamivudine		2	0,00	0,00	7,67	0	0	0
112	CGQ	S0133604-1	W24	China	180 mcg PEG-IFN + Lamivudine		2	0,00	0,00	2,29	0	0	0
113	XMZ	S0066454-2	Bl	China	180 mcg PEG-IFN + Lamivudin	C	1	3371,60	62,85	0,00	98,14	1,86	0,00
114	XMZ	S0121432-6	W12	China	180 mcg PEG-IFN + Lamivudin	C	1	6653,11	690,90	3,97	89,56	10,38	0,06

Appendix Table 5 Appendix Table 1 Part 5 of Data and Results of entire ELISA- Analysis

		Kohorte chronisch Infizierter Patientin mit pegINFa-Therapie																							
		Blut-				Response to	Western Blot Analyse			Western Blot Analyse			Western Blot Analyse									Western Blot Analyse					
		Entnahme				Therapy	Angaben in Prozent Anteil			absolute gemessene Intensitäten			absolute gemessene Intensitäten						Summe	prozentueller Anteil						
ID	Code	Zeitpunkt	Country	Behandlung	Genotype	1=NR, 2=R	LHBS	MHS	SHBS	LHBS	MHBS	SHBS	LHBsglyc	LHBs	MHBglyc	MHBs	SHBsglyc	SHBs		LHBsglyc	LHBs	MHBglyc	MHBs	SHBsglyc	SHBs
1	YCF	BI	China	90 mcg PEG-IFN 48 weeks	B	2	X																		
2	YCF	W12	China	90 mcg PEG-IFN 48 weeks	B	2	6,07	0,09	93,84	11546	163	178400	10600	946		163	14400	164000	190109	5,58	0,50	0,00	0,09	7,57	86,27
3	YCF	W24	China	90 mcg PEG-IFN 48 weeks	B	2	31,98	3,35	64,67	12393	1300	25060	12200	193		1300	2260	22800	38753	31,48	0,50	0,00	3,35	5,83	58,83
4	YZJ	BI	China	90 mcg PEG-IFN 48 weeks	B	1	26,40	17,20	56,40	505000	329000	1079000	316000	189000	117000	212000	523000	556000	1913000	16,52	9,88	6,12	11,08	27,34	29,06
5	YZJ	W12	China	90 mcg PEG-IFN 48 weeks	B	1	17,63	7,78	74,59	232800	102800	985000	163000	69800	32600	70200	426000	559000	1320600	12,34	5,29	2,47	5,32	32,26	42,33
6	YZJ	W24	China	90 mcg PEG-IFN 48 weeks	B	1	17,41	17,70	64,89	362000	368000	1349000	234000	128000	164000	204000	591000	758000	2079000	11,26	6,16	7,89	9,81	28,43	36,46
7	ZZH	BI	China	180 mcg PEG-IFN 24 weeks	B	1	19,98	8,84	71,18	279000	123400	994000	185000	94000	30200	93200	351000	643000	1396400	13,25	6,73	2,16	6,67	25,14	46,05
8	ZZH	W12	China	180 mcg PEG-IFN 24 weeks	B	1	19,41	6,81	73,78	227000	79700	863000	156000	71000	20300	59400	326000	537000	1169700	13,34	6,07	1,74	5,08	27,87	45,91
9	ZZH	W24	China	180 mcg PEG-IFN 24 weeks	B	1	21,04	2,43	76,53	100900	11630	367000	54400	46500	3910	7720	118000	249000	479530	11,34	9,70	0,82	1,61	24,61	51,93
10	XX	BI	China	180 mcg PEG-IFN 24 weeks	B	1	13,75	14,82	71,43	464000	500000	2410000	264000	200000	170000	330000	500000	1910000	3374000	7,82	5,93	5,04	9,78	14,82	56,61
11	XX	W12	China	180 mcg PEG-IFN 24 weeks	B	1	20,56	13,56	65,87	354000	233500	1134000	202000	152000	75500	158000	435000	699000	1721500	11,73	8,83	4,39	9,18	25,27	40,60
12	XX	W24	China	180 mcg PEG-IFN 24 weeks	B	1	17,10	9,19	73,71	204400	109800	881000	126000	78400	29000	80800	255000	626000	1195200	10,54	6,56	2,43	6,76	21,34	52,38
13	XX	W48	China	180 mcg PEG-IFN 24 weeks	B	1	19,91	18,77	61,33	122900	124400	642900	53900	69000	42700	81700	59900	583000	890200	6,05	7,75	4,80	9,18	6,73	65,49
14	YJ	BI	China	90 mcg PEG-IFN 24 weeks	B	2	8,20	7,80	84,00	410000	390000	4200000	259000	151000	114000	276000	1250000	2950000	5000000	5,18	3,02	2,28	5,52	25,00	59,00
15	YJ	W12	China	90 mcg PEG-IFN 24 weeks	B	2	9,45	3,77	86,78	107900	43000	990800	81800	26100	39300	3700	57800	933000	1141700	7,16	2,29	3,44	0,32	5,06	81,72
16	YJ	W24	China	90 mcg PEG-IFN 24 weeks	B	2	13,61	15,37	71,02	450000	508000	2348000	269000	181000	128000	380000	298000	2050000	3306000	8,14	5,47	3,87	11,49	9,01	62,01
17	YJ	W48	China	90 mcg PEG-IFN 24 weeks	B	2	8,19	12,34	79,47	144500	320100	2339000	56500	88000	73100	247000	559000	1780000	2803600	2,02	3,14	2,61	8,81	19,94	63,49
18	KW	BI	China	90 mcg PEG-IFN 48 weeks	B	1	11,81	15,14	73,05	668000	856000	4130000	451000	217000	271000	585000	1250000	2880000	5654000	7,98	3,84	4,79	10,35	22,11	50,94
19	KW	W12	China	90 mcg PEG-IFN 48 weeks	B	1	19,41	18,29	62,31	607000	572000	1949000	383000	224000	150000	422000	499000	1450000	3128000	12,24	7,16	4,80	13,49	15,95	46,36
20	KW	W24	China	90 mcg PEG-IFN 48 weeks	B	1	16,41	9,27	74,31	222200	125500	1006000	145000	77200	30000	95500	217000	789000	1353700	10,71	5,70	2,22	7,05	16,03	58,28
21	ZYH	BI	China	180 mcg PEG-IFN 48 weeks	B	2	12,57	6,12	81,30	237100	115400	1533000	157000	80100	29400	86000	313000	1220000	1885500	8,33	4,25	1,56	4,56	16,60	64,70
22	ZYH	W12	China	180 mcg PEG-IFN 48 weeks	B	2	13,27	8,87	77,86	290000	193900	1702000	174000	116000	48900	145000	392000	1310000	2185900	7,96	5,31	2,24	6,63	17,93	59,93
23	ZYH	W24	China	180 mcg PEG-IFN 48 weeks	B	2	x																		
24	HZP	BI	China	180 mcg PEG-IFN 48 weeks	C	1																			
25	HZP	W12	China	180 mcg PEG-IFN 48 weeks	C	1																			
26	HZP	W24	China	180 mcg PEG-IFN 48 weeks	C	1																			
27	HBJ	BI	China	90 mcg PEG-IFN 48 weeks	B	2	17,41	5,81	76,78	21070	7030	92900	7770	13300	1920	5110	34600	58300	121000	6,42	10,99	1,59	4,22	28,60	48,18
28	HBJ	W12	China	90 mcg PEG-IFN 48 weeks	B	2	26,60	7,82	65,58	58700	17260	144700	26900	31800	4460	12800	53100	91600	220660	12,19	14,41	2,02	5,80	24,06	41,51
29	HBJ	W24	China	90 mcg PEG-IFN 48 weeks	B	2	29,82	6,83	63,35	54000	12370	114700	28100	25900	3800	8570	47200	67500	181070	15,52	14,30	2,10	4,73	26,07	37,28
30	LRH	BI	China	180 mcg PG-IFN 48 weeks	B	1	9,40	9,86	80,74	98800	103700	849000	45700	53100	36300	67400	234000	615000	1051500	4,35	5,05	3,45	6,41	22,25	58,49
31	LRH	W12	China	180 mcg PG-IFN 48 weeks	B	1	20,42	5,83	73,76	24500	6990	88500	12500	12000	1450	5540	26900	61600	119990	10,42	10,00	1,21	4,62	22,42	51,34
32	LRH	W24	China	180 mcg PG-IFN 48 weeks	B	1	14,21	5,24	80,55	24010	8860	136100	14600	9410	2160	6700	65300	70800	168970	8,64	5,57	1,28	3,97	38,65	41,90
33	LJ	BI	China	180 mcg PG-IFN 48 weeks	B	1	8,48	8,78	82,74	172700	179000	1686000	85600	87100	51000	128000	656000	1030000	2037700	4,20	4,27	2,50	6,28	32,19	50,55
34	LJ	W12	China	180 mcg PG-IFN 48 weeks	B	1	15,27	14,01	70,72	112300	103000	520000	67800	44500	34900	68100	144000	376000	735300	9,22	6,05	4,75	9,26	19,58	51,14
35	LJ	W24	China	180 mcg PG-IFN 48 weeks	B	1	11,83	11,34	76,83	88500	84900	575000	52300	36200	33000	51900	203000	372000	748400	6,99	4,84	4,41	6,93	27,12	49,71

Appendix Table 6 Appendix Table 1 Part1 of Data and Results of entire Western-Blot- Analysis

ID	Code	Blut-Entnahme Zeitpunkt	Country	Behandlung	Genotype	Response to Therapy 1=NR, 2=R	Western Blot Analyse Angaben in Prozent Anteil			Western Blot Analyse absolute gemessene Intensitäten			Western Blot Analyse absolute gemessene Intensitäten						Summe	Western Blot Analyse prozentueller Anteil					
							LHBS	MHS	SHBS	LHBS	MHBS	SHBS	LHBsglyc	LHBs	MHBsglyc	MHBs	SHBsglyc	SHBs		LHBsglyc	LHBs	MHBsglyc	MHBs	SHBsglyc	SHBs
64	WN	Bl	China	180 mcg PEG-IFN + Placebo	B	1	x																		
65	WN	W12	China	180 mcg PEG-IFN + Placebo	B	1	2,52	4,84	92,64	6400	12310	235500	2200	4200	5850	6460	99500	136000	254210	0,87	1,65	2,30	2,54	39,14	53,50
66	WN	W24	China	180 mcg PEG-IFN + Placebo	B	1	9,55	2,68	87,77	11290	3170	103800	3220	8070	1480	1690	59100	44700	118260	2,72	6,82	1,25	1,43	49,97	37,80
67	HAY	Bl	China	180 mcg PEG-IFN + Placebo	C	2	x																		
68	HAY	W12	China	180 mcg PEG-IFN + Placebo	C	2	1,35	1,17	97,47	1003	870	72200	386	617	358	512	62000	10200	74073	0,52	0,83	0,48	0,69	83,70	13,77
69	HAY	W24	China	180 mcg PEG-IFN + Placebo	C	2	6,79	2,26	90,94	4400	1466	58910	1510	2890	256	1210	53700	5210	64776	2,33	4,46	0,40	1,87	82,90	8,04
70	LL	Bl	China	180 mcg PEG-IFN + Placebo	C	1	12,71	24,22	63,07	161400	307600	801000	104000	57400	78600	229000	389000	412000	1270000	8,19	4,52	6,19	18,03	30,63	32,44
71	LL	W12	China	180 mcg PEG-IFN + Placebo	C	1	2,88	14,15	82,97	16290	80000	469000	6950	9340	21800	58200	203000	266000	565290	1,23	1,65	3,86	10,30	35,91	47,06
72	LL	W24	China	180 mcg PEG-IFN + Placebo	C	1	2,27	10,92	86,81	13680	65800	523000	5310	8370	16700	49100	222000	301000	602480	0,88	1,39	2,77	8,15	36,85	49,96
73	ZLP	Bl	China	180 mcg PEG-IFN + Placebo	B	1	17,60	24,62	57,78	451000	631000	1481000	337000	114000	324000	307000	1060000	421000	2563000	13,15	4,45	12,64	11,98	41,36	16,43
74	ZLP	W12	China	180 mcg PEG-IFN + Placebo	B	1	29,70	8,92	61,38	199000	59800	411300	142000	57000	19400	40400	69300	342000	670100	21,19	8,51	2,90	6,03	10,34	51,04
75	ZLP	W24	China	180 mcg PEG-IFN + Placebo	B	1	19,78	7,83	72,39	170800	67600	625000	114000	56800	19900	47700	185000	440000	863400	13,20	6,58	2,30	5,52	21,43	50,96
76	CZP	Bl	China	180 mcg PEG-IFN + Placebo	C	2	x																		
77	CZP	W12	China	180 mcg PEG-IFN + Placebo	C	2	x																		
78	CZP	W24	China	180 mcg PEG-IFN + Placebo	C	2	12,90	2,03	85,07	29300	4610	193200	21100	8200	1810	2800	108000	85200	227110	9,29	3,61	0,80	1,23	47,55	37,51
79	CGJ	Bl	China	180 mcg PEG-IFN + Placebo	C	1	2,91	22,97	74,12	304700	2405000	7760000	56700	248000	245000	2160000	3080000	4680000	1E+07	0,54	2,37	2,34	20,63	29,42	44,70
80	CGJ	W12	China	180 mcg PEG-IFN + Placebo	C	1	2,93	11,84	85,23	57000	230400	1658000	33400	23600	15400	215000	757000	901000	1945400	1,72	1,21	0,79	11,05	38,91	46,31
81	CGJ	W24	China	180 mcg PEG-IFN + Placebo	C	1	4,62	13,50	81,88	36000	105200	638000	18200	17800	19200	86000	172000	466000	779200	2,34	2,28	2,46	11,04	22,07	59,80
82	ZTG	Bl	China	180 mcg PEG-IFN + Placebo	B	2	9,83	15,26	74,92	362000	562000	2760000	339000	23000	269000	293000	1130000	1630000	3684000	9,20	0,62	7,30	7,95	30,67	44,25
83	ZTG	W12	China	180 mcg PEG-IFN + Placebo	B	2	9,67	13,34	77,00	281200	388000	2240000	203000	78200	195000	193000	1020000	1220000	2909200	6,98	2,69	6,70	6,63	35,06	41,94
84	ZTG	W24	China	180 mcg PEG-IFN + Placebo	B	2	9,80	12,47	77,74	260100	331000	2064000	210000	50100	138000	193000	614000	1450000	2655100	7,91	1,89	5,20	7,27	23,13	54,61
85	KI	Bl	German	180 mcg PEG-IFN + Placebo	D	2	10,45	9,01	80,54	114400	98700	882000	91300	23100	22700	76000	337000	545000	1095100	8,34	2,11	2,07	6,94	30,77	49,77
86	KI	W12	German	180 mcg PEG-IFN + Placebo	D	2	11,26	8,07	80,67	133300	95500	955000	93900	39400	46200	49300	270000	685000	1183800	7,93	3,33	3,90	4,16	22,81	57,86
87	KI	W24	German	180 mcg PEG-IFN + Placebo	D	2	5,10	4,81	90,09																
88	NJ	Bl	German	180 mcg PEG-IFN + Placebo	A	2	14,03	17,45	68,52	72300	89900	353000	46100	26200	32300	57600	171000	182000	515200	8,95	5,09	6,27	11,18	33,19	35,33
89	NJ	W12	German	180 mcg PEG-IFN + Placebo	A	2	7,96	14,69	77,35	10440	19260	101400	6520	3920	8360	10900	49500	51900	131100	4,97	2,99	6,38	8,31	37,76	39,59
90	NJ	W24	German	180 mcg PEG-IFN + Placebo	A	2	11,65	28,77	59,59	26600	65700	136100	13800	12800	26100	39600	63500	72600	228400	6,04	5,60	11,43	17,34	27,80	31,79
91	ISC	Bl	German	180 mcg PEG-IFN + Placebo	D	1	3,89	4,94	91,18	15860	20139	372000	7410	8450	239	19900	167000	205000	407999	1,82	2,07	0,06	4,88	40,93	50,25
92	ISC	W12	German	180 mcg PEG-IFN + Placebo	D	1	4,80	26,61	68,60	88500	491000	1266000	60200	28300	121000	370000	739000	527000	1845500	3,26	1,53	6,56	20,05	40,04	28,56
93	ISC	W24	German	180 mcg PEG-IFN + Placebo	D	1	1,98	9,54	88,48	1818	8760	81220	886	932	5440	3320	7220	74000	91798	0,97	1,02	5,93	3,62	7,87	80,61

Appendix Table 71 Part 2 of Data and Results of entire Western-Blot- Analysis

ID	Code	Blut-Entnahme Zeitpunkt	Country	Behandlung	Genotype	Response to Therapy 1=NR, 2=R	Western Blot Analyse Angaben in Prozent Anteil			Western Blot Analyse absolute gemessene Intensitäten			Western Blot Analyse absolute gemessene Intensitäten						Summe	Western Blot Analyse prozentueller Anteil					
							LHBS	MHS	SHBS	LHBS	MHBS	SHBS	LHBsglyc	LHBs	MHBsglyc	MHBs	SHBsglyc	SHBs		LHBsglyc	LHBs	MHBsglyc	MHBs	SHBsglyc	SHBs
94	TL	BI	Poland	180 mcg PEG-IFN + Placebo	A	1	4,72	18,41	76,87	51600	201400	841000	18500	33100	69400	132000	412000	429000	1094000	1,69	3,03	6,34	12,07	37,66	39,21
95	TL	W12	Poland	180 mcg PEG-IFN + Placebo	A	1	6,57	24,78	68,65	51400	193800	537000	32500	18900	76800	117000	374000	163000	782200	4,15	2,42	9,82	14,96	47,81	20,84
96	TL	W24	Poland	180 mcg PEG-IFN + Placebo	A	1	4,50	26,43	69,07	97300	354000	925000	58200	39100	183000	171000	547000	378000	1376300	4,23	2,84	13,30	12,42	39,74	27,46
97	WL	BI	China	180 mcg PEG-IFN 24 weeks	B	2	10,07	40,56	366,26	19570	675	111900	15100	4470	206	469	66800	45100	132145	11,43	3,38	0,16	0,35	50,55	34,13
98	WL	W12	China	180 mcg PEG-IFN 24 weeks	B	2	2,42	9,73	87,85	13310	53600	484000	7690	5620	31600	22000	303000	181000	550910	1,40	1,02	5,74	3,99	55,00	32,85
99	WL	W24	China	180 mcg PEG-IFN 24 weeks	B	2	x																		
100	MK	BI	Poland	180 mcg PEG-IFN + Placebo	A	2	18,30	24,68	57,02	468000	631000	1458000	337000	131000	324000	307000	1080000	378000	2557000	13,18	5,12	12,67	12,01	42,24	14,78
101	MK	W12	Poland	180 mcg PEG-IFN + Placebo	A	2	32,06	10,50	57,43	208800	68400	374000	142000	66800	19900	48500	54000	320000	651200	21,81	10,26	3,06	7,45	8,29	49,14
102	MK	W24	Poland	180 mcg PEG-IFN + Placebo	A	2	19,99	10,41	69,61	174600	90900	608000	114000	60600	35000	55900	185000	423000	873500	13,05	6,94	4,01	6,40	21,18	48,43
103	JC	BI	Poland	180 mcg PEG-IFN + Lamivudin	A	1	5,10	30,39	64,51	216000	1286000	2730000	105000	111000	446000	840000	1620000	1110000	4232000	2,48	2,62	10,54	19,85	38,28	26,23
104	JC	W12	Poland	180 mcg PEG-IFN + Lamivudin	A	1	5,66	29,39	64,95	174500	906000	2002000	126000	48500	312000	594000	1440000	562000	3082500	4,09	1,57	10,12	19,27	46,72	18,23
105	JC	W24	Poland	180 mcg PEG-IFN + Lamivudin	A	1	8,09	33,83	58,08	104800	438000	752000	73400	31400	135000	303000	425000	327000	1294800	5,67	2,43	10,43	23,40	32,82	25,25
106	XXF	BI	China	180 mcg PEG-IFN + Lamivudin	C	2	x																		
107	XXF	W12	China	180 mcg PEG-IFN + Lamivudin	C	2	2,90	14,63	82,47	14300	72000	406000	7620	6680	19900	52100	155000	251000	492300	1,55	1,36	4,04	10,58	31,48	50,99
108	XXF	W24	China	180 mcg PEG-IFN + Lamivudin	C	2	2,43	4,88	92,69	342	4490	85300		342		4490	31000	54300	90132		0,38		4,98	34,39	60,24
109	XXF	W48	China	180 mcg PEG-IFN + Lamivudin	C	2	3,07	4,68	92,25	243	4020	45800		243		4020	13400	32400	50063		0,49		8,03	26,77	64,72
110	CGQ	BI	China	180 mcg PEG-IFN + Lamivudine		2	x																		
111	CGQ	W12	China	180 mcg PEG-IFN + Lamivudine		2	9,26	12,26	78,48	3480	4610	29500		3480		4610	21900	7600	37590		9,26		12,26	58,26	20,22
112	CGQ	W24	China	180 mcg PEG-IFN + Lamivudine		2	4,51	5,69	94,31	2830	3570	59198		2830		3570	58500	698	65598		4,31		5,44	89,18	1,06
113	XMZ	BI	China	180 mcg PEG-IFN + Lamivudin	C	1																			
114	XMZ	W12	China	180 mcg PEG-IFN + Lamivudin	C	1																			
115	XMZ	W24	China	180 mcg PEG-IFN + Lamivudin	C	1																			

Appendix Table 8 Part 3 of Data and Results of entire Western-Blot- Analysis

Ethik-Votum

JUSTUS-LIEBIG UNIVERSITÄT GIESSEN

FACHBEREICH 11 MEDIZIN

Ethik-Kommission, Klinikstr. 29 (Alte Chirurgie), D-35385 Gießen

Prof. Dr. D. Glebe
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ETHIK-KOMMISSION
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Vorsitz: Prof. H. Tillmanns

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Gießen, 12. April 2017
Dr. Kr./

AZ.: **56/17**

Titel: *HBsAG as biomarker for optimized management of patients with hepatitis B virus (HBV) infection.*

Sehr geehrter Herr Prof. Glebe, Lieber Herr Glebe,

das genannte Projekt wurde außerhalb der Sitzung der Ethikkommission am 10.04.17 zusammen mit einem weiteren Mitglied der Kommission, Herrn Apotheker Brumhard, diskutiert.

Es handelt sich um eine Untersuchung an bereits vorhandenen Proben von Verlaufsseren (1 ml), die von der Universität Bonn und der Medizinischen Hochschule Hannover im Rahmen einer Kooperation zur Verfügung gestellt werden. Es soll mittels ELISA und Western-Blot die Zusammensetzung der HBV-Oberflächenproteine charakterisiert werden mit dem Ziel, neue Biomarker für die Therapiekontrolle zu entwickeln. Die Voten der Ethikkommissionen der MHH und der Universität Bonn zur Probengewinnung und –aufbewahrung im Rahmen einer Biobank liegen vor. In Gießen werden keine neuen Proben gewonnen.

Die Kommission hat keinerlei Einwände gegen die genannte Verwendung des Probenmaterials im Zuge der Kooperation mit Bonn und Hannover und wünscht gutes Gelingen.

Mit freundlichen Grüßen

Prof. Dr. H. Tillmanns
Vorsitzender

JUSTUS-LIEBIG UNIVERSITÄT GIESSEN

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Gießen, den 21. Januar 2019
Dr. Kr./

Votum der Ethik-Kommission AZ 257/18

Sehr geehrter Herr Prof. Dr. Glebe,

das Projekt (AZ **257/18:** *Molekularbiologische Charakterisierung von HBV/HDV-Isolaten aus Patientenproben sowie Untersuchung und Charakterisierung der Antigen- bzw. Antikörperverläufe im Rahmen von Infektionen oder HBV-Impfungen. Evaluierung und Verbesserung neuer und bestehender diagnostischer Nachweisverfahren zur Charakterisierung der Hepatitis B und D.)* wurde außerhalb der Sitzung der Ethikkommission begutachtet.

Hintergrund:
Infektionen mit dem pandemischen Hepatitis-B-Virus (HBV) sind nach Angaben der WHO immer noch eine der häufigsten Infektionskrankheiten weltweit mit geschätzten 257 Millionen chronisch HBV-infizierten Patienten. Trotz einer wirksamen Schutzimpfung sterben jährlich ca. 880.000 Menschen weltweit an den Folgen einer HBV-Infektion. Deutschland gehört zwar zu den Niedrigprävalenzländern, zwischen 0,3% und 0,5 % der Bevölkerung zeigen jedoch diagnostische Marker einer aktiven HBV-Infektion.
Prävention, Impfung, Diagnostik und Therapie sind daher die Grundpfeiler einer wirksamen Eindämmung der HBV-Infektionen. Die frühzeitige Identifikation viraler Varianten mit Immune-Escape-Mutationen gegenüber der HBV-Impfung oder den diagnostischen Nachweisverfahren sowie Therapie-assoziierter antiviraler Resistenzmutanten sind hierbei essentiell.
Das Nationale Referenzzentrum für Hepatitis-B- und -D-Viren (NRZ) ist am Institut für Medizinische Virologie der JLU Gießen beheimatet. Hier werden im Auftrag des Robert Koch-Instituts (RKI) und des Bundesministeriums für Gesundheit (BMG) u.a .die in Deutschland aktuell zirkulierenden HBV- und HDV-Stämme untersucht, und HBV-/HDV-begleitende virologisch-diagnostische Nachweisverfahren optimiert und getestet, die Schutzwirkung von Antikörpern nach HBV-Impfung untersucht sowie die Wirksamkeit antiviral wirksamer Medikamente zur HBV-/HDV-Therapie gegenüber viralen Mutanten analysiert.
Am Standort Gießen sollen daher anonymisierte HBV-/HDV-relevante Patientenproben aus der diagnostischen Abteilung des Instituts für Medizinische Virologie (Prof. Dr. med. Ziebuhr, Dr. med. Schüttler, Dr. med. Slanina) mit virologisch-molekularbiologischen Verfahren über die stattgefundene Routinediagnostik hinausgehend näher charakterisiert werden.
Im Einzelnen umfaßt dies die Isolierung und phänotypische, molekularvirologische Charakterisierung von HBV-/HDV-Isolaten sowie die Charakterisierung der virusassoziierten

Antigenverläufe und Antikörper-Antworten im Serum/Plasma nach Infektion bzw. HBV-Impfung.

Je nach Aufkommen wird mit ca. 100 bis 150 Patientenproben/Jahr aus Gießen gerechnet.

Einschlußkriterien: Ergebnis des serologischen Befundes und/oder der genotypischen Ergebnisse von HBV bzw. HDV.

Die Patientenproben werden auf der Grundlage der in der Diagnostikabteilung des Instituts für Medizinische Virologie erhobenen serologischen Befunde und/oder auf der Grundlage der Genotypisierungsergebnisse der entsprechenden Hepatitis-B-/-D-Isolate ausgewählt. Die weiterführende Untersuchung erfolgt anonymisiert.

Die Kommission stimmt der Durchführung des klinisch wichtigen Projektes ohne Einwände zu und wünscht gutes Gelingen.

Mit freundlichen Grüßen

Prof. Dr. H. Tillmanns
Vorsitzender der Ethik-Kommission

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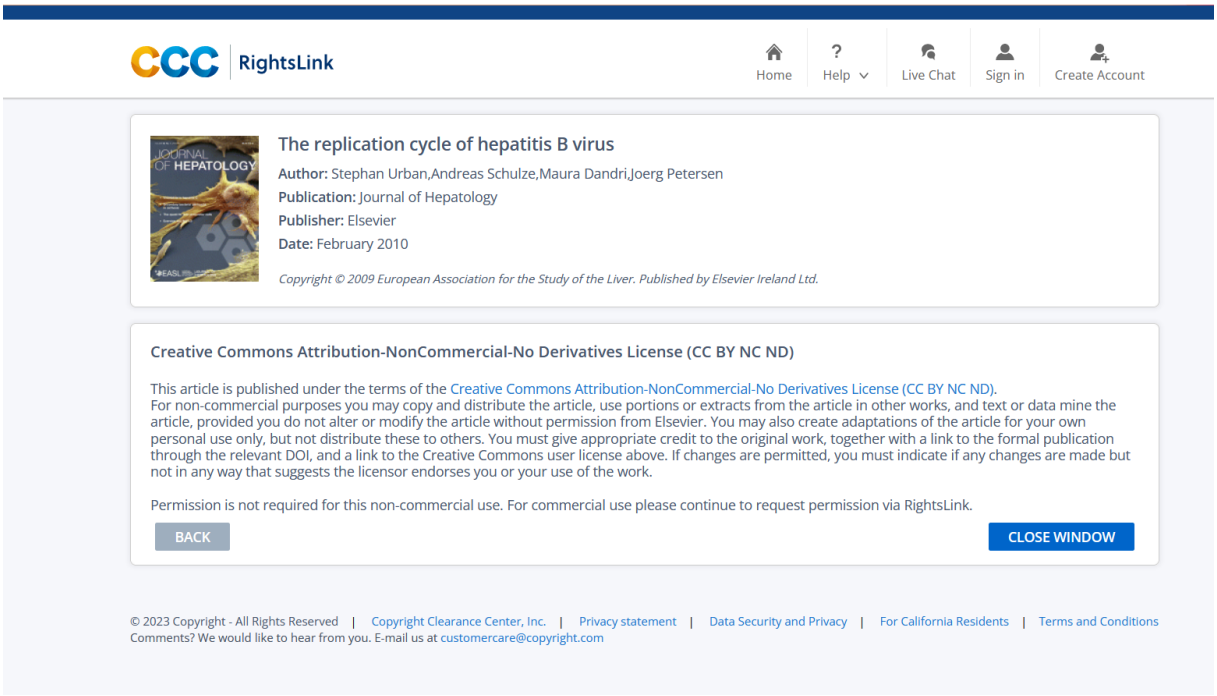

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Acknowledgements

This journey would have been impossible without the steadfast support and guidance of my thesis advisor, Prof. Dr.Dieter Glebe . His belief in my potential, coupled with his expertise and patience, has been the guiding light throughout this endeavour. I am profoundly grateful for his mentorship. I have great appreciation for my second supervisor and tutor Corinna Bremer who taught me profound research skills and took me patiently and kindly through every step of my research development.

I am deeply indebted my lab colleagues and lab friends, and research collaboration partners, Pia Seiz, Alexander König, and especially Sigrid Broehl (thank you for the supply and the caring hand) at the University of Giessen, Franziska Rinker in Hannover and Maria Pfefferkorn in Leipzig, who have been a source of inspiration and camaraderie throughout my doctoral journey. I am grateful for the enriching interactions, intellectual exchanges, and unwavering support of my peers and mentors while conducting my research for this doctorate thesis. To my colleagues and collaborators, thank you for the countless conversations, brainstorming sessions, and shared moments of discovery. Your insights and perspectives have been instrumental in shaping this research and broadening my horizons.

To my family, whose love, encouragement, and sacrifices have sustained me through the highs and lows of the doctoral study, I offer my deepest gratitude. Most of all, I would like to thank my parents Jane and Hardmuth Schröder, my sister Sarah and my brother in law Simon, my brother Stefan and my sister in law Jasmin and my dear Aunty Carol for their prayers, love and encouragement throughout my life. I would like to extend my gratitude to my best friend Marsela Vid who cheered me on and gave me motivation to do my best and Adelaide Lönnberg who carried me through with her prayers and kindness especially in the last phases of completing the thesis and preparing for the oral defence.

Lastly, I give thanks and honour to God, who has been the source of every blessing, strength, peace, hope, faith and joy.