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Study of antiviral mechanisms induced by interferon in HDV infection using human liver chimeric mice

Dissertation

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1 Introduction

1.1 Epidemiology

In 1977, Mario Rizzetto first identified the hepatitis delta antigen and antibody in patients infected with chronic hepatitis B. At first, the newly discovered delta antigen was thought to be just another hepatitis B virus (HBV) antigen. However, in subsequent experiments with chimpanzees, the delta antigen was characterized in more detail and identified as a distinct component of an infectious particle that was encapsulated by HBV envelope proteins. The newly discovered RNA virus was then classified as a member of the genus Deltaviridae and added to the group of already known hepatitis viruses (Rizzetto et al., 1977, 1980).

When the hepatitis D Virus (HDV) was discovered in Italy in the late 1970s, it was found to be endemic during major HBV outbreaks in the Mediterranean region. Studies performed at that time reported 25% of HBsAg positive patients to be anti-HDV positive (Rizzetto & Ciancio, 2012). Since then, HDV has always been a global health problem, with various widespread outbreaks in different countries around the world. Today, it is still highly endemic in Mediterranean countries, Central Africa, the Middle East, Mongolia, Pakistan and northern parts of South America, especially in the Amazon Basin (Hughes et al., 2011). Through the direct link to hepatitis B infection, HDV is transmitted mainly through parenteral exposure (by blood, blood derived products and sexual interference) (Niro et al., 1999) and is therefore present in many poor and underdeveloped regions of the world, where in precarious medical conditions, the existing vaccine cannot be accessed easily (Hughes et al., 2011; Noureddin & Gish, 2014). In North America and Western Europe high anti-HDV prevalence is confined to certain high-risk-groups, in which drug abusage has the greatest impact (Heidrich et al., 2009; Zachou et al., 2010). It is estimated that more than 240 million people worldwide carry the HBs-antigen, of which 15-20 (6-8%) million people are anti-HDV positive (Stockdale et al., 2020a; Wedemeyer & Manns, 2010). This number has been challenged recently by a meta-analysis, postulating a global HDV seroprevalence of 62-72 million people worldwide (Chen et al., 2019), which would be twice as high as the human immunodeficiency virus (HIV)

prevalence (estimated in 2019 by the world health organization (WHO)). However, the methodical approach and use of non-standardised screening practices of this particular analysis are still very much debated (Stockdale et. al., 2020b). In general, accurate estimates of the global HDV prevalence should be treated with caution, since many endemic regions are inaccessible for proper testing (Stockdale et al., 2020a).

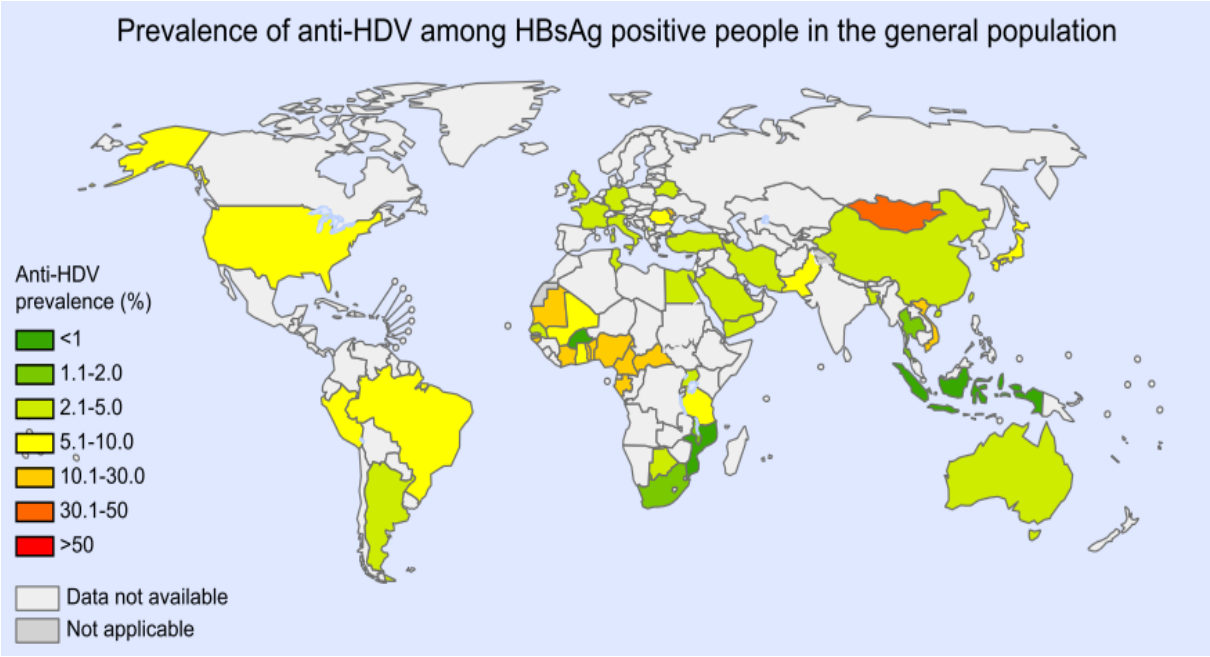


Figure 1: Prevalence of anti-HDV among HBsAg positive people in the general population. *Country-levelled estimation of the HDV seroprevalence (which is indicated by the colour). Areas where no reliable data are available are coloured grey.* (Stockdale et al., 2020a)

Although HBV vaccination programs and upraised socio-economic standards have improved prevalence rates in some western European countries, HDV infection is by any means completely eradicated in industrialized countries. Due to increasing migration flows in the course of globalization, HDV still is a significant health burden in central Europe (Heidrich et al., 2009; Wedemeyer & Manns, 2010). Several studies described high HDV prevalence in the European immigrant population (Coppola et al., 2019). In France, the prevalence rates of HDV infection therefore even increased over the last 15 years (Servant-Delmas et al., 2014). The German foundation of hepatitis estimates that approximately 10.000 – 30.000 people are

infected with HDV in Germany, while new infections are rare (RKI, 2020). To this day, infection with HDV virus still remains a global health problem, as it was decades ago.

1.2 HDV genotypes

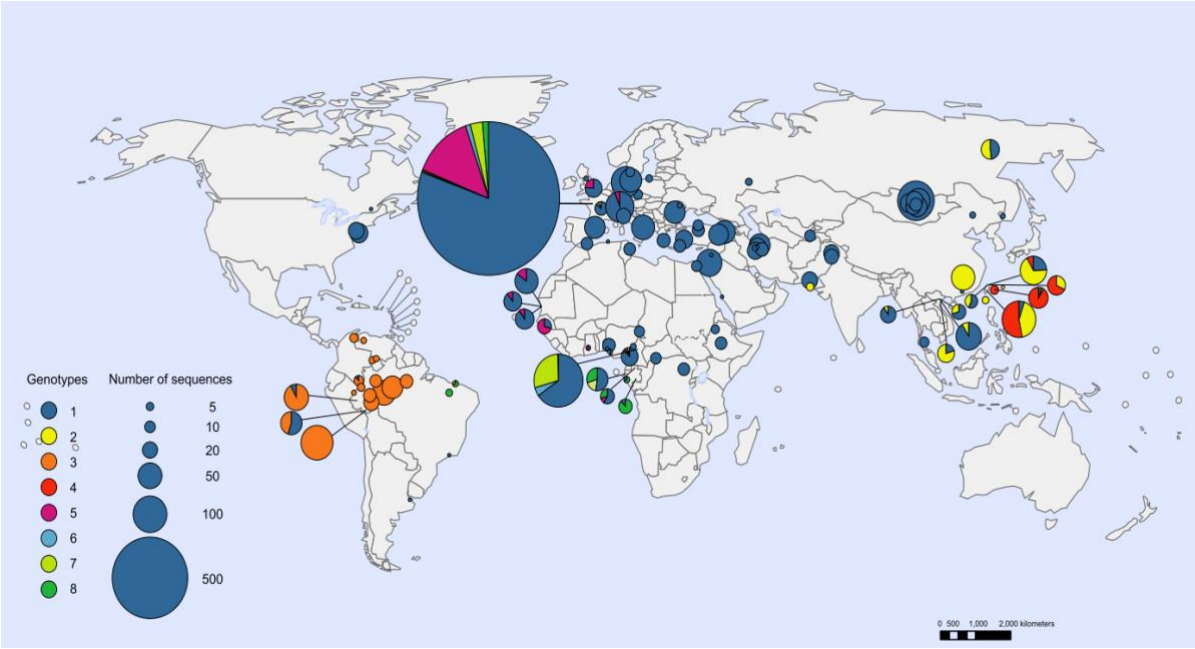


Figure 2: Geographic distribution of HDV genotypes. *Each circle represents a sample of HDV sequences from publicly available databases (GenBank, European Nucleotide Archive Database). The area of the circle is proportional to the size of the sample. (Stockdale et al., 2020b)*

HDV is sub-classified into eight genotypes with distinct clinical outcomes and geographical distribution (Botelho-Souza et al., 2017; Delfino et al., 2018; Dény, 2006; Le Gal et al., 2006). Genetic analysis revealed a sequence heterogeneity among the HDV genotypes of approximately 40% over the full RNA genome and 35% for the amino acid sequence of HDAg (Sureau & Negro, 2016). HDV genotype 1 (HDV-1) is the most widespread worldwide. It is mainly distributed in Europe, the Middle East, North America and North Africa. HDV genotype 3 (HDV-3) is found exclusively in South America, especially in the Amazon Basin (Casey et al., 1993, 1996). Genotype 2 is found in Asia and Russia, genotype 4 in Taiwan and Japan

while genotypes 5-8 are seen in different regions of Africa (Dény, 2006; Stockdale et al., 2020a). A precise bioinformatic analysis of the HDV full-length-genome has characterized the genetic variability in more detail and suggests a grouping of the eight known HDV genotypes into three genogroups based on their similarities (Figure 3) (Delfino et al., 2018).

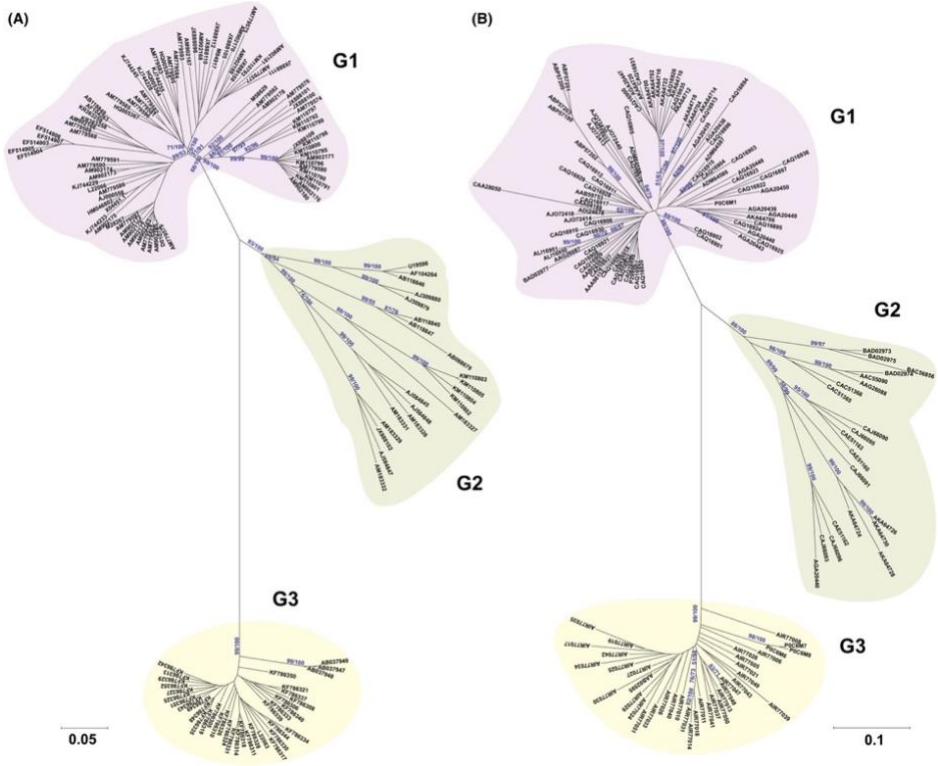


Figure 3: The phylogenetic clusters of hepatitis delta virus. *A phylogenetic tree with 144 HDV nucleotide sequences (A) or with 144 L-HDAg amino acid sequences (B) was generated. Both trees were reconstructed using Neighbor-Joining (NJ, MEGA) and the Bayes method (Mr. Bayes, CIPRES server). The trees map the evolutionary distance between sequences using branch lengths (NJ, MEGA). The analyses show that both the nucleotide and amino acid sequences form three main clusters (or groups, G1, G2, G3): G1, which includes HDV-1 sequences, G3, which corresponds to HDV-3, and G2, to which are clustered all the remaining HDV genotypes, HDV-2, HDV-4-8, which seem to share a close relationship. G1 (HDV-1) and G3 (HDV-3) show major genetic and evolutionary differences. (Delfino et al., 2018)*

It is important to note, that the HDV genotype contributes to different clinical outcomes in infected patients. HDV-1 can cause a broad spectrum of pathogenicity (Su et al., 2006), whereas an infection with HDV-3 is generally associated with a particularly severe course of disease (Bensabath, 1987; Gomes-Gouvêa et al., 2009; Gomes-Gouvêa et al., 2008). There is no evidence that a specific HDV genotype only infects patients of a certain HBV genotype. However, there is some evidence suggesting that different mutations of the hepatitis delta virus are better associated with certain HBV genotypes (Kay et al., 2014).

1.3 Viral structure

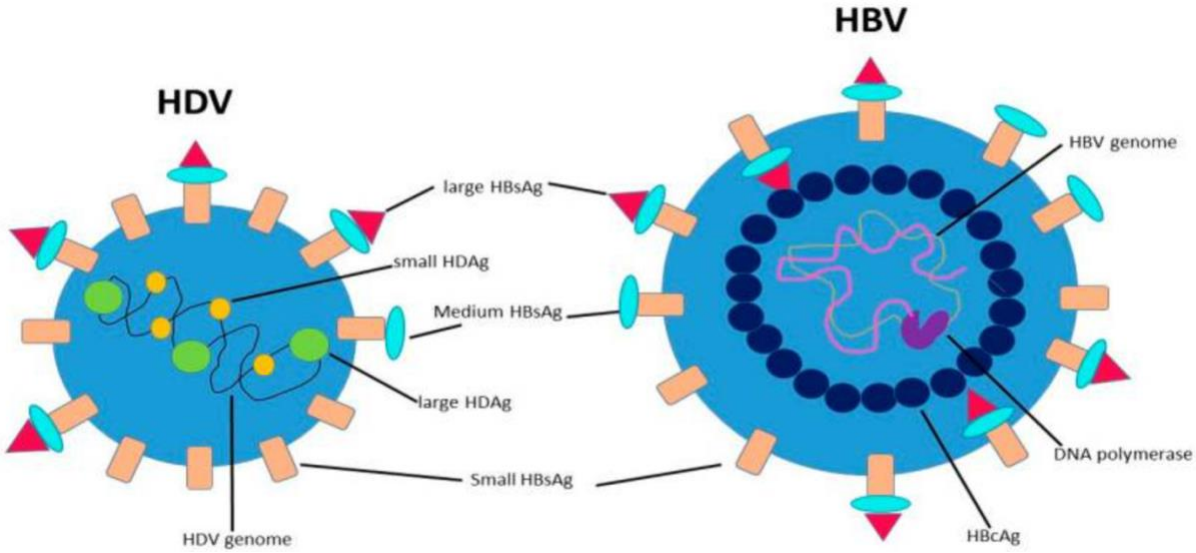


Figure 4: HBV and HDV virions. *The HBV capsid is formed by its core protein and contains the partially double-stranded DNA genome (rcDNA) and viral polymerase. Both viruses utilize HBV surface proteins (S-, M- and L-HBsAg). The HDV genome contains a single-stranded RNA genome that is folding into a rod-like structure due to its high sequence complementarity. The RNA genome is associated with the S-HDAg and L-HDAg forming the ribonucleoprotein (RNP).* (Sagnelli et al., 2021)

HDV is the smallest human pathogenic virus known. Its viral genome is a circular, single-stranded RNA genome (G RNA) of negative polarity (Wang et al., 1986). The hepatitis D virion has a particle size of approximately 36 nm, containing HDV RNA and delta antigen. The inner nucleocapsid contains close to 1700 nucleotides and has a single active open reading frame encoding the HDAg. The sequence self-

complementarity is high and HDV adopts a sort of double-stranded conformation through 74% internal base pairing that permits folding into a so called “rod-like structure”, which has been demonstrated by electron microscopy (Kos et al., 1987).

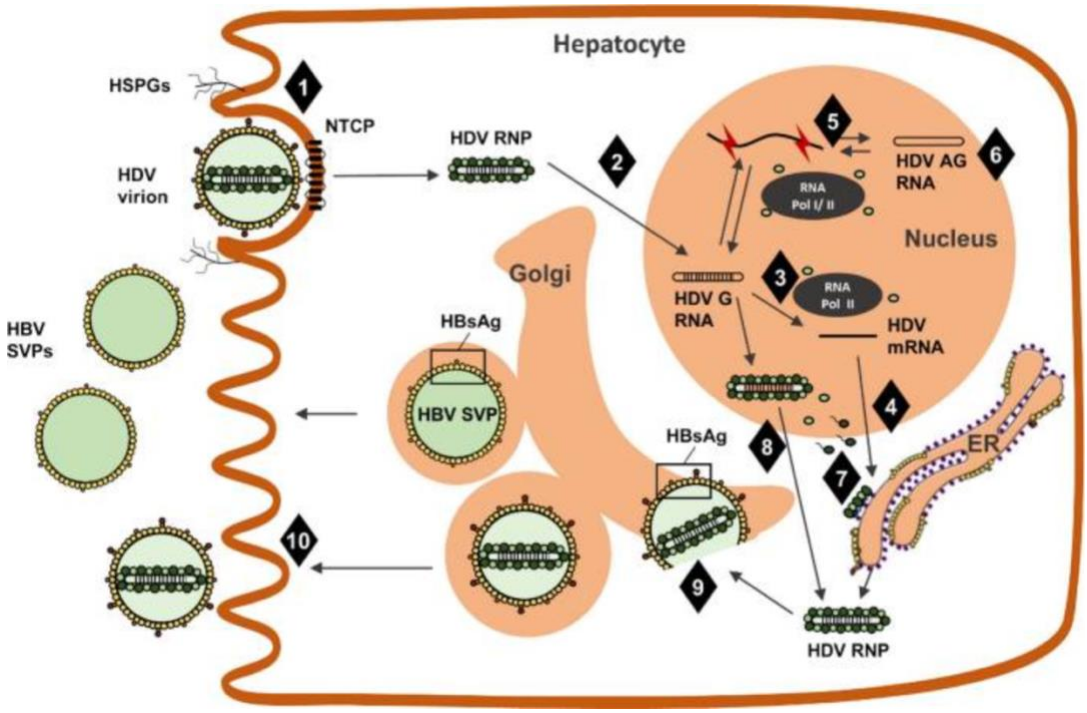


Figure 5: HDV life cycle. *HDV entry (step 1) is mediated by a first attachment step, resulting from viral interaction with heparan sulfan proteaglutans (HSPGs), and later specific interaction of L-HBsAg with the viral receptor, NTCP. HDV entry is facilitated through viral interaction with HSPGs and L-HBsAg with the NTCP receptor (step 1). The viral RNP is transported to the nucleus (step 2) where it releases the viral genome that serves as template for HDV mRNA (step 3), from which HDAg is later translated (step 4). The replication of viral RNA (step 5) is mediated by a DNA-dependent RNA polymerase in the presence of S-HDAg, through a double-rolling-circle mechanism. Antigenomic RNA can be edited by ADAR1 (step 6), allowing the expression of L-HDAg. Farnesylation of L-HDAg (step 7). The newly built HDV RNPs are assembled in the nucleus (step 8), exported and then encoated by HBV surface proteins (step 9) through the specific interaction of L-HDAg with HBsAg. (Mentha et al., 2019)*

1.4 HDV life cycle

HDV shares similarities with plant viroids (Sureau & Negro, 2016; Taylor & Pelchat, 2010), which are also small infectious pathogens, that are composed of nucleic acid, but do not encode for any proteins. In fact, the hepatitis delta virus is not entirely fulfilling the proper definition of a virus, since, as a sub-viral infectious agent, it appears to be an obligate satellite of the hepatitis B virus (HBV). More precisely, HDV is a defective virus that does not code for its own surface proteins, but instead requires the HBV envelope proteins to functionally propagate in human host (Freitas et al., 2014; Wang et al., 1991). The surface antigen of HBV (HBsAg) facilitates the coating of HDV for complete replication and enables the transmission to new hepatocytes (Rizzetto et al., 1980). Hence, active HDV infection occurs either upon simultaneous co-infection with HBV or as a super-infection in patients already infected with HBV. Recently, it has been reported that also HBV unrelated viruses can induce HDV spreading in vitro (Perez-Vargas et al., 2019).

HDV primarily targets hepatocytes, although it was shown in vitro that HDV replication can take place in different human cell types (Taylor et al., 2009). This suggests a strict hepatotropism to be receptor dependent. Both, HBV and HDV enter human hepatocytes via the sodium taurocholate co-transporting polypeptide (NTCP) (Yan et al., 2012). This receptor takes up bile acids at the basolateral membrane of hepatocytes. An essential myristoylation site near the N-terminus of the L-HBsAg (in the PreS1 domain) enables the interaction with the NTCP receptor (Li & Urban, 2016). Interestingly, new findings suggest that also the epidermal growth factor receptor (EGFR) may critically be involved in the viral internalization process in HBV and HDV infection (Iwamoto et al., 2019). To this day, the post-entry steps, such as the release of the HDV RNP, transcription and replication of the RNA genome are not completely understood. Replication takes place the nucleus and is independent of HBV. HDV lacks an own viral polymerase and is therefore essentially dependent on host enzymes to replicate via a double rolling-circle amplification process (Lai, 2005). This leads to the accumulation of two additional RNAs: the antigenomic RNA (AG RNA), which is an exact complement of the G RNA and the smaller linear mRNA encoding for the only viral protein, the hepatitis delta antigen (HDAg). Both, the genomic and antigenomic RNA contain a sequence that can act

as self-cleaving ribozymes (Kuo et al., 1988; Sharmeen et al., 1989). It is estimated to find around 300.000 copies of genomic, 50.000 copies of antigenomic and 1000 copies of mRNA in an infected hepatocyte (Chen et al., 1986). HDAg exists in two different isoforms: the small (S-) HDAg (24 kDa) is crucial for virus replication and accumulation of HDV RNAs, whereas the large (L-) variant (27 kDa), which is transcribed as a result of an adenosine deaminase acting on RNA (ADAR)-mediated RNA editing event, inhibits replication but promotes virion assembly (Chang et al., 1991; Wang et al., 1991). The small HDAg strongly supports replication, whereas the large HDAg acts as a negative inhibitor (Chao et al., 1990).

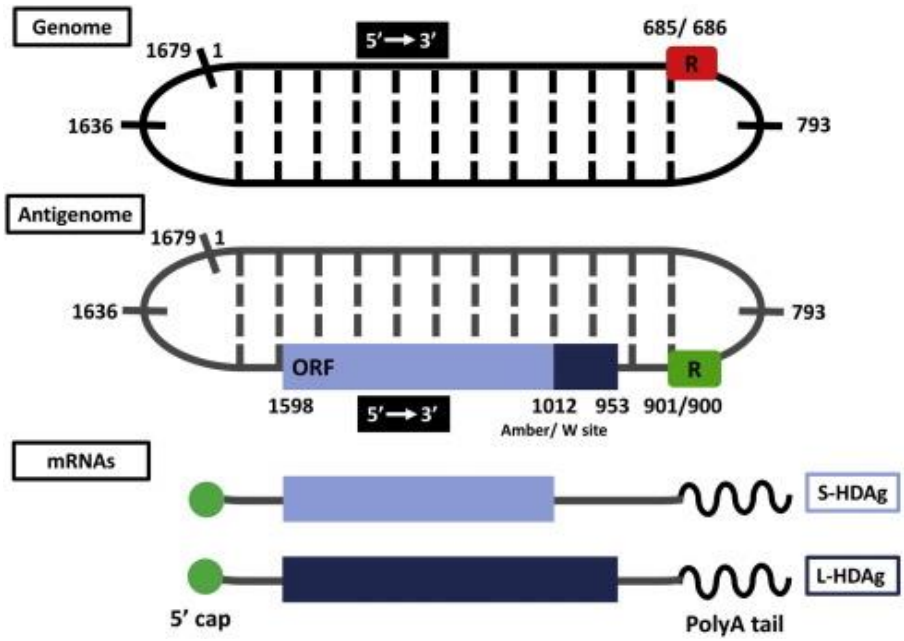


Figure 6: HDV RNAs and protein structures. *There are different forms of HDV RNA, the genomic (G RNA), antigenomic (AG RNA) and messenger (mRNA). The AG RNA contains the only open reading frame (ORF), which encodes for the HDAg, which is translated through mRNA in the cytoplasm. For the synthesis of mRNA G RNA serves as a template for synthesis of HDAg mRNA by the RNA polymerase II. The mRNA is then modified by the addition of a 5'-cap and a 3'-polyA tail. The S-HDAg is translated from the unedited RNA. Host adenosine deaminase acting on RNA 1 (ADAR1) is a key regulator of HDV replication cycle. ADAR1 leads to the change of UAG codon for the S-HDAg to UGG codon and ultimately to the formation of the L-HDAg.* (Alfaiate et al., 2015)

1.5 Course of infection, diagnosis and treatment

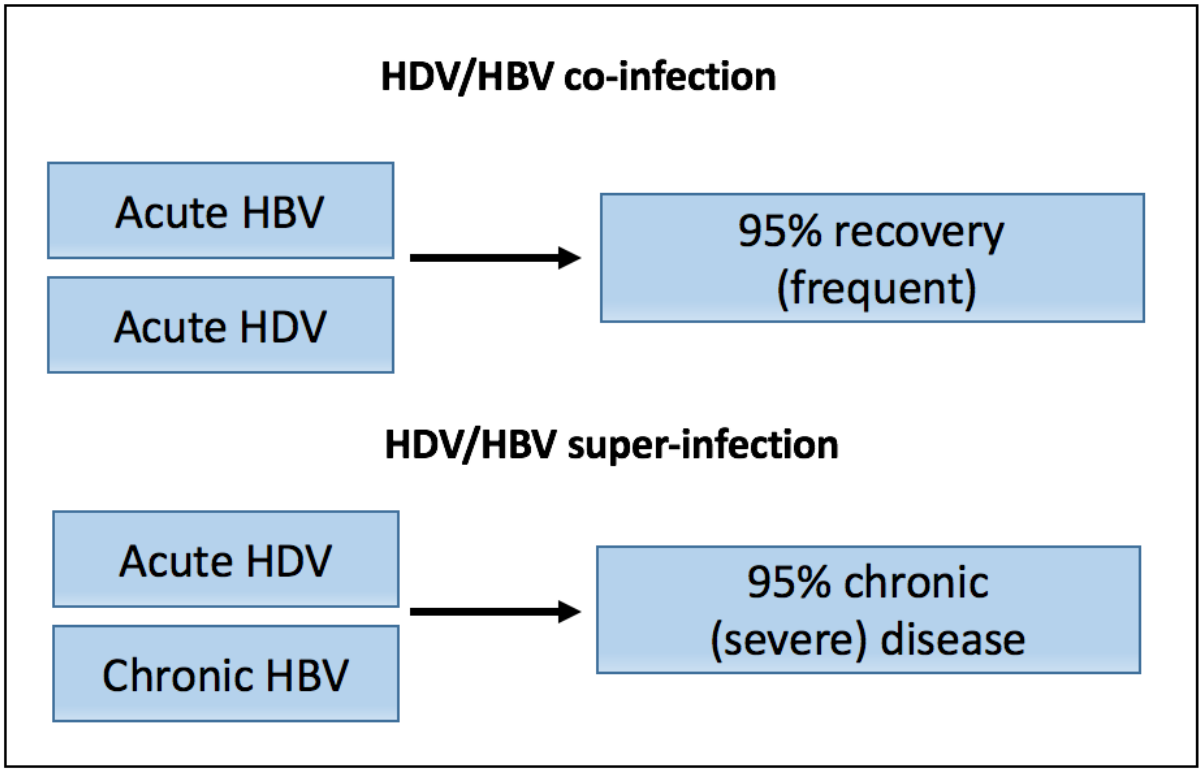


Figure 7: Course of HDV infection. *HDV infection occurs only in HBsAg positive individuals either as acute co-infection or super-infection in chronic HBV patients* (Wedemeyer & Manns, 2010).

1.5.1 Course of disease – Acute and chronic HBV/HDV co-infection

HDV infection occurs only in HBsAg positive patients either as acute co-infection or as super-infection in patients that are already chronically infected with HBV.
The co-infection with HDV and HBV leads to an acute hepatitis D and acute hepatitis B infection. Histopathological analysis of livers of chimpanzees show that the co-infection with HBV/HDV results in severe liver damage compared to the HBV mono-infection (Dienes et al., 1990). Also, in immunocompetent adults the acute co-infection regularly displays more severe symptoms than the HBV mono-infection, but nonetheless, since HDV is a defective virus depending on the HBsAg, the course of disease in the acute co-infection depends very much on the viral kinetics of the concomitant HBV. High HBV titers usually result in a shorter incubation time and a

typical bi-phasic course of elevated aminotransferase levels, which is caused by an initial HBV propagation and a second HDV spread. In contrast, low HBV titers can result in a longer incubation periods and mono-phasic peak. Non-specific symptoms like lethargy, fatigue and nausea, as well as asymptomatic courses of disease can occur. Although, the vast majority of acute HBV/HDV co-infections are self-limited in 90% of the cases, acute co-infection frequently leads to severe hepatitis with the risk of emerging into a fulminant course with liver failure. (Farci & Niro, 2012; Rizzetto, 2009; Zachou et al., 2010)

The course of disease and the clinical outcome in patients super-infected with HDV is noticeably different to the acute co-infection setting. Patients that are chronic carriers of the HBsAg enable the immediate spread and infection establishment by taking advantage of the pre-existing HBV infection. Around 95% HDV super-infected patients endure a severe hepatitis that progresses into a chronic stage of infection (Negro, 2014). In this scenario, HDV is rarely cleared spontaneously. The chronic HBV/HDV hepatitis is considered the most severe form of all hepatitis and is associated with an accelerated course of fibrosis progression, cirrhosis, liver decompensation and hepatocellular carcinoma (HCC) (Farci & Niro, 2012). Without a doubt, liver cirrhosis is a main risk factor for the incidence of the HCC, however it is controversially discussed whether HDV is inherently contributing to cancer development. There are studies that depicted no difference in the number of HCC between patients with chronic HBV/HDV co-infection and HBV cirrhosis alone (Niro et al., 2010; Romeo et al., 2009). However, in multiple cohort studies that were performed recently the risk appeared to be as much as nine times higher (Béguelin et al., 2017; Fattovich et al., 2000; Ji et al., 2012; Kushner et al., 2015).

1.5.2 Diagnosis of HDV infection

The European Association of the Liver (EASL) guidelines of 2017 recommend testing every HBV-infected patient for HDV. However, countries like the United States rarely test for anti-HDV Immunoglobulins (Ig), as they only screen certain “risk groups” that include migrants from endemic regions, intravenous drug usage, high risk sexual behaviour, patients infected with HIV and/or hepatitis C virus (HCV) and patients with elevated aminotransferases with low or undetectable HBV DNA. (Safaie et al., 2018). Interestingly, recent publications show that the actual US HDV prevalence might be a substantially higher than previously estimated. (Patel et al., 2019). Recent studies covering new HDV treatment strategies underline the need to identify chronic HDV infected patients and show the value of general anti-HDV screenings of all HBsAg positive patients. This could result in early therapeutic intervention, as more patients would be diagnosed in early stages of infection. As a result, estimations on the global HDV prevalence would be more accurate.

There are different markers that can be used in HDV diagnostics. Firstly, HBsAg positive patients should be tested for anti-HDV antibodies (IgM, IgG) by ELISA to detect HDV infection. Supporting evidence shows that anti-HDV IgM correlates with the activity of disease (Wranke et al., 2014). To finally confirm infection, a test for active HDV replication by quantitative real time PCR (qPCR) (measuring HDV RNA) allows to distinguish between past and chronic infection. Furthermore, treatment response can be evaluated more precisely. The fact that anti-HDV antibodies cannot be detected in the first week of infection are a limiting factor in HDV diagnostics. Also, the standardisation for HDV PCR results in comparison between laboratories is still an issue, since the HDV genus is characterized by a very high genetic variability. However, the WHO 1st international HDV RNA standard and new commercial kits, viable for all genotypes, allow comparable reports of results. (Le Gal et al., 2016, 2017)

1.5.3 Treatment of HDV infection

HDV infection is the most severe form of any viral hepatitis in humans and yet its treatment options remain highly unsatisfactory. Clearance of HDV with concomitant seroconversion of HBV infection, elimination of the persistent HBV DNA form, the cccDNA (covalently closed circular DNA), and recovery of liver transaminases sets the optimal therapeutic endpoint. Unfortunately, no antiviral treatment reliably achieves a sustained viral clearance in the majority of the patients and ultimately liver transplantation remains the only option for many patients with end stage acute liver failure (Grazia Anna Niro et al., 2005). The idea of targeting the reverse transcriptase of HBV with nucleoside/nucleotide analogues (NUCs) to suppress HBV and consequently also target HDV failed in various clinical trials. Nevertheless, if HBV viral loads are high, the combination of NUCs and peg-IFNα can be considered (Abbas et al., 2016; Wolters et al., 2000; Yurdaydin & Idilman, 2015). Until today, peg-IFNα (weekly injections for 12-18 months) remains the only recommended therapy in HDV infection (Lampertico et al., 2017; Terrault et al., 2018). Clinical studies reported that the pegylated form of IFNα (prolonged plasma half-life) had fewer side effects and was more effective than the conventional IFNα (Abbas et al., 2011). No difference in effectiveness was detected between peg-IFNα 2a and 2b (Abbas & Abbas, 2015). A randomised study in 2011 reported that treatment with peg-IFNα 2a resulted in sustained viral clearance in about 25-30% of the patients (Wedemeyer et al., 2011). Trials that evaluated the effect of peg-IFNα in shorter treatment durations from three to six months showed a clear suppression of HDV replication, but also a relapse of HDV infection in all cases (Di Bisceglie et al., 1990; Porres et al., 1989). Furthermore, it has been shown that prolonged treatment is not of beneficial value to the majority of patients (Wedemeyer, Yurdaydin, et al., 2019), however, there are particular cases where prolonged treatment provides an advantage. (Lampertico et al., 2017; Terrault et al., 2018; Yurdaydin et al., 2019)

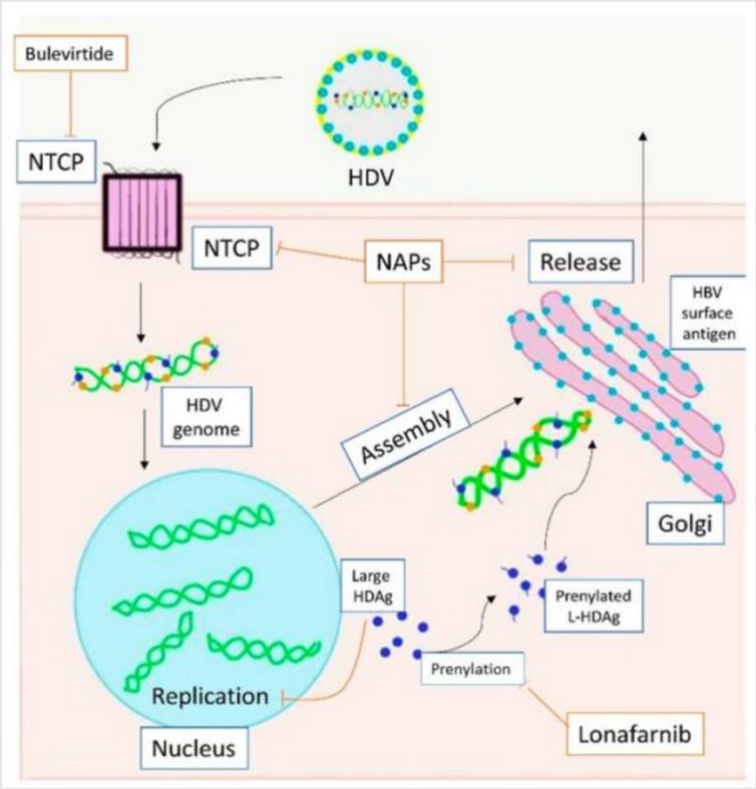


Figure 8: Where HDV therapeutic agents act on the HDV life cycle. *Both HDV and HBV enter human hepatocytes via the NTCP receptor. HDV replication takes place in the nucleus of the cell. The large HDAg is farnesylated in the cytoplasm. Viral assembly is completed with the interaction of HBsAg and HDAg in the Golgi complex. Bulevertide is a first-in-class entry inhibitor blocking the NTCP-receptor and therefore viral entry. Lonarfarnib inhibits farnesylation (assembly).* (Sagnelli et al., 2021)

Advances in detailed characterisation of the viral life cycle facilitated the development of new HDV targeting molecules. A very promising drug is Bulvertide, which has been on the market in Europe since July 2020 under the trade name Hepcludex (formerly Myrcludex B). The substance had been developed as an entry inhibitor, which directly targets the bile acid transporter sodium taurocholate cotransporting peptide (Ni et al., 2014). The myristoylated lipopeptide that derived from the preS1 domain of the HBV envelope, was shown to hinder HDV infection in vivo in humanized uPA/SCID mice (Lütgehetmann et al., 2012). The substance is very well tolerated by patients, as no significant side effects occurred during clinical trials, besides minor asymptomatic and reversible increase in bile acids (Yurdaydin et al., 2019). With overall good tolerability, efficacy against HDV has been

demonstrated as monotherapy and in combination therapy with peg-IFN-α (Wedemeyer et al., 2018).

A different approach in HDV therapy is to target the protein interaction between the L-HDAg and the HBsAg, which is facilitated through farnesylation, a post-translational modification (PTM) step. This PTM is key to enable the final particle formation and the completion of the viral life cycle. Lonafarnib (LNF) is a farnesyl-transferase inhibitor that effectively prevents the interaction between the HDV RNP and the HBsAg. Both, in vitro and in vivo experimental studies showed a clear inhibitory effect on HDV (Bordier et al., 2003). First clinical trials reported reduced HDV RNA levels in patients treated with Lonafarnib for 28 days (Koh et al., 2016), in which the anti-HDV levels correlated with serum drug levels. However, due to strong gastrointestinal side effects, LNF was combined with ritonavir, an inhibitor of CYP3A4 which is mainly metabolizing LNF. This allowed lower dosages and reduced side effects. Recent studies that combined LNF and ritonavir with peg-IFNα achieved a more substantial HDV RNA reduction compared to treatment with peg-IFNα alone.

Since treatment with peg-IFNα causes a broad range of side effects, especially in prolonged treatment, the need for new medication is urgent. Pegylated IFN lambda (peg-IFNλ) is currently tested in clinical studies and represents an attractive therapeutic alternative to peg-IFNα. Binding a different receptor, that is only expressed on specific cell types (Boisvert & Shoukry, 2016), side effects are reduced compared to treatment with peg-IFNα (Muir et al., 2010). For both drugs, a comparable strong serological and intrahepatic anti-HDV effect has been shown in humanized mice infected with patient derived (pd) HDV-1 (Giersch et al., 2017).

1.6 Immune response towards viral infection

Interferons (IFN) are effectors of innate immunity that are specifically directed against intracellular replicating pathogens. They were named after their ability to "interfere" with the replication of the influenza virus in cell-culture.In vivo, interferons show similar antiviral properties. They inhibit viral replication, enhance the cellular immune response and thus prevent new infections in non-infected cells (Goodbourn et al., 2000). Based on sequence homologies and depending on binding to specific receptors, interferons can be divided into different groups - type I, II, III interferons. The best known and most studied type I IFNs are IFNα and IFNß, while Interferon gamma (IFNγ**)** is the only type II IFN. Type III interferons represent a newer family of IFNs. The occurrence of type III IFN receptors is restricted to fewer cells, in contrast to type I IFNs and IFNγ. (Ivashkiv & Donlin, 2014)

1.6.1 Recognition of viruses and interferon production

The synthesis and induction of type I interferons is not restricted to cells of the immune system alone. Almost all cell types can produce IFNα and IFNß in response to viral infection, usually in answer to an innate stimulus mediated by pattern recognition receptors (PRRs). One such stimulus of type I IFN induction is double-stranded RNA, which is not present in human cells but may be part of the genome of several viruses. The single-stranded RNA virus HDV also has a double-stranded-like conformation due to its unique secondary structure. Membrane-bound Toll-like receptor (TLR) family receptors (such as TLR3) and cytoplasmic RNA sensors (such as RIG-I and MDA-5) are able to detect double-stranded RNA. This interaction with specific RNA patterns can result in a change of conformation, which exposes the Caspase activation and recruitment domain (CARD) to interact with the mitochondrial antiviral signalling protein (MAVS). This signalling pathway is forwarded in two different ways: (1) Initiating the release of proinflammatory cytokines through NF-kB activation and (2) a strong type I IFN induction. (Ivashkiv & Donlin, 2014; Jung et al., 2020)

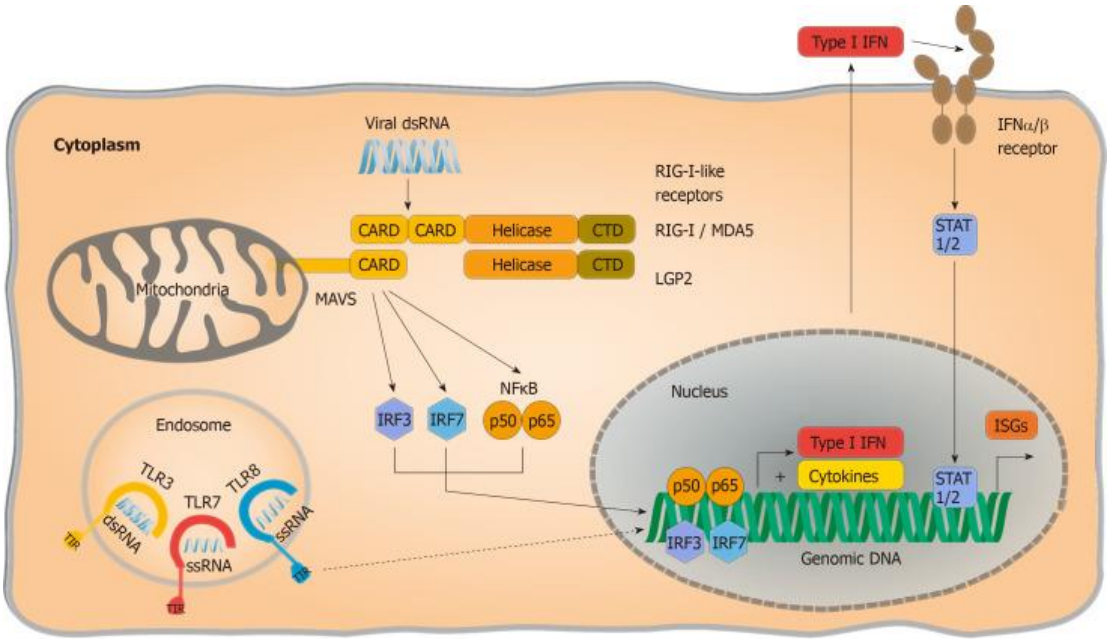


Figure 9: Pattern recognition of viruses. *Viral dsRNA is sensed by TLRs and cytoplasmatic RNA sensors like RIG-1 and MDA5. Binding of RNA to these receptors induces a change in conformation and leads to the exposure of CARD domains which enables the binding of the MAVS protein. Two pathways are activated: (1) proinflammatory cytokine production through NFkB induction and (2) a strong IFN type-1 induction leading to an antiviral state of cells through ISG-induction.* (Jung et al., 2020)

The IFN signalling subsequently induce viral infection control, both on infected- and on uninfected cells. This is done by first IFNα or IFN*ß* binding to the common IFN receptor (IFNAR), which consists of two subunits (IFNAR1, IFNAR2). These subunits are associated with the cytoplasmic tyrosine kinases of the Janus family (JAK1, Tyk2). These kinases themselves directly phosphorylate the signal transducing transcriptional activators (STAT1 and STAT2), leading to rapid induction of certain genes. The factors STAT1 and STAT2 can dimerize and therefore exist as homo- or heterodimers. The heterodimer forms the interferon stimulated gene factor 3 (ISGF3) complex with interferon regulatory factor 9 (IRF9). This complex can translocate to the nucleus and activate the promoters of various interferon-stimulated genes (ISGs). STAT1 homodimers, on the other hand, bind to gamma activated sequences (GAS) and thus induce pro-inflammatory genes. (López de Padilla & Niewold, 2016)

The antiviral effect is mediated by different mechanisms. On one hand, by activating ISGs, IFN triggers the synthesis of various proteins such as oligoadenylate synthetase and Mx proteins, which can inhibit viral replication and translation. On the other hand, increased cytokine production (CXCL9, CXCL10, and CXCL11) results in upregulation of the expression of MHC class I molecules in all cell types. Thus, killing of infected cells by cytotoxic CD8 T cells is promoted. Through these mechanisms, an antiviral status is established in the cells. This stimulation of innate immune cells can then have a strong impact on the adaptive immune response. Thus, antibody production by B cells can be increased and effector functions of T cells can be enhanced (Figure 10). However, in addition to inducing antiviral defence mechanisms, these multifunctional cytokines have further characteristics. They influence the regulation of cell growth and the activation of the adaptive immune system and thus also have antiproliferative, antitumoral and immunomodulatory effects. (López de Padilla & Niewold, 2016)

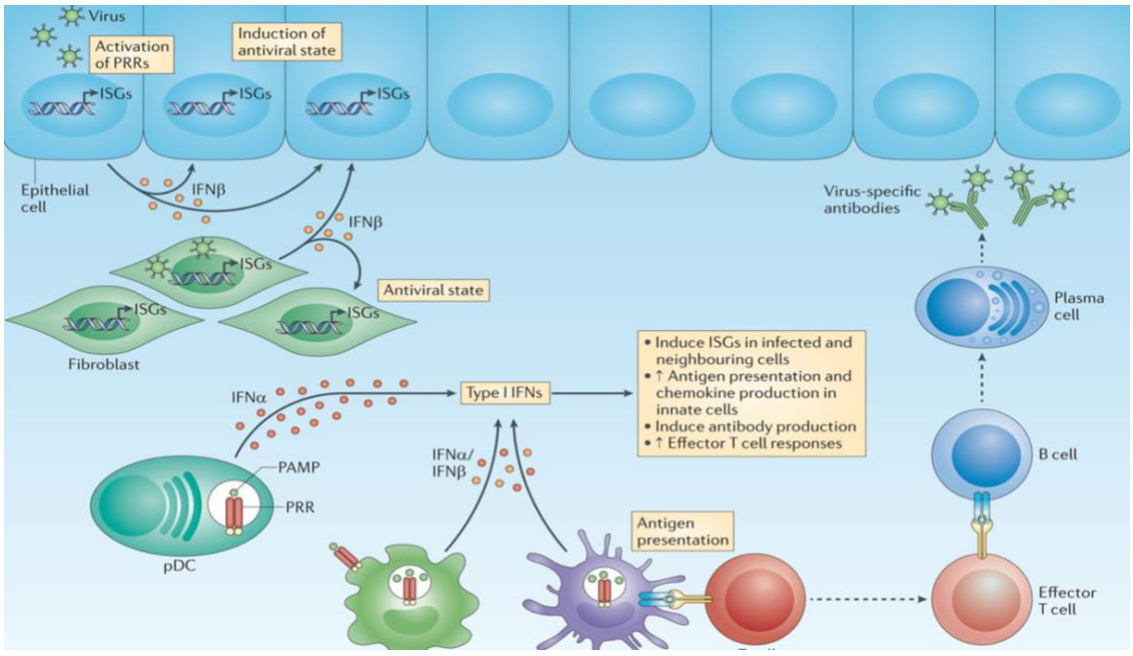


Figure 10: Type-I Interferons – regulation of innate and adaptive Immune system *(Ivashkiv & Donlin, 2014) When pathogens are detected, the infected cells produce type 1 Interferons (IFNs) and induce an antiviral status. Innate immune cells produce sense pathogens through pattern-recognition receptors (PRRs), fibroblasts and other non-immune cells produce IFN beta (IFNß). Type 1 IFNs predominantly induce IFN stimulated Genes (ISGs) which is limiting the pathogen spread, induce B cells to produces antibodies and augment the effector function of T cells.*

1.7 Intrinsic IFN-response

1.7.1 Intrinsic IFN-response in HDV infection

In the last few years, further insights into the interaction of HDV with the host immune system have been gained. Suarez et al. recently identified MAVS-protein (engli. mitochondrial antiviral signaling protein) as the essential part of the signaling pathway for the induction of intrinsic interferons during HDV infection in a mouse model (Suárez-Amarán et al., 2017). MAVS contains an N-terminal CARD domain that interacts with the associated CARD domains of intracellular receptors such as TLR3, MDA5, and RIG-I (Kato et al., 2006). Zhang et al. subsequently performed knockout experiments of these same receptors in cell culture (HepaRGntcp) and identified MDA-5 (melanoma differentiation antigen 5) as the crucial receptor to recognize HDV RNA and activate MAVS (Zhang et al., 2018). These findings show that the immune response via the MDA-5/MAVS signaling pathway in HDV infection is mainly carried out through IFN beta (IFNß), but not IFNα. This is also consistent with previously published studies (Alfaiate et al., 2016; Giersch et al., 2015; He et al., 2015). Moreover, induction of IFNλ has also been described in humanized mice co-infected with HBV and HDV (Giersch et al., 2015). Interestingly, a strong induction of IFNß was shown to have no effect on HDV replication in in vitro experiments, which has also been described in previous work (P. J. Chen et al., 1986; Zhang et al., 2018). While a potent IFN response with induction of many classical ISGs has been described in HDV infection, the exact molecular bases and specific interaction between IFN-mediated effector molecules and HDV is still unknown and remains the subject of current research.

1.8 Pathogenesis of chronic Hepatitis D infection

The cellular innate immune response is the first defense against pathogens and is an essential component for the induction of the adaptive immune response (Gasteiger & Rudensky, 2014; Jain & Pasare, 2017). It is therefore crucial for the progression of infections, i.e., successful control or infection-persistence (which is often associated with chronic inflammation). Infection with HDV, unlike HBV infection (Mutz et al., 2018), results in a strong response of the innate immune system with induction of type I interferons, which are a potent antiviral component of the innate immune system (Randall & Goodbourn, 2008). Moreover, HDV/HBV co-infection is associated with a particularly severe clinic and poorer prognosis compared to other viral hepatitis. Experimental in vivo studies from our own research group have already shown that in the setting of HBV/HDV co-infection, antiviral ISGs and cytokines are more strongly induced in human hepatocytes than in HBV mono-infection (Giersch et al., 2015). Among other explanations, this could very well be one underlying pathomechanism of the more severe clinical expression of HDV/HBV infection, as it is already known from other viral diseases that a strong induction of the innate immune response, i.e., an increased pro-inflammatory status of the cells, can contribute significantly to the immunological pathogenesis. For instance, in SARS-CoV-induced pneumonia, it has been demonstrated in a mouse model that an over-regulated induction of type I interferons is responsible for the resulting lung damage (Channappanavar et al., 2016). Although some in vitro studies have attributed a direct cytopathic effect to HDV (Cole et al., 1991; Macnaughton et al., 1990), this is more likely not due to the HDAg itself, as transgenic mice expressing the HDAg do not manifest any liver damage (Guilhot et al., 1994). As of today, the pathomechanism in chronic HDV-infection, i.e., the precise interactions between the virus and the host innate immune system, are not well characterized. Thus, it is currently presumed that both innate and adaptive immune responses contribute to the pathogenesis of HDV infection. Since in the vast majority of CHD patients the adaptive immune response is unable to control infection, many authors suggest an increasingly important role of the innate immune system and IFN signaling pathways (Abbas & Afzal, 2013).

1.9 Animal models

HDV research has brought up various different in-vitro and in-vivo models to study the HDV life cycle and test therapeutic options. In general, the use of in-vitro models offers some natural advantages over in-vivo models, for instance lower costs, lower effort (compared to maintain an animal facility) and less ethical concerns. However, any in-vitro model is artificial and far from reflecting real conditions in HDV patients. Hepatocytes in cell-culture systems are not anchored in an organic structure with surrounding parenchyma cells and connection to the blood flow and therefore can only remotely copy important factors such as an immune system, viral spreading and pharmacodynamics of therapeutic agents. To fully understand the interactions between a virus and its host, HDV and HBV, HDV and the immune system, and various therapeutic options, specific animal models are needed that are closer to the conditions in real patients. (Allweiss et al., 2016)

1.9.1 Human liver chimeric mice

Within different species, the natural enzyme machinery and intracellular signaling pathways differ, which is why in vivo models based on human hepatocytes are of great importance in HDV research. (Allweiss et al., 2016). One of these models is the human-liver-chimeric-mouse model. This particular urokinase-type plasminogen activator (uPA) transgenic mouse (Dandri et al., 2001) is widely used and well characterized in the field of HDV research. In this mouse model, a uPA transgene is overexpressed in the mouse via an albumin promoter. The high plasma levels of uPA lead to acute liver failure. These uPA mice are crossed with immunodeficient mice, i.e.with severe combined immunodeficiency (SCID) (B- and T-cell depletion) or with SCID/beige (NK-cell depletion) mice (short USB-mice). The immunodeficiency of the mice allows transplantation of cryopreserved human hepatocytes. The human cells repopulate the defective mouse liver and maintain their natural function. This is of great importance for HDV research, as these cells remain susceptible to infection with various human hepatotropic viruses.

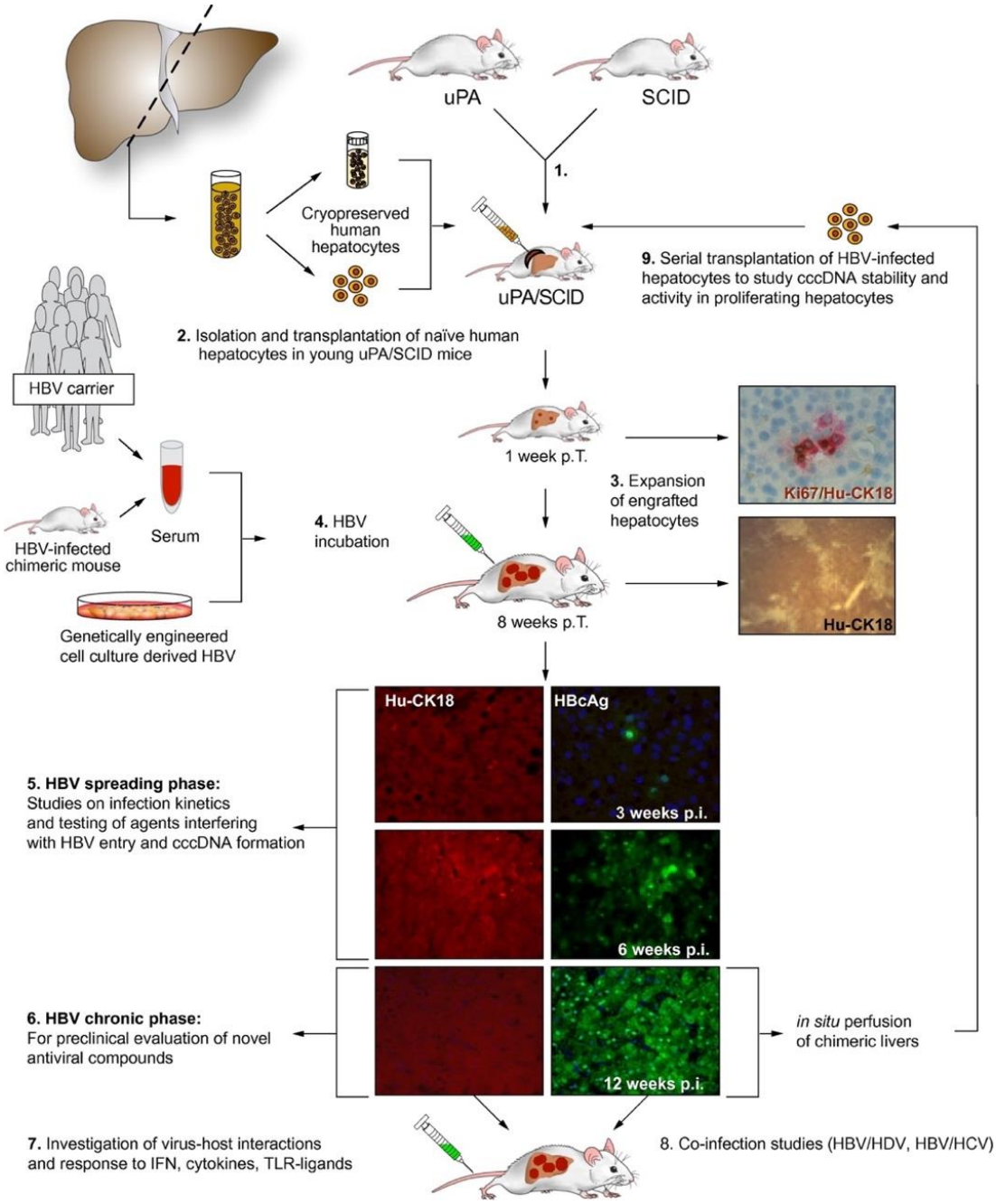


Figure 11: Generation of human-liver-chimeric-mice and infection analysis. *uPA and SCID mice are crossed. Cryopreserved human hepatocytes then get transplanted into uPA/SCID mice to repopulate the mice livers. An Expansion of newly engrafted hepatocytes usually lasts 8 weeks. Mice can be infected with patient-, human liver-chimeric mice sera or cell culture derived Viruses. The spreading of HBV in this model lasts at least 12 weeks until establishment of a so called 'chronic' infection* (Allweiss et al., 2016).

This animal model offers the possibility of mono- or co-infection with different HBV and/or HDV strains and is the basis for the experimental trials undertaken in this work. After an infection is established, serological and intrahepatic measurements can be obtained, IF or RNA in situ hybridization staining can be performed and as a result the infection behavior and treatment success can be monitored. Thus, viral entry, replication, assembly and cell exit can be studied in this model.

This particular mouse model of human liver chimeric mice undoubtedly offers unique opportunities in HDV research, but also has its own limitations. On the one hand, the process of human hepatocyte transplantation is technically demanding and very complex and expensive. Thus, the number of experimental animals is usually limited and often rather small groups can be compared. Consequently, statistical significance is often lower in small experimental groups. On the other hand, the human donors of the hepatocytes may cause heterogeneous experimental groups, but it is not clear to what extent this affects the results. However, with careful distribution of the different human cell batches, a potentially confounding effect due to donor or mouse background can almost be ruled out. However, this is often not the case in in vitro experiments, where model-related influences often cannot be identified with certainty. Another quite obvious limitation is the lack of an adaptive immune system. On the other hand, the effect of direct-acting substances can be studied independently of adaptive immunity without confounding factors.

2 Aim of work

There are few therapeutic options for the treatment of HDV infection. Despite major advances in HDV therapy, such as the market authorization of the HBV/HDV entry inhibitor Hepcludex (Bulevertide/Myrcludex-B) (Bogomolov et al., 2016; Kang & Syed, 2020) as the first specific drug in HDV therapy, peg-IFNα remains a key treatment option in off-label therapy. Unfortunately, the therapeutic outcome of peg-IFNα therapy is still highly unsatisfactory, as sustained viral clearance can only be achieved in about 25-30% of patients treated according to guidelines (Wedemeyer et al., 2011). IFNs act as immunomodulatory agents and can induce an antiviral state via upregulation of numerous ISGs. Nevertheless, the exact mode of action of IFN on HDV infection remains the subject of ongoing research. (Zhang & Urban, 2020)

To date it is believed that host genetics play some role in IFN responsiveness, whereas the effect of the HDV genotype is scarcely understood. As predictors of response to IFN treatment, viral genetics might be very relevant, as recent studies showed that patients mainly infected with HDV-1 achieved a sustained viral response only in about 25-30% when treated with IFNα (Heidrich et al., 2013). In contrast, in a study including patients infected with HDV-3, peg-IFNα therapy achieved 95% SVR in CHD patients, 62% SVR was achieved in patients infected with HDV genotype 5 (HDV-5) given peg-IFNα mono-therapy, suggesting that HDV-3 and HDV-5 might be ‘easier to treat’ genotypes compared to HDV-1 (Borzacov et al., 2016; Spaan et al., 2020). Cell culture experiments showed that exogenous IFNα treatment exerted no significant effect on HDV replication once infection was already established. In contrast, early IFNα treatment resulted in a moderate inhibitory effect on HDV replication (Zhang et al., 2018). This is consistent with previous results from cell culture experiments by Taylor et al. (2011). (Han et al., 2011; Ilan et al., 1992). In these studies, pre-treatment with IFNα was able to inhibit the establishment of HDV infection, but an already established infection could not be affected by the treatment. It is important to note, that in these studies only one specific HDV-1 strain (AJ000558) was used (referred as cc-HDV-1 in this work) - which is almost exclusively used in experimental HDV research. Interestingly, a strong antiviral effect (1.4 log) on established HDV/HBV co-infection was previously

observed by treating humanized mice infected with a different HDV-1 strain, obtained from a patient who later achieved SVR under interferon therapy. In this case, these mice were treated with both peg-IFNα and peg-IFNλ for 4 weeks (Giersch et al., 2017).

Overall, these observations clearly indicate HDV genotype and even strain-specific differences to IFN treatment. Therefore, the aim of this work was:

1) To comparatively assess the sensitivity of ccHDV-1 and ccHDV-3 to peg-IFNα and peg-IFNλ treatment using human liver chimeric mice.
2) To further elucidate how IFN affects HDV in the early stages of infection, humanized mice have been pre-treated with peg-IFNα to compare infection establishment of ccHDV-1 and ccHDV-3 to peg-IFNα and investigate whether not only intracellular HDV replication but also the entry steps are affected by peg-IFNα pre-treatment.
3) In addition, a novel HDV strain, originated from a patient who has reached SVR under peg-IFNα therapy was here sequenced, to further understand the virus-specific differences of distinct HDV-strains. Full-genome sequencing is also fundamental to clone this novel HDV strain for its use in HDV research.

3 Material and Methods

3.1 Material

3.1.1 Table 1: Instruments

Device	Producer	Country
Centrifuge Galaxy Mini	VWR	USA
Centrifuge Mini Spin	Eppendorf	Germany
Centrifuge 5415R	Eppendorf	Germany
Centrifuge 5417C	Eppendorf	Germany
Geneious Bioinformatics Software	Biomatters Ltd	New Zealand
Graph Pad Prism 6 Software	GraphPad	USA
Light Cycler Software 3.5	Roche Diagnostics	Switzerland
ACD HybEZ oven	ACD	USA
Microscope Keyence X710	Keyence	Japan
Mini Trans-Blot Electrophoretic Transfer cell	BioRAD Laboratories	USA
Nanodrop 2000 spectrophometer	NanoDrop Technologies	USA
Power Supply PAC300	BioRAD Laboratories	USA
QuantityOne Software	BioRAD Laboratories	USA
QubitFluorometer 3.0	Thermo Fisher	USA
Thermocycler Veriti 96-well fast	Applied Biosystem	USA
ViiA™ 7 System	Life Technologies GmbH	Germany
ViiA™ 7 Software	Life Technologies GmbH	Germany
Vortexer MS2 Minishaker	IKA	Germany
Vortexer Reax Top	Heidolph	Germany
GFL 1083 (Water bath)	Thermolab	Germany

3.1.2 Table 2: General reagents

Reagents	Producer	Country
ABI Fast 1-Step Virus Master	Applied Biosystems	USA
ABI Fast Advanced Master	Applied Biosystems	USA
Acetone	Th. Geyer GmbH & Co	Germany
Anchored-oligo(dT) primer (cDNA)	Hoffmann-La Roche	Switzerland
ATP 100mM	Biozym Scientific GmbH	Germany
AW1 buffer	Qiagen	Netherlands
AF2 buffer	Qiagen	Netherlands
Buffer AL	Qiagen	Netherlands
Dako Mounting Medium	Dako	Denmark
Desoxynucleotide mix (DNAse Kit)	Hoffmann-La Roche	Switzerland
Ethanol 100%	Th. Geyer GmbH & Co	Germany
GelRed	GeneON GmbH	Germany
Glycogen	Hoffmann-La Roche	Switzerland
Hoechst	Hoechst AG	Germany
Isopopyl alcohol	Baxter International	USA
QIAGEN Protease	Qiagen	Netherlands
RDD buffer	Qiagen	Netherlands
Reaction buffer	Epicentre	USA
RLT buffer	Qiagen	Netherlands
RNAse free water	Qiagen	Netherlands
RPE buffer	Qiagen	Netherlands
RW1 buffer	Qiagen	Netherlands
RW2 buffer	Qiagen	Netherlands
Transcriptor Reverse Transcriptase	Hoffmann-La Roche	Switzerland
Transcriptor RT Reaction buffer	Hoffmann-La Roche	Switzerland

3.1.3 Table 3: Kits

Kits	Producer	Country
ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit	Applied Biosystems	USA
Architect HBeAg assay	Abbott Ireland Diagnostics	Ireland
Architect HBsAg assay	Abbott Ireland Diagnostics	Ireland
MasterPure DNA Purification Kit	Epicentre	USA
MinElute PCR Purification Kit	Qiagen	Netherlands
QIAmp MinElute Virus Spin Kit	Qiagen	Netherlands
QIAquick Gel Extraction Kit	Qiagen	Netherlands
RNeasy RNA Mini Kit	Qiagen	Netherlands
Taqman Gene Expression Assays	Applied Biosystems	USA
Transcriptor First Strand cDNA Synthesis Kit	Hoffmann-La Roche	Switzerland
TSA Fluorescein System Perkin	Elmer	Germany

3.1.4 Table 4: Antibodies for immunohistochemistry

Antibody	Dilution	Producer	Country
Anti-Cytokeratin 18 (DC, sc-6259) (Maus)	1:400	Santa Cruz Biotechnology	USA
Alexa Fluor® 488 (grün) Ziege anti-Kaninchen IgG (H+L) (A-11034)	1:400	Life Technologies	USA
Alexa Fluor® 546 (rot) Ziege anti-Maus	1:800	Invitrogen	USA
Alexa Fluor® 555 (rot) Ziege anti-Maus	1:800	Invitrogen	USA

3.1.5 Table 5: Primer and probes - Viral measurements

Assay	Primer/Probe	Sequenz 5'-3'
HDV	FW	GCG CCG GCY GGG CAA C
	RV	TTC CTC TTC GGG TCG GCA TG
	Probe	CGC GGT CCG ACC TGG GCA TCC G
HDV-1p	FW	AGG AGT AAG ATC ATA GCG ATA
	RV	CTG CTC TCT TTG CTT TCC
	Probe	CGC CTC GGT /ZEN/CTC CTC TAA CTC
ccHDV-1	FW	TCA CGG TAA AGA GCA TTG
	RV	TCA CGG TAA AGA GCA TTG
	Probe	CGT CCG CTT /ZEN/CCT GAG ACC TC
ccHDV-3	FW	GGT CCG TCG TTC CAT
	RV	GTA GCT CCC TCG GAT CGT TG
	Probe	CTT ACC TCG TGG CCG GC
HBV-pgRNA	FW	GGT CCC CTA GAA GAA GAA CTC CCT
	RV	CAT TGA GAT TCC CGA GAT TGA GAT
	Probe	TCT CAA TCG CCG CGT CGC AGA

The HDV-assay primers and probes were taken from the Ferns et al. 2011 publication. The ccHDV-3 primers are from the thesis (PhD thesis Hermanussen). The ccHDV-1 and HDV-1p primers and probe were redesigned as part of this thesis. The HBV pg-RNA primer and probe are from the publication by Malmström et al 2012. All primers and probes shown in the table were made by MWG-Biotech. The probe is dye labeled (FAM) at the 5' end and has a minor groove binder (MGB) and a non-fluorescent quencher (NFQ) at the 3' end.

3.1.6 Table 6: TaqMan genexpression assays

Gene	Assay-ID
HBV-DNA	Pa03453406_s1
HBV-RNA	Pa03453406_s1
Hβ-Globin	Hs00758889_s1
hGAPDH	Hs99999905_m1
hIP10/hCXCL10	Hs00171042_m1
hMX1	Hs00895608_m1
hOAS1	Hs00973637_m1

The TaqMan® gene expression assays were purchased from Thermo Fischer Scientific (USA). Assays consist of a pair of unlabeled PCR primers and a TaqMan probe. The probe is dye-labeled (FAM) at the 5' end and has a minor groove binder (MGB) and a non-fluorescent quencher (NFQ) at the 3' end.

3.2 Methods

3.2.1 Generation of humanized USB mice and viral infection

Human liver chimeric USB (urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID)/beige) mice were generated by transplanting one million thawed cryo-preserved human hepatocytes obtained from two different human donors (both with intermediate interferon responsive C/T IL28 locus) into homozygous USB mice, as previously reported (Lütgehetmann et al., 2012). All mice were maintained under specific pathogen free conditions in accordance with institutional guidelines under approved protocols. Primary human hepatocytes were isolated from rejected explant livers using protocols approved by the Ethical Committee of the city and state of Hamburg (OB-042/06) and accorded to the principles of the Declaration of Helsinki. Repopulation rates were estimated by human serum albumin (HSA) levels in mouse sera (Bethyl Laboratories, Biomol GmbH, Hamburg, Germany) and confirmed at sacrifice by determining human cell contents by histology and qRT-PCR using the beta-globin gene kit (Roche DNA control Kit; Roche Diagnostics) (Lütgehetmann et al., 2012). Animals displaying high levels of human chimerism (>2 mg/ml HSA in serum) were used for the study. All animal experiments were conducted in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the City of Hamburg, Germany.

3.2.2 Establishment of viral infection

To establish an HBV/HDV co-infection, human chimeric USB mice received a single peritoneal injection of 100 µl HBV positive, patient derived serum (1×10^7) HBV DNA copies/mouse, genotype D, HBeAg-positive, provided by Dieter Glebe, Universität Gießen, Germany) together with HDV of genotype 1 and 3 (HDV-1 and HDV-3) either derived from cell culture or patient sera. For HDV-3 infection, one particular cell culture-derived virus was used (provided by John Casey, Washington D.C., USA (John L. Casey & Gerin, 1998), 1×10^7 HDV RNA copies/mouse, which will now be referred to as “ccHDV-3”. For HDV-1 infection, either cell culture-derived virus

(provided by John Taylor, Fox Chase Cancer Center, USA, 1 x 10^7 HDV RNA copies/mouse (Kuo et al., 1989) which will now be referred to as “ccHDV-1” or serum from an HDV-1/HBV genotype D co-infected patient, which will now be referred to as “HDV-1p” were used for infection establishment. The inoculation with 1 x 10^7 viral copies corresponded to an MOI (multiplicity of infection) of approximately 0.3 (Dandri et al., 2008). To establish HDV mono-infection, humanized USB mice received a single peritoneal injection of 100µl of either ccHDV-1 or ccHDV-3 virions (1 × 10^8) HDV RNA copies/mouse). The humanized mice were co-infected with either HBV + ccHDV-1 or HBV + ccHDV-3. Blood samples were taken at different time-points, as indicated in the results. The mice were sacrificed at the end of treatment after 13 weeks. Liver specimens collected at sacrifice were cryo-conserved in chilled isopentane for further histological and molecular analyses.

3.2.3 Antiviral treatments

Stably HBV/HDV co-infected (9 weeks post virus inoculation) human-chimeric USB mice were treated with peg-IFNα (provided by Hoffmann-La Roche Inc., Basel, Switzerland) (n=6) or peg-IFNλ (provided by Bristol-Myers Squibb, CT US) (n=8) for four weeks. All co-infected animals were sacrificed 24 hours after their last interferon injection for intrahepatic analyses. The IFNs were injected subcutaneously twice a week (each 25 ng/g body weight) (Lena Allweiss et al., 2014). In humans, 180 µg of peg-IFNα is used for a single injection, which would be an equivalent of 50ng/20 g mouse. In line with previous reports and according to commonly used dose scaling to adjust human doses to mouse equivalent doses we used 500ng/20g mouse (25ng/g body weight) in this study (Allweiss et al., 2014; Nakagawa et al., 2013).

In the setting of HDV mono-infection with either ccHDV-1 or ccHDV-3 the mice were treated with peg-IFNα (25ng/g body weight). The mice received Myrcludex-B (Myr-B, kindly provided by prof. Urban, Germany, Heidelberg) daily (2µg/g body weight), starting 24h after infection. The mice were killed after 1-week post infection. For all experiments control mice were infected in parallel but left untreated.

3.2.4 Virological measurements and intrahepatic quantification

Viral DNA and RNA were extracted from serum samples using the QiAmp MinElute Virus Spin kit (Qiagen, Hilden, Germany) and from liver tissues using the RNeasy RNA purification kit (Qiagen, Hilden, Germany) and the MasterPureTM complete DNA Purification Kit (Epicentre, Madison, Wisconsin, USA). For RNA and DNA isolation from mouse serum, 5 µl were used and purified over the columns according to the manufacturer's instructions. The isolated RNA/DNA was then eluted in 25 µl of water and stored at -80°C. To isolate RNA and DNA from frozen liver tissue, small pieces (approximately 30 mg) were separated with a razor blade and placed on dry ice in a 1.5 ml tube containing 420 µl of RTL buffer (RNEasy® Mini Kit). The liver pieces were then pounded until an almost homogeneous liquid was obtained. 200 µl were used for DNA extraction and the rest for RNA extraction, so that virological DNA and RNA parameters could be determined from the same liver piece. RNA extraction was performed according to the manufacturer's instructions using the RNeasy® Mini Kit and without further modifications. For subsequent qRT-PCRs, samples with RNA concentrations of 100-500 ng/µl (determined with Nanodrop 2000 Spectrophometer, Thermo Scientific, USA) were used and higher concentrations were diluted accordingly. DNA extraction was performed using the QiAmp MinElute Virus Spin Kit according to the manufacturer's instructions and without further modification. DNA concentrations were measured using a Qubit® flurometer and the Qubit® dsDNA BR buffer (Thermo Fisher, USA). Samples with concentrations of 100-300ng/µl were used for subsequent qRT-PCRs.

3.2.4.1 HDV measurements

HDV viremia and intrahepatic HDV RNA levels were determined by reverse transcription and qRT-PCR using the ABI Fast 1-Step Virus Master (Applied Biosystems, Carlsbad, USA) and HDV specific primers and probes using the ABI Viia7 PCR system (Applied Biosystems, Carlsbad, USA) (Table 5) (Ferns et al., 2012). In detail, RNA extracted from 5 µl mouse serum or 1 µl liver derived RNA were denatured at 95 °C for 10 min, immediately cooled down to −4 °C and reverse transcribed at 50 °C for 5 min. After inactivation of the reverse transcriptase at 95 °C for 20 s, amplification was performed under the following conditions: initial step 95 °C 20 s, 40 cycles at 95 °C for 3 s and 60 °C for 30 s. HDV viremia is shown as median log change from median baseline levels.

3.2.4.2 HBV measurements

HBV viremia was determined as reported (Dandri et al., 2008). Known amounts of HBV or HDV containing plasmids were used as standards for quantification and hGAPDH and hRPL30 for expression normalization (Chen et al., 1986). HBsAg quantification was performed using the Architect HBsAg assay (Abbott Ireland Diagnostics, Sligo, Ireland). HBV viremia is shown as median log change from median baseline levels.

For HBV DNA measurement, 4.5 µl of isolated serum DNA was added to 5 µl of ABI Fast Advanced Master and 0.5 µl of probes/primer (500 µM). Amplification was performed under these conditions: preliminarily 95°C for 20 seconds, then 40 cycles of 95°C for 1 second and 60°C for 20 seconds. For HBV quantification in serum, known amounts of a plasmid containing HBV were used. To measure HBV pgRNA, 1µl of isolated liver RNA was added to 2.5µl of ABI Fast 1-Step Virus Master (Applied Biosystems, Carlsbad, USA), 1.0µl of primers (1 mM) and 0.5µl of probe (500 µM). Then, reverse transcription (50°C for 5 min, then 95°C for 20 seconds to inactivate reverse transcriptase) and amplification (40 cycles: 95°C for 3 seconds, 60°C for 30 seconds) were performed in one step. Levels of intrahepatic pg HBV RNA were normalized to human-specific hGAPDH (Taqman Gene Expression Assay Hs999905_m1, Applied Biosystems).

3.2.5 Immunohistochemistry

Cryostat sections of chimeric mouse livers were stained as previously described (Lütgehetmann et al., 2012). Briefly, sections were fixed with acetone for 10 minutes and incubated with mouse anti-CK18 (1:400; Dako, Glostrup, Denmark) and human anti-Delta (anti-HDAg-positive human serum, 1:8,000). Specific signals were visualized with Alexa 488- or 555-labeled secondary antibodies (Invitrogen, Darmstadt, Germany). Nuclear staining was achieved by Hoechst 33258 (1:2,000 diluted, Invitrogen, Darmstadt, Germany). Stained sections were then mounted with fluorescent mounting media (Dako, Glostrup, Denmark) and analyzed with the fluorescence microscope BZ9000 (Keyence, Osaka, Japan) using the same settings for the different experimental groups. The percentages of HDAg positive human hepatocytes were estimated by using 4 visual fields (displaying approx. 2000 human hepatocytes) per mouse. Cell counting of HDAg-positive cells was performed by using the BZ-II Analyzer Software (Keyence).

3.2.6 Expression of human ISGs and cytokines

To determine expression levels of genes related to IFN signalling (ISGs and cytokines) in human hepatocytes repopulating the mouse liver, primers specifically recognizing human transcripts and not cross-reacting with murine genes were used (Taqman Gene Expression Assays, Applied Biosystems). We analysed hOAS1 (Hs00973637_m1), hCXCL10 (Hs00171042_m1) and hMxA (Hs00895608_m1). For determining the expression of ISGs and cytokines, RNA was extracted as described above, cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) using oligo-dT primer according to the manufacturer's instructions and qRT-PCR was performed with the ABI Fast Advanced Master (Applied Biosystems, Carlsbad, USA) in an ABI Viia7 (Applied Biosystems, Carlsbad, USA). Expression levels were calculated relative to the median of hGAPDH and human hRPL30 and are depicted as log change from median values obtained from untreated control mice.

3.2.7 Sequencing

For HDV full genome sequencing of the amber/W site (position 1012) cDNA was synthesized with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) using random hexamer primer according to the manufacturer's instructions. Five overlapping PCR products were amplified by using cDNA, respective primers (Table 1, primer pairs: WHO-F5 & WHO-R4, WHO-F5 & Tx-R2, 879F1 & WHO-R4, 1160FNew & 430R and 300F & 1400R) (Pyne et al., 2017) and a Red-Taq Polymerase (Sigma-Aldrich, St. Louis, USA). Gradient PCR was used in order to determine the optimal annealing temperature. A gradient of 59 to 68°C was set. PCR cycling consisted of 30 seconds of initial denaturation at 98°C, 35 cycles: 10 seconds at 98°C, gradient annealing temperatures (59-68°C), 40 seconds 72°C. PCR product length was analyzed on a 1,5% agarose gel and DNA fragments were extracted with the MinElute PCR Purification Kit (Qiagen) as recommended by the manufacturer. The forward and reverse strand was sequenced with Sanger sequencing (Mix2seq kit) performed by Eurofins Genomics (Ebersberg, Germany).

Primer Pairs	RV- and FW Primer	Sequenz 5'-3'
PP-1	WHO-F5	CATGAGCCAAGTTCCGAGCG
	WHO-R4	CTCGAACTTGGGCGGCGGGT
PP-2	WHO-F5	CATGAGCCAAGTTCCGAGCG
	Tx-R2	CAAGAGTTGTCGACCCCAGTG
PP-3	879F1	GGTGGAGATGCCATGCCG
	WHO-R4	CTCGAACTTGGGCGGCGGGT
PP-4	1160F NEW	GCGGGCCGGCTACTCTTCTTT
	430R	CTAGCCCCGTTGCTTTCTTTGCTTT
PP-5	300F	ACCTCCAGAGGACCCCTTCAGCGAA
	1400R	GAGGGAGCTCCCCCGGCGAAGAG

Table 7: Primer Pairs for HDV-1p Sequencing (Pyne et al., 2017)

4 Results

The overall results of this thesis are based on a number of separate experimental approaches to gain deeper insight into the mechanisms of interferon responsiveness in the treatment of HDV infection. Three different sets of experiments can be subdivided. First, the comparative study of two interferons (peg-**IFNα** and peg-**IFNλ**), which were used to treat humanized mice infected with either ccHDV-1 or ccHDV-3 in the setting of stable co-infection with HBV. Second, the study of interferon pre-treatment in the setting of HDV mono-infection to obtain more detailed information about the effect of interferon treatment on HDV infection establishment and its mode of action. And third, sequencing of an interferon sensitive HDV-1 strain and subsequent establishment of a strain specific qPCR.

4.1 Comparative analysis of peg-IFNα and λ in HBV/HDV co-infection

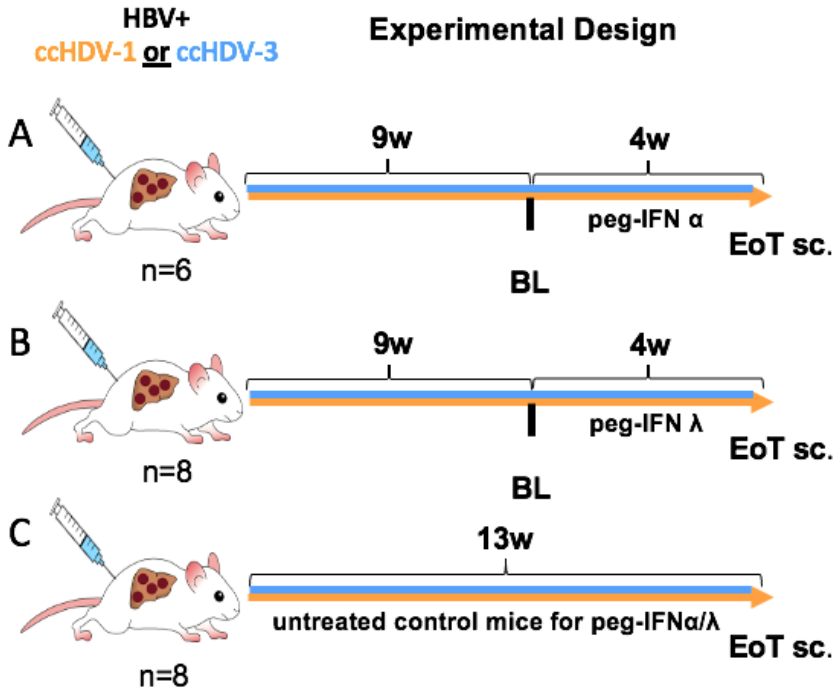


Figure 12: Comparative analysis of peg-IFNα/λ in chronic HBV/HDV co-infection. *To study the interferon responsiveness in a stable co-infection setting – humanized mice were co-infected with HBV (GT-D) and either ccHDV-1 or ccHDV-3 for 9 weeks until titers were stable. The mice were then treated for 4 weeks with peg-IFNα (25ng/g body weight, n=3) (A) or peg-IFNλ (25ng/g body weight, n=4) (B)*

subsequently. For both HDV genotypes a corresponding control group with comparable viremia levels was included (n=4) (C). (EoT: End of treatment)

Experimental Setup. As shown in Figure 12 - humanized USB mice were co-infected with HBV and ccHDV-1 (n=11) or ccHDV-3 (n=11). After 9 weeks, when titers were stable, peg-IFNα and peg-IFNλ treatment of ccHDV-1- (ccHDV-1 + peg-IFNα: n=3, ccHDV-1 + peg-IFNλ: n=4) and ccHDV-3- (ccHDV-3 + peg-IFNα: n=3, ccHDV-3 + peg-IFNλ: n=4) infected mice was performed for a period of 4 weeks. A control group (ccHDV-1: n=4, ccHDV-3: n=4) remained untreated. Serum samples were collected from all infected mice both before the start of treatment (BL) and at two and four weeks after the start of interferon treatment. Before treatment started, mice were grouped based on HDV titers and human serum albumin to minimize potential bias.

4.1.1 HDV - Serological analysis

In humanized mice treated with peg-IFNα a strong serological reduction of ccHDV-3 (median -1.7-log change) could be determined. Interestingly, we could not observe a clear antiviral effect against ccHDV-1 in infected mice (median -0.3-log) compared to untreated control mice over the treatment period of 4 weeks (Figure 13 A/C). Regarding the treatment of mice with peg-IFNλ, a similar antiviral effect on ccHDV-3 viremia (median -1.5-log change) compared to treatment with peg-IFNα was seen.

We were surprised to find that treatment of ccHDV-1 infected mice achieved a clear antiviral effect after 2 weeks of peg-IFNλ treatment (median -1.4-log change), as peg-IFNα did not affect this ccHDV-1 strain. However, ccHDV-1 viremia was refractory since in 3 out of 4 mice titers were raising again at the end of the 4-week treatment period (median -0.5-log change) compared to untreated control mice (Figure 13 B/D). These data revealed a differential response of the two HDV strains used to both peg-IFNα and peg-IFNλ at the end of the 4-week treatment period, as both, peg-IFNα and peg-IFNλ, exert strong antiviral serological effects against ccHDV-3, but not against ccHDV-1 after 4 weeks of IFN-treatment.

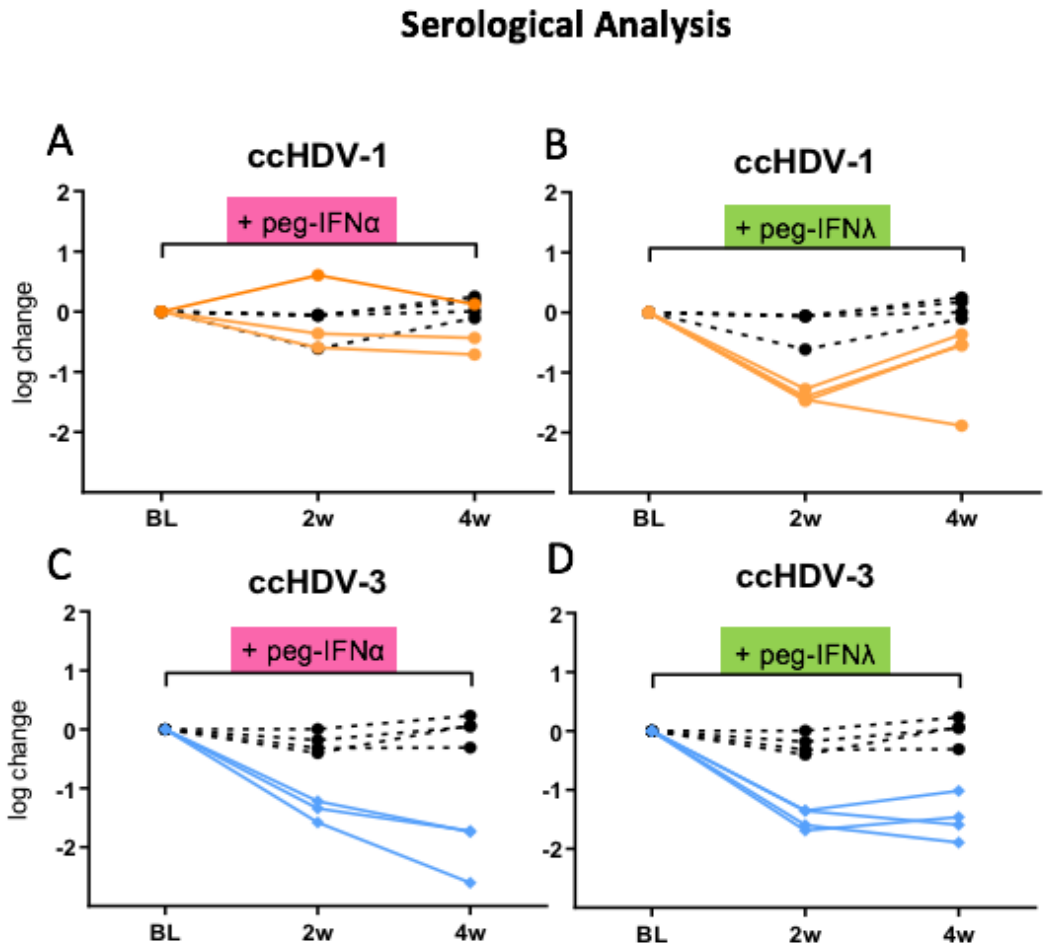


Figure 13: ccHDV-1 and ccHDV-3 viremia over 4-weeks of peg-IFN α/λ treatment. *HDV viremia is shown in peg-IFNα (pink) and peg-IFNλ (green) treated and untreated HBV/ccHDV-1- (orange) or HBV/ccHDV-3 (blue) co-infected mice as median log change of each mouse from baseline (BL) levels to the end of 4 weeks. Over the treatment period of 4 weeks serum samples were taken at BL, 2 and 4 weeks, respectively. Control mice remained untreated (dashed line).*

4.1.2 HDV - Intrahepatic analysis

In addition to HDV viremia, the intrahepatic levels of HDV RNA were analysed after 4 weeks of treatment with peg-IFNα and peg-IFNλ. The intrahepatic results are in line with the serological analysis, as treatment with either peg-IFNα or λ did not affect intrahepatic ccHDV-1 RNA levels when compared to the untreated control group (median -0.16 and 0.12-log change) (Figure 14 A). In contrast, median RNA levels in cc-HDV-3 infected mice clearly decreased over the 4-week treatment period when treated with either peg-IFNα or peg-IFNλ (median -0.99-log and 0.93-log change) (Figure 14 B).

Immunofluorescence staining (IF) also confirmed strain specific intrahepatic differences to peg-IFNα treatment. On the one hand, regarding infection efficacy, we detected considerably more HDV-positive cells in ccHDV-3 infected mice (60%), compared to ccHDV-1 (35%), which is in line with previous findings of our group (Giersch et al., 2017). Regarding the effect of the two interferons used for treatment, the strong antiviral effect of peg-IFNα/λ on serological and intrahepatic parameters in ccHDV-3 infected mice, as well as no effect in ccHDV-1 infected mice, was also evident in the immunofluorescence staining (Figure 14 C). Overall, the amount of HDAg positive cells in the co-staining of HDAg and CK18 in ccHDV-3 infected mice was clearly lower in peg-IFNα treated mice (30%) compared to the corresponding control group (60%). In contrast, ccHDV-1 infected mice showed a similar amount of HDAg positive cells (32%) at the end of the 4-week treatment period, compared to untreated controls (35%) when treated with peg-IFNα. (Figure 14 C). Treatment with peg-IFNλ also revealed a clear reduction of ccHDV-3 HDAg positive cells (25%) compared to controls (60%). Interestingly, peg-IFNλ also did not reduce HDAg positive cells in HDV-1 infected mice (37%). In fact, there was even a small increase in HDV positive cells noted compared to the control group. Consistent with previous results, this intrahepatic analysis underlines a significantly different behavior of the commonly used ccHDV-1 strain, by not responding to IFN-treatment, from other strains, such as ccHDV-3 when treated with IFNs. (Figure 14 C). (Giersch et al., 2017; Han et al., 2011; Ilan et al., 1992; Zhang et al., 2018)

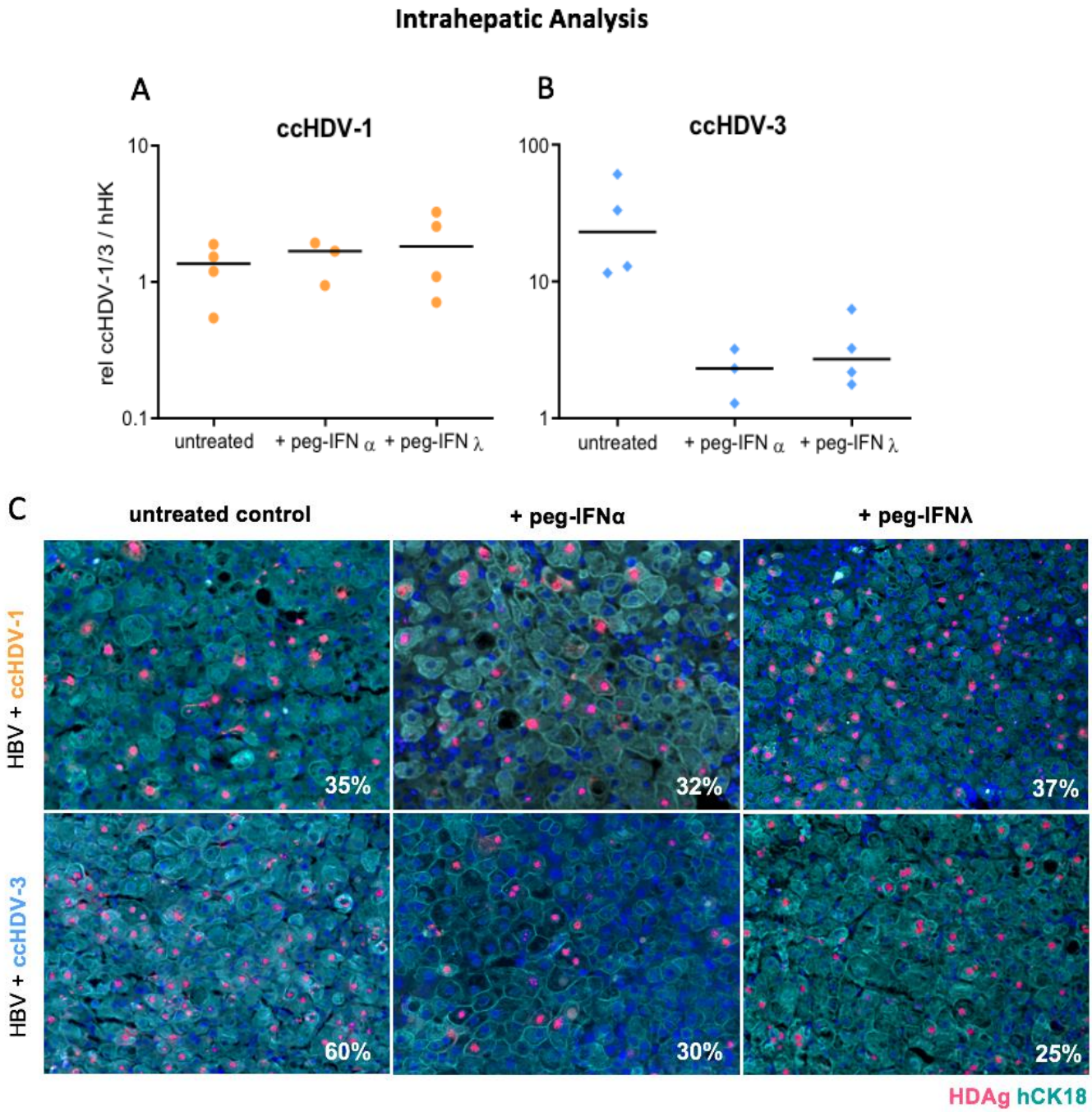


Figure 14: Intrahepatic analysis. *Intrahepatic HDV RNA levels relative to hGAPDH, in peg-IFNα/λ treated mice and untreated control mice (A, B). Immuno-fluorescence staining of mouse livers after 4 weeks of treatment with peg-IFNα//λ and in control mice. Nuclei blue, HDAg red, human CK18 aqua (C). The median percentage of HDV-infected human hepatocytes of all counted IF images is indicated in the specific representative images.*

4.1.3 HBV - Serological and intrahepatic analysis

In order to investigate the effect of peg-IFNα and peg-IFNλ on HBV, serological and intrahepatic HBV parameters were evaluated over the 4 weeks of treatment.

HBsAg levels are a main predictor of treatment response in patients, since it ultimately defines the point of cure in chronic HBV-infection (Papatheodoridis et al., 2012). It is a discussed whether HBsAg levels can serve as a marker to revise the duration of treatment in CHD patients (Zachou et al., 2010). The analysis of serum HBsAg levels revealed a small decrease of a median 0.5-log and 0.4-log change in ccHDV-1 and ccHDV-3 infected mice, respectively, over the 4-week treatment period (Figure 15). Similarly, peg-IFNλ also reduced HBsAg by -0.3-log and -0.5-log in ccHDV-1 and ccHDV-3 infected mice. Notably, in ccHDV-3 infected mice the HBsAg levels were consistently around one log lower compared to ccHDV-1 infected mice (untreated controls and treated mice), indicating that ccHDV-3 is able to suppress HBV more than ccHDV-1. Both, peg-IFNα and peg-IFNλ were able to reduce all intrahepatic HBV parameters (Figure 16).

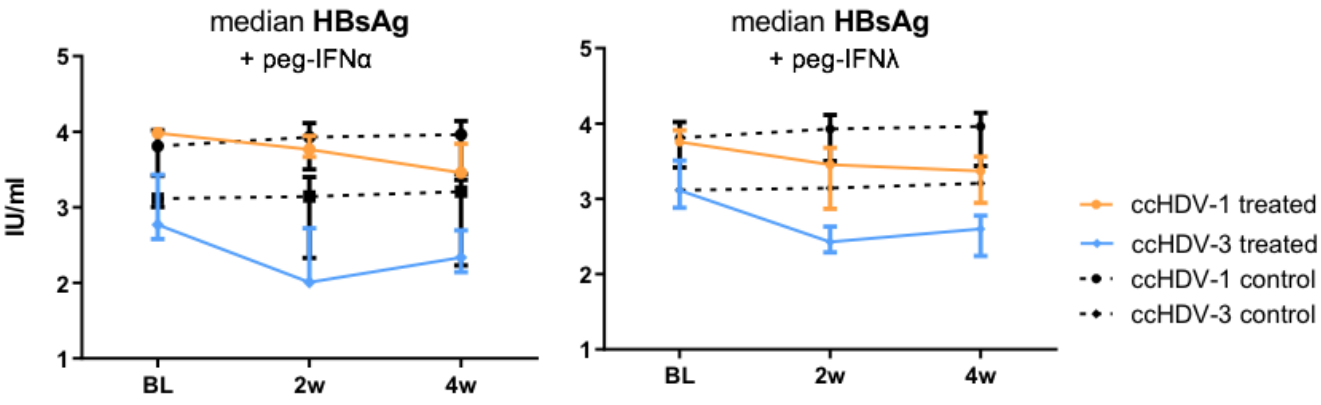


Figure 15: Median HBsAg level. *The figure shows the median HBsAg (IU/ml) level at BL, after 2 and 4 weeks of treatment with either peg-IFNα (left) or peg-IFNλ (right) in humanized mice infected with either ccHDV-1 (orange) or ccHDV-3 (blue).*

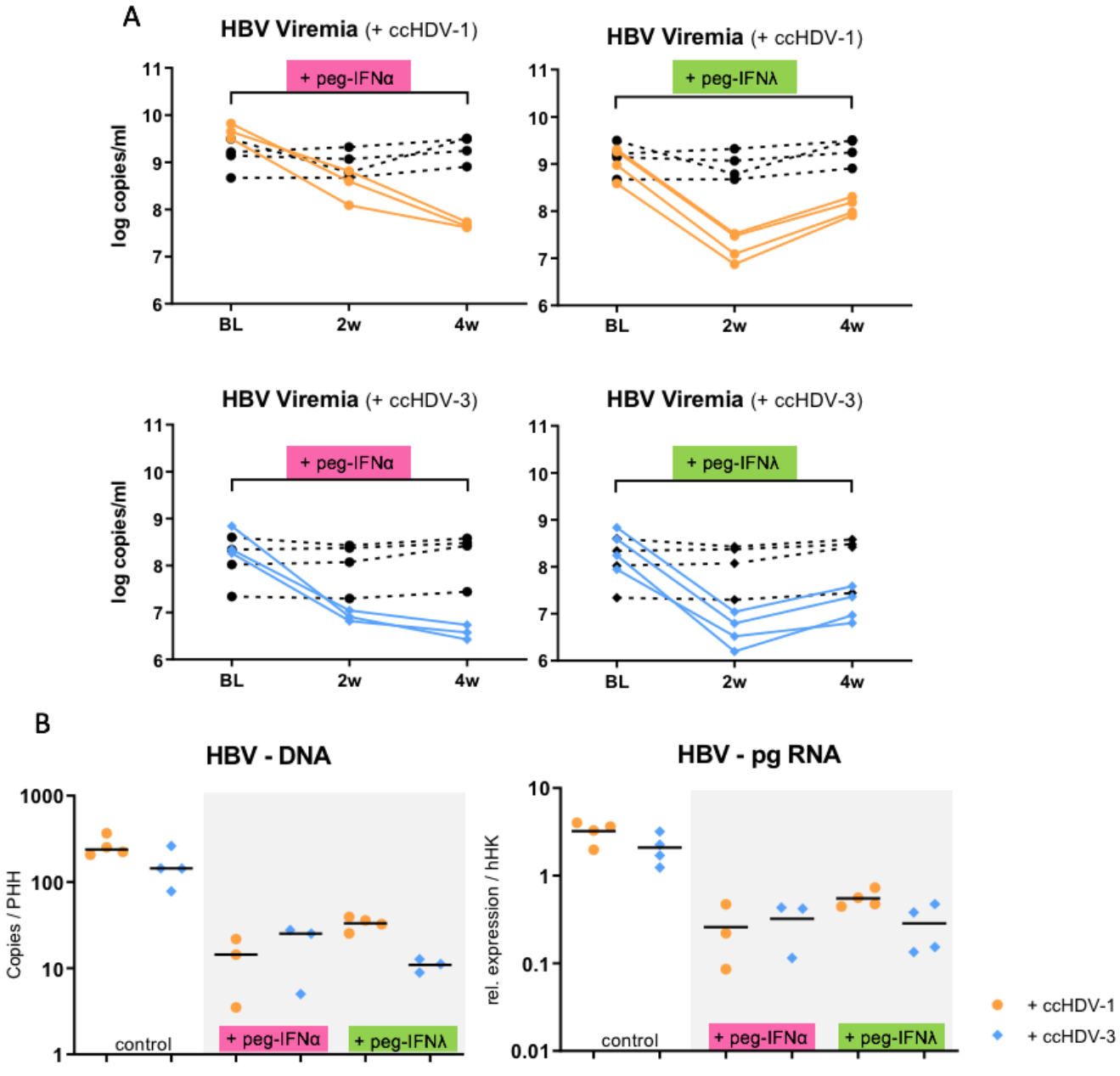


Figure 16. Serological and intrahepatic HBV parameters. *The effect of peg-IFNα and peg-IFNλ on serological and intrahepatic HBV parameters in either co-infection with ccHDV-1 or ccHDV-3. A: HBV viremia is shown here over the 4-week treatment period. Controls are shown as dashed line, HBV/ccHDV-1 infected mice in orange and HBV/ccHDV-3 infected mice in blue. Peg-IFNα treatment on left, peg-IFNλ treatment on right. B: intrahepatic HBV DNA in copies/primary human hepatocytes (PHH) (ß-globin) and intrahepatic HBV pg-RNA relative to human GAPDH after 4 weeks of interferon treatment.*

In ccHDV-1 infected mice median titers of $4.5x10^9$ HBV DNA copies/ml were reached at baseline, whereas the HBV titers in ccHDV-3 infected mice were lower, showing a median level of $2.9x10^8$ HBV DNA copies/ml. At the end of antiviral treatment (4 weeks), peg-IFNα induced a median 1.9-log and 1.7-log decrease in HBV viremia in mice infected with either ccHDV-1 or ccHDV-3 (Figure 16 A). Interestingly, peg-IFNλ achieved a strong reduction of HBV viremia after 2 weeks in ccHDV-1 and -3 infected mice (-1.7-log and -1.8-log), but surprisingly the effect of treatment diminished, as HBV viremia increased again at the end of the 4-week treatment period (0.8-log and 1,2-log) compared to stably infected untreated controls (Figure 16 A). Intrahepatic analysis revealed a strong decrease of HBV DNA and HBV-pg-RNA levels after 4 weeks of treatment with either peg-IFNα or peg-IFNλ in co-infection with both ccHDV-1 and ccHDV-3 compared to controls (Figure 16 B). In sum, both interferons showed a clear antiviral effect on HBV, which was to be expected as this is in line with previous data (Giersch et al., 2017).

The measurement of human serum albumin in humanized mice is an important parameter to assess the level of repopulation. To ensure comparability of individual mice between different HDV strains and interferon treatment, a balanced distribution of albumin levels in treated and untreated mice is very important and allows accurate analysis between groups. Monitoring of albumin levels is required before infection with HDV to ensure an equal and equitable distribution of mice before infection. Albumin levels were examined before and after IFN-treatment to determine whether IFNs have cytotoxic effects on human hepatocytes. During peg-IFNα administration, treated mice showed a slight decrease in serum albumin levels over the entire course of the 4-week therapy period. In the ccHDV-1 infected animals that received peg-IFNα, it was shown that a slight decrease in serum albumin occurred in these animals even before peg-IFNα treatment. Serum albumin levels of mice treated with peg-IFNλ also showed a decrease in the first 2 weeks of treatment, which reversed after 4 weeks, but still remained below baseline levels. The sudden loss of effect is surprising and requires further analysis, since there is initially no obvious explanation. In the discussion part of this work, some potential reasons for the loss of efficacy of the antiviral therapy will be addressed in detail. (Figure 17)

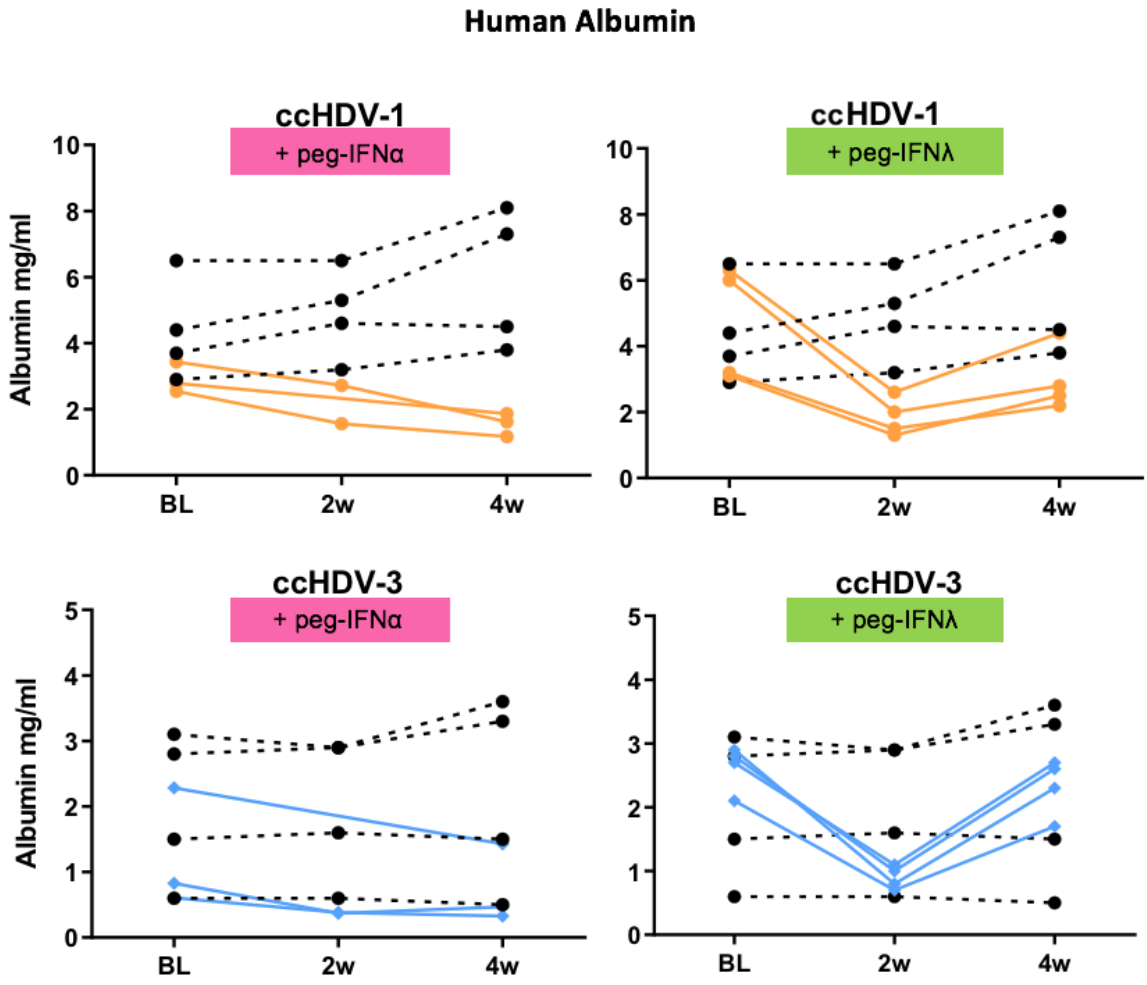


Figure 17: Serological human serum albumin. *Human serum albumin (mg/ml) in HBV/ccHDV-1 or HBV/ccHDV-3 co-infected mice from BL (start of treatment,2 and 4 weeks after IFN treatment. A single control group served for both IFN treatments. Colour coding indicates which of the ccHDV-1 (in orange) and ccHDV-3 (in blue) received peg-IFNα (left) or peg-IFNλ (right) treatment. Control group is indicated by the dashed line.*

4.1.4 Intrahepatic ISG-induction after peg-IFNα/λ treatment

The results obtained above raised the question if the resistance of ccHDV-1 to peg-IFN treatment is based on the lack of induction of certain IFN-stimulated genes (ISGs) or ISG-related pathways and thus caused by a direct interference with the innate immunity of human hepatocytes. Therefore, intrahepatic human ISGs (hMxA, hOAS1, hISG15) and cytokine RNA (hCXCL10) levels were analysed by qPCR in all mouse groups. The expression levels were normalized to human housekeepers (hGAPDH, hRPL30). Surprisingly, a similar induction of human ISGs was detected upon infection with either ccHDV-1 or ccHDV-3 and when treated with either peg-IFNα or peg-IFNλ (Figure 18).

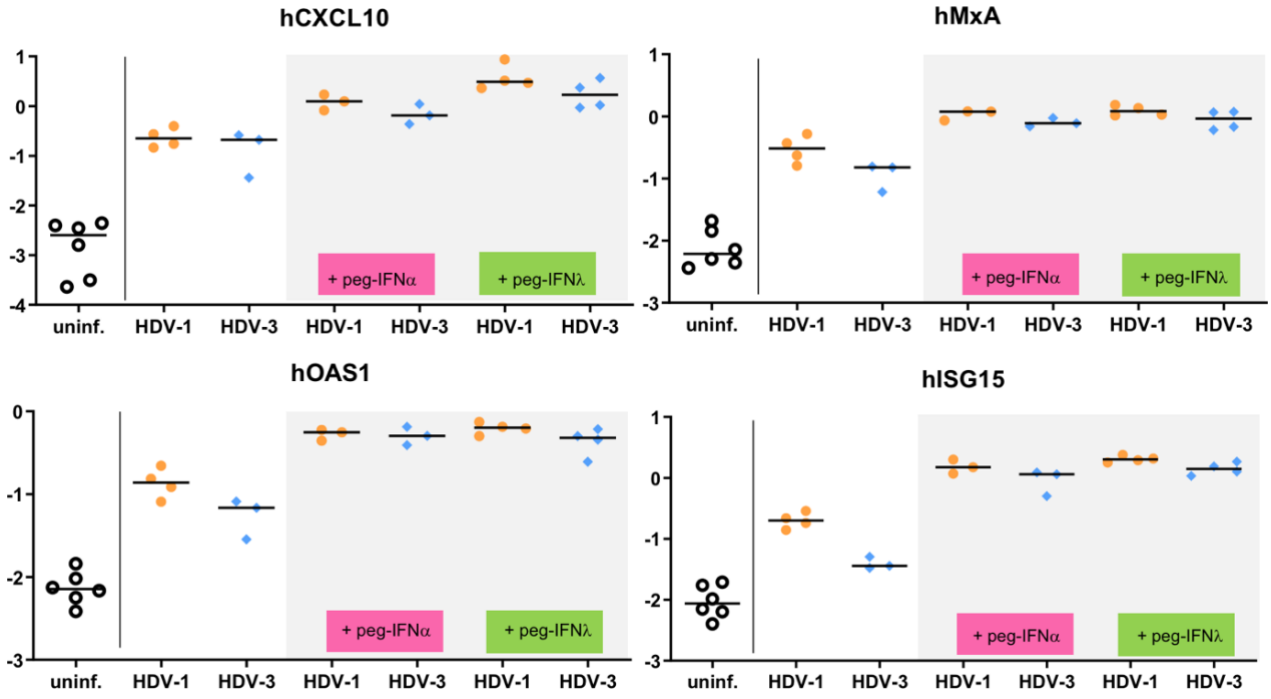


Figure 18: Intrahepatic ISG-Induction. *hISGs in HDV/HBV-coinfected mice after treatment with peg-IFNα and peg-IFNλ: Expression of hISGs relative to hGAPDH and hRPL30 in uninfected human liver chimeric mice (black), in untreated and peg-IFNα/λ-treated ccHDV-1/HBV- (in orange) or ccHDV-3/HBV-coinfected mice (in blue).*

4.2 Pre-treatment with peg-IFNα in HDV mono-infection

The aim was to investigate whether not only a stable (established) HDV infection, but also infection establishment (cell entry and/or early steps of infection) is affected by peg-IFNα treatment. To study the early steps of infection in humanized USB mice, the setting of an HDV mono-infection was used. The absence of HBV prevents HDV from spreading, which is why the number of infected cells is low. Our group also showed that HDV mono-infection remained similar for at least 6 weeks post infection (Giersch et al., 2014). An inhibitory effect on virus entry would therefore be reflected in a lower number of HDV-infected human hepatocytes and lower intrahepatic HDV RNA levels than in untreated mice. This is particularly interesting with regard to the ccHDV-1 strain resistant to the peg-IFNα/λ treatment, which showed no significant response to interferon therapy in the stable co-infection setting.

4.2.1 Experimental setup

Humanized mice were mono-infected with either ccHDV-1 or ccHDV-3 and pre-treated with peg-IFNα 24h before infection. To ensure that treatment only affected the entry phase mice received the entry inhibitor Myrcludex-B daily, starting 24h after infection and were sacrificed 9 days later (Figure 19 A). Our group has previously shown that the ISG induction through peg-IFNα and peg-IFNλ after a single shot administration reached its peak around 24h post injection (Figure 19 B). The exact time of infection was therefore chosen to be 24 hours after the single administration (pre-treatment) of peg-IFNα.

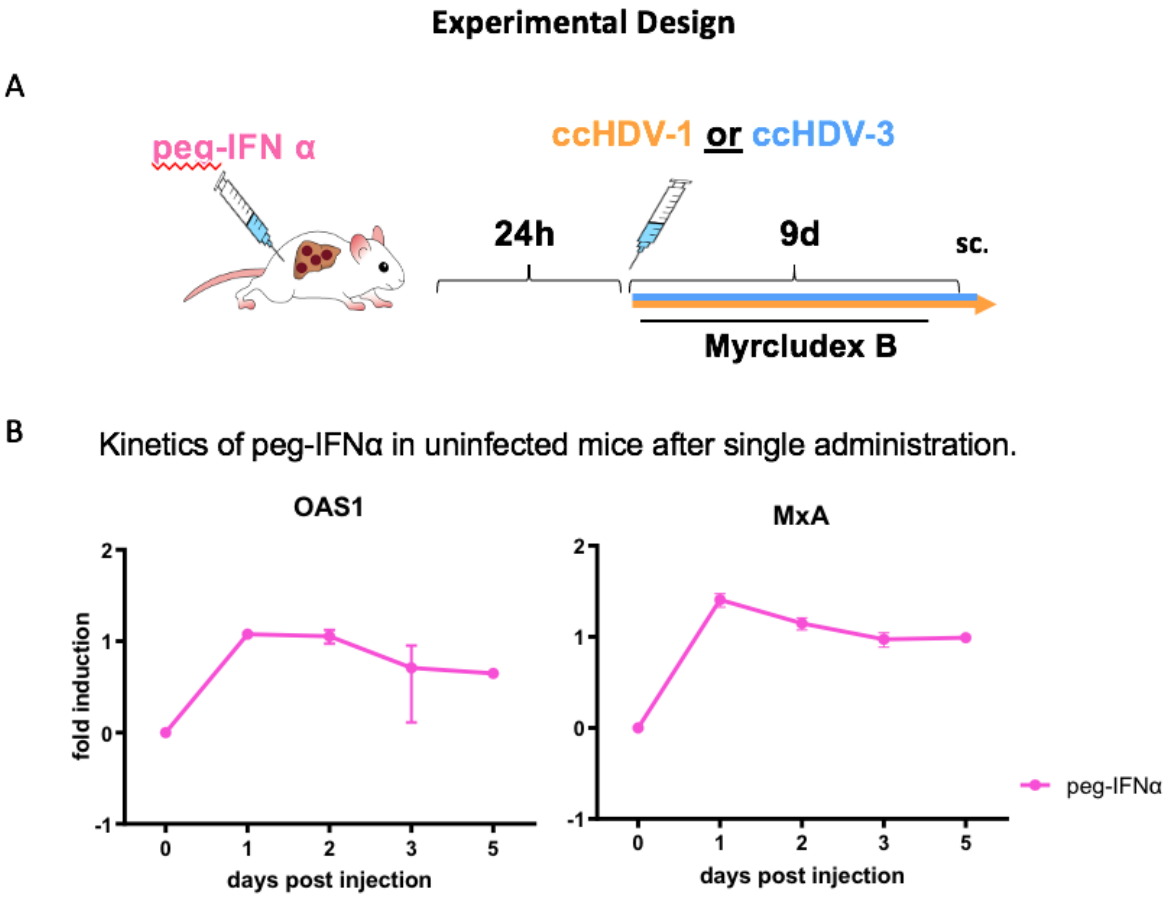


Figure 19: Experimental design of HDV-mono-infection. *Pre-treatment of humanized mice with peg-IFNα 24h before mono-infection with either ccHDV-1 or ccHDV-3. The mice received Myrcludex-B daily (first dose 24h after HDV-infection) and were sacrificed 9 days later. (ccHDV-1 (n=3) and control group (n=3), ccHDV-3 (n=3) and control group (n=3)) (A). Intrahepatic hISG induction in untreated mice before and after inoculation with either peg-IFNα or peg-IFNλ (*25ng/g body weight) (B).

4.2.2 Intrahepatic analysis

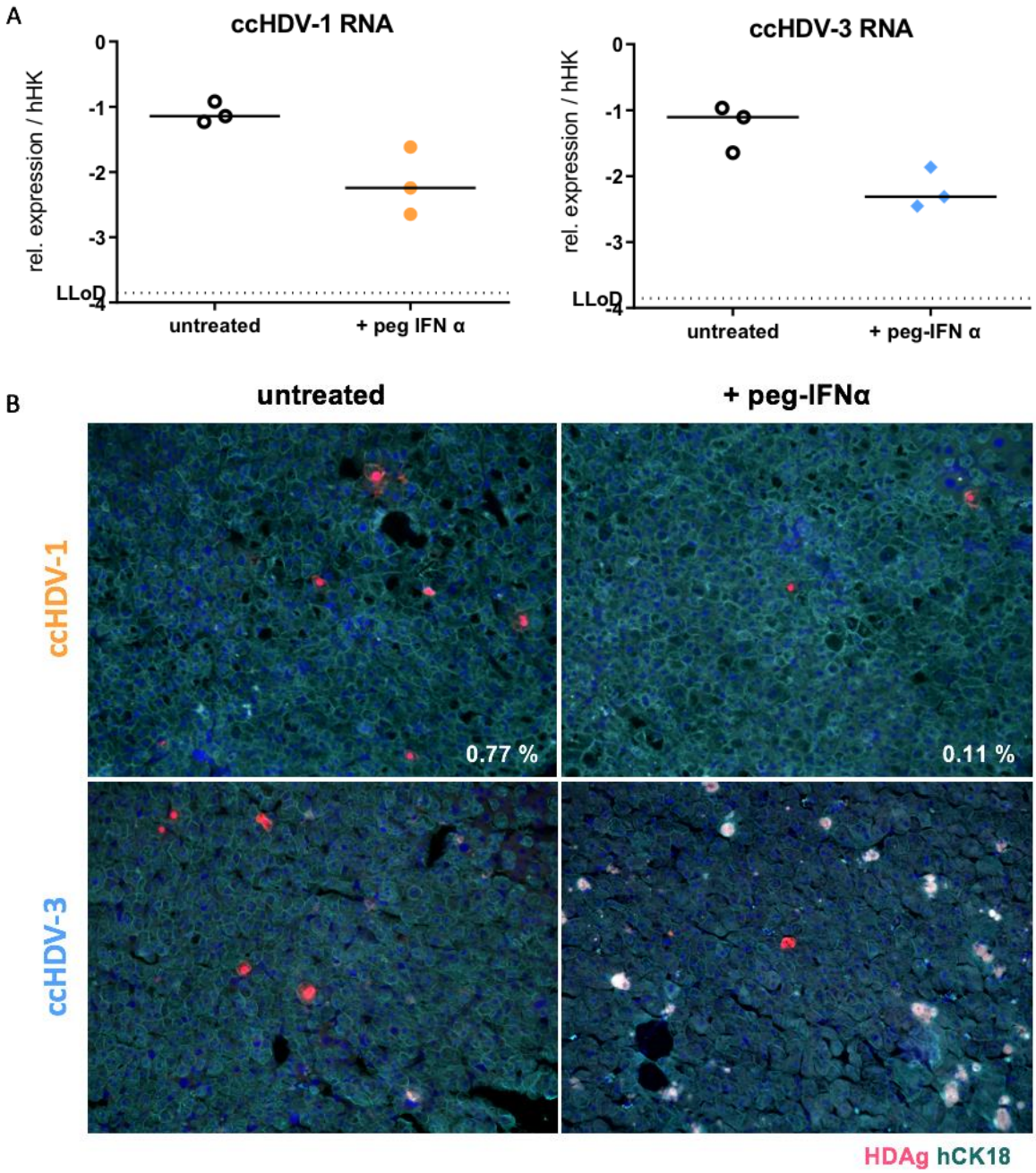


Figure 20: **Intrahepatic changes upon pre-treatment with peg-IFNα.** *Intrahepatic HDV RNA levels relative to hGAPDH in peg-IFNα pre-treated and untreated control mice after 9 days post infection with either ccHDV-1 (orange) or ccHDV-3 (blue). Immunofluorescence staining of mouse livers either infected with ccHDV-1 or ccHD-3 in untreated control mice (left) or mice that received pre-treatment with peg-IFNα. Nuclei blue, HDAg red, human CK18 aqua.*

A clear effect of peg-IFNα pre-treatment on early steps of infection was observed in both ccHDV-1 and -3 infected mice as the intrahepatic analysis revealed a clear reduction of 1.1-log and 1.2-log of ccHDV-1 and ccHDV-3 HDV RNA levels (Figure 20 A).

Notably, peg-IFNα did not affect ccHDV-1 intracellular levels in the prior experiments (setting of stable co-infection). In line with previous experiments, we were to find this particular ccHDV-1 strain to be sensitive to peg-IFNα treatment in early steps of infection. In order to ensure that the entry phase was actually inhibited (less cells infected) we performed an immunofluorescence staining of HDAg and CK18. In both, ccHDV-1 and -3 mono-infection a clear reduction of HDAg positive cells (relative to PHH) was observed, confirming the previous intrahepatic analysis (Figure 20 B).

4.3 Sequencing of HDV-1p (peg-IFNα sensitive strain)

Today, there are very few HDV strains that find use in experimental research. The ccHDV-1 strain mainly used in this work is resistant to treatment with interferon alpha in the setting of a stable HBV/HDV co-infection. It is one of the most used strains in experimental HDV research. In order to contribute to more comparative analysis using different HDV strains and to unveiling the mode of action of IFNs, a particular HDV a particular HDV-1 strain (HDV-1p), derived from a patient who later achieved a sustained viral response under interferon alpha treatment, was sequenced. (Bockmann et al., 2020; Giersch et al., 2017).

4.3.1 Full genome sequence

The unique structure of the HDV genome with its rod-like structure and self-complementary regions can complicate the binding potential of primers and probes. *Pyne et al.* used 5 different primer pairs to sequence different HDV-1 genomes, which cover the whole genome and overlap in many sequence regions (Pyne et al., 2017) (Figure 21 A). These primers were now used for sequencing of the IFN-sensitive HDV-1p strain. For sequencing, HDV-infected serum and liver samples were collected from mice that were infected with the patient serum. After RNA extraction from the samples, the optimal temperature for the primers (sequences are shown in the methods section) was established by gradient PCR and then applied to an agarose gel to obtain the best genetic output (Figure 21 B). After purification of the PCR products, the products were analyzed by SANGER sequencing. The different parts of the HDV genome sequences were then aligned into a full genome consensus sequence by using the Geneious bioinformatics software (Biomatters Ltd, New Zealand) (Figure 22). Notably, the sequences of HDV already differ significantly within closely related HDV-1 strains. Surprisingly, the sequences of primer pairs 1 and 2 failed to bind properly and did not lead to any result in the sequencing. Primer pair 3, on the other hand, worked only partially. After full genome sequencing, an alignment of the new sequence of HDV-1p with primer pairs 1, 2 and 3 used for sequencing revealed several base pair mismatches. Therefore, the final sequence of HDV-1p is mainly derived from results of primer

pairs 4 and 5 and partially 3. Primers adapted to the new sequence have already been designed for future sequencing.

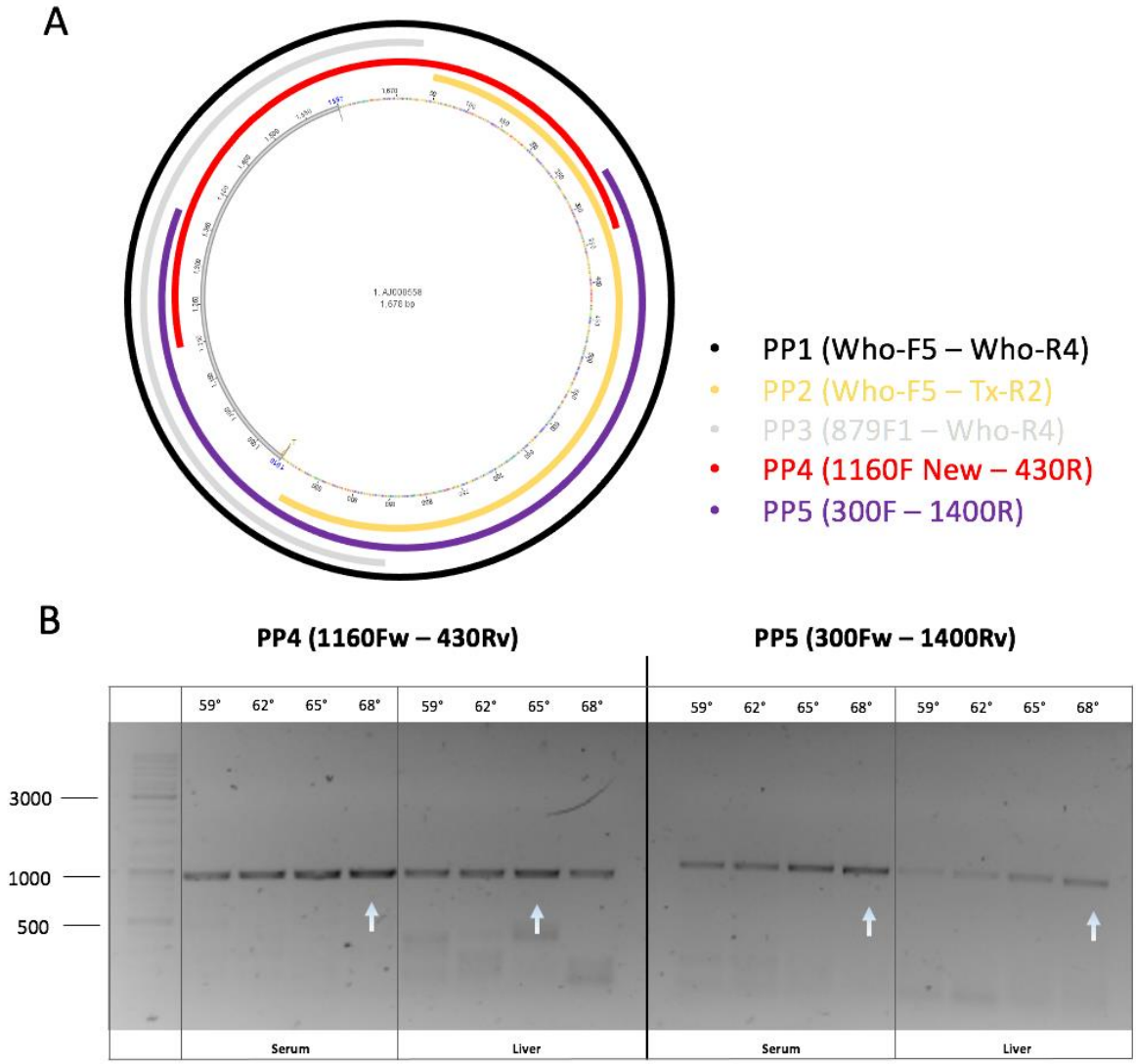


Figure 21. Method of HDV Sequencing. *Schematic representation of the HDV genome and the regions covered by the 5 different primer pairs used (Pyne et al., 2017) (A). Experimental determination of optimal annealing temperature by gradient PCR (a gradient was set between 59° and 68°C) of serum and liver samples, applied to an agarose gel (as described in the methods section). The bands marked with an arrow were purified and later sent on for genome sequencing.*

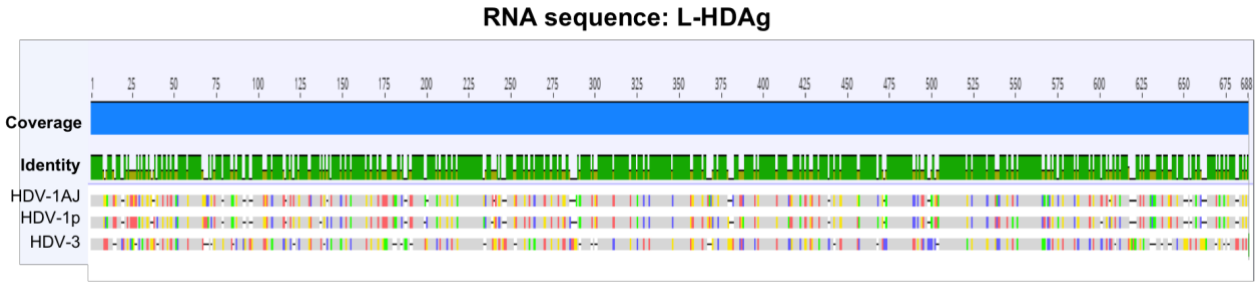
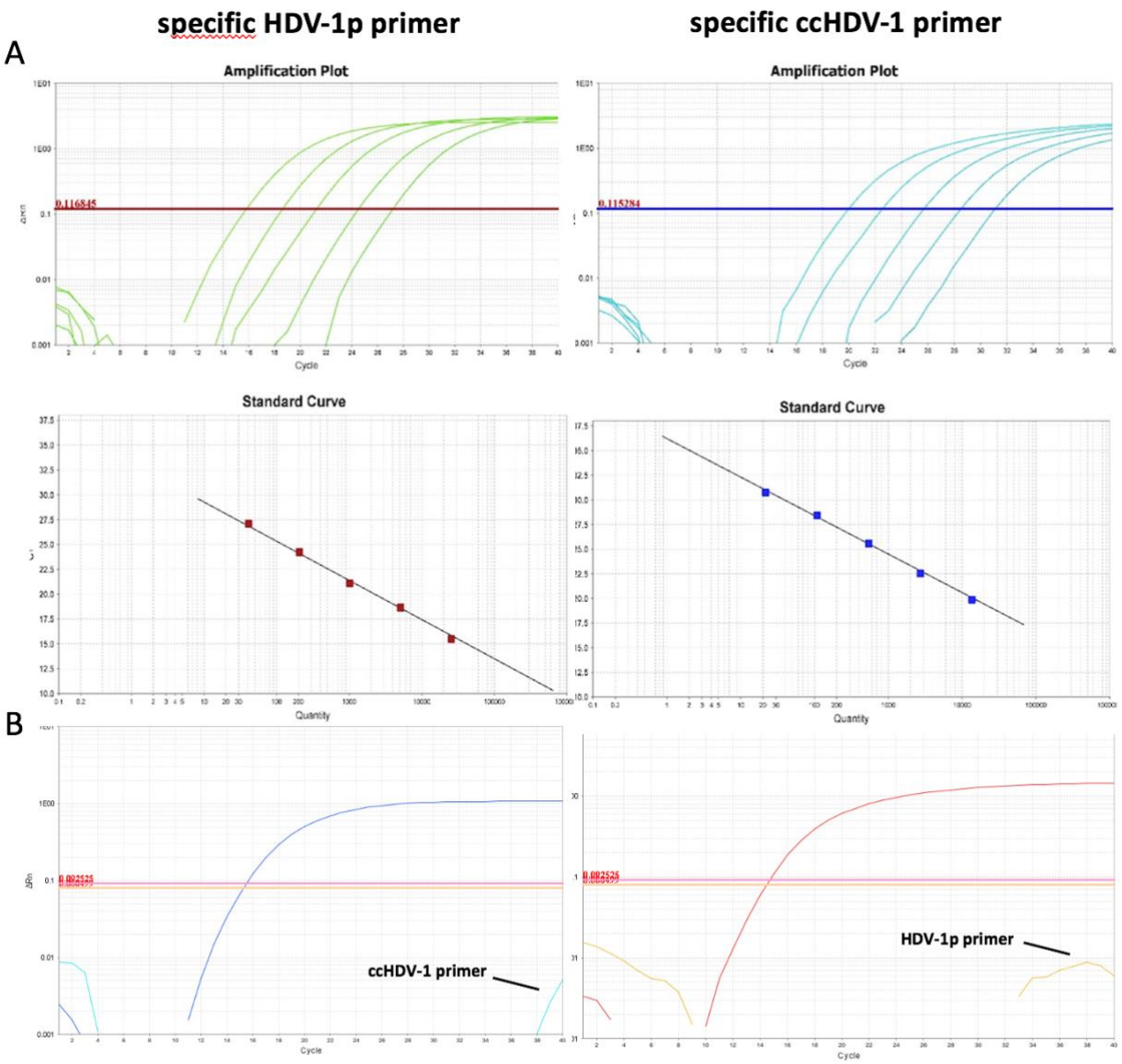


Figure 22: HDV RNA genome sequencing. Genome sequencing of the open reading frame encoding for the large HDAg of cchDV-1, HDV-1p and ccHDV-3 infected humanized mice.

With the newly obtained consensus sequence of the HDV-1p strain, further genetic analyses can be performed. The central question is whether virus-specific factors influence the response to IFN therapy. Figure 22 shows the alignment of the sequence of the open reading frame of L-HDAg comparing the ccHDV-1, ccHDV-3 and HDV-1p strain. This provides the basis for future comparative genetic analysis (e.g., of the already known post-translational modification sites) to highlight differences between the strains.

4.3.2 Establishment of specific HDV-1p and ccHDV-1 primers

Based on the HDV-1p sequence, specific primers and probes were designed to provide strain specific serological and intrahepatic measurements of this HDV-1p strain. The main goal was to make the binding of primers specific enough to ensure reliable differentiation of HDV-1p and ccHDV-1 strains in future studies. Thus, a new qPCR assay based on the new HDV-1p consensus sequence and on the known ccHDV-1 sequence (accession number: AJ000558) was designed using the Geneious Bioinformatics software (primer and probe sequences in Material and Methods). Serum samples of either HDV-1p or ccHDV-1 infected mice were then analyzed with the newly designed qPCR-assays. Figure 23 shows that the qPCR assay with the strain-specific primers are linear (A) and specificity of the new assays is provided, so that the two HDV strains can be distinguished from each other (B).



Name	Slope	Y-intercept	R2	Efficiency %
HDV-1p	-3.94	33.21	0.998	79.394
HDV-1cc	-3.90	36.20	0.995	80.434

Figure 23: ***Standard curves*** *of the qRT-PCR HDV-1p and ccHDV-1 assays (A). Specificity of primers. Measurement of ccHDV-1 and HDV-1p RNA from serum samples of ccHDV-1/HBV and HDV-1p/HBV co-infected mice. The assays confirmed the specificity of both HDV-1 primers (B).*

5 Discussion

After years of research peg-IFNα has remained the therapy of choice in HDV infection. Unfortunately, peg-IFNα is unable to achieve a sustained viral clearance in the majority of the CHD patients, as only 25-30% of patients are sensitive to peg-IFNα treatment (Deterding & Wedemeyer, 2019; Urban et al., 2021; Wedemeyer et al., 2011). Prolonged treatment may be beneficial in some cases, but also increases the time during which the patient might be exposed to strong side effects. In fact, only a small number of patients are actually cured. Currently, the main problem is the absence of precise surrogate markers for HDV therapy, which could help to predict treatment success (Yurdaydin et al., 2019). Recent findings suggest that viral genetics might play a relevant role to predict the clinical outcome of patients, as it is known that the diverse geographical distribution and great sequence divergence among HDV isolates is accompanied by distinct clinical outcomes in patients treated with peg-IFNα. Current experimental research and pre-clinical drug evaluation is directed at further characterizing the Interferon sensitivity of different HDV strains in IFN treatment.

Today, there are only very few HDV strains available for experimental research. This is critical to consider when interpreting experimental and preclinical results in HDV research, since in recent years it has become somehow more recognized that response to treatment can vary greatly upon the HDV genotype and even by individual HDV strains targeted. In the field of experimental HDV research, a certain HDV-1 strain was almost exclusively used in the past decades. This particular HDV-1 strain (AJ000558, ccHDV-1) has a peculiar history, as it was obtained from an infected patient, serially passaged through chimpanzees and woodchuck and then cloned (Kuo et al., 1988). This particular ccHDV-1 strain has been shown to be resistant to IFNα therapy in experimental research in the past. In cell culture experiments, Zhang et al. showed that exogenous IFNα treatment exerted no significant effect on this particular ccHDV-1 strain once infection was already established. Similarly, in cDNA-uPA/SCID mice, only very modest efficacy was achieved (Ye et al., 2019). In contrast, treatment with peg-IFNα and peg-IFNλ in human liver chimeric mice that were co-infected with HBV and a patient derived HDV-1 strain (HDV-1p) showed a clear antiviral effect (Giersch et al., 2015). This is

in contrast to the previous experimental results, where the HDV-1 genotype was found to be highly resistant to IFNα therapy. The differential response of two HDV-1 strains may indicate strains specific treatment response due to genetic alteration or simply be explicable by different experimental models used in these experiments. Therefore, in this work, the IFN-resistant ccHDV-1 strain was examined in more detail under the same conditions as the IFN-sensitive HDV-1p strain in the work by Giersch et al., 2017 (human liver chimeric mouse model, treatment period and dose of peg-IFNα and λ) (Giersch et al., 2017). In addition, another HDV genotype (HDV-3) was added in this work, since it has been known from clinical data that HDV-1 and HDV-3 differ very clearly from one another in terms of the course of infection and response to IFNα treatment (Borzacov et al., 2016). For that reason, two different HDV genotypes were treated with either peg-IFNα or peg-IFNλ.

5.1 Differential response of HDV strains to peg-IFNα/λ treatment

In this work, the treatment with either peg-IFNα or peg-IFNλ of the ccHDV-3 strain exerted a strong antiviral effect on serological and intrahepatic parameters in the setting of chronic co-infection. The rate of HDAg positive cells was significantly lower after treatment than in untreated control mice. This is in line with previous findings and clinical data that suggest HDV-3 to be an “easier to treat” genotype then HDV-1 (Borzacov et al., 2016). Regarding the peg-IFNα treatment of the ccHDV-1 strain, there was no antiviral effect revealed after the 4-week treatment period, which is consistent with previous results of cell culture and humanized mice experiments as already described before. There was also no reduction of HDAg positive cells seen. In general, these results were to be expected based on previous experiments. However, with regard to the HDV-1p sensitive strain, a model-related resistance of ccHDV-1 towards IFN treatment can now most probably be ruled out and a strain-specific resistance must be assumed.

So far, little is known about the efficacy of peg-IFNλ responsiveness for ccHDV-1, nor data from human liver chimeric mice are available with this particular strain. However, peg-IFNλ was already shown to be a very potent alternative to peg-IFNα in the treatment of HDV-1p in humanized mice. Clinical research studies on the efficacy of peg-IFNλ have also been conducted. In a phase 2 study, the Lambda

Interferon mono-therapy - Hepatitis Delta Virus study (LIMT-2 HDV), conducted by Eiger BioPharmaceuticals, initial results were promising. After 48 weeks of peg-IFNλ monotherapy 23% of the included patients were HDV-RNA negative and 55% showed an improved Histology Activity Index (HAI). This is comparable to treatment results achieved with peg-IFNα. However, there were significantly fewer side effects compared to treatment with peg-IFNα. Currently, the ongoing phase 3 LIMT-HDV study with 150 included patients is ongoing to evaluate and confirm the efficacy of peg-IFNλ also in larger groups of patients over a period of 48 weeks. In this study, the inclusion criteria did not include any sequencing of the HDV genotype (Eiger BioPharmaceuticals, 2020).

In this study the treatment of the ccHDV-3 strain with peg-IFNλ also showed a clear antiviral effect, comparable to peg-IFNα over the 4-week treatment period. In contrast to peg-IFNα, peg-IFNλ treatment also achieved a clear antiviral effect at first in the treatment of ccHDV-1 mice, which was then refractory in 3 of the 4 mice after 4 weeks. Thus, the initial strong effect unfortunately ceased after 2 weeks of peg-IFNλ treatment. The sudden loss of effect is surprising and the underlying reasons need to be determined in future studies. The basic rationale behind IFNλ as a therapeutic alternative for HDV infection in patients is the reduction in side effects with similar potency to peg-IFNα. IFNλ binds a different receptor, which unlike the IFNα receptor is not ubiquitously present, but is expressed only in epithelial cells of the lung, liver and intestine (Hemann et al., 2017).
Why a sudden loss of efficacy occurred in 3 of 4 mice is not clear and needs to be investigated in further experiments. Different approaches to better understand the loss of efficacy are conceivable. Since a very strong effect was seen after 2 weeks, more detailed hepatic analyses could be performed at this time to compare histological and intrahepatic analyses from 2- and 4-week timepoints. It would also be interesting to see if the peg-IFNλ administration of 2 times per week over a period of 4 weeks might not have been sufficient to achieve a long-term effect, since the effect lasted only in 1 of 4 mice. It would be interesting to tighten the treatment intervals and administer peg-IFNλ more than 2x per week and compare treatment success in future experiments.

In conclusion of the results obtained from chronic co-infection, the ccHDV-1 strain is resistant to peg-IFNα treatment in the setting of chronic co-infection. Peg-IFNλ achieved a very strong short-term effect, which however, was almost completely refractory in 3 out of 4 mice. Thus, after 4 weeks, complete resistance to peg-IFNα- and partial resistance to peg-IFNλ in the treatment of ccHDV-1 was observed. In the following, potential underlaying resistance mechanisms of the ccHDV-1 will be discussed.

5.1.1 Interplay between HDV and IFN treatment response

In recent years, further insights have been gained into the interaction of HDV with the host immune system. As discussed in detail in the introduction, there are now further insights into how HDV provokes an intrinsic interferon response. Two recent studies showed that the immune response via the MDA-5/MAVS pathway in HDV infection is mainly carried out by IFN beta (IFNß), but not by IFNα (Alfaiate et al., 2016; Giersch et al., 2015; He et al., 2015). In humanized mice, induction of IFN-lambda has also been described (Giersch et al., 2015). Strong IFN-ß induction was shown to have no effect on replication of HDV in vitro, which has also been described in previous work (Chen et al., 1986; Zhang et al., 2018).

Interestingly, HDV persistence is not only achieved through HDV dependent de-novo infection. Cell division-mediated HDV spread also contributes to HDV persistence and was reported both in human liver chimeric mice and different cell culture lines (Giersch et al., 2019; Zhang et al., 2019). Zhang et al. (2021) recently stated that HDV infection is sensitive to peg-IFNα/λ treatment mainly during cell-division, as they demonstrated a strong inhibition of HDV spread in HDV-1 infected NTCP-expressing HuH7 and H7NB2.7 cell lines. The authors propose HDV RNA to be targeted when nuclei divide and viral RNA is exposed to induced ISGs through interferon treatment during cell division (Zhang & Urban, 2021). Since monotherapy with peg-IFNα is not effective in most HDV-infected patients, a combined treatment with direct-acting antivirals could be of interest. Furthermore, a synergistic effect of peg-IFNα suppressing HDV spread through cell-division and the additive value of direct-acting antivirals targeting primarily de-novo infection (Bulevertide, Lonarfarnib) is currently undergoing promising clinical trials. However, in this work cell-division most likely does not play a determining factor for HDV spread, since

human hepatocyte engraftment was mostly completed at time of treatment and cell division is low (Wedemeyer, Schöneweis, et al., 2019).

Since the intrinsic interferon response has a limited effect on HDV persistence, the virus must somehow escape the triggered IFN response. The resistance of ccHDV-1 to IFNα could be related to direct interference with interferon signaling pathways, which is still controversially discussed. To date, exact molecular escape mechanisms are not fully understood, but in principle, viral resistance to interferon-mediated effector molecules can occur either by avoidance or blockage (including of IFN signaling pathways). There are two basic mechanisms of how HDV avoids the innate immune system. First, replication itself takes place in the nucleus, which is why cytoplasmic receptors fail to interfere here. In addition, the special molecular conformation of the HDV RNA (circular RNP complex) hinders the recognition of certain receptors such as RIG-1 (Wesselhoeft et al., 2020).

Whether HDV interferes directly with IFNα and IFNß signaling pathways is discussed controversially. For instance, Giersch et al. described reduced activation of STAT1 in HDAg-positive hepatocytes in humanized mice (in stable HDV/HBV co-infection) (Giersch et al., 2015). Moreover, as early as 2009, Pugnale et al. showed that HDV inhibits IFN signaling pathways in cell culture and described a reduced induction of several classical ISGs such as Mx1 and OAS1 (Pugnale et al., 2009). This interference with IFN signaling pathways could explain a poor response of IFN therapy in chronic HDV patients. However, since numerous studies show contrary results, this hypothesis is opposed. There is a particular study by Zhang et al. that shows a strong immune activation via MDA-5, which argues against the interference of HDV on IFN signaling pathways (Zhang et al., 2018). This is consistent with other studies showing that IFN-induced Mx1 suppresses HBV replication and that the L-HDAg directly induces the Mx1 protein (Williams et al., 2009). These findings also go in line with data from humanized mice, where it has been shown that in the setting of HDV/HBV co-infection ISGs are more strongly induced than in HBV mono-infection. This could explain that the development of HBV viremia and cccDNA is suppressed more strongly in the setting of HBV/HDV co-infection, which indicates that a highly induced antiviral status affects HBV replication (Allweiss et al., 2012; Giersch et al., 2015). However, it has not yet been clarified whether patients who are chronically infected with HDV actually exhibit increased IFN production. In this

work, it is demonstrated that both peg-IFNα and peg-IFNλ treatment of the interferon-resistant ccHDV-1 and the IFN-sensitive ccHDV-3 strains strongly induce ISGs. Thus, the interference with IFN signaling pathways of either ccHDV-1 or cchDV-3 does not appear to be the basis for the resistance to IFN treatment of ccHDV-1. Interestingly, peg-IFNα induced levels of human ISGs comparably strong, irrespective of the therapy outcome. In summary, the innate immune response cannot be the sole determining factor in the response to interferon treatment in HDV infection. It is more likely that the different HDV strains display other virus-specific characteristics that have a strong impact on the treatment response. Since the mice lack an adaptive immune system, further investigations were not performed in this direction. Nevertheless, the adaptive immune system generally has little opportunity to target the infection directly, since HDV only produces two proteins in the form of the S- and L-HDAg. Thus, studies show that in HDV-infection the overall CD8+-epitopes presenting HDV derived peptides was very low, compared to other hepatotropic viruses. Furthermore, it has also been described, that certain mutations in the RNA genome of HDV allow an escape detection by CD8+ T-cells resulting in a persistent infection. (Karimzadeh et al., 2018, 2019)

In the context of IFN treatment, the question may arise to what extent the treatment exerts cytotoxic effects, including cell death of human hepatocytes. Human liver chimeric mice, which were used exclusively in this study, produce human albumin and can therefore indicate the synthesis capacity of hepatocytes (vitality) on the one hand and give a good estimate of the degree of repopulation on the other. It is essential to detect toxic side effects on human hepatocytes and exclude the possibility that viral parameters may be biased by the IFN treatment and thus get misinterpreted. When interpreting albumin levels in human liver chimeric mice, it is important to note that HDV infection alone does not lead to a decrease in albumin levels compared with uninfected mice in general. Thus, there is no evidence for significant cell death due to HDV infection, which would be reflected in lower albumin levels. In the setting of chronic co-infection with either ccHDV1 or ccHDV-3 and HBV, there is a decrease in serum albumin levels in peg-IFNα treated mice in the 4-weeks treatment period. In contrast, albumin level in untreated control mice remained stable with a rather increasing tendency. A slight loss of human hepatocytes was observed upon peg-IFNα treatment. However, since ccHDV-1

parameters also remained stable during the entire 4-weeks treatment period, lower serum albumin level cannot provide a sufficient explanation for the differential strain specific response to IFN therapy. Treatment with peg-IFNλ also led to similar results in the first two weeks. The subsequent recovery of serum albumin levels in treated mice is compatible with the loss of effect, which has already been discussed above.

5.2 Pre-treatment with peg-IFNα exerts strong antiviral effect

In order to further characterize the resistance of ccHDV-1, which has already been discussed in detail, a pre-treatment with peg-IFNα was now performed in the setting of HDV mono-infection. The aim was to see how the early stages of infection are affected by pretreatment. The results here clearly show that pre-treatment of the cells induced a significant antiviral effect. The cells must therefore have been shifted into an antiviral status, which inhibited the early stages of infection very effectively and prevented an infection establishment to a large extent. It should be highlighted here that peg-IFNα pre-treatment of the ccHDV-1 strain, which was resistant to treatment in the chronic co-infection with HBV, can achieve a significant antiviral effect. This is in line with previous in vitro data, which also showed an antiviral effect of interferon pre-treatment of the ccHDV-1 strain resulted in a moderate inhibitory effect on HDV replication (Zhang et al., 2018). This is also consistent with previous results from cell culture experiments by Taylor et al. (2011). In these studies, pre-treatment with IFNα was able to inhibit the establishment of HDV infection by 80%, but an already established infection could not be affected by the treatment (Han et al., 2011; Ilan et al., 1992). Also, in HepNB2.7 cells (contain NTCP and HBV envelope proteins through transgenic expression), which produces infectious HDV virions and closely resembles the actual life cycle of HDV, IFNα pre-treatment of the cells achieved an antiviral effect only at very high doses (1400IU/ml) and was not able to completely clear or block HDV infection (Lempp et al., 2019). Overall, the findings clearly show that HDV genotype- and even strain-specific differences majorly impact the results of clinical research and drug evaluation. The comparative use of different HDV strains, especially upon treatment and drug evaluation is therefore strongly recommended. In general, pre-clinical drug evaluation should include a wide range of HDV genotypes and strains. Therefore, the results

emphasize the relevance of the great sequence heterogeneity among the different HDV isolates regarding the treatment outcome in patients.

5.3 Sequencing of interferon sensitive HDV-1 strain

The HDV-1p strain sequenced in this work originates from an infected patient who was able to achieve SVR under peg-IFNα therapy. Thus, HDV-1p appears to differ from ccHDV-1 in important properties such as interferon sensitivity, as is evident in this work. Since the sequence of the IFN resistant strain ccHDV-1 has been known for a long time, it was now essential to also obtain the HDV-1p sequence. The sequences of both HDV-1 strains provide the basis for further research. Consequently, the HDV-1p strain could be cloned and serve as an alternative to the ubiquitously used ccHDV-1. It is clear from this work that in experimental therapy of HDV, whether in cell culture or animal studies, the spectrum of different HDV strains should be expanded. In this way, the understanding about certain therapy resistances can be classified even better and scientific results will get a higher validity. Of course, this would require further preliminary work. If cloning is successfully accomplished and the viruses are confirmed as functional and infectious, an interferon sensitive HDV strain would then be available for experimental research. Novel therapies could be tested on an interferon-sensitive HDV-1 strain. Since HDV-1 is the most prevalent worldwide, this would be an advance for experimental HDV research in general.

In addition to the possibility of cloning a new HDV strain, sequencing also offers further experimental options in HDV research. Genetic analysis of IFN sensitive (ccHDV-3 and HDV-1p) and resistant HDV strains (ccHDV-1) can now be performed by comparative alignments. This could reveal specific sequence overlaps and differences between the strains at the molecular level and provide a deeper insight into a possible mechanism of IFN resistance. Overall, all possible mutations in the HDV ribozyme and HDAg coding regions could be analyzed in more detail. In addition, all known post-translational modification sites in the HDAg open reading frame could be comparatively assessed. This could provide an explanation for the existing IFN resistance of the ccHDV-1 strain. With regard to the interferon

resistance of the ccHDV-1 strain, the production of chimeric HDV-1 viruses with the newly sequenced HDV-1p and ccHDV-1 strain would also be conceivable in the future to better understand infection kinetics and response to interferon therapy. A potential approach would be the exchange of the L-HDAg between the HDV strains to detect any L-HDAg mediated resistance.

Alongside comparative genetic analysis in experimental research and the resulting idea of generating chimeric viruses, there are also clinical approaches to further characterize the resistance of HDV strains to IFN therapy. As the response to IFN therapy is also very poor in patients, it would be of great importance to sequence the HDV-strains in infected patients and document the response of therapy. Determining HDV strains prior to initiation of therapy in an HDV infected patient could also be of help to predict whether a patient might benefit from IFNα therapy in an in the upcoming therapy. Thus, in the best case, every patient infected with HDV should be sequenced and the success of the therapy should be continuously documented.

Declaration regarding my own contribution within my doctoral thesis

As part of my dissertation I isolated, examined, and evaluated DNA and RNA from serum and liver samples of mice and stained and analyzed liver tissue sections using various methods. I was not directly involved in the work with mice, neither the breeding nor in the process of transplantation of human hepatocytes, infection or treatment of the mice.

This results section and the corresponding interpretation were developed in close collaboration with Katja Giersch and Lennart Hermanussen. Katja Giersch and Lennart Hermanussen characterized certain mice and groups of the ccHDV-1/HBV and ccHDV-3/HBV coinfection experiments in human liver chimeric mice and performed serological and intrahepatic analyses. In partial collaboration, Lennart Hermanussen and I performed the serological and intrahepatic analysis of peg-IFNα treated mice. My contribution to these results consists primarily of the serological and intrahepatic analysis of the peg-IFNλ mice, the interferon pretreatment with peg-IFNα in HDV-1 and HDV-3 mono-infected mice, and the sequencing and successive primer establishment of the HDV-1p interferon sensitive strain. These data are therefore presented with the kind and explicit permission of Katja Giersch and Lennart Hermanussen.

6 Abstract

Peg-IFNα is commonly used for the treatment of HDV infection. However, even after standard treatment for 48 weeks, complete remission of HDV RNA is observed in only 20-35% of patients (Deterding & Wedemeyer, 2019). IFNλ has achieved comparable results to IFNα in early clinical trials and has therefore been discussed as an alternative treatment option due to its fewer side effects.

In this work, the two HDV strains ccHDV-1 and ccHDV-3 showed a different response to treatment with peg-IFNα and peg-IFNλ in human liver chimeric mice in the setting of a chronic HBV/HDV co-infection over the period of 4 weeks. Herein, the two interferons were able to strongly suppress all virological parameters of the ccHDV-3 infected humanized mice. Conversely, the ccHDV-1 showed clear resistance under peg-IFNα treatment, which has also been described previously in cell culture experiments. Although peg-IFNλ was initially able to induce a partial effect in the treatment of ccHDV-1, it was predominantly refractory throughout the treatment period. The exact mechanism of resistance to peg-IFNα and peg-IFNλ is not well understood to date and further investigation is needed. Pre-treatment of human liver chimeric mice with peg-IFNα of ccHDV-1 and ccHDV-3 in HDV mono-infection showed a clear antiviral effect. Thus, the early phases of infection of both HDV strains are sensitive to IFN treatment. Therefore, peg-IFNα resistance of ccHDV-1 is only seen after an infection has already established. Based on these results and generally growing evidence for the relevance of HDV genotypes and strains with regard to the treatment response of IFN therapy, there should be more use of distinct HDV strains besides ccHDV-1 in experimental research. Therefore, in this work a particular HDV strain, originated from a patient who has reached SVR under peg-IFNα therapy, was sequenced. Cloning of this strain is the next step to apply alternative HDV strains in experimental research. In addition, a genetic comparative analysis of the resistant ccHDV-1 and sensitive HDV-1p might provide further insights into the mode of action of IFNα on HDV.

Zusammenfassung

Peg-IFNα wird in der Regel zur Behandlung der HDV Infektion eingesetzt. Nach einer 48-wöchigen Standardbehandlung wird eine vollständige Remission jedoch nur bei 20-35% der Patienten beobachtet (Deterding & Wedemeyer, 2019). IFNλ hat in den ersten klinischen Studien bisher vergleichbare Ergebnisse wie IFNα erzielt und wird daher als alternative Behandlungsoption diskutiert.

In dieser Arbeit zeigten die beiden HDV-Stämme ccHDV-1 und ccHDV-3 ein unterschiedliches Ansprechen auf die Behandlung mit peg-IFNα und peg-IFNλ in humanisierten Mäusen im Rahmen einer chronischen HBV/HDV Ko-infektion über einen Zeitraum von 4 Wochen. Dabei waren die beiden Interferone in der Lage, alle virologischen Parameter der mit ccHDV-3 infizierten humanisierten Mäuse stark zu unterdrücken. Im Gegensatz dazu wies der ccHDV-1 Stamm unter der peg-IFNα-Behandlung eine deutliche Resistenz auf, die auch schon in Zellkulturexperimenten beschrieben wurde. Obwohl peg-IFNλ bei der Behandlung von ccHDV-1 zunächst eine partielle Wirkung erzielen konnte, war diese nach Abschluss der gesamten Behandlungsdauer überwiegend refraktär. Der genaue Mechanismus der Resistenz gegen peg-IFNα und peg-IFNλ ist bis heute nicht gut verstanden und bedarf weiterer Untersuchungen. Die Vorbehandlung von chimären Mäusen mit peg-IFNα von ccHDV-1 und ccHDV-3 in der HDV-Monoinfektion zeigte eine deutliche antivirale Wirkung. Somit sind die frühen Phasen der Infektion beider HDV-Stämme empfindlich gegenüber einer IFN-Behandlung. Eine peg-IFNα-Resistenz von ccHDV-1 tritt daher erst auf, nachdem sich eine Infektion bereits etabliert hat. Aufgrund dieser Ergebnisse und der allgemein zunehmenden Belege für die Relevanz von HDV-Genotypen und -Stämmen im Hinblick auf das Ansprechen auf eine IFN-Therapie sollten in der experimentellen Forschung neben ccHDV-1 auch andere HDV-Stämme verstärkt eingesetzt werden. In dieser Arbeit wurde daher ein ausgewählter HDV-1 Stamm sequenziert, der von einem Patienten stammt, welcher unter einer peg-IFNα-Therapie eine vollständige Remission erzielte. Das Klonen dieses Stammes ist der nächste Schritt, um alternative HDV-Stämme in der experimentellen Forschung einzusetzen. Darüber hinaus könnte eine vergleichende genetische Analyse des resistenten ccHDV-1 und des empfindlichen HDV-1p weitere Erkenntnisse über die Wirkungsweise von IFNα auf HDV liefern.

7 List of abbreviations

ADAR1 ..... *adenosin deaminase acting on RNA 1*
AG RNA ..... *antigenomic RNA*
EASL ..... *European Association of the Liver*
EGFR ..... *Epidermal Growth Factor Receptor*
G RNA ..... *genomic RNA*
G1 ..... *Genogroup 1*
G2 ..... *Genogroup 2*
G3 ..... *Genogroup 3*
HBsAg ..... *Hepatitis B surface Antigen*
HBV ..... *Hepatitis B Virus*
HCC ..... *Hepatocellular carcinoma*
HCV ..... *Hepatitis C Virus*
HDAg ..... *Hepatitis Delta Antigen*
HDV ..... *Hepatitis D Virus*
HDV-1 ..... *HDV genotype 1*
HDV-2 ..... *HDV genotype 2*
HDV-3 ..... *HDV genotype 3*
HDV-4-8 ..... *HDV genotypes 4-8*
HIV ..... *Human Immunodeficiency Virus*
HSPGs ..... *heparan sulfan proteoglutans*
IF ..... *Immunofluorescence staining*
IFNß ..... *interferon beta*
IFNα ..... *Interferon alpha*
IFNγ ..... *interferon gamma*
IRF9 ..... *interferon regulatory factor 9*
ISGs ..... *interferon stimulated genes*
NFQ ..... *non-fluorescent quencher*
NJ ..... *Neighbor-Joining*
NTCP ..... *sodium taurocholate co-transporting polypeptide*
NUCs ..... *nucleoside/nucleotide analogues*
ORF ..... *open reading frame*
peg-IFNα ..... *pegylated interferon alpha*
peg-IFNλ ..... *pegylated interferon lambda*
PRRs ..... *pattern recognition receptors*
qPCR ..... *quantitative real-time polymerase chain reaction*
rcDNA ..... *relaxed circular DNA*
RNP ..... *Ribonucleoprotein*
SCID ..... *severe combined immunodeficiency*
STAT1 ..... *signal transducing transcriptional activator 1*
SVR ..... *sustained viral response*
TLR ..... *toll-like receptor*
uPA ..... *urokinase-type plasminogen activator*
WHO ..... *World Health Organisation*

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9 Appendix

9.1 List of figures

9.2 List of tables

9.3 Danksagung

Die vorliegende Dissertation wurde in der Arbeitsgruppe Virushepatitis unter der Leitung von Frau Prof. Dr. Maura Dandri an der I. medizinischen Klinik des Universitätsklinikums Eppendorf angefertigt.

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9.4 Lebenslauf

Der Lebenslauf wurde aus datenschutzrechtlichen Gründen aus der elektronischen Kopie entfernt.

10 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....