Development and Evaluation of Diagnostic Methods for Hepatitis Delta Virus (HDV): Molecular and Serological Approaches

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Table of contents

1. Working hypothesis and research question

Diagnosing infections caused by the hepatitis delta virus (HDV) presents significant challenges, particularly in the reliable detection and quantification of HDV ribonucleic acid (RNA) using nucleic acid amplification tests (NAAT). Despite these difficulties, HDV RNA detection remains crucial for risk assessment and monitoring treatment efficacy, especially as new therapeutic options for HDV emerge (Mangia et al. 2023, Wedemeyer et al. 2025). The extreme heterogeneity of the HDV genome, which encompasses high diversity across and within the eight proposed genotypes, along with its complex secondary structure and high guanine-cytosine content, complicate the design of effective polymerase chain reaction (PCR) assays (Brichler et al. 2014, Le Gal et al. 2017, Osiowy et al. 2024). Although the first international HDV RNA standard was introduced in 2013, substantial variability persists in quantitative results across laboratories (Wedemeyer et al. 2025).

Moreover, recent discoveries of HDV-like viruses that can infect various species without the presence of Hepadnaviridae, along with evidence of viruses other than the hepatitis B virus (HBV) facilitating the egress of infectious HDV *in vitro,* challenge the assumption that HDV infection is exclusively dependent on HBV in humans (Chang et al. 2019a, Perez-Vargas et al. 2019, Pérez-Vargas et al. 2021). In a recent study, the authors demonstrated that the hepatitis C virus (HCV), among other human pathogenic viruses, can facilitate the packaging and egress of HDV *in vitro* and in a mouse model (Perez-Vargas et al. 2019). Based on these findings, we hypothesized that HCV may enable HDV infections in humans.

Consequently, this dissertation aimed to i) improve molecular diagnostics for HDV to reduce variability in test results across laboratories and ii) investigate HBV-independent propagation of HDV in humans through two primary objectives:

- Development and validation of a pan-genotype reverse transcription polymerase chain reaction (*RT-qPCR*) assay for HDV on a fully automated platform that complies with European guidelines.
- Evaluation of HCV RNA-positive and hepatitis B surface antigen (HBsAg)-negative samples for the presence of antibodies against HDV (anti-HDV) and HDV RNA within a comprehensive patient cohort.

2. Introduction

2.1. The hepatitis Delta virus

2.1.1. Genomic structure and life cycle

In 1977, Prof. Mario Rizzetto and his group discovered a novel antigen in the liver tissue of patients infected with HBV (Rizzetto et al. 1977). Initially, it was hypothesized that this discovery represented a novel hepatitis B virus antigen, designated as the delta antigen. However, subsequent investigations revealed that it was, in fact, a distinct virus, later named HDV and categorized within the genus Deltavirus (Magnius et al. 2018, Rizzetto 1983). Since then, further research has provided a more detailed understanding of the viral genomic structure, the mechanisms of HDV infection, and its medical implications.

The genome of HDV consists of around 1,700 ribonucleotides (1672–1697, depending on the HDV genotype (GT)) and is considered the smallest known virus that can infect humans (Lai 1995, Thiyagarajah et al. 2023). Its genome is a negative-sense, single-stranded circular RNA with a complex secondary structure described as unbranched rod-like and exhibits an extremely high level of intramolecular base pairing (>70 %) (Wang et al. 1986).

A single open reading frame (ORF) has been identified within the HDV genome, which encodes for the sole known protein of HDV, the hepatitis delta antigen (HDAg) (Weiner et al. 1988). HDAg exists in two distinct variations that differ in the length of the amino acids, the large and small HDAg (S-HDAg, L-HDAg). Together with the genomic HDV RNA, the S-HDAg and L-HDAg form the ribonucleoprotein (RNP) core complex (Hughes et al. 2011, Ryu et al. 1993). However, HDV, often described as a “defective virus”, cannot provide a coating for its RNPs. Thus, HDV relies on other viruses, specifically HBV, to provide envelope proteins (Freitas et al. 2014). **Figure 1 (A)** depicts the viral structure of both HBV and HDV.

Consequently, HBV determines the tropism of HDV, and both viruses use the same mechanisms to enter the target cell (hepatocyte). Upon binding to the basolateral membrane of human hepatocytes, an important step for the internalization of these viruses is the high-affinity binding to the sodium taurocholate cotransporting polypeptide (NTCP) (Yan et al. 2012).

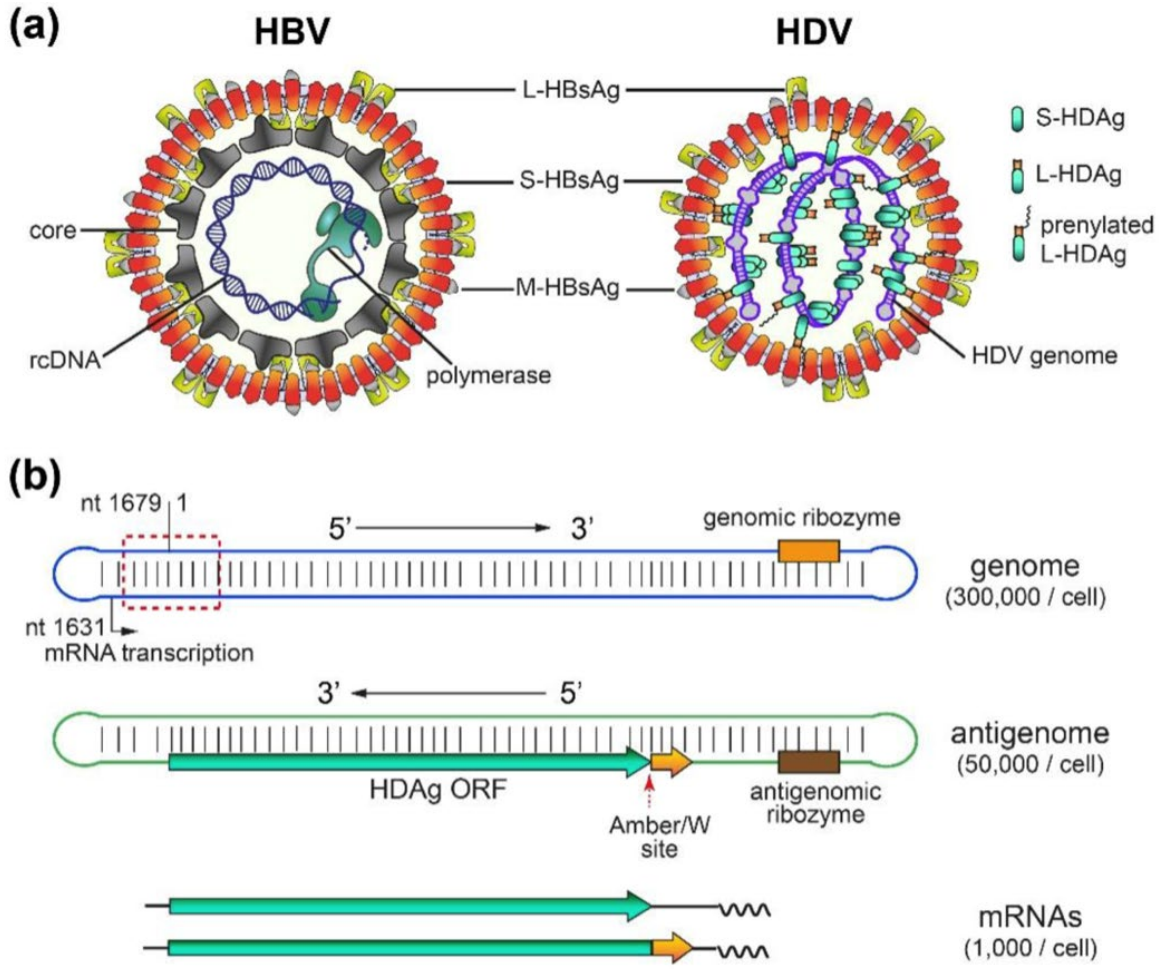


Figure 1: Structure of HBV and HDV virions and genomic and antigenomic HDV RNA

(A) Both the envelopes of HBV and HDV consist of HBV antigens, namely the small, medium, and large versions of the HBsAg. HDV particles contain genomic HDV RNA and the small and large HDAg, which form the ribonucleoprotein core complex. **(B)** Inside the nucleus of infected cells, genomic and antigenomic HDV RNA are synthesized, along with HDV mRNA. Both the genomic and antigenomic HDV RNAs contain a ribozyme motif, which is essential during the rolling circle amplification of HDV, as it exhibits self-cleaving activity. The editing of the antigenomic HDV RNA at the amber/W /W site (red arrow) results in the modification of a stop codon. Subsequently, this process results in the production of the large HDAg, which differs from the small HDAg by 19-20 additional amino acids, depending on the HVD GT. This figure was originally published by Zhang et al. in a review article in the Journal Viruses titled "Figure 1" (Zhang and Urban 2020). The figure has not been edited.

Abbreviations: HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HDV, hepatitis D virus; HDAg, hepatitis delta virus antigen; GT, genotype.

Once HDV particles have entered the cell, a nuclear localization sequence that is present within the S-HDAg promotes the translocation of the viral RNPs to the nucleus, where HDV replication takes place (Alves et al. 2008). Synthesis of genomic and antigenomic RNA is carried out by the host deoxyribonucleic acid (DNA)-dependent RNA polymerase (polymerase II (pol II)) (Lai Michael 2005). Interestingly, this indicates that HDV is capable of hijacking a host polymerase that is typically DNA-dependent to function properly using an RNA template. The hypothesis of how HDV manages to "persuade" pol II is primarily focused on the secondary structure of the HDV genome mimicking a quasi-double-stranded DNA, but the exact mechanism remains elusive (Greco-Stewart et al. 2007).

The genomic HDV RNA serves as a template for the production of antigenomic RNA and vice versa through rolling-circle amplification (Macnaughton et al. 2002). **Figure 1 (B)** shows a schematic overview of the structure of both genomic and antigenomic HDV RNA. The multimeric RNA molecules generated during this process are subsequently cleaved by the HDV self-cleaving ribozyme, which has been identified in both the genomic and antigenomic HDV RNAs (Wadkins and Been 2002).

The antigenomic RNA acts as a template for messenger RNA (mRNA) transcription from which S-HDAg is produced (Modahl and Lai 1998). In addition, the antigenomic RNA is edited by a host enzyme called the adenosine deaminase acting on RNA (ADAR) (Jayan and Casey 2002). This posttranscriptional modification of the antigenomic HDV RNA leads to the conversion of adenine to inosine through deamination at the amber/W site (Casey 2006, Wong and Lazinski 2002). Through this process, a stop UAG codon in the small HDAg ORF is changed to a UGG codon, which subsequently leads to the transcription of mRNA encoding the L-HDAg (Luo et al. 1990).

Tough only differing by a few amino acids (19-20 additional amino acids at the N-terminus of the protein; HDV GT dependent), the S- and L-HDAg serve distinct functions in the HDV life cycle (**Figure 2**). For one, S-HDAg is necessary for HDV replication, whereas L-HDAg has been shown to have an inhibitory effect on the synthesis of genomic/antigenomic HDV RNA and HDV mRNA (Lee et al. 1993, Sato et al. 2004). Inhibition of antigenomic HDV RNA and HDV mRNA is only observed when L-HDAg is present to a significantly greater extent than S-HDAg, while repression of the synthesis of genomic HDV RNA can be observed even with small amounts of L-HDAg (Modahl and Lai 2000). This observation

favors HDV replication in the early phases of the HDV life cycle. Conversely, in later stages, the accumulated amount of L-HDAg tends to promote the assembly of new viral particles, as the L-HDAg contains a nuclear export signal and a farnesylation site (Lee et al. 1994, Wang et al. 2005). The farnesylation of the L-HDAg is essential for the interaction of the L-HDAg and HBsAg and thereby promotes viral assembly and the egress of infectious particles from hepatocytes (Glenn 2006, Glenn et al. 1992, Thiyagarajah et al. 2023).

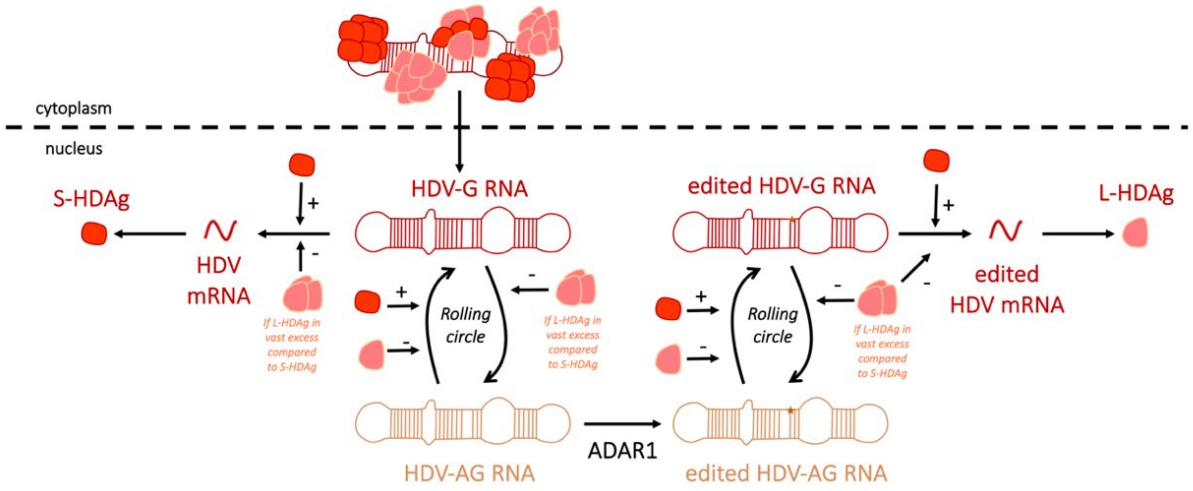


Figure 2: HDV life cycle: The role of S- and L-HDAg in the replication of genomic and antigenomic HDV RNA and the transcription of HDV mRNA

Upon translocation into the nucleus of infected hepatocytes, the synthesis of genomic and antigenomic HDV RNA is carried out by the host pol II (DNA-dependent RNA polymerase). Through posttranscriptional editing by the adenosine deaminase acting on RNA 1 (ADAR1) a stop codon within the open reading frame of the S-HDAg (depicted in bright red) is changed. Consequently, the edited antigenomic HDV RNA encodes for the L-HDAg (represented in light pink) instead of the S-HDAg. Further, the figure shows how S- and L-HDAg influence replication and transcription of genomic/antigenomic HDV RAN and transcription of HDV mRNA. This figure was originally published by Lucifora and Delphin in a review article in the Journal Antiviral Research titled “Fig. 4. Schematic representation of HDV RNAs synthesis” (Lucifora and Delphin 2020). The figure has not been edited.

Abbreviations: ADAR1, adenosine deaminase acting on RNA 1; HDV-AG RNA, hepatitis delta virus antigenomic RNA; HDV-G RNA, hepatitis delta virus genomic RNA; L-HDAg, large hepatitis delta antigen; S-HDAg, small hepatitis delta antigen.

2.1.2. Transmission pathways and mechanisms of HDV infection: HBV/HDV super- and coinfection

The transmission of HDV is similar to that of HBV and occurs through contact with blood or blood products containing intact HDV particles. Potential routes of transmission include needle sharing, tattooing, and the transfusion of inadequately tested blood products (Caviglia et al. 2022, Davaalkham et al. 2006). Additionally, the spread of HDV via sexual contact is possible, and transmission from mother to child has been reported, although it is a rare event (Sellier et al. 2018).

Infections with HDV occur either simultaneously with an HBV infection, a condition referred to as co-infection, or as superinfection with HDV in individuals who are HBsAg positive. Co-infection with HBV and HDV may lead to acute hepatitis with the potential for acute liver failure (Smedile et al. 1982). Symptoms exhibited by affected patients include general malaise or fatigue, fever, loss of appetite or nausea, upper abdominal discomfort, and muscle and/or joint pain (CDC 2024, WHO 2025). Additionally, clinical manifestations may include a darkening of the urine, elevated liver enzymes, and jaundice (WHO 2025). A small proportion of approximately 5 % of HBV/HDV co-infected patients develop chronic hepatitis, while the majority can clear both viruses spontaneously (**Figure 3**) (Negro 2014).

In contrast, infection with HDV in the presence of an existing HBV infection is associated with the development of chronic hepatitis, as only a small proportion of patients can spontaneously clear HDV in this context (**Figure 3)** (Negro 2014, Yurdaydın et al. 2010). These patients often exhibit a higher HDV viral load compared to HBV DNA, a phenomenon that may be attributed, at least in part, to HDV's ability to influence HBV replication (Coghill et al. 2018). The mechanisms underlying the HDV-mediated impairment of HBV replication remain incompletely understood. Previous studies have shown that the HDV antigen proteins can suppress HBV enhancers (enhI and II) and stimulate the MxAa gene, which belongs to the group of interferon-inducible genes (Williams et al. 2009). Moreover, it has been found that L-HDAg can inhibit pol II, which is involved in the replication of both HBV and HDV (Modahl and Lai 2000). However, instances of HBV predominating over HDV and low detectable viral loads of both viruses have also been documented and longitudinal studies demonstrating that viral loads fluctuate over time (Schaper et al. 2010).

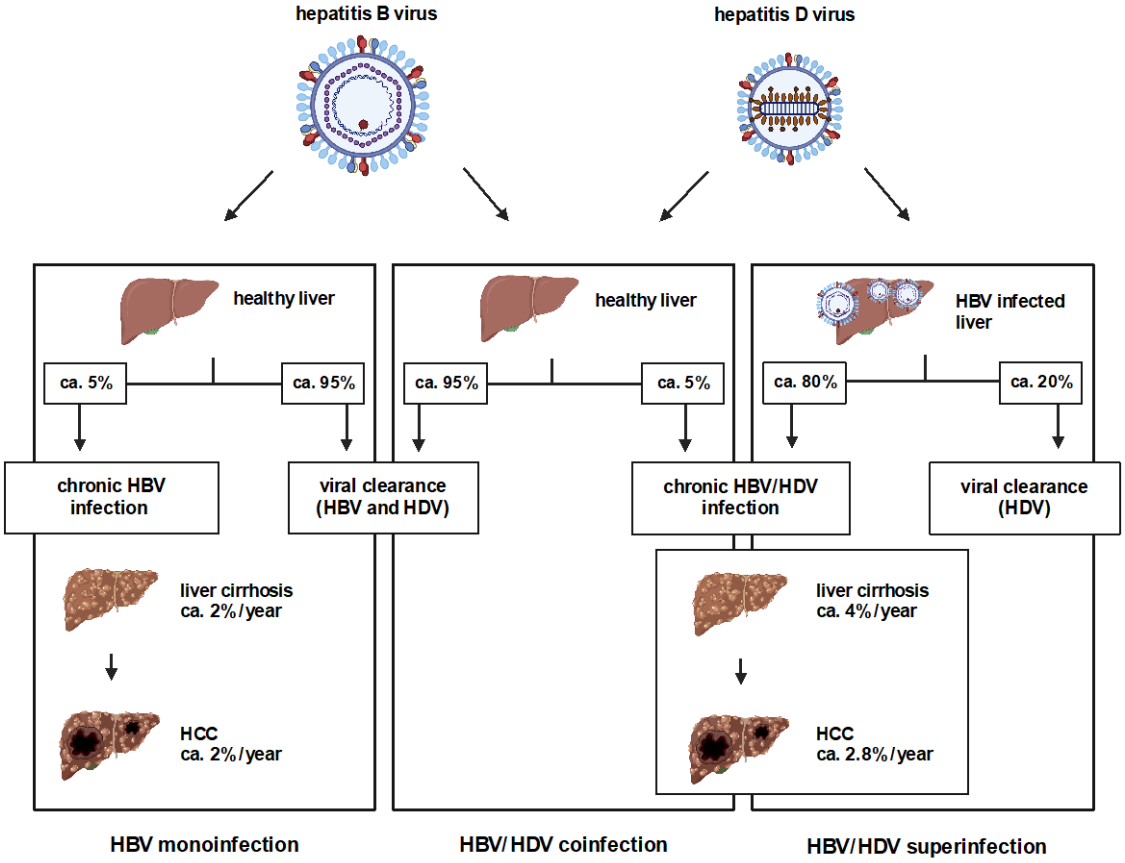


Figure 3: HDV/HBV co- and super-infection

In an HBV/HDV co-infection setting, most patients clear HDV spontaneously, while in the case of HDV super-infection of an HBV mono-infected patient, the majority of patients develop chronic hepatitis. These chronic HBV/HDV infections are associated with a higher progression rate to liver cirrhosis and an increased risk of the development of HCC compared to HBV mono-infection. The figure was made using BioRender and adapted from the publication by Turon-Lagot et al., published in the Journal of Clinical Medicine entitled “Figure 4. Natural history of HBV mono-infection and HDV co- and super-infection” (Turon-Lagot et al. 2020).

Abbreviations: CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HDV, hepatitis delta virus.

Patients with chronic HBV/HDV infection face a significantly higher risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) compared to those who are HBV monoinfected (Alfaiate et al. 2020, Bockmann et al. 2020, Romeo et al. 2009). Moreover, these patients exhibit a faster disease progression and face a greater likelihood of needing a liver transplant (Muhammad et al. 2021, Wranke et al. 2023).

2.1.3. Diagnostic tools and approaches in the diagnosis of HDV infection

In general, serological assays and NAAT are currently used to diagnose HDV infection. Firstly, to screen for an HDV infection in HBsAg-positive patients, anti-HDV can be assessed. Next, an NAAT can be performed in anti-HDV reactive patients to determine the HDV RNA load and confirm active HDV infection (EASL 2017, Terrault et al. 2018). This approach facilitates the identification of viremic patients, which is a pivotal aspect of risk stratification. Studies comparing the outcomes of HDV non-viremic and viremic HDV/HBV-infected patients have demonstrated a considerably elevated risk for the development of complications in those with HDV viremia (Kamal et al. 2020). Moreover, the development of new treatment options for HDV infection in recent years has made reliable detection and quantification of HDV RNA increasingly important for identifying patients eligible for HDV treatment and for monitoring during therapy and clinical trials (Wedemeyer et al. 2025). Clinical studies evaluating the efficacy of new therapeutic agents favor virological and laboratory parameters as reliable surrogate markers of treatment efficacy and alternative endpoints. This preference is partly due to the fact that hard endpoints, such as a reduction in the development of liver cirrhosis, liver decompensation, HCC, and overall HDV-related death, require long-term observational studies and ongoing follow-up of patients. For instance, the following endpoints were employed in the Phase III efficacy study on bulevirtide, a new promising drug for the treatment of HDV infection (discussed in greater detail below): i) undetectable HDV RNA levels for 48 weeks, or ii) a 2-$\log_{10}$ reduction in HDV viral load in international units (IU)/ milliliter (ml) compared to baseline in conjunction with iii) normalization of alanine transaminase (ALT) levels (Wedemeyer et al. 2023).

As previously discussed, the diagnosis of HDV infection relies on a combination of serologic assessment, typically a chemiluminescent immunoassay or enzyme-linked immunosorbent assay that detects both IgG and IgM antibodies against HDV, along with the evaluation of HDV RNA in serum or EDTA plasma samples. Two distinct approaches exist for determining the appropriate timing of HDV diagnostics: the universal and risk-based screening approach. The universal screening approach states that every HBsAg-positive patient should be tested for anti-HDV at least once to exclude the possibility of HBV/HDV coinfection. In contrast, the risk-based screening approach recommends testing only HBsAg-positive patients who are at specific risk for HBV/HDV coinfection. Criteria for initiating

HDV screening include elevated liver enzymes, the presence of HIV-/HCV coinfection, HBV DNA <2,000 IU/ml, and behavioral factors such as intravenous drug use (Terrault et al. 2018). Regardless of the screening approach used, confirmatory testing with a NAAT - preferably a quantitative assay - is recommended when a reactive anti-HDV test result is obtained.

Recommendations concerning which screening approach should be used vary from country to country and between leading organizations drafting guidelines. In Germany, the guideline of the European Association of the Study of the Liver (EASL) is followed, which favors the universal screening approach (EASL 2023).

2.1.4. Challenges in the detection and quantification of HDV RNA

Overall, the detection and quantification of HDV RNA have historically shown considerable variability between laboratories and across different HDV GT (Anolli et al. 2025, Illescas-López et al. 2024). This variability is partly due to the challenges involved in detecting and reliably quantifying HDV RNA using NAAT, especially related to assay design (Le Gal et al. 2016). The circular HDV genome has a high content of cytosine and guanine (around 60%) and exists in an unbranched rod-like structure due to its exceptionally high level of intramolecular base pairing of about 70% (Wang et al. 1986). This complex secondary structure requires long denaturation times and high annealing temperatures to ensure adequate oligonucleotide binding (Homs et al. 2014).

Next, the eight proposed HDV GT, with GT 1 being the most prevalent in Germany, display a high genomic diversity of approximately 20 - 40% among GT (Casey et al. 1993, Wu et al. 1998). Further, a significant degree of heterogeneity is observed within the GT (approximately 16% divergence), and the existence of quasispecies within a single patient, potentially resulting from poor RNA proofreading quality, has been documented (Dény 2006, Homs et al. 2016, Hughes et al. 2011, Shakil et al. 1997). These factors underline the challenges associated with adequate primer design to achieve i) reliable quantification and ii) inclusivity for all GT.

In 2013, a significant step was taken to enhance the standardization of HDV quantitative RT-qPCR assays with the development of the first international HDV WHO standard. This standard includes lyophilized HDV RNA derived from an HBV/HDV-infected individual (HDV GT 1), enabling the reporting of quantitative values in IU/ml for the first time (Chudy et al. 2013). Although the introduction of a WHO standard marked a crucial advancement in reducing the variability of test results using RT-qPCR, the test results and limits of detection for HDV PCR assays still vary significantly among different laboratories (Le Gal et al. 2016, Stelzl et al. 2021). A study conducted across 17 countries evaluated the quantification of HDV in 28 distinct laboratories (Le Gal et al. 2016). A dilution panel of the HDV WHO standard was distributed to each participating site to facilitate standardized reporting of test results in IU/ml (Le Gal et al. 2016). The findings of the study revealed that over 50% of the laboratories were unable to detect at least one of the HDV RNA-positive samples, and quantitative test results varied by more than 3 $\log_{10}$, particularly for certain HDV GT (Le Gal et al. 2016).

Further steps toward achieving adequate standardization may be accomplished through increased automation of workflows. This approach can significantly enhance the comparability of quantitative test results, particularly due to greater standardization of nucleic acid extraction and purification (Pauly et al. 2019). Another crucial aspect of high-quality HDV diagnosis by NAAT is the use of internal controls (IC; preferably RNA templates), which are processed using the same workflow as the samples, including extraction and purification (Wedemeyer et al. 2025).

Unfortunately, the availability of commercial assays that meet the high-quality requirements (inclusivity for all HDV GT, calibration to the HDV WHO standard, and use of IC) is scarce. Currently, no CE-marked *in vitro* diagnostic (CE-IVD) or U.S. Food and Drug Administration (FDA)-approved assay is commercially available that fulfills these requirements and can run on a fully automated platform (FDA 2025, Wedemeyer et al. 2025).

2.1.5. The burden of HDV infection: Epidemiological aspects

The World Health Organization (WHO) estimates that 296 million people are living with chronic HBV infection, and 1.5 million people (1.1 – 2.6 million people) were newly infected in 2019 (World Health Organization 2021). The number of HBV-related deaths, estimated at 800,000 in 2019, is higher than the number of deaths caused by human immunodeficiency virus (HIV) infections in that year (estimate: 700,000 HIV-related deaths (World Health Organization 2021)).

Estimating the prevalence of HBV/HDV coinfections is challenging, as testing for HDV coinfection in HBsAg-positive patients is not conducted consistently worldwide, leading to a lack of sufficient epidemiological data (Kushner et al. 2015, Stockdale et al. 2020), Moreover, recommendations regarding screening strategies for HDV infection vary significantly. The American Association for the Study of Liver Diseases (AASLD) and the National Institutes of Health (NIH) in the United States (U.S.) recommend risk-based screening, while the EASL and the Asian Pacific Association for the Study of the Liver (APASL) state that all HBsAg-positive patients should be tested for HDV infection at least once (Da et al. 2021, EASL 2023, Terrault et al. 2018).

Additional contributing factors include suboptimal molecular testing techniques and the lack of availability of a testing platform (Le Gal et al. 2016). Therefore, HDV prevalence may be systematically underestimated, and existing estimates should be interpreted cautiously. A recent systematic review and meta-analysis that included 282 studies demonstrated that HDV prevalence was 4.5% (95% confidence interval (CI): 3.6-5.7) in HBsAg positive patients, rising to 16.4% (95% CI: 14.6–18.6) when examining hepatology clinic attendees (Stockdale et al. 2020). The global distribution of the prevalence of anti-HDV among the two groups is shown in **Figure 4** for those countries where data was available.

The epidemiological data clearly show that, despite the development of the first vaccine against HBV, which also protects against HDV infections, in the early 1980s, these infections continue to pose a significant health problem worldwide and are far from eradicated.

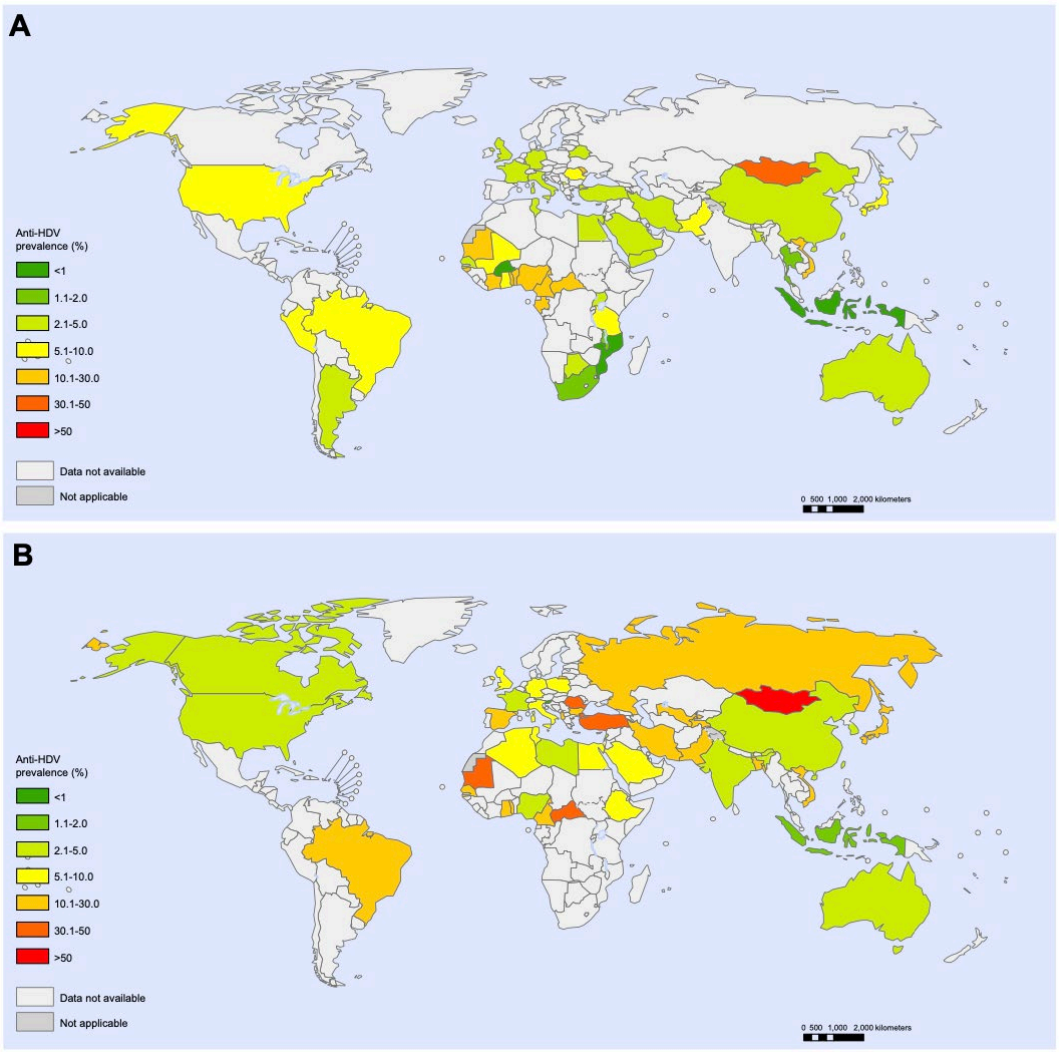


Figure 4: Global anti-HDV prevalence

The Figure illustrates the global prevalence of anti-HDV positive patients by color in (**A**) the general population and (**B**) in hepatology clinic attendees. This figure was originally published by Stockdale et al., in the Journal of Hepatology titled "Fig. 4. Country-level estimates of anti-HDV prevalence among HBsAg-positive people" (Stockdale et al. 2020). The figure has not been edited.

Abbreviations: Anti-HDV, antibodies against the hepatitis delta virus.

2.2. Beyond interferon: Novel treatment options for chronic HDV infection

Until recently, the only available treatment for HDV infection was the off-label use of type one interferon (INF), with PEGylated interferon alpha (pegINFα) being the preferred agent.

The mechanism of pegINFα on HDV is still not well understood. Current hypotheses suggest that pegINFα activates the JAK/STAT pathway, which subsequently leads to the transcription of interferon-stimulated genes and establishes a cellular antiviral state (Giersch et al. 2017). Moreover, INF has been shown to interfere with the cell-division-mediated spread of HDV (Zhang et al. 2022). However, pegINFα is not an optimal therapeutic option for managing HDV infection. Alongside the numerous adverse effects reported by patients, this agent has shown only a 30% success rate in reducing HDV RNA levels (Wedemeyer et al. 2011, Wedemeyer et al. 2019). In addition, relapse is frequently observed even years after treatment ends, indicating that INF treatment is insufficient for establishing sustained viral control (Heidrich et al. 2014, Wranke et al. 2020).

The recent discovery of novel therapeutic agents for HBV/HDV infections marks the start of a new era in treating HDV infection. The innovative approaches discussed in greater detail below show considerable promise in improving care for patients with chronic HDV infection. **Figure 5** offers an overview of some of the new therapeutic agents. Overall, these advancements come after a period of relative stagnation in HDV therapy and highlight the need for reliable diagnostic tools to diagnose HDV infection and monitor treatment response.

2.2.1. Bulevirtide

The most promising candidate amongst the novel agents is bulevirtide, a drug that inhibits NTCP, the entry receptor of HBV, and thus HDV (Liu et al. 2024). Additionally, bulevirtide was recently approved for the treatment of HDV infections by the European Medicines Agency, while the other putative agents are still undergoing clinical trials (Kang and Syed 2020).

Bulevirtide consists of a peptide that specifically targets the pre-S1 domain of NTCP, the binding site of HBV, on the basolateral membrane of differentiated hepatocytes (Liu et al. 2024). Randomized controlled trials evaluating the efficacy of bulevirtide have thus far shown excellent results. In phase III of clinical trials, the authors chose a combined primary endpoint that included i) undetectable levels of HDV RNA after 48 weeks (or a reduction of at least 2 $\log_{10}$ from baseline) and ii) normalization of AST (Wedemeyer et al. 2023). The results of this study indicated a favorable outcome of bulevirtide treatment (2 mg and 10 mg

groups) compared to the control group (no treatment) (Wedemeyer et al. 2023). More than 45% of patients assigned to the bulevirtide treatment groups reached the primary endpoint, whereas this was observed in only 2% of the control group (Wedemeyer et al. 2023). Serious adverse events did not occur during this study (Wedemeyer et al. 2023). Nevertheless, it has been demonstrated that bulevirtide cannot achieve long-term suppression of HDV RNA in all patients, as HDV RNA can recur even after prolonged periods of HDV suppression (Jachs et al. 2023). Still, further studies are required to determine the optimal dosage and therapy duration, as well as to assess the long-term efficacy of bulevirtide in the treatment of chronic HDV infection.

2.2.2. Lonafarnib

Lonafarnib, a farnesyltransferase inhibitor and potential candidate for treating HDV infections, blocks the assembly of viral particles, a crucial step in the life cycle of HDV (Bordier et al. 2003). Treatment with lonafarnib, combined with a drug that inhibits CYP3A4 (ritonavir), allows for a reduction in the dosage of lonafarnib, thus minimizing side effects and demonstrating a dose-dependent response in conducted studies (Yurdaydin et al. 2018, Yurdaydin et al. 2022). However, a large randomized controlled trial (double-blinded, multicenter) that enrolled 407 patients and compared i) lonafarnib + ritonavir, ii) lonafarnib + ritonavir + pegINFα, iii) pegINFα monotherapy, and iv) placebo did not discover significant differences when comparing composite endpoint (Etzion et al. 2023). The endpoint consisted of i) a reduction of HDV RNA decline of at least 2log and ii) normalization of ALT at 48 weeks (Etzion et al. 2023).

2.2.3. Nucleic acid polymers

Nucleic acid polymers (NAP) elicit antiviral effects for which polymer length and hydrophobicity are particularly important. It has been established that NAP can exert this antiviral effect against a wide range of viruses, including HCV, HBV, and HDV (Basu et al. 2007, Beilstein et al. 2018, Schöneweis et al. 2018).

The antiviral effect of NAP on HBV/HDV infections remains incompletely understood. However, the antiviral effects seem to depend, at least in part, on interference with the

assembly and/or release of subviral particles and the inhibition of viral entry into hepatocytes (Guillot et al. 2017, Noordeen et al. 2015). A phase II clinical trial evaluated the efficacy and safety of the NAP REP 2139 in combination with pegINFα for the treatment of chronic HBV/HDV infection (Bazinet et al. 2017). The combination therapy of REP 2139 and pegINFα showed promising results regarding the reduction of liver enzymes and HBV as well as HDV markers (HBV DNA, HDV RNA, and HBsAg levels) (Bazinet et al. 2017). In this study, 11 out of 12 patients were shown to be HDV RNA negative during the treatment period, with 9 patients remaining HDV RNA negative at the end of the trial (Bazinet et al. 2017). The results indicate that NAP may represent a potential therapeutic option for chronic HBV/HDV in the future. Nevertheless, larger studies are needed to ascertain the potential of NAP as a treatment for HBV/HDV infections.

2.2.4. Monoclonal antibodies against HBsAg and small-interfering RNA

The use of small interfering RNA (siRNA) and monoclonal antibodies directed against HBsAg represents an additional approach to treating HBV/HDV infection. SiRNA interferes with intracellular RNA and, when aimed at HBV-specific mRNA transcripts, has been shown to reduce HBsAg levels (Gane et al. 2023). Monoclonal antibodies exert their potential through neutralization and opsonization of HBsAg. Particularly when combined, monoclonal antibodies and siRNA have been shown to effectively lower HDV RNA and ALT levels in chronically infected patients (Park et al. 2025). Data demonstrating this effect using tobevibart (VIR-3434, monoclonal antibody) and elebsiran (VIR-2218, siRNA) was presented during the EASL conference in 2024 (Asselah et al. 2024). Furthermore, the concept of reduced HDV RNA and HBsAg levels in patients treated with monoclonal antibodies has been shown for multiple monoclonal antibodies (BJT-778 and HH-003), which are directed against HBsAg (Agarwal et al. 2024, Wang et al. 2023). It remains to be seen whether these remarkable findings will be confirmed by later studies.

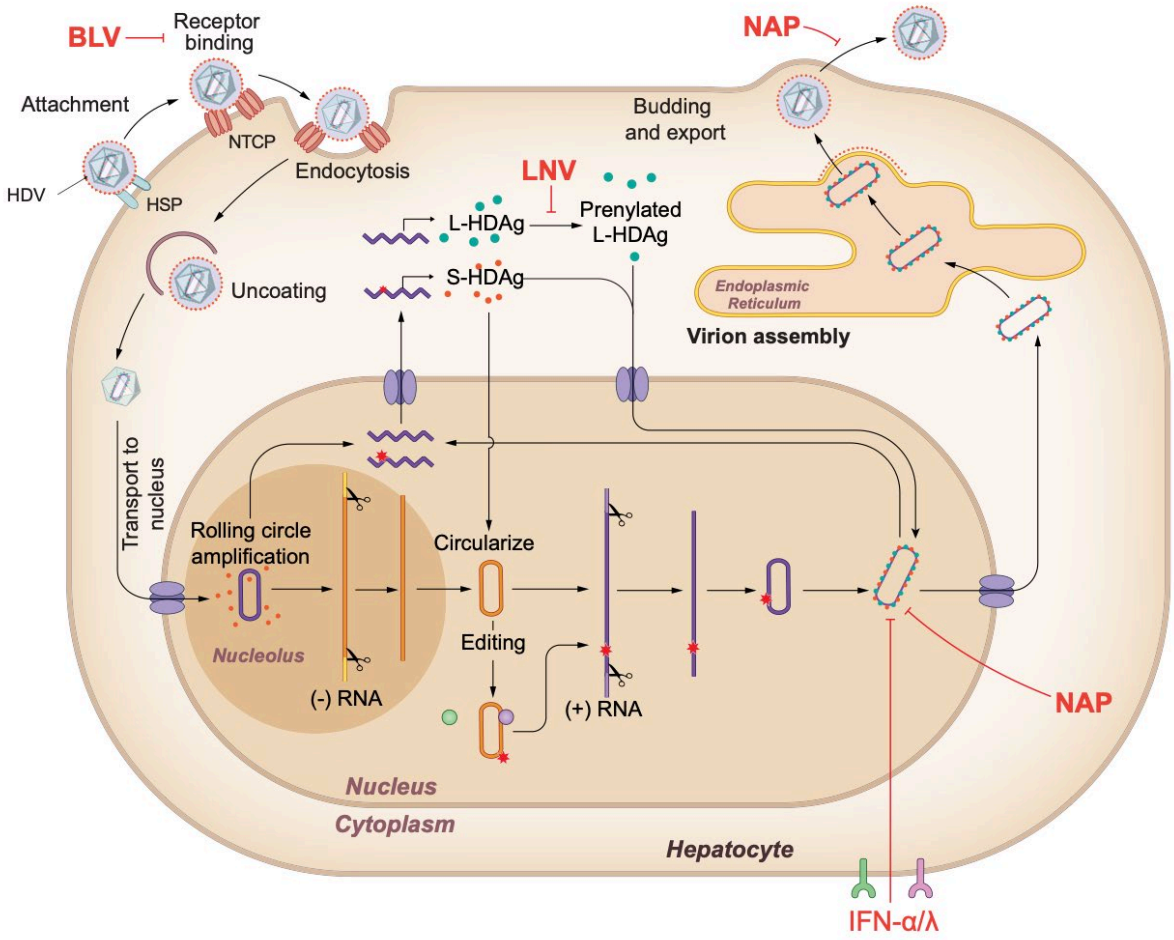


Figure 5: Treatment options for HDV infection

Overview of novel and existing therapies for HDV infection and their target within the HDV life cycle. BLV blocks the NTCP, which is the entry receptor for HBV and HDV. LNV, a farnesyltransferase inhibitor, prevents the farnesylation of L-HDAg and thus interferes with viral assembly. This figure was originally published by Ghany et al., in the Journal of Hepatology titled "Fig. 3 HDV life cycle and drug targets" (Ghany et al. 2023). The figure has not been edited.

Abbreviations: BLV, bulevirtide; HDV, hepatitis delta virus; HSP, heparan sulfate proteoglycans; IFN-α/χ; interferon alpha/lambda; L-HDAg, large version of the hepatitis delta virus antigen; LNV; lonafarnib; NAP, nucleic acid polymers; NTCP, natrium taurocholate cotransporting polypeptide; S-HDAg, small version of the hepatitis delta virus antigen.

2.3. Discovery of HDV-like viruses

HDV-like agents were recently found in snakes (Hetzel et al. 2019, Wille et al. 2018), birds (Chang et al. 2019b), fish, and mammals (Paraskevopoulou et al. 2020). Most of which can cause infection without the presence of Hepadnaviridae (Pérez-Vargas et al. 2021). The range of putative helper viruses of HDV-like agents is broad and includes next to others Flaviviridae, Herpesviridae, and Retroviridae (Pérez-Vargas et al. 2021). **Table 1** shows an overview of discovered HDV-likes agents and their putative helper viruses.

Table 1: HDV-like viruses

Deltavirus	Host	CXXQ Motif	Putative Co-Infecting Viruses	Tested Helper Viruses (In Vitro)	HBsAg Usage (In Vitro)
HDV	Human	Yes	HBV	[a] HBV,[b] VSV, [c] HCV, [d] DENV, WNV, [e] LCMV, [f] HMPV	Yes
aDeV	ducks	no	Influenza A virus	nd	nd
RDeV	rodent *P. semispinosus*	no	Hepacivirus	nd	nd
SDeV	Boa constrictor	no	Reptarenavirus, hartmanivirus	[g] UHV-2, UGV-1 [h] HISV-1 [e] LCMV, JUNV [f] PUUV	no
tgDeV	Zebra finch *Taeniopygia guttata*	no	none	nd	no
mmDeV	Eastern woodchuck *Marmota monax*	no	WHV, herpesvirus, flavivirus, retrovirus	nd	no
DrDeV	Vampire bats *D. rotundus*	no	Herpesvirus, flavivirus, retrovirus	nd	nd
OvirDeV	White-tailed deer *Odocoileus virginianus*	no	Herpesvirus, flavivirus, retrovirus	nd	nd
PmacDeV	Lesser dog-like bat *Peropteryx macrotis*	no	Herpesvirus, flavivirus, retrovirus	nd	nd

The table shows an overview of recently discovered HDV like viruses and their putative "helper-viruses". The presence of the CXXQ motif, which is located within the C-terminus of the L-HDAg and is required for the farnesylation, is indicated. This table was originally published by Pérez-Vargas et al., in the Journal Viruses titled "Table 1. Summary of Deltavirus infections" (Pérez-Vargas et al. 2021). The table has not been edited.

Abbreviations: DENV, dengue virus; HBV, hepatitis B virus; HBsAg, hepatitis B virus surface antigen; HCV, hepatitis C virus; HISV-1, hartmanivirus 1; HMPV, human metapneumovirus; JUNV, Junin virus; LCMV, lymphocytic choriomeningitis virus; nd, not determined; PUUV, Puumala virus; TBHBV, tent-making bat hepatitis B virus; UHV-2, university of Helsinki virus 2; UGV-1, univierust of Gießen virus 1; VSV, vesicular stomatitis virus; WHV, woodchuck hepatitis virus; WNV, west nile virus; WMHBV, wooly monkey hepatitis B virus.

Several similarities have been identified between HDV and discovered HDV-like agents. First, the genome is comparable in size, ranging from 1500 to 1700 nucleotides, and is single-stranded, circular, and folded into a rod-like structure (Pérez-Vargas et al. 2021). Furthermore, the genome of the HDV-like agents, like the human counterpart, encodes a protein similar to HDAg (13-55% amino acid identity), and HDV-like agents have been found to harbor a self-cleaving motif (Webb and Lupták 2011). The presence of the HDV ribozyme motif has been reported for HDV-like agents found in amniotes, while for other HDV-like agents, self-cleaving motifs from the hammerhead class were discovered (de la Peña et al. 2021, Hetzel et al. 2019, Wille et al. 2018).

The discovery of agents similar to HDV may provide insight into the origin of HDV, which remains uncertain to this day. Some theories about the origin of HDV focus on the numerous similarities between HDV and viroids. These similarities include that many viroids consist of small circular RNA genomes and utilize a rolling cycle amplification mechanism (Branch and Robertson 1984, Flores et al. 2008). Viroids have been shown to replicate using host polymerases, with some displaying ribozyme motifs characteristic of the hammerhead class (Flores et al. 2008, Schindler and Mühlbach 1992). Another hypothesis proposes that HDV may have evolved from a combination of viroid-like structures and host mRNA elements. A cellular protein, the delta-interacting protein (DIPA), which shows 24% similarity to the protein sequence of HDAg, has been put forward as a potential precursor of HDAg (Brazas and Ganem 1996). These findings have sparked the discussion about whether HDV has originated from the human transcriptome. Salehi-Ashtiani et al. identified a ribozyme located in the intron of the CPEB3 gene that is structurally similar to the HDV self-cleaving ribozyme (Salehi-Ashtiani et al. 2006). However, the discovery of HDV-like agents suggests that the evolutionary history of HDV may be considerably longer than previously assumed and indicates that the evolution of HDV may not be restricted to the human organism.

2.4. Propagation of HDV by human pathogenic viruses other than HBV

For a long time, HDV was thought to be an obligate satellite virus of HBV. Interestingly, a recent study revealed that HDV can be enveloped by other human pathogenic viruses beyond HBV (Perez-Vargas et al. 2019). The authors demonstrated that various viruses, including non-hepatotropic ones, could package HDV and facilitate the egress of infectious particles

in vitro and in a humanized mouse model, all without the presence of an innate immune system (Perez-Vargas et al. 2019). Notably, HCV, varicella-zoster virus (VZV), and Dengue Virus (DENV) were found to act in a similar manner and pattern as HBV (Perez-Vargas et al. 2019).

The mechanism underlying the formation of HDV particles enveloped by the glycoproteins (GP) of non-HBV viruses is not yet fully understood. In the assembly of HDV RNP particles coated with HBsAg, the farnesylation of L-HDAg has been identified as an important factor (O'Malley and Lazinski David 2005). The farnesylated version of L-HDAg serves to anchor the HDV RNP to the membrane of the endoplasmic reticulum (ER) (Netter et al. 2021). The ER is a cell organelle located in the cytosol where the GP of various viruses are synthesized. This mechanism also seems to play a significant role in the formation of HDV particles that utilize the GP of VZV and HCV as a coating (Perez-Vargas et al. 2019). Indeed, Perez-Vargas et al. demonstrated that the production of these VZV/HCV-coated HDV particles could be inhibited *in vitro* when lonafarnib, a farnesyltransferase inhibitor, was used (Perez-Vargas et al. 2019).

Finally, the authors demonstrated that Huh-7.5 cells expressing HDV RNA produced HDV particles five days after inoculation with HCV grown in cell culture (Perez-Vargas et al. 2019). The HDV particles contained full-length genomic HDV RNA, as measured by qPCR in the supernatant of the cells, and were shown to be infectious (Perez-Vargas et al. 2019). In contrast, no HDV RNA was detected in the supernatant of cells without prior inoculation with HCV (Perez-Vargas et al. 2019). Furthermore, the authors confirmed these findings in a liver-humanized mouse model (Perez-Vargas et al. 2019).

Overall, these observations indicate that other human pathogenic viruses can fulfill the necessary functions to package HDV and promote the egress of infectious HDV particles without HBV and suggest that HDV mono-infection can be rescued following HCV infection. These findings challenge the accuracy of the long-held belief that HDV infection occurs only in HBsAg-positive patients. If HDV infection can indeed be established in humans without HBV, the implications could be significant, especially since the glycoproteins on the virus's envelope determine their tropism (Banerjee and Mukhopadhyay 2016, Dandri and Petersen 2016, Ding et al. 2014). Consequently, it is possible that HDV is capable of infecting a much broader range of tissues and organs, potentially contributing to

a more severe course of infection, for example, in the case of HCV-mediated HDV infection. In light of these recent findings, it is becoming increasingly evident that the question of whether HDV infections in HBsAg-negative patients are overlooked simply due to a lack of testing needs to be addressed.

3. Materials and Methods

3.1. Inclusion criteria for the selection of clinical samples

Records of patients administered to our center (University Medical Center Hamburg-Eppendorf) were screened and remnant clinical samples were selected according to the following criteria.

For the establishment and validation of the HDV RT-qPCR assay:

- Anti-HDV immunoglobin G (IgG) positive serum samples (screening period: 2008 - 2020).

For the evaluation of HCV/HDV coinfection:

- HCV PCR positive and HBsAg negative serum samples (screening period: 2010 - 2019).

In total, n=110 anti-HDV positive and n=323 HCV PCR positive and HBsAg negative samples were included in the experiments conducted for this dissertation. Samples were stored at or below -20° C. All samples were de-identified before use. For both projects separate positive approvals were obtained from the local ethics committee (Freie und Hansestadt Hamburg: PC-5626 and WF-036/20)

3.2. Establishment of the HDV RT-qPCR

3.2.1. PCR assay design and cycling conditions

A primer/probe set as previously described was selected to amplify a 90 base pair (bp) target within the highly conserved region of the HDV genome, the hepatitis delta ribozyme self-cleaving site (Coller et al. 2018).

Modifications of the primer/probe set for use on the cobas6800 platform included O'methylation close to the 3' end of the forward and reverse primer to reduce the formation of cross- and self-primer dimers within the utilized master mix (MMRX-2, Roche, Rotkreuz,

Switzerland). 3' modification of the respective TaqMan-probe included a minor groove binder to enhance binding stability and a black hole quencher to effectively reduce background fluorescence. Sequences of the primer/probe set are listed in **Table 2**. The oligonucleotides were custom-made and ordered from IDT DNA Technologies (Coralville, Iowa, USA) and biromers.net GmbH (Ulm, Germany).

Table 2: Sequences of oligonucleotides used within the HDV_UCT

HDV_UCT oligonucleotides				
target	**oligonucleotide**	**sequence (5' to 3')**	**conc. [nM]**	**reference**
HDV ribozyme self-cleaving site	forward primer	CTC CCT TWG CCA TCC (2'0-Meth-G)AG	1,000	Coller et al. (2018)
	reverse primer	CTC TTC GGG TCG GCA T(2'0-Meth-G)G	1,000	
	probe	HEX- ATG CCC AGG TCG GAC CRC -MGB -BMN-Q535	50	

Sequences of oligonucleotides for detection and quantification of HDV as adapted for the use on the cobas6800. The listed concentrations refer to the final concentration within the reaction mix. A "W" in the sequence indicates that either adenine or thymine and an "R" indicates that either adenine or guanine is added during the manufacturing of the oligonucleotide.

Abbreviations: BMN-Q535, quencher from biomers.net; conc., concentration; HDV, hepatitis delta virus; HDV_UCT, hepatitis delta virus Utility channel test; HEX, fluorophore: hexachloro-flurescein; nM, nanomolar; MGB, minor grove binder; R, purine (adenine or guanine); W, weak (adenine or thymine); 2'0-Meth-X, 2'O-methylation of the respective base.

For the setup of the PCR on the automated platform, a new analysis package including adapted PCR cycling conditions was programmed on the open channel of the device (Utility channel, **Table 3**). The relative fluorescence increase minimum (RFI min.) was selected to allow automated calling of test results. An addition of an IC was not necessary, as IC are spiked in automatically during extraction on the device, thus acting as a full process control. The sequence-specific primers and probe are added to the respective master mix when preparing a new reagent cassette (Roche, Rotkreuz, Switzerland) for testing.

Table 3: PCR cycling conditions

	PCR cycling parameters				
	incubation step	pre-PCR	1st measurement	2nd measurment	cooling step
step	1	2	3	4	5
protocol	predifined	55 °C for 120 s 60 °C for 360 s 65 °C for 240 s	95 °C for 5 s 55 °C for 30 s	91 °C for 5 s 58 °C for 25 s	predifined
no. of cycles		1	5	45	
data aquasititon	n/a	n/a	end of each cycle	end of each cycle	n/a

The table depicts the PCR cycling conditions as programmed using the Utility channel software for the HDV_UCT. The first and last steps cannot be modified.

Abbreviations: HDV_UCT, hepatitis delta virus Utility channel test; n/a, not applicable; no., number; PCR, polymerase chain reaction; s, seconds.

3.2.2. In silico analysis of assay inclusivity for all HDV GT and evaluation of possible oligonucleotide interactions

To confirm the inclusivity of the HDV Utility channel test (HDV_UCT) for all HDV GT and to assess the possibility of false-negative PCR results due to mismatches within the primer or probe regions, a comprehensive in silico analysis was conducted. Genotyped sequences of the HDV genome spanning the respective targeted region of the PCR assay were selected and downloaded from NCBI (selected database: nucleotide; accessed: May – June 2023). The sequences were then grouped according to HDV GT and analyzed separately. Geneious (version 2019.2 or higher, Biomatters Ltd., Boston, Massachusetts, USA) was used to align primers and probes to the selected sequences using “clustal omega” as the alignment method. The alignment was manually edited to remove gaps.

To evaluate potential oligonucleotide interaction, all primers and probes were analyzed using the Oligo Analyzer software (IDT). Critical interactions were predefined as binding energy below the preselected cutoff (delta G: < -10 kcal/mol). Special emphasis was placed on the formation of primer dimers and changes in Tm after oligonucleotide modification.

3.2.3. Analytical performance evaluation: Limit of detection and linearity

The lower limit of detection (LoD) was determined by serial dilution of the WHO HDV standard (Paul-Ehrlich Institute (PEI) code: 7657/12; PEI; Langen; Germany; 2-fold; 10 steps, n=21 repeats/step) and a set of HDV RNA negative samples (n=21). All samples were tested on the same day and device under standardized conditions. The WHO HDV standard was resuspended according to manufacturer instructions and stored at -80° C until use.

Linearity was determined using the WHO HDV standard and cell-culture-derived virus (HDV GT 1-8) to evaluate possible differences in PCR performance depending on the HDV GT used as a template. The cell-culture derived viruses were provided by Prof. Dieter Glebe (Justus-Liebig-Universität Gießen, Hessen, Germany) as a contribution to the project. The dilution series (10-fold) consisted of 5 replicas per step each. For all experiments, commercially available serum negative for HBsAg and antibodies against the hepatitis B virus surface antigen (anti-HBs; ARCHITECT HBsAg Manual Diluent (6C36-40), Abbott, Abbott Park, Illinois, USA) was used as diluent.

3.2.4. Technical performance evaluation: Precision, stability, and exclusivity

To assess precision, the HDV WHO standard was utilized. Three concentrations (10-fold dilution, starting concentration: 5,750 IU/ml (1:100)) and a negative set were evaluated over three consecutive days and in different runs to determine the within-run and between-day variability of the assay. Each set consisted of five technical replicates. Target values for the inter- and intra-run variability were established before testing at ± 0.5 cycle threshold (ct) and ± 1 ct, respectively.

Next, the stability of the testing reagents and the reproducibility of test results were evaluated over a six-month period. Again, a 10-fold dilution of the HDV WHO standard (three different concentrations) and an HDV RNA negative sample were examined (n=3 technical replicates per concentration). All samples were tested on the day of preparation of the reagent cassette and after 1, 3, and 6 months. The reagents were stored at 4°C to determine whether their stability was comparable to that of commercially available reagent kits for the cobas X800 series. The dilutions of the HDV WHO standard were kept at -20°C and thawed at room temperature before testing.

The exclusivity of the assay was verified using a set of clinical samples from patients administered to our center (University Medical Center Hamburg-Eppendorf) that tested positive by qPCR for either HBV, HCV, Epstein-Barr virus (EBV), HIV-1, BK-polyomavirus (BKPyV), or cytomegalovirus (CMV). For each exclusivity panel member, n=10 serum samples with different viral loads were selected.

3.2.5. Clinical performance evaluation: Comparison to a CE-IVD assay, external quality assessment samples, and evaluation of viral kinetics over time

To evaluate clinical performance, a CE-IVD assay (RoboGene HDV RNA Quantification kit, Analytik Jena, Jena, Germany) was chosen as the comparator assay. Nucleic acids were extracted manually using the INSTANT virus RNA/DNA kit (Analytik Jena, Jena, Germany) and the PCR was performed on a LightCycler 480 II (Roche, Rotkreuz, Switzerland) utilizing 5 µl of eluate each. All steps were performed according to the respective manufacturer's instructions.

For further evaluation, a set of external quality assessment samples (EQA; n=20; INSTAND, Düsseldorf, Germany) and a set of samples from HDV RNA positive patients that were collected over time at our center (n=4 patients; 3-7 different time points per patient) were subjected to the HDV_UCT and the comparator assay.

3.3. Evaluation of HCV-mediated HDV propagation in HCV mono-infected patients

3.3.1. Parameters for the determination of patient characteristics

For patients meeting the inclusion criteria for the evaluation of HCV-mediated HDV infections as described above (see Materials and Methods: 3.1. Clinical samples) the medical records were screened to obtain the following clinical parameters. Clinical data was anonymized directly after collection and saved on a secure platform.

General patient characteristics:

- Age in years at the time of blood sampling
- Sex (female/male/divers)

Data related to the HCV infection:

- Time in years since the first diagnosis of HCV infection
- HCV viral load in IU/ml at the time of blood sampling as determined using the current HCV PCR assay at that time (see below: Materials and Methods: 3.3.2.)
- HCV GT

Parameters of liver injury:

- Aspartate transaminase (AST)
- Alanine transaminase (ALT)

3.3.2. Methods and assay specifications for the detection of HCV/HDV RNA and serological parameters

To exclude the possibility of missed HDV infection in HCV mono-infected patients, n=323 HCV PCR positive and HBsAg negative serum samples were included in this study. Before 2016, the presence of HCV RNA within patients' serum samples was detected using the COBAS AmpliPrep/COBAS TaqMan quantitative HCV Test (CE-IVD, Roche, Rotkreuz, Switzerland). After 2016, quantification of HCV RNA was performed using the cobas HCV test (CE-IVD; Roche, Rotkreuz, Switzerland) that amplifies a target in the highly conserved 5'non-translated region of the HCV genome.

For the detection and quantification of HDV RNA, the RT-qPCR assay (HCV_UCT) which was established and validated as part of this dissertation was used. All performed RT-qPCR reactions (HCV and HDV) included a full-process control. Testing of the serology parameters (HBsAg, anti-HBs, antibodies against the hepatitis B core antigen (anti-HBc) IgG, and immunoglobin M (IgM)) was performed on the Centaur XL system (before 2018; Siemens Healthcare, Erlangen, Germany) or the Alinity I (after 2018; Abbott, Abbott Park, Illinois, USA) and anti-HDV was tested on the Liaison XL (Diasorin, Saluggia, Italy). A detailed description of the assay specifications is shown in **Table 4**.

Table 4: Assay specifications

assay specifications: HCV RT-qPCR							
assay name	**manufacturer**	**detection of**	**nucleic acid extraction**	**amplification and**	**LoD [IU/ml]**	**linear range**	**inclusivity**
COBAS AmpliPrep/COBAS TaqMan quantitative HCV Test (version 2.0)	Roche	HCV RNA	COBAS AmpliPrep	COBAS TaqMan 48	15	15 - $1.7*10^6$	HCV GT 1-6
cobas HCV test	Roche	HCV RNA	cobas6800	cobas6800	8.46	15 - 10^8	HCV GT 1-6

assay specifications: serology testing						
assay name	**manufacturer**	**detection of**	**performed on**	**detection method**	**cutoff**	**result**
ADVIA Centauer HBsAg Assay*	Siemens Healthcare	HBsAg	Centauer XP (Siemens Healthcare)	CLIA	1.00 Index Value	qualitative
ADVIA Centauer anti-HBs2 Assay*	Siemens Healthcare	anti-HBs	Centauer XP (Siemens Healthcare)	CLIA	10 mIU/ml	qunatitaive
ADVIA Centauer HBc Total-Test*	Siemens Healthcare	anti-HBc IgG & IgM	Centauer XP (Siemens Healthcare)	CLIA	0.50 Index Value	quantitative
HBsAg Next Qualitative	Abbott	HBsAg	Alinity I (Abbott) / Centaur XL (Siemens Healthcare)	CMIA	1.00 S/CO	qualitative
Anti-HBs	Abbott	anti-HBs	Alinity I (Abbott) / Centaur XL (Siemens Healthcare)	CMIA	n/a	quantitative
Anti-HBc II	Abbott	anti-HBc IgG & IgM	Alinity I (Abbott) / Centaur XL (Siemens Healthcare)	CMIA	1.00 S/CO	qualitative
LIAISON XL murex Anti-HDV	Diasorin	anti-HDV IgG & IgM	Liaison XL (Diasorin)	CLIA	1.00 AU/ml	qualitative

Legend corresponding to:
Table 4: Assay specifications

The table lists the assay specifications of the used HCV RT-qPCR and serology assays as provided by the manufacturer.

Abbreviations: Anti-HBc, antibodies against the hepatitis B core antigen; anti-HBs, antibodies against the hepatitis B surface antigen GT, anti-HDV, antibodies against the hepatitis delta virus; CLIA, chemiluminescent-immunoassay; CMIA, chemiluminescent-microparticle-immunoassay; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HDV, hepatitis delta virus; IgG, immunoglobulin G antibodies; IgM, immunoglobulin M antibodies; LoD, limit of detection; RT-qPCR, quantitative reverse transcription polymerase chain reaction.

3.4. Statistical analysis

Statistical analysis was conducted using GraphPad Prism (version 8 or higher; San Diego, California, USA) and the Validation Manager software (Finbiosoft, Espoo, Finland). For the assessment of 95% LoDs, a probit analysis was performed. Correlation was assessed using a simple linear regression analysis and Pearson's correlation coefficients (r^2). For comparison of the HDV_UCT assay to the comparator assay, a Bland-Altman analysis was conducted. A Fisher's exact test was performed to test for rejection of the null hypothesis. To compare anti-HDV levels of HCV PCR positive to HBV/HBV coinfected patients, the Mann-Whitney test was chosen. P-values < 0.05 were regarded as statistically significant. **Figure 3, 8,** and **12** were created or assembled using BioRender.com.

4. Results

4.1. Establishment of the HDV RT-qPCR assay and validation of clinical and analytical performance

4.1.1. Confirmation of the inclusivity of the HDV_UCT for the eight HDV GT and minimal risk for oligonucleotide interactions

A total of 632 sequences were selected, covering the entire HDV PCR amplicon and all 8 HDV GT (GT 1: n=400; GT 2: n=64; GT 3: n=83; GT 4: n=1; GT 5: n=17; GT 6: n=15; GT 7: n=47; GT 8: n=5). Alignments with the respective primer/probe set indicated that 91.61 % (n=679/632), 98.58 % (n=623/632), and 98.89 % (n=625/632) of the sequences were identical to the target regions of the forward primer, reverse primer, and probe, respectively (analysis included all HDV GT). The percentage of sequences exhibiting more than two mismatches within a single oligonucleotide was < 0.33 % for all oligonucleotides (forward primer: 2/632; reverse primer: 2/632; probe: 1/632). A total of 5/632 (0.79 %) sequences could be identified with mismatches across 2/3 oligonucleotides, while no HDV sequence was found with mismatches in all oligonucleotides. The most prevalent mutation involved the substitution of guanine to adenine at the 5'-end of the binding region of the forward primer (n=42/632 sequences; 6.65 %), which was most frequent in GT 7 (n=39/42).

These observations were consistent with the published alignment results of the respective primer/probe set from the original publication (Coller et al. 2018). In their study, the authors aligned n=291 sequences of various HDV GT using "MUSCLE" as the preferred alignment tool. 92.4 % (n=269/291) of the sequences were identical to the target region of the primer/probe set (Coller et al. 2018). Predominantly single nucleotide mutations were detected, with a prevalence of no more than 3,4 % within the aligned sequences (Coller et al. 2018). Based on this analysis, it was determined that no further modification of the primer/probe set was needed to improve the inclusivity of the assay. Detailed alignment results are shown in **Table 5**.

Table 5: Genotype-specific alignment of HDV sequences and oligonucleotides

genotype-specific alignment												
genotype	forward primer				probe				reverse primer			
	accession-no.	sequence CTCCCTTWGCCATCCGAG	no. of sequences	in %	accession-no.	sequence ATGCCCAGGTCGGACCRC	no. of sequences	in %	accession-no.	sequence CTCTTCGGGTCGGCATGG	no. of sequences	in %
GT 1	n/a	.....	391/400	97.75	n/a	.....	399/400	99.75	n/a	.....	397/400	99.25
	MG711695	A.....	2/400	0.50	MG711701*	.G.....	1/400	0.25	OK142941*	..A..A....A....U..	1/400	0.25
	OK142865								OK142939*	......U.U.....	1/400	0.25
	OK142941*	........U......G.U	1/400	0.25					EF514906	AGA.GC.....	1/400	0.25
	OK142939*	...A.....	1/400	0.25								
	MG711701	.G.....	1/400	0.25								
	MN984407	.....U..	1/400	0.25								
	KJ744233	........G.....	1/400	0.25								
	KJ744232	........G......U..	1/400	0.25								
	KJ744245	.....U	1/400	0.25								
GT 2	n/a	.....	64/64	100	n/a	.....	62/64	96.8	n/a	.....	63/64	98.4
					AB118836	.....G.....	1/64	1.6	LT604952	..A.....	1/64	1.6
					AF309420	.....G.	1/64	1.6				
GT 3	n/a	.....	79/83	95.18	n/a	.....	80/83	96.4	n/a	.....	78/83	94
	KF786309*	.....UCC.....A....	1/83	1.2	KF786309*	.....G.....	2/83	2.41	KC590319	A.....	5/83	6
	KF786324	.....G.....	1/83	1.2	AB118837				AB118821			
	KF786347*	.....A....	1/83	1.2	KF786339	.....C.....	1/83	1.2	KF786347*			
	KF786320	......G.....	1/83	1.2					KF786329			
									KF786336			
GT 4	MT050453	.....	1/1	100	MT050453	.....	1/1	100	MT050453	.....	1/1	100
GT 5	n/a	.....	17/17	100	n/a	.....	16/17	94.1	n/a	.....	17/17	100
					JX888107	.....CAA.....	1/17	5.9				
GT 6	n/a	.....	15/15	100	n/a	.....	15/15	100	n/a	.....	15/15	100
GT 7	n/a	A.....	39/47	82.98	n/a	.....	47/47	100	n/a	.....	47/47	100
	MG711768	.....	7/47	14.89								
	MG711682											
	LT604969											
	MG711783											
	MG711754											
	MG711789											
	MG711772											
	LT604971	A.....G.....	1/47	2.13								
GT 8	n/a	.....	5/5	100	n/a	.....	5/5	100	n/a	.....	5/5	100

Legend corresponding to:

Table 5: Genotype-specific alignment of HDV sequences and oligonucleotides

All oligonucleotides are listed from 5' to 3' end. Dots represent agreement with the sequence of the respective oligonucleotides to the binding region. Mismatches are shown as letters corresponding to the respective base present in the HDV sequence at that position. Sequences that have mismatches within 2/3 oligonucleotides are marked with a "*". No sequence had mismatches within all three oligonucleotides.

Abbreviations: A, adenine; C, cytosine; G, guanine; GT, genotype, HDV, hepatitis delta virus; HDV_UCT, hepatitis delta virus Utility channel test; n/a, not applicable; no., number; U, uracil

Moreover, no critical oligonucleotide interactions could be determined (cutoff of the binding energy: delta G < -10 kcal/mol) using the IDT Oligo Analyzer Software, indicating that the formation of self- or cross-primer/ probe dimers is minimal within the reaction mix. Thus, no further modification of the utilized oligonucleotides was performed. Detailed results are listed in **Table 6**.

Table 6: Oligonucleotide interactions

Oligonucleotide interactions			
	forward primer	reverse primer	probe
forward primer	-3.61	-8.26	-9.83
reverse primer	-8.26	-5.38	-9.64
probe	-9.83	-9.64	-6.68

Analysis of oligonucleotide interactions conducted using the IDT Oligo Analyzer Software. Delta G values of the binding energy in kcal/mol are shown. The cutoff for possible critical interaction was selected to be < -10 kcal/mol.

4.1.2. High sensitivity and confirmation of the linearity of the HDV_UCT across all HDV GT

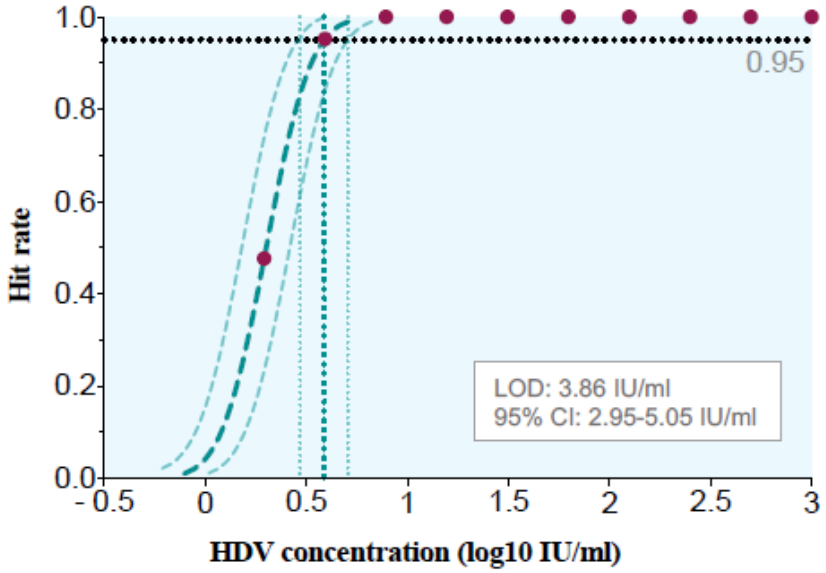
The results presented in this and the following three paragraphs (4.1.2. – 4.1.5.) were published listing me as the first author during my time as a doctoral candidate in the Journal JHEP reports (Pflüger et al. 2021).

The assay validation was performed according to the European guidelines (Regulation 2017/746 EU IVDR). The minimum RFI was set at 1.25 for the automated calling of test results. All experiments were conducted on the same device and under standardized conditions.

To determine the LoD of the assay, serial dilutions of the HDV WHO standard (2-fold, 10 steps, n=21 repeats per step), with the highest concentration being 1,000 IU/ml and the lowest concentration at 1.95 IU/ml, were tested. A probit analysis established the 95% LoD as 3.86 IU/ml (95% CI: 2.95 – 5.05 IU/ml), confirming the assay's high sensitivity (see **Figure 6)** (Pflüger et al. 2021).

Next, the linearity of the assay was evaluated using the HDV WHO standard (GT 1) and cell-culture-derived viruses (GT 1-8) in serial dilution (10-fold, n=5 repeats/step). The assay demonstrated excellent linearity for all HDV GT. Testing of the HDV WHO standard (4 steps) revealed a slope of -3.422 and an r^2 of 0.998 (Pflüger et al. 2021). The cell-culture-derived viruses (HDV GT 1-8) were tested within the range of 10^{1} to $10^{4\text{-}8}$ IU/ml. Slopes varied from -3.481 to -4.134, and r^2 was > 0.918 for all HDV GT (**Figure 7**) (Pflüger et al. 2021). Examples of amplification curves (HDV GT 1 - 4) from the linearity assessment are shown in **Figure 8**.

A



B

Limit of detection - HDV WHO standard				
step	conc. [IU/ml]	positive results [n]	positive results [%]	mean ct value
1	1000	21/21	100	29.47
2	500	21/21	100	30.50
3	250	21/21	100	31.38
4	125	21/21	100	32.22
5	62.5	21/21	100	33.13
6	31.25	21/21	100	34.08
7	15.63	21/21	100	35.37
8	7.81	21/21	100	36.47
9	3.91	20/21	95.23	37.85
10	1.95	10/21	47.61	39.02
neg. set	0	0/21	0	n/a

Figure 6: Limit of detection

The limit of detection was determined using serial dilutions of the HDV WHO standard. A probit analysis determined the 95% LoD at 3.86 IU/ml (**A**). Detailed results of testing of the serial dilutions of the HDV WHO standard (2-fold, 10 steps, n=21 repeats/step) are shown in (**B**). The figure was adapted from a figure published by Pflüger et al., in the Journal JHEP Reports titled “Fig. 1. Probit analysis and linearity for HDV genotype 1 and test results of the new HDV_UCT assay compared to the CE-trademarked IVD RoboGene assay” (Pflüger et al. 2021).

Abbreviations: conc., concentration; ct, cycle threshold; HDV, hepatitis delta virus; IU/ml, international units per milliliter; n/a, not applicable; neg., negative; WHO, World Health Organization.

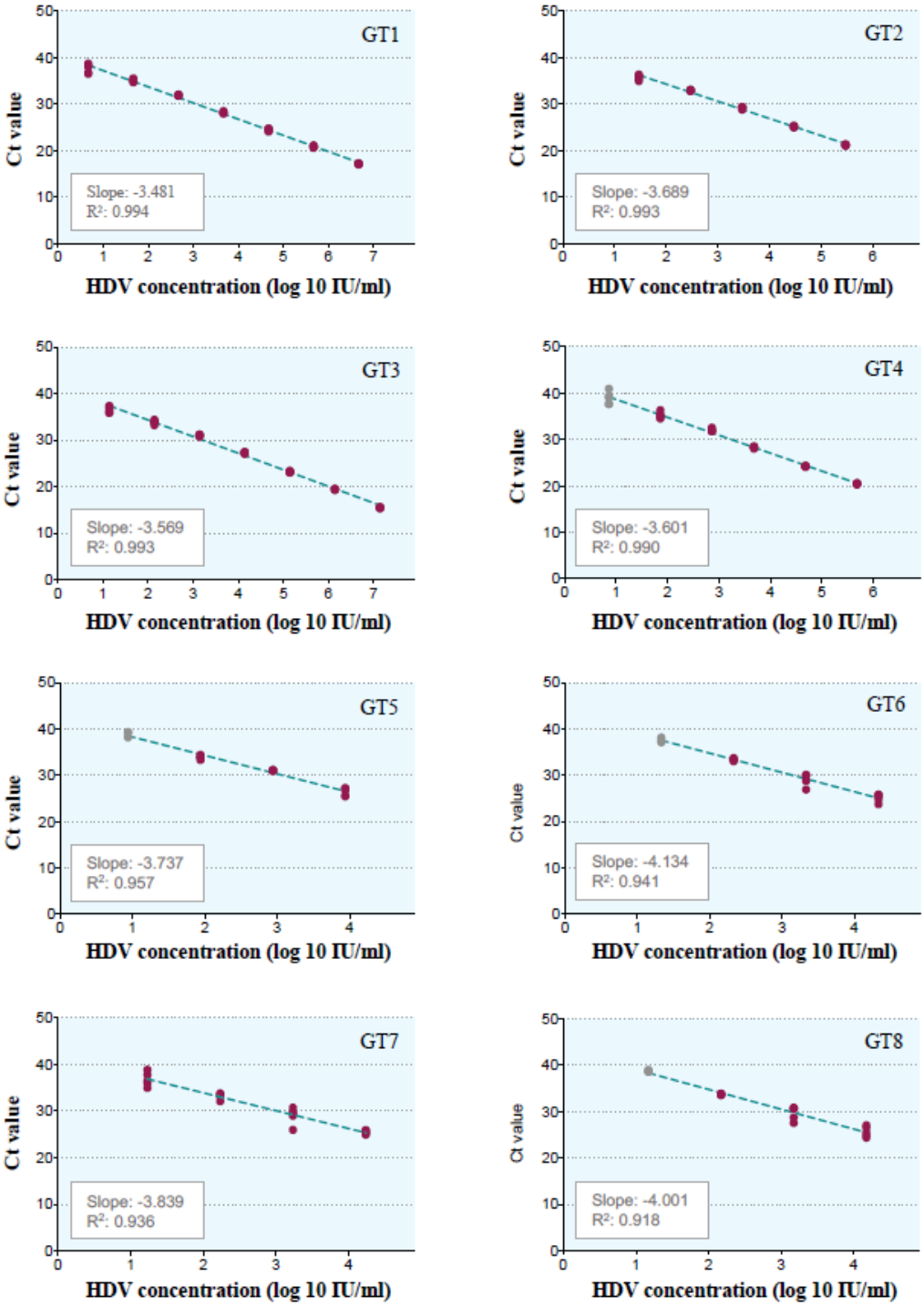


Figure 7: Linearity

Linearity was assessed using serial dilutions (10-fold) of cell-culture-derived viruses (HDV GT 1-8). HDV concentrations are log-transformed. The figure was adapted from a figure published by Pflüger et al., in the Journal JHEP Reports titled "Fig. 2. Linearity and inclusivity of cell culture-derived HDV genotypes 1-8" (Pflüger et al. 2021).

Abbreviations: Ct, cycle threshold; HDV, hepatitis delta virus; IU/ml, international units per milliliter.

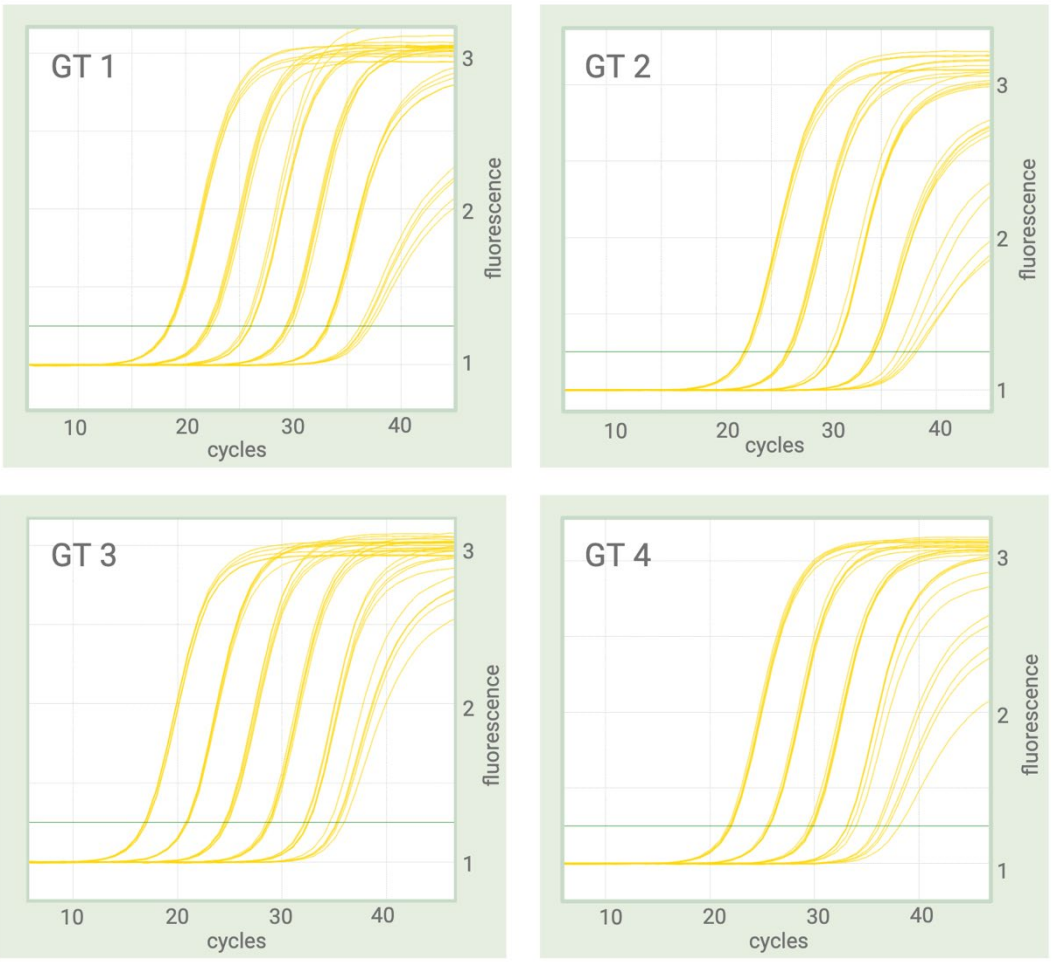


Figure 8: Amplifications curves

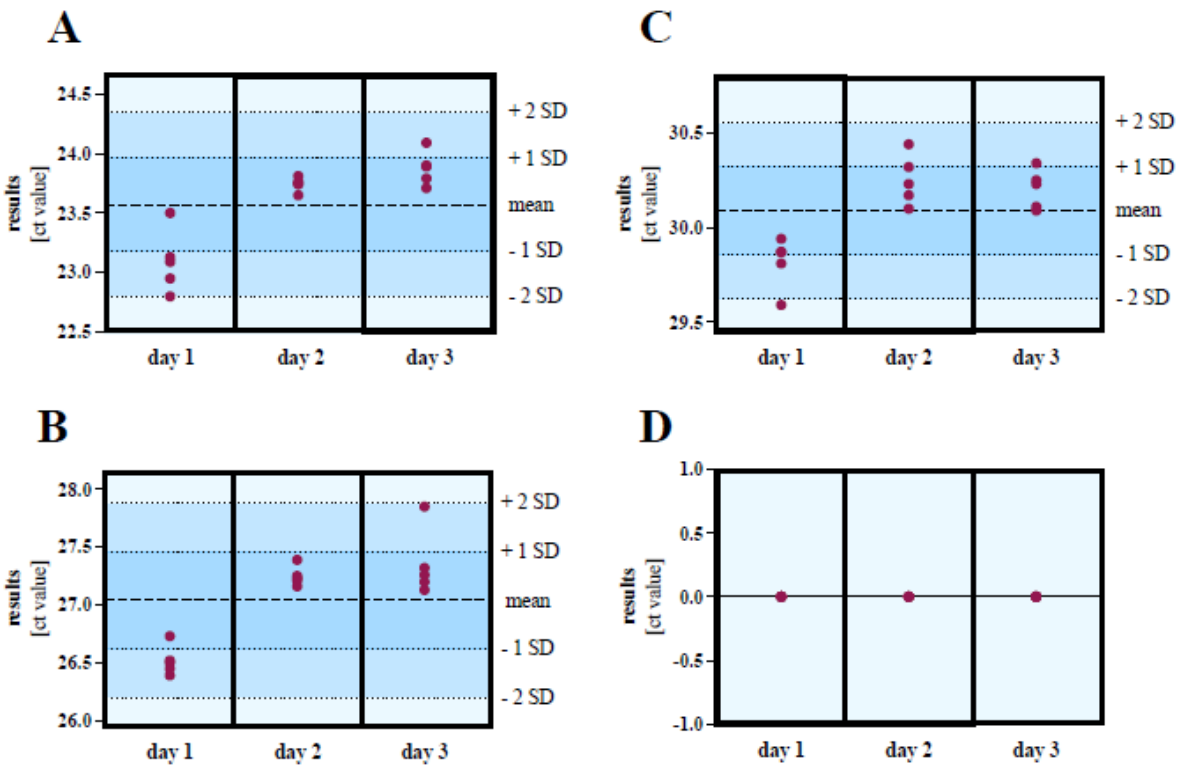
Examples of amplification curves from dilution series (2-fold, 5 repeats/step) of cell-culture-derived viruses (HDV GT 1 - 4) used to determine linearity for the HDV_UCT tested on the cobas6800. This figure was assembled using BioRender.

Abbreviations: GT, genotype; HDV_UCT, hepatitis delta virus Utility channel test.

4.1.3. High precision and stability of the HDC_UCT and confirmation of assay exclusivity

Precision was evaluated over three days, including three different concentrations of the HDV WHO standard (10-fold dilution; 5 repeats per step; starting concentration, 5,750 IU/mL) and an HDV RNA-negative sample. Analysis of the test results demonstrated that the pre-selected target values were achieved with a within-run variability of < 0.2 ct and a between-

day variability of < 0.44 ct, indicating high reproducibility of test results (**Figure 9**) (Pflüger et al. 2021).



E

	Precision							
	HDV WHO standard 1:100		HDV WHO standard 1:1,000		HDV WHO standard 1:10,000		negative set	
	SD	95 % CI	SD	95 % CI	SD	95 % CI	SD	95 % CI
within-run variability	0.165	0.118 - 0.272	0.197	0.141 - 0.325	0.124	0.089 - 0.205	0	n/a
between-day variability	0.402	0.207 - 2.82	0.439	0.225 - 3.14	0.233	0.118 - 1.76	0	n/a

Figure 9: Precision

Precision was evaluated on three consecutive days using 10-fold dilutions of the HDV WHO standard and an HDV RNA negative sample. Test results are displayed in Levey-Jennings-charts (**A-D**). All results were within the target ranges of less than 0.5 and 1 ct SD for within-run and between-day variability (**E**).

Abbreviations: CI, confidence interval; ct, cycle threshold; HDV, hepatitis delta virus; n/a, not applicable; SD, standard deviations; WHO, World Health Organization.

In addition, excellent reproducibility of test results and high stability of the reagents were confirmed over a six-month period by measuring dilutions of the HDV WHO standard (10-fold, three steps, starting concentration of 57,500 IU/ml). All Samples were tested in technical replicates of 5 at four different time points (the day of the preparation of the reagent cassette, and after 1, 3, and 6 months). The within-run and between-day variability were below 0.5 and 1 ct, respectively (**Table 7**).

Table 7: Stability

Stability								
time since preparation of the cassette	HDV WHO standard 1:100		HDV WHO standard 1:1,000		HDV WHO standard 1:10,000		HDV RNA negative set	
	ct value	IU/ml	ct value	IU/ml	ct value	IU/ml	ct value	IU/ml
none: day of the preparation	23.09	82,939	26.50	8,361	29.94	826	negative	n/a
	22.95	91,132	26.52	8,249	29.81	901	negative	n/a
	23.50	62,943	26.45	8,647	29.59	1,045	negative	n/a
	23.13	80,736	26.39	9,003	29.87	865	negative	n/a
	22.88	95,527	26.73	7,162	29.87	865	negative	n/a
one month	23.52	62,101	27.90	3,259	30.66	508	negative	n/a
	24.28	37,240	27.78	3,533	30.94	421	negative	n/a
	24.58	30,432	27.68	3,779	30.52	559	negative	n/a
	24.35	35,526	27.60	3,988	30.74	482	negative	n/a
	24.58	30,432	27.57	4,069	30.99	407	negative	n/a
three month	23.86	49,402	27.93	3,194	30.91	430	negative	n/a
	24.59	30,228	28.32	2,457	31.42	305	negative	n/a
	24.62	29,624	27.98	3,088	31.38	313	negative	n/a
	24.69	28,261	28.35	2,407	31.57	275	negative	n/a
	25.46	16,833	28.43	2,281	31.75	244	negative	n/a
six month	25.30	18,747	28.51	2,162	31.96	212	negative	n/a
	25.43	17,177	28.49	2,191	31.85	228	negative	n/a
	25.36	18,005	28.48	2,206	31.87	225	negative	n/a
	25.35	18,127	28.36	2,391	31.83	231	negative	n/a
	25.50	16,386	28.50	2,176	32.06	198	negative	n/a
variability	SD	95% CI	SD	95% CI	SD	95% CI	SD	95% CI
within-run	0.38	0.28 - 0.58	0.15	0.11 - 0.23	0.20	0.15 - 0.31	0	n/a
between-day	0.94	0.53 - 3.74	0.86	0.49 - 3.25	0.89	0.51 - 3.43	0	n/a

Test results (ct and quantitative values) of the assessment of the reproducibility of test results and the stability of reagents over six months. Within-run and between-day variability was calculated using the Validation Manager software (Finbiosoft, Espoo, Finland).

Abbreviations: CI, confidence interval; ct, cycle threshold; HDV, hepatitis delta virus; IU/ml, international units per milliliter; SD, standard deviation; WHO, World Health Organization.

The exclusivity of the assay was confirmed as none of the panel members yielded a positive result with the HCV_UCT assay. Each panel comprised a total of 10 clinical specimens, including samples from patients infected with HBV, HCV, EBV, HIV-1, BKPyV, or CMV (Pflüger et al. 2021).

4.1.4. Excellent clinical performance of the HDV_UCT in comparison to a CE-IVD assay

A total of 110 anti-HDV reactive serum samples were selected and used for clinical performance evaluation of the HDV_UCT. The HDV PCR positivity rate within this cohort was 52.7%, as determined by screening of the records (Pflüger et al. 2021). All samples were stored at or below -80°C and tested on the same day they were thawed.

To determine the clinical performance of the HDV_UCT, the selected anti-HDV reactive samples were tested using the newly established assay and a commercially available CE-IVD comparator assay for quantification of HDV RNA. Of the 110 samples tested, 61 (55.45%) were positive for HDV RNA using the HDV_UCT, while 58 (52.7%) yielded positive test results with the comparator assay (Pflüger et al. 2021). The median HDV viral loads were 53,197.51 (range: 2.14 – 295,053.41) and 37,381.73 (range: 23.78 – 37,381.73) with the HDV_UCT and the CE-IDV assay, respectively (Pflüger et al. 2021). The median of the differences between quantitative test results was 0.28 log (Pflüger et al. 2021). In 43/58 samples, quantitative results were higher with the HDV_UCT tested on the cobas6800 and in 15/58 of patients the RoboGene assay yielded a higher quantitative test result (Pflüger et al. 2021). 5/110 samples were identified where quantitative test results varied > 1 $\log_{10}$ (Pflüger et al. 2021). In four out of five samples, the quantification of the HDV_UCT was higher compared to the CE-IVD assay whereas in only one out of five samples the comparator assay showed a higher quantitative test result (**Table 8**) (Pflüger et al. 2021).

In total, three out of 110 samples yielded a positive result with the HDV_UCT but were tested non-reactive with the commercial assay (Pflüger et al. 2021). The ct-values for the discordant results were all above 35.6, and the quantitative test results with the HDV_UCT assays were 18.2, 7.34, and 2.14 IU/ml (Pflüger et al. 2021). No sample yielded a positive

result when tested with the comparator assay, but was negative for HDV RNA when tested with the HDV_UCT (Pflüger et al. 2021).

Table 8: Comparison of test results between the HDV_UCT and the comparator assay: Sample with quantitative values differing more than 1 log

Discrepency of test results > 1 log_{10} of the HDV_UCT and RoboGene assays					
sample	HDV_UCT [ct value]	RoboGene [ct value]	HDV_UCT [IU/ml]	RoboGene [IU/ml]	difference HDV_UCT and RoboGene [log10 IU/ml]
1	27.45	31.28	4,412	194	1.36
2	22.39	25.22	132,837	10,711	1.09
3	14.36	18.82	29,505,341	737,720	1.60
4	14.39	16.80	28,915,708	2,805,552	1.01
5	28.41	23.59	2,312	31,476	1.13

Detailed results of samples where quantitative values differed more than 1 log between the HDV_UCT and the comparator assay.

Abbreviations: CE-IVD, CE-marked in-vitro diagnostic assay; ct, cycle threshold; HDV_UCT, hepatitis delta virus utility channel test; IU/ml, international units per milliliter.

The sensitivity and specificity of the HDV_UCT assay were determined to be 100 % (95 % CI: 93.84 – 100 %) and 94.55 % (95 % CI: 84.88 – 98.86 %), respectively (Pflüger et al. 2021). Positive predictive value and negative predictive value were calculated using a disease prevalence of 52.7 % (determined HDV PCR positivity rate within the preselected study cohort identified by screening of the records) and were 95.33 % (95 % CI: 87.17 – 98.4 %) and 100 % (95 % CI: 93.15 % - 100 %), respectively (Pflüger et al. 2021).

To further compare the two assays, a linear regression analysis was performed which demonstrated a strong correlation between the quantitative test results in IU/ml from the HDV_UCT and the comparator assay, reaching a Person correlation coefficient (r) of 0.9232 (95% CI: 0.8732 - 0.9540) and an r^2 of 0.8524 (**Figure 10 (A)**) (Pflüger et al. 2021). In addition, a Bland-Altman analysis was conducted that confirmed a good agreement between the two methods (mean: -0.2997; +1.96 standard deviation (SD): 0.6914; -1.96 SD: -1.29) (Pflüger et al. 2021). Out of the analyzed samples, only three were outside of the 95% Bland-Altman limits of agreement (± 1.96 SD; **Figure 10 (B)**) (Pflüger et al. 2021).

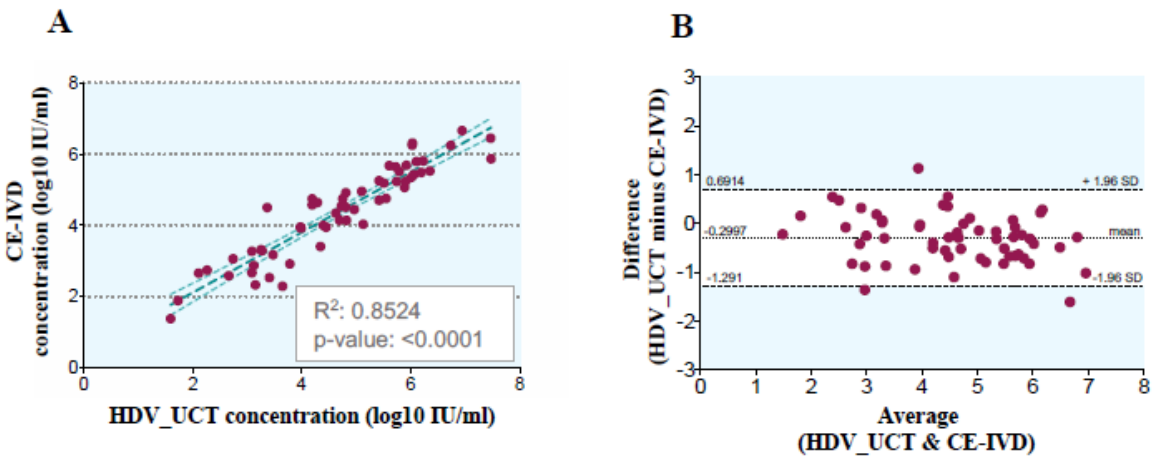


Figure 10: Linear regression analysis and Bland-Altman plot

(**A**) depicts the linear regression analysis of positive test results of the HDV_UCT and the comparator assay (CE-IVD). The linear regression line is plotted as a green dashed line and the corresponding 95 % CIs are displayed as thin green dashed lines. (**B**) Bland-Altman analysis of the respective compared assays (HDV_UCT and the CE-IVD RoboGene HDV RNA Quantification kit). HDV concentrations were log_{10}-transformed. The figure was adapted from a figure published by Pflüger et al., in the Journal JHEP Reports titled "Fig. 1. Probit analysis and linearity for HDV genotype 1 and test results of the new HDV_UCT assay compared to the CE-trademarked IVD RoboGene assay" (Pflüger et al. 2021).

Abbreviations: CE-IVD, CE-marked in-vitro diagnostic assay; HDV_UCT, hepatitis delta virus utility channel test; IU/ml, international units per milliliter.

4.1.5. Consistency of the HDV_UCT with external quality assessment samples and viral kinetics over time

Commercially available EQA samples (n=20) underwent the HDV_UCT assay and were compared to the manufacturer's expected results. The qualitative comparison showed 100% agreement, with 17 out of 17 samples accurately identified as HDV RNA positive by the HDV_UCT assay and 4 out of 4 yielding the expected negative results (Pflüger et al. 2021). Furthermore, all quantitative test results fell within the respective ranges (n=4 out of 4)

(Pflüger et al. 2021). The deviation from the target value did not exceed 0.4 $\log_{10}$ for all tested samples (**Table 9**) (Pflüger et al. 2021).

Patient samples from HDV-infected individuals, collected at various time points during active HDV infection, were tested using the HDV_UCT and the commercial comparator assay. One patient received pegINFα treatment, while another was diagnosed with acute HDV infection. Test results of HDV RNA exhibited a similar pattern over time for all tested patients (n=4) with both assays **(Figure 11)** (Pflüger et al. 2021)**.** However, quantitative values varied by as much as 1 $\log_{10}$ (Pflüger et al. 2021).

Table 9: External quality assessment samples

EQA samples - qualitative agreement			
EQA ID-numbers	**positive/ expected positive**	**negative/ expected negative**	**number of samples**
400013-400016 400021-400024 400033-400044	16/16	4/4	20
EQA samples - quantitative agreement			
EQA ID-numbers	**expected result [IU/ml]**	**excepted range [IU/ml]**	**HDV_UCT result [IU/ml]**
40041	5376	538 - 53760	2849
40042	2447	245 - 24470	1038
40043	8080	808 - 80800	3989
40044	17543	1754 - 1755430	15320

Qualitative and quantitative agreement of the established HDV assay and EQA samples (INSTAND, Düsseldorf, Germany). The table was adapted from a figure published by Pflüger et al., in the Journal JHEP Reports in the supplementary material titled "Fig. S1. Test results of the EQA panel from 2020 an of the comparison of the HDV_UCT and CE-IVD assay test results for four different HDV-RNA+ patients tested art different time points" (Pflüger et al. 2021).

Abbreviations: EQA, external quality assessment; HDV_UCT, hepatitis delta virus utility channel test; ID, identification; IU/ml, international units per milliliter.

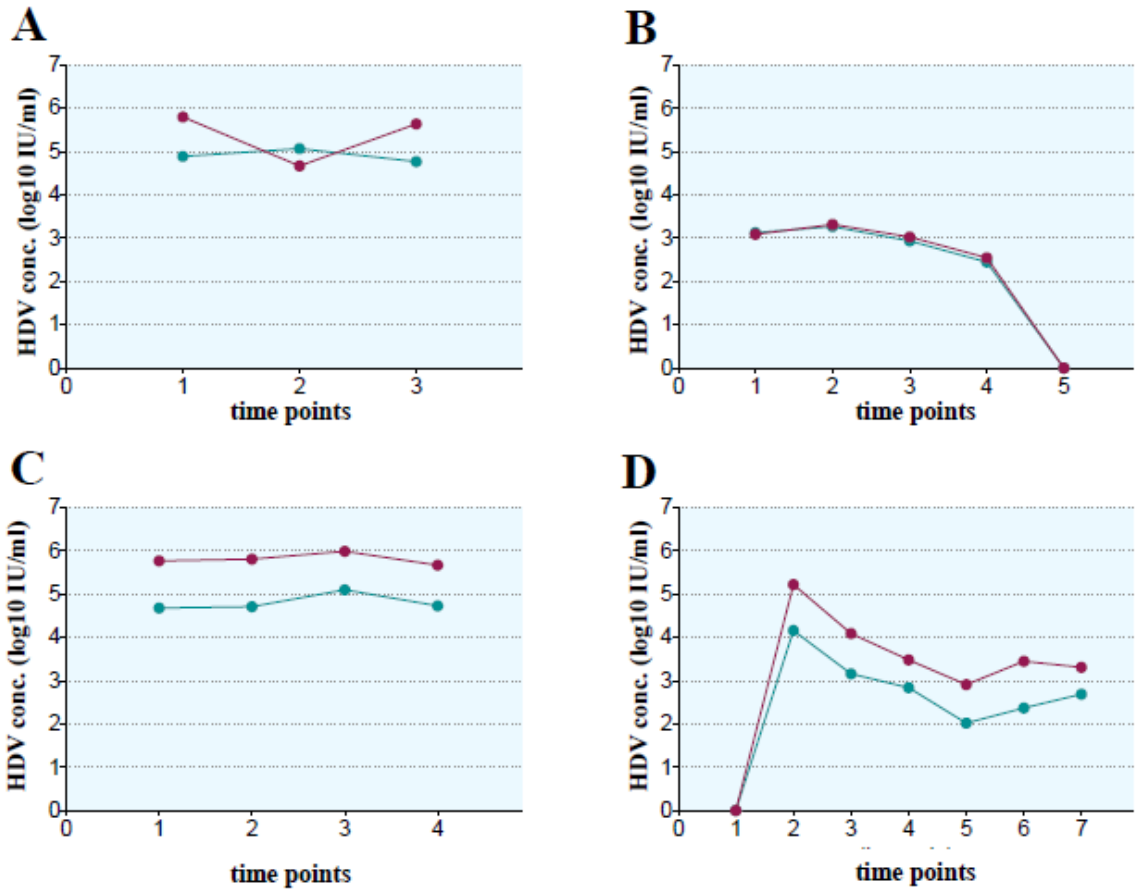


Figure 11: Viral kinetics of active HDV infection: Comparison of quantitative test results of the HDV_UCT and comparator assay

Quantitative test results (log_{10}-transformed) of patient samples with active HDV infection collected at different time points. The violet dots represent the quantitative test results obtained with the HDV_UCT assay whereas the green dots display those of the comparator assay. Patient (**B**) was treated with PEGylated interferon alpha and patient (D) was diagnosed with acute HDV infection. The figure was adapted from a figure published by Pflüger et al., in the Journal JHEP Reports in the supplementary material titled "Fig. S1. Test results of the EQA panel from 2020 an of the comparison of the HDV_UCT and CE-IVD assay test results for four different HDV-RNA+ patients tested art different time points" (Pflüger et al. 2021).

Abbreviations: CE-IVD, CE-marked in-vitro diagnostic assay; HDV_UCT, hepatitis delta virus utility channel test; IU/ml, international units per milliliter.

4.2. Evaluation of HCV-mediated, HDV propagation in HCV mono-infected patients

4.2.1. Patient characteristics

The results presented in this and the following paragraph (4.2.1. and 4.2.2.) were published listing me as the first author during my time as a doctoral candidate in the Journal of Viral Hepatitis (Pflüger et al. 2020).

A total of n=323 serum samples from different patients with active HCV mono-infection, defined by HCV PCR positivity and undetectable levels of HBsAg, were included in this study. The patient cohort consisted of n=125/323 (38.7%) female patients and n=196/323 (60.7%) male patients, respectively. For n=2/323 (0.6%), the sex was identified as either "divers" or "not classified" (Pflüger et al. 2020). The median age of the study cohort was 52 years, and the median HCV viral load was determined to be 30,100 IU/ml (range: <15 to 60,000,000 IU/ml) (Pflüger et al. 2020). A detailed list of patient characteristics, as identified by screening of the medical records, is shown in **Table 10**.

Table 10: Patient characteristics

Patient characteristics					
		n	%	median	range
age [years]		323		52	6 – 92
sex	female	125	38.7		
	male	196	60.7		
	divers	2			
HCV load [IU/ml]		323		301000	< 15 – 60000000
HCV GT	1	12	3.7		
	1a	67	20.7		
	1b	73	22.6		
	2	24	7.4		
	3	85	26.3		
	4	18	5.6		
	5	2	0.6		
	6	2	0.6		
	n/a	40	12.4		
time since diag. [years]		284		5.9	0.3 – 40.0
AST [ULN]		238		1.2	0.1 – 45.5
ALT [ULN]		239		1.6	0.4 – 37.6

Legend corresponding to:

Table 10: Patient characteristics

Patient characteristics as assessed by screening of the medical records. The table was adapted from a table published by Pflüger et al., in the Journal of Viral Hepatitis titled "Table 1 Clinical and virological patient characteristics" (Pflüger et al. 2020).

Abbreviations: AST, aspartate transaminase; ALT, alanine transaminase; diag,, diagnosis; HCV, hepatitis C virus; GT, genotype; IU/ml, international units per milliliter; ULN, upper limit of normal

4.2.2. No molecular or serological evidence of HCV-mediated, HDV propagation in HCV mono-infected patients

All samples meeting the inclusion criteria (n=323; see Materials and Methods: 2.1. Clinical samples) were screened for HDV RNA using the HDV_UCT that was established and validated as part of this dissertation. As a first result, the patient cohort exhibited no evidence of HCV-mediated HDV replication, as all samples tested negative for HDV RNA (n=0/323) (Pflüger et al. 2020). All samples with sufficient residual material (n=316) were screened for previous HDV infection. The majority of sera were non-reactive for anti-HDV (n=308/316) (Pflüger et al. 2020). Interestingly, anti-HDV was detected in n=8/316 patients, with a median concentration of 3.25 arbitrary units (AU)/ml (range: 1.12 – 8.64 AU/ml), suggesting that HCV-mediated HDV infection may have occurred in the past (Pflüger et al. 2020). However, the overall antibody levels observed were lower compared to those of HBV/HDV coinfected patients at the University Medical Center Hamburg-Eppendorf (median: 8.53 AU/ml; range: 1.22 – 82 AU/ml; analysis of n=38 patients diagnosed in 2020; Mann-Whitney test: p-value: 0.044) (Pflüger et al. 2020).

To screen for HBsAg-negative HBV infection, all samples with sufficient residual volume (n=316) were tested for anti-HBc IgG and IgM. In all anti-HDV reactive patients (n=8/316), anti-HBc IgG/IgM could be detected (median: 6.88 signal to cutoff (S/CO); range: 3.85 – 7.88 S/CO), indicating a past, currently immune-controlled HBV infection (Pflüger et al. 2020). Anti-HBs was positive in 50% of the anti-HDV-positive patients (n=4/8; median: 19.5 mIU/ml; range: 12.31 - 80.98 mIU/ml; **Table 11**) (Pflüger et al. 2020). None of the anti-HBc-negative patients (n=225/316) tested positive for anti-HDV (Fisher`s exact test: p-

value: 0.0001) (Pflüger et al. 2020). **Figure 12** shows a flowchart of the testing regime and qualitative results.

Table 11: HDV PCR and HBV/HDV serology test results of anti-HDV positive, HCV negative, and HBsAg negative patients

HDV PCR and HBV/HDV serology test results						
sample	age at time of blood sampling	HDV-UCT	anti-HDV	anti-HBc IgG and IgM QL	anti-HBc IgM QL	anti-HBs QL
1	47	negative	reactive (8.64 AU/ml)	reactive	non-reactive	reactive
2	56	negative	reactive (6.57 AU/ml)	reactive	n/a	reactive
3	42	negative	reactive (7.03 AU/ml)	reactive	non-reactive	reactive
4	56	negative	reactive (4.91 AU/ml)	reactive	non-reactive	reactive
5	66	negative	reactive (1.22 AU/ml)	reactive	non-reactive	non-reactive
6	50	negative	reactive (1.12 AU/ml)	reactive	non-reactive	non-reactive
7	47	negative	reactive (1.27 AU/ml)	reactive	non-reactive	non-reactive
8	55	negative	reactive (1.58 AU/ml)	reactive	non-reactive	non-reactive

The table shows the testing results of the assessment of HDV RNA, anti-HDV, anti-HBc, and anti-HBs for the HCV-infected, HBsAg non-reactive patients that tested reactive using a commercial anti-HDV assay.

Abbreviations: Anti-HBc, antibodies against the hepatitis B core antigen; anti-HBs, antibodies against the hepatitis B surface antigen; anit-HDV, antibodies against the hepatitis delta virus; AU/ml, arbitrary units per milliliter; HBsAg, hepatitis B virus surface antigen, HBV, hepatitis B virus; HCV, hepatitis C virus, HDV, hepatitis delta virus; HDV_UCT, hepatitis delta virus utility channel test, PCR, polymerase chain reaction.

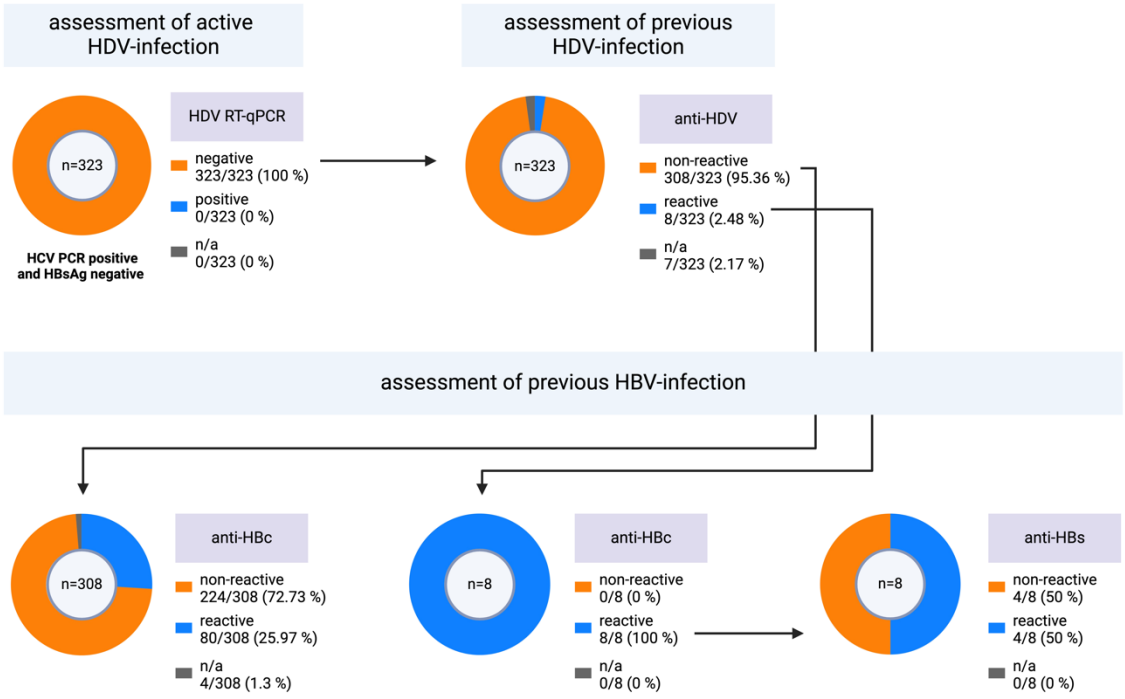


Figure 12: Assessment of active and previous HDV infection in HCV PCR positive and HBsAg negative patients.

Flowchart depicting the testing regimen and corresponding test results of the assessment of active and past HDV infection as well as previous HBV infection in a cohort of HCV PCR positive and HBsAg negative individuals (n=323). Not all 323 patient samples contained sufficient residual material to perform the subsequent analyses. These samples were labeled as 'not applicable' (n/a) in the respective categories. The figure was created using BioRender and adapted from a figure published by Pflüger et al., in the Journal of Virial Hepatitis titled "Figure 1" (Pflüger et al. 2020).

Abbreviations: Anti-HBc, antibodies against the hepatitis B core antigen; anti-HBs, antibodies against the hepatitis B surface antigen; anti-HDV, antibodies against the hepatitis delta virus; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus.

5. Discussion

Reliable detection and quantification of HDV RNA play an increasingly important role in diagnosing HDV infections. The EASL and APASL guidelines recommend a universal screening approach, which states that every patient with a reactive HBsAg test should be tested at least once for anti-HDV (EASL 2023, Sarin et al. 2016). Compared to the risk-based screening approach, implementing the universal screening approach has led to a notable increase in identified HDV infections (Yardeni et al. 2025). For instance, in a study conducted in Spain, the universal approach resulted in a five-fold increase in the number of HDV diagnoses (Palom et al. 2022). No risk factors could be identified in 60% of the patients included in the study, indicating that they would not have been subjected to testing under a risk-based screening approach (Palom et al. 2022). Testing for HDV RNA in anti-HDV reactive patients facilitates precise and prompt diagnosis of active HDV infection and helps identify patients who may benefit from therapeutic intervention. The EASL guidelines state that a sensitive and standardized RT-PCR assay should be used to test all anti-HDV reactive patients (EASL 2023). This approach is supported by the high rates of HDV RNA positivity (around 60-70%) detected in recently published studies (Bockmann et al. 2020, Kamal et al. 2024, Osiowy et al. 2022).

Additionally, testing patients for the presence of HDV RNA, even when a reactive serological result is absent, should be performed in cases with high clinical suspicion. Interestingly, data presented at the second Deltacure meeting demonstrated that 4.1% of seronegative patients (mostly HDV GT 5-8) tested positive for HDV RNA (Ceesay 2023). These patients would have been misdiagnosed if no HDV PCR assay had been performed.

Furthermore, reliable quantification of HDV is becoming an increasingly important tool for treatment monitoring due to the recently developed therapies for chronic HDV (Degasperi et al. 2023, EASL 2023). For a long time, the only available treatment option for HBV/HDV-infected patients was the off-label therapy with pegINFα, a drug associated with numerous side effects and a high rate of relapse among patients post-therapy (EASL 2017, Elazar and Glenn 2022, Wedemeyer et al. 2011, Wedemeyer et al. 2019). Fortunately, after more than three decades in which pegINFα was the sole treatment option, new therapeutic alternatives, particularly bulevirtide and lonafarnib, have now been identified that demonstrate, for the first time, promising results in treating chronic HDV infection.

Both bulevirtide and lonafarnib have been shown to effectively reduce HDV RNA-emia and have the potential to lower liver transaminases, while their side effects compare favorably to pegINFα monotherapy, especially when combined with other antiviral agents (Khan et al. 2021, Lampertico et al. 2022, Olsen et al. 2023, Wedemeyer et al. 2023, Yurdaydin et al. 2018, Yurdaydin et al. 2022).

For assessing patients undergoing treatment with these novel therapies, it is essential to have access to a reliable and robust method for quantifying HDV RNA (Stelzl et al. 2021). This need is particularly important since HDV PCR plays a significant role in evaluating treatment response, as hard endpoints such as overall survival, reduced liver-related complications, and prevention of disease progression require long-term studies and comprehensive patient follow-up. Recently, a paper was published following the 2022 AASLD-EASL HBV-HDV Treatment Endpoints Conference, providing guidance on selecting adequate endpoints for studies assessing the efficacy of novel HDV therapies (Ghany et al. 2023). The authors agreed that HDV RNA < lower limit of quantification in conjunction with sustained HBsAg loss 24 weeks off treatment is the preferred endpoint for this purpose (Ghany et al. 2023).

Next, it is also important to acknowledge the limited availability of data concerning the long-term efficacy of therapy with the recently developed HDV drugs. In a long-term study investigating the outcomes of patients treated with bulevirtide, HDV RNA was detected in the blood of some patients following a prolonged period of HDV RNA suppression (Jachs et al. 2023). The long-term efficacy and potential for establishing sustained viral control of these emerging therapies require further evaluation. This is particularly relevant given that HDV can replicate independently through cell division, without the need for a helper virus (Giersch et al. 2019). Thus, the possibility of HDV RNA reoccurring years after the conclusion of treatment cannot be excluded, and patients should be systematically screened for HDV reoccurrence using PCR assays (Alqahtani et al. 2025).

As a result, a dependable method for quantifying HDV RNA in patient samples, with excellent comparability across laboratories and testing sites, is a crucial resource for diagnosing HDV infection, monitoring patients during and after therapy, and conducting future efficacy studies of novel or existing combination therapies. Thus, one of the objectives

of this dissertation was to establish and validate a highly sensitive, quantitative HDV PCR on a fully automated system that accommodates all HDV genotypes.

However, detecting and quantifying HDV RNA in patient samples requires careful assay design and a detailed analysis of inclusivity for HDV GT, as HDV is highly heterogeneous, with over 20 to 40% variance between strains and around 16% genomic variance within HDV GT (Dény 2006, Hughes et al. 2011). A study conducted across multiple countries and various laboratories used an external quality assessment panel consisting of dilutions of the HDV WHO standard and a panel of patient samples from different HDV GT to evaluate HDV PCR performance (Le Gal et al. 2016). The authors demonstrated that quantification (reported in IU/ml) significantly differed, particularly for HDV GT 5 and 8, and over 50% failed to detect at least one HDV RNA-positive sample (Le Gal et al. 2016). To address this issue, a highly conserved region of the HDV genome, the delta ribozyme, was selected as the PCR target for the HDV_UCT, and a comprehensive in silico analysis, including over 650 HDV sequences covering the respective PCR amplicon and all HDV GT, was conducted as part of this dissertation. Furthermore, the assay's performance was evaluated using cell-culture-derived viruses for all HDV GT, resulting in excellent PCR performance across all HDV GT. This is particularly important because most evaluations of existing/published HDV PCR assays are performed using HDV GT 1, and data on non-GT1 HDV strains are scarce (Wedemeyer et al. 2025). Subsequently, HDV sequencing should be performed more comprehensively worldwide, especially in countries with a higher prevalence of non-GT1 HDV strains, to increase the availability of non-GT1 sequences and thereby enhance assay performance across all HDV GT.

Furthermore, standardizing PCR assay results and their reporting is a key factor in reducing variability in test results across different laboratories and countries. Therefore, the WHO HDV standard was employed to enable standardized reporting of test results in IU/ml during the evaluation of the LoD and linearity of the assay described here. The LoD of the HDV_UCT was determined to be 3.9 IU/ml, making it the most sensitive assay compared to other commercial HDV PCR assays and published laboratory-developed tests (LDT) that meet the same quality standards (calibrated to the WHO HDV standard, inclusivity for all HD GT, and utilization of an IC; **Table 12**). (Wedemeyer et al. 2025)

Table 12: Overview of HDV PCR assay calibrated to the WHO HDV standard, with inclusivity for all HDV GT and use of an internal control

Assay name	Manufacturer/ provider	Target site	LoD (IU/mL)	LLoQ (IU/mL)	ULoQ (IU/mL)	Regulation	RNA extraction method	Detection equipment (cycler)	Comments
EurobioPlex HDV	Eurobio Scientific	HDAg	10	562	3.16E+08	CE-IVD	m2000sp	CFX96	
RoboGene HDV RNA Quantification Kit 2.0	Roboscreen GmbH	HDAg	6	60	1.00E+08	CE-IVD	Instand Virus RNA/DNA Kit	Several options	
HDV QNP 2.1 Real-Time PCR Kit	Iontec	Proprietary	400	1000	1.00E+10	CE-IVD	Fluorion i12, i24/ i12 Kit	Several options	*no information on GT8
AltoStar HDV RT-PCR Kit 1.5	altona Diagnostics	Proprietary	< 10*	100*	1.00E+06	RUO**	AltoStar AM16r	CFX96	*Still under verification. **The kit is CE-IVDR ready
RealStar HDV RT-PCR Kit 1.0	altona Diagnostics	Proprietary				RUO	Several options	Several options	LoD depend on extraction/detection method
SYSTAAQ HDV Real-time PCR Kit	SYSTAAQ	Proprietary	10	10	8.00E+06	RUO	Several options	Not specified	LoD depend on extraction/detection method
HDV RNA, Quantitative Real-Time PCR	Quest Diagnostics	Proprietary	5	40	1.00E+07	LDT	MagNA Pure 96	ABI 7500	
Pflüger (LDT)		Ribozyme	3.9	10	1.00E+08	LDT	cobas 6800	cobas 6800	
Olivero (LDT)		Ribozyme	9.2	10	1.00E+06	LDT	EZ1 Advanced XL	CFX96 QX200	

The table lists all HDV PCR assays for quantification of HDV RNA in patient samples that were calibrated to the HDV WHO standard, are inclusive for all HDV GT, and utilize an internal control. The assay established as part of this dissertation is listed under the assay name “Pflüger (LDT)”. This table was originally published by Wedemeyer et al., in the Journal of Hepatology titled “Table 3 Detailed performance characteristics for assays and products calibrated to WHO IS, detect GT1-8, and used IC for RNA extraction” (Wedemeyer et al. 2025). The table has not been edited.

Abbreviations: CE-IVD, CE-marked in virto diagnostics; GT, genotype; HDAg, hepatitis delta virus antigen; HDV, hepatitis delta virus; IU/ml, international units per milliliter; LDT, laboratory developed test; LLoQ, lower limit of quantification; LoD, limit of detection; RUO, research use only; ULoQ, upper limit of quantification.

However, it is important to note that the HDV WHO standard is derived from a patient infected with HDV GT 1. Therefore, calibrating test results solely to the HDV WHO standard might lead to the incorrect quantification of other HDV GT. Additionally, recently published data demonstrated that quantitative test results using different commercial PCR assays (all calibrated to the HDV WHO standard) and extraction methods (manual vs. automated) varied by up to 2 $\log_{10}$ IU/ml in some clinical samples (Sandmann et al. 2025). Consequently, utilizing HDV RNA tests calibrated exclusively based on the HDV WHO standard may lead to considerable quantitative differences when clinical samples are tested. The reasons for and implications of this recent finding require further investigation and should be considered when interpreting test results.

The assay described here for detecting and quantifying HDV RNA was validated on a fully automated, high-throughput system, the cobas6800, as these systems exhibit high accuracy and reproducibility. A study conducted at three different testing sites evaluated the performance of the cobasX800 series for HBV, HCV, and HIV-1 (Tschäpe et al. 2022). The authors demonstrated excellent reproducibility between testing sites with an r^2 of 0.999 (Tschäpe et al. 2022). Moreover, manual steps are drastically reduced when using these sample-to-result platforms, making the workflow less susceptible to human error. Additionally, performing nucleic acid extraction and purification under standardized conditions enhances reproducibility, as the selected extraction methods and their execution have been shown to influence test results dramatically (Stelzl et al. 2021). Accordingly, the evaluation of assay reproducibility conducted as part of this dissertation demonstrated excellent precision. Consequently, the assay can serve as a powerful tool to enhance the standardization of HDV test results across different laboratories and countries, thus representing a valuable contribution to HDV diagnostics and further epidemiological or treatment efficacy studies.

Another important factor in improving assay quality is the use of an internal control (IC), which not only ensures technical control of the PCR procedure but also monitors nucleic acid amplification and extraction (Wedemeyer et al. 2025). The cobas6800 employs ICs that are automatically spiked in during extraction, thus serving as a full-process control. Furthermore, the UNG incubation, which is the first step of every assay run on the system, reduces the likelihood of amplifying previous PCR products during the subsequent amplification cycles. The inclusion of both positive and negative controls in each PCR run thereby facilitates robust quality control of test results using the HDV_UCT.

Limitations of the established HDV_UCT include a lack of sufficient HDV genome sequences across all HDV GT to establish robust in silico analysis (less than 20 sequences available for GT 4, 5, 6, and 8). However, when using cell culture-derived viruses to assess inclusivity and linearity, the assay demonstrated comparable performance to GT 1, the most prevalent GT in Germany (n=400 sequences analyzed). Next, an analysis of the assay's performance using a comprehensive patient cohort (n=110 anti-HDV reactive patient samples) and a comparison of quantitative test results to a CE-IVD assay demonstrated a high correlation between the two methods (r^2: 0.85). Thus, the excellent analytical performance of the assay was confirmed in a routine laboratory setting within an HDV GT-

1 dominant setting. Still, larger studies should be conducted in high-prevalence regions for HDV, encompassing non-GT 1 strains, to further evaluate assay performance across all HDV GT.

As of this writing, no FDA-approved or CE-IVD HDV PCR assay exists that can be run on a fully automated system (FDA 2025, Wedemeyer et al. 2025). The assay described here was comprehensively validated in accordance with current European guidelines (regulation 2017/746 EU IVDR), and its performance was confirmed in a large cohort of patients (n=110 anti-HDV reactive patient samples). The excellent analytical and clinical performance, along with the utilization of a fully automated system, make the HDV_UCT a powerful tool for the routine diagnosis of HDV infection and therapy monitoring. Furthermore, the capability for dynamic scaling of testing provided by most high-throughput platforms has proven beneficial, especially in recent years when laboratories worldwide faced constantly changing demands during the coronavirus disease 2019 (COVID-19) pandemic. However, if the LDT is used for diagnostic purposes at other laboratories, verification and validation experiments are necessary, as regulations and guidelines differ significantly between countries.

Another question to address is whether the diagnosis of HDV infection could be improved by introducing screening for HDV RNA and/or anti-HDV in the absence of HBsAg, as recent findings indicate that HDV may not exclusively depend on HBV. Firstly, HDV-like viruses have recently been discovered in various species that can cause infection without the presence of Hepadnaviridae (Chang et al. 2019a, Pérez-Vargas et al. 2021, Szirovicza et al. 2019). Secondly, the findings of Pérez-Vargas et al. demonstrate that HDV can be packaged by human pathogenic viruses other than HBV *in vitro* and in a humanized mouse model (Perez-Vargas et al. 2019). The possibility of HBV-independent HDV propagation in the human host and the occurrence of non-HBV related HDV infections have been overlooked due to a lack of testing, which needs to be addressed. Subsequently, the second part of this dissertation focuses on evaluating HCV-mediated, HBV-independent HDV infections in humans.

To address this question, 323 samples from HCV mono-infected individuals - defined by HCV PCR positivity and the absence of HBsAg - were selected and tested for HDV RNA using the HDV_UCT and anti-HDV. Samples that yielded a reactive anti-HDV test result were further analyzed for the presence of HBV serology markers (anti-HBc and anti-HBs).

As a main result, not a single patient (n=0/323) was identified who was both positive for HCV DNA and HDV RNA simultaneously. Moreover, all sera that were reactive with the anti-HDV assay showed a history of prior but currently immune-controlled HBV infection (n=8/8 anti-HBc reactive). These results imply that the possibility of *in vivo* propagation of HDV in HCV mono-infected patients, analogous to the results of animal models (Perez-Vargas et al. 2019), could not be confirmed in the current cohort and must be - if it occurs at all - an extremely rare event that epidemiologically does not play a significant role. However, these observations do not exclude the remote possibility of transient HCV-mediated propagation of HDV in the absence of HBsAg *in vivo*.

The results of the current dissertation align with other recent publications. Most studies could not detect any HCV PCR or antibodies against the hepatitis C virus (anti-HCV) in patients who were positive for HDV RNA without the presence of HBsAg (Cappy et al. 2021, Roggenbach et al. 2021). However, Chemin et al., who conducted their study in a region with a high prevalence of HDV infections (Venezuela), identified two patients (n=2/160) that were anti-HDV positive (tested with the Liaison murex XL Anti-HDV assay; 1.96 AU/ml and 7.38 AU/ml, respectively) (Chemin et al. 2021). HBV serology markers and HBV DNA were absent in both cases (Chemin et al. 2021). Interestingly, low levels of HDV RNA (GT 1) were detected (Chemin et al. 2021). The negative test results for HBsAg, anti-HBc, anti-HBs, and the undetectable HBV DNA levels for this specific patient reasonably excluded an occult HBV infection; however, no liver tissue was available for further examination (Chemin et al. 2021).

Furthermore, one study demonstrated that in 2 out of 21 anti-HDV positive patients, HCV and HDV RNA could be detected without evidence of active HBV infection (HBsAg and HBV DNA not detected) (Roggenbach et al. 2021). These observations indicate that HDV replication and the dissemination of HDV RNA can occur *in vivo* without detectable levels of HBsAg. Details of the studies examining HDV infection in HCV-infected individuals are outlined in **Table 13**.

Table 13: Overview of studies on HCV-mediated HDV infection

overview of studies on HCV-mediated HDV infection					
study	sample characteristics	anti-HDV +	anti-HBc +	HDV PCR +	conducted in
Pflüger et al. 2021	n=323 HCV PCR +; HBsAg -	8/323 2.5 %	8/8 100 %	0/8 0 %	Germany
Cappy et al. 2021	n=2,123 anti-HCV +	41/2,123 1.9 %	27/41 65.9 %	0/41 0 %	France
Chemin et al. 2021	n=160 HCV PCR +	2/160 1.3 %	0/2 0 %	1/2 50 %	Venezuela
Roggenbach et al. 2021	n=247 HBV mono-infected	23/257 9.3 %	n/a	13/23 56.5 %	China and Germany
	n=107 HBV/HCV coinfected	34/107 31.8 %	n/a	18/27 58.1 %	
	n=1,033 HCV mono-infected, HBsAg -; anti-HBc +	21/1,033 2 %	n/a	4/21 19 %	
	n=365 HCV mono-infected, HBsAg -; anti-HBc -	0/365 0 %	n/a	n/a	

Summary of studies that evaluated HCV-mediated HDV infection in humans as published at the time of writing this dissertation. "+" and "-" indicate positive and negative results, respectively.

Abbreviations: Anti-HDV; antibodies against the hepatitis delta virus; anti-HCV, antibodies against hepatitis C virus; anti-HBc, antibodies against the hepatitis B virus core antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; n/a, not applicable; PCR, polymerase chain reaction.

HDV replication occurs independently of HBV and can take place not only in hepatocytes but also in various other cells (Polo et al. 1995). In contrast, the formation of infectious particles can only happen through interaction with HBsAg following the posttranslational modification of L-HDAg in the form of farnesylation (Glenn et al. 1992, Komla-Soukha and Sureau 2006). This process does not require active HBV replication, as it has been demonstrated that HDV can replicate without signs of active HBV infection due to HBsAg expressed from integrated HBV DNA in the human genome (Freitas et al. 2014). HDV can spread through cell mitosis, and HDV RNA can persist in hepatocytes for several weeks in the absence of HBV in a humanized mouse model (Giersch et al. 2019). Therefore, HDV

does not seem to be entirely reliant on “helper viruses” for dissemination. The spread of HDV through HBV-independent pathways warrants further investigation. The implications of these findings indicate that active HDV infection is likely rare in HCV mono-infected patients, although HDV replication and dissemination can occur in a tripe infection setting (HBV/HCV/HDV) without markers for active HBV infection (HBsAg and HBV DNA-emia).

Further research into the possibility of HCV-mediated HDV propagation in humans is necessary to better understand the role and interaction of HDV with putative “helper viruses,” especially in regions with a high prevalence of HDV infection. Additionally, the potential for other human pathogenic viruses, such as DENV and VZV, to propagate and spread HDV in humans should be explored. These findings may offer greater insight into whether HDV can depend on viruses other than HBV to establish infection in humans and might even enhance the understanding of HDV's origin.

In conclusion, the RT-qPCR assay described here for quantifying HDV RNA loads in serum samples, validated according to current European guidelines, demonstrated excellent technical and clinical performance. The HDV_UCT enables the quantification of all proposed HDV GT and, with a limit of detection (LoD) of 3.86 IU/ml, is the most sensitive HDV assay reported to date. Therefore, the assay can be utilized as a tool for diagnosing active HDV infection and for monitoring patients in clinical practice, as well as for epidemiological studies and treatment response evaluations, which are increasingly important due to the growing number of treatment options. Moreover, the findings presented in this dissertation suggest that HCV-mediated HDV propagation is unlikely to occur independently of HBV infection, at least in non-endemic regions for HDV infections. As other studies concur with this conclusion (Cappy et al. 2021, Chemin et al. 2021, Roggenbach et al. 2021), these findings support the notion that screening for HDV infection should only be performed in patients exhibiting signs of HBV infection (HBsAg or HBV PCR positive) in alignment with current clinical guidelines (EASL 2017, 2023). Part of the results presented in this dissertation were published listing me as the first author (Pflüger et al. 2021, Pflüger et al. 2020).

6. Zusammenfassung

English version:

Background & Aim: Detecting the hepatitis D virus (HDV) RNA via PCR is challenging due to the heterogeneity of genotypes (GT), high GC content (approximately 60%), and significant levels of intramolecular base pairings. HDV can self-replicate, but it relies on the hepatitis B virus (HBV) for viral assembly. Recent studies have shown, both *in vitro* and in a mouse model, that viruses other than HBV, such as the hepatitis C virus (HCV), can package HDV and facilitate the release of infectious particles. The aim of this dissertation was I) to establish and validate an RT-qPCR assay for the detection and quantification of all 8 HDV GT on a fully automated, high-throughput platform (cobas6800) and II) to evaluate HCV-mediated HDV propagation in HCV mono-infected patients in Germany. *Methods:* Previously published primers were adapted for use on a fully automated platform. Serial dilutions of the HDV WHO standard were employed to assess the technical performance of the assay (lower limit of detection, linearity, and precision). Inclusivity was evaluated using cell culture-derived viruses, and anti-HDV positive sera (n=323) were included to compare clinical performance with a CE-IVD assay. HCV PCR-positive and hepatitis B antigen-negative serum samples (n=110) were utilized to investigate HDV PCR and anti-HDV positivity in HCV mono-infected patients. Additionally, serology markers for HBV infection were determined. *Results:* The RT-qPCR assay demonstrated excellent technical and clinical performance, with a lower limit of detection of 3.86 IU/ml (95% CI: 2.95 – 5.05 IU/ml), inclusivity, and linearity for all HDV GT (slopes: -3.48 to -4.413; $r^2 > 0.92$), as well as high precision. A comparison with a CE-IVD assay revealed a strong correlation (r^2: 0.87) and an overall agreement of 97.3%. The evaluation of HCV/HDV co-infection showed that no HDV PCR-positive patients could be identified (n=0/323) and all anti-HDV positive patients (n=8/316) exhibited signs of past but currently immune-controlled HBV infection (n=8/8 anti-HBc positive). *Conclusion:* In conclusion the excellent performance observed demonstrates that the HDV RT-qPCR assay can be used as a powerful tool for diagnosing active HDV infection and monitoring patients. Furthermore, the results suggest that the likelihood of HCV-mediated HDV infection without signs of previous HBV infection is low, at least in regions with low HDV prevalence.

Deutsche Version:

Hintergrund & Ziel: Der Nachweis des Hepatitis-D-Virus (HDV) RNA mittels PCR ist aufgrund der Heterogenität der Genotypen (GT), des hohen GC-Gehaltes und der zahlreichen intramolekularen Basenpaarungen eine Herausforderung. HDV kann zwar selbstständig replizieren, ist aber darüber hinaus auf die Hüllproteine des Hepatitis-B-Virus (HBV) angewiesen. Neuere Studien zeigten jedoch, dass neben HBV auch andere Viren, z.B. das Hepatitis-C-Virus (HCV), HDV verpacken und zur Freisetzung infektiöser Partikel *in vitro* und im Mausmodell führen können. Das Ziel dieser Dissertation war daher I) die Etablierung und Validierung eines quantitativen RT-qPCR-Assays zum Nachweis aller 8 HDV GT auf einer vollautomatisierten Plattform (cobas6800=) und II) die Evaluierung von HCV-vermittelten HDV Infektionen bei HCV-monoinfizierten Patient:innen in Deutschland. *Methoden:* Bereits publizierte Primer wurden für die Verwendung auf einer vollautomatisierten Plattform angepasst. Zur Bewertung der technischen Leistungsfähigkeit (untere Nachweisgrenze, Linearität und Präzision) wurden serielle Verdünnungen des HDV-WHO-Standards verwendet. Die Inklusivität wurde mit aus Zellkulturen gewonnenen Viren bewertet, und Anti-HDV-positive Seren (n=323) wurden zur Bewertung der klinischen Leistung im Vergleich zu einem CE-IVD-Test einbezogen. HCV-PCR-positive und Hepatitis-B-surface-Antigen-negative Serumproben (n=110) wurden verwendet, um die HDV-PCR- und Anti-HDV-Positivität bei HCV-monoinfizierten Patient:innen zu untersuchen. Zusätzlich wurden serologische Marker einer HBV-Infektion bestimmt. *Ergebnisse*: Der RT-qPCR-Assay zeigte eine hervorragende technische Performance mit einer unteren Nachweisgrenze von 3,86 IU/ml (95% CI: 2,95 - 5,05 IU/ml), Inklusivität und Linearität für alle HDV GT (Steigungen: -3,48 bis -4,413; r2 > 0,92) sowie eine hohe Präzision. Der Vergleich mit einem CE-IDV-Assay ergab eine starke Korrelation (r2: 0,87) und eine Gesamtübereinstimmung von 97,3 %. Keine einzige Probe war HDV PCR positiv (n=0/323) und alle Anti-HDV-positiven Patient:innen (n=8/316) wiesen Anzeichen einer früheren, derzeit immunkontrollierten HBV-Infektion auf (n=8/8 Anti-HBc positiv). *Zusammenfassung*: Die hervorragende technische und klinische Performance des HDV-RT-qPCR-Assays unterstreicht, dass sich dieser als leistun gsstarkes Instrument für die Diagnostik einer aktiven HDV-Infektion sowie für die Verlaufsdiagnostik eignet. Darüber hinaus konnte gezeigt werden, dass die Wahrscheinlichkeit einer HCV-vermittelten HDV-Infektion ohne Anzeichen einer HBV-Infektion, zumindest in Regionen mit niedriger HDV-Prävalenz, gering ist.

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8. Abbreviations

AASLD *American Association for the Study of Liver Diseases*
ADAR *adenosine deaminase acting on RNA*
ALT *alanine transaminase*
anti-HBc *antibodies against the hepatitis B virus core antigen*
anti-HBs *antibodies against the hepatitis B virus surface antigen*
anti-HCV *antibodies against the hepatitis C virus*
anti-HDV *antibodies against the hepatitis delta virus*
APASL *Asian Pacific Association for the Study of the Liver*
AST *Aspartate transaminase*
AU *arbitrary units*
BKPyV *BK-polyomavirus*
bp *base pair*
CE-IVD *CE-marked in vitro diagnostic*
CI *confidence interval*
CMV *cytomegalovirus*
COVID-19 *coronavirus disease 2019*
ct cycle threshold
DENV *Dengue virus*
DIPA delta-interacting protein
DNA *desoxyribonucleic acid*
EASL *European Association of the Study of the Liver*
EBV *Epstein-Barr virus*
EQA *external quality assessment samples*
ER *endoplasmic reticulum*
FDA Food and Drug administration
GP *glycoproteins*
GT genotype
HBsAg *hepatitis B virus surface antigen*
HBV *hepatitis B virus*
HCC *hepatocellular carcinoma*
HCV *hepatitis C virus*
HDAg *hepatitis delta antigen*
HDV *hepatitis delta virus*
HDV_UCT *HDV Utility channel test*
HIV *human immunodeficiency virus*
IC *internal control*
IgG *immunoglobin G*
IgM *immunoglobin M*
interferon *INF*
IU *international units*
LDT *laboratory developed tests*
L-HDAg *large hepatitis delta virus antigen*
LoD *limit of detection*
ml *milliliter*
mRNA *messenger RNA*
NAAT *nucleic acid amplification test*
NIH *National Institutes of Health*
NTCP *sodium taurocholate cotransporting polypeptide*
ORF *open reading frame*
PCR *polymerase chain reaction*

pegINFα *PEGylated interferon alpha*

PEI *Paul-Ehrlich Institute*

pol II *polymerase II*

r^2 *Pearson's correlation coefficients*

RFI min. *relative fluorescence increase minimum*

RNA *ribonucleic acid*

RNP *ribonucleoprotein*

RT-qPCR reverse transcription polymerase chain reaction

S/CO *signal to cutoff*

SD *standard deviation*

S-HDAg *small hepatitis delta virus antigen*

U.S. *United States*

VZV *varicella-zoster virus*

WHO *World Health Organization*

10. Table of Figures

11. Table of Tables

12. Publications

Giersch K, Nörz N, Grunwald M, Pfefferle S, Pflüger LS, Fischer N, Aepfelbacher M, Lütgehetmann M (2025) Adaptation and validation of a gastrointestinal panel to detect diarrheal virus pathogens on a high-throughput qPCR system. Med Microbiol Immunol. 214(1):28. doi: 10.1007/s00430-025-00837-z.

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Pflüger LS[#], Bannasch JH[#], Brehm TT, Pfefferle S, Hoffmann A, Nörz D, van der Meirschen M, Kluge S, Haddad M, Pischke S, Hiller J, Addo MM, Lohse AW, Schulze Zur Wiesch J, Peine S, Aepfelbacher M, Lütgehetmann M (2020) Clinical evaluation of five different automated SARS-CoV-2 serology assays in a cohort of hospitalized COVID-19 patients. J Clin Virol. 130: 104549. doi: 10.1016/j.jcv.2020.104549..

shared authorship

13. Erklärung des Eigenanteils

Das Thema, die Konzeptualisierung, die Entwicklung der Fragestellungen sowie die Planung der in dieser Dissertation enthaltenen Studien und Experimente wurden durch mich und federführend von Herrn Prof. Dr. Julian Schulze zur Wiesch und Herrn Dr. Marc Lütgehetmann erarbeitet.

Die aus Zellkultur gewonnenen Viren (HDV Genotyp 1-8), die für die Validierung der PCR verwendet wurden, wurden von Herrn Prof. Dr. Dieter Glebe (Justus-Liebig-Universität Gießen) und Mitarbeitenden seiner Arbeitsgruppe für die Experimente zur Verfügung gestellt.

Die in dieser Dissertation beschriebenen Experimente wurden von mir eigenständig durchgeführt und dokumentiert. Die Analyse und Interpretation der Daten inklusive der statistischen Auswertung wurden ebenfalls von mir unter Absprache mit und Beratung von Herrn Prof. Dr. Julian Schulze zur Wiesch und Herrn Dr. med. Marc Lütgehetmann erarbeitet. Für die statistische Auswertung wurde das Programm GraphPad Prism verwendet.

Die in dieser Dissertation enthaltenen Tabellen und Abbildungen, die die Darstellung der Ergebnisse dieser Dissertation präsentierten, wurden unter der Verwendung der Programme BioRender und GraphPad Prism eigenständig von mir erstellt. Insbesondere in der Einleitung sowie im Diskussionsteil sind Abbildungen und Tabellen enthalten, die aus bereits publizierten Veröffentlichungen andere Autor:innen stammen. Diese sind stets klar als solche gekennzeichnet und mit der entsprechenden Quellenangabe versehen.

Die für diese Dissertation durchgeführte Literaturrecherche, die Zusammenstellung der Ergebnisse und die Diskussion dieser wurden von mir durchgeführt. Ebenso wurde die vorliegende Dissertationsschrift von mir eigenständig verfasst.

Teile der in dieser Dissertation gezeigten Ergebnisse wurden zudem von mir als Erstautorin in Pubmed-gelisteten Journals (inklusive Peer Review Prozess) während meiner Zeit als Doktorandin veröffentlicht (Pflüger et al. 2021, Pflüger et al. 2020). Die Manuskripte für diese Veröffentlichungen wurde von mir persönlich verfasst und von den Koautor:innen der

Studie überarbeitet. Alle Abbildungen und Tabellen, die in den jeweiligen Publikationen gezeigt werden, habe ich erstellt. Ebenso wurden die Auswertungen und statistischen Berechnungen von mir durchgeführt. Wesentliche Unterstützung in Form von Überarbeitungen, Korrekturen und Hilfe bei der Konzeptualisierung wurde von Prof. Dr. Schulze zur Wisch und Dr. Marc Lütgehetmann geleistet.

14. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe, insbesondere ohne entgeltliche Hilfe von Vermittlungs- und Beratungsdiensten, 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. Das gilt insbesondere auch für alle Informationen aus Internetquellen.

Soweit beim Verfassen der Dissertation KI-basierte Tools („Chatbots“) verwendet wurden, versichere ich ausdrücklich, den daraus generierten Anteil deutlich kenntlich gemacht zu haben. Die „Stellungnahme des Präsidiums der Deutschen Forschungsgemeinschaft (DFG) zum Einfluss generativer Modelle für die Text- und Bilderstellung auf die Wissenschaften und das Förderhandeln der DFG“ aus September 2023 wurde dabei beachtet.

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 damit einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Datum Unterschrift

15. Danksagung

Zunächst einmal möchte ich meinem Doktorvater, Prof. Dr. Julian Schulze zur Wiesch, meinen herzlichen Dank für seine ständige Unterstützung und hervorragende Betreuung ausdrücken, ohne die diese Dissertation nicht möglich gewesen wäre.

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Abschließend danke ich meiner Familie, insbesondere meinem Großvater Pus, und meinen Freunden für die großartige Unterstützung und ihr Verständnis.