Study of the interplay between hepatitis B and hepatitis delta viruses and evaluation of investigational anti-HDV immuno-modulators in superinfection cell culture models

Dulce Alfaiate

THESE DE DOCTORAT DE L'UNIVERSITE DE LYON

Délivrée par

UNIVERSITE CLAUDE BERNARD LYON 1

ECOLE DOCTORALE BIOLOGIE MOLECULAIRE INTEGRATIVE ET CELLULAIRE

Numéro d'ordre: 123-2015

Study of the interplay between hepatitis B and hepatitis delta viruses and evaluation of investigational anti-HDV immuno-modulators in superinfection cell culture models

Soutenue publiquement le Vendredi 25 Septembre 2015 par

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Réalisée au sein de

CENTRE DE RECHERCHE EN CANCÉROLOGIE DE LYON

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“La seule façon de lutter contre la peste, c'est l'honnêteté.

Qu'est ce que l'honnêteté?(…)

Je ne sais pas ce qu'elle est en général. Mais dans mon cas, je sais qu'elle consiste à faire mon metier.”

In **La peste**, Albert Camus

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Abstract

Study of the interplay between hepatitis B and hepatitis delta viruses and evaluation of investigational anti-HDV immuno-modulators in superinfection cell culture models

HDV/HBV superinfection is the most aggressive form of chronic viral hepatitis and is estimated to affect 15-20 million patients worldwide. HDV is not susceptible to available direct anti-HBV drugs and sustained response to IFNα therapy occurs in less than 1/4 of patients. Despite the faster progression of liver disease, most HDV/ HBV infected patients present a suppression of HBV replication. The details of the interactions between HDV, HBV and the host cell innate immune response remain largely unexplored and research efforts have been limited by the lack of infection models.

The aims of this thesis work were: i) to study HDV infection and the interplay with the host innate immune response; ii) to identify novel therapeutic strategies for the inhibition of HDV; iii) to further explore HDV/ HBV interference.

The experimental strategy was based on infection of dHepaRG cells, which are known to be permissive to both HBV and HDV full replicative cycles and to present physiological innate immune responses.

We observed that: i) HDV infection is associated with a strong, yet transient replication, a potent induction of the expression of ISGs; ii) IFN-α treatment of HDV-infected cells does not induce a further increase of ISG expression and has a modest antiviral activity. Conversely, some PRR agonists, in particular those inducing the NF-kB pathway, induce a strong decline in HDV replication; iii) despite the low number of coinfected cells, HDV as well as its encoded proteins exert a repressive effect on HBV replication.

Our work opens an array of perspectives on the pathogenesis of hepatitis delta and the identification of novel immune modulatory therapeutic strategies.

Key words: Hepatitis D virus; hepatitis B virus; viral interference; IFN response; immune-modulators.

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Résumé

Étude des interactions entre les virus des hépatites B et delta et évaluation de nouveaux immuno-modulateurs anti-HDV dans des modèles cellulaires de surinfection

La surinfection par HDV/ HBV est la forme la plus grave d'hépatite virale chronique et affecte entre 15-20 millions de patients au niveau mondial. HDV n'est pas susceptible aux traitements anti-HBV et le taux de réponse à l'IFNα est <25%. Malgré une progression plus rapide de la maladie hépatique, la majorité des patients présente une suppression de la réplication du HBV. Les détails des interactions entre HDV, HBV et le système immunitaire inné des cellules infectées restent inconnus.

Les objectifs de ces travaux de thèse ont été: i) l'étude de l'infection par HDV et son interaction avec la réponse innée cellulaire; ii) l'identification de nouvelles stratégies thérapeutiques anti-HDV; iii) l'exploration de l'interaction entre HDV et HBV.

L'approche expérimentale a été basée sur l'infection de cellules dHepaRG, capables d´entretenir des cycles réplicatifs complets de HBV et HDV et ayant une réponse immunitaire innée physiologique.

Nous avons observé que: i) l'infection par HDV est associée à un réplication forte dans un nombre limité de cellules, et à une induction de l'expression des ISGs; ii) le traitement des cellules infectées par HDV avec de l'IFNα ne conduit pas à une induction accrue des ISGs et a une faible activité antivirale. Quelques agonistes de PRR, notamment activant la voie NF-kB, induisent une forte diminution de la réplication de HDV; iii) malgré le faible nombre de cellules infectées, HDV et ses protéines induisent une diminution de la réplication de HBV.

Ces travaux ouvrent des perspectives importantes concernant la caractérisation de la pathogénèse de l'hépatite delta et l'identification de nouvelles stratégies thérapeutiques immuno modulatrices.

Mots clé: virus de l'hépatite D; virus de l'hépatite B; interférence virale; réponse IFN; immuno-modulateurs.

Résumé substantiel

Étude des interactions entre les virus des hépatites B et delta et évaluation de nouveaux immuno-modulateurs anti-HDV dans des modèles cellulaires de surinfection

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Introduction:

L'infection par les virus de l'hépatite B (HBV) et de l'hépatite delta (HDV) représente la forme la plus agressive d'hépatite virale chronique. Le nombre estimé de patients affectés au niveau mondial est de 15-20 millions. Le virus de l'hépatite delta est un satellite du HBV. Deux modes d'acquisition sont décrits : soit une infection simultanée par les deux virus (coinfection) qui conduit à une guérison dans la majorité des cas ; soit une infection delta d'un patient porteur du HBV (surinfection), qui est associé à une progression vers la chronicité et le développement de complications associées à une hépatite D chronique (dont la cirrhose et le carcinome hepato-cellulaire). De façon intéressante, chez les patients infectés par HDV, malgré la progression plus agressive de la maladie hépatique, il y a souvent une répression de la réplication du HBV. Ceci a été vérifié par l'étude de biopsies hépatiques de patients, mais aussi dans des modèles expérimentaux *in vivo* et *in vitro.* Des mécanismes de régulation de la transcription du génome HBV par les protéines du HDV ont été proposés, mais les détails de cette interaction ne sont pas connus.

Il est connu que l'infection par HBV n'induit pas une réponse immunitaire innée de type interféron dans les cellules infectées (de plus en plus d'évidences suggèrent même une inhibition active de cette réponse). En ce que concerne HDV, une modulation de la réponse interféron a également été proposée, mais les études précédentes n'ont pas été conclusives.

Il est important de noter que la réplication d'HDV n'est pas affectée par les inhibiteurs spécifiques d'HBV actuellement disponibles. De plus, le traitement prolongé avec

l'interféron (IFN) alfa pégylé entraîne une réponse soutenue dans moins d'un quart des cas et est associé à un nombre significatif de rechutes tardives, soulignant l'urgence de trouver de nouvelles options thérapeutiques contre HDV.

Pour cela, il est fondamental d'étudier la réplication des deux virus et leurs interactions avec la réponse immunitaire de l'hôte dans des cellules infectées. Cette étude a été limitée au cours du temps par un manque de modèles d'infection adéquats. Les cellules HepaRG sont sensibles à l'infection par ces deux virus hépatotropes et, jouant d'une réponse immunitaire innée fonctionnelle, représentent un modèle stable et alternatif aux hépatocytes primaires humains (PHH).

Objectifs du projet de recherche :

Cette thèse a eu comme objectifs : 1) l'établissement et caractérisation d'un modèle *in vitro* de surinfection par HBV/ HDV ; 2) la caractérisation et étude des mécanismes d'interaction entre les deux virus ; 3) l'évaluation de la réponse immunitaire innée dans les hepatocytes infectés par HDV et 4) l'utilisation du modèle établi pour l'évaluation de nouvelles stratégies thérapeutiques contre HDV.

Matériel et méthodes:

Des cellules HepaRG différenciées ont d'abord été infectées par HDV à différentes multiplicités d'infection (MOI), et les paramètres de la réplication virale ainsi que la réponse immunitaire cellulaire ont été suivies au cours du temps.

Pour l'évaluation d'un contexte de surinfection (plus physiologique), les cellules ont été infectées (ou non) par HBV puis surinfectées (ou non) par HDV. Les marqueurs de la réplication de chaque virus et de la réponse immunitaire cellulaire ont été suivis dans chaque condition par qPCR, RT-qPCR, Northern blot, ELISA, Western blot et immunofluorescence.

Les cellules infectées ont aussi été utilisées pour l'évaluation de l'effet anti-HDV de molécules immuno-modulatrices, notamment des agonistes de senseurs de l'immunité innée ('Pattern Recognition Receptors'; PRRs). Leur effet sur la réplication de HDV a été évalué par qRT-PCR et Northern Blot.

Résultats :

Les cellules HepaRG supportent une forte mono-infection transitoire par HDV en l'absence d'HBV. Comme attendu, si la réplication d'HDV était similaire en présence ou en absence d'infection par HBV, la sécrétion de virions HDV n'a été observée qu'en co-infection. Ce résultat confirme la coexistence des deux virus dans une même cellule, malgré le faible pourcentage de cellules infectées. La surinfection par HDV était associée à une diminution des paramètres de réplication d'HBV, sauf de l'ADNccc, confirmant un certain degré d'interférence virale dans ce modèle.

L'infection par HDV était associée à une forte induction de l'expression des gènes de la réponse interféron ('Interferon Stimulated Genes'; ISGs), aussi bien en mono qu'en surinfection. Cette induction est associée à l'augmentation du MOI de HDV et suit la cinétique d'accumulation de l'ARN du HDV dans les cellules.

Nous avons identifié 4 agonistes de PRRs présentant un effet antiviral significatif sur la réplication de HDV: Pam3, Imiquimod, Poly (d :A ; dT) et Poly (I :C)).

Conclusions et perpectives:

Nous avons mis en place un modèle cellulaire original de surinfection HBV/HDV qui conduit à une meilleure caractérisation moléculaire des interactions entre HBV et HDV. Nous confirmons dans ce modèle le rôle suppresseur du HDV sur la réplication du HBV et nous avons aussi pu démontrer que l'infection par HDV est associée à une forte induction de la réponse interféron dans les cellules infectées. Par ailleurs, et de façon originale, nous avons identifié des molécules immuno modulatrices avec un effet répressif sur la réplication de HDV.

Ce modèle est donc pertinent pour étudier et identifier de nouvelles cibles thérapeutiques, évaluer de nouveaux antiviraux à action directe, et définir les molécules immuno-modulatrices spécifiques. Ça permettra dans un futur proche d'approfondir l'étude des mécanismes d'interaction entre HDV et HBV, bien que de mieux caractériser la réponse immunitaire à l'infection par HDV et le mécanisme d'action des immuno-modulateurs identifiés.

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List of abbreviations

Aa – aminoacid

AAV – adeno-associated virus

AIM2 – absent in melanoma 2

ALT – alanine aminotransferase

ASC - apoptosis-associated speck-like protein containing a caspase recruitment domain

BCP – basal core promoter

bp – base pair

cART – combined antiretroviral therapy

cccDNA – covalently closed circular DNA

CHB – chronic hepatitis B

CHC – chronic hepatitis C

CHD – chronic hepatitis D (delta)

ChIP – Chromatin immunoprecipitation

CLR – C-type lectin receptor

CTD – C-terminal domain

CTL – cytotoxic T lymphocytes

DAA – direct acting antivirals

DAMP – damage associated molecular pattern

DC – dendritic cell

DENV – dengue virus

DHBV – duck hepatitis B virus

DR – direct repeat

DSL – double stranded linear

dsRNA – double-stranded RNA

EBV - Epstein Barr Virus

ER – endoplasmic reticulum

ERGID - ER-Golgi Intermediate compartment

HAP - heteroaryldhydropyrimidines

HAT – histone acetyltransferase

HBIG – hepatitis B immunoglobulin

HBSP – hepatitis B spliced protein

HBV – hepatitis B virus

HCMV – human cytomegalovirus

HCC – hepatocellular carcinoma

HDAC – histone deacetylase

HDAg – hepatitis delta antigen

HDV – hepatitis D (delta) virus

HHV – human herpes virus

HIV – human immunodeficiency virus

HPV – human papilloma virus

HSPG – heparin sulfate proteoglycans

HSV – Herpes Simplex virus

ICTV – International Committee on Taxonomy of Viruses

IFNa – interferon alpha

IFNAR – type I interferon receptor

IRF – interferon-regulatory factor

ISG – interferon stimulated genes

ISRE - interferon-stimulated response element

IVDU – intravenous drug use

JEV – Japanese encephalitis virus

KSHV – Kaposi associated herpes virus

LCMV - lymphocytic choriomeningitis virus

LPS - lipopolysaccharide

LRR – leucine-rich repeat

LSEC - liver sinusoidal endothelial cells

LTR – long terminal repeat

MHC – major hystocompatibility complex

MOI – multiplicity of infection

MVB – multivesicular body

NAP – nucleic acid polymer

NK cells – natural killer cells

NLR – nod like receptor

NLS – nuclear localization signal

NOD – nucleotide binding oligomerization domain

NTCP – sodium taurocholate cotransporting peptide

NTD – N-terminal domain

ORF – open reading frame

PAMP – pathogen associated molecular pattern

PCH – primary chimpanzee hepatocytes

Peg-IFNa – pegylated interferon alpha

pgRNA – pregenomic RNA

PHH – primary human hepatocytes

PRR – Pattern Recognition Receptor

rcDNA – relaxed-circular DNA

RIG-I - retinoic acid inducible gene-I

RLR – rig-I like receptor

RNP - ribonucleoprotein

RT – reverse transcriptase

STAT - signal transducer and activator of transcription

SVP – subviral particules

TA – tetracycline transactivator

TDP – tyrosyl-DNA-phosphodiesterase

TGF - transforming growth factor

TLR – toll like receptor

TP – terminal protein

TR – tetracycline repressor

TRAIL - tumour necrosis factor related apoptosis inducing ligand

Vge – viral genome equivalent

VVI – virus-virus interaction

WHO – World Health Organization

WHV – woodchuck hepatitis virus

YFV – yellow fever virus

Chapter I. Background

1. Overview

Currently, 250 million people are infected by Hepatitis B Virus (HBV). Among them, 15-20 million are estimated to be co-infected the Hepatitis Delta Virus (HDV). Chronic hepatitis delta (CHD) is considered to be the most severe form of chronic viral hepatitis and still poses major challenges to both clinicians and researchers.

In order to approach this problem, a thorough knowledge of the characteristics of both diseases and viruses is warranted. Moreover, lessons learnt from other cases of viral interactions and a wider perspective of the host response to viral infection need to be applied in the interpretation of this very particular clinical and virological entity.

2. Hepatitis B

2.1. Introduction

2.1.1. Discovery of Hepatitis B virus

The history of Hepatitis B virus identification goes hand in hand with the major milestones in the timeline of hepatitis research, as represented in

Figure 1. Descriptions of epidemic jaundice, although very likely associated not only with hepatitis but also with other diseases such as malaria and leptospirosis, date from ancient times, and it was Hippocrates who first used the expression “icterus”. The hypothesis of a transmissible cause for jaundice was first issued in the 19th century (associated to disease outbreaks following mass vaccination procedures). The first suggestion that some forms of jaundice could be due to viral infection was made 10 years after the discovery of the first virus and consequent foundation of virology by Beijerink in 1898. The development of the liver biopsy technique and the discovery of the inflammatory nature of the disease led to the introduction of the term “viral hepatitis”, by the time of the 2nd World War. Two types of hepatitis were then described, based on different transmission pathways and clinical courses: **epidemic hepatitis** or hepatitis A (acute, fecal-oral transmission) and **serum hepatitis** or

hepatitis B (related mainly to vaccination and parenteral procedures, with a longer incubation period and evolution to chronicity) (Thomas et al., 2015; Trepo, 2014).

Two major discoveries in the history of HBV followed: in 1965 Baruch Blumberg identified the Australia Antigen (now known as HBsAg) and five years later David Dane described the HBV virion (Dane's particle) (Blumberg et al., 1965; Dane et al., 1970). These discoveries soon led to the full characterization of the virus and the development of screening and immunization strategies with a huge public health impact. Indirectly, they also led to the identification of a second form of serum hepatitis (non-A, non-B) that opened the research field of Hepatitis C.

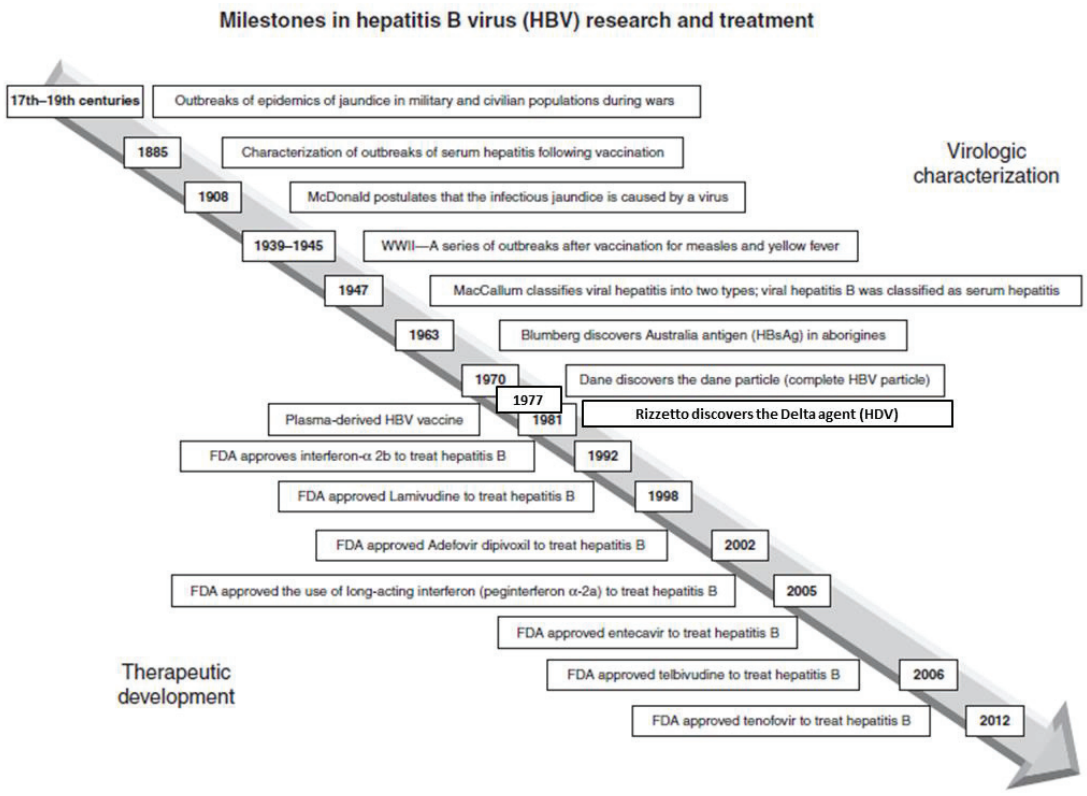

Figure 1. Timeline of major advancements in HBV research and treatment. Adapted from (Thomas et al., 2015)

2.1.2. Clinical and virological relevance

From a clinical standpoint, and despite the existence of an effective vaccine, hepatitis B still is a particularly challenging entity. Firstly, vaccination is still costly and not universally accessible, being particularly limited in some of the regions with highest

incidence of the disease. Secondly, the current pool of chronically infected patients remains very significant and, although effective antivirals exist, no single treatment ensures a cure and a life-long therapy is warranted. Finally, HBV infection is the major cause of hepatocellular carcinoma (HCC) and death (an estimated 786,000 people die each year as a result of HBV infection) (Locarnini et al., 2015).

In terms of virological characteristics, HBV (and hepadnaviruses in general) display some particular and challenging features, including: i) similarities with retroviruses (as the presence of a reverse transcription step); ii) the establishment of a permanent viral form in the infected cells (circular-covalently-closed DNA or cccDNA) with fascinating properties; and iii) a unique capability of silencing the host cell immune response.

2.2. The virus

2.2.1. Viral classification

HBV is a partially double-stranded DNA virus belonging to group VII of the Baltimore classification (double-stranded DNA viruses that replicate through a RNA intermediate). Following the International Committee on the Taxonomy of Virus (ICTV) classification, HBV belongs to the *Hepadnaviridae* family (standing for hepatotropic DNA virus). The members of this family have in common their hepatotropism, genetic organization, viral morphology and replication mechanism (that includes a retro-transcription step). The family is divided into two genera, depending on the species specificity of the viruses: the genus **orthohepadnavirus** includes HBV and other mammalian viruses (as woodchuck hepatitis virus [WHV], ground squirrel hepatitis virus, woolly monkey hepatitis virus and bat hepatitis virus), while the genus **avihepadnavirus** includes avian virus, as the duck hepatitis B virus (DHBV) and heron HBV (Schaefer, 2007).

2.2.2. Viral structure

Virions and subviral particles

Two major types of particles have historically been characterized in the serum of HBV infected patients, as represented in Figure 2:

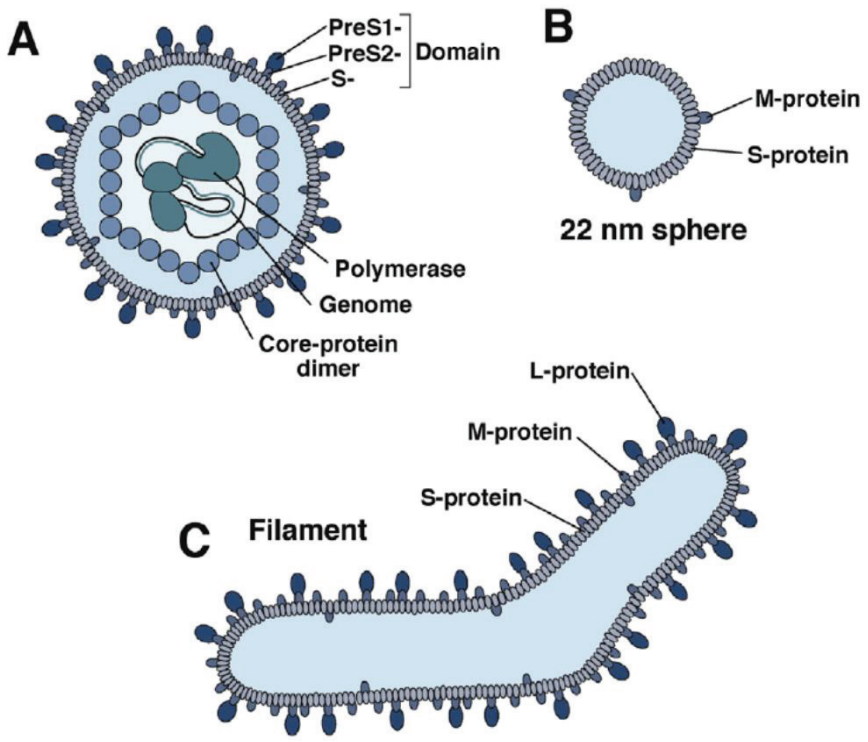

Figure 2. **HBV virion and empty subviral particles**. **A**. HBV virion (Dane particle). **B**. Spherical SVP. **C**. Filamentous SVP. Reproduced from (Urban et al., 2014)

1. Complete virions, also named **Dane particles**, are the infectious particles and can be present in the serum of patients in titers as high as 10^{10} genome-copies/mL. They are spherical, 42nm in diameter, have an inner icosahedral symmetry, and a buoyant density of 1,24-1,27 g/mL in CsCl (Dane et al., 1970; Hruska and Robinson, 1977). HBV virions consist of (from the surface to the interior): a) a lipoprotein membrane with the three forms of HBsAg (S-HBsAg, M-HBsAg and L-HBsAg) with a ratio of 4:1:1; b) a nucleocapsid featuring 120 dimers of the core/capsid protein (HBcAg or HBc; triangulation number T=4; a smaller proportion of “empty” virions has 90 dimers and T=3); c) the capsid

contains one single copy of HBV DNA in its relaxed-circular form (rcDNA), covalently linked to the viral polymerase (Howley, 2013);

2. **Subviral non-infectious particles** (SVP) are constituted only by envelope proteins embedded in the bilayer membrane. They have approximately 22nm and with a buoyant density of 1,18-1,19 g/mL and circulate in 1000-10000 fold excess relative to virions in patients' sera (Urban et al., 2014). Two types are known: spherical particles, with an octahedral symmetry; and asymmetrical, rod-like (or filamentous) particles with variable length and width. Whereas filamentous SVP have a ratio of surface proteins similar to virions, spheres do not have L-HBsAg and have a 4:1 ratio of S-HBsAg: M-HBsAg. Their precise role in HBV infection is still debated, although they may favor viral infection through a saturation of the humoral response to HBV and hence play a role in immune subversion mechanisms.

Besides these HBsAg containing particles, identified from patients' sera, two other sub-viral forms have been described:

1. **Non-enveloped nucleocapsids**, found to be secreted *in vitro* by HBV replicating hepatoma cell lines and suggested to play a role in virus spreading (Cooper and Shaul, 2006; Watanabe et al., 2007). Their existence and potential role *in vivo* is still to be demonstrated;

2. More recently, **enveloped empty capsids** have also been described in patients' sera, but their proper relevance and function is yet to be determined (Luckenbaugh et al., 2015).

Viral DNAs

HBV genome is found both in the viral particle and inside mature capsids in the cytoplasm of infected cells. It is a partially double-stranded circular DNA molecule of around 3,2kb, also called **rcDNA**. The complete strand, from which transcription will

occur, is of negative polarity and is covalently linked to the viral polymerase at its 5' extremity. The positive, incomplete, strand contains a gap and a 3' extremity of variable length (Summers et al., 1975) and is associated with an RNA oligomer derived from pregenomic RNA (pgRNA), that serves as a template for negative strand completion. The circularity of the molecule is guaranteed by a 200 nucleotide-long region in the positive strand that overlaps the 5' and 3' extremities of the negative strand. It comprises direct repeat (DR) 1 and 2 sequences, necessary for viral replication (Howley, 2013). Being very compact, the HBV genome is also highly complex. It contains 4 open reading frames (ORF), 4 promoters, 2 enhancer elements and a single polyadenylation signal to regulate the expression of all viral RNAs. All nucleotides have coding functions for at least one protein (as a result of the presence of overlapping ORFs) and transcription regulation regions overlap with coding information (Nassal, 2015). A schematic representation of HBV genome organization is shown in Figure 3.

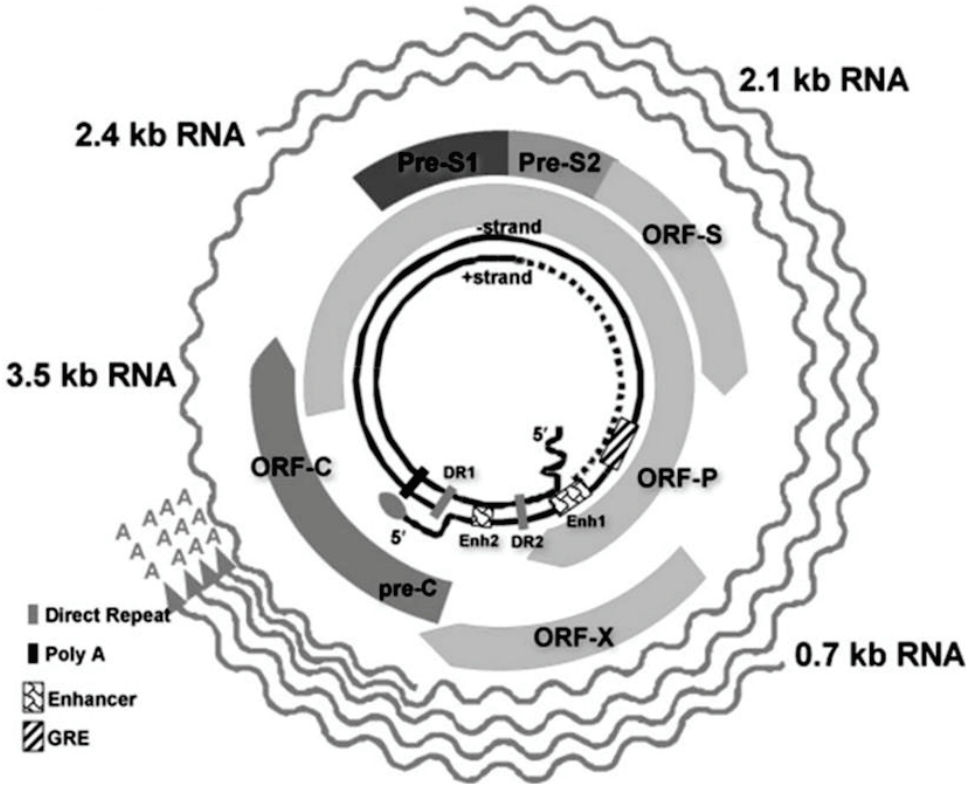

Figure 3. HBV genome organization, including key regulatory elements, open reading frames and RNA transcripts. Reproduced from (Liang, 2009).

The overlapping ORFs (all in the same direction and encompassing the whole HBV genome), which code for the 7 viral proteins, are:

- **Polymerase ORF** – it's the largest ORF, accounting for 80% of the viral genome, and codes for the viral polymerase;
- **PreS1/ PreS2/ S ORF** – contained within the Polymerase ORF, contains three in-phase starting codons and codes for the three forms of the envelope protein (S-, M- and L-HBsAg, which stand for small, medium and large HBV surface antigen, respectively);
- **PreCore/ Core ORF** – contains two in-phase initiation codons and codes for the secreted HBeAg and the HBcAg (core antigen);
- **X ORF** – codes for the X protein.

Transcription is regulated by 4 promoters (Core, PreS, S and X), 2 enhancer elements (Enh1 and Enh2) and cis-acting negative regulatory elements. These elements are only functional in the nucleus, after the conversion of rcDNA to cccDNA (Moolla et al., 2002). The organization and functions of the regulatory regions will be discussed in further detail in the Transcriptional regulation of the cccDNA section.

CccDNA is the form of HBV DNA responsible for virus persistence in infected cells. Although isolated reports have suggested its existence in other cells, it has only been conclusively demonstrated in the nucleus of infected hepatocytes (Howley, 2013). It is an episomal, plasmid-like, replicative intermediate that resides in the nucleus of the infected cells. In physiological conditions, cccDNA molecules are organized into a chromatin-like structure, forming a viral minichromosome, associated with histones and other host and viral proteins (Levrero et al., 2009). The organization into nucleosomal structure has been described for several other DNA viruses that cause human disease and is commonly associated with the establishment of latent infections. Examples are polyomaviruses, the simian virus 40, adenoviruses, herpes viruses (namely herpes simplex type 1 [HSV1], the gammaherpesvirus Kaposi

associated herpes virus [KSHV] and Epstein Barr virus [EBV]) and papillomaviruses (Lieberman, 2006).

HBV cccDNA serves as the only template for transcription of the viral RNAs and constitutes a stable pool in infected cells (mean copy number/ cell = 1,8 [0,008-54] in HBeAg positive patients and 0,09 [0,01-15] in HBeAg negative patients) (Volz et al., 2007). The regulation of the cccDNA pool has been a subject of debate and research throughout time. It is known that cccDNA persists in quiescent hepatocytes, with no effect on their viability. However, cccDNA half-life and its persistence through mitosis are still controversial (Howley, 2013). Furthermore, in chronic infection, the eradication of cccDNA is a rare event, although its clearance (at least partial) exists notably in the acute infection setting. Several mechanisms have been proposed to explain the clearance: i) the "curing" or non-cytolytic elimination model postulates that cccDNA may be directly eliminated by the action of cytokines, with no need for hepatocyte death or regeneration; ii) the alternative "killing" model proposes that cccDNA may only be eliminated by hepatocyte death. A third model proposes that in the absence of cccDNA neo-formation (as happens patients under nucleot(s)ide analogues [NA] therapy), the existing pool may be progressively eliminated by a combination of hepatocyte death and mitotic loss (Levrero et al., 2009; Nassal, 2015).

RNA transcripts

HBV replication occurs, as is characteristic of hepadnaviruses, through RNA intermediates. Transcription is carried out by the cellular RNA-polymerase II, using cccDNA as a template. Five transcripts (2 genomic and 3 sub-genomic, all possessing a 5' cap and a shared polyA tail) can be identified in infected cells:

1) **Pregenomic RNA** (pgRNA; 3,5 kb) – serves as a template for translation of polymerase and core proteins and is the template for *de novo* synthesis of viral DNA genome by reverse transcription;
2) **PreCore mRNA** (3,5 kb) is the template for translation of the precore protein, that is ultimately secreted as HBeAg;
3) **PreS1 mRNA** (2,4 kb) codes for the L-HBsAg;

4) **PreS2/S mRNA** (2,1 kb) and 5), that codes for S- and M-HBsAg;
5) **X mRNA** (0,7 kb) that codes for the X protein (Moolla et al., 2002).

These RNAs are detected as 3 major bands on Northern blot (3,5 kb; 2,4 kb and 2,1 kb). The X transcript is only occasionally detected in infected tissues (Howley, 2013). These major transcripts and the encoded protein products are represented in Figure 4.

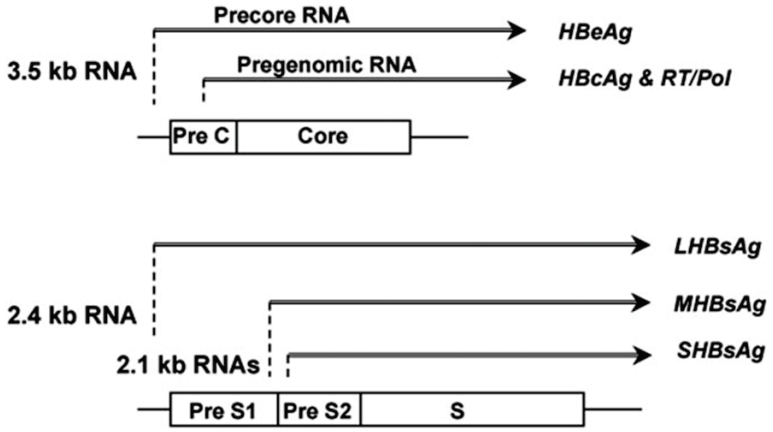

Figure 4. **Major HBV RNA transcripts and their respective protein products**. X mRNA (not represented) is only occasionally identified in infected cells. Reproduced from (Liang, 2009).

Viral proteins

HBV genome codes for 7 proteins: HBV polymerase, Core protein (HBcAg), PreCore (later cleaved to originate HBeAg), large, medium and small envelope proteins (L-, M- and S-HBsAg respectively) and X protein (Seeger and Mason, 2015). The role of an eighth protein, resulting from alternative splicing of pgRNA, hence called hepatitis B spliced protein (HBSP), identified in patients' liver biopsies is still debated (Soussan et al., 2003).

Envelope proteins (HBsAg)

As previously mentioned, three forms of envelope proteins (clinically detected as circulating or intracellular HBsAg) are translated from the PreS1/PreS2/S ORF, from 3 different ATG codons. They are synthetized in the endoplasmic reticulum (ER) and,

after oligomerization, are secreted as the envelope of HBV virions and subviral proteins. Their characteristics are summarized below and represented in Figure 5.

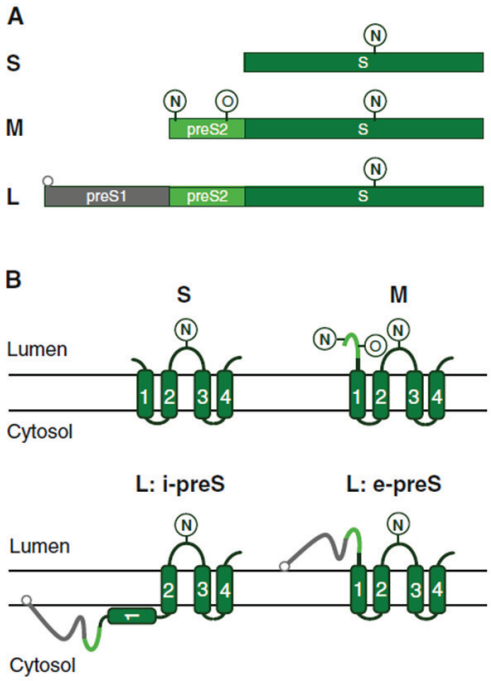

Figure 5. HBV envelope proteins. **A.** The three forms of HBsAg share the same C-terminus (S). As translation initiates in different in-frame sites, besides S (the only domain of S-HBsAg), M-HBsAg contains PreS2 and L-HBsAg further contains the 108 aa of PreS1. **B.** Schematic representation of membrane inserted HBsAg, showing the four putative transmembrane domains (1-4) and the two conformations of L-HBsAg, i-PreS1 and e-PreS1 (see text for details). Circled N and circled O represent glycosylation modifications. Reproduced from (Prange, 2012).

- **S-HBsAg** (226 amino acids [aa]; 24-27 kDa, depending on the glycosylation status) – is the most abundant envelope protein and contains the major antigenic determinants that led to the identification of the Australia antigen. It is considered to have 4 putative hydrophobic transmembrane domains (I-IV). The first and last two domains (I-II and III-IV) are separated by cytosolic loops (facing the interior of the viral particle), whereas between domains II and III lies an antigenic loop (called "a" determinant) that is exposed in the outside of the viral particle and is the target of neutralizing antibodies. The antigenic loop is an infectivity determinant (Abou-Jaoudé and Sureau, 2007) and its

amino acid sequence was used to classify HBV into 4 serotypes, as discussed below;

- **M-HBsAg** (281 aa; 31 kDa) – differs from S-HBsAg by an additional 55 amino acids at its N-terminus (PreS2 domain) and is always glycosylated. It represents 10-15% of the total HBsAg contained in infected cells. Its function is still not clear as it does not seem to be necessary for either assembly or infectivity (Fernholz et al., 1991);

- **L-HBsAg** (389 aa; 42 kDa) – contains, in addition to M-HBsAg, the PreS1 domain (108 aa), that becomes myristoylated and is essential for both viral assembly and infectivity (Gripon et al., 1995). It is a minor form in the infected cells (1-2%), but is much enriched in virions. It is not present in the spherical SVPs. L-HBsAg has 2 conformations in virions: half of the PreS1 (i-PreS1) N-terminal domains is located in the cytosol (viral interior) and mediate the interaction with the nucleocapsid; for the other half (e-PreS1), the N-terminus is exposed on the outside of virions and mediates the interaction with the viral receptor. The major infectivity determinants are located in the 75 N-terminal aa (Seeger and Mason, 2015; Urban et al., 2014).

Core protein (HBcAg)

The Core protein (HBcAg) is composed by 183aa, has a molecular weight of 21 kDa and corresponds to the structural unit of the viral nucleocapsid, as depicted in Figure 6. It has two main domains: the N-terminal domain (NTD), involved in capsid dimerization and envelopment, and the C-terminal "protamine" domain (CTD), involved in pgRNA encapsidation and DNA replication. The level of phosphorylation of the CTD is associated with capsid maturation, as DNA synthesis is accompanied by core dephosphorylation and conformational re-organization of the CTD. The kinases and phosphatases involved in this process are not definitely identified. Such changes expose binding sites for the envelope proteins leading to assembly of the mature (but not immature) nucleocapsids into virions (Mabit and Schaller, 2000; Perlman et al., 2005). Furthermore, the CTD possesses a nuclear localization signal (NLS) that mediates nuclear transport of the entering and part of the newly synthetized capsids

(a phenomenon called recycling) (Yeh et al., 1990). HBcAg is highly immunogenic. Although in HBV-infected patients HBcAg can only be detected in the liver, circulating anti-HBc antibodies are a relevant clinical marker of current and past infection.

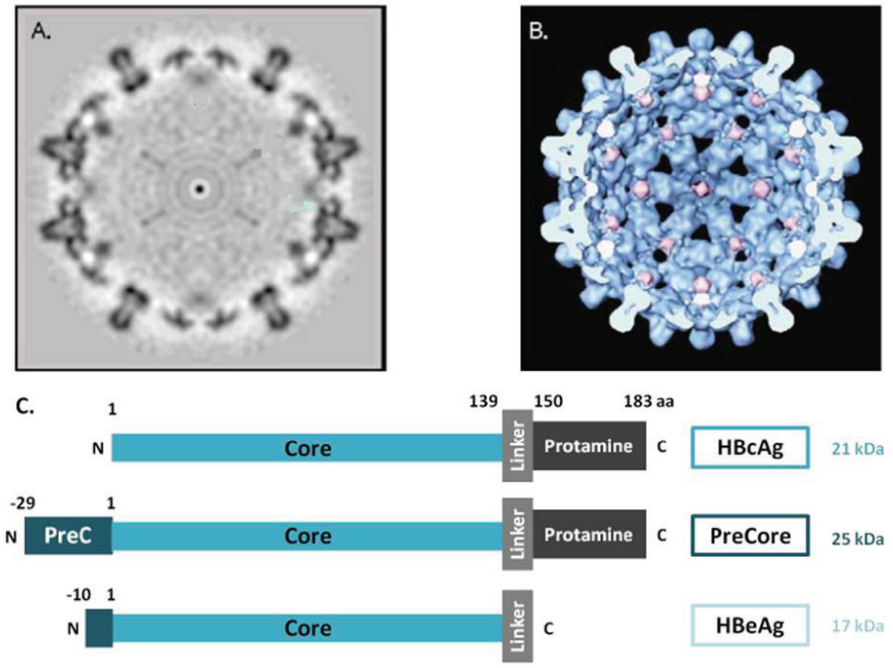

Figure 6. HBV core structure. Central section (**A**) and an interior view (**B**) of the T = 4 capsid, obtained by cryo-electron microscopy. **C**. Representation of HBcAg, PreCore and HBeAg domain organization. Adapted by Isorce N from (Steven et al., 2005)

PreCore protein and HBeAg

The translation of the PreCore mRNA leads to the synthesis of a precursor protein of 25 kDa that has, in addition to the domains previously described for the Core protein, an N-terminal extension of 29 aa (Yeh et al., 1990). This protein is signaled to the ER and enters the secretory pathway, where, after sequential cleavage, it is secreted as the 17 kDa HBeAg, a soluble protein (Standring et al., 1988). Although most of their primary amino acid sequence is identical, HBcAg and HBeAg display different antigenic properties (Salfeld et al., 1989). Represenatation of their organization is shown in Figure 6. HBeAg identification in the serum of infected patients is used as a surrogate marker of active replication, although it has no obvious role in HBV replication within hepatocytes. HBeAg to anti-HBeAg seroconversion may be associated with the end of active viral replication and the beginning of clinical resolution. However, in a particular

subset of patients, HBeAg loss is associated with the selection of mutants bearing either PreCore stop codon mutations or BCP (i.e. basal core promoter) mutations that abrogate its synthesis. These patients albeit having no circulating HBeAg, may have a continuous disease activity (Volz et al., 2007)

Polymerase

The polymerase (830 aa; 90 kDa) is the only enzyme encoded by the HBV genome and the target of currently available direct-acting antiviral (DAA) drugs. Significant body of evidence regarding its structure and function has been obtained in DHBV infection models. Up until now, no direct structural data are available for HBV polymerase. Sequence analysis identifies three functional domains (depicted in Figure 7): terminal protein (TP), the reverse transcriptase (RT) and RNAse-H. A spacer (with no clear function, except keeping away the two other domains) links TP and RT (Nassal, 2008).

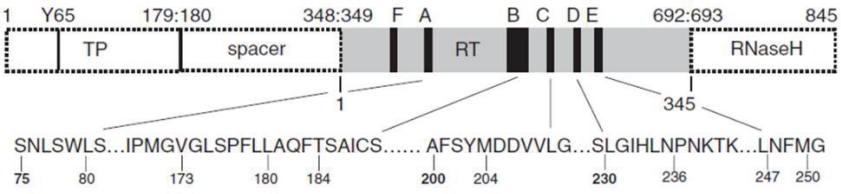

Figure 7. HBV polymerase domain organization. The four functional domains are represented from N- to C-terminus: TP, terminal protein, RT, reverse transcriptase, and RNase H. Position Y65 is necessary for priming of reverse transcription while RT position 204 (part of the YMDD conserved motif) is frequently mutated in cases of nucleos(t)ide analogue resistance. Reproduced from (Howley, 2013).

The **TP domain** is essential for the interaction with the "epsilon" (Ɛ) packaging signal of pgRNA and confers template specificity to the polymerase activity. Reverse transcription is primed from the Y65 residue of the TP, resulting in the formation of a covalent link between the polymerase and the nascent minus-strand DNA (Nassal, 2008; Weber et al., 1994) It has recently been suggested that the TP domain could contribute to the nuclear import of HBV genome, after the identification of a NLS (Lupberger et al., 2013).

The **RT domain** is associated with two enzymatic functions throughout HBV replicative cycle: the retro-transcription of pgRNA into the minus-strand DNA and the DNA-dependent DNA synthesis of the HBV DNA positive strand from the minus-strand DNA template. RT activity is dependent on a conserved YMDD motif, where the first RT-inhibitor resistance mutation was identified (rtM204V) (Gish et al., 2012). The retro-transcriptase has no 3'-5' exonuclease activity, which makes it error-prone, contributing to HBV variability (1.4-3.2 x 10^{-5} substitutions per site per year) (Okamoto et al., 1987).

The **RNAse-H** domain is responsible for the pgRNA degradation following the synthesis of the minus-strand of HBV DNA, as well as for the generation of the short RNA primer that is required for DNA positive strand synthesis (Wei and Peterson, 1996). RNase-H inhibitors were shown to block HBV replication and it is tempting to consider them as potential therapeutic options in the future (Tavis and Lomonosova, 2015).

X protein (HBx)

X is the smallest of HBV proteins (154 aa; 17 kDa) and the most enigmatic. It is known to be required for HBV replication *in vivo* and to act as a trans-activator of a number of cellular promoters. The exact mechanism of its activity is still elusive, as it varies greatly depending on the model system used (Bouchard and Schneider, 2004).

Its essential role in the establishment of HBV infection has been demonstrated both *in vitro* and *in vivo*, in the woodchuck model (Lucifora et al., 2011; Zoulim et al., 1994). It has been suggested that this effect is mediated by a role in the epigenetic activation of cccDNA (Belloni et al., 2009). Regulation of the nucleocapsid phosphorylation status by X has also been postulated to be important for the promotion of HBV replication (Melegari et al., 2005). Furthermore, X has been shown to trans-activate all HBV promoters (Moolla et al., 2002), particularly from extrachromosomal DNA templates, and not from integrated DNA (van Breugel et al., 2012).

The interaction of HBx with the host cell signaling pathways and its role in the pathogenesis of HBV infection have been a matter of debate throughout the years. Although it does not directly bind DNA, it can interact with transcription factors and hence regulate expression of host genes (Lara-Pezzi et al., 1998). Its interaction with

histone modification enzymes suggests a role in epigenetic regulation of gene expression (Tian et al., 2013). Finally, roles in cell cycle regulation, apoptosis, DNA-damage response and in the modulation of innate immune response have also been proposed (Wei et al., 2010a, 2010b). Through its multiple interactions with cell signaling pathways, HBx has been proposed to be involved in the development of HBV-associated HCC, although this is still debated (Fallot et al., 2012)

2.2.3. Viral life cycle

The complete HBV life cycle has been described to occur only in differentiated hepatocytes from a narrow range of species. For HDV, as explored in the corresponding chapter, hepatotropism and species specificity seem to be related only to the viral receptor (as, after entry or genome delivery, the virus can effectively replicate in a wide range of mammal cells, including murine). Conversely, for HBV, genome delivery is not sufficient and there seem to be other host factors essential for viral replication. Host and liver-specific transcription factors may notably be involved in RNA transcription from cccDNA (see Transcriptional regulation of the cccDNA below), although their role in earlier phases of HBV life cycle, previous to cccDNA establishment (as nucleocapsid migration and conversion of rcDNA to cccDNA) cannot be excluded (Seeger and Mason, 2015). Unlike HDV, HBV does not establish an infection in murine cells expressing human sodium taurocholate cotransporter peptide (hNTCP), the putative HBV/ HDV receptor, and, although it replicates in transgenic mouse models, no cccDNA can be detected (Guidotti et al., 1995; Yan et al., 2013). If such a restriction is due to the lack of specific factors or the presence of inhibitory elements has not been fully explored.

Given the very narrow range of susceptible and permissive hosts to HBV infection, a limited number of models is available for the study of HBV life cycle. *In vitro* studies have relied on transfection of hepatoma cell lines (not susceptible to natural infection due to the lack of viral receptor), primary hepatocytes (mainly human and tupaia) and, alternatively, on the HepaRG model (that, upon differentiation, allows a full replicative cycle, including the entry steps) (Gripon et al., 2002; Zeisel et al., 2015). An important body of evidence was collected *in vivo* using other hepadnaviruses, namely DHBV and

WHV. A discussion of the models currently available for the study of HBV and HDV infection is made in the HDV chapter.

Viral entry

Mechanisms of viral attachment and receptor-mediated entry are thought to be shared by HBV and HDV and are described in further detail in the HDV chapter.

The steps of HBV internalization after binding to the receptor are not completely clear. A pH-independent endocytosis mechanism has been proposed as likely, and both caveolin, on the one hand, and clathrin on the other have been suggested to be involved (Huang et al., 2012; Macovei et al., 2013).

Nuclear import and cccDNA formation

After release of the nucleocapsid in the hepatocyte cytoplasm, it is transported to the nucleus using the microtubular system and crosses the nuclear pore upon interaction of the core NLS with importins α and β. This model, depicted in Figure 8, postulates that the capsid disintegrates within the nuclear pore and releases rcDNA into the nucleus. Only mature, hypophosphorylated capsids would disintegrate, guarantying that only rcDNA, and not pgRNA, are imported into the nucleus (Kann et al., 2007).

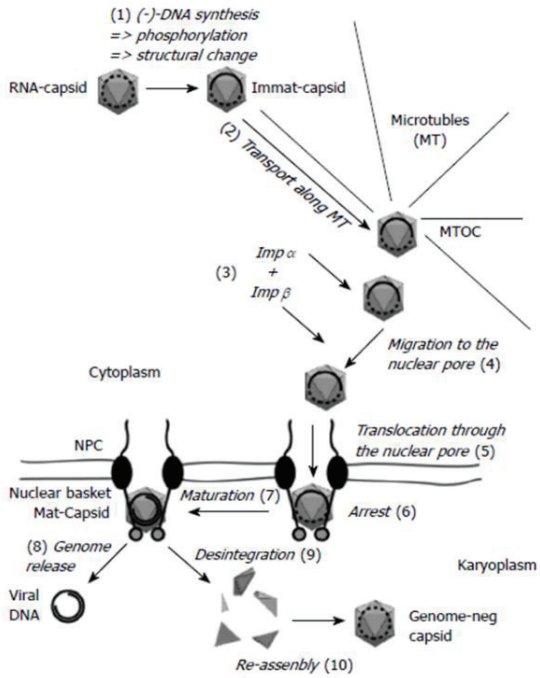

Figure 8. Nuclear trafficking of hepadnaviral nucleocapsids. Capsids are drawn as grey icosahedra. The nucleic acid found within the capsids is depicted as a dotted line (RNA) or a full line (DNA). Immat-Capsid, immature capsid; Mat-Capsid, mature capsid. Reproduced from (Kann et al., 2007).

Available evidence supports a model where cccDNA is synthetized from rcDNA (from both incoming virions and recycled capsids). For the formation of cccDNA, the rcDNA must undergo several steps, still not fully under understood (Levrero et al., 2009; Nassal, 2015), including (as represented in Figure 9):

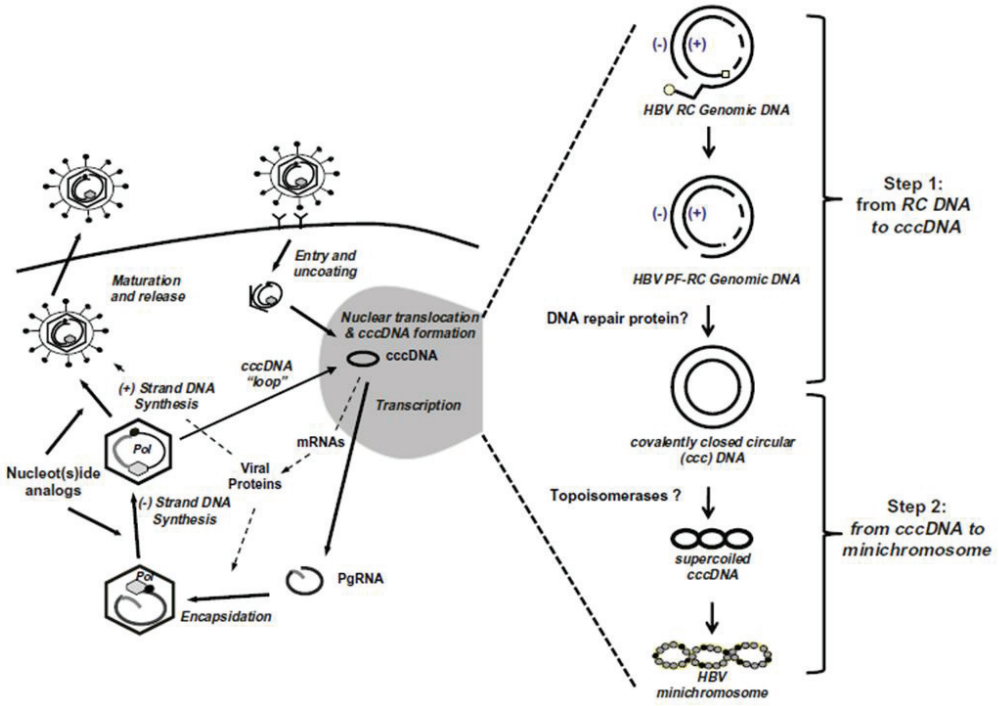

Figure 9. HBV cccDNA in the viral life-cycle. Reproduced from (Levrero et al., 2009).

1. Removal of the HBV polymerase, covalently linked to the 5' extremity of the negative strand of rcDNA. A recent study suggests that this step may be mediated by the host cell DNA repair machinery and Tyrosyl-DNA-phosphodiesterase 2 (TDP2) was shown to be one of the (but possible not the only) enzyme involved in this cleavage (Königer et al., 2014). The existence of an intermediate protein-free rcDNA (PF-rcDNA) has been suggested *in vitro* (Levrero et al., 2009);

2. Removal of the RNA primer covalently linked to the 5' end of the positive strand of rcDNA;
3. Removal of one copy of the terminal redundancy of the minus strand;
4. Completion of the plus strand to originate a full length double-stranded DNA. This step is not fully understood as it could be mediated by either HBV RT or a cell polymerase. The evidence that RT inhibitors do not preclude cccDNA synthesis, suggests that need for cellular factors (Seeger and Mason, 2015);
5. Ligation of the ends of both strands, also likely to be mediated by a cell enzyme.

Unlike retroviruses, HBV replication is independent from the integration of viral DNA into the host genome. However, integrated HBV DNA has been demonstrated in patients (it is thought to be present in 10-100% of hepatocytes from chronic carriers by the time they develop HCC). Integration seems to originate from a double-stranded linear DNA form – DSL DNA - that arises during rcDNA synthesis. The involvement of randomly integrated HBV in HCC development has been proposed but is not established (Seeger and Mason, 2015).

pgRNA transcription, encapsidation and retro-transcription

HBV RNAs are transcribed from cccDNA by the cellular DNA-dependent RNA polymerase II and hence all contain a 5' cap and a common polyA tail. They are next translated as cellular RNAs by the ER-associated ribosomes.

The binding of the polymerase to the Ɛ loop on the 5' end of the pgRNA from which it was translated, triggers the co-packaging of both molecules into nucleocapsids, through the interaction between the pgRNA molecule and the CTD of the core protein (Bartenschlager et al., 1990; Nassal, 2015). The polymerase conformational change induced by the interaction with the core protein would favor the initiation of retro-transcription. Retro-transcription of pgRNA first gives rise to the negative strand of rcDNA, followed by RNAse digestion of the template (with the exception of a small oligomer at the 5' extremity of pgRNA that will serve as a primer for the synthesis of the positive strand), as represented in Figure 10. The complete negative strand is then

used as a template for the synthesis of the positive strand. The reason why the positive strand is less than one genome long is unknown, but may be related to the depletion of nucleotides inside the capsid (Beck and Nassal, 2007). DSL DNA results from a defective retro-transcription process.

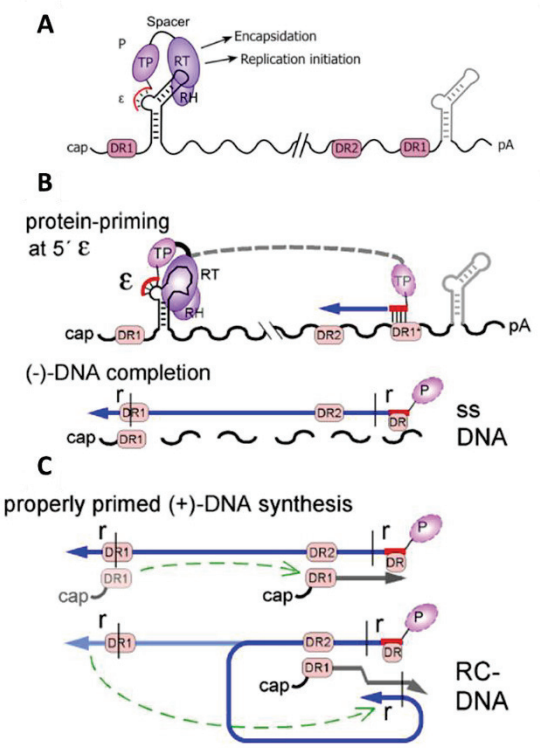

Figure 10. Essential steps of rcDNA synthesis. A. pgRNA organization and its interaction with the polymerase. Binding of the polymerase (P) to 5´ Ɛ, but not 3´ Ɛ, initiates pgRNA encapsidation and replication. **B.** Transfer of the RNA primer to DR2 is necessary for proprer DNA synthesis. **C.** Elongation. After reaching the 5' end of the (-)-DNA template, the small terminal redundancy (r) enables exchange against the sequence-identical 3' r sequence such that (+)-strand synthesis can go on to generate rcDNA. Reproduced from (Beck and Nassal, 2007; Nassal, 2015).

Retro-transcription is closely linked to capsid maturation. Capsids containing pgRNA are hyperphosphorylated (immature) while the retro-transcription process is associated with a progressive dephosphorylation of the core protein (mature capsids). This process has two direct consequences: on the one hand, the conformational changes induced by dephosphorylation allow only the mature capsid to interact with HBsAg, guarantying a correct viral secretion; on the other, phosphorylated immature capsids are stable and do not disintegrate upon interaction with nuclear pores, ensuring that only rcDNA is delivered to the nucleus for cccDNA synthesis, upon re-

circulation (Bruss, 2007; Kann et al., 2007). The regulation of the fate of mature capsids (viral assembly versus re-circulation) depends on the amount of L-HBsAg being synthesized (Summers et al., 1990).

Assembly and secretion

Viral envelope proteins, synthesized in the ER, face two major possibilities: they can either be used for nucleocapsid assembly (a process that is dependent on the presence of L-HBsAg) or be secreted as subviral particles. The cell secretion pathways involved seem to be different. For SVP secretion, envelope proteins are thought to be directed to the ER-Golgi intermediate compartment (ERGID) and from then to the Golgi secretory pathway. Secretion of virions was shown to occur through the multivesicular body (MVB) pathway (Huovila et al., 1992; Watanabe et al., 2007), as depicted in Figure 11.

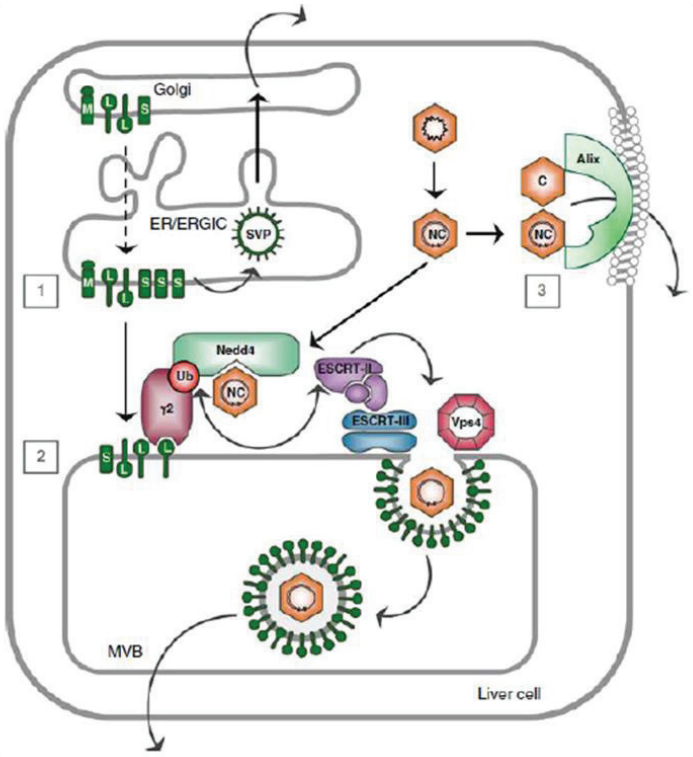

Figure 11. Hypothetical model of HBV particle morphogenesis, showing the release of SVP (through the Golgi - 1), virions (via MVB - 2) and naked capsids (3). Reproduced from (Prange, 2012).

Transcriptional regulation of the cccDNA

HBV DNA contains, as stated above, several regulatory sequences, including four promoters and 2 enhancers, as represented above in Figure 3, page 30.

Enhancer I (Enh I), a 270-bp major element of transcriptional regulation, is situated between ORFs PreS1/PreS2/S and X, partially overlapping the X promoter region. It particularly induces the activation of Core and X and more modestly that of PreS and S promoters. It has binding sites not only for ubiquitary transcription factors, as AP-1 and NF-κB, but also to liver-specific transcription factors, as HNF1, HNF3 and HNF4 (Moolla et al., 2002). An interferon stimulated response element (ISRE) has been described in the EnhI sequence and may underly a mechanism for interferon-induced regulation of HBV transcription, through the binding of STAT1 and STAT2 (Belloni et al., 2012; Tur-Kaspa et al., 1990). STAT3 has also been shown to fixate to EnhI and increase its activity.

Enhancer II (Enh II) is 105-bp long and is located upstream of the Core promoter and mostly induces PreS, S and X activity and to a lesser extent Core promoter activity. HNF3, HNF4 and Sp1 have be shown to also bind this region (Moolla et al., 2002). Interestingly the Core and X promoters seem to be stronger than the S1 and S2 promoters, likely reflecting their proximity to the enhancer elements.

Besides direct regulation of the enhancer and promoter regions, cccDNA transcription if further regulated through **epigenetic modifications** of both the DNA molecule and the attached histones. As previously mentioned, cccDNA is subject to chromatinization. It was seen to display the characteristic “beads-on-a-string” arrangement on electronic microscopy and to be organized into nucleosomes, although with a reduced spacing when compared to host cell chromatin (Bock et al., 2001; Newbold et al., 1995). The Core protein is a structural component of the HBV nucleosome. Histone proteins H3 and H2B were the most prominent species identified, but lower levels of histone proteins H4, H2A, and H1 were also detectable by immunoblotting. A number of cellular transcription factors (CREB, ATF YY1 STAT1

and STAT2) and chromatin modifying enzymes (PCAF, p300/CBP, HDAC1, SIRT1 and EZH2) have been shown, by Chromatin Immunoprecipitation (ChIP), to bind to the cccDNA in human hepatoma cells containing replicating HBV (Belloni et al., 2009; Pollicino et al., 2006). For some of the transcription factors known to interact *in vitro* with HBV enhancer elements, the association with the cccDNA minichromosome remains to be proven (Levrero et al., 2009). The amino-terminal tails of the four core histones on the nucleosome surface are subjected to a variety of enzyme-catalyzed, post-translational modifications, including lysine acetylation, lysine and arginine methylation, serine phosphorylation, ubiquitination and SUMOylation, associated with specific patterns of gene expression. Briefly, and as represented in Figure 12, histone acetylation is associated with transcription initiation, whereas reduced acetylation of histones H3 and H4 N-terminal leads to the recruitment of histone methyl-transferase and DNA methyl-transferase enzymes that results in repression of gene expression and heterochromatinization.

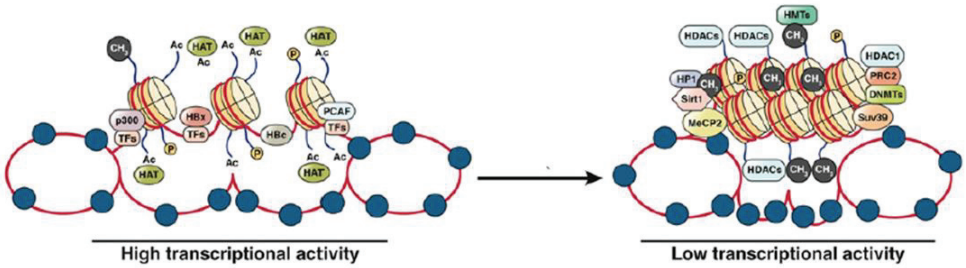

Figure 12. Proteins involved in cccDNA transcriptional regulation. CccDNA is organized as a minichromosome in the nucleus of the infected cells and is subject to epigenetic regulation. High transcriptional activity is associated a relaxed structure, histone acetylation and with the recruitment of proteins such as transcription factors (TFs), histones acetyltransferases (HAT), HBc and HBx. Conversely, during periods of low transcriptional activity, the chromatin is condensed, histones and DNA are methylated and there is recruitment of histone deacetylases (HDAC), histone methyltransferases (HMTs) and DNA methyltransferases (DNMTs). Adapted from (Zoulim et al., 2013).

The transcriptional activity of cccDNA has been show to vary throughout the course of the disease and to be modulated by immune factors, as interferon administration, and by the binding of X protein (Belloni et al., 2009, 2012; Levrero et al., 2009). Besides post-translational histone modifications, methylation of the CpG islands on HBV DNA also contributes to the regulation of HBV gene expression. At least six CpG islands have been identified in the HBV genome and their methylation status has been associated

with transcriptional repression, both *in vitro* and *in vivo* (Zhang et al., 2013). The most common chromatin modifications are summarized in Table I.

Table I. cccDNA epigenetic modifications. Type, site and function of known chromatin marks. Numbers within brackets identify the amino acid residues involved in specific modifications. Adapted from (Levrero et al., 2009)

Chromatin modifications		
Mark (DNA or histones)	**Site**	**Function**
DNA methylation		
Methylated cytosine (meC)	CpG islands	Repression
Histone Post-Translational modifications		
Acetylated lysine (K Ac)	H3 (9, 14, 18, 56) H4 (5, 8, 13, 16) H2A, H2B	Activation
Phosphorylated serine/threonine (S/T)	H3 (3, 10, 28) H2A, H2B	Activation
Methylated arginine (R Me)	H3 (17, 23), H4 (3)	Activation
Methylated lysine (K Me)	H3 (4, 36, 79) H3 (9, 27), H4 (20)	Activation
Ubiquitylated lysine (K Ub)	H2B (120) H2A (119)	Activation
Sumoylated lysine (K Su)	H2B (6/7), H2A (126)	Repression

2.3. The disease

2.3.1. Epidemiology

2.3.1.1. Prevalence and geographic distribution

HBV infection, both in its acute and chronic forms, is estimated to be responsible for 780000 deaths per year worldwide (being ranked 15th in the Global Burden of Disease Study) (Locarnini et al., 2015). It is estimated that 248 million people are chronically infected with HBV (translating into a global prevalence of 3,61%) (Schweitzer et al., 2015). Depending on HBsAg prevalence, endemicity is categorized as low (<2%), low-intermediate (2-4,9%), high-intermediate (5-7,9%) and high (≥8%), with a distribution that can be observed in

Figure **13**.

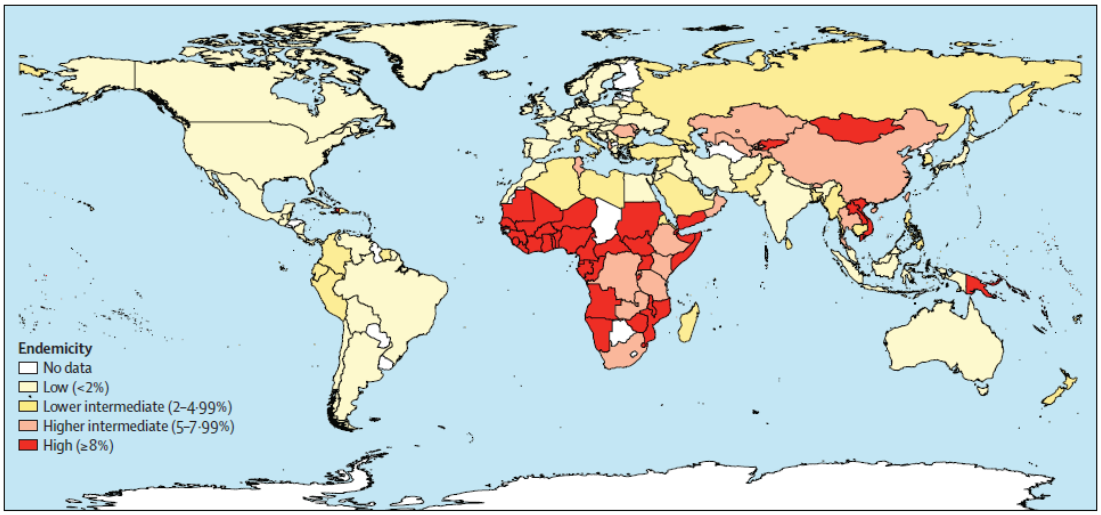

Figure 13. HBsAg global prevalence (1957-2013). Reproduced from (Schweitzer et al., 2015).

2.3.1.2. Molecular epidemiology and genotypes

The first classification of HBV was based on the antigenicity of the extracellular loop of HBsAg (antigenic loop) and it has been largely used for epidemiological studies. Briefly, it considers the major "a" determinant (residues 124-147) and two pairs of mutually exclusive determinants, d/y and r/w, to define 4 major serotypes (Dény and Zoulim, 2010; Norder et al., 1992).

Extensive sequence analysis has led to the definition of viral genotypes. HBV is considered to be divided into 8 genotypes (A-H), based on a nucleotide sequence dissimilarity of more than 7,5%. Two other Asian genotypes (I and J) have been proposed, but not yet approved by the ICTV. Genotypes can further be divided into sub-genotypes (dissimilarity greater than 4%) and recombinants have also been described (Dény and Zoulim, 2010; Locarnini et al., 2013). Geographical distribution of the genotypes is represented Figure 14.

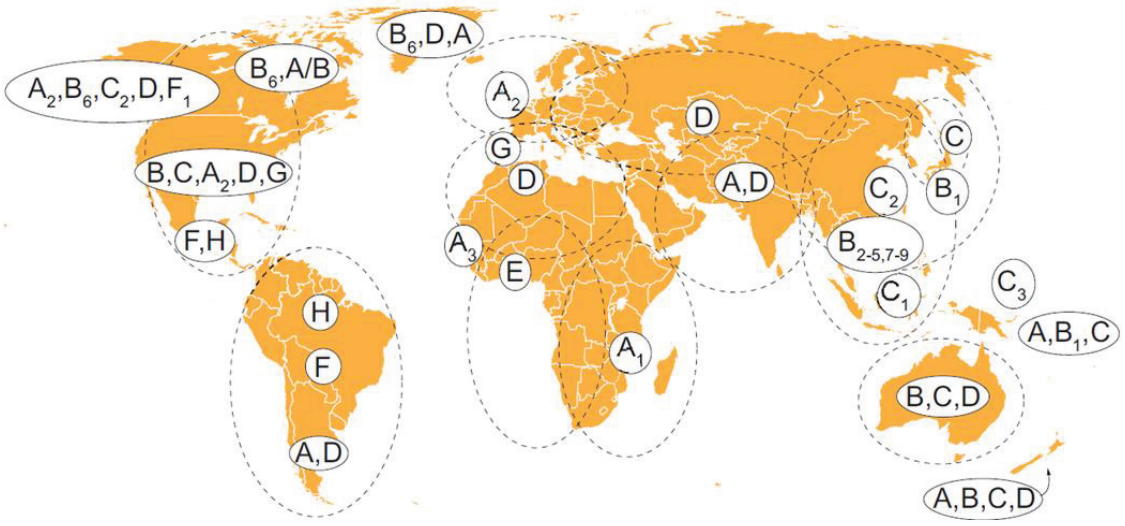

Figure 14. Geographical distribution of the HBV genotypes and sub-genotypes. Genotype I and J are not shown as they have not been ratified by the ICTV; genotype I is found in Southern China and Vietnam whilst genotype J was identified from a Japanese World War II person who lived in Borneo. Reproduced from (Locarnini et al., 2015)

Correlations between genotypes, clinical progression and treatment response have been established, although data are not clear enough to attribute to each genotype a severity predictive value. Genotype D has been particularly associated with mutations in the PreCore region that abolish the secretion of HBeAg (Dény and Zoulim, 2010; Howley, 2013). Finally, it should be noted that no correlation exists between the serotyping and genotyping classifications.

2.3.2. Transmission pathways

Two major patterns of HBV transmission are recognized: vertical and horizontal (Dény and Zoulim, 2010):

- **Vertical transmission** (mother to infant) is particularly important in the perpetuation of infection in highly endemic areas. It occurs during the peripartum or in the initial months after birth. No significant transplacental transmission has been demonstrated. Acute HBV infection in the third trimester, high viral load and HBeAg positivity are associated with the highest risk of transmission;

- **Horizontal transmission**, important in low endemicity areas, occurs mainly by parenteral exposure to infected blood (namely through intravenous drug use, occupational exposure, tattooing, blood transfusion or other medical procedures) or intimate contacts with exchange of bodily fluids (as sexual contacts) (Kidd-Ljunggren et al., 2006).

However, in about 40% of the adults with acute HBV infection, no specific route of transmission can be identified, reflecting the high infectivity of the virus.

2.3.3. Clinical features and natural history

After contamination, the incubation period ranges from 1 to 6 months.

Acute hepatitis B (lasting 1-2 weeks) is asymptomatic in 60-80% of the cases. Less than one third of the patients will display hyperbilirubinemia-related signs and symptoms. Fulminant hepatitis (acute liver failure) occurs in less than 1% of the cases (this proportion is increased in the setting of HDV coinfection as described below). Following acute infection, more than 90% of the children and 5% of the adults will progress to chronicity (Lai and Yuen, 2007).

Chronic hepatitis B (CHB) is defined by the persistence of HBsAg for more than 6 months. It can be schematically divided into 5 phases of variable duration and not necessarily sequential (European Association For The Study Of The Liver, 2012; Locarnini et al., 2015):

1. **''Immune tolerant''** phase is most common and more prolonged in patients infected perinatally or in early childhood. It is characterized by high viral replication (as evidenced by HBeAg positivity and high HBV viremia) in the absence of liver damage (normal alanine aminotransferase [ALT] levels, minimal liver histological activity). The concept of immune tolerance has been challenged, as evidence suggests that immune mediated liver damage, albeit

low level, exists during this phase (Bertoletti and Kennedy, 2015). The pathology of this phase is particularly difficult to analyze, given that, in the absence of markers of liver damage, liver biopsies are seldom performed;

2. **"Immune reactive"** phase corresponds to the maturation of a specific anti-HBV immune response, clinically translated by a decrease in viremia and an increase of ALT levels, as a consequence of hepatic necro-inflammatory activity. This phase culminates with HBe seroconversion;

3. **"Inactive carrier state"**. It's a low replicative phase following HBeAg to anti-HBe antibody seroconversion. It is characterized by very low or undetectable serum HBV DNA levels (below 2000 IU/ml), and normal serum aminotransferases, accounting for minimal hepatic necro-inflammatory activity. It is associated with a favourable clinical course. HBsAg loss and seroconversion to anti-HBs antibody may occur spontaneously in 1–3% of cases per year. However, viral infection is not resolved and progression to CHB (and its associated complications), may still occur;

4. **"HBeAg negative chronic hepatitis B"** represents a later immune reactive phase in the natural history of CHB. It is characterized by a pattern of fluctuating HBV DNA and aminotransferases levels and active hepatitis. HBeAg remains undetectable, due to a selection of pre-core and basal core promoter mutants;

A fifth stage or "HBsAg negative phase" can be considered, although these patients can be included in the broader definition of occult HBV infection. **Occult HBV infection** refers to the presence of HBV DNA in the liver (with or without HBV DNA detectable in serum) in the absence of HBsAg, as determined by the currently available assays (Kwak and Kim, 2014). In some patients this presentation may result from HBs escape mutants not detected by serologic assays, being associated with high HBV DNA titers. However, occult hepatitis B is most commonly a result of low HBV replicative activity, that may be associated with cccDNA epigenetic silencing (Levrero et al., 2009).

Untreated CHB patients will progress to develop fibrosis, cirrhosis and HCC, at variable rates, depending on disease activity. In the inactive carrier state, progression is slow, <1% per year, while in the immune reactive and HBeAg negative hepatitis phases, progression to cirrhosis can be as high as 2-10% per year. Overall, in CHB patients a cumulative risk of cirrhosis development ranges from 8 to 20%.

Cirrhotic patients present an increased risk of liver decompensation, HCC and death (European Association For The Study Of The Liver, 2012; Locarnini et al., 2015):

- The 5-year cumulative incidence of **hepatic decompensation** is approximately 20% for untreated patients with compensated cirrhosis. The overall 5-year mortality of HBV cirrhotic patients is 15%. Untreated patients with decompensated cirrhosis, however, have a 5-year mortality as high as 65-86%
- In cirrhotic patients, the 5-year cumulative risk of **HCC** varies depending of the region, being as high as 17% in East Asia and 10% in Western Europe and USA. The main risks factors for progression for HCC are HBeAg positivity (3,6 times higher incidence than HBeAg negative patients) and HBV DNA levels (identified as the main determinant of progression in the Taiwanese REVEAL-HBV study);
- Overall, the outcome of CHB is determined by both viral (HBV DNA levels, genotype, and particular mutation patterns) and host factors (age, gender, genetic background, immune status) (Liang, 2009).

2.3.4. Diagnosis

The diagnosis of HBV infection, both acute and chronic, is based on a combination of clinical signs and symptoms with biochemical, serologic, virologic and histological parameters. Most commonly used serologic and virologic markers and their significance are represented in Table II, whereas their evolution through the phases in infection is summarized in Figure 15.

Primary diagnosis of HBV infection relies on a combination of HBsAg and anti-HBc antibodies. If both are negative, infection by a wild type virus can be excluded, while, if at least one is positive, further evaluation is warranted.

Table II. Serologic and virologic markers used in the diagnosis of HBV infection. Adapted from (Liang, 2009).

HBV marker	Meaning
HBsAg	HBV infection (acute or chronic)
HBeAg	High-level HBV replication and infectivity; serologic marker for treatment response
Anti-HBc (IgM)	Acute HBV infection (high levels); could be seen in flare of chronic hepatitis B (low levels)
Anti-HBc (IgG)	Chronic or recovered HBV infection
Anti-HBs	Recovered HBV infection or marker of HBV vaccination; immunity to HBV infection
Anti- HBe	Low-level HBV replication and infectivity; marker for treatment response
HBV DNA	Level of HBV replication; virologic marker for treatment response
Combination of markers	**Meaning**
Anti-HBc (IgG) + anti-HBs	Past HBV infection; anti-HBs can be lost
Anti-HBc (IgG) + HBsAg	Chronic HBV infection
Anti-HBc (IgG) + absent HBsAg +/- anti-HBs +/- HBV DNA	Latent or occult HBV infection

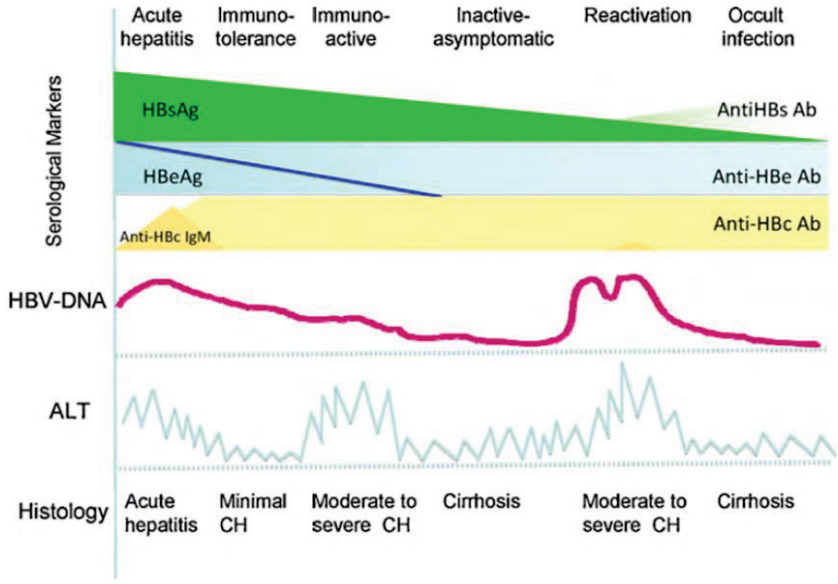

Figure 15. Natural course of chronic hepatitis B infection – evolution of serologic and virologic markers throughout the course of the disease. CH: chronic hepatitis. Reproduced from (Dény and Zoulim, 2010).

In a patient with signs, symptoms or laboratory features of acute hepatitis and positive HBsAg, the diagnosis of acute hepatitis B is confirmed by a high level anti-HBc IgM in serum (low levels of circulating IgM anti-HBc can be detected during periods of

reactivation of chronic infection). Serologic evaluation may be complemented by serum HBV DNA quantification by qPCR. Evaluation of a possible HDV coinfection is indicated at this point, as well as exclusion of other forms of acute viral hepatitis.

CHB is usually asymptomatic and often found during screening procedures, by detection of HBsAg. HBeAg, anti-HBe antibodies and viremia will vary, depending on the phase of the disease as mentioned above. Although HBe serologic status may be informative regarding the phase of the disease, decisions to treat are based in three factors that must be determined in all CHB patients: ALT levels, serum HBV DNA levels and the severity of liver disease. As for acute infection, exclusion of other forms of viral hepatitis is warranted, in particular HDV co- or superinfection (that will greatly influence treatment decisions), as is screening for Human Immunodeficiency Virus (HIV) infection (European Association For The Study Of The Liver, 2012).

Liver disease severity can be accessed by both invasive (liver biopsy) and non-invasive (FibroTest, FibroScan®) methodologies. Basic indicators of liver function include bilirubin, albumin, prothrombin time and platelet counts and will be abnormal in the presence of cirrhosis. The use of biochemical fibrosis scores and non-invasive methods for liver stiffness evaluation, as means of avoiding liver biopsy, has attracted much interest, although further evaluation and validation of their use in HBV infected patients are needed (Branchi et al., 2014). Liver biopsy is still useful in cases where diagnosis or treatment indications are not clear. However it is usually not required in patients with clinical evidence of cirrhosis or in those in whom treatment is indicated irrespective of the grade of activity or the stage of fibrosis (European Association For The Study Of The Liver, 2012).

2.3.5. Pathogenesis

Progression to HCC

The association between HBV infection and HCC has been known since the early days that followed HBV discovery (Blumberg et al., 1975). HBV infected patients were estimated to have a 233 fold increased risk of HCC, when compared to healthy

individuals (Beasley et al., 1981). Posterior data supported this link and led to the classification of HBV as an oncogenic virus.

The process conducting to the development of HCC in CHB patients is multifactorial and both immune response (associated with hepatocyte death and regeneration) and viral factors are involved. The latter would include: i) the transactivator role of HBx, and its involvement on the epigenetic regulation of cell transcription ; ii) the induction of oxidative stress in the infected cells, associated with the accumulation of envelope proteins (notably in the presence of PreS2 mutations, that lead to the synthesis of a truncated M-HBsAg); iii) the random integration of viral DNA in the host cell genome, that was shown to occur in transcriptionally active regions, in the proximity of cell genes (Fallot et al., 2012; El-Serag, 2012). These factors can help explaining the few cases of HCC that occur in HBV infected patients in the absence of cirrhosis. However, in the majority of the patients, HCC does develop after decades of infection and is associated with liver fibrosis, underscoring the importance of liver inflammation and regeneration in the pathogenic process. NF-kB activation, oxidative stress, cytokine production and hepatocyte killing by cytotoxic T lymphocytes (CTL) (Howley, 2013) are involved. Importantly, it is worth noting that nucleos(t)ide analogue-based therapies, which are associated with long-lasting viro-supression (*i.e.* no detectable viremia, but almost no effect of intrahepatic cccDNA and transcription) in CHB patients, do not abrogate the risk of developing HCC. Indeed, in large Asiatic cohorts of patients it was shown that the incidence was reduced and delayed but did not return to baseline (Hosaka et al., 2013).

Immune response to HBV infection

HBV infection is associated with a modulation of both innate and adaptive immune responses. Throughout the years, research has focused mainly on the role of the adaptive immune response in the control of HBV and no induction of innate immune responses was thought to occur in response to the infection. Recent data now challenge the concept that HBV is a stealth virus.

Modulation of the innate immune response modulation by HBV

The control of HBV infection mostly lays in the induction of a cellular and adaptive response. However, the importance of the innate immune response to HBV infection was first suggested by an *in vivo* study in the chimpanzee model, showing that acute infection was cleared by a non-cytolytic mechanism before the establishment of an adaptive response (Guidotti et al., 1999). A subsequent study from the same group further indicated that, in HBV infected chimpanzees, in contrast to HCV and HIV infected animals, no activation of an innate immune response could be detected (Wieland et al., 2004). Furthermore, only low levels of interferon (IFN) were shown to be present in the serum of patients with acute HBV infection (Dunn et al., 2009). Overall, these observations led to the definition of HBV as being a "stealth virus", not recognized by the innate immune system (Wieland and Chisari, 2005).

Over the years, it has, however, become clear that, more than not being recognized, HBV could in fact inhibit the host antiviral immune response at several levels:

- Data obtained *in vitro* by the use of a baculovirus containing HBV genome have suggested that HBV replication may indeed induce an interferon response, implying that inhibitory mechanism must be in place in the case of natural infection (Luangsay et al., 2015a; Lucifora et al., 2010);
- Recent data from the humanized mouse model, suggests a low, yet detectable, basal rate if ISG expression by HBV (Lütgehetmann et al., 2011);
- Early data suggested that HBc was able to suppress IFNβ expression in infected cells, as well as inhibit the expression of ISGs. These data have recently been approached in our laboratory, further suggesting the active inhibitory role of HBV core protein (Fernández et al., 2003; Gruffaz et al., 2013; Twu et al., 1988);
- Inhibition of Jak-STAT signaling has been proposed as a mechanism of interferon resistance (Christen et al., 2007). Such modulation has been proposed to be mediated by the viral polymerase (Wu et al., 2007);
- The viral polymerase has further been suggested to inhibit IFNβ expression in response to TLR3 stimulation (Wang and Ryu, 2010);

- A similar mechanism of inhibition has been proposed to be mediated by HBx, inhibiting IFN production in response to RLR stimulation (Wei et al., 2010a).

The concept of "invisibility" of HBV is hence slowly being replaced by a model of early recognition and active inhibition of the innate immune response by the virus.

2.4. Prevention

The history of the HBV vaccine goes hand in hand with the history of HBV discovery itself. After the identification of HBsAg containing SVPs, purification and inactivation of carriers' plasma led to the development of a first vaccine. The first clinical trials were completed by 1981, demonstrating safety, effective induction of an humoral response (in nearly 100% of the patients) and protection against HBV infection in comparison to non-vaccinated controls (Szmuness et al., 1981). Three years after, the first wide-range vaccination program was started in Taiwan (an area of high HBV endemicity, associated with vertical transmission), It was based on universal screening of pregnant women for HBV infection and vaccination of neonates born to HBsAg mothers (Chen et al., 1987).

Given the growing difficulties in fulfilling the high plasma requirements for the production of the inactivated vaccine, an alternative strategy led to the development of a recombinant vaccine (produced in yeast) that was licensed in 1986 and that is currently used (Hilleman, 2000).

Global HBV vaccination coverage with 3 doses of hepatitis B vaccine is estimated at 81% of the children worldwide and a total of 183 countries had already introduced a nationwide hepatitis B vaccination program for infants by the end of 2013 (http://www.who.int/mediacentre/factsheets/fs378/en/), representing an amazing evolution in the fifty years since the identification of the virus. The Taiwanese experience offers an invaluable proof of the impact of vaccination with 91% decrease in HBsAg carriage in people aged <25 years old, 80% decrease in the incidence of HCC in patients younger than 30 years old, and 90% decrease in the infant mortality from fulminant hepatitis (Locarnini et al., 2015).

Prevention of vertical transmission is based on the combination of passive (hepatitis B immunoglobulin - HBIg) and active immunization (HBV vaccination) of the neonate at the time of birth (European Association For The Study Of The Liver, 2012). Such a strategy has led to a significant decrease in vertical transmission rates with an impact in the number of infections and complications (as stated above). However, a proportion of newborns from highly viremic (serum HBV DNA $>10^6$–10^7 IU/ml), mostly HBeAg positive mothers, still present a >10% risk of vertical HBV transmission despite HBIg and vaccination (that can increase to more than 30% with maternal viremia > 10^9 IU/ml) (Chen et al., 2012; Wen et al., 2013). It has been shown that the initiation of antiviral therapy in the third trimester in highly viremic women leads to a further decrease in vertical transmission in comparison to immunization alone (Han et al., 2011a). Current treatment guidelines hence postulate initiation of antiviral therapy in the third trimester for women with viral load> 10^6 IU/ml (European Association For The Study Of The Liver, 2012).

Besides the strategies for transmission prevention just discussed, prevention of HBV reactivation or disease exacerbation is warranted in the setting of predictable immune suppression (as the initiation of immune modulatory therapies, anti-cancer chemotherapy and transplantation) and is achieved by the instauration of pre-emptive therapy with DAAs. Furthermore, for HBV infected patients subjected to liver transplantion, prevention of graft infection may be achieved by a combination of antiviral treatment and passive immunization (with HBIg) (European Association For The Study Of The Liver, 2012).

2.5. Treatment

2.5.1. Current available therapeutic strategies

Whereas no specific therapy is indicated for acute hepatitis B, current CHB treatment is based on the utilization of 7 available drugs, including two formulations of IFNα (conventional and pegylated) and 5 nucleos(t)ide polymerase inhibitors (lamivudine,

telbivudine, adefovir dipivoxil, tenofovir disoproxil fumarate and entecavir). Data on the efficacy of antiviral drugs are compiled in Table III.

Table III. Outcomes of the most commonly used HBV therapies, evaluated at 48 weeks of treatment. Adapted from (Zoulim and Durantel, 2015).

	Entecavir	Tenofovir	PEG-IFN α-2a
HBeAg positive	n = 354	n = 176	n = 271
HBV DNA undetectable	67%	76%	25%[a]
HBeAg seroconversion	21%	21%	27%
ALT normalization	68%	68%	39%
HBsAg loss	2%	3.2%	2.9%[b]
HBeAg negative	n = 325	n = 250	n = 177
HBV DNA undetectable	90%	93%	63%[a]
ALT normalization	78%	76%	38%
HBsAg loss	0.3%	0%	0.6%[b]

IFNα has long been known to have an effect on HBV infection (Greenberg et al., 1976) and has been approved for the treatment of CHB in the early nineties. It is currently recommended for HBeAg positive patients with low viral loads and persistent liver necro-inflammatory activity (as evidenced by elevated aminotransferases).

The definitive mechanism of inhibitory effect of IFNα on HBV infection is not established and a number of possibilities have been suggested. Replication inhibition has been reported to be mediated by (non-exhaustive list):

- blockage of RNA-containing core particle formation (Wieland et al., 2000);
- accelerated decay of replication-competent core particles (Xu et al., 2010);
- degradation of the pgRNA (Li et al., 2010);
- MxA mediated inhibition of nuclear export of HBV mRNAs (Gordien et al., 2001);
- inhibition of HBV transcriptional activity, associated with repression of enhancer 1 activity (Rang et al., 1999; Tur-Kaspa et al., 1990; Uprichard et al., 2003);
- epigenetic regulation of cccDNA transcriptional activity (Belloni et al., 2012; Liu et al., 2013);

- non-hepatotoxic degradation of cccDNA (Lucifora et al., 2014);

Besides a direct effect on hepatocytes, IFNα was shown to activate natural killer (NK) and NK-T cells in HBV infected patients (Micco et al., 2013), while an induction of specific TCD8 + cells was noted in another study (Chen et al., 2010).

DAAs directly inhibit HBV polymerase by mimicking natural nucleotides and are incorporated into newly synthesized HBV DNA leading to chain termination (Koumbi, 2015). They contribute to a very slow (*i.e.* decades) decrease of the amounts of cccDNA, hypothesized to be associated with the impairment in mature nucleocapsid recirculation, with no impact on the establishment of the initial pool of cccDNA (that will not be degraded) (Werle-Lapostolle et al., 2004). These molecules can be classified into 3 groups, according to their structure (Zoulim and Durantel, 2015):

- **L-nucleosides** as lamivudine (3TC) and telbivudine;
- **Acyclic phosphonates**, including adefovir dipivoxil (ADF) and tenofovir disoproxil fumarate (TDF);
- **Cyclopentanes**, represented by entacavir (ETV).

Contrary to interferon therapy, that is limited in time (recommended duration of 1 year), no current stopping strategy has been possible for NAs and relapses (associated with clinical exacerbations) follow therapeutic interruption, meaning that NAs treatment with must be kept lifelong. The problem of cumulative resistance mutations, first evidenced with 3TC, seems to have been circumvented with the development of ETV and TDF, as shown on Figure 16.
Currently, and based on their potency and favorable resistance profile, entecavir and tenofovir are the main choices (European Association For The Study Of The Liver, 2012).

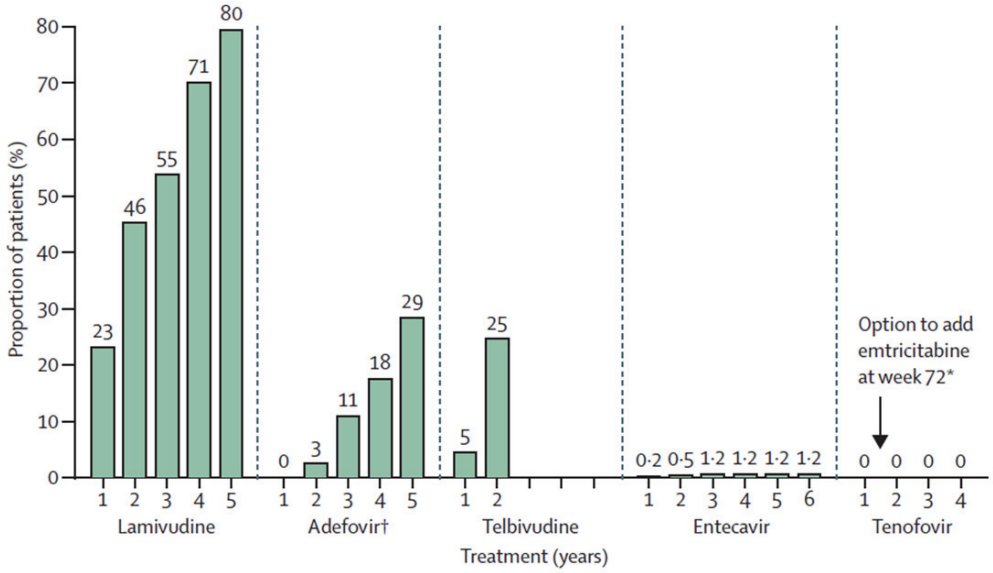

Figure 16. Cumulative annual incidence of resistance among patients who are nucleos(t)ide analogue naïve. Reproduced from (Gish et al., 2012).

2.5.2. Investigational drugs in preclinical/ clinical development

Despite the existence of an effective and safe vaccine and several therapeutic options that manage to control viral replication, a main challenge remains: no current therapy leads to viral eradication and the rates of HBs seroconversion (translating a "functional cure") remain very low (Zeisel et al., 2015). With increasing evidence of success of HCV new therapeutic approaches and the development of new and exciting study models, the prospect of developing curative treatment strategies for HBV infection is now being vigorously approached (Block et al., 2015).

The main focus is on the inhibition of cccDNA, either by preventing its formation, promoting its degradation or silencing its transcription. However, molecules targeting other steps of the viral cycle and new polymerase inhibitors are also in development. Drugs undergoing clinical and preclinical development are summarized in Table IV.

Table IV. Drugs undergoing clinical and preclinical development for the treatment of HBV infection. Reproduced from (Zeisel et al., 2015).

	Targets	Compounds	Stage of development
DAAs	HBV capsid	Phenylpropenamide derivatives Heteroaryldihydropyrimidines	Predinical and early dinical phase Morphothiadine mesilate (GLS4) in phase II
	rcDNA-cccDNA conversion	Disubstituted sulfonamide	Predinical
	cccDNA	DNA cleavage enzymes	Predinical
	HBV RNA	siRNA antisense	ARC-520 in phase II ISIS-HBVRx in phase I
HTAs	NTCP	HBV preS1-derived lipopeptide cyclosporine A, ezetimibe	Myrcludex-B in phase II FDA approved but not tested for HBV
	Host factors involved in HBV secretion and budding	Iminosugar derivatives of butyldeoxynojirimycin and related glycolipids	Predinical
		α-glucosidase inhibitors	Predinical
		triazol-o-pyrimidine derivatives	Predinical
		benzimidazole derivative	Predinical
		phosphorothioate oligonucleotides	REP 9 AC in phase II
	Innate immune responses	LTβR agonists	Predinical
		TLR7 agonists	Phase II
		thymosin α1	Phase IV
		Nitazoxanide	Phase I
		interleukin-7	Phase I/II
		IFN-λ	Phase II
	Adaptive immune responses	PD1 blockade	Phase I/II for HCC
		X-S-Core proteins (antigen-based vaccine) HBV DNA (DNA-based vaccine)	GS-4774 in phase II, DV-601 in phase I DNA vaccine pCMVS2.S in phase I/II

Entry inhibition

Myrcludex® B has been developed as a myristoylated peptide that competitively blocks the interaction between HBV and HDV envelope and hNTCP. As it has a mechanism of action targeting both HBV and HDV, it will be further discussed in the HDV chapter.

cccDNA destabilization

Molecules targeting cccDNA formation or mediating its degradation have been identified in preclinical studies and it is tempting to think that they may evolve into clinical applications. Examples are:

- Disubstituted sulfonamide compounds were shown to specifically inhibit cccDNA formation in vitro (Cai et al., 2012);
- Enzymes and cytokines capable of inducing non-cytolytic cccDNA degradation have been identified. Examples include zinc-finger nucleases and the CRISP/

Cas9 system, and agonists of the lymphotoxin beta receptor, all still in preclinical evaluation (Lucifora et al., 2014; Weber et al., 2014).

Transcriptional repression

As evidence accumulates that cccDNA transcription is subject to epigenetic regulation, the hypothesis of using small molecules targeting chromatin modifying enzymes is tempting, even more as some compounds, like valproate, are already used in the clinics for other indications. Strategies can include induction of histone hypoacetylation and induction of DNA and specific histone methylation (Levrero et al., 2009).

Another possible strategy involves the use of siRNAs targeting HBV transcripts that would be delivered to hepatocytes in polymer formulations (Wooddell et al., 2013).

Encapsidation inhibition

The HBV nucleocapsid plays a central role in HBV replication. Besides being essential for HBV genome packaging, is also necessary for effective reverse transcription and intracellular trafficking of viral pgRNA and rcDNA and consequently the maintenance of chronic infection from the cccDNA pool (resulting from genome recirculation). Its use as a therapeutic target has been explored for more than one decade, and two classes of molecules have been developed so far:

- **Heteroaryldhydropyrimidines** (HAP) – HAPs have been described to have a complex mechanism of action that varies depending on the used dose. At low doses these molecules accelerate assembly and induce the formation of aberrant particles, whereas at higher doses they accelerate capsid degradation by the proteasome (Bourne et al., 2008). Efficacy of these compounds has been demonstrated in the humanized mouse model and the first developed molecule has entered clinical evaluation (Brezillon et al., 2011). No cross resistance with currently available NAs has been demonstrated;

- **Phenylpropenamide derivatives** – these molecules specifically target pgRNA encapsidation, by promoting capsid formation in the absence of pgRNA/polymerase complexes. Despite having no effect on the overall levels of core protein function, an effect is noticed in the amount of cccDNA. Furthermore, a synergistic effect with 3TC has been suggested (Feld et al., 2007; King et al., 1998).

Viral assembly inhibition

Several molecules are in preclinical evaluation regarding their effect on the inhibition of viral assembly and release (Block et al., 2015). A particular attention should be drawn to the clinical development of nucleic-acid-polymers (NAPs) that, in preclinical models were shown to inhibit HBsAg secretion and hence viral particle formation (Noordeen et al., 2013a, 2013b). Further details are discussed in the 3. Hepatitis delta chapter.

Immunomodulation

Several immunomodulatory strategies are undergoing clinical evaluation for the control of HBV infection and include interferons, cytokines, Pattern Recognition Receptors (PRR) agonists and therapeutic vaccines (Isorce et al., 2015).

Besides IFNα, inhibition of HBV replication can be achieved *in vitro* and animal models with both interferon gamma and lambda (Pagliaccetti et al., 2010; Shi et al., 2012). Several cytokines have been considered for HBV treatment and are at diverse phases of preclinical and clinical evaluation. IL-1β has been shown to have a potent anti-viral effect against HBV (Isorce et al., 2014; Watashi et al., 2013). IL-12, in combination with 3TC, was shown to increase HBV-specific T cell activity and to induce HBeAg seroconversion (Rigopoulou et al., 2005).

Tymosin-alpha-1 is a synthetic polypeptide that has immunomodulating activity and has been shown to promote T cell activity, IFN gamma and IL12 production, as well as NK-induced cytotoxicity. Preclinical and clinical studies have been conducted on anti-HBV effects and addition of thymosin-α1 seems to improve the response to 3TC

therapy, but adds no benefit to IFNα monotherapy (Kim et al., 2012; Zhang et al., 2009).

Active clinical and preclinical research is currently underway to explore the role of PRR stimulation in the inhibition of HBV infection. This strategy may be associated with a double effect, as it will not only lead to the establishment of an antiviral state in the infected hepatocytes, but also to the activation of the innate immune cells, like NK and dendritic cells. A first report of efficacy was made for the activation of Toll-like Receptor (TLR) 3 by poly (I:C) in the transgenic mouse model (Uprichard et al., 2003) and followed by the identification of an antiviral effect of the activation of other TLRs (notably TLR2, TLR4, 5, 7 and 9) (Isogawa et al., 2005; Zhang et al., 2012). In parallel to TLR activation, stimulation of intracellular RIG-I like receptors (RLRs) was also seen to have an inhibitory effect on HBV replication.

Very promising results are currently being obtained with TLR7 agonizing strategies. GS-9620 has been shown to induce a significant decrease of HBV replicative intermediates (including cccDNA and HBsAg) and to have a good safety and tolerability (Fosdick et al., 2014; Lanford et al., 2013; Menne et al., 2015; Roethle et al., 2013). Recruitment for a phase II clinical trial is now underway (NCT02166047).

Finally, several strategies are being developed to modulate acquired immune response, namely PD-1 blockage by the administration of monoclonal antibodies, aiming for restoration of T-cell function and therapeutic vaccines, either antigen- or DNA-based are undergoing clinical development (Koumbi, 2015; Zeisel et al., 2015).

3. Hepatitis delta

The text and the original illustrations contained in this chapter constitute a review article that has been published in *Antiviral Research* – see pdf in Appendix 1.

3.1. Introduction

3.1.1. Discovery of Hepatitis D virus

Hepatitis D virus (HDV), the virus causing hepatitis delta, was first identified in 1977 by Mario Rizzetto and colleagues, in a cohort of HBV infected patients who experienced a severe hepatitis (Rizzetto et al., 1977). Some of these patients' liver biopsies were found positive after staining by anti-sera from some HBsAg positive patients, in the absence of HBV Core protein (HBcAg), as evidenced by electron microscopy and negative staining with monoclonal specific antibodies. This novel 'antigen-antibody system' (called δ antigen/anti-δ antibodies), although associated with HBV infection, was immunologically different from HBsAg, HBeAg, and HBcAg. The δ antigen (HDAg) isolated from these initial patients was then instrumental to screen the sera of other patients of diverse geographic origins and associate the presence of circulating anti-δ antibodies to chronic liver disease (Rizzetto et al., 1979, 1980a). The δ antigen was later confirmed to be associated with a transmissible pathogenic 'delta agent', now called HDV. Indeed, the serum from patients with δ-positive hepatitis could infect chimpanzees in the presence of its helper HBV both in co- or superinfection conditions (Rizzetto et al., 1980b). In these chimpanzees, δ antigen was shown to circulate as particles, containing HBsAg and a low molecular weight RNA molecule, raising for the first time the hypothesis of HDV being an RNA satellite virus of HBV (Rizzetto et al., 1980c).

3.1.2. Clinical and virological relevance

Almost 40 years after discovery, HDV and hepatitis D remain challenging entities to both clinicians and researchers. The impact of the disease, mostly present in low-

income countries, is largely neglected. Despite being considered as the most severe form of chronic hepatitis, HDV infection is still under diagnosed, either by lack of awareness or appropriate diagnostic tools, and available treatments are still largely ineffective. Although significant advances have been made in understanding the cellular and molecular virology of HDV, they have failed to translate into the development of effective therapeutic strategies. Explanations stem from the peculiarity of HDV life cycle, which features no virally-encoded enzymatic activity and completely relies, not only on cell machinery as other viruses, but also on HBV for spreading.

3.2. The virus

3.2.1. Origin of HDV

HDV is a 35-37 nm diameter virus featuring a small single-stranded, circular RNA genome of 1672-1697 nucleotides, the smallest among mammalian viruses. Its particular characteristics still pose unanswered questions regarding its origin. Currently, two main theories are considered: HDV may have evolved from plant viroids or/and host cell pre-mRNA via the splicing machinery (Taylor, 2014).

Plant viroids are considered the lowest entities in the biological scale in terms of genome complexity. More than 30 species have been described so far, that have in common being constituted of a small single stranded, often circular, RNA molecule (250-400 nucleotides), having no coding capacity, and being replicated by host cell enzymes. Two families of viroids are considered: *Pospiviroidae* (represented by the potato spindle tuber viroid) and *Avsunviroidae* (represented by the avocado sunblotch viroid). HDV has RNA structural and replicative features in common with both families: its rod-like RNA structure and nuclear replication relate to *Pospiviroidae,* whereas the existence of a ribozyme and the symmetrical rolling circle mechanism remind *Avsunviroidae.* These common features, added to the fact that RNAs from HDV and plant viroids interact with homologous cellular proteins, and the experimental preliminary, yet not clearly confirmed by others, evidence that HDV might replicate and spread after inoculation of tomato seedlings' leaves, suggest an analogy between

HDV and plant viroids. This hypothesis, however, leaves unanswered questions such as the origin of the delta antigen and the relation between HDV and HBV, its helper virus (Flores et al., 2012).

A second theory, which can be complementary to the first one, suggests that HDV may have evolved from the host cell transcriptome. This vision was supported by studies showing that ribozyme RNA with similar secondary structure and biochemical properties to the HDV ribozyme can be found in human cells (e.g. within an intron of the CPEB3 gene). However, pseudoknot ribozymes were later found in all life kingdoms (except Archaea) and in insect viruses (Salehi-Ashtiani et al., 2006; Webb et al., 2009). A host cell origin has also been proposed to the delta antigen. Indeed a protein, named DIPA (as delta interacting protein A), was initially described as a potential candidate. Although the actual relevance of its homology has later been dismissed, DIPA might still be a cellular HDAg partner (Brazas and Ganem, 1996; Long et al., 1997; Taylor and Pelchat, 2010).

An integrated model suggests that HDV might have arisen from a re-combination event between a viroid like element and a cellular pre-mRNA/mRNA (Robertson, 1996).

3.2.2. Viral structure

Role of HBV envelope proteins in HDV assembly

Firstly characterized from sera of chimpanzees experimentally infected with the serum from an Italian chronic carrier, HDV was shown to be a HBsAg enveloped particle of 35 to 37 nm (Figure 17), with a buoyant density of 1,25g/cm^3 in cesium chloride gradient and a sedimentation coefficient value intermediate between the one from the HBsAg empty SVPs (22 nm) and the HBV virion (Dane particle, 42 nm) (Rizzetto et al., 1980c).

HDV is indeed a defective virus-like particle that uses an envelope composed of the three forms of HBV glycoproteins (small (S or S-HBsAg), medium (M or M-HBsAg) and large (L or L-HBsAg) HBV surface proteins) to egress from and re-enter into hepatocytes, thus conferring the same tropism to both viruses.

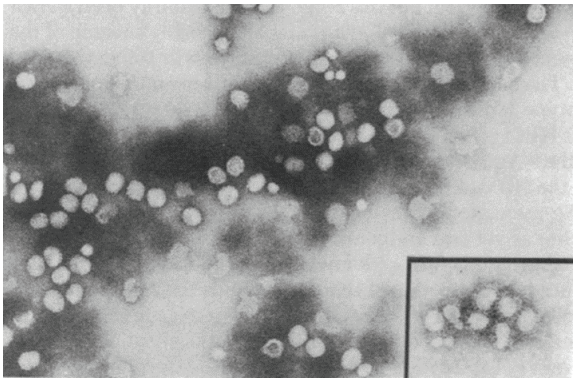

Figure 17. Electron microscopy of δ-associated particles. Both a predominant 35- to 37-nm particle and a few 22-nm forms of HBsAg were observed (magnification 70500X). Reproduced from (Rizzetto et al., 1980c).

The three forms of HBsAg share a common C-terminus, as discussed in the HBV Viral proteins section, and represented in Figure 18.

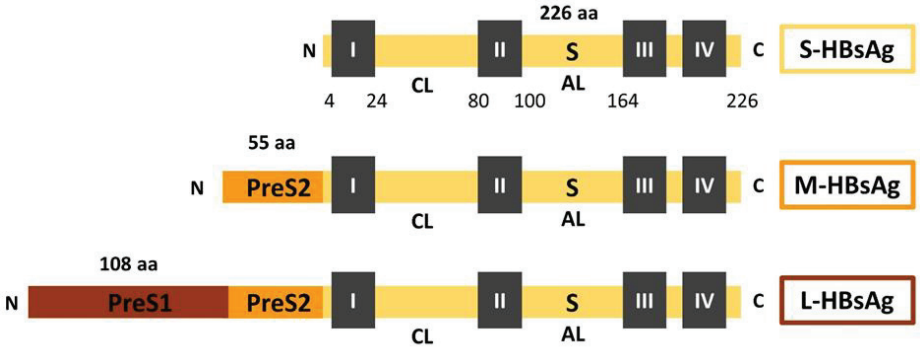

Figure 18. HBV envelope proteins. The three forms of HBsAg share the same C-terminus (S) containing four putative transmembrane domains (filled grey rectangles I-IV), a cytosolic loop (CL) and an antigenic loop (AL). As translation initiates in different in-frame sites, besides S (the only domain of S-HBsAg), M-HBsAg contains PreS2 and L-HBsAg further contains the 108 aa of PreS1. HDV assembly is dependent on the contact of the CL with HDV RNP, whereas HBV's capsid's contact with HBsAg is established with the PreS region.

In addition to the S domain, M-HBsAg contains an N-terminal hydrophilic domain named PreS2, and, relative to M-HBsAg, L-HBsAg has an additional domain named PreS1 (Urban et al., 2014). L-HBsAg is essential for the assembly and infectivity of HBV, although not sufficient, and S-HBsAg is needed for the release of particles from cells (Gudima et al., 2007). In contrast, HDV can be assembled by S-HBsAg alone, but without L-HBAgs, the particle is not infectious (Sureau et al., 1993). These distinct

budding requirements are explained by different binding domains of the cytosolic loops of the envelope proteins to HBV nucleocapsid and HDV ribonucleoprotein (Sureau et al., 1994). A recent study has shown that the assembly (and infectivity) of HDV genotype 1 (HDV1) is not restricted to a particular HBV genotype (Freitas et al., 2014). It can also occur with woodchuck, bat and woolly monkey hepadnavirus envelopes (Barrera et al., 2004; Drexler et al., 2013; Ryu et al., 1992).

Viral RNAs

The HDV virion contains a circular single-stranded negative RNA genome, with a strong secondary flexible-quasi double-stranded RNA conformation. During replication in infected cells, two other main viral RNAs can be detected: the genomic complementary molecule, called antigenome, and the HDV mRNA (Figure 19). Constituted of only 1672-1697 nt, the HDV genome is the smallest of all known mammalian viruses and presents some similarities with plant viroid counterparts. It has a high C+G content (60%) and about 74% intra base pairing allowing it to fold into a rod-like structure (Kuo et al., 1988a; Wang et al., 1986). Infected cells may contain about 300,000 molecules of HDV genome, divided between nucleus and cytoplasm, thus indicating a high level of replication (Chen et al., 1986; Macnaughton and Lai, 2002).

The HDV antigenomic RNA is a replicative intermediate of positive polarity, complementary to the genome sequence and contains the coding sequence for HDAg. It is 5-22 times less abundant than the genome, found exclusively in the nucleus of infected cells and therefore not packaged into virions (Chen et al., 1986; Macnaughton and Lai, 2002). HDV proteins are translated from a specific 800nt length HDV mRNA (Lo et al., 1998), which is transcribed by host DNA-dependent/RNA-polymerase II and matured (*i.e.* capped and polyA-tailed), as cellular mRNAs (Gudima et al., 2000; Hsieh et al., 1990).

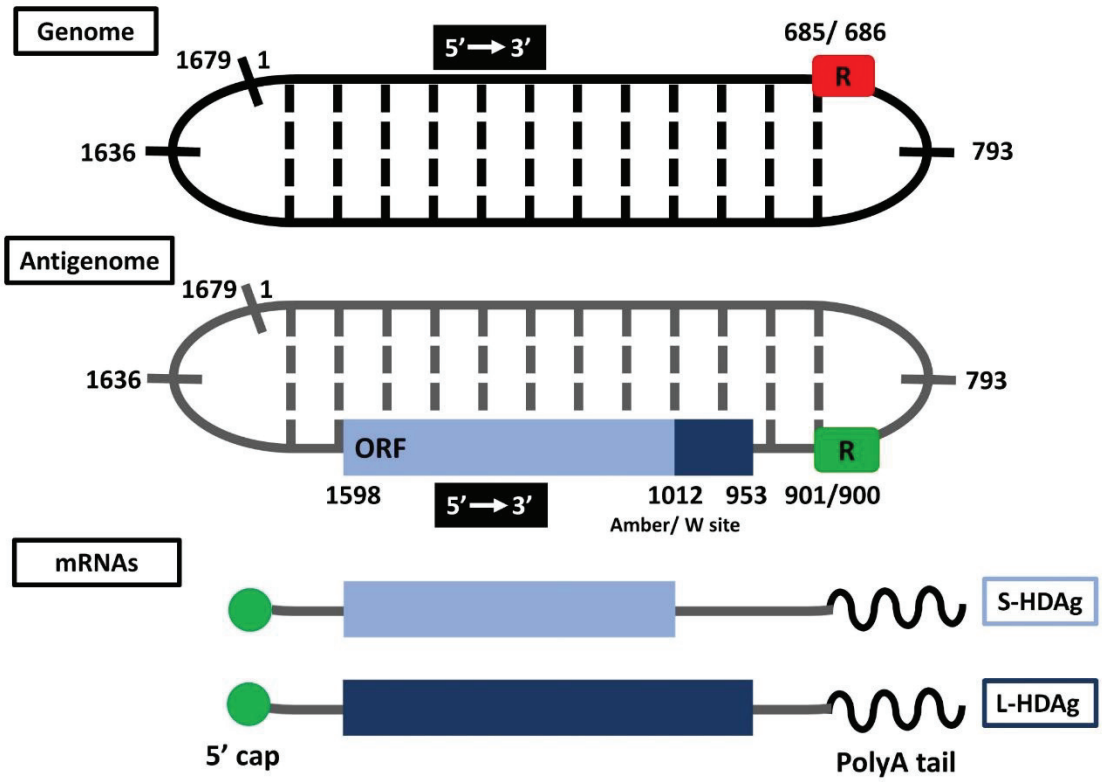

Figure 19. Structure of HDV RNAs. Both genome and antigenome form an unbranched rod-like structure of ~1700 bp, due to extensive intramolecular base-pairing, and contain a self-cleaving ribozyme (R). Although antigenomic RNA contains the open-reading frame (ORF) for HDAg, the antigen is translated from the ~800bp mRNA. HDV mRNA is transcribed form genome by RNA-Pol II and matured as a host cell mRNA (with a 5' cap and a 3' polyA tail). S-HDAg is translated from un-edited mRNAs whereas antigenome editing of the Amber/ W site by ADAR1 leads to the emergence of L-HDAg.

Ribozyme

Small self-cleaving RNA sequences (of about 85 contiguous nucleotides) were identified in both HDV genome and antigenome (Kuo et al., 1988b; Wu et al., 1989). These ribozymes, whose sequences are very well conserved among HDV genotypes, are responsible for the cleavage of multimeric RNA molecules that arise during transcription into unit-long genome or antigenome sequences.

The HDV ribozyme has unique structural and functional characteristics (Figure **20**) and is distinct from viroid ones (Serganov and Patel, 2007). Several crystal structures of HDV ribozyme have been obtained and allowed to uncover a pseudoknot-like

mechanism of cleavage (Riccitelli and Lupták, 2013). As previously mentioned, recent studies have identified HDV-like ribozyme sequences in host-cell genomes.

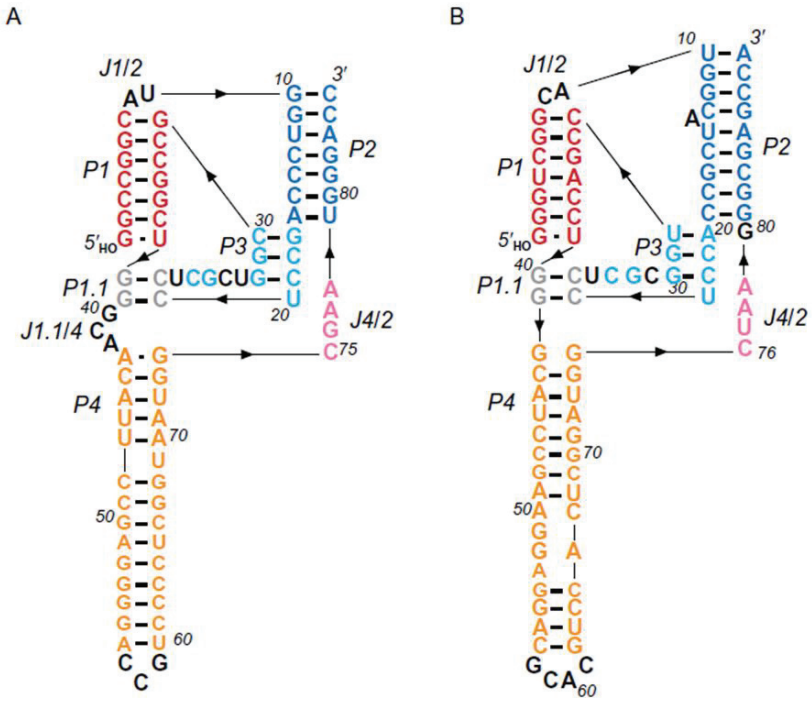

Figure 20. Secondary structures of (A) genomic and (B) antigenomic HDV ribozymes. The single-stranded portion containing the catalytic cytosine is pink. Black nucleotides denote unconserved, single-stranded joining regions. Arrows connecting nucleotides indicate the direction of the strands, and the 5' OH marks the 5' end of the ribozyme following self-scission. Reproduced from (Riccitelli and Lupták, 2013).

Viral proteins and editing

Early cloning and sequencing suggested the existence of 9 putative ORFs in HDV RNA (4 in the HDV genome and 5 in the antigenome). Although a small polypeptide resulting from an alternative ORF (*i.e.* ORF-K) has been described (Bichko et al., 1996a), the virus produces one main viral protein - HDAg (Wang et al., 1986). The coding region for HDAg is contained in the antigenome molecule, but translation occurs from a linear HDV mRNA. While originating from the same ORF, two isoforms of HDAg are translated, *i.e.* S-HDAg (for 'small', 24 kDa) and L-HDAg (for 'large', 27 kDA). S-HDAg can be directly translated from the first round of HDV genome transcription and is the earlier form to arise in infected cells. During replication, as depicted in Figure 23, the 'Adenosine Deaminase Acting on RNA' protein (ADAR1)

catalyses adenosine-to-inosine editing on the antigenomic HDV RNA (adenosine 1012; the amber/W site is changed from UAG to UIG) (Polson et al., 1996). This gives rise to a genome that has a complementary ACC instead of the AUC and ultimately leads to the appearance of mRNA forms where the UAG (amber) stop codon is replaced by a UGG (tryptophan) codon. This modification allows translation to continue until the next stop codon, leading to the appearance of a protein 19-20 amino-acids longer, *i.e*. L-HDAg (Casey, 2012).

Based on relative levels of expression and knock-down experiments, ADAR1 has been shown to be the specific cellular enzyme acting on antigenomic HDV RNA (Wong and Lazinski, 2002). ADAR1 has two isoforms – small (ADAR1-S) and large (ADAR1-L) - with a common C-terminus. ADAR1-S is the most abundant form, constitutively expressed, and has a nuclear localization, whereas ADAR1-L is found mainly in the cytoplasm and its expression is stimulated by interferon. Preliminary works indicated that ADAR1-L was very efficient in editing transcripts in the cytoplasm, but it was later shown that HDV RNA editing occurs in the nucleus and is mediated by ADAR1-S (Wong and Lazinski, 2002). However, recent works suggested an increased in HDV RNA editing following interferon treatment that could be attributed to ADAR1-L (Hartwig et al., 2004, 2006).

The two forms of HDAg share the 195 amino acids of N-terminus and present several common functional domains, including RNA binding motifs, a coiled-coil domain, a nuclear localization signal and a helix-loop-helix motif (Figure 21). The 19 extra amino acids of L-HDAg are proline rich, feature a viral assembly signal, a nuclear export signal (NES), and a prenylation site (Lee et al., 2001).

HDAg undergoes post-translational modifications that can impact HDV replication, namely serine phosphorylation, lysine acetylation, arginine methylation and lysine sumoylation (Lai, 2005). Prenylation of the Cys211 residue of L-HDAg, by a cellular farnesyl transferase is involved in particle assembly (Glenn et al., 1992). S-HDAg and L-HDAg have different roles during the viral life cycle and may contribute to different pathogenic mechanisms. S-HDAg, while having no polymerase activity, is necessary for the initiation of HDV replication, RNA Pol II elongation and for the accumulation of HDV RNAs during the cycle (Kuo et al., 1989; Yamaguchi et al., 2001). L-HDAg acts as a dominant negative inhibitor of HDV replication, by specifically inhibiting genome,

but not antigenome, synthesis and is essential for virion assembly (Chang et al., 1991; Chao et al., 1990; Modahl and Lai, 2000).

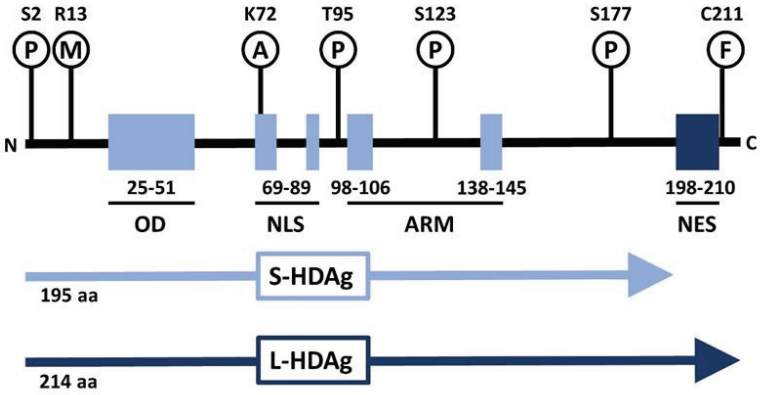

Figure 21. Functional domains and post-translational modifications of HDAg. Both forms of HDAg contain and oligomerization domain (*OD*), a nuclear localization signal (*NLS*) and an arginine-rich motif (*ARM*). L-HDAg is 19 aa longer the S-HDAg and further contains a nuclear export signal (*NES*). Post-translational modifications that have been described for both forms of HDAg are represented by circles (*P*, phosphorylation; *M*, methylation; *A*, acetylation; *F*, farnesylation).

Ribonucleoprotein

The genomic HDV RNA associates with HDAg to form a ribonucleoprotein (RNP) present both in viral particles and infected cells. This RNP is essential, not only for virion assembly, but also for the nucleus/cytoplasm trafficking of HDV RNA (Tavanez et al., 2002). The exact structure and stoichiometry of this RNP have been debated. Early studies suggested that, in the virion, the genome molecule was associated to 70 HDAg molecules, whereas in the nucleus of infected cells, both genome and antigenome formed RNP structures featuring an average of 30 HDAg molecules (Ryu et al., 1993). A more recent study suggested a molar ratio of 200 molecules of HDAg per genome molecule both in virions and infected cells (Gudima et al., 2002). However these numbers have been questioned by studies suggesting the oligomerization of delta antigen to attach to the HDV RNA molecule, which would be compatible with the smaller proportions previously proposed (Alves et al., 2010; Lin et al., 2010).

Importantly, the binding specificity of HDAg to the genome seems to be dictated by its secondary structure rather than its primary sequence (Griffin et al., 2014).

3.2.3. Viral life cycle

An overall representation of HDV life cycle in a hepatocyte co-infected by HBV is depicted in Figure 22.

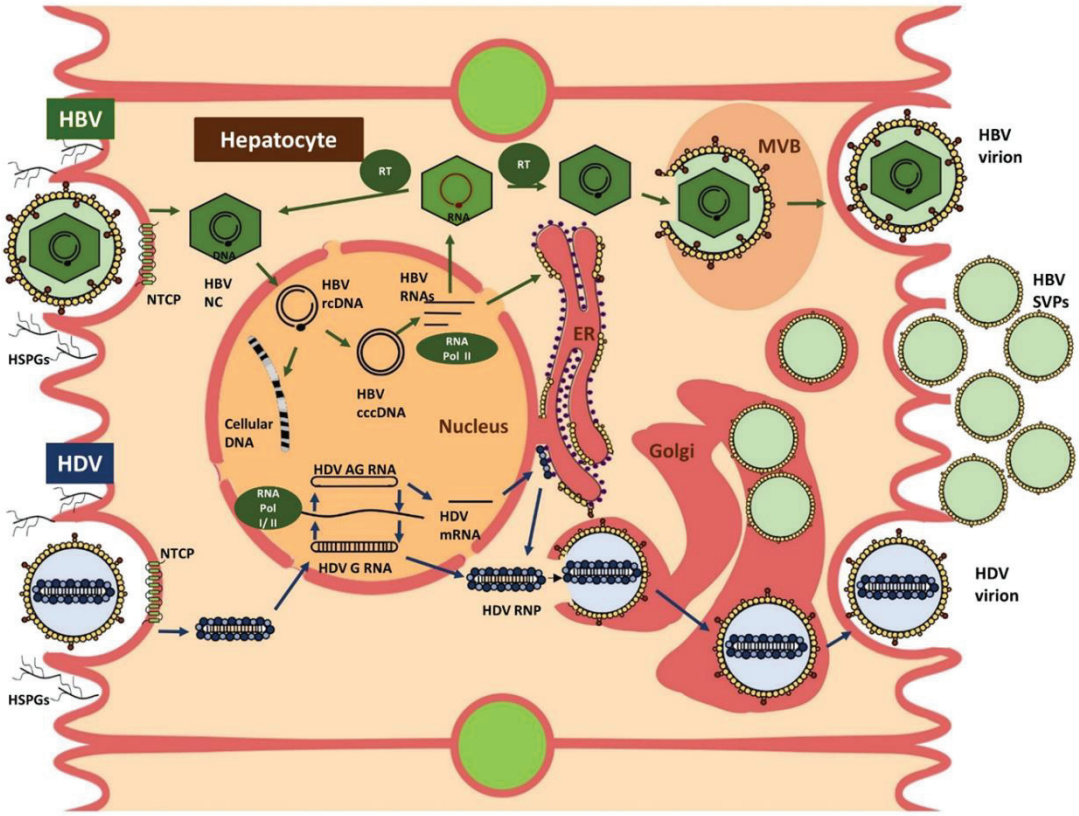

Figure 22. HDV life cycle. For a productive HDV viral cycle, HBV infection of the same cell is necessary. For both viruses, entry is mediated by NTCP binding of the preS1 domain of HBsAg, preceded by virion attachment to HSPGs. Apart from entry, replication of HBV and HDV seems to be completely independent. HBV nucleocapsid (*NC*) is transported to the nucleus where viral relaxed circular DNA (*rcDNA*) is converted to covalently- closed circular DNA (*cccDNA*), a mini chromosome-like structure that is permanently kept in the infected cell. Fragments of HBV DNA are integrated in the host cell genome. HBV RNA transcription from cccDNA is mediated by the cell RNA Pol II and gives rise to mRNA transcripts that can either be included in immature nucleocapsids and undergo retro-transcription by the viral retro-transcriptase (*RT*) to form new genomes; or translated into viral structural proteins. Virions are assembled and released through the multivesicular body (*MVB*) pathway (whereas subviral particles – *SVPs* – are thought to be released via Golgi secretory pathway). Replication of HDV genome (*HDV G RNA*) is exclusively nuclear, occurs through a double rolling circle mechanism, involving

the formation of antigenome (*HDV AG RNA*) and multimeric RNA intermediates and the recruitement of host cell RNA polymerases. HDV ribonucleoprotein (*RNP*) is enveloped in the Golgi by HBsAg. HDV secretion is thought to occur via Golgi. ER, endoplasmic reticulum; *HSPG*, heparan sulfate proteoglycans; *NTCP*, sodium taurocholate cotransporting polyeptide.

3.2.3.1. Attachment and entry

Hepatotropism. The hepatotropism of HDV and its capacity to ensure a productive cycle are related to the entry process and HBV coinfection of cells. While a productive cycle requires the expression of HBV surface glycoproteins in the same cell to allow assembly of HDV virions, other aspects of the two virus replication cycles seem completely independent, as no other component of HBV seems to contribute to HDV replication and *vice versa* (Bichko et al., 1996b). In contrast to HBV, which requires liver-specific transcription factors, HDV genome replication may occur in various mammalian cell types provided that its genome is experimentally delivered to cells (Taylor, 2009). Given the shared envelope structure of both virus, attachment and entry mechanisms are thought to be similar between HBV and HDV, and most of the current knowledge on HBV entry mechanisms has been obtained in HDV infection models (Sureau, 2010).

*Viral factors***.** For both viruses, the presence of the L-HBsAg envelope protein is essential for infectivity. Specific mutations in the N-terminal 75 amino acids of the Pre-S1 domain or inhibition of myristoylation can block infectivity (Blanchet and Sureau, 2007; Gripon et al., 1995). S-HBsAg antigenic loop domain and pattern of glycosylation also seem to play an role, as specific mutations in this domain may inhibit infection, independently of the PreS1 domain (Le Duff et al., 2009; Julithe et al., 2014).

*Host factors***.** Viral attachment on the cell surface precedes receptor-specific entry for HBV and is mediated by cellular heparan sulphate proteoglycans (HSPGs) (Leistner et al., 2008; Schulze et al., 2007). This was recently confirmed for HDV (Lamas Longarela et al., 2013; Sureau and Salisse, 2013). The exact HSPGs involved in HBV/ HDV viral attachment are still to be identified, even though Glypican-5 was recently reported to be preponderant in this process (Verrier et al., 2015). This step, although necessary, is not sufficient for infection, as the entry of HSPG-attached viruses can still be inhibited (Han et al., 2011b). A potential role for purinergic receptors (i.e. P2YRs, P2XRs) in the

attachment process of both HDV and HBV has also been suggested from the reported inhibitory effect of suramin (Lamas Longarela et al., 2013; Taylor and Han, 2010).

Recently, a ground-breaking study from Yan and colleagues led to the identification of hNTCP, encoded by *SLC10A1*, as a functional receptor for HBV and HDV (Yan et al., 2012). NTCP is located in the basolateral membrane of hepatocytes and participates in the enterohepatic circulation of bile salts. Viral infection seems to be supported by amino acids involved in bile acid binding (and not those involved in sodium binding) (Yan et al., 2014). The interaction between NTCP and HBV/HDV seems to be mediated by the viral PreS1 N-terminal 75 amino acids and a NTCP binding region located on helix 5 in the outer leaflet of the cell membrane (Urban et al., 2014). Backing up the fact that HDV can replicate into many cell types (*i.e.* not only human hepatocytes), if its genome is correctly delivered, an hNTCP-trangenic mice was recently shown to be infectable by HDV (He et al., 2015). Interestingly, a polymorphism of the *SLC10A1 g*ene (S267F), affecting NTCP's bile acid transport function is thought to be present in 9% of the East Asian population was shown to impair HBV and HDV uptake by hepatocytes (Yan et al., 2014).

3.2.3.2. Uncoating and nuclear transport

HBV is thought to be internalized through clathrin-dependent endocytosis, via early and late endosomal compartments, independently of acidification and protease activity (Huang et al., 2012; Macovei et al., 2013). Such evidence is unavailable for HDV, although it may be supported by the identification of L-HDAg as a clathrin-adaptor-like protein (Huang et al., 2007). The steps that mediate post-fusion nuclear transport of HDV RNP and virus genome uncoating are not fully understood. Trafficking of HDV RNP between cytoplasm and nucleus would involve HDAg and its interaction with importins (Chou et al., 1998; Tavanez et al., 2002). Further understanding of this phase of the viral cycle would be crucial to understand in particular the innate immune response against the virus.

3.2.3.3. Replication

Throughout replication, antigenome RNA is limited to the nucleus, whereas genomic RNA molecules can either enter another nuclear replicative cycle or be exported to the cytoplasm for assembly into new infectious particles (Macnaughton and Lai, 2002). A summary of the currently accepted steps involved in HDV replication is represented in Figure 23.

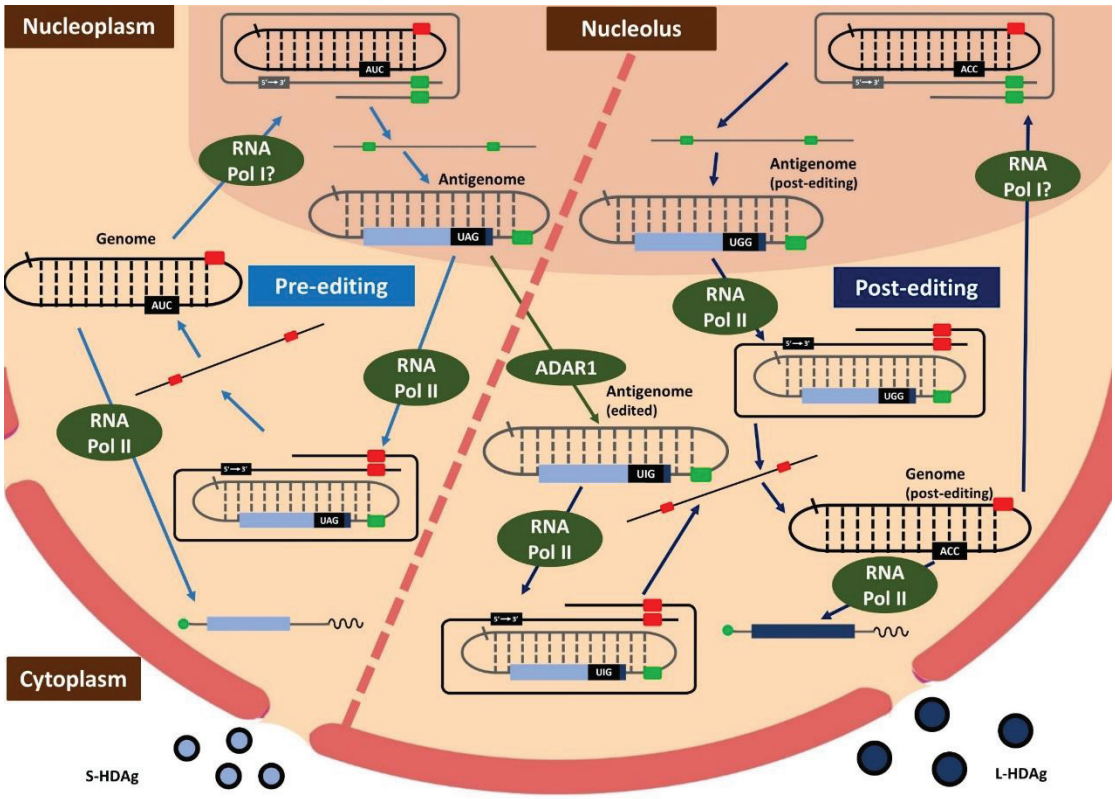

Figure 23. Model of HDV RNA replication. HDV replication is limited to the nucleus and involves antigenome and multimeric RNA molecules as replicative intermediates. Genome is transcribed in the nucleolus by cellular polymerase(s). The generation of multimeric RNAs is necessary for the self-cleavage and ligation activities of HDV ribozyme. The cycle is completed by transcription of antigenome by RNA Pol II in order to form a new genome molecule. Genome can either enter a new replicative cycle, initiate transcription of HDV mRNA or be exported to the cytoplasm as HDV RNA for virion production. In parallel, antigenome molecules can enter a novel replicative cycle after editing of the Amber/ W site by ADAR1, ultimately leading to the appearance of mRNA coding for L-HDAg (see text for details). *ADAR*, adenosine deaminase acting on RNA.

Double Rolling circle model of replication. It is now accepted that HDV has an RNA-dependent RNA replication in a "double rolling-circle mechanism", which involves the

recruitment of host-cell DNA-dependant RNA polymerases and likely their switch in template specificity (Lai, 2005; Taylor, 2009). The double rolling-circle replication (similar to plant viroids symmetrical rolling-circle replication, but modified to include mRNA synthesis step) relies on two circular RNA templates of inverse polarity, genome and antigenome, and the generation of intermediate multimeric linear transcripts.

For the replication of HDV RNA, three enzymatic activities are needed: a polymerase to synthetize oligomeric strands from circular templates, a ribozyme-dependant RNAse activity to cleave them into unit-length strands, and a ligase to circularize monomers.

Transcription. Unlike some RNA viruses with larger genomes, HDV does not possess its own RNA-dependent RNA-polymerase. Moreover, in contrast to other satellite viruses (*e.g.* satellite tobacco necrosis virus), it does not use the polymerase of its helper virus, and therefore fully relies on host-cell properties (Lai, 2005). Several lines of evidence support the fact that RNA polymerase II (Pol II) would be involved in HDV replication: firstly, the HDV mRNA displays a 5' cap and 3' poly-A tail as cellular mRNAs (Gudima et al., 2000; Hsieh et al., 1990); secondly, HDV RNA transcription has been shown to be inhibited by low doses of α-amanitin (Chang et al., 2008); and finally, RNA pol II has been shown to be able to bind to HDV RNA of both genomic and anti-genomic polarity (Chang et al., 2008; Greco-Stewart et al., 2007). In other reports, antigenome synthesis seemed to be somehow resistant to α-amanitin, suggesting that Pol I could be also involved (Modahl et al., 2000). This possibility is supported by two other studies suggesting that both Pol I and Pol III could interact with HDV RNA (Greco-Stewart et al., 2009), and that genome and antigenome synthesis could take place in distinct nuclear areas (Li et al., 2006). Although several hypotheses prevail and the exact role of the various polymerases is still to be clarified, all studies agree that HDV is able to redirect an otherwise DNA-dependent RNA-polymerase to its RNA template. The underlying molecular mechanism is however largely unknown.

The role ofS-HDAg. S-HDAg is an important candidate to explain this hijacking of RNA pol II. It is a nuclear protein known to bind to HDV RNA, which presents structural similarities to transcription factors (e.g. NELF-A), and can undergo acetylation and methylation as other transcription regulatory proteins (Lai, 2005). S-HDAg can bind to

RNA Pol II and enhance transcription either by direct stimulation of elongation or by reversion of inhibitory effects (Yamaguchi et al., 2001). Furthermore, S-HDAg has been shown to biochemically interact with 9 out of 12 subunits of the RNA pol II in a combined proteomic-RNA interference screening (Cao et al., 2009). This interaction may not be restricted to Pol II, as S-HDAg has been shown to interact and/ or co-localize with nucleolar proteins (e.g. B23 and nucleolin), which could further support a Pol I involvement (Huang et al., 2001).

Motifs for transcription and replication. The presence of transcription initiation sites or promoters on HDV RNA is also debated. It seems to be the case for HDV mRNA (Gudima et al., 2000), and a recent study has suggested that the 5'-end region of the HDAg mRNA, which coincides with one extremity of the rod-like genomic RNA and displays a complex secondary structure, may play an important role in HDV replication (Beeharry et al., 2014).

Cleavage. Genome and antigenome molecules are processed from more than unit-length linear precursors, needing a cleaving catalytic activity. This activity is exerted by the ribozyme auto-cleaving sequences present in both genome and antigenome that have to be transcribed at least twice from the circular templates to give rise to one unit length, justifying the presence of multimers.

Ligation. A ligase activity would be needed to circularize the linear monomers into genome and antigenome molecules. While a study has suggested the recruitment of a host-cell ligase, as ligation of HDV RNA occurred only in mammalian cells (Reid and Lazinski, 2000), another has shown self-ligating properties of HDV ribozyme sequences (Sharmeen et al., 1989).

3.2.3.4. Viral assembly and release

To form HDV virions, the HDV ribonucleoprotein is enveloped by at least S- and L-HBsAg, meaning that, in a natural infection, HDV can only be released from cells co-infected with HBV. Many questions remain unanswered regarding HDV assembly and release. The newly developed infection models should help uncovering this step of HDV life cycle in parallel with a better understanding of HBV assembly.

In contrast to HBV, the cytoplasmic domain of HBsAg, spanning the junction between PreS1 and PreS2, does not seem crucial for HDV release; hence it has been suggested that HDV would mostly use the subviral particle release pathway (through the Golgi) rather than the multivesicular body pathway, involved in assembly of infectious HBV virions (Taylor, 2012; Watanabe et al., 2007; Zeisel et al., 2015). As for post-entry trafficking of HDV RNP, clathrin could also be involved in viral particle export (Huang et al., 2009). Regarding viral components, the farnesylation of the C-terminal sequence of L-HDAg is necessary for HDV envelopment, as it mediates the interaction with the S region of HBsAg (Hwang and Lai, 1993). Farnesylation involves the fixation of a 15 carbon chain to the C_{211}XXQ box peptide motif that is present at the carboxy-terminal end of the L-HDAg and conserved among all HDV genotypes.

3.2.4. Experimental models for the study of HDV

Since the discovery of HDV, both *in vitro* and *in vivo* models have been established to perform molecular and cellular studies.

In vitro models

Unlike HBV, HDV replication is not restricted to hepatocytes, and the virus can replicate to high levels in a wide range of mammalian cells upon delivery of viral RNA (and antigen) or cDNA. *In vitro* models of transfection of hepatic cancer cell lines (e.g. Huh7, HepG2) have allowed major contributions to the study of viral replication. However, as viral assembly depends on the presence of HBV envelope proteins, viral particle production can only be achieved in the presence of concomitant HBV transcription or by the co-transfection of a plasmid encoding HBV envelope proteins (Gudima et al., 2007; Sureau, 2010).

Until recently only differentiated primary human (PHH), chimpanzee (not often used), or tupaia (PTH) hepatocytes, as well as the non-transformed, bipotent progenitor differentiated-HepaRG cell line were susceptible to HDV (and HBV) infection (Urban et al., 2014). These cells are difficult to obtain, to work with, and can be confronted (with the exception of HepaRG) to batch-to-batch variability (both genetically and

physiologically), thus impairing reproducibility of experiments. The recent identification of hNTCP as an essential receptor for HDV and HBV has changed this scenario, as its constitutive or inducible overexpression in clonal transformed hepatic cell lines (e.g. HepG2 and Huh7) renders them susceptible to high level of infection in a more reproducible manner (Urban et al., 2014).

In vivo models

Although natural HDV infection seems to occur only in humans, a limited number of susceptible mammalian hosts has been identified and used for research purposes. HDV infection has hence been extensively studied in chimpanzees (with HBV as a helper virus) and woodchucks (in the presence of WHB) (Ponzetto et al., 1984; Rizzetto et al., 1980b). Tree shrew species *Tupaia bengaleri* (susceptible to HBV), woolly monkeys (with WMHBV as helper virus), and more recently bats have also been used for the study of HDV infection, although their use is limited by lack of tools. Interestingly, Peking ducks' hepatocytes, although a useful model for the study of avihepadnavirus (DHBV), do not sustain HDV replication (Liu et al., 2001).

Many mice models, which are more or less relevant, have been developed to date. First of all, it is worth noting that a straight HDV injection of wild type mice does not lead to an efficient infection, with < 1% of infected hepatocytes; this finding is compatible with the current knowledge on species specificity of HDV infection mediated by hNTCP (Netter et al., 1993; Yan et al., 2013). Gene delivery strategies based on hydrodynamic transfection of plasmids (with trimeric or dimeric constructs) led to measurable intrahepatic HDV replication in mice and to the establishment of HDV viremia, when HBV-transgenic mice are used (Bordier et al., 2003; Chang et al., 2001). This approach is interesting to study potential antiviral targeting HDV in an immune competent model. In a less flexible approach, a transgenic mouse expressing a replication competent HDV genome dimer has also been generated. Interestingly, as the transgene was not only expressed in hepatocytes, it was found that HDV could replicate in other tissues than liver, supporting the fact that HDV hepatotropism is mediated by an entry restriction (Polo et al., 1995). More recently a transgenic mouse model expressing hNTCP was developed and shown to support acute HDV infections

(He et al., 2015). But in this model coinfection or superinfection with HBV cannot be studied. It is worth noting that transgenic mice expressing either L-HDAg or S-HDAg have been developed and have proven useful to exclude a direct pathogenic/carcinogenic role in this setting (Guilhot et al., 1994).

All mice models described so far do not allow co- or superinfection with HBV in a context in which cccDNA is present. To overcome this problem, humanized liver mice models (initially used for the study of HCV and HBV infections) have been validated for HDV (Dandri and Lütgehetmann, 2014). Several models have been developed (notably uPA SCID, FRG and TK-NOG mice), having in common a human reconstituted liver (from the engraftment of PHH) in the absence of an immune system (Bissig et al., 2010; Dandri et al., 2001; Kosaka et al., 2013). Notably, the uPA SCID mouse model has been used for the preclinical validation of Myrcludex B®, an entry inhibitor for both HBV and HDV (Lütgehetmann et al., 2012). More recently, mouse models displaying not only a humanized liver but also a human immune system, have been developed and reported to be susceptible to HBV infection. Although a promising tool for the study of virus-host interactions, their potential contribution for the study of HDV infection is yet to be established (Bility et al., 2014; Gutti et al., 2014; Washburn et al., 2011).

3.3. The disease

3.3.1. Epidemiology

3.3.1.1. Prevalence and geographic distribution

HDV has a worldwide distribution (Figure 24), with variable prevalence rates, which surprisingly do not exactly match the distribution of chronically HBV-infected (CHB) patients (Ponzetto et al., 1985; Rizzetto et al., 1980a). HDV prevalence may be underestimated by non-standardized and heterogeneous screening practices and the inaccessibility to testing in many highly endemic areas. In the 80's, the number of HDV infected patients was estimated at 15 to 20 million worldwide, with an overall prevalence rate of 5% of all HBsAg carriers (Ciancio and Rizzetto, 2014). Areas with

high endemicity were at that time identified in Africa and South America, with prevalence as high as 85% of HBsAg positive patients in the Amazon basin. Countries of the Mediterranean basin were considered of intermediate endemicity (with a prevalence of 25% in Italy and Turkey), whereas in low endemic countries (Northern Europe and USA), the disease seemed to be restricted to intravenous drug users (IVDU) (Ponzetto et al., 1985; Rizzetto and Ciancio, 2012).

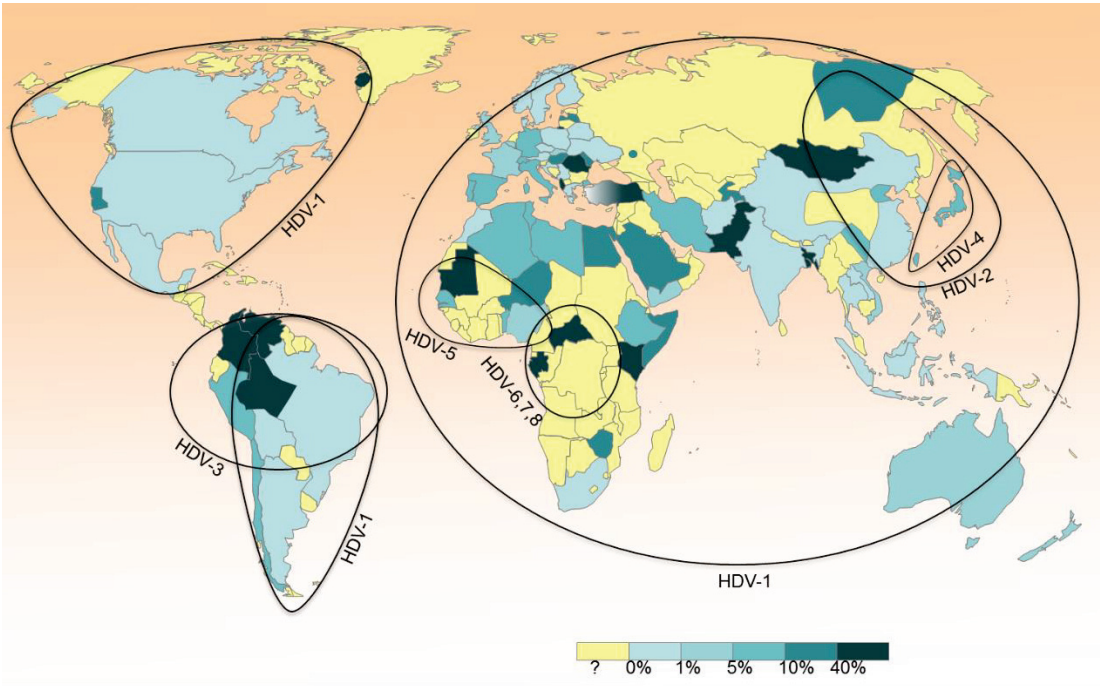

Figure 24. Prevalence of Hepatitis delta virus infection among HBV carriers. The scale indicates the suspected proportion of HDV co- or superinfection of HBV-infected patients. Yellow colour: insufficient data. The black circles indicate preferential area diffusion of HDV genotypes, HDV-1 being ubiquitous. Due to globalization, human migration or travel during chronic carriage, some strains geographically defined, can extend outside their initial spreading area. Therefore, diagnosis has to be adapted for all different HDV genotypes. Note that some prevalence remained from the nineties, while others correspond to more recent works.

The case of low endemicity regions

In the 90's, the decrease in prevalence of HBV infection (due to improvements in sanitation, behavioural changes and, mostly, to the introduction of an HBV vaccine) was accompanied by a decrease in HDV prevalence in Europe (Rizzetto and Ciancio, 2012). But for the last 15 years, no further decline has been observed. In Italy, where an HDV epidemic spreading occurred during the 80's, there was a steady decrease

during the two following decades, but recent data suggest that no further decrease was achieved, with steady prevalence rates of 8,1% of HBsAg carriers in 2008 (Sagnelli et al., 2014; Stroffolini et al., 2009). In France, the latest data suggest that the prevalence of HDV infection among HBsAg carriers is also stable, if not increasing (Servant-Delmas et al., 2014). This trend seems to be associated with the increasing number of migrants, originating mainly in Africa and Eastern Europe (Le Gal et al., 2007). Similar data were reported in Germany, with a current prevalence of 7,4 -11,3% of HBsAg carriers, with a significant proportion of patients originating in Turkey and Eastern Europe. (Reinheimer et al., 2012; Wedemeyer et al., 2007).

A recent re-assessment of HDV prevalence in London (UK) reported a decrease in the prevalence rate (2.1% of all the HBsAg carriers, while the previous reported prevalence was 8,5% in 2008) and attributed a significant proportion of the positive cases to IVDUs (22,7%) and to patients of African origin (28-50%) (Cross et al., 2008; William Tong et al., 2013). In the USA, the diagnosis of HDV infection has for long been neglected. Recent studies indicate that the number of affected patients is comparable to the European reality (8% of the HBsAg positive patients) and not restricted to IVDUs (26% of the patients).(Gish et al., 2013)

In summary, three subgroups of patients can be described nowadays in Western countries: a) older patients infected during the eighties epidemic (namely in the Mediterranean basin), currently suffering from advanced liver disease; b) younger IVDUs, often co-infected with HCV and/or HIV; and c) patients migrating from highly endemic countries (Ciancio and Rizzetto, 2014).

The case of high endemicity regions

In the early 90's, an outbreak of severe acute hepatitis delta (mostly due to coinfection) was reported in the IVDUs of the Russian region of Samara. During the outbreak, 39% of acute hepatitis B patients had a positive HDV IgG (48% among the fulminant hepatitis cases) (Flodgren et al., 2000). High prevalence of chronic hepatitis delta (CHD) is still reported in central Europe: 47.6% of HBsAg positive patients in Romania and 13,9% in Hungary (Pár et al., 1992).

Although Brazil as a whole does not have a high prevalence of HDV infection, the Amazonian basin was soon recognized as an endemic region (Galizzi F et al., 2010;

Ponzetto et al., 1985). In this region, HDV-3 has been associated with “Labrea fever”, a form of fulminant hepatitis that affected indigenous populations (Bensabath et al., 1987). Recent evaluations of HDV prevalence are consistent with high endemicity, with HDV seropositivity rates ranging from 8,5% of HBsAg-positive asymptomatic blood donors to 65% in HBsAg-positive hospital outpatients (Crispim et al., 2014). High HDV prevalence rates are also reported in the Amazonian areas of Venezuela and Colombia, where the infection is, as in Brazil, caused by HDV-3 and associated with fulminant hepatitis outbreaks (Alvarado-Mora and Pinho, 2013).

In Africa, HDV infection affects mainly countries in the western and central areas. In Mauritania a prevalence rate of 33.1% among HBsAg positive patients was recently reported, and HDV was associated with more aggressive disease than HBV infection alone (Lunel-Fabiani et al., 2013). Other studies show wide differences in sero-prevalence in HBsAg positive liver patients, ranging from 1.3% in a Nigerian centre, to 50% in Central African Republic and 66% in Gabon (Andernach et al., 2014; Makuwa et al., 2009). However, it should be kept in mind that data on anti-HDV seroprevalence are lacking from several African areas. In Egypt, a seroprevalence of HDV of 20% was described among the HBsAg positive individuals (mostly patients with liver disease) (Saudy et al., 2003).

The situation is Asia is particularly interesting: in some places, the distribution of HDV infection does not match the high endemicity of HBV in the region. In Taiwan, a classic case of high HBV prevalence and an example of HBV vaccination success, HDV infection is frequent in the IVDU population (66,7% prevalence) and frequently associated with HIV infection, but remains rare in non-IVDUs (6%) (Chang et al., 2011). In Mongolia a prevalence of HDV antibodies as high as 82% has been reported in HBV infected patients with advanced liver disease (Oyunsuren et al., 2006). Finally, a recent report from the Pacific region also suggests high prevalence rates (37% among HBsAg positive patients) in isolated Micronesian islands (Han et al., 2014).

In summary, HDV infections are far from being on the way to eradication, and remain a major health problem in developing and low-resource countries where the diagnosis is sub-optimal. There is an urgent need to standardize RNA testing procedures (Detection of the virus section) and implement them in countries of high endemicity

and surrounding ones to better monitor evolution of prevalence, while remaining vigilant in the rest of the word, due to increasing travel habits.

3.3.1.2. Molecular epidemiology and genotypes

HDV evolution seems to result from 3 main mechanisms: mutation, editing and, as recently described, recombination (Chao, 2007). The substitution rate for HDV, determined *in vivo* by the longitudinal evaluation of patient samples was estimated to be of 5.9×10^{-4} to 3.0×10^{-2} substitutions per site per year, varying with the phase of the infection (higher in the acute phase), the genome location (higher in the less conserved regions than in the conserved regions such as ribozyme) (Chao et al., 1994), and increasing under therapeutic pressure (Dény, 2006; Lee et al., 1992). These values seem to be higher than for the majority of the RNA virus that have estimated rates between 1×10^{-5} and 3.4×10^{-3}; as an example, HCV is estimated to have a substitution rate of 7.9×10^{-4} and HIV 2.5×10^{-3} (Jenkins et al., 2002). A long term *in vitro* study identified a substitution rate of 2×10^{-2} and suggested that 70% of this substitutions may be linked to editing events (Chang et al., 2005a). These evidences of genetic diversity led to the proposition that, in the same patient, HDV, as also described for other RNA virus, circulates as a quasi-species (Lee et al., 1992).

Recombination was firstly suggested from a patient sample analysis in 1999, then confirmed to occur in mixed genotype infections, both *in vivo* and *in vitro,* and recently re-visited in a patient suffering from acute hepatitis in Vietnam (Sy et al., 2015; Wang and Chao, 2005; Wu et al., 1999). Template-switching driven by the host RNA polymerase has been proposed as the most likely mechanism of HDV RNA recombination (Chang and Taylor, 2002; Gudima et al., 2005).

From the phylogenetic analysis of worldwide HDV sequences, eight genotypes have been proposed, associated with distinct geographic distribution (represented in Figure 24) and clinical features (Le Gal et al., 2006; Radjef et al., 2004). Sequence differences within a genotype are smaller than 20% but can be as high as 35% between different genotypes (Dény, 2006). Interestingly, among HDV isolates, South American HDV-3 sequences are the most divergent ones, matching the tree topology of the local helper HBV genotype F among HBV genetic variability, suggesting common cause of viral evolution in South American natives.

3.3.2. Transmission

3.3.2.1. Transmission pathways

HDV transmission is considered to occur mainly through a parenteral route. The main transmission route however may vary between low and high prevalence zones. In Europe and in the USA transmission occurs mainly through contact with infected blood, particularly in IVDUs, frequently in association with HIV and HCV. There is evidence that both homosexual and heterosexual transmission of HDV can occur and may be particularly relevant in highly endemic areas (Braga et al., 2012; Brook, 1998). Intra-familial transmission, presumably involving unapparent exchange of body fluids, occurs in low-income communities and has been proven by sequence analysis in an Italian population in the nineties (Niro et al., 1999). Vertical transmission of HDV has not been described and is possibly disfavoured by HDV's repression of HBV replication lowering HBV mother-to-infant transmission at birth.

3.3.3. Clinical features

3.3.3.1. Forms of acquisition

Being a satellite virus, HDV can establish a productive infection and spread only in cells concomitantly infected by its helper virus, HBV. Clinically, this translates into two possible forms of HDV acquisition: (i) simultaneously with HBV, corresponding to **coinfection** or (ii) by infection of a patient already chronically infected by HBV, corresponding to **superinfection**. In the transplantation setting a third form of acquisition has been suggested as "helper-independent hepatitis delta".

Coinfection translates into acute hepatitis, ranging from mild to severe, or even fulminant, and usually leads in 90-95% of cases to resolution of both HBV and HDV infections; this means that the rate of progression to chronicity is as low as for HBV, in immune-competent adults. The proportion of fulminant hepatitis in this setting can be as high as 17%, which is far higher than that observed in acute HBV infection (Yurdaydın et al., 2010). Superinfection, on CHB setting, may also translate into an acute hepatitis episode and may lead to fulminant hepatitis (Farci and Niro, 2012).

Although some patients experience an HBsAg sero-conversion upon HDV superinfection, the vast majority progresses to chronic infection by both viruses (Caredda et al., 1985). A summary of the available data on infection progression is proposed in Figure 25.

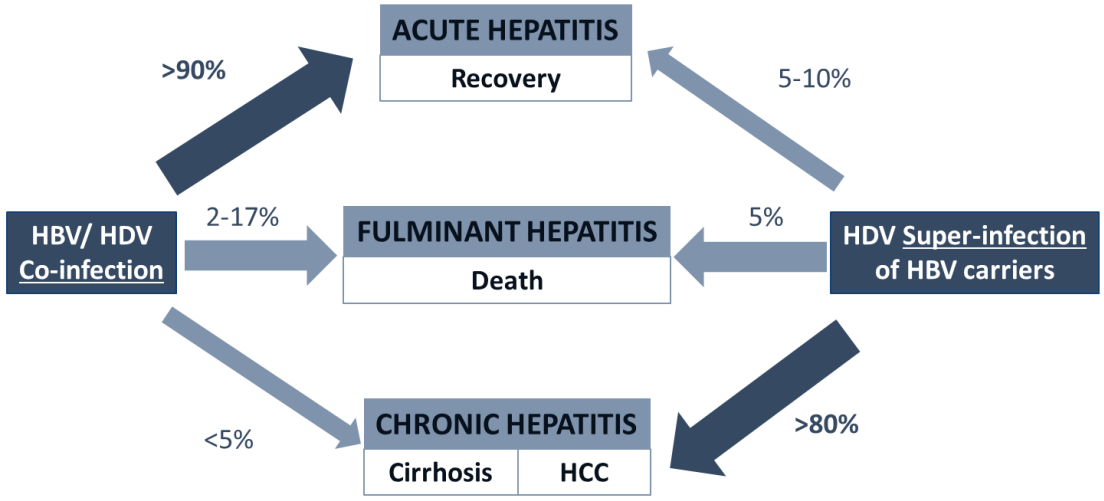

Figure 25. Progression of HDV infection depending on the acquisition pattern.

In liver-transplanted HBV/HDV patients a "**helper independent HDV infection**" or "latent" HDV infection has been suggested with markers of HDV replication identified in the liver and serum in the absence of HBV markers (Ottobrelli et al., 1991). Re-evaluation of some of these cases, using more sensitive PCR techniques, has shown that HDV infection was maintained in parallel with a very low-level HBV replication, ruling out the possibility of an isolated HDV infection (Smedile et al., 1998).

3.3.3.2. Clinical course and serologic patterns

Acute HBV/HDV coinfection is in most cases clinically indistinguishable from acute HBV infection. However, a subset of patients (15% in some cohorts) can present a severe clinical course with a pattern of biphasic hepatitis, associated with a re-increase of transaminase levels after a first period of improvement and that is thought to be caused by sequential spreading of HBV followed by HDV (Moestrup et al., 1983). HDV acute superinfection of a CHB patient is clinically characterized by an auto-limited

flare of hepatitis (clinically evident in half of the patients), that can be mistakenly considered to be an hepatitis B reactivation, and is usually followed by progression to chronicity of both infections. During acute hepatitis delta, HDV markers do not allow a reliable distinction between co- and superinfection. Diagnosis is established based on the presence of anti-HBc IgM antibodies (directed against the capsid of HBV) that will be positive in the setting of an acute HBV infection (as is the case in coinfection) and remain negative or at a very low titre in super-infected patients. Fulminant hepatitis (acute liver failure in a patient with no prior liver disease) is more frequent in HDV infected patients than in HBV mono-infected patients and, without liver transplantation, is associated with a mortality of 80%. Chronic hepatitis D (CHD) is clinically indistinguishable from chronic HBV infection, although with a faster progression to cirrhosis and hepatocellular carcinoma (HCC) (Farci and Niro, 2012).

3.3.4. Diagnosis

Detection of the virus

Given the high prevalence of HDV infection in some parts of the world and the absence of decrease in Europe and North America, there is an increasing consensus to suggest that all HBV infected patients (including inactive carriers) should be screened, at least at the diagnostic of CHB, for HDV infection (Hughes et al., 2011; Noureddin and Gish, 2014). This is already argued in European HBV treatment guidelines (EASL), but not in the North American counterpart; indeed AASLD's recommendations still restrict HDV screening to HBV infected patients originating from endemic countries or with a history of IV drug use (European Association For The Study Of The Liver, 2012; Lok and McMahon, 2009).

Anti-HDV 'total' antibody (*i.e.* anti HDV-IgM plus anti-HDV IgG) testing should be used as a first approach to hepatitis delta diagnosis, given its early appearance after HDV infection and its persistence throughout the course of the disease (Olivero and Smedile, 2012). There are two limitations to this approach: first, in the first days after infection, anti-HDV antibodies may be undetectable, and patients presenting with acute HBsAg positive hepatitis must be re-evaluated for the appearance of anti-HDV

antibodies; secondly, anti-HDV IgG may persist after HDV infection resolution. A positive anti-HDV serology should be confirmed, ideally by the detection of serum HDV RNA. In the settings where HDV PCR is unavailable, anti-HDV IgM may be considered as a surrogate marker of HDV active infection and its clearance is correlated with therapeutic response (Wranke et al., 2014). Serum HDAg measurement is of limited utility, as in immunocompetent patients it is only transiently detected just before the acute phase of HDV infection, preceding the rising of anti-HDV antibodies. In the chronic phase of disease it can only be occasionally detected in immunocompromised patients (Grippon et al., 1987).

As for other viral infections, nucleic acid quantification plays a crescent role in the diagnosis and most importantly therapeutic monitoring of HDV infected patients. However, unlike HBV, HCV or HIV, there is no fully standardized PCR technique. HDV molecular diagnosis mostly relies on *in house* techniques developed in academic centres. Given the wide genetic variability of the viral RNA, quantification of non-genotype 1 or African-specific genotype 1 samples has yet to be optimized (Brichler et al., 2013). The number of published qRT-PCR techniques has increased over the last years, based on both two-steps and one-step protocols (Ferns et al., 2012; Le Gal et al., 2005; Homs et al., 2014; Mederacke et al., 2010; Scholtes et al., 2012; Yamashiro et al., 2004). Significant technical challenges associated with HDV amplification arise from the high CG content and complementarity of viral RNA (that may limit the RT efficiency) and the high genetic variability of HDV that requires a careful design of primers (and probes) (Le Gal et al., 2005). Utilization of a robust PCR standard and internal control are also essential for a reliable HDV RNA quantification. Given the need for international quality assessment and control, an international PCR standard has now been validated by the World Health Organization to allow result comparison between the available PCR techniques (Chudy et al., 2013).

It is worth noting that the correlation between viral load and disease severity is not clear, being suggested in some cohorts, but not in others, possibly as a result of distinct patient characteristics, viral genotypes and errors in quantification (Braga et al., 2014; Brichler et al., 2013; Romeo et al., 2014; Wedemeyer and Manns, 2010).

Evaluation of liver disease

Liver disease grading and staging must be performed in all patients with HDV infection, in order to weight risks and benefits of interferon therapy. Liver biopsy, although not needed to establish the diagnosis of HDV infection, remains the gold standard for the staging of liver disease and delta antigen detection and is performed in most centres before treatment consideration (Wedemeyer and Manns, 2010). While non-invasive methods for fibrosis evaluation are increasingly used for other types of hepatitis (mostly HCV infection), their use in hepatitis delta still awaits validation. Liver elastography has been used by different groups for non-invasive fibrosis evaluation of HBV/ HDV/ HIV infected patients, although validation studies in HDV infected patients need to be performed (Castellares et al., 2008; Soriano et al., 2014). Furthermore, clinical scores currently used as indirect markers of fibrosis and predictors of outcome, are not validated in hepatitis delta patients. Part of these scores has shown poor performance in one hepatitis delta cohort, and a new Baseline Event Anticipation score (BEA) has been proposed, based on age, gender, geographical origin, International Normalized Ratio, platelet count and bilirubin (Calle Serrano et al., 2014).

3.3.5. Natural history and pathogenesis

Natural history

CHD is often considered as the most severe form of chronic viral hepatitis. HDV infected patients have a more aggressive clinical course than HBV mono-infected patients, with a three times higher risk of developing cirrhosis and a faster progression (Fattovich et al., 1987). The risk of cirrhosis increases overtime and has been reported to be of 23%, 41% and 77% after respectively 10, 20 and 30 years of infection (Yurdaydın et al., 2010). Clinical decompensation has been described as the major complication in cirrhotic CHD patients, with a more than 2 fold increased risk when compared to HBV mono-infection (Fattovich et al., 2000). The course of disease may be influenced by HDV genotype, with predominant genotype 1 being associated with more severe disease than genotypes 2 and 4 (Su et al., 2006). Genotype 3 has been

associated with a particularly aggressive evolution (Casey et al., 1996). A role of HBV genotypes in the course of disease has also been suggested.

The association between CHD and HCC is still a matter of debate. Three major studies identified liver decompensation and death, and not HCC, as the major complication of CHD, and associated the development of HCC with persistent HBV replication (Buti et al., 2011; Niro et al., 2010; Romeo et al., 2009). However, in the Eurohep cohort, an increased risk of 3,2 fold was shown in CHD patients in comparison to CHB ones, with a 5-year risk of 13% (Fattovich et al., 2000). A more recent Swedish study also suggested a 6-fold increase in the risk of HCC in CHD patients (Ji et al., 2012). More studies are yet needed to further evidence the link between CHD and HCC.

Insights into fibrosis and carcinogenesis mechanisms

The pathogenesis of liver damage during HDV infection is still incompletely understood, and the relative contribution of direct pathogenic effect of HDV or immune-mediated injury is a matter of debate. A direct cytopathic effect of HDV antigens seems unlikely given the absence of liver damage upon its expression in transgenic mice (Guilhot et al., 1994). However, cell death in the presence of high levels of replication (replication-associated cytopathogenicity) has been observed *in vitro* and has been suggested to contribute to cases of acute/fulminant hepatitis, where cell death induced by high levels of replication might precede synthesis of L-HDAg (Chang et al., 2005b). A direct implication of HDV proteins in liver pathogenesis can however be supported by studies that showed an interaction with cellular proteins and/or a modulation of cell signalling pathways. For a comprehensive review of HDV cell interactants refer to Greco-Stewart and colleagues (Greco-Stewart and Pelchat, 2010). Large transcriptomic and proteomic studies highlighted several possibilities. A combined proteomic RNAi screening identified 100 putative cell partners of S-HDAg, mostly involved in transcription and HDV replication, but consequences of such interactions for the host cell cannot be ruled out (Cao et al., 2009). A direct role of L-HDAg in fibrogenesis has been proposed, as L-HDAg was shown to activate TGF-β pathway. Moreover, this activation was dependent on isoprenylation and synergistic with HBx-mediated TGF-β and AP1 signal transduction

(Choi et al., 2007). A similar synergism between HBx protein and L-HDAg has been described in the activation of the serum responsive element- dependent pathway (Goto et al., 2003).

The direct effect of HDV on cell proliferation and cancer has been long debated. Analysis of cell proteome changes in the presence of delta antigen or viral genome replication led to the identification of differentially expressed proteins mostly associated to cell cycle regulation and pyruvate metabolism and the suggestion of direct involvement of HDV in carcinogenesis promotion (Mendes et al., 2013; Mota et al., 2008, 2009). Using another screening approach, clusterin expression was shown to be up-regulated upon S-HDAg expression and HDV replication, possibly through histone hyper-acetylation, and to be associated with increased cell survival. These findings support an oncogenic potential of HDV, given that clusterin is not only involved in oncogenic pathways, but was also shown to be overexpressed in HCC tissues (Liao et al., 2009). NF-κB activity has also been shown to be modulated by HDV. Several mechanisms have been proposed, including L-HDAg direct interaction with TRAF-2 and potentiation of TNF-α induced NF-κB transcriptional activation (Park et al., 2009), induction of endoplasmic reticulum stress (Huang et al., 2006), and production of reactive oxygen species upon expression of L-HDAg (Williams et al., 2012).

Immune response and its modulation

As for other aspects of hepatitis delta, much remains to be explored regarding the immune response to HDV infection. Indeed, immune responses, both innate and adaptive, are believed to play an important role in liver damage and disease progression.

Regarding the innate immune response, results are conflicting between studies and the details of the interaction between HDV and hepatocyte innate immune response are only partially explored. Several studies support an activation of interferon signalling pathways by HDV: *in vitro*, HDV replication was first shown to be associated with increased expression of IFNβ (McNair et al., 1994); later the expression of HDAg was associated with an induction of MxA (an Interferon Stimulated Gene – ISG)

(Williams et al., 2009) and recently misfolded HDV RNA was shown to directly activate PKR (Heinicke and Bevilacqua, 2012). *In vivo* HDV was also associated with an increased expression of ISGs, both in the hNTCP transgenic mouse and in the humanized mice model (Giersch et al., 2015; He et al., 2015). Paradoxically, in a study by Negro and colleagues, HDV replication was not associated with the expression of ISGs, but with an inhibition of the cell response to interferon, by interference with an early step of the JAK-STAT signalling pathway (Pugnale et al., 2009). Interestingly, this inhibition of interferon pathway was also suggested (although not thoroughly explored) in the aforementioned study in the humanized mouse model (Giersch et al., 2015).

The role of NK cells during HDV infection and treatment has recently been addressed. The number of NK cells was found to be increased in CHD patients in comparison to healthy controls, however, as for HBV and HCV, they presented a less activated phenotype and were less prone to stimulation (Lunemann et al., 2014). Little is also known about T-cell responses in HDV infection. HDV infected patients have a higher frequency of perforin-positive TCD4+ cells than patients with HBV or HCV, independently of the level of liver inflammatory activity, which may be a possible explanation for the faster progression of liver disease in CHD (Aslan et al., 2006). Specific HDAg epitopes were shown to induce TCD4+ cells expansion, but only in patients with inactive disease (Nisini et al., 1997). Interestingly, HDV CD8+ cytotoxic T lymphocyte responses were also only identified in patients with inactive disease (Huang et al., 2004). In the HIDIT-1 trial patients, a specific pattern of cytokines was shown to decrease during interferon treatment, being related to treatment response. Specific T cell response restoration was only observed in treatment responders (Grabowski et al., 2011).

Interplay between HDV and HBV

In the early characterization of HDV infection, it became evident that, despite its dependency on HBV for its envelope, HDV suppressed HBV replication in both humans and chimpanzees (Hadziyannis et al., 1985; Rizzetto et al., 1980b). The same effect was observed in WHV infected woodchucks and more recently in the HBV-infected

humanized mouse model (Lütgehetmann et al., 2012; Negro et al., 1989). A study of HBV replication in the liver of HDV infected patients, indicated a significant decrease in the levels of HBV replicative intermediates, but not in the synthesis of envelope proteins, in comparison to HBV mono-infected patients (Pollicino et al., 2011). Interestingly, longitudinal follow-up of patients by qPCR, suggests that this pattern of interference is more complex, as viruses may alternate patterns of dominance over time (Schaper et al., 2010). As HBV viremia is an important predictor of disease progression in these patients, a close monitoring of both viral replication markers is warranted.

The mechanisms of viral interference in HBV/HDV infection are still incompletely known. This phenotype was confirmed *in vitro*, indicating its independence from the acquired immune system, and HBV suppression could be reproduced upon the isolated expression of HDAg (Wu et al., 1991). Indeed, one study proposes that both forms of delta antigen might inhibit HBV replication by decreasing HBV enhancer effect (L-HDAg could decrease Enh1 activation by 60-80%). Furthermore, the induction of MxA expression, whose product is known to be a repressor of HBV replication, that has been shown both *in vitro* and in the humanized mouse, could contribute to this phenotype (Giersch et al., 2015; Williams et al., 2009).

3.4. Prevention

Transmission of HDV can be greatly reduced by measures targeting the main exposure risks. As for other blood-borne virus, public health measures focused on a strict screening of blood and its fractionated products for HBV, practices aiming at reducing transmission among IVDUs and hygiene measures in limited resources settings, play an important role in limiting HDV transmission.

HBV vaccine is highly effective in preventing HDV transmission in naïve patients and should be proposed to all the close contacts of HDV infected patients. But no immunoprophylactic strategy exists for the prevention of HDV superinfection in HBV carriers; HBV vaccine does not elicit anti-HBs antibody production in these patients. Unfortunately, antibodies directed to HDAg have no strong neutralizing capacity in HDV infection, thus precluding any use of HDAg based vaccine. However, it has been

shown for other viruses that, even though not providing sterilizing protective immunity, immunization with internal viral proteins can produce a T-cell response and prevent virus spreading. *In vitro* results were promising as stimulation of CD8 T cells with HDV peptides led to an increase on IFNγ production and cytotoxic activity (Roggendorf, 2012). However, in woodchucks, vaccination (either by HDV proteins, DNA or recombinant viruses) was only effective in preventing HDV infection of naïve animals but not in WHV chronic carriers and, although the course of infection was modified, its utility is still to be proven (Fiedler et al., 2001, 2013).

3.5. Treatment

3.5.1. Current available therapeutic strategies

There are currently no specific antiviral treatments for HDV. This is mainly due to the fact that the virus does not encode enzymatic activities, and fully relies, even more than other viruses, on host-cell machinery for its replication. A better understanding of the molecular and cellular biology HDV is yet required to identify viro-modified host cell functions that could be targeted.

In the setting of acute hepatitis delta, no specific treatment has proven useful and patient management relies on monitoring and general support measures, or referral for liver transplantation in patients who progress to fulminant hepatitis (Niro et al., 2005).

Interferon alpha (IFNα) remains the only drug recommended by international guidelines for the treatment of CHD (European Association For The Study Of The Liver, 2012; Lok and McMahon, 2009). Both conventional and pegylated interferon (Peg-IFNα) have been shown to suppress HDV viremia in a subset of patients. Sustained virologic response (SVR) rates vary between 14 to 50% for conventional IFNα and 17 to 44% for Peg-IFNα (Yurdaydin, 2012). However, comparison between studies is difficult as methods, treatment schedules, doses and duration used were highly variable and the number of patients included is very small. Several questions relative to interferon treatment remain unanswered. Firstly, the mechanism of action of IFNα on the control of HDV infection is not completely understood. *In vitro* studies have

failed to demonstrate an potent effect of IFNα on HDV replication (McNair et al., 1994), whereas in the hNTCP transgenic mouse model a suppression of IFN endogenous IFNα/β stimulation was associated with an increased viral replication, suggesting an antiviral effect of the molecule (He et al., 2015). Indeed, IFNα could play an indirect role in the regulation of HDV replication through ADAR-mediated editing (Hartwig et al., 2004). More recent evaluations have suggested that IFNα may also mediate its action via inhibition of viral entry. This was also inferred from mathematical modelling based on the kinetics of the IFNα effect (Goyal and Murray, 2014; Han et al., 2011b). Secondly, unlike what has been described for HCV infection, no impact of IL-28b polymorphisms in treatment response has been demonstrated (Visco-Comandini et al., 2014; Yilmaz et al., 2014). Thirdly, the optimal duration of treatment has not been defined. It is currently advised to treat HDV infected patients for at least one year. Although cases have been reported of patients under interferon treatment for up to 12 years, the benefit of prolonged IFN therapy is still debated. The only study that compared 1 versus 2 years treatment duration found no advantage on treatment prolongation but was limited by the small size of the study population (Ormeci et al., 2011). Recently, two large clinical trials addressed the question of late relapses after interferon treatment of CHD patients. In the first study (HIDIT-1), patients were treated for 48 weeks with Peg-IFNα plus adefovir or either drug alone. At 24 weeks post-treatment, 28% of interferon treated patients had undetectable HDV viremia (Wedemeyer et al., 2011). However, long term follow-up of these patients showed that late HDV RNA relapses occurred in 56% of the patients, compromising the use of the 24 weeks SVR end-point in CHD patients (Heidrich et al., 2014). A second study (HIDIT-2) aimed to evaluate a possible benefit of prolonging therapy in order to avoid relapses. Patients were treated for 96 weeks with Peg-IFNα (associated or not with tenofovir). Although at the end of treatment 33 *versus* 47% of the patients receiving Peg-IFNα (without and with tenofovir respectively; non-significant difference) had undetectable viremia, at 24 weeks post treatment, this proportion significantly decreased to 23-30%. The fact that this values are comparable to the ones obtained for HIDIT-1 (48 weeks treatment) and that adverse events occurred in more than 30% of patients, suggests that extending IFN therapy for more than 1 year does not have a clear benefit (Wedemeyer et al., 2014).

Another unresolved issue refers to the utility of anti-HBV direct-acting antivirals (DAA) in the management of chronic hepatitis delta patients. Although anti-HBV DAAs do not seem to affect HDV replication (Alfaiate et al., 2014), an impact on HDV infection can be expected from the continuous suppression of HBsAg production, leading to a blockage of HDV spreading. However, the persistence of HDV infection in the absence of its helper virus is still debated. Data from different experimental models suggest that HDV monoinfection is self-limiting, in the absence of spreading. Such a profile has been described both *in vitro*, in PHH, and *in vivo*, either following hydrodynamic injection of HDV cDNA into wild type mice or, more recently, following HDV infection of transgenic mice expressing hNTCP (Chang et al., 2001; Gudima et al., 2007; He et al., 2015). It is tempting to postulate that, in the absence of viral particle egress, accumulation of L-HDAg throughout time ultimately leads to replication inhibition (Modahl and Lai, 2000). Furthermore, it has been suggested that, in the absence of particle budding, continuous genome editing, associated with the accumulation of mutations, may also contribute to such abrogation of replication over time (Chang et al., 2005a). Such scenario, combined with the lack of evidence supporting latent HDV infection in transplanted patients (Smedile et al., 1998), supports an essential role for HBV in the maintenance of a chronic HDV infection and would support the utility of HBV DAAs in patients with CHD. However, data exist suggesting that HDV can indeed establish a latent infection in the absence of HBV. Following *in vitro* HDV infection of both primary human and chimpanzee hepatocytes, in particular culture conditions, HDV replication was kept at stable levels for more than one month (Barrera et al., 2004; Sureau et al., 1991). More recently, in the humanized liver mouse model, HDV infection was shown to be kept latent for at least 6 weeks, in the absence of HBV (Giersch et al., 2014). In this scenario, the utility of HBV DAAs in the control of HDV infection would be more limited, as in the presence of continuous HDV replication, the virus would only be eliminated by the turnover of infected hepatocytes overtime. Patient data confirm a limited utility of HBV DAAs in the control of HDV infection, in association with modest HBsAg decrease rates. Lamivudine, adefovir and entecavir did not prove effective against HDV (Yurdaydin, 2012). However, studies from HIV/HBV/HDV co-infected patients suggested that long-treatment with tenofovir not only suppressed HBV but also led to a decrease of HDV replication parameters and

improvement of liver disease (Boyd et al., 2013; Soriano et al., 2014). The aforementioned HIDIT-1 and 2 trials failed to show a significant increase in Peg-IFNα treatment effectiveness upon association with adefovir or tenofovir, respectively. A beneficial effect of the addition of adefovir on the levels of HBsAg levels was observed in HIDIT-1 but not confirmed with tenofovir in HIDIT-2. Furthermore, no significant decrease in the levels of transaminases was observed during DAA treatment, suggesting that, HDV replication needs to be specifically tackled in order to prevent liver damage (Wedemeyer et al., 2011, 2014). Other antiviral molecules like ribavirin and clevudine also failed to prove effective in human clinical studies, despite promising results in experimental models (Yurdaydin, 2012).

Management of chronic hepatitis delta hence remains mostly empiric and should be decided for each individual patient. As for HBV, only HBsAg sero-conversion (and not undetectable HDV viremia, even if persistent) is synonymous of hepatitis delta cure, making close monitoring of HDV and HBV infection markers mandatory during and after treatment.

3.5.2. Investigational drugs in preclinical/ clinical development

Given the particular characteristics of HDV, replication remains difficult to target. Ongoing development of anti-HDV drugs is hence focusing on other steps of the viral cycle, namely entry and assembly. Novel strategies based on immune-stimulation with cytokines or PRR agonists may also play a role in the future management of HDV infection.

Entry inhibition

As for other viruses, several therapeutic strategies can be used to target HDV attachment and cell entry and hence prevent the infection of new cells. After identification of myristoylation of the PreS1 region as essential for viral entry, peptides mimicking the myristoylated N-terminal 47 aminoacids of L-HBsAg were synthetized and shown to potently and specifically, inhibit HBV entry *in vitro* (Gripon et al., 2005). Later on, a lead substance consisting of the consensus sequence of all known primate

HBV genotypes (later named Myrcludex-B®) showed a very potent inhibition of HBV infection (IC50 80pM) *in vitro* (Schulze et al., 2010). Studies in the humanized mouse model confirmed the inhibitory effect of Myrcludex-B® both in the establishment of *de novo* HBV and HDV infections and in the spreading of previously established infections (Lütgehetmann et al., 2012; Volz et al., 2013). Besides blocking viral entry, Myrcludex-B® also blocks bile acid transport (NTCP's physiological function). Although the clinical repercussions of this side effect remain to be identified, they may not be significant as the EC_{50} of the drug for viral entry inhibition is almost 1000 times lower than the EC_{50} for inhibition of bile salt uptake (Nkongolo et al., 2014). After safety and tolerability testing first in chimpanzees and then in a phase I clinical trial, Myrcludex-B® is currently undergoing a phase IIa trial. Preliminary results showed a decrease in both HBV and HDV viremia at week 24 of treatment (Bogomolov et al., 2014).

Other molecules have been shown to inhibit HBV/HDV NTCP-mediated entry *in vitro*. Cyclosporin A has recently been identified by two different teams as an inhibitor of HBV/HDV entry through binding to NTCP, in a cyclophilin-independent manner (Nkongolo et al., 2014; Watashi et al., 2014). As cyclosporine A is often a part of the immunosuppressor combination used in the post-liver transplantation setting, it is tempting to consider its potential role to prevent the graft re-infection. Other NTCP inhibitors already approved for other indications, as ezetimibe, irbesartan and ritonavir, have been shown to inhibit early stages of HDV infection, although the mediation through NTCP for this effect is still to be demonstrated (Blanchet et al., 2014).

Anti-viral strategies also explore the interaction between HBsAg and HSPGs. Suramin would be a candidate but its use is limited by toxicity (Urban et al., 2014). A new class of agents has recently been shown to be effective, not only against HBV, but also HCV, HSV 1 and 2 and HIV (Krepstakies et al., 2012).

Assembly inhibition

As previously described, HDV assembly depends on the prenylation (more specifically farnesylation) of the last four amino acids of L-HDAg (CXXQ motif) (Glenn et al., 1992). Prenylation inhibitors have been used as anticancer drugs, as oncogenic forms of Ras

are dependent on farnesylation. These molecules were shown to inhibit HDV assembly and secretion both *in vitro* and in a mouse model capable of producing HDV viremia (Bordier et al., 2002, 2003). Results from a phase 2a clinical trial conducted in adult patients with CHD have recently been reported. The patients received the drug for 28 days and were then followed for 6 months. During treatment, lonafarnib was associated with a dose-dependent, albeit modest (-1,54 log IU/ml for the higher dose), decrease in HDV viremia. However, treatment had no effect on transaminases and, once stopped, a rebound of HDV viremia was observed in all patients, underscoring the need for further optimization and development of anti-HDV therapeutic strategies (Koh et al., 2015)

As for entry inhibition strategies, progress can arise from HBV research. Promising results have been reported for the inhibition of DHBV infection by nucleic-acid-polymers (NAPs). These oligonucleotides interact with structurally conserved amphipathic alpha-helical protein domains found in a variety of virus and other infectious agents and are known to concentrate in the liver. These molecules have been shown to inhibit DHBV infection *in vitro* and *in vivo* (Noordeen et al., 2013a, 2013b). Preliminary works suggest an effect on HBV infection. The mechanism of action, although not fully elucidated, seems to be associated with both the inhibition HBsAg-mediated entry and release, and opens a perspective for their use in hepatitis delta (Noureddin and Gish, 2014).

Immunomodulation

New immune-modulatory agents, such as interferon lambda and PRR agonists (Zeisel et al., 2015), are proving effective against HBV infection. TLR7 agonist GS9620 is particularly promising as it has been shown to have a potent antiviral response against HBV (with HBsAg loss and reduced amounts of cccDNA), both in the chimpanzee and in the woodchuck model (with WHV) (Lanford et al., 2013; Menne et al., 2015). A phase II clinical trial is currently underway (clinicaltrials.gov NCT2430181). Although its action on HDV infection is for the moment unknown, it is tempting to speculate that these drugs may have a role in the future treatment of hepatitis delta.

3.6. Conclusion

In spite of its severity, the overall high number of infected patients, and the viral characteristics that make HDV a unique model of study, HDV infection can unfortunately be considered as a neglected tropical and non-tropical disease. Many questions are still unanswered regarding clinical issues, as well as more basic knowledge regarding the virus life cycle and its interactions with the host. Novel interest on HDV seems to be arising, as new antiviral strategies are being developed against HBV infection. Hopefully the newly developed animal and cell culture models will boost research on the mechanisms of HDV infection and pathogenesis, and will contribute to the development of novel HDV-specific antiviral strategies.

4. Viral coinfections and virus-virus interactions

4.1. Introduction

In order to approach the possible mechanisms of interaction between HDV and HBV, a broader evaluation of the known mechanisms of virus-virus interactions (VVIs) is essential. Viral coinfections are found in every branch of life, from unicellular organisms (bacteria in particular) to animals, including humans, passing through plants. In human pathology, coinfections may modulate disease expression, regulate pathogenicity, and lead to greater cumulative immunosuppression in the host (Singh, 2005).

Many definitions of viral interaction have been proposed ranging from broad and vague to restrictive interpretations of the designation. DaPalma and colleagues proposed a systematic approach to VVI and define it as "the **measurable difference** in the course of infection of one virus as a result of a concurrent or prior infection by a different species or strain of virus. A **concurrent infection** may include infection of the same cell by two or more virus species, or two viruses may infect different cell types within one organism and produce measurable VVI. Measurable differences include changes in tissue permissiveness or tropism, viral replication, patterns of progeny production and release, latency, pathology including immunopathology, and immunological responses" (DaPalma et al., 2010). This stringent definition includes not only the influence of coinfection on virus life cycle, but also the modulation of the host response to infection.

In order to study the mechanisms of viral interaction, a systematic classification must be used, so that similar viral strategies can be grouped together and compared among them. A simple classification splits VVIs, according to their outcome, into facilitative and antagonistic. **Facilitative interactions** involve a benefice for both or at least one of the viral partners involved. It comprises *synergistic interactions* that are reflected by increased replication and exacerbated disease progression and *helper dependent interactions*, where there is a facilitation of the dependent virus by its helper. In

antagonistic interactions only one of the viruses is beneficiary and its presence and activity lower the fitness of the other (Syller, 2012).

A more comprehensive classification has been proposed considering the known mechanisms involved in the interaction. It splits VVIs into three main categories: **direct** interactions, **environmental** interactions and **immune mediated** interactions. Table V presents common examples of the main groups of interactions.

Table V. Classification of viral interactions according to the mechanism involved. Examples include only viruses known to be associated with human disease. Adapted from (DaPalma et al., 2010)

Categories	Examples (virus causing human disease)
Direct interactions	
Helper-dependent viruses	AAV and HSV-1; AAV and Adenovirus; HDV and HBV
Pseudotype viruses	HTLV and CMV; HIV-1 and HSV-1
Superinfection exclusion	
Genomic recombination	Influenza, HIV, Poliovirus
Embedded viruses	
Heterologous transactivation	CMV and HIV-1; EBV and HCV; HSV-1 and HIV-1
Environmental interactions	
Indirect transctivation of genes	HHV6 and EBV; HERV-K and HSV1; CMV and HIV; EBV and HERV K18
Breakdown of physical barriers	HSV-2 and HIV-1
Altered receptor expression	HHV-6 and HIV; HHV-7 and HIV
Heterologous activation of pro-drugs	HSV-1 and HIV-1
Modification of the IFN-induced anti-viral state	HSV-1 and SV40
Immune effects	
Altered immune cell activation	HIV-1 and CMV; GBV-C and HIV-1; HBV and HCV (?)
Induction of auto-immunity	Enterovirus
Antibody-dependent enhancement of infection	DENV, YFV, WNV, JEV
Heterologous Immunity	Influenza and LCMV

To contextualize the research work discussed in this thesis manuscript, we will proceed with a brief and non-exhaustive discussion of relevant examples of virus-virus interference in human disease. Although a number of human viral coinfections has been described, as seen previously in Table V, we decided to explore the most frequent ones and/ or those associated with a clear modification of the natural history of the infection. As such, the discussion will be focused on hepatotropic viruses and HIV.

4.2. Coinfections between hepatotropic viruses

4.2.1. HBV/ HCV

HBV and HCV infect a considerable number of patients worldwide (around 240 and 150 million people, respectively), have common transmission modes and share the capacity to induce a chronic infection. It is hence not surprising that their combined infection is fairly frequent, particularly in highly endemic areas and among subjects with a high risk of parenteral infections (e.g. drug users). The estimated prevalence of HBV/HCV dual infection is approximately 5%-20% in HBsAg positive patients and 2%-10% in HCV positive patients, although variable rates are reported depending on the geographic region. In these patients, acute infection is frequently asymptomatic. Three main patterns of acquisition can occur: i) **HBV/HCV coinfection** that, as previously described for HDV, often has a self-limiting, benign course with complete recovery from one or both infections; ii) **acute HCV superinfection** of an HBV chronic carrier or, finally, iii) **acute HBV superinfection** of a chronic hepatitis C (CHC) patient. In all the settings, patterns of viral dominance have been described, mostly related to the chronology of infection, with a repression of the chronic infection by the acutely acquired virus. In cases of HCV superinfection, suppression of HBV replication is commonly reported, although often transient. In cases of HBV superinfection, HCV has been described to be definitively cleared. (Liaw et al., 2000, 2004; Sagnelli et al., 2002, 2006). Longitudinal studies have shown that, in chronic coinfection, the patterns of viral dominance are not stable and may vary over time in the same patient. It should be noted that cases of occult hepatitis B are particularly common in the HBV/HCV

coinfection setting and, despite absent or low HBV replicative activity, these patients still have a more aggressive progression than mono-infected CHC patients (Squadrito et al., 2013). Moreover, in patients with an HCV replicative dominance, completion of IFNα treatment may be associated to ulterior HBV relapse (Konstantinou and Deutsch, 2015).

Clinically, chronic HBV/HCV coinfection is indistinguishable from either chronic mono-infection. However the prognosis of these patients is more severe: coinfection is associated with a higher prevalence of liver cirrhosis and hepatic decompensation as compared with HBV or HCV mono-infection and was it demonstrated to be an independent predictor of HCC development (several studies report an increased risk of HCC in these patients, yet meta-analysis have not been conclusive) (Caccamo et al., 2014; Cho et al., 2011).

For long, the study of interactions between the two viruses has been limited by the absence of adequate experimental models (both *in vivo* and *in vitro*). *In vitro* studies were at first based on heterologous overexpression of viral proteins and have yielded conflicting results. Two independent studies based on Huh7 cells (that allow the full life cycle of HCV [JFH1 strain] and HBV replication [after transfection]), have excluded direct viral interference, as neither virus had its replication affected by the other (Bellecave et al., 2009; Eyre et al., 2009). The same results have been recently replicated in a new cell line supporting the complete life cycles of both HBV and HCV (Yang et al., 2014). However, an immunological interaction between the two viruses cannot be excluded *in vivo* and has recently been supported by evidence of HBV superinfection restriction in chimpanzees chronically infected by HCV (Wieland et al., 2014).

4.2.2. HBV/ HCV/ HDV

Triple infections by HBV, HCV and HDV are common in populations at risk of parenteral transmission, as IVDUs, frequently in association with HIV infection. Although the epidemiology, natural history, viral kinetics, and treatment strategies for each single infection have been extensively studied, less is known about the specific features of multiple hepatitis virus coinfections. Moreover, clinical trials conducted in patients

with viral hepatitis commonly exclude triple infections. Consequently, most of the data available derive from retrospective studies conducted on a limited number of patients or from epidemiologic surveys. It is known that multiple infections aggravate liver injury, particularly fibrosis, and increase the risk of HCC, although the long-term clinical outcome in these patients is still largely undefined (Farci and Niro, 2012).

Most reports on patterns of viral dominance are based on cross-sectional studies and results seem to depend on geographical location: whereas western studies documented a suppressive effect of HDV on both HBV and HCV, studies from Taiwan have demonstrated a suppressive role of HCV on HBV and HDV (Jardi et al., 2001; Liaw, 1995; Lu et al., 2003; Mathurin et al., 2000). Similarly to the other multiple infections, longitudinal studies indicate that viremia levels of each virus fluctuate over time (Raimondo et al., 2006). The mechanisms underlying such interactions are widely unknown.

4.2.3. Interactions between hepatitis viruses and other viral agents

HBV

Important data has emerged from the Chisari's laboratory concerning the regulation of HBV replication and gene expression in the presence of other viruses, notably **adenovirus**, **lymphocytic choriomeningitis virus** (**LCMV)** and **mouse cytomegalovirus (CMV)**. Taken together, these successive studies in the HBV-transgenic mouse model, evidenced a suppression of HBV replication upon infections with other viral agents. Notably, it was shown that: i) adenovirus infection was associated with a decrease in HBV DNA but not RNA levels; ii) LCMV and mouse CMV infections lead to a decrease in both DNA and RNA; iii) while LCMV post-transcriptionally decreases the levels of HBV RNA, MCMV is associated with transcriptional inhibition. Furthermore, it was demonstrated that interference was immune mediated, being dependent on TNFα and/or IFNα/β signalling pathways and therefore NF-kB and ISGF3 transcriptions factors. A role for IFNγ in post-transcriptional regulation of HBV replication was also suggested (Cavanaugh et al., 1998; Guidotti et al., 1996; Uprichard et al., 2003). An *in*

vitro study alternatively suggested a direct inhibitory effect of adenoviral EIA protein in the inhibition of HBV Enh I activity (Chen et al., 1992).

In spite of the clear interest of such findings to understand HBV transcriptional regulation, the impact of such coinfections in humans is not clear. It was only possible to identify an isolated case-report on the consequences of coinfection by HBV and CMV. The clinical course was particularly serious with development of hemophagocytic syndrome (a hyperinflammatory condition), but the patient ultimately progressed to resolution of both infections (Halfon et al., 2009).

The viral interaction can also be positive. HBx protein was recently reported to increase the expression of a transgene delivered to cells by Adeno-associated virus (AAV) vectors (Hösel et al., 2014). Indeed, HBx is a transactivating protein particularly efficient at up-regulating genes bore by extra-chromosomal DNA (van Breugel et al., 2012). This applies to AAV genome and opens interesting opportunities to better deliver anti-HBV effector genes into HBV infected cells.

HCV

In the setting of organ transplantation, significant interactions have been found between HCV and herpes viruses, leading to the exacerbation of liver pathology. A number of independent studies have shown that **CMV** infection significantly increased the risk of fibrosis and allograft cirrhosis in patients undergoing liver transplantation because of HCV. Additionally, this effect seems to be abolished in patients receiving anti-CMV pre-emptive therapy. The mechanism of such aggravation is unknown (Singh, 2005).

Interactions between HCV and **EBV** have been postulated to be reciprocal: on the one hand, HCV, by its association with mixed cryoglobulinemia, seems to be associated with an increased risk of B cell non-Hodgkin's lymphoma in EBV infected patients; in the other hand, EBV has been found in 37% of analysed CHC tissues from patients with HCV. Results from studies evaluating the interactions between the two viruses are contradictory, as interactions are not always found. A role for EBV EBNA protein in enhancing HCV replication *in vitro* has been proposed (Challine et al., 2002; Sugawara et al., 1999).

4.3. Coinfections in the setting of HIV infection

4.3.1. HIV/ hepatitis coinfections

Due to similar routes of transmission, coinfection of HIV with other sexual and blood-borne viruses such as HBV, HCV and/or HDV is relatively common. The average estimated risk of transmission for HBV and HCV and HIV based on mode of transmission is depicted in Table VI.

Table VI. **Average estimated risk of transmission for parenteral agents**. The risk of HBV vertical transmission varies depending of the mother's HBeAg serostatus (higher for HBeAg positive mothers with HBV viremia > 10^6 IU/ml). HBV risk of transmission by sexual contact also depends on viremia levels. Adapted from (Lacombe and Rockstroh, 2012)

	Average transmission risk (%)				
Transmission	**HIV**	**HBV**	**HCV**	**HBV-HIV**	**HCV-HIV**
Perinatal	10-20	10-90	<2-7	10-90	10-20
Sexual	<1	<90	<1	<90	<1-3
Needle stick with cannula	0,3	30	0,3	?	?

Of the 35 million people living with HIV worldwide, around 20% (~7 million) are estimated to have chronic hepatitis C (Figure 26). This population is mainly represented by individuals with past history of IVDU, haemophiliacs and recipients of contaminated blood. With respect to HBV, the situation is slightly different, with rates of HBV chronicity in HIV+ patients ranging from 5% in Western countries to 20% in some HBV endemic regions of Sub-Saharan Africa and South East Asia. Around 15-50% of HBV–HIV co-infected patients worldwide are superinfected by the delta virus (Lacombe and Rockstroh, 2012; Soriano et al., 2010).

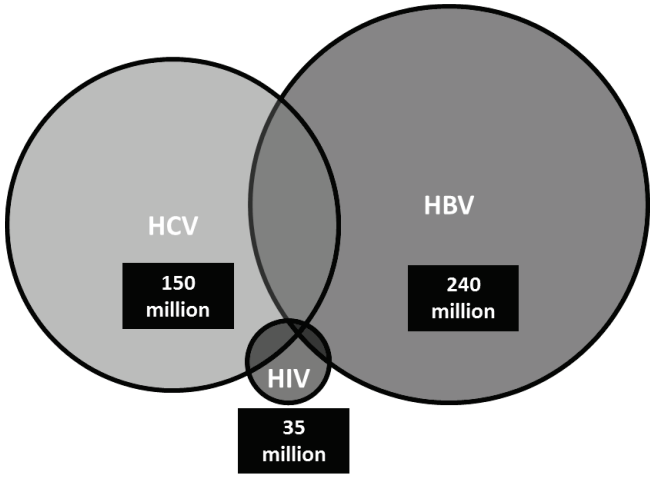

Figure 26. Estimated number of individuals with HIV, HBV and HCV worldwide. Adapted from (Soriano et al., 2010).

4.3.1.1. Impact of HIV infection on viral hepatitis progression

Despite the immune suppression it induces, HIV infection is associated with accelerated liver fibrosis progression in patients with chronic viral hepatitis (Farci and Niro, 2012). The prevalence of cirrhosis differs among HIV co-infected patients depending on the aetiology of hepatitis: HCV, 19.2%; HBV, 6.1%; HBV⁄ HCV, 41.7%; and HBV⁄ HCV⁄ HDV, 66.7% (Castellares et al., 2008).

In these patients, as in mono-infected patients, variable patterns of hepatitis virus dominance have been described (Boyd et al., 2010; Maida et al., 2008; Morsica et al., 2009).

HCV

HIV infection is associated with an aggravated clinical course of HCV infection, as summarised in Table VII.

Briefly, HCV/HIV coinfected patients have increased levels of HCV viremia, a higher probability of progression to chronicity following acute HCV infection (80% versus <70% in HIV negative patients), a faster progression of liver fibrosis (development of cirrhosis occurs 12–16 years earlier than in patients infected with HCV alone) and a worse response to interferon treatment (Joshi et al., 2011; Soriano et al., 2010; Thomas, 2002). Interestingly, effective HIV viremia reduction under combined antiretroviral therapy (cART) has been shown to be associated with a reversion of

progression rates (Bräu et al., 2006). However, an ulterior meta-analysis has shown that this reversion was only partial (Thein et al., 2008).

Table VII. Characteristics of HCV infection in HIV infected patients.
Adapted from (Thomas, 2002)

Difference	Comment
Higher prevalence of HCV infection	Overall 25%, much higher when HIV by parenteral route
Increased viral persistence	Probably increased 2-fold, but race and gender could bias studies
Increased HCV RNA level	Was only observed after HIV seroconversion and in some studies with declining CD4 lymphocyte count; unknown significance
Increased risk of cirrhosis	Variable risks reported, 2-fold increase
Increased seronegative infection	Overall 2% to 6%; low rates in injection drug users; 20% Iowa City HIV cohort
Decreased response to interferon alpha	No true comparative studies taking into account genotype and race. Very low effectivity if interferon if TCD4 <200cells/mcl
Increased drug-drug interactions	Ribavirin may interact with several antiretroviral drugs

The mechanisms underlying the faster progression of hepatitis C in HIV-infected patients seem to be multifactorial, both through direct involvement of HIV or via modulation of the immune response:

i. in patients with HIV infection, weakened adaptive immune responses to HCV infection, reduced CD8+ cell responses and reduced intrahepatic secretion of IFNγ, TNFα and IL-10, can contribute to HCV persistence (Bruno et al., 2008; Joshi et al., 2011);
ii. HIV can bind hepatic stellate cells and hepatocytes through its coreceptors, CXCR4 and CCR5. Although the virus does not enter hepatocytes, binding of gp120 to CXCR4 has been shown to increase tumour necrosis factor related

apoptosis inducing ligand (TRAIL) production, resulting in cellular apoptosis, and to upregulate production of transforming growth factor (TGF)-β1, which promotes further stellate cell activation and HCV replication (Babu et al., 2009; Lin et al., 2008);

iii. Direct activation of hepatic stellate cells by HIV triggers a pro-inflammatory cascade, leading to myofibroblastic differentiation via enhanced production of a-smooth muscle actin, collagen and monocyte chemoattractant protein 1 (MCP-1) (Tuyama et al., 2010);

iv. decreased interleukin-10 expression by intrahepatic TCD4+ cells exacerbates the profibrotic milieu (Blackard et al., 2006; Joshi et al., 2011);

v. markers of microbial translocation (lipopolysaccharide, lipopolysaccharide binding protein, CD14, and fucose-binding lectin) are raised in individuals coinfected with HIV and HCV, and strongly correlate with HIV-related depletion of CD4+ cells and progressive HCV-related liver disease (Joshi et al., 2011);

vi. Finally, it is important to recognize that there are several additional factors that could lead to more severe liver disease in HIV/HCV coinfected patients, including drug related hepatotoxicity, concurrent use of drugs and alcohol, and steatosis related to metabolic syndrome (Hernandez and Sherman, 2011). It will be important to further evaluate the efficacy of new anti-HCV DAAs in HIV infected patients and their potential interactions with antiretrovirals drugs.

HBV

As for HCV, HBV progression also seems to be affected by HIV coinfection. Briefly, HIV coinfection is associated with a 5-fold increased progression to chronicity after an acute HBV infection in comparison to non-HIV infected adults, a decreased rate of HBe and HBs seroconversion (which can be restored with cART initiation), an increased risk of liver disease progression and death as well as an increased risk of HCC (in association with higher levels of HBV viremia in coinfected patients) (Lacombe and Rockstroh, 2012; Soriano et al., 2010). See Table VIII for a summary.

Table VIII. Effects of HIV on the natural history of adult-acquired HBV infection.
Adapted from (Thio, 2009).

Difference	Comments
Increased risk of chronicity	Studies in men who have sex with men. Lower CD4+ T-cell count with higher risk of chronicity.
Decreased rate of HBeAg clearance	
Increased HBV replication	Higher HBV DNA levels
Increased risk of anti-HBs antibody loss	Increased risk associated with lower CD4 T cell counts
Decreased inflammatory response	Lower ALT levels
Increased liver disease progression	More frequent cirrhosis and higher liver-related mortality
Increased risk of HCC	Overall 33% increased risk per 100 TCD4 cell decrease. Risk greater among men who have sex with men than in injection drug users.

Mechanisms previously mentioned for fibrosis progression in CHC can also apply to HBV infection. Additional mechanisms underlying the altered progression of HBV infection (extensively reviewed by Iser and Lewin, 2009) could include:

i. Reduced NK cytotoxicity is associated with failure to clear acute HBV infection in HIV patients;
ii. Decreased expression and TLR dysfunction induced by HIV infection may limit HBV clearance;
iii. HBV-specific TCL and TCD4+ responses are decreased in HIV infected patients, in comparison to HBV mono-infected patients and an increase is verified in patients starting cART;

Finally, HBV infected patients, even with occult HBV infection, are susceptible to disease aggravation once HIV mediated immune suppression is reversed, namely by cART (the so-called “immune reconstitution syndrome”). This increase in inflammatory response, translated by an increase of liver necroinflammatory activity, may or may not be associated with a decrease of HBV replication. It is thought to be a

consequence of the restoration of anti-HBV adaptive immune response (Iser and Lewin, 2009).

HDV

The prevalence of anti-delta antibodies in HIV infected patients with HBsAg ranges from 15% to 50%, depending on geographical region and risk group category. In Western countries, HDV is more frequent in IVDU than among persons infected with HIV by sexual contact.

In a sub-analysis of the EuroSIDA cohort, HDV was found to infect 14,5% of the HIV/ HBV infected patients (a proportion that is higher than the one observed in non-HIV infected patients in Europe), more significantly associated to transmission in IDVUs (42%). The majority of patients had detectable HDV viremia. Dominance of HDV over HBV was documented and HDV was mainly acquired as a result of superinfections rather than coinfections with HBV (Soriano et al., 2010). Compared to HBV/HDV infected patients, HBV/ HDV/ HIV infected patients presented higher level of transaminases, frequent presence of circulating HDAg and higher incidence of hepatic decompensation (De Pouplana et al., 1995).

The mechanisms by which HIV facilitates liver disease progression in CHD patients are unknown.

4.3.1.2. Impact of viral hepatitis on HIV progression

HCV

Clinical studies comparing the evolution of HIV infection in patients with or without infection by hepatitis viruses have documented, in some cases, a more serious clinical course in coinfected patients.

Before the introduction of cART, no differences in progression to AIDS, death, or decline in CD4+ cell counts were recorded between patients infected with HIV alone and those co-infected with HIV and HCV. Furthermore, progression to AIDS and rate of reduction in CD4+ cell counts did not differ between HCV-positive patients acquiring HIV and those with HIV infection alone, suggesting that HCV coinfection did not affect HIV disease in the absence of cART. However, after the introduction of cART, evidence

suggests that HCV coinfection does affect HIV progression. In the Swiss HIV Cohort Study, HIV/ HCV coinfection was associated with faster progression to AIDS and slower TCD4+ recovery than in patients with HIV infection alone. Posterior studies have shown contradictory results, as an impaired response is not always documented in HCV infected patients. However, in a review of 8 studies, it was demonstrated that HIV/ HCV coinfected patients are more likely to have a worse immunological response to antiretroviral therapy than HIV monoinfected patients. Moreover, in a group of HIV natural viral suppressors (HIV-1 patients with the ability to suppress HIV viral loads to less than 400 copies) chronic HCV infection had statistically significant reduction of TCD4+ cell count and TCD4+ percentage, suggesting once more a possible detrimental effect of HCV on HIV infection (Hernandez and Sherman, 2011).

The mechanisms by which chronic HCV replication could have a deleterious effect on TCD4+ cell count reconstitution remain unexplored, but may include ongoing T cell activation related to HCV infection and direct infection of TCD4+ cells by HCV that has been shown to be lymphotropic in the setting of HIV coinfection (Thomas, 2002).

HBV

Recent data suggest that HBV coinfection might be associated with an increase in overall mortality in HIV infected patients. These patients have a 10 times higher risk of dying from liver related causes compared with HIV or HBV mono-infected patients (Lacombe and Rockstroh, 2012).

In an African cohort, HBV has been associated with an unfavourable course of HIV infection, reflected not only by increased levels of viremia and lower TCD4+ lymphocyte counts previous to cART institution, but also by a weaker immunological recovery during therapy (Ladep et al., 2013). However, these results have not always been reproduced by other long-term outcome studies where HBV did not affect HIV response to cART (Joshi et al., 2011). No exact mechanism has been proposed to this facilitative effect of HBV on HIV infection. Several possibilities can be evoked for both direct and immune-mediated interactions, namely HBx capacity to increase the transcription of HIV long terminal repeats (LTR) *in vitro* and an effect of HBV on the function and maturation of dendritic cells (DCs) (Iser and Lewin, 2009).

GBV-C

GB virus type C (GBV-C) was identified in 1995 in the serum of a non-A non-B hepatitis patient and variants of the same virus were subsequently discovered in the serum of other patients. It was initially considered as a possible cause of hepatitis, however no causal association was ever established between GBV-C and viral hepatitis or any other human pathology.

GBV-C is an enveloped positive-sense, single stranded RNA virus of about 9400nt. It belongs to the *Flaviviridae* family (unassigned genus). It bears sequence similarities with HCV. GBV-C tropism is a matter of discussion, with *in vivo* and *in vitro* reports of both lymphotropism and hepatotropism.

Shortly after its discovery, screening of HIV patients has shown high prevalence of coinfection (GBV-C viremia detected in 17-27% of the patients) in this population. Interestingly, a possible modulation of HIV infection in these patients was suggested. Most of the subsequent studies confirmed an association of GBV-C coinfection with lower HIV viremia, higher TCD4+ cell counts, slower progression to AIDS, improved response to cART and reduced mortality. An inverse relationship between GBV-C and HIV viral loads was suggested and the possibility of inhibition of HIV replication has since been explored (Giret and Kallas, 2012) .

The exact mechanisms of interference remain obscure, despite the identification of several possible pathways, most of them related to immune regulation (Shankar et al., 2011):

i. GBV-C down-regulates chemokine receptors (CCR5 and CXCR4, which function as HIV-1 co-receptors) to interfere with HIV-1 binding to T-cell surface;
ii. GBV-C effectively prevents the onset of immune activation by preserving the Th1 cytokine responses to effectively control viral levels;
iii. GBV-C infection in HIV-infected patients leads to the activation of ISGs;
iv. GBV-C replication is associated with lower T-cell activation;
v. GBV-C could interfere with apoptosis in the T cells of HIV-coinfected patients, by modulating Fas expression;

Moreover, direct mechanisms of inhibition have also been suggested, namely (Giret and Kallas, 2012; Schwarze-Zander et al., 2012):

i. Direct inhibition of HIV entry by binding of GBV-C E2 protein to HIV particles, blocks their interaction with the receptor;
ii. A direct inhibition of HIV replication by GBV-C proteins has also been suggested, although details of such interaction remain unexplored.

4.3.2. Others

HIV infection is associated with increased susceptibility and accelerated progression of a number of viral infections, as a consequence of the generalized immune-depression it induces. As this is not a specifically related to the pathogen, we will focus on examples of viruses that have, conversely, been shown to interfere with the course of HIV infection.

Herpesviruses

A number of studies, particularly in the transplantation setting, have shown that HIV infection progression could be modulated by herpes virus coinfection. Most of these mechanisms are related to transactivation of HDV LTR by herpes viral proteins.

Clinically, most studies have focused on the interactions between **HSV-2** and HIV, mainly due to the shared transmission by sexual contact. HSV-2 is thought to infect half of HIV infected adults. Support for viral interference arises from two clinical observations (Barnabas et al., 2011; Tan et al., 2013):

i. HSV-2 infection is associated with increased HIV plasma viral load. In a meta-analysis performed in 2011 an overall increase of 0,18 log10 copies/ ml was found in HIV viremia in patients with a positive HSV-2 serology. Moreover, an increase of 0,4 log10 copies/ ml was evidenced in patients having detectable genital HSV-2 DNA. Nonetheless, results on the impact of HSV-2 infection on CD4+ cell counts are contradictory and mainly support the absence of an effect;

ii. Patients under acyclovir suppressive therapy demonstrate a modest attenuation of HIV disease progression (-0,28 log10 copies/ ml). This effect is not reproduced in patients treated episodically. It must be considered that the observed trend may be a result of acyclovir antiviral effect on HIV replication and not of HSV-2 control.

It has been shown that herpes viruses can directly influence HIV translation by transactivating its LTR sequence, hence supporting the existence of direct interference (Singh, 2005).

An additional form of modulation of HIV infection by HSV-2, concerns the potentiation of its transmission. HSV-2 infected patients have a higher susceptibility to HIV infection and, when infected, display a higher possibility of transmission to other persons. This is associated with the ability of HSV-2 to cause genital mucosal breaks and to recruit TCD4+ cells to the lesions (Celum, 2004; Sheffield et al., 2007).

HHV-6 has been proposed to be a cofactor in HIV progression, as it can, not only transactivate HIV LTR, but also upregulate CD4 expression (Singh, 2005).

An association between **HHV-8** (Kaposi associated Herpes virus, KSHV, itself a opportunistic pathogen of HIV-infected patients) and enhanced HIV replication was also evidenced both *in vivo* and *in vitro* (Mercader et al., 2001). A direct interference mechanism has been suggested (Sun et al., 2005).

Interestingly, **HHV-7** coinfection has been suggested to inhibit HIV infection progression, through competition for CD4 binding, thus interfering with HIV entry (Lusso et al., 1994).

Human T-cell lymphotropic virus (HTLV)

HTLV 1 and 2 are retroviruses that share the same transmission routes as HIV and are simultaneously prevalent in several parts of the world, so coinfection is not rare (rates vary depending on geographical distribution).

HTLV-1, which is prevalent in the southern hemisphere, has a tropism for TCD4+ cells.

Coinfection with HTLV-1 seems to be detrimental to HIV-1 patients, with accelerated disease progression and association with neurologic and haematologic complications. HTLV-1 Tax protein has been demonstrated to be involved in the outcome of coinfection (Brites et al., 2009; Casoli et al., 2007).

HTLV-2 predominates in Europe and in the USA and is tropic for TCD8+ cells. Interestingly, in HIV-1 infected patients, HTLV-2 coinfection may confer an immunologic and survival benefit and is frequently found in long-term non-progressers. Underlying mechanisms seem to involve regulation of chemokine production and TCD8+ cell expansion (Casoli et al., 2007).

5. Innate immune sensing of pathogens and the antiviral interferon response

5.1. Introduction

The role of the immune system is to distinguish between self and non-self, the latter designating not only pathogens but also cancer cells, and to eliminate it. Classically, three levels of protection against pathogens have been described: i. **physical barriers** to infection (the skin being the most important); ii. **innate immune response**; iii. **acquired or adaptive immune response**. Viral infection poses a particular problem to the host immune responses, as, on the one hand, virus are fast-replicating intracellular organisms and therefore more difficult to detect and eliminate, and, in the other, they have evolved mechanisms of immune escape and modulation (a characteristic that is particularly common in viruses, like hepatitis, that can cause chronic infections).

Clinical consequences of a viral infection are dictated by the interplay between the virus and the immune system. For many infections, the line between immunity and immunopathology can be thin, as most of the clinical consequences may result from the immune response and not the virus infection itself (Rouse and Sehrawat, 2010).

Understanding the immune response against viral infection can allow not only the identification of new therapeutic strategies but also a clarification of important aspects of viral pathogenesis. A deductive approach, may allow the exploitation of general mechanisms into particular cases of infection.

We will focus hereinafter on the innate immune responses to viral infection.

5.2. Pathogen recognition

Innate immunity is the first barrier of defence against a pathogen and is hence both rapid and non-specific. The basic machineries underlying innate immune recognition are highly conserved among species (from plants and fruit flies to mammals). It relies on the recognition of particular conserved motifs that arise during pathogen life cycle, known as pathogen associated molecular patterns (**PAMPs**) that can be nucleic acids,

proteins, lipids or carbohydrates. Common examples are double-stranded RNA (dsRNA), for some viruses, or lipopolysaccharide (LPS) of gram negative bacteria. These molecules are difficult for the microorganism to alter, as they are essential for its survival. They are sensed by the host's pattern recognition receptors (**PRRs**). It has been suggested that, in order to distinguish pathogenic from non-pathogenic (e.g. commensal) microorganisms (that will also display PAMPs), a second stimulus is needed to activate the innate immune response. This stimulus would be conveyed by host molecules resulting from tissue damage, hence called damage associated molecular patterns (DAMPs) and may play an essential role in immune-tolerance (Seong and Matzinger, 2004).

PRRs possess two major common characteristics: firstly, they are expressed constitutively in the host and detect the pathogens regardless of their life-cycle stage; and secondly, they are germline encoded, non-clonal, expressed on all cells of a given type, and independent of antigen presentation and immunologic memory. Different PRRs react to specific PAMP stimulation, show distinct expression patterns, activate specific signalling pathways, and lead to distinct anti-pathogen responses. PRRs are commonly expressed on i) bone marrow-derived immune cells such as myeloid dendritic cells (DCs), macrophages, and neutrophils, or ii) lymphoid B cells and specific types of T cells, but also iii) on liver-resident cells, such as hepatocytes, liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (Akira et al., 2006; Kumar et al., 2011; Protzer et al., 2012).

Several classes of PRRs have been described, namely Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CTR), NOD-like receptors (NLRs) and DNA receptors (cytosolic sensors for DNA) (Kumar et al., 2011).

Effective sensing of PAMPs by PRRs results in the rapid (within minutes or hours) activation of cascade signalling pathways that culminate in the induction of immune responses mediated by various interferons and cytokines/chemokines. IFNs have direct and indirect actions: they can induce the expression of ISGs that encode proteins bearing antiviral properties within infected cells, as well as contribute to the activation/differentiation of other immune cells, both innate and adaptive ones. Other cytokines/chemokines will help recruiting immune cells to the site of infection and contribute to their activation, proliferation and differentiation. Furthermore,

activation of the innate immune system leads to the initiation of more time-consuming adaptive immunity, which is antigen-specific, long-lasting and has immunological memory (Lester and Li, 2014).

5.2.1. Toll-like receptors

Human **TLR** identification and characterization was preceded by the discovery of Toll receptor, involved in *Drosophila melanogaster*'s defence against fungal infection (Lemaitre et al., 1996). Ten human homologs of Toll (hence called TLRs 1-10) have so far been identified and characterized (although the characterization of TLR-10 is still very incomplete).

TLRs are type I transmembrane glycoproteins composed of: i) an N-terminal ectodomain containing varying numbers of leucine-rich repeat (LRR) motifs, responsible for PAMP recognition; ii) a transmembrane domain; iii) a C-terminal cytoplasmic domain, homologous to that of the interleukin-1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain, that activates downstream signal transduction (Lester and Li, 2014). Their expression is variable according to the cell type. Hepatocytes have been shown to express functional TLR1, 2, 3, 4, 5, and 6, whereas TLR7, 8, and 9 were not found active in these cells (Luangsay et al., 2015b).

Several classifications can be proposed for TLRs, based on structural characteristics, sub-cellular localization, molecules sensed, etc. TLRs 1, 2, 4, 5 and 6 are primarily found on the cell surface and recognize PAMPs (namely lipids) derived from bacteria, fungi and protozoa, whereas TLRs 3, 7, 8 and 9 are exclusively found within endocytic compartments and primarily recognize nucleic acid PAMPs derived from viruses as well as bacteria, as summarized below in Table IX.

Table IX. TLR cellular location and known ligands. Adapted from (Kumar et al., 2011)

TLR	Cellular localization	Ligands
TLR1/2	Cell surface	Triacyl lipopeptides
TLR2	Cell surface	Peptidoglycan, lipoarabinomannan, hemagglutinin, phospholipomannan, glycosylphosphophatidyl Inositol, mucin, zymosan
TLR3	Endosome	ssRNA virus, dsRNA virus, respiratory syncytial virus
TLR4	Cell surface	Lipopolysaccride, mannan, glycoinositolphospholipids, envelope and fusion proteins from mammary tumor virus and respiratory syncytial virus, respectively, endogenous oxidized phospholipids produced after H5N1 avian influenza virus infection, pneumolysin from *Streptococcus pneumoniae*, paclitaxel
TLR5	Cell surface	Flagellin from flagellated bacteria
TLR6/2	Cell surface	Diacyl lipopeptides from *Mycoplasma*, lipoteichoic acid
TLR7	Endolysosome	ssRNA viruses, purine analog compounds (imidazoquinolines),RNA from bacteria.
TLR8	Endolysosome	ssRNA from RNA virus, purine analog compounds (imidazoquinolines)
TLR9	Endolysosome	dsDNA viruses, herpes simplex virus, CpG motifs from bacteria and viruses, hemozoin from *Plasmodium*.

TLR downstream signal transduction is mediated by the recruitment of four major adaptor/coadaptor molecules: myeloid differentiation factor 88 (**MyD88**), TIR-associated protein (**TIRAP**)/ MyD88-adaptor-like (**MAL**), TIR-domain-containing

adaptor inducing interferon (IFN)-β (**TRIF**, also known as TICAM1) and TRIF-related adaptor molecule (**TRAM**). The differential responses triggered by distinct TLR ligands can be explained in part by the selective usage of these adaptor molecules and their activation of distinct signalling pathways. MyD88 and TRIF are commonly associated with the production of pro-inflammatory cytokines and type I interferons, respectively. The production of proinflammatory cytokines is mediated by IRF-5, AP-1 and NF-kB transcription factors. IFNβ and ISGs expression is stimulated by TLRs either via a TRIF-dependent pathway (as is the case for TLR3 and 4), leading to the recruitment of IRF3 and IRF7 on ISREs, or, upon stimulation of TLR7 and 9, via a MyD88-dependent pathway leading to IRF7 recruitment (Akira et al., 2006; Kumar et al., 2011). Figure 27 summarises the main aspects of TLR signalling in mammalian cells (comprising both human and murine TLRs).

5.2.2. RIG-I-like-receptors

The **RIG-I-like-receptor family** groups several cytoplasmic RNA sensors, namely RIG-I, MDA5, DDX3 and LGP2. Small double-stranded RNAs are preferentially recognized by RIG-I, while MDA5 is particularly involved in the recognition of longer RNAs, which explains their selectivity for the recognition of particular viruses (Kato et al., 2008). Their activation is associated with the induction of type I and type III IFNs, but also proinflammatory responses. This is mediated by RLRs' interaction with IPS-1 adaptor (also called MAVS, CARDIF or VISA), located in mitochondria (where it will induce the recruitment of IRF3) and peroxisomes (where it leads to recruitment of IRF1) (Kumar et al., 2011; Reikine et al., 2014).

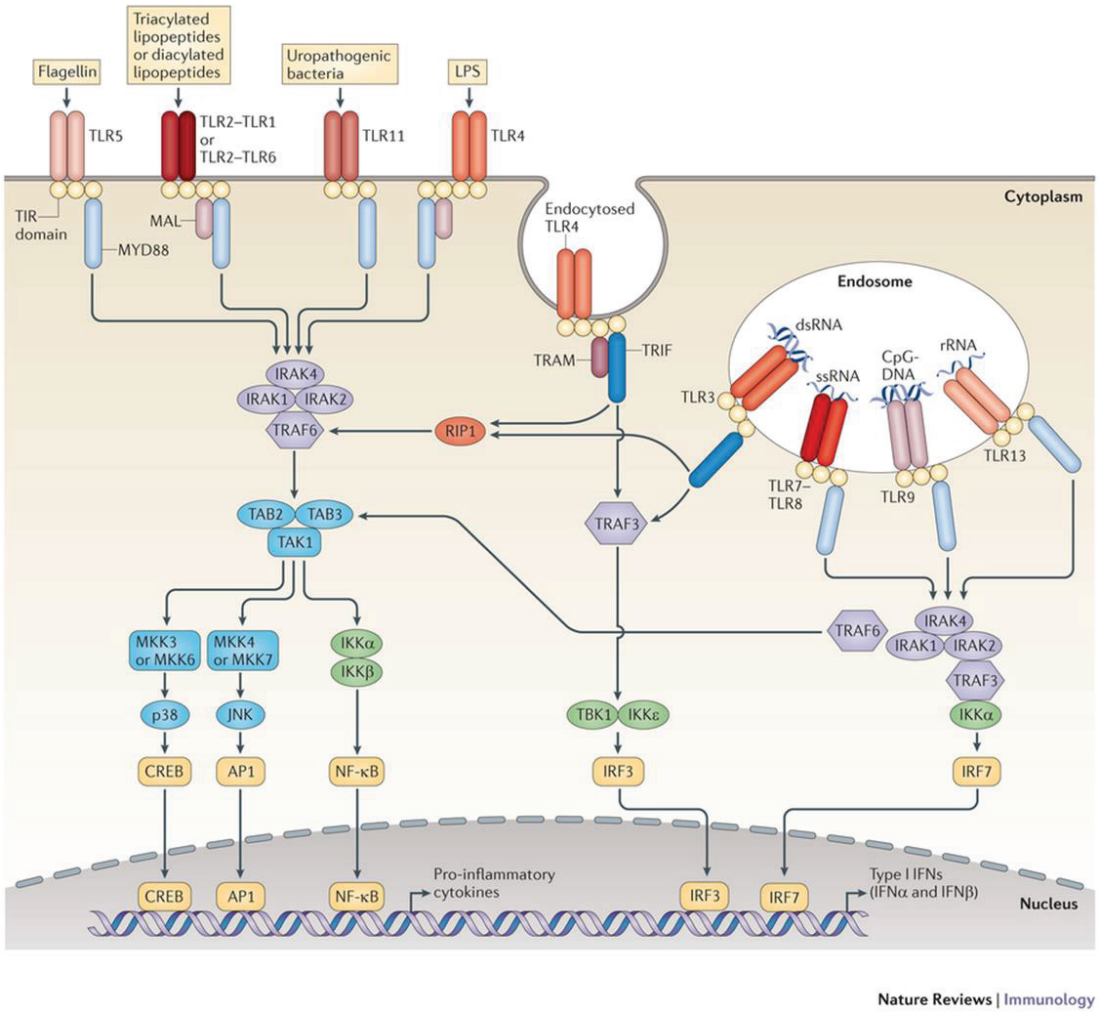

Figure 27. TLR signalling in mammalian cells. TLR5, TLR11, TLR4, and the heterodimers of TLR2–TLR1 or TLR2–TLR6 bind to their respective ligands at the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 localize to the endosomes, where they sense microbial and host-derived nucleic acids. TLR4 localizes at both the plasma membrane and the endosomes. TLR signalling is initiated by ligand-induced dimerization of receptors. Following this, the Toll–IL-1-resistence (*TIR*) domains of TLRs engage TIR domain-containing adaptor proteins (either myeloid differentiation primary-response protein 88 (***MYD88***) and MYD88-adaptor-like protein (***MAL***), or TIR domain-containing adaptor protein inducing IFNβ (***TRIF***) and TRIF-related adaptor molecule (***TRAM***)). TLR4 moves from the plasma membrane to the endosomes in order to switch signalling from MYD88 to TRIF. Engagement of the signalling adaptor molecules stimulates downstream signalling pathways that involve interactions between IL-1R-associated kinases (*IRAKs*) and the adaptor molecules TNF receptor-associated factors (*TRAFs*), and that lead to the activation of the mitogen-activated protein kinases (*MAPKs*) JUN N-terminal kinase (*JNK*) and p38, and to the activation of transcription factors. Two important families of transcription factors that are activated downstream of TLR signalling are nuclear factor-κB (***NF-κB***) and the interferon-regulatory factors (***IRFs***), but other transcription factors, such as cyclic AMP-responsive element-binding protein (*CREB*) and activator protein 1 (*AP1*), are also important. A major consequence of TLR signalling is the induction of pro-inflammatory cytokines, and in the case of the endosomal TLRs, the induction of type I interferon (IFN). *dsRNA*, double-stranded RNA*; IKK*, inhibitor of NF-κB kinase; *LPS*, lipopolysaccharide; *MKK*, MAP kinase kinase; *RIP1*, receptor-interacting protein 1; *rRNA*, ribosomal RNA; *ssRNA*, single-stranded RNA; *TAB*, TAK1-binding protein; *TAK*, TGFβ-activated kinase; *TBK1*, TANK-binding kinase 1. Reproduced from (O'Neill et al., 2013).

5.2.3. Cytoplasmic DNA sensors

DNA, usually confined to the nucleus and mitochondria of eukaryotic cells, can be a potent immune stimulant when recognized as non-self. The presence of cytosolic DNA, either through infections (viral or bacterial) or cellular damage, triggers robust immune responses, including inflammasome activation and type I IFN induction (Cai et al., 2014).

Several **cytoplasmic DNA sensors** have been described thus far. The first one to be identified was DNA-dependent activator of IFN-regulatory factors (DAI), which has a cytoplasmic location and leads to the induction of and IFN type I response (Kumar et al., 2011). DAI, however, was shown not to be essential to the IFN production in response to DNA, suggesting the existence of other sensors, whose number and description have exploded recently (i.e. DDX41, IFI16, DHX9, DHX36, LRRFIP1...). Very recently, a new significant pathway has been identified, based on the interaction between cytosolic GAMP synthase (**cGAS**) and stimulator of IFN genes (**STING**, located on the ER membrane). Cytosolic DNA activates cGAS to form a dimeric cGAS-DNA complex which synthesizes 2'3'- cyclic di-GMP-AMP (cGAMP) from ATP and GTP. 2'3'-cGAMP binds and activates STING. Activation of STING ultimately leads to nuclear translocation of IRF3 and NF-kB and the subsequent production of type I IFNs and other cytokines (Cai et al., 2014).

5.2.4. C-type lectin receptors

C-type lectin receptors (CLRs) comprise a transmembrane receptor family involved in the recognition of carbohydrate structures on microorganisms such as viruses, bacteria, and fungi. CLRs are expressed mainly in DCs, macrophages and monocytes. Their stimulation leads not only to the production of proinflammatory cytokines through the activation of MAP-kinases and NF-kB and regulation of TLR signaling, but also to internalization of the pathogen, its degradation and subsequent antigen presentation (Geijtenbeek and Gringhuis, 2009; Takeuchi and Akira, 2010).

5.2.5. Nod-like receptors and Inflammasomes

NOD-like receptors (NLRs) are a family of molecules that sense mainly bacterial components (peptidoglycans) within the cytoplasm of cells. Upon recognition of PAMPs, these sensors either induce production of inflammatory cytokines (through NF-κB or MAP-kinases activation) or activate the inflammasome, leading to the maturation and production of inflammatory cytokines, such as IL-1β and IL-18 or cell death (Kumar et al., 2011; Takeuchi and Akira, 2010). Moreover, recent studies have revealed a role for NLRs in type I IFN production as well (Kanneganti, 2010)

Inflammasomes are cytoplasmic protein complexes in which the scaffolding and sensing proteins comprise both members of the NLR family (e.g., NALPs) and non-NLR proteins, absent in melanoma 2 (AIM2) and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). Three inflammasomes have been described to be involved in the response to viral infection: the NLRP3 inflammasome, the RIG-I inflammasome and the AIM2 inflammasome. Their activation ultimately leads to the proteolytic activation of the proinflammatory cytokines IL-1β and IL-18, mediated by caspases (Martinon et al., 2009).

Caspases are proteases produced in cells as catalytically inactive zymogens and usually undergo proteolytic processing during activation. Inflammasomes activate a class of caspases known as inflammatory caspases that include caspase 1, 4, 5, 11 and 12 that have as main substrate inflammatory cytokines, such as IL-1β, IL-18, and possibly IL-33 (Martinon et al., 2009). The bipartite, 22-kDa adaptor ASC provides a link between NLRP3, RIG-I or AIM2 and the pro-form of caspase-1. Activated caspase-1 cleaves pro-IL-1β and pro-IL-18 into biologically active IL-1β and IL-18. After secretion from the cell (through a Golgi-independent mechanism), IL-1β and IL-18 induce various biological effects that are associated with infection, inflammation and autoimmune processes (Kanneganti, 2010).

Inflammasomes are involved not only in inflammatory and immune responses to pathogens, but also in the so-called danger response (recognition of tissue damage).

5.3. Interferon response

The antiviral potential of interferon molecules has been known for more than 50 years (Isaacs and Lindenmann, 1957). Interferon molecules are produced in response to the detection of PAMPs by PRRs and constitute a first line of defence against viral infection by inducing an antiviral state within infected cells, as well as in non-infected cells by auto/paracrine mechanisms. The essential role played by interferon in the antiviral response has been well demonstrated by the increased susceptibility of knock-out mice models to viral infection and can further be reinforced by the observation that patients with specific inborn defects in the IFN signalling pathways have an increased risk of viral infection in (Schoggins, 2014).

There are three distinct interferon families:

- The **type I IFN** family is a multi-gene cytokine family, whose main representatives are IFNα (13 partially homologous subtypes known in humans) and IFNβ. Several other gene products have been described but are still poorly defined (IFNε, IFNτ, IFNκ, IFNω, IFNδ and IFNζ). IFNα and IFNβ can be produced by almost every nucleated cell in the human body, mainly in response to PRR activation. A broad range of PRRs (cell surface, endosomal and cytoplasmic) lead to IFNs' production, through distinct downstream signalling pathways that converge in key molecules such as the IFN-regulatory factor (**IRF**) family. In most cases, IRF3 and IRF7 are the fundamental IRFs required, although others, like IRF1, IRF5 and IRF8, can also induce *Ifn-α/β* gene transcription. IFNβ and all of the IFNα subtypes bind to and signal through a heterodimeric transmembrane receptor (**IFNAR**), composed of subunits IFNAR1 and IFNAR2 that, upon dimerization, can trigger distinct downstream signalling pathways, ultimately leading to the selective activation of IFN-stimulated genes (ISGs). In the canonical pathway of IFNα/β-mediated signalling, activated JAK1 and TYK2 phosphorylate signal transducer and activator of transcription 1 (**STAT**1) and STAT2 in the cytosol, leading to their dimerization, nuclear translocation and binding to IRF9 to form the ISG factor 3 (ISGF3) complex. This complex then binds to IFN-stimulated response

elements (ISRE) in ISG promoters and activates ISG transcription, a large number of which function to induce an antiviral state within the cell (McNab et al., 2015);

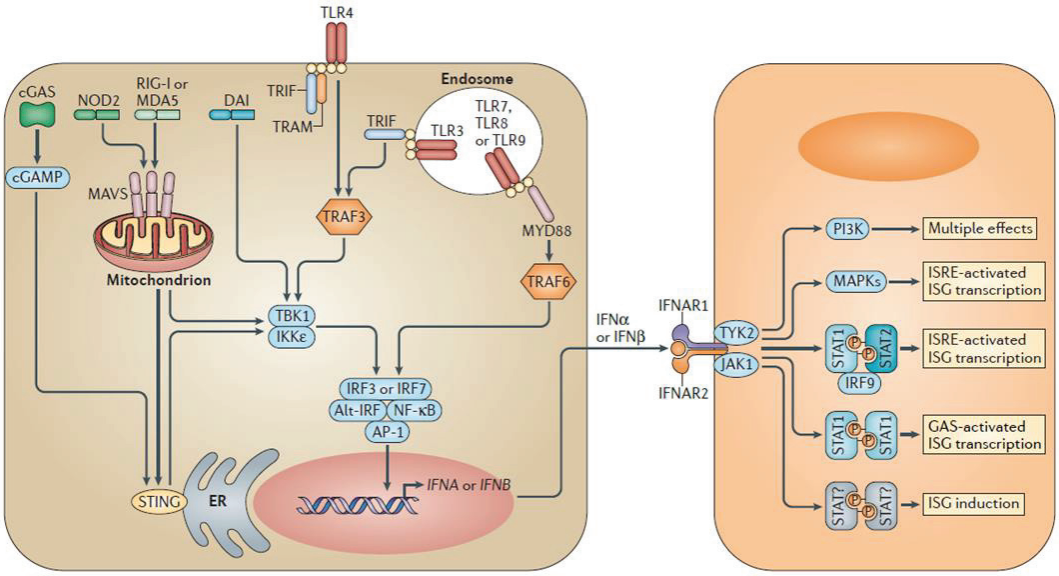

Figure 28. Pathways of type I interferon induction and receptor signalling. Recognition of microbial products by a range of cell-surface and intracellular pattern recognition receptors, including Toll-like receptors (*TLRs*) and retinoic acid-inducible gene I (*RIG-I*), can lead to induction of the genes encoding type I interferons (*IFNs*), which is mediated by several distinct signalling pathways. On the binding of type I IFNs to their receptor *(IFNAR*), multiple downstream signalling pathways can be induced, leading to a diverse range of biological effects. The canonical signal transducer and activator of transcription 1 (*STAT1*)–*STAT2*–IFN-regulatory factor 9 (*IRF9*) signalling complex (also known as the IFN-stimulated gene factor 3 (*ISGF3*) complex) binds to IFN-stimulated response elements (*ISREs*) in gene promoters, leading to induction of a large number of IFN-stimulated genes (*ISGs*). Type I IFNs can also signal through STAT1 homodimers, which are more commonly associated with the IFNγ-mediated signalling pathway. Other STAT heterodimers and homodimers may also be activated downstream, including STAT3, STAT4 and STAT5. Other signalling pathways that do not rely on Janus kinase (*JAK*) and/or STAT activity may also be activated, including mitogen-activated protein kinases (*MAPKs*) and the phosphoinositide 3-kinase (*PI3K*) pathway, thereby leading to diverse effects on the cell. Alt-IRF, IRFs other than IRF3 or IRF7; *AP-1*, activator protein 1; *cGAMP*, cyclic di-GMP-AMP; *cGAS*, cytosolic GAMP synthase; *DAI*, DNA-dependent activator of IRFs; ER, endoplasmic reticulum; *GAS*, γ-activated sequence; *IKKε*, IκB kinase-ε; *MAVS*, mitochondrial antiviral signalling protein; *MDA5*, melanoma differentiation-associated gene 5; *MYD88*, myeloid differentiation primary response protein 88; *NF-κB*, nuclear factor-κB; *NOD2*, NOD-containing protein 2; *STING*, stimulator of IFN genes; *TBK1*, TANK-binding kinase 1; *TRAF*, TNF receptor-associated factor; *TRAM*, TLR adaptor molecule (also known as TICAM2); *TRIF*, TIR domain-containing adaptor protein inducing IFNβ; *TYK2,* tyrosine kinase 2. Reproduced from (McNab et al., 2015)

- The **type II IFN** family consists of a single gene product, IFNγ. Type II IFN is predominantly produced by T cells and natural killer (NK) cells, and can act on a broad range of cell types that express the IFNγ receptor (IFNγR). It has a weak antiviral effect. It is involved in the regulation of innate immune cells activity. Unlike type I and III IFNs that lead to the fixation of regulatory factors on ISRE sequences, IFNγ leads to regulation of genes possessing GAS elements (IFNγ activation site);

- The **type III IFN** family comprises IFNλ1, IFNλ2 and IFNλ3 (also known as IL-29, IL-28A and IL-28B, respectively) and the recently identified IFNλ4. Type III IFNs signal through a heterodimeric cell surface receptor composed of two chains: IFN-λR1, which is specific to IFN-λ, and IL-10RB, which is shared by other IL-10-related cytokines. IFN-λR dimerization, as IFNAR, triggers the JAK-STAT transduction pathway and induces the upregulation of ISGs. Type III interferons IFNs hence share intracellular signaling functions with cytokines of the type I IFN family. Differences in IFNλ activity spectrum may be explained by its expression dynamics, as it is only secreted by a subset of immune cells such as NK cells and pathogen-specific T cells as well as some epithelial cells (including hepatocytes). In addition, the IFN-λR is less widely expressed as compared to IFNAR, thus conferring a perspectives of better toxicologic profile of the therapeutic utilization of IFNλ. Furthermore, IFNλ also demonstrates activity at the interface of innate and adaptive immunity (Egli et al., 2014; Hermant and Michiels, 2014; Hoffmann et al., 2015).

Details on specific signalling pathways used by the distinct IFN families are conveyed by Figure 29.

As previously described, newly synthesized type I and type III IFNs are secreted from the infected cells and upon autocrine or paracrine binding to their respective receptors (IFNAR and IFN-λR), lead to the development of an antiviral response, via activation of the JAK/ STAT pathway. The result is the transcriptional induction of ISGs,

which encode direct antiviral effectors or molecules with the potential to positively and negatively regulate IFN signalling and other host responses (Schoggins, 2014).

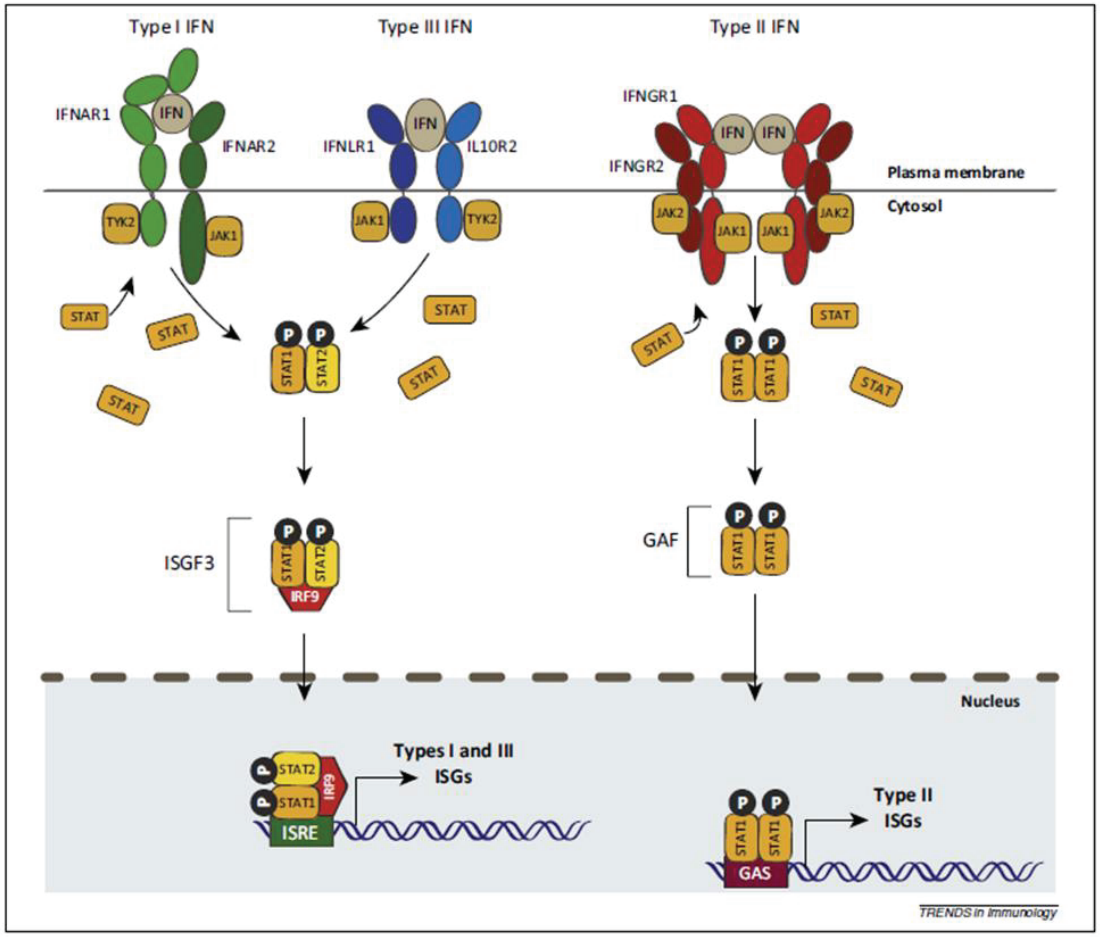

Figure 29. Signalling pathways used by the distinct IFN families. Once type I or III IFNs bind their receptors at the cell surface, individual receptor chains are brought into close proximity. As a result, intracellular receptor-associated tyrosine kinases of the Janus kinase (JAK) family of proteins become activated. Activated JAK proteins subsequently phosphorylate (P) members of the STAT family of proteins, ultimately leading to the transcriptional activation of ISGs. Inasmuch as is currently known, after receptor engagement, type I and III IFNs signal through the same pathway: activation of the two JAK proteins, JAK1 and TYK2, results in the phosphorylation of conserved tyrosine residues on STAT1 and STAT2, followed by formation of a heterotrimeric complex with IFN-regulatory factor 9 (IRF9). This complex translocates to the nucleus and binds to a DNA sequence known as the IFN-stimulated response element (ISRE) in the promoters of ISGs. As a result, hundreds of ISGs are transcriptionally regulated. *GAF*, g-interferon activation factor; *GAS*, g-interferon activation site. Reproduced from (Hoffmann et al., 2015).

Depending on cell type, IFN dose, and time of treatment, microarray studies have identified 50–1000 ISGs, with 200–500 genes typical of many cell types (Schoggins and Rice, 2011). However, of the hundreds of known ISGs, relatively few have been characterized *in vivo* for their role in antiviral immune responses. A major contribution

was made in 2011, through a comprehensive overexpression screen in which more than 380 genes were tested for antiviral activity against six viruses: HCV, HIV-1, yellow fever virus (YFV), West Nile Virus (WNV), Venezuelan equine encephalitis virus (VEEV), and chikungunya virus (CHIKV). At least 25 genes were shown to have antiviral activity against one or several viruses (Schoggins et al., 2011). The antiviral activity of these genes is variable and, as more effectors are uncovered, it is becoming clear that a gradient of antiviral activity exists, with many gene products acting as only modest inhibitors. Although it would be tempting to think that the most highly induced ISGs (during infection or IFN treatment) are those that most effectively control viral replication, magnitude of ISG induction does not always correlate with the strength of antiviral effector function (Schoggins, 2014).

As for their mechanism of action, most ISGs may have not only a direct effect on virus replication, but may also be involved in feedback into antiviral pathways, as molecules involved in pathogen recognition and IFN signalling, can themselves be regulated by interferon. Such is the case of major IFN signalling components like RIGI-like receptors, IRFs, and STAT1.

ISG products play diverse roles in the modulation of viral life cycles, as represented in Figure **30**. Some of the most common and best characterized examples of ISGs include:

- 'Classical ISGs' like interferon-induced, double-stranded RNA-activated protein kinase (**PKR**, encoded by *Eif2ak2*), the myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (**MX1** or **MXA**, encoded by *Mx1*) and the 2',5'-oligoadenylate synthetase/ RNase L system (**OAS1**, **OAS2**, **OAS3**, encoded by *Oas1, Oas2, Oas3*). **PKR** is a known inhibitor of cellular and viral mRNA translation and is involved in a variety of cellular processes, including inflammation and apoptosis. It inhibits translation via the phosphorylation of eIF2α. Several members of the **OAS** enzyme family are activated by double-stranded RNA to catalyze the formation of 2',5'-oligoadenylates, which activate cellular RNase-L to degrade viral genomes. **MX1** is a dynamin-like GTPase that appears to target viral nucleocapsids at an early post-entry step,

resulting in viral inhibition prior to the establishment of replication (Schneider et al., 2014; Schoggins, 2014);

- Interferon stimulated gene 15 (**ISG15**, encoded by *Isg15*) is a small, ubiquitin-like molecule and is one of the most highly induced ISGs. ISG15 has pleiotropic antiviral functions including inhibition of virus release, ISGylation of both viral and host proteins, and immunomodulatory cytokine-like properties in its unconjugated form;

- **Viperin** (encoded by *Rsad2*) is a radical SAM domain-containing molecule with diverse antiviral activities. It can be induced by at least two different innate immune pathways: via JAK-STAT signaling or via direct activation by IRF1/3. It normally resides in the ER and in ER-derived lipid droplets. Most of its antiviral activities appear are related to lipid biosynthetic or fatty acid metabolic pathways. *In vitro*, viperin has been implicated in controlling a number of viruses, including human cytomegalovirus, influenza A virus, Sindbis virus (an alphavirus), and flaviviruses such as WNV and DENV. Viperin inhibits HIV-1 and influenza A virus budding at the host cell membrane by inhibiting farnesyl diphosphate synthase (FPPS), an enzyme involved in isoprenoid biosynthesis; viperin inhibits RNA replication of HCV subgenomic replicons (that takes place in the membranous web, closely associated with lipid droplets); its effect on the inhibition of DENV replication its thought to occur via the same mechanism. Interestingly, viperin was shown to be directly induced by HCMV and not only was its antiviral role inhibited, but a proviral effect was demonstrated as a result of hijacking into the mitochondria mediated by a viral protein (Helbig and Beard, 2014; Schneider et al., 2014; Schoggins, 2014);

- IFN-inducible retrovirus restriction factors include **APOBEC3**, **Samhd1**, **Bst2/tetherin** and the recently characterized **MxB** protein (Haller, 2013). Humans have seven APOBEC3 genes, with APOBEC3G being the best characterized with respect to antiviral function. These are cytidine deaminases that introduce deleterious mutations into the viral matrix. APOBEC3A and 3B

have recently been shown to have an anti-HBV antiviral activity and to induce cccDNA degradation (Lucifora et al., 2014; Schoggins, 2014).

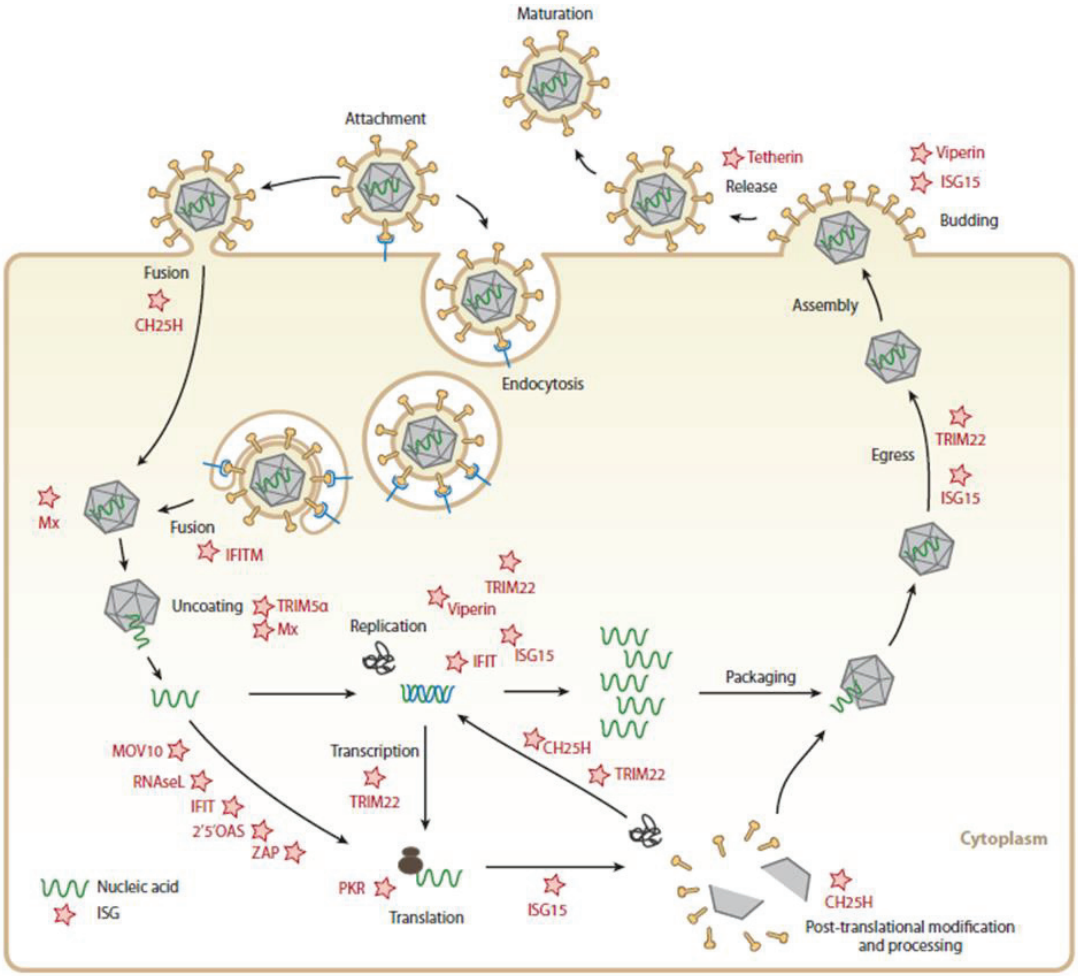

Figure 30. ISG products interference with different stages of viral life cycles. Particular proteins are represented by stars next to the viral life cycle steps with which they have been demonstrated to interfere. Refer to text for details. Reproduced from (Schneider et al., 2014)

If the interferon response is effective in controlling viral infection, viruses have in turn developed a variety of IFN antagonist proteins to counteract host defence, using diverse strategies. Such strategies can be divided into two main categories: inhibition of IFN induction (that includes inhibition of the recognition of viral RNAs by RLRs, modulation of downstream signalling or inactivation of transcription factors IRF3/7) and inhibition of IFN signalling (including dephosphorylation and degradation of STAT proteins). Viruses without such abilities to suppress the host type I IFN responses generally have low pathogenicity (Hoffmann et al., 2015; Koyama et al., 2008).

5.4. Regulation of innate and adaptive cellular immune responses

The effects of IFNs on the host response to viruses are not limited to the acute, cell-intrinsic antiviral response described above. IFNα/β have effects on both the innate and adaptive cellular immune responses that include (McNab et al., 2015):

- Enhanced action of **dendritic cells and monocytes**. IFNα/β seem to have an activating effect on immature committed DCs, enhancing the cell-surface expression of Major Hystocompatibility Complex (MHC) molecules and co-stimulatory molecules, such as CD80 and CD86, which is associated with an increased ability to stimulate T cells. IFNα/β promote the ability of DCs to cross-present antigens during viral infections. IFNα/β may also promote the migration of DCs to lymph nodes, through up-regulation of chemokine receptor expression, thus promoting T cell activation;

- **Enhancement of NK cell responses**. NK cells display a wide array of antiviral functions that include cytolytic targeting of infected cells (mediated by perforins), regulation of DC antigen presenting functions and also secretion of IFNγ. IFNα/β promote the function and survival of NK cells, through both direct and indirect means. While in some viral infections the direct action of IFNα/β on NK cells is required for the activation and expression of cytolytic effector functions and the production of IFNγ, in others, signalling through STAT1 is required for NK cell accumulation and cytolytic function, but not for IFNγ production. The ability of IFNα/β to induce or restrict IFNγ production by NK cells is related to differential STAT1 and STAT4 signalling. High levels of STAT1-dependent signalling inhibit IFNγ production by NK cells, whereas high basal levels of STAT4 prime NK cells for IFNγ production;

- **Promotion of CD4+ and CD8+ T cell responses**. IFNα/β have been described to have both inhibitory and stimulatory effects on T cell survival and proliferation, cytokine (IFNγ) production, cytotoxic function and memory formation. As for

NK cells, these diverse outcomes are controlled by differential levels and differential activation of STAT molecules downstream of IFNAR;

- **Enhancement of B cell responses.** As it is the case for viral antigens, IFNα/β can promote B cell activation and antibody responses, including class switching, during viral infection.

The main cellular types involved in the antiviral response (and the resulting immunopathology) are represented in

Figure **31**.

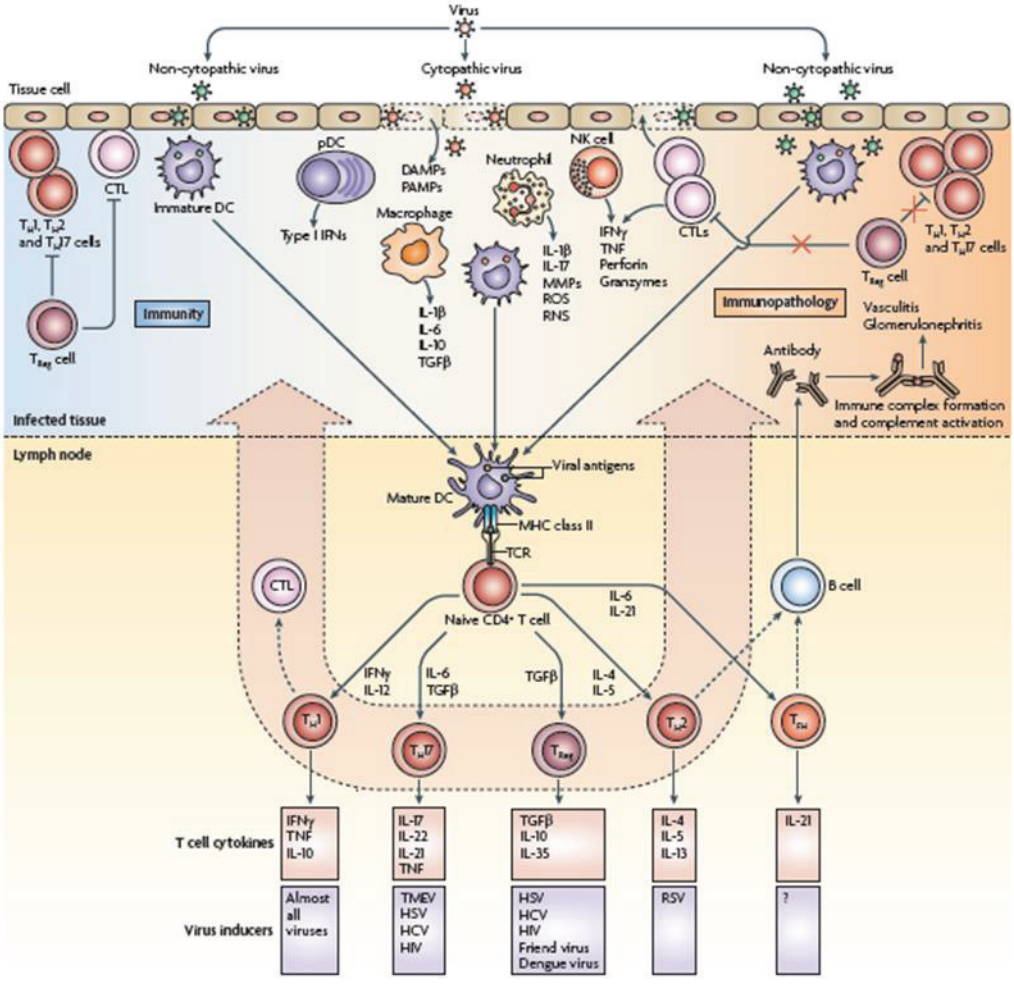

Figure 31. Cell types involved in the immune response to viral infection. Reproduced from (Rouse and Sehrawat, 2010)

Chapter II. Research project

1. Questions, objectives and experimental strategy

Despite the existence of an effective vaccine, hepatitis B remains an important cause of cirrhosis and hepatocellular carcinoma worldwide. Current therapies are still unable to warrant a cure, as they do not target cccDNA.

Co- or superinfection by HDV is known to significantly change the progression of hepatitis B. Firstly, it is known that a minor subset of CHB patients, when super-infected by HDV, progress to eradicate both infections; secondly, patients chronically infected by both viruses, although presenting dynamic patterns of viral dominance often have a suppression of HBV replication evidenced both by decreased levels HBV viremia and by a decrease of liver cell replicative intermediates. Finally, HDV superinfection of CHB patients has been associated with faster progression of liver disease, with a higher incidence of decompensations and hepatocellular carcinoma.

It is hence clear that, in spite of being a satellite virus, HDV is able to modulate HBV infection, both by interfering with its replication and by potentiating the pathogenic effect on the infected cell.

However, little is known about the mechanisms of interaction between the two viruses. An important body of research was produced over the years concerning the shared processes of viral entry into the hepatocyte and viral assembly. Apart from these steps, it is considered that HBV and HDV have fully independent replication cycles.

Two major hypothesis may be considered regarding the interaction between HDV and HBV that ultimately leads to an inhibition of HBV replication: HDV replication or viral components may exert a direct effect on the HBV replicative cycle or HDV, being an RNA virus with a pseudo-double stranded structure, may be associated with an intense immune response that counteracts the silencing of interferon response that HBV installs in the infected cells and hence ultimately limits its replication.

As for the first hypothesis, a few arguments have been evoked over the years to suggest that a direct effect of HDV and its proteins may at least partially be responsible for an inhibition of HBV replication. Very recent data have recently emerged

suggesting that HDV induces a strong interferon response. The link between such response and the inhibition of HBV replication has not been clearly established, although some of the ISGs induced by HDV have been described to inhibit HBV replicative cycle.

Furthermore, HDV research over the years has been hampered by the lack of appropriate infection models, with a direct repercussion on the limited number of therapeutic options available to these patients.

Our aims hence were:

i) to study HDV infection, replication and interplay with the host innate immune reponse;
ii) to identify possible innovative therapeutic strategies for the inhibition of HDV infection;
iii) to further explore HDV/ HBV interference and characterize the mechanisms involved.

In order to do so, we first adapted the HepaRG cell model, frequentlty used for the study of HBV and HDV monoinfections, with the objective of establishing HBV/ HDV superinfections.

The first part of the work concerns the optimization of culture and infection conditions as well as HDV detection techniques in HepaRG cells and the characterization of HDV infection (with or without its helper HBV) in this model. It is presented as a submitted paper awaiting for review.

After the characterization of the model and the identification of immune activation and interference phenotypes, it was our aim to study the mechanisms underlying such observations. The work performed so far is compiled in the form of a chapter of additional results, as it is still being validated and further explored.

2. Submitted article

This part is presented as a manuscript and has been submitted to *Journal of Hepatology* – see submission confirmation in Appendix 2.

HDV replication is associated with HBV repression in superinfection, induction of interferon response, and can be inhibited by immune-modulators

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Manuscript information:

Electronic word count:

Number of table: 1

Number of figures: 8

Number of supplementary tables: 1

Number of supplementary figures: 3

List of abbreviations:

CHD, chronic hepatitis delta; HBV, hepatitis B virus; PEG, polyethylene glycol; IFN, interferon; HDV, hepatitis D virus; HDAg, Hepatitis delta antigen; ADAR, adenosine deaminase acting on RNA; aa, amino acid; hNTCP, human sodium taurocholate cotransporting polypeptide; WHV, woodchuck hepatitis virus; ISG, interferon stimulated genes; cccDNA, circular covalently closed DNA; dHepaRG, differentiated HepaRG; PRR, pathogen recognition receptor; VGE, virus genome equivalent; SRB, sulforhodamine B; n.s., non-significant; MOI, multiplicity of infection; p.i., post-infection; pgRNA, pregenomic RNA; TLR ,toll-like receptor; HIV, human immunodeficiency virus; PHH, primary human hepatocytes; PAMP, pathogen-associated molecular pattern.

Key Words:

Hepatitis D virus; hepatitis B virus; viral interference; IFN response; immune-modulators.

Conflict of interest:

No conflict of interest to declare on this work.

Financial support and acknowledgements:

The authors would like to thank Dr Alan Kay for the kind gift of anti-HDAg antibodies. Dulce Alfaiate was supported by grants from Fundação Calouste Gulbenkian and Fundação para a Ciência e a Tecnologia. DD, JL, FZ, and PD were supported by grants from ANRS (French national agency for research on AIDS and viral hepatitis; several grants from CSS4), FINOVI (Foundation for innovation in infectiology; project call n°#4), and by INSERM core grants. PD was supported by an INSERM Interface contract. DD and FZ were also supported by FRM (Foundation for medical research; DEQ20110421327), and DEVweCAN LABEX (ANR-10-LABX-0061) of the “Université de Lyon”, within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR).

Author’s contributions:

- study concept and design: DA, FZ, PD and DD
- acquisition of data: DA, MM, NAS, SM, JL
- analysis and interpretation of data: DA, JL, PD and DD
- writing of the manuscript: DA, CS, FZ, JL, PD and DD

- statistical analysis: BT, DA

- technical, or material support: JCC and CS

Abstract

Background & Aims: Hepatitis D virus (HDV) superinfection of Hepatitis B virus (HBV)-infected patients is the most aggressive form of viral hepatitis. HDV infection is not susceptible to direct anti-HBV drugs, and only suboptimal antiviral responses are obtained with interferon (IFN)-alpha-based therapy. Our aim was to get insights on HDV replication and interplay with HBV in physiologically-relevant hepatocytes and evaluate the potential anti-HDV effect of immune-modulators.

Methods: Differentiated HepaRG (dHepaRG) cells were infected with HDV both in the presence and absence of a previous HBV infection and viral markers were extensively analyzed. IFN response to HDV was monitored by measuring pro-inflammatory and interferon-stimulated gene (ISG) expression. In addition, infected cells were treated with various pathogen recognition receptors (PRR) agonists to evaluate their anti-HDV activity.

Results: Both mono- and superinfected dHepaRG cells supported a strong but transient HDV replication, accompanied by the secretion of HDV virions only in the coinfection setting. Upon HDV superinfection, HBV replication markers including HBeAg, total HBV-DNA and pregenomic RNA were significantly decreased, confirming viral interference of HDV on HBV. No decrease of circular covalently closed HBV DNA (cccDNA) and HBsAg levels occurred. Concomitant to the peak of HDV-RNA accumulation and the onset of interference on HBV replication, a strong type-I IFN response was observed at day-6 post HDV infection. Importantly, PRR agonists were found more efficient than IFN-alpha to impede HDV replication, the best being Pam3CSK4 engaging the NF-kappaB pathway via TLR1/2.

Conclusions: We established a relevant cellular model to further characterize HBV/HDV direct and/or indirect interplay and test novel HDV inhibitors, including immune-modulators. A suppressive role of HDV on HBV replication could convincingly be confirmed, whereas an IFN response to HDV infection was clearly evidenced. This model is contributing to assess molecular and immunological mechanisms of this viral interference.

Introduction

Chronic hepatitis delta (CHD) is estimated to affect 15-20 million people worldwide (5-10% of the Hepatitis B virus (HBV) infected patients) (1). It is considered to be the most aggressive form of chronic viral hepatitis, with an accelerated progression towards fibrosis and cirrhosis and an increased risk of liver decompensation, hepatocellular carcinoma and premature death (2). Pegylated-interferon (Peg-IFN)-alpha remains the single therapeutic option for these patients, with virological response rates of less than 30% at 24 weeks post-treatment and high rates (>50%) of relapse (3). HBV reverse transcriptase inhibitors have no effect on Hepatitis D virus (HDV) replication. The stretch of investigational drugs against HDV infection remains limited due to the fact that i) HDV does not encode enzymatic activities and uses a cellular RNA polymerase for its replication, ii) there are remaining gaps in the knowledge of the viral life-cycle, and iii) no appropriate *in vitro* model of super/co-infection exists for to screening antiviral drugs.

HDV is a subviral agent, satellite of HBV, and its genome is the smallest known among mammalian viruses, having similarities to plant viroids. It is a single-stranded circular RNA of ~1680 bp, with a high intramolecular base pairing, allowing a rod-like structure folding. Its complementary strand includes the *SHD* gene that codes for a single protein, the hepatitis delta antigen (HDAg), or small, 24 kDa, protein (S-HDAg), which is essential for HDV RNA replication. At a later phase of the HDV replication cycle, *SHD* stop codon editing catalysed by Adenosine Deaminase acting on RNA-1 (ADAR-1), leads to the synthesis of a 19-20 amino-acid (aa) carboxy-terminal extended isoform of HDAg. This large, 27kDa, protein (L-HDAg), thwarts HDV RNA replication and is involved in particle assembly. The viral genome encodes no polymerase activity and HDV fully relies on the recruitment of host cell DNA-dependent RNA polymerases (particularly RNA Pol II) for the nuclear replication of its RNA (4,5).

To ensure propagation, HDV relies on HBV, as HDV virions are assembled by HBV envelope proteins. Furthermore, the entry of HDV in human hepatocytes is mediated by the interaction of the large HBV envelope protein (L-HBsAg) with the recently discovered cell surface HBV receptor, *i.e.* the human sodium taurocholate cotransporting polypeptide (hNTCP) (6). Both clinical and experimental data support

the existence of viral interference between HDV and HBV. In the clinical setting, most patients infected with both HBV and HDV feature a pattern of HDV dominance, with a significant decrease in HBV-DNA viral load, when compared to monoinfected patients (7–9). Moreover, studies on liver biopsies from chronically HDV-infected patients have shown a decreased level of HBV replicative intermediates in the liver (10). Finally, this negative interference has been confirmed *in vivo,* in super- or co-infection conditions, using HBV-infected chimpanzees, woodchuck hepatitis virus (WHV)-infected woodchucks, and more recently HBV-infected humanized mice (11–14).

To understand the molecular basis of HDV interference on HBV, relevant infection-based *in vitro* models are essential. Viral interference has been observed in Huh7 cells by transfection of DNA vectors expressing HBV and HDV (or either HDAg isoforms) (15). Direct inhibition of HBV enhancer-1 and activation of *MxA* gene, an interferon stimulated gene (ISG) known to suppress HBV replication, have been documented in the same model (16). However, transfection models have limitations and protein overexpression may lead to inaccurate assumptions. In order to analyse better HBV/ HDV interference, a cell culture model featuring both cccDNA formation and a competent innate immunity is required. Until recently, the innate immune response knowledge to HDV infection remained scarce. After *in vitro* studies suggesting a modulation of the IFN response (17,18), recent data from mouse models (both the humanized uPA-SCID mouse and the hNTCP transgenic mouse) revealed a strong induction of the intra-hepatocyte ISG expression (19,20). Further knowledge on the interactions between HDV and the innate immune system should allow for a better understanding of the interplay between HDV and its helper and the identification of potential therapeutic pathways.

The aim of this study was to establish and take benefit of a relevant cellular model of HDV superinfection, to characterize HBV/HDV interactions and evaluate the host-cell innate immune system in response to infection. This model could furthermore allow an evaluation of the effect of immune-modulatory drugs on HDV replication. Using the differentiated HepaRG (dHepaRG) cells, which are immune-competent (21), we confirmed a suppression of HBV replication and showed that HDV infection is associated with induction of ISGs, but not of NF-kappaB regulated genes. We further

showed that some pattern recognition receptors (PRR) agonists lead to a more significant reduction of HDV replication than IFN-alpha treatment.

Material and Methods

Production of HBV and HDV virions

Both HBV and HDV virions were produced *in vitro* as described in supplementary material and schematically in **Supplementary Fig. 1.**

HepaRG cell culture and infection

The human liver progenitor HepaRG cells were cultured, differentiated and infected overnight with either HBV or HDV as previously described (21–23). For superinfection experiments, cells already infected with HBV (100 viral genome equivalents (vge)/cell, unless otherwise indicated) for 6 days were exposed to HDV overnight as indicated above (100 vge/cell, unless otherwise specified).

Nucleic acid quantification: qPCR, RT-qPCR and Northern Blot

Viral and intracellular DNA and RNA extractions are detailed in supplementary material. All primers and probes are listed in **Supplementary Table 1**. HDV quantification was performed by one-step RT-qPCR (Express One-Step SYBR Greener, Life Technologies) using the primers described by Scholtes et al (24) and the following cycling conditions: 50°C for 20 min (retro-transcription - RT), 95°C for 5 min and then 40 cycles of 95°C for 30 s, 60°C for 20 s, and 72°C for 20 s. PCR was run in the Roche LightCycler 480. Further details are described in supplementary materials and **Sup. Fig. 2**.

HBV DNA/RNA and innate immune gene expression were performed as previously described (23). For all intracellular gene expression analysis, the comparative cycle threshold (Ct) method was applied and results are displayed as a ratio (2^-DDCt), to a control sample (described for each experiment) (25).

Northern blot for HDV and HBV RNA detection was essentially performed as previously described (26,27), with slight modifications, detailed in supplementary material.

Elisa, immunofluorescence and WB

These techniques are detailed in supplementary materials.

Antiviral treatment

For evaluation of the antiviral effect of PRR agonists, dHepaRG cells were infected with HDV (10 vge/cell) and treated at days 3 and 6 post-infection. Supernatants for cytokine dosage were collected 24 hours after the beginning of treatment (i.e. day-4 post-infection). Cells were lysed for RNA extraction at day-9 post-infection. The list and concentrations of PRR agonists used for treatment are given in **Table 1**. All ligands were purchased from Invivogen. IFN-alpha (1000 IU/ml; Roche) was used as a positive control for anti-HDV activity.

Cell viability testing

Cell viability was sequentially evaluated by neutral red uptake and sulforhodamine (SRB) assays, as detailed in supplementary material.

Statistical analysis

Results were computed with Microsoft Office Excel and Prisma Graph Pad softwares. Sample groups were first evaluated for the presence of outliers with Dixon test. Statistical analysis was subsequently performed with Mann-Whitney test for single comparisons and Kruskal-Wallis test with Dunns correction for multiple comparisons. The *p*-values are represented according to the following convention: $p>0.05$ (non-significant, n.s); $p<0.05$ (*); $p<0.01$ (**); $p<0.001$(***).

Results

In a monoinfection setting, dHepaRG cells support a strong but transient HDV replication

To assess the conditions of HDV inoculation, differentiated dHepaRG cells were either mock-infected or infected with HDV at multiplicities of infection (MOI) ranging from 1 to 500 vge/cell. At day-6 post-infection (p.i.), intracellular HDV RNA could be detected by RT-qPCR from the lowest MOI tested (i.e. 1 vge/cell), with a linear increase up to 50 vge/cell, reaching a plateau for a MOI ranging between 50 and 500 vge/cell (**Fig. 1A**). Northern blot analysis confirmed RT-qPCR findings and, using a genomic sense probe, indicated *de novo* formation of HDV replicative antigenomic RNA through the initiation of a replicative cycle (**Fig 1B**). Comparing HDV MOI 50 and 100, while an increase in amount of both isoforms of HDAg occurred, it did not match an increase in the number of infected cells (**Fig. 1C and 1D**).

To get insights on infection kinetics, dHepaRG cells were infected 10 vge/cell and total RNA and proteins were collected at different time points. As a negative control, cells were treated with the entry inhibitor Myrcludex® (100nM) from 2 hours before infection to the end of viral inoculation, as shown in **Fig. 2A**. A steep rise in HDV intracellular RNA accumulation was detected from day 2 post-infection (p.i.) by RT-qPCR, reaching a peak at day 6 and a subsequent decrease (**Fig. 2B and 2C**). At day 21 p.i., HDV RNA remained detectable by both RT-qPCR and northern blot, indicating a residual amount of replicative intermediates or a low replication persistence. As expected, no increase in HDV intracellular RNA occurred in the Myrcludex®-treated conditions, confirming the efficacy of the drug and the fact that the detected replication was associated with the specific hNTCP receptor-mediated entry. Increase in HDV RNA levels was associated with an increase in the expression of HDAg, with both forms (S-HDAg and L-HDAg) being clearly detectable from day 3 p.i (**Fig. 2D**). The pattern of expression was slightly delayed as compared to HDV RNA, but followed the same bell-shaped curve. At later time points both HDAg isoforms decreased but remained detectable at least until day 21 post-infection. By indirect immunofluorescence staining, HDAg was detected in a maximum of 3% of cells (**Fig. 2E**).

In already HBV-infected cells, HDV superinfection is associated with a decreased HBV replication.

To set up the superinfection model, dHepaRG cells were first inoculated with HBV (100 vge/cell), and, at day 6, at the plateau of HBV replication, (28); cells were inoculated with HDV (100 vge/cell) (**Fig. 3A**). No difference in intracellular HDV RNA accumulation was observed between HDV mono- and superinfected conditions over the follow-up kinetics (**Fig. 3B**). HBV replication was monitored by measuring HBeAg and HBsAg secreted in the supernatant, intracellular HBV DNA and total and pregenomic RNA (pgRNA) levels. Compared to HBV monoinfected cells, HDV superinfected cells displayed a significant decrease of both HBeAg secretion and intracellular HBV DNA (34 and 39% decrease, $p<0.001$ and $p<0.05$, respectively) (**Fig. 3C and 3E**). Increasing HDV MOI (up to 1000 vge/cell) further inhibited HBeAg secretion, indicating the relevance of this marker to monitor viral interference (**Sup. Fig. 3**). In contrast, there was no significant difference in HBsAg secretion levels between HBV monoinfected and HDV superinfected cells (**Fig. 3D and Sup. Fig. 3**). Although no significant difference was observed in the amount of total HBV RNA (as measured by RTqPCR; **Fig. 3F)**, pgRNA level seemed to decrease over time in HDV superinfected cells (24% decrease as compared to HBV monoinfection at D15; $p<0.05$) (**Fig. 3G and 3H**).

To further investigate the impact of MOI on viral interference, cells were superinfected at variable MOIs for both HBV (0, 100 and 500 vge/cell) and HDV (0, 10 and 100 vge/cell) (**Fig. 4A**). Replication parameters were analysed at day-15 post-HBV inoculation (9 days post-HDV superinfection). As expected, a significant decrease in both HBeAg secretion and intracellular HBV DNA accumulation was observed upon HDV superinfection when cells had been infected with HBV at MOIs of 100 or 500, and the decrease was more pronounced with increasing HDV MOIs (**Fig. 4B and 4E**). Concomitantly, in the supernatant, HBV-DNA level decreased in the presence of HDV; this effect was dominating with low (MOI 100; $p<0.05$) rather than high HBV infectious titer (MOI 500; $p=0.08$, n.s.) (**Fig. 4D**). In such conditions, no reduction of HBsAg was observed (**Fig. 4C**), which correlated with the absence of variation on cccDNA levels (as measured by qPCR; **data not shown**). Considering low HBV MOI conditions (100 vge/cell), the decrease of HBV pgRNA correlated with the HDV MOI (24% reduction -

p<0.05) (**Fig. 4F and 4G**). In contrast, no variation in the amount of total HBV RNA was observed even at high HDV MOI. As expected, HDV intracellular RNA levels significantly increased by incrementing HDV MOI (**Fig. 4H**). Surprisingly, total HDV RNA levels also paralleled HBV MOI increase, although this tendency was not statistically significant (13% increase at HBV MOI 500 *versus* MOI 100; p=0.39).

Secretion of HDV particles during superinfection demonstrates HBV/HDV coinfected cells.

In the superinfection setting, three patterns could be identified, upon labelling with anti-HBcAg and anti-HDAg antibodies: HBcAg positive/HDAg negative cells, HBcAg negative/HDAg positive cells and HBcAg positive/HDAg positive cells (**Fig. 5A**). This suggested that cells were either monoinfected by either HBV or HDV, or co-infected cells, respectively. The same observation was obtained when HBsAg immunostaining was used instead of HBcAg labelling (data not shown). The proportion of infected cells expressing either antigen remained below 3% for either HBV or HDV markers, whereas co-labelling occurred in approximately 1% of the total dHepaRG cells (**Fig. 5B**).

Interestingly, despite the low number of detectable co-labelled cells, $1.3x10^7$ vge/mL of HDV RNA was quantified in the supernatant of HBV-HDV superinfected cells (**Fig. 5C**). This result was obtained without detectable cell toxicity (**data not shown**), suggesting a non-cytolytic release of viral particles. Indeed, we could exclude cell lysis as the origin of the detection of HDV viral RNA in the supernatant since extracted HDV RNA was under the limit of quantification in both HDV monoinfected and mock-infected cell supernatants. Furthermore, an increase in HDV secretion not only significantly matched an increase in HDV MOI (p<0.05); but it also matched an increased HBV MOI from 100 to HBV MOI 500 vge/cell, although the difference was not significant (6-fold HDV RNA increase: p=0.1). Collectively these results demonstrate that some HepaRG cells can be infected by both viruses. Moreover, despite the low number of HBcAg/HDAg-positive cells, and likely due to the high efficiency of HDV-replication per cell (29), in the HepaRG HBV/ HDV superinfection setting, secreted HDV virions could be quantified.

HDV induces a strong expression of ISGs both in the mono- and superinfection settings

In contrast to hepatoma cells, differentiated HepaRG cells express functional innate immune sensors, namely PRRs, and are therefore relevant to study antiviral response in hepatocytes (21). We aimed to decipher IFN response to HDV infection in this model. Upon HDV monoinfection, increased expression of several *ISGs* could be detected. Interestingly, *ISGs* expression peaked at day-6 p.i. and correlated with HDV RNA replication kinetics (**Fig. 6**). Since no *ISGs* induction occurred during the first 3 days p.i., nor in the presence of Myrcludex®, it may suggest that the IFN response matched HDV RNA replication and neosynthesis rather than the incoming viral RNA material. In comparison to non-infected cells, highest expressions were detected for *RSAD2* (i.e. *VIPERIN*; mean fold change 469,4) and *IFI78* (i.e. *MXA*; mean fold change 157,7). Other evaluated genes included *ISG15* (mean fold change 44,6), *DDX58* (i.e. *RIGI*; mean fold change 26,6), *OAS1* (mean fold change 26,3), *MDA5* (mean fold change 11,2) and *IFN-beta* (mean fold change 6,9). For all studied time points, no significant difference was found in *IFN-alpha* and *IL-6* expression levels between HDV infected cells and mock or Myrcludex® treated controls.

The expression of innate immune related genes was also evaluated in superinfection setting at day-15 post-HBV infection (day-9 post-HDV infection, corresponding to the time point where interference was detected). As previously determined, HBV alone did not induce any innate gene expression (**Fig. 7**). In contrast, HDV infection was clearly associated with a strong induction of all studied ISGs, which was HDV MOI dependent but HBV independent. Consistent with HDV monoinfection, superinfection (HBV MOI 500 + HDV MOI 100), induced preferentially *RSAD2* and *MXA*, with a respective 110.3 and a 50.5 fold increased gene expression. Finally, no induction of NF-□B induced genes was identified in HDV infections, as exemplified for *IL-6* (**Fig. 7**), *IL-8* and *IL-1*□ (**data not shown**). Collectively, these data indicate that in dHepaRG cells, HDV infection induces a strong IFN response at the peak of RNA replication, independently of both HBV infection and NF-kappaB pathway.

Study of the anti-HDV effect of pathogen recognition receptor (PRR) agonists

Having found that, in dHepaRG cells, HDV activates IFN response without inducing pro-inflammatory response via NF-kappaB, we subsequently sought to examine the antiviral effect of some PRR agonists, which are known to induce IFN and/or cytokine production via IRFs and/or NF-kappaB transcription factors in this model (21). dHepaRG cells were inoculated with HDV (10 vge/cell) and subjected to two 72h treatments with PRR agonists, as displayed on **Fig. 8A**. Doses and treatment schedule were selected based on previous toxicity data on dHepaRG cells (30). An array of PRR agonists was tested, with the exception of Toll-like receptors (TLR) 8 and 9 ligands, as corresponding receptors have been previously shown to be non-functional in hepatocytes (21). In addition, we also tested imiquimod, a TLR7 agonist shown to have a strong anti-HBV effect irrespective of innate immunity (30). Interferon toxicity in dHepaRG cells having been previously described (31), we sequentially evaluated cell viability after each PRR agonist treatment by neutral red and sulforhodamine assays and found no significant cell toxicity for each drug (**Fig. 8B)**. To measure PRR functional response, cytokine secretion was evaluated 24h after the beginning of treatment: IL-6 relies upon NF-kappaB pathway activation, whereas IP-10 reflects type-1 interferon response (**Fig. 8C**).

As shown in **Fig. 8D**, relative to the non-treated condition, a significant decrease (60%) of intracellular HDV RNA was obtained using Pam3CSK4 (i.e. Pam3; a TLR1/2 agonist) and imiquimod ($p<0.01$). Treatments with Poly (I:C) and Poly (dA:dT) led also to a significant decrease in HDV RNA (40 and 50% respectively; $p<0.05$). As expected, stimulation by Pam3CSK4 was associated with IL-6 secretion, whereas treatment with poly (I:C) and led to IP-10 secretion. Despite its significant antiviral effect, no apparent cytokine secretion was induced by imiquimod treatment. Under our experimental conditions, IFN-alpha treatment (1000 UI/ml) resulted only in a modest decrease in HDV RNA (25%); furthermore, this effect was found non-significant in comparison to the non-treated condition, thus emphasizing the important anti-HDV effect of some PRR agonists/ligands.

Discussion

Despite leading to the most severe form of chronic viral hepatitis and infecting a pool of individuals as high as half of human immunodficiency virus (HIV)-infected population, HDV remains a neglected pathogen. Understanding the fundamental issues associated with the complexity of dual HBV/HDV infections may ultimately translate into the development of much needed new therapeutic strategies against HDV.

Since its discovery, the HepaRG cell line has proven to be a very useful *in vitro* model to study HBV biology (21). Indeed, before the era of hNTCP-expressing hepatoma cell lines, HepaRG cells were the best alternative to PHH cultures to study HBV infection, as a full replication cycle occurred in this model (28). Similarly to primary human hepatocytes (PHH) but in contrast to widely-used HepG2 and Huh7 cells, HepaRG cells are able to express most of the innate immunity sensors (21) and are functional for IFN response (32). Furthermore, cccDNA can be formed in HBV-infected HepaRG cells, and has been shown to be degraded in an APOBEC3A/B-dependent manner by activation of either IFN-alpha or LR-beta receptors (29). Therefore, the HepaRG model is suitable to study the interplay between HBV and HDV and hepatocyte-specific innate immunity, and explore new therapeutics, especially by evaluating the antiviral effect of immune modulators. This latter approach was recently implemented for exploring anti-HBV effect of PPR agonists (30). With respect to HDV biology, the HepaRG model has mostly been used for studying the entry step (26).

HDV was here confirmed to specifically infect HepaRG cells, as entry was blocked by Myrcludex®, a drug competing with hNTCP viral attachment (33). If the proportion of HDV infected cells was rather low (3%) and could not be enhanced by increasing HDV MOI beyond 50 vge/cell, the level of replication per cell was significant and could be detected, without amplification, by northern blot, which has a limit of detection of 10^6 vge. This is further sustained by the fact that, in a superinfection setting, despite the low proportion of co-infected cells (1%), as determined by IF assays, the HDV virion egress titer reached 10^7 vge/mL. The rather low proportion of infected cells could be due, at least in part, to cell polarization and accessibility of hNTCP in the basolateral

membrane of hepatocytes (34). Interestingly, HDV replication, in both monoinfection and superinfection conditions, seemed to be controlled, while not fully suppressed after a peak of replication at day-6 post inoculation. Similar decrease over time has also been described in mice injected with a HDV cDNA construct and, more recently, in the hNTCP transgenic mouse model (20, 35). At least two hypotheses might explain such phenotype: the infection is limited in time either by the decrease of available factor needed for replication, such as S-HDAg or some cellular components, or due to accumulation of inhibitors, such as L-HDAg or cellular negative factor(s). Alternatively, active antiviral innate immune response, temporally related to the peak of RNA accumulation, could also contribute to such inhibition.

Besides mediating virion assembly, L-HDAg inhibits viral replication (36). However, L-HDAg may not play a major role in the decrease of HDV RNA at day-9 post-inoculation in dHepaRG as: i) the ratio of S-HDAg and L-HDAg remained consistent throughout the kinetics of HDV RNA replication from day 3, and ii) in the superinfection setting, in which HDV virions release is observed, the decline of HDV RNA accumulation after day-6 p.i. is still observed. In line with our results, the inhibitory activity of L-HDAg on HDV replication has been discussed (37), and may be restricted to trans-expression during early steps of viral replication. On the other hand, we showed that the induction of some ISGs expression occurs at the peak of HDV RNA accumulation, suggesting that neo-synthesised HDV-replicative intermediates, rather than inoculum RNA, act as a pathogen-associated molecular pattern (PAMP). Whether the activation of IFN response could control HDV replication is still unknown in dHepaRG, but this was not confirmed in the transgenic hNTCP mouse model (20).

In this cellular superinfection model, we were able to confirm that HDV can interfere with HBV replication. The observations that HDV superinfection is associated with a decreased secretion of HBeAg, HBV virions, intracellular HBV DNA and pgRNA, although not HBsAg, total HBV RNA or cccDNA, are in agreement with what has been described in HDV-infected patients (10). This is part of the originality of this satellite infection that may often overcome its helper replication, while maintaining its budding trans-complementation. This might also argue for a modulation of cccDNA transcriptional activity (10, 15). In reporter systems and exogenous expression of HD

proteins, direct inhibition of both HBV enhancers, especially by L-HDAg has been previously suggested (16), however further characterization of the differential expression of HBV transcripts is needed. Indeed, this cellular superinfection model may contribute to determine at which step (e.g. cccDNA transcription, viral mRNA export and/or stability) this inhibition occurs.

Another explanation may be linked to the HDV-induced IFN response. Our results indicate that an increase in HDV MOI was associated with a more significant decrease of HBV replicative intermediates and a dose-dependent increase in ISGs expression. Previous works on HBV have shown that type-I IFN response modulates transcriptional regulation of cccDNA, decreasing pgRNA synthesis through modifications in histone acetylation status and recruitment of chromatin modifying enzymes (38). Whether HDV superinfection could induce such response remains to be explored.

During HDV infection we identified a pattern of gene activation suggesting the induction of an IFN response, without any effect on NF-kappaB regulated genes. The induction of ISG expression by HDV is fully in agreement with results obtained in in both the humanized and hNTCP transgenic mice (19, 20) and could be responsible for the limited clinical response to IFN-alpha, as described in chronic hepatitis C (39). Furthermore, our results suggest that a strong induction of innate immune responses by the stimulation of PRRs may induce a decrease of HDV RNA replication. The strongest replication reduction (60% inhibition) follows stimulation of TLR 1/2 by Pam3CSK4, activating NF-kappaB pathway and secretion of IL-6, suggesting that the activation of this pathway, independently of the natural HDV infection, may represent an antiviral strategy. The fact that poly (I:C) (i.e. TLR3-L activation) and poly (dA:dT) (AIM2-L and indirect RIGI/MDA5-L activation) induce a stronger antiviral response than IFN-alpha itself, also opens new perspectives. Finally, the antiviral activity of Imiquimod raises a new set of questions: HepaRG treatment with this molecule does not lead to cytokine production, which is consistent with the absence of a functional TLR7 in these cells (30). Exploration of an alternative mechanism for its antiviral activity is needed, especially considering that analogous drugs such as GS9620 are entering clinical evaluation with promising results to control HBV infection (40).

In summary, we demonstrated here the usefulness of the differentiated HepaRG cell line model for the study of HDV infection and could show that a robust HDV replication occurs in these cells and is associated with a strong induction of ISG expression. Moreover, upon HDV superinfection of HBV-infected cells, viral interference and the production of HDV particles could be confirmed. Finally, the screening and study of PRR agonists led to the identification of four molecules an antiviral activity against HDV, opening promising therapeutic perspectives.

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Figure legends

Figure 1. HDV monoinfection in dHepaRG cells: impact of the MOI. dHepaRG were infected with HDV at different MOIs (ranging from 1 to 500 vge/cell). At day-6 post-infection, intracellular HDV RNA was quantified by RT-qPCR (**A**; representative graph out of 3 independent experiments with 3 different viral batches), and Northern Blot, using a genomic probe, for antigenome detection (**B**). HDAg expression was evidenced by immunofluorescence (**C.i.**; magnification 200X); the percentage of labelled cells is represented in panel **C.ii.** (average counting of three 200X fields, normalized to the total number of nuclei) and western blot **(D**).. *MOI*, multiplicity of infection; *AG*, antigenome; *L-HDAg*, large hepatitis delta antigen; *S-HDAg*, small hepatitis delta antigen; *n.s.*, non-significant.

Figure 2. Kinetics of HDV monoinfection in dHepaRG cells. dHepaRG cells were infected with HDV at 10 vge/cell and infection was followed up to different time points. Maximum inoculation time was 24 hours. As controls, cells were either mock-infected or treated with Myrcludex® at 100nM for 2h pre-infection and during HDV inoculation (**A**). For each point, intracellular HDV RNA was quantified by RT-qPCR (**B**), and Northern Blot, using a genomic probe (**C**); HDAg expression was evaluated by Western Blot (**D**) and immunofluorescence (**E.i.**; magnification 200X). The percentage of labelled cells is represented in panel **E.ii.** (average of three 200X fields, normalized to the total number of nuclei). *Myr*, Myrcludex®; *AG*, antigenome; *L-HDAg*, large hepatitis delta antigen; *S-HDAg*, small hepatitis delta antigen.

Figure 3. Kinetics of HDV superinfection of HBV-infected dHepaRG cells. **A)** Cells were either mock or infected with HBV at 100 vge/cell for 6 days, then either mock- or superinfected with HDV (100 vge/cell), and viral replication parameters were monitored at indicated times p.i. **B)** HDV total intracellular RNA, all values are normalized to HDV infected cells at day 12 post-HBV infection (corresponding to D6 post-HDV infection); **C)** HBeAg ELISA and **D)** HBsAg ELISA (values are normalized to the level detected in HBV infected cells at day 6 post-HBV infection); **E)** HBV total

intracellular DNA; **F)** HBV total intracellular RNA; **G)** HBV pregenomic RNA (all values are normalized to the corresponding HBV monoinfection value); **H)** HBV Northern blot.

Figure 4. HDV superinfection of HBV infected HepaRG cells: impact of variations of the MOI of both viruses. **A)** Cells were infected with HBV at 0, 100 or 500 vge/cell and, at day-6, each HBV-infected condition was superinfected by HDV at 0. 10 or 100 vge/cell. At day-15 post-HBV infection, supernatant was collected and cells were lysed for nucleic acid extraction and subsequent analysis of viral replicative parameters. **B)** HBeAg ELISA; **C)** HBsAg ELISA; **D)** HBV secreted DNA (supernatant); **E)** HBV total intracellular DNA; **F)** HBV total intracellular RNA; **G)** HBV pregenomic RNA. All HBV-related values are normalized to HBV cells infected at MOI 100. **H)** HDV intracellular RNA (normalized to cells infected at HDV MOI 100).

Figure 5. Characterization of co-infection by IF and RT-qPCR assays. After HBV infection (100 vge/cell) and HDV superinfection at day-6 (100 vge/cell), cells were labelled with anti-HBcAg or anti-HDAg antibodies at day-10 and -14 post-HBV inoculation. **A.i)** Immunofluorescence at day-14 (upper panels – magnification 200X; lower panels – magnification 600X); **A.ii)** Percentage of labelled cells (average of four 200X fields, normalized to the total number of nuclei). **B)** Quantification of HDV RNA in the supernatant (normalized to cells infected with HBV MOI 100 plus HDV MOI 100).

Figure 6. Kinetics of *IL-6*, *IFNs*, and *ISGs* expression in cells inoculated with HDV alone. dHepaRG cells were inoculated with HDV at 10 vge/cell and cells were harvested at different time points post-inoculation. *IL-6*, *type-1 IFNs* and *ISG* expressions were evaluated by RT-qPCR. Controls included mock-infected cells, and cells treated with Myrcludex® (initiated 2 hours before and kept during HDV inoculation, as previously shown on Fig. 2.A). Results are presented as a ratio to the mock condition at each day.

Figure 7. Expression of *IL-6*, *IFNs* and *ISGs* after HBV and HDV infection. Cells were inoculated with HBV at 0. 100 or 500 vge/cell and, and 6 days later, exposed to HDV at 0. 10 or 100 vge/cell. *IL-6*, *type-1 IFNs* and *ISGs* expressions were evaluated by RT-qPCR at day-15 post-HBV infection. Results are presented as a ratio to mock condition.

Figure 8. Evaluation of the effect of PRR agonists on HDV replication. A) dHepaRG were infected with HDV at 10 vge/cell and submitted to two 72h-treatments with PRR agonists or not. **B)** Toxicity was evaluated by neutral red assay and SRB assay in dHepaRG cells submitted to two 72-hours treatments with PRR agonists. Results displayed are normalized to the non-treated condition. Cytokine production was evaluated by IP-10 (**Ci**) and IL-6 (**Cii**) secretion determined by ELISA evaluation of the cell culture supernatant 24 hours after the first treatment. **D)** HDV replication was evaluated by RT-qPCR at the end of the second treatment (day-9 post-infection). Results are presented as a ratio to the non-treated condition. *SRB*, sulforhodamine B.

Tables

Table 1. List of PRR agonists, the respective targets and doses used.

Molecule	Target PRR	Dose used
LTA	TLR 2	1 µg/ ml
Pam3CSK4	TLR 1/2	0.5 µg/ ml
Poly (I:C) HMW	TLR 3	5 µg/ml
LPS	TLR 4	0.5 µg/ ml
FLA-BS	TLR 5	1 µg/ ml
FSL-1	TLR 2/6	1 µg/ ml
Imiquimod	TLR 7	5 µg/ml
Poly (I:C) (HMW)/ Lyovec™	RIGI/ MDA5	0.01 µg/ ml
Poly (dA:dT)/ Lyovec™	RIGI/ MDA5	0.1 µg/ ml

Figures

Figure 1.

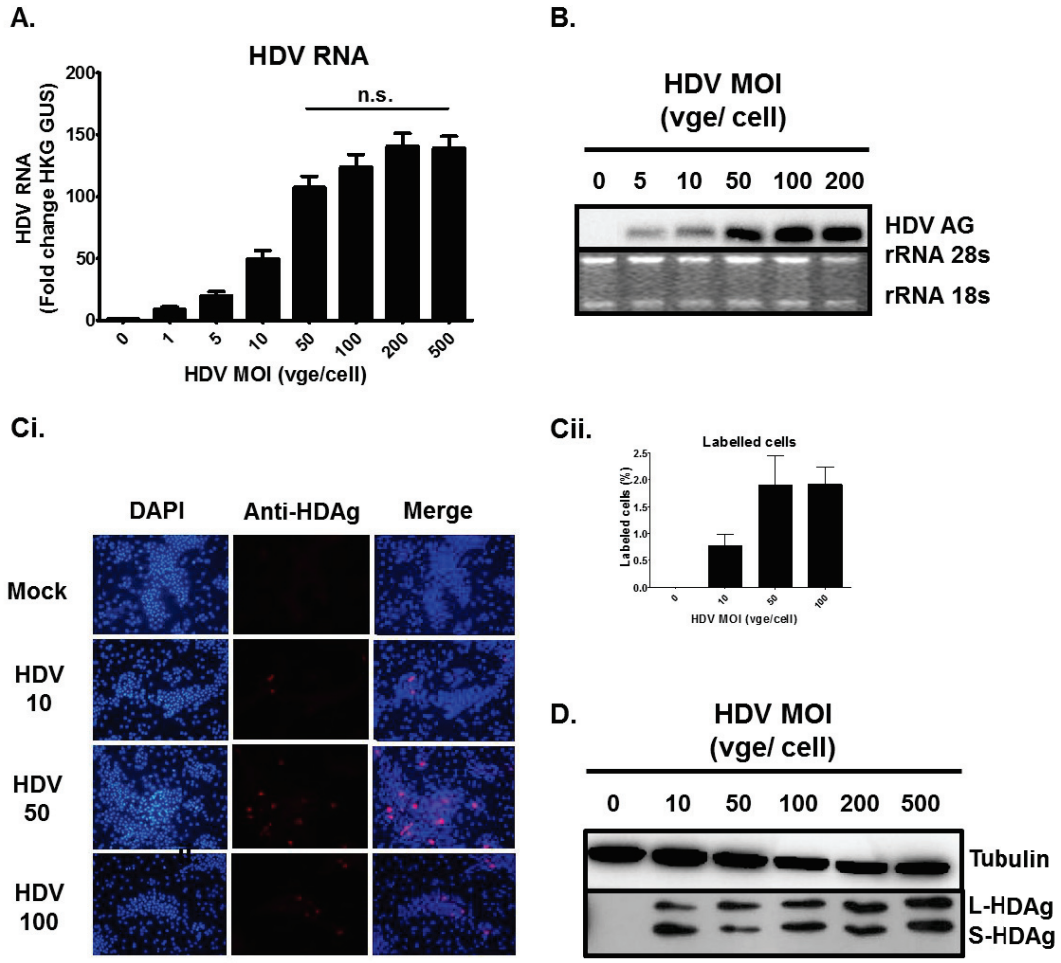


Figure 2.

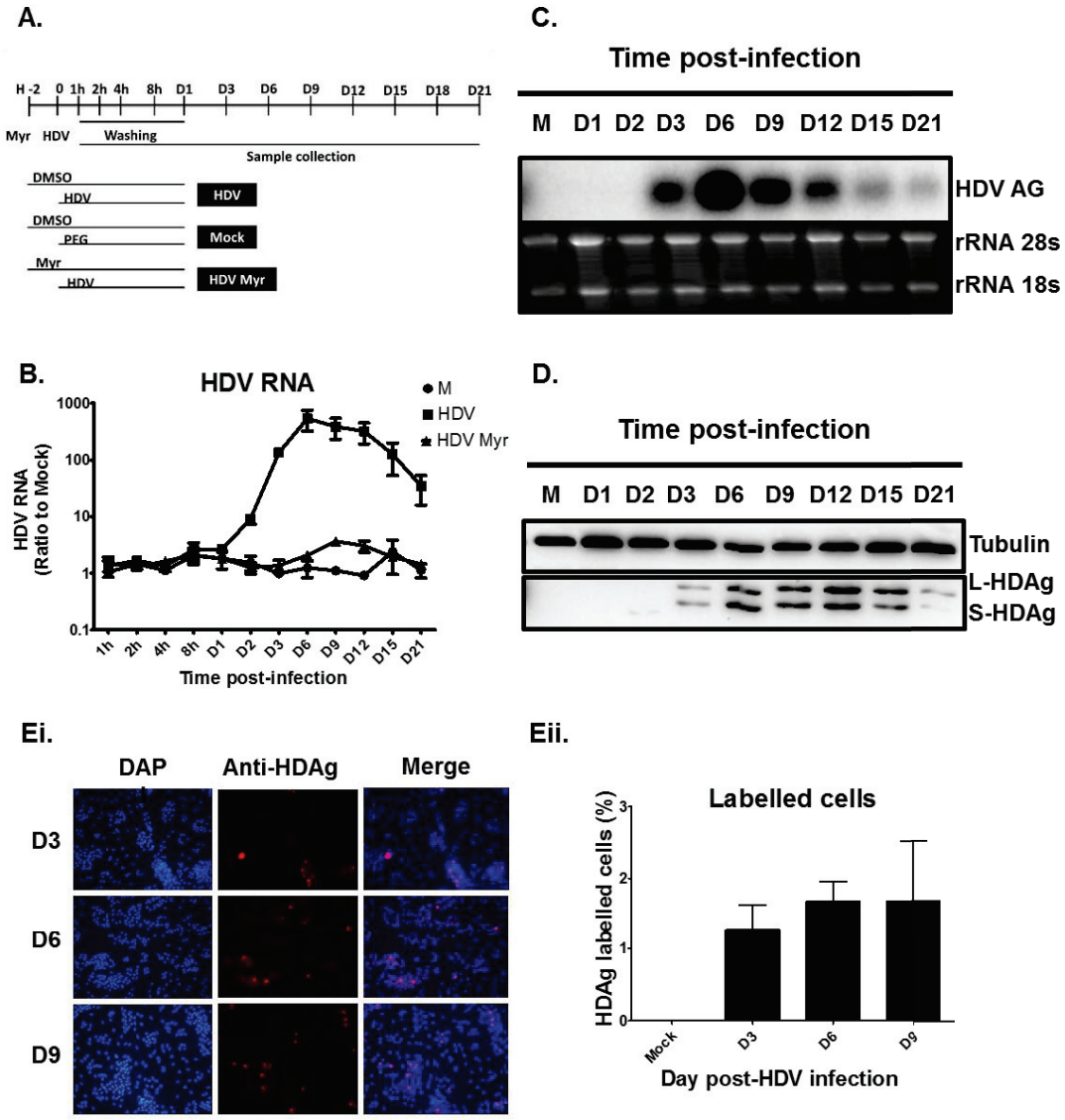

A.
H -2 0 1h 2h 4h 8h D1 D3 D6 D9 D12 D15 D18 D21
Myr HDV
Washing
Sample collection
DMSO
HDV
HDV
DMSO
PEG
Mock
Myr
HDV
HDV Myr
B.
HDV RNA
M
HDV
HDV Myr
HDV RNA (Ratio to Mock)
1000
100
10
1
0.1
1h 2h 4h 8h D1 D2 D3 D6 D9 D12 D15 D21
Time post-infection
C.
Time post-infection
M D1 D2 D3 D6 D9 D12 D15 D21
HDV AG
rRNA 28s
rRNA 18s
D.
Time post-infection
M D1 D2 D3 D6 D9 D12 D15 D21
Tubulin
L-HDAg
S-HDAg
Ei.
DAP
Anti-HDAg
Merge
D3
D6
D9
Eii.
Labelled cells
HDAg labelled cells (%)
3
2
1
0
Mock
D3
D6
D9
Day post-HDV infection

Figure 3.

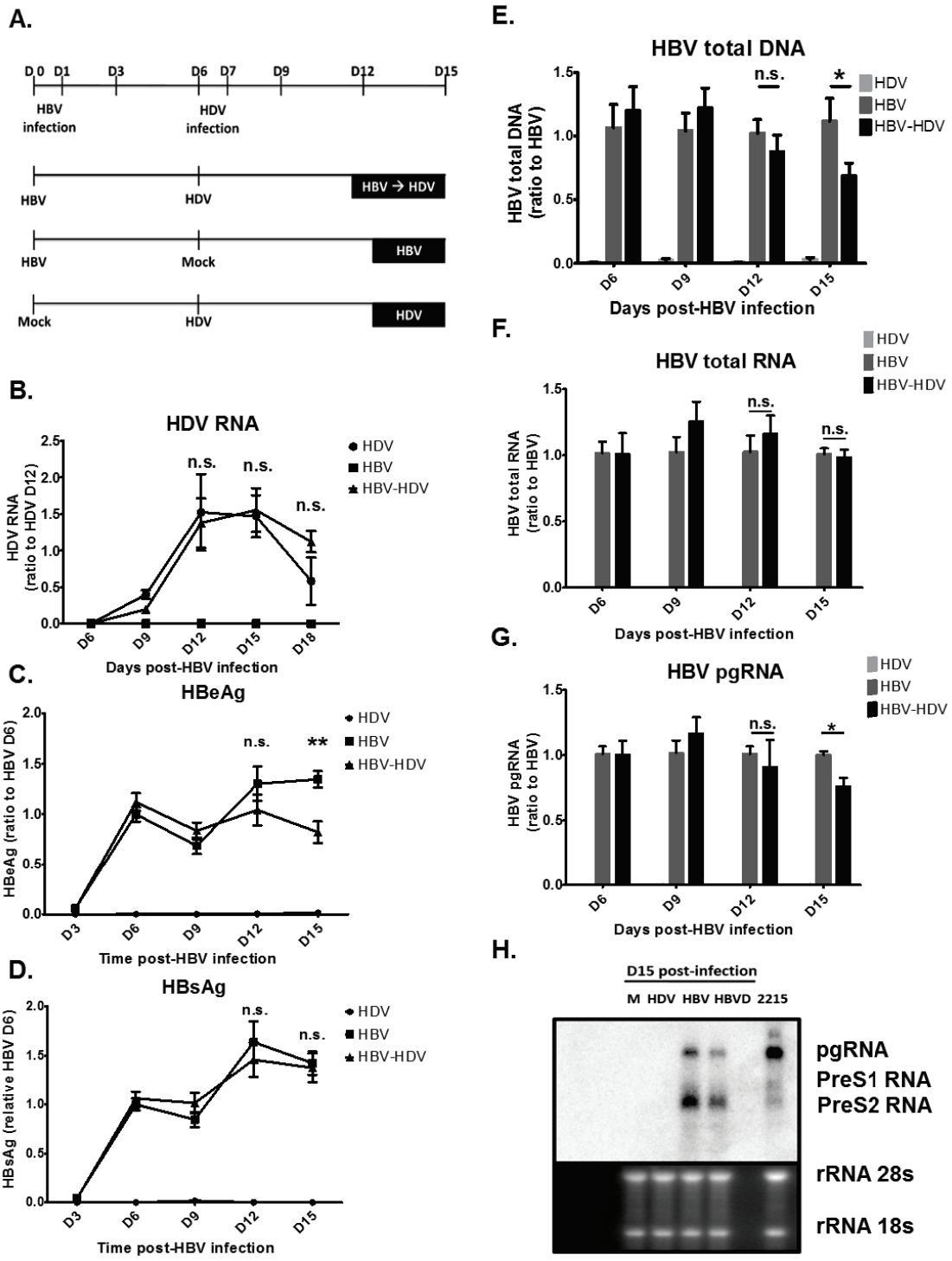

A.
D 0 D1 D3 D6 D7 D9 D12 D15
HBV infection
HDV infection
HBV → HDV
HBV
HDV
HBV
Mock
Mock
HDV
B.
HDV RNA
HDV RNA (ratio to HDV D12)
n.s.
n.s.
n.s.
HDV
HBV
HBV-HDV
D6 D9 D12 D15 D18
Days post-HBV infection
C.
HBeAg
HBeAg (ratio to HBV D6)
n.s.
**
HDV
HBV
HBV-HDV
D3 D6 D9 D12 D15
Time post-HBV infection
D.
HBsAg
HBsAg (relative HBV D6)
n.s.
n.s.
HDV
HBV
HBV-HDV
D3 D6 D9 D12 D15
Time post-HBV infection
E.
HBV total DNA
HBV total DNA (ratio to HBV)
n.s.
*
HDV
HBV
HBV-HDV
D6 D9 D12 D15
Days post-HBV infection
F.
HBV total RNA
HBV total RNA (ratio to HBV)
n.s.
n.s.
HDV
HBV
HBV-HDV
D6 D9 D12 D15
Days post-HBV infection
G.
HBV pgRNA
HBV pgRNA (ratio to HBV)
n.s.
*
HDV
HBV
HBV-HDV
D6 D9 D12 D15
Days post-HBV infection
H.
D15 post-infection
M HDV HBV HBVD 2215
pgRNA
PreS1 RNA
PreS2 RNA
rRNA 28s
rRNA 18s

Figure 4.

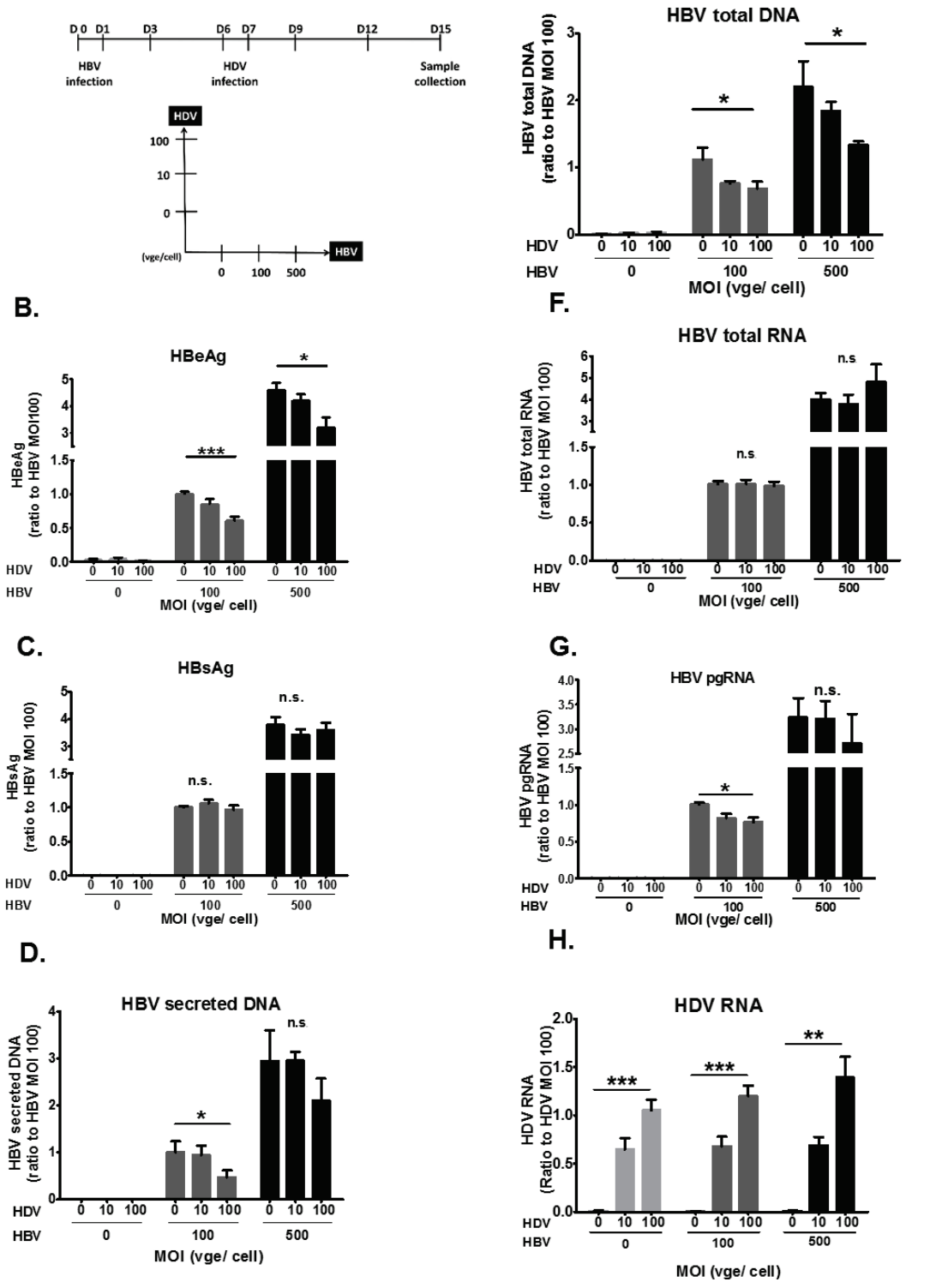

D 0 D1 D3 D6 D7 D9 D12 D15
HBV infection
HDV infection
Sample collection
HDV
100
10
0
(vge/cell)
HBV
0 100 500
B.
HBeAg
HBeAg (ratio to HBV MOI100)
HDV
HBV
MOI (vge/ cell)
C.
HBsAg
HBsAg (ratio to HBV MOI 100)
n.s.
D.
HBV secreted DNA
HBV secreted DNA (ratio to HBV MOI 100)
F.
HBV total DNA
HBV total DNA (ratio to HBV MOI 100)
G.
HBV total RNA
HBV total RNA (ratio to HBV MOI 100)
H.
HBV pgRNA
HBV pgRNA (ratio to HBV MOI 100)
HDV RNA
HDV RNA (Ratio to HDV MOI 100)

Figure 5.

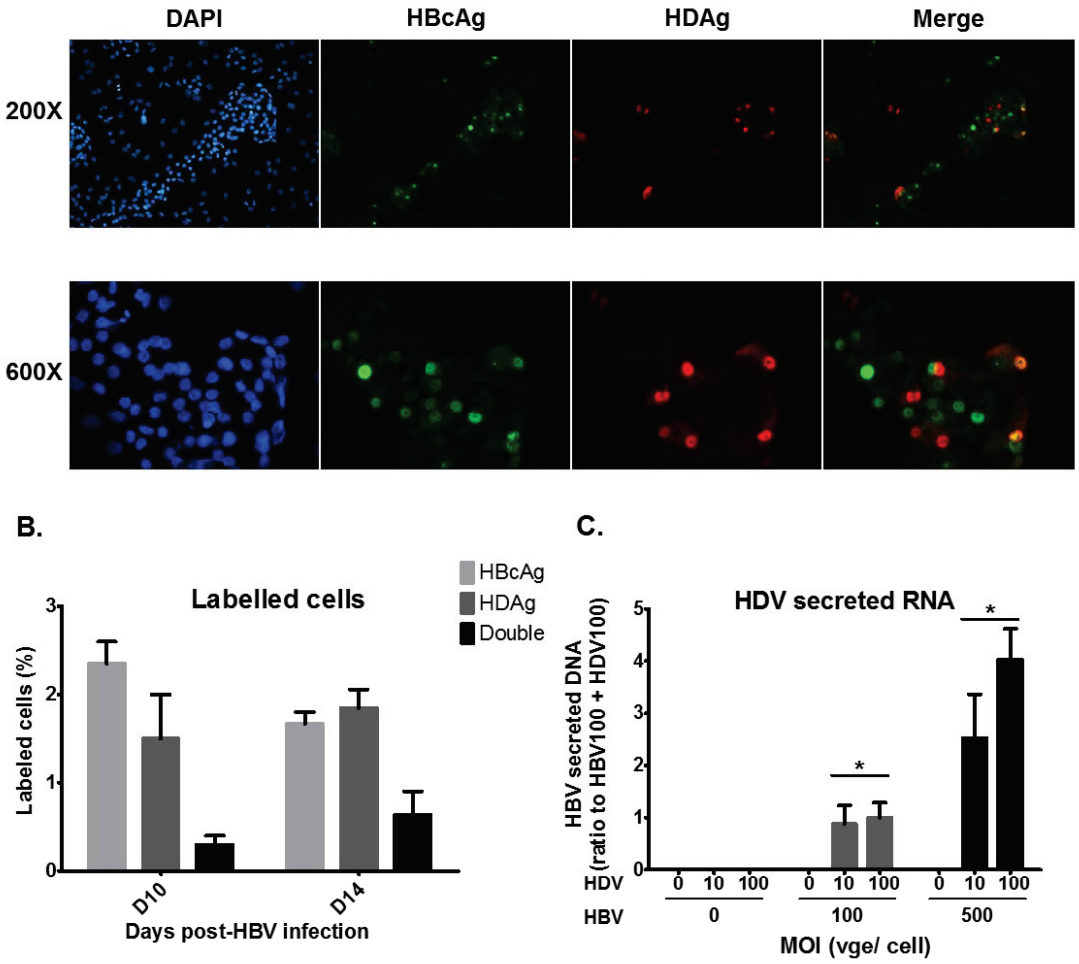

DAPI
HBcAg
HDAg
Merge
200X
600X
B.
Labelled cells
HBcAg
HDAg
Double
Labeled cells (%)
D10
D14
Days post-HBV infection
C.
HDV secreted RNA
HBV secreted DNA (ratio to HBV100 + HDV100)
HDV
HBV
0 10 100 0 10 100 0 10 100
0 100 500
MOI (vge/ cell)

Figure 6.

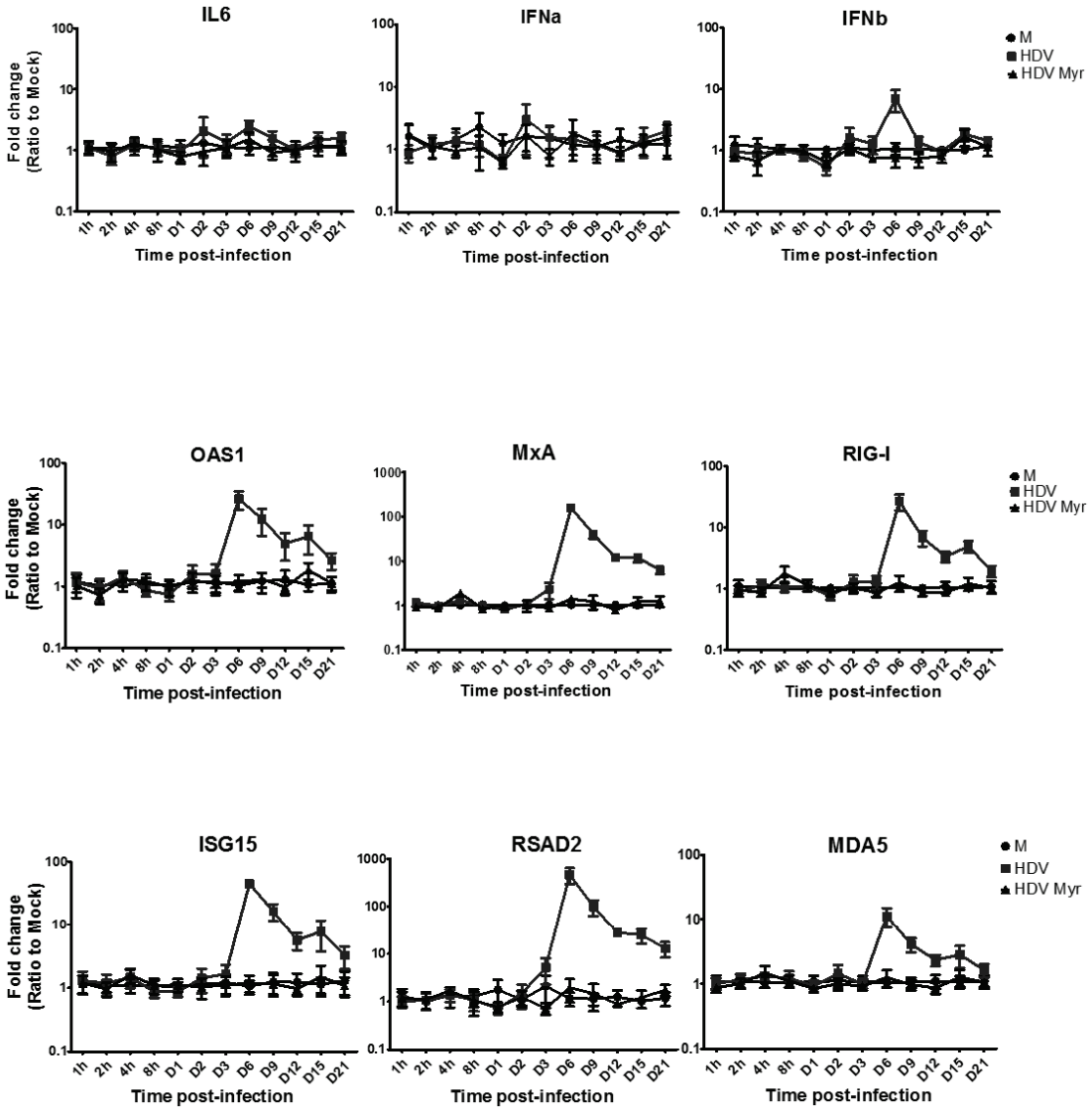

IL6
IFNa
IFNb
OAS1
MxA
RIG-I
ISG15
RSAD2
MDA5
Fold change (Ratio to Mock)
Time post-infection
1h 2h 4h 8h D1 D2 D3 D6 D9 D12 D15 D21
M
HDV
HDV Myr

Figure 7.

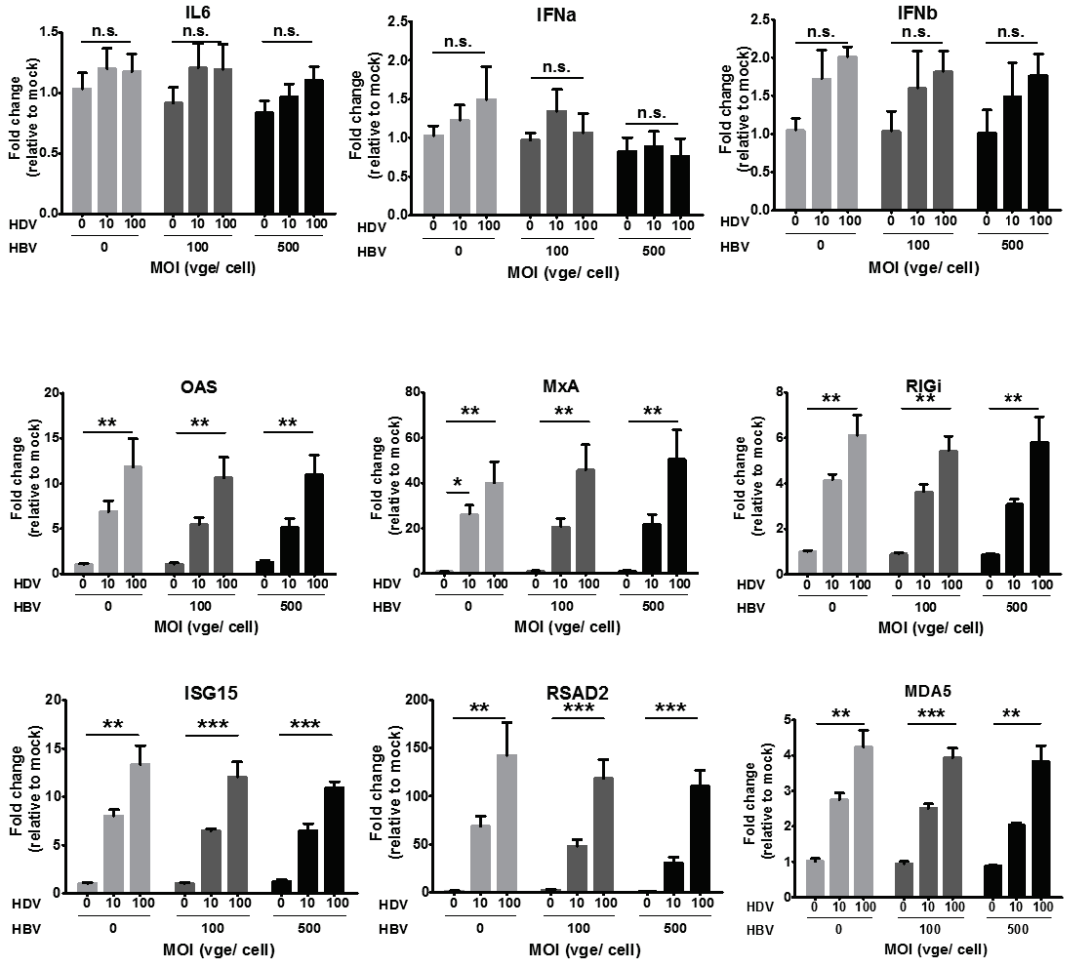
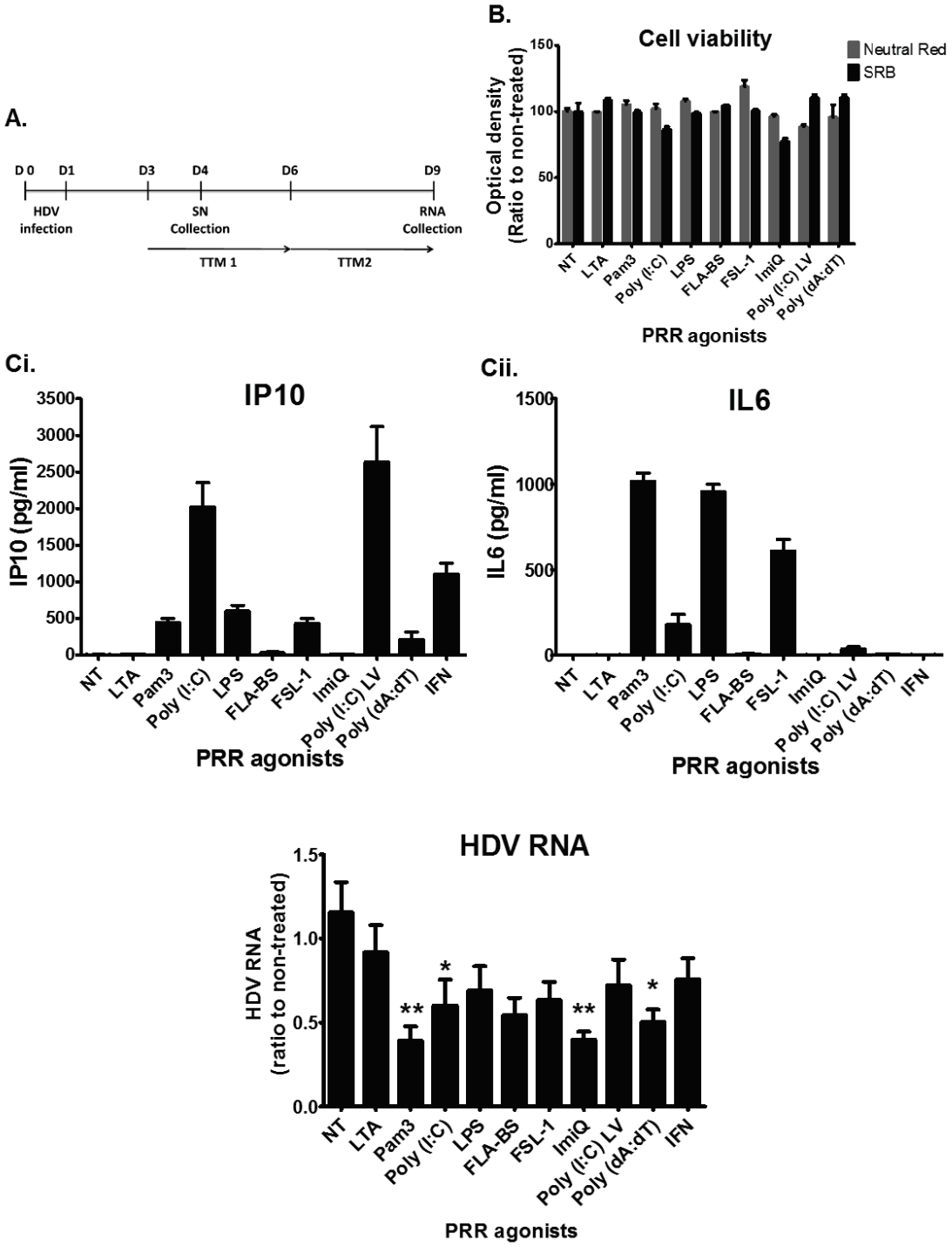

IL6
IFNa
IFNb
OAS
MxA
RIGi
ISG15
RSAD2
MDA5
Fold change (relative to mock)
n.s.
*
**
***
HDV
HBV
0 10 100
0 100 500
MOI (vge/ cell)

Figure 8.


A.
D 0
D1
D3
D4
D6
D9
HDV infection
SN Collection
RNA Collection
TTM 1
TTM2
B.
Cell viability
Neutral Red
SRB
Optical density (Ratio to non-treated)
150
100
50
0
NT
LTA
Pam3
Poly (I:C)
LPS
FLA-BS
FSL-1
ImiQ
Poly (I:C) LV
Poly (dA:dT)
PRR agonists
Ci.
IP10
IP10 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
NT
LTA
Pam3
Poly (I:C)
LPS
FLA-BS
FSL-1
ImiQ
Poly (I:C) LV
Poly (dA:dT)
IFN
PRR agonists
Cii.
IL6
IL6 (pg/ml)
1500
1000
500
0
NT
LTA
Pam3
Poly (I:C)
LPS
FLA-BS
FSL-1
ImiQ
Poly (I:C) LV
Poly (dA:dT)
IFN
PRR agonists
HDV RNA
HDV RNA (ratio to non-treated)
1.5
1.0
0.5
0.0
**
*
**
*
NT
LTA
Pam3
Poly (I:C)
LPS
FLA-BS
FSL-1
ImiQ
Poly (I:C) LV
Poly (dA:dT)
IFN
PRR agonists

Supplements

HDV replication is associated with HBV repression in superinfection, induction of interferon response and can be inhibited by immune-modulators

Dulce Alfaiate[1,2], Julie Lucifora[1,2], Natali Abeywickrama-Samarakoon[1,2,*], Maud Michelet[1,2,*], Sarah Maadadi[1,2], Barbara Testoni[1,2], Jean-Claude Cortay[1,2], Camille Sureau[4], Fabien Zoulim[1,2,5,6], Paul Dény[1,3], David Durantel[1,2,5]

Table of contents

Supplementary Methods

Production of HBV and HDV virions

High-titer HBV supernatants were produced from HepG2.2.15 cells (1) as previously described (2). HDV particles were produced according to Sureau et al. (3), and as schematically described in **Figure Sup 1**. Both HBV and HDV supernatants were concentrated with PEG 8000 at 8% final (Sigma-Aldrich). All virus preparations were tested for the absence of endotoxin (Lonza). Viral titrations were performed by qPCR/qRT-PCR as described below.

Nucleic acid extraction

For HBV titration, DNA was extracted with the QiAmp Ultrasens Virus kit (Qiagen) and submitted to qPCR, as described below. HDV was titrated by qRT-PCR (see below) after RNA extraction with the NucleoSpin RNA Virus kit (Macherey-Nagel) and DNAse I (Life Technologies) digestion 1h at 37°C followed by 20 min at 70ºC, to eliminate residual plasmid DNA.

Supernatants from infected dHepaRG cells were used for viral RNA and DNA quantification. In order to remove free nucleic acid, clarified supernatants were submitted to DNAse and RNAse digestion (Roche) followed by overnight precipitation with PEG 8000 8% (Sigma-Aldrich). After centrifugation, pellets were re-suspended in

PBS 1X and nucleic acids were extracted with the Nucleospin 96 Virus kit (Macherey Nagel). The resultant nucleic acids were submitted to qPCR and RT-qPCR for HBV and HDV quantification, respectively. All viral titers are expressed in viral genome equivalents per mL (vge/mL).

For HBV intracellular total DNA quantification, DNA extraction was performed using the Master Pure Complete DNA and RNA extraction kit (Epicentre) or the Nucleospin 96 tissue kit (Macherey Nagel). Intracellular total RNA was extracted with the NucleoSpin RNA kit (Macherey Nagel), which includes a DNAse digestion step.

RT-qPCR HDV

Serial dilution of quantified full length HDV-1 RNA (obtained from *in vitro* transcription of a pCDNA-3-derived plasmid containing a monomeric full length HDV-1 cDNA insert) was used as a quantification standard.

For viral titration, extraction was performed in triplicate and 10-fold serial dilution of each sample were tested in order to ensure correct RT-qPCR efficiency. Intracellular RNA quantification was performed on 10ng RNA/ reaction and *GUS* was used as a housekeeping gene. For each experiment diluted HDV standard RNA (see **Fig. Sup. 2**) and four serial dilutions of one of the samples were used to ensure RT-qPCR linearity. RT-qPCR reactions omitting the reverse transcriptase were performed in parallel to exclude DNA contamination.

qPCR cccDNA

For cccDNA quantification, total DNA was submitted to digestion with plasmid-safe DNAse (Epicentre) for 4hours at 37ºC, followed by 30 minutes of heat inactivation. Quantification was performed by FRET-based qPCR as previously described (4) *Beta globin* was used as a house-keeping gene.

Northern blot

Purified RNA was denatured at 50°C for one hour with glyoxal reagent (Life Technologies) and then subjected to electrophoresis through a phosphate, 1.2% agarose gel and transferred to a nylon membrane (Amersham N+, GE). Membrane-bound RNA was hybridized to 32P-labeled full genome HDV or HBV-specific

probes.The membrane was washed twice in low-stringency wash buffer (1× SSC; 0.1% SDS) for 30 min at room temperature and twice in high-stringency wash buffer (0.1× SSC, 0.1% SDS) for 30 min at 65°C. Quantitative analysis of HDV RNA was achieved by phosphorimager scanning (Typhoon Fla 9500, GE) and 18S and 28S rRNA quantification was were used as loading control.

ELISA

Commercial immunoassay kits (Autobio Diagnostics Co., China) were used for HBsAg and HBeAg quantification in the cell culture supernatant. Results are presented as a ratio to a control sample, described for each experiment. Cut-offs for these ELISA were 1 NCU/mL (i.e. 1 NCU ≈ 13 ng) for HBeAg and 2.5 ng/mL for HBsAg.

IL-6 and IP-10 were quantified in the cell culture supernatants by ELISA (R&D Systems), according to the manufacturer's recommendations.

Immunofluorescence

Cells were fixated with paraformaldehyde 4% and permeabilized by Triton 0.3%. Labeling was done using the following antibodies: HBcAg – monoclonal mouse antibody from Abcam (8637 – 1/200 dilution); HDAg – polyclonal in-house rabbit antibody (1/200 dilution). Secondary labeling was performed with Alexa Flour fluorescent antibodies (wavelengths 555 and 488) and cell nuclei were stained with 4.6-diamidino-2-phenylindole (DAPI). All images were obtained by epifluorescence microscopy (Nikon eclipse TE2000-E; Nikon) and processed ImageJ software. Labeling were quantified by a combination of automatic nuclei counting provided by the software and manual counting of labeled cells. Displayed results correspond to the average of at least three fields (200X magnification).

Western Blot

Cell lysis was performed with M-PER reagent (Pierce) in the presence of protease inhibitors. Western blots were performed with standard procedures using in-house polyclonal rabbit anti-HDAg antibodies (kind gift from Alan Kay) and anti-tubulin mouse monoclonal antibody (Sigma Aldrich). Detection was performed with Gel Doc XR+ System (BioRad) and images were analyzed with ImageJ software.

Cell viability testing

Cell viability was sequentially evaluated by neutral red uptake and sulforhodamine B (SRB) assays. Neutral red assay was adapted from Repetto et al (5). Briefly, cells were first incubated for 2h at 37ºC, 5% CO2 with pre-warmed 40 µg/mL neutral red solution diluted in complete culture medium containing DMSO. Then, neutral red medium was removed and, after PBS washing, cells were de-stained with 50% absolute ethanol, 49% deionized water and 1% glacial acetic acid. After homogenization, triplicate samples of the destaining solution were collected per condition and optical densities were measured at 540 nm.

After neutral red uptake assay, cells were washed with deionized water, and then dried for further staining adapted from Vichai and Kirtikara, 2006 (6). Dried cells were incubated with SRB solution for 5 min at room temperature. The solution was then removed, and cells were rapidly washed three times with 1% acetic acid solution and completely dried at 37 °C. Finally, 10 mM Tris solution (pH 10.5) was added into each well and shaken during 5 min. After homogenization, the solution was harvested in triplicates for each condition and optical density was measured at 490 nm. All results are displayed as a ratio to the non-treated condition.

Supplementary figures

Figure S1.

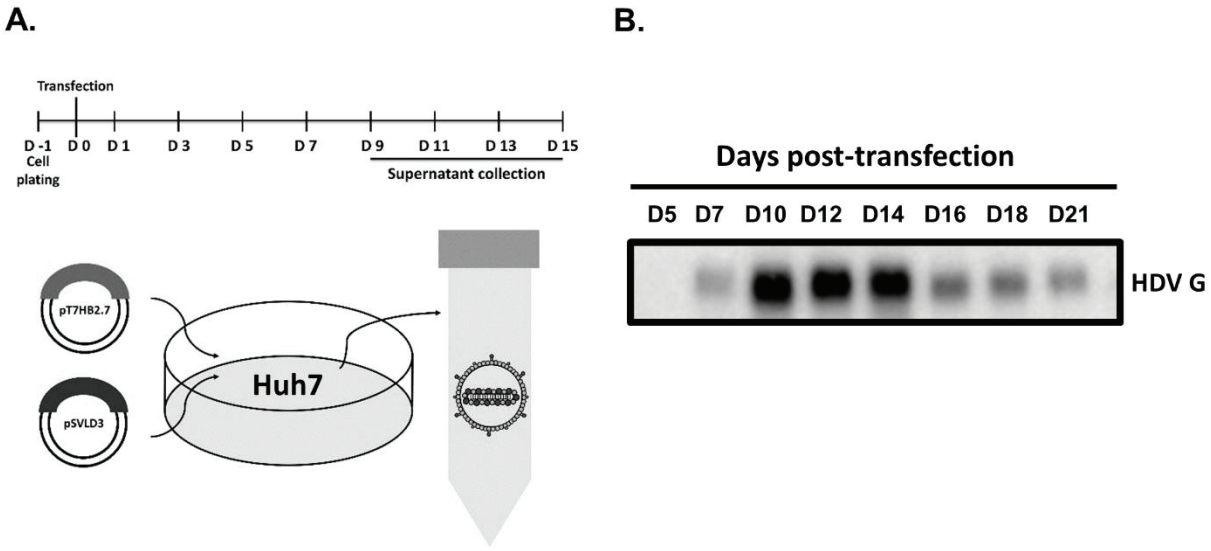


Figure S1. HDV production. A) HDV was produced in vitro by co-transfection of Huh7 cells with T7HB2.7 (coding for PreS1-PreS2-S from HBV) and pSVLD3 (containing a trimer of HDV genotype 1 genome). Supernatant was collected every other day from day-9 to day-15 post-transfection. **B)** Northern blot analysis (with a full-length anti-genomic probe, for detection of HDV genome) of HDV RNA in the supernatant of transfected cells throughout time. *G*, genome.

Figure S2.

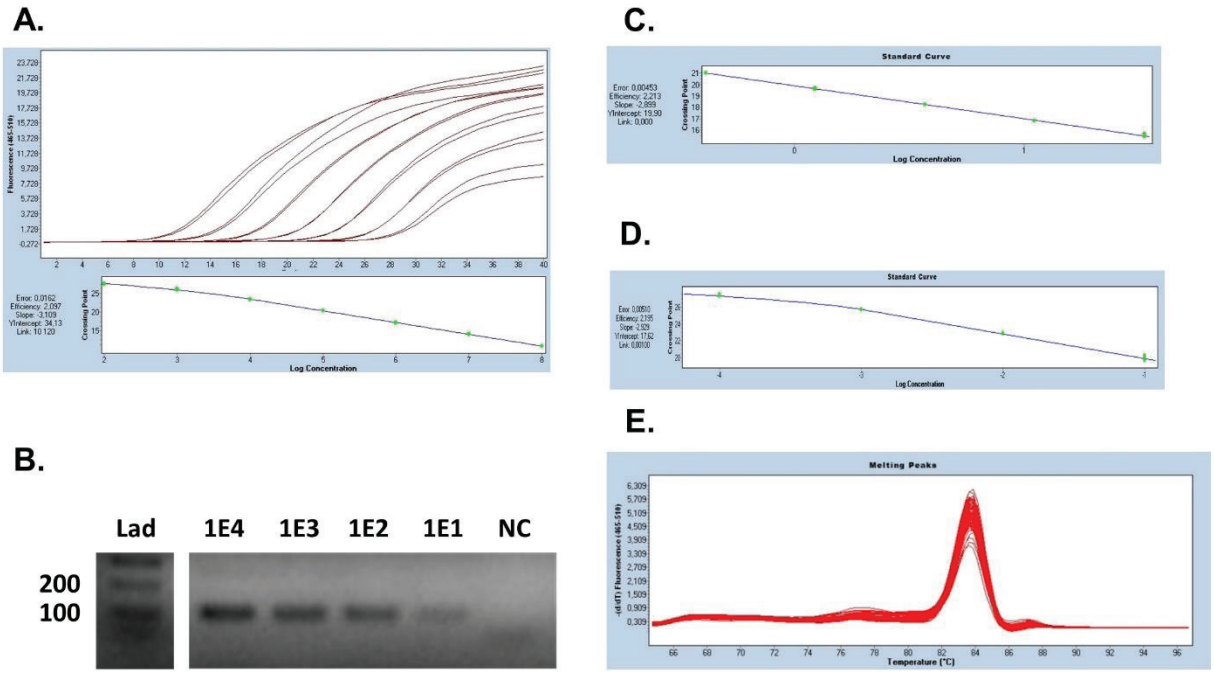


Figure S2. RT-qPCR HDV. A) Serial 10-fold dilutions of a full-length HDV-1 RNA genomic transcript are used as a quantification standard, confirm PCR linearity within a range of 10^2 to 10^8 copies/ reaction with a PCR efficiency of ~2,1; **B)** Electrophoresis of PCR products evidences a unique band located between 100 and 200bp, consistent with the predicted 129bp amplicon; **C)** Serial 10-fold dilutions of RNA extracted from cell culture supernatant, confirm PCR linearity between $6X10^1$ and $6X10^5$ copies/ reaction, with a PCR efficiency of ~2,2; **D)** Serial 3-fold dilutions of intracellular total RNA confirm a linear HDV amplification within a range of 0.4-33,3 ng of total RNA per PCR reaction; **E)** Melting curve plot of HDV infected samples, confirming one single Tm peak consistently found at 84ºC. *Lad*, DNA ladder (100bp, New England Biolabs); *NC*, negative control; *Tm*, melting temperature.

Figure S3.

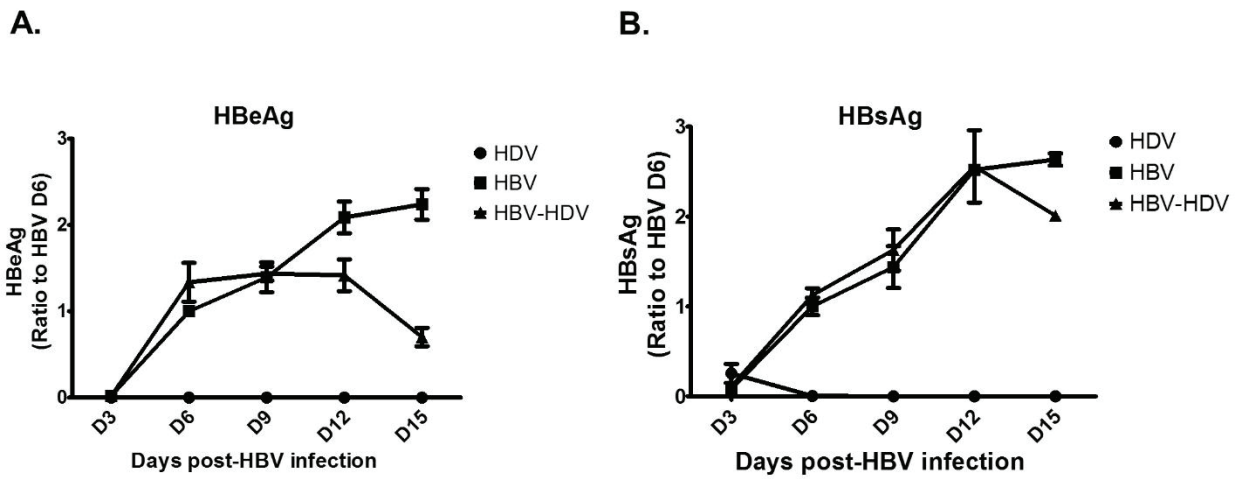


Figure S3. Kinetics of HDV super-infection of HBV-infected dHepaRG cells. Evaluation of HBeAg (**A**) and HBsAg (**B**) secretion of HBV cells super-infected or not by HDV, as described for **Fig. 3**. at an estimated HDV MOI of 1000. A 70% decrease of HBeAg, but not HBsAg, secretion can be observed in HDV super-infected cells.

Supplementary tables

Table S1. List of primers and probes used for qPCR

Designation		Sequence (5'-3')
HDV	Forward Primer	CGGGCCGGCTACTCTTCT
	Reverse Primer	AAGGAAGGCCCTCGAGAACA
HBV total	Forward Primer	GCT GAC GCA ACC CCC ACT
	Reverse Primer	AGG AGT TCC GCA GTA TGG
HBV pgRNA	Forward Primer	GGA GTG TGG ATT CGC ACT CCT
	Reverse Primer	AGA TTG AGA TCT TCT GCG AC
HBV cccDNA	Forward Primer	CTC CCC GTC TGT GCC TTC T
	Reverse Primer	GCC CCA AAG CCA CCC AAG
	Probe	GTT CAC GGT GGT CTC CAT GCA ACG T
	Probe	AGG TGA AGC GAA GTG CAC ACG GAC C
GUS	Forward Primer	CGTGGTTGGAGAGCTCATTTGGAA
	Reverse Primer	ATTCCCCAGCACTCTCGTCGG
RPLP0	Forward Primer	CAC CAT TGA AAT CCT GAG TGA TGT
	Reverse Primer	TGA CCA GCC CAA AGG AGA AG
B-globin	Forward Primer	ACA CAA CTG TGT TCA CTA GC
	Reverse Primer	CAA CTT CAT CCA CGT TCA CC
	Probe	CAA ACA GAC ACC ATG GTG CAC CTG ACT CCT GAG GA

	Probe	AAG TCT GCC GTT ACT GCC CTG TGG GGC AA
IL6	Forward Primer	ACCCCTGACCCAACCACAAAT
	Reverse Primer	AGCTGCGCAGAATGAGATGAGTT
IFNa	Forward Primer	GTGAGGAAATACTTCCAAAGAATCAC
	Reverse Primer	TCTCATGATTTCTGCTCTGACAA
IFNb	Forward Primer	GCCGCATTGACCATGTATGAGA
	Reverse Primer	GAGATCTTCAGTTTCGGAGGTAAC
OAS1	Forward Primer	AGGTGGTAAAGGGTGGCTCC
	Reverse Primer	ACAACCAGGTCAGCGTCAGAT
ISG15	Forward Primer	ATGGGCTGGGACCTGACG
	Reverse Primer	GCCAATCTTCTGGGTGATCTG
MxA	Forward Primer	GGTGGTCCCCAGTAATGTGG
	Reverse Primer	CGTCAAGATTCCGATGGTCCT
RSAD2	Forward Primer	CTTTGTGCTGCCCCTTGAG
	Reverse Primer	TCCATACCAGCTTCCTTAAGCAA
RIG-I	Forward Primer	GCTGATGAAGGCATTGACATTG
	Reverse Primer	CAGCATTACTAGTCAGAAGGAAGCA
MDA5	Forward Primer	CCCATGACACAGAATGAACAAAA
	Reverse Primer	CGAGACCATAACGGATAACAATGT

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2. Luangsay S, Gruffaz M, Isorce N, Testoni B, Michelet M, Faure-Dupuy S, et al. Early Inhibition of Hepatocyte Innate Responses by Hepatitis B Virus. J Hepatol. 2015 Jul 24;

3. Sureau C. The use of hepatocytes to investigate HDV infection: the HDV/HepaRG model. Methods Mol Biol Clifton NJ. 2010;640:463–73.

4. Werle-Lapostolle B, Bowden S, Locarnini S, Wursthorn K, Petersen J, Lau G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology. 2004 Jun;126(7):1750–8.

5. Repetto G, del Peso A, Zurita JL. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc. 2008;3(7):1125–31.

6. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc. 2006;1(3):1112–6.

3. Additional results

The first part of the work, presented above as 2. Submitted article, concerned the set-up and characterization of a model of HDV infection, either in the absence (monoinfection) or presence (superinfection) of its helper virus, HBV. From these results, it was possible to identify two main phenotypes:

i) the existence of a robust, HDV replication, although limited in time, associated with an IFN response, which was evidenced by the induction of the expression of some ISGs;
ii) the confirmation of an interference phenotype, with an inhibition of HBV replication as a consequence of HDV superinfection.

To get further, albeit preliminary, insights into the molecular and immunological mechanisms underlying such phenotypes, further experimentations have been conducted and are yet being performed. The results obtained so far are presented in this chapter.

3.1. Evaluation of HDV infection in other cell models

3.1.1. Introduction

Having characterized the phenotype of HDV infection in HepaRG cells, we proceeded to evaluate its reproducibility in other models. We hence focused on two additional cell models:

i. Primary Human Hepatocytes (PHH) that are permissive to a complete life cycle of both HBV and HDV and are considered to be the most physiological *in vitro* model for the study of these infections;
ii. HepG2 hNTCP cells that were obtained following stable transduction of HepG2 cells (a transformed cell line isolated from hepatoblastoma tissue) with a vector coding for the sequence of the putative human HBV/ HDV receptor -

hNTCP - and were previously shown to support both HBV and HDV infections (Ni et al., 2014).

3.1.2. Material and methods

Primary hepatocyte and HepG2 hNTCP cell culture

Primary human hepatocytes (PHH) were prepared from surgical liver resections (kindly provided by Pr. Michel Rivoire, Centre Léon Bérard and INSERM U1032, Lyon), as previously described (Lecluyse and Alexandre, 2010). One day post-seeding, cells were thoroughly washed and placed into Williams medium devoid of FCS for 24 hours to counter select for endothelial and fibroblastic cells, and afterwards cultivated, and infected in medium containing 5% FCS and 2% DMSO.

HepG2 hNTCP cells were kindly provided by Dr. Stephan Urban (Heidelberg University Hospital). Cells were cultured in Dulbecco's modified Eagle medium (Invitrogen), supplemented with 5% FCS and 2.5 µg/mL puromycin (Invivogen), GlutaMAX™, sodium pyruvate and penicillin/streptomycin. They were seeded in the same medium (without puromycin) at a density 100,000 cells per cm^2, and further cultivated and infected in medium containing 2.5% DMSO. All cells were maintained at 37 °C and 5% CO2.

HBV and HDV infections, ELISAs for HBeAg and HBsAg detection in supernatants, DNA and RNA extraction, q(RT)-PCR and Northern Blot were done as previously described (Material and Methods section of the 2. Submitted article chapter)

3.1.3. Results

HDV infection of PHH results in a peak of replication followed by a subsequent decline

In order to validate the main findings obtained in the HepaRG cell line, we proceeded to the study of HDV infection (both in the presence and absence of HBV) in PHH. For all the experiments shown, cell viability and morphology were monitored by

microscopy observation and only experiments where hepatocyte morphology was maintained throughout time were considered.

We first evaluated the kinetics of HDV mono-infection. PHH were either mock or HDV infected with MOI of 10 vge/cell and HDV RNA was collected at every third day and analysed by qRT-PCR and Northern Blot. As seen in **Fig. 1A.i**, representative of 2 independent experiments done with cells from 2 donors, HDV replication was maximal between D6 and D9 post-infection and then experienced a five-fold decline. As far as we could evaluate while keeping PHHs in good shape, no further decline was observed. The qRT-PCR trend was confirmed to be associated with a decrease in the anti-genomic RNA by Northern Blot, as shown in **Figure 1A.ii**.

HDV interference on HBV replication could be confirmed in PHH

To further characterize HDV infection in PHH, we proceeded to superinfection evaluation. Cells were first either mock or HBV infected with a MOI of 100 vge/cell, then 6 days after, either mock or super-infected by HDV at MOIs of 10 or 100 vge/cell. At day-15 post-HBV infection (9 days after HDV superinfection), HBsAg and HBeAg were quantified in the supernatants and cells were lysed for DNA extraction followed by quantification of intracellular total HBV DNA by qPCR (**Fig. 1B.i**). The results correspond to one experiment with two biological replicates. As previously described for HepaRG, and represented in **Fig. 1B.ii**, a decrease could be observed in the secretion of HBeAg (but not HBsAg) in cells super-infected with HDV. Furthermore, this trend was more evident with the increase of the HDV MOI (in comparison to HBV mono-infection, cells super-infected with HDV 10 vge/ cell had an 11% decrease in HBeAg secretion, while superinfection with HDV 100 vge/ cell was associated with a 19% decrease; neither difference was statistically significant). Intracellular total HBV DNA amounts were not affected by HDV infection at low MOI (10 vge/ cell), but decreased by 60% in cells super-infected with HDV 100 vge/ cell (**Fig. 1B.iii**), thus confirming viral interference in this model.

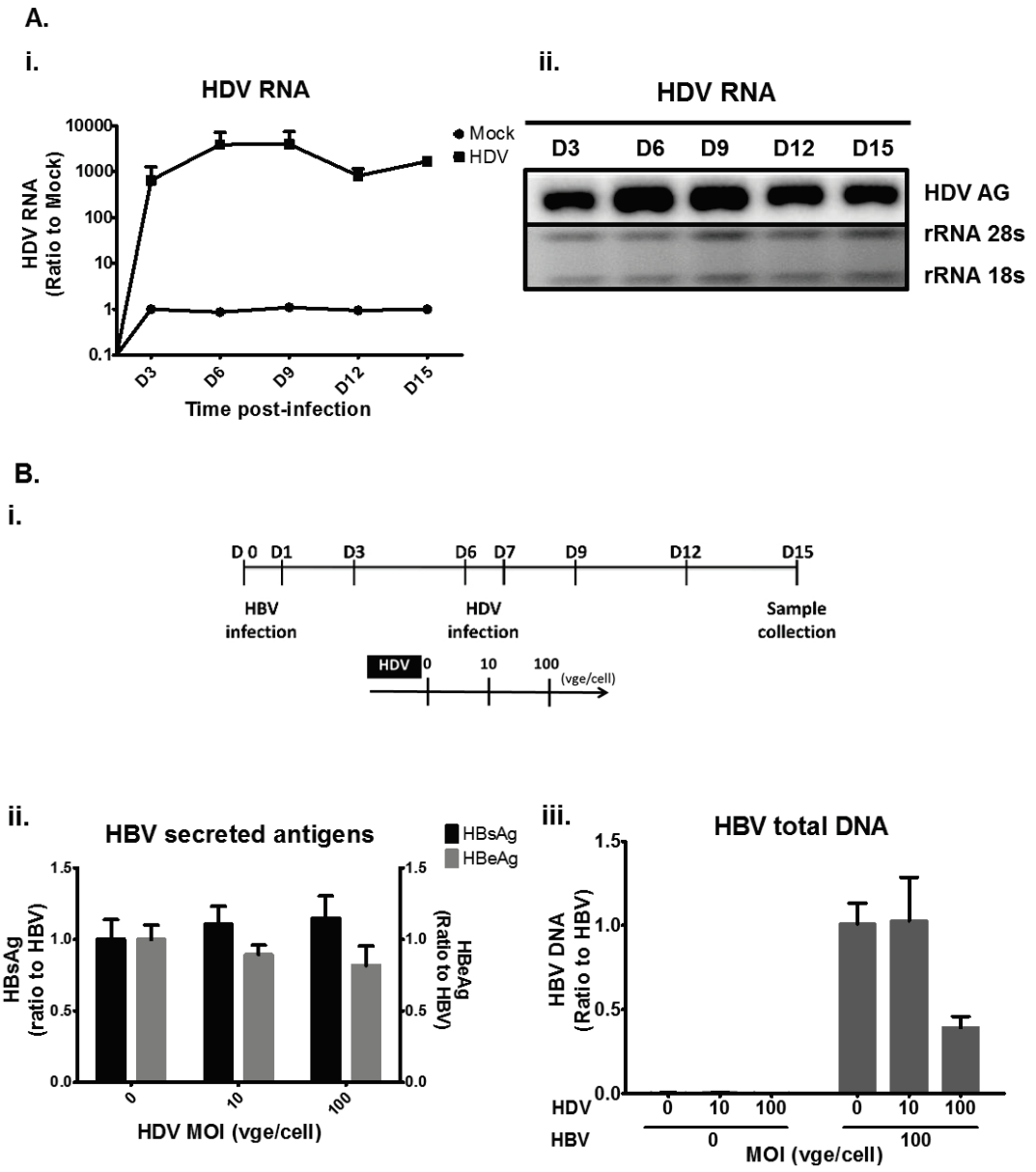

Figure 1. Evaluation of HDV infection in primary human hepatocytes. HDV monoinfection of PHH was performed at MOI 10 vge /cell and followed for 15 days. HDV RNA was quantified by qRT-PCR HDV (**A.i**) and Northern blot (**A.ii**) performed with a full-length genomic probe for detection of antigenome. Results correspond to two independent experiments. **B.i**. HDV superinfection (10 or 100 vge/ cell) of HBV infected cells (100 vge/ cell) was performed at day 6 and followed until day 15. Supernatants were used for HBeAg and HBsAg quantification by ELISA (**B.ii**) and intracellular HBV DNA was analysed by qPCR (**B.iii**). The results correspond to one experiment with two replicates.

HDV interference on HBV replication is not observed in HepG2 hNTCP cells

A first evaluation of HDV infection in HepG2 hNTCP cells was performed in a superinfection setting. Cells were first either mock or infected with HBV at MOIs of

100 or 500 vge/cell, then HDV superinfection (MOI 10 or 100 vge/ cell) was performed at day-6 post HBV infection and controlled by mock infection, as previously described and represented in **Fig. 2A**. It was possible to keep cells in culture for 12 days post-infection, as a significant change in cell morphology was observed beyond this time point. Supernatants and total intracellular DNA were collected at the end of the experiment. The results correspond to two independent experiments, each with biological duplicates. As can be observed in **Figs. 2B** and **2C**, no significant variation was evidenced in the secreted levels of either HBeAg or HBsAg upon HDV superinfection. In cells infected with a high HBV dose (500 vge/ cell), there is a decreasing trend of intracellular total HBV DNA levels, upon HDV superinfection (45 and 60%, for HDV MOIs 10 and 100, respectively). However, due to a high variability between the two experiments, this difference was not statistically significant (**Fig. 2D**).

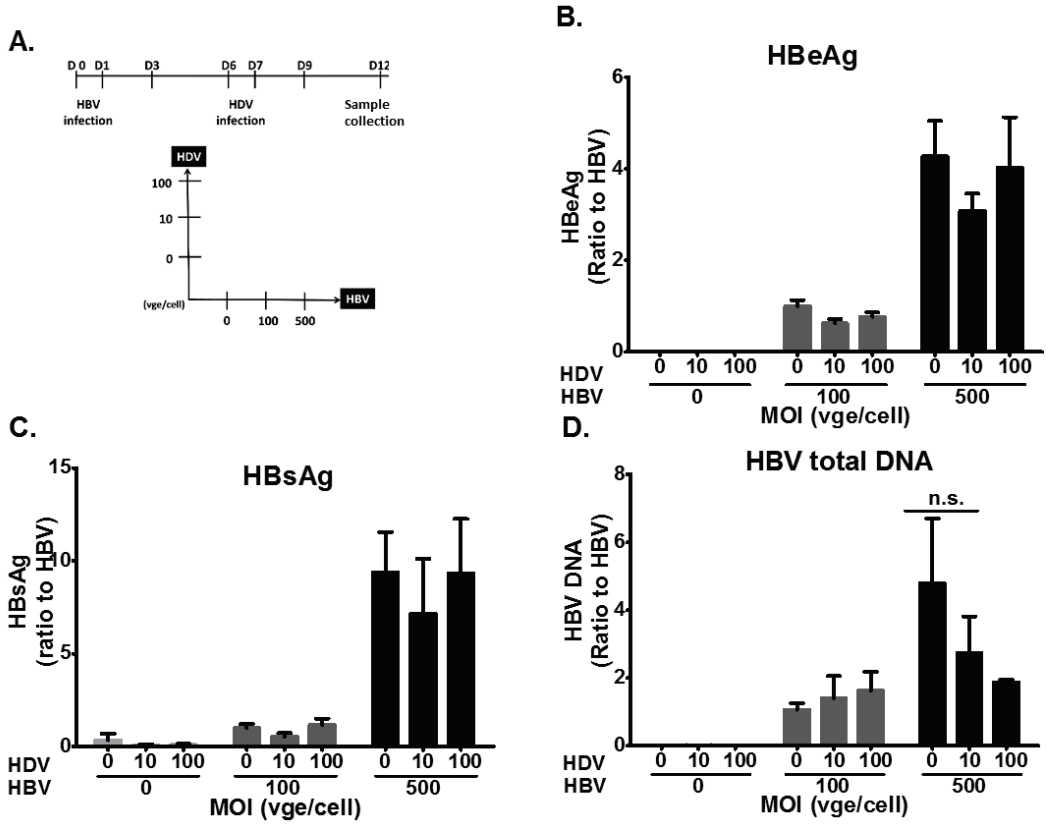

Figure 2. Evaluation of HDV/ HBV superinfection in HepG2 hNTCP. A. HepG2 hNTCP were infected with diferent HBV MOIs (0, 100 and 500 vge/ cell) and superinfected 6 days later with HDV (0, 10 or 100 vge/ cell). Experiment was stopped at day 15. Supernatants were analysed HBeAg (**B**) and HBsAg ELISA (**C**) and total HBV intracellular DNA was quantified by PCR (**D**). The results correspond to two independent experiments, each with two replicates. *n.s.,* non-significant (p> 0,05).

3.1.4. Discussion

Primary hepatocytes, from human, primate or woodchuck donors, have been an invaluable model for the study of both HBV and HDV infections. In our study conditions, we were able to demonstrate that HDV mono-infection of PHH led to a peak of replication between days 6 and 9 post-infection followed by a subsequent decline. Although less pronounced, this decline reproduced the observations made in HepaRG.

Previous studies of HDV infection of PHH have demonstrated variable profiles: while Gudima and colleagues described a peak of replication followed by a decline, similar to that we observed in dHepaRG, studies from the Lanford's group, both in human and primate hepatocytes (primary chimpanzee hepatocytes, PCH), identified a plateau of replication. A striking difference can be identified in the culture setting, as in the latter case cells were cultivated in particular conditions, with a serum-free, but very rich medium, which was associated with long-term cultivation periods (more than 40 days) and eventually the capability of PHH/ PCH to sustain or control viral infection (Barrera et al., 2004; Gudima et al., 2007; Sureau et al., 1991). It would be interesting to use such conditions in the culture of dHepaRG cells to investigate a possible change in the kinetic profile of HDV infection and further investigate if the observed decline in viral replication is associated with the lack of an essential factor for viral replication or an active antiviral response.

Although further reproduction and characterization is warranted, the replication of an interference phenotype in PHH allows us to validate the observations made in the HepaRG model, and its usefulness as an alternative and convenient model to further study HDV/ HDV superinfection and understand underlying molecular and/ or immunological mechanisms at work to explain viral interference. Conversely, the fact that such phenomenon was not reproduced in the HepG2 hNTCP cells rises important questions, not only relative to their use as a model, but ultimately related to the mechanisms of HDV/ HBV interference. In a first approach it will be essential to have a more thorough characterization of the kinetics of infection in this model. An important technical difference must be noticed: in HepG2 hNTCP, evaluation of superinfection was possible 12 days after HBV infection, while in HepaRG the decrease

in HBV viral parameters was only significant 3 days later. We cannot exclude the need for a longer period of superinfection in order to detect interference. Although HepG2 hNTCP are known to be susceptible to HDV infection (Ni et al., 2014), and replication could be detected in our experiments (data not shown), a thorough characterization of the kinetics of HDV RNA and protein accumulation and an assessment of the number of infected cells is warranted for further discussion of these results.

3.2. Evaluation of HDV mono-infection

3.2.1. Introduction

Results obtained in the first part of this thesis work, and described in the previous chapter revealed that, in HepaRG cells, after a peak of replication, HDV infection suffered a steep decrease (2. Submitted article, **Fig. 2**). Coincidently, this kinetic profile correlated with a sharp increase in the expression of ISGs. This observation raised the question of whether an effective IFN response could be leading to the control of HDV replication in this model.

In order to evaluate such possibility, two strategies were used:

i. Assessment of HDV replication in a context of blockage of the type I interferon response. This was performed by reproducing the evaluation of HDV replication kinetics in the presence of a neutralizing antibody to human Interferon (Alpha, Beta and Omega) Receptor (IFNAR) 2;
ii. Evaluation of HDV replication in the presence of HBV proteins, previously shown to modulate IFN response. Previous works from the laboratory (Gruffaz et al., 2013) have demonstrated that HBV core protein is associated with an early inhibition of the IFN response in HBV infected cells. Hence, in order to evaluate a possible effect of such inhibition on HDV replication, we monitored HDV infection in cell lines expressing selected HBV proteins.

Furthermore, beyond the observation that HDV was associated with a strong induction of ISG expression, we also evidenced that treatment with exogenous IFN

was only modestly effective (2. Submitted article, **Fig.8**). These observations led to the hypothesis, previously approached in a different cell model (Pugnale et al., 2009), that HDV infected cells may be resistant to further IFN stimulation. We hence proceeded to determine if IFN treatment was effective in further inducing ISG expression in HDV infected cells.

3.2.2. Material and methods

Neutralization of IFNα/β signalling

Differentiated HepaRG cells were subjected to HDV mono-infection as previously described. Briefly, as represented in **Fig. 3A**, cells were inoculated with HDV 10 vge/cell for 24 hours (as a negative control, cells were treated with the entry inhibitor Myrcludex [100 nM] from 2 hours before infection and until the end of viral inoculation). At the end of the inoculation cells were thoroughly washed with PBS and anti-human IFNAR2 antibody (PBL Assay Science) was added to the culture medium at a concentration of 500 ng/ml and kept throughout the experiment. RNA was collected at different time points and analysed by RT-qPCR for HDV quantification or subjected to RT and subsequent qPCR for ISG expression evaluation as described above (2. Submitted article, Material and methods).

Interferon treatment of HDV infected cells

HDV infection of dHepaRG cells was performed as already described. IFNα (Roche) 1000 IU/ml was added to the cell culture medium at day-5 post-infection and cells were collected for RNA extraction and qRT-PCR analysis after 24 hours of treatment (at day-6 post-infection). Mock infected and non-treated cells were used as controls (**Fig. 4A**).

Establishment of HepaRG TR-HBc, TR-HBe, TR-HBs and TR-HBx cell lines

Recombinant HepaRG cells were previously generated in the laboratory for the expression of selected HBV proteins: HBc, HBe, S-HBs and HBx (Gruffaz M, *in preparation*). Briefly, each HepaRG cell line was established using the ViraPower T-Rex Lentiviral Expression System from Invitrogen. Stable overexpression of the

tetracycline repressor (TR) in HepaRG cells was obtained after transduction with a lentivirus (multiplicity of infection of 5) produced upon cotransfection of HEK-293 cells with pLenti6-TR plasmid and packaging vectors, according to the manufacturer's indications. Blasticidin-resistant HepaRG-TR cells were then transduced with a second lentivirus conferring zeocin resistance and coding for either HBc, HBe, S-HBs and HBx (HBV genotype D, serotype ayw, Galibert's strain), produced upon cotranfection of HEK-293 cells with packaging vectors and with corresponding pLenti4/TO plasmids, in which transgene are under the control of a tetracycline-regulated promoter. Cells were co-selected by blasticidin and zeocin and cultivated as previously described for HepaRG.

HDV infection and qRT-PCR were performed as previously described.

3.2.3. Results

IFNα/β signalling blockage is associated with a decrease, but not abrogation, of ISG expression

In HDV infected cells, the blockage of IFNα/β signalling by an anti-IFNAR neutralizing antibody was associated with a decline of ISG peak expression (at day-6 post-infection), as depicted in **Fig. 3B**. Such decrease was observed for all the tested ISGs and it ranged from 32% (for MDA5) to 62% (for RSAD2).

IFNα/β signalling blockage does not prevent the decline in HDV replication

In the presence of anti-IFNAR antibody, the peak of HDV replication is attained later, at day-9 and not day-6 post-infection, and is slightly inferior (20%) to that observed in the control condition. However, HDV replication still declines after attaining the peak, following the same kinetics as the control condition (**Fig. 3C**), suggesting that other factors than IFN α/β may be involved in such decrease.

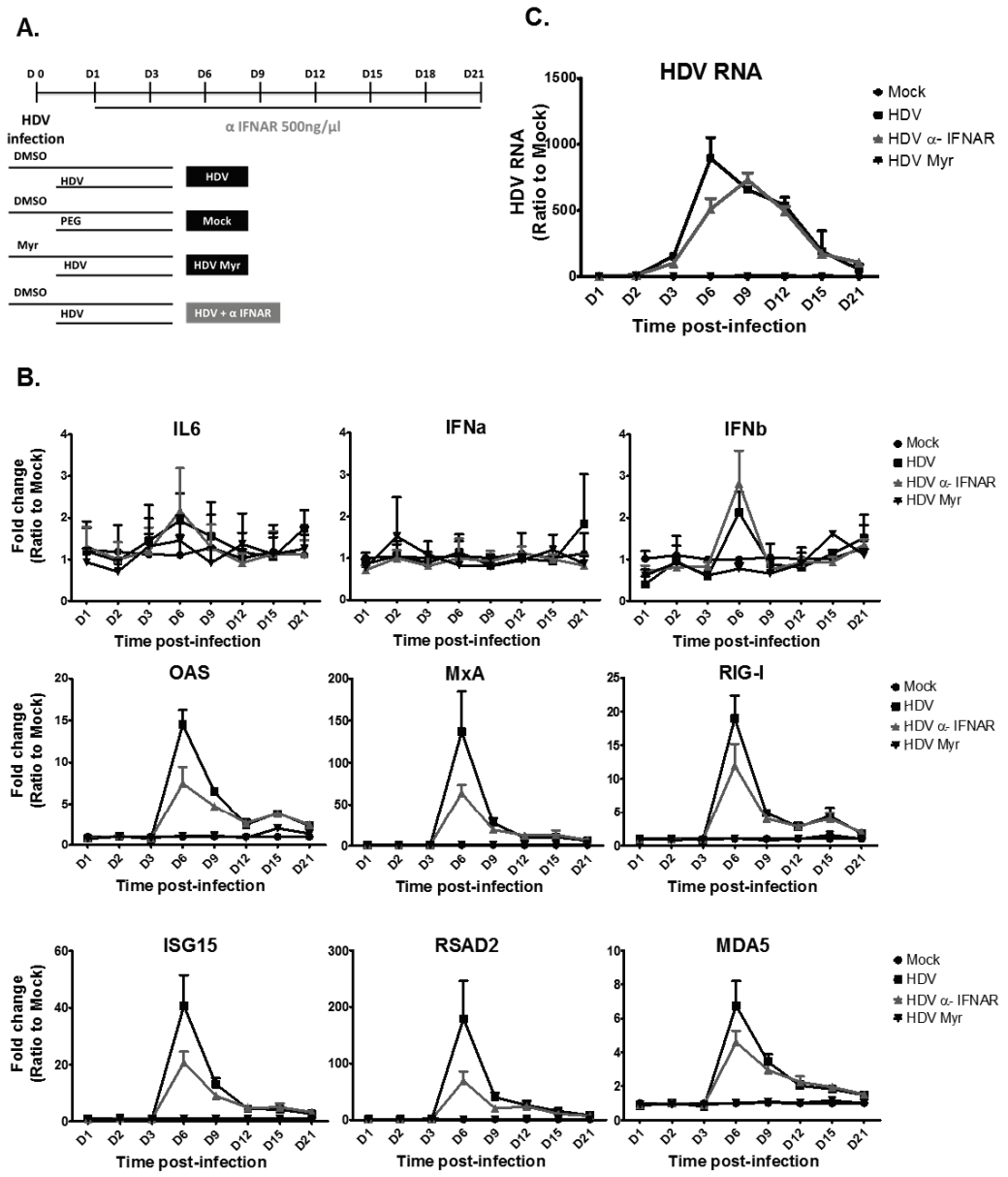

Figure 3. Evaluation of the effect of IFNα/β signalling blockage on HDV replication. **A.** HDV monoinfection was established and after inoculation cells were treated or not with anti-IFNAR2 neutralizing antibody, that was kept until the end of the experiment, when cells were collected for RNA extraction and qRT-PCR. The results represent two replicates (wells) in the same experiment. **B.** RNA quantification of selected ISGs. **C.** HDV RNA quantification by qRT-PCR.

Interferon alpha treatment of HDV infected cells does not lead to a further increase of ISG expression

As discussed before, HDV replication in HepaRG cells is associated with a strong induction of ISG expression. To evaluate the responsiveness of HDV infected cells to IFNα treatment, a 24 hour IFN treatment was done, and HDV replication and ISG

expression were assessed at their peaks (previously determined to occur at day-6 post-infection) – **Fig. 4A**.

As seen in **Fig. 4B**, HDV replication was not affected by the 24-hour IFNα treatment.

As expected, and can be observed in **Fig. 4C** in mock infected cells, IFNα treatment was associated with a strong increase in ISG expression ranging from 20x for RIG-I to 2298x for RSAD2. A strong induction of ISG expression by HDV infection alone was once again confirmed and attained levels were comparable to those induced by treatment with IFNα 1000 IU/ml. Moreover, treatment of infected cells with IFNα did not induce a further increment in ISG expression (maximum increase was observed in RIG-I expression: 1,5 fold). **Table 1** represents the average fold increase in ISG expression after interferon treatment of both mock and HDV-infected cells.

Table 1. Fold change variation of ISG's RNA in response to IFNa treatment

	Fold change (relative to non-treated)	
	Mock	**HDV**
OAS1	36	1,1
ISG15	115	1,05
MxA	434	1,27
RSAD2	2298	1,45
RIG-I	59	1,5
MDA5	20,1	1,22

HDV replication is increased in cells expressing HBc

HepaRG cell lineages expressing HBc, HBe, HBs and HBx under the control of a tetracycline inducible promoter were constructed and characterized in the laboratory beforehand. A summary of their functional evaluation is presented in **Fig. 5**.

We first evaluated HDV replication in the HepaRG TR-HBc lineage. Cells were plated and differentiated as described for HepaRG cells. Transgene expression was induced by addition of tetracyclin (10 μg/ml) to the cell culture medium and 3 days after cells were infected by HDV (MOI 1vge/ cell). RNA was collected at days 1, 4, 8, 11 and 14 post-infection.

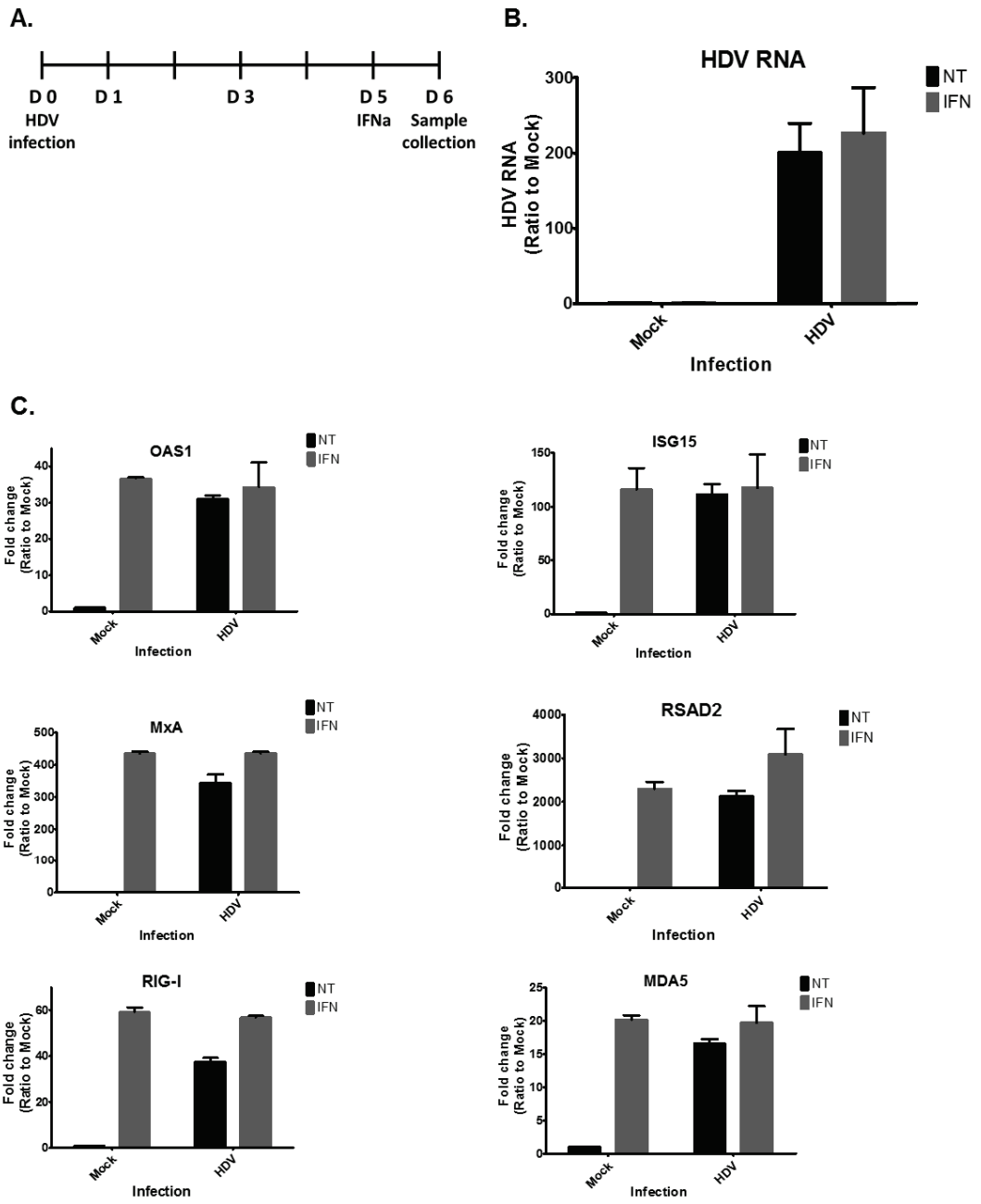

Figure 4. Assessment of the effect of IFNα treatment of HDV infected cells. A. dHepaRG cells were infected with HDV 10 vge/ cell and after 5 days treated with IFNα 1000 IU/ml. Cells were harvested at day 6 post-infection (corresponding to 24 hours of IFNα treatment) and RNA was analysed by qRT-PCR HDV (**B**) and RT-qPCR for selected ISGs (**C**). The displayed results correspond to the average of two biologic replicates in one experiment.

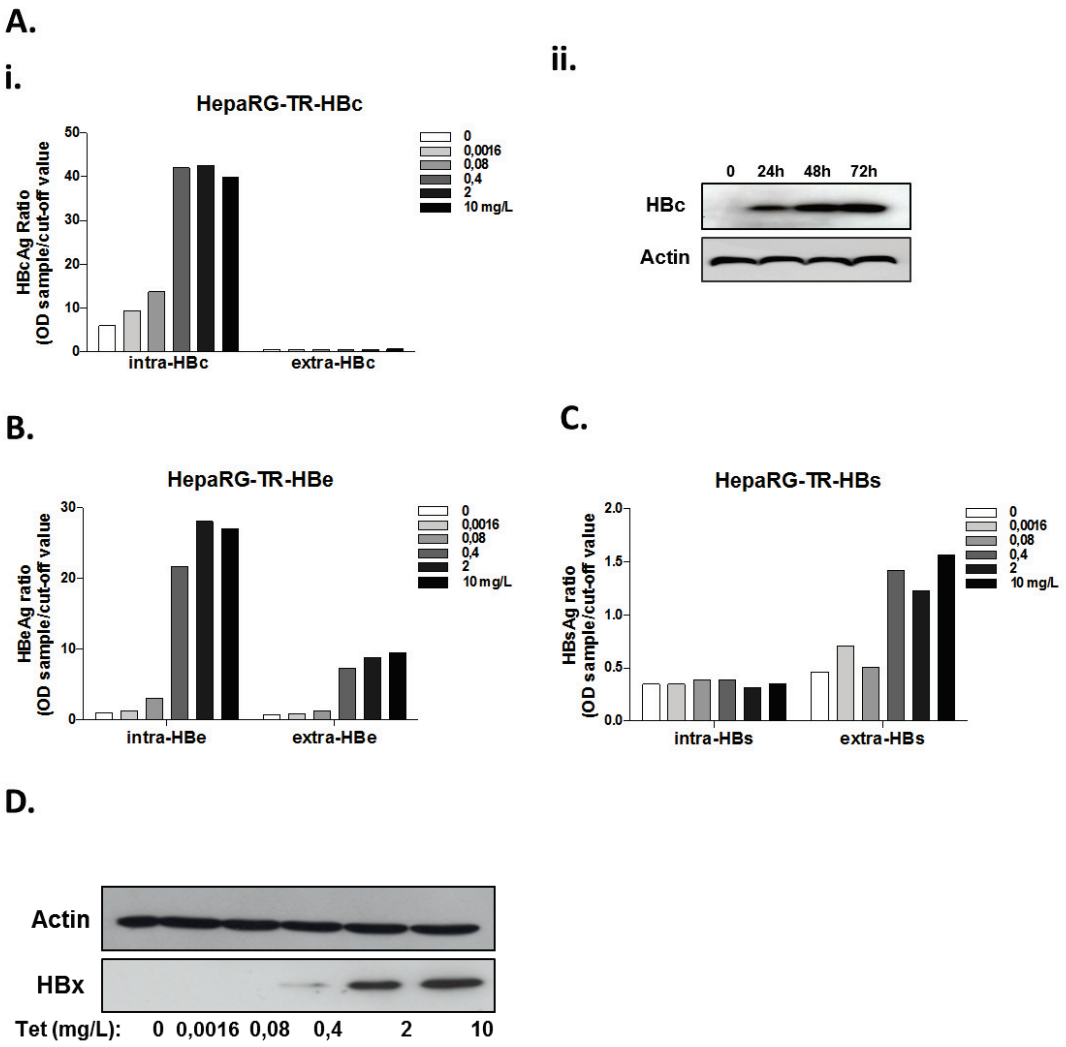

Figure 5. Characterization of HepaRG TR-HBc, -HBe, -HBs and -HBx cell lines. For each lineage, protein expression was evaluated in function of tetracycline dose **A**. HBc expression was evaluated by intracellular and extracellular HBcAg ELISA and by Western Blot. **B**. HBe expression was verified by intracellular and extracellular HBeAg ELISA. **C**. HBsAg expression was also quantified by ELISA. **D**. HBx expression was assessed by western blot.

As seen in **Fig. 6A.i**, corresponding to the result of three independent experiments, HDV replication is favoured in the cells expressing HBc (labeled Tet+), although a statistically significant difference was not attained (maximum 1,7 fold increase). Transgene expression was confirmed by RT-qPCR using primers designed for PreC/C /pgRNA detection (2. Submitted article, Table 1) as shown in panel **Fig. 6A.ii**.

In order to confirm the specificity of the HBc effect on HDV replication and to evaluate possible interactions of other HBV proteins, the experiment was repeated with HepaRG TR-HBc, TR-HBe, TR-HBs and TR-HBx. Differentiated cells were pre-treated

with tetracyclin for 3 days (10µg/ml) and then infected with HDV (MOI 5 vge/ cell). As HBe and HBs are secreted in the cell culture supernatant, inoculation was performed using the conditioned supernatants. RNA was collected at days 3, 6, 9, 12 and 15 post-infection. As seen in **Fig. 6B** (representative of one experiment), a favourable effect of core expression on HDV replication was confirmed. Furthermore its specificity could be verified as neither HBe nor HBx seem to interfere with HDV replication. A slight increase of HDV RNA was detected with HBs expression and must be confirmed.

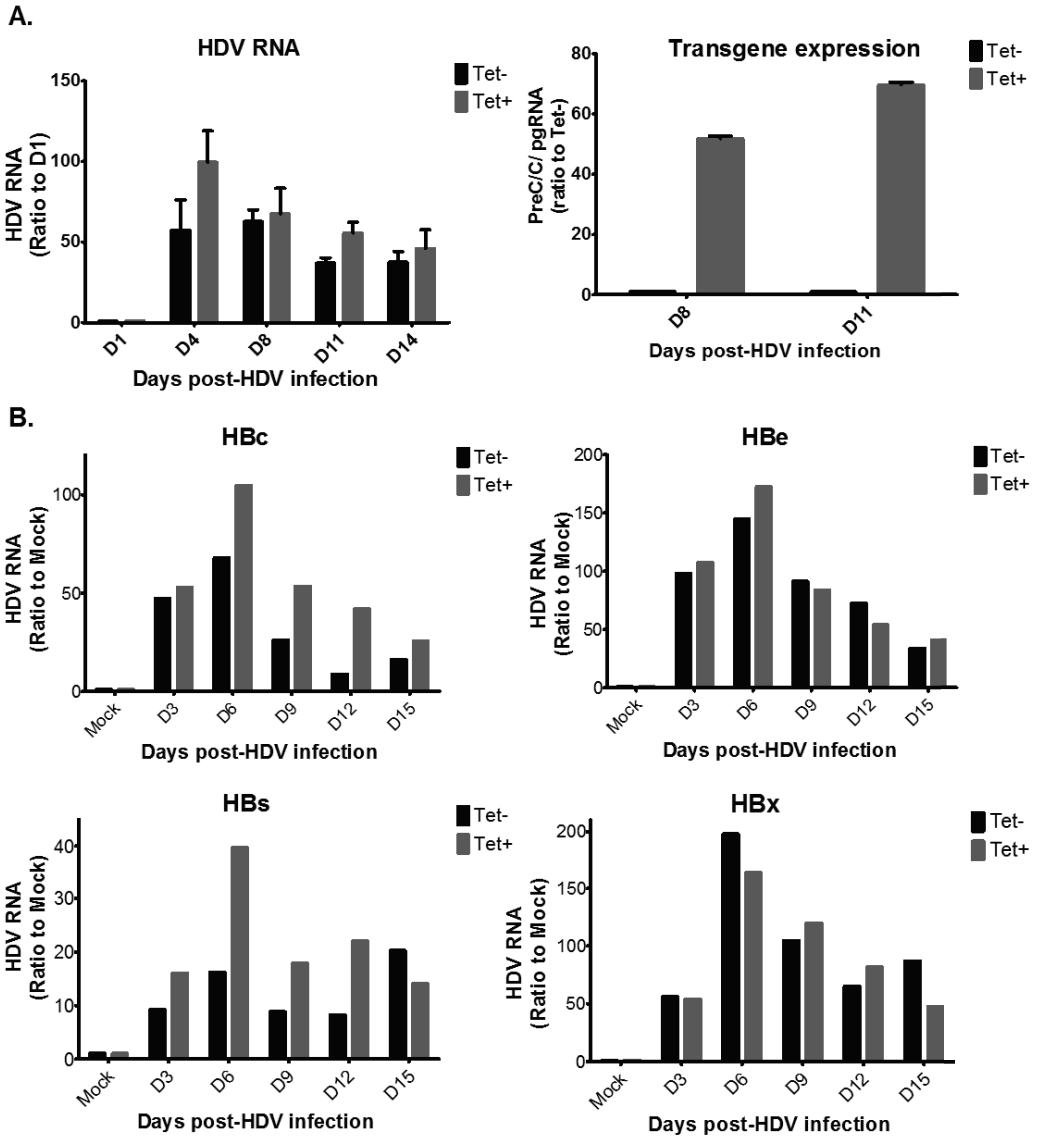

Figure 6. Evaluation of HDV mono-infection in HepaRG cell lines expressing HBV proteins. A.i. Evaluation of HDV replication (estimated by RT-qPCR) in HepaRG TR-HBc cells treated or not with tetracycline. **A.ii.** Assessment of transgene expression in response to tetracycline stimulation, by RT-qPCR using specific PreC/C/ pgRNA primers. **B.** Evaluation of HDV replication in HepaRG TR-HBc, -HBe, -HBs and –HBx.

3.2.4. Discussion

Results obtained regarding HDV monoinfection of HepaRG cells reveal a temporal coincidence between the peak of HDV replication (reflected by maximum amounts of HDV antigenomic RNA) and the greatest levels of ISG induction. This observation raises questions regarding the causal link between the two observations, most importantly, concerning the possible influence of the IFN response on the control of HDV replication. Such an antiviral effect is only partially comforted by clinical data as, while the administration of exogenous IFNα is associated with a decrease in HDV viremia, it occurs in a minority of patients and evidence of a direct effect of IFN on HDV replication is scarce (other mechanisms as entry inhibition have been suggested) (Han et al., 2011b; McNair et al., 1994; Wedemeyer et al., 2011). Furthermore, it has been suggested that HDV directly inhibits type I IFN signalling, which would justify the absence of response observed in most of the patients (Pugnale et al., 2009). Our data are in line with such possibility, as no further increase in ISG expression is observed in HDV infected cells treated with IFN. However, in contradiction to our findings, in the aforementioned work, the authors did not find a basal ISG induction in the absence of IFN stimulation. Hence, whether, in our model, this absence of response is due to an active inhibition of IFN signalling or a mere reflection of a state of response saturation remains to be elucidated.

To verify the hypothesis that, if an IFN response is responsible for the control of HDV replication in HepaRG, the observed decline in RNA amounts should be abrogated or at least attenuated in settings where such response in blocked, we set-up two types of experiments.

We first evaluated the possibility that autocrine or paracrine type I IFN stimulation may be involved in the control of HDV replication, using an anti-IFNAR neutralizing antibody. It has previously been shown that HepaRG cells produce low doses of IFN-β upon PRR agonisation - less than 10 IU/mL with RIGI/MDA5 agonists (Luangsay et al., 2015b). Its neutralization is associated with increased HBV replication in the case of delivery with baculoviral vectors (Lucifora et al., 2010). In HDV infected cells, IFNAR2 neutralization was associated with a decrease, but not a complete suppression, of ISG

expression. Surprisingly, this was correlated with a slight decrease in the maximum levels of HDV RNA and the replication peak was delayed. However, the subsequent decline in replication was similar to that observed in the non-treated cells, thus suggesting that either ISG protein production is still high enough to lead to anti-HDV effect or that a part of the ISG protein production could be independent of auto/ paracrine effect (i.e. independent of the activation of ISGF3). The meaning of such findings is for the moment difficult to explore, as these experiments have yet to be reproduced to become convincing. Moreover, although the decrease in ISG expression constitutes an evidence for IFN type I response inhibition (at least partial), the neutralization conditions need further optimization. It will be essential to verify a dose-response effect of the neutralizing antibody, although the doses used should be neutralizing, given the small quantity of IFN produced by HepaRG cells (even after PRR stimulation). Interestingly, similar results have recently been reported in the hNTCP transgenic mouse model, suggesting that HDV replication control in that model may be independent of the type I IFN response (He et al., 2015).

We next evaluated HDV replication in a cell line with an inducible expression of HBV core protein (HBc). HDV replication was repeatedly found to be increased upon its expression (and not when HBe or HBs were expressed). HBc expression in these cells has previously been shown to lead to a blockage of type I IFN response triggered by PRR agonists or exposition to viruses (e.g. Sendai), an argument in favour of the immune-modulation effect of HBV core protein (Gruffaz et al., 2013). However, the fact that the replicative profile of HDV in this cells is distinct from that observed in association of IFNAR neutralization, raises the question of whether this apparent proviral effect of HBV core protein on HDV replication is indeed mediated through an abrogation of the IFN response or another mechanism.

3.3. Evaluation of HDV-HBV interference

3.3.1. Introduction

In the HepaRG cell model, HDV superinfection of cells persistently infected with HBV was associated with a decrease in HBV replicative parameters.

Previous works have suggested that this interference phenotype could be associated with a direct effect of HDAg on the regulation of HBV enhancers and/or mediated by the induction of MxA expression, as it is a known restriction factor for HBV RNA nuclear export (Williams et al., 2009).

It was our purpose to evaluate both the possibility of a direct effect of HDV proteins in HBV replication repression, namely through epigenetic cccDNA transcriptional regulation and to ascertain the importance of the interferon response induced by HDV in this interference phenotype.

3.3.2. Material and methods

Establishment of HepaRG TA-SHDAg and HepaRG TA-LHDAg cell lines

In order to study the effects of HDV antigens on viral replication, we generated two recombinant cell lines, through lentiviral transduction of HepaRG cells, to allow the tetracycline/doxycycline-inducible expression of S-HDAg and L-HDAg respectively.

S-HDAg and L-HDAg coding sequences (derived from cDNA obtained from a patient infected with HDV genotype 1, studied in the setting of an outbreak of fulminant hepatitis (Dény P, unpublished data) and containing a Strep-tag at their N-terminus were cloned into pEN_Tmcs entry vectors (Addgene) and then, through a LR recombination, inserted into a pSLIK-Hygro vector (Addgene), which expresses the transgene under the control of a tetracycline/doxycycline-activated promoter, and a tetracyclin trans-activator (TA). Lentiviruses were generated upon cotransfection of HEK 293T cells with the pSLIK plasmid and lentiviral packaging vectors following Invitrogen protocols. HepaRG cells were transduced at a multiplicity of infection of at least 1. HepaRG TA-SHDAg and HepaRG TA-LHDAg polyclonal cell lineages were derived upon hygromycin selection. A similar procedure was followed for the generation of a control HepaRG TA-GFP cell line.

HepaRG cell culture and induction of transgene expression

HepaRG cells were cultured as previously described in the Submitted Paper.

Induction of transgene expression was achieved by addition of doxycycline (Sigma) to the cell culture medium in the concentrations and time period specified for each experiment.

Subcellular fractionation

Differentiated HepaRG TA- SHDAg and TA-LHDAg cells were subjected to subcellular fractionation using the "subcellular protein fractionation kit" for cultured cell (Thermo Scientific). After 48 h of induction of transgene expression by addition of doxycycline (80 ng) to the cell culture medium (or not), cells were harvested with trypsin-EDTA and centrifuged at 800g for 8 min at 20°C. For extraction of the cytoplasmic proteins enriched fraction: ice cold cytoplasmic extraction buffer (CEB) containing protease inhibitors (1X) was added to the cell pellet and incubated for 10 min at 4°C with gentle mixing. The cytoplasmic lysate was centrifuged at 500g for 5 min and supernatant enriched with cytoplasmic proteins was transferred to a pre-chilled microfuge tube. The resulting pellet was used for membrane associated proteins extraction by addition of Ice cold membrane extraction buffer (MEB) containing protease inhibitors (1X), incubation at 4°C for 10 min with gentle mixing and centrifugation at 3000g for 5 min. The supernatant enriched with membrane-associated proteins were transferred to a pre-chilled microfuge tube. The ice cold nuclear extraction buffer containing protease inhibitors was added to the pellet obtained lastly, vortexed vigorously and was incubated at 4°C for 30 min with gentle mixing. The nuclear lysate was centrifuged at 5000g for 5 min and the supernatant, enriched with nuclear associated proteins, was transferred to a pre-chilled microfuge tube. Finally, chromatin-associated proteins were recovered by addition of room temperature nuclear extraction buffer containing protease inhibitors, 5 µl of 100 mM CaCl2 and 3 µl of Micrococcal Nuclease to the pellet obtained after the last step. The sample was vortexed vigorously and was incubated at 37°C for 5 min. After incubation, the lysates enriched with protein extracts were vortexed vigorously and centrifuged at 16,000g for 5 min. The supernatant was transferred to a pre-chilled tube.

10 µg of protein of each compartment was subjected to western blot.

Transcomplementation assay

Proliferative HepaRG TA-S-HDAg and TA-L-HDAg cells were transfected with pSVL(D2m), a plasmid that contains a dimer of a mutated HDV-1 cDNA, under the control of an SV40 promoter. This mutant has a two-nucleotide deletion within the delta antigen sequence, which leads to abortive translation of the S−HDAg protein and hence, given the need of S-HDAg for HDV replication, is replication incompetent. As a control, cells were alternatively transfected with pSVLD3 that contains a trimer of wild-type HDV-1 cDNA (also under the control of an SV40 promoter) and is replication competent. Mock transfection was also established. Transfection was made with Mirus LT1 reactive, following the manufacturer's protocol. Transfection medium was removed 24h hours of contact with the cells. The next day (D2 post-transfection), transgene expression was induced by the addition of doxycycline to the culture medium. Cell culture medium was changed every other day, with addition of doxycycline, and cells were lysed for RNA extraction at D8 post-transfection. HDV replication was evaluated by Northern Blot (using a full length genomic probe for detection of anti-genome).

Western blot

Western blot analysis was carried as previously described using as primary antibodies: Anti Strep−Tag® (Qiagen), Anti-Tubuline (Santa Cruz), Anti-Histone H3 (Cell Signalling).

HBV infection, HBeAg and HBsAg ELISA, RNA and DNA extraction qPCR and qRT-PCR HBV, Immunofluorescence, Northern Blot and neutral red assay were carried as previously described.

cccDNA specific chromatin immunoprecipitation

ChIP analysis was based on the protocol described by Testoni et al. (Testoni et al., 2011) with minor modifications. Briefly, cells (2-5X10^6 per condition) were washed in phosphate-buffered saline (PBS) and cross-linked for 10 min with 1% formaldehyde, followed by quenching with Glycine 0.125 M. Cells were then submitted to nuclear lysis and light sonication. Immunoprecipitations were performed with Dynabeads® Protein G (100.03D, Life Technologies) and 5–10 µg of the following antibodies:

Monoclonal mouse antibody anti-Strep-Tag (Qiagen) and Polyclonal rabbit antibody anti-HDAg (kind gift from Alan Kay). After immunoprecipitation, washes and reverse cross-linking, the samples were extracted with phenol/chloroform/isoamyl alcohol and ethanol precipitated in the presence of 3 M sodium acetate and 20 µg of glycogen (Sigma Aldrich). For cccDNA quantification, total DNA was submitted to digestion with plasmid-safe DNAse (Epicentre) for 4hours at 37ºC, followed by 30 min of heat inactivation. Quantification was performed by FRET-based qPCR as previously described (Werle-Lapostolle et al., 2004). Results are expressed as a ratio of the cccDNA copy number of the precipitated samples to the corresponding input.

Neutralization of IFNα/β signalling

Differentiated HepaRG cells were first infected with HBV 100 vge/cell, and at day-6, which corresponds to the plateau of HBV replication with established levels of all viral parameters (including cccDNA), cells were super-infected by HDV with 100 vge/cell. At the end of HDV inoculation, cells were washed and fresh medium was added containing or not anti-human IFNAR2 antibody (PBL Assay Science) at a concentration of 500 ng/ml. The neutralizing antibody was kept until the end of the experiment and HBV replication was monitored at different time points by HBeAg quantification by ELISA and at day-15 by HBV DNA qPCR.

3.3.3. Results

HepaRG TA-SHDAg and TA-LHDAg have a good expression of the transgene upon induction with doxycycline

As seen in **Fig. 7A**, HepaRG TA-SHDAg and TA-LHDAg stimulation with increasing doxycycline doses leads to a stepwise increase in protein expression. L-HDAg expression is detectable from 8h post-stimulation and reaches a plateau from 48 hours on, while S-HDAg expression is present after 24h of doxycycline treatment and does not increase beyond 72 hours (**Fig. 7B**). Immunofluorescence does not show a significant system leak for any of the lineages, as evidenced by the absence of labelled cells without stimulation (**Fig. 7C**).

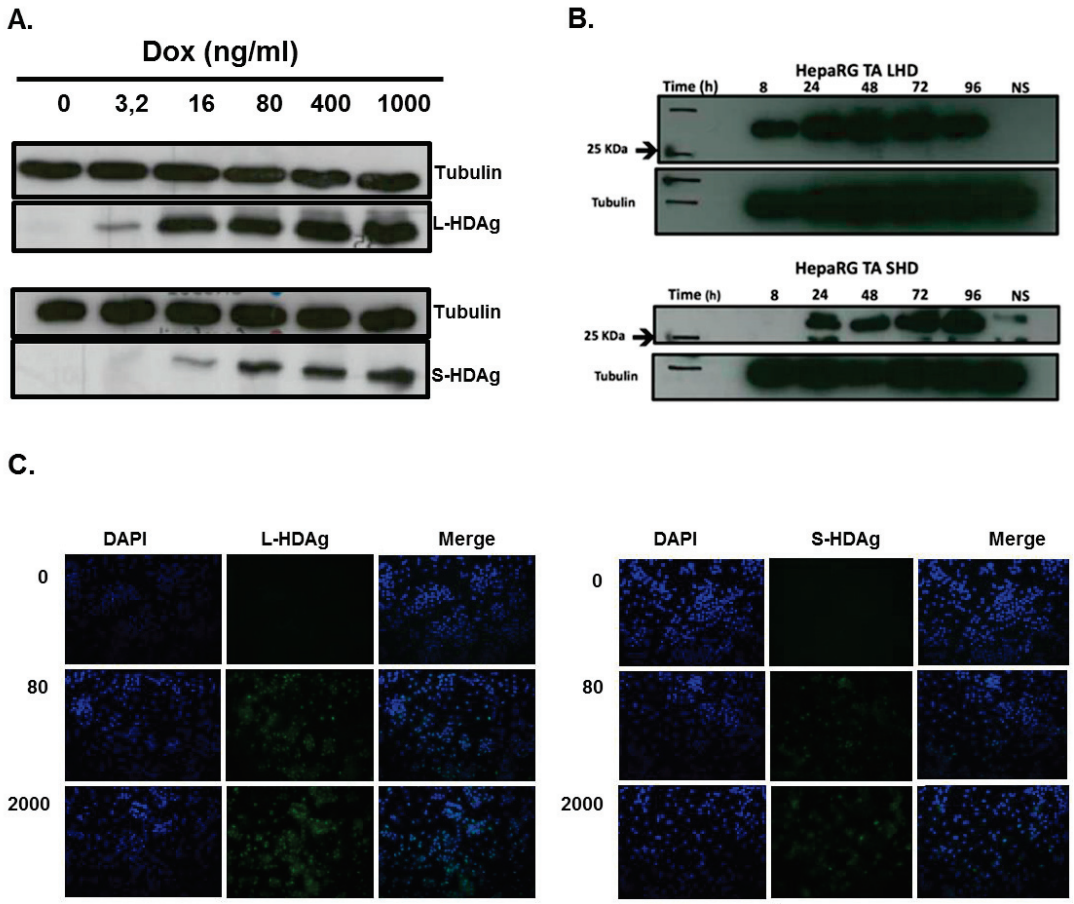

Figure 7. Characterization of HepaRG TA-LHDAg and TA-SHDAg cell lines. Cells were differentiated and treated with rising doxycycline doses for 72 hours (**A**) of with doxycycline 80 ng/ ml for different time periods (**B**). Evaluation was performed by western blot or immunofluorescence labelling (**C**).

As expected SHDAg has a predominant nuclear localization and is competent in trans-complementing a replication defective plasmid, whereas LHDAg is distributed between nucleus and cytoplasm and may be associated with replication inhibition

To ensure that the presence of the N-terminal Strep-Tag sequence had no influence in the physiologic role of the proteins, verification of their subcellular distribution and functionality was warranted for validation of the model.

As first suggested by immunofluorescence and further evaluated by western blot of subcellular protein fractions (**Fig. 8A**), L-HDAg is distributed between all the protein fractions, while S-HDAg has a predominantly nuclear localization and can be found mostly in the chromatin-associated fraction.

For evaluating protein functionality, a trans-complementation assay was set-up based on the following principles: i) transfection of a plasmid containing a trimer of wild type

HDV-1 cDNA (pSVLD3) starts a replicative cycle, with formation of the replication intermediate antigenomic RNA; ii) as S-HDAg has been shown to be essential for HDV replication initiation (Kuo et al., 1989), a plasmid containing a dimer of HDV-1 cDNA, with a point mutation in the HDAg ORF leading to abortive S-HDAg expression, with not be replication competent; iii) this replicative deficit can be overcome by trans expression of S-HDAg. Furthermore, L-HDAg expression may be associated with a decreased replicative activity of pSVLD3, as this form of the protein has been shown to have an inhibitory role during HDV replication (Chao et al., 1990).

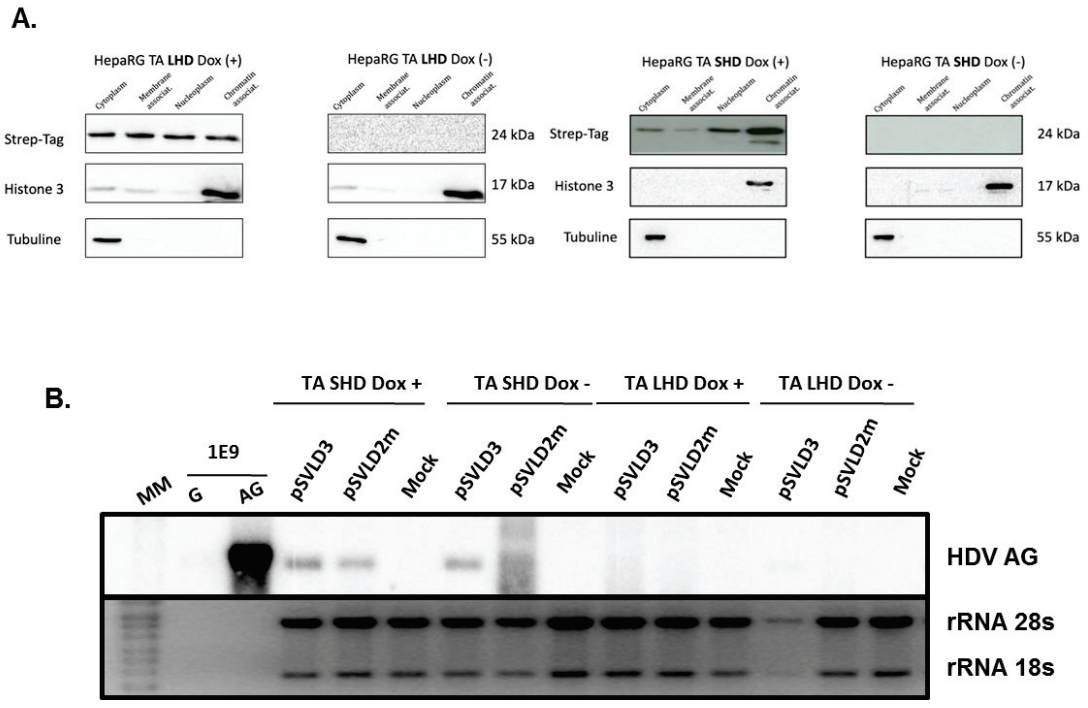

Figure 8. Evaluation of HDAg localization and functionality in HepaRG TA-LHDAg and TA-SHDAg cells. A. Analysis of S-HDAg and L-HDAg by western blot of subcellular protein fractions **B.** Northern blot analysis of intracellular RNA obtained from a transcomplementation assay. *HDV AG*, HDV antigenome; *G*, genome; *rRNA*, ribosomal RNA; *MM*, millennium marker.

We hence evaluated HDV replication through Northern Blot detection of HDV anti-genome, following transfection of both cell lines with either pSVLD3 or pSVLD2m, followed or not by induction of transgene expression. As shown in **Fig. 8B**, when HepaRG TA-SHDAg cells were treated with doxycycline to induce transgene expression, both pSVLD3 and pSVLD2 transfection led to detectable HDV antigenome. However, in the absence of doxycycline, no specific band could be observed following

pSVLD2m transfection, consistent with the absence of HDV replication, while antigenome was still detected for pSVLD3. As expected, no antigenomic RNA was detected in HepaRG TA-LHDAg either in the presence or absence of transgene induction. Interestingly, pSVLD3 transfection led to no detectable anti-genome in the presence of L-HDAg expression, suggesting an inhibition of replication by L-HDAg. However, as only a faint band could be seen in the control condition (non-induced HepaRG TA-LHDAg cells), due to a loading problem, these results must be interpreted with caution.

Transgene expression induces a decrease in HBV infection markers in a dose-dependent manner

Once the functionality of the cell lineages was established, we proceeded to the evaluation of a possible effect of HDAg expression on HBV replication. To this end, both HepaRG TA-LHDAg and TA-SHDAg and the control cell line HepaRG TA-GFP were differentiated in the presence of DMSO, as previously described, and then subjected to HBV infection (100 vge/ cell). In order to replicate the conditions used for HDV superinfection, transgene expression was induced by the addition of doxycycline to the cell culture medium at day-6 post-infection and kept throughout the experiment. Growing concentrations (from 3,2 to 2000 ng) were used to induce increasing levels of protein expression (as already described in **Fig. 7A**). Supernatants were collected for HBeAg and HBsAg quantification by ELISAs, and at endpoint (day-15 post-infection) cell viability was evaluated by neutral red assay and cells were lysed for DNA, RNA and protein extraction (**Fig. 9A**). The results presented correspond to a total three independent experiments.

We first verified if the prolonged doxycycline treatment had had an impact on cell viability, by neutral red assay. As can be seen in **Fig. 9B**, doxycycline treatment induced a moderate decrease in cell viability at the highest doxycycline doses (15% in HepaRG TA-LHDAg cells and 22% in HepaRG TA-SHDAg cells). Dose-dependent transgene expression was confirmed by western blot, as depicted in **Fig. 9C**.

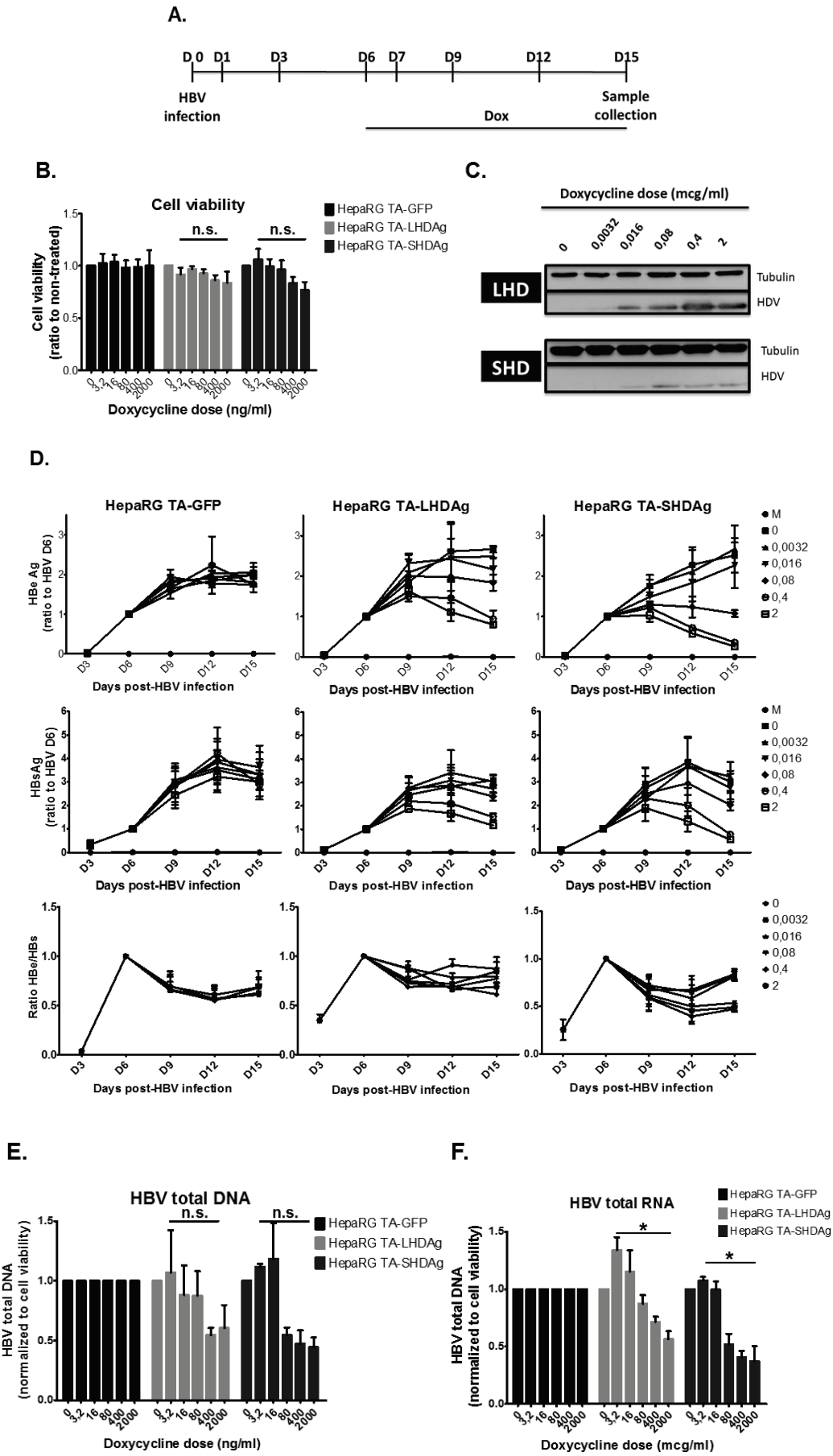
A.
D 0 D1 D3 D6 D7 D9 D12 D15
HBV infection
Dox
Sample collection
B.
Cell viability
n.s.
n.s.
HepaRG TA-GFP
HepaRG TA-LHDAg
HepaRG TA-SHDAg
Cell viability (ratio to non-treated)
Doxycycline dose (ng/ml)
C.
Doxycycline dose (mcg/ml)
0 0,0032 0,016 0,08 0,4 2
LHD
SHD
Tubulin
HDV
D.
HepaRG TA-GFP
HepaRG TA-LHDAg
HepaRG TA-SHDAg
HBe Ag (ratio to HBV D6)
HBsAg (ratio to HBV D6)
Ratio HBe/HBs
Days post-HBV infection
M
0
0,0032
0,016
0,08
0,4
2
E.
HBV total DNA
n.s.
n.s.
HepaRG TA-GFP
HepaRG TA-LHDAg
HepaRG TA-SHDAg
HBV total DNA (normalized to cell viability)
Doxycycline dose (ng/ml)
F.
HBV total RNA
*
*
HepaRG TA-GFP
HepaRG TA-LHDAg
HepaRG TA-SHDAg
HBV total DNA (normalized to cell viability)
Doxycycline dose (mcg/ml)

Figure 9. Evaluation of HBV replication in HepaRG TA-LHDAg and TA-SHDAg cells. A. Cells were infected with HBV for 6 days, before induction of transgene expression with rising doses of doxycycline. **B.** cell viability was evaluated by neutral red assay at the end of the experiment (results are displayed as a ratio to the non-treated condition). **C.** the expression of the transgene was assessed by western blot. **D.** Supernatants collected throughout the experiment were submitted to HBeAg and HBsAg ELISA (results are displayed as a ratio to the non-treated condition). Intracellular nucleic acids were quantified by qPCR for HBV total DNA and RT-qPCR for HBV total RNA. Results are normalized to the percentage of cell viability. *n.s.* non-significant; *p< 0,05.

As seen in **Fig. 9D**, following the addition of doxycycline, HBeAg secretion decreased over time in both HepaRG TA-LHDAg and TA-SHDAg cells, but not in the control HepaRG TA-GFP cells. For HepaRG TA-LHD, significant differences were observed for the two highest doxycycline doses (400 and 2000 ng), when compared to non-induced cells (65 and 70% decrease, respectively; p< 0,05). A significant difference was also documented in S-HDAg cells treated with 2000 ng doxycycline (90% average decrease; p<0,05). A lesser, yet significant, decline was detected in HBsAg secretion when HepaRG TA-LHDAg cells (but not TA-SHDAg or TA-GFP cells) were treated with the highest doxycycline dose (63% decrease in comparison to non-treated cells; p< 0,05). As seen in the lower panels of **Fig. 9D**, greater doses of doxycycline are associated with a more important decline in HBeAg than in HBsAg secretion, both in HepaRG TA-LHD and particularly HepaRG TA-SHD, but not in the control lineage. Such a finding allows the exclusion of a non-specific reduction of antigen secretion and suggests a differential regulation of HBeAg secretion in the presence of HDAg.

HBV replication in these cells was further characterized by dosing intracellular HBV total RNA and DNA (**Fig. 9E and 9F**). Following normalization, to account for differences in cell viability, an important decrease in both parameters was observed in the both lineages expressing HDAg, but not in the control cell line. RNA but not DNA decreases were significant (at the maximal doxycycline dose: HepaRG TA-LHDAg 45% decrease, p< 0,05; HepaRG TA-SHDAg 62% decrease, p< 0,05).

Inhibition of HBV replication may be associated with SHDAg recruitment on cccDNA

We previously documented that SHDAg, and to a lesser extent LHDAg, has a preferential nuclear (chromatin bound) localization and a greater impact on the decrease of HBV replication. Furthermore, in superinfection, HDV has been shown to

preferentially decrease HBeAg and not HBsAg, with no decrease in the amounts of cccDNA. This may suggest an underlying mechanism of differential transcriptional regulation of HBV minichromosome, possibly associated with epigenetic modifications.

We hence first focused on a possible interaction between SHDAg and HBV cccDNA. To this end, differentiated HepaRG TA-SHDAg cells were infected with HBV, and transgene expression was induced at day-6 post-infection as previously described. Based on the previously described kinetics of transgene expression in this system (with SHDAg being detected as soon as after 8 hours of doxycycline stimulation), doxyclycline treatment was performed for 24 hours (as shown in **Fig. 10A**). At that time point, cells were cross-linked and then subjected to ChIP analysis. Mock-infected and non-induced cells were used as controls. We performed, for the moment, two independent experiments. In the first experiment, chromatin present in the nuclear fraction was immune-precipitated with a monoclonal anti-Strep-Tag antibody. As seen in **Fig. 10B** a 20 fold increase in cccDNA precipitation with S-HDAg was observed in the doxycycline induced condition in comparison to the non-induced. A second experiment, using the same settings, was performed in order to validate the results using both strep-tag and HDAg immunoprecipitation (with a polyclonal anti-HDAg antibody). Results displayed in **Fig. 10C** confirm the cccDNA enrichment in the induced condition, upon anti-Strep-Tag immunoprecipitation, however with a lower magnitude than the verified in the previous experiment (3,2 fold change). Furthermore, the same tendency was confirmed with HDAg precipitation (2,6 fold change).

Blockage of IFNα/β signalling is associated with partial reversion of the HDV/HBV interference phenotype in HepaRG cells

As shown in **Fig. 11A**, HBV infected HepaRG cells were super-infected by HDV and subsequently treated with anti-IFNAR2 antibody or not. Results displayed correspond to the average of 3 biological replicates from a single experiment. Viral interference was once more translated by a decrease in HBeAg but not HBsAg secretion that could be partially reversed by the addition of anti-IFNAR antibody (**Fig.11B and 11C**),

although the observed differences are small, probably as a consequence of a low level HBV infection in this experiment.

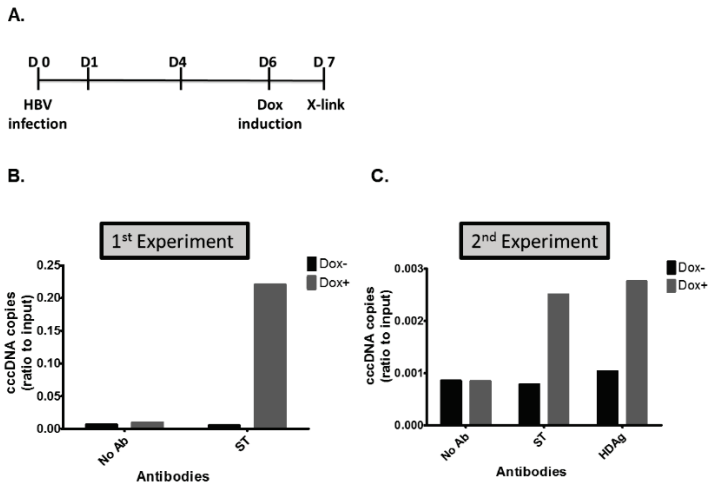

Figure 10. CccDNA specific chromatin immunoprecipitation. A. Cells were infected with HBV for 6 days, before doxycycline induction of transgene expression. Cross-link and ChIP analysis were performed after 24 hours of induction. Panels (**B**) and (**C**) represent two independent experiments. CccDNA is represented as a ration between the number of copies precipitated and that of the input. *No Ab*, no antibody (negative control); *ST*, anti-Strep-Tag antibody; *HDAg*, anti-delta antigen antibody.

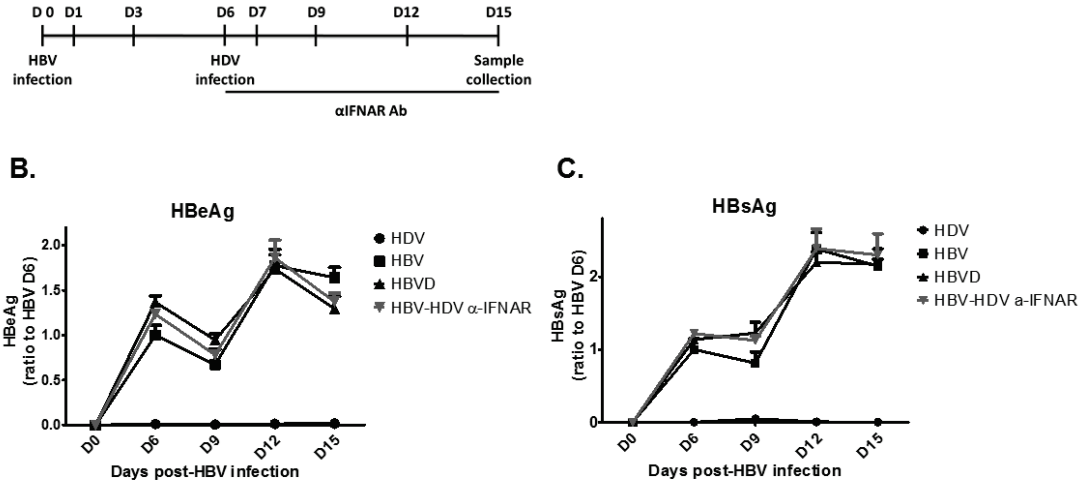

Figure 11. Evaluation of the effect of IFNa/b signalling blockage on HDV/HBV superinfection. A. HDV superinfection of HBV infected cells was established as described above. At the end of HDV inoculation, anti-IFNAR2 neutralizing antibody was added to the cell culture medium and kept until the end-point at day 15 post-HBV infection. HBeAg (**B**) and HBsAg (**C**) secretion in the supernatant were followed by ELISA. The displayed results are an average of three replicates of one single experiment.

3.3.4. Discussion

Having confirmed a previously described phenomenon of HDV/HBV interference in HepaRG cells (and later in PHH), we focused on the assessment of two different, yet not mutually exclusive, possible mechanisms to explain this phenotype. First, we evaluated the hypothesis of a down-regulation of HBV cccDNA transcriptional activity directly mediated by HDAg. In parallel, we were interested in appraising a possible involvement of IFN type I signalling in the restriction of HBV replication.

In order to approach the first possibility, we first constructed HepaRG cell lines, with an inducible expression of either form of HDAg. The strategy used involved insertion of an N-terminal Strep-Tag, in order to facilitate protein detection and eventual precipitation. To ensure that tagged proteins were biologically and functionally relevant, we proceeded to extensive characterization of each cell line. Both proteins were found to localize intracellularly as previously described (Bichko and Taylor, 1996; Han et al., 2009), with an important enrichment of S-HDAg in the chromatin associated fraction. Moreover, strep-tagged S-HDAg was capable of restoring replication of an HDAg defective plasmid, confirming its functionality (Kuo et al., 1989). For L-HDAg, that assertion was less clear as it is more difficult to verify its functionality in the regulation of HDV replication. That could eventually be achieved by demonstrating its capability to permit HDV assembly and secretion. Such an experimental setting is not straightforward as it would involve the need to have in the same cell (in this case the HepaRG TA-LHDAg lineage) an HDV replication-competent construction, with an abrogated LHD expression (that could be achieved by a mutation on the amber/w editing site), and a replicating HBV genome (or a construction expressing its surface proteins). Detection of HDV genome in the supernatant upon transgene expression (and not in its absence) would confirm functionality of strep-tagged L-HDAg in the interaction with HBsAg and consequent assembly and release of HDV virion. We were however able to have an indirect indication of L-HDAg functionality, as HDV replication was not detected when L-HDAg expression was induced, implying the conservation of its described dominant-negative role in HDV replication (Chao et al., 1990).

An inhibitory effect of both forms of HDAg on HBV replication was described early in the characterization of HDV infection (Wu et al., 1991) and was later suggested to be

related to a direct, yet partial, inhibition of HBV replication, through interaction with its enhancer sequences (Williams et al., 2009). In our cell lines, we confirmed that both forms of HDAg were associated with a decrease in HBV replication, as evidenced by diminished HBeAg (and to a lesser extend HBsAg) secretion, HBV total intracellular DNA and RNA. A limitation can be pointed out in this study, as, with time, doxycycline treatment was associated with a detectable (yet not significant) decline in cell viability, potentially underestimating the number of HBV infected cells. However, the verified inhibition was more important than the decrease in cell viability, excluding it as the underlying cause. Moreover, loss of cell viability would have led to a comparable decline of both HBV secreted antigens, which was not the case. The same argument can be used to exclude a decrease in the amounts cccDNA (that does not occur in the superinfection setting in HepaRG cells). Overall these findings may suggest a differential regulation of HBV transcriptional activity.

Regulation of HBV replication can be achieved through epigenetic control of cccDNA transcription, as described in response to interferon treatment (Belloni et al., 2012). Furthermore, viral proteins such and HBx (and HBc) have been immune-precipitated with cccDNA and shown to play a role in the recruitment of transcription factors and histone modifying enzymes (Belloni et al., 2009; Bock et al., 2001). S-HDAg, as a nuclear protein, preferentially chromatin associated, is suggested to be a transactivator of cellular genes. Moreover, although no DNA binding properties have clearly been demonstrated, its RNA binding domain is a homologous of the HMG box of SRY (a DNA binding domain) (Veretnik and Gribskov, 1999). Furthermore, S-HDAg has been suggested to be involved in the epigenetic modification of clusterin, a cellular gene, with a role in carcinogenesis (Liao et al., 2009). We hence hypothesized that, like HBx and HBc, S-HDAg could directly o indirectly interact with cccDNA and eventually regulate its transcriptional activity. Two independent cccDNA specific chromatin immunoprecipitation experiments confirmed, yet with distinct magnitudes, co-precipitation of cccDNA with S-HDAg, indicating a possible interaction of the HDV protein with HBV minichromosome. The specificity of the cccDNA ChIP technique is warranted by: i) a nuclear extraction procedure, to eliminate the major pool of rcDNA, located in the cytoplasm; ii) a mild sonication step, optimized to keep cccDNA integrity, while promoting chromosomal DNA fractioning; iii) a plasmid safe

digestion of the obtained DNA; iv) a specific PCR (Werle-Lapostolle et al., 2004), based on FRET technlogie, with primers and probes desiged for hybridation in the 'gap' of the positive strand of rcDNA. PCR specificity was further validated by the lack of amplification of DNA extracted from a high titer viral inoculum.

These ChIP results, although being suggestive of an interaction between S-HDAg and cccDNA, do not allow for the moment to affirm its involvement in epigenetic regulation. Further validation is needed, as well as evaluation of possibly associated epigenetic modifications (namely histone alterations and recruitement modifying enzymes).

A possible dependence of the interference phenotype on type I IFN signalling, cannot not be excluded from the analysis of a preliminary experiment with the use of an anti-IFNAR antibody (**Fig. 11**). Although the results are difficult to interpret due to a weak HBV infection in this single experiment, the decline in HBeAg secretion upon HDV superinfection is partially reversed by the blockage of type I IFN signalling, supporting the need for confirmation and further exploration of such hypothesis.

4. Discussion and perspectives

In the present study we characterized virological and immunological aspects of HDV infection using *in vitro* models, most particularly HepaRG cells. We first focused on the optimization of the infection conditions and the characterization of HDV infection parameters and later on the interactions of HDV with its helper HBV and the hepatocyte innate immune response. In this cellular model, we were able to demonstrate, or confirm, that:

i. HDV mono-infection is associated with a strong, yet transient replication in a limited number of cells and is associated with a potent induction of the expression of interferon stimulated genes;
ii. IFN-α treatment of HDV-infected cells does not induce a further increase of ISG expression and has a modest antiviral activity. Conversely, some PRR agonists, in particular those inducing the NF-κB pathway, induce a strong decline in HDV replication;
iii. In a setting of superinfection, despite the low number of coinfected cells, HDV as well as its encoded proteins/antigens exert a repressive effect on HBV replication.

A number of new questions arises from the identification and characterization of these phenotypes.

Characteristics of the experimental model

HepaRG cells have been demonstrated to be a useful model for studying either HBV or HDV infection. The functional characterization of these cells has shown physiological properties close to primary human hepatocytes. Notably, i) they display a physiologic metabolism, with relevant detoxifying properties (Gómez-Icazbalceta et al., 2013); ii) they have the ability to produce VLDL, a characteristic of differentiated hepatocyte, not present in hepatoma cell lines (e.g. Huh7 and HepG2 cells) (Jammart et al., 2013); iii) they express functional innate immune sensors and display functional interferon responses (Luangsay et al., 2015b; Maire et al., 2008; Parent et al., 2004).

Since their identification, they have been an invaluable tool for the study of HBV infection (Gripon et al., 2002; Hantz et al., 2009) and constituted, until recently, the only alternative to PHH for the study of a full HBV life cycle, including NTCP-mediated entry (Ni et al., 2014), and the biology of cccDNA synthesis and degradation (Lucifora et al., 2014).

Their utilization for the study of HDV infection has mostly been limited to the characterization of viral entry steps, as HDV is an easier to manage surrogate model for the study of HBV envelope functions (Sureau, 2010; Urban et al., 2014).

For the interpretation of results obtained in these cells, two main limitations are to keep in in mind: i) likely due to differences in polarization, the pool of cells permissive to HBV or HDV infection is limited (Schulze et al., 2012); and ii) despite the production of infectious particles, no propagation is observed, *i.e.* only one cycle of infection occurs (Hantz et al., 2009).

The aforementioned characteristics of this cell model may partially explain our findings, namely the reduced number of infected cells and the saturation profile despite the increase in the multiplicity of infection. Moreover, the fact that the pool of susceptible, correctly polarized, cells is rather limited, may also explain that, upon HDV superinfection, the proportion of coinfected cells is higher that the number predicted by probability calculation, being associated with significant levels of HDV particle secretion.

Features of HDV monoinfection

The particular kinetic profile of HDV infection in HepaRG cells has to be interpreted based not only on the characteristics of the model, but also on data collected from other models. The exploitation of our results can be useful for a more comprehensive understanding of the kinetics of HDV replication at a tissue level. It is important to acknowledge that HepaRG cells are not the only model where HDV replication is limited in time. Indeed, both our results and previous reports from Gudima and colleagues seem to suggest that a limitation of HDV replication occurs as well in PHH (Gudima et al., 2007). Furthermore, such a profile has been described *in vivo*, first following hydrodynamic injection of HDV cDNA into wild type mice and recently following HDV infection of transgenic mice expressing hNTCP (Chang et al., 2001; He

et al., 2015). All these models have in common the establishment of an HDV mono-infection in the absence of an HBV-mediated propagation or an external source of transcription (as is the case in cell transfection or transgenic mice expressing HDV cDNA). Full data on the kinetics of mono-infection in the uPA SCID model with humanized liver are missing, as no evaluation of the early intracellular levels of HDV RNA has been reported; therefore, a similar profile cannot be excluded (Giersch et al., 2014). HDV infection in the presence of its helper virus, to our knowledge, has only been studied *in vivo*. In such models, including chimpanzee, woodchuck and the uPA SCID mouse with humanized liver, no decrease in replication overtime has been described. Overall, these data may suggest that HDV infection would only be maintained overtime in the presence of its helper virus. Two main hypotheses arise concerning the contribution of HBV: i) either the assembly and exit of viral particles is crucial to the maintenance of HDV replication at a single cell level; or ii) at the tissue level, preservation of a stable HDV infection is dependent on viral propagation and infection of new cells, more than on persistently infected cells.

The first hypothesis can be physiologically sustained by the dominant negative role of the L-HDAg during HDV replication (Modahl and Lai, 2000). Indeed, it is tempting to postulate that, in the absence of viral particle egress, persistent editing leads to an accumulation of L-HDAg throughout time, ultimately leading to replication inhibition. This hypothesis can however be disputed based on our data, considering that, in HDV monoinfection of HepaRG we have shown that S-HDAg/ L-HDAg ratio remains rather consistent throughout the kinetics of infection, with no evidence of L-HDAg over-accumulation. Moreover, we observe a similar HDV RNA decline in the superinfection setting, in which HDV secretion is made possible.

The second hypothesis would imply that HDV infection is maintained in time, not by stable replication at a single cell level, but by viral spreading and infection of new cells. It would explain the different phenotype observed between experimental models, but not experimental data from the Lanford's group, showing a persistent replication of HDV in PHH for several weeks. Indeed, based on this hypothesis, it is hard to explain why HDV mono-infection is effectively kept for a long time, in the absence of HBV, suggesting that no or very slow decline in replication is being induced at the single-cell level (Barrera et al., 2004). The validity of such a hypothesis will ultimately be verified

in the clinical evaluation of entry inhibitors. If propagation is indeed necessary for the maintenance of a stable viral infection, Myrcludex B® treatment might be able to lead to an effective inhibition of HDV infection over time. In this respect preliminary results of a phase IIb clinical trial seem to confirm this hypothesis (Bogomolov et al., 2014).

The mechanism underlying the control of HDV replication in infected cells is still not clear. As a general principle, it could be due either to a lack of a factor needed for replication (as is the case in avian cells) or due to the active inhibition of replication. As previously mentioned, our data do not support the possibility that HDV replication decrease is due to an imbalance of the ratio S-HDAg/L-HDAg, not only by the reasons evoked above, but also because HDV replication starts decreasing while the amount of S-HDAg is still stable, excluding S-HDAg depletion as the cause of such decline. Moreover, unlike HBV, HDV replication is not liver specific, ruling out the role of possible hepatocyte dedifferentiation over time in the limitation of replication. It is not possible, from the available data to exclude a possible role of the depletion of a yet unknown factor in the decline of HDV replication.

Interaction with the innate immune response and its use for the identification of novel therapeutic strategies

The fact that the maximal HDV replicative level is coincident with the peak of ISG expression raises the possibility of a role of an IFN response in the control of HDV replication. However, our data show that neutralization of type-I IFN signalling, in this background, does not prevent the decline of HDV replication indicating that other mechanism(s) may be involved. Further possibilities will have to be evaluated, namely the hypothesis that replication control may be associated with direct activation of intracellular effectors, independently of IFNAR signalling, i.e. bypassing ISGF3 activation. A possible involvement of type-II and III IFN responses in the control of HDV replication must also be ascertained.

Another question that requires further exploration concerns the identification of the HDV PAMP sensed by the infected cell. We have shown that for the first 72 hours following infection no induction of ISGs could be observed. The expression of these

genes arises simultaneously with the peak of antigenome production, supporting the possibility that the viral inoculum induces no antiviral response, which seems to be elicited by neo-synthesis of viral components during replication. As is the case for most viruses, it is tempting to consider viral RNAs to be responsible for the induction of the observed innate immune response. Indeed, in spite of being single stranded, HDV RNA presents a high degree of base pairing and could hence be sensed as a pseudo-double stranded RNA (namely by TLR3 or the cytoplasmic sensors RIG-I or MDA5). However, this hypothesis is not clear-cut, as the current knowledge of HDV infection suggests that there is no free RNA in the cytoplasm or in the endosomes of infected cells in any phase of the viral cycle. Indeed, the replication is exclusively nuclear and RNA shuttling between the nucleus and the cytoplasm is mediated by the formation of the HDV RNP. Although far less likely, it will be important to exclude the role of DNA-sensing PRR (such as IFI16, which has nuclear localization). On the other hand, the potential role of HDAg in the eliciting of the innate interferon response or directly modulating ISG expression cannot be excluded from our results, as no temporal distinction was observed between maximum RNA accumulation and protein expression. A previous study has indeed suggested that the MxA promoter could be activated by L-HDAg (although no direct interaction was evidenced) (Williams et al., 2009)

Interestingly, in our conditions, HDV infection is rather resistant to IFN treatment, in line with *in vivo* observations, as previously discussed in point 3.2.4. This resistance correlated with a failure of IFN-α to induce ISGs beyond the levels already induced by HDV replication itself. A parallel can be drawn here with HCV infection, where a basal up-regulation of ISG expression was found to be related, not only with particular polymorphisms of the IL28b gene, but most importantly, with a worsened response to IFN-α-based treatments (Abe et al., 2011; Honda et al., 2010). Very interestingly, a recent work in our laboratory has shown that, in HCV infected patients, the antiviral contribution of ribavirin is associated with a reversion of the up-regulation of a set of ISGs through epigenetic regulation of their expression (Testoni et al., 2015). It would hence be tempting to propose a possible role for the addition of ribavirin to IFN in the therapy of CHD. Unfortunately, such a strategy has previously been tested in a clinical

trials and was not associated with an improved outcome as compared to mono-treatment with IFN-α (Gunsar et al., 2005; Niro et al., 2006). In order to elucidate the clinical relevance of our findings, it will be essential to perform a thorough evaluation of the baseline hepatic gene expression profile of HDV infected patients (with a particular focus on innate immunity related genes), and to eventually establish a correlation between patterns of expression and outcomes of IFN therapy. Such an evaluation will only be possible through a multicentre collaborative effort, given the restricted number of patient samples available.

The molecular mechanisms of interaction between the virus and IFN signalling pathways also need further clarification. It has been suggested that HDV could inhibit a crucial step in the Jak/STAT signalling pathway and hence the response to exogenous IFN (Pugnale et al., 2009). Furthermore, the pattern of ISGs it induces does not seem to be associated with an antiviral effect and may actually favour viral replication (as suggested by the finding of a reduced replication peak following IFNAR neutralization). Such a mechanism of viral adaptation and even hijacking of host-antiviral effectors to its own profit, has previously been described in the virologic world. An example is the induction of expression of viperin (coincidentally, the most up-regulated ISG in our study) by cytomegalovirus and the diversion of its mechanism of action in favour of viral infectivity (Seo et al., 2011). The existence of such mechanisms in the case of HDV infection needs further characterization, as, by adding to the current understanding of IFN-resistance, it may allow the design of complementary therapeutic strategies involving IFN sensitizing.

In contrast to IFN, we observed a strong antiviral activity of poly (I:C) (i.e. TLR3-L) and poly (dA:dT) (associated with AIM2-L and indirectly RIGI/MDA5-L stimulation) to HDV monoinfected cells. This antiviral response, being stronger than the one elicited by IFNα itself, raises interesting questions regarding the possibility of further stimulating endogenous IFN production. Clarification of the role of ISGs and autocrine/ paracrine IFNα /IFNβ signalling in this antiviral response would be a first step towards the understanding of its mechanism.

Nonetheless, the strongest antiviral effect was obtained with the stimulation of TLR-1/2 by Pam3CSK4 (an analog of the acylated amino-terminus of bacterial lipopeptides), which is associated with the activation of the NF-κB pathway and secretion of pro-inflammatory cytokines, such as IL-6. This is an interesting finding as this pathway does not seem to be modulated by the virus itself. Moreover, two recent contributions highlight the role of the inflammatory response in the control of HBV infection through the strong antiviral effect of both IL-1β and a lymphotoxin-receptor-β agonist (Isorce et al., 2014; Lucifora et al., 2014; Watashi et al., 2013). A screening of the antiviral effect of pro-inflammatory cytokines is hence warranted to further explore the potential role of NF-κB signalling in the control of HDV replication.

A strong antiviral effect was also documented for imiquimod, opening a new set of interesting questions with respect to a mechanism of action possibly independent of TLR7 signalling. This is particularly exciting, as analogous drugs, such as GS9620, are entering clinical evaluation with very promising results in the control of HBV infection (Menne et al., 2015). The mechanisms of its potential activity against HDV must be evaluated. Validation of the imiquimod antiviral effect by the use of the analogous molecule GS-9620 could represent a major contribution to a therapeutic field with rather limited efficient options.

HDV/ HBV interaction

Besides providing new data on aspects of HDV infection and therapy, by confirming and exploring its interactions with the helper virus, our results open new perspectives on the regulation of HBV replication itself. This interference phenomenon has previously been well characterized in liver samples from CHD patients (Pollicino et al., 2011) and the pattern of expression of HBV replication intermediates found is compatible with a modulation of HBV transcriptional activity in the presence of HDV. Other model systems have been used to reproduce the interference phenomenon, both *in vivo* as *in vitro*. To the best of our knowledge, our work represents the first characterization of an HDV/HBV interplay in a cellular model of infection. As discussed in section 3.3.4, we focused on the evaluation of a possible direct mechanism of interference involving a role of S-HDAg in the epigenetic regulation of cccDNA

transcriptional activity. We have so far been able to demonstrate a co-precipitation of cccDNA with S-HDAg, suggesting a possible fixation of this protein on HBV minichromosome. However, up until now, it was not possible to identify the possible epigenetic changes associated with such an interaction and to correlate them with the observed phenotype. It would be expected for HDV infection to be associated with a decrease of activation marks (as histone 3 acetylation), an enhancement of repressive marks and possibly a recruitment of histone modifying enzymes on the cccDNA (as HDAC). Furthermore, it is not possible to exclude the indirect regulation of cccDNA by IFN-regulated transcription factors (Belloni et al., 2012). The characterization of such epigenetic modifications has been hampered in our model by the the low amounts of cccDNA, near to the lower limits of detection of the ChIP procedure. This limitation can be circumvented by the use of cell models allowing a more significant accumulation of cccDNA. Indeed, an *in vitro* study based on transfection of HepG2 cells, whose preliminary results were reported by Raffa et al, was able to the evidence a spectrum of cccDNA epigenetic modifications that is compatible with the hypothesis of an inhibitory effect of delta proteins, but has not been confirmed in an infectious model (Raffa et al., 2011). HepG2-hNTCP cells may constitute a valuable option to perform further ChIP assays, as the amounts of cccDNA accumulated during HBV infection are significantly increased in comparison to HepaRG. Furthermore, contrary to PHH (that also accumulate significant cccDNA amounts), they can be transduced and selected in order to achieve an inducible expression of HDV proteins. Alternatively, in the eventuality of persistent technical limitations related to the ChIP procedure and cccDNA detection, a proof of concept would consist in the evaluation of the effect of drugs acting on histone modifying enzymes and study its effect on the reversion of the interference phenotype. The definite proof of the validity of this mechanism would have invariably to be obtained *in vivo*, by ChIP analysis of liver samples from CHD patients.

In summary, by having adapted and explored an existing model for the study of HDV infection and having identified particular phenotypes associated with HDV replication, the work here discussed opens an array of new perspectives to a better characterization of the pathogenesis of hepatitis delta.

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Acknowledgements

Although a doctoral thesis is, in the end, an individual process, along the almost 4 years of work, many were the persons and institutions that participated in or, in any way, facilitated this work.

I would like to start by thanking Professor **Francesco Negro** and Doctor **Mirjam Zeisel** for their availability to review this work and for helpful comments and suggestions on the manuscript. I thank Doctor **Camille Sureau**, for having followed my work throughout the last 4 years, for receiving me in his laboratory and above all for his availability and generosity in sharing his vast knowledge on HDV. I'm grateful to Professor **Christian Trépo** and Doctor **François-Loic Cosset** for accepting the invitation to participate in the thesis defence and to Doctors **Ana Salvetti** and **Patrice André** for their helpful participations in my thesis evaluation comitees.

I'm particularly grateful to **Fundação para a Ciência e para a Tecnologia** for funding my PhD work and to **Fundação Calouste Gulbenkian**, notably to Professors **António Coutinho**, **Leonor Parreira** and **João Ferreira**, for the opportunity (the privilege!) and stimulus to embrace this challenge. No matter of where the future may take me, PFMA opened the door.

I'm very grateful to Doctor **Caroline Scholtes** for her contribution for the optimization of the HDV qRT-PCR.

I'd like to acknowledge the help of **all the people in the laboratory, without exception,** with whom I had the chance to discuss, to ask for advice, exchange ideas, protocols, worries, problems, complaints and smiles. I'm particularly grateful to **Fabien**, for accepting me in the laboratory and for the very helpful discussions throughout the years. To **Paul**, for sharing his knowledge and tools on hepatitis delta and for the challenges along the way. To **David** for accepting and supporting my project proposal from day one and to be willing to continue after the thesis defence.

Thank you for the great scientific discussions, the motivation, the enthusiasm, the constant flow of ideas, the kindness and strength that kept me going on the hard days. It was an honor to have you as a mentor. To **Christian** who first showed me the ways of the lab. To **Romain** for the great talks on science and life. To **Olivier** for his generosity in sharing his great knowledge on HBV. To **Isabelle** for welcoming me into the team and **Claude** for always being available to dicuss. To **Jean Claude** for the long talks on delta, travels, biochemestry and food. To **Judith** for having adopted me and never being too far and **Maud** for all her help, her good mood and her example of efficiency. To **Barbara**, **Marion** and **Souphalone** for all the discussions, ideas and guidance and above all for making me feel a part of the team and allowing me to proudly become an LMNC girl. To **Mariama** for patiently introducing me to the delta universe and, by showing me her vision of the world, becoming a good friend and an example.To **Julie** for her ideas, comments, help and enthusiasm and the will to continue this work. To **Maelle**, **Suzanne** and **Océane**, for being just amazing and for, together with **Patricia**, **Laetitia** and **Nadim** ensuring the continuity of the good vibes in the "annexe". To all the persons to whose formation I tried to contribute along the way: **Yannick**, **Antoine**, **Ana**, **Ioana**, **Charlotte**, **Chloe**, **Emma** and **Aurélie**. Speacially to **Natali** for her trust, patience and help. To all the past and current members of the HBV Innate Immunity team, **Malika**, **Noemie**, **Valentina**, **Sarah**, **Marc**, **Fanny**, **Pascal**, **Adrien**, and **Ahmed**. To **Boyan** for the very nice discussions in the lab and his companionship in London. To **Sumy** for his great humour and **Marilou** and **Aurélie** for their support.

To **Nathalie** for being side by side on the trenches, through sunny and rainy days. For sharing stress, time, plans, travels, thoughts, food, tea, beer (and gin!), chocolate, music, activities, motivation, her house, her friends, her family, her roomates. Because a hug a day keeps all the problems away! And only true friends know how to do it.

To **Clément**, **Pierre**, **Clémence** and **Nora** (and **Nathalie** again…) for teaching so many nice French expressions and such kind and fraternal jokes on the Portuguese. The dream team will last wherever we are! To all the previous students in the lab, **Chachou**, **Anaïs**, **Mae**, **Thomas**, for the good times we shared.

A particular thank you to **Marie Agnès** for always being there, ready to listen, and for the great hints on outdoors activities, **Isabelle** and **Françoise** for discretely keeping the laborarory working and **Lydie** for all her work with PHH isolation.

But these almost four years have been about so much more than science! It has been about personal challenge, evolution and discovery. I'm grateful to all the people that, beyond science, allowed me to discover the world in Lyon. To **Jean Pierre**, that, in spite of his unorthodox methods, made me leave the confort bubble and did nothing less than change my vision of life. To **Qian** for all the storms we went through together. To **Natalia** for being my Brazilian sister and making me "um tiquim mineira". To **Daphna**, **Catha**, **Ana**, **Belen**, **Maria Jose**, **Paula**, **Lucia** and **Mireia**, because it's great to be a Lulu. To **Nadège** for all the great scientific and non-scientific moments.

Finally, because we can only discover the world, when we know where home is:

To Doctor **Kamal Mansinho**, Doctor **Fernando Borges** for helping me to fly but still allow me to come back to the confrort of the nest. To **Ana Cláudia**, **Susana**, **Cláudia** and **Diana**, for showing me how good it feels to work with friends.

To **Pedro**, **Lili**, **Tiago** and **Ana** for being my family away from home.

To **Cris**, **Angie**, **Binha**, **Sónia**, **Diogo**, **Daniel** and **Bruno**, for being there throughout the years, no matter the distance and always reminding me of where home is.

To **Sara** and **Paula** for almost making me forget that I'm not there.

To **Patrícia**, for being a friend and a true inspiration with the brightness, the motivation, the *joie de vivre* and the strength that push me to try to always be better.

To **Rita**, because it was also about hot chocolate, smiles, hugs and silent words… Because girls, we will always love GFP!

To **Lúcia** and **Inácio**, porque nunca nada teria sido possível sem o vosso interminável amor e apoio. To **Maria Amélia** and **Joca** (because I know you're always there), and the all the family for all the support.

Appendices

1. Review (published version)

Antiviral Research 122 (2015) 112–129

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Review

Hepatitis delta virus: From biological and medical aspects to current and investigational therapeutic options

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ARTICLE INFO

Article history:
Received 11 June 2015
Revised 10 August 2015
Accepted 11 August 2015
Available online 11 August 2015

Keywords:
Hepatitis D virus
HBV/HDV co-infections
Pathogenesis
Viral interference
Interferon alpha

ABSTRACT

An estimated 15–20 million individuals are co-infected by hepatitis B and hepatitis D virus worldwide and are at high risk of developing end-stage liver disease, including hepatocellular carcinoma. While HBV viremia can now be controlled in the vast majority of individuals by nucleoside analogs, leading to a delay of disease progression, HDV treatment has for long relied on the relatively inefficient and not well-tolerated interferon-alpha. While the epidemiology and pathogenesis of the disease remain to be precisely determined, using adequate diagnostic tools and well-designed cohort studies, basic research efforts have led to interesting progress in the understanding of HDV biology, which is not yet sufficient to identify specific antiviral targets. More resources now need to be devoted to the HDV field to achieve therapeutic breakthroughs. In this manuscript, we carefully review the literature regarding the biology of hepatitis D virus, the disease, its prevention, current treatments and investigational strategies. This article forms part of a symposium in Antiviral Research on "An unfinished story: from the discovery of the Australia antigen to the development of new curative therapies for chronic hepatitis B."

Contents

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http://dx.doi.org/10.1016/j.antiviral.2015.08.009
0166-3542/

1. Introduction

1.1. Discovery of hepatitis D virus

Hepatitis D virus (HDV), the virus causing hepatitis delta, was first identified in 1977 by Mario Rizzetto and colleagues, in a cohort of Hepatitis B virus (HBV) infected patients who experienced a severe hepatitis (Rizzetto et al., 1977). Some of these patients' liver biopsies were found positive after staining by antisera from some HBsAg positive patients, in the absence of HBV core protein (HBcAg), as evidenced by electron microscopy and negative staining with monoclonal specific antibodies. This novel 'antigen–antibody system' (called δ antigen/anti-δ antibodies), although associated with HBV infection, was immunologically different from HBsAg, HBeAg, and HBcAg. The δ antigen (HDAg) isolated from these initial patients was then instrumental to screen the sera of other patients of diverse geographic origins and associate the presence of circulating anti-δ antibodies to chronic liver disease (Rizzetto et al., 1979, 1980a). The δ antigen was later confirmed to be associated with a transmissible pathogenic "delta agent" now called HDV. Indeed, the serum from patients with δ-positive hepatitis could infect chimpanzees in the presence of its helper HBV both in co- or super-infection conditions (Rizzetto et al., 1980b). In these chimpanzees, δ antigen was shown to circulate as particles, containing HBsAg and a low molecular weight RNA molecule, raising for the first time the hypothesis of HDV being an RNA satellite virus of HBV (Rizzetto et al., 1980).

1.2. Clinical and virological relevance

Almost 40 years after discovery, HDV and hepatitis D remain challenging entities to both clinicians and researchers. The impact of the disease, mostly present in low-income countries, is largely neglected. Despite being considered as the most severe form of chronic hepatitis, HDV infection is still under diagnosed, either by lack of awareness or appropriate diagnostic tools, and available treatments are still largely ineffective. Although significant advances have been made in understanding the cellular and molecular virology of HDV, they have failed to translate into the development of effective therapeutic strategies. Explanations stem from the peculiarity of HDV life cycle, which features no virally-encoded enzymatic activity and completely relies, not only on cell machinery as other viruses, but also on HBV for spreading.

2. The virus

2.1. Origin of HDV

HDV is a 35–37 nm diameter virus featuring a small single-stranded, circular RNA genome of 1672–1697 nucleotides, the smallest among mammalian viruses. Its particular characteristics still pose unanswered questions regarding its origin. Currently, two main theories are considered: HDV may have evolved from plant viroids or/and host cell pre-mRNA via the splicing machinery (Taylor, 2014).

Plant viroids are considered the lowest entities in the biological scale in terms of genome complexity. More than 30 species have been described so far, that have in common being constituted of a small single stranded, often circular, RNA molecule (250–400 nucleotides), having no coding capacity, and being replicated by host cell enzymes. Two families of viroids are considered: *Pospiviroidae* (represented by the potato spindle tuber viroid) and *Avsunviroidae* (represented by the avocado sunblotch viroid). HDV has RNA structural and replicative features in common with both families: its rod-like RNA structure and nuclear replication relate to *Pospiviroidae*, whereas the existence of a ribozyme and the symmetrical rolling circle mechanism of replication remind *Avsunviroidae*. These common features, added to the fact that RNAs from HDV and plant viroids interact with homologous cellular proteins, and the experimental preliminary, yet not clearly confirmed by others, evidence that HDV might replicate and spread after inoculation of tomato seedling leaves, suggest an analogy between HDV and plant viroids. This hypothesis, however, leaves unanswered questions such as the origin of the delta antigen and the relation between HDV and HBV, its helper virus (Flores et al., 2012).

A second theory, which can be complementary to the first one, suggests that HDV may have evolved from the host cell

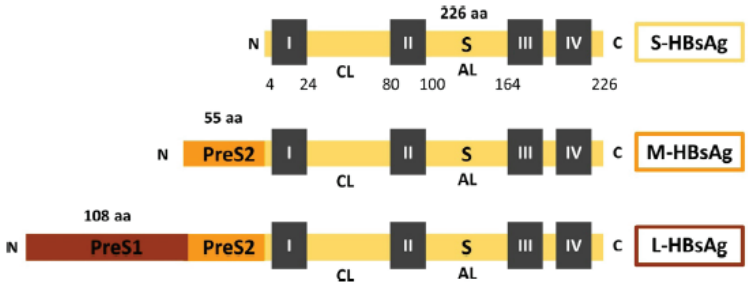

Fig. 1. HBV envelope proteins. The three forms of HBsAg share the same C-terminus (S) containing four putative trans-membrane domains (filled gray rectangles I–IV), a cytosolic loop (CL) and an antigenic loop (AL). As translation initiates in different in-frame sites, besides S (the only domain of S-HBsAg), M-HBsAg contains PreS2 and L-HBsAg further contains additional 108–119 aa of PreS1 (depending on genotype). HDV assembly is dependent on the contact of the CL with HDV ribonucleoprotein (RNP), whereas the contact between HBV nucleocapsid and HBsAg is established with the PreS region.

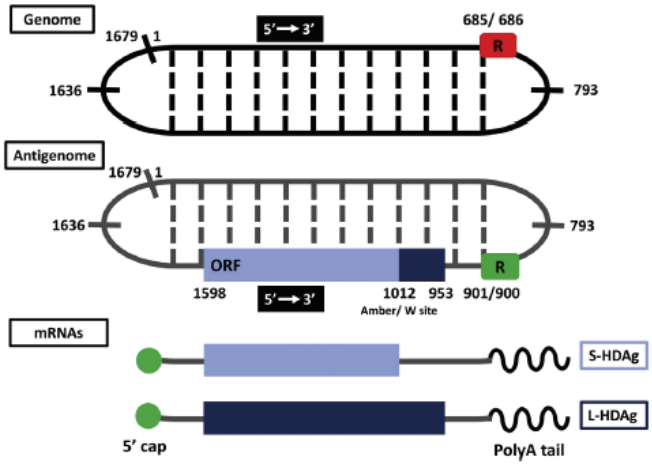

Fig. 2. Structure of HDV RNAs. Both genome and antigenome form an unbranched rod-like structure of ~1700 bp, due to extensive intramolecular base-pairing, and contain a self-cleaving ribozyme (R). Although antigenomic RNA contains the open-reading frame (ORF) for HDAg, the antigen is translated from the ~800 bp mRNA. HDV mRNA is transcribed form genome by RNA-Pol II and matured as a host cell mRNA (with a 5′ cap and a 3′ polyA tail). S-HDAg is translated from un-edited mRNAs whereas antigenome editing of the Amber/W site by ADAR1 leads to L-HDAg.

transcriptome. This vision was supported by studies showing that ribozyme RNA with similar secondary structure and biochemical properties to the HDV ribozyme can be found in human cells (*e.g.* within an intron of the *CPEB3* gene). However, pseudoknot ribozymes were later found in all life kingdoms (except Archaea) and in insect viruses (Webb et al., 2009; Salehi-Ashtiani et al., 2006). A host cell origin has also been proposed to the delta antigen. Indeed a protein, named DIPA (*i.e.* "delta interacting protein A"), was initially described as a potential candidate. Although the actual relevance of its homology has later been dismissed, DIPA might still be a cellular HDAg partner (Taylor and Pelchat, 2010; Brazas and Ganem, 1996; Long et al., 1997).

An integrated model suggests that HDV might have arisen from a re-combination event between a viroid like element and a cellular pre-mRNA/mRNA (Robertson, 1996).

2.2. Viral structure

2.2.1. Role of HBV envelope proteins in HDV assembly

Firstly characterized from sera of chimpanzees experimentally-infected with the serum of an Italian chronic carrier, HDV was shown to be a HBsAg enveloped particle of 35–37 nm, with a buoyant density of 1.25 g/cm^3 in cesium chloride gradient and a sedimentation coefficient value intermediate between the one from the HBsAg empty subviral particles (22 nm) and the HBV virion (Dane particle, 42 nm) (Rizzetto et al., 1980). HDV is indeed a defective virus-like particle that uses an envelope composed of the three forms of HBV glycoproteins (small (S or S-HBsAg), medium (M or M-HBsAg) and large (L or L-HBsAg) HBV surface proteins) to egress from and re-enter into hepatocytes, thus conferring the same tropism to both viruses.

The three forms of HBsAg share a common C-terminus (Fig. 1). In addition to the S domain, M-HBsAg contains an N-terminal hydrophilic domain named PreS2, and, relative to M-HBsAg, L-HBsAg has an additional domain named PreS1 (Urban et al., 2014). L-HBsAg is essential for the assembly and infectivity of HBV, although not sufficient, and S-HBsAg is needed for the release of particles from cells (Gudima et al., 2007). In contrast, HDV can be assembled by S-HBsAg alone, but without L-HBAgs, the particle is not infectious (Sureau et al., 1993). These distinct budding requirements are explained by different binding domains of the cytosolic loops of the envelope proteins to HBV nucleocapsid and

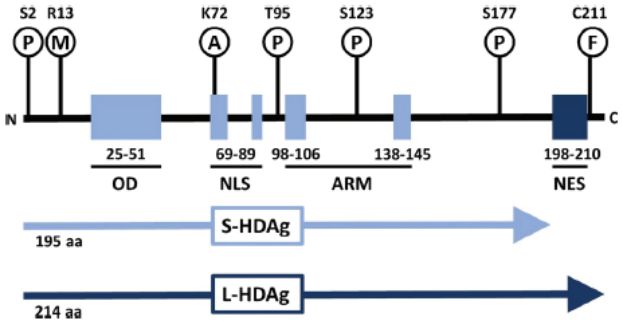

Fig. 3. Functional domains and post-translational modifications of HDAg. Both forms of HDAg contain an oligomerization domain (OD), a nuclear localization signal (NLS) and an arginine-rich motif (ARM). L-HDAg is 19 aa longer than S-HDAg and further contains a nuclear export signal (NES). Post-translational modifications that have been described for both forms of HDAg are represented by circles (P, phosphorylation; M, methylation; A, acetylation; F, farnesylation).

HDV ribonucleoprotein (Sureau et al., 1994). A recent study has shown that the assembly (and infectivity) of HDV genotype 1 (HDV1) is not restricted to a particular HBV genotype (Freitas et al., 2014). It can also occur with woodchuck, bat and wooly monkey hepadnavirus envelopes (Ryu et al., 1992; Drexler et al., 2013; Barrera et al., 2004).

2.2.2. Viral RNAs

The HDV virion contains a circular single-stranded negative RNA genome, with a strong secondary flexible quasi-double-stranded RNA conformation. During replication in infected cells, two other main viral RNAs can be detected: the genomic complementary molecule, called antigenome, and the HDV mRNA (Fig. 2). Constituted of only 1672–1697 nt, the HDV genome is the smallest of all known mammalian viruses and presents some similarities with plant viroid counterparts. It has a high C + G content (60%) and about 74% intra base pairing allowing it to fold into a rod-like structure (Wang et al., 1986; Kuo et al., 1988). Infected cells may contain about 300,000 molecules of HDV genome, divided between nucleus and cytoplasm, thus indicating a high level of replication (Chen et al., 1986; Macnaughton and Lai, 2002).

The HDV antigenomic RNA is a replicative intermediate of positive polarity, complementary to the genome sequence and contains the coding sequence for HDAg. It is 5–22 times less abundant than the genome, found exclusively in the nucleus of infected cells and therefore not packaged into virions (Chen et al., 1986; Macnaughton and Lai, 2002). HDV proteins are translated from a specific 800 nt length HDV mRNA (Lo et al., 1998), which is transcribed by host DNA-dependent/RNA-polymerase II and matured (*i.e.* capped and polyA-tailed), as cellular mRNAs (Gudima et al., 2000; Hsieh et al., 1990).

2.2.3. Ribozyme

Small self-cleaving RNA sequences (of about 85 contiguous nucleotides) were identified in both HDV genome and antigenome (Kuo et al., 1988; Wu et al., 1989). These ribozymes, whose sequences are very well conserved among HDV genotypes, are responsible for the cleavage of multimeric RNA molecules that arise during transcription into unit-long genome or antigenome sequences.

The HDV ribozyme has unique structural and functional characteristics and is distinct from viroid ones (Serganov and Patel, 2007). Several crystal structures of HDV ribozyme have been obtained and allowed to uncover a pseudoknot-like mechanism of cleavage (Riccitelli and Lupták, 2013). As previously mentioned, recent studies have identified HDV-like ribozyme sequences in host-cell genomes.

2.2.4. Viral proteins and editing

Early cloning and sequencing suggested the existence of 9 putative ORFs in HDV RNA (4 in the HDV genome and 5 in the antigenome). Although a small polypeptide resulting from an alternative ORF (*i.e.* ORF-K) has been described (Bichko et al., 1996), the virus produces one main viral protein – HDAg (Wang et al., 1986). The coding region for HDAg is contained in the antigenome molecule, but translation occurs from a linear HDV mRNA. While originating from the same ORF, two isoforms of HDAg are translated, *i.e.* S-HDAg (for "small", 24 kDa) and L-HDAg (for "large", 27 kDa). S-HDAg can be directly translated from the first round of HDV genome transcription and is the earlier form to arise in infected cells. During replication, as depicted in Fig. 5, the 'adenosine deaminase acting on RNA' protein (ADAR1) catalyzes adenosine-to-inosine editing on the antigenomic HDV RNA (adenosine 1012; the amber/W site is changed from UAG to UIG) (Polson et al., 1996). This gives rise to a genome that has a complementary ACC instead of the AUC and ultimately leads to the appearance of mRNA forms where the UAG (amber) stop codon is replaced by a UGG (tryptophan) codon. This modification allows translation to continue until the next stop codon, leading to the appearance of a protein 19–20 amino-acids longer, *i.e.* L-HDAg (Casey, 2012).

Based on relative levels of expression and knock-down experiments, ADAR1 has been shown to be the specific cellular enzyme acting on antigenomic HDV RNA (Wong and Lazinski, 2002). ADAR1 has two isoforms – small (ADAR1-S) and large (ADAR1-L) – with a common C-terminus. ADAR1-S is the most abundant form, constitutively expressed, and has a nuclear localization, whereas ADAR1-L is found mainly in the cytoplasm and its expression is stimulated by interferon. Preliminary works indicated that ADAR1-L was very efficient in editing transcripts in the cytoplasm, but it was later shown that HDV RNA editing occurs in the nucleus and is mediated by ADAR1-S (Wong and Lazinski, 2002). However, recent works suggested an increased in HDV RNA editing following interferon treatment that could be attributed to ADAR1-L (Hartwig et al., 2004, 2006).

The two forms of HDAg share the 195 amino acids of N-terminus and present several common functional domains, including RNA binding motifs, a coiled-coil domain, a nuclear localization signal and a helix-loop-helix motif (Fig. 3). The 19 extra amino acids of L-HDAg are proline rich, feature a viral assembly signal, a nuclear export signal (NES), and a prenylation site (Lee et al., 2001).

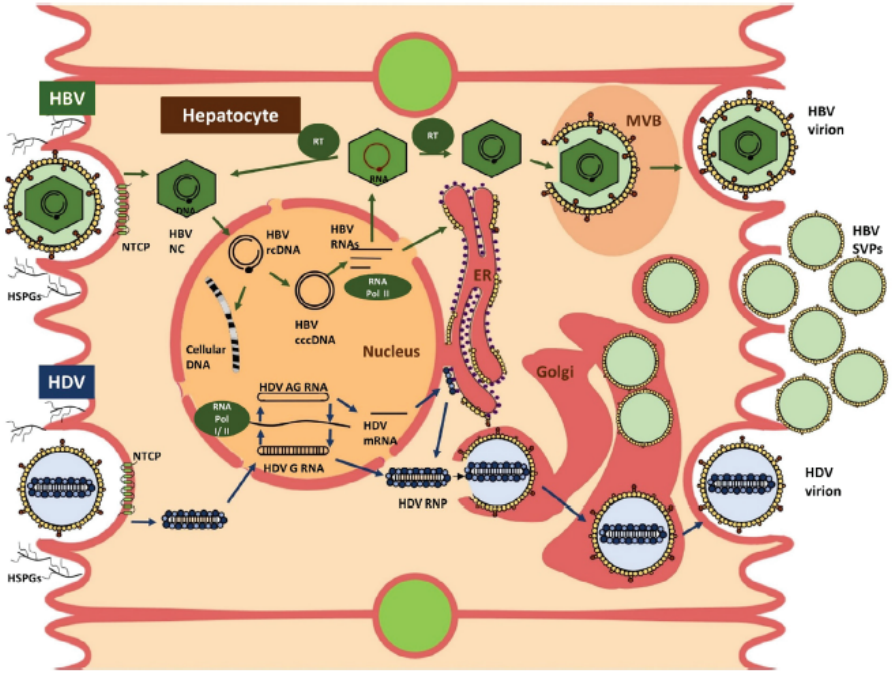

Fig. 4. HDV life cycle. For a productive HDV viral cycle, HBV infection of the same cell is necessary. For both viruses, entry is mediated by NTCP binding of the preS1 domain of HBsAg, preceded by virion attachment to HSPGs. Apart from entry, replication of HBV and HDV seems to be completely independent. HBV nucleocapsid (NC) is transported to the nucleus where viral relaxed circular DNA (rcDNA) is converted to covalently-closed circular DNA (cccDNA), a mini chromosome-like structure that is permanently kept in the infected cell. Fragments of HBV DNA are integrated in the host cell genome. HBV RNA transcription from cccDNA is mediated by the cell RNA Pol II and gives rise to mRNA transcripts that can either be included in immature nucleocapsids and undergo retro-transcription by the viral retro-transcriptase (RT) to form new genomes; or translated into viral structural proteins. Virions are assembled and released through the multivesicular body (MVB) pathway (whereas subviral particles – SVPs – are thought to be released via Golgi secretory pathway). Replication of HDV genome (HDV G RNA) is exclusively nuclear, occurs through a double rolling circle mechanism, involving the formation of antigenome (HDV AG RNA) and multimeric RNA intermediates and the recruitement of host cell RNA polymerases. HDV ribonucleoprotein (RNP) is enveloped in the Golgi by HBsAg. HDV secretion is thought to occur via Golgi. *ER*, endoplasmic reticulum; *HSPG*, heparan sulfate proteoglycans; *NTCP*, sodium taurocholate cotransporting polyeptide.

HDAg undergoes post-translational modifications that can impact HDV replication, namely serine phosphorylation, lysine acetylation, arginine methylation and lysine sumoylation (Lai, 2005). Prenylation of the Cys211 residue of L-HDAg, by a cellular farnesyl transferase is involved in particle assembly (Glenn et al., 1992). S-HDAg and L-HDAg have different roles during the viral life cycle and may contribute to different pathogenic mechanisms. S-HDAg, while having no polymerase activity, is necessary for the initiation of HDV replication, RNA Pol II elongation and for the accumulation of HDV RNAs during the cycle (Kuo et al., 1989; Yamaguchi et al., 2001). L-HDAg acts as a dominant negative inhibitor of HDV replication, by specifically inhibiting genome, but not antigenome, synthesis and is essential for virion assembly (Chang et al., 1991; Chao et al., 1990; Modahl and Lai, 2000).

2.2.5. Ribonucleoprotein

The genomic HDV RNA associates with HDAg to form a ribonucleoprotein (RNP) present both in viral particles and infected cells. This RNP is essential, not only for virion assembly, but also for the nucleus/cytoplasm trafficking of HDV RNA (Tavanez et al., 2002). The exact structure and stoichiometry of this RNP have been debated. Early studies suggested that, in the virion, the genome molecule was associated to 70 HDAg molecules, whereas in the nucleus of infected cells, both genome and antigenome formed RNP structures featuring an average of 30 HDAg molecules (Ryu et al., 1993). A more recent study suggested a molar ratio of 200 molecules of HDAg per genome molecule both in virions and infected cells (Gudima et al., 2002). However these numbers have been questioned by studies suggesting the oligomerization of delta antigen to attach to the HDV RNA molecule, which would be compatible with the smaller proportions previously proposed (Lin et al., 2010; Alves et al., 2010). Importantly, the binding specificity of HDAg to the genome seems to be dictated by its secondary structure rather than its primary sequence (Griffin et al., 2014).

2.3. Viral life cycle

An overall representation of HDV life cycle in a hepatocyte co-infected by HBV is depicted in Fig. 4. In the following text, details are given regarding important steps of the life cycle.

2.3.1. Attachment and entry

2.3.1.1. Hepatotropism. The hepatotropism of HDV and its capacity to ensure a productive cycle are related to the entry process and HBV co-infection of cells. While a productive cycle requires the expression of HBV surface glycoproteins in the same cell to allow assembly of HDV virions, other aspects of the two virus replication cycles seem completely independent, as no other component of

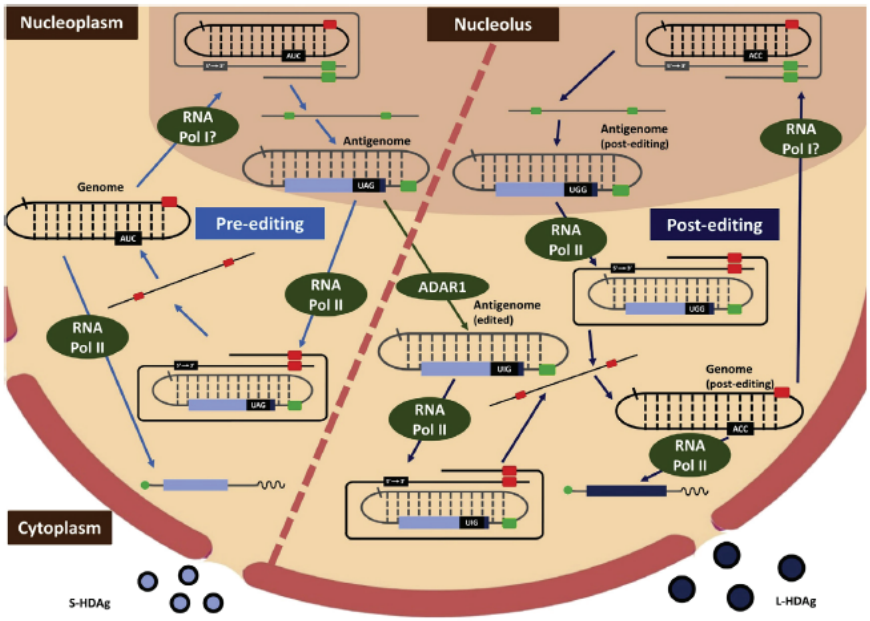

Fig. 5. Model of HDV RNA replication. HDV replication is limited to the nucleus and involves antigenome and multimeric RNA molecules as replicative intermediates. Genome is transcribed in the nucleolus by cellular polymerase(s). The generation of multimeric RNAs is necessary for the self-cleavage and ligation activities of HDV ribozyme. The cycle is completed by transcription of antigenome by RNA Pol II in order to form a new genome molecule. Initiation sites for the transcription of multimeric linear molecules from genome and antigenome are still unkown (the represented starting points of linear multimers are arbitrary). Genome can either enter a new replicative cycle, initiate transcription of HDV mRNA or be exported to the cytoplasm as HDV RNA for virion production. In parallel, antigenome molecules can enter a novel replicative cycle after editing of the Amber/ W site by ADAR1, ultimately leading to the appearance of mRNA coding for L-HDAg (see text for details). *ADAR*, adenosine deaminase acting on RNA.

HBV seems to contribute to HDV replication and *vice versa* (Bichko et al., 1996). In contrast to HBV, which requires liver-specific transcription factors, HDV genome replication may occur in various mammalian cell types provided that its genome is experimentally delivered to cells (Taylor, 2009). Given the shared envelope structure of both virus, attachment and entry mechanisms are thought to be similar between HBV and HDV, and most of the current knowledge on HBV entry mechanisms has been obtained in HDV infection models (Sureau, 2010).

2.3.1.2. Viral factors. For both viruses, the presence of the L-HBsAg envelope protein is essential for infectivity. Specific mutations in the N-terminal 75 amino acids of the Pre-S1 domain or inhibition of myristoylation can block infectivity (Gripon et al., 1995; Blanchet and Sureau, 2007). S-HBsAg antigenic loop domain and pattern of glycosylation also seem to play an role, as specific mutations in this domain may inhibit infection, independently of the PreS1 domain (Le Duff et al., 2009; Julithe et al., 2014).

2.3.1.3. Host factors. Viral attachment on the cell surface precedes receptor-specific entry for HBV and is mediated by cellular heparan sulfate proteoglycans (HSPGs) (Schulze et al., 2007; Leistner et al., 2008). This was recently confirmed for HDV (Sureau and Salisse, 2013; Lamas Longarela et al., 2013). The exact HSPGs involved in HBV/ HDV viral attachment are still to be identified, even though Glypican-5 was recently reported to be preponderant in this process (Verrier et al., 2015). This step, although necessary, is not sufficient for infection, as the entry of HSPG-attached viruses can still be inhibited (Han et al., 2011). A potential role for purinergic receptors (*i.e.* P2YRs, P2XRs) in the attachment process of both HDV and HBV has also been suggested from the reported inhibitory effect of suramin (Lamas Longarela et al., 2013; Taylor and Han, 2010).

Recently, a ground breaking study from Yan and colleagues led to the identification of human sodium taurocholate cotransporting peptide (hNTCP, encoded by *SLC10A1*) as a functional receptor for HBV and HDV (Yan et al., 2012). NTCP is located in the basolateral membrane of hepatocytes and participates in the enterohepatic circulation of bile salts. Viral infection seems to be supported by amino acids involved in bile acid binding (and not those involved in sodium binding) (Yan et al., 2014). The interaction between NTCP and HBV/HDV seems to be mediated by the viral PreS1 N-terminal 75 amino acids and a NTCP binding region located on helix 5 in the outer leaflet of the cell membrane (Urban et al., 2014). Backing up the fact that HDV can replicate into many cell types (*i.e.* not only human hepatocytes), if its genome is correctly delivered, an hNTCP-transgenic mice was recently shown to be infectable by HDV (He et al., 2015).

2.3.2. Uncoating and nuclear transport

HBV is thought to be internalized through clathrin-dependent endocytosis, via early and late endosomal compartments, independently of acidification and protease activity (Huang et al., 2012; Macovei et al., 2013). Such evidence is unavailable for HDV, although it may be supported by the identification of L-HDAg as a clathrin-adaptor-like protein (Huang et al., 2007). The steps that mediate post-fusion nuclear transport of HDV RNP and virus genome uncoating are not fully understood. Trafficking of HDV RNP

between cytoplasm and nucleus would involve HDAg and its interaction with importins (Tavanez et al., 2002; Chou et al., 1998). Further understanding of this phase of the viral cycle would be crucial to understand in particular the innate immune response against the virus.

2.3.3. Replication

Throughout replication, antigenome RNA is limited to the nucleus, whereas genomic RNA molecules can either enter another nuclear replicative cycle or be exported to the cytoplasm for assembly into new infectious particles (Macnaughton and Lai, 2002). A summary of the currently accepted steps involved in HDV replication is represented in Fig. 5.

2.3.3.1. Double rolling-circle model of replication. It is now accepted that HDV has an RNA-dependent RNA replication in a "double rolling-circle mechanism", which involves the recruitment of host-cell DNA-dependant RNA polymerases and likely their switch in template specificity (Lai, 2005; Taylor, 2009). The double rolling-circle replication (similar to plant viroids symmetrical rolling-circle replication, but modified to include a mRNA synthesis step) relies on two circular RNA templates of inverse polarity, genome and antigenome, and the generation of intermediate multimeric linear transcripts.

For the replication of HDV RNA, three enzymatic activities are needed: a polymerase to synthetize oligomeric strands from circular templates, a ribozyme-dependent RNAse activity to cleave them into unit-length strands, and a ligase to circularize monomers.

Unlike some RNA viruses with larger genomes, HDV does not possess its own RNA-dependent RNA-polymerase. Moreover, in contrast to other satellite viruses (*e.g.* satellite tobacco necrosis virus), it does not use the polymerase of its helper virus, and therefore fully relies on host-cell properties (Lai, 2005). Several lines of evidence support the fact that RNA polymerase II (Pol II) would be involved in HDV replication: firstly, the HDV mRNA displays a 5′ cap and 3′ poly-A tail as cellular mRNAs (Gudima et al., 2000; Hsieh et al., 1990); secondly, HDV RNA transcription has been shown to be inhibited by low doses of α-amanitin (Chang et al., 2008); and finally, RNA pol II has been shown to be able to bind to HDV RNA of both genomic and anti-genomic polarity (Chang et al., 2008; Greco-Stewart et al., 2007). In other reports, antigenome synthesis seemed to be somehow resistant to α-amanitin, suggesting that Pol I could be also involved (Modahl et al., 2000). This possibility is supported by two other studies suggesting that both Pol I and Pol III could interact with HDV RNA (Greco-Stewart et al., 2009), and that genome and antigenome synthesis could take place in distinct nuclear areas (Li et al., 2006). Although several hypotheses prevail and the exact role of the various polymerases is still to be clarified, all studies agree that HDV is able to redirect an otherwise DNA-dependent RNA-polymerase to its RNA template. The underlying molecular mechanism is however largely unknown.

2.3.3.2. Role of S-HDAg. S-HDAg is an important candidate to explain this hijacking of RNA pol II. It is a nuclear protein known to bind to HDV RNA, which presents structural similarities to transcription factors (*e.g.* NELF-A), and can undergo acetylation and methylation as other transcription regulatory proteins (Lai, 2005). S-HDAg can bind to RNA Pol II and enhance transcription either by direct stimulation of elongation or by reversion of inhibitory effects (Yamaguchi et al., 2001). Furthermore, S-HDAg has been shown to biochemically interact with 9 out of 12 subunits of the RNA pol II in a combined proteomic-RNA interference screening (Cao et al., 2009). This interaction may not be restricted to Pol II, as S-HDAg has been shown to interact and/ or co-localize with nucleolar proteins (*e.g.* B23 and nucleolin), which could further support a Pol I involvement (Huang et al., 2001).

2.3.3.3. Motifs for transcription and replication. The presence of transcription initiation sites or promoters on HDV RNA is also debated. It seems to be the case for HDV mRNA (Gudima et al., 2000), and a recent study has suggested that the 5′-end region of the HDAg mRNA, which coincides with one extremity of the rod-like genomic RNA and displays a complex secondary structure, may play an important role in HDV replication (Beeharry et al., 2014).

2.3.3.3.1. Cleavage. Genome and antigenome molecules are processed from more than unit-length linear precursors, needing a cleaving catalytic activity. This activity is exerted by the ribozyme auto-cleaving sequences present in both genome and anti-genome that have to be transcribed at least twice from the circular templates to give rise to one unit length, justifying the presence of multimers.

2.3.3.3.2. Ligation. A ligase activity would be needed to circularize the linear monomers into genome and antigenome molecules. While a study has suggested the recruitment of a host-cell ligase, as ligation of HDV RNA occurred only in mammalian cells (Reid and Lazinski, 2000), another has shown self-ligating properties of HDV ribozyme sequences (Sharmeen et al., 1989).

2.3.4. Viral assembly and release

To form HDV virions, the HDV ribonucleoprotein is enveloped by at least S- and L-HBsAg, meaning that, in a natural infection, HDV can only be released from cells co-infected with HBV. Many questions remain unanswered regarding HDV assembly and release. The newly developed infection models should help uncovering this step of HDV life cycle in parallel with a better understanding of HBV assembly.

In contrast to HBV, the cytoplasmic domain of HBsAg, spanning the junction between PreS1 and PreS2, does not seem crucial for HDV release; hence it has been suggested that HDV would mostly use the subviral particle release pathway (through the Golgi) rather than the multivesicular body pathway, involved in assembly of infectious HBV virions (Watanabe et al., 2007; Zeisel et al., 2015; Taylor, 2012). As for post-entry trafficking of HDV RNP, clathrin could also be involved in viral particle export (Huang et al., 2009). Regarding viral components, the farnesylation of the C-terminal sequence of L-HDAg is necessary for HDV envelopment, as it mediates the interaction with the S region of HBsAg (Hwang and Lai, 1993). Farnesylation involves the fixation of a 15 carbon chain to the $C_{211}XXQ$ box peptide motif that is present at the carboxy-terminal end of the L-HDAg and conserved among all HDV genotypes.

2.4. Experimental models for the study of HDV

Since the discovery of HDV, both *in vitro* and *in vivo* models have been established to perform molecular and cellular studies.

2.4.1. In vitro models

Unlike HBV, HDV replication is not restricted to hepatocytes, and the virus can replicate to high levels in a wide range of mammalian cells upon delivery of viral RNA (and antigen) or cDNA. *In vitro* models of transfection of hepatic cancer cell lines (*e.g.* Huh7, HepG2) have allowed major contributions to the study of viral replication. However, as viral assembly depends on the presence of HBV envelope proteins, viral particle production can only be achieved in the presence of concomitant HBV transcription or by the co-transfection of a plasmid encoding HBV envelope proteins (Gudima et al., 2007; Sureau, 2010).

Until recently only differentiated primary human (PHH), chimpanzee (not often used), or tupaia (PTH) hepatocytes, as well as the

non-transformed, bipotent progenitor differentiated-HepaRG cell line were susceptible to HDV (and HBV) infection (Urban et al., 2014). These cells are difficult to obtain, to work with, and can be confronted (with the exception of HepaRG) to batch-to-batch variability (both genetically and physiologically), thus impairing reproducibility of experiments. The recent identification of hNTCP as an essential receptor for HDV and HBV has changed this scenario, as its constitutive or inducible overexpression in clonal transformed hepatic cell lines (*e.g.* HepG2 and Huh7) renders them susceptible to high level of infection in a more reproducible manner (Urban et al., 2014).

2.4.2. In vivo models

Although natural HDV infection seems to occur only in humans, a limited number of susceptible mammalian hosts has been identified and used for research purposes. HDV infection has hence been extensively studied in chimpanzees (with HBV as a helper virus) and woodchucks (in the presence of WHB) (Rizzetto et al., 1980b; Ponzetto et al., 1984). Tree shrew species *Tupaia bengaleri* (susceptible to HBV), wooly monkeys (with WMHBV as helper virus), and more recently bats have also been used for the study of HDV infection, although their use is limited by lack of tools. Interestingly, Peking ducks' hepatocytes, although a useful model for the study of avihepadnavirus (DHBV), do not sustain HDV replication (Liu et al., 2001).

Many mice models, which are more or less relevant, have been developed to date. First of all, it is worth noting that a straight HDV injection of wild type mice does not lead to an efficient infection, with <1% of infected hepatocytes; this finding is compatible with the current knowledge on species specificity of HDV infection mediated by hNTCP (Netter et al., 1993; Yan et al., 2013). Gene delivery strategies based on hydrodynamic transfection of plasmids (with trimeric or dimeric constructs) led to measurable intrahepatic HDV replication in mice and to the establishment of HDV viremia, when HBV-transgenic mice are used (Chang et al., 2001; Bordier et al., 2003). This approach is interesting to study potential antiviral targeting HDV in an immune competent model. In a less flexible approach, a transgenic mouse expressing a replication competent HDV genome dimer has also been generated. Interestingly, as the transgene was not only expressed in hepatocytes, it was found that HDV could replicate in other tissues than liver, supporting the fact that HDV hepatotropism is mediated by an entry restriction (Polo et al., 1995). More recently a transgenic mouse model expressing hNTCP was developed and shown to support acute HDV infections (He et al., 2015). But in this model co-infection or superinfection with HBV cannot be studied. It is worth noting that transgenic mice expressing either L-HDAg or S-HDAg have been developed and have proven useful to exclude a direct pathogenic/carcinogenic role in this setting (Guilhot et al., 1994).

All mice models described so far do not allow co- or superinfection with HBV in a context in which cccDNA is present. To overcome this problem, humanized liver mice models (initially used for the study of HCV and HBV infections) have been validated for HDV (Dandri and Lütgehetmann, 2014). Several models have been developed (notably uPA SCID, FRG and TK-NOG mice), having in common a human reconstituted liver (from the engraftment of PHH) in the absence of an immune system (Dandri et al., 2001; Bissig et al., 2010; Kosaka et al., 2013). Notably, the uPA SCID mouse model has been used for the preclinical validation of Myrcludex B®, an entry inhibitor for both HBV and HDV (Lütgehetmann et al., 2012). More recently, mouse models displaying not only a humanized liver but also a human immune system, have been developed and reported to be susceptible to HBV infection. Although a promising tool for the study of virus-host interactions, their potential contribution for the study of HDV infection is yet to be established (Washburn et al., 2011; Gutti et al., 2014; Bility et al., 2014).

3. The disease

3.1. Epidemiology

3.1.1. Prevalence and geographic distribution

HDV has a worldwide distribution (Fig. 6), with variable prevalence rates, which surprisingly do not exactly match the distribution of chronically HBV-infected (CHB) patients (Rizzetto et al., 1980a; Ponzetto et al., 1985). HDV prevalence may be underestimated by non-standardized and heterogeneous screening practices and the inaccessibility to testing in many highly endemic areas. In the 80's, the number of HDV infected patients was estimated at 15–20 million worldwide, with an overall prevalence rate of 5% of all HBsAg carriers (Ciancio and Rizzetto, 2014). Areas with high endemicity were at that time identified in Africa and South America, with prevalence as high as 85% of HBsAg positive patients in the Amazon basin. Countries of the Mediterranean basin were considered of intermediate endemicity (with a prevalence of 25% in Italy and Turkey), whereas in low endemic countries (Northern Europe and USA), the disease seemed to be restricted to intravenous drug users (IVDU) (Ponzetto et al., 1985; Rizzetto and Ciancio, 2012).

3.1.1.1. The case of low endemicity regions. In the 90's, the decrease in prevalence of HBV infection (due to improvements in sanitation, behavioral changes and, mostly, to the introduction of an HBV vaccine) was accompanied by a decrease in HDV prevalence in Europe (Rizzetto and Ciancio, 2012). But for the last 15 years, no further decline has been observed. In Italy, where an HDV epidemic spreading occurred during the 80's, there was a steady decrease during the two following decades, but recent data suggest that no further decrease was achieved, with steady prevalence rates of 8.1% of HBsAg carriers in 2008 (Stroffolini et al., 2009; Sagnelli et al., 2014). In France, the latest data suggest that the prevalence of HDV infection among HBsAg carriers is also stable, if not increasing (Servant-Delmas et al., 2014). This trend seems to be associated with the increasing number of migrants, originating mainly in Africa and Eastern Europe (Le Gal et al., 2007). Similar data were reported in Germany, with a current prevalence of 7.4–11.3% of HBsAg carriers, with a significant proportion of patients originating in Turkey and Eastern Europe. (Wedemeyer et al., 2007; Reinheimer et al., 2012).

A recent re-assessment of HDV prevalence in London (UK) reported a decrease in the prevalence rate (2.1% of all the HBsAg carriers, while the previous reported prevalence was 8.5% in 2008) and attributed a significant proportion of the positive cases to IVDUs (22.7%) and to patients of African origin (28–50%) (William Tong et al., 2013; Cross et al., 2008). In the USA, the diagnosis of HDV infection has for long been neglected. Recent studies indicate that the number of affected patients is comparable to the European reality (8% of the HBsAg positive patients) and not restricted to IVDUs (26% of the patients) (Gish et al., 2013).

In summary, three subgroups of patients can be described nowadays in Western countries: (a) older patients infected during the eighties epidemic (namely in the Mediterranean basin), currently suffering from advanced liver disease; (b) younger IVDUs, often co-infected with HCV and/or HIV; and (c) patients migrating from highly endemic countries (Ciancio and Rizzetto, 2014).

3.1.1.2. The case of high endemicity regions. In the early 90's, an outbreak of severe acute hepatitis delta (mostly due to co-infection) was reported in the IVDUs of the Russian region of Samara.

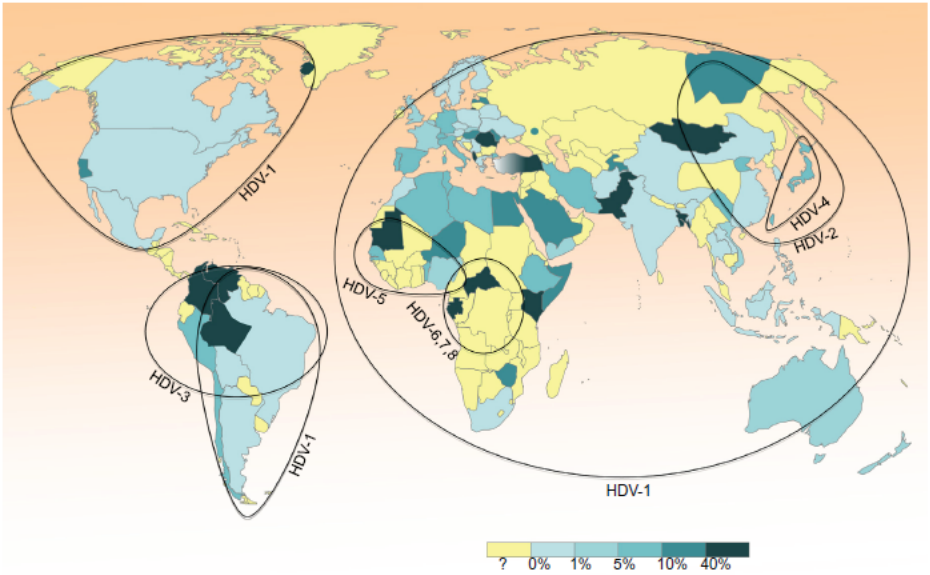

Fig. 6. Prevalence of hepatitis delta virus infection among HBV carriers. The scale indicates the suspected proportion of HDV co- or super-infection of HBV-infected patients. Yellow color: insufficient data. The black circles indicate preferential area diffusion of HDV genotypes, HDV-1 being ubiquitous. Due to globalization, human migration or travel during chronic carriage, some strains geographically defined, can extend outside their initial spreading area. Therefore, diagnosis has to be adapted for all different HDV genotypes. Note that some prevalence remained from the nineties, while others correspond to more recent works.

During the outbreak, 39% of acute hepatitis B patients were anti-HD IgG positive (48% among the fulminant hepatitis cases) (Flodgren et al., 2000). High prevalence of chronic hepatitis delta (CHD) is still reported in central Europe: 47.6% of HBsAg positive patients in Romania and 13.9% in Hungary (Pár et al., 1992) (Fig. 6).

Although Brazil as a whole does not have a high prevalence of HDV infection, the Amazonian basin was soon recognized as an endemic region (Ponzetto et al., 1985; Galizzi et al., 2010). In this region, HDV-3 has been associated with "Labrea fever", a form of fulminant hepatitis that affected indigenous populations (Bensabath et al., 1987). Recent evaluations of HDV prevalence are consistent with high endemicity, with HDV seropositivity rates ranging from 8.5% of HBsAg-positive asymptomatic blood donors to 65% in HBsAg-positive hospital outpatients (Crispim et al., 2014). High HDV prevalence rates are also reported in the Amazonian areas of Venezuela and Colombia, where the infection is, as in Brazil, caused by HDV-3 and associated with fulminant hepatitis outbreaks (Alvarado-Mora and Pinho, 2013).

In Africa, HDV infection affects mainly countries in the western and central areas. In Mauritania a prevalence rate of 33.1% among HBsAg positive patients was recently reported, and HDV was associated with more aggressive disease than HBV infection alone (Lunel-Fabiani et al., 2013). Other studies show wide differences in sero-prevalence in HBsAg positive liver patients, ranging from 1.3% in a Nigerian center, to 50% in Central African Republic and 66% in Gabon (Andernach et al., 2014; Makuwa et al., 2009). However it should be kept in mind that data on anti-HDV sero-prevalence are lacking from several African areas. In Egypt, a sero-prevalence of HDV of 20% was described among the HBsAg positive individuals (mostly patients with liver disease) (Saudy et al., 2003).

The situation is Asia is particularly interesting: in some places, the distribution of HDV infection does not match the high endemicity of HBV in the region. In Taiwan, a classic case of high HBV prevalence and an example of HBV vaccination success, HDV infection is frequent in the IVDU population (66.7% prevalence) and frequently associated with HIV infection, but remains rare in non-IVDUs (6%) (Chang et al., 2011). In Mongolia a prevalence of HDV antibodies as high as 82% has been reported in HBV infected patients with advanced liver disease (Oyunsuren et al., 2006). Finally, a recent report from the Pacific region also suggests high prevalence rates (37% among HBsAg positive patients) in isolated Micronesian islands (Han et al., 2014).

In summary, HDV infections are far from being on the way to eradication, and remain a major health problem in developing and low-resource countries where the diagnosis is sub-optimal. There is an urgent need to standardize RNA testing procedures (see dedicated section) and implement them in countries of high endemicity and surrounding ones to better monitor evolution of prevalence, while remaining vigilant in the rest of the word, due to increasing travel habits.

3.1.2. Molecular epidemiology and genotypes

HDV evolution seems to result from 3 main mechanisms: mutation, editing and, as recently described, recombination (Chao, 2007). The substitution rate for HDV, determined *in vivo* by the longitudinal evaluation of patient samples was estimated to be of 5.9×10^{-4} to 3.0×10^{-2} substitutions per site per year, varying with the phase of the infection (higher in the acute phase), the genome location (higher in the less conserved regions than in the conserved regions such as ribozyme) (Chao et al., 1994), and increasing under therapeutic pressure (Lee et al., 1992; Dény, 2006). These values seem to be higher than for the majority of the RNA virus that have estimated rates between 1×10^{-5} and 3.4×10^{-3}; as an example, HCV is estimated to have a substitution rate of 7.9×10^{-4} and HIV 2.5×10^{-3} (Jenkins et al., 2002). A long

term *in vitro* study identified a substitution rate of 2×10^{-2} and suggested that 70% of this substitutions may be linked to editing events (Chang et al., 2005). These evidences of genetic diversity led to the proposition that, in the same patient, HDV, as also described for other RNA virus, circulates as a quasi-species (Lee et al., 1992).

Recombination was firstly suggested from a patient sample analysis in 1999, then confirmed to occur in mixed genotype infections, both *in vivo* and *in vitro*, and recently re-visited in a patient suffering from acute hepatitis in Vietnam (Wu et al., 1999; Wang and Chao, 2005; Sy et al., 2015). Template-switching driven by the host RNA polymerase has been proposed as the most likely mechanism of HDV RNA recombination (Gudima et al., 2005; Chang and Taylor, 2002).

From the phylogenetic analysis of worldwide HDV sequences, eight genotypes have been proposed, associated with distinct geographic distribution and clinical features (Radjef et al., 2004; Le Gal et al., 2006). Sequence differences within a genotype are smaller than 20% but can be as high as 35% between different genotypes (Dény, 2006). Interestingly, among HDV isolates South American HDV-3 sequences are the most divergent ones, matching the tree topology of the local helper HBV genotype F among HBV genetic variability, suggesting common cause of viral evolution in South American natives.

3.2. Transmission

HDV transmission is considered to occur mainly through a parenteral route. The main transmission route however may vary between low and high prevalence zones. In Europe and in the USA transmission occurs mainly through contact with infected blood, particularly in IVDUs, frequently in association with HIV and HCV. There is evidence that both homosexual and heterosexual transmission of HDV can occur and may be particularly relevant in highly endemic areas (Brook, 1998; Braga et al., 2012). Intrafamilial transmission, presumably involving unapparent exchange of body fluids, occurs in low-income communities and has been proven by sequence analysis in an Italian population in the nineties (Niro et al., 1999). Vertical transmission of HDV has not been described and is possibly disfavoured by HDV's repression of HBV replication lowering HBV mother-to-infant transmission at birth.

3.3. Clinical features

3.3.1. Forms of acquisition

Being a satellite virus, HDV can establish a productive infection and spread only in cells concomitantly infected by its helper virus, HBV. Clinically, this translates into two possible forms of HDV acquisition: (i) simultaneously with HBV, corresponding to co-infection or (ii) by infection of a patient already chronically infected by HBV, corresponding to super-infection. In the transplantation setting a third form of acquisition has been suggested as "helper-independent hepatitis delta".

Co-infection translates into acute hepatitis, ranging from mild to severe, or even fulminant, and usually leads in 90–95% of cases to resolution of both HBV and HDV infections; this means that the rate of progression to chronicity is as low as for HBV, in immune-competent adults. The proportion of fulminant hepatitis in this setting can be as high as 17%, which is far higher than that observed in acute HBV infection (Yurdaydın et al., 2010). Super-infection, on CHB setting, may also translate into an acute hepatitis episode and may lead to fulminant hepatitis (Farci and Niro, 2012). Although some patients experience an HBsAg sero-conversion upon HDV super-infection, the vast majority progresses to chronic infection by both viruses (Caredda et al., 1985).

In liver-transplanted HBV/HDV patients a "helper independent HDV infection" or "latent" HDV infection has been suggested with markers of HDV replication identified in the liver and serum in the absence of HBV markers (Ottobrelli et al., 1991). Re-evaluation of some of these cases, using more sensitive PCR techniques, has shown that HDV infection was maintained in parallel with a very low-level HBV replication, ruling out the possibility of an isolated HDV infection (Smedile et al., 1998).

3.3.2. Clinical course and serologic patterns

Acute HBV/HDV co-infection is in most cases clinically indistinguishable from acute HBV infection. However, a subset of patients (15% in some cohorts) can present a severe clinical course with a pattern of biphasic hepatitis, associated with a re-increase of transaminase levels after a first period of improvement and that is thought to be caused by sequential spreading of HBV followed by HDV (Moestrup et al., 1983). HDV acute super-infection of a CHB patient is clinically characterized by an auto-limited flare of hepatitis (clinically evident in half of the patients), that can be mistakenly considered to be an hepatitis B reactivation, and is usually followed by progression to chronicity of both infections. During acute hepatitis delta, HDV markers do not allow a reliable distinction between co- and super-infection. Diagnosis is established based on the presence of anti-HBc IgM antibodies (directed against the capsid of HBV) that will be positive in the setting of an acute HBV infection (as is the case in co-infection) and remain negative or at a very low titer in super-infected patients. Fulminant hepatitis (acute liver failure in a patient with no prior liver disease) is more frequent in HDV infected patients than in HBV mono-infected patients and, without liver transplantation, is associated with a mortality of 80%. Chronic hepatitis D (CHD) is clinically indistinguishable from chronic HBV infection, although with a faster progression to cirrhosis and hepatocellular carcinoma (HCC).

3.4. Diagnosis

3.4.1. Detection of the virus

Given the high prevalence of HDV infection in some parts of the world and the absence of decrease in Europe and North America, there is an increasing consensus to suggest that all HBV infected patients (including inactive carriers) should be screened, at least at the diagnostic of CHB, for HDV infection (Noureddin and Gish, 2014; Hughes et al., 2011). This is already argued in European HBV treatment guidelines (EASL), but not in the North American counterpart; indeed AASLD's recommendations still restrict HDV screening to HBV infected patients originating from endemic countries or with a history of IV drug use (European Association for the Study of the Liver, 2012; Lok and McMahon, 2009).

Anti-HDV "total" antibody (*i.e.* anti HDV-IgM plus anti-HDV IgG) testing should be used as a first approach to hepatitis delta diagnosis, given its early appearance after HDV infection and its persistence throughout the course of the disease (Olivero and Smedile, 2012). There are two limitations to this approach: first, in the first days after infection, anti-HDV antibodies may be undetectable, and patients presenting with acute HBsAg positive hepatitis must be re-evaluated for the appearance of anti-HDV antibodies; secondly, anti-HDV IgG may persist after HDV infection resolution. A positive anti-HDV serology should be confirmed, ideally by the detection of serum HDV RNA. In the settings where HDV PCR is unavailable, anti-HDV IgM may be considered as a surrogate marker of HDV active infection and its clearance is correlated with therapeutic response (Wranke et al., 2014). Serum HDAg measurement is of limited utility, as in immunocompetent patients it is only transiently detected just before the acute phase of HDV infection, preceding the rising of anti-HDV antibodies. In the chronic

phase of disease it can only be occasionally detected in immunocompromised patients (Grippon et al., 1987).

As for other viral infections, nucleic acid quantification plays a crescent role in the diagnosis and most importantly therapeutic monitoring of HDV infected patients. However, unlike HBV, HCV or HIV, there is no fully standardized PCR technique. HDV molecular diagnosis mostly relies on *in house* techniques developed in academic centers. Given the wide genetic variability of the viral RNA, quantification of non-genotype 1 or African-specific genotype 1 samples has yet to be optimized (Brichler et al., 2013). The number of published qRT-PCR techniques has increased over the last years, based on both two-steps and one-step protocols (Yamashiro et al., 2004; Le Gal et al., 2005; Mederacke et al., 2010; Scholtes et al., 2012; Ferns et al., 2012; Homs et al., 2014). Significant technical challenges associated with HDV amplification arise from the high CG content and complementarity of viral RNA (that may limit the RT efficiency) and the high genetic variability of HDV that requires a careful design of primers and probes (Le Gal et al., 2005). Utilization of a robust PCR standard and internal control are also essential for a reliable HDV RNA quantification. Given the need for international quality assessment and control, an international PCR standard has now been validated by the World Health Organization to allow result comparison between the available PCR techniques (Chudy et al., 2013).

It is worth noting that the correlation between viral load and disease severity is not clear, being suggested in some cohorts, but not in others, possibly as a result of distinct patient characteristics, viral genotypes and errors in quantification (Brichler et al., 2013; Wedemeyer and Manns, 2010; Romeo et al., 2014; Braga et al., 2014).

3.4.2. Evaluation of liver disease

Liver disease grading and staging must be performed in all patients with HDV infection, in order to weight risks and benefits of interferon therapy. Liver biopsy, although not needed to establish the diagnosis of HDV infection, remains the gold standard for the staging of liver disease and delta antigen detection and is performed in most centers before treatment consideration (Wedemeyer and Manns, 2010). While non-invasive methods for fibrosis evaluation are increasingly used for other types of hepatitis (mostly HCV infection), their use in hepatitis delta still awaits validation. Liver elastography has been used by different groups for non-invasive fibrosis evaluation of HBV/HDV/HIV infected patients, although validation studies in HDV infected patients need to be performed (Castellares et al., 2008; Soriano et al., 2014). Furthermore, clinical scores currently used as indirect markers of fibrosis and predictors of outcome, are not validated in hepatitis delta patients. Part of these scores has shown poor performance in one hepatitis delta cohort, and a new Baseline Event Anticipation score (BEA) has been proposed, based on age, gender, geographical origin, International Normalized Ratio, platelet count and bilirubin (Calle Serrano et al., 2014).

3.5. Natural history and pathogenesis

3.5.1. Natural history

CHD is often considered as the most severe form of chronic viral hepatitis. HDV infected patients have a more aggressive clinical course than HBV mono-infected patients, with a three times higher risk of developing cirrhosis and a faster progression (Fattovich et al., 1987). The risk of cirrhosis increases overtime and has been reported to be of 23%, 41% and 77% after respectively 10, 20 and 30 years of infection (Yurdaydın et al., 2010). Clinical decompensation has been described as the major complication in cirrhotic CHD patients, with a more than 2-fold increased risk when compared to HBV mono-infection (Fattovich et al., 2000). The course of disease may be influenced by HDV genotype, with predominant genotype 1 being associated with more severe disease than genotypes 2 and 4 (Su et al., 2006). Genotype 3 has been associated with a particularly aggressive evolution (Casey et al., 1996). A role of HBV genotypes in the course of disease has also been suggested.

The association between CHD and HCC is still a matter of debate. Three major studies identified liver decompensation and death, and not HCC, as the major complication of CHD, and associated the development of HCC with persistent HBV replication (Romeo et al., 2009; Niro et al., 2010; Buti et al., 2011). However, in the Eurohep cohort, an increased risk of 3.2-fold was shown in CHD patients in comparison to CHB ones, with a 5-year risk of 13% (Fattovich et al., 2000). A more recent Swedish study also suggested a 6-fold increase in the risk of HCC in CHD patients (Ji et al., 2012). More studies are yet needed to further evidence the link between CHD and HCC.

3.5.2. Insights into fibrosis and carcinogenesis mechanisms

The pathogenesis of liver damage during HDV infection is still incompletely understood, and the relative contribution of direct pathogenic effect of HDV or immune-mediated injury is a matter of debate. A direct cytopathic effect of HDV antigens seems unlikely given the absence of liver damage upon its expression in transgenic mice (Guilhot et al., 1994). However, cell death in the presence of high levels of replication (replication-associated cytopathogenicity) has been observed *in vitro* and has been suggested to contribute to cases of acute/fulminant hepatitis, where cell death induced by high levels of replication might precede synthesis of L-HDAg (Chang et al., 2005). A direct implication of HDV proteins in liver pathogenesis can however be supported by studies that showed an interaction with cellular proteins and/or a modulation of cell signaling pathways. For a comprehensive review of HDV cell interactants refer to Greco-Stewart and colleagues (Greco-Stewart and Pelchat, 2010). Large transcriptomic and proteomic studies highlighted several possibilities. A combined proteomic RNAi screening identified 100 putative cell partners of S-HDAg, mostly involved in transcription and HDV replication, but consequences of such interactions for the host cell cannot be ruled out (Cao et al., 2009). A direct role of L-HDAg in fibrogenesis has been proposed, as L-HDAg was shown to activate TGF-β pathway. Moreover, this activation was dependent on isoprenylation and synergistic with HBx-mediated TGF-β and AP1 signal transduction (Choi et al., 2007). A similar synergism between HBx protein and L-HDAg has been described in the activation of the serum responsive element- dependent pathway (Goto et al., 2003).

The direct effect of HDV on cell proliferation and cancer has been long debated. Analysis of cell proteome changes in the presence of delta antigen or viral genome replication led to the identification of differentially expressed proteins mostly associated to cell cycle regulation and pyruvate metabolism and the suggestion of direct involvement of HDV in carcinogenesis promotion (Mota et al., 2008, 2009; Mendes et al., 2013). Using another screening approach, clusterin expression was shown to be up-regulated upon S-HDAg expression and HDV replication, possibly through histone hyper-acetylation, and to be associated with increased cell survival. These findings support an oncogenic potential of HDV, given that clusterin is not only involved in oncogenic pathways, but was also shown to be overexpressed in HCC tissues (Liao et al., 2009). NF-κB activity has also been shown to be modulated by HDV. Several mechanisms have been proposed, including L-HDAg direct interaction with TRAF-2 and potentiation of TNF-α induced NF-κB transcriptional activation (Park et al., 2009), induction of endoplasmic reticulum stress (Huang et al., 2006), and production of reactive oxygen species upon expression of L-HDAg (Williams et al., 2012).

3.5.3. Immune response and its modulation

As for other aspects of hepatitis delta, much remains to be explored regarding the immune response to HDV infection. Indeed, immune responses, both innate and adaptive, are believed to play an important role in liver damage and disease progression.

Regarding the innate immune response, results are conflicting between studies and the details of the interaction between HDV and hepatocyte innate immune response are only partially explored. Several studies support an activation of interferon signaling pathways by HDV: *in vitro*, HDV replication was first shown to be associated with increased expression of IFNβ (McNair et al., 1994); later the expression of HDAg was associated with an induction of MxA (an Interferon Stimulated Gene – ISG) (Williams et al., 2009) and recently misfolded HDV RNA was shown to directly activate PKR (Heinicke and Bevilacqua, 2012). *In vivo* HDV was also associated with an increased expression of ISGs, both in the hNTCP transgenic mouse and in the humanized mice model (He et al., 2015; Giersch et al., 2015). Paradoxically, in a study by Negro and colleagues, HDV replication was not associated with the expression of ISGs, but with an inhibition of the cell response to interferon, by interference with an early step of the JAK–STAT signaling pathway (Pugnale et al., 2009). Interestingly, this inhibition of interferon pathway was also suggested (although not thoroughly explored) in the aforementioned study in the humanized mouse model (Giersch et al., 2015).

The role of NK cells during HDV infection and treatment has recently been addressed. The number of NK cells was found to be increased in CHD patients in comparison to healthy controls, however, as for HBV and HCV, they presented a less activated phenotype and were less prone to stimulation (Lunemann et al., 2014). Little is also known about T-cell responses in HDV infection. HDV infected patients have a higher frequency of perforin-positive CD4+ T cells than patients with HBV or HCV, independently of the level of liver inflammatory activity, which may be a possible explanation for the faster progression of liver disease in CHD (Aslan et al., 2006). Specific HDAg epitopes were shown to induce CD4+ T cells expansion, but only in patients with inactive disease (Nisini et al., 1997). Interestingly, HDV CD8+ cytotoxic T lymphocyte responses were also only identified in patients with inactive disease (Huang et al., 2004). In the HIDIT-1 trial patients, a specific pattern of cytokines was shown to decrease during interferon treatment, being related to treatment response. Specific T cell response restoration was only observed in treatment responders (Grabowski et al., 2011).

3.5.4. Interplay between HDV and HBV

In the early characterization of HDV infection, it became evident that, despite its dependency on HBV for its envelope, HDV suppressed HBV replication in both humans and chimpanzees (Rizzetto et al., 1980b; Hadziyannis et al., 1985). The same effect was observed in WHV infected woodchucks and more recently in the HBV-infected humanized mouse model (Lütgehetmann et al., 2012; Negro et al., 1989). A study of HBV replication in the liver of HDV infected patients, indicated a significant decrease in the levels of HBV replicative intermediates, but not in the synthesis of envelope proteins, in comparison to HBV mono-infected patients (Pollicino et al., 2011). Interestingly, longitudinal follow-up of patients by qPCR, suggests that this pattern of interference is more complex, as viruses may alternate patterns of dominance over time (Schaper et al., 2010). As HBV viremia is an important predictor of disease progression in these patients, a close monitoring of both viral replication markers is warranted.

The mechanisms of viral interference in HBV/HDV infection are still incompletely known. This phenotype was confirmed *in vitro*, indicating its independence from the acquired immune system, and HBV suppression could be reproduced upon the isolated expression of HDAg (Wu et al., 1991). Indeed, one study proposes that both forms of delta antigen might inhibit HBV replication by decreasing HBV enhancer effect (L-HDAg could decrease Enh1 activation by 60–80%). Furthermore, the induction of MxA expression, whose product is known to be a repressor of HBV replication, that has been shown both *in vitro* and in the humanized mouse, could contribute to this phenotype (Williams et al., 2009; Giersch et al., 2015).

4. Prevention

Transmission of HDV can be greatly reduced by measures targeting the main exposure risks. As for other blood-borne virus, public health measures focused on a strict screening of blood and its fractionated products for HBV, practices aiming at reducing transmission among IVDUs and hygiene measures in limited resources settings, play an important role in limiting HDV transmission.

HBV vaccine is highly effective in preventing HDV transmission in naïve patients and should be proposed to all the close contacts of HDV infected patients. But no immunoprophylactic strategy exists for the prevention of HDV super-infection in HBV carriers; HBV vaccine does not elicit anti-HBs antibody production in these patients. Unfortunately, antibodies directed to HDAg have no strong neutralizing capacity in HDV infection, thus precluding any use of HD-Ag based vaccine. However, it has been shown for other viruses that, even though not providing sterilizing protective immunity, immunization with internal viral proteins can produce a T-cell response and prevent virus spreading. *In vitro* results were promising as stimulation of CD8 T cells with HDV peptides led to an increase on IFN gamma production and cytotoxic activity (Roggendorf, 2012). However, in woodchucks, vaccination (either by HDV proteins, DNA or recombinant viruses) was only effective in preventing HDV infection of naïve animals but not in WHV chronic carriers and, although the course of infection was modified, its utility is still to be proven (Fiedler et al., 2013, 2001).

5. Treatment

5.1. Current available therapeutic strategies

There are currently no specific direct antiviral treatments for HDV. This is mainly due to the fact that the virus does not encode enzymatic activities, and fully relies, even more than other viruses, on host-cell machinery for its replication. A better understanding of the molecular and cellular biology HDV is yet required to identify viro-modified host cell functions that could be targeted.

In the setting of acute hepatitis delta, no specific treatment has proven useful and patient management relies on monitoring and general support measures, or referral for liver transplantation in patients who progress to fulminant hepatitis (Niro et al., 2005).

Interferon alpha (IFNα) remains the only drug recommended by international guidelines for the treatment of CHD (European Association for the Study of the Liver, 2012; Lok and McMahon, 2009). Both conventional and pegylated interferon (Peg-IFNα) have been shown to suppress HDV viremia in a subset of patients. Sustained virologic response (SVR) rates vary between 14 to 50% for conventional IFNα and 17 to 44% for Peg-IFNα (Yurdaydin, 2012). However, comparison between studies is difficult as methods, treatment schedules, doses and duration used were highly variable and the number of patients included is very small. Several questions relative to interferon treatment remain unanswered. Firstly, the mechanism of action of IFNα on the control of HDV infection is not completely understood. *In vitro* studies have failed to demonstrate a potent effect of IFNα on HDV

replication (McNair et al., 1994), whereas in the hNTCP transgenic mouse model a suppression of IFN endogenous IFNα/β stimulation was associated with an increased viral replication, suggesting an antiviral effect of the molecule (He et al., 2015). Indeed, IFNα could play an indirect role in the regulation of HDV replication through ADAR-mediated editing (Hartwig et al., 2004). More recent evaluations have suggested that IFNα may also mediate its action via inhibition of viral entry. This was also inferred from mathematical modeling based on the kinetics of the IFNα effect (Han et al., 2011; Goyal and Murray, 2015). Secondly, unlike what has been described for HCV infection, no impact of IL-28b polymorphisms in treatment response has been demonstrated (Visco-Comandini et al., 2014; Yilmaz et al., 2014). Thirdly, the optimal duration of treatment has not been defined. It is currently advised to treat HDV infected patients for at least one year. Although cases have been reported of patients under interferon treatment for up to 12 years, the benefit of prolonged IFN therapy is still debated. The only study that compared 1 *versus* 2 years treatment duration found no advantage on treatment prolongation but was limited by the small size of the study population (Ormeci et al., 2011). Recently, two large clinical trials addressed the question of late relapses after interferon treatment of CHD patients. In the first study (HIDIT-1), patients were treated for 48 weeks with Peg-IFNα plus adefovir or either drug alone. At 24 weeks post-treatment, 28% of interferon treated patients had undetectable HDV viremia (Wedemeyer et al., 2011). However, long term follow-up of these patients showed that late HDV RNA relapses occurred in 56% of the patients, compromising the use of the 24 weeks SVR end-point in CHD patients (Heidrich et al., 2014). A second study (HIDIT-2) aimed to evaluate a possible benefit of prolonging therapy in order to avoid relapses. Patients were treated for 96 weeks with Peg-IFNα (associated or not with tenofovir). Although at the end of treatment 33 *versus* 47% of the patients receiving Peg-IFNα (without and with tenofovir respectively; non-significant difference) had undetectable viremia, at 24 weeks post treatment, this proportion significantly decreased to 23–30%. The fact that this values are comparable to the ones obtained for HIDIT-1 (48 weeks treatment) and that adverse events occurred in more than 30% of patients, suggests that extending IFN therapy for more than 1 year does not have a clear benefit (Wedemeyer et al., 2014).

Another unsolved issue refers to the utility of anti-HBV direct-acting antivirals (DAA) in the management of chronic hepatitis delta patients. Although anti-HBV DAAs do not seem to affect HDV replication (Alfaiate et al., 2014), an impact on HDV infection can be expected from the continuous suppression of HBsAg production, leading to a blockage of HDV spreading. However, the persistence of HDV infection in the absence of its helper virus is still debated. Data from different experimental models suggest that HDV mono-infection is self-limiting, in the absence of spreading. Such a profile has been described both *in vitro*, *i.e.* in PHH, and *in vivo*, either following hydrodynamic injection of HDV cDNA into wild type mice or, more recently, following HDV infection of transgenic mice expressing hNTCP (Gudima et al., 2007; He et al., 2015; Chang et al., 2001). It is tempting to postulate that, in the absence of viral particle egress, accumulation of L-HDAg throughout time would ultimately lead to the inhibition of HDV replication (Modahl and Lai, 2000). Furthermore, it has been suggested that, in the absence of particle budding, continuous genome editing, associated with the accumulation of mutations, may also contribute to such abrogation of replication over time (Chang et al., 2005). Such scenario, combined with the lack of evidence supporting latent HDV infection in transplanted patients (Smedile et al., 1998), supports an essential role for HBV in the maintenance of a chronic HDV infection and would support the utility of HBV DAAs in patients with CHD. However, data suggesting that HDV can indeed establish a latent infection in the absence of HBV have also been reported. Following *in vitro* HDV infection of both primary human and chimpanzee hepatocytes, in particular culture conditions, HDV replication was kept at stable levels for more than one month (Barrera et al., 2004; Sureau et al., 1991). More recently, in the humanized liver mouse model, HDV infection was shown to be kept latent for at least 6 weeks, in the absence of HBV (Giersch et al., 2014). In this scenario, the utility of HBV DAAs in the control of HDV infection would be more limited, as in the presence of continuous HDV replication, the virus would only be eliminated by the turnover of infected hepatocytes overtime.

Data from patients confirm a limited utility of HBV DAAs in the control of HDV infection, in association with modest HBsAg decrease rates. Lamivudine, adefovir and entecavir did not prove effective against HDV (Yurdaydin, 2012). However, studies from HIV/HBV/HDV co-infected patients suggested that long-treatment with tenofovir not only suppressed HBV but also led to a decrease of HDV replication parameters and improvement of liver disease (Soriano et al., 2014; Boyd et al., 2013). The aforementioned HIDIT-1 and 2 trials failed to show a significant increase in Peg-IFNα treatment effectiveness upon association with adefovir or tenofovir, respectively. A beneficial effect of the addition of adefovir on the levels of HBsAg levels was observed in HIDIT-1 but not confirmed with tenofovir in HIDIT-2. Furthermore, no significant decrease in the levels of transaminases was observed during DAA treatment, suggesting that, HDV replication needs to be specifically targeted in order to prevent liver damage (Wedemeyer et al., 2011, 2014).

Other antiviral molecules like ribavirin and clevudine also failed to prove effective in human clinical studies, despite promising results in experimental models (Yurdaydin, 2012).

Management of chronic hepatitis delta hence remains mostly empiric and should be decided for each individual patient. As for HBV, only HBsAg sero-conversion (and not undetectable HDV viremia, even if persistent) is synonymous of hepatitis delta cure, making close monitoring of HDV and HBV infection markers mandatory during and after treatment.

5.2. Investigational drugs in preclinical/ clinical development

Given the particular characteristics of HDV, replication remains difficult to target. Ongoing development of anti-HDV drugs is hence focusing on other steps of the viral cycle, namely entry and assembly. Novel strategies based on immune-stimulation with cytokines or Pattern Recognition Receptor (PRR) agonists may also play a role in the future management of HDV infection.

5.2.1. Entry inhibition

As for other viruses, several therapeutic strategies can be used to target HDV attachment and cell entry and hence prevent the infection of new cells. After identification of myristoylation of the PreS1 region as essential for viral entry, peptides mimicking the myristoylated N-terminal 47 aminoacids of L-HBsAg were synthetized and shown to potently and specifically, inhibit HBV entry *in vitro* (Gripon et al., 2005). Later on, a lead substance consisting of the consensus sequence of all known primate HBV genotypes (later named Myrcludex-B®) showed a very potent inhibition of HBV infection (EC_{50} 80 pM) *in vitro* (Schulze et al., 2010). Studies in the humanized mouse model confirmed the inhibitory effect of Myrcludex-B® both in the establishment of *de novo* HBV and HDV infections and in the spreading of previously established infections (Lütgehetmann et al., 2012; Volz et al., 2013). Besides blocking viral entry, Myrcludex-B® also blocks bile acid transport (NTCP's physiological function). Although the clinical repercussions of this side effect remain to be identified, they may not be significant as the EC_{50} of the drug for viral entry inhibition is

almost 1000 times lower than the EC_{50} for inhibition of bile salt uptake (Nkongolo et al., 2014). After safety and tolerability testing first in chimpanzees and then in a phase I clinical trial, Myrcludex-B® is currently undergoing a phase IIa trial. Preliminary results showed a decrease in both HBV and HDV viremia at week 24 of treatment (Bogomolov et al., 2014).

Other molecules have been shown to inhibit HBV/HDV NTCP-mediated entry *in vitro*. Cyclosporin A has recently been identified by two different teams as an inhibitor of HBV/HDV entry through binding to NTCP, in a cyclophilin-independent manner (Nkongolo et al., 2014; Watashi et al., 2014). As cyclosporine A is often a part of the immunosuppressor combination used in the post-liver transplantation setting, it is tempting to consider its potential role to prevent the graft re-infection. Other NTCP inhibitors already approved for other indications, as ezetimibe, irbesartan and ritonavir, have been shown to inhibit early stages of HDV infection, although the mediation through NTCP for this effect is still to be demonstrated (Blanchet et al., 2014).

Anti-viral strategies also explore the interaction between HBsAg and HSPGs. Suramin would be a candidate but its use is limited by toxicity (Urban et al., 2014). A new class of agents has recently been shown to be effective, not only against HBV, but also HCV, HSV 1 and 2 and HIV (Krepstakies et al., 2012).

5.2.2. Assembly inhibition

As previously described, HDV assembly depends on the prenylation (more specifically farnesylation) of the last four amino acids of L-HDAg (CXXQ motif) (Glenn et al., 1992). Prenylation inhibitors have been used as anticancer drugs, as oncogenic forms of Ras are dependent on farnesylation. These molecules were shown to inhibit HDV assembly and secretion (but not replication) both *in vitro* and in a mouse model capable of producing HDV viremia (Bordier et al., 2003, 2002). Results from a phase 2a clinical trial conducted in adult CHD patients have recently been reported. The patients received the drug for 28 days and were then followed for 6 months. During treatment, lonafarnib was associated with a dose-dependent, albeit modest (−1.54 log IU/ml for the higher dose), decrease in HDV viremia. However, treatment had no effect on transaminases and, once stopped, a rebound of HDV viremia was observed in all patients, emphasizing the need for further optimization and development of anti-HDV therapeutic strategies (Koh et al., 2015).

As for entry inhibition strategies, progress can arise from HBV research. Promising results have been reported for the inhibition of DHBV infection by nucleic-acid-polymers (NAPs). These oligonucleotides interact with structurally conserved amphipathic alpha-helical protein domains found in a variety of virus and other infectious agents and are known to concentrate in the liver. These molecules have been shown to inhibit DHBV infection *in vitro* and *in vivo* (Noordeen et al., 2013a,b). Preliminary works suggest an effect on HBV infection. The mechanism of action, although not fully elucidated, seems to be associated with both the inhibition HBsAg-mediated entry and release, and opens a perspective for their use in hepatitis delta (Noureddin and Gish, 2014).

5.2.3. Immunomodulation

New immune-modulatory agents, such as interferon lambda and PRR agonists (Zeisel et al., 2015), are proving effective against HBV infection. TLR7 agonist GS9620 is particularly promising as it has been shown to have a potent antiviral response against HBV (with HBsAg loss and reduced amounts of cccDNA), both in the chimpanzee and in the woodchuck model (with WHV) (Lanford et al., 2013; Menne et al., 2015). A phase II clinical trial is currently underway (clinicaltrials.gov NCT2430181). Although its action on HDV infection is for the moment unknown, it is tempting to speculate that these drugs may also have a role in the future treatment of hepatitis delta.

6. Conclusion

In spite of its severity, the overall high number of infected patients, and the viral characteristics that make HDV a unique model of study, HDV infection can unfortunately be considered as a neglected tropical and non-tropical disease. Many questions are still unanswered regarding clinical issues, as well as more basic knowledge regarding the virus life cycle and its interactions with the host. Novel interest on HDV seems to be arising, as new antiviral strategies are being developed against HBV infection. Hopefully the newly developed animal and cell culture models will boost research on the mechanisms of HDV infection and pathogenesis, and will contribute to the development of novel HDV-specific antiviral strategies.

Acknowledgements

We would like to thank Frédéric Le Gal, Natali Abeywickrama Samarakoon, Jean-Claude Cortay and Camille Sureau for helpful discussions. Dulce Alfaiate was supported by Grants from Fundação Calouste Gulbenkian and Fundação para a Ciência e a Tecnologia. DD and PD were supported by Grants from ANRS (French national agency for research on AIDS and viral hepatitis; several Grants from CSS4), FINOVI (Foundation for innovation in infectiology; project call n°#4), FRM (Foundation for medical research; DEQ20110421327), Hoffmann-La-Roche Ltd (Switzerland division) and by INSERM core Grants and interface contract (PD). DD is also supported by the DEVweCAN LABEX (ANR-10-LABX-0061) of the "Université de Lyon", within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR).

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2. Research paper submission

Elsevier Editorial System(tm) for Journal of Hepatology

Manuscript Draft

Manuscript Number:

Title: HDV replication is associated with HBV repression in superinfection, induction of interferon response and can be inhibited by immune-modulators

Article Type: Original Article

Keywords: Hepatitis D virus; hepatitis B virus; viral interference; IFN response; immune-modulators.

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Abstract: Background & Aims: Hepatitis D virus (HDV) superinfection of Hepatitis B virus (HBV)-infected patients is the most aggressive form of viral hepatitis. HDV infection is not susceptible to direct anti-HBV drugs, and only suboptimal antiviral responses are obtained with interferon (IFN)-alpha-based therapy. Our aim was to get insights on HDV replication and interplay with HBV in physiologically-relevant hepatocytes and evaluate the potential anti-HDV effect of immune-modulators.

Methods: Differentiated HepaRG (dHepaRG) cells were infected with HDV both in the presence and absence of a previous HBV infection and viral markers were extensively analyzed. IFN response to HDV was monitored by measuring pro-inflammatory and interferon-stimulated gene (ISG) expression. In addition, infected cells were treated with various pathogen recognition receptors (PRR) agonists to evaluate their anti-HDV activity.

Results: Both mono- and superinfected dHepaRG cells supported a strong but transient HDV replication, accompanied by the secretion of HDV virions only in the coinfection setting. Upon HDV superinfection, HBV replication markers including HBeAg, total HBV-DNA and pregenomic RNA were significantly decreased, confirming viral interference of HDV on HBV. No decrease of circular covalently closed HBV DNA (cccDNA) and HBsAg levels occurred. Concomitant to the peak of HDV-RNA accumulation and the onset of interference on HBV replication, a strong type I IFN response was observed at day-6 post HDV infection. Importantly, PRR agonists were found more efficient than IFN-α to impede HDV replication, the best being Pam3CSK4 engaging the NF-κB pathway via TLR1/2.

Conclusions: We established a relevant cellular model to further characterize HBV/HDV direct and/or indirect interplay and test novel HDV

inhibitors, including immune-modulators. A suppressive role of HDV on HBV replication could convincingly be confirmed, whereas an IFN response to HDV infection was clearly evidenced. This model is contributing to assess molecular and immunological mechanisms of this viral interference.

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conflict of interest due to similar resaerch programs

3. Congress presentations

1. EASL Monothematic Conference – Translational Research on Chronical Viral Hepatitis

Lyon, 28-30 November 2014

Poster: FULMINANT HEPATITIS B AND DELTA HEPATITIS OUTBREAK IN THE MID-EIGHTIES IN CENTRAL AFRICAN REPUBLIC (CAR): FOCUSING ON SHDAG

Dulce Alfaiate, Natali Abeywickrama-Samarakoon, Sumantra Ghosh, Gina Laghoe, Ségolène Brichler, Christian Trépo, Emmanuel Gordien, Fabien Zoulim, Narcisse P. Komas, David Durantel, Jean Claude Cortay, & Paul Dény

2. 14ème Réunion du Réseau National Hépatites de l'ANRS

Paris, 23-24 January 2014

Poster: STUDYING RECIPROCAL VIRAL INTERPLAY BETWEEN HBV AND HDV IN CO-INFECTED HEPARG CELLS

Dulce Alfaiate, Natali Abeywickrama-Samarakoon, Barbara Testoni, Marion Gruffaz, Jean Claude Cortay, Fabien Zoulim, David Durantel and Paul Dény

3. EASL 2014 International Liver Congress

London, 9-13 April 2014

Poster: STUDYING RECIPROCAL VIRAL INTERPLAY BETWEEN HBV AND HDV IN CO-INFECTED HEPARG CELLS

Dulce Alfaiate, Natali Abeywickrama-Samarakoon, Barbara Testoni, Marion Gruffaz, Jean Claude Cortay, Fabien Zoulim, David Durantel and Paul Dény

4. 2nd Silibinin Workshop

Cologne, 23 May 2014

Oral Communication: SILIBININ AS AN ENTRY INHIBITOR OF HEPATITIS B AND DELTA VIRUSES: WHAT DO WE LEARN FROM HEPATITIS C VIRUS INFECTION?

Julie Blaising, Claire Gondeau, Dulce Alfaiate, Chloé Montès, Emma Reungoat, Camille Sureau, Fabien Zoulim, Eve-Isabelle Pécheur

5. HBV International Meeting 2014

Los Angeles, 3-6 September 2014

Poster: STUDY OF THE VIRAL INTERPLAY BETWEEN HEPATITIS B VIRUS (HBV) AND HEPATITIS DELTA VIRUS (HDV) AND EVALUATION OF ANTIVIRAL THERAPIES IN HEPARG CELL LINE SUPER-INFECTION MODEL

Dulce Alfaiate, Natali Abeyvikrama-Samarakoon, Barbara Testoni, Julie Lucifora, Fabien Zoulim, Jean Claude Cortay, Paul Dény and David Durantel

Poster: MID-EIGHTIES HEPATITIS B AND DELTA FULMINANT HEPATITIS OUTBREAK IN CENTRAL AFRICAN REPUBLIC (CAR): REASSESSMENT OF VIRAL STRAINS FROM A LARGE COHORT AND COMPARISON WITH 2010 STRAINS (ANRS 12202)

Sumantra Ghosh, Dulce Alfaiate, Natali Abeywickrama Samarakoon, Ségolène Brichler, Gina Laghoe, Mariama Abdou-Chekaraou, Jean-Omer Ouavene, Jean Louis Lesbordes, Christian Trépo, Olivier Hantz, Alan Kay, David Durantel, Fabien Zoulim, Emmanuel Gordien, Jean-Claude Cortay,

Narcisse P. Komas* & Paul Dény*

6. 75èmes Journées Scientifiques de l'AFEF

Paris, 24-26 September 2014

Oral communication: MODELE CELLULAIRE DE SUPER-INFECTION HBV/HDV POUR L'ETUDE MOLECULAIRE DES MECANISMES D'INTERFERENCE VIRALE ET L'EVALUATION D'ANTIVIRAUX

Dulce Alfaiate, Natali Abeyvikrama-Samarakoon, Barbara Testoni, Julie Lucifora, Fabien Zoulim, Jean Claude Cortay, Paul Dény and David Durantel

7. 3rd Antivirals Congress

Amsterdam, 12-14 October 2014

Poster: MODEL OF HBV/HDV SUPER-INFECTION TO STUDY MECHANISMS OF VIRAL INTERPLAY AND ANTIVIRALS

Dulce Alfaiate, Natali Abeyvikrama-Samarakoon, Barbara Testoni, Julie Lucifora, Fabien Zoulim, Jean Claude Cortay, Paul Dény and David Durantel

8. AASLD - The Liver Meeting 2014

Boston, 7-11 November 2014

Poster: MODEL OF HBV/HDV SUPER-INFECTION TO STUDY MECHANISMS OF VIRAL INTERPLAY AND ANTIVIRALS

Dulce Alfaiate, Natali Abeyvikrama-Samarakoon, Barbara Testoni, Julie Lucifora, Fabien Zoulim, Jean Claude Cortay, Paul Dény and David Durantel

9. XII Congresso Nacional de Doenças Infecciosas e Microbiologia Clínica

Lisbon, 1-3 December 2014

Oral communication: ABORDAGEM EXPERIMETAL DA SUPERINFECÇÃO PELOS VIRUS DA HEPATITE B E HEPATITE DELTA

Dulce Alfaiate, Natali Abeywickrama-Samarakoon, Barbara Testoni, Julie Lucifora, Fabien Zoulim, Jean-Claude Cortay, Paul Dény and David Durantel

9. 15th International Symposium on Viral Hepatitis and Liver Diseases

Berlin, 26-28 July 2015

Oral Communication: STUDY OF THE SUPERINFECTION BY HBV AND HDV IN THE HEPARG CELL MODEL

Dulce Alfaiate, Natali Abeywickrama-Samarakoon, Barbara Testoni, Julie Lucifora, Fabien Zoulim, Jean-Claude Cortay, Paul Dény and David Durantel

4. Curriculum vitae

Dulce Alfaiate
PhD candidate - Virology
MD - Infectious Diseases

42 avenue Félix Faure
69003 Lyon, France
+33 6 49 10 15 64
dulcealfaiate@gmail.com
https://fr.linkedin.com/in/dulcealfaiate

Professional Experience

Research Experience

Nov 2011- Present - Cancerology Research Center of Lyon (CRCL), Lyon, France
PhD Student
Subject: Study of the interactions between hepatitis B (HBV) and hepatitis Delta viruses (HDV) and the hepatocytic innate immune system
Supervision: Professor Paul Dény and Doctor David Durantel

Jan 2010 - Mar 2010 - INSERM U871, Lyon, France
Research Intern
Subject: Phenotypical characterization of HBV resistance mutants
Supervision: Professor Fabien Zoulim

2009 – Molecular biology laboratory, Hospital de Egas Moniz, Lisbon, Portugal
Medical resident
Subject: Evaluation of HIV drug-resistance mutations in clinical isolates
Supervision: Doctor Ricardo Camacho

Sep 2005- Jul 2011 – Infectious Diseases Department, Hospital de Egas Moniz, Lisbon, Portugal
Co-investigator
Multicenter international phase II and phase III clinical trials.

Clinical Experience

Jul 2011- Nov 2011 – Hospital de Egas Moniz, Lisbon, Portugal
Infectious Diseases Consultant
Certified by the Infectious Diseases Board of the Portuguese Medical Association in July 2011.

Sep 2005- Jul 2011 – Lisbon, Portugal and Lyon, France
Infectious Diseases Resident
Rotations in Internal Medicine, Infectious Diseases, Emergency Medicine, Intensive Care, Dermatology, Microbiology, Virology and Hepatology.

Jan 2004- Sep 2005 - Hospital de Egas Moniz, Lisbon, Portugal
Medical Intern
Rotations in Internal Medicine, Infectious Diseases, General Surgery, Pediatrics, Obstetrics and Gynecology and Primary Care.
Registered as a physician to the Portuguese Medical Association in October 2003 (#42952).

Teaching Experience

Sep 2005 - Oct 2011 – Department of Physiology, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal
Invited Lecturer
Responsible for Physiology practical classes, seminars and evaluation of 2nd year medical students (12 hours per week).

Skills

Scientific practice

- ***Experiment*** design, execution and interpretation;
- Project and paper ***writing***;
- Result discussion and ***communication***;
- ***Interaction*** with collaborators;
- Student ***training***.

Laboratory techniques

- ***Cell biology and virology:*** cell culture of immortalized and primary hepatic cells, isolation and culture of liver non-parenchymal cells, generation of inducible cell lineages by lentiviral transduction, *in vitro* production and characterization of HBV and HDV virions, establishment and follow-up of in vitro infection;
- ***Molecular biology and biochemistry:*** nucleic acid extraction, qPCR, qRT-PCR, Northern, Southern and Western Blot, ChIP, Immunofluorescence;
- ***Antiviral drug testing***.

Clinical practice

- ***Patient management*** (ward, consultation and ER);
- Management of ***HIV infection*** and opportunistic diseases, chronic viral ***hepatitis***, ***tuberculosis***, nosocomial infections and ***tropical diseases***;
- Patient enrolment and follow-up in ***clinical trials***;
- ***Training*** of younger residents, interns and medical students.

Languages

- ***Portuguese:*** native language;
- ***English:*** proficient user (C);
- ***French:*** proficient user (C);
- ***Spanish:*** independent user (B);
- ***German:*** beginner (A).

Informatics

- Good command of Windows and Mac operative systems, MS Office tools, Prisma GraphPad, ImageJ;
- Basic knowledge of SPSS.

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Dulce Alfaiate, MD, PhD candidate

Education

Most relevant

Oct 2010 – Mar 2011 - Fundação Calouste Gulbenkian, Lisbon, Portugal
Doctoral program for physicians
Full-time courses (1082 hours) with portuguese and foreign researchers on: Mollecular and Cell Biology, Structural Biology, Genetics, Immunology, Oncology, Infection, Neuroscience, Regenerative Medicine, Evolution, Biostatistics, Epidemiology, Computational Biology and Scientific Integrity. Evaluation by a board of academic referees, based on the proposition of a PhD project and the identification of a host laboratory.

Oct 1997- Oct 2003 – Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal
Medical Degree
Cum laude

Others

Apr 2009 – Harvard Medical School/ Massachusetts General Hospital, Boston, USA
Post-Graduate Course: Infectious Diseases of Adults

July 2008 – European Society of Clinical Microbiology and Infectious Diseases, Regensburg, Germany
7th ESCMID Summer School

May 2008 - July 2008 – Institute of Hygiene and Tropical Medicine, New University of Lisbon, Lisbon, Portugal
Post-Graduate Course: Medical Parasitology

Feb 2007- July 2007 – Institute of Hygiene and Tropical Medicine, New University of Lisbon, Lisbon, Portugal
Post-Graduate Course: Clinics of Tropical Diseases

Publications

Alfaiate D, Dény P, Durantel D. Hepatitis Delta virus: from biological and medical aspects to current and investigational therapeutic options. Antiviral Research 2015 Aug 11; 122: 112-129.

Submitted

Alfaiate D, Michelet M, Samarakoon N, Maadadi S, Cortay JC, Sureau C, Zoulim F, Lucifora J, Dény P, Durantel D. HDV replication is associated with repression of HBV markers and induction of interferon response and can be inhibited by immune-modulators.

Others

Four papers published in portuguese peer-reviewed journals;

Invited author of one ***book chapter***.

Oral presentations (most relevant)

Jun 2015 – *International Symposium on Viral Hepatitis and Liver Diseases*, Berlin, Germany
Sep 2014 - *75èmes Journées Scientifiques de l'AFEF*, Paris, France
Jun 2008 - 7th ESCMID Summer School; Regensburg, Germany

Poster presentations (most relevant)

Nov 2014 – *AASLD The Liver Congress 2014*, Boston, USA
Oct 2014 – *3rd Antivirals Congress*, Amsterdam, Netherlands
Sep 2014 - *HBV International Meeting 2014*, Los Angeles, USA
Apr 2014 - *EASL 2014 International Liver Congress*, London, UK
Jan 2014 - *14ème Réunion du Réseau National Hépatites de l'ANRS*, Paris, France
Nov 2013 - *EASL Monothematic Conference -Translational Research on Chronic Viral Hepatitis*, Lyon, France
Nov 2010 - *10th International Congress on Drug Therapy in HIV Infection*, Glasgow, UK
May 2009 – *8th Congress of the European Federation of Internal Medicine*, Istanbul, Turkey
Mar 2008 - *6th European HIV Drug Resistance Workshop*, Budapest, Hungary

Achievements

Fellowships

Jan 2012 – Fundação para a Ciência e Tecnologia, Lisbon, Portugal
PhD studentship
4-year full-time fellowship

Oct 2010 – Fundação Calouste Gulbenkian, Lisbon, Portugal
PhD research grant

May 2009 - Portuguese Group for the study of HIV/ Hepatitis co-infection, Lisbon, Portugal
Resident research fellowship

Congress awards and grants

Jun 2015 – *International Symposium on Viral Hepatitis and Liver Diseases*, Berlin, Germany
Young investigator bursary

Dec 2014 – *Congresso Nacional Doenças Infecciosas e Microbiologia Clínica*, Lisbon, Portugal
Best poster award

Sep 2014 – *75èmes Journées Scientifiques de l'AFEF*, Paris, France
Young investigator bursary

Sep 2014 – *HBV International Meeting 2014*, Los Angeles, USA
Young investigator bursary

Apr 2014 – *EASL 2014 International Liver Congress*, London, UK
Young investigator registration bursary

Nov 2013 - *EASL Monothematic Conference on Translational Research on Chronic Viral Hepatitis*, Lyon, France
Young investigator registration bursary

Organization

Portuguese Group for the study of HIV/ Hepatitis co-infection
Co-founder

CRCL Young researchers committee
Board member

Other interests

Voluntary work **with the French Red Cross;**

Passionate by *travelling*, *reading*, learning *languages* and discovering other *cultures* and *foods*;

Jogging **and *trekking*.**