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Exosomes as a potential novel mechanism for HDV transmission independent of HBV infection

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Abstract

Hepatitis delta virus (HDV) is the smallest known human-infecting RNA virus, first described in the late 1970s. HDV infection requires the Hepatitis B virus (HBV) for its replication, with the HBV/HDV superinfection being the most severe form of chronic viral hepatitis due to more rapid progression towards liver-related death. Hepatitis viral infection increases the risk of developing Hepatocellular Carcinoma (HCC) and is a major etiological factor in a large proportion of HCC cases – it was estimated that HBV/HDV co-infection is responsible for nearly 20% of liver disease and liver cancer cases. The association between these two viruses is explained by the fact that HDV does not encode envelope proteins for packaging and relies on the HBV surface glycoproteins for virion assembly and cellular transmission. However, HDV RNA genome can efficiently replicate in different tissues and species, raising the possibility that HDV can be transmitted independently of HBV. It was recently demonstrated that enveloped viruses distinct from HBV can induce HDV spread *in vivo*, suggesting the existence of alternative pathways for HDV to disseminate.

In this work, we analysed the existence of exosomes containing HDV components that would allow HDV to infect liver cells in the absence of HBV. Exosomes are membrane-derived nanovesicles secreted by both normal and malignant cells that are currently recognized as critical structures in intercellular communication through horizontal transfer of information via their cargo, which includes selective proteins, mRNAs and miRNAs. Interestingly, exosomes present in the sera of chronic HBV patients contain HBV nucleic acids and proteins and are able to deliver HBV to healthy hepatocytes in an active manner.

Two human cell lines, Huh7-D12 and its parental cell line, the human hepatocarcinoma Huh7 were used to purify exosomes. Huh7-D12 replicates the HDV genome and constitutively expresses all HDV components. Supernatants of HDV-expressing cells were transferred to human cells that do not express HDV and HDV expression was analysed by PCR. Analysis of the RNA and protein composition of these exosomes was also performed, including a proteomic analysis of Huh7-D12-derived HDV-containing exosomes.

Supernatants from exponentially grown Huh7-D12 and Huh7 cells were used to isolate and purify exosomes using standard commercial kits and protocols. We show that HDV genomic RNA is present in exosomes from Huh7-D12 cells, along with important human RNAs (GAPDH and snRNPs). Exosome-containing Huh7-D12 supernatants were transferred to non-expressing HDV cells, which were subsequently observed to express HDV components.

Exosomes participate in cell-to-cell communication by transporting molecules to recipient cells near or distant from the original cells. Besides being involved in normal physiological processes, they are also associated with the development and progression of many diseases including cancer. Hepatitis viruses use exosomes to transfer viral genetic material, signaling-competent proteins and functional RNAs to uninfected cells to facilitate viral spread. We conclude that Huh7-D12 cells secrete exosomes that contain the HDV genomic RNA and that HDV-containing exosomes may constitutive an alternative mechanism of HDV dissemination independent of HBV.

Keywords: **Hepatitis Delta Virus, Exosomes, Viral Dissemination, Hepatocellular Carcinoma.**

Resumo

O Vírus da Hepatite Delta (HDV) é o mais pequeno vírus de RNA conhecido capaz de infetar humanos. A infeção por HDV requer a presença do Vírus da Hepatite B (HBV) para a sua transmissão, sendo a superinfeção por HBV/HDV a forma mais severa de hepatite viral crónica devido à rápida progressão para morte relacionada com doença do fígado. A infeção por hepatite viral aumenta o risco de desenvolver Carcinoma Hepatocelular (HCC) e é o maior fator etiológico na elevada proporção de casos de HCC – estima-se que a coinfecção HBV/HDV é responsável por aproximadamente 20% dos casos de doença hepática e cancro do fígado. A associação entre estes dois vírus explica-se pelo facto de o HDV não codificar proteínas do envelope para o empacotamento e depender das glicoproteínas de superfície do HBV para a montagem do virião e transmissão celular. Contudo, o genoma de RNA do HDV consegue replicar-se eficientemente em diferentes tecidos e espécies, levantando a possibilidade de que o HDV pode ser transmitido independentemente do HBV. Foi recentemente demonstrado que vírus envelopados distintos do HBV podem induzir a propagação do HDV *in vivo*, sugerindo a existência de vias alternativas para a disseminação do HDV.

Neste trabalho, analisamos a existência de exossomas contendo componentes de HDV e que permitiriam ao HDV infetar células hepáticas na ausência do HBV. Exossomas são nanovesículas membranares secretadas por células normais e malignas e são atualmente reconhecidos como estruturas críticas na comunicação intercelular por meio da transferência horizontal de informação através da sua carga, que inclui proteínas seletivas, mRNAs e miRNAs. Curiosamente, exossomas presentes no soro de pacientes cronicamente infetados com HBV contêm ácidos nucleicos e proteínas de HBV, e são capazes de transmitir o vírus a hepatócitos saudáveis.

Para a purificação de exossomas foram usadas duas linhas celulares humanas, Huh7-D12 e a sua linha celular parental, Huh7. Huh7-D12 replica o genoma do HDV e expressa constitutivamente todos os seus componentes. Sobrenadantes de células que expressam o HDV foram transferidos para células humanas que não expressam HDV e a expressão do vírus foi analisada por PCR. A análise do RNA e da composição proteica desses exossomas foi também realizada.

Sobrenadantes de células Huh7-D12 e Huh7 crescidas exponencialmente foram usados para isolar e purificar exossomas usando *kits* e protocolos comerciais padrão. Mostramos que o RNA genómico de HDV está presente em exossomas de Huh7-D12, assim como importantes RNAs humanos (GAPDH e snRNPs). Os sobrenadantes de Huh7-D12 contendo HDV foram transferidos para células que não expressam HDV, as quais foram posteriormente observadas a expressar componentes de HDV.

Os exossomas participam na comunicação célula a célula transportando moléculas para células recetoras. Além de estarem envolvidos em processos fisiológicos normais, também estão associados ao desenvolvimento e progressão de muitas doenças, incluindo cancro. Os vírus de hepatite usam exossomas para transferir material genético viral, proteínas sinalizadoras e RNAs funcionais para células não infetadas, facilitando a propagação viral. Concluímos que células Huh7-D12 secretam exossomas que contêm o RNA genómico de HDV e que exossomas contendo HDV podem constituir um mecanismo alternativo de disseminação do HDV independente do HBV.

Palavras-chave: **Vírus da Hepatite Delta, Exossomas, Disseminação Viral, Carcinoma Hepatocelular.**

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Abbreviation list

˚C – Degrees Celsius

a.a. – Amino acid

ADAR1 – Double-stranded RNA-specific adenosine deaminase 1

Ago2 – Argonaut RISC catalytic component 2

bp – Base pairs

CCD – Coiled-Coil Domain

CCND1 – Cyclin D1

cDNA – Complementary DNA

CHD – Chronic Hepatitis Delta

cm – Centimetre

cm^2 – Square centimetre

CO_2 – Carbon dioxide

DNA – Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

ER – Endoplasmic Reticulum

***et al* –** *et alia*

FBS – Foetal Bovine Serum

Fwd – Forward

GAPDH – Glyceraldehyde-3-phosphate Dehydrogenase

h – Hour

HBsAgs – Hepatitis B surface Antigens

HBV – Hepatitis B Virus

HCC – Hepatocellular Carcinoma

HCV – Hepatitis C virus

HDAgs – Hepatitis Delta Antigens

HDV – Hepatitis Delta Virus

hnRNP K – Heterogeneous nuclear ribonucleoprotein K

HRP – Horseradish peroxidase

HSP90 – Heat shock protein 90

HSPGs – Heparin Sulphate Proteoglycans

Huh7 – Hepatocellular carcinoma cell line

Huh7-D12 – Hepatocellular carcinoma cell line stably transfected with pSVL(D3) plasmid.

IFNα – Interferon alpha

kDa – Kilo Dalton

LB – Luria-Bertani

L-HBsAg – Large Hepatitis B Surface Antigen

L-HDAg – Large Hepatitis Delta Antigen

LMNA – Lamin

$MgCl_2$ – Magnesium chloride

M-HBsAg – Middle Hepatitis B Surface Antigen

miRNA – Micro RNA

mL – Millilitre

mM – milimolar

mRNA – Messenger RNA

Myr B – Myrcludex B

NES – Nuclear Export Signal

ng – Nanogram

NLS – Nuclear Localization Signal

nm – Nanometre

nt – Nucleotides

NTCP – Sodium-taurocholate co-transporting polypeptide

ORF – Open Reading Frame

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PEG – Pegylated

PEG-IFNα – Pegylated Interferon alpha

Poly(A) – Polyadenylated tail

pSVL-S-HDAg – Small Hepatitis Delta Antigen Plasmid

PTM – Post-translational Modification

RBD – RNA-binding Domain

Rev – Reverse

RNA – Ribonucleic acid

RNA pol I – RNA polymerase I

RNA pol II – RNA polymerase II

RNP – Ribonucleoprotein

rpm – Rotations per minute

SC – Spectral Counts

SDS – Sodium Dodecyl Sulphate

SDS-PAGE – Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis

SF3B1 – Splicing factor 3B subunit 1

S-HBsAg – Small Hepatitis B Surface Antigen

S-HDAg – Small Hepatitis Delta Antigen

snRNA – Small nuclear ribonucleic acid

snRNP – Small nuclear ribonucleoprotein

STAT3 – Transducer and Activator of Transcription 3

TAE – Tris-acetate EDTA

TEE – Total Exosome Extraction

TRA2β – Transformer-2 protein homolog beta

TSG 101 – Tumour Suppressor Gene 101

UAG – Amber stop codon

v/v – Volume/volume percentage

δ – Delta

µg – Microgram

µL – Microliter

1. INTRODUCTION

1.1. HDV discovery

Hepatitis Delta Virus (HDV) is part of the Deltaviridae family being the only member known so far. This virus was first described by Mario Rizzetto and co-workers in 1977, with the authors reporting the identification of a new antigen present in liver biopsies from patients seropositive for Hepatitis B Virus (HBV), to what they called the δ antigen (HDAg). This antigen was detected by immunofluorescence and was found exclusively in the nuclei of hepatic cells from infected patients with HBV (Rizzetto *et al.*, 1977). Further investigation revealed that what they thought to be a new HBV surface antigen, was instead an antigen belonging to a novel infectious agent that required the helper function of HBV – the HDV. This was shown through experimental infection, demonstrating that HDAg in association with a RNA molecule gave rise to an internal portion of a virus other than HBV (Rizzetto, Canese, *et al.*, 1980). Later, Bonino found out that this particle is organized into a virion-like form with the δ antigen and RNA as internal components within a coat of hepatitis B surface antigens (HBsAgs) (Bonino *et al.*, 1984). Along these lines, HDV is defined as a defective and a satellite virus of the HBV (Sureau and Negro, 2016).

As a result of its particular characteristics, it has been hypothesized that HDV originated from cellular circular RNAs or plant viroids (Mentha *et al.*, 2019). HDV and viroid RNAs share several common features that include compact folding, circular structure and replication via a rolling-circle mechanism (Flores, Owens and Taylor, 2016).

1.2. The biology of HDV

Biologically, HDV is the smallest known virus infecting mammals with a circular RNA genome of about 1,700 nucleotides, for which humans are the only reservoir (Taylor, 2014).

1.2.1. Viral structure and assembly

To form infectious particles and spread along the hepatocytes, HDV requires the envelope proteins of HBV, originating a virion of 35-37 nm. These particles are composed of a ribonucleoprotein core (RNP) and the HBV envelope proteins (Figure 1). The HBV envelope consists of an outer lipid membrane and the three HBV surface proteins. They all have a common carboxyl-terminus and are designated by their sizes in small (S-HBsAg), middle (M-HBsAg) and large (L-HBsAg) hepatitis B surface antigens (Mentha *et al.*, 2019). These three forms are transcribed from a single open reading frame (ORF), which has three different initiation codons (AUG). S-HBsAg has 226 amino acids (a.a.) shared with the three forms (S domain). M-HBsAg contains an N-terminal hydrophilic domain corresponding to an additional 55 a.a. (pre-S2 domain). Compared to M-HBsAg, L-HBsAg N-terminal consists of 119 additional a.a. (pre-S1 domain) (Urban *et al.*, 2014). The RNP, present both in viral particles and infected cells, is a spherical, core-like structure consisting of one copy of the RNA genome and roughly 200 copies of the only virus encoded protein, the delta antigen (Cunha, Tavanez and Gudima, 2015). This complex is essential for viral assembly and for the nuclear trafficking of HDV RNA (Tavanez *et al.*, 2002).

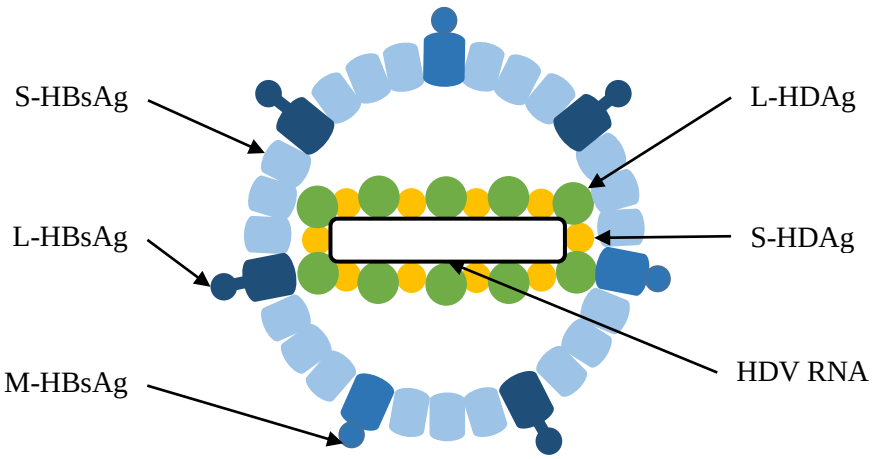

Figure 1. Schematic representation of HDV virion structure. The surface proteins (S-, M- and L-HBsAg) are from HBV capsid. HDV viral particles contain one copy of the circular single-stranded RNA genome, associated with both forms of HDAg – large and small delta antigen (S- and L-HDAg) – making the viral ribonucleoprotein (RNP).

1.2.2. HDV genome and RNAs

HDV genome is a circular single-stranded RNA molecule and has been estimated to contain about 1,700 nucleotides (nt) and negative polarity. Important features such as high G+C content (~60%) resulting in the formation of a stable secondary structure with a significant degree of internal base-pairing (~70%) have been described (Wang *et al.*, 1986; Cunha, Tavanez and Gudima, 2015).

Besides the genome, during replication, a complementary strand is synthetized, the antigenome. This antigenome contains the ORF for the only protein encoded in the HDV genome. However, the antigen is not translated from the circular antigenomic RNA but rather from a third synthetized linear RNA, with antigenomic polarity and approximately 800 nt, a 5'-cap structure and a 3'-polyadenylation tail consistent with the role of an mRNA (Figure 2) (Alves, Branco and Cunha, 2013).

The delta antigen has two isoforms that can be found in virions, Small (S-HDAg, 195 a.a.) and Large (L-HDAg, 213 or 214 a.a., depending on the genotype) hepatitis delta antigens. Both isoforms of the HDAg share the same functional domains excluding the L-HDAg-specific C-terminal extension (Cunha, Tavanez and Gudima, 2015).

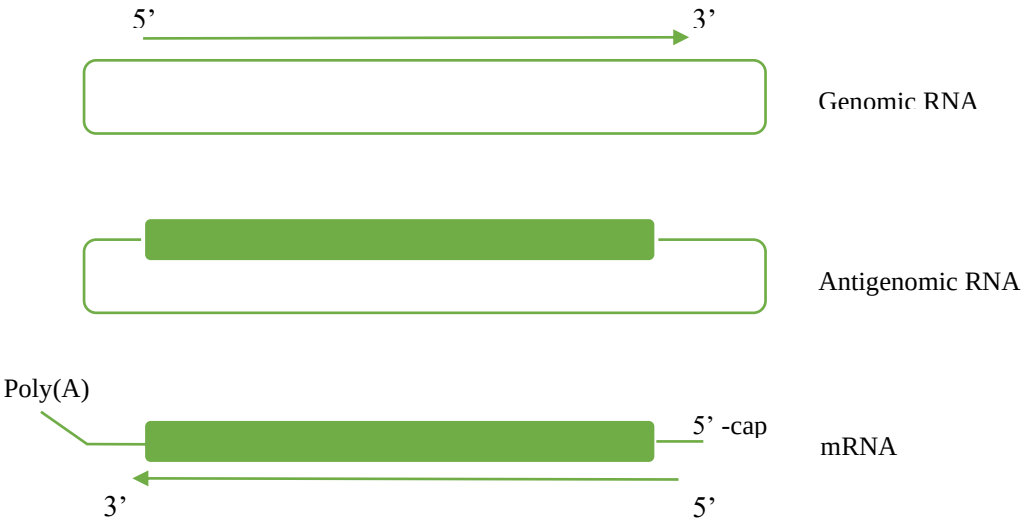

Figure 2. Representation of the three main HDV RNA species – genomic and antigenomic RNA and mRNA. Antigenomic RNA is the exact complement of the genomic RNA. Also indicated in the antigenomic RNA is the open reading frame for the delta antigen, represented by a green box. The antigen is translated from the mRNA, which has a 5'-cap and 3'-poly(A) tail.

1.2.3. HDV delta antigens

Both HDAg isoforms are synthetized from HDV mRNA and have important and distinct roles on the HDV life cycle.

The S-HDAg (195 a.a. and 24 kDa) is expressed early in the replication cycle being essential for initiation of HDV genome replication and accumulation of HDV RNA-directed RNA transcripts. This protein has functions described such as regulation of HDV RNA editing, promotion of nuclear import of HDV RNPs, facilitation of ribozyme cleavage and may also be involved in sequestering and manipulating host cell components to facilitate HDV replication (Cunha, Tavanez and Gudima, 2015).

As replication proceeds, the HDV antigenomic RNA is recognized by a host double-stranded RNA-dependent adenosine deaminase 1 (ADAR1), which catalyses an editing mechanism, converting an amber STOP codon for the S-HDAg – UAG –, into a tryptophan codon – UGG – by changing the adenosine to inosine. As a consequence, the reading frame is extended by 19 a.a. and L-HDAg is synthetized (Taylor, 2015). The large form of the antigen, a 214 a.a. protein with a molecular weight of 27 kDa, is known to be a dominant negative inhibitor of replication supported by the S-HDAg and essential for viral packaging, most probably via interaction with HBV envelope proteins (Cunha, Tavanez and Gudima, 2015). Along these lines, the two HDAgs share 195 a.a. and differ only in the additional 19 a.a. on the C-terminus in the large form. As such, these two proteins share the majority of the several functional domains that have been identified in the delta antigens over the years. Both antigens contain a coiled-coil domain (CCD) – 12 to 60 a.a.; a nuclear localization signal (NLS) – 66 to 75 a.a.; and an RNA binding domain (RBD) – 97 to 146 a.a. Within its extra sequence, L-HDAg has a nuclear export signal (NES) – 198 to 210 a.a. – and a farnesylation signal designated by CXXX box – 210 to 214 a.a (Alves, Branco and Cunha, 2013). The functional domains of the HDV antigens are represented in Figure 3.

Both HDV proteins undergo post-translational modifications (PTMs) critical for their respective functions. So far, delta antigens have been shown to be phosphorylated, methylated, acetylated and more recently sumoylated (Alves, Branco and Cunha, 2013; Taylor, 2015). Note that, phosphorylation of S-HDAg in serine-2 and -117 residues, allows interactions with the cellular RNA polymerase II, enabling the replication of HDV RNA (Hong and Chen, 2010). It is still unclear which, if any, of these PTMs have impact

in intracellular HDAgs functionality and/or localization. In contrast, there is a modification that is exclusive and essential to the L-HDAg. This PTM corresponds to a farnesylation and occurs at a specific cysteine residue located in position 211, the fourth last a.a. of the L-HDAg sequence. A farnesylation signal in the additional 19 a.a. enables a farnesyl lipid group to be added covalently to the cysteine and the three amino-terminal a.a. are removed in the process. This farnesylated form of L-HDAg contributes to its ability to inhibit S-HDAg-supported replication and has a role in virus assembly because farnesylated L-HDAg displays higher affinity to the HBV surface antigens (Taylor, 2015; Mentha *et al.*, 2019). In fact, agents that inhibit the addition of the farnesyl lipid group to the C-terminus of the L-HDAg have been shown to interfere with its interaction with HBsAgs, consequently preventing HDV virion assembly and secretion (Mentha *et al.*, 2019).

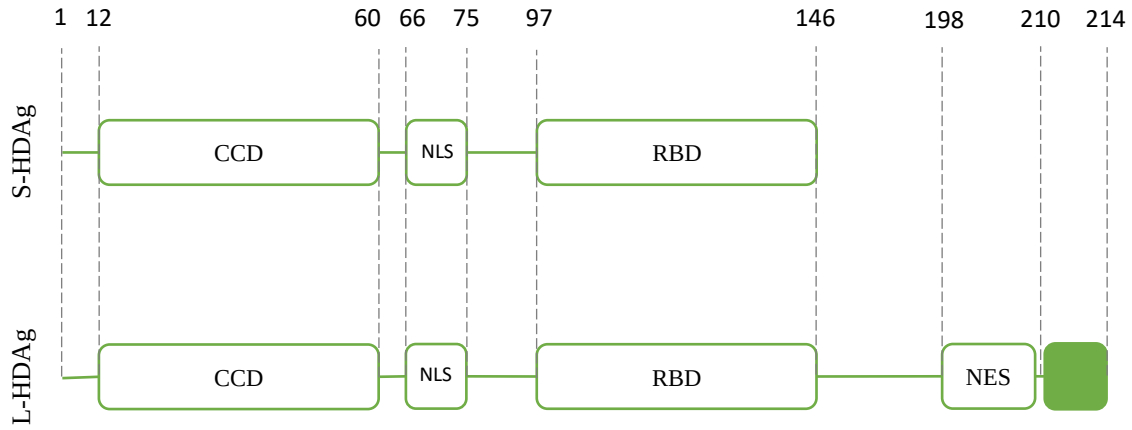

Figure 3. S- and L-HDAg functional domains. Except for the 19-amino-acid extension at the C-terminal of L-HDAg, the delta antigens share all of their sequence. Within the common sequence, both antigens contain a coiled-coil domain (CCD), a nuclear localization signal (NLS) and an RNA binding domain (RBD). L-HDAg has an additional nuclear export signal (NES) and a farnesylation signal, CXXX box, represented by the green square. The numbers indicate the a.a. residue position.

1.2.4. HDV replication and life cycle

Since both HDAgs and HDV RNA are localized mainly in the nucleus, HDV genome replication occurs in the nucleus of infected cells and is completely independent of HBV (Chou *et al.*, 1998). However, as is well known, HDV requires the HBV envelope proteins to disseminate (Sureau and Negro, 2016). Therefore, upon entry into the host's bloodstream, the virion binds to cells in an unspecific way, mediated by heparin sulphate proteoglycans (HSPGs) interaction – glycoproteins found on dermal and endothelial cells as well as in hepatocytes in higher density. When the viral particle binds to a hepatocyte, it attaches to the sodium-taurocholate co-transporting polypeptide (NTCP), through which the HDV enters the cell. Note that this connection, although highly specific, can only be achieved and give rise to a successful infection if virions contain the L-HBsAg. The process is identical for both HBV and HDV (Krause, Haberkorn and Mier, 2018).

Subsequently to the uncoating of the virions, due to the NLS domains present in both delta antigens, the HDV RNPs are transported to the nucleus where HDV genome replication takes place (Cunha, Tavanez and Gudima, 2015). Replication of the RNA viral genome occurs through a double rolling-circle mechanism producing multimeric anti-genomic and genomic molecules, similar to the proposed for plant viroids (Kuo *et al.*, 1988). Briefly, in accordance with the rolling-circle model, genomic RNA serves as a template for multimeric antigenomic RNA synthesis. The growing transcript is then subjected to self-cleavage by the activity of a ribozyme, intrinsic to both HDV genome and antigenome RNAs, originating antigenome monomers which then re-circularize. The circularized HDV antigenome then serves as a template to new genomic RNA transcripts by a similar mechanism to the one described (Chen *et al.*, 1986; Kuo *et al.*, 1988; Krause, Haberkorn and Mier, 2018). During HDV antigenome synthesis, a different RNA species with the same 5' end as the antigenome and with 800 nt in size, is also synthetized. This 800 nt RNA then undergoes mRNA processing – 5'-capping and 3'-polyadenylation (poly(A)) – originating HDV mRNA. Thereafter, during HDV replication, RNA editing occurs at the antigenome leading to the production of L-HDAg (Gudima *et al.*, 2000; Taylor, 2009). Note that, since S-HDAg is essential to initiate replication, presumably the first event in the HDV replication cycle is the transcription of S-HDAg mRNA. Normally, in infected hepatocytes, there are five to twenty times less copies of antigenome compared to genome and an even smaller amount of mRNA (Chen *et al.*, 1986). This replication

model is supported by the detection of 1,700 nt multiple-length HDV RNAs species in liver biopsies and cell cultures, corresponding to replicative intermediates (Chen *et al.*, 1986). However, it is important to consider that HDV codifies to a protein and thus, the HDV replication is certainly even more complex than the one based on the rolling circle mechanism.

Another interesting fact about the HDV replication consists in the fact that HDV does not possess its own RNA-dependent RNA polymerase neither uses the polymerase of HBV and so, it must use the host cell DNA-dependent RNA polymerases, namely cellular RNA polymerase II (RNA pol II) (Modahl *et al.*, 2000). Mammalian cells do not codify for RNA-dependent RNA polymerases, which means that HDV has the ability to redirect an RNA polymerase that normally is DNA-dependent, to translate its viral genome (Fu and Taylor, 1993). Several evidences suggest the participation of RNA pol II in the three HDV RNAs synthesis. It was known that the HDV mRNA contains a 5'-cap structure (Gudima *et al.*, 2000) and a 3'-poly(A) tail (Hsieh *et al.*, 1990), consistent with RNA pol II transcription. Additional studies show that, after treatment with low concentration of α-amanitin, expression of HDV genomic RNA and mRNA was inhibited, showing the involvement of RNA pol II in HDV replication (Fu and Taylor, 1993; Chang *et al.*, 2008). Nevertheless, the participation of other RNA polymerases in the HDV life cycle is still open to debate. Because expression of the antigenomic RNA has been shown to be resistant to treatments with high doses of α-amanitin, these studies suggest that RNA pol I activity is also relevant (Modahl and Lai, 2000; Macnaughton *et al.*, 2002).

After replication and translation of both HDAgs, viral RNPs are assembled in the nucleus of hepatic cells. The RNP is then exported to the cytoplasm due to the NES domain present in L-HDAg. The presence of this domain allows the RNP to reach the endoplasmic reticulum (ER) where the viral particles will be assembled with the envelope protein of HBV. In addition, the farnesyl group of the L-HDAg serves as an anchor so that the RNP binds to the ER membrane, allowing its interaction with the HBsAgs and, consequently, the assembly of virions which will then be released (Tavanez *et al.*, 2002; Koh *et al.*, 2015).

1.3. Pathogenesis, epidemiology, transmission and treatment of HDV

1.3.1. Pathogenesis

HDV is the hepatotropic virus that causes the least common but most severe and rapidly progressive form of chronic viral hepatitis, which is the most likely to lead to cirrhosis and is correlated with increased risk of hepatocellular carcinoma (HCC) (Diaz *et al.*, 2018; Koh, Heller and Glenn, 2019). From an histological point of view, HDV infection is identical to other forms of viral hepatitis, with very similar pathological changes comprising severe inflammation and extensive hepatocellular necrosis as principal clinical manifestations (Rizzetto, Hoyer, *et al.*, 1980). The clinical features of HDV infection can be mistaken with other viral hepatitis, although they tend to be more severe. After an incubation period of 21 to 49 days, indicated by active HDV replication, the first nonspecific clinical symptoms start to appear, such as fatigue, lethargy, nausea and anorexia along with biochemical evidence of hepatitis associated with a decrease in viral replication. Later, the icteric phase takes place, with dark urine, clay-coloured stools, yellowing of the skin or whites of the eyes, due to the excess of bilirubin pigment in the serum (Farci and Niro, 2012).

As mentioned before, HDV requires the presence of HBV to cause productive infection and HDV spread as they share the same protein envelope. A person with anti-HB antibodies is immune to HBV infection and therefore immune to HDV. However, in non-immune individuals, HDV infection can occur in two modalities: concurrently with HBV infection – coinfection –, or in chronically infected individuals with HBV – superinfection (Smedile, Rizzetto and Gerin, 1994). Both HBV coinfection and superinfection can lead to acute HDV infection (Koh, Heller and Glenn, 2019).

The simultaneous acute infection of HBV and HDV in a susceptible individual is described to be a coinfection. This infection begins only after HBV has infected hepatocytes and the clinical course is similar to acute HBV infection. Since HBV is essential for HDV spread, the rate of progression to chronicity is similar to acute hepatitis B, between 2 and 5% (Botelho-Souza *et al.*, 2017; Koh, Heller and Glenn, 2019). However it is important to note that coinfection causes more severe disease, with an increased risk of acute hepatic failure (Koh, Heller and Glenn, 2019).

If an individual is chronically infected with HBV and later infected by HDV, this condition is called superinfection and can be mistaken for a HBV flare (Botelho-Souza *et al.*, 2017). In this setting, HDV promptly establishes infection using the HBsAgs from pre-existing HBV infection rather than those provided by HBV present in the infectious inoculum, as this pre-existing HBsAgs status provides the perfect biological scenario for the rapid expression of the defective hepatitis delta virus (Farci and Niro, 2012). Compared to acute coinfection, acute superinfection of HDV has a more severe clinical course, with a relatively short period of incubation and particularly high risk of acute liver failure (Koh, Heller and Glenn, 2019). Superinfection can cause fulminant hepatitis and chronicity rates are above 80%, which is associated to a higher risk of early development of cirrhosis and HCC. In fact, in asymptomatic HBsAgs patients it can lead to acute hepatitis, while in patients with chronic active hepatitis B, it can lead to exacerbation of symptoms, with liver decompensation and progress to chronic hepatitis in nearly 90% of cases (Botelho-Souza *et al.*, 2017).

The correlation between HDV and HCC is still under debate. Chronic hepatitis D (CHD) is considered the most severe form of chronic viral hepatitis, with rapid progression towards cirrhosis and with higher risk of decompensation and mortality. Although the most common complication of CHD is the decompensation of chronic liver disease rather than HCC and despite the belief that HDV-infected patients are at increased risk of HCC, several studies have recently shown that this risk may indeed be up to nine times higher than in HBV mono-infected patients (Mentha *et al.*, 2019).

1.3.2. Epidemiology

The World Health Organization estimates that, in 2015, 257 million people were living with chronic hepatitis B infection (WHO, 2019a). Based on these estimations, at least 5% of individuals with chronic HBV infection are co-infected with HDV, which results in a total of 15 to 20 million induviduals infected with HDV worldwide (WHO, 2019b). However, recent studies proposed that 62-72 million people may live with HDV globally, a prevalence nearly two-times greater than that of human immunodeficiency virus (Chen *et al.*, 2019). These observations imply a disease burden much greater than previously considered. In fact, the exact overall prevalence of HDV infection remains

unknown, since many countries do not report HDV data, screening practices are heterogeneous and non-standard and testing inaccessibility remains a reality in many endemic areas (Mentha *et al.*, 2019; WHO, 2019b). HDV global distribution, as it can be seen in Figure 4, does not follow a uniform pattern. The areas of high prevalence include: Central Africa, West Africa, the Mediterranean basin, the Middle East, Eastern Europe, certain areas of Southeast Asia, the Amazon basin and Northern Asia (Chen *et al.*, 2019; Koh, Heller and Glenn, 2019).

For the last 20 years, universal vaccination programs have been controlling HBV infection, changing the scenario of HDV in the industrialized world. In Western Europe, the HDV circulation has been decreasing, with the remaining individuals that were infected long ago being now older, with terminal stages of hepatitis D (Rizzetto, 2016; Mentha *et al.*, 2019). In Southern Europe, with better public health standards, better socioeconomic conditions and mass vaccination campaigns against HBV, levels of HDV infection have been decreasing, as is the case of Italy, which had high rates soon after HDV identification. The decline of HDV infection in the last decades led some investigators to believe that it was no longer a relevant medical problem and that the eradication of HDV infection in Southern Europe was possible. Despite the optimistic perspective, this assumption has caused negligence in virus screening as awareness has been weakened (Rizzetto, 2015). In fact, HDV has not disappeared from Europe, on the contrary, the prevalence of HDV in Europe has been increasing due to immigration of individuals from highly endemic regions and without health control policies, it may lead to a novel spread of HDV (Rizzetto, 2015, 2016).

Until now, eight HDV genotypes have been identified, with up to 60% sequence similarity as result from three main mechanisms: mutation, editing and recombination. Genotype 1 is associated with severe and mild disease and can be found worldwide being the prevalent genotype in Europe and North America. Genotype 3 has been associated with a form of fulminant hepatitis and a higher risk of acute liver failure that affect indigenous populations and seems to be found in South America, confined to the Amazon region (Alfaiate, Dény and Durantel, 2015). Genotypes 2 and 4 are associated with milder liver disease when compared to genotype 1 and are prevalent in East Asia. Genotypes 5 to 8 are mainly found in Africa (Mentha *et al.*, 2019).

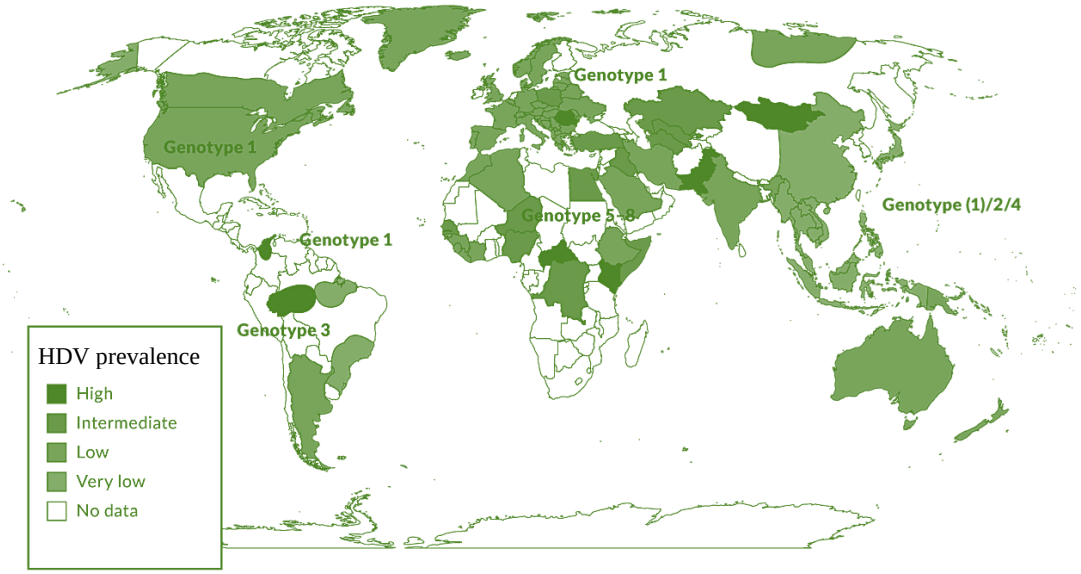

Figure 4. Schematic representation of the worldwide HDV prevalence and genotype distribution adapted from (Wedemeyer, 2020).

1.3.3. Transmission

HBV and HDV share the same transmission mechanisms, with both viruses being transmitted by blood-derived products and sexual contact. In endemic areas, the parenteral route is considered to be the main source of HDV transmission, although it can also occur due to intra-familial and iatrogenic dissemination, associated with poor hygiene conditions. In regions with low endemicity, such as Europe and the USA, transmission occurs mainly through unprotected sexual intercourse and contact with infected blood, especially in intravenous drug users. Vertical transmission however, is uncommon and not well described (Alfaiate, Dény and Durantel, 2015; Mentha *et al.*, 2019).

1.3.4. Treatment and new approaches

Currently there is no established effective therapy for HDV infection available mainly because HDV is a host-dependent virus devoid of any potentially druggable enzyme encoded in its small genome. HBV immunization is the most effective and safest way to prevent infection, prior to contact with HBV, as it reduces the available pool for

HDV infection. The vaccine is eligible for children immediately after birth and for adults but is ineffective if the disease is already settled (Goyal and Murray, 2014; Krause, Haberkorn and Mier, 2018).

Therapeutically approaches for CHD infection are scarce. A therapy based on a PEG-modified interferon-alpha has proved to be the only with anti-viral activity specific for HDV, though its efficacy is rather limited (Kabaçam *et al.*, 2012; Heller *et al.*, 2014; Goyal and Murray, 2015). It is an important public health challenge to search for novel therapies, specific drugs, new molecular targets and improved treatment strategies. Novel effective therapies are crucial to significantly reduce the number of CHD patients progressing to cirrhosis and HCC.

As HDV has a peculiar life cycle, with its dependence on host cellular enzymes, the development of direct-acting antiviral agents for possible treatment of CHD is limited. Authors have suggested that the ideal treatment for HDV infection should include a functional control of HBV replication, encompassing the complete suppression of the production of HBV envelope protein (Lempp, Ni and Urban, 2016). In fact, it is easy to think that, since HDV needs the protein envelope of HBV, the elimination of HBV must prevent HDV infection. However, drugs used to treat HBV, such as nucleosides and nucleotides analogues, for example, Lamivudine, Famciclovir and Ribavirin, have been ineffective in treating HDV (Wedemeyer and Manns, 2010).

In extreme cirrhosis caused by HDV, the only treatment available is liver transplant. To prevent HBV reinfection, the patient is immunized with the HBV vaccine, becoming anti-HBs positive (Rosenau *et al.*, 2007). Despite being a last resource intervention, liver transplantation for treatment of CHD has a survival rate, after 5 years, higher than transplantation for other types of chronic hepatitis (Niro, Rosina and Rizzetto, 2005).

Alpha-interferon

One of the first therapies used for HDV treatment was interferon alpha (IFNα), which was first described in 1957 (Isaacs and Lindenmann, 1957). Although the mechanism of IFNs agents is not fully understood, it is associated with anti-proliferative, immunomodulatory and antiviral activities, which have been used in several malignancies and several viral infections, as is the case of chronic viral hepatitis caused by HBV, HCV and HDV (Bahcecioglu and Sahin, 2017). At the beginning of the century, clinical trials

were carried out to demonstrate the beneficial effects of IFNα therapy for chronic patients, however, despite the promising results, the recurrence rate was high after treatment interruption (Farci *et al.*, 2004; Niro, Rosina and Rizzetto, 2005). Under IFNα treatment, HDV viral particles production rate was reduced by 74.9% and HDV-RNA clearance occurred in approximately 25% of patients (Guedj *et al.*, 2014; Bogomolov *et al.*, 2016). Also, at a dose of 3 to 6 million units three times a week for 6 to 12 months treatment, controlled liver enzymes were only observed in no more than 20-25% of the patients (Lempp *et al.*, 2019). A different study showed that, with a prolonged treatment of about two years, 50% of patients reached undetectable levels of HDV RNA; however, only 20% of patients remained negative at the end of the follow-up (Elazar, Koh and Glenn, 2017). Another important fact to note is that, as mentioned, IFNα has anti-proliferative and immunomodulatory properties, and this latter activity can not only aggravate but also induce new autoimmune diseases (Aslam *et al.*, 2015).

PEG-interferon

A subsequent therapeutic approach consisted in a pegylated version of interferon alpha (PEG-IFNα), which has an improved safety profile and increased half-life (Elazar, Koh and Glenn, 2017). Several studies have shown negativity levels of HDV RNA of 17 to 47% with no HBsAgs seroconversion (Lempp, Ni and Urban, 2016). For example, Elazar and co-workers demonstrated that HDV RNA negativity was achieved in 57% of patients with one year of PEG-IFNα treatment, which dropped to 43% at end of follow-up. On the other hand, a treatment of 5 years resulted in 42% of patients with HDV RNA negative, with only 25% remaining negative by the end of 5 years (Elazar, Koh and Glenn, 2017). Moreover, other studies, in which a combined treatment of PEG-IFNα with adefovir or tenofovir was administrated, did not considerably improve the HDV RNA response. Instead, it leads to low response rate and high frequency of adverse events such as fatigue, weight loss and psychiatric alterations (Lempp, Ni and Urban, 2016).

New approaches – Myrcludex B and Farnesyltransferase inhibitors

Currently, two new therapeutic approaches are being investigated in clinical trials: Myrcludex B, which specifically blocks NTCP-mediated entry of HDV and HBV into hepatocytes, and Lonafarnib, an orally administered farnesyltransferase inhibitor (Lempp, Ni and Urban, 2016).

- Myrcludex B

Current therapeutic strategies aim to deprive the HDV of critical functions provided by the helper HBV or by the infected host. Myrcludex B (MyrB) is a myristoylated lipopetide that inhibits the entry of HBV and HDV into hepatocytes by blocking the binding of the HBsAgs to the NTCP receptor. The main disadvantage of MyrB is associated with the blockage of bile acids, which use the same cellular transporter, NTCP; however, some preclinical studies have shown that the concentration of MyrB that blocks viral entry is 100 times lower than that needed to inhibit the transport of bile acids, which indicates that the antiviral effect can occur without interfering with the metabolic functions of NTCP (Lempp, Ni and Urban, 2016; Mentha *et al.*, 2019). Under monotherapy, MyrB showed a strong effect on serum HDV RNA levels, with no dose limiting toxicity, it was well tolerated and, according to Bogomolov and co-workers, 25% of monotherapy patients have become clear for HDV RNA (Bogomolov *et al.*, 2016; Rizzetto, 2018).

- Farnesyltransferase Inhibitors

L-HDAg farnesylation is an important PTM, as it plays a vital role in the interaction of HDV RNP with the HBV envelope. Lonafarnib is a farnesyltransferase inhibitor, originally tested in humans for treatment of Hutchinson-Gilford progeria syndrome (Gordon *et al.*, 2012, 2014; Young *et al.*, 2013). Progeria is an ultra-rare segmental premature aging disease, characterized by a point mutation in the Lamin (*LMNA*) gene (Kubben and Misteli, 2017).

Lonafarnib has the ability to block viral production, since it directly interferes with HDV particle release, acting in a similar way to HBV inhibitors. Some studies have shown that Lonafarnib reduces HDV serum levels when administrated in patients with CHD (Bogomolov *et al.*, 2016; Lempp, Ni and Urban, 2016). In fact, a clinical trial from 2015 demonstrated that Lonafarnib can decrease serum HDV levels in a dose-dependent manner during 28 days of therapy (Koh *et al.*, 2015). It is still important to note that it is not clear whether this reduction results in elimination of infected hepatocytes (Bogomolov *et al.*, 2016; Lempp, Ni and Urban, 2016). Lonafarnib can ablate HDV in a dose dependent manner; however, treatment up to 24 weeks achieved only a reduction of HDV RNA, being necessary prolonged therapies to decrease serum HDV RNA to undetectable levels (Rizzetto, 2017). Although Lonafarnib is a well-tolerated drug,

gastrointestinal side effects and adverse effects associated with enzymatic inhibition of farnesyltransferase for long-term treatments are still challenges to be considered in forthcoming studies (Lempp, Ni and Urban, 2016; Mentha *et al.*, 2019). In addition, studies show that, although Lonafarnib has the ability to inhibit virus secretion, it leads to a substantial intracellular accumulation of L-HDAg (eightfold) and replicative intermediates (twofold) along with the induction of the innate immune response (Lempp *et al.*, 2019). This is in line with the strong decline of HDV serum RNA levels observed in the Lonafarnib clinical trials (Koh *et al.*, 2015). However, if Lonafarnib enhances the intrahepatic replication, it raises the question of whether HDV RNA can be cleared from the liver.

1.4. Viral spread

HDV has been described as an HBV satellite virus, a strictly hepatic human pathogen. This may be the reason why HDV has been exclusively detected in the liver of HBV-infected patients, since HBV provides RNP envelopment and transmission functions (Perez-Vargas *et al.*, 2019). Considering the shuttle of RNP HDV from cytoplasm to nucleus, an idea of a dynamic virus is beginning to be created. In fact, evidence suggest that HDV RNA contains a putative element that interacts with cellular nuclear export elements (Tavanez *et al.*, 2002). Cosset and co-workers suggest that HDV RNPs can exploit assembly functions provided by viruses from several alternative families and genera, including Flavivirus, Vesiculovirus, Hepacivirus among other enveloped viruses. This has led to the belief that HDV RNA may be transmitted through unorthodox means, as it appears that it can self-replicate in a much broader variety of cell types and species. Through the test of glycoproteins from ten different virus genera they demonstrated that HDV RNPs could be enveloped by glycoproteins from six of those non-HBV particles and could produce infectious HDV particles. Noteworthy, vesicular stomatitis virus can induce the formation of infectious particle virus as well as HCV. This may indicate that HDV can be a satellite of a virus genus totally unrelated to HBV. Probably, in nature, HDV could be associated with different virus types, which includes human viral pathogens (Perez-Vargas *et al.*, 2019). Moreover, viruses closely related to HDV have been detected in non-human species in the absence of any Hepadnarivirus and,

also, patients with primary Sjögren's syndrome reported the presence of HDAgs and HDV RNA in salivary glands without the presence of HBV antibodies or HBsAgs (Weller *et al.*, 2016; Perez-Vargas *et al.*, 2019). This information brings into question the HDV transmission scenarios and its pathogenicity, questioning what other types of cells can be infected by HDV and what other forms of transmission HDV can take advantage of.

1.5. Cellular communication – exosomes

Most cell types produce constitutively extracellular vesicles – exosomes and microvesicles – containing both mRNAs and non-coding RNAs and also selective proteins. This mechanism of horizontal gene transference has been recently described and has a significant impact on natural physiological processes. On the other hand, this natural ability might facilitate the spread of disease through the delivery of genetic material and/or pathogenic proteins (Lee, El Andaloussi and Wood, 2012).

Exosomes are endogenous nanovesicles that originate from multivesicular bodies with a bilayer membrane that contains several proteins, lipids and nucleic acids, ranging from 30 to 150 nm in size. These vesicles can be detected in blood, urine, other body fluids and cell culture supernatant and play an important role in many biological processes (Yang *et al.*, 2017). Over the last years, evidence has been showing that exosomes mediate indirect cell-to-cell communication through the transfer of bio-macromolecules, functional proteins and RNAs between cells (Bukong *et al.*, 2014; Yang *et al.*, 2017).

In general, these extracellular vesicles contain proteins from the plasma membrane, the cytosol and endosomes, some components from the nucleus, endoplasmic reticulum, mitochondria and Golgi apparatus. The cell type from which they are secreted defines the exosomes protein content and TSG101, CD63 and CD81 are some of the specific biomarkers of these nanovesicles. Exosomes macromolecular components play an important role in cellular functions and pathological states, for example, in inflammation, angiogenesis, neurodegenerative diseases, immune responses, cell death and cancer (Gurunathan *et al.*, 2019).

Although isolation of exosomes is critical to the success of these vesicles' characterization, there is no standard method to extract and isolate these nanovesicles. Several conventional methods have been employed to isolate exosomes, such as

ultrafiltration, precipitation, immunoaffinity separation, etc. (Gurunathan *et al.*, 2019). After isolation, exosomes can be analysed for the presence of proteins and nucleic acids.

1.5.1. The role of exosomes in HCV and HBV

Some viruses can hijack host exosomes and incorporate viral constituents into them. Infected cells release infected exosomes that transport several vital components to neighbouring cells, helping to regulate cellular responses and producing infections (Li *et al.*, 2019). Recent studies have been reported the presence of Hepatitis C Virus (HCV) RNA in exosome-like structures isolated from the plasma of HCV-infected patients and in the supernatant of HCV infected cells. Furthermore, evidence demonstrate that HCV virus can take advantage of host exosomes to successfully transmit infection to naïve hepatocytes in a receptor-independent manner, even in the presence of potent blocking anti-HCV receptor antibody treatments (Bukong *et al.*, 2014; Yang *et al.*, 2017). Evidence also indicates that a higher level of HCV transmission to hepatocytes is shown in infections involving HCV exosomes, when compared with the multiplicity of infection of free HCV particles (Shen *et al.*, 2017). In fact, exosomes mediate higher HCV viral particles transmission because they contain a complex, consisting of Ago2, HSP90 and miR-122. This protein complex was demonstrated to enhance HCV RNA stability and viral replication (Bukong *et al.*, 2014; Shen *et al.*, 2017).

Regarding exosomes in HBV infection, it is not clear whether exosomes derived from HBV-infected hepatocytes mediate the transmission of HBV infection. Research suggests that serum exosomes from patients with chronic hepatitis B contain HBV proteins and HBV nucleic acids, consequently the exosomes act as carriers of HBV virus, as observed in HCV. These nanovesicles may serve as important regulators of HBV transmission, since they transport HBV to uninfected hepatocytes as efficiently as infection by free-virus (Yang *et al.*, 2017). Another interesting discovery is that HBsAgs particles can provide housing to miRNAs from hepatocytes, releasing them from HBV infected cells into the bloodstream (Novellino *et al.*, 2012).

All this information starts to indicate different possibilities for the spread of HDV and gives rise to the idea that it may have the ability to enter other cells types, besides

hepatocytes, taking use of the envelope proteins of other virus or even human nanovesicles, as exosomes.

1.1. Main objective

The main goal of the present work was to analyse the existence of an exosome-based mechanism by which HDV infection could be propagated in the absence of HBV.

1.1.1. Specific Objectives

To achieve the main objective, five specific objectives were defined:

- Establishment and optimization of an exosome isolation protocol.
- Purification of exosomes from a human hepatocarcinoma cell line that constitutively expresses all HDV components.
- Characterization of the RNA and protein composition of the purified exosomes.
- Analysis of the presence of HDV components in Huh7-D12 derived exosomes.
- Analysis of the potential of the Huh7-D12 derived exosomes to serve as vehicles to transmit HDV components from infected cells to non-infected cells.

2. MATERIALS AND METHODS

2.1. Cell lines

In this study, two human hepatocarcinoma cell lines Huh7 and Huh7-D12 were used. The first was originally obtained from a liver tumour in a 57-years-old Japanese male (Nakabayashi *et al.*, 1982).

The Huh7-D12 cell line originated from a stable transfection of Huh7 cells with a plasmid containing a full HDV cDNA trimer, pSVL (D3). This plasmid resulted from the reverse transcription of the total liver RNA from an HDV-infected woodchuck by cloning into the eukaryotic expression vector pSVL (Kuo *et al.*, 1988) After stable transfection, the result was a cell line containing multiple copies of HDV cDNA integrated into the genome, being able to express both delta antigens and all HDV life cycle-associated RNA transcripts (Cunha *et al.*, 1998).

2.2. Cell culture

Huh7 cells were seeded in monolayer in RPMI-1640 medium (Sigma-Aldrich, USA), supplemented with 10% (vol/vol) Foetal Bovine Serum (FBS, Gibco, USA) and 1X penicillin/streptomycin.

Huh7-D12 cells were also seeded in monolayer in RPMI-1640 medium (Sigma-Aldrich, USA), supplemented with 10% FBS (Gibco, USA) and 200µg/mL geneticine (G418, Sigma-Aldrich, USA).

For exosome extraction procedure, cells were incubated for 24 hours in RPMI complete medium, then medium was exchanged to RPMI 10% FBS exosome depleted (Gibco, USA).

2.3. Thawing cells

Huh7 and Huh7-D12 cell lines were cryopreserved at -80˚C in a vertical liquid nitrogen freezer (SANYO, Ultra-low temperature freezer, model: MDF-U3086S). First, the cryotube (Sarstedt, Germany) was heated, without submerging it, in a thermostatic bath at 37˚C (Medingen, model: W12) until the thawing of the cryopreservation medium was completed. The entire cell volume was inoculated into 10 mL of complete medium previously placed in a 75 cm^2 capped filter culture flask (Sarstedt, Germany). After 24h

incubation at 37˚C and 5% CO_2 (Nuaire, US autoflow), the medium was replaced with fresh medium.

2.4. Maintenance of cultured cells

Both cell lines were individually kept growing in 75 cm^2 culture flasks (Sarstedt, Germany), in complete RPMI-1640 culture medium (Sigma-Aldrich, USA). The culture flasks were incubated at 37˚C, in a humidified atmosphere containing 5% CO_2 and with regular control of possible contaminations.

Whenever cell confluence greater than 90% was observed, cells were transferred from the culture flask to a new flask, often at 1:3 dilutions. The cell culture maintenance process included culture medium removal, followed by washing with a phosphate buffered saline solution 1X (PBS, Gibco, USA) and further incubation for 1 to 2 minutes with 0.5 mL trypsin-EDTA 0.25% (Sigma-Aldrich, USA).

Trypsin inactivation was done through its dilution into 9.5 mL of fresh RPMI cell culture medium (Sigma-Aldrich, USA), making up a total volume of 10 mL, in which cells were resuspended and then transferred to a new culture flask at the desired dilution.

2.5. Cell culture in six-well plate

Cells were inoculated into six-well plates, each well with 3.5 cm diameter (Sarstedt, Germany). Confluence of 70 to 90% was required to subsequent RNA extraction. After washing, trypsinization and resuspension of cultured cells, the six-well plates were prepared with a final volume of 1.5 mL per well and with a volume of cell suspension between 0.3 and 0.5 mL in complete medium. Plates were incubated for 24 to 48 hours at 37˚C and 5% CO_2 (Nuaire, US autoflow), until the desire confluence was reached.

2.6. Bacterial transformation and plasmid purification

Plasmids used were eukaryotic expression vectors that contained a resistance gene against ampicillin and a DH5α competent *E. coli* strain was used, to perform transformation. 10 ng of plasmid DNA was added to 100 μL of bacteria, the

transformation tube was incubated on ice for 30 minutes and then subjected to a heat shock at 42˚C for 45 seconds (Medingen, modelo: W12). The solution was cooled on ice for 1 minute, supplemented with 900 µL Luria-Bertani medium (LB; Gibco, USA) and incubated at 37˚C for 1 hour with agitation of 200 rotations per minute (rpm). Transformed bacteria were plated in LB agar plates (Invitrogen, USA) supplemented with ampicillin (Thermo Fisher, USA) and left overnight incubating at 37˚C.

After incubation, small-scale liquid cultures using well-isolated colonies were prepared in 8 mL LB medium supplemented with ampicillin and left incubating for 16 hours at 37˚C with agitation. Following incubation, cultures were centrifuged at 8,000 rpm and recombinant vectors were extracted using the commercial kit QIAprep Spin Miniprep Kit (Qiagen, Germany) and following the manufacturer instructions.

Briefly, pellet was resuspended in 250 µL P1 buffer, followed by alkaline bacterial lysis by addition of 250 µL P2 buffer. Neutralization occurred by adding 350 µL N3 buffer and then centrifuging at 13,000 rpm for 10 minutes, with the pellet being subsequently discarded. Supernatant was transferred to a Qiaprep 2.0 column (Qiagen, Germany) and centrifuged at 13,000 rpm for 1 minute, discarding the flow-through. 750 µL of PE buffer was added and samples were then subjected to two successive centrifugations at maximum speed for 1 minute. Column was then transferred to a 1.5 mL tube, 50 µL nuclease-free water was added and left incubating at room temperature for 2 minutes. To elute plasmid DNA, samples were centrifuged at maximum speed for 2 minutes. After NanoDrop 1000 quantification (Thermo Fisher, USA) and electrophoresis analysis, samples were stored at -20˚C until further use.

2.7. Transient transfection of Huh7 cell line

To perform transient transfection of Huh7 cells, FUGENE 6 (Promega, USA) reagent was used according to the manufacturer instructions. Briefly, 3 µL FUGENE 6 (Promega, USA) was diluted in 100 µL RPMI-1640 medium (Sigma-Aldrich, USA) and incubated at room temperature for 5 minutes. Another tube was then prepared containing 1 µg of plasmid DNA to which the transfection mixture previously prepared was transferred. After 15 minutes of incubation at room temperature, the mixture was poured into the six-well plate dropwise. Plates were then incubated at 37˚C and 5% CO_2 (Nuaire,

US autoflow) for 24 hours. Transient transfections were done using the plasmid that promote the expression of Small HDV antigen (pSVL-S-HDAg).

2.8. Exosome extraction

Huh7 and Huh7-D12 cells were seeded in 25 cm^2 culture flasks with RPMI-1640 complete medium (Sigma-Aldrich, USA). 24h after, the medium was exchanged to RPMI-1640 10% FBS exosome depleted medium (Gibco, USA) and left incubating for 48 hours. Cell culture supernatants were collected and further subjected to two successive centrifugations of 2,000 G for 20 minutes at 4˚C to remove cells and cellular debris. Supernatant was collected and 0.5 volumes of the Total Exosome Extraction reagent (TEE, Invitrogen, USA) was added. Samples were left to incubate overnight at 4˚C.

On the following day, samples were centrifuged at 10,000 G for 1 hour at 4˚C. Supernatant was discarded and the pellet was resuspended in nuclease-free water (Ambion, USA) or SDS sample buffer (Invitrogen, USA), for RNA and protein extraction, respectively, and stored at -20˚C. Samples were then RNA and protein extracted (see subchapter 2.9 and 2.15).

2.9. Total RNA extraction

RNA extraction and purification were performed using RNA extraction commercial kit, RNeasy Mini (Qiagen, USA), following the manufacturer instructions, in accordance with the centrifugation protocol indicated for animal cells in monolayer.

Briefly, the cell culture medium was removed, and cells were washed with PBS 1X. Then, the cells were disrupted using RLT buffer supplemented with β-mercaptoethanol, with subsequent scrapping of the plate surface and the lysate was passed through a 20-gauge needle at least 10 times. Samples were homogenised in 350 µL 70% ethanol, the total volume was transferred to a mini-RNeasy column and centrifuged at 10,000 rpm for 15 seconds, discarding the flow-through. Subsequently, 350 µL RW1 buffer was added and, again, centrifuged at 10,000 rpm for 15 seconds, discarding the flow-through.

Samples were treated with RNase-Free DNase Set (Qiagen, USA), previously dissolved in 550 µL RNase-free water supplied in the kit. 10 µL DNase was added

together with 70 μL buffer RDD (supplied in the kit), directly onto the RNeasy silica-gel membrane. Samples were left incubating for 20 minutes at room temperature.

After this incubation, 350 μL of RW1 buffer was added and samples were centrifuged at 10,000 rpm for 15 seconds, discarding the flow-through and then 500 μL RPE buffer was added and centrifuged at 10,000 rpm for 15 seconds, discarding the flow-through. This last step was repeated, with a centrifugation at the same speed, only for 2 minutes. The collection tube was then changed and centrifuged at full speed for 1 minute, discarding the flow-through.

Finally, to elute the RNA, the RNeasy column was placed in a 1.5 mL tube and 40 μL RNase-free water (supplied in the kit) was added directly onto the membrane. Samples were left incubating for 1 minute and then centrifuged at 10,000 rpm for 1 minute. The column was discarded and the volume that crossed the membrane was quantified on the NanoDrop 1000 (Thermo Fisher, USA). After this step, samples were stored at -20°C, until further use.

2.10. Exosome RNA isolation

Exosome RNA isolation was performed by phenol-chloroform RNA extraction. Briefly, dried pellet from exosome extraction was resuspended in water. Then, 1 volume of phenol-chloroform was added, samples were vortexed for 1 minute and centrifuged at 13,000 rpm for 5 minutes at room temperature. The upper phase was carefully transferred to a fresh tube and 2 volumes of ice cold 100% ethanol and 0.1 volumes of sodium acetate 3M pH 5.2 were added. After vortexing, nucleic acids were precipitated by placing the samples at -20˚C overnight.

On the following day, samples were centrifuged at 13,000 rpm for 30 minutes at 4˚C. Supernatant was discarded, pellets were washed with ice cold 70% ethanol and further centrifuged at 13,000 rpm for 5 minutes at 4˚C. Supernatants were discarded and the pellet was resuspended in nuclease-free water (Ambion, USA).

Finally, samples were incubated for 30 minutes at 4˚C with RQ1 DNase (Promega, USA). After the DNase treatment, samples were subjected to another phenol-chloroform RNA extraction, following the same steps descried before. In the end, pellets were resuspended in nuclease-free water and quantified using the NanoDrop 1000 (Thermo Fisher, USA).

2.11. RNA quantification

1 µL of RNA extraction sample was placed into the NanoDrop 1000 spectrophotometer (Termo Fisher, USA). The degree of purity was evaluated through the ratio between the absorbance measured at 260 nm and 280 nm.

2.12. Complementary DNA synthesis

Complementary DNA (cDNA) was synthetized from the RNA extraction samples using NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal) according to manufacturer instructions. Each reaction occurred in a total volume of 20 µL – 10 µL NZYRT 2X Master Mix, 2 µL NZYRT Enzyme Mix, volume up to 1 µg RNA and nuclease-free water (Ambion, USA). After homogenization, samples were incubated for 10 minutes at room temperature. Samples were then transferred to MJ MiniTM thermal cycler (BioRad, USA) for 30 minutes at 50˚C, followed by 5 minutes at 85˚C and briefly chilled on ice. 1 µL NZY RNase H was added and placed back in the thermal cycler for 20 minutes at 37˚C.

Samples were stored at -20˚C until further utilization.

2.13. Polymerase Chain Reaction (PCR)

The polymerization chain reactions took place in a Gene Pro thermal cycler (Bioer, China), according to the conditions described in Table I. The final volume of each reaction was 25 µL, being 12.5 µL Red Taq DNA Polymerase 2x Master Mix with 1.5 mM $MgCl_2$ (VWR, USA), 0.5 µL of each primer (Forward (Fwd) and Reverse (Rev)) at 20 µM, 2 µL cDNA and 9.5 µL nuclease-free water (Ambion, USA). Negative controls were also prepared, with the cDNA being replaced by the addition of 2 µL nuclease-free water.

Excepting stated otherwise, the primers used in this study – described in Table II – were designed in the laboratory using the free software Primer3 (v.0.4.0) (Untergasser *et al.*, 2012).

Table I. PCR steps, emphasizing temperature and duration for each step. Every PCR occurred following the same conditions.

Step	Time (min)	Temperature (˚C)	Number of cycles
Initial denaturation	5	95	1
Denaturation	1	95	29
Annealing	1	56	
Extension	1	72	
Final extension	5	72	1
Storage	Indefinite	-20	

Table II. 5'-3' primers sequences used to gene amplification by PCR. The exons where the primers are located are also indicated, as well as the size of the PCR product obtained (in base pairs – bp).

Gene	Exon (ex.)	Sequence	Size - bp
U1	Fwd	GATACCATGATCACGAAGGTGGTT	113
	Rev	CACAAATTATGCAGTCGAGTTTCC	
U2	Fwd	TTTGGCTAAGATCAAGTGTAGTATCTGTTC	86
	Rev	AATCCATTTAATATATTGTCCTCGGATAGA	
U4	Fwd	GCGCGATTATTGCTAATTGAAA	79
	Rev	AAAAATTGCCAATGCCGACTA	
U5	Fwd	GGTTTCTCTTCAGATCGCATAAATC	90
	Rev	CTCAAAAAATTGGGTTAAGACTCAGA	
U6	Fwd	CTCGCTTCGGCAGCACA	100
	Rev	AACGCTTCACGAATTTGCGT	
GAPDH	Fwd	CAATGACCCCTTCATTGACC	156
	Rev	GATCTCGCTCCTGGAAGATG	
HDV genomic RNA	Fwd	GGACCCCTTCAGCGAACA	100
	Red	CCTAGCATCTCCTCCTATCGCTAT	
HDV1	Fwd	GAGAGGGGATGTCACGGTAA	218
	Rev	TGGGTGCTACTCAACCCTTC	
HDV2	Fwd	GAAGGGGAAAGAGGAAGGTG	797
	Rev	TGAATAAAGCGGGTTTCCAC	
HDV3	Fwd	ACCCGAAGAGGAAAGAAGGA	478
	Rev	GAACGGACCAGATGGAGGTA	
CCND1	Fwd ex. 1	CCCTCGGTGTCCTACTTCAA	149
	Rev ex. 2	AGGAAGCGGTCCAGGTAGTT	
FOS	Fwd ex. 1	AGCAGTGACCGTGCTCCTAC	285
	Rev ex. 2	GTGACCGTGGGAATGAAGTT	
ADAR1	Fwd ex. 3	GTCATCAATGGCCGAGAGTT	78
	Rev ex. 3	GGCTTTCATAGCTGCATCCT	
STAT3	Fwd ex. 3	TCCTGGGAGAGATTGACCAG	184
	Rev ex. 4	GTGGCTGCAGTCTGTAGAAGG	

2.14. Agarose gel electrophoresis

PCR reaction products were loaded into an agarose gel (2%) in Tris-acetate-EDTA buffer (TAE). For gel preparation, 2 grams of agarose powder was dissolved in 100 mL boiling TAE buffer 1X. After cooling, 5 µL of RedGel dye (Biotium, Canada) was added. Then, the solution was poured into a casting tray with a well-former template with the appropriate number of wells. After gel solidification, 12.5 µL of each PCR sample was loaded into the wells, along with 1.5 µL Low Range DNA Ladder (Thermo Fisher, USA). Electrophoresis was performed at 80 V for approximately 90 minutes.

Obtained results were analysed using a transilluminator for image capture using the QuantityOne 4.6.1 software (BioRad, USA).

2.15. Protein extraction

For total extract preparation, cells were directly resuspended in Sodium Dodecyl Sulphate (SDS) sample buffer 1X (Invitrogen, USA) and benzonase 1:10 (Sigma-Aldrich, USA) and incubated for 10 minutes at room temperature. Samples were then boiled at 95˚C for 5 minutes and stored at -20˚C until use. For exosome protein extraction, dried pellet was subjected to the same extraction protocol.

2.16. SDS-PAGE and western blot analysis

Protein samples were separated by 10% SDS-PAGE gel (BioRad, USA) and proteins were transferred to a nitrocellulose membrane (BioRad, USA) using a semi-dry system (BioRad, USA). After transferring the protein from the gel to the membrane, the membrane was blocked with 5% low fat milk powder in PBS 1X for 1 hour at room temperature. Then, the membrane was incubated with primary antibodies diluted in 2.5% low fat milk powder in PBS 1X for 2 hours at room temperature, and after washing with PBS 1X supplemented with 0.05% Tween-20 for 45 minutes, the membrane was incubated with secondary antibodies conjugated with Horseradish Peroxidase (HRP, BioRad, USA) in a 1:3000 dilution in 2.5% low fat milk powder in PBS 1X for 1 hour at room temperature. Following the last wash with PBS 1X supplemented with 0.05%

Tween-20 for 1 hour, membranes were developed using a chemiluminescent system (ECL Western Blotting Detection Reagent, GE Healthcare, USA) in a dark room.

The following primary antibodies were used in this study: anti-heterogeneous nuclear ribonucleoprotein K (hnRNP K) monoclonal antibody (1:200; Santa Cruz Biotechnology, USA) and anti-HDAg polyclonal antibody (B3) (1:500; Santa Cruz Biotechnology, USA).

2.17. Silver staining

In order to silver stain exosome protein extracts in polyacrylamide gels, we made use of the SilverQuest Silver Staining Kit (Invitrogen, USA) following manufacturer instructions (Gharahdaghi *et al.*, 1999).

After the electrophoresis, the gel was removed from the cassette and washed briefly with ultrapure water in a clean staining tray. Then, the gel was fixed with 40% methanol / 10% acetic acid and left in agitation for 20 minutes. The gel was then washed with 30% ethanol, left incubating in sensitizer solution for 10 minutes, followed by two washes, one in ethanol and the other in water for 10 minutes each. The gel was incubated in staining solution for 20 to 60 seconds and developed in developing solution until the desired band intensity was reached. After the appropriate staining intensity was achieved, development was stopped by the addition of a stopper solution directly onto the gel.

2.18. Supernatant transference

Huh7 cells were seeded in six-well plates (Sarstedt, Germany). When a 50% confluence was reached, 1.5 mL of Huh7-D12 supernatant was poured into each well. Huh7-D12 supernatant was subjected to three successive centrifugations at 2,000 G for 10 minutes at room temperature. After three days of incubation with Huh7-D12 supernatant, RNA and proteins from the Huh7 cells were collected for further use.

2.19. Mass spectrometry analysis

Proteins were extracted from vesicles by adding SDS buffer (Invitrogen, USA) and subjecting them to a short migration on a denaturing SDS-PAGE gel (Hartmann *et*

al., 2014). Peptides were generated by in-gel trypsin proteolysis and were identified by tandem mass spectrometry. NanoLC-MS/MS experiments were performed on a Q-Exactive HF high resolution tandem mass spectrometer (Thermo Fisher, USA) coupled to an UltiMate 300 LC system (Dionex-LC Packings, France) as previously described (Malard *et al.*, 2012; Hartmann *et al.*, 2014).

3. RESULTS

3.1. Huh7-D12 cell line constitutively expresses HDV

To ensure that the cellular model we wanted to use was working, the first thing to be done was to prove that the Huh7-D12 cell line was constitutively expressing HDV. For this purpose, Huh7 and Huh7-D12 cells were plated in six-well plates of 3.5 cm diameter and incubated for 24h. Huh7-D12 cell line results from a stable transfection of Huh7 cells with the pSVL (D3) plasmid. Following stable transfection, this cell line expresses both delta antigens and all RNA transcripts associated with the HDV life cycle (Cunha *et al.*, 1998). After 24h, total RNA was extracted, cDNA was synthetized and HDV genomic RNA expression levels were evaluated by PCR. PCR products were resolved on a 2% agarose gel.

Results show the presence of a PCR product of approximately 100 bp in the Huh7-D12 cell line samples, product that is not amplified in the samples of Huh7 cell line, which serves as a negative control (Figure 5, A panel). The same samples were subjected to an additional PCR, using specific primers for U6 small nuclear RNA (snRNA), an endogenous control, which serves as a loading control, testing for the absence of errors in the amount of RNA used for the cDNA reaction and in the amount of cDNA used in the PCR. The results show that the levels of U6 snRNA expression are similar between the two samples analysed, not suggesting the occurrence of any handling error (Figure 5, panel B).

Using the same experimental procedure, total protein extraction from Huh7-D12 cells was performed to analyse the expression of delta antigens by western blot. Protein extracts were separated by SDS-PAGE, transferred to a nitrocellulose membrane and incubated with anti-HDAg antibody (B3), a polyclonal serum that recognizes epitopes common to both delta antigens (Tavanez *et al.*, 2002) and an anti-hnRNP K monoclonal antibody, which serves as a loading control for the amount of proteins used in the assay. western blot results with anti-HDAg antibody (B3) show the detection of two specific bands in the Huh7-D12 cells sample, with molecular weights of 27 and 24 kDa, corresponding to L-HDAg and S-HDAg, respectively (Figure 6, panel A). Regarding the results of western blot analysis with anti-hnRNP K antibody, these show identical levels for this protein in the two samples analysed – Huh7 and Huh7-D12 (Figure 6, panel B).

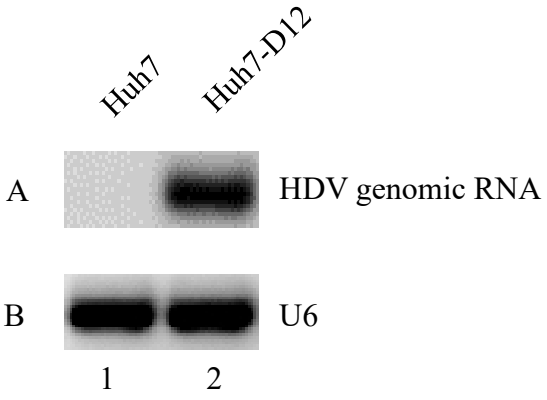

Figure 5. Expression levels of HDV genomic RNA in Huh7 and Huh7-D12 cells. The levels of HDV genomic RNA (A) and U6 snRNA (B) were analysed by PCR and resolved on a 2% agarose gel. Lane 1 corresponds to Huh7 cell line samples. Lane 2 corresponds to samples from Huh7-D12 cell line.

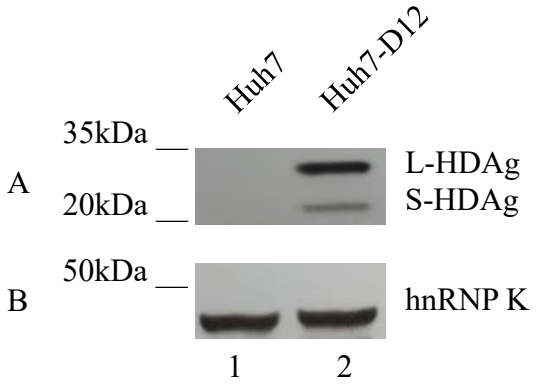

Figure 6. Expression levels of S-HDAg and L-HDAg in Huh7 and Huh7-D12 cells. Protein levels of S-HDAg and L-HDAg (A) were analysed by western blot using the anti-HDAg polyclonal antibody (B3). The levels of hnRNP K protein were detected by western blot using the monoclonal antibody against hnRNP K (B). Lane 1 corresponds to Huh7 cell line samples. Lane 2 corresponds to Huh7-D12 cell line samples.

3.2. Huh7-D12 cell line secretes exosome-like vesicles

Exosomes are described to play a role in mediating cell-to-cell communication and to be associated with various biological functions. These vesicles contain several proteins, mRNAs and microRNAs, and have been suggested to have roles in liver cirrhosis and HCC. Additional evidence shows that HBV and HCV use exosomes to transfer functional microRNAs and signalling-competent proteins to uninfected cells (Bility *et al.*, 2014; Shen *et al.*, 2017; Wang *et al.*, 2018).

Given our interest in investigating the capacity of Huh7 and Huh7-D12 cell line to produce and secrete exosomes, it was necessary to establish a protocol for the extraction of these vesicles. The protocol, schematically depicted in Figure 7, mainly consists of two successive centrifugations to remove cells and cellular debris, the addition of Total Exosome Extraction reagent to precipitate exosomes, one additional centrifugation and resuspension of pellets. After the implementation of the protocol, vesicles were isolated and eluted samples were finally analysed by PCR or western blot.

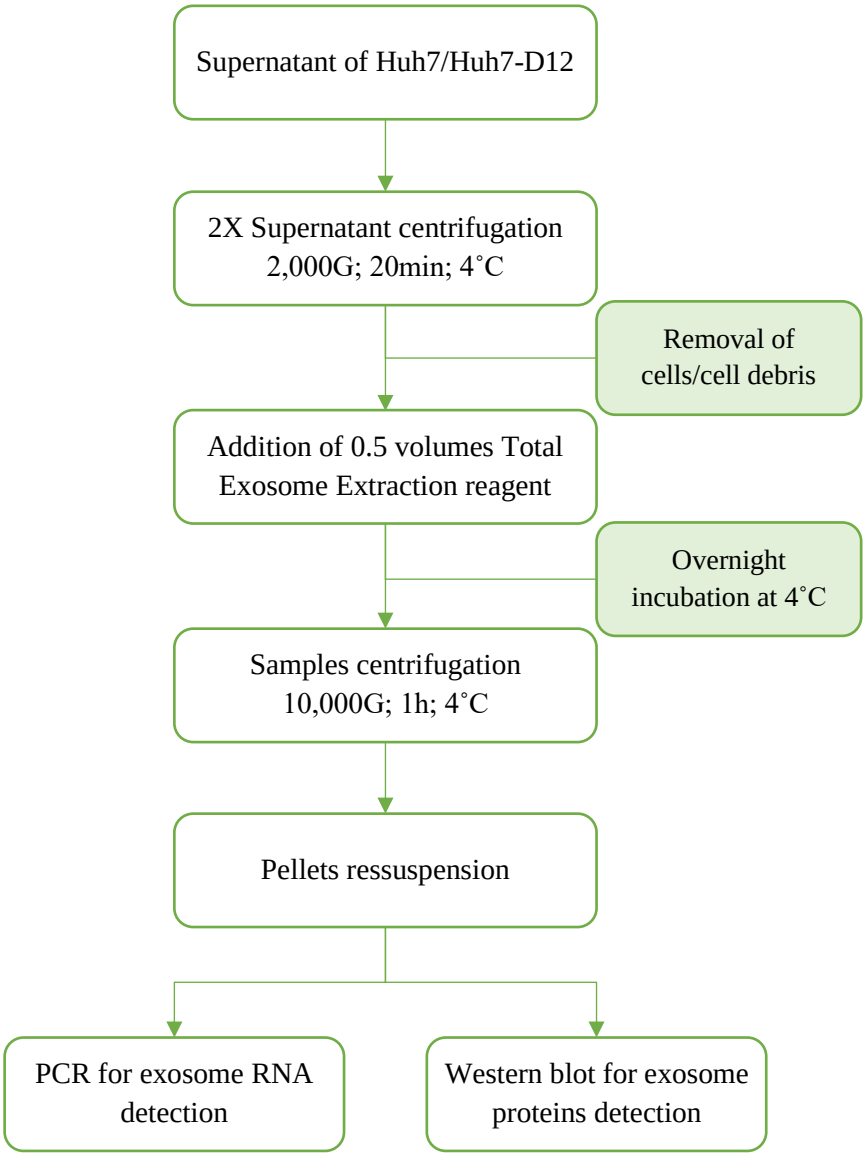

Figure 7. Representative flowthrough of the protocol designed for exosome extraction.

3.3. Exosome RNA characterization

Exosomes play a role in intercellular communication, transferring proteins, microRNAs and mRNAs from cell to cell. As explained earlier, Huh7-D12 cell line constitutively expresses HDV components, however, it is unable to secrete HDV since it needs the usage of HBV envelope proteins to form fully infective particles (Cunha *et al.*, 1998). In this part of the results, we aimed to show that Huh7-D12 can secrete HDV RNPs via exosomes. Exosomal and total RNA were extracted as previously described, cDNA was synthetized and PCR was performed using primers designed to amplify different regions of the HDV genomic RNA with different amplicon sizes – 200, 500 and 800 base pairs, approximately. Simultaneously, U6 snRNA was used as an endogenous control.

Results show that all different sizes of HDV genome are present in exosome samples from Huh7-D12 cells (Figure 8, panels A, B, C), as well as in samples of total RNA extraction of Huh7-D12 (Figure 8, lanes 3 and 4). On the other hand, HDV is not detected in samples from Huh7, which is in line with the expected, since this cell line is not capable of expressing HDV components (Figure 8, lanes 1 and 2). As expected, U6 is present in all samples and show expression levels similar between controls and exosome samples, respectively (Figure 8, panel D).

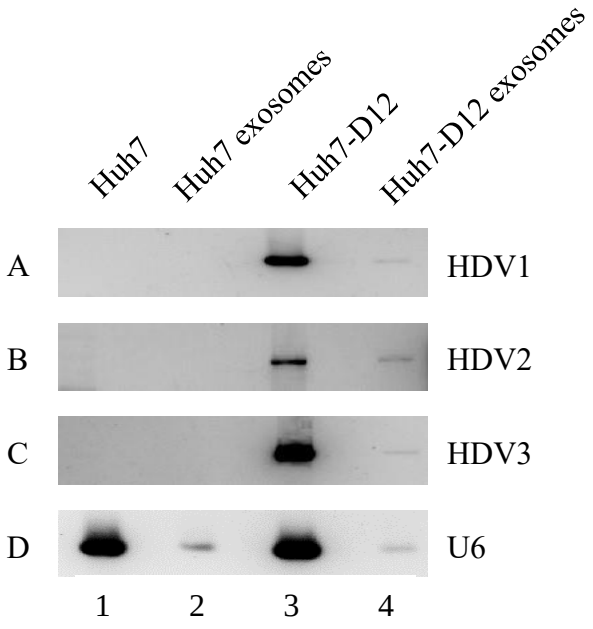

Figure 8. Huh7-D12 exosome samples contain HDV RNA. The RNA present in the nanovesicles of the Huh7 and Huh7-D12 cell line was analysed by PCR and resolved on a 2% agarose gel. Different regions of the HDV genomic RNA (A, B, C) were detected in Huh7-D12 cell line and in exosomes derived from

Huh7-D12; in contrast, HDV genomic RNA was not detected in Huh7 cell line or in exosomes derived from Huh7. U6 snRNA (D) was used as housekeeping gene and it was detected in all the samples analysed. Lanes 1 and 3 correspond to control samples from Huh7 and Huh7-D12 cell lines, respectively. Lanes 2 and 4 correspond to exosome samples extracted from Huh7 and Huh7-D12 cell lines, respectively.

To validate the specificity of the HDV RNA and U6 snRNA detected, and also to rule out the possibility of contamination with genomic DNA in exosome samples, we performed several PCRs for specific genes studied in our laboratory, such as Cyclin D1 (CCND1), FOS, Adenosine Deaminase Acting on RNA 1 (ADAR1) and Signal Transducer and Activator of Transcription 3 (STAT3), mRNAs that we did not expect to be present in exosomes. Figure 9 shows that the above-mentioned mRNAs are detected only in the total RNA extraction samples, which indicates that the Huh7 and Huh7-D12 exosomes do not contain these mRNAs.

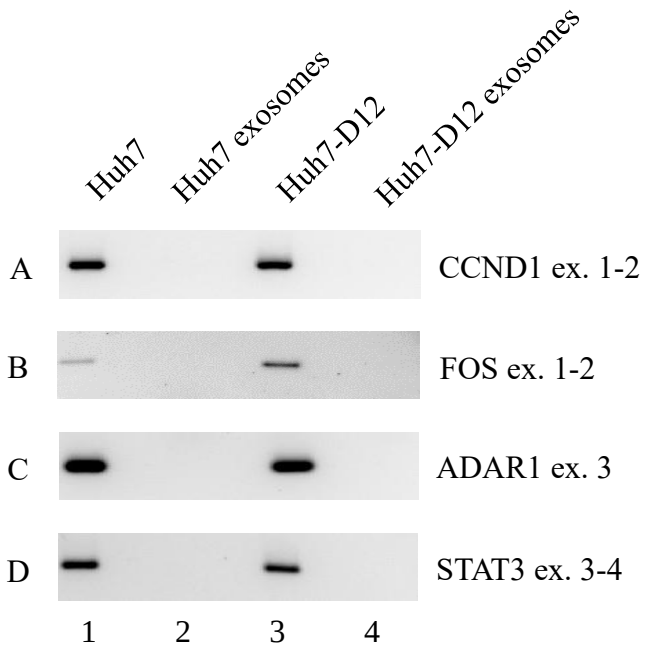

Figure 9. Huh7 and Huh7-D12 exosomes do not contain RNA from CCND1, FOS, ADAR1 or STAT3. The RNA present in the nanovesicles of the Huh7 and Huh7-D12 cell lines was analysed by PCR and resolved on a 2% agarose gel. RNAs like CCND1 (A), FOS (B), ADAR1 (C) and STAT3 (D) were not detected in exosomes derived from Huh7 and Huh7-D12; in contrast, these RNAs were detected in Huh7 and Huh7-D12 cell line samples. Lanes 1 and 3 correspond to control samples from Huh7 and Huh7-D12 cell lines, respectively. Lanes 2 and 4 correspond to exosome samples extracted from Huh7 and Huh7-D12 cell lines, respectively.

After the identification that exosomes from Huh7-D12 contain HDV RNA and that our samples were not contaminated with genomic DNA, we tested our samples for the presence of reference genes known to be present in extracellular vesicles. Two transcripts, namely the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the U6 small nuclear RNA, are among the most conserved transcripts identified in exosome vesicles in a variety of conditions (Patel *et al.*, 2019). snRNP U6 is the most common spliceosomal nuclear RNA found in cells and is a highly conserved non-coding fragment of the ribonuclear protein responsible for mediating pre-mRNA splicing. GAPDH is a metabolic enzyme commonly used as a housekeeping gene, such as U6 (Gouin *et al.*, 2017). Following exosome purification according to the protocol explained above and showed in Figure 7 and RNA extraction, cDNA was synthesised and used to analyse for the presence of U6 and GAPDH RNA by PCR, using two pairs of primers specific for these mRNAs.

Results depicted in Figure 10 show that both exosomes extracted from Huh7 and Huh7-D12 contain GAPDH and U6 transcripts, in line with what is described in the literature and validating that our purification protocol is indeed optimized in terms of exosomal isolation.

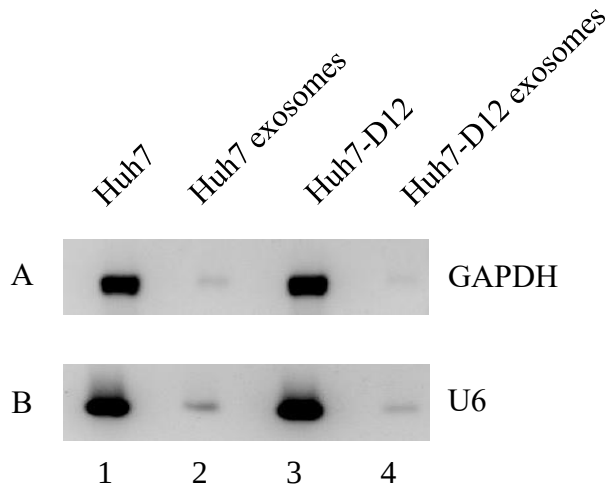

Figure 10. Huh7 and Huh7-D12 exosomes contain GAPDH and U6 RNA. The RNA present in the nanovesicles of the Huh7 and Huh7-D12 cell lines was analysed by PCR and resolved on a 2% agarose gel. GAPDH (A) and U6 (B) RNAs were detected in exosomes from Huh7 and Huh7-D12. Lanes 1 and 3 correspond to control samples from Huh7 and Huh7-D12 cell lines, respectively. Lanes 2 and 4 correspond to exosome samples extracted from Huh7 and Huh7-D12 cell lines, respectively.

After the identification of U6 snRNA, we decided to investigate whether the additional snRNAs part of the spliceosome were also present in vesicles extracted from Huh7 and Huh7-D12 cells. For this, we designed primers for the U1, U2, U4 and U5 snRNAs and tested for their presence by PCR. As it can be seen in Figure 11, U1, U2, U4 and U5 snRNAs are also detected in exosomes from Huh7 and Huh7-D12 cells, as well as in the respective control samples of total RNA.

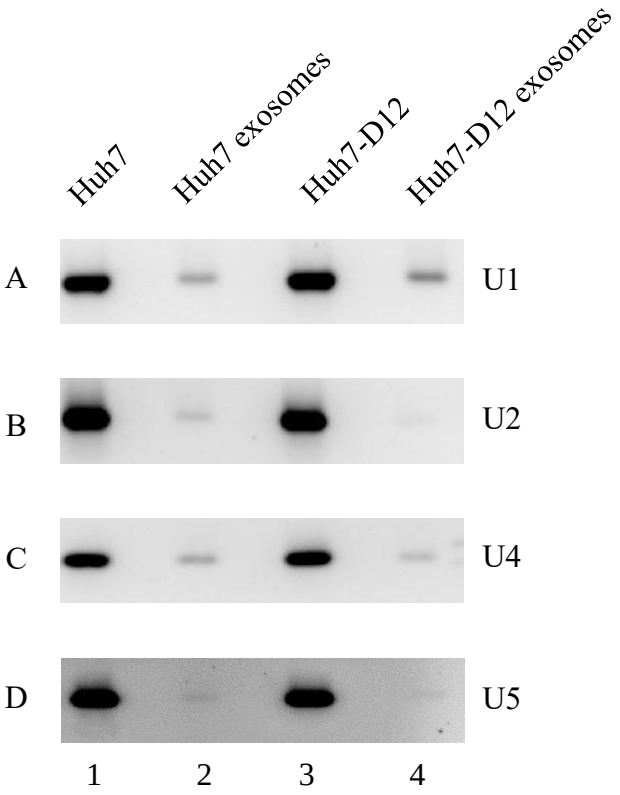

Figure 11. All small nuclear RNAs of the spliceosome are present in Huh7 and Huh7-D12 exosomes content. The RNA present in exosomes derived from Huh7 and Huh7-D12 cell lines was analysed by PCR and resolved on a 2% agarose gel. U1 (A), U2 (B), U4 (C) and U5 (D) snRNAs were detected in exosomes derived from Huh7 and Huh7-D12 cell lines. Lanes 1 and 3 correspond to control samples from Huh7 and Huh7-D12 cell lines, respectively. Lanes 2 and 4 correspond to exosome samples extracted from Huh7 and Huh7-D12 cell lines, respectively.

3.4. Exosome protein characterization

Before characterizing the exosome protein content, we analysed the amount of protein present in exosome samples from Huh7 and Huh7-D12 cell lines. For this a silver staining was performed, as explained in Material and Methods section. Briefly, proteins were denatured, separated in a polyacrylamide gel and after repeated washing with water, the gel was incubated in a silver nitrate solution. Silver ions bind to negatively charged side chains of the proteins. This procedure stains the sites where proteins are present, brown to black. As it can be seen in Figure 12, Huh7 and Huh7-D12 total extracts present high amounts of proteins, shown by the saturation of the lane (Figure 12, lane 1 and 2). Samples of exosomes extracted from cell culture medium show no proteins, which is as expected (Figure 12, lane 3 and 4). Huh7 exosome samples show higher amounts of proteins when compared with Huh7-D12 exosome samples (Figure 12, lane 5 to 8).

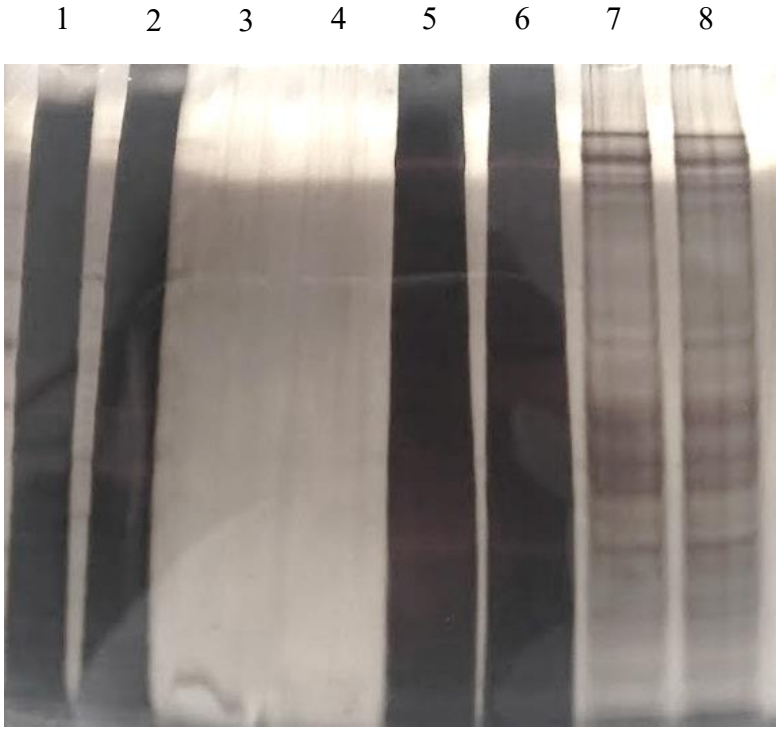

Figure 12. Huh7-D12 and Huh7 exosomes have proteins in their content. SDS-PAGE was performed on a 10% polyacrylamide gel. Lanes 1 and 2 correspond to total protein extraction from Huh7 and Huh7-D12 cell line, respectively. Lanes 3 to 8 correspond to samples submitted to exosome extraction protocol in duplicate. Lanes 3 and 4 correspond to exosome extraction from cell-free cell culture medium. Lanes 5 and 6 correspond to exosome extraction from Huh7 cells supernatant. Lanes 7 and 8 correspond to exosome extraction from Huh-D12 cells supernatant.

To characterize the protein content of isolated exosomes from Huh7 and Huh7-D12 cell lines we prepared protein extracts and a mass spectrometry analysis was performed. In total, 31 949 peptides were identified, which allowed to identify 3175 unique proteins and to validate 2381 proteins with at least two different peptide sequences. For each protein, the number of spectra assigned, spectral counts (SC), were counted as proxy of their abundances.

There were different amounts of proteins when comparing the samples of Huh7 and Huh7-D12 isolated exosomes, with an average of 46 175 and 29 002 SC, respectively. For this reason, a direct comparison between the two conditions was rather difficult, however, since there are less SC for Huh7-D12, when a greater number of SC is detected in the Huh7-D12 samples in comparison with SC of Huh7 samples, it is possible to infer that there are higher levels of the protein in question.

Table III shows the list of some of the proteins that were identified by mass spectrometry. It highlights three family proteins that we considered to be the most interesting, Exosomal markers, Splicing factors and Heparin-binding proteins. Also, some transcriptional factors, proteins related with the inflammatory response and with the cell cycle are also described on Appendix I.

Concerning exosome protein markers, some are described as being specifically found in the exosome layer, for example, Tumour susceptibility gene 101 protein (TSG101), CD63 and CD81, as can be seen in Table III. Exosomes originating from a variety of different cells share common structural and functional proteins. Besides, exosomes contain proteins from endosomes, the plasma membrane, and cytosol, as well as some components from some organelles, such as nucleus, mitochondria, Golgi apparatus and endoplasmic reticulum (Gurunathan *et al.*, 2019).

Table III. Huh7 and Huh7-D12 protein exosomal content. Proteins obtained by mass spectrometry analysis and divided in three categories: Exosomal markers, Splicing factors and Heparin-binging proteins.

Protein family	Protein	Protein accession	Spectral counts (SC)	Huh7 SC	Huh7-D12 SC
Exosomal markers	CD63 antigen	CD63_HUMAN	19	1	9
	Tumour susceptibility gene 101 protein	TS101_HUMAN	16	4	4
	CD81 antigen	CD81_HUMAN	12	1	5

Splicing factors	U1 small nuclear ribonucleoprotein A	SNRPA_HUMAN	20	9	2
	U2 small nuclear ribonucleoprotein A'	RU2A_HUMAN	17	9	0
	U2 small nuclear ribonucleoprotein B"	RU2B_HUMAN	12	6	0
	U2 snRNP-associated SURP motif-containing protein	SR140_HUMAN	22	11	0
	Splicing factor U2AF 65 kDa subunit	U2AF2_HUMAN	12	4	2
	U5 small nuclear ribonucleoprotein 200 kDa helicase	U520_HUMAN	121	49	21
	116 kDa U5 small nuclear ribonucleoprotein component	U5S1_HUMAN	117	49	10
	U4/U6.U5 tri-snRNP-associated protein 2	SNUT2_HUMAN	12	6	0
	U4/U6 small nuclear ribonucleoprotein Prp4	PRP4_HUMAN	22	11	1
	U4/U6 small nuclear ribonucleoprotein Prp31	PRP31_HUMAN	8	4	0
	Pre-mRNA-processing-splicing factor 8	PRP8_HUMAN	58	29	0
	Pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16	PRP16_HUMAN	7	4	0
	Pre-mRNA-processing factor 19	PRP19_HUMAN	88	25	19
	Splicing factor 1	SF01_HUMAN	67	23	11
	Splicing factor 3A subunit 1	SF3A1_HUMAN	199	67	33
	Splicing factor 3A subunit 2	SF3A2_HUMAN	37	14	5
	Splicing factor 3A subunit 3	SF3A3_HUMAN	66	25	9
	Splicing factor 3B subunit 1	SF3B1_HUMAN	200	71	9
	Splicing factor 3B subunit 2	SF3B2_HUMAN	140	62	8
	Splicing factor 3B subunit 3	SF3B3_HUMAN	130	54	12
	Splicing factor 3B subunit 4	SF3B4_HUMAN	13	5	2
	Heterogeneous nuclear ribonucleoprotein F	HNRPF_HUMAN	57	18	11
	Heterogeneous nuclear ribonucleoprotein L	HNRPL_HUMAN	185	68	25
	Heterogeneous nuclear ribonucleoprotein M	HNRPM_HUMAN	207	90	14

Heterogeneous nuclear ribonucleoprotein R	HNRPR_HUMAN	110	43	12
Heterogeneous nuclear ribonucleoprotein D-like	HNRDL_HUMAN	91	32	14
Heterogeneous nuclear ribonucleoprotein L-like	HNRLL_HUMAN	19	10	0
Heterogeneous nuclear ribonucleoprotein U-like protein 1	HNRL1_HUMAN	71	36	0
Heterogeneous nuclear ribonucleoprotein U-like protein 2	HNRL2_HUMAN	27	13	1
Serine/arginine-rich splicing factor 1	SRSF1_HUMAN	84	26	16
Serine/arginine-rich splicing factor 2	SRSF2_HUMAN	23	11	1
Serine/arginine-rich splicing factor 4	SRSF4_HUMAN	24	6	7
Serine/arginine-rich splicing factor 6	SRSF6_HUMAN	62	16	15
Serine/arginine-rich splicing factor 9	SRSF9_HUMAN	10	5	0
ATP-dependent RNA helicase A	DHX9_HUMAN	164	76	7
Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	DHX15_HUMAN	175	70	18
Probable ATP-dependent RNA helicase DDX17	DDX17_HUMAN	211	88	18
Spliceosome RNA helicase DDX39B	DX39B_HUMAN	80	29	11
RNA-binding protein 10	RBM10_HUMAN	31	11	5
SNW domain-containing protein 1	SNW1_HUMAN	12	6	0
Protein PRRC2A	PRC2A_HUMAN	13	7	0
Ribonucleoprotein PTB-binding 1	RAVR1_HUMAN	9	5	0
Protein Red	RED_HUMAN	8	4	0
Non-POU domain-containing octamer-binding protein	NONO_HUMAN	244	78	44
Heterogeneous nuclear ribonucleoprotein A1	ROA1_HUMAN	257	100	29
Cell cycle and apoptosis regulator protein 2	CCAR2_HUMAN	100	42	8
RNA-splicing ligase RtcB homolog	RTCB_HUMAN	46	22	1

	Serine-threonine kinase receptor-associated protein	STRAP_HUMAN	57	23	6
	Heterogeneous nuclear ribonucleoprotein A/B	ROAA_HUMAN	70	22	14
	Gem-associated protein 5	GEMI5_HUMAN	19	8	2
	Poly(U)-binding-splicing factor PUF60	PUF60_HUMAN	21	8	3
	WW domain-binding protein 11	WBP11_HUMAN	15	7	1
	WD40 repeat-containing protein SMU1	SMU1_HUMAN	18	8	1
	Small nuclear ribonucleoprotein Sm D2	SMD2_HUMAN	19	9	1
	Small nuclear ribonucleoprotein-associated proteins B and B'	RSMB_HUMAN	35	13	5
	Transformer-2 protein homolog beta	TRA2B_HUMAN	13	1	6
	RNA helicase aquarius	AQR_HUMAN	15	8	0
Heparin-binding	Syndecan-4	SDC4_HUMAN	30	2	14
	Hepatic triacylglycerol lipase	LIPC_HUMAN	14	1	6
	Beta-2-glycoprotein 1	APOH_HUMAN	22	0	11
	Glia-derived nexin	GDN_HUMAN	12	0	6
	Antithrombin-III	ANT3_HUMAN	12	0	6
	CCN family member 1	CCN1_HUMAN	10	0	5
	Laminin subunit gamma-2	LAMC2_HUMAN	5	0	3

3.5. Huh7-D12 cells can infect naïve Huh7 cells by supernatant transference

As already mentioned, some studies reported the presence of HDAgs and HDV RNA in salivary glands in patients with primary Sjögren's syndrome, without the presence of HBV antibodies and HBsAgs (Weller et al., 2016; Perez-Vargas et al., 2019). On this basis, we decided to investigate whether HDV has the ability to infect Huh7 naïve cells without the presence of HBV or its envelope proteins. For this purpose, Huh7 naïve cells were seeded in 6-well plates and incubated overnight, followed by transient transfection with pSVL-S-HDAg. On the third day, cells were incubated for 48h either with Huh7 or Huh7-D12 supernatants from exponentially grown Huh7-D12 and Huh7 cells. Results show that HDV can be detected in total extract samples from Huh7-D12 control sample and in Huh7 cells incubated with Huh7-D12 supernatants, subjected or not to transfection with pSVL-S-HDAg (Figure 13).

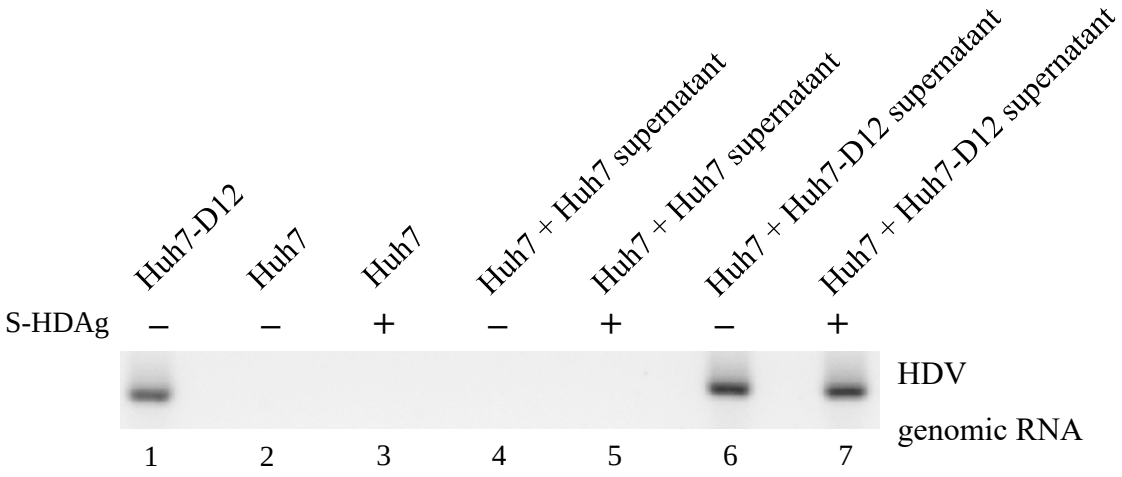

Figure 13. HDV genomic RNA is detected in Huh7 naïve cells incubated with Huh7-D12 supernatant and transfected with pSVL-S-HDAg. RNA from naïve Huh7 cells was analysed by PCR and resolved on a 2% agarose gel. HDV was detected in samples incubated with Huh7-D12 supernatant and transfected with pSVL-S-HDAg. Samples were incubated with fresh culture medium, Huh7 cells supernatant or Huh7-D12 cells supernatant, with or without transfection with pSVL-S-HDAg. Lanes 1, 2 and 3 correspond to control samples from Huh7-D12 and Huh7 incubated with fresh medium. Lane 3 was also transfected with pSVL-S-HDAg. Lanes 4 and 5 correspond to Huh7 cells incubated with Huh7 cells supernatant. Lane 5 was also transfected with pSVL-S-HDAg. Lanes 6 and 7 correspond to Huh7 cells incubated with Huh7-D12 cells supernatant. Lane 7 was also transfected with pSVL-S-HDAg.

We show that exosomes from infected cells can transport the complete molecule of HDV genomic RNA that now becomes detected in Huh7 naïve cells. Regarding the presence of HDAgs in exosome content, their incorporation seems to be either absent or present in low amounts as we could not detect them when submitting these extracts to western blotting experiments using an anti-HDAgs polyclonal antibody (data not shown). In order to complement these previous set of experiments, protein extracts were obtained from an experiment similar to the one described earlier. Huh7 naïve cells were seeded in 6-well plates and incubated overnight. The following day, cells were transfected with pSVL-S-HDAg and the day after, incubated with either Huh7 or Huh7-D12 supernatants. Our results show that S-HDAg is expressed in samples transfected with pSVL-S-HDAg, as expected (Figure 14, lanes 1, 5 and 7). Both antigens were detected, S-HDAg and L-HDAg, in the Huh7-D12 total extract positive control, as well as in Huh7 cell samples incubated with Huh7-D12 supernatant and transfected with pSVL-S-HDAg (Figure 14, lanes 3 and 7). This supports the idea that exosomes have in their content the entire HDV genomic RNA, and that the exogenous expression of S-HDAg promotes the initiation of the HDV replication cycle, leading to the expression of the L-HDAg.

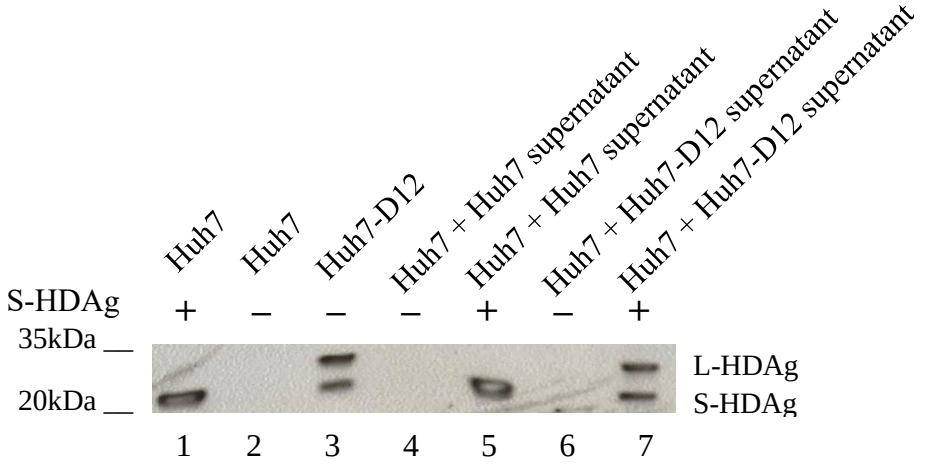

Figure 14. HDV antigens detected in Huh7 naïve cells incubated with Huh7-D12 cells supernatant. Protein levels of S-HDAg and L-HDAg were analysed by western blot using the anti-HDAg polyclonal antibody. Both HDAgs were detected in samples incubated with Huh7-D12 supernatant and transfected with pSVL-S-HDAg. Samples were incubated with fresh culture medium, Huh7 cells supernatant or Huh7-D12 cells supernatant, with or without transfection with pSVL-S-HDAg. Lane 1 corresponds to control Huh7 cell line samples transfected with pSVL-S-HDAg. Lanes 2 and 3 correspond to control samples from Huh7 and Huh7-D12 cell lines, respectively. Lanes 4 and 5 correspond to Huh7 cells incubated with Huh7 supernatant. Lane 5 was also transfected with pSVL-S-HDAg. Lanes 6 and 7 correspond to Huh7 cells incubated with Huh7-D12 supernatant. Lane 7 was also transfected with pSVL-S-HDAg.

4. DISCUSSION

Hepatitis Delta Virus was first described in 1977 by Mario Rizzetto and co-workers (Rizzetto *et al.*, 1977). Most severe viral hepatitis results of HBV/HDV superinfection, that not only increases the risk of hepatic disease, but also induces early development of hepatocellular carcinoma, the fifth cancer most common worldwide and one of the major complications associated with viral hepatitis (Koh, Heller and Glenn, 2019). HDV represents a global public health problem with statistic data suggesting that there are approximately 400 million people chronically infected with HBV and the most recent estimates suggest that 5% of these individuals are co-infected with HDV (WHO, 2019b).

The association between HDV and HBV is explained by the fact that HDV does not encode envelope proteins for packaging and relies on the surface glycoproteins of HBV for virion assembly and cellular transmission (Cunha, Tavanez and Gudima, 2015). HDV has the same transmission mechanisms as HBV, being transmitted by blood-derived products and sexual contact (Alfaiate, Dény and Durantel, 2015). Upon entry into the host's bloodstream, the HDV virion binds to cells by heparin sulphate proteoglycans interaction, a reaction that only occurs if L-HBsAg is part of the viral coating and that is identical for both HBV ad HDV (Krause, Haberkorn and Mier, 2018).

Entry of HBV and HDV into a host cell represents the initial step of infection. This process requires multiple steps, including the low-affinity attachment of the virus to the cell surface, followed by high-affinity attachment to specific receptors, and subsequent endocytosis-mediated internalization. Within the viral envelope, the preS1 region is involved in receptor binding. Recently, sodium taurocholate co-transporting polypeptide (NTCP) has been identified as an entry receptor of HBV and HDV by affinity purification using a preS1 peptide. NTCP is mainly or exclusively expressed in the liver, and this membrane protein is at least one of the factors determining the narrow species specificity and hepatotropism of HBV and HDV. Interestingly however, the HDV RNA genome can efficiently replicate in different tissues and species, raising the possibility that HDV is able to be transmitted independently of HBV. Some reports have been questioning the viral transmission mechanisms, not only for HDV but for other different viruses. It was recently demonstrated that enveloped viruses distinct from HBV can induce HDV dissemination *in vivo*, suggesting the existence of alternative pathways for HDV to propagate. In addition, it has been suggested that HDV ribonucleoproteins can

exploit assembly functions provided by other virus genus, for example, Flavivirus, Vesiculovirus, Hepacivirus among other enveloped viruses (Perez-Vargas *et al.*, 2019). Finally, one very interesting study identified the presence of HDV RNA and HDAgs in the salivary glands of Sjögren syndrome patients, without the presence of HBsAgs or HBV antibodies (Weller *et al.*, 2016). With all the information described above, we decided to evaluate whether HDV would be able to infect naïve cells without the presence of HBV coating or any other viral coating.

Novel findings in exosome biology have shown that some viruses can disseminate by this form of intercellular communication. Exosomes are membrane-derived nanovesicles secreted both by normal and malignant cells and currently recognized as critical structures in intercellular communication through horizontal transfer of information via their cargo, which includes selective proteins, mRNAs and miRNAs (Yang *et al.*, 2017). Macromolecules transported by exosomes may play a role in important cellular functions, such as transcription regulation, splicing, inflammation, cell signalling, apoptosis and cancer. Our organism contains several different types of extracellular vesicles which may be separated based on density, size, or surface markers. Exosomes are fairly easy to identify and differentiate because of the presence of the exosomal membrane markers TSG101, CD63 and CD81 (Gurunathan *et al.*, 2019).

Recent studies suggest that some viruses can hijack host's exosomes, loading viral constituents on their interior and infecting other cells through this type of intercellular communication (Li *et al.*, 2019). Several reports have demonstrated that HBV and HCV can be found in exosome-like vesicles isolated from serum and plasma, respectively, from HBV- and HCV-infected patients. In the case of HCV, it was shown that HCV is capable of successful transmission into naïve hepatocytes in a receptor-independent manner, taking advantage of host's exosomes. As for HBV, isolated exosomes from the sera of chronic HBV patients have been described to contain HBV nucleic acids and proteins, and to deliver HBV to healthy hepatocytes in an active manner, as observed for HCV (Bility *et al.*, 2014; Yang *et al.*, 2017; Wang *et al.*, 2018).

Our goal with this work was to establish a protocol for isolation and purification of exosomes and to analyse the presence of the HDV genome and HDV components in exosomes of HDV-expressing cells that would allow HDV to infect naïve human liver cells in the absence of HBV. Exosomes were purified from two human cell lines, Huh7-

D12 and its parental cell line, the human hepatocellular carcinoma Huh7. Huh7-D12, which was obtained by stable transfection of Huh7 with HDV genomic cDNA, replicates the HDV genome and constitutively expresses all HDV components. Exosome isolation and purification protocol was established based on exosome isolation and purification protocols described in the literature. For our experiments, we performed successive centrifugations followed by the addition of a Total Exosome Extraction reagent to cell supernatants of Huh7 and Huh7-D12 cell lines. Purified exosomes and supernatants of HDV-expressing cells were transferred to human cells that do not express HDV and HDV expression was analysed by PCR. Analysis of the RNA and protein content of these exosomes was also performed.

After establishment and optimization of the exosome isolation and purification protocol, we began our analysis by characterizing the exosomes RNA composition. The first question that we raised was whether the Huh7-D12 exosomes have HDV genomic RNA in their interior. For this, three pairs of primers with amplicon sizes of approximately 200, 500 and 800 base pairs were designed, pairing different regions of the genomic HDV RNA. We showed that the exosomes of Huh7-D12 cell line most likely contain the entire HDV genomic RNA in its content since we detected amplification of all the above-mentioned amplicons, which have different sizes and correspond to different sequences covering different regions of the HDV genome. In contrast, and in agreement with what was expected, these amplicons were not amplified when the starting material was the purified RNA from Huh7 cells exosomes.

We further addressed the RNA composition of these exosomes by analysing for the presence of transcripts of genes frequently identified in exosomes in other conditions. Two of the most common transcripts found in exosomes correspond to the transcripts of GAPDH and U6snRNA, which we were able to identify in exosome samples from both Huh7 and Huh7-D12 cell lines as expected. Particularly interesting was the identification of U6snRNA, which is part of the U6 small nuclear ribonucleoprotein (U6snRNP) and has been described to be present in other hepatic virus exosomes like HBV and HCV(Li *et al.*, 2015; Zhao *et al.*, 2020). U6 snRNP plays an essential role in pre-mRNA splicing, a process that is catalysed by a multi-megadalton ribonucleoprotein complex called spliceosome, that is composed of five snRNPs, U1, U2, U4, U5 and U6 (Will and Lührmann, 2011). After the identification of the U6 transcript in our exosome samples,

we raised the possibility that additional snRNA transcripts could also be identified. To test this hypothesis, specific primers for U1, U2, U4 and U5 were used and interestingly, all snRNAs analysed were present in exosomes from Huh7-D12 cells. Given the recent findings from our laboratory showing that HDV expression induces changes in the splicing of human genes, specifically in genes involved in cell cycle control pathways (Tavanez *et al.*, 2020), one attractive possibility to explain these observations is that HDV exosomes selectively incorporate splicing elements. Incorporation of specific splicing components in these exosomes, such as snRNPs, snRNPs-associated splicing factors and splicing factors not associated to snRNPs, would allow the establishment of specific splicing reactions and specific gene expression profiles in the cells targeted by these exosomes, which in turn could facilitate viral replication and dissemination for instance. In the future, it would be very interesting to determine the complete RNA composition of these exosomes, both in terms of coding and non-coding RNA, and this can be easily achieved by the use of RNA sequencing methodologies. It would also be extremely interesting to analyse whether the RNAs we have identified are also detected in patient samples, which would strengthen the biological meaning of our observations.

After our initial observation that the HDV genomic RNA is present in the HDV exosomes, and because HDV antigens interact with the HDV genomic RNA, the next logical question that we raised was whether HDV antigens were also part of the exosome content. Protein extracts from purified exosomes of Huh7 and Huh7-D12 cells were isolated and subjected to mass spectrometry analysis. From the 31 949 total peptides identified, 3175 proteins were identified and 2381 proteins were validated. A direct comparison between Huh7 and Huh7-D12 proteins was challenging to perform, as there was a significant difference in the protein concentration between the two cell lines. However, there are some empirical conclusions that can be taken, for example, when the spectral counts of proteins from Huh7-D12 exosomes are higher compared to the spectral counts registered for Huh7 exosomes, it is possible to infer that these protein levels are preferentially detected in exosomes of HDV-infected cells. Enrichment analysis of the proteins identified in Huh7 and Huh7-D12 exosome samples was performed and Table III and Appendix I present the most significant protein families identified. Exosomal markers such as CD63, Tumour Susceptibility Gene 101 (TSG101) and CD81 were identified in our mass spectrometry analysis, in the exosomes of both cell lines as

expected. On the other hand, HDV antigens were not identified both in the mass spectrometry analysis and in western blot analysis, which may be due to two reasons: first, HDAgs are not a cargo loaded in the HDV exosomes; second, HDAgs are loaded in the HDV exosomes in relatively low amounts, undetectable by the methodologies and techniques that we used.

After exosome RNA and protein characterization, exosome-containing Huh7-D12 supernatants were transferred to non-expressing HDV cell lines. The purpose of this experiment was to analyse whether HDV in the Huh7-D12 exosomes was capable to infect naïve Huh7 cells without the presence of HBV antigens. Naïve cells were subsequently incubated with this exogenous supernatant for three days, followed by total RNA and protein extractions in order to detect the presence of HDV components. Whereas the HDV genomic RNA was detected in Huh7 naïve cells after Huh7-D12 supernatant transference, neither of the HDV antigens were detected in the same conditions. However, when we performed the same type of experiment transferring Huh7-D12 supernatants to Huh7 cells that were previously transfected with S-HDAg, we were able to detect the HDV genomic RNA and both HDV antigens. This is a very important result because detection of the L-HDAg indicates the presence of the entire HDV RNA genome in these HDV exosomes. Our overall interpretation of these results is that HDV exosomes contain the entire HDV RNA genome and, most likely, very low levels of both HDV antigens that we could not detect with the methodologies we used in this study. When these exosomes are transferred to Huh7 cells that are expressing high levels of exogenous S-HDAg, the high levels of the exogenous HDV small antigen positively contribute to the initiation of complete HDV replication cycles, including expression of the additional HDV RNA elements (antigenomic RNA and mRNA) and RNA editing mediated by ADAR-1, ultimately leading to the expression of the L-HDAg.

Overall, Huh7 and Huh7-D12 exosomes composition is highly similar, as mass spectrometry analysis show almost identical protein entities in the exosome preparations from both cell lines. One of the most interesting observation of this study was the identification of a significant number of splicing factors in the exosomes from both cell lines, an observation that most likely reflects the incorporation of all five snRNPs. In fact, snRNPs being loaded into exosomes may be important to increase the incorporation of specific splicing factors. As explained earlier, our laboratory has recently reported that

HDV infection induces changes in alternative splicing of human genes dependent on SF3B155, which is a component of the U2 snRNP complex, essential for the early recognition of 3' splice sites in the pre-mRNA (Tavanez *et al.*, 2020). Interestingly, SF3B155 was one of the splicing factors that we identified in our mass spectrometry analysis. It is thus tempting to speculate whether HDV-containing exosomes can also modulate alternative splicing and whether HDV exosomes are part of a diverse viral strategy to modulate the host gene expression of target cells, facilitating viral infection.

Splicing factors are involved in constitutive and alternative splicing, being responsible for intricate and complex combinations of exon skipping and inclusion patterns, important for the correct modulation of gene expression and protein diversity. Alternative splicing can quickly regulate the expression of several groups of proteins, and defects in this mechanism are the basis of several human diseases and are often found in human tumours.

The mass spectrometry analysis that we performed led to the identification of several splicing factors in our exosome purifications. Out of the identified splicing factors, we must highlight the identification of TRA2β, a serine-arginine-rich-like protein that is the splicing factor that we found to be more present in Huh7-D12 exosomes than in exosomes of Huh7. Tumours frequently present altered levels of serine-arginine-rich proteins that consequently lead to the formation of RNA isoforms involved in tumour biology, in addition, these altered levels may affect downstream targets and alternative splicing events. TRA2β regulates alternative splicing and is essential for embryonic development; reported overexpression and downregulation of TRA2β is associated with several tumours and cancers such as lung, breast, ovarian, cervical, prostate, colon, central nervous system, renal and thyroid (Urbanski, Leclair and Anczuków, 2018; Leclair *et al.*, 2020). Although a role for TRA2β in HDV or in HCC biology is yet to be identified, it is a very well-described splicing factor that is known to be involved in tumour progression and it is possible that TRA2β may be important for the splicing regulation of genes important for HDV, in terms of the HDV replication cycle or HCC progression, for example. To test this hypothesis, we intend to perform additional studies based on overexpression and downregulation of TRA2β, to understand whether this particular splicing factor plays a role in the HDV life cycle.

A second family of proteins of great interest corresponded to proteins involved in inflammatory response, some of which were identified in higher levels in the exosomes of Huh7-D12 cells. One of the most common used mechanism shared between HBV, HCV and HDV to take over the infected cells is persistent liver inflammation and immune mediated oxidative stress damage, which may unintentionally lead to HCC development. HDV has different mechanisms to modulate the immune system when compared to HBV and HCV due to the permanent presence of co-infection. In addition, HDV can indirectly mediate hepatocarcinogenesis by innate immune response modulation and induction of adaptive immune responses, with this non-resolving inflammation being a hallmark of cancer that contributes significantly to the development and progression of HCC (D'souza *et al.*, 2020). There is a growing number of reports indicating that exosomes can modulate gene expression and cell function, playing an important role in various processes, for example, in inflammation and immune response that are involved in a large number of pathological conditions, including cancer. Also, exosomes are important in shaping the inflammatory microenvironment of tumours, which is extremely essential for initiation and progression of liver cancer (Console, Scalise and Indiveri, 2019).

Heparin-binding proteins seem to be exclusive of Huh7-D12 exosomes, which is interesting because both HBV and HDV bind to cells in an unspecific way upon entry into the bloodstream mediated by heparin sulphate proteoglycans interaction ((Krause, Haberkorn and Mier, 2018). One of the reasons why Huh7-D12 exosomes incorporate heparin-binding proteins as opposed to exosomes of Huh7 cells may be related to the need to induce proto-receptor formation in non-hepatic cells to facilitate the viral spread. Interestingly, some studies have demonstrated that HSPGs have an important role in exosome uptake and also, the uptake route dependent on HSPG is very relevant for the biological response evoked by exosomes in target cells (Christianson *et al.*, 2013). As reported, the viral biogenesis and release converge with exosomes pathways, as shown in this work, which suggests an evolutionary conserved system of virus-exosomes co-dependency.

With this work, we conclude that Huh7-D12 cells secrete exosomes that contain the entire HDV genomic RNA, and we propose a novel mechanism of HDV dissemination independent of HBV and that allows the virus to spread to neighbouring hepatic cells and, probably, to non-hepatic cells. We have shown that HDV-associated

exosomes contain HDV genomic materials and serve as vehicles to transmit HDV RNA from infected cells to bystander cells. Indeed, HDV genomic materials could be released from infected hepatocytes into peripheral blood in the form of circulating exosomes, and these molecules could exploit the fusogenic capabilities of the exosomes with other cells to transmit HDV infection, similar to the mechanisms recently described for HCV and HBV infections. Our results illustrate a possible mechanism of viral immune evasion: i.e., exosomal package and secretion of viral RNA from infected cells may represent an optimal pathogen–host interaction and immune evasion machinery that serves as a viral strategy to transmit infection in a cell contact-independent manner and HBV-independent manner. Further characterization of the virus-associated exosomes, in splicing regulation, host inflammatory response and regulation of the host immunity for instance, will enhance our understanding of the mechanisms of virus persistence and treatment failure in chronic viral infection in humans.

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Appendix

Appendix I. Huh7 and Huh7-D12 protein exosomal content. Proteins obtained by mass spectrometry analysis.

Protein family	Protein	Protein accession	Spectral counts (SC)	Huh7 SC	Huh7-D12 SC
Transcription factors	5'-3' exoribonuclease 2	XRN2_HUMAN	51	25	1
	Activated RNA polymerase II transcriptional coactivator p15	TCP4_HUMAN	39	19	1
	APOBEC1 complementation factor	A1CF_HUMAN	49	22	3
	AT-rich interactive domain-containing protein 3A	ARI3A_HUMAN	69	34	1
	Basic leucine zipper and W2 domain-containing protein 1	BZW1_HUMAN	15	8	0
	Bromodomain-containing protein 3	BRD3_HUMAN	10	5	0
	CCR4-NOT transcription complex subunit 1	CNOT1_HUMAN	88	40	5
	CREB-binding protein	CBP_HUMAN	7	4	0
	DNA-directed RNA polymerase I subunit RPA1	RPA1_HUMAN	10	5	0
	DNA-directed RNA polymerase II subunit RPB1	RPB1_HUMAN	51	26	0
	DNA-directed RNA polymerase II subunit RPB2	RPB2_HUMAN	52	26	0
	Elongator complex protein 2	ELP2_HUMAN	11	6	0
	Eukaryotic translation initiation factor 4 gamma 1	IF4G1_HUMAN	231	113	3
	Far upstream element-binding protein 1	FUBP1_HUMAN	117	36	23
	General transcription factor 3C polypeptide 4	TF3C4_HUMAN	6	3	0
	General transcription factor II-I	GTF2I_HUMAN	41	21	0
	Hepatocyte nuclear factor 1-alpha	HNF1A_HUMAN	8	4	0
	Histone-arginine methyltransferase CARM1	CARM1_HUMAN	18	7	2
	Interferon regulatory factor 2-binding protein 2	I2BP2_HUMAN	10	5	0
	Leucine-rich PPR motif-containing protein, mitochondrial	LPPRC_HUMAN	6	3	0
	Matrin-3	MATR3_HUMAN	108	52	2
	Metastasis-associated protein MTA1	MTA1_HUMAN	8	4	0
	Methylosome protein 50	MEP50_HUMAN	15	6	2
	Mothers against decapentaplegic homolog 4	SMAD4_HUMAN	9	5	0

	Myb-binding protein 1A	MBB1A_HUMAN	8	4	0
	Nascent polypeptide-associated complex subunit alpha, muscle-specific form	NACAM_HUMAN	13	5	2
	Negative elongation factor A	NELFA_HUMAN	7	4	0
	Negative elongation factor B	NELFB_HUMAN	10	5	0
	Nucleoside diphosphate kinase B	NDKB_HUMAN	6	3	0
	Paired amphipathic helix protein Sin3a	SIN3A_HUMAN	8	4	0
	Protein arginine N-methyltransferase 5	ANM5_HUMAN	52	24	2
	Protein argonaute-2	AGO2_HUMAN	15	8	0
	Ribosomal protein S6 kinase alpha-3	KS6A3_HUMAN	181	78	13
	RNA polymerase II-associated protein 1	RPAP1_HUMAN	9	5	0
	RNA polymerase-associated protein LEO1	LEO1_HUMAN	12	5	1
	RNA polymerase-associated protein RTF1 homolog	RTF1_HUMAN	8	4	0
	RNA-binding motif protein, X chromosome	RBMX_HUMAN	37	18	1
	RNA-binding protein EWS	EWS_HUMAN	70	25	11
	SAP domain-containing ribonucleoprotein	SARNP_HUMAN	11	4	2
	Scaffold attachment factor B1	SAFB1_HUMAN	87	36	8
	Signal transducer and activator of transcription 1-alpha/beta	STAT1_HUMAN	91	38	8
	Signal transducer and activator of transcription 2	STAT2_HUMAN	10	5	0
	Signal transducer and activator of transcription 3	STAT3_HUMAN	11	6	0
	SWI/SNF complex subunit SMARCC2	SMRC2_HUMAN	16	8	0
	TATA element modulatory factor	TMF1_HUMAN	7	3	1
	TATA-binding protein-associated factor 172	BTAF1_HUMAN	7	4	0
	Telomeric repeat-binding factor 2-interacting protein 1	TE2IP_HUMAN	9	5	0
	Transcription activator BRG1	SMCA4_HUMAN	31	16	0
	Transcription elongation factor A protein 1	TCEA1_HUMAN	13	7	0
	Transcription elongation factor SPT5	SPT5H_HUMAN	85	36	7
	Transcription elongation factor SPT6	SPT6H_HUMAN	116	44	15
	Transcription elongation regulator 1	TCRG1_HUMAN	60	21	10

	Transcription factor p65	TF65_HUMAN	9	4	1
	Transcription intermediary factor 1-alpha	TIF1A_HUMAN	18	9	0
	Transcription intermediary factor 1-beta	TIF1B_HUMAN	250	96	29
	Transducin-like enhancer protein 1	TLE1_HUMAN	11	5	1
	Transforming acidic coiled-coil-containing protein 1	TACC1_HUMAN	17	8	1
	YLP motif-containing protein 1	YLPM1_HUMAN	16	7	2
Inflammatory response	YTH domain-containing family protein 1	YTHD1_HUMAN	9	5	0
	Cytokine receptor-like factor 1	CRLF1_HUMAN	6	0	3
	Nucleolar RNA helicase 2	DDX21_HUMAN	8	4	0
	Complement factor I	CFAI_HUMAN	20	0	10
	Complement C1r subcomponent	C1R_HUMAN	20	0	10
	Complement C1s subcomponent	C1S_HUMAN	15	0	8
	Complement C3	CO3_HUMAN	885	29	414
	Complement C4-A	CO4A_HUMAN	9	0	5
	Complement C5	CO5_HUMAN	771	85	301
	Complement factor H	CFAH_HUMAN	575	135	153
	Galectin-3-binding protein	LG3BP_HUMAN	53	1	26
	Mannan-binding lectin serine protease 1	MASP1_HUMAN	34	4	13
	Spondin-2	SPON2_HUMAN	27	1	13
	Chitinase domain-containing protein 1	CHID1_HUMAN	11	1	5
	Serine/threonine-protein kinase TBK1	TBK1_HUMAN	11	6	0
	NudC domain-containing protein 1	NUDC1_HUMAN	11	6	0
	Serine/threonine-protein phosphatase 6 regulatory subunit 2	PP6R2_HUMAN	31	14	2
	HLA class I histocompatibility antigen, A alpha chain	HLAA_HUMAN	11	0	6
	Tyrosine-protein kinase CSK	CSK_HUMAN	8	4	0
	E3 ubiquitin-protein ligase Itchy homolog	ITCH_HUMAN	6	3	0
Cell cycle	Insulin-like growth factor-binding protein 2	IBP2_HUMAN	65	1	32
	G2/mitotic-specific cyclin-B1	CCNB1_HUMAN	20	4	7
	Protein RCC2	RCC2_HUMAN	50	23	2
	Serine/threonine-protein kinase Nek9	NEK9_HUMAN	23	11	1

	Ribosomal protein S6 kinase alpha-1	KS6A1_HUMAN	46	23	0
	Hepatoma-derived growth factor-related protein 2	HDGR2_HUMAN	32	16	0
	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	PP1G_HUMAN	17	9	0
	Serine/threonine-protein phosphatase PP1-beta catalytic subunit	PP1B_HUMAN	12	6	0
	Kinesin-like protein KIF11	KIF11_HUMAN	9	5	0
	Proto-oncogene tyrosine-protein kinase Src	SRC_HUMAN	7	4	0
	14-3-3 protein gamma	1433G_HUMAN	12	6	0
	Protein FAM98B	FA98B_HUMAN	9	5	0
	Fanconi anemia group D2 protein	FACD2_HUMAN	9	5	0
	MAGUK p55 subfamily member 6	MPP6_HUMAN	9	5	0
	Retinoblastoma-associated protein	RB_HUMAN	9	5	0
	DNA ligase 1	DNLI1_HUMAN	7	4	0
	Fanconi anemia group I protein	FANCI_HUMAN	7	4	0
	DCC-interacting protein 13-alpha	DP13A_HUMAN	8	4	0
	Wings apart-like protein homolog	WAPL_HUMAN	8	4	0
	CLIP-associating protein 1	CLAP1_HUMAN	7	4	0
	DNA ligase 3	DNLI3_HUMAN	7	4	0
	Disks large-associated protein 5	DLGP5_HUMAN	7	4	0
	Anaphase-promoting complex subunit 1	APC1_HUMAN	6	3	0
	Band 4.1-like protein 2	E41L2_HUMAN	98	41	4
	Host cell factor 1	HCFC1_HUMAN	57	26	3
	Prominin-1	PROM1_HUMAN	69	2	33
	Scaffold attachment factor B2	SAFB2_HUMAN	61	27	4
	GMP synthase [glutamine-hydrolyzing]	GUAA_HUMAN	48	24	1
	Cell division cycle and apoptosis regulator protein 1	CCAR1_HUMAN	43	20	2
	Nuclear migration protein nudC	NUDC_HUMAN	65	24	9
	KH domain-containing, RNA-binding, signal transduction-associated protein 1	KHDR1_HUMAN	78	32	7
	N-alpha-acetyltransferase 25, NatB auxiliary subunit	NAA25_HUMAN	28	13	1

	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	PP1A_HUMAN	57	21	8
	Mitotic checkpoint protein BUB3	BUB3_HUMAN	40	13	8
	Centromere/kinetochore protein zw10 homolog	ZW10_HUMAN	25	12	1
	Cyclin-dependent kinase 1	CDK1_HUMAN	24	10	2
	Cell division cycle 5-like protein	CDC5L_HUMAN	18	9	1
	Clathrin light chain A	CLCA_HUMAN	27	8	6
	Neural Wiskott-Aldrich syndrome protein	WASL_HUMAN	12	6	1
	Serine/threonine-protein phosphatase 6 catalytic subunit	PPP6_HUMAN	19	7	3
	Golgin subfamily A member 2	GOGA2_HUMAN	12	4	3
	Ras-related protein Rab-11A	RB11A_HUMAN	13	5	2
	Sperm-associated antigen 5	SPAG5_HUMAN	9	4	1
	Pre-mRNA-processing factor 40 homolog A	PR40A_HUMAN	9	4	1
	Nuclear autoantigenic sperm protein	NASP_HUMAN	13	4	3
	mRNA export factor	RAE1L_HUMAN	8	4	1
	Kinesin-like protein KIF2A	KIF2A_HUMAN	7	3	1