The crosstalk between Nrf1 activity, lipids and hepatitis C virus

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1 Introduction

Hepatitis C virus (HCV) is majorly responsible for chronic liver disease worldwide following acute infection, where 75% of people fail to clear the virus resulting in the chronic state (Blach et al. 2017; Esmaeili et al. 2018; Lanini et al. 2016). Chronic Hepatitis C infection is a major cause of liver-related morbidity and mortality (Global hepatitis report, 2017 2017). When the chronic infection is left untreated, progression can occur leading to cirrhosis and hepatocellular carcinoma (Alter H. J. and Seef L.B. 2000). The dual therapy, which is a combination between pegylated interferon (IFN) alpha and ribavirin (PEG IFN/riba), is still till recently in some third-world countries the golden standard to treat chronic hepatitis C patients. It has achieved a sustained virological response. With only 50% of HCV genotype 1 infected-patients (the more common one) in comparison with the 80% genotype 2 or 3, despite being costly and prolonged (e.g. 24-48 weeks) with numerous harsh side effects, which are difficult to tolerate (National Institutes of Health Consensus Development Conference Statement: Management of hepatitis C: 2002--June 10-12, 2002 2002). Identifying the JFH1 isolate was considered a major breakthrough in HCV treatment by some in vitro studies of HCV. That lead to a better understanding of the HCV life cycle and the 3D structures of viral proteins. Based on this insight, the first direct-acting antivirals (DAAs) could be developed that specifically affect viral proteins. The two protease inhibitors (PI) telaprevir and boceprevir inhibit the viral NS3-4A protease and were approved in 2011 as combination therapy with PegIFN-α and ribavirin, which increased the SVR to 67-75% (Pawlotsky et al. 2015).
To develop more effective and better-tolerated therapies, we urgently need to gain a better understanding of the HCV lifecycle.

1.1 History and Classification of Hepatitis C Virus (HCV)

In 1975 Feinstone and colleagues first fully recognized the existence of the Hepatitis C virus after they realized that the majority of the transfusion-associated hepatitis cases were associated with neither hepatitis A nor hepatitis B virus (Feinstone et al. 1975). Therefore, it was concluded that such a disease will be called non-A, non-B hepatitis

(Feinstone et al. 1975; Choo et al. 1989; Bartenschlager et al. 2011). Later, in some transmission studies done in chimpanzees, it was shown that this newly described viral infection of the liver was caused by a small enveloped agent (Farci et al. 2007; Houghton 2009). But it took time to characterize the virus and its lifecycle because of the inability to cultivate HCV *in vitro.* Due to the fact that there was no animal model available, an efficient and robust in vitro HCV replication system was required and this took almost a decade from the first cloning of an HCV genome (Choo et al. 1989) before the first reliable cell culture model (Huh7 cells) was developed (Lohmann et al. 1999). This model was followed by the development of these highly permissive cells (Huh7.5) that were obtained by curing cell clones containing subgenomic replicon with IFN-α, allowing Huh-7.5 cells to be highly permissive to HCV replication independently from the adaptive mutational changes to NS3-5B region (Blight et al. 2002). With the development of the highly permissive hepatoma cell line Huh7.5 and the isolation of the HCV clone JFH1 from a Japanese patient with fulminant hepatitis, it was possible for the first time to develop a cell culture system in which the complete life cycle of HCV can be understood (Blight et al. 2002; Lindenbach et al. 2005; Wakita et al. 2005).

Hepatitis C virus is a hepatotropic RNA virus that belongs to genus Hepacivirus within the family of Flaviviridae, other pathogenic members of this family are the yellow fever virus (from which the family name is derived) (flavus = yellow), the dengue virus and the Zika virus (Blázquez et al. 2014). HCV is classified into seven genotypes and more than 60 subtypes that have a different geographical distribution and drug sensitivity (Kuiken and Simmonds 2009; Smith et al. 2014; Simmonds 2004).

1.2 Epidemiology and pathogenesis

The worldwide spread of HCV infected people depending on anti-HCV antibodies detection has been predicted at around 1.6%. This correlates to ~ 115 million individuals (Manns et al. 2017), also, comparable data were published by the WHO (World Health Organization. 2016). Nonetheless, a minority of these cases are infected with HCV at present, as they have cleared the virus spontaneously or after treatment. Consequently, the blood-borne viral spread worldwide (where HCV RNA is detected) is low at

approximately 1% (range: 0.8–1.14%) or around 71 million (range: 62–79 million) cases with HCV infection. These percentages are concluded after a number of generalizable study reports from more than 95 countries (Manns et al. 2017).

Due to insufficient or non-existent documentation on HCV infection cases in many developing countries, robust global data is limited and thus the global HCV prevalence differs slightly (Messina et al. 2015). Only 29% of the developing countries and 60% of developed countries reporting HCV prevalence. The accuracy of the HCV worldwide distribution data announced also varies from one country to another (Manns et al. 2017).

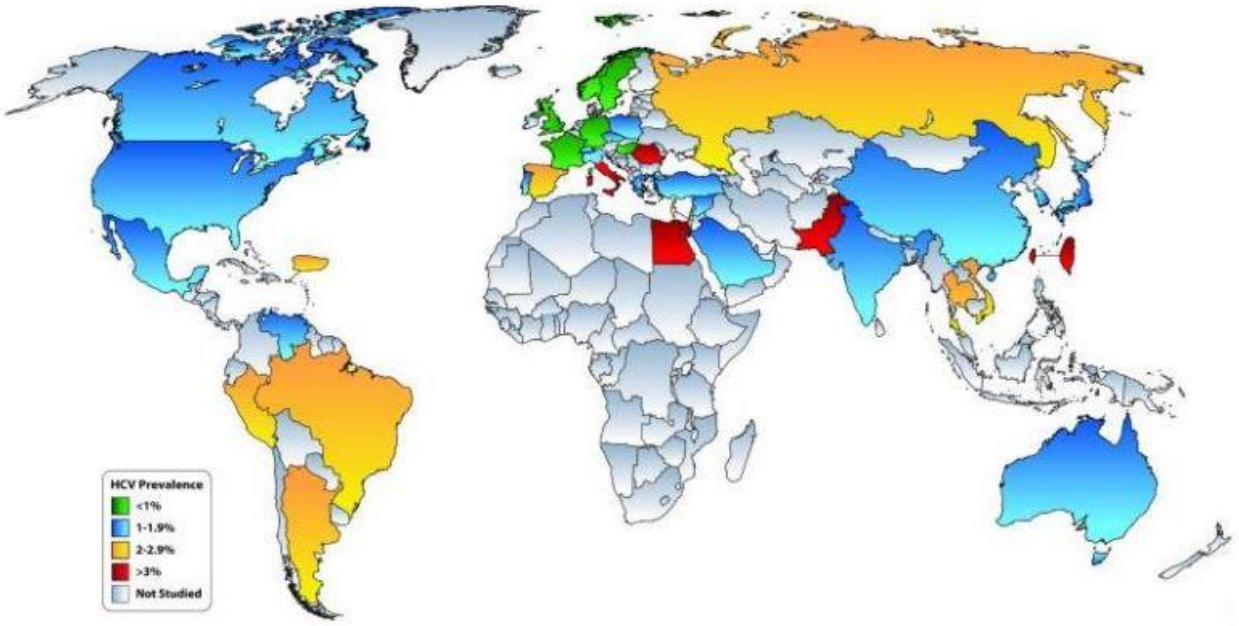


Figure 1: Global Prevalence of Hepatitis C Source: Center of Disease Analysis.

The ubiquity of HCV infection exhibits a significant variation worldwide, with countries with a former or current history of physician-induced infections possessing the highest infection percentage (FIG. 1). The estimation of current studies is that 115 million (1.6%) or 185 million (2.8%) are chronically infected people (Messina et al. 2015; Gower et al. 2014). The lowest HCV prevalence (<0.1%) was reported in Scandinavian countries, while in North America and Western Europe it is estimated at 1%, 3-4% in Asia and 20% in Central Africa (Alter 2007; Wasley and Alter 2000). The countries with the highest prevalence of HCV are Egypt (15%), Pakistan (4.8%) and China (3.2%), where in China, Pakistan, Nigeria, Egypt, India, and Russia more than 50% of infected people lives (Gower et al.

2014). More than 5% of the adult population in many 3rd world countries such as Gabon, Nigeria, and Georgia were infected with HCV. The major cause of this situation in this population is because of the physician-induced or iatrogenic infection. In Egypt, intravenous treatment of Bilharziasis infection is thoroughly reported and documented as the main source of HCV infection in the '60s & '70s (Manns et al. 2017). The western developed countries with more awareness and high educational levels contribute to them representing a small part of the world HCV epidemic. On the other hand, the HCV epidemic in countries like Egypt, China and India contributes to ~50% of the total global HCV infection (Manns et al. 2017).

In certain countries, age is an important factor determining the distribution of HCV infection, where countries such as Finland or Russia have a high rate of drug abuse through injections in blood at a young age. HCV infection peaks in the population age between 30 and 40 years. This deviates from the normal peak pattern that occurs at the age between 50 and 60 years in those countries where physician-induced infections were the main cause of infection before the '90s where HCV diagnosis was available (Razavi et al. 2014; Manns et al. 2017). Also, HCV genotype distribution varies from one region to another (FIG. 2). Genotype 1 is the most prevalent with 66% of HCV infections worldwide, followed by genotype 3 (22%) and genotypes 2 and 4 (13% each). Only subtype 1b is responsible for 22% of all HCV infections, whereas only 3% of patients are infected with genotypes 5 and 6 (Gower et al. 2014). To date, there is only one documented case of infection of genotype 7 (Messina et al. 2015). The geographical distribution of the different HCV genotypes is shown in Figure 3. Genotype 1 is widespread worldwide and has the highest prevalence in Western industrialized countries. Genotype 2 occurs mainly in West Africa, while genotypes 4 and 5 have the highest prevalence in South Africa. Genotypes 3 and 6 are mainly found in Asia (Messina et al. 2015).

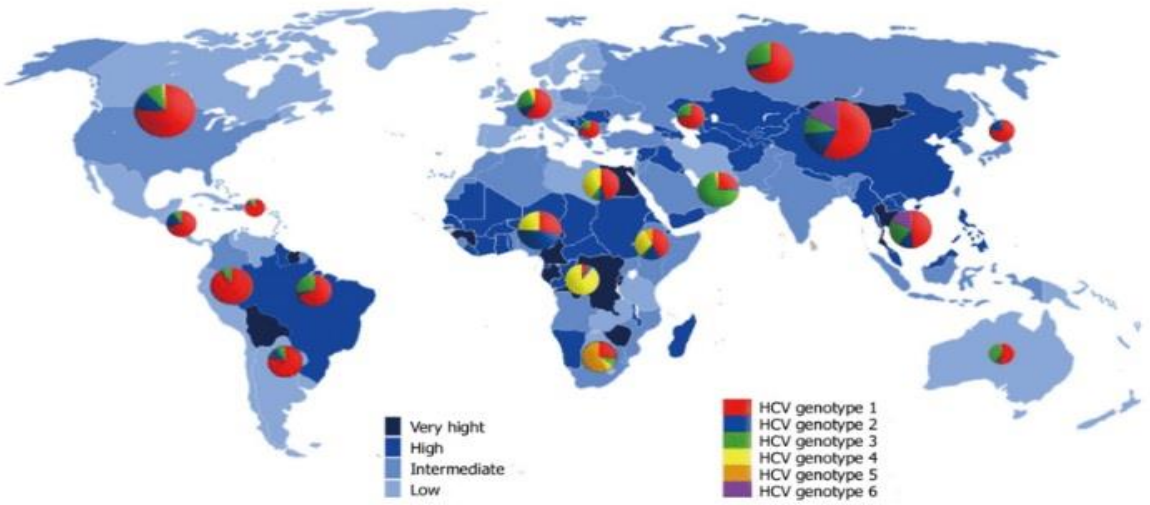


Figure 2: Relative prevalence of different HCV-Genotypes (Messina et al. 2015).

This genotypic prevalence has an influence on the clinical course, treatment requirements, and drug development. Pan-genotypic drugs are required in the developed poor countries where genotype 1 is responsible for less than 50% of the total infections as shown in (fig.2) (Manns et al. 2017). About 27% of cirrhotic and 25% of HCC diagnoses are due to HCV infection and about 500,000 people die each year on the consequences of a chronic HCV infection (Alter 2007; World Health Organization. 2016). The diagnosis of HCV-mediated HCC is an indication for liver transplantation (Testino G, Sumberaz A, Leone S, Borro P 2013). The transmission of HCV is parenteral, by direct blood contact, bypassing the intestine. The most efficient transfer occurs during blood transfusion by blood products from infected donors and injection drug use (Alter 2007; Frank et al. 2000). The iatrogenic risk of infection is virtually eliminated since the introduction of HCV tests and virus inactivation of blood products. Other, but less efficient, transmission pathways include tattooing, hemodialysis, mother-child infections during childbirth, and sexual contact with infected people (Alter 2007; Frank et al. 2000; Sy and Jamal 2006; Roberts and Yeung 2002; Clarke and Kulasegaram 2006; Terrault 2002; Kamili et al. 2007). Since HCV virions remain stable and infectious even after dehydration and for a long time at room temperature, transmission via cross-contamination due to a lack of hygiene in the medical sector is not excluded (Paintsil et al. 2014).

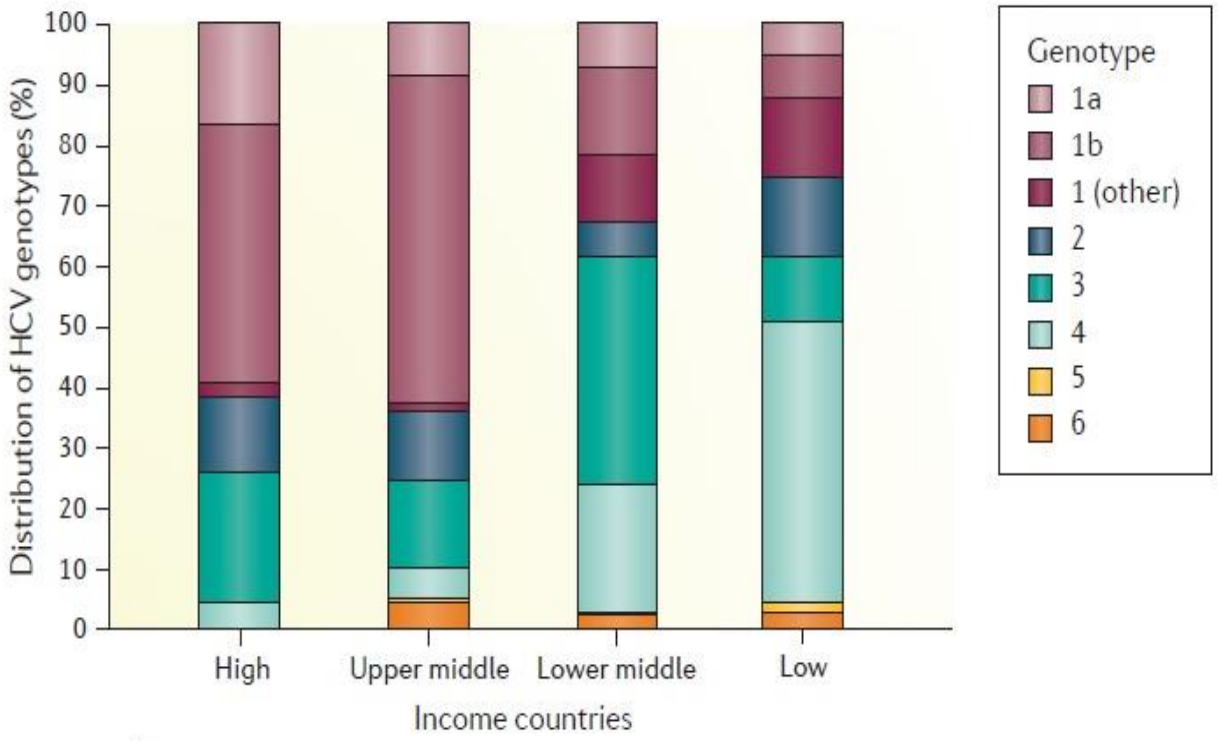


Figure 3: HCV genotype distribution between countries according to the World Bank income categories (Manns et al. 2017).

HCV has no cytopathic effect. It enters the liver cell and directly undergoes replication causing cell necrosis by several mechanistic pathways (Fig.4) including immune-mediated cytolysis. Additionally, various other metabolic-mediated phenomena such as hepatic steatosis, oxidative stress, and insulin resistance that enhance or promote indirectly the virus infection and pathogenesis through the high-stress levels on the liver and accumulation of fat (fatty liver) within the liver (Irshad et al. 2013).

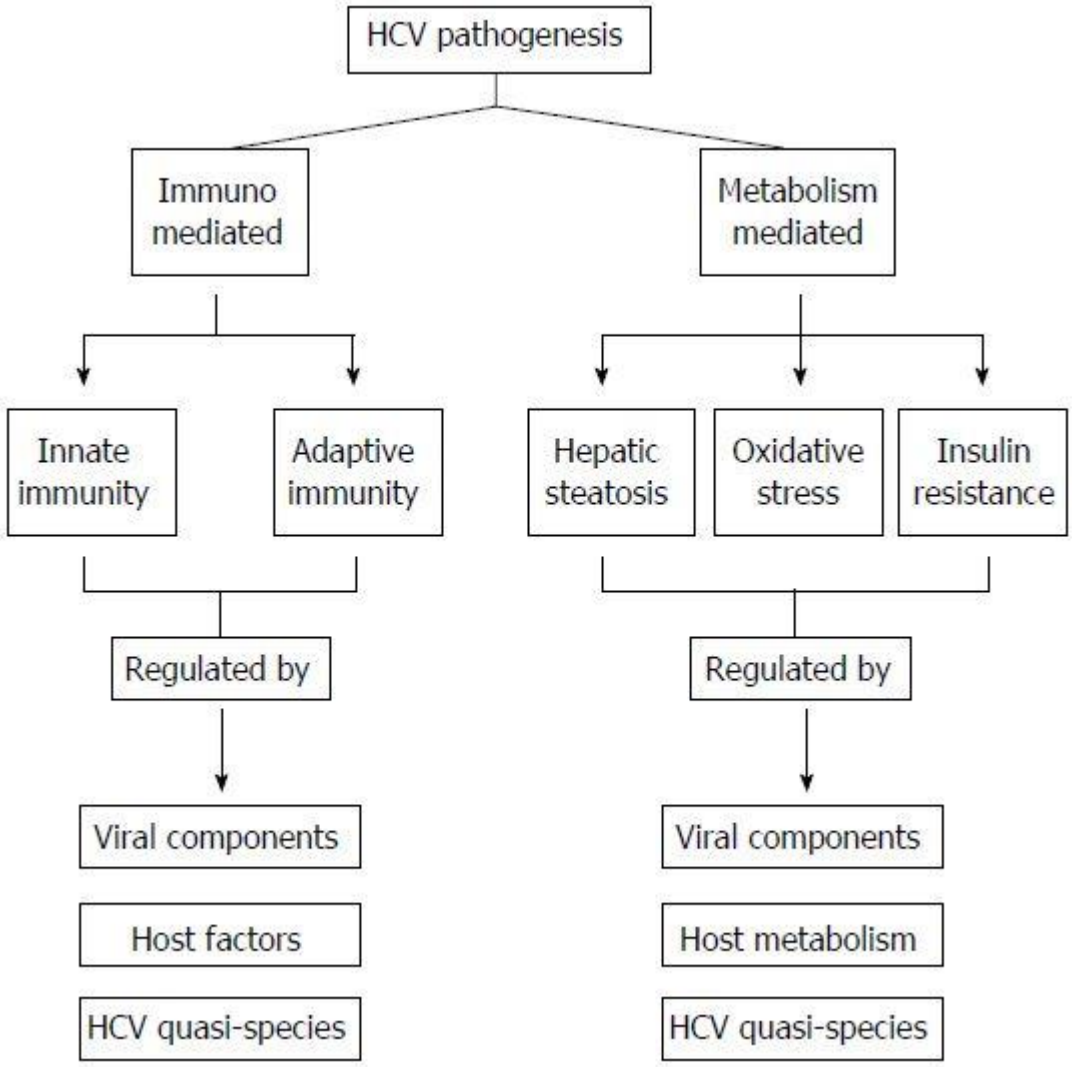


Figure 4: Regulation of hepatitis C virus pathogenesis by host immunity and metabolic factors (Irshad et al. 2013).

The incubation time of HCV differs significantly. HCV RNA can be detected in the blood of those affected after a few days up to 8 weeks after infection (Hoofnagle 1997; Nawaz et al. 2015) prior to the increase in liver enzymes. An acute HCV infection often goes almost asymptomatic as it causes no or flu-like symptoms. In ~ 15% of the acute infection, it heals by itself by a strong immune response, i.e. natural or cell-mediated immunity after a few months (Nawaz et al. 2015). The remaining 85% of people with acute infection will develop a chronic state, where HCV RNA can still be detected 6 months after infection (Alter 1997).

Due to the fact that the inflammatory activity of the immune system of the infected persons is persistent, the immunopathogenesis of HCV is initiated. Infected cells are recognized and eliminated by natural killer cells and cytotoxic T lymphocytes, followed by the replacement of these cells with newly formed hepatocytes during liver regeneration (Hiroishi et al. 2008). But this immune defense and cell replacement by regeneration leads to the formation of liver fibrosis, which is the replacement of functional liver tissue by connective tissue (Rockey and Bissell 2006). If the chronic hepatitis C infection in its early asymptomatic stage is left untreated, cirrhosis (which takes up to 30 years) could develop. This can result in complete liver failure. In addition, cirrhotic patients have a greatly increased risk of developing Hepatocellular Carcinoma (Fig.5), which is the third most common tumor-induced fatal cause without liver transplantation (Blonski and Reddy 2008).

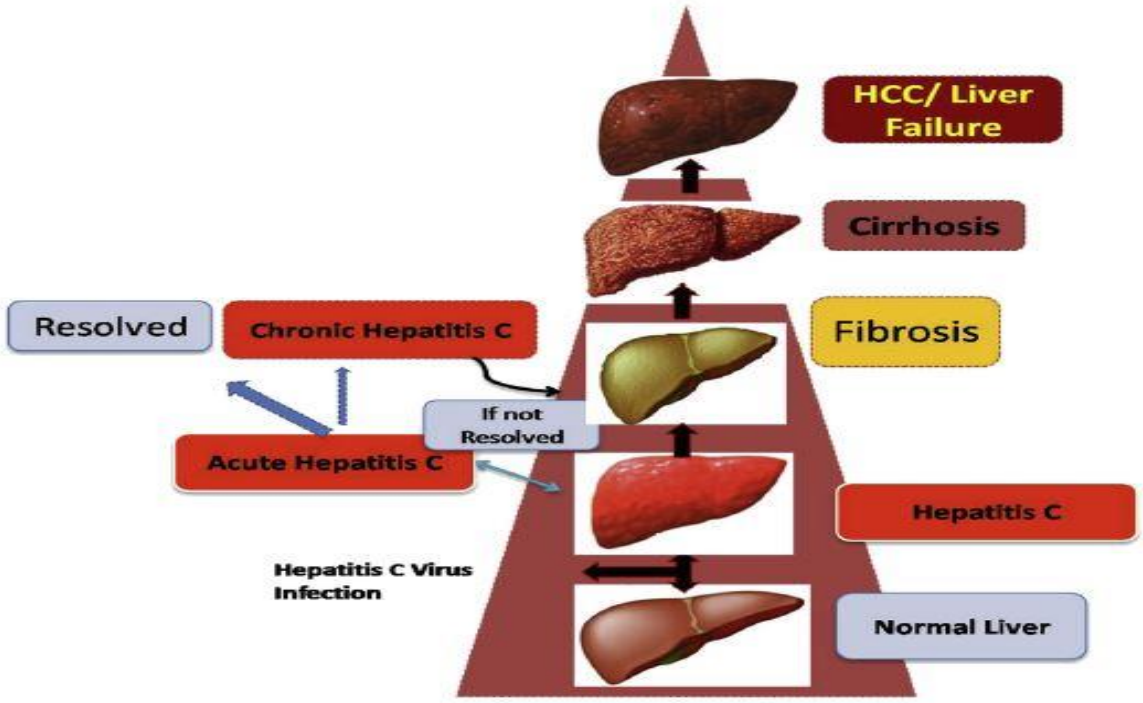


Figure 5: Stages of liver damage.

This figure shows how normal healthy liver develops into hepatitis C, fibrosis, cirrhosis, and even hepatocellular carcinoma or liver failure. (Nawaz et al. 2015)

1.3 Diagnosis and Therapy

HCV infection can be diagnosed and controlled by several virological tools. Four different generation of kits have been established to date for HCV detection. First, the c100-3 (which is a recombinant antigen) was used as the first-generation Enzyme Immunoassay approach to identify anti-NS4 antibodies. Second was the use of core, NS3 & NS4 regions -related antigen (c22-3), (c33c) & (c100-3), respectively, in a ratio (5:1:1). Third, EIA included an additional NS5 region plus reconfigured core and NS3 region-related antigen. Fourth, which was recently established, in which additional nonstructural protein parts were incorporated for the detection of the HCV infection. The third generation is the one that is currently in use to identify indirectly the HCV presence in serum (Irshad et al. 2013; Kish et al. 2017; Manns et al. 2017). These tests are sensitive and specific, can be fully automated and are relatively inexpensive. Nonetheless, there was a disadvantage with these approaches as they produce frequently false results and are not very sensitive in identifying the anti-HCV antibodies in the early stages. This window period also varies and ranges on average between 2 and 8 weeks (Irshad et al. 2013). Thus, early infection might be overlooked by testing only for anti-HCV antibodies. Anti-HCV antibodies then persist in patients who develop chronic infection (Manns et al. 2017). Since these antibody-dependent assays were unable to differentiate between current, old or chronic infections. A qualitative and quantitative detection of HCV RNA is beneficial to diagnose active infection, spot patients with an indication for therapy and assess the response to antiviral treatment, and identify when patients start to develop resistance against DAAs (Manns et al. 2017). HCV RNA detection and quantification are based on real-time PCR or transcription-mediated amplification methods, which are both sensitive and specific, where results are expressed in IU (international units) per ml. As shown in the case of genotype 1, subtype identification is required to guide the treatment regimen, the duration of therapy and the combination therapy with ribavirin (Manns et al. 2017), where the reference method is phylogenetic analysis of the nucleotide sequence of part of the viral genomic material, which is achieved by population (direct) sequencing. In clinical practice, standardized methods based on direct sequence analysis or reverse hybridization (line probe assay) are also used (Chevaliez et al. 2012). The HCV core antigen-epitopes that are expressed on the HCV nucleocapsid surface are an alternative marker of HCV

replication that can be identified and quantified in the patient serum. Thus, it can be used as a backup plan to HCV RNA assays in the diagnosis of infection and antiviral treatment monitoring (Chevaliez et al. 2014; Bouvier-Alias et al. 2002).

Medical treatment regimens for patients with chronic persistent HCV infection have progressed greatly in recent years. Historically, HCV was treated with interferon-alpha (IFN-α) “monotherapy” with low cure rates. After that, it was the IFN-α in combination with ribavirin given for 24 weeks or 48 weeks. The later resulted in an intermediate mediocre sustained virological response (SVR) with cure rates of 40% to 50%. Unfortunately, these different treatments were accompanied by various strong intolerable side effects, such as flu-like symptoms, hemolytic anemia, and psychiatric disturbances. Finally, IFN free therapy with higher cure rates was used (Pawlotsky et al. 2015). This deviation from the usual treatment models occurred in late 2013, early 2014 with the approval of simeprevir and sofosbuvir from (Olysio, Janssen) (Sovaldi, Gilead Sciences), respectively, within weeks of one another. These antiviral agents were the first oral treatments administered once-daily that had well-tolerated side effects and SVR cure rates greater than 90%, either combined or with the addition of PEG-IFN plus RBV in selected genotypes (Kish et al. 2017). The newer DAA antiviral agents target different points of the HCV viral replication cycle. These drugs affect major structures or the replication process either by directly binding to replicase complex components or by initiating the termination of the RNA chain. Medications that target the NS3/4A protease have the suffix “-previr”, while medications that inhibit the NS5B polymerase have the suffix “-buvir”, and last but not least NS5A inhibitors have the suffix “-asvir”. (Kish et al. 2017).

Nonetheless, present drug regimens optimization, resistance mutation’s problem limitation, designing an individualized therapy and the high price therapy remain a challenge (Pawlotsky 2016; Pawlotsky et al. 2015; Sarrazin 2016). However, the presence of no vaccine is considered the major challenge for worldwide control of HCV (Bukh 2016).

1.4 Structure and Composition of the HCV Particle

The HCV viral particles range in diameter from 50-60 nm and are icosahedral in structure. The particle is surrounded by a host-cell derived envelope composed of a lipid bilayer membrane. It contains two glycoproteins E1 and E2, which encloses a nucleocapsid that is formed from the communication between the homo-oligomerized core protein and the viral RNA (Dubuisson et al. 2002; Wakita et al. 2005; Popescu et al. 2014).

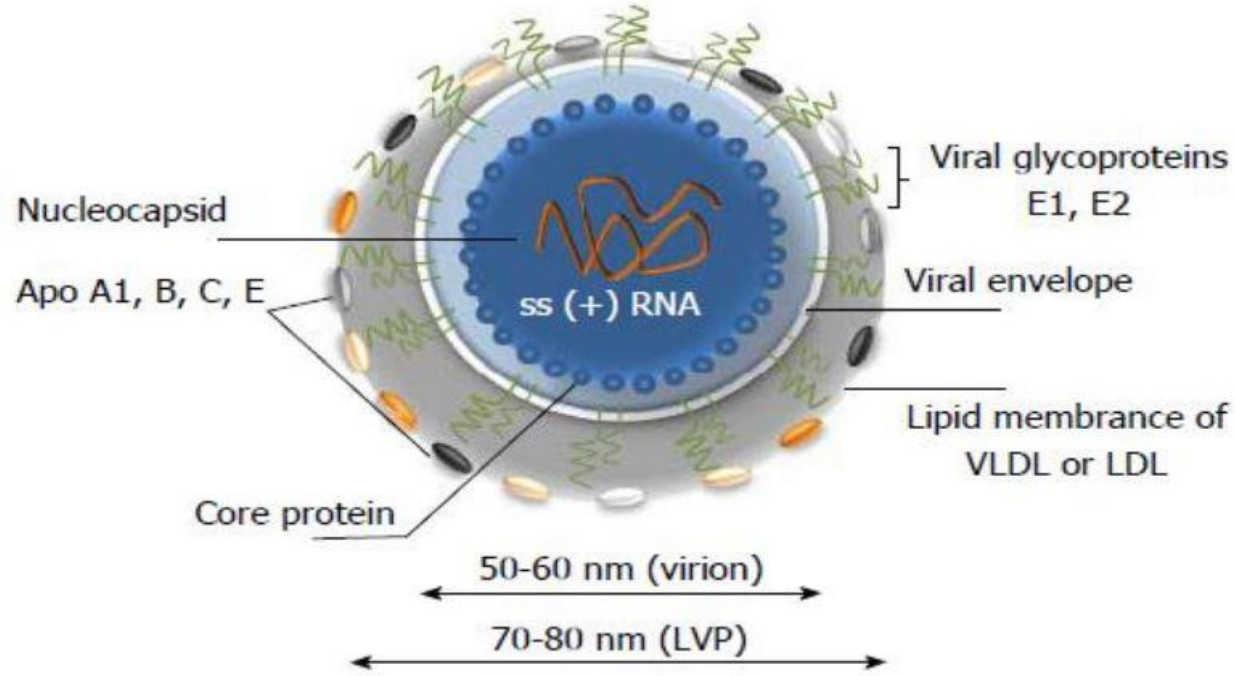


Figure 6: Model of the hepatitis C virus lipoviral particle.

Lipid membrane formed by low-density lipoproteins (LDL) and very-low-density lipoproteins (VLDL) on the surface of the virion (given in grey). Viral core is given in blue and viral RNA is shown in orange. Heterodimers of glycoproteins E1 and E2 are partially embedded in the lipid bilayer and are forming 6 nm long spikes (projections) on the surface of the virion. (Morozov and Lagaye 2018)

HCV viral particles exhibit a notable low density and over a wide range of densities ranging from 1.03 g/cm3 to 1.20 g/cm3 are distributed when tested using sucrose gradient. HCV dynamics and density in plasma of chronically infected patients are altered surprisingly by the dietary triglycerides (Popescu et al. 2014). These unexpected biophysical features are due to the existence of HCV particles-associated lipoproteins (Nielsen et al. 2006). Surely, apolipoproteins (apo) such as apoE, apoB, apoA1, and several apoC proteins can be found in association with HCV particles (Wakita et al. 2005). Also, it was found that

cholesteryl esters constitute more than half of the total HCV lipids. This is similar to the lipid content of VLDL and low-density lipoproteins (LDL) (Popescu et al. 2014), which was also recognized in the HCVcc system, thus, termed lipoviral particles (Chang 2007). Nonetheless, it must be mentioned that there is a difference in physical properties between HCV particles produced in vitro and those produced *in vivo* due to the complexity of the patient's serum, antibodies and other serum components (Bartenschlager 2013).

1.5 Genome Organization and Protein Biosynthesis

Like all Flaviviridae, HCV has a positively-oriented single-stranded RNA genome that is approximately 9.6k nucleotides long. It contains two regions (5'-UTR and 3'-UTR), untranslated regions that are highly conserved and are located at both ends of an only one open reading frame (ORF). This single ORF encodes for an approximately ~3K amino acid polyprotein, which is co-translated by the help of both host and viral proteases into ten viral proteins (Fig.7). The highly conserved 5'-UTR is required to control both genome replication and polyprotein translation processes. This region is composed of 341 nucleotides in length and features four characteristic domains (I, II, III & IV), where the first two domains are 125 nucleotides long and are essential in the genome replication, while domains (III & IV) constitute internal ribosomal entry site (IRES), which is essential for the binding of the viral RNA to the host ribosome to initiate the translation of the viral polyprotein in a manner independent from the usual 5'-end cap region (Morozov and Lagaye 2018). On the other end, the 3 'UTR consists of a short variable region, a poly (U / UC) region of 80 nucleotides and the conserved X-tail of 98 nucleotides (Moradpour et al. 2007).

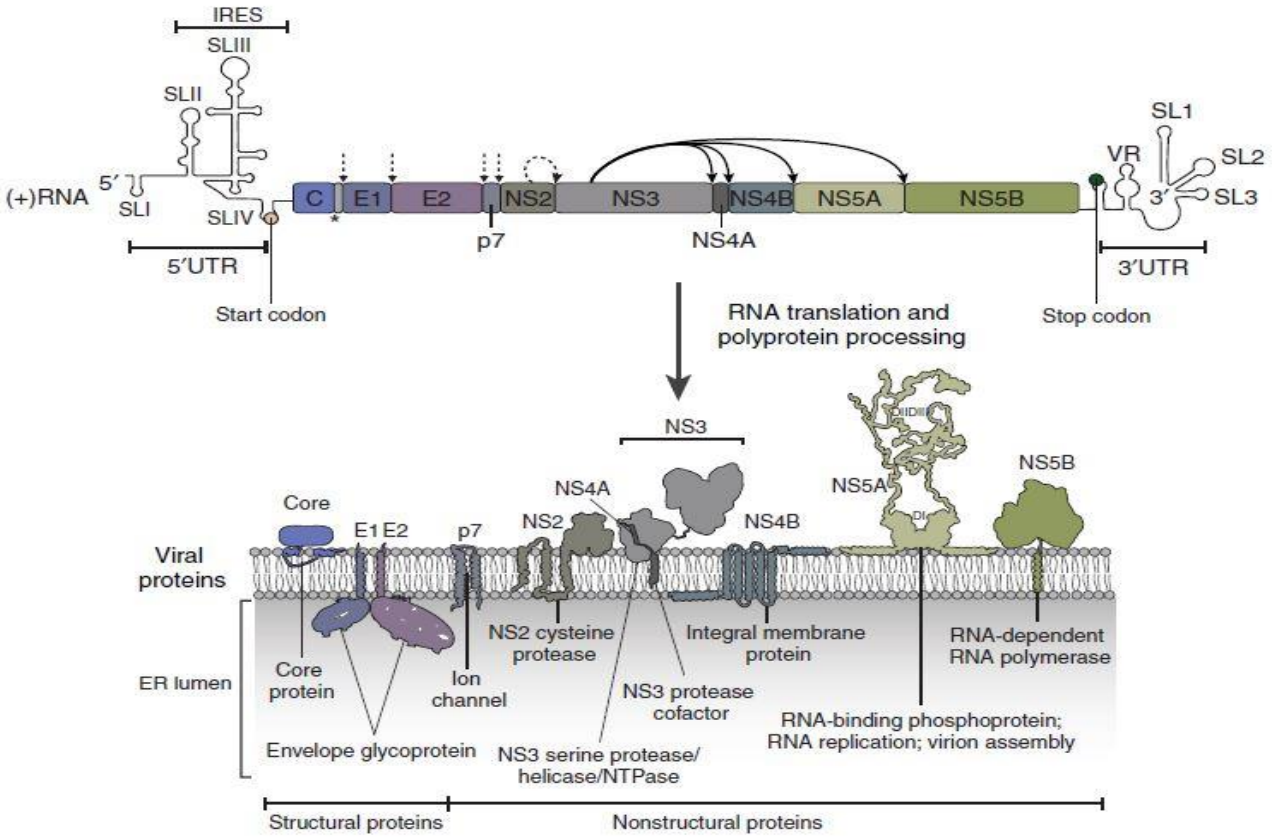


Figure 7: HCV translation and proteolytic processing.

Schematic presentation of the 9.6-kb positive-strand RNA genome (top). The RNA secondary structures in the 5'- and 3'-non-coding regions (NCRs) and the core gene, as well as the NS5B stem-loop 3 cis-acting replication element (5B-SL3) are shown. Internal ribosome entry site (IRES)-mediated translation yields a polyprotein precursor that is processed into the mature structural and non-structural proteins. Amino-acid numbers are shown above each protein (HCV H strain; genotype 1a; GenBank accession number AF009606). Solid diamonds denote cleavage sites of the HCV polyprotein precursor by the endoplasmic reticulum signal peptidase. The open diamond indicates further C-terminal processing of the core protein by signal peptide peptidase. Arrows indicate cleavages by the HCV NS2–3 and NS3–4A proteases. Dots in E1 and E2 indicate the glycosylation of the envelope proteins (4 and 11 N-linked glycans, respectively, in the HCV H strain). (Tabata et al. 2019)

On the other end, the 3'-UTR region is located, which is 400 nucleotides in length and is thought to play a key role in HCV genome replication. This highly conserved region is divided into three functional parts: A 40 nucleotides long variable sequence, a 30 to 80 nucleotides variable internal poly (U/UC) rich tract (depending on the HCV strains), followed by a highly conserved 98-nucleotide X-tail containing three stable stem-loop (SL) structures designated: 3'-(SL1, SL2 & SL3) (Fig. 7) (Morozov and Lagaye 2018; Fraser and Doudna 2007). The N-terminal end of the HCV genome encodes for the proteins (Core, E1, and E2) that are involved in the structural formation of the HCV particles and

are therefore called the structural proteins, while (NS3, NS4A, NS4B, NS5A, and NS5B) are the nonstructural proteins, and they are the building units of the replication complex, which is necessary for replication of the virus and also regulate some cellular processes (Bartenschlager et al. 2013). The ion channel p7 and the non-structural protein NS2 support the formation of the viral structure without being part of the viral particles (Bartenschlager et al. 2011). The assembly of the ribosomal pre-initiation complexes is required for the initiation of HCV protein synthesis. The process starts with the binding of the small ribosomal subunit (40S) to a messenger RNA (IRES). Afterwards, the 40S subunit starts scanning for the initiation codon followed by the binding of the bigger ribosomal subunit 60S to form an active 80S ribosome leading to the beginning of the cap-independent translation (Fraser and Doudna 2007) (Morozov and Lagaye 2018).

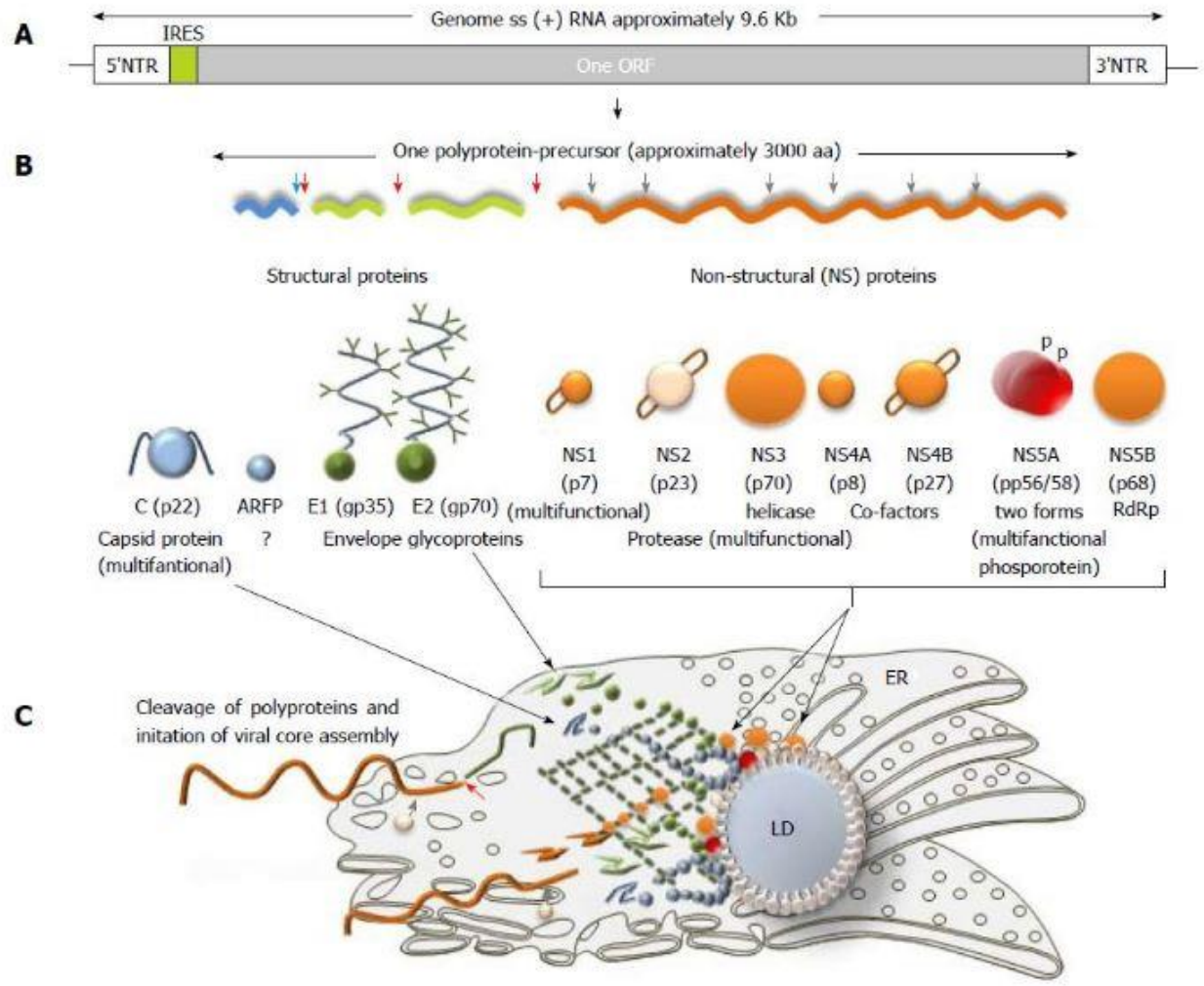


Figure 8: Hepatitis C virus genome, polyprotein precursor and the initial steps of core assembly in the endoplasmic reticulum.

A) Being structurally identical, the genomes of seven hepatitis C virus (HCV) genotypes demonstrated approximately 30% of sequence diversity. Two non-translated regions (5'- and 3'-NTR) are flanking a single open reading frame (ORF) shown in grey; **B)** Polyprotein precursor composed of about 3000 amino acids translated from a single ORF. Three structural proteins - a core protein (shown in blue) and two envelope proteins (shown in green) and seven non-structural (NS) proteins NS1, NS3, NS4A, NS4B, NS5A, and NS5B (shown in orange) that are involved in cleavage, assembly, transcription, and some other functions are shown. Alternative reading frame protein (ARFP) that overlaps with the core protein sequence is given in blue. Serine protease NS2 is given in pale yellow. Transmembrane fragments in non-structural proteins are shown as staples; p- phosphoprotein; **C)** A model of initial steps of the virion core assembly in the endoplasmic reticulum (membranous web is given in dark green) on lipid droplet (LD). Polyprotein precursor is cleaved by cellular C-terminal signal peptidase (red arrow) and cellular signal peptidase (blue arrow) to release capsid protein. NS3-NS4A serine protease cleaves the remaining proteins, while NS2-NS3 (grey arrow) protease cleaves itself. Pre-assembled cores are transported to the LD, where the final steps of assembly take place (Morozov and Lagaye 2018).

All HCV proteins harbor transmembrane domains (TMD) or amphipathic α-helices with intracellular membranes. Depending on their function, they are subdivided into structural and non-structural proteins. The membrane topology and major functions of each protein are shown in (Fig. 8).

1.5.1 Structure proteins

1.5.1.1 Core Protein

HCV core is the viral nucleocapsid protein with a various number of functions such as RNA binding. In the beginning, the HCV core protein is translated to a 22 kDa protein (immature form), which is composed of 191 amino acids (aa). It is cut out from the polyprotein in the endoplasmic reticulum (ER) via a cellular signal peptidase (SP) (Morozov and Lagaye 2018). Afterwards, the immature protein undergoes additional cleavage that results in the mature 21 kDa core protein. The core protein is composed of three main domains. The first domain covers 117 aa (aa 1-117) of the N-terminal region and is mostly made of basic residues and two short hydrophobic regions. This domain functions in the binding to the viral RNA. The second domain covers the region between aa 118 and 174 is characterized by being less basic and more hydrophobic, and it plays a role in the interaction with the lipid droplet (LD). The intracellular lipids are stored within a spherical structure known as lipid droplets (Fig. 8C). The aa 175 and aa 191 sequence

which represents the third domain is the region responsible for signaling the E1 ectodomain translocation to the ER membrane (Fig. 9) (Morozov and Lagaye 2018).

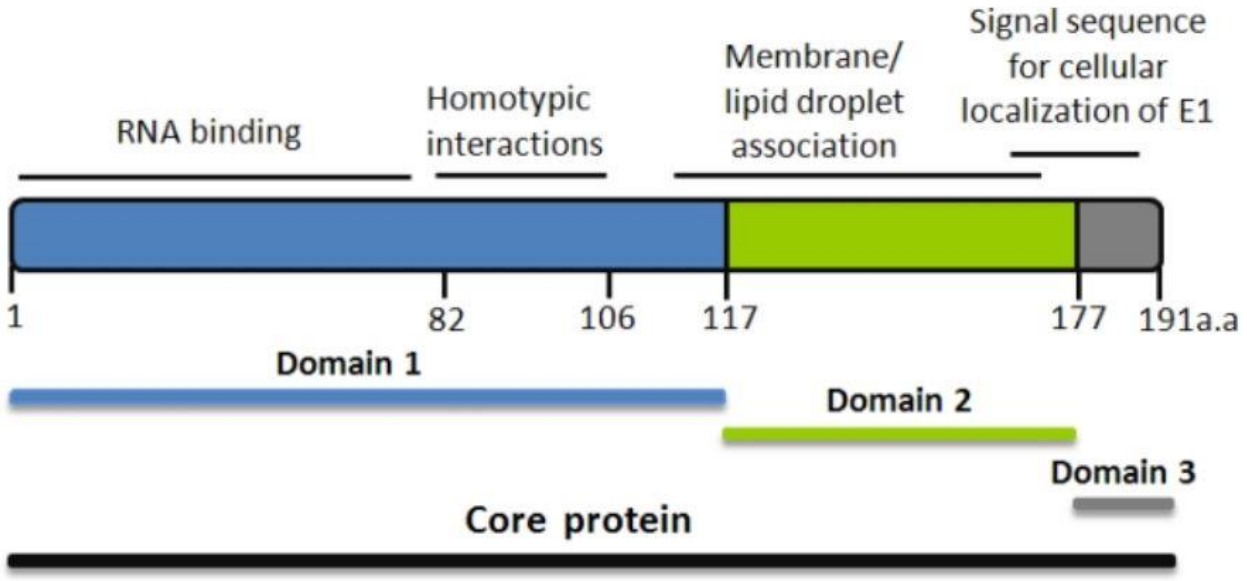


Figure 9: Structural and Functional domains of the HCV core protein (Strosberg et al. 2010).

HCV core protein interacts with HCV RNA during the viral capsid assembly. Also, the core protein is involved in regulatory functions during the RNA translation. Analyses of HCV core protein levels indicate that additionally it may be involved in several processes in cells such as immune modulation, oncogenic potential, cell signaling, autophagy apoptosis and the development of HCC (Kim and Chang 2013; Morozov and Lagaye 2018). Also noteworthy is the effect of the HCV core on the miR-122. miR-122 is a liver-specific microRNA that represents 70% of the total cellular miRNA content. It is important for liver metabolism and development. It was found that HCV core downregulates the spread of miR-122 by destabilizing it through the GLD-2 (a non-canonical cytoplasmic poly(A) polymerase) inhibition. This leads to impairment in the miR-122-regulated functions. Thus, promoting HCV replication (proviral) (Kim et al. 2016)

1.5.1.2 E1 and E2 Envelope Glycoproteins

HCV envelope glycoproteins (E1&E2) are type 1 transmembrane proteins that are forming the envelope glycoproteins surrounding the viral particles (Kim and Chang 2013). E2 is an HCV envelope subunit that mediates the binding with hepatocytes and initiates infection. E1 has attributed fusogenic properties that allow fusion of the viral and endosomal membranes to release the nucleocapsid into the infected cell cytoplasm (Ashfaq et al. 2011). The two hypervariable regions (HVR) HVR1 and HVR2 in E2 are under constant selection by mutations. This allows the virus to escape from neutralizing antibodies and to develop a chronic infection. Also worth mentioning is that HVR1 is engaged in cell infection (Ashfaq et al. 2011). E1 and E2 are produced from the cleavage of a precursor protein located in the ER by the cellular SP. The glycoprotein part is rich in large hydrophilic ectodomains and 30 aa long transmembrane domains (TMD). The TMD plays a major role in the anchoring of the envelope proteins to the endoplasmic reticulum membrane and their ER retention. The glycoproteins ectodomains were to be heavily glycosylated where E1 has 4-5 and the E2 - 11 presumably N-glycosylation sites, respectively. Interestingly, these sites are rather conserved. These E1-E2 heterodimer complexes are crucial for the interaction with cellular receptors and help to promote the fusion between virus and cell.

1.5.2 Non-Structural proteins

1.5.2.1 NS1 (P7)

P7 is a polypeptide of 63 aa with two transmembrane domains. It oligomerizes in the ER membrane to a cation channel that is essential for infection in vivo and the generation of mature virions (Steinmann et al. 2007). It is located between E2 and NS2 proteins and it is linked to both structural and non-structural proteins. However, up till now the exact role of this protein in the HCV life cycle remains unclear. Also, recent studies reported that P7 plays a key role in the production of HCV particles by preventing acidification (Kim and Chang 2013).

1.5.2.2 NS2 (p23)

NS2 is a 21-23 kDa hydrophobic cysteine autoprotease protein with four transmembrane domains (Morozov and Lagaye 2018). It is not essential for viral replication, but plays a role in viral assembly and nonetheless the generation of mature virions (Ashfaq et al. 2011; Kim and Chang 2013). It also initiates the cleavage of its bond with the NS3 zinc-binding domain via the NS3 metalloprotease activity. This protease enzyme is produced from the C-terminal region in the cytoplasm through the interaction with NS3 (Lorenz et al. 2006). In fact, NS2 expression leads to the up-regulation of the fatty acid synthase transcription, which may suggest the role of NS2 in the elicit HCV induced steatosis (Morozov and Lagaye 2018).

1.5.2.3 NS3 (p70)

The NS3 is a 70 kDa protein. Its N-terminal domain encompasses a serine protease enzyme. It plays an important role in the viral polyprotein processing by cleaving after the NS3 at 4 different locations (between NS3/4A, NS4A/4B, NS4B/NS5A, NS5A/NS5B). Additionally, it cleaves the TRIF (TLR3 adaptor protein) as well as the MAVS (mitochondrial antiviral signaling protein), therefore blocking the cellular induction of IFN (type-1) pathway (Morozov and Lagaye 2018; Kim and Chang 2013). In addition, NS3 is able to modulate the innate immune response of an infected cell by disrupting the RIG-I signaling pathway through cleavage of IPS-1 (Loo et al. 2006). NS3 also plays an important role in the HCV-mediated disruption of the Nrf2/Keap1-signaling pathway in cells with HCV-induced oxidative stress. In which, HCV core mediates the delocalization of sMAF proteins away from the nucleus and near the replication organelle where it binds to the NS3 for later fishing of the released Nrf2 (Bender and Hildt 2019).

1.5.2.4 NS4A (p8)

NS4A is an 8 kDa protein that acts as a cofactor of the NS3 protease by forming a stable complex with it (Kim and Chang 2013). It was shown via a deletion analysis study that

NS4A is essential for the NS3-ER targeting. Furthermore, NS5A phosphorylation was found to require interaction with NS4A. The deletion analysis studies have shown that NS4A is essential for the ER targeting of NS3. In addition, the NS4A interaction with NS5A is important for the NS5A phosphorylation (Morozov and Lagaye 2018). NS4A is able to change some cellular functions, thus, considered to be involved in the viral pathogenesis. Surprisingly, it was found that NS4A exists not only on the ER, but also on the mitochondria, either alone or accompanied with NS3 in the form of NS3/4A polyprotein. It has been shown that the mitochondrial distribution was altered by the NS4A expression, damaging it and leading to host cell apoptosis (Egger et al. 2002; Nomura-Takigawa et al. 2006; Morozov and Lagaye 2018).

1.5.2.5 NS4B (p27)

The NS4B is a 27 kDa protein. It binds to the ER through its four TMDs, which play a role in recruiting more non-structural viral proteins (Egger et al. 2002; Popescu et al. 2014). It can be seen under the electron microscope that it induces the formation of a tight structure known as the membranous web, which is a cellular structure formed by lipid droplets and ER membranes (Hügle et al. 2001). By interacting with NS4A, it indirectly interacts with NS3 and NS5A to form the replication complex (RC) (“factory”) of HCV in the membranous web in HCV infected cells (Morozov and Lagaye 2018; Egger et al. 2002). This requires the interaction with the monomeric GTPase Rab5 (Stone et al. 2007). NS4B is probably not involved in the replication of the virus, but could play a role in the assembly and the release of the virus particles.

1.5.2.6 NS5A (p56/ p58)

NS5A is a 56 kDa dimeric zinc-binding metallophospho-protein (Figure 9b), which binds the viral RNA as well as various host factors close to HCV core and lipid droplets (Kim and Chang 2013). This protein, when examined in HCV infected cells, was found in two different phosphorylated forms, hypophosphorylated (56 kDa) and hyperphosphorylated (58 kDa). The phosphoprotein NS5A interacts with many cellular proteins, most importantly the apoE, and TIP47 (Ploen et al. 2013). apoE is a human protein representing

a major component of lipoproteins enriched with cholesterol. It is involved in the metabolism of fats in the body. In cells infected with HCV, apoE was found to interact via the α-helix domain in its C-terminal with NS5A protein. This apoE-NS5A interaction was found to be essential for HCV production (Cun et al. 2010). TIP47 is a tail interacting protein 47 kDa in size (Ploen et al. 2013). It is associated with lipid droplets. It is important for the LD maturation as it contains a domain that resembles apoE. It has been identified as a factor that plays a role in cellular cytoplasmic sorting. (Bulankina et al. 2009). It was reported that TIP47 binds with NS5A. This TIP47-NS5A interaction (complex) directs the synthesized viral genome bound to the replication complex to the LD (Ploen et al. 2013). In addition to viral proteins thereby contributing to the regulation of HCV replication, cell signaling modification, virus pathogenesis, virus propagation and the interferon response (Ashfaq et al. 2011).

NS5A protein has an amphipathic helix located at the N-terminal and is conserved in many genotypes and plays a key role in membrane localization (Singh et al. 2003). NS5A was found to disrupt several signal transduction pathways through both of its terminal domains. NS5A protein levels were found to activate more the PI3K-AKT-BAD pathway through the direct interaction between the NS5A N-terminal and the p85 subunit of PI3K. This leads to downstream regulation of apoptosis. This apoptotic modulation confers the survival of the viral infection and persistence and assures the viral pathogenesis (He et al. 2002). On the other hand, the NS5A through its C-terminal SH3-binding motif that is rich in proline interacts directly with the adaptor protein of the growth factor receptor-binding protein 2 (Grb2). This deactivates the extracellular signal-regulated kinases 1 and 2 (ERK1/2) through the epidermal growth factor (EGF) and downregulates the Ras-Raf-MEK-MAPK pathway, which promotes the viral persistence and the IFN response (He et al. 2001; He et al. 2002). Besides this NS5A goes through various mutations that improve the capability of the subgenomic HCV RNA replication in cell culture systems (Tamura et al. 2011). As mentioned in the beginning, it possesses a Zinc-coordination motif, that influences the HCV replication (Macdonald et al. 2004; Morozov and Lagaye 2018). Additionally, it was noticed that NS5A plays an essential role in mediating the IFN response through a ~39 mm region called “interferon-α sensitivity determining region” (ISDR) (Carranza-Rosales et al. 2010), which can be used to estimate the sensitivity and resistance of HCV to interferon treatment. It was also found that NS5A is coupled with a variety of cell signaling

pathways such as apoptosis (Macdonald et al. 2004). This reflects on how critical the NS5A roles to the HCV survival and spread, which is why it is already targeted with direct-acting antiviral (DAA) drugs.

1.5.2.7 NS5B (p66-68)

NS5B is a 66-68 kDa protein located in the C-terminus of the precursor. It represents the RNA-dependent RNA polymerase (RdRp), containing an active motif known as the GDD in its active site (Lesburg et al. 1999). It is essential for enzyme activity. The enzyme uses a template, which is the (+) RNA genome for the synthesis of a negatively oriented RNA and synthesizes immediately thereafter another (+) RNA genome. Since NS5B does not have a proofreading function, replication leads to an increase in mutations leading to a large amount of quasispecies. Due to its key role in the replication of the virus, the NS5B protein is considered as a potential target for an antiviral drug such as NM283 (Francesco and Migliaccio 2005) and Sofosbuvir (Stedman 2014).

1.6 Viral Life Cycle

After the primary infection HCV particles circulate in the blood and eventually come into contact with the basolateral surface of hepatocytes, where principal cell surface interaction between the virus and host cell binding receptors is initiated (Dubuisson and Cosset 2014; Morozov and Lagaye 2018). This first process involves at least four cellular entry factors and two specific receptors that are required for a successful virus entry (Fig. 10). This includes the scavenger receptor class B member 1 (SRB1) and the heparan sulfate proteoglycans (HSPGs) syndecan-1 and syndecan-4, tetraspanin CD81 and the tight junction proteins Claudin 1 (CLDN1) and occludin (OCLN) (Shi et al. 2013; Zeisel et al. 2013; Dubuisson and Cosset 2014), and also the glycosaminoglycans (GAGs).

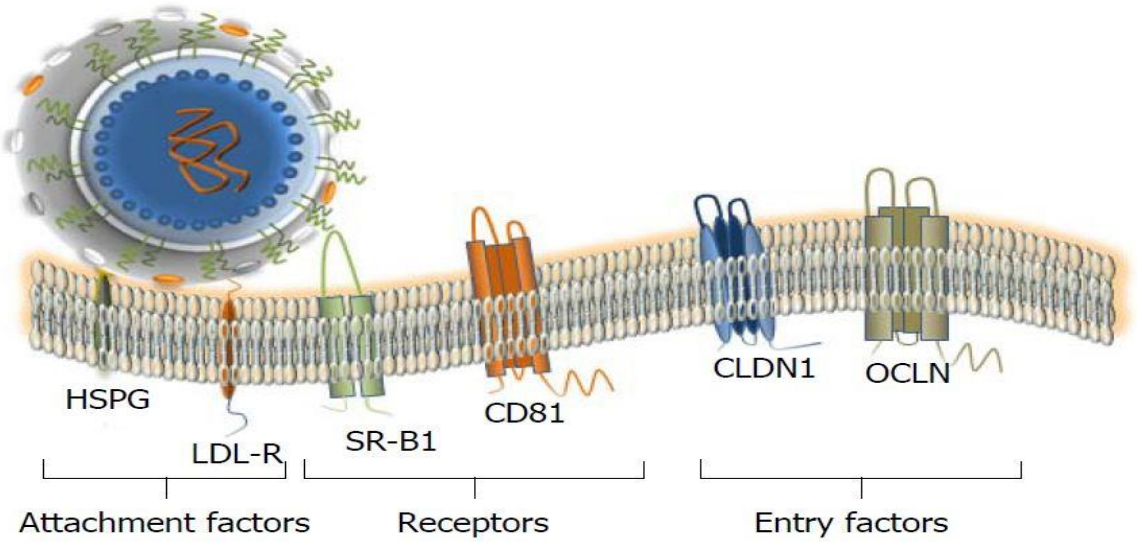


Figure 10: Attachment factors, receptor and entry factors utilized by the hepatitis C virus.

At least six membrane proteins are essential for virus attachment and entry. Heparan sulfate proteoglycan (HSPG) - a type of glycosaminoglycan (GAG) and low-density lipoproteins (LDL)-receptor that is promoting the LDL endocytosis are considered as binding factors for hepatitis C virus (HCV). Scavenger Receptor class B member 1 (SR-B1) and CD81 that are ubiquitously expressed on the cell surface, are considered as true receptors. The receptor–viral cargo complex is then moving to the cell-cell contacts (not shown), where the interaction with the tight-junction proteins Claudin 1 (CLDN1) and Occludin (OCLN) takes place. Both proteins are required at the late stage of the entry. Additional interaction partners that are likely engaged in the HCV entry are described in the text. The docking of the LVP to the cellular membrane is shown. (Morozov and Lagaye 2018).

Such interaction is mediated in case of the HCV (as it interacts with lipoproteins) through apoE, which seems to be similar to that of many other viruses and it is the reason why apoE is contained in the lipoviral particles (LVPs) which allow the later to bind to low-density lipoprotein receptor (LDLR) (Dubuisson and Cosset 2014; Morozov and Lagaye 2018). After the docking process is completed, viral particle interacts with specific cellular receptors, the scavenger receptor B1 (SR-B1), and with CD81 via its envelope glycoprotein E2, where CD81 already interacts with CLDN1 to form a co-receptor complex. This allows the virion E2 to interact with the CLDN1, such an interaction is mediated through the activation of the HRas / MEK / ERK pathway via EGFR (epidermal growth factor receptor) and EphA2 (ephrin type-A receptor 2) (Zona et al. 2013; Lupberger et al. 2011). This CD81-OCLN interaction is crucial in determining host-cell affinities (tropism) of HCV, in this case against human hepatocytes (Dorner et al. 2011). Additionally, the cholesterol transporter NPC1L1 (Niemann-Pick C1-like) and the transferrin receptor 1 were both identified and classified as entry factors (Martin and

Uprichard 2013; Morozov and Lagaye 2018). Last but not least is the transferrin receptor1 (TfR1). It acts as a specific receptor for the HCVE1/E2 protein during the entrance of the HCV to the cells, where efficient binding occurs between the HCV particles and TfR1 (208–212 amino acid residues) directly without the involvement of other receptors. When antibody-mediated neutralization of TfR1 occurred that led to the repression of the HCV infection. But for this to happen the CD81 receptor has to remain intact and active. This may suggest that the importance of TfR1 in HCV entry through adhesion and endocytosis CD81 dependent (Liang 2018). All the above-mentioned entry factors are not expressed solely on hepatocytes, but maybe also found in other tissues, but what is exclusive to the hepatocytes is the combined presence of these protein factors that define hepatocytes as host cells for HCV (Reynolds et al. 2008). Bound viral particles or lipoviral particles are then engulfed by the liver cells combined with the CD81-CLDN1 receptor complex via clathrin-dependent endocytosis (Blanchard et al. 2006) and are transported via actin filaments retrograde to early endosomes (Dubuisson and Cosset 2014; Morozov and Lagaye 2018). Early endosomes undergo acidification, which is believed to induce a conformational change in the E1E2 complex that exposes a region in E1 that is responsible for the fusion between the viral envelop and the endosomal membrane, leading to the discharging of the nucleocapsid into the cytoplasm (Dubuisson and Cosset 2014). Noteworthy, HCV spreads via direct cell contact with most of the entry factors mentioned being involved (Morozov and Lagaye 2018). The released nucleocapsid will be degraded to release the RNA which is utilized in both the replication and polyprotein translation (Fig. 11), where the later is initiated through the binding of the viral 5`UTR (IRES) to the ribosomal subunits. This process takes place on the endoplasmic reticulum (ER) membrane surface. This results in the production of the polyprotein precursor that is 3000 a.a. long which is afterward cleaved by the host and viral proteases producing the structural and non-structural proteins (Dubuisson and Cosset 2014; Morozov and Lagaye 2018). It was shown in many studies that the HCV infection process results in ER stress and autophagy responses, where the latter is regulated by HCV. As a matter of fact, the autophagy machinery was found essential for the HCV replication and that any autophagy suppression results in the inhibition of viral replication (Rice 2011; Morozov and Lagaye 2018). Noteworthy, it has been illustrated

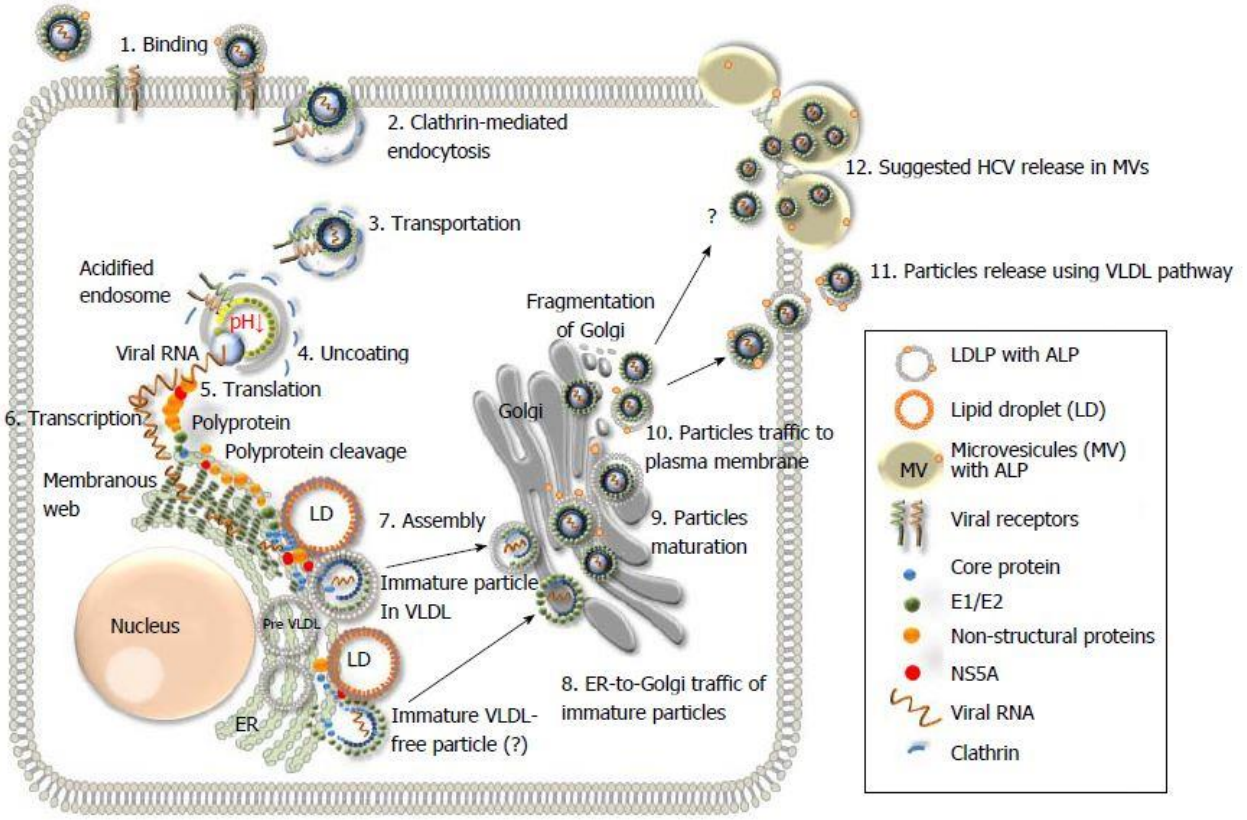


Figure 11: Schematic representation of the hepatitis C virus life cycle. (Morozov and Lagaye 2018).

that through Class III PI3K-independent pathway, HCV can induce autophagosomes formation and uses afterwards its membranes as sites for its RNA replication (Rice 2011). These are used for translation and replication or packaged in virions (Bartenschlager et al. 2013). Once the viral proteins and RNA are produced, viral assembly follows which is a multi-step process. It occurs in the ER close or directly on the LD (Lindenbach and Rice 2013). NS5A initiates an early phase of the virion formation following the protease-mediated cleavage of the polyprotein precursor, through the interaction with its C—terminal serine cluster and the core protein. The newly synthesized and released RNA is directed to the replication complex, which is by then localized in the membranous web, where it is encapsulated within the nucleocapsid. These newly assembled immature viral particles are transported from the ER to the Golgi, where maturation of these particles takes place. Afterwards, these nucleocapsids become enveloped by budding from the Golgi, followed by its secretion through the plasma membrane.

1.7 Model Systems for the Analysis of HCV

The milestones done in HCV research are dependent completely on model systems. The most frequently used models (Fig. 12) for the investigation of HCV are described below (Morozov and Lagaye 2018). The HCV genome was first identified by cloning it from an infected chimpanzee in 1989, because the HCV amount in humans was undetectably low. Until the first full-length viral cDNA from genotype 1a (strain-H77) was cloned that was able to infect chimpanzee upon intrahepatic injection (Yanagi et al. 1997; Morozov and Lagaye 2018). However, the inability of these HCV replicons to replicate in vitro remained a problem, until 1999 when the research group of Prof. Bartenschlager was able to construct subgenomic replicons. A subgenomic replicon is a bicistronic RNA containing the sequences of the non-structural proteins NS3 to NS5B and a neomycin resistance cassette for selection (Moradpour et al. 2007). These could be stably transfected into cells to study the intracellular steps of HCV replication and antiviral effects. One of these stable cell lines is the clone Huh 9-13 (Lohmann et al. 1999). However, infectious particles are not formed by the subgenomic replicon systems, because of the absence of the sequence of the structural proteins (Lohmann 2009). For this reason, cell entry of HCV was initially investigated with recombinant HCV-like particles or E1E2-pseudotyped retroviruses (HCVpp viruses) (Baumert et al. 1998; Bartosch et al. 2003). Then the research group of Prof Bartenschlager was able to confirm these replicons' ability to reproduce in many cell lines. The most permissive being the hepatocarcinoma Huh7 cell line with further improvement being noticed upon the interferon treatment of the inoculated Huh7 cells. The cured cells now became highly permissive to both the subgenomic clones and the full-length genome. These sub-cell lines were called Huh7.5 & Huh7.5.1 (Morozov and Lagaye 2018).

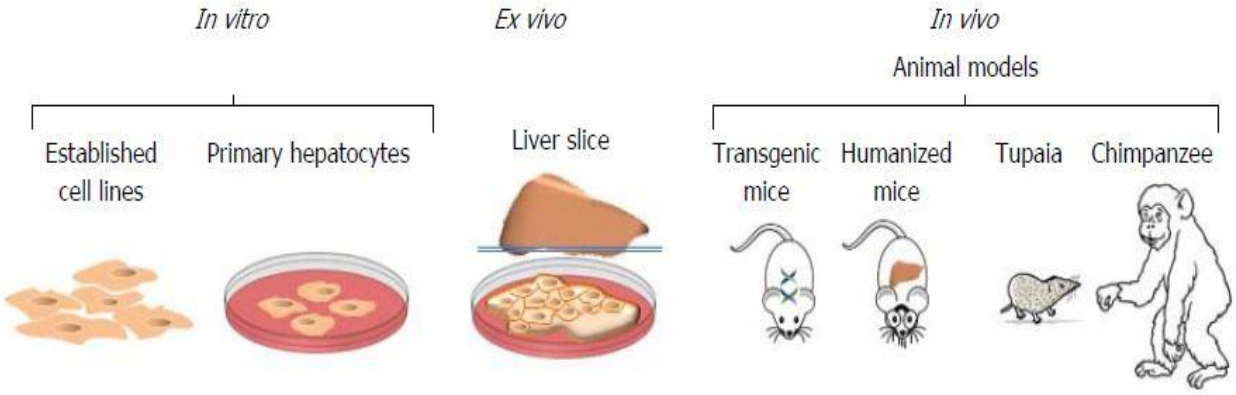


Figure 12: In vitro, ex vivo and in vivo models to study the hepatitis C virus. (Morozov and Lagaye 2018)

Thankfully this problem was solved by a breakthrough discovery of the g(2a) JFH1 isolate in 2005. This genotype 2a isolated from a Japanese fulminant hepatitis patient was able to replicate in Huh-7 cells, independent from any adaptive mutations. It forms infectious HCVcc particles in vitro and vivo. This was the first time that the full life cycle of HCV has been studied in vitro (Morozov and Lagaye 2018). Based on this infectious clone, many different genotypes chimeric clones such as (genotype 1a, 1b, 2a, and 3b) were constructed, where the NS2-NS5B region was replaced by those of JFH-1 shown to be infectious in Huh-7 cells. The most efficient construct is the genotype 2a/2a clone which consists of J6 and JFH-1 derived sequence (JC1). Based on the Jc1 construct, a bicistronic reporter virus encoding an IRES-dependent Renilla luciferase whose enzyme activity is proportional to viral replication was prepared (Koutsoudakis et al. 2006). The integration of fluorescent proteins into permissive sites of different viral proteins has been used to study the dynamics of the viral structure in infected cells. An NS5A-GFP variant of Jc1 allows visualization of the replicon complex, while E1-mCherry viruses allow one to observe the movement of assembled virions (Moradpour et al. 2004). The efficiency of replication and virion formation was further enhanced by the highly permissive Huh7.5 Huh7.5.1 sub-cell line. Also, by a point mutation of the catalytic motif GDD (GLyAspAsp) of the RdRP NS5B to GND (GlyAsnAsp) (Fig. 13), it was possible to generate a

replication-deficient virus, which can be used as a negative control (Lindenbach et al. 2005; Wakita et al. 2005).

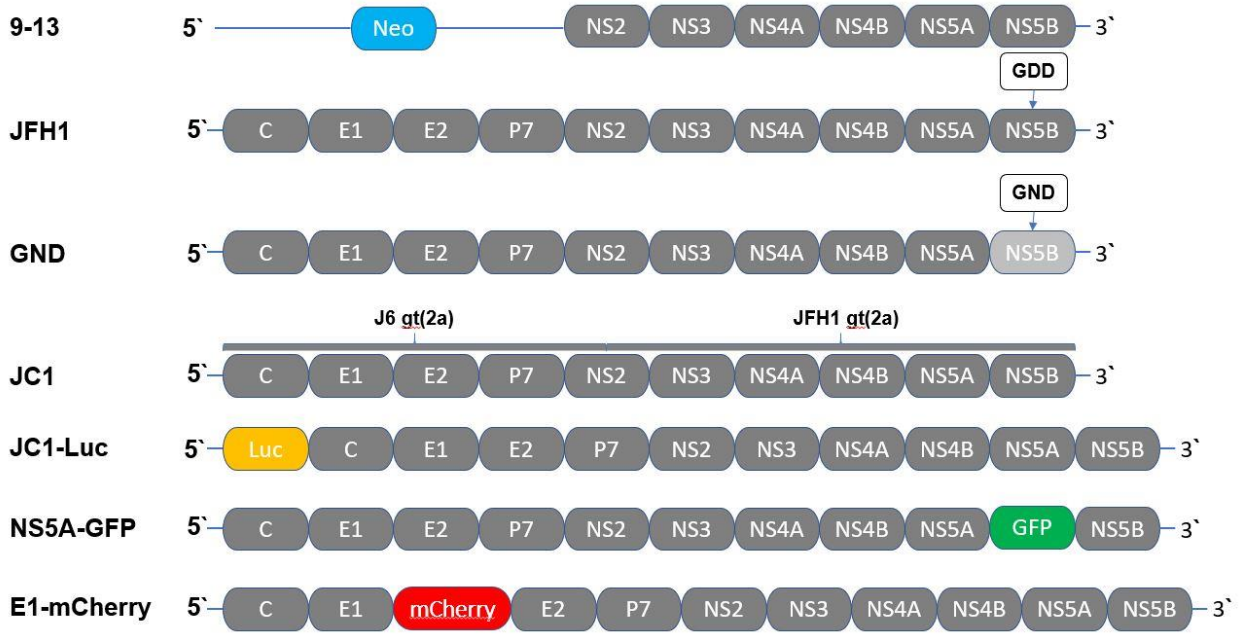


Figure 13: Schematic representation of different HCV constructs.

However, there is a difference between the cell lines and bona fide hepatocytes. For example, these cells lack the ability to grow in a polarized monolayer and do not secrete full-fledged VLDLs, which is why the HCV particles produced in cell culture differ from those produced by LVPs in vivo (Ploss et al. 2010; Podevin et al. 2010; Yamamoto et al. 1987). Samples were also collected via partial hepatectomies from healthy liver tissue from which primary human hepatocytes can be obtained. These can be cultured in special tissue culture plates and medium for some time. They can be infected with HCVcc, but the replication of the virus is so low that it can only be detected with highly sensitive RT-qPCR methods (Zeisel et al. 2011). The only animal models of HCV to date are chimpanzees, transgenic mice and humanized mice (Lindenbach et al. 2006).

1.8 Hepatitis C and Autophagy

Autophagy “Self-eating” is a highly conserved process that confers the cell homeostasis by breaking down or catabolizing intracellular components. It is very much regulated by damaged organelles, ER stress, oxygen stress in addition to some pathogens and also lack of nutrients to keep the cellular homeostasis in balance (Kroemer et al. 2010). It was found that nutrient deficiency leads to the inhibition of mTOR1 kinase (mammalian target of rapamycin), which in turn activates autophagy (Jung et al. 2010).

The autophagy process starts with an initial membrane called the IM isolation membrane originally called Phagophore (Bender and Hildt 2019). It surrounds the catabolisable material, which can be soluble cell constituents, membranes, and even whole organelles, and grow into closed double vesicle vesicles, the autophagosomes. A frequently used marker of autophagosomes is the protein LC3 (microtubule-associated protein 1 light chain 3), which is processed and lipidated by a strictly regulated ubiquitin-dependent reaction (Fujita et al. 2008). Another important marker protein in autophagy is the ubiquitously expressed cellular protein p62, that directly interacts with LC3 on the phagophore surface. Both then accumulate in the autophagosomal membrane and become incorporated into autophagosomes for degradation via lysosomal (Bjørkøy et al. 2009). However, it was found that p62 protein interacts with the Keap1-Nrf2 binding site facilitating the release and induction of Nrf2. Nrf2 (nuclear factor erythroid 2-related factor 2) is another key transcription factor that belongs to the CNC-bZIP family and plays a crucial role in regulating the oxidative stress, which is expressed ubiquitously in many different cell types and tissues (Moi et al. 1994). Moreover, HCV infection was found to induce autophagy, either through the viral replication that takes place at the membranous web, which cause ER stress by the concentration of the E1E2 dimers in the ER membrane (Choukhi et al. 1998; Bartosch et al. 2009; Asselah et al. 2010) and is followed by unfolded protein response. Also, HCV core was found to mediate the delocalization of sMaf proteins from the nucleus to the replication complex sites in the cytoplasm, where it is trapped by the NS3 (Bender and Hildt 2019). This leads to fishing the Nrf2 after its release from the Keap1, preventing the Nrf2/AREs interaction. Therefore, inhibiting the expression of the cytoprotective genes. This leads to an increase in ROS levels. This cumulatively results in the induction of autophagy (Bartosch et al. 2009). Noteworthy, it was reported that

NS4B with Rab5 was able to directly interact with mTOR1 directly which results also in the induction of autophagy (Li et al. 2010).

1.9 Hepatitis C and cellular lipid metabolism

Cellular lipid metabolism and cholesterol equilibrium, in particular, were found to be affected by HCV infection. It was also shown that HCV was able to modulate lipid metabolism leading to an improved replication of virus RNA. It was frequently recognized that HCV infection is accompanied in patients with liver steatosis, and low blood-free cholesterol, LDL, HDL and Non-HDL-Cholesterol in addition to a frequent increase in triglycerides (Dubuisson et al. 2002; Popescu et al. 2014), and upon interferon-alpha plus ribavirin treatment lipoprotein and cholesterol levels were restored in patient sera (Popescu et al. 2014). Activation of lipid metabolism affects both the essential lipid components' abundance and membrane fluidity. It allows the formation of the HCV replication complex. Saturated and monounsaturated fatty acids, that are responsible for maintaining the membrane structure and fluidity, stimulate HCV replication, they are responsible for maintaining the membrane structure and fluidity. While on the other hand, polyunsaturated fatty acids inhibit the HCV replication by negatively affecting the membrane fluidity (Kapadia and Chisari 2005). HCV has the ability to affect the cellular lipid metabolism in three directions that would favor its survival by increasing the lipogenesis, alter the degradation and export of lipids.

1.10 Nuclear factor erythroid 2-related factor 1 (Nrf1)

Cellular responses and metabolic adaptations are responsible for the homeostasis inside cells and tissues. However, when cells are chronically exposed to a flow of nutrients, it can render the cells susceptible to the accumulation of nutrients. This causes a number of metabolic diseases by stressing the adaptive system (Hotamisligil 2017; Widenmaier et al. 2017). The endoplasmic reticulum (ER) is the organelle representing the adaptive systems that are responsible for maintaining the cellular homeostasis. This is via acting as a platform for enzymes that bind substrates, or stress signals with regulated changes in protein synthesis, folding and secretion. In addition to lipid metabolism and trafficking

(Fu et al. 2012; Widenmaier et al. 2017). Noteworthy, excess cellular cholesterol metabolism is indeed regulated via its conversion into bile, esterification, or oxysterol formation by the endoplasmic reticulum. It is considered pathogenic in steatosis, neurodegeneration, and diabetes, when exposed to excess dietary cholesterol or if the circulating cholesterol levels are elevated, where the cellular cholesterol thresholds are surpassed and the excess starts depositing on the ER-membrane (Chang et al. 2006; Lange et al. 2014). Since the ER is the cellular organelle responsible for defending homeostasis, it is essential to sustain metabolism through the ER adaptation to cholesterol excess. One way is through an enzyme called cholesterol acyltransferase (ACAT), which functions by detoxifying cholesterol through cholesterol esters formation (Chang et al. 2006). Due to the fact that this enzyme doesn't remove excess cholesterol, but rather stores it, it has its limitations. Another factor for adaptation are the liver X receptors (LXRs), which operate by exporting cholesterol (Bensinger and Tontonoz 2008). Nonetheless, LxR still doesn`t bind to cholesterol, but rather bound to the genomic DNA (Watanabe et al. 2003). Stress sensors are very much required to go across membranes to achieve adaptation. For example, (UPR) unfolded protein response or the (SREBP2) sterol response element-binding protein 2, which initiates inflammatory reactions and a number of metabolic pathways to restore and maintain homeostasis (Widenmaier et al. 2017).

Nrf1 was identified as a predominantly expressed ER-membrane spanning protein. one of the Cap'N'Collar (CNC) family members that functions as a transcription factor. It is involved in the cellular adaptation to stress as well as detoxification (Sykiotis and Bohmann 2010). Along with Nrf2, they represent the primary factors that heterodimerize with the small Maf proteins in the nucleus and bind to the antioxidant response element (ARE) (Bugno et al. 2015). Although, newly synthesized Nrf1 is ER membrane-bound, cleavage near the N terminus releases Nrf1 from the ER to regulate transcription through (AREs) in the genome (Radhakrishnan et al. 2004). Deglycosylation process is required for the Nrf1 regulation and also proteolysis mainly by proteasomal degradation (Bugno et al. 2015).

It is necessary to know that the Nrf1 function is accurately adjusted by keeping a balance between the translation of the transcripts to produce the CNC-bZIP protein simultaneously with the processing following both transcription and translation. A single Nrf1 gene is

differentially expressed to generate from 1.5 kb and 5.8 kb of mRNA transcripts. This consequently leads to the production of different isoforms or proteoforms between 25 kDa and 140 kDa (Chan et al. 1993; Luna et al. 1994; Caterina et al. 1994; Murphy and Kolstø 2000; Bugno et al. 2015). They have distinct functions but in the end, they provide strong cytoprotection against various cellular stress via the regulation of developmental and homeostatic genes. It is worth mentioning that the expression of these important cytoprotective genes is mediated through the antioxidant response elements (AREs) with or without other cis-adjusting consensus sequences (Bugno et al. 2015).

It is of crucial importance to know the dynamic intercommunication between Nrf1 (NFE2L1) and the endoplasmic reticulum to be able to understand the form, production, and function of Nrf1.

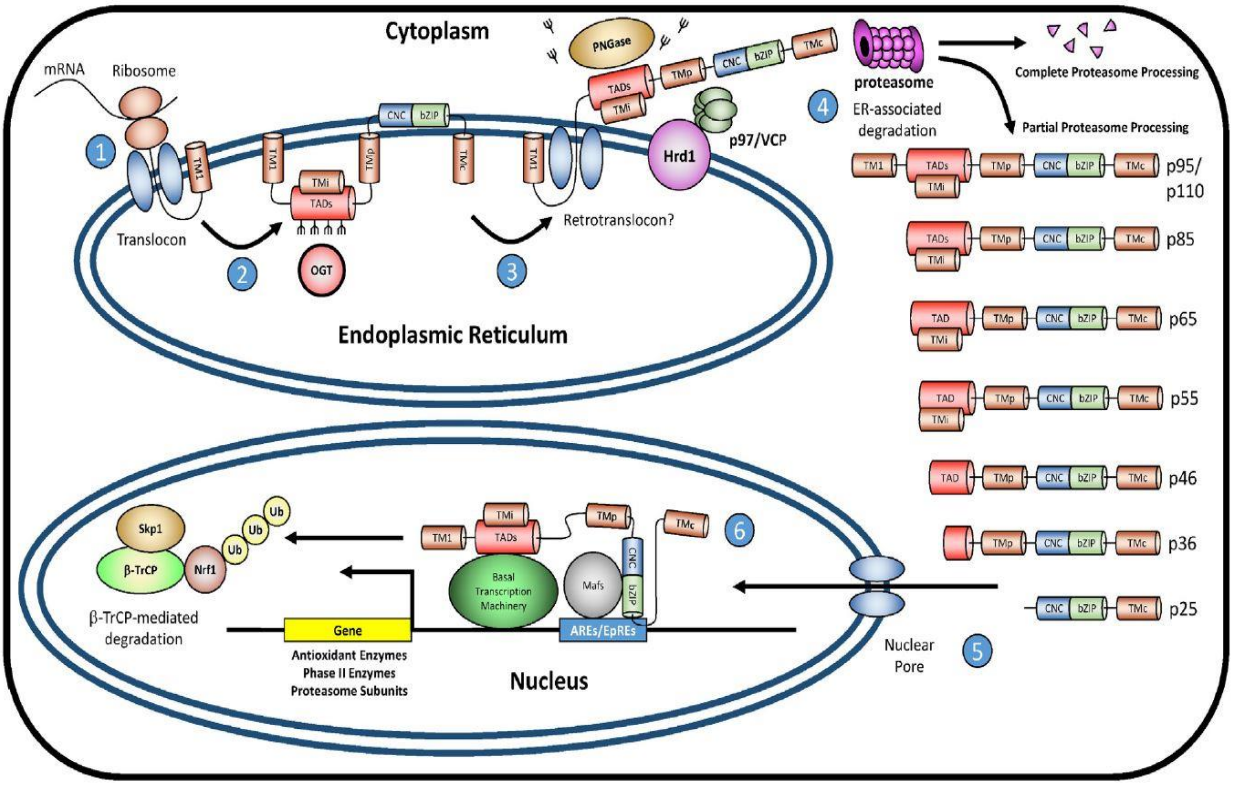


Figure 14: Proposed mechanisms illustrating Nrf1 modulation, processing, and localization (Bugno et al. 2015)

It starts when the Nrf1 mRNA migrates to the cytoplasm, where it binds to the ribosomes located on the surface of the endoplasmic reticulum to be translated. It was shown that

the newly synthesized Nrf1 protein localization is mediated through the N-terminal domain. This occurs after the fusion of residues (1-86) of Nrf1 to nucleoplasmin (a nuclear protein) (Sha and Goldberg 2014). Further studies showed that the produced Nrf1 is anchored in the ER membrane with the help of the N-terminal homology box 1 residues (11–30) (Zhang and Hayes 2013). Then it moves through translocon. After that, it is directly inserted into the ER membrane. The transactivation domain (TAD) in addition to the NST domain of the Nrf1 is translocated transiently inside of the ER lumen, where glycosylation occurs to form the inactive full-length p120 Nrf1 (Bugno et al. 2015). Retrotranslocation then follows, which takes place through the ER membrane, where it undergoes PNGase-mediated deglycosylation to form the active p95 Nrf1. Simultaneously, the full-length Nrf1 was proposed to associate itself with (ERAD); endoplasmic reticulum-associated degradation. By accompanying the ERAD complexes (p97 / VCP and Hrd1), where it undergoes incomplete or partial degradation generating the deglycosylated, now truncated forms of the Nrf1 full-length (p95, p85, p65, p55, p46, p36, and p25). These isoforms afterward are translocated into the nucleus through the nuclear pore. Through there CNC-bZIP domains they bind to the small Maf proteins. Followed by the interaction with AREs located in many cytoprotective genes triggering their expression. In the end, Nrf1 through the ubiquitination and nuclear proteosomes is degraded inside the nucleus. It is worth mentioning that PNGaseF revealed in different cellular fractions in vitro modification, that only cytosolic full-length TCF11 treatment resulted in a molecular weight shift in TCF11 (Bugno et al. 2015).

It was interesting to know a fact that Nrf1 possesses cholesterol recognition amino acid consensus (CRAC) domains that could identify Nrf1 as a cholesterol sensor that helps the ER maintain the cellular homeostasis (Zhang et al. 2014).

2 Aim of this Study

It was reported previously how HCV infection affects lipid metabolism and cholesterol homeostasis in particular, and also recently the relationship between the lipids and the Nrf1 activity. So, we wanted to study and understand the crosstalk and relationship between Hepatitis-C Virus and Nrf1 activity and the effect of cholesterol level modulation on both of them and what happens to HCV upon modulating Nrf1.

Despite the significant advances in HCV therapy, drug resistance and genotype-specific efficacy are still issues to be considered. That´s why targeting this new crosstalk could hold a promising therapeutic opportunity that will likely emerge upon identifying the complete spectrum of ER adaptive systems, and how they are integrated during metabolic stress.

3 Materials

3.1 Cells

3.1.1 Prokaryotic cells

Strain	Genotype	Source
E. coli DH5α	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK-mK+), λ-	Invitrogen, Karlsruhe, DE

3.1.2 Eukaryotic cells

Strain	Description	Source
Huh7.5.1	Human hepatoma cell line derived from Huh7 cells.	Prof. Dr. Ralf Bartenschalger, University of Heidelberg
Primary human Hepatocytes	Primary human hepatocytes	Isolates from tissue samples obtained from partial hepatectomy

3.2 Plasmids

3.2.1 Plasmids containing HCV genome

The JFH1 plasmids were kindly provided by Prof. Dr. Ralf Bartenschlager (University Hospital Heidelberg, Institute for Virology).

Plasmid	Description	Source
pFK-JFH1/GND	Replicationdeficient HCV-JFH1-Mutant due to a singlepoint mutation in NS5A	Wakita et al., 2005
pFK-Jc1	A chimera of HCV genotype 1b and 2a that	Pietschmann

	produces higher titers of infectious particles	et al., 2006

3.2.2 Expression plasmids

Plasmid	Description	Source
pFlag-Nrf1-HA	Nrf1 expression construct with N-terminal 3xFlag and C-terminal HA tag.	Addgene, Watertown, MA USA
pGF1-LXRE	LxR reporter construct with GFP tag.	System Bioscience, Eching, DE
pPUC18	Empty expression vector	Invitrogen, Karlsruhe, DE

3.3 Oligonucleotides

Name	Number	Sequence **(5´→** 3´)
JFH1-fwd	#26	atg acc aca agg cct ttc g
JFH1-rev	#27	cgg gag agc cat agt gg
Nrf1-fwd	#1400	gct gga cac cat cct gaa tc
Nrf1-rev	#1401	cct tct gct tca tct gtc gc
ABCA1-fwd	#1404	gaa gta cat cag aac atg ggc
ABCA1-rev	#1405	gat caa agc cat ggc tgt ag
HMGCS1-fwd	#1410	gag ggc ttc gtg gga cac ata
HMGCS1-rev	#1411	gcc act ggg cat gga tct tt
HMGCR-fwd	#1485	cct tag tgg ctg aaa cag ata ccc
HMGCR-rev	#1486	ctg gat gat ctc agc atc act aag g

LDLR-fwd	#1412	gcc cag cga aga tgc gaa gat at
LDLR-rev	#1413	tgc tga tga cgg tgt cat agg aa
hRPL27cDNA-fwd	#835	aaa gct gtc atc gtg aag aac
hRPL27cDNA-rev	#836	gct gtc act ttg cgg ggg tag

3.3.1 qRT-PCR primer

3.4 Antibodies

3.4.1 Primary Antibodies

Antibody	Species / Clonality	Dilution (WB/IF)	Manufacturer
Anti-HCV-core	Mouse, monoclonal	1:1000 / 1:200	Thermo Scientific, Karlsruhe, DE
Anti-HCV-NS5A	Rabbit, polyclonal	1:1000 / 1:200	selfmade (Bürckstümmer et al., 2006)
Anti-Flag	Rabbit, monoclonal	1:1000 / 1:200	Sigma-Aldrich, Seelze, DE
Anti-ß-actin	Mouse, monoclonal	1:10.000 /-	Sigma-Aldrich, Seelze, DE
Anti-Nrf1 (N-terminal)	Rabbit, monoclonal	1:500 / 1:50	Cell Signaling, Frankfurt am Main, DE
Anti-Lamin-A	Rabit, polyclonal	1:300 / -	Santa Cruz Biotech, US
Anti-GAPDH	Rabit, polyclonal	1:600 / -	Santa Cruz Biotech, US

3.4.2 Secondary Antibodies

Antibody	Species / Clonality	Dilution (WB/IF)	Manufacturer
Anti-mouse IgG-Alexa488	Donkey, polyclonal	- / 1:1000	Thermo Scientific, Karlsruhe, DE
Anti-rabbit IgG-Alexa488	Donkey, polyclonal	- / 1:1000	Thermo Scientific, Karlsruhe, DE

Anti-mouse IgG-Cy3	Donkey, polyclonal	- / 1:400	Jackson ImmunoResearch Europe, Suffolk, UK
Anti-rabbit IgG-Cy3	Donkey, polyclonal	- / 1:400	Jackson ImmunoResearch Europe, Suffolk, UK
Anti-mouse IgG-Cy5	Donkey, polyclonal	- / 1:400	Jackson ImmunoResearch Europe, Suffolk, UK
Anti-rabbit IgG-Cy5	Donkey, polyclonal	- / 1:400	Jackson ImmunoResearch Europe, Suffolk, UK
Anti-mouse IRDye800CW	Donkey, polyclonal	1:10000 / -	LI-COR Biosciences GmbH, Bad Homburg, DE
Anti-mouse IRDye800CW	Donkey, polyclonal	1:10000 / -	LI-COR Biosciences GmbH, Bad Homburg, DE
Anti-mouse IRDye680CW	Donkey, polyclonal	1:10000 / -	LI-COR Biosciences GmbH, Bad Homburg, DE
Anti-rabbit IRDye680CW	Donkey, polyclonal	1:10000 / -	LI-COR Biosciences GmbH, Bad Homburg, DE

3.5 Fluorescent Dyes

Dye	Dilution	Manufacturer
DAPI (0,1 mg/ml Stock in PBS)	1:1000	Carl-Roth, Karlsruhe
BODIPY 498/503 (1 mg/ml Stock in Ethanol)	1:100	Invitrogen, Karlsruhe
Filipin III	1:100	Sigma-Aldrich, Sleeze

3.6 Reagents for cell culture

Reagent	Manufacturer
DMEM High Glucose (4.5 g/l glucose)	Biowest, Nuaillé - France
LB- Medium	Paul-Ehrlich-Institut, Langen, DE
Fetal calf serum superior (FCS)	Biochrom AG, Berlin, DE
L-glutamine	Biochrom AG, Berlin, DE
Penicillin/Streptomycin	Paul-Ehrlich-Institut, Langen, DE
Trypsin/EDTA	Paul-Ehrlich-Institut, Langen, DE
PBS without Ca2+ and Mg2+	Paul-Ehrlich-Institut, Langen, DE

3.7 Molecular weight markers

3.7.1 DNA-Marker

DNA marker	Manufacturer
GeneRuler 1 kb DNA Ladder	Fermentas, St.-Leon Rot, DE

3.7.2 Protein-Marker

Protein marker	Manufacturer
PageRuler Prestained Protein Ladder	Fermentas, St.-Leon Rot, DE

3.8 Enzymes

Enzyme	Manufacturer
RevertAid H Minus Reverse transcriptase	Thermo Fisher Scientific, Schwerte
AseI	NEB, Frankfurt am Main
XbaI	NEB, Frankfurt am Main
RQ1 RNase-Free DNase	Promega, Fitchburg, USA
T7 RNA-Polymerase	Thermo Fisher Scientific, Schwerte
PNGase F	NEB, Frankfurt am Main

3.9 Inhibitors

3.9.1 Protease inhibitors

Inhibitor	Target	Manufacturer
Aprotinin	Serine proteases (1 µM)	Sigma-Aldrich, Sleeze, DE
Leupeptin	Serine-, cysteine- protease (4 µM)	Sigma-Aldrich, Sleeze, DE
Peptastin	Acid, aspartatic proteases (1 µM)	Sigma-Aldrich, Sleeze, DE
PMSF	Serine proteases (1 mM)	Carl-Roth, Karlsruhe, DE

3.9.2 Protein synthesis inhibitor

Inhibitor	Target	Manufacturer
Cycloheximide (CHX)	Interferes with the translocation step in protein synthesis	Sigma-Aldrich, Sleeze, DE

3.10 Fine chemicals

Chemical	Manufacturer
Random hexamer primer	Fermentas, St. Leon-Rot, DE
Glycerol 99.5 %	GERBU Biotechnik, Gaiberg, DE
Agarose	Genaxxon, Biberach, DE
dNTP mix	Fermentas, St. Leon-Rot, DE
Ethidium bromide	Applichem, Darmstadt, DE
Dimethyl sulfoxide (DMSO)	Genaxxon, Biberach, DE
N,N,N’,N’- Tetramethylethylenediamine (TEMED)	Merck, Darmstadt, DE
Ethylendiaminotetraacetic acid (EDTA)	Serva, Heidelberg, DE
Triton X-100	Sigma-Aldrich, Seelze, DE
Bovines serum albumin (BSA) Fraction V	Applichem, Darmstadt, DE

Mowiol	Sigma Aldrich, Seelze, DE
Tween-20	Genaxxon, Biberach, DE
Phenol	Applichem, Darmstadt, DE
peqGOLD TriFast	PeqLab, Erlangen, DE
Polyethylenimine (PEI)	Polysciences, Eppelheim, DE
RQ1 DNase 10x Reaction Buffer	Promega, Mannheim, DE
RQ1 DNase Stop solution	Promega, Mannheim, DE
5x reverse transcriptase buffer	Thermo Scientific, Karlsruhe, DE
Dithiothreitol (DTT)	Biomol, Hamburg, DE
Roti-Block	Carl-Roth, Karlsruhe, DE
Bradford Reagent	Sigma-Aldrich, Sleeze, DE
5x Reaction Buffer for RT	Thermo Fisher Scientific, Schwerte, DE
10x T7-Scribe Transcription Buffer	Biozym, Hessisch Oldendorf, DE
Acetone	Carl-Roth, Karlsruhe, DE
Ampicillin	Carl-Roth, Karlsruhe, DE
APS	Carl-Roth, Karlsruhe, DE
Roti-C/I	Carl-Roth, Karlsruhe, DE
Bortezomib	Selleckchem, DE
Bromphenolblau	Merck, Darmstadt, DE
BSA (Bovines Serum Albumin)	PAA, Linz Austria
Butanol	Merck, Darmstadt, DE
Chloroform	Carl-Roth, Karlsruhe, DE
Ethanol	Carl-Roth, Karlsruhe, DE
Ethidium bromide	Applichem, Darmstadt, DE
Formaldehyd 37,5%	Carl-Roth, Karlsruhe, DE
GenAgarose	Genaxxon, Biberach, DE
Glycerol	Gerbu Biotechnik, Hedelberg, DE
Luciferin	Sigma-Aldrich, Sleeze, DE
Luminata Western HRP Substrate	Merck Millipore, DE
Skimmed milk powder	Carl-Roth, Karlsruhe, DE
Maxima Probe SYBR Green qPCR Master Mix	Thermo Fisher Scientific, Schwerte, DE
Methanol	Carl-Roth, Karlsruhe, DE
Sodium chloride	Carl-Roth, Karlsruhe, DE
NEBuffer 2.1	NEB, Frankfurt am Main, DE

NEBuffer 3.1	NEB, Frankfurt am Main, DE
PrestoBlue® Cell Viability Reagent	Thermo Fisher Scientific, Schwerte, DE
Random Hexamer Primer	Thermo Fisher Scientific, Schwerte, DE
Hydrochloric acid	Carl-Roth, Karlsruhe, DE
Sodium dodecyl sulfate (SDS)	Carl-Roth, Karlsruhe, DE
Tris	Carl-Roth, Karlsruhe, DE
β-Mercaptoethanol	Sigma-Aldrich, Sleeze, DE
NP-40	Sigma-Aldrich, Sleeze, DE
Cholesterol-Water Soluble	Sigma-Aldrich, Sleeze, DE
Methyl-β-cycloheximide	Sigma-Aldrich, Sleeze, DE

3.11 Buffers and solutions

Buffer	Composition
6x DNA loading dye	1mM Tris-HCl pH 7.6 0.03 % Bromophenol blue 0.03% Xylene cyanol 60% Glycerol 60mM EDTA
TAE buffer (50x)	2M Tris base 1M NaAc 50mM EDTA pH 8.0
Separation gel buffer	1.5M Tris-HCl 0.4% SDS (w/v) pH 8.8
Stacking gel puffer	0.5M Tris-HCl 0.4% SDS (w/v) pH 6.7
Anode buffer I	20% Ethanol (v/v) 300mM Tris base
Anode buffer II	20% Ethanol (v/v) 25mM Tris base
Cathode buffer	20% Ethanol (v/v) 40mM 6-Aminohexanoic acid

4 x SDS loading buffer	4% SDS (w/v) 125mM Tris-HCl pH 6.8 10% Glycerol (v/v) 10% ß-Mercaptoethanol (v/v) 0.02% Bromphenol blue (w/v)
SDS running buffer (10x)	0.25 M Tris 2 M Glycin 1 % SDS (w/v) pH 8.3
Phosphate buffered saline (PBS) 10x	80.0 g NaCl 2.0 g KCl 14.4 g Na2HPO4 2.4 g KH2PO4 pH 7.1 ad 1L H2O
Mounting medium Mowiol 10 %	10% Mowiol (w/v) 25% Glycerol (w/v) 2.5% DABCO 100mM Tris-HCl pH 8.5
Luciferase Lysis-Buffer	25mM Tris-HCl 2mM DTT 2mM EGTA 10% Glycerol (v/v) 1% TritonX-100 (v/v)
Nuclear Fractionation Lysis buffer-A	10mM Hepes (pH 7.9) 1.5mM MgCl2 10mM KCl 0.5mM DTT 0.05% NP40 Protease Inhibitors Cocktail
Nuclear Fractionation Lysis buffer-B	5mM Hepes (pH 7.9) 1.5mM MgCl2 0.2mM EDTA 0.5mM DTT 26% Glycerol 4.6M NaCl

Luciferase-Substrate	20mM Tris-HCl pH 7.8 5mM MgCl2 0,mM EDTA 33.3 mM DTT 47µM Luciferin 530 µM ATP
Deglycosylation Lysis buffer	PBS Protease inhibitors Cocktail 1% NP-40
Glycoprotein Denaturing Buffer 10X	0.5% SDS 40 mM DTT
GlycoBuffer 2 Buffer 10X	50 mM Sodium Phosphate (pH 7.5 @ 25°C)

3.12 Kits

Kit	Manufacturer
artus® HCV RG RT-PCR Kit	Qiagen, Hilden, DE
Qiagen Plasmid Maxi Kit	Qiagen, Hilden, DE
QIAamp Viral RNA mini Kit	Qiagen, Hilden, DE
Maxima SYBR Green qPCR master mix	Thermo Fisher Scientific, Schwerte, DE
PNGase F Deglycosylation Kit	NEB, Frankfurt am Main, DE

3.13 Devices

3.13.1 PCR cyclers

PCR cycler	Manufacturer
LightCycler 480 Instrument II	Roche, Mannheim, DE
Rotor-Gene Q	Qiagen, Hilden, DE

3.13.2 Electrophoresis

System	Manufacturer
Horizontal electrophoresis system HE 33	BioRad, München, DE
Mighty small multiple gel caster SE 200	GE Healthcare, Freiburg, DE
Mighty small II vertical electrophoresis system SE 250	GE Healthcare, Freiburg, DE

Semiphor TE70 semi-dry transfer unit blotter	GE Healthcare, Freiburg, DE
Amersham ECL Semi-Dry Blotter	GE Healthcare, Freiburg, DE
Hoefer Electrophoresis power supply EPS 301	GE Healthcare, Freiburg, DE
Standard power pack P25	Biometra, Göttingen, DE
Horizontal electrophoresis system HE 33	BioRad, München, DE

3.13.3 Microscopy

System	Manufacturer
Confocal laser scanning microscope LSM 510	Zeiss, Jena, DE
Eclipse Ti	Nikon, Tokio, Japan

3.13.4 Imaging

Product	Manufacturer
INTAS imaging system (gel documentation)	Intas, Göttingen, DE
Odyssey imaging system	LI-COR, Bad Homburg, DE
AGFA Curix60 film developer	AGFA, Köln, DE

3.13.5 Centrifuges

Product	Manufacturer
Heraeus Multifuge 1S-R	Thermo Fisher Scientific, Schwerte, DE
Heraeus Fresco 17 Centrifuge	Thermo Fisher Scientific, Schwerte, DE
Heraeus Sepatech Cryofuge 8500	Thermo Fisher Scientific, Schwerte, DE
Optima L-80 XP Ultracentrifuge	Beckman Coulter, Krefeld, DE
Microcentrifuge	Carl-Roth, Karlsruhe, DE
Optima Max-up Ultracentrifuge	Beckman Coulter, Krefeld, DE
Heraeus Multifuge X3 FR	Thermo Fisher Scientific, Schwerte, DE

3.13.6 Other devices

Device	Manufacturer
Nanodrop One	Thermo Fisher Scientific, Schwerte, DE
Orion II microplate Luminometer	Berthold Sys., Bad Wildbad
Nanophotometer P300	Implen, München, DE
Stuart Roller Incubator SRT9	Bibby Scientific, UK
Incubator series B	Binder, Tuttlingen, DE
Innova® 44/44R Incubator Shaker	Eppendorf, Hamburg, DE
Sonopuls HD 2200	Bandelin GmbH, Berlin, DE
IKAMAG® universal hot plate magnetic stirrer	IKA, Staufen,DE
S20 - SevenEasy™ pH	Mettler Toledo, DE
Satorius balance LP 6000 200S	Satorius, Göttingen, DE
Satorius universal analytical balance	Satorius, Göttingen, DE
Rocking platform WT16	Biometra, Göttingen, DE
Thermomixer compact	Eppendorf, Hamburg, DE
Thermomixer 5436	Eppendorf, Hamburg, DE
Vortex®Genie 2	Scientific Industries, US
SterilGARD III Advance	The Baker Company, US
CO2-Incubator BBD 6220	Thermo Scientific, Karlsruhe, DE
Hemocytometer	Carl Roth, Karlsruhe, DE
Water bath1228-2F	VWR, Darmstadt, DE
Vacuum pump	Progen, Heidelberg, DE
Gene Pulser MXcell™	BioRad, US
Infinite M1000	Tecan, Männedorf, Switzerland

3.13.7 Relevant materials

Material	Manufacturer
Whatman filter papers	GE Healthcare, Freiburg, DE
Hybond-P, PVDF membrane	Millipore, Schwalbach, DE
Hyperfilm ECL	GE Healthcare, Freiburg, DE

Fixer type F 1–2	C & L GmbH, Planegg, DE
Developer type E 1–3	C & L GmbH, Planegg, DE
HypercassetteTM	GE Healthcare, Freiburg, DE
Falcon tubes (15 ml, 50 ml)	Greiner, Frickenhausen, DE
Graduated pipettes (1, 5, 10, 25 ml)	Greiner, Frickenhausen, DE
Pipette tips (10 µl, 100 µl, 1ml)	Sarstedt, Nümbrecht, DE
Filtered pipette tips (10 µl, 100 µl, 1ml)	4titude, Berlin, DE
Safe-lock micro test tubes (1.5ml, 2 ml)	Sarstedt, Nümbrecht, DE
Microscope slides	Carl Roth, Karlsruhe, DE
Coverslips	Carl Roth, Karlsruhe, DE
Cell scrapers	A.Hartenstein GmbH, Würzburg, DE
Cell culture flasks (T25, T75, T175)	Greiner, Frickenhausen, DE
Cell culture plates (6, 12, 24, 96 wells)	Greiner, Frickenhausen, DE
Phase lock gel heavy, 2 ml	5Prime, Hilden, DE
Electroporation cuvettes, 4mm	VWR, Darmstadt, DE
MF-MilliporeTM Membrane filters (0.025 µm)	Merck Millipore, Schwalbach, DE

3.13.8 Software

Software	Manufacturer
LightCycler 480 Software version 1.5	Roche, Mannheim, DE
Rotor-Gene Q 2.2.3.11	Qiagen, Hilden, DE
ZEN 2012	Zeiss, Jena, DE
Image Studio Lite	LI-COR, Bad Homburg, DE
GraphPad Prism	GraphPad, US
Citavi 6	Swiss Academic Software GmbH, CH
MS Office	Microsoft, US

4 Methods

4.1 Cell biology

4.1.1 Prokaryotic cell culture

E.coli bacterial strain *DH5α* cells were cultivated for 16 h (overnight) in 100 µg/ml ampicillin-containing LB medium (at 37 °C with constant shaking (200 rpm) in Erlenmeyer flasks). Frozen stocks were stored in 30 % (v/v) glycerol at -80.

4.1.2 Eukaryotic cell culture

In this study, the highly permissive HCV cured human hepatoma cell line Huh7.5.1 was used. The cells were grown in DMEM (4.5 g/l glucose) containing 10% FCS, 0.1 U/ml penicillin, 100 *µg*/ml streptomycin and 2 mM L-glutamine (DMEM complete) at 37°, 5% CO2 and ≥ 90% humidity. After lentiviral transduction for selection of stable cells overexpressing Nrf1 50 *µ*g/ml, Hygromycin was added to the medium. Passaging of adherent cells was performed using 1x trypsin/EDTA-solution for 2-5 min at 37°C. Prior to trypsinization cells were washed with PBS. The activity of trypsin was stopped by adding 7 ml DMEM complete. Cells were resuspended carefully and seeded in fresh medium at different dilutions (1:2, 1:10) depending on the cell line in order to obtain optimal growth. All experiments with infectious HCV were performed under safety level 3 (S3) conditions.

4.1.3 Electroporation of Huh7.5.1 Cells

Huh7.5.1 cells were harvested as described in (4.1.2) when they reached a confluence of 80-90 %. Cells were washed twice with ice-cold PBS and resuspended to a final concentration of 10 x10^6 cells/ml in PBS. Then, 800 µl cells suspension and 10 µg in vitro transcribed RNA (see 4.2.6) were mixed and added into a 4 mm cuvette. Cells were immediately electroporated by using Gene Pulser MXcell™ (BioRad, USA) that was adjusted to deliver one pulse at 300 V and 950 µF. After 10 min incubation at room temperature, the electroporated cells were diluted in 12 ml complete DMEM and seeded into appropriate culture plates. The medium was changed 4 h post electroporation.

4.1.4 Transfection of eukaryotic cells

4.1.4.1 Transfection of expression plasmids

For transfection, the linear polyethylenimine (PEI) (1 mg/ml) was used. Briefly, (0.5-1 µg/ml) plasmid-DNA, as well as 6 µl PEI, were separately re-suspended in 100 µl PBS each, afterward, they were mixed together (200µl in total) followed by vortexing for 15 sec. After 20 min incubation at room temperature, the transfection mix was added dropwise to 2 ml medium in a 6-well plate. 24 h post-transfection (with PEI), the medium was changed. 72 h after transfection (electroporation), cell lysate was harvested or treatment followed by cell lysate harvesting takes place. Where gene expression was analyzed by Western blot, qRT-PCR, and immunofluorescence microscopy.

4.1.5 Infection of primary human hepatocytes

PHHs were received from Hanover University Hospital in either a 6 or 12 -well plate.1-2 ml supernatant derived from HCV JC1-replicating Huh7.5.1 cells was added to one well and incubated for 72 h. Afterward, cells were washed once with PBS and harvested, total RNA was isolated (see chapter 5.2.7) and analyzed by qRT-PCR (see chapter 4.2.10).

4.1.5.1 Protein Lysates

For Western blot analysis, total cell lysates from Huh7.5.1 cells were used. Therefore cells were washed once with PBS and lysed by adding 150 *µ*l RIPA buffer supplemented with protease inhibitors (see chapter 3.9.1). After 10 min incubation on ice, lysed cells were scraped from the wells, transferred to Eppendorf tubes, sonicated and centrifuged for 5 min at full speed at 4°C. The supernatant was subjected to protein quantification (see chapter 4.3.1).

4.1.5.2 Luciferase Lysates

For luciferase reporter-gene assay, cells were lysed 72 h pe. After that cells were washed once with PBS and lysed by adding 250 *µ*l luciferase lysis buffer. After 10 min incubation on ice, lysed cells were scraped from the wells, transferred to Eppendorf tubes and centrifuged for 10 min at 13.000 rpm at 4°C. The supernatant was subjected

to protein quantification (see chapter 4.3.1) and luciferase reporter gene assay (see chapter 4.1.6.3).

4.1.6 Luciferase Reporter Gene Assay

To monitor the LxR activity in HCV +ve & -ve replicating Huh7.5.1 cells, cells were first electroporated with the JC1 & GND construct, after 24hrs cells were transfected with a pGF1-LXRE plasmid that is coupled to the firefly luciferase (*Phontinus pyralis*). Lysates (50 µl) were analyzed by the automated addition of 50 µl luciferase substrate and subsequent determination of chemiluminescence by an Orion II microplate Luminometer. Measurements were carried out at in triplicate. Luciferase levels (RLUs) were referred to the values of a Bradford assay that was performed with the same lysates for each luciferase assay.

4.1.7 Cell Viability Assay

To investigate the cytotoxic effects of a substance, the PrestoBlue cell viability assay was performed. For this purpose, 10,000 cells per well were seeded in a 96-well microtiter plate and the corresponding experiment carried out (transfection, treatment). At the time the experiment was stopped, the cells were incubated for 1 hour at 37 ° C with DMEM complete supplemented with 10% PrestoBlue. The fluorescence of the plate was then read in the Tecan Reader (excitation wavelength: 560 nm, 10 nm bandwidth, and emission wavelength: 590 nm, 10 nm bandwidth). Lower fluorescence correlates with the lower metabolic activity which means more cytotoxicity.

4.2 Molecular biology

4.2.1 Agarose Gel Electrophoresis

This method is used to analyze DNA-plasmid and RNA *in vitro* transcripts. Depending on the size of the fragments 0.75-2% (w/v) agarose (Genaxxon, Biberach) was resuspended in 1x TAE and dissolved by heating. After cooling the solution to be hand warm, 0.1 µg/ml ethidium bromide was added and the liquid agarose was poured into a horizontal gel chamber that was stuffed with a comb. Once the gel has become solid, it was given into a gel chamber containing 1x TAE. The DNA or RNA samples were supplemented with a 6x loading buffer and separated at 100 Volt by electrophoresis. DNA or RNA bands were visualized by the means of UV light (254/365 nm) and documented with an INTAS-imaging system.

4.2.2 Determination of Nucleic Acid Concentration

The determination of nucleic acids was done spectrophotometrically by using a Nanodrop One (Thermo Fisher Scientific). Nucleic acids can be quantified by measuring the absorbance at λ = 260 nm due to their aromatic groups. At this wavelength, an absorbance/extinction of 1.0 corresponds to a concentration of 50 µg/ml double-stranded DNA or 40 µg/ml RNA. The purity of DNA/RNA preparations was assayed by measuring the absorbance at λ = 280 nm at the same time. Pure samples should obtain an OD260/OD280 ratio of 1.8-2.0 and OD 260/ OD 230 ca. 2.0–2.2. Lower values indicate a protein or phenol contamination.

4.2.3 Isolation of Plasmid DNA

Plasmid DNA isolation was performed according to the manufacturer's protocol of QIAGEN Plasmid Maxi Kit. Plasmid DNA was extracted from 500−1000 ml bacterial culture for maxi scale isolation. The final purification of the DNA involves the precipitation by the means of isopropanol. The precipitate was washed and air-dried before being resuspended in ddH2O and stored at -20 °C.

4.2.4 Restriction Endonuclease Digestion

This method was used to linearize circular plasmids containing HCV genomes. Therefore, 15*µ*g pFK-JFH1/J6 were linearized using *AseI* and pUC-JFH1_GND using the *Xba*I restriction enzyme. DNA was digested according to the enzyme requirements listed by the manufacturer.

4.2.5 Phenol-/Chloroform Extraction of Nucleic Acids

Phenol-/Chloroform extraction is performed to extract nucleic acids from solutions containing proteins, especially after enzymatic reactions. Therefore, the aqueous solution was mixed with 1/10 volume 3 M sodium acetate (pH 5.2) and one volume phenol and centrifuged for 5 min at 13.000 rpm/4°C. The aqueous solution was transferred into a prepared *Phase lock* tube and mixed with one volume chloroform: isoamyl alcohol (24:1). After additional centrifugation for 5 min at 13.000 rpm/4°C the aqueous phase was collected in a new reaction tube. The nucleic acids were

precipitated by adding 2.5 volumes ice-cold absolute ethanol (or 0.8 volumes absolute isopropanol) and centrifugation for 30 min at 13.000 rpm/4°C.

4.2.6 In vitro T7-Transcription

In vitro T7 transcription was used to generate HCV RNA genomes from linearized DNA plasmids containing a T7 promotor for electroporation purposes. The reaction (Tabel 4.1) was incubated for 2 h at 42 °C. Afterward, 1 µl RNase-Free DNaseI (1U/ µl) was added to digest the input DNA with 15 min incubation at 37 °C. After phenol-chloroform extraction (see chapter 5.2.5), the purified transcribed RNA was resuspended in DEPC water adjusted to (1µg/µl) and frozen at 10 µg aliquots at -80 °C.

Tabel 4.1: T7 transcription

T7 transcription reaction mixture Reagent	Amount
RNase-Free Water	x µl
Linearized template DNA with T7-RNAP promotor	4 µg
10x T7-Scribe Transcription buffer	2 µl
100 mM ATP	1.5 µl
100 mM CTP	1.5 µl
100 mM UTP	1.5 µl
100 mM GTP	1.5 µl
100 mM DTT	2 µl
ScriptGuard RNase Inhibitor	0.5 µl
T7-Scribe Enzyme Solution	2 µl
Total Reaction volume	20 µl

4.2.7 RNA isolation

Total RNA was isolated using peqGOLD TriFast according to the manufacturer's protocol. RNA was resuspended in DEPC water and the quality was controlled by running an agarose gel.

4.2.8 cDNA synthesis

5 µg total RNA was digested by DNaseI in a final volume of 10 µl to remove the remaining DNA. The reaction was incubated at 37 °C for 1 h, and the activity of DNase was stopped by adding 1 µl stop solution and incubate at 65 °C for 10 min. cDNA synthesis was performed with RevertAid H Minus reverse transcriptase according to the manufacturer's protocol using random hexamer primer.

4.2.9 HCV genome isolation from the cell culture supernatant

The QIAamp Viral RNA Mini Kit was used for the isolation and purification of viral nucleic acids from cell culture supernatant according to the manufacturer's instructions. Isolated HCV genomes IU were determined by Rotor-Gene RT-PCR assay on (see chapter 5.2.11).

4.2.10 Quantitative reverse transcription PCR (qRT-PCR)

The specific RNA was quantified by LightCycler 480 system using Maxima SYBR Green qPCR-Kit. The quantification is based on the intercalation of the DNA-binding fluorescent dye SYBR Green to double-stranded DNA during PCR reaction. The increasing amount of amplified DNA leads to an increased fluorescence intensity, which was measured after each cycle. The PCR reaction was prepared according to the manufacturer's protocol. In brief, the reaction mix contained 5 µl 2x Maxima SYBR Green qPCR Master Mix, 3 µl cDNA template synthesized from total RNA, and 0.25 µl of each primer (10 µM) and was filled up to 10 µl with ultrapure ddH2O. The relative amount of specific RNA was determined by referring to the housekeeping gene Ribosomal Protein L27 (RPL27) and control without reverse transcriptase was used. The qRT-PCR program is described in Tabel 4.2.

Tabel 4.2: qRT-PCR program

Program	Temperature (°C)	Hold time (sec)	Slope (°C/sec)	Cycles
denaturating	95	600	20	1
cycling	95	15	20	45
	56	30	20	
	72	30	5	
melting	95	60	20	1
	60	30	20	
	95	0	0.1	
cooling	40	30	20	

4.2.11 Quantification of HCV genomes by RT-PCR

Quantitation of HCV genomes from cell culture supernatant was done with the artus HCV RG RT-PCR Kit on the Rotor-Gene Q instrument according to the manufacturer's protocol. In brief, 10 µl of the eluted sample RNA was added to the master mix (6 µl Hep. C Virus RG Master A and 9 µl Master B) for RT-PCR on Rotor-Gene. Correspondingly, the quantitation standards (Hep. C Virus RG QS 1–4) were detected. The Hep. C Virus RG Master A and B contain reagents and enzymes for the reverse transcription and specific amplification of a 240 bp region of the HCV genome and for the direct detection of the specific amplicon in fluorescence channel Cycling Green of the Rotor-Gene Q. The test can quantitate HCV RNA over the range of 65–1 x 10^6 HCV IU/ml.

4.3 Protein Biochemistry

4.3.1 Protein quantification by Bradford assay

The protein amount in cellular lysates was quantified with Bradford reagent which shifts the light absorption maximum from 465 nm to 595 nm after the binding of proteins to the dye Coomassie Brilliant Blue G-250. Protein quantification was performed according to the manufacturer's instructions. In brief, 5 µl cellular lysate was mixed with 100 µl Bradford reagent and the absorption was measured with Tecan reader (Infinite M1000).

4.3.2 Half-life determination

Huh7.5.1 Cells were treated with 142 µg/ml cycloheximide (CHX) to inhibit protein synthesis and harvested (0, 30, 60, 120 & 240 min). Untreated cells served as control. Cellular lysates from different time points (0−4 h) were analyzed by Western blot and the half-life was calculated from the non-linear regression equation.

4.3.3 SDS-PAGE electrophoresis

SDS-PAGE electrophoresis was used to separate a protein mixture depending on the molecular weight (MW) of proteins (Laemmli 1970). The gel is composed of a stacking gel and a separating gel. The polymer density of the stacking gel is 4 %, while the density of the separating gel varied due to the MW of target proteins (8−14 %). The

composition of gels is summarized in Tabel 4.3. Equal volumes of the sample lysate (150µl) were denatured in a 1x SDS-PAGE sample buffer by heating for 10 min at 95 °C and separated at 80−120 V.

Tabel 4.3: Composition of the stacking and separating gels

Stacking gel	4 %	Separating gel	**8** %	12 %
Stacking gel buffer (4x)	15 ml	Separating gel buffer (4x)	20 ml	20 ml
Rotiphorese 40 (29:1)	6 ml	Rotiphorese 40 (29:1)	16 ml	20 ml
H2O	45 ml	H2O	44 ml	40 ml
TEMED	60 µl	TEMED	80 µl	80 µl
APS (10 %)	600 µl	APS (10 %)	800 µl	800 µl

4.3.4 Western blot

Proteins separated by SDS-PAGE were transferred onto a methanol-pretreated PVDF membrane in an electric field of 1.3 mA/cm² for 1 h, using a discontinuous buffer system and a semi-dry blotting chamber (Towbin et al. 1979). After blotting, the membrane was incubated with 10 % (v/v) Roti in dH2O for 1 h to block unspecific binding of antibodies. Then, the membrane was incubated with primary antibody diluted in blocking buffer overnight at 4 °C. After washing the excess unbound primary antibody with 1x TBST buffer, the membrane was incubated with a fluorescent-labeled secondary antibody for an additional hour at room temperature. The unbound antibody was removed by washing with a 1x TBST buffer. Finally, proteins were detected by the Odyssey imaging system.

4.3.5 Nuclear Fractionation

To detect the location of the Nrf1 isoforms inside the cells whether it is in the cytosolic or nucleosolic fractions, nuclear fractionation was performed. Briefly, Huh7.5.1 cells from at least 3wells of a 6 wp format were washed 1x with PBS. Cells were harvested using lysis buffer-A (see chapter 3.11) on ice after scraping in one Eppendorf tube. Centrifugation at 3.000 rpm at 4°C for 20min. Supernatant represent the Cytosolic fraction. Pellet is washed a couple of times and re-suspended in lysis buffer-B. Homogenization with 20 strokes in a Douncer on ice followed by ice incubation for 30 min. Centrifugation at 55.000 rpm for 20 min. Supernatant represents the nucleosolic

fraction. Discard the pellet. Both fractions were prepared for SDS-PAGE electrophoresis followed by Western Blot (see chapters 4.3.3 & 4.3.4).

4.3.6 Deglycosylation assay

To study the degree of glycosylation of a protein of interest PNGase F deglycosylation assay was carried out. Briefly under denaturing-reaction condition cells from a single well of a 6 wp format in a 100µl lysis buffer. 1-20µg of glycoprotein approximately (9µl) in duplicates were taken. One without the deglycosylation enzyme “as a control” and the other with the enzyme. Add 1µl of Glycoprotein Denaturing Buffer (10X) (see chapter 3.11) and H2O if necessary to make a 10 µl total reaction volume. Afterward, glycoproteins were denatured by heating at 100°C for 10 min, followed by incubation on ice. Centrifugation at maximum speed for 10 seconds. A total reaction volume of 20µl by adding 2µl GlycoBuffer-2 (10X) (see chapter 3.11), 2µl of 10% NP-40 and 6µl H2O was prepared. 1µl of PNGase F enzyme was added to the sample followed by gentle mixing. Incubation for 1hr. Finally, the sample together with the control is prepared for SDS-PAGE and Western Blot (see chapters 4.3.3 & 4.3.4).

4.4 Immunological Methods

4.4.1 Indirect Immunofluorescence Microscopy

Immunostaining was performed with fluorescent-labeled antibodies in order to analyze the distribution of proteins and also intracellular localization. Cells were grown on 15 mm coverslips in a 12-well plate were washed once with PBS and fixed with 4% formaldehyde for 20 min at room temperature. After washing with PBS twice, coverslips were blocked for 1 h with 1% BSA in PBS to prevent unspecific binding of the antibodies. Then cells were incubated with specific primary antibodies in a humid chamber for 1h and washed 3 times with PBS to remove unbound antibodies. Afterward, cells were incubated with fluorescent-labeled secondary antibodies, nuclei stain DAPI (4,6- diamidino-2-phenylindole) solution (1µg/µl) and BODIPY 493/503 (1:100 dilution in PBS) for lipid droplets staining all in diluted in the blocking buffer also in a humid dark chamber for 1 h. Finally, the coverslip was sealed with Mowiol after being washed 3 times with PBS. Immunofluorescence staining was analyzed by a confocal laser scanning microscope (see chapter 4.5.1).

4.5 Microscopy

4.5.1 Confocal laser scanning microscopy (CLSM)

CLSM was used to detect fluorescent-labeled proteins in a defined layer within a cell. The analysis was performed using an LSM 510 microscope and LSM image browser software. 40x and 100x objectives were used.

4.6 Statistical analyses

Results are described as mean ± SEM. The bars represent the standard error of the mean (SEM). The significance of results was analyzed by ratio t test using GraphPad Prism 8. (ns: $p > 0,05$; *: $0,05 \geq p \geq 0,01$; **: $0,01 \geq p \geq 0,001$; ***: $p < 0,001$).

5 Results

5.1 Effect of Hepatitis C virus on Nrf1

We wanted to study the effect of HCV on the Nrf1 protein amount and subcellular localization. To do so, Western Blot analysis and confocal immunofluorescence staining were carried out.

First of all, cellular lysates from a 6 well cell culture plate 72hrs post electroporation of Huh7.5.1 cells with JC1 (J6/JFH1) and the corresponding negative control (JFH1/GND) were harvested for the Western blot to measure the Nrf1 protein amount (Fig.15c). Also, cells were grown on coverslips and later fixed for the immunofluorescence staining to analyze the Nrf1 localization (Fig.15c).

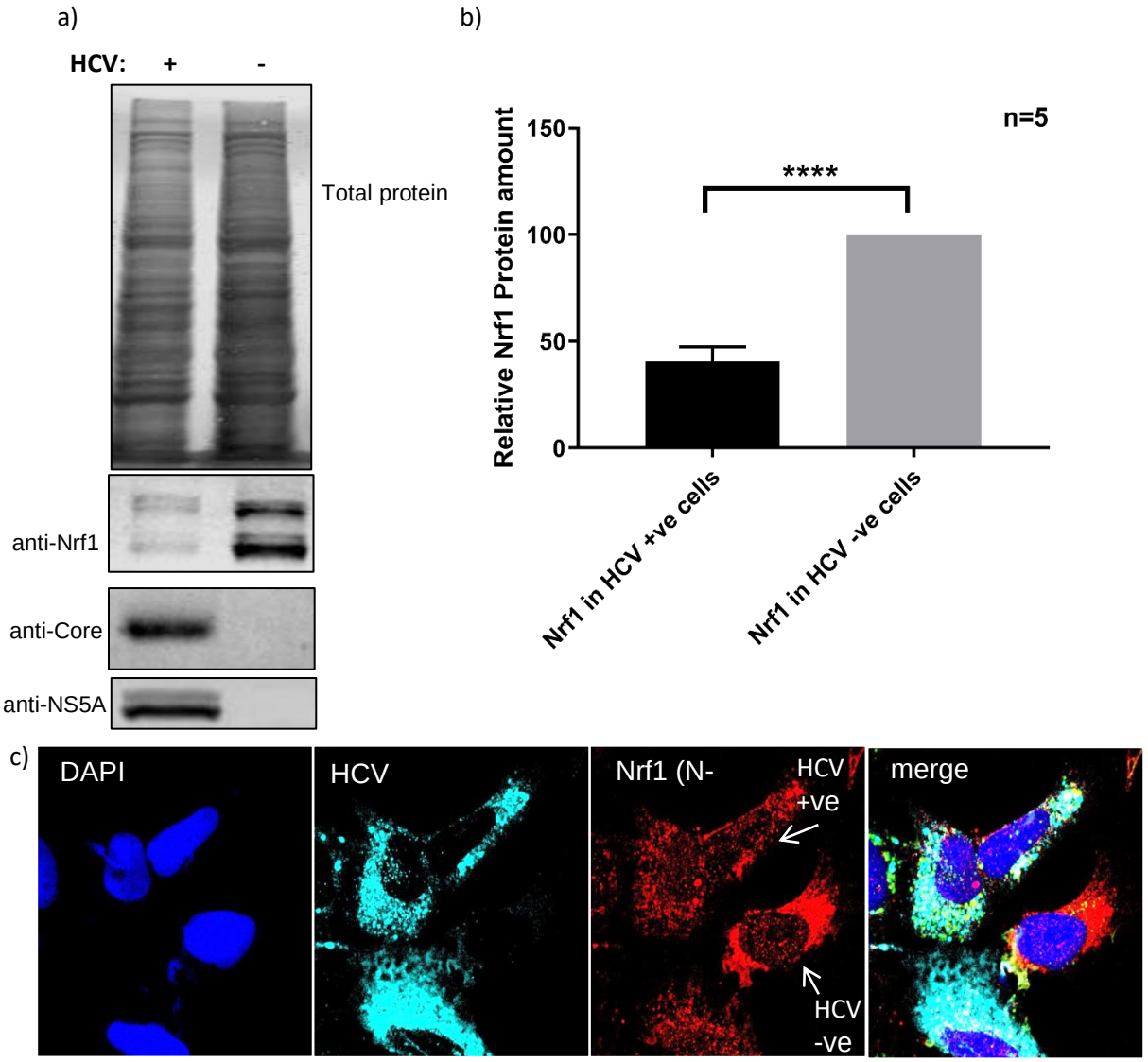


Figure 15: a reduction in the Nrf1 protein levels in the Hepatitis C virus transfected Huh7.5.1 cells.

a) 10x 10^6 Huh7.5.1 cells were electroporated with 10 µg RNA of the replicon JFH1/J6 or the replication-deficient JFH1/GND construct (negative control), harvested after 72hrs and analyzed by Western blot. For detection Nrf1, NS5A-, and core-specific antibodies were used; as a reference, total protein stain was used. **b)** The graph shows the quantification of the Nrf1-specific signal referred to the total protein in the lysate. **c)** Confocal immunofluorescence analysis showing that the HCV (turquoise) indeed reduces the Nrf1 (red) intracellular protein levels.

It was observed that a significant reduction in the Nrf1 protein levels was detected in the HCV replicating Huh7.5.1 cells compared to HCV non-replicating ones.

At the same time, it was also observed in the immunofluorescence staining that in the HCV replicating Huh7.5.1 cells, the Nrf1 subcellular distribution and localization were different from the non-replicating ones. In the HCV +ve cells there was a cytoplasmic distribution, while in the HCV -ve cells it was localized around the nucleus.

Furthermore, to confirm the effect of HCV on the Nrf1 protein levels, we tested it in a lentiviral transduction-mediated stable Huh7.5.1 cell overexpressing Nrf1 (Fig.16) and we can see the same reducing effect.

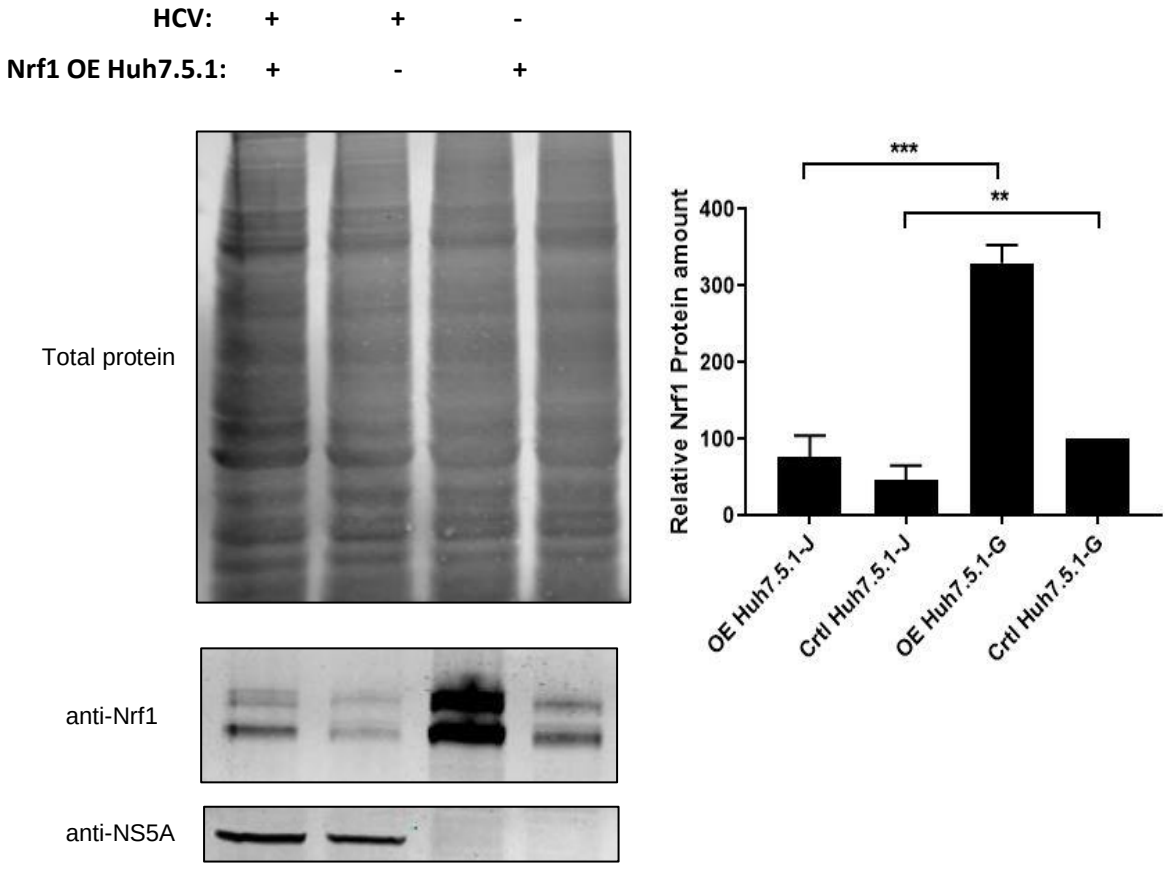


Figure 16: A reduction in the Nrf1 protein levels in the Hepatitis C virus transfected Huh7.5.1 cells as well as in Nrf1-overexpression Huh7.5.1 cells. 10x10^6 Huh7.5.1 cells and Nrf1 overexpressed Huh7.5.1 cells were electroporated with 10 µg RNA of the replicon JFH1/J6 or the replication-deficient JFH1/GND construct (negative control), harvested after 72hrs and analyzed by Western blot. For detection, Nrf1 and NS5A-specific antibodies were used; as a reference, total protein stain was used.

In light of this, it can be concluded that HCV indeed reduces Nrf1 protein amount and affects its subcellular localization.

In line with this, to further study the effect of HCV on the expression of the Nrf1 gene, total RNA was isolated from HCV-positive and the corresponding control cells. After reverse transcription to cDNA, the amount of Nrf1-specific transcripts was quantified by real-time (RT) PCR using Nrf1 specific oligonucleotides (# 1400, # 1401). All Nrf1 levels were referenced to the house-keeping gene 60S ribosomal protein L27 (RPL27) using RPL27 specific oligonucleotides (# 835, # 836). The RT-PCR shows that in HCV-replicating cells Nrf1 gene expression was significantly increased compared to HCV negative cells (Fig. 17).

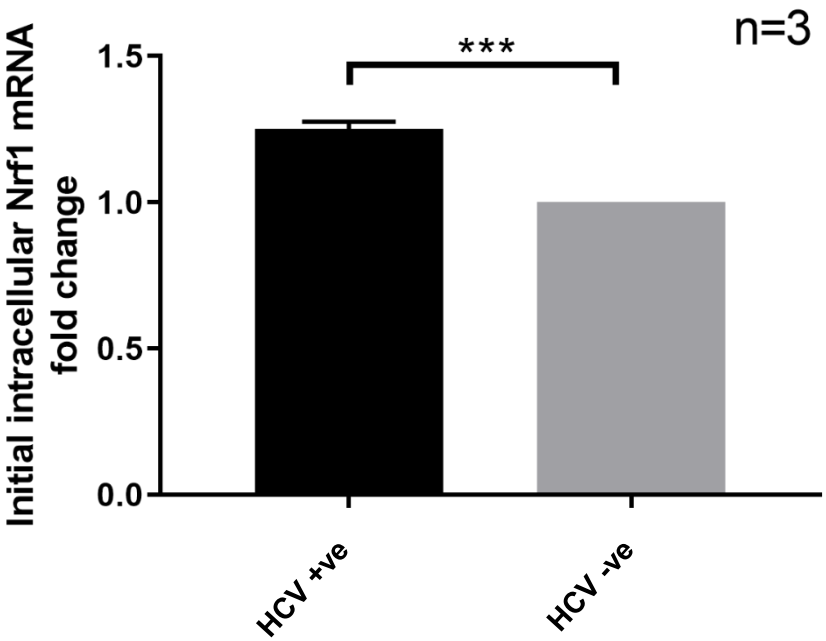


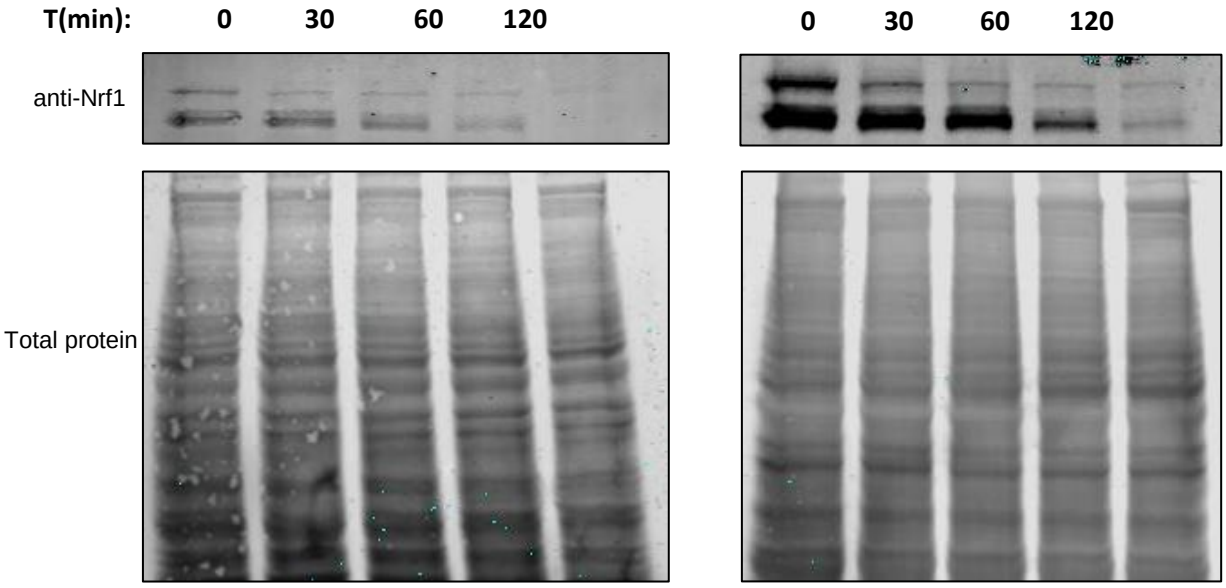
Figure 17: An increase in the expression of Nrf1 in HCV positive Huh7.5.1 cells.

Quantification of Nrf1-specific mRNA by RT-PCR. Total RNA was isolated from HCV-replicating Huh7.5.1 cells transfected with JFH1/J6 replicon 72 h after electroporation. JFH1/GND served as a control. The levels of Nrf1-specific transcripts are referenced to the RPL27. The graphs represent the mean value of three independent experiments.

In light of this, these data indicate that in HCV-replicating cells the intracellular protein levels of Nrf1 are decreased compared to in HCV-negative control cells, while on the other hand the level of Nrf1 mRNA is increased.

This suggests that the increased amount of Nrf1 gene is for the sake of compensating the reduction in the Nrf1 protein levels.

Furthermore, Cycloheximide treatment whose function is to inhibit the De novo protein synthesis, enabled us to measure the Half-life of the Nrf1 protein (Fig. 18). We were able to detect that in HCV replicating cells the half-life of the intracellular Nrf1 protein (t1/2=242.1min) was decreased by almost 3-fold compared to the HCV negative Huh7.5.1 cells (t1/2=631,3min).



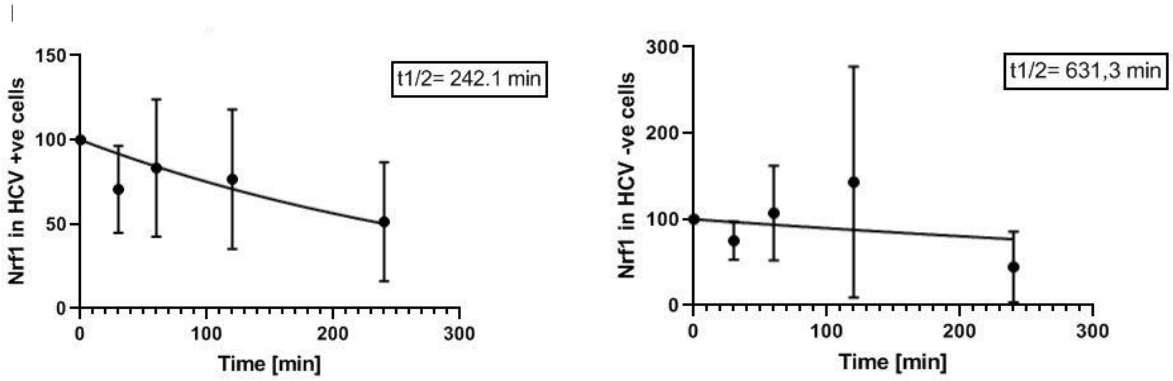
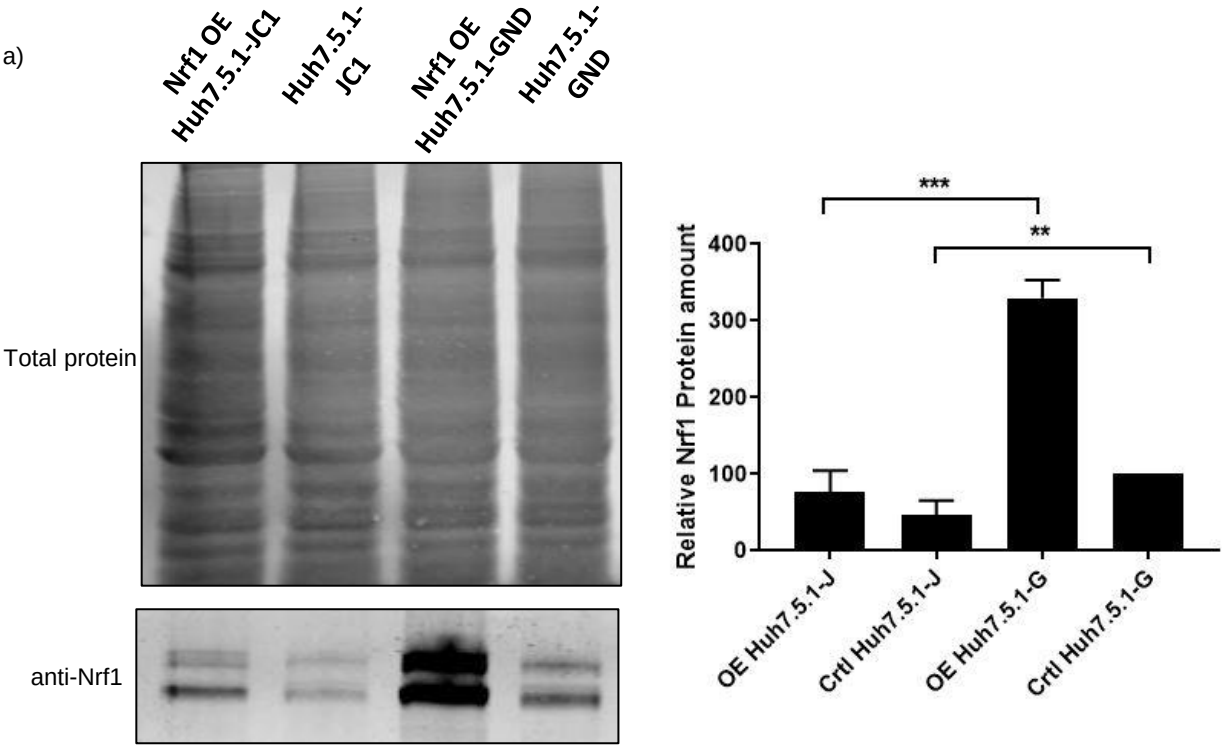


Figure 18: The half-life of Nrf1 protein in HCV positive and negative Huh7.5.1 cells. 10x10^6 Huh7.5.1 cells were electroporated with 10 µg RNA of the replicon JFH1/J6 or the replication-deficient JFH1/GND construct (negative control). 72 hrs post electroporation cells were treated with 142 µM for different time intervals (0, 30, 60, 120, and 240min). At each time point, cells were washed with PBS, harvested and analyzed by means of Western blot. For detection Nrf1 -specific antibody was used; as a reference, total protein stain was used.

5.2 Effect of Nrf1 overexpression on Hepatitis C virus

Furthermore, we wanted to study and see if there is any effect of Nrf1 on HCV. To do so, lentiviral transduction-mediated stable Huh7.5.1 cells overexpressing Nrf1 were transfected also by means of electroporation with JC1 as well as GND as the negative control. A significant increase in the HCV core protein levels can be seen compared to normal Huh7.5.1 cells, by means of Western blot and confocal immunofluorescence staining (Fig. 19).



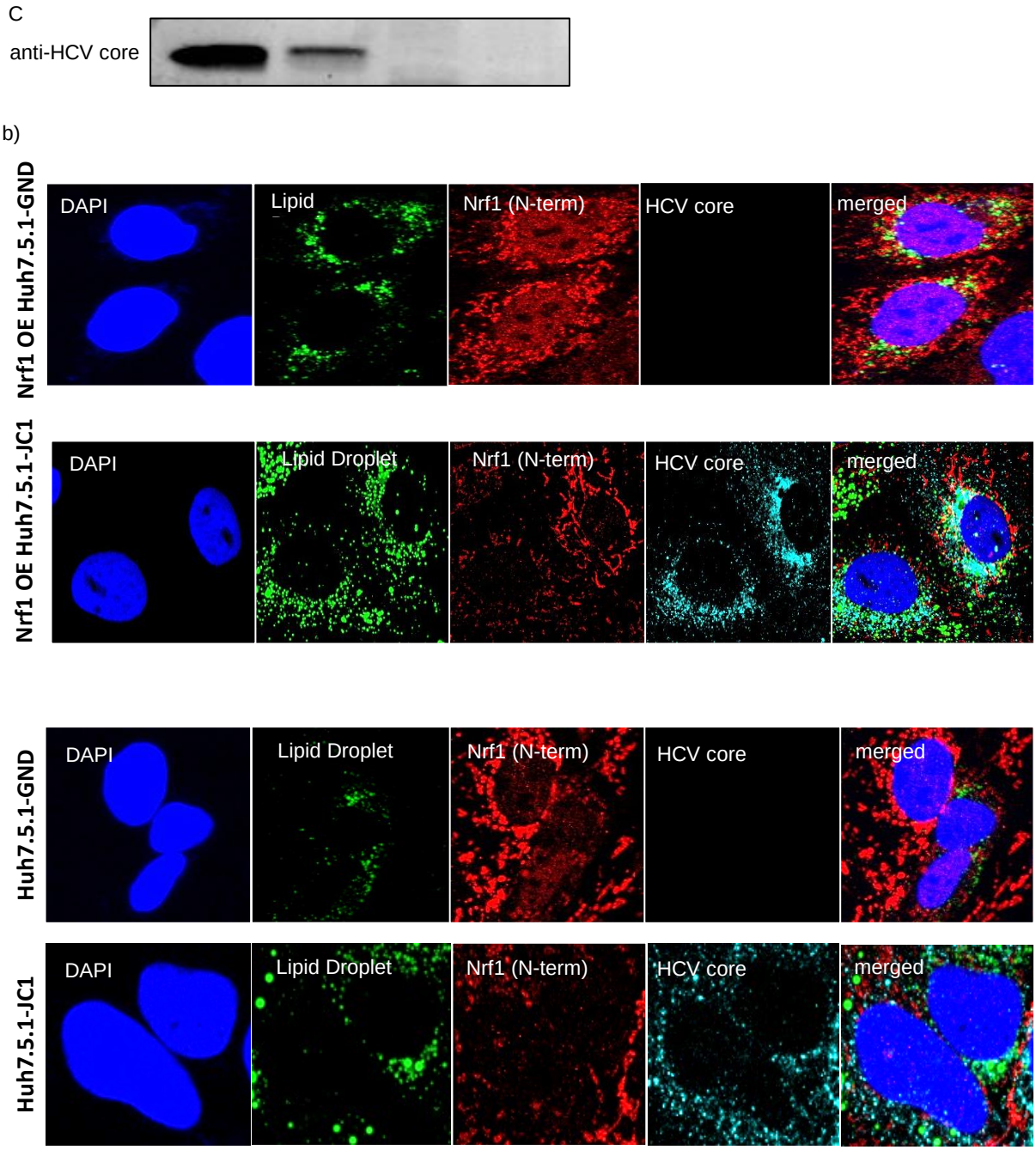


Figure 19: a) Western blot & b) Confocal immunofluorescence microscopy of HCV-replicating Nrf1-OE Huh7.5.1 cells vs HCV-replicating Huh7.5.1.

a) Western blot analysis of cellular lysates and normalization. HCV replicating Nrf1-OE and normal Huh7.5.1 cells were generated by electroporation with 10 μg RNA of the replicon JC1. 72h pe, the cells were lysed and the lysate was analyzed by Western blot using Nrf1-, and core-specific antisera, all referenced to the total protein from two separate experiments. b) Using an HCV core-specific antiserum (turquoise) and an Nrf1-specific monoclonal antibody (red). Lipid droplets (LDs) were visualized by BODIPY 498/503 (green), nuclei by DAPI (blue).

In light of this, we can conclude that overexpressing the cellular Nrf1 indeed helps and favors the HCV intracellular existence and propagation.

Furthermore, after looking for the Nrf1 overexpression effect on the HCV core protein levels, we wanted to see if there was also an effect from overexpression Nrf1 on the HCV genome amount. We were able to observe a significant increase in the HCV mRNA amount after overexpressing the Nrf1 (Fig. 20).

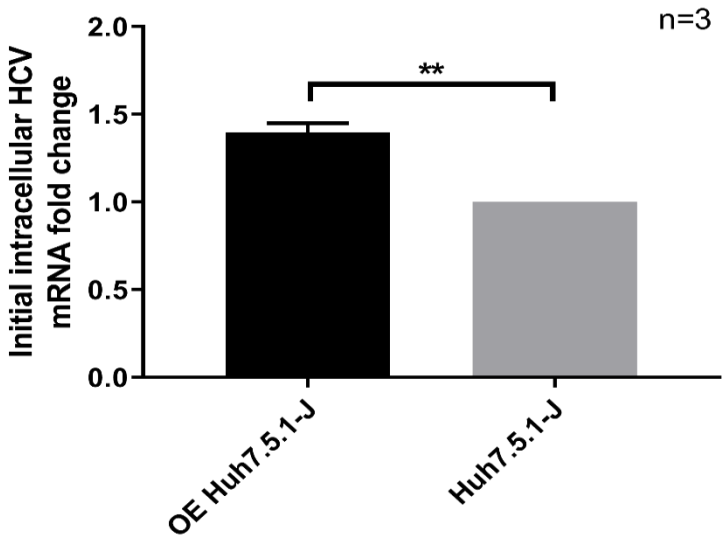


Figure 20: Overexpressing of Nrf1 leads to an increase in the HCV genome compared to normal Huh7.5.1 cells.

10x10^6 Huh7.5.1 and Nrf1-OE Huh7.5.1 cells were electroporated with 10 μg RNA of the replicon JFH1/J6 and seeded in 6 wp. Total RNA was isolated 72 h pt and the HCV-specific RNA was quantified by rtPCR. As a reference, the amount of RPL27-specific transcripts was determined.

In line with the observation of the induced effect of Nrf1 overexpression on the intracellular HCV genomes (Fig. 20), we wanted to see, how the Nrf1 overexpression affects the extracellular genomes as well. This was achieved by isolating RNA 72 hrs pt from the media from which the viral genome amount was quantified using qPCR. We were able to observe a decrease in the egression of the HCV genome, which could reflect a block in the release process (Fig. 21).

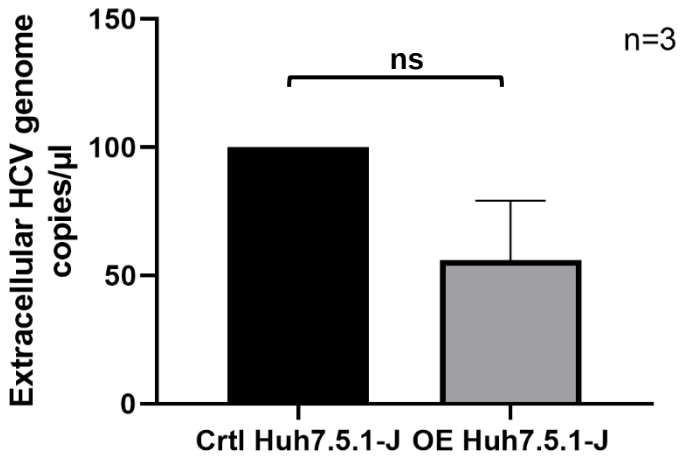


Figure 21: Nrf1 overexpression reduces the HCV genome released compared to the HCV released from the normal HCV-replicating Huh7.5.1 cells.

10x10^6 Huh7.5.1 and Nrf1-OE Huh7.5.1 cells were electroporated with 10 *µ*g RNA of the replicon JFH1/J6 and then seeded in a 6 well plate. Total RNA was isolated 72 h pt from the media and examined by Rotor gene qPCR for the HCV copies/µl released.

Meanwhile, we wanted also to look at other effects that could result from overexpressing the Nrf1. One of these were the effect of Nrf1 overexpression on the liver-X receptor factor (LxR) (responsible for the initiation of the cholesterol removal program) target gene; ATP-binding cassette transporter (ABCA1) and sterol regulatory element-binding protein 2 (SREBP2) (responsible for the initiation of the cholesterol production program) target genes; 3-Hydroxy-3-Methylglutaryl-CoA Synthase 1 (HMGCS1), HMG-CoA reductase (HMGCR), & low-density lipoprotein receptor (LDLR) in HCV replicating and non-replicating Huh7.5.1 cells. From two independent experiments it can be observed that the Hepatitis C virus was able to decrease the fold change of these target genes. But when the Nrf1 was overexpressed, we can see that this overexpression overcame this virus-mediated decrease. In another meaning, there was an increase in these target genes amount (Fig. 22).

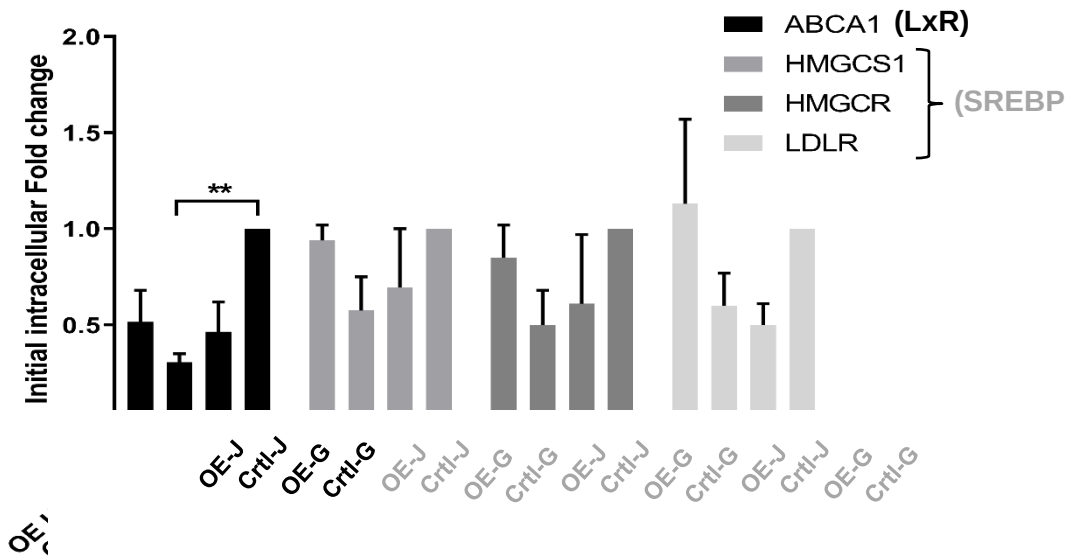


Figure 22: Nrf1 overexpression was able to reverse the HCV mediated reduction in the LxR and SREBP2 target genes compared to normal Huh7.5.1 cells

10x 10^6 Huh7.5.1 and Nrf1-OE Huh7.5.1 cells were electroporated with 10 μg RNA of the replicon JFH1/J6 & JFH1/GND and then seeded in a 6 well plate. Total RNA was isolated 72 h pt and the LXR (and SREBP2 target genes specific RNA was quantified by real-time PCR (rtPCR). As a reference, the amount of Ribosomal Protein L27 (RPL27) -specific transcripts was determined in two separate experiments.

In line with this, we wanted to see if the viral-mediated reduction effect on for example the ABCA1 was perhaps due to the viral effect on the liver-x receptor that targets the ABCA1 and other genes. To do so, luciferase assay using the LxR reporter construct was carried out, where LxR is a cellular factor controlling the cellular cholesterol removal program and it is according to (Widenmaier et al. 2017) regulated by the Nrf1.

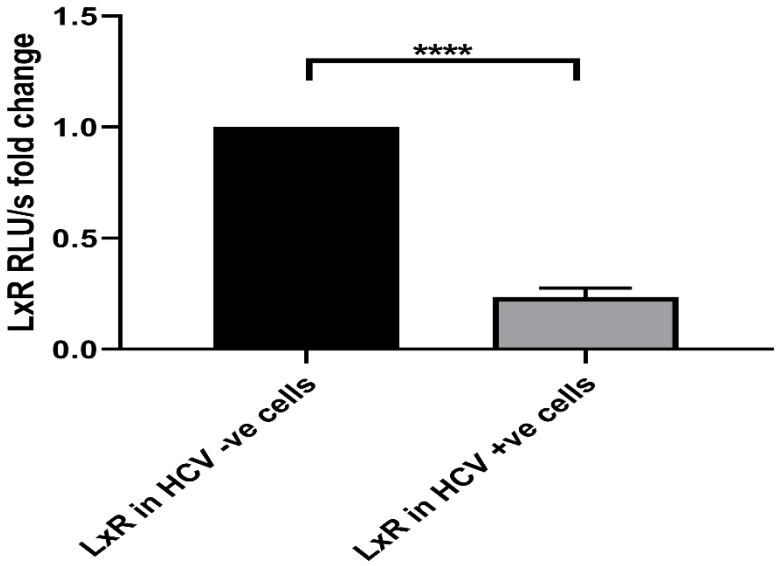


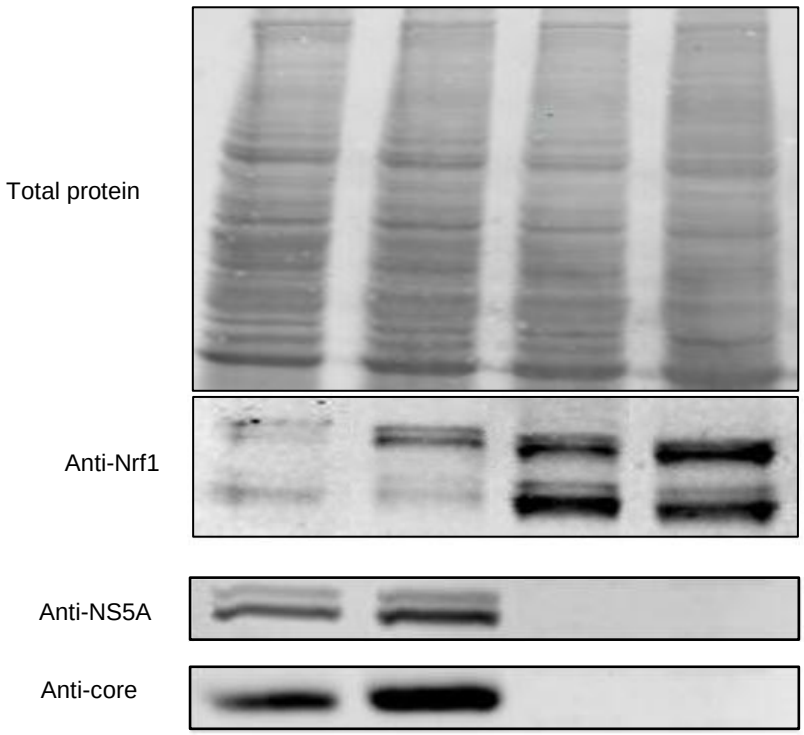
Figure 23: Luciferase assay carried out using the LxR reporter construct that was introduced via polyethylenimine (PEI) to the already (JC1 & GND) transfected Huh7.5.1 cells.

We were able to clearly observe that the LxR transcription rate was significantly reduced in HCV replicating Huh7.5.1 cells in comparison with the HCV negative ones (Fig. 23). This indeed reflects on the fact that cholesterol is important for the HCV infection, because, as mentioned earlier, that the LxR is responsible for initiating the cellular cholesterol removal program.

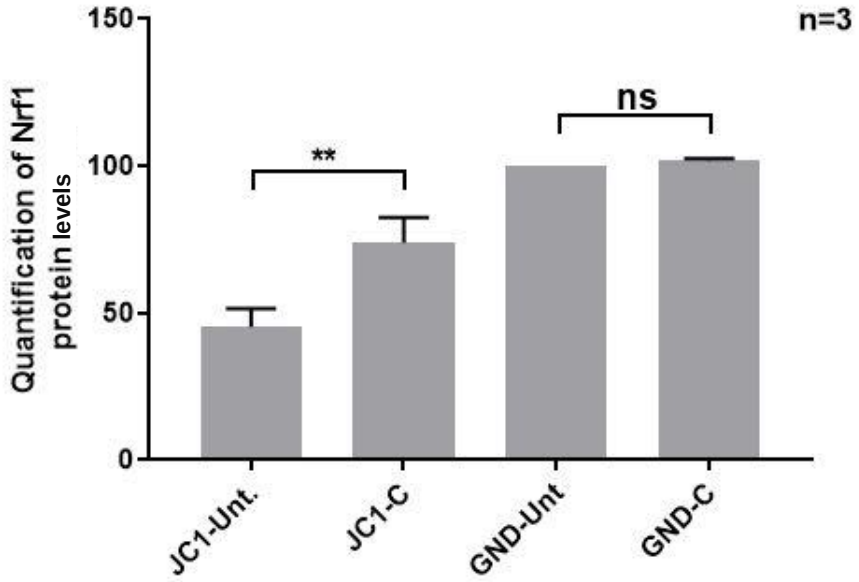
5.3 Effect of high cholesterol on Nrf1 and Hepatitis C virus

To study the relevance of high cholesterol to the HCV life cycle and Nrf1, intracellular cholesterol was increased through the treatment of Huh7.5.1 cells with a complex. This complex is composed of only cholesterol loaded on methyl beta cyclodextrin mβCD that acts as a carrier to deliver the cholesterol into the cells. This was evaluated with Western blot (Fig. 24) and confocal immunofluorescence (Fig. 25).

a)


HCV + + - -
Complex - + - +
Total protein
Anti-Nrf1
Anti-NS5A
Anti-core

b)


n=3
Quantification of Nrf1 protein levels
150
100
50
0
**
ns
JC1-Unt.
JC1-C
GND-Unt
GND-C

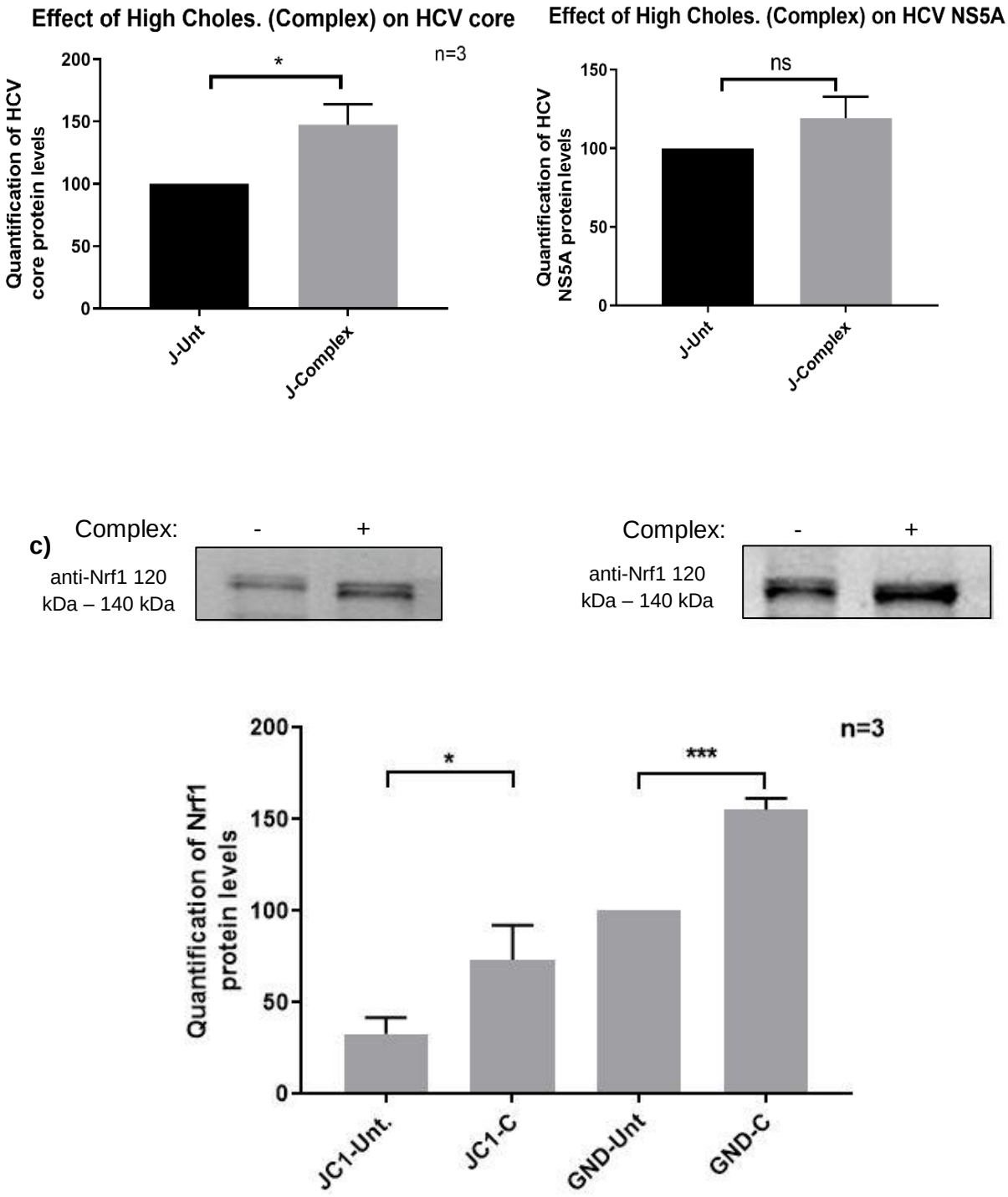


Figure 24: An increase in the Nrf1 protein levels as well as in the HCV core upon increasing the intracellular cholesterol.

Huh7.5.1 cells were electroporated with JC1 and GND constructs and seeded in 6 well plates. 72hrs post electroporation cells were treated with the cholesterol (100µM/ml) in the form of the complex compound for 4hrs. **a)** lysates were collected and analyzed with Western blot using Nrf1-, core- and NS5A anti-sera **b)** showing the quantification of the Nrf1, core and NS5A signal after normalization to the total protein of the lysate. Confocal immunofluorescence staining of Huh7.5.1 cells **c)** showing the effect of cholesterol on Nrf1 full-length in HCV replicating Huh7.5.1 cells compared to non-HCV replicating ones

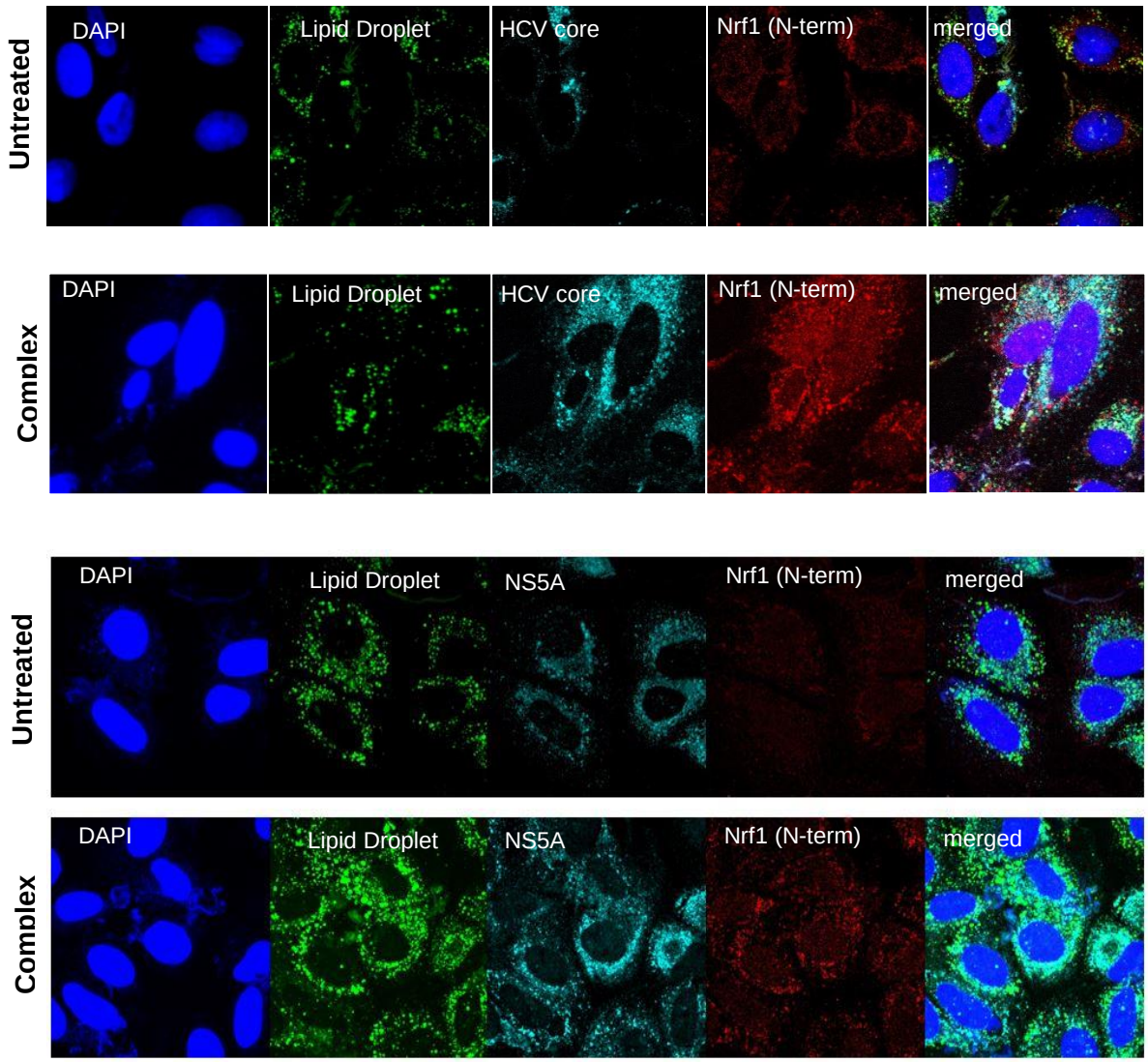


Figure 25: An increase in the Nrf1 protein levels as well as in the HCV core upon increasing the intracellular cholesterol.

Huh7.5.1 cells were electroporated with JC1 and GND constructs and seeded in 6 well plates with coverslips. 72hrs post electroporation cells were treated with the cholesterol (100µM/ml) in the form of the complex compound for 4hrs. It shows the effect of cholesterol on the HCV core and NS5A. Using an HCV core-specific antiserum, NS5A antisera (turquoise) and an Nrf1-specific monoclonal antibody (red). Lipid droplets (LDs) were visualized by BODIPY 498/503 (green), nuclei by DAPI (blue).

It was observed that upon increasing the intracellular cholesterol levels (noticeable by the increase in the lipid droplet amount), after the complex treatment of HCV replicating Huh7.5.1 cells, there was a significant induction in the Nrf1 protein levels compared to the untreated ones, which according to (Widenmaier et al. 2017) is due to the increase in the amount of the Nrf1 full length that act as a ER-sensor for cholesterol in the cytoplasm (Fig. 24c). Also, increasing the cholesterol leads to a significant increase in the amount of the HCV core protein, while the NS5A protein levels more or less remained the same.

In line with this, we wanted to study if increasing the intracellular cholesterol levels has an effect on the HCV genome level (Fig. 25). We were able to observe a significant increase in the number of intracellular HCV genomes compared to untreated Huh7.5.1 cells.

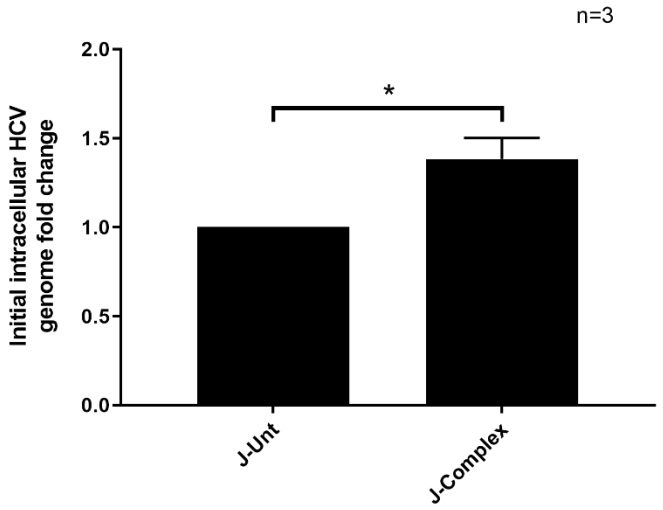


Figure 26: Effect of increasing the cellular cholesterol on the HCV genome amount.

Total RNA isolated from HCV replicating Huh7.5.1 cells that were treated with complex to increase the intracellular cholesterol were analyzed using HCV core-specific primers (#26 & #27) via RT-qPCR analysis.

Furthermore, we wanted to study the effect of high cholesterol levels after complex treatment on the HCV egression. We can observe that increasing cholesterol leads to a significant reduction in the HCV copies egressed (Fig. 26).

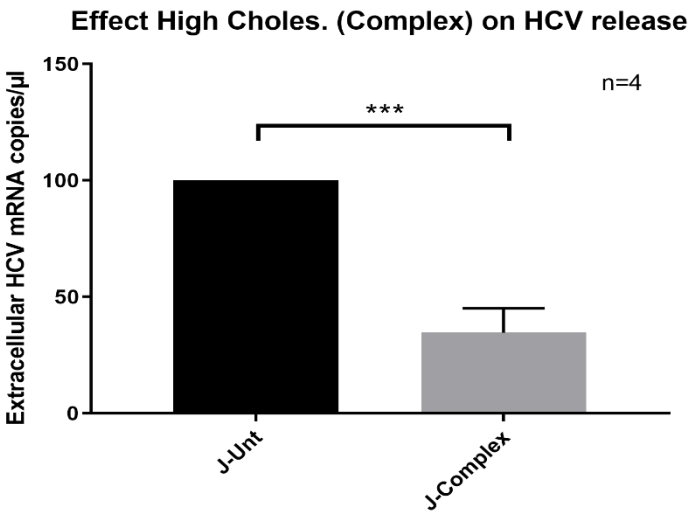


Figure 27: A decrease in the egression of the HCV upon increasing intracellular cholesterol levels

10x10^6 Huh7.5.1 cells were electroporated with 10 *µ*g RNA of the replicon JFH1/J6 and then seeded in a 6 well plate. Total RNA was isolated 72 h pt from the media and examined by Rotor gene qPCR for the HCV copies/µl released.

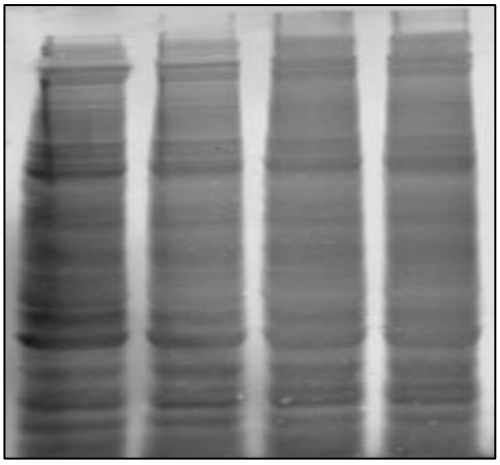
Taken together, these data indicate that in HCV-replicating cells treated with complex (which increases the intracellular cholesterol levels), although having an increase in both HCV genome number and HCV core protein levels, HCV genome egression was reduced.

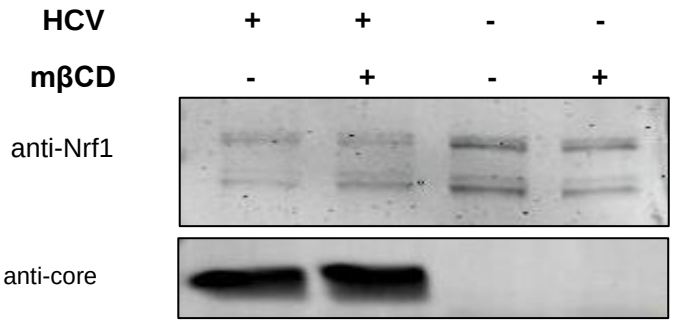
5.4 Effect of low cholesterol on Nrf1 and Hepatitis C virus

Next, we wanted to study the effect of low intracellular cholesterol levels on the Nrf1 and the Hepatitis C virus. This was carried out by electroporating Huh7.5.1 cells with the JC1 & GND (negative control) constructs through electroporation. After 72hrs pe cells were treated with methyl beta-cyclodextrin (mβCD) 2mg/ml for 4 hrs, which withdraw the intracellular cholesterol from the cells. Lysates were collected for the Western blot analysis and cells fixed on coverslips were used for confocal immunofluorescence imaging. We can observe that Nrf1 protein levels in the HCV replicating Huh7.5.1 cells treated with (mβCD) were increased due to the increase in the Nrf1 cleaved form (Fig. 28c), while in HCV negative cells, Nrf1 protein levels were decreased after (mβCD) treatment (Fig. 28).

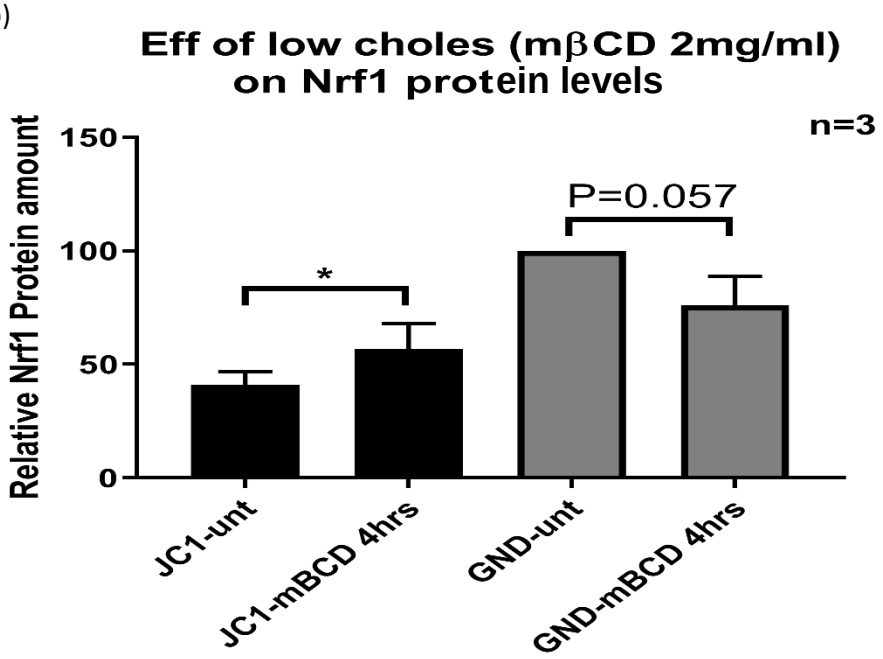
a)

Total protein stain




HCV + + - -
mβCD - + - +
anti-Nrf1
anti-core

b)


Eff of low choles (mβCD 2mg/ml)
on Nrf1 protein levels
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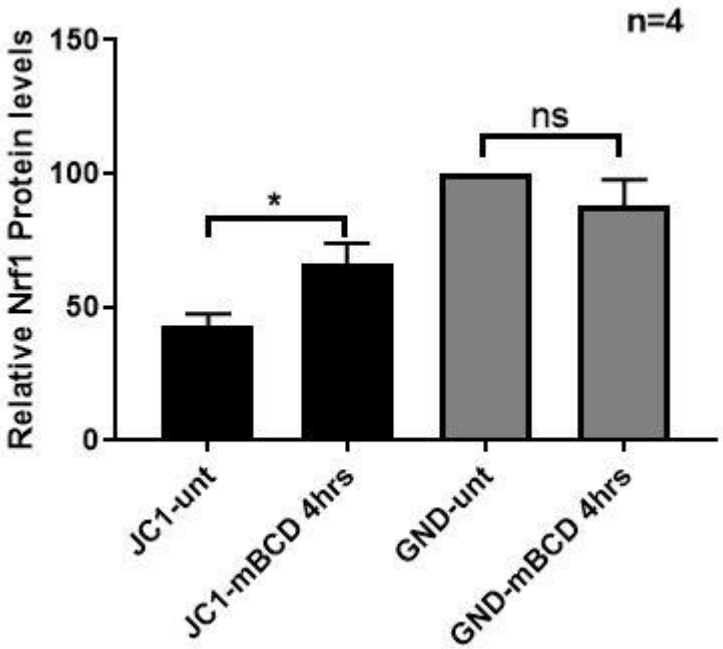
Relative Nrf1 Protein amount
150
100
50
0
P=0.057
*
JC1-unt
JC1-mBCD 4hrs
GND-unt
GND-mBCD 4hrs

c)


anti-Nrf1 cleaved form
95kDa


n=4
Relative Nrf1 Protein levels
150
100
50
0
*
ns
JC1-unt
JC1-mBCD 4hrs
GND-unt
GND-mBCD 4hrs

d)

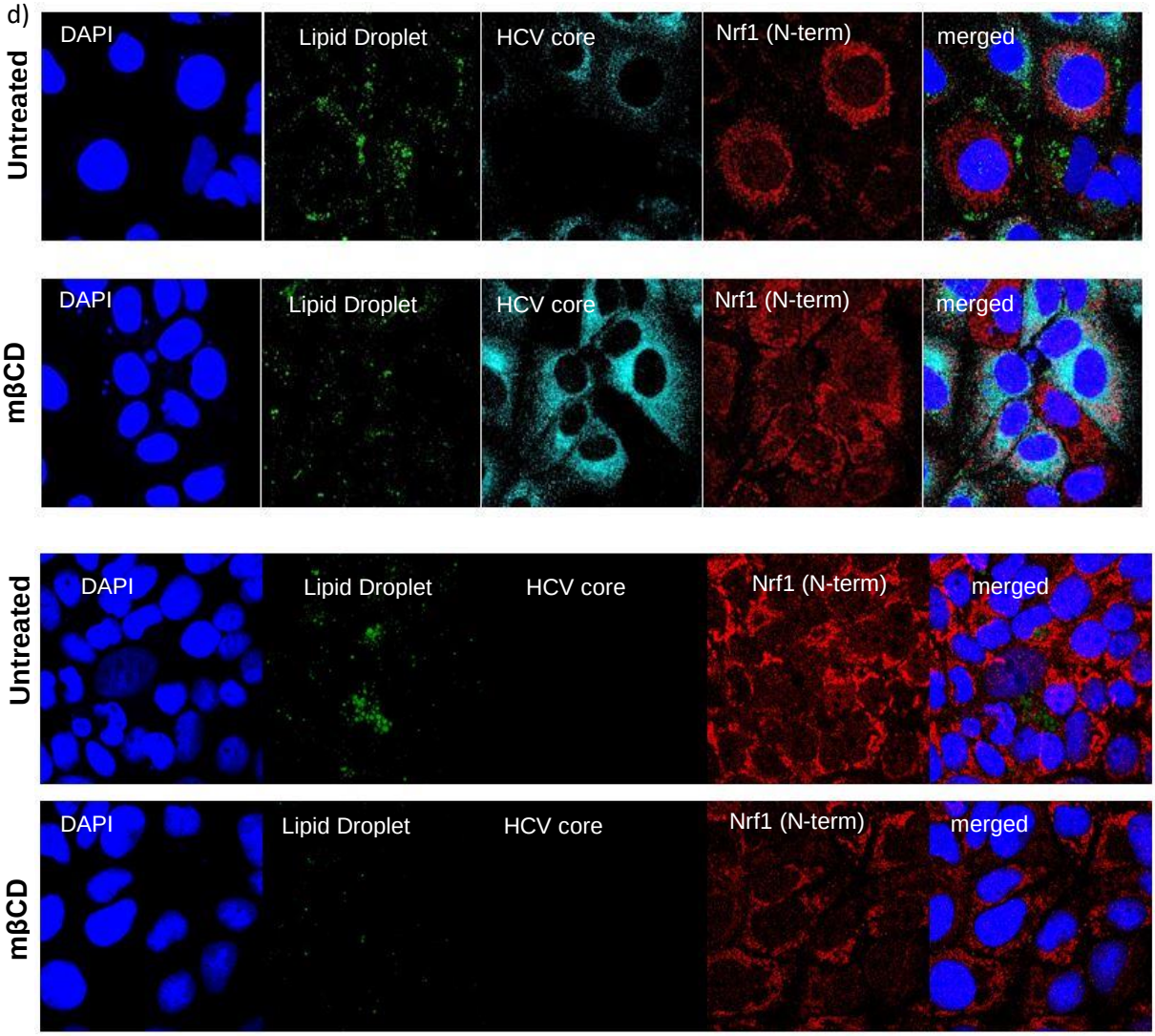
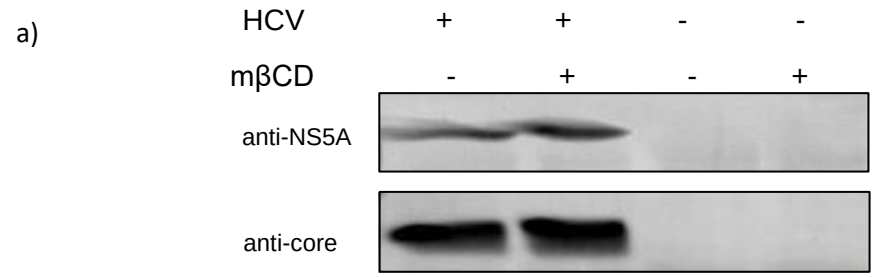
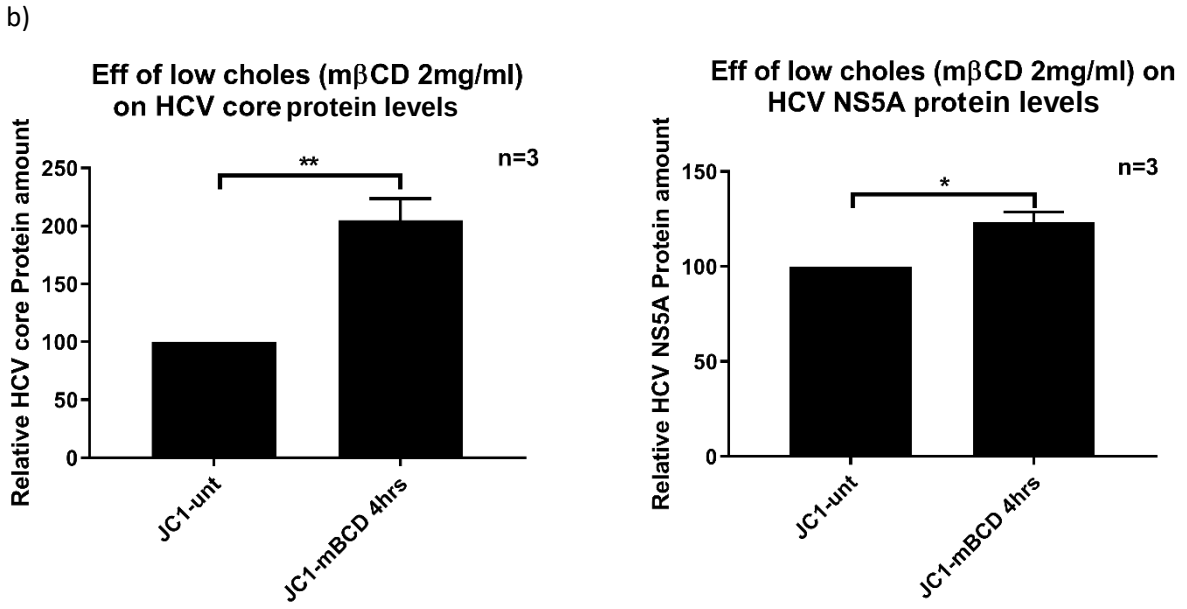

Untreated
DAPI
Lipid Droplet
HCV core
Nrf1 (N-term)
merged
mβCD
DAPI
Lipid Droplet
HCV core
Nrf1 (N-term)
merged
Untreated
DAPI
Lipid Droplet
HCV core
Nrf1 (N-term)
merged
mβCD
DAPI
Lipid Droplet
HCV core
Nrf1 (N-term)
merged

Figure 28: An increase in the Nrf1 protein levels mainly in the cleaved form as well as in the HCV core upon decreasing the intracellular cholesterol.

10x10^6 Cells were transfected by means of electroporation seeded in a 6 well plate. 72hrs pe cells were treated with 2mg/ml mβCD for 4 hrs **a)** Western blot membrane showing the Nrf1 protein expressed in both HCV replicating cells and the negative control cells and also both after treatment using Nrf1 anti-sera and signal was referred to the total lysate protein. **b)** Quantification of the Nrf1 protein bands. **c)** Western blot analysis and quantification showing the increased effect after treatment on Nrf1 cleaved form (95 kDa) in HCV +ve cells while showing a decreased effect in HCV -ve cells. **d)** Confocal immunofluorescence staining showing the effect of mβCD on Nrf1 and HCV core in HCV +ve and -ve Huh7.5.1 cells. HCV core- (turquoise) and an Nrf1-specific monoclonal antibody (red). Lipid droplets (LDs) were visualized by BODIPY 498/503 (green), nuclei by DAPI (blue).

Moreover, we wanted to study if decreasing the cholesterol after mβCD treatment will affect the Hepatitis C virus. We can observe that after treatment both HCV core and NS5A protein levels have increased compared to the untreated cells (Fig. 29).





c)

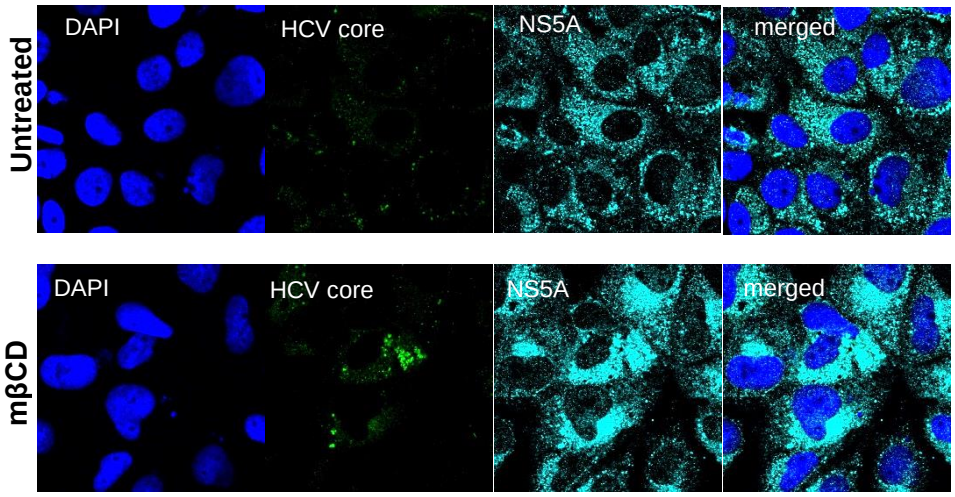


Figure 29: An increase in both HCV core and NS5A protein levels after mβCD treatment.

a) Western blot of the Huh7.5.1 cells lysate for HCV replicating cells and the negative control cells both treated with mβCD, where HCV core- and NS5A- antisera were used. b) Bands quantification after referral to the total lysate protein. c) Confocal immunofluorescence imaging **upper panel** showing untreated HCV replicating Huh7.5.1 cells **lower panel** showing the HCV replicating cells after mβCD treatment using an HCV core-specific antiserum, NS5A anti-sera (turquoise) and an Nrf1-specific monoclonal antibody (red). Lipid droplets (LDs) were visualized by BODIPY 498/503 (green), nuclei by DAPI (blue).

Following up on our investigation of the low cholesterol-mediated effect on the protein levels of both Nrf1 and HCV we wanted to study if lowering or withdrawing the cellular cholesterol levels using the mβCD would have an effect on the Nrf1 mRNA as well as the HCV genome expression levels. So, we analyzed this using qPCR, where we first isolated the RNA from the Huh7.5.1 cells lysate that were transfected by JC1 and GND respectively and observed that there was more or less no effect on the Nrf1 mRNA levels in both HCV +ve and -ve Huh7.5.1 cells as well as on the HCV genome amount after lowering the cellular cholesterol levels (Fig. 30).

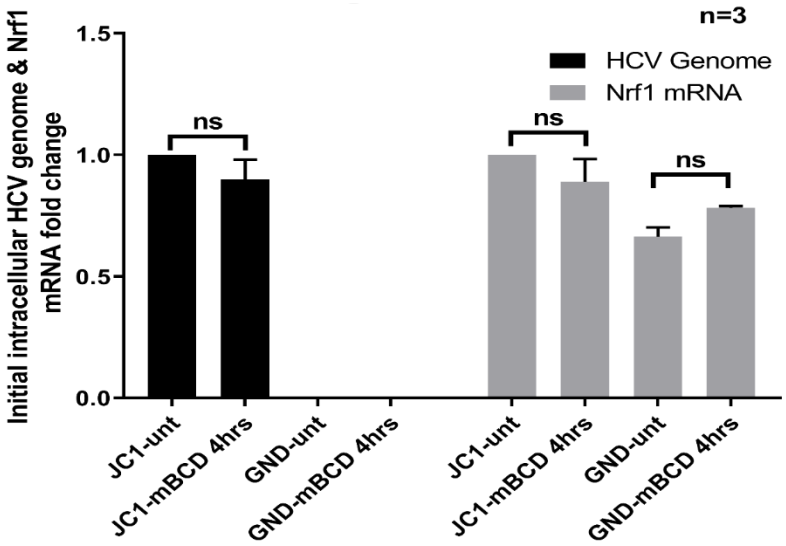


Figure 30: Effect of low cellular cholesterol levels on Nrf1 mRNA and HCV genome amount.

10x 10^6 Huh7.5.1 cells were electroporated with JC1 and GND constructs and seeded in 6 well plates. 72hrs post electroporation cells were treated with the mβCD (2mg/ml) for 4 hrs. After the treatment of both HCV replicating cells and control negative cells total RNA from the cells lysate was isolated and analyzed with qPCR using HCV core-specific primers (#26 & #27) and Nrf1 specific primers (#1400 &#1401). As a reference, the amount of RPL27-specific transcripts was determined.

In light of these results, we can conclude that lowering or withdrawing the cellular cholesterol indeed increased the Nrf1 amount in HCV +ve cells, but not in HCV-ve cells. Also, lowering the cholesterol levels led to an increase in the HCV core and NS5A protein levels, but had no effect on the Nrf1 mRNA levels and the number of HCV genome.

Furthermore, we wanted to see if the mβCD-mediated lowering of the cholesterol would affect the Nrf1 regulated genes (LxR and SREBP2 target genes). We observed that indeed the treatment has affected the expression of these target genes, where the LxR (responsible for initiating the cholesterol removal program) target gene (ABCA1) expression was significantly reduced in both HCV +ve and -ve Huh7.5.1 cells. Moreover, SREBP2 (responsible for initiating the cholesterol removal program) target genes (HMGCS1, HMGCR & LDLR) expression levels were induced in HCV+ve Huh7.5.1 cells as well as the HCV-ve ones. In light of this, we can conclude that the regulation of these genes is not affected by the presence of the Hepatitis C virus.

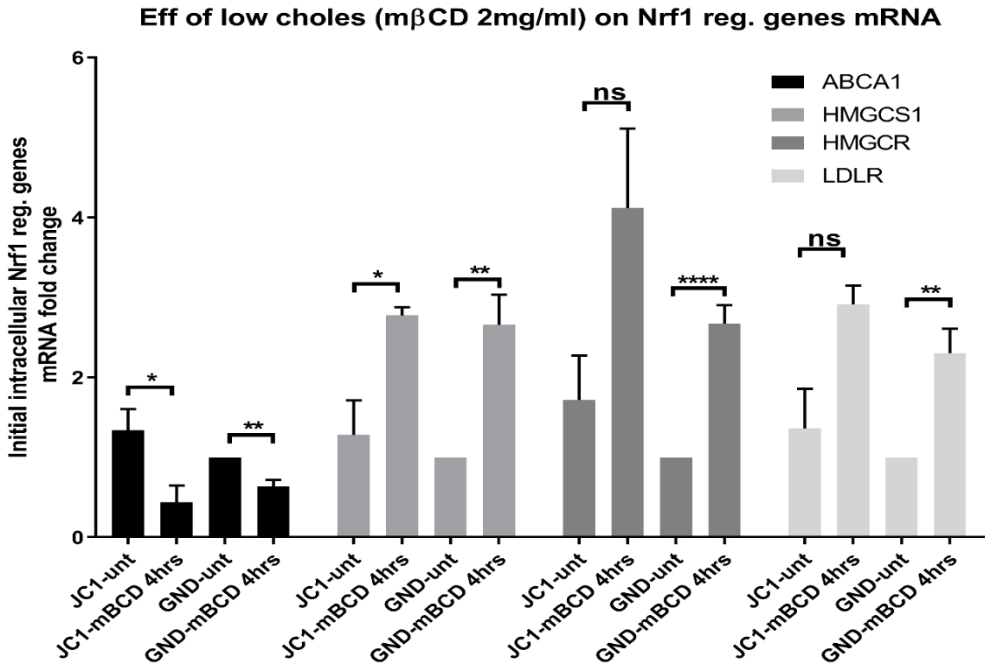


Figure 31: qPCR for the total RNA isolated from Huh7.5.1 cells after treatment with mβCD and the ABC1, HMGCS1 HMGCR & LDLR primers were used and RPL27 expression was measured as reference for the other genes.

In line with the fact that we are investigating the crosstalk relations among cholesterol, Nrf1, and the HCV, we wanted to test the effect of the mβCD treatment on the HCV released. We observed after the treatment that HCV copies released were very much reduced compared to the untreated cells (Fig. 31).

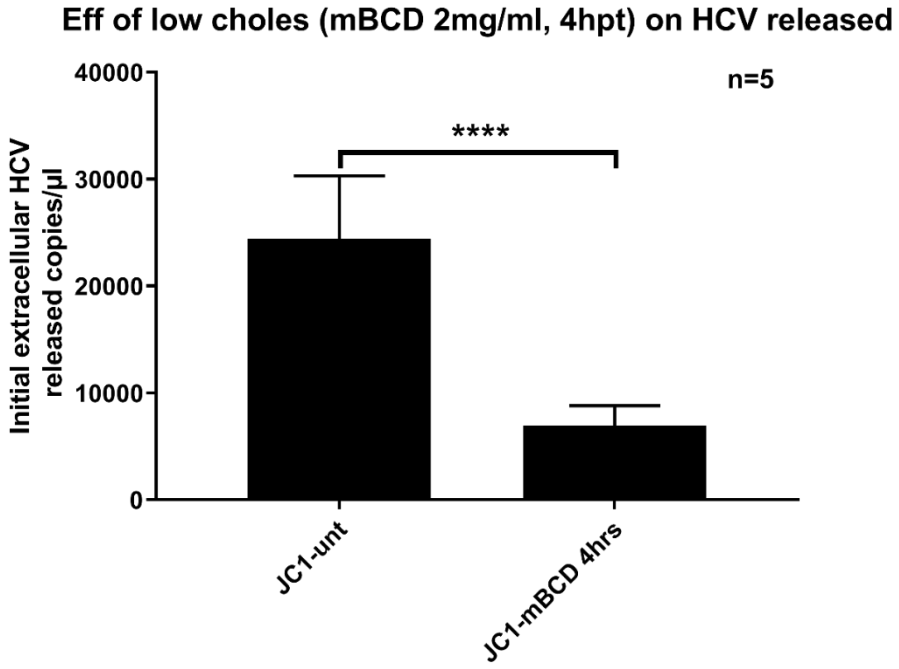


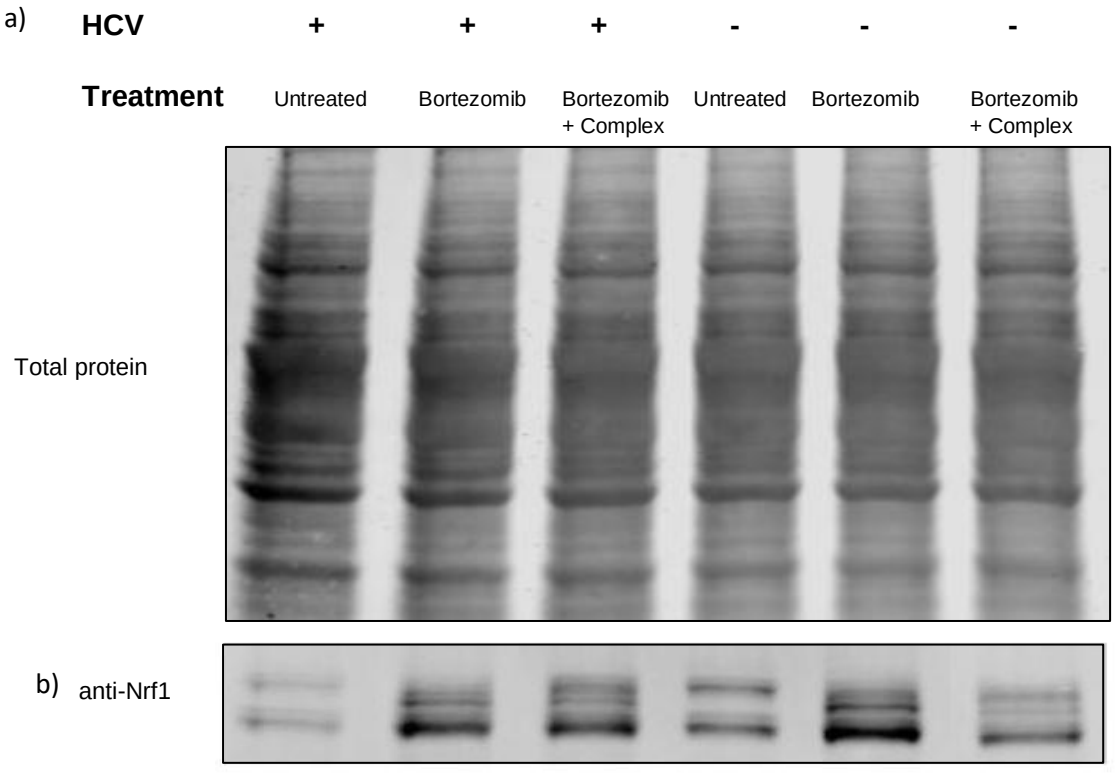
Figure 32: The effect of lowering the cellular cholesterol levels on the released HCV genome amount.

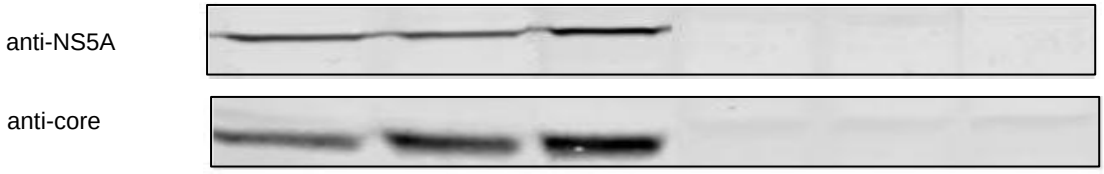
10x10^6 Huh7.5.1 cells were transfected by means of electroporation with the JC1 (JFH1/J6) and GND (JFH1/GND) constructs. 72hrs pe cells were treated with mβCD for 4hrs. Followed was the isolation of the total RNA isolation from the supernatant of the cells, where the released HCV genomes were analyzed by means of Rotor gene qPCR.

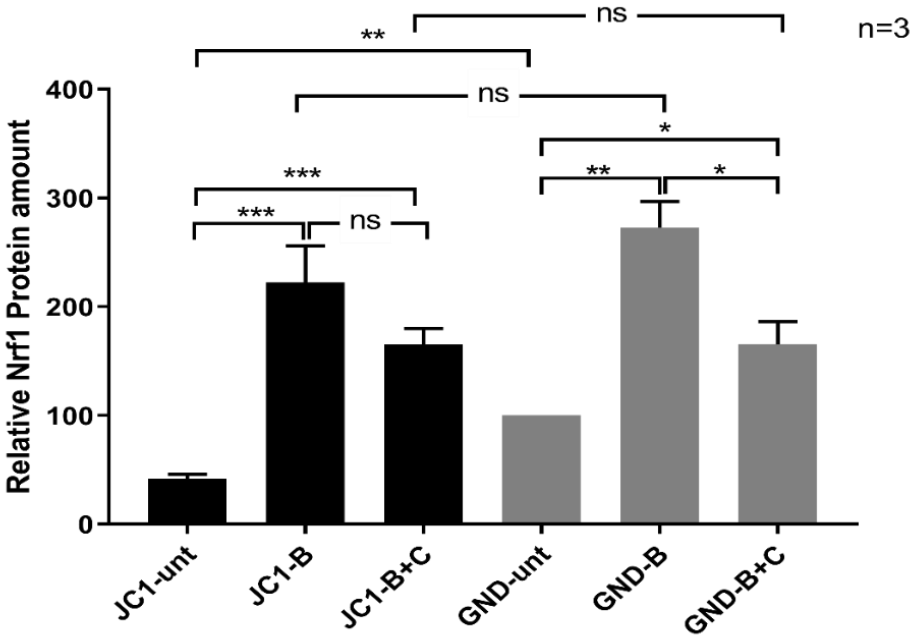
In light of this, we can conclude that withdrawing cholesterol from the HCV-replicating cells leads to a significant reduction in the released HCV genome amount.

5.5 Effect of Nrf1 processing modification on the Hepatitis C virus

Since Nrf1 undergoes rapid proteasome-mediated degradation (Biswas et al. 2011), we wanted to study whether the effect of modifying this process using bortezomib would have an effect on Nrf1 turnover and HCV in the presence and absence of high cholesterol. This was examined with Western blot analysis of the lysates from HCV replicating Huh7.5.1 cells that were harvested 72 h pe, in addition to the confocal immunofluorescence staining Western blot and confocal immunofluorescence (Fig. 32).




anti-NS5A
anti-core


n=3
ns
**
ns
*
**
*
***
***
ns
Relative Nrf1 Protein amount
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200
100
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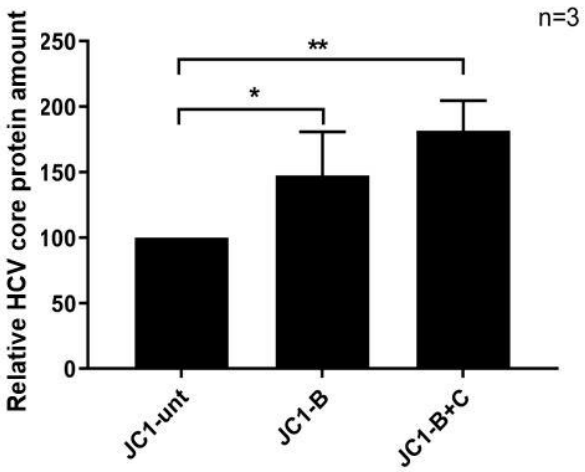
JC1-B
JC1-B+C
GND-unt
GND-B
GND-B+C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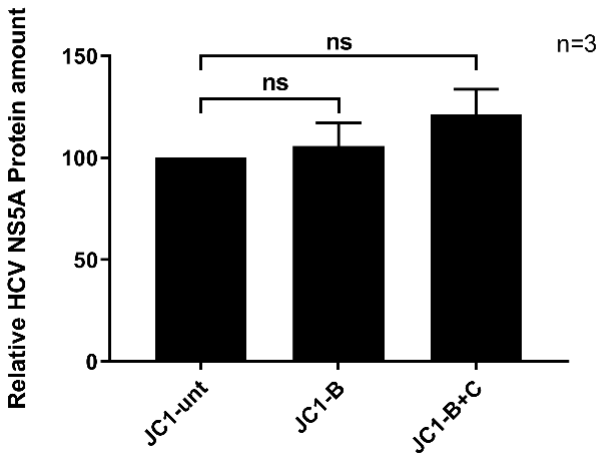
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Relative HCV NS5A Protein amount
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JC1-B
JC1-B+C

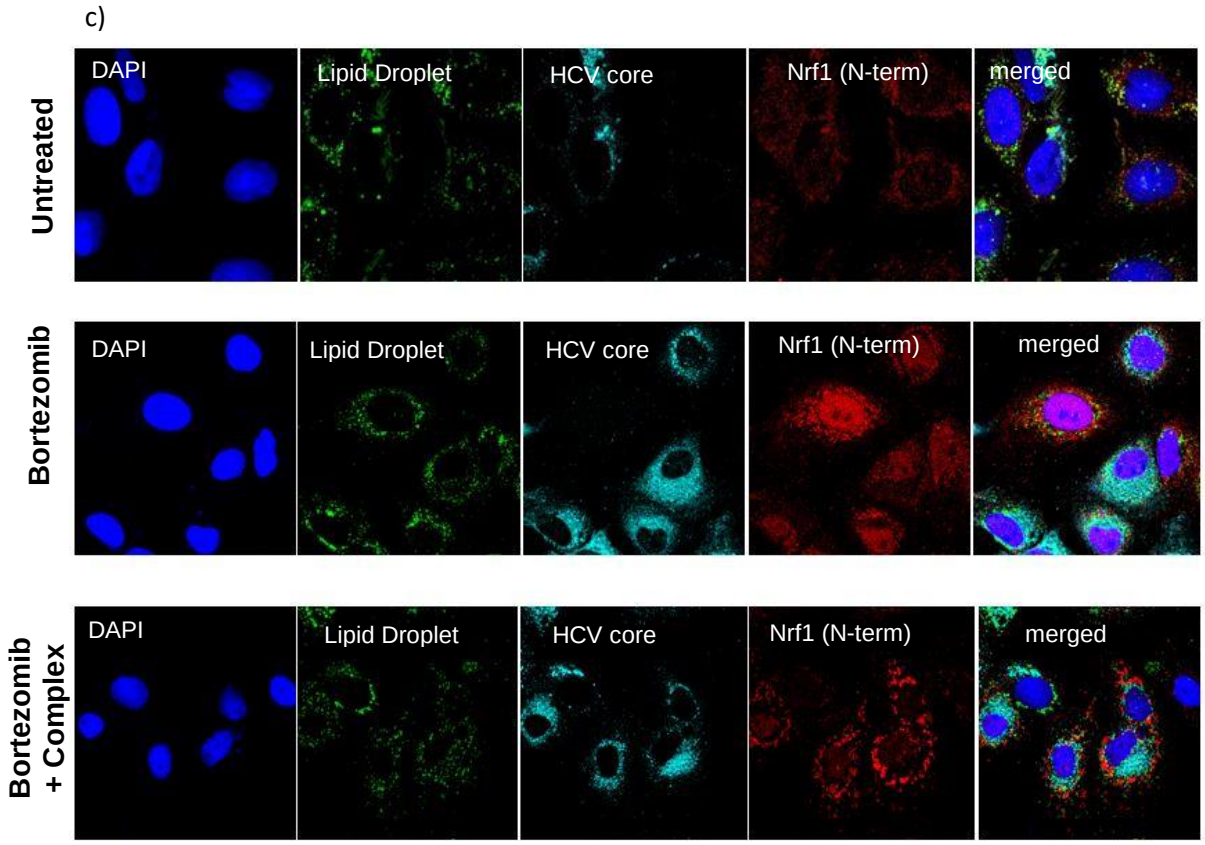

Figure 33: Western blot analysis.

Huh7.5.1 cells were transfected simultaneously with JC1 and GND the negative control. 72hrs post electroporation bortezomib treatment (10µM/ml) was applied for 4hrs in the presence and the absence of high cholesterol. Lysates were collected for **a)** western blot analysis where Nrf1- and HCV core-antisera were used. **b)** Quantification of the signal referring it to the total lysate protein. **c)** confocal immunofluorescence staining **upper panel:** untreated cells, **Middle panel:** cells treated with bortezomib, **Lower Panel:** cells treated with bortezomib + cholesterol (complex) using an HCV core-specific antiserum (turquoise) and an Nrf1-specific monoclonal antibody (red). Lipid droplets (LDs) were visualized by BODIPY 498/503 (green), nuclei by DAPI (blue).

We observed that the bortezomib treatment leads to the induction of the Nrf1 protein levels mainly due to the induction of the Nrf1 cleaved forms. This induction was attenuated in the presence of high cholesterol, which can be clearly seen in the immunofluorescence images (Fig. 32c). Also, we observed an increase in the HCV core protein levels, while the HCV NS5A remain more or less the same (Fig. 32).

In line with this, we wanted to study if there was an effect of such modification on the HCV genome and Nrf1 gene expression. We observed that upon the treatment with bortezomib a clear increase in the HCV genome number took place, while Nrf1 mRNA expression levels remained the same in HCV +ve and -ve Huh7.5.1 cells.

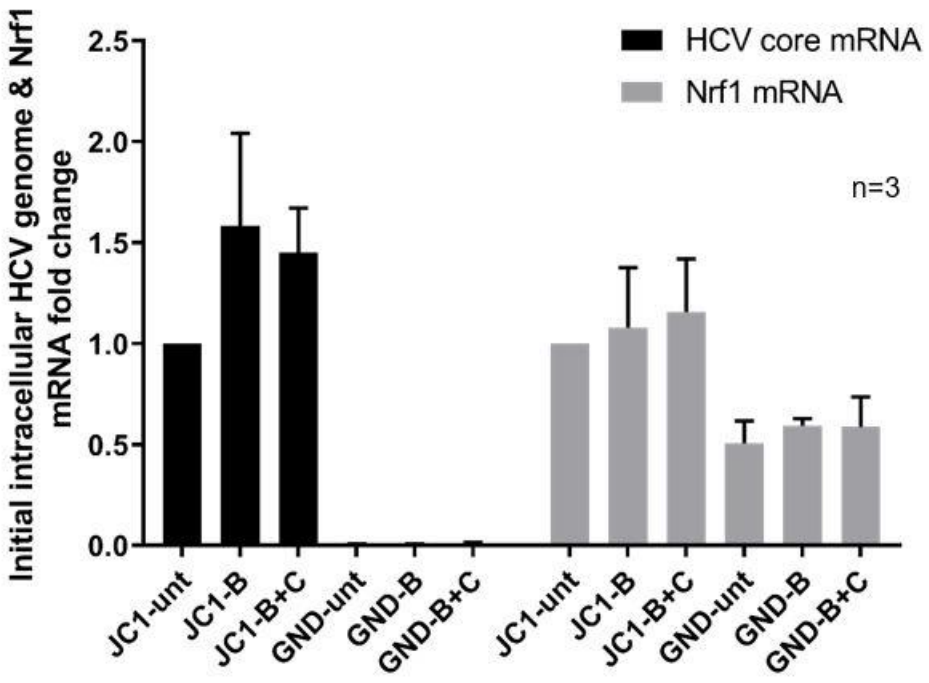

Figure 34: Effect of modifying Nrf1 processing on Nrf1 mRNA and HCV genome fold change.

After the treatment of both HCV replicating cells and control negative cells with bortezomib in the presence and the absence of cholesterol. Total RNA from the cells lysate was isolated and analyzed with SYBR Green qPCR using HCV core-specific primers (#26 & #27) and Nrf1 specific primers (#1400 &#1401) As a reference, the amount of RPL27-specific transcripts was determined.

Following up on the investigation on whether the inhibition of the proteasome-mediated Nrf1 degradation affects the Nrf1 mRNA expression levels, we wanted to know if such a modification affects the expression of the Nrf1 regulated genes.

These genes are the LxR and SREBP2, where the first is the cellular factor responsible for initiating the cholesterol removal program and it targets a couple of genes one of them is the (ABCA1), while the latter is the (cellular factor responsible for initiating the cholesterol production program) and it targets a couple of genes some of them are the (HMGCS1, HMGCR & LDLR) (Fig. 34).

First, we observed that ABCA1 expression in untreated HCV +ve Huh7.5.1 cells is higher than that in HCV -ve ones. Upon treatment of cells with bortezomib, ABCA1 expression was reduced in both HCV +ve and -ve cells. This can be explained by the

fact that the inhibition of the proteasome-mediated Nrf1 degradation using bortezomib leads to more Nrf1 cleaved forms that enter the nucleus inhibiting LxR.

When Huh7.5.1 cells were treated with both bortezomib and complex, we can observe that in both HCV +ve and -ve cells ABCA1 expression was more induced compared to the untreated cells, which also can be explained by the fact that more cholesterol led to more full-length Nrf1 than cleaved ones, this means less cleaved Nrf1 going into the nucleus. Thus, no inhibition to the LxR.

Furthermore, it was also observed that HMGCS1, HMGCR & LDLR expression in untreated HCV +ve Huh7.5.1 cells was higher than that in HCV -ve ones. This can be explained due to the fact that all of these genes play a crucial role in cellular lipid metabolism, especially in cholesterol synthesis, which as mentioned earlier is very important for the HCV life cycle.

When HCV -ve Huh7.5.1 cells were treated with bortezomib, an increase in the HMGCS1, HMGCR & LDLR expression was observed. This can be interpreted by the fact that the inhibition of the proteasome-mediated Nrf1 degradation using bortezomib leads to more Nrf1 cleaved forms that enter the nucleus and promotes the SREBP2 factor that targets and upregulates the previously mentioned genes.

However, when HCV +ve Huh7.5.1 cells were also treated with bortezomib, it was observed that HMGCS1 & HMGCR expression was decreased, while LDLR expression was increased. One explanation for the decrease in the HMGCS1 & HMGCR expression could be that, when the Nrf1 was cleaved, it was trapped in the cytoplasm through the HCV-mediated delocalized sMAF preventing it from entering the nucleus.

However, the increase in the LDLR expression could be explained by the following. Since HMGCR (the rate-limiting enzyme for cholesterol synthesis) was decreased, this triggers the induction in the LDLR expression that codes for the LDL receptor protein which is localized on the cells surface, especially the liver cells, where it binds to LDLs (which are the primary carriers of cholesterol in the blood) and transport them into the cell. Thus, compensating the downregulation that has happened to the HMGCS1 & HMGCR genes.

When Huh7.5.1 cells were treated with both bortezomib and complex, we can observe that in both HCV +ve and -ve cells the increase in the HMGCS1, HMGCR & LDLR expression was more reduced compared to the untreated cells. This can be explained by the fact that more cellular cholesterol levels will lead to negative feedback inhibition to the genes involved in the cholesterol producing program (Fig. 35).

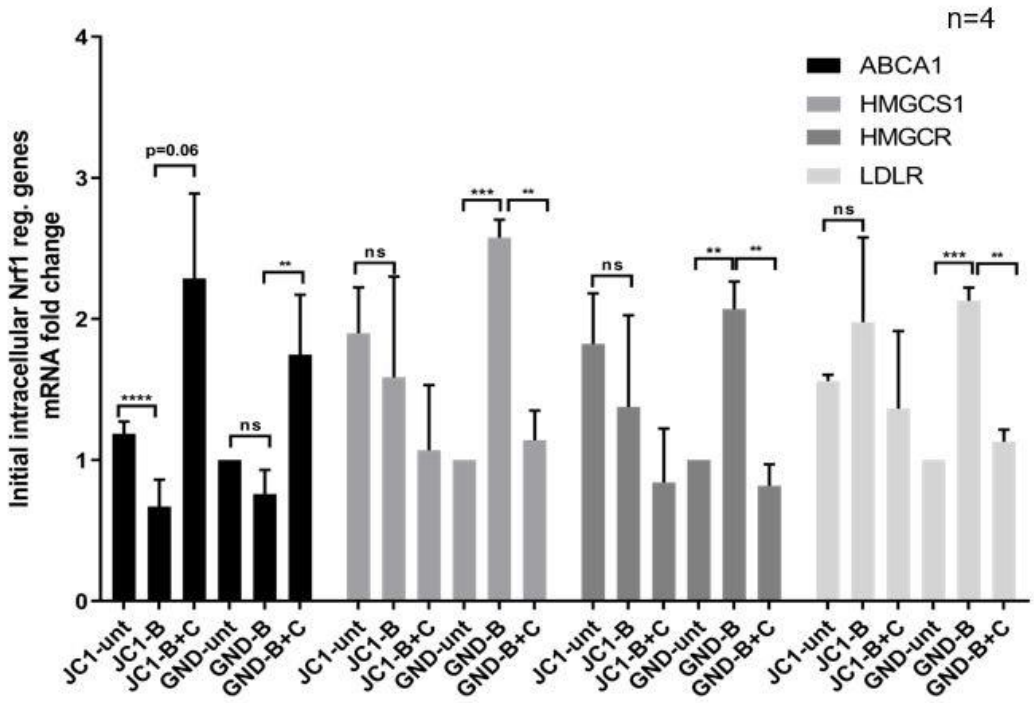

Figure 35: Nrf1 regulated genes fold change.

After the treatment of both HCV replicating cells and control negative cells with bortezomib in the presence and the absence of cholesterol. Total RNA from the cells lysate was isolated and analyzed with SYBR Green qPCR using the designated specific primers for each gene. As a reference, the amount of RPL27-specific transcripts was determined.

Moreover, we also wanted to know if there was an effect of modifying the Nrf1 turnover using the bortezomib in the presence and the absence of cholesterol on the HCV release (Fig. 35).

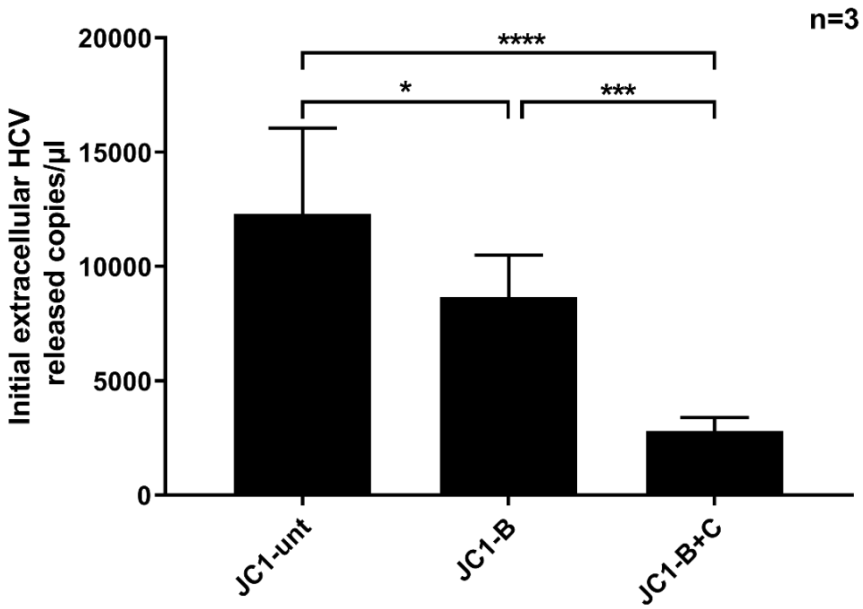

Figure 36: Effect of this modification on HCV genome egression.

This was done by transfecting the Huh7.5.1 cells by means of electroporation with the JC1 (JFH1/J6) and GND (JFH1/GND) constructs. 72hrs pe cells were treated with bortezomib in the presence and absence of high cholesterol. Followed was the isolation of the total RNA isolation from the supernatant of the cells and analyzed by means of Rotor gene qPCR.

We can observe that inhibiting the proteasome-mediated degradation leads to a reduction in the HCV egressed in absence of high cholesterol, while in the presence of high cholesterol the HCV genome egression was reduced even more.

Summary:

It is estimated that approximately 71 million people worldwide are chronically infected and are living with HCV infection, and it is estimated that around 400,000 have died from HCV-related liver disease in 2016 particularly due to the development of liver cirrhosis and liver tumors. Despite the wide variation in the prevalence estimates and the epidemiologic data quality, the most recent worldwide assessment shows that the viremic spread of HCV infection (prevalence of HCV RNA) in most developed countries is less than 1.0%, including the United States (www.cdc.gov/hepatitis/HCV). In some countries in Eastern Europe such as Latvia (2.2%) or Russia (3.3%) and certain countries in Africa Egypt (6.3%) and Gabon (7.0%) or Middle East Syria (3.0%), the prevalence is remarkably higher. Sharing of drug preparation and drug-injection equipment (needles) are considered the most frequent current mode of transmission in the United States and most developed countries, while the predominant mode of transmission in countries where HCV infection spread is higher relative to the developed countries is from health care exposures where infection-control practices are poor and unsafe injections.

It is clear that present drug regimens optimization, limitation in the mutation resistance problem, designing individualized therapy, accessibility to these therapeutic antiviral-drugs, and their high price remain a challenge (Pawlotsky 2016; Pawlotsky et al. 2015; Sarrazin 2016). However, the presence of no vaccine is considered the major challenge for worldwide control of HCV (Bukh 2016). That is why it is important to continue understanding more about the HCV life cycle and the factors that may affect its replication and overall lifecycle for the sake of developing efficient, high quality, and most importantly easily accessible treatments for everyone worldwide.

It was reported previously how HCV infection affects lipid metabolism and cholesterol homeostasis in particular, and also recently the relationship between the lipids and the Nrf1 activity. So, we wanted to study and understand the crosstalk and relationship between Hepatitis-C Virus and Nrf1 activity and the effect of cholesterol level modulation on both of them and what happens to HCV upon modulating Nrf1.

Despite the significant advances in HCV therapy, drug resistance and genotype-specific efficacy are still issues to be considered. That´s why the aim of this study, which is shedding the light on this new crosstalk, could hold a promising therapeutic opportunity that will likely emerge upon identifying the complete spectrum of ER adaptive systems, and how they are integrated during metabolic stress.

After carrying out this study, it can be demonstrated that for the first time it could be demonstrated that the Hepatitis C virus indeed has an effect on the Nrf1 at the protein and mRNA levels. On the other hand, It was also shown that overexpressing the Nrf1 favors the HCV intracellular survival and propagation, but at the same time reduces the HCV genome amount released, which could be due to some sort of blocking or disturbance in the assembly of the viral particle. It was also concluded that increasing the intracellular cholesterol levels indeed increases the Nrf1 protein levels due to the increase in the Nrf1 full-length and at the same time it also increased the HCV genome amount and core protein amount, while not affecting NS5A protein amount. But surprisingly led to a decrease in the HCV genome released. It was also concluded that lowering cholesterol levels also led to an increase in the Nrf1 protein levels in the HCV replicating cells and not in the non-replicating ones. However, this time was due to the increase in the Nrf1 cleaved form, which at the same time led to an increase in the HCV core and NS5A protein levels. Although there was no effect on the HCV genome amount or the Nrf1 mRNA. It was also realized that HCV had no direct influence on the Nrf1 target genes (ABCA1, HMGCS1, HMGCR, and LDLR after cholesterol withdrawal. It was also demonstrated that the inhibition of the Nrf1 proteasome-mediated led to an increase in the Nrf1 mainly the cleaved form and that, when this inhibition occurred at high cholesterol conditions, the amount of Nrf1 protein decreased, but a decrease in the cleaved form and increase in the full-length one can be seen. But HCV had no further significant influence on Nrf1 in the cells in which proteasome-mediated Nrf1 degradation was inhibited and cholesterol was elevated compared to non-replicating cells.

Taken together, these data all together indicate that there is indeed a cross-talk between Nrf1 activity, lipids radicals and hepatitis C virus and a potential link between the Nrf1 regulation and that of the HCV which requires further and deeper investigation.

6 Discussion

Chronic hepatitis C is a leading cause of hepatocellular carcinoma (HCC) worldwide (Hoshida et al. 2014). There are approximately 71 million people chronically infected, and this number can increase due to the presence of no vaccine and that the available current therapy either generates sustained response in only ~55% of the patients or direct antiviral agents (DAAs) with a higher sustained response, but are costly to patients of average economical status. The pathogenic mechanisms for liver injury and the outcome of cancer development are more or less unclear. That is why it is important to continue exploring the different aspects of the hepatitis C virus life cycle in addition to its crosstalk with other cellular factors. This would definitely help future researchers understanding more about HCV and help develop an efficient and more importantly accessible treatment worldwide.

It is well established that many aspects of the Hepatitis C virus life cycle are closely related and dependent on cellular lipid metabolism. In addition to the association of apolipoproteins with the viral particles, and how it influences the way the virus binds to the liver cells in particular in the entry stage (Popescu et al. 2014). Nrf1 was first described in 1993 by Kan et al. The Nrf1 which is “cap'n'collar" bZIP transcription factor is known to regulate specifically the pathways in amino acid metabolism, lipid metabolism, proteasomal degradation, mitochondrial respiratory chain, and the citric acid cycle (Bugno et al. 2015). It was also shown that the level of cellular cholesterol indeed regulates Nrf1 turnover, localization, Nrf1-dependent transcription, and processing (Widenmaier et al. 2017). In light of this, we wanted to address in this thesis the crosstalk between the Hepatitis C virus, Nrf1, and lipids.

6.1 Effect of Hepatitis C virus on Nrf1

A huge rearrangement of the membranes derived from the endoplasmic reticulum is known to be associated with HCV infection, which subsequently leads to the disturbing the homeostatic condition of the host cell´s ER-protein. Moreover, it was reported that infection with HCV leads to the induction of ER-stress and the unfolded protein response (UPR) (Dash et al. 2016; Paillet and Kroemer 2019; Ríos-Ocampo et al. 2019). This leads to the induction of ER protein quality control that maintains the proper protein folding. Another example of cellular homeostasis is maintaining moderate ROS

(reactive oxygen species) levels within the cell through Nrf2/Keap1 signaling pathway. However, this signaling pathway is also interrupted during the HCV infection leading to elevated ROS levels. Thus, causing ER-stress that triggers autophagy which favors the release of the virus (Bender and Hildt 2019).

Also, as mentioned before, a lot of aspects of the HCV life cycle are closely related to and dependent on the host cellular lipid metabolism and how the HCV circulates the blood as lipoviroparticles considering that it is rich in lipids. At the same time, HCV causes within the infected host cell a serious lipid disorder, resulting in a common histopathological condition known as hepatic steatosis in chronic hepatitis-C patients and a decrease in the blood cholesterol (Simon and Butt 2015). Furthermore, it was demonstrated that Nrf1 acts as an ER-membrane sensor that senses the cellular cholesterol via binding it through the CRAC (cholesterol recognition amino acid consensus) domain (Widenmaier et al. 2017). Thus, playing a role in defending the cellular cholesterol homeostasis. In light of this, we wanted to study the possible influence of HCV on Nrf1.

In this study, it was shown that the HCV has the capacity to impair the Nrf1 protein production levels as it significantly decreased the Nrf1 protein levels. This was confirmed with Western blot analysis of the lysates from HCV replicating Huh7.5.1 cells that were harvested 72 h pe and also the confocal immunofluorescence staining (Fig. 15). In line with this, we wanted to examine the HCV effect on Nrf1-specific RNA transcript and it was found that, although the protein amount in HCV replicating cells were strongly decreased, nonetheless, the Nrf1 gene expression was significantly increased (Fig. 17).

This HCV effect on Nrf1 was also confirmed and reproduced using Western blot in HCV-replicating Nrf1 overexpressing Huh7.5.1 cells (Fig. 16).

Moreover, it was also found that in HCV replicating Huh7.5.1 cells, HCV can indeed affect one of the Nrf1 regulated genes (LxR). This gene is responsible for initiating the cholesterol removal program. This was confirmed with luciferase assay, where it is shown that its activity was significantly decreased compared to the non-HCV replicating Huh7.5.1 cells (Fig.23).

Next, we wanted to see if the HCV affects the Nrf1 half-life. It can be seen in HCV replicating Huh7.5.1 cells that the Nrf1 half-life (t1/2=242.1min) is decreased by almost threefold compared to non-HCV replicating Huh7.5.1 cells (t1/2=631,3min). (Fig. 18).
Taken together, these data indicate that HCV indeed downregulates significantly the Nrf1 protein amount, maybe due to an increase in the proteasomal activity and that the infected cells simultaneously upregulate the Nrf1 mRNA gene expression levels for the sake of compensating the reduction or down-regulation in the Nrf1 protein amount compared to non-HCV replicating Huh7.5.1 cells.

6.2 Effect of Nrf1 overexpression on Hepatitis C virus

We also wanted to see the other way around, which is the effect of overexpressing the Nrf1 on HCV. It can be observed that, when Nrf1 was overexpressed in HCV replicating Huh7.5.1 cells, there was an accumulation in HCV core protein. This is confirmed with Western blot analysis of the lysates from HCV replicating Nrf1-overexpression Huh7.5.1 cells that were harvested 72 h pe and also in the confocal immunofluorescence microscopy compared to normal HCV replicating Huh7.5.1 cells (Fig. 19). In line with this, we can also detect a significant increase in the intracellular HCV genome fold change (Fig. 20).
Moreover, it was also observed in the immunofluorescence that upon overexpressing the Nrf1 in HCV replicating cells lipid droplets were produced in large numbers, but small in size, while in cells normally expressing the Nrf1 lipid droplets were less in number, but bigger in size (Fig. 19), but the reason behind the variation in the lipid droplets size/number ration is not clear.
It was also observed via luciferase assay using LxR-reporter construct that LxR (cholesterol removal program involving factor) activity was significantly reduced in normal HCV replicating Huh7.5.1 cells compared to non-HCV replicating Huh7.5.1 cells (Fig. 23). This was also confirmed by the significant reduction in the ABCA1 (Nrf1-regulated LxR-target gene (which is responsible for the initiation of the cholesterol removal programs)) gene expression (Fig. 22). This goes in line with the fact that cholesterol is indeed important for the HCV life cycle (Felmlee et al. 2013). However, when Nrf1 was overexpressed, in HCV replicating Huh7.5.1 cells there was a slight increase in the ABCA1 expression which may be due to less cleaved-form Nrf1 entering the nucleus (due to the HCV mediated Nrf1 trap), thus, stimulating the LxR activity, therefore, upregulating the ABCA1 gene expression. However, in non-HCV replicating cells, there was a reduction which may be because more cleaved Nrf1 are

entering the nucleus leading to the inhibition of the LxR activity, therefore, downregulating the ABCA1 gene expression(Fig. 22) based on the study done by Widenmaier and colleagues. This might be explained by the fact that more Nrf1 are available to trigger the initiation of the cholesterol removal program to defend the cellular cholesterol homeostasis, which is not in favor of the HCV infection. In line with this, we wanted to see if overexpressing the Nrf1 will affect the HCV egressed and we indeed observed that it has reduced the HCV released. It is also recommended to take a closer look at the released HCV to see if there are infectious particles via measuring the TCID50 of the extracellular and intracellular lysates in addition to the analysis of the lipoviroparticle composition as well.

6.3 Effect of high cholesterol on Nrf1 and Hepatitis C virus

Hepatitis C infection is known to be associated with lipid and lipoprotein metabolism disorders such as hepatic steatosis, and hypocholesterolemia. Moreover, it was also reported that virus production is dependent on the formation of VLDL (hepatic very-low-density lipoprotein). Thus, the virions circulating the blood are physically complexed with lipoproteins, therefore, termed lipoviroparticles (Felmlee et al. 2013). HCV virions or the lipoviroparticles were found to be not exclusively accompanying apoB-100-containing triglyceride-rich lipoprotein of liver origin, but are also found to be accompanied with apoB-48-containing lipoprotein of an intestine origin. This subpopulation existence of LVP is a sign of how virions alternate between lipoproteins (Diaz et al. 2006; Felmlee et al. 2010). It has also been described recently that HCV commandeers proteins responsible for lipid-transfer for the sake of the formation of the cholesterol-enriched membranous web (MW) and double-membrane vesicles (DMVs), which is featured commonly in positive-strand RNA viruses to maintain the viral replication (Stoeck et al. 2018). Thus, assuring the extreme importance of lipids to the HCV. So, we wanted to study the effect of high intracellular cholesterol on Nrf1 and HCV.

So, after increasing the intracellular levels of cholesterol using a complex containing cholesterol as the only source of lipid we were able to see a significant increase in the Nrf1 protein levels in HCV replicating Huh7.5.1 cells, while in the non-HCV replicating Huh7.5.1 cells it was more or less the same. But when we took a closer look, we realized that the increase was due to the increase in the Nrf1 full-length form (Fig. 24c). That remains as described in the literature bound to the endoplasmic reticulum. This is confirmed with Western blot analysis of the lysates from HCV replicating Huh7.5.1

cells that were harvested 72 h pe, and also in the confocal immunofluorescence staining (Fig. 25). However, it was difficult to contradistinguish between endogenous/exogenous Nrf1 in the IF. According to the work of Widenmaier et al. 2017, Nrf1 acts as a cholesterol sensor spanning the endoplasmic reticulum to detect any change in cellular cholesterol. Therefore, defending the cholesterol homeostasis and that cholesterol indeed regulates the Nrf1 localization and processing. Lipid droplets (LDs) on the other hand which are non-adipocytes rich in triglycerides (TG) as well as cholesterol esters (CE) in different ratios. It was found that TG-rich lipid droplets are produced when unsaturated fatty acids are administered, but the conditions causing an induction in CE-rich LD formation are less well characterized (Suzuki et al. 2012). However, in our study it was noticed that upon increasing the intracellular cholesterol using the complex, it led to the increase in the lipid droplet formation which should be the CE-rich type in this case Furthermore, we can see that high intracellular cholesterol levels lead to a significant increase in HCV core protein levels, while NS5A protein levels were non-significantly increased (Fig. 24). In line with this, we could also observe a significant increase in the HCV genome fold change (Fig. 26). We also can see that increasing the intracellular cholesterol leads to a significant decrease in the HCV egression. This can be confirmed by Rotorgene qPCR of the supernatant-isolated viral-RNA to measure the released HCV genome copies/µl in the supernatant (Fig. 27). In light of this, one explanation could be that the cholesterol, although important for the HCV, but alone was not enough for the assembly and release of the virus and that other components as important as the cholesterol are required as well. It is also known that cholesterol can modulate the phospholipid (PL) bilayer structure of membranes, by altering the biological membrane thickness, compressibility. Thus, altering its fluidity and permeability, which also can cause the integral membrane proteins to change in conformation and distribution in the membrane (Yang et al. 2016). This could also be a factor affecting the release of the virus. It would be also interesting to see if increasing the intracellular cholesterol levels affects the levels of Niemann-Pick-type C1 (NPC1), which was reported to mediate the cholesterol transfer from late endosome (LE), and the lysosome (LY) to the replication organelles (RO) (Stoeck et al. 2018). It was shown also that, when HCV-replicating cells were treated with the amphiphatic inhibitor U18666A that inhibits the intracellular lipid transport by directly inhibiting NPC1 protein leading to accumulation of cholesterol in lysosomal storage structures. This leads to the impairment in the viral release without affecting the viral replication in addition to a

slight accumulation in the HCV core (Elgner et al. 2016). However, in the current study, we were able to observe that increasing the intracellular cholesterol, which may have led also to intracellular cholesterol accumulation caused also an impairment in the viral release and in addition to the significant accumulation in the HCV core protein which was obvious in western blot and immunofluorescence microscopy, there was a significant increase in the HCV genome replication. But it would be interesting to check if this increase in HCV core protein and genome corresponds to an accumulation of an assembled viral particles in the late endosomes and if these particles are indeed infectious through analyzing the intracellular TCID50. Also, if increasing the intracellular cholesterol using the complex treatment would shift the viral density gradient.

6.4 Effect of low cholesterol on Nrf1 and Hepatitis C virus

As already discussed before the importance of the cholesterol to the HCV life cycle. We wanted to study the effect of lowering the cellular cholesterol on Nrf1 and HCV to better understand the crosstalk between the three of them. When the intracellular cholesterol levels were lowered via withdrawing it using methyl beta cyclodextrin (mβCD), it was observed that Nrf1 protein levels has increased significantly in HCV replicating Huh7.5.1 cells. However, in non-HCV replicating cells it has almost significantly decreased (P=0.05). This is confirmed with Western blot analysis of the lysates from HCV replicating Huh7.5.1 cells that were harvested 72 h pe, as well as in the confocal immunofluorescence microscopy (Fig. 28a&b). This time the increase in the Nrf1 protein levels in HCV +ve cells was due to the increase of the Nrf1 cleaved form (95 kDa) (Fig. 28c). As according to Widenmaier and colleagues in 2017 when the cells face a low cholesterol situation, Nrf1 is cleaved and the cleaved form enters the nucleus, where it initiates the expression of SREBP2-regulated cytoprotective genes (HMGCS1, HMGCR, and LDLR) that take part in the cholesterol producing program to compensate the loss of it (Fig. 30). Thus, defending the cellular cholesterol homeostasis. But there are many Nrf1 isoforms (Zhang and Xiang 2016) and the role of each isoform in this process is still not clear. These results also raise a question concerning the HCV role in allowing the cleaved Nrf1 forms to enter the nucleus without binding to it as it is the case with the Nrf2 (Carvajal-Yepes et al. 2011) and raises the question of the HCV-Nrf1 binding criteria.

Furthermore, we wanted to see if the treatment can affect HCV. It was observed a significant increase in both HCV core and NS5A protein levels upon treatment This is

confirmed with Western blot analysis of the lysates from HCV replicating Huh7.5.1 cells that were harvested 72 h pe in addition to the confocal immunofluorescence staining (Fig. 28a, b, and c). In line with this, we wanted to see if there was an effect on the HCV RNA replication or the Nrf1 gene expression. It could not be observed a significant change in them. Although according to (Simon and Butt 2015) expression of LDLR (which in our study was shown to be increased upon treatment of HCV replicating Huh7.5.1 cells.) leads to the build-up of HCV RNA within the hepatocytes.
In the end, we wanted to examine if lowering the intracellular cholesterol affects the HCV egression and it was observed that there was a significant decrease in the HCV released (Fig. 31). One explanation could be that withdrawing the cellular cholesterol could have led to the disturbing of the major lipid species-synthesis that is essential for the lipid droplets formation (Liefhebber et al. 2014). This can also be observed in the confocal immunofluorescence imaging (Fig. 28d) where lipid droplet formation was significantly reduced after withdrawing the intracellular cholesterol using mβCD. This could disturb the intracellular lipid transport, causing functional inhibition of late endosomes and formation of MLBs (Elgner et al. 2016), which would be interesting to examine if this led to an accumulation of the LVPs in late endosomes and MLBs as both HCV core and NS5A were significantly accumulated, which will then explain the reduction in the HCV genome copies released. Also, it would be interesting to examine if the apoE accumulates following the withdrawal of the intracellular cholesterol that would reflect an impairment in the endosome-dependent release of VLDLs. As it was found also that after cholesterol withdrawal the number of released HCV genome copies in the supernatant were significantly reduced.

It was also shown in a previous study that upon short-term NPC1-inhibitor treatment the prevalent effect was a decrease in the virus production with no effect on the RNA replication (Stoeck et al. 2018). It was shown also that inhibiting the intracellular lipid trafficking in HCV-replicating cells using the amphiphatic inhibitor U18666A, which directly inhibits the NPC1 protein leading to accumulation of cholesterol in lysosomal storage structures, impaired the viral release without affecting the viral replication in addition to a slight accumulation in the HCV core (Elgner et al. 2016). Thus, it would be interesting to see the effect of mβCD on the NPC1 function.

It would also be recommended to check the infectivity of the released particles through TCID50 as well as the intracellular particles. Also, if withdrawing the intracellular cholesterol using mβCD would shift the viral density gradient.

It was shown in a previous study that the statins-mediated inhibition of HMGCR (HMG-CoA reductase), that is a major regulator in the cholesterol synthesis pathway, caused an impairment in the HCV RNA replication (Ikeda et al. 2006), and in our study we observed that mβCD treatment for 4hrs led to an elevation in the HMGCR levels, but didn't affect the HCV genome replication. Thus, it would be interesting to see a comparative study on the HCV replication.

6.5 Effect of Nrf1 processing modification on the Hepatitis C virus

Ubiquitinated Nrf1 is rapidly degraded via proteasomes (Sha and Goldberg 2014). Since bortezomib is known to be a proteasome inhibitor (Paramore and Frantz 2003). Also, taking into consideration that HCV can affect the proteasomal activity, as the later was reported to be regulated by intracellular oxidant, which multiple agents in liver cells, including viral proteins, in particular, the core protein, enhance their generation (Osna et al. 2008).

We wanted to study if modifying the Nrf1 processing using bortezomib in the presence and absence of high cholesterol (complex) in Huh7.5.1 cells would affect Nrf1 as well as the HCV virus,

We could observe in non-HCV replicating cells that there was a significant increase in the Nrf1 protein levels in the absence of high cholesterol. Mostly due to the increase in the Nrf1 cleaved form (Fig.33a), as inhibiting the rapid proteasome-mediated Nrf1 degradation allows the Nrf1 to be cleaved or partially proteolyzed and thus enters the nucleus regulating the transcription of many cytoprotective genes. In the case of the presence of high cholesterol, this increase was attenuated, as the ER located full-length Nrf1 remains in the cytoplasm to bind the cholesterol. Additionally, in a previous study (Radhakrishnan et al. 2010), it was reported that the Nrf1 independent from Nrf2, is essential for proteasome gene transcription induction in mouse embryonic fibroblasts (MEFs) via the transcriptional feedback loop mechanism. In line with this, it was observed in our study that in both the HCV-replicating cells and non-HCV-replicating ones, the bortezomib treatment led to a significant induction in the Nrf1, while in the presence of high cholesterol the induction was attenuated. This correlates with a previous study (Widenmaier et al. 2017) which reported that cholesterol excess

suppresses Nrf1 cleavage and indeed attenuated the induction effect on Nrf1 after epoxomicin treatment, but since the same pattern was observed in both HCV +ve and -ve cells, therefore, suggesting that the HCV may have no influence on this. This is confirmed with Western blot analysis of the lysates from HCV replicating Huh7.5.1 cells that were harvested 72 h pe. In addition to the confocal immunofluorescence staining where the nucleus-translocated cleaved Nrf1 can be observed, while after excess cholesterol treatment it can be observed how the Nrf1 full-length in this case was retained in the cytoplasm and remained bound to the ER (Fig. 33b&c). Moreover, Bortezomib was reported in a previous study (Sowa et al. 2008) to have had an antiviral effect on HCV replication, which was accompanied by elevated IFN-β production. Conversely in our results, it was observed that there was a non-significant increase in the HCV genome and also no effect on the Nrf1 gene expression (Fig. 34). However, in our study, it was also observed that Nrf1 processing modification via bortezomib treatment led to an increase in the HCV core protein levels, while in combination with high cholesterol has significantly enhanced it even further. Nonetheless, there was no change in the NS5A protein levels. However, it was interesting to observe the synergistic effect of bortezomib and cholesterol on HCV core, and also would be interesting to take a closer look and see if this synergistic effect was also associated with increased IFN-β production, and whether this as yet unrecognized property of bortezomib may be of therapeutic value in the treatment of chronic HCV infection remains to be clarified.

In the same context, we wanted to test the effect of the different treatments on the Nrf1 regulated genes (Fig. 35). In the case of (ABCA1), it was significantly suppressed. This gene is targeted and regulated by the cholesterol-removal program initiating factor (LxR) (Widenmaier et al. 2017). It is in itself inhibited when more of the cleaved Nrf1 form enters the nucleus. However, when combined with cholesterol, we observed a significant induction in it, since less cleaved Nrf1 enters the nucleus as it remains ER-bound in the cytoplasm sensing and binding to the excess cholesterol. This shows that the treatment has worked, although, there was no difference between the HCV-replicating and non-replicating Huh7.5.1. This shows that the virus does not affect this gene. In other cases with the (HMGCS1, HMGCR, & LDLR) genes that are targeted and regulated by the cholesterol-producing program initiating factor SREBP2 (Widenmaier et al. 2017). It is in itself promoted when more of the cleaved Nrf1 form

enters the nucleus. We observed that after the bortezomib treatment in non-HCV replicating Huh7.5.1 cells, there was as expected an induction in these genes which upon cholesterol treatment was reduced. However, after the bortezomib treatment in HCV-replicating Huh7.5.1 cells, we observed a change in the expression pattern of these genes. Their expression levels were reduced, one explanation could be that similar to the Nrf2 after Nrf1 was cleaved from the ER-membrane, it could have been trapped to the viral proteins via the virus-mediated delocalized sMAF protein. Thus, raising the question if the Nrf1 also colocalizes with the sMAF proteins in the cytoplasm.

Furthermore, we also wanted to study the influence of the Nrf1 processing modification on the HCV released. We could see that it has significantly reduced the release of the virus and this reduction was even enhanced more in the case of the high cholesterol levels (Fig. 35), which correlates with the antiviral effect of the Bortezomib on HCV reported earlier (Sowa et al. 2008). Although it was reported that cancer patients under bortezomib treatment suffered episodes of HCV reactivation (HCVr) (Torres et al. 2018). But since HCV core was also reported to be degraded via proteasome-mediated degradation either through Ubiquitin-Dependent or PA28γ-Dependent pathways (Suzuki et al. 2009). So it would be interesting to see the effect of the Nrf1-knockout on the HCV core and release in a more clear way.

The knowledge generated in this study gives insights into the understanding of the crosstalk and relationship between Hepatitis-C Virus and Nrf1 activity and the effect of cholesterol level modulation on both of them and what happens to HCV upon modulating Nrf1 and also opens up a lot of new question to be addressed in the future for a better understanding of the complete spectrum of ER adaptive systems, and how they are integrated during metabolic stress, that may aid in a promising therapeutic opportunity to emerge.

7 Zusammenfassung

Schätzungen zufolge sind weltweit etwa 71 Millionen Menschen chronisch mit dem Hepatitis-C-Virus (HCV) infiziert. Im Jahre 2016 sind rund 400.000 Menschen an einer HCV-bedingten Lebererkrankung gestorben, insbesondere aufgrund der Entwicklung von Leberzirrhose und Lebertumoren. Trotz der großen Unterschiede in den Prävalenzschätzungen und der Qualität der epidemiologischen Daten zeigt die jüngste weltweite Bewertung, dass die virämische Ausbreitung der HCV-Infektion (Prävalenz der HCV-RNA) in den meisten Industrieländern, einschließlich der USA, weniger als 1,0% beträgt (www .cdc.gov / Hepatitis / HCV). In einigen osteuropäischen Ländern wie Lettland (2,2%) oder Russland (3,3%) und bestimmten Ländern in Afrika, Ägypten (6,3%) und Gabun (7,0%) oder im Nahen Osten Syriens (3,0%) ist die Prävalenz bemerkenswert höher. In den USA und den am weitesten entwickelten Ländern gilt die gemeinsame Nutzung von Werkzeugezur Herstellung von Arzneimitteln und zur Injektion von Medikamenten (Nadeln) als die häufigste derzeitige Übertragungsart. Die vorherrschende Übertragungsart in Ländern, in denen die Ausbreitung von HCV-Infektionen im Vergleich zu den Industrieländern höher ist, beruht jedoch auf schlechten Methoden zur Infektionskontrolle und unsicherer Handhabung von Injektionsnadeln.

Wenn die chronische Infektion unbehandelt bleibt, kann sich im fortschreitenden Verlauf eine Zirrhose oder ein hepatozelluläres Karzinom bilden (Alter H. J. und Seef L. B. 2000). Die Doppeltherapie, bei der es sich um eine Kombination aus pegyliertem Interferon-α (PEG IFNα) und Ribavirin (riba) handelt, war in einigen Ländern der Dritten Welt bis vor kurzem der goldene Standard für die Behandlung von Patienten mit chronischer Hepatitis C und hat eine anhaltende virologische Reaktion erzielt. Mit nur 50% der mit HCV-Genotyp 1 infizierten Patienten (der häufigere) im Vergleich zu 80% mit Genotyp 2 oder 3, obwohl sie kostspielig und langwierig sind (z. B. 24-48 Wochen) und zahlreiche harte Nebenwirkungen aufweisen, die schwer zu bekämpfen sind tolerieren (Erklärung der National Institutes of Health Consensus Development Conference: Management von Hepatitis C: 2002 - 10.-12. Juni 2002 2002). Die Identifizierung des JFH1 (japanische fulminante Hepatitis Typ 1) -Isolats wurde in einigen *in vitro*-Studien zu HCV als wichtiger Durchbruch bei der HCV-Behandlung angesehen. Die Verwendung dieses Isolats führte nachfolgend zu einem besseren

Verständnis des HCV-Lebenszyklus und der 3D-Strukturen der viralen Proteine. Basierend auf dieser Erkenntnis konnten die ersten direkt wirkenden antiviralen Mittel (DAAs) entwickelt werden, die spezifisch virale Proteine beeinflussen. Die beiden Proteasehemmer (PI) Telaprevir und Boceprevir hemmen die virale NS3-4A-Protease und wurden 2011 als Kombinationstherapie mit PEG IFNα und Ribavirin zugelassen, was die anhaltende virologische Reaktion auf 67-75% erhöhte (Pawlotsky et al. 2015).

Die Optimierung der gegenwärtigen Arzneimittelregime, die Einschränkung des Problems der Mutationsresistenz, die Gestaltung einer individualisierten Therapie, der Zugang zu diesen therapeutischen antiviralen Arzneimitteln und ihr hoher Preis bleiben weiterhin eine Herausforderung (Pawlotsky 2016; Pawlotsky et al. 2015; Sarrazin 2016). Die Entwicklung eines Impfstoffs wird jedoch als größte Herausforderung für die weltweite Kontrolle von HCV angesehen (Bukh 2016). Aus diesem Grund ist es wichtig, weiterhin mehr über den HCV-Lebenszyklus und die Faktoren zu erfahren, die sich auf die Replikation und den gesamten Lebenszyklus auswirken können, um effiziente, qualitativ hochwertige und vor allem leicht zugängliche Behandlungen für alle Menschen weltweit zu entwickeln.

Der Lipidstoffwechsel und insbesondere das Cholesteringleichgewicht werden durch die HCV-Infektion beeinflusst. Die Korrelation zwischen Lipidstoffwechsel und HCV wurde klinisch seit langem beobachtet. In den Leberbiopsien von mit HCV infizierten Patienten wurde ein Anstieg der in den Lipidtröpfchen im Cytosol akkumulierten neutralen Lipide festgestellt (Dienes et al. 1982). Das Hepatitis-C-Virus wurde auch von Hypobetalipoproteinämie, Hypocholesterinämie und Lebersteatose begleitet (Schaefer und Chung 2013). Die Leber ist der primäre Ort für die Synthese, Speicherung und Oxidation von Lipiden und anderen Makromolekülen. Daher ist der Fettstoffwechsel in der Leber für die Aufrechterhaltung der systemischen Nährstoffhomöostase von wesentlicher Bedeutung. Eine Dysregulation des Leberlipidstoffwechsels ist ein Kennzeichen mehrererKrankheiten wie Diabetes, alkoholische und nichtalkoholische Fettlebererkrankungen sowie parasitäre und virale Infektionen, einschließlich einer HCV-Infektion. (Erklärung der National Institutes of Health Consensus Development Conference: Management von Hepatitis C: 2002 - 10.-12. Juni 2002 2002; Fon Tacer und Rozman 2011; Chen et al. 2013; Reddy und Rao 2006; Visser et al. 2013; Wu und Parhofer 2014)

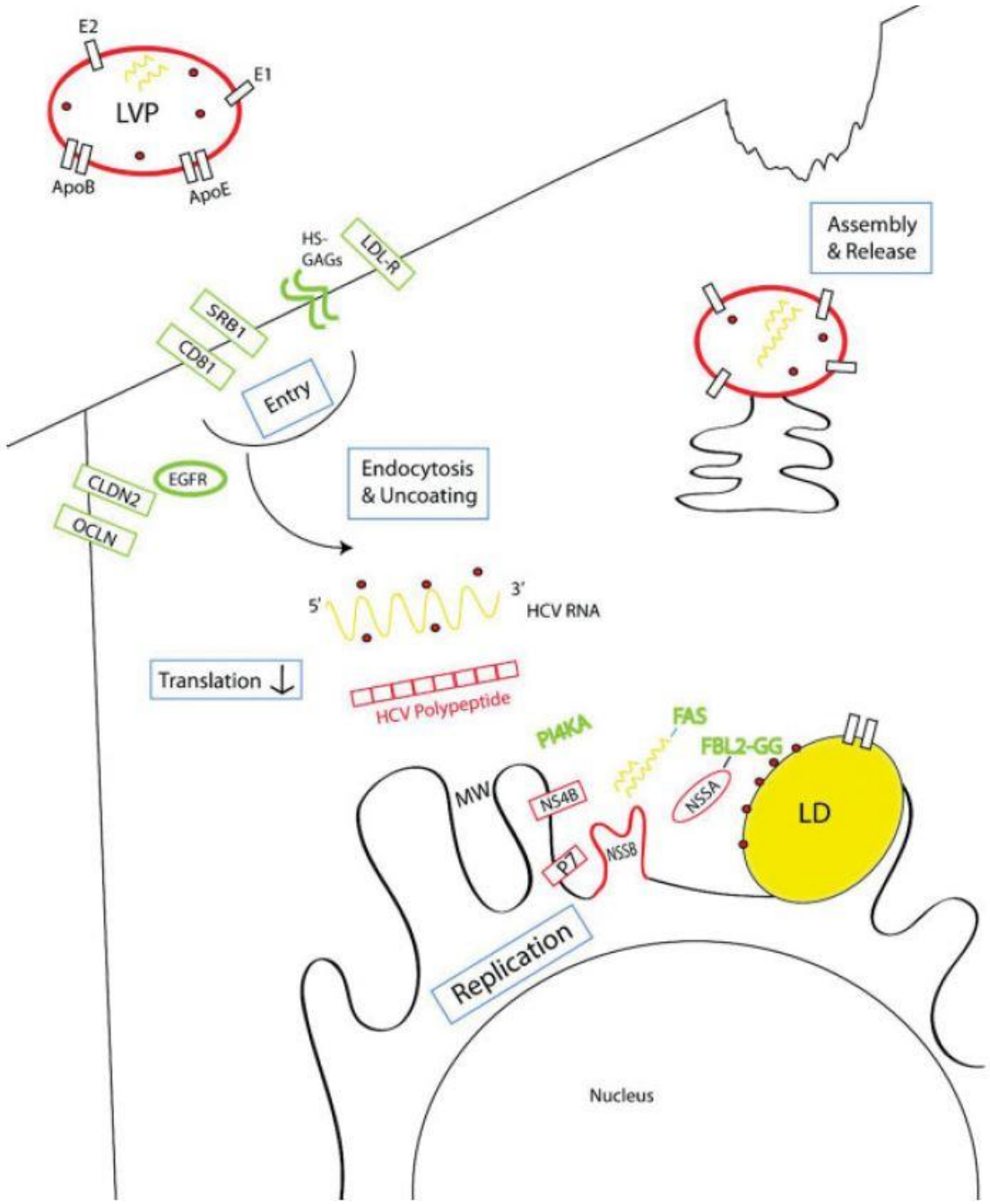

Figure 37: Virusreplikation des Hepatitis C-Virus (HCV).

Der HCV-Viruslebenszyklus und der Lipidstoffwechsel sind eng miteinander verbunden. HCV zirkuliert als stark lipidiertes lipovirales Partikel (LVP), angereichert mit den Apolipoproteinen B und E (apoB, apoE) zusammen mit den Kernzellen der viralen Strukturproteine E1 und E2. Das LVP gelangt über mehrere erforderliche Eintritts-Cofaktoren in die Zelle. Dazu gehören Heparansulfatglycosaminoglycane (HS-GAGs), CD81, Scavenger-Rezeptor B1 (SRB1), Claudin1 (CLDN1), Occludin (OCLN), LDL-Rezeptor (LDL-R) und epidermaler Wachstumsfaktorrezeptor (EGFR). Nach dem Eintritt löst sich das Virus und enthüllt das nackte HCV-Genom, das von der Wirts-Translationsmaschinerie in ein virales Polypeptid übersetzt wird. Das Polypeptid

wird durch eine Kombination von Wirts- und Virusproteasen gespalten; Der Replikationskomplex bildet sich auf einem spezialisierten membranösen Netz (MW) aus dem endoplasmatischen Retikulum, das eng an Lipidtröpfchen (LD) anliegt, die zusammen mit apoB und apoE im HCV-Kern angereichert sind. Die Replikation erfordert Wirtsfaktoren, die am Lipidstoffwechsel beteiligt sind, einschließlich Phosphatidylinositol-4-phosphat (PI4KA), Fettsäuresynthase (FAS) und Geranylgeranylierung des Wirtsproteins FBL2 (FBL-GG). Das Virus usurpiert dann Lipoproteinsekretionsschritte mit sehr geringer Dichte, um neue LVPs zusammenzusetzen und freizusetzen.

Kürzlich wurde auch über den Zusammenhang zwischen Lipiden und der Nrf1-Aktivität berichtet. Aus diesem Grund sollte im Rahmen dieses Projekts die Beziehung zwischen dem Hepatitis-C-Virus und der Nrf1-Aktivität sowie die Auswirkung der Cholesterinspiegelmodulation auf beide untersucht werden, um zu verstehen welche Effekte eine Modulation von Nrf1 auf HCV ausübt.

Trotz der signifikanten Fortschritte in der HCV-Therapie sind Arzneimittelresistenzen und genotypspezifische Wirksamkeit immer noch zu berücksichtigen. Aus diesem Grund sollte das Ziel dieser Studie sein, welche Licht auf diesen neuen *Crosstalk* wirft, eine vielversprechende therapeutische Chance zu bieten, die sich wahrscheinlich aus der Identifizierung des gesamten Spektrums ER-adaptiver Systeme und ihrer Integration bei metabolischem Stress ergeben wird.

Im Zuge dieser Studie konnte beobachtet werden, dass das Hepatitis-C-Virus tatsächlich einen Einfluss auf Nrf1 auf Protein- und mRNA-Ebene hat. Andererseits wurde auch gezeigt, dass eine Überexpression von Nrf1 das intrazelluläre Überdauern und die Replikation von HCV begünstigt, gleichzeitig aber die freigesetzte HCV-Genommenge verringert, was auf eine Art Blockierung oder Störung beim Zusammenbau des Viruspartikels zurückzuführen sein könnte. Es wurde ebenso beobachtet, dass eine Erhöhung des intrazellulären Cholesterinspiegels tatsächlich die Nrf1-Proteinmenge aufgrund der Zunahme an Nrf1-Protein voller Länge erhöht und gleichzeitig auch die HCV-Genommenge und die Coreproteinmenge erhöht, ohne die NS5A-Proteinmenge zu beeinflussen. Aber überraschenderweise führt dies gleichzeitig zu einer Abnahme des freigesetzten HCV-Genoms. Es konnte zudem festgestellt werden, dass eine Senkung des Cholesterinspiegels zu einem Anstieg der Nrf1-Proteinmenge in den HCV-replizierenden Zellen aber nicht in den nicht-replizierenden Zellen führt.

In diesem Fall konnte der Anstieg der Nrf1-Proteinspiegel jedoch auf den Anstieg der Nrf1-gespaltenen Form zurückgeführt werden

Anstieg die gleichzeitig zu einer Zunahme des HCV-Core- und NS5A-Proteinspiegels führte. Obwohl hierbei keine Auswirkungen auf die HCV-Genommenge oder die Menge an Nrf1-mRNA beobachtet werden konnten. Es wurde auch festgestellt, dass HCV keinen direkten Einfluss auf die Nrf1-Zielgene (ABCA1, HMGCS1, HMGCR und LDLR nach Cholesterinentzug) hatte. Zudem wurde beobachtet, dass die Hemmung derProteasom-vermittelten Degradation von Nrf1 zu einem Anstieg von Nrf1 führte, in überwiegend verkürzter Form, und dass, wenn diese Hemmung bei Bedingungen mit hohem Cholesterinspiegel auftrat, die Menge an Nrf1-Protein abnahm, aber eine Abnahme der gespaltenen Form und eine Zunahme der Proteine voller Länge zu sehen ist. Aber HCV hatte keinen weiteren signifikanten Einfluss auf Nrf1 in der Zellen, in denen der Proteasom-vermittelte Nrf1-Abbau gehemmt und das Cholesterin im Vergleich zu nicht-replizierenden Zellen erhöht war.

Zusammenfassend weisen diese Daten darauf hin, dass ein Zusammenhang zwischen der Nrf1-Aktivität, den Lipidradikalen und dem Hepatitis-C-Virus sowie zwischen der Nrf1-Regulation und der des HCV besteht, der weiterer und eingehenderer Untersuchungen bedarf.

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9 Abbreviations

abbreviation	Explanation
ACAT	acetyl-CoA C-acetyltransferase
9–13	Huh7 I377/ NS3–3'/wt/9–13
aa	amino acid
ABCA1	ATP-binding cassette transporter
AIDS	acquired immunodeficiency syndrome
apoE	apolipoprotein E
APS	ammonium persulfate
ARE	antioxidative response element
ARFP	Alternative reading frame protein
ATP	Adenosine triphosphate
BODIPY	boron-dipyrromethene
BSA	bovines Serum-Albumin
CD81	cluster of differentiation 81
cDNA	complementary DNA
CHX	cycloheximide
CLDN1	claudin-1
CLSM	confocal laser scanning microscope
CNC	Cap'N'Collar
CNC-bZIP	Cap'n' collar basic leucine zipper
CRAC	cholesterol recognition amino acid consensus
DAA	direct-acting antiviral drugs
DABCO	1,4-diazabicyclo[2.2. 2]octane
DAPI	4′,6-diamidino-2-phenylindole
ddH2O	double-distilled water
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxid
DMVs	double membrane vesicles
DNA	deoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	1,4-Dithiothreitol
E. coli	Escherichia coli
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EGTA	egtazic acid
EIA	enzyme immunoassay approach
ER	Endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ERK	extracellular-signal-regulated kinase
FAS	fatty acid synthase
FCS	Fetal calf serum
GAG	glycosaminoglycan

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GLD-2	germ Line development 2
Grb2	growth factor receptor-binding protein 2
GTPase	guanosine diphosphate enzyme
h pe	hours post electroporation
HCC	hepatocellular carcinoma
HCVcc	hepatitis C virus cell culture
HDL	high-density lipoprotein
HMGCR	HMG-CoA reductase
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1
Hrd1	3-hydroxy-3-methylglutaryl reductase degradation
HRP	horseradish peroxidase
HS-GAGs	heparan sulfate glycosaminoglycans
HSPGs	heparan sulfate proteoglycans
Huh7.5.1	human hepatoma cell line
HVR	hypervariable region
IFN	interferon
IgG	immunoglobulin G
IRES	internal ribosome entry site
IU	International unit
JFH-1	Japanese fulminant hepatitis 1
kDa	kilodalton
kb	kilobase
LC3	microtubule-associated protein light chain 3
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
LDs	lipid droplets
LVP	Lipo-Viro-Particles
LVP	lipo-viro-particle
LxR	liver-X receptor factor
mA	milliampere
MAPK	mitogen activated protein kinase
MAVS	mitochondrial antiviral signaling protein
MLB	multilamellar bodies
MW	membranous web
mβCD	methyl-beta-cyclodextrin
NFE2L1 (Nrf1)	nuclear factor erythroid-2-like 1
NPC1	niemann-Pick disease, type C1
PAGE	polyacrylamid-Gelelektrophorese
PBS	phosphate buffered saline
PEI	polyethylenimine
PEG IFN/riba	pegylated interferon alpha and ribavirin
PI	Protease inhibitor
PI3K-AKT	phosphatidylinositol 3-kinase
PI4KA	phosphatidylinositol 4-phosphate
PNGase	peptide:N-glycosidase F
PVDF	polyvinylidene difluoride
RdRp	RNA-dependent RNA polymerase

RLUs	relative luminometer units
RPL27	ribosomal protein L27
SDS	Sodium dodecyl sulfate
SL	stem-loop
sMAF	small Maf proteins
SREBP2	sterol regulatory element-binding protein 2
TBST	tris-buffered saline and Polysorbate 20 (Tween 20)
TAD	transactivation domain
UPR	unfolded protein response
VCP	valosin-containing protein
VLDL	very-low-density lipoprotein

10 Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne unerlaubte Hilfe angefertigt und andere als die in der Arbeit angegebenen Hilfsmittel nicht benutzt habe. Alle Stellen, die wörtlich oder sinngemäß aus anderen Schriften entnommen sind, habe ich als solche kenntlich gemacht. Diese Arbeit hat in gleicher oder ähnlicher Form noch keiner Prüfungsbehörde vorgelegen. Des Weiteren bin ich mit der späteren Ausleihe meiner Doktorarbeit an die Fachbereichsbibliothek einverstanden.

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