

Hepatitis C virus: Morphogenesis, infection and therapy

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Author contributions: Morozov VA and Lagaye S equally contributed to this review.

Conflict-of-interest statement: The authors have no conflict of interest to declare.

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Manuscript source: Invited manuscript

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Received: December 4, 2017

Peer-review started: December 5, 2017

First decision: December 11, 2017

Revised: January 11, 2018

Accepted: February 7, 2018

Article in press: February 7, 2018

Published online: February 27, 2018

Abstract

Hepatitis C virus (HCV) is a major cause of liver

diseases including liver cirrhosis and hepatocellular carcinoma. Approximately 3% of the world population is infected with HCV. Thus, HCV infection is considered a public healthy challenge. It is worth mentioning, that the HCV prevalence is dependent on the countries with infection rates around 20% in high endemic countries. The review summarizes recent data on HCV molecular biology, the physiopathology of infection (immune-mediated liver damage, liver fibrosis and lipid metabolism), virus diagnostic and treatment. In addition, currently available *in vitro*, *ex vivo* and animal models to study the virus life cycle, virus pathogenesis and therapy are described. Understanding of both host and viral factors may in the future lead to creation of new approaches in generation of an efficient therapeutic vaccine.

Key words: Hepatitis C virus; Transmission; Molecular biology; Pathogenesis; *In vitro* and *ex vivo* models of hepatitis C virus infection; Treatment

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Core tip: Brief overviews on epidemiology of hepatitis C virus (HCV), virus morphology and the virus life cycle are presented. A special attention was focused on *in vitro* and *in vivo* models that are currently used to study the HCV infection. In fact, extensive use of existing models and creating a new ones is a way to reveal important events in the virus-cell interaction. In particular, the models might shed light on the mechanisms behind virus induced pathogenesis and chronicity, and by that contribute to the development of new drugs and prophylactic vaccine. Recently, multiple therapies with a pan-genotypic activity appeared on the market. The new agents (third generation) and new inhibitors (entry inhibitors, release inhibitors) being studied, should allow to cure most of the patients in the mid-term, if they will have equal access to the therapy.

Morozov VA, Lagaye S. Hepatitis C virus: Morphogenesis,

infection and therapy. *World J Hepatol* 2018; 10(2): 186-212
Available from: URL: <http://www.wjgnet.com/1948-5182/full/v10/i2/186.htm> DOI: <http://dx.doi.org/10.4254/wjh.v10.i2.186>

INTRODUCTION

The hepatitis C virus (HCV) is a major blood borne human pathogen. There are approximately 120-130 million or 3% of the total world population that are HCV infected (Figure 1). According to World Health Organization (WHO), annually there are about 3-4 million new cases of infection^[1,2]. HCV is considered a major public health issue, since the virus is the etiological factor of chronic hepatitis that frequently progress to a cirrhosis and hepatocellular carcinoma (HCC). In developed countries, the most important route of HCV transmission is intravenous drug abuse, whereas in resource-poor countries invasive procedures or injection-based therapies with contaminated instruments are the predominant source of new infections^[3].

Without treatment, most of the acute infections progress to chronic ones, followed by liver disease, such as cirrhosis and HCC. Alcohol abuse and the metabolic syndrome are the main cofactors influencing the progression to advanced liver disease and HCC^[4]. Each year, about a third of liver transplantations are performed on patients with complications associated with the HCV infection, with decompensated cirrhosis or HCC^[5]. In the next decade, an increase in the burden of hepatitis C is expected because of aging of the currently infected population^[6,7]. During that period, the number of HCV-related cirrhosis cases is estimated to increase by 31% and HCC by approximately 50%^[6] with an additive effect due to the occurrence of the metabolic syndrome^[8,9]. Thus, HCV infection represents a major public health issue that should be addressed with strong policy interventions to effectively identify and treat HCV infected patients.

There are seven genotypes (gt 1-7) and numerous subtypes of HCV. The rate of infection and subtype prevalence are country depend. The difference between the infection rate in low and high endemic countries is about 20% (Figure 1)^[10,11]. For example, North America, Western Europe and North and Australia's have the lowest HCV prevalence. Conversely, in Asian and African countries the virus prevalence is high. The highest virus prevalence was registered in Egypt, where 22% of population is infected^[12]. It has been postulated that the epidemic has been caused by extensive iatrogenic transmission during the era of parenteral-antischistosomal-therapy mass-treatment campaigns before 1985^[13].

MOLECULAR BIOLOGY OF HCV

HCV morphology and parameters

HCV is a small, enveloped, positive single-stranded RNA virus that belongs to the *Flaviviridae* family, genus *Hepacivirus*. Analysis of viruses from plasma and

from cell culture supernatant indicated that enveloped particles are icosahedral and 56-65 nm^[14,15] in diameter, while and the viral core is about 45 nm^[14,15]. Viral spikes on the membrane of the virion are about 6 nm^[14] and they are formed by heterodimers of E1 and E2 glycoproteins. In fact, the population of the extracellular HCV particles is heterogeneous. Particles are pleomorphic and size, buoyant density, and infectivity might differ significantly^[14,15]. A large majority of particles is non-infectious. Interestingly, the buoyant densities of infectious particles isolated from serum and from cell culture medium are different. A significant amount of the particles are associated with cellular lipoproteins making that a hallmark mark of HCV^[16,17]. The pattern of virus-associated lipoproteins might differ and several of those are associated with HCV most frequently: low density lipoproteins (LDL), very low-density lipoproteins (VLDL) and apolipoproteins (Apo) A1, B, C and E (Figure 2)^[18]. The viral particles associated with lipoproteins are called "lipoviral particles (LVP)". More details on LVP are given in "Virus assembly and release" section. Detailed update on HCV-associated lipoproteins is given in the review by Grassi *et al.*^[19].

Viral genome

The genome of HCV is approximately 9600 nucleotides long (Figure 3A). It contains two highly conserved untranslated regions (UTR) 5'-UTR and 3'-UTR that are flanking a single open reading frame (ORF). Dependent on the genotype, the ORF might contain from 9030 to 9099 nucleotides and it is coding for a single polyprotein precursor of 3010 to 3033 amino acids (aa), respectively (Figure 3B and C)^[20,21]. Translation occurs in the endoplasmic reticulum and it is initiated by IRES at the 5' UTR^[22].

5'-untranslated region

The 5'-UTR is highly conserved including special site to control the HCV genome replication and the viral polyprotein translation. The region is 341 nucleotides long and it contains four distinct domains (I-IV). The first 125 nucleotides of 5'UTR spanning the domains I and II have been shown to be essential for the viral RNA replication. The domains III-IV composes an internal ribosomal entry site (IRES) involved in ribosome binding which can initiate viral polyprotein translation in a cap-independent manner^[20]. The initiation of HCV protein synthesis requires the ordered assembly of ribosomal pre-initiation complexes, beginning with the association of the small (40S) ribosomal subunit with a messenger RNA (mRNA). The cap-independent translation begins with the 40S ribosome binding and the scanning to the initiation codon which is followed by association with the 60S ribosomal subunit to form an active 80S ribosome^[21,22]. The HCV IRES is folded into highly structured domains which are called II to IV. The mutational analysis of the IRES domains suggested that a structural integrity was necessary for an efficient protein synthesis both *in vitro* and *in vivo*^[23-25].

Table 1 Overview of the size of hepatitis C virus proteins

Protein	No. of aa	aa position n ref. seq.	MW of protein, kDa
Core immature	191	1-191	23
Core mature	174	1-174	21
F protein of ARF protein	126-161		Approximately 16-17
E1	192	192-383	35
E2	363	384-746	70
p7	63	747-809	7
NS2	217	810-1026	21
NS3	631	1027-1657	70
NS4A	54	1658-1711	4
NS4B	261	1712-1972	27
NS5A	448	1973-2420	56
NS5B	591	2421-3011	66

No. of aa: Number of amino acid; MW: Molecular weight; kDa: KiloDalton; ref.seq.: Reference sequence (HCV strain H77 ; accession number NC_0041).

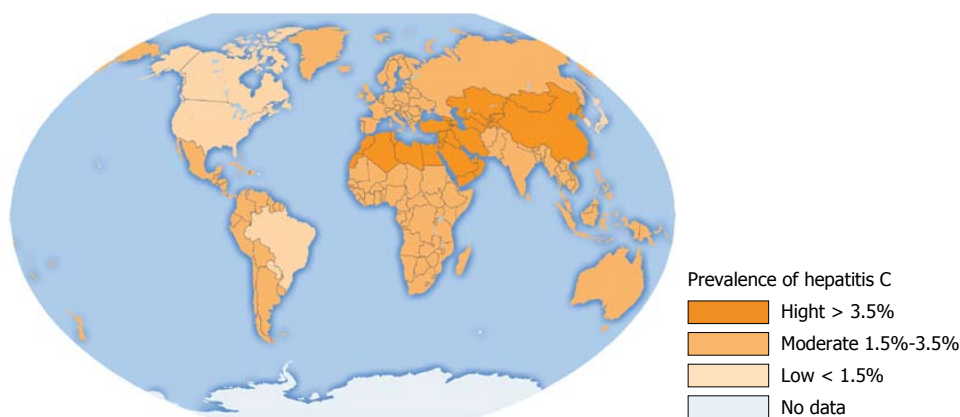


Figure 1 Hepatitis C virus infection in the World. Analysis of seroprevalence^[2].

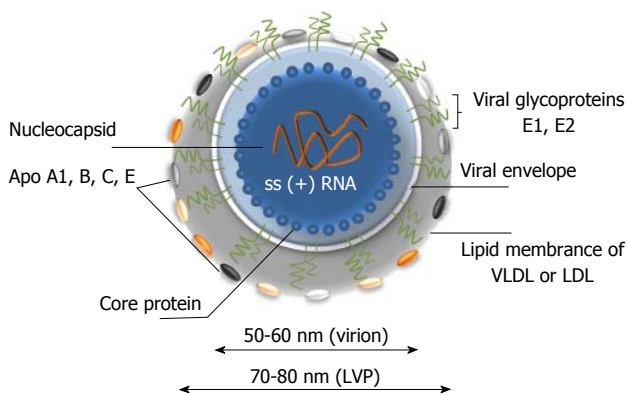


Figure 2 A model of hepatitis C virus lipoviral particle. Lipid membrane formed by low density lipoproteins (LDL) and very low-density lipoproteins (VLDL) on the surface of the virion (given in grey). Viral core is given in blue and viral RNA is shown in orange. Heterodimers of glycoproteins E1 and E2 are partially embedded in the lipid bilayer and are forming 6 nm long spikes (projections) on the surface of the virion^[14-19]. As a result of association with LDL and VLDL, the morphology of the virion is not icosahedral. Depending on the viral source, the shape and size of the particles might vary.

3'-untranslated region

The 400 nucleotides long 3'-UTR region is thought to playing a crucial role in the HCV replication. The region is highly conserved and it is divided into three functionally parts: A variable sequence of 40 nucleotides, a variable

internal poly (U/UC) rich tract of 30 to 80 nucleotides (depending on the HCV strains), which is followed by a highly conserved 98-nucleotide X-tail containing three stable stem loop (SL) structures called: 3'SL1, 3'SL2, 3'SL3^[25].

Viral proteins

The translation occurs in the endoplasmic reticulum and it is initiated by IRES at the 5'UTR^[25]. A single polyprotein precursor is processed by cellular and viral proteases into ten proteins (Figure 3B). Three structural proteins (core, E1, E2) are located at the amino-terminal part of the polyprotein and are essential components of the virions. Seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) are located in the remaining part of the polyprotein and these proteins are involved in particle morphogenesis, RNA replication and in regulation of cell functions (Table 1). It is worth mentioning, that the structural and non-structural proteins of HCV are multifunctional. A brief characterization of the protein is given below.

Core protein (p22)

The core protein of HCV is translated as an immature protein of 22 kDa, that it is composed of 191 amino acids (aa). It is excised from the polyprotein in the

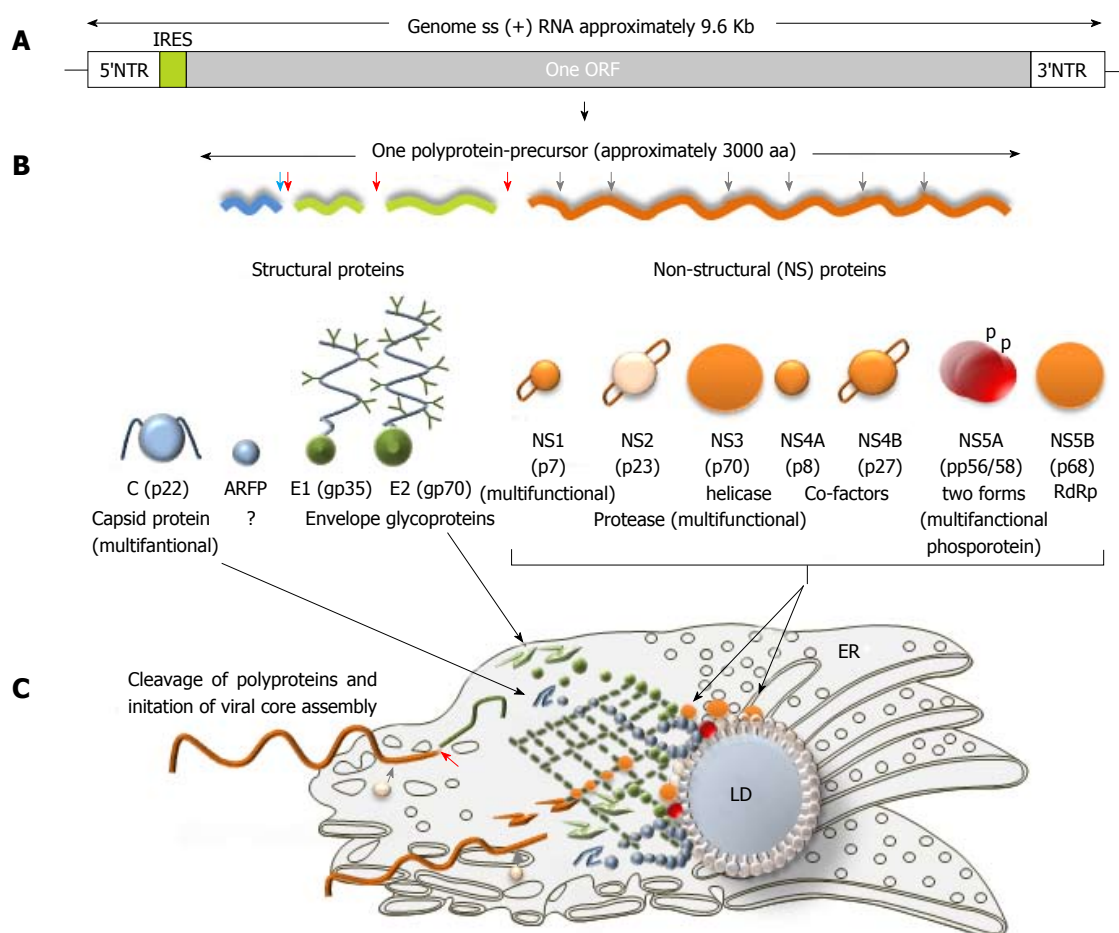


Figure 3 Hepatitis C virus genome, polyprotein precursor and the initial steps of core assembly in endoplasmic reticulum. A: Being structurally identical, the genomes of seven hepatitis C virus (HCV) genotypes demonstrated approximately 30% of sequence diversity^[140]. Two non-translated regions (5'- 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^[183].

endoplasmic reticulum (ER) by a cellular signal peptidase (SP)^[26]. An additional cleavage of the immature protein results in the mature 21 kDa core protein. The core protein contains three domains. The first domain spans the N-terminal region of 117 aa (aa 1-117). It contains mostly basic residues and there are two short hydrophobic regions. This domain is involved in binding to the viral RNA. The second domain between the aa 118 and 174 is more hydrophobic and less basic. This domain is engaged in the creation of links with the lipid droplet (LD). LDs are intracellular structures that are used for the lipid storage (Figure 3C)^[18]. The third domain is localized between the aa 175 and aa 191 and it contains the signal sequence for the ER membrane translocation of E1 ectodomain^[27]. *In vivo*, the mature core proteins are believed to form homo-multimers that are accumulated mainly at the ER membrane and can be self-assembly into the HCV-like particles^[28]. During

the viral capsid assembly, the protein also interacts with the HCV RNA. Besides that, the core protein poses regulatory functions during the RNA translation. Analyses of HCV core protein expression indicate that additionally it may be involved in several processes in cells such as apoptosis, lipid metabolism [HCV-related (mainly gt 3) fatty liver] and the development of HCC^[29]. Interestingly, the core protein can induce the redistribution of LDs by the regeneration and the regression of these organelles in specific intracellular domains^[30]. This phenomenon is also linked to the assembly of HCV particles. LDs are associated with the HCV core protein at the second domain and the interaction seems to trigger steatosis^[30]. It has been shown, that if the core protein is mutated, the association with LDs might be disrupted. A decrease of this association has a negative effect on the HCVcc production. The HCV core protein was also shown to be involved in the Ca²⁺ regulation^[31]. Thus, it is not only a

basic structural element of HCV, but it is an active player in a significant amount of additional processes, including signaling pathways regulation^[17,30,31].

Envelope proteins E1 (gp35) and E2 (gp70)

Glycoproteins E1 (gp35 kDa, 192 aa long) and E2 (gp70 kDa, 363 aa long) are type 1 transmembrane proteins that are forming the envelope of the virus particle (Figures 2 and 3B). E1 and E2 are cleaved from the precursor in ER by the cellular SP. The glycoproteins contained large hydrophilic ectodomains and 30 aa long transmembrane domains (TMD). The TMD is responsible for an anchoring of the envelope proteins in the membrane of the ER and their ER retention^[32]. The ectodomains of both glycoproteins are heavily glycosylated. The E1 has 4-5 and the E2 - 11 putative N-glycosylation sites, respectively. Interestingly, the glycosylation sites are rather conserved in each genotype^[33], but the number of glycosylation sites varies between the genotypes. It's worth mentioning, that some of the glycan's are engaged in folding and formation of the E1-E2 heterodimer complexes on the surface of the virion. These complexes are essential for the interaction with cellular receptors and helps to promote the virus-to-cell fusion^[34].

The HCV E2 contains two hypervariable regions HVR 1 and HVR2 which are the most mutable parts of the HCV genome. The first 27 amino acids of E2 are attributed to the HVR1 sequence. It is suggested that a prominent heterogeneity of the region could help the virus to evade from the immune system pressure and to develop a chronic infection^[35]. It is worth mentioning, that an infectious clone lacking the HVR1 was still capable to infect chimpanzee, but with a largely reduced efficiency, thus supporting the HVR1 engagement in the cell infection^[36]. The HVR2 includes seven aa (91-97) and this region was shown to contribute to the HCV E2 receptor binding^[37]. The high variability of the HVRs reflects the exposure of these domains to HCV-specific antibodies. In fact, the E2-HVR1 is the most frequent target for neutralizing antibodies^[38,39]. However, the combination of a viral mutation with the selective pressure of the humoral immune response leads to the viral escape *via* epitope alterations^[40]. Moreover, the association of virions with lipoproteins and the presence of a glycan shield (possessed by the E1-E2 heterodimers), reduce the antibody access to the "sensitive" neutralizing epitopes^[24,41,42]. This protection is representing a serious obstacle on the way to obtain broadly neutralizing antibodies.

Non-structural protein NS1 (p7)

The NS1 (also called "p7") is a small 63 aa long protein. It is located between E2 and NS2 proteins and it is linked to both structural and non-structural proteins. Its cleavage is mediated by the cellular SP. Interestingly, the E2-p7-NS2 precursor can also be detected^[43,44]. It is suggested, that such a precursor may regulate the

kinetic of the HCV infection; however, up to now the precise role of this protein in the HCV life cycle remains obscure. NS1 has two transmembrane domains (TMDs) embedded in the ER membrane and the C-terminal TMD of NS1 can act as a signal sequence to promote the translocation of NS2 to the ER lumen^[45]. The protein can also form the ion channel which has an important role in the HCV infection. This channel can be blocked by the antiviral drug^[45]. The recent studies demonstrate that NS1 acts to prevent the acidification which is required for the production of HCV particles^[46]. In addition, the protein appears to be essential during assembly and release of infectious particles as shown on different genotypes^[47]. Based on that, NS1 may be considered as a potential target for new antivirals^[48,49].

Non-structural protein NS2 (p23)

The NS2 is a hydrophobic transmembrane protein of 23 kDa composed of 217 aa. It is a cysteine protease which is required for the HCV infectivity^[50]. The N-terminal residues of NS2 can form 3 or 4 transmembrane helices which are inserted in the ER membrane. The crystal structure has been determined. The C-terminal residues of NS2 with the 181 aa long N-terminal domain of NS3 play an important role in the function of the NS2/3 cysteine protease^[51]. The NS2/3 protease is spanning from the amino acid 810 to amino acid 1206. It has been shown that the NS3 zinc-binding domain could stimulate the activity of the NS2 protease^[52]. The well-known function of NS2 is the auto-cleavage at the NS2/3 site^[53]. Oem *et al.*^[53] suggested that the expression of HCV NS2 results in the up-regulation of the fatty acid synthase transcription. In fact, this may implicate the role of NS2 in the prompting HCV induced steatosis^[54]. There data are in favor of the protein engagement in the viral assembly and release^[54].

Non-structural protein NS3 (p70)

The NS3 is a 70 kDa protein composed of 631 aa. It is cleaved at its N-terminus by the viral NS2/NS3 autoprotease. The C-terminal part of NS3 (442 aa) has an ATPase/helicase activity, and it catalyses the binding and unwinding of the viral RNA genome during the viral replication^[55,56]. The N-terminal part (189 aa) of the NS3 protein has both serine protease and NTPase activities^[56]. The NS3 along with the non-structural protein NS4 is involved in cleavage of the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions^[57,58]. A direct interaction between NS3 and NS5B is mediated through the protease domain of NS3. It is suggested, that these proteins may act together during the HCV replication^[59]. The HCV protease NS3/4A can also cleave some cellular targets involved in the innate immunity, such as MAVS (an antiviral signaling protein), which can indirectly activate the nuclear factor-kappa B (NF- κ B) and the IFN regulatory factor 3 to induce type I interferon^[60,61]. The protein NS3/NS4A can interfere with the adaptive immunity. In addition, the NS3/NS4A protease is

essential for the viral infectivity, thus, it is a promising target for antivirals^[61-64]. In 2011, two potent NS3/NS4A inhibitors, boceprevir^[61] and telaprevir^[62] were approved by FDA and EMA and were used in combination with IFN α and ribavirin. However, several resistance-associated mutations within the NS3/NS4A coding region have been observed.

Non-structural protein NS4A (p8)

The NS4A is an 8 kDa proteins that is composed of 54 aa. As indicated above, it is a co-factor required for the NS3 protease activity. The N-amino-terminal domain of NS4A is hydrophobic and the deletion analysis shows that NS4A was required for the ER targeting of NS3^[65-68]. In addition the NS4A interaction with NS5A is essential for the phosphorylation of NS5A^[68]. Besides its important role in HCV replication, NS4A can also contribute to the viral pathogenesis by influencing some cellular function^[66]. Interestingly, NS4A was detected not only on the ER, but also on the mitochondria either alone or together with NS3 in the form of NS3/4A polyprotein. It has been shown, that the NS4 expression altered the distribution of mitochondria and caused damage which leads to the host cell apoptosis^[66].

Non-structural protein NS4B (p27)

The NS4B is a 27 kDa protein of 217 aa. It has four membrane spanning domains which are important in recruitment of the other non-structural viral proteins^[68]. By electron microscopy, it has been shown, that NS4B induced a tight structure termed "membranous web". All viral proteins were found to be associated with this membranous web that formed a viral replication complex ("factory") in HCV infected cells^[67]. The polymerization activity and lipid modification of NS4B are important for the induction of the specialized membrane structure involved in the viral RNA replication^[68,69]. NS4B is likely not engaged in the virus replication, but may contribute to the assembly and the release of the virus particles^[69].

Non-structural protein NS5A (p56/p58)

The NS5A is a 56 kDa phosphoprotein of 485 aa (Figure 3). In HCV infected cells, the protein is present in two differently phosphorylated forms: phosphorylated (56 kDa) and hyper phosphorylated (58 kDa). NS5A is multifunctional and it contributes to the HCV replication, virus pathogenesis, modulation of cell signaling pathways, virus propagation and the interferon response^[70,71]. The N-terminal amphipathic helix of the NS5A protein is conserved in different genotypes and it is necessary for the membrane localization^[72-76]. Mutations in NS5A enhanced the capacity of the sub genomic HCV RNA replication in cell culture systems^[77]. It was shown that the N-terminal part of NS5A contained a new zinc-coordination motif which affected the HCV replication^[70]. NS5A has a potential role in mediating the IFN response. It contains a so-called "interferon- α sensitivity-determining region" (ISDR, aa 237-276)^[78]. ISDR can

be used to predict the resistance and the sensitivity of HCV to the IFN treatment. NS5A was also showed to be associated with a variety of cellular signaling pathways including apoptosis^[70]. NS5A is already used as a target for the direct acting antivirals.

NS5B protein (p66-68)

The NS5B is a 591 aa long protein and it is located at the C-terminus of the precursor. NS5B is a RNA-dependent RNA polymerase containing the GDD motif in its active site^[79]. The NS5B initiates synthesis of the HCV negative-strand RNA. The crystal structure of NS5B showed a typical 'right hand' polymerase shape with finger, palm and thumb sub domain. Since NS5B lacks the "proof-reading" function^[79], numerous mutants might be generated during transcription. Because of its key role in the virus replication, the NS5B protein is considered as a potential target for the antiviral drug^[80].

F protein, ARFP

In addition to ten proteins described above, the frameshift (F) or alternate reading frame protein (ARFP), or "core+1" protein has been reported^[81-83]. The ARFP is the result of a -2/+1 ribosomal frameshift between codons 8 and 14 of the adenosine-rich region encoding the core protein. ARFP ends have different stop codons depending on the genotype. Thus, its length may vary from 126 to 161 amino acids. In the case of genotype 1a, the protein contains 161 amino acids. However, the situation with ARFP is more complicated, since alternative forms of this protein such as ARFP/DF (double-frame shift) in genotype 1b, and ARFP/S (short form) were recently described^[83]. ARFP is a short-living protein located in the cytoplasm^[84] in associated with the endoplasmic reticulum^[85]. Detection of anti-ARFP antibodies in sera of HCV-positive subjects indicates that the protein is expressed during infection^[86], but is likely not involved in the virus replication. Some findings suggested engagement of ARFP in the modulation of dendritic cells function and stimulation of the T cell responses^[87]. The implication of ARFP in the viral life cycle remain to be elucidated.

HCV TRANSMISSION ROUTES

The HCV in blood and blood products is the main source of infection. However, the transmission routes of HCV might be different and country dependent. The iatrogenic transmissions are: the blood transfusion of unscreened products^[88], the transfusion of clotting factors or other blood products^[89,90], the organ transplantation, the reuse of medical instruments used in invasive settings (*e.g.*, needles, infusion sets, syringes, catheters) in Egypt before 1985, hemodialysis, endoscopy, intravenous drug use^[91-95]. The sexual way of transmission is controversial. Nevertheless, the risk may increase when favoring conditions such as sexually transmitted infections, the frequencies and the type of sexual activity are taking

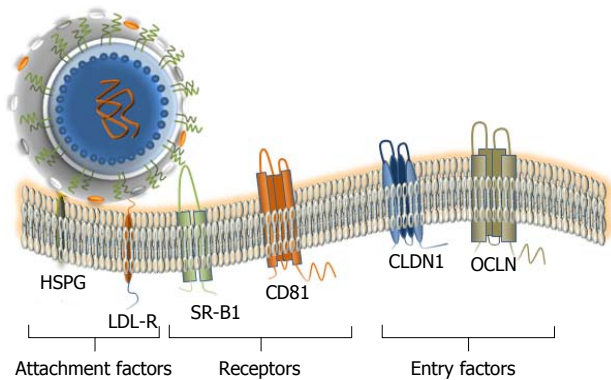


Figure 4 Attachment factors, receptor and entry factors utilized by hepatitis C virus. At least six membrane proteins are essential for the virus attachment and entry. Heparan sulfate proteoglycan (HSPG) - a type of glycosaminoglycan (GAG) and low density lipoproteins (LDL)-receptor that is promoting 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.

place^[94-101].

The rate of HCV mother-to-child transmission is about 4.3%, but it is much higher, 22.1% among the human immunodeficiency virus (HIV) co-infected mothers^[102,103]. The mother-to-child transmission generally occurs at delivery, but also *in utero*, if associated with high risk factors, such as high maternal HCV RNA levels and/or HIV co-infection. Interestingly, vaginal vs cesarean section delivery, amniocentesis and breast-feeding did seem to increase the transmission risk^[102-108]. Because of a passive transfer of anti-HCV antibodies at, or after 18 mo of ages, perinatal transmission diagnosis as serum HCV RNA and/or anti-HCV antibodies should be performed twice in infants (6 mo and 1 year of age)^[109] during this period, as recommended by the European pediatric HCV network^[110]. It should be emphasized, that the INFL3 CC (IL28B) genotype is likely associated with a spontaneous clearance of the HCV genotype 1(gt1) infection^[104].

According to the World Health Organization, thousands of new cases of the HCV infection (as a result of an occupational exposure *via* skin injury), are registered annually^[111]. Most of these cases occurred during surgery in emergency departments, and routine medical procedures^[112]. Seroconversion rates in infected individuals are ranging from 0% to 10.3% (+/- 0.75%)^[113,114]. For instance, after accidental needle stick, seroconversion rate was reported as 1.8%^[115]. During a 5-year period (2008-2012), a total of 16 HCV outbreaks resulting in 160 outbreak-associated cases and more than 90000 at-risk persons notified for screening were reported by the Centers for Disease Control (CDC) and Prevention^[115]. Among dentists^[116] and surgeons^[117], an eye protection should be reinforced because of possible HCV transmission

by splashes of blood and other body fluids^[117]. Cosmetic procedures and/or acupuncture, as well as circumcision are extensively associated with HCV transmission, but the risk seems to be small^[118,119]. Lacking of sterile techniques and/or nonprofessionally performed tattoos or piercings, especially before the mid-1980s, raised the HCV transmission rate significantly^[120-126].

HCV GENOTYPES/SUBTYPES DEPENDENT TRANSMISSION

The HCV genome demonstrated a prominent genetic diversity. Seven genotypes (gt 1-7) and 67 subtypes (a, b, c, etc.) have been described. The genotypes are characterized by a distinct geographic distribution and clinical manifestations^[127,128]. For example, the genotype 1 is prevalent in Americas, Japan and Europe^[129]. Indeed, the HCV genotype prevalence also differs according to the transmission route and the age of infected individuals. For instance, the HCV gt 3a and 1a are highly represented among intravenous drug users and the HCV gt 1b is frequent among patients who received blood transfusions^[130-134]. In Japan, the HCV gt 1b is the most prevalent^[1] while, the infected population is generally older than in the United States^[2], and an iatrogenic transmission is a predominant risk factor for the HCV acquisition.

HCV LIFE CYCLE

Binding and entry

The HCV entry initiates the viral replication cycle. Virus that is a complex and multi-step process that is not completely understood. However, the main principal steps and the principal cell surface interaction partners are known. At least, four cellular entry factors and two specific receptors are required for a successful virus entry (Figure 4). Since many interactions should take place, the binding to the cell surface is a relatively slow process which should be well coordinated^[135-141]. The initiation of HCV entry seems to be similar to that of many other viruses, that utilize for attachment the glycosaminoglycans (GAGs)^[141]. Earlier, it was proposed that E1 and E2 are involved in these interactions^[141]. However, the HCV particle interacts with lipoproteins and in this regard, it is more likely that the apolipoprotein E is responsible for the interaction with GAGs^[141]. Furthermore, due to the nature of the viral lipoprotein particle, it was proposed that the LDL receptor is required in the initial stage of attachment. However, this interaction leads the particle to a degradation pathway^[141]. After the completion of docking, the viral particle interacts with specific cellular receptors. It was shown that the envelope glycoprotein E2 interacts with two receptors a scavenger receptor B1 (SR-B1), and with CD81 (tetraspanin family protein -TSPAN28)^[142-144]. It is likely, that interaction of HCV with SR-B1 comes first^[142]. In this regard, it remains to be determined whether SR-B1

and CD81 form a complex that facilitates the entry. It is noteworthy that the interaction with CD81 alone can activate signaling pathways that may be important for the viral infectious cycle^[141] and the interaction between CD81 and the E2 glycoprotein appears to be essential for initiating the adsorption^[141]. Next, the receptor complex with attached virion is moving to the tight junction, where the interaction with the proteins claudin-1 (Cldn1) and occludin (OCLN) is likely taking place. However, there is no experimental evidence indicating a direct interaction between the viral particle and the tight junction proteins. Thus, these proteins cannot be considered as classical receptors. Finally, other cellular factors such as epidermal growth factor receptor^[145] and the Niemann-Pick C1-like 1 cholesterol uptake receptor^[146] are likely involved in the HCV entry. It has been shown that CD81 and Cldn1 proteins may interact with the surface of hepatocytes^[147], suggesting that CD81-Cldn1 form a complex that is implicated in the HCV entry. Although the tight junction proteins are involved in the HCV entry, the role of cell polarization in viral entry remains controversial. Interestingly, a live imaging of the fluorescent HCV particle in Huh7.5 cells showed that the particle was first attached on filopodia and then migrated towards the main body of the cell using presumably an actin-dependent transport mechanism^[148]. It is important to note that the hepatocyte is a polarized cell with multiple apical and basolateral poles presenting a unique organization that is observed only in the liver. Another characteristic of HCV is its ability to spread by cell-to-cell contacts^[149]. The essential role of the E1, E2 transmembrane domains in the viral entry^[150] and the involvement of different regions of the ectodomain of E2 in the viral assembly were demonstrated^[151]. It has been shown that glycans on the envelope proteins may be involved in different stages of the viral infectious cycle^[42,152,153]. Furthermore, the N-linked glycan shield of the viral glycoproteins is essential to protect against the neutralization of the virus by antibodies^[154].

An additional interaction partner of the CD81 receptor, called "EWI-2wint" was identified^[155]. It was demonstrated that EWI-2wint is a natural inhibitor of the HCV entry, which by an interaction with the CD81 receptor can prevent CD81-E2 interaction. Later on, the regions involved in the interaction between CD81 and EWI-2wint were identified^[156] and studies in living cells at a single molecule level proved that EWI-2wint reduces the mobility of CD81 at the plasma membrane^[157,158]. Finally, it was reported that the enrichment of the plasma membrane in ceramides has an inhibitory effect on the viral entry^[159].

Post-entry events

It was shown by a traffic monitoring that after binding to the cells surface, the virus particles enter into the cells using clathrin dependent endocytic pathway^[160,161]. The viral particles are transported to the early endosomes

expressing RAB5A where the merger took place. This process requires an acidification of the compartment^[162]. The virus capsid is then released and destroyed, while the viral RNA is released to the cytoplasm. The RNA is used for both processes the replication and the polyprotein translation (Figure 5). The RNA translation occurs in the endoplasmic reticulum (ER) and it is initiated by a binding of the 5'UTR IRES to the ribosome. The primary translation product is approximately 3000 amino acid long polyprotein precursor which contains structural and non-structural proteins of the HCV. Then, the polyprotein is cleaved by the host and viral proteases into structural and non-structural proteins.

Several studies evidenced that the HCV infection results in ER stress and autophagy responses and that HCV can regulate the autophagy pathway. In fact, the autophagy machinery is required to initiate the HCV replication and suppression of autophagy inhibits this process^[163-171]. Interestingly, it has been demonstrated that HCV induces autophagosomes *via* a Class III PI3K-independent pathway and uses autophagosomal membranes as sites for its own RNA replication^[171]. For the HCV RNA replication, polarized positive HCV RNA genome synthesizes a negative strand by the NS5B RNA-dependent RNA polymerase. The newly synthesized negative RNA strand may further act as a template to synthesize the positive strand of the viral RNA^[172,173].

Viral assembly and release

An assembly of the virion is another multi-step process and certain steps of it remains obscure. It is known that the particle assembly occurs within the ER in a close proximity or directly on the surface to LD^[170] (Figure 5). After the proteolytic cleavage of the polyprotein precursor, NS5A initiates the early phase of the viral particle formation by the interaction with the core protein and its C-terminal serine cluster determines the NS5A-core protein interaction^[174].

The viral RNA released from the viral core is recruited to the replication complex within the membranous web. The HCV RNA-dependent RNA polymerase (NS5B) has no proofreading mechanism to correct errors during the strand synthesis. This propensity for error during replication results in an accumulation of the HCV quasispecies, those are closely related, but genetically somehow distinct. The newly synthesized positive-sense viral RNA is transported to the site of the core assembly and is encapsulated. However, the mechanism that engaged in RNA delivery to the capsid during assembly is not known. The nucleocapsids with RNA are presumably enveloped by budding into the lumen of the ER. So, after the nucleocapsid formation, it is associated with the envelope protein forming an immature particle and secreted from cell through the cytoplasmic membrane^[173,174]. However, it remains unclear how the coupling with lipoproteins and apolipoproteins is regulated, since the pattern of lipoproteins on the virus particle might differ significantly. Next, the virions are

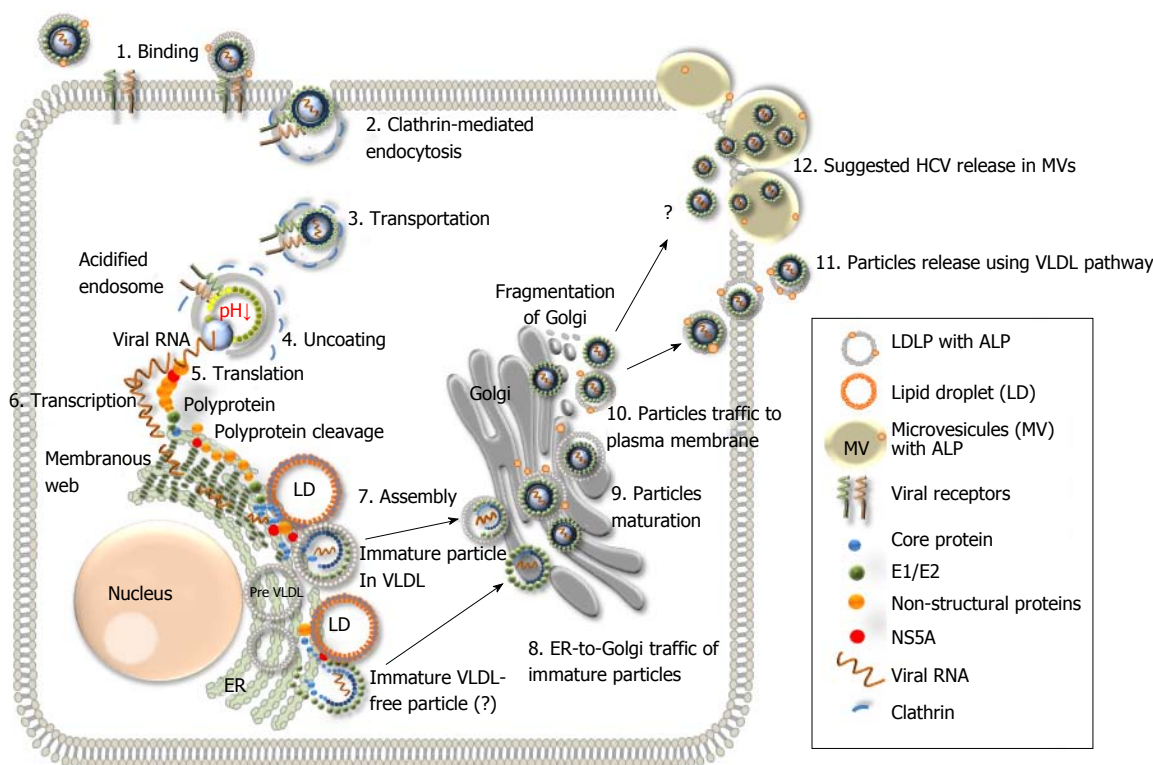


Figure 5 Schematic representation of the hepatitis C virus life cycle. (1) The lipoviral particles (LVP) binds to entry factors and receptors on the surface of hepatocyte; (2) The virus enters into the cell by a clathrin-mediated endocytosis; (3) Transportation of the virus in endosome; (3) Acidification of endosome, uncoating of the virion and dissociation of the viral core; (4) Release of the viral RNA; (5 and 6) Translation and replication of the viral RNA in the ER in the convoluted membrane structure called the membranous web (shown in dark green); (7) Cleavage of the protein precursor by cellular and viral proteases, assembly of the core on the surface of the lipid droplet (LD) and recruitment of the newly synthesized viral RNA to the viral core during the formation. The mechanism of the viral RNA recruitment to the site of the assembly is not known. It is also unclear whether the core maturation is finalized at this stage; (8) The viral nucleocapsid egresses into the lumen side of the ER, likely interacted with very low-density lipoproteins (VLDL) and translocated to Golgi. It is not clear if some VLDL-free particles can also be produced (?). Details of traffic machinery are not well defined; (9) Final maturation of viruses in Golgi and virus induced partial fragmentation of Golgi; (10) The hepatitis C virus (HCV) complex with VLDL is directed to the plasma membrane using the VLDL secretory pathway; (11) Release of particles from the cell (adapted from Ref.^[16,19,135]); and (12) The HCV release in MV cannot be excluded as another pathway for the virus release during active replication.

transported through the Golgi compartment to finalize the maturation. Recently, it has been shown, that HCV can induce the fragmentation of Golgi^[175]. Thus, it might be another important indication of the virus-induced pathogenesis. However, if the fragmentation of Golgi is significant, it may cause not only destructions of Golgi, but also provoke the cell death. Definitely, it might be not advantageous for the virus. It cannot be excluded, that this phenomenon might take place predominantly during the active replication of the virus. In this regard, it might be useful to investigate the HCV-induced Golgi destructions, as a possible trigger of liver necrosis.

Virus release and extracellular particles

The detail mechanism of the HCV release requires more comprehensive investigation. It is well established that secretion of the HCV particle depends on both the VLDL and the apolipoprotein (apoB) presence^[176-187]. So, when the maturation is finished, the virions are transported to the plasma membrane using the VLDL pathway^[145]. Particles isolated from cell-cultures and from patient sera are pleomorphic, immature and most of them are non-infectious^[170]. In this regard, in the

virus preparations from human plasma examined by electronic microscopy (EM), it was difficult to reveal "perfect" mature particle^[170]. The association with fragments of cellular membranes (or other cellular debris) and/or an irregular lipoprotein content might be the reasons of the problem. Anyhow, it is evident, that a classical "assembly-maturation-release" processing line of HCV is facing problems. A diverse buoyant density of infectious particles is reflecting the problems. The analysis of LVP from plasma by sucrose gradient centrifugation demonstrated, that the buoyant density of the virus-contained fractions is diverse (1.10 g/mL to 1.16 g/mL)^[170]. Positioning of virus-containing fraction on the gradient depends on the initial material that was used for the virus isolation. The infectious particles isolated by isopycnic centrifugation from cell-culture were mostly present in the fraction with a density 1.14 g/mL and examined by cryo EM, when were about 60 nm in diameter^[170]. Significantly more virus-associated cellular impurities including lipoproteins, are associated with viruses isolated from patient sera and that influence the virus density and the particles are significantly less dense^[177,178]. It is considered that the observed density

fluctuation is a result of irregular content of lipoproteins tightly associated with virion. In fact, this diversity might have an additional reason. It cannot be excluded that *in vivo* the virus particles might in addition be transported by extracellular vesicles. In fact, the size of putative transporters might be large enough to carry not a single, but numerous viral particles. For example, membrane derived microvesicles (MVs) can be 500-1000 nm in diameter, so as large as lipid droplets^[184,185]. MVs are present in the fractions of different densities, indicating a diversity of diameter and/or cargo^[186].

Analysis of cell supernatant by gradient density centrifugation demonstrated that the MVs subpopulations are different, depending on the cell line. It is known, that MVs are intensively released by primary hepatocytes and immortalized cells of hepatic origin^[186]. The release from hepatocytes might be increased by an undergoing lipotoxicity^[187]. Thus, MVs are actively used by the hepatocytes as delivery system and the amounts of MVs might be increased in response to lipotoxicity and likely other complications. In this regard, we speculate that during acute phase of infection (or high rate of replication) the virus can trigger the MV release and that might be an additional pathway for the virus. How MVs containing virus particles can influence HCV distribution along the density gradient? In fact, MV containing different amount of viruses may contribute to observed diversity of buoyant densities of HCV, especially when plasma (or serum) is investigated.

It is known, that the diffusion of spherical particle through a viscous liquid is dependent on the particle diameter. It is described by Stokes-Einstein equation:

$$D = \frac{kT}{6\pi\eta r}$$

D = diffusion constant, k = Boltzmann constant, T = temperature (K), η = solvent viscosity, r = radius of spherical particle.

Another parameter that influences the diffusion is the mass of spherical particle and the equation counting these parameters looks as follows:

$$nD = \frac{6\pi\eta\alpha}{M}$$

where: D = diffusion; α = radius of the spherical particle; M = mass of the particle; η = viscosity of the medium.

So, the diffusion of a spherical particle is decreased with the increase of diameter and mass. Thus, if MVs besides the regular cargo (mRNA, miRNA, modulators, peptides, proteins) have in addition a different particle load, it might explain why MVs of the same sizes might be recovered in fractions with different densities, and vice versa. Thus, when supernatants (or plasma) are examined by density gradient centrifugation, MVs of different size and different virus loaded, can increase the amount of virus-containing fractions. The expected distribution of lipoproteins, LVP from human plasma

in a sucrose gradient and the suggested positioning of MVs with different virus load are given below (Figure 6, adapted from^[170] with modifications).

What might be the advantage, for the virus to use the MVs secretory pathway? Since the MV are released by budding from the plasma membrane, it allows the virus to acquire additional protection from the "rough environment". Second, if MV is loaded with numerous particles, it might be the way to increase the probability of a successful infection of distant cells by creating a high "local MOI" after MV destruction. That effect might be even more prominent, if the MV loaded with viruses would be endocytosed by the cell. We suggest, that the MV pathway might be used during active virus replication of the virus. The possible implication of MVs for the virus delivery might be interesting to investigate using *in vitro* models.

MODELS TO STUDY HCV

The progress in HCV research is completely dependent on model systems^[188]. The most frequently used models (Figure 7) for investigation of HCV are described below.

HCV replicon systems

The HCV genome was identified in 1989 by cloning it from infected chimpanzee, while in humans the amounts were too low for detection^[189]. The first complete full-length HCV cDNA clone was constructed from the HCV strain H77 (genotype 1a). The HCV RNA transcribed from this clone was found to be infectious after intrahepatic injection in a chimpanzee. The HCV viremia was detected at week 1 and increased from 1×10^2 genomes/mL to 1×10^6 genomes/mL at week 8^[190,191]. Then, several full-length HCV RNAs were synthesized and were shown to be infectious in chimpanzees^[190-193]. However, these HCV clones were found to replicate inefficiently *in vitro*. This limitation was resolved by the group of Prof. Ralf Bartenschlager, when subgenomic HCV replicon, cloned from the HCV genome was constructed^[73]. After transfection of this subgenomic clone into the Huh7 cells, it was found that in drug-resistant cells a high-level of the HCV RNA replication occurred. The replication of the HCV subgenomic replicon was confirmed in several cell lines, but the hepatocarcinoma cell line Huh7 was the most permissive. The interferon treatment of the replicon inoculated Huh7 cell clones, made them more permissive to support both subgenomic and full-length HCV replication, (these so-called cured cell lines are Huh7.5 or Huh 7.5.1 cells)^[15,194]. Afterwards, several studies demonstrated that the virus and host factors were important for the HCV replication in cells. Some mutations in the wide-type (wt) consensus sequence efficiently enhanced the HCV replicon replication, some mutations in the non-structural protein efficiently contributed to the replication and the adaptation to the host cells^[195-198]. The mechanism of improved replication caused by adaptive mutations is still unknown. Although

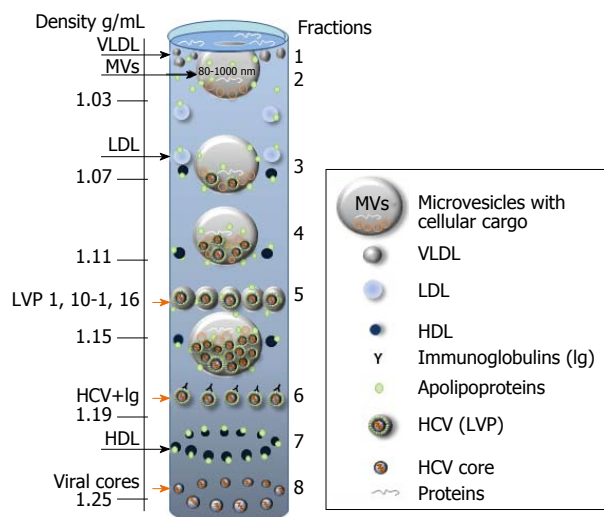


Figure 6 Putative distribution of lipoproteins (LDL, VLDL, HDL), lipoviral particles and viral cores after density gradient centrifugation of plasma from infected individual. The picture is based on the known buoyant densities of analyzed elements^[15,170,184-187]. The density values (g/mL) are given on the left. The initial material that has been used for the virus isolation, contributes greatly to the associated lipid content of the virions and, as a consequence, it influences the buoyant density^[177,183-187]. Because of an irregular protein to cholesterol amounts, high density lipoproteins (HDL) of 5-15 nm in diameter can be detected in the different fractions (1.06-1.21 g/mL). The family of HDL is given as dark blue spheres. The buoyant density of microvesicles (MVs) may vary from 1.02 g/mL to 1.16 g/mL, depending on MVs diameter and cargo (miRNA, tRNA, fragments of mRNA and proteins). If hepatitis C virus (HCV) is released in MVs with similar diffusion parameters (size and mass), the buoyant density of vesicle should be viral cargo-dependent. Fractions 1: Single cellular or viral proteins “floating” on the surface, very low-density lipoproteins (VLDL, diameter 50-75 nm, density 0.95-1.006 g/mL); Fraction 2: Low density lipoproteins (LDL, diameter 18-25 nm, density 1.019-1.06 g/mL) and virus-free MVs with a low cellular cargo content; Fraction 3: MVs that carry few viral particles, possible overlap with LDL and HDL; Fraction 4: MVs with a higher viral load, possible overlaps with HDL and with densely loaded virus-free MVs; Fraction 5: Lipoviral particles (LVP, diameter 60-80 nm, density 1.10-1.16 g/mL) and MVs with a significant viral and/or mixed load. Overlaps with HDL, virus-loaded MVs and “dense” virus-free MVs are possible; Fraction 6: Lipid-free virions with attached immunoglobulins, possible overlap with HDL; Fractions 7: HDL with a high protein load; Fraction 8: Non-enveloped viral cores that might overlap with dense HDL. Small grey spheres - VLDL. Light blue spheres - LDL. Orange arrows indicated fractions of the gradient that are most likely to contain viruses and viral cores.

these replicons with adaptive mutations could replicate with a high efficiency, they were not able to produce infectious particles *in vitro*.

This problem was solved with development of the genotype 2a infectious clone JFH-1. A selectable HCV replicon was constructed containing the full-length HCV cDNA and showed to produced infectious particles *in vitro* and *in vivo*^[199]. Based on this infectious clone, several different genotypes chimeric clones (genotype 1a, 1b, 2a and 3b) where the non-structural genes have been replaced by those of JFH-1 were constructed and were shown to be infectious in Huh-7 cells. The most efficient construct is the genotype 2a/2a clone which consists of J6CF and JFH-1 derived sequence^[200]. The HCV replicon is remarkably valuable for studying the HCV replication and for testing of new antiviral drugs.

The HCV subgenomic replicons containing reporter genes (luciferase, secreted alkaline phosphatase and chloramphenicol transferase) facilitated the study of the HCV infection. This high-throughput screening assay allowed the visualization and tracking of the HCV replication complex in living host cells without affecting the HCV replication^[47,201].

HCV pseudotype virus particles

The HCV pseudotyped particles were constructed with chimeric genes expressing HCV (genotype 1a) envelope E1 and E2 proteins (HCVpp) and the transmembrane and cytoplasmic tail of vesicular stomatitis virus G protein. The pseudotyped particles allowed a detailed study of the role of HCV receptors in the early steps of HCV infection (adsorption, and viral entry) in Huh7 cells and primary human hepatocytes^[202-204]. The system is useful for testing of new antiviral drugs^[203].

The HCV subgenomic replicon and the HCV pseudotyped particles (HCVpp) have markedly improved studies on the HCV infection. However, the high replication rate in cells was not correlated with amount of released infectious virions. However, the main disadvantage of these *in vitro* systems is that the viruses released from these cells are not infectious. The major reason of that might be that the adaptive mutations enhancing HCV replication rates are deleterious for HCV particles assembly and release. The problem was solved when a genotype 2a subgenomic replicon was established in the Huh-7 cell lines. It contained a full-length genotype 2a clone JFH-1 derived from serum of a Japanese patient with fulminant hepatitis. The replication rate was about 20 times greater than Con1 (gt1b) when transfected into Huh-7 cells. In addition, this replicon could replicate efficiently without amino acid mutations^[47]. In 2005, Wakita *et al.*^[200] continued to transfect this JFH-1 clone into Huh-7 cell lines and found high levels of intracellular HCV RNA replication and protein expression. By means of passages in infected cells, they demonstrated that JFH-1 transfected cells had continuous HCV replication. In this study, it was also shown, that the HCV viral particles (HCVcc) secreted in the culture supernatant had a density of approximately 1.15-1.17 g/mL. The released particles have a spherical morphology and a diameter of about 55 nm. The JFH-1 infectivity could be neutralized by anti-CD81 antibody, suggesting the important role of CD81 in HCV entry. In addition it was found that the secreted particles could also infect chimpanzee^[191]. Later, several groups observed that JFH-1 could efficiently infect and replicate without adaptive gene mutation in different cell types and HCV infectious particles could be produced in the culture supernatant^[25,199,205-209]. Zhong *et al.*^[192] established a robust highly infectious *in vitro* system with JFH-1 and Huh-7.5.1 cells. The advantage of this model, compared to that established by the group of Prof. Wakita, was the possibility of HCV to undergo serial

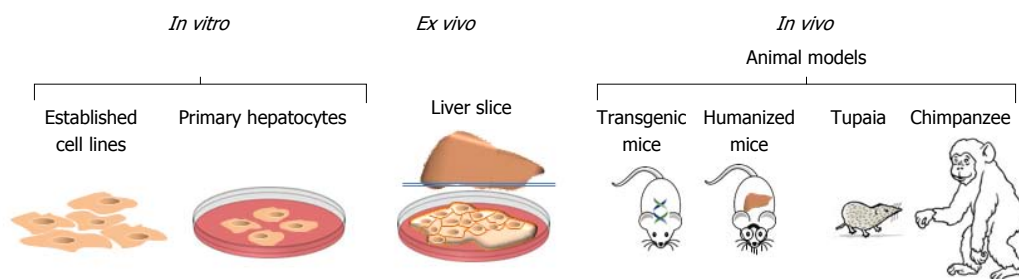


Figure 7 *In vitro*, *ex vivo* and *in vivo* models to study hepatitis C virus.

passages without losing of infectivity. Although JFH-1 *in vitro* system provides a powerful tool to study antiviral drugs and vaccines, it has some limitations: HCV JFH-1 derived from an exceptional case of HCV-related fulminant hepatitis belongs to genotype 2a which is not the dominant genotype worldwide. That is why since 2005, different chimeric JFH-1 clones were constructed to transfect Huh-7.5 cells, based on intergenotypic genotype replicons such as J6/JFH-1 (genotype 2a/2a), S52/JFH1 (genotype 3a/2a) and sa13/JFH-1 (genotype 5a/2a)^[209,210], but they are less infectious than HCV JFH-1. The reason why JFH-1 is more permissive to Huh-7.5 cell lines than other genotypes is still not fully understood. In summary, the HCVcc model allowed to understand the entire life cycle of HCV and that serves as a background for development of numerous antivirals^[211].

Animal models

Chimpanzee could be a good model to study the HCV infection^[194,212]. However, these animals are rare, difficult to handle, very costly and limited in their use by ethical issues^[212]. Few data obtained using chimpanzee model indicated a specific immune response at the acute phase of infection, resulting in inconsistent specific neutralization and viral eradication.

Tupaia (Treeshrew, *Anathana ellioti*) is potentially a good model to study the HCV infection, but the instability of the infection and its low level limit the use of these animals.

The immunotolerized rat model supports the HCV replication. Histological and biochemical evidence of infection were present, but viremia was relatively low as compared with viral load in humans^[188].

Mouse models compared with other animal models, have some advantages, such as producing animals in a short time (short gestation period), lower breeding cost and their small size making them easy to manipulate^[188,213-217]. Heterotopic liver graft mouse model seemed to be suitable to evaluate the putative effect of anti-HCV drugs, but the short and low viremia and loss of the liver graft were limiting factors. Hepatic repopulation mouse model as the chimeric urokinase-type plasminogen activator/severe combined immunodeficient disorder mouse model has many similarities to human systems, and so is the most successful small animal

model for HCV infection. The main limitation of the model is the absence of a normal immune system, which may be overcome by combining the model with a human hemato-lymphoid system, the value and reproducibility of which has yet to be established^[218].

A mouse combined human immune system/human liver chimeric Rag2-g(c) model was developed in order to study immune system and liver pathogens. This 'Hu-HEP' model was used to study hepatocytes derived from human induced pluripotent stem cells. The main limitations of these models are the low number of animals that can be generated and the high cost^[219-221].

Recent study demonstrated that hepatocyte-like cells differentiated from human embryonic stem cells and patient-derived induced pluripotent stem cells could be engrafted in the liver parenchyma of immune-deficient transgenic mice carrying the urokinase-type plasminogen activator gene driven by the major urinary protein promoter. This efficient engraftment and *in vivo* HCV infection of human stem cell-derived hepatocytes provide a model to study chronic HCV infection in patient-derived hepatocytes, action of antiviral therapies, and the biology of HCV infection^[222-225].

In vitro models

Established human hepatocarcinoma cell line Huh-7 and its derivatives support the HCV replication. However, as every transformed cell line, they resemble the primary cells only partially. Thus, results of experiments performed on these cells might not be always appropriated^[222].

The primary human hepatocytes and human fetal hepatocytes are more clinically and physiologically relevant. Thus, they are used to test the susceptibility of HCV to drugs and drug-metabolizing enzymes. Another *in vitro* model, the micropatterned co-cultures of primary human hepatocytes surrounded by a supportive stroma that expressed all known HCV entry factors. The cells could be infected by HCV pseudotyped particles and HCVcc, albeit with low viral titers. Using this method in combination with the highly sensitive luminescence-based and fluorescence reporter systems, the efficiency of anti-HCV therapeutics has been evaluated^[222].

Recent studies show that both embryonic^[223] and induced pluripotent^[224,225] stem cells can be differentiated into hepatocytes, that are phenotypically similar to

human fetal liver. Study of genetic defects that impact the HCV infection could be performed in a human iPS-derived hepatocyte-like cell-based model^[225]. Induced human liver-like cells supported the entire life cycle of HCV genotype 2a reporter virus. Produced infectious particles were able to infect HuH-7.5 cells and secretion of TNF- α , and IL-28B/IL-29 was detected in cell culture supernatants. Thus, the evaluation of antiviral drugs using these cells was possible^[222].

Ex vivo model

Analysis of precision-cutting adult human liver slices from infected or non-infected individuals represents another promising model. In fact, this model allows to maintain the tridimensional structure of liver and analyse gene and protein expression. It is worth mentioning, that using this model, it was demonstrated for the first time, the ability of primary isolates (as well as JFH-1, H77/C3, Con1/C3) to undergo *de novo* viral replication with the production of high titer infectious virus. Thus, this approach allowed to validate the efficiency of the new antiviral drugs^[226,227].

PATHOGENESIS

Immune-mediated liver damage

The HCV causes damage to the liver cells, but the exact mechanism of this phenomenon is unknown. It is believed, that the damage is largely mediated by the host immune response. In immunocompetent and immunocompromised patients with little, or no intrahepatic damage, including inflammation, high levels of the HCV replication have been reported^[133,228,229]. In about 30% of HCV liver transplanted patients, despite the high levels of HCV replication, a recurrent hepatitis is developed one year after transplantation^[230]. However, high levels of an intrahepatic HCV replication are usually tolerated by the host immune system. A lympho-mononuclear infiltrate represented mainly by CD8⁺ T cells expected to play a major role in the viral containment, though other subsets, such as CD4⁺ T and natural killer (NK) cells, and regulatory T cells (Treg) are considered^[231]. The intrahepatic CD4⁺ and CD8⁺ T cells can recognize HCV structural and nonstructural antigens^[232,233]. However, why in most patients the immune response cannot resolve the infection remains obscure. In fact, cytotoxic CD8⁺ T cell-mediated killing could be blunt by a predominant Treg response^[234].

The liver fibrosis is caused by inflammatory cells of the intrahepatic infiltrate secreting cytokines and chemokines to activate hepatic stellate cells (HSC) to secrete collagen^[235]. HSCs may exist as several different phenotypes with distinct molecular and cellular functions and features, each of which contributes significantly to the liver homeostasis and the disease. The quiescent stellate cells are critical to the normal metabolic functioning of the liver. The liver injury provokes the transdifferentiation of quiescent stellate

cells to their activated phenotype, leading to a metabolic reprogramming. That increases the autophagy (to fuel the metabolic demands), the amplification of parenchymal injury and the development of 'classic' phenotypic features of activated HSCs/myofibroblasts. Through these changes, the activated stellate cells drive the fibrotic response to injury and the development of cirrhosis. As liver injury subsides, the activated stellate cells can be eliminated by one of three pathways: Apoptosis, senescence or reversion to an inactivated phenotype. The senescent stellate cells are more likely to be cleared by the NK cell-mediated cell death while the inactivated stellate cells remain 'primed' to respond to further liver injury. Reduction in the number of activated stellate cells contributes to the regression of fibrosis or cirrhosis and the liver repair in most, but not all patients. The relative inputs of these three pathways on the fibrosis regression are not clearly defined^[233]. The HCV-specific CD8⁺ T cells expressing PD-1, a marker for exhaustion, were found at the time of the acute phase^[236]. The acquisition of a memory phenotype and the recovery of an efficient CD8⁺ T cell function declined with HCV infections PD-1 expression, whereas, when HCV persisted and the HCV-specific CD8⁺ cells remained dysfunctional, high levels of PD-1 were maintained, the PD-1 ligation likely provides an overall inhibitory signal to Tregs cells shown in a study of PD-1 expression on Tregs in humans during the chronic HCV infection. In response to HCV antigens, the PD-1 blockade enhanced an interleukin-2 (IL-2)-dependent proliferation of intrahepatic Tregs and enhanced the overall ability of Tregs to inhibit T-effector cells. An increase in Treg proliferation with PD-1 blockade was linked to this effect^[237]. A typical model of the wound-healing response to a persistent liver injury is the hepatic fibrosis occurring in the chronic hepatitis C^[238]. Cytokines and chemokines capable of activating hepatic stellate cells to secrete collagen are secreted by the inflammatory cells of the intrahepatic infiltrate^[239,240]. Thus, the fibrogenesis seems to be linked to the HCV expression through indirect mechanisms, mediated by a virally driven inflammation, but the direct role of viral factors in the disease progression should be investigated in more details. The cell injury, such as an oxidative stress and a steatosis, may be induced specifically by several viral proteins alone which could directly activate the hepatic stellate cells^[241,242]. These observations may explain why some patients with chronic hepatitis C with normal liver enzymes and a minimal/mild inflammation may present a significant liver fibrosis as shown by the histology of liver biopsies. Overall, up to 70% of patients with a HCV cirrhosis will demonstrate a reversibility on follow-up biopsies^[243-248]. Moreover, a reduced portal pressure and a decreased all-cause mortality are improved when the reversal occurs^[245]. It is to point out that approximately 10% of HCV patients present a persistent or even progressive fibrosis following SVR, which might reflect other concurrent underlying liver diseases, especially a

nonalcoholic fatty liver disease (NAFLD)^[248]. The stellate cells can respond to cytokines and growth factors after priming stimuli. Then the proliferation, contractility, fibrogenesis, matrix degradation and proinflammatory signaling are enhanced. Now, it is clear that there are disease-specific pathways of fibrosis, without all activated cytokine pathways^[249,250]. This is especially relevant to NAFLD, where there are many convergent pathogenic routes^[248-250]. Importantly, different families of inflammatory cell types and their subsets may either promote or inhibit fibrosis^[249-252].

Alterations of lipid metabolism

Lipids are required for the HCV replication and particles assembly. As mentioned above, HCV can modify the host serum lipid profile and this(ese) modification(s) can provoke the steatosis^[253]. The steatosis is more frequent and more severe in patients with HCV gt 3 and it is correlated with a high HCV RNA levels. On one hand, in HCV-infected patients, the steatosis can be considered as a marker of the liver disease progression^[254] and, on the other hand, as an indication of the reduced response to therapy^[254]. However, if it is not metabolic or alcoholic steatosis, an efficient antiviral therapy is capable to reduce it^[255,256].

Extrahepatic replication of HCV

The apparent presence of the HCV genomes in extrahepatic sites of patients infected with HCV has been shown. Using sensitive PCR assays, the HCV was revealed in leukocytes and there are evidences that these cells may represent a reservoir of the virus after treatment^[257,258]. Interestingly, the pool of the HCV quasi-species differs between the plasma and peripheral blood monocytes, suggesting an independent spread of HCV within different cell types^[259,260]. The infection of B-cells from non-Hodgkin's lymphoma by HCV has also been demonstrated. A cell line established from transformed lymphocytes supported the HCV replication and also enable production of infectious viral particles capable to infect peripheral blood B cells^[261]. The significance of these extrahepatic HCV reservoirs is not well understood, although one could speculate that the leukocyte compartments might represent an additional route by which HCV can directly manipulate the immune system and also another means by which the virus avoids the eradication.

Extrahepatic manifestations associated with the HCV infection

At least, one clinically significant extrahepatic manifestation occurs in 38% to 76% of HCV infected patients with chronic HCV^[262,263]. The most frequent associated pathology is a mixed cryoglobulinemia. It is detected in 19% to 50% of HCV infected patients, while only 15% of them are symptomatic. The cryoglobulins are immunoglobulins which precipitate at a temperature below 37 °C. They are produced by HCV activated B cells.

The cryoglobulins deposited in small and medium vessels are the cause of systemic vasculitis which can manifest in level joint, skin, renal or peripheral nerves^[262]. Other observed extrahepatic manifestations are the following: lymphoma, thyroid disorders, diabetes, xerostomia and xerophthalmia^[263-265]. The HCV infection cure leads to a gradual decrease of the cryoglobulin level in serum, followed by the remission of cryoglobulin-related symptoms and pathologic lesions^[265]. Interestingly, as a result of treatment the incidence of type 2 diabetes is also reduced by approximately two thirds^[264,265].

CLINICAL MANIFESTATION OF HCV INFECTION

Acute hepatitis

The acute hepatitis C is asymptomatic in 90% of infected people^[266]. In some cases, asthenia, fever and muscle, and joint pain can appear. While, signs of jaundice are not frequent. Acute hepatitis C is characterized by a transient increase in the rate of serum transaminases. The first detectable virus marker is viral RNA that appears one to two weeks after exposure. Then seven-eight weeks later, the anti-HCV IgG response can be detected^[266-268]. In 20% of cases, hepatitis C is resolved spontaneously through the innate and adaptive immunity^[269]. The viral RNA becomes undetectable within three to four months after infection. Various factors could promote the viral clearance. Similarly, hepatitis acute symptoms would reflect a significant immune response of the host. The gene polymorphism of interleukin (IL) 28B also influences the host immune response^[268,269]. The fulminant hepatitis C is exceptional^[268].

Chronic infection

In about 80% of cases^[268], the immune system is not capable to eradicate the HCV during the acute phase of infection. When the viral replication persists for more than six months after acute infection, the hepatitis is considered chronic. At the stage of chronic hepatitis, most patients are asymptomatic and may have no non-specific symptoms such as fatigue, arthralgia or myalgia. The transaminase levels may be moderately increased or even normal^[270]. The long-term evolution of chronic infection is variable. The factors that accelerate the disease progression are the following: acquisition of more than 40 years, male gender, co-infection by HIV, higher body mass index, fatty liver and alcohol consumption^[269]. After 10 to 30 years, about 20 to 30% of patients develop a cirrhosis. The cirrhosis may be associated with a liver failure, as a decompensation following a portal hypertension (ascites, gastrointestinal bleeding, etc.). In cirrhotic patients, the risk of death from complications is 4% per year, and their risk of developing an hepatocellular carcinoma (HCC) is 1 to 5% per year. Thirty-three percent of patients with HCC die within one year after diagnosis^[269,270]. Overall, among patients with cirrhosis, the 5-year survival rate is 50%.

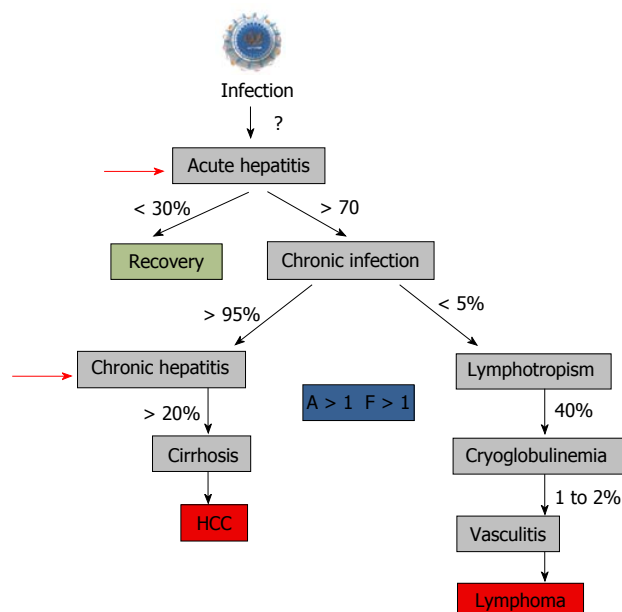


Figure 8 Natural history of hepatitis C virus infection and start to treat. The recommendation to treat chronic hepatitis is usually a “significant” fibrosis as defined by a Child–Pugh score (A to C) and a fibrosis grade (F) greater than 1 by the Metavir scoring system, with usually a significant necrotic-inflammation as defined by an activity stage greater than 1 by the Metavir scoring system^[288,289]. The Child–Pugh score employs five clinical measures of liver disease: Total bilirubin, Serum albumin, Prothrombin time, Ascites, Hepatic encephalopathy. The letter F refers to the scars of the liver caused by the aggression. It is classified from F0 to F4: F1, F2 are minimal to moderate fibrosis, F3 corresponds to a pre-cirrhotic stage and F4 corresponds to cirrhosis. Red arrows indicated the time to start treatment^[290].

In fact, the decompensated cirrhosis is the leading cause of liver transplantation^[268,269] (Figure 8).

DIAGNOSTIC

For HCV diagnosis both serologic and nucleic acid-based tests were developed^[270,271]. Serologic tests are sufficient when chronic hepatitis C is expected, with a sensitivity of more than 99% if using the 3rd generation assays. Positive serologic results require additional HCV RNA or (with slightly reduced sensitivity) HCV core antigen measurements in order to differentiate between chronic hepatitis C and resolved HCV infection from the past. When an acute hepatitis C is considered, a serologic screening alone is insufficient, because mature anti-HCV antibodies are developed late after transmission of the virus.

Morphological methods like immunohistochemistry, *in situ* hybridization or PCR from liver specimens play no relevant role in the diagnosis of hepatitis C because of their low sensitivity, poor specificity and low efficacy compared to serologic and nucleic acid-based approaches.

HCV core antigen assay

Recently, a new quantitative HCV core antigen assay (Architect HCV Ag, Abbott Diagnostics) was approved

by the EMA. This assay comprises 5 different antibodies targeted the HCV core. The test is highly specific (99.8%), equally effective for different HCV genotypes, and shows a relatively high sensitivity for the determination of chronic hepatitis C (corresponding to 600-1000 IU/mL HCV RNA). However, HCV core antigen correlated well, but not fully linearly, with HCV RNA serum levels, and false-negative results might be obtained in patients with an impaired immunity^[271-273]. Another study has shown that the HCV core antigen quantification could be an alternative to the HCV RNA quantification for on-treatment antiviral response monitoring^[274]. Here, a HCV core antigen below the limit of quantification at treatment 1 wk was strongly predictive of RVR, whereas patients with a less than 1 log₁₀ decline in HCV core antigen at treatment 12 wk had a high probability of achieving nonresponse. The new HCV core antigen assay could be a cheaper, though somewhat less sensitive, alternative for nucleic acid testing.

Nucleic acid testing for HCV

Since the HCV RNA is detectable within a few days of infection; the nucleic acid-based tests are efficient in an early diagnostic of acute hepatitis C and should be considered as mandatory. The HCV RNA measurement is furthermore important in determination of the HCV genotype, selection of treatment strategy, therapy duration and evaluation of the treatment success^[274]. For a number of antiviral combination therapies, the HCV RNA follow-up studies are essential to define the outcome of the treatment and further therapeutic strategies, if necessary. Traditionally, the tests should be repeated 24 wk after treatment completion to assess whether a sustained virologic response (SVR) has been achieved. However, as the probability of a virologic relapse is similar after 12 and 24 wk, the new time point for assessment of final virological treatment outcome is 12 wk after the end-of-treatment^[275,276]. Both qualitative and quantitative PCR-based detection assays are available. Qualitative PCR tests are sensitive and are used for initial diagnostic of hepatitis C, for screening of blood and organ donations and for confirming SVR after treatment completion (Table 2). Quantitative reverse transcriptase (RT) real-time PCR-based assays can detect and quantify the HCV RNA over a very wide range, from approximately 10 IU/mL to 10 million IU/mL. The measurements are essential in the treatment monitoring when the virus load is gradually reducing.

HCV genotyping

HCV genotyping is mandatory for every patient who considers antiviral therapy^[277]. For DAA-based therapies, the determination of HCV genotypes and even subtypes is important because of significantly distinct barriers to resistance on the HCV subtype level. However, the importance for the HCV genotyping may decline with the availability of highly and broadly effective all oral combination therapies in the future. Both direct sequence

Table 2 Commercially available hepatitis C virus RNA detection assays

Assay	Distributor	Technology	Detection limits (IU/mL)	Genotypes	Approval status
Qualitative HCV RNA detection assays					
Amplicor™ HCV 2.0	Roche molecular Systems	PCR	50	All	FDA, CE
Versant™ HCV	Siemens Medical Solutions Diagnostics	TMA	5-10	All	FDA, CE
Quantitative HCV RNA detection					
Amplicor™ HCV Monitor 2.0	Roche molecular Systems	PCR	500 to approximately 5.10 ⁵	All	CE
HCV SuperQuant™	National Genetics institute	PCR	30 to 10 ⁷	All	U.S only
Versant ^R HCV RNA 3.0	Siemens Medical Solutions Diagnostics	bDNA	615 to 8.10 ⁶	All	FDA, CE
Cobas ^R AmpliPrep/High pure system/Cobas ^R TaqMan ^R	Roche molecular Systems	Real-time PCR	15 to 10 ⁷	All	FDA, CE
Abbot RealTime™ HCV	Abbot Diagnostics	Real-time PCR	10 to 10 ⁷	All	FDA, CE
Artus HCV QS-RGQ assay	Qiagen	Real-time PCR	34 to 10 ⁸	All	CE
Versant ^R HCV 1.0 kPCR assay	Siemens	Real-time PCR	10 to approximately 10 ⁷	-	CE

HCV: hepatitis C virus; TMA: Transcription-mediated amplification of RNA; bDNA: Branched DNA hybridization assay.

analysis and reverse hybridization technology allow the HCV genotyping. Initial assays were designed to analyze exclusively the 5'UTR, which was burdened with a high rate of misclassification especially on the subtype level. Current assays were improved by additionally analyzing the coding regions, in particular the genes encoding core protein and the NS5B, both of which provide non-overlapping sequence differences between the genotypes and subtypes^[278,279].

TREATMENT

All people with confirmed chronic HCV infection should be offered a high-quality care as soon as possible. Simultaneously, the screening and management of alcohol use is essential to prevent the progression to cirrhosis^[278,280]. The modelling suggests that the treatment early in the course of HCV disease for all or specific populations could prevent the disease progression and onward transmission^[281-283]. In fact, not all people with chronic HCV infection will progress to fibrosis^[284]. So, medical authorities of a country to should decide when to start anti-HCV treatment. It should be emphasized that the transmission has been documented among people recently cured who lacked access to prevention services^[285]. More research is needed to determine cost-effective eligibility criteria for both key and other populations that maximize reductions in the HCV-related morbidity, mortality, and transmission in different epidemiological contexts.

The WHO guidelines recommend the prioritizing treatment among people with an advanced fibrosis or cirrhosis in order to prevent a liver cancer^[286]. The HCV co-infection in people living with HIV increases mortality while HIV has been shown to accelerate the progression of HCV disease^[287,288]. Therefore, people with HIV/HCV co-infection may also warrant treatment prioritization. The stage of HCV disease could be estimated through liver biopsy (METAVIR system) identifying individuals needing an immediate treatment^[288]. However, the liver biopsy test is expensive and can lead to complications such as infections, excessive bleeding, pain, or accidental

injury to other organs. The use of liver function tests and platelet counts to determine the degree of liver fibrosis, such as the aminotransferase-to-platelet ratio index and the Fibrosis-4 score could be a non-invasive alternative and a more useful approach for gauging treatment eligibility across different tiers of health systems^[289,290]. Monitoring systems should be put in place for people who do not initiate treatment immediately.

In the course of the last two decades, treatment of the HCV infection has significantly improved. For nearly 15 years, the combination of pegylated interferon alfa and ribavirin (PR) allowed a moderate sustain virologic response (SVR). Cure of the infection was achieved in 45% of genotype 1 and 65% of genotype 3 and around 85% of genotype 2 infected patients^[291]. Development of the direct-acting antiviral drugs (DAAs) targeting viral proteins (NS3/4A protease, NS5B polymerase with nucleotide and non-nucleotide inhibitors, NS5A viral replication complex) was achieved due to the better understanding of the HCV life cycle. It revolutionized the treatment of chronic hepatitis C^[292,293]. The classes of oral anti-HCV inhibitors and their characteristics are shown (Table 3). The combination of PR with the first-generation protease inhibitors demonstrated a high antiviral effectiveness (75% of SVR, but restricted to genotypes 1) with substantial adverse effects for the first-generation protease inhibitors. In 2011, telaprevir and boceprevir obtained a market approval. In 2012, the recommendations were approved for their use in HCV mono- and in 2013 for HCV/HIV co-infected infected patients. Then, SVR rates increased from 75% to 90% while reducing treatment duration, adverse effects, and the number of pills, thanks to the combination of PR with second-generation protease inhibitors^[63,294,295]. From 2015 the standard of care is a combination of DAAs. Sofosbuvir, daclatasvir and the sofosbuvir/ledipasvir combination are part of the preferred regimens in the WHO guidelines, and can achieve cure rates above 95%^[63]. These medicines are much more effective, safer and better-tolerated than the older therapies. In the first studies, excellent results were obtained on a group of so-called "easy-to-treat patients" and in

Table 3 Selected directly acting antiviral agents and host targeting agents in the pipeline^[63]

Drugs name	Company	Target/active site	Phase
NS3/4A protease inhibitors			
Vaniprevir (MK-7009)	Merck	Active site/macrocylic	III
Voxilaprevir GS-9857	Gilead	Active site	III
Glecaprevir (ABT-493)	Abbvie	Active site	III
IDX21437	Idenix	Active site	II
Sovaprevir (ACH-1625)	Achillion	Active site/macrocylic?	II
Nucleoside analog NS5B polymerase inhibitors (NI)			
MK-3682 (formerly IDX20963)	Merck	Active site	II
ACH-3422	Achillion/Janssen	Active site	II
Non- Nucleoside analog NS5B polymerase inhibitors (NNI)			
Beclabuvir (BMS-791325)	Bristol-Myers Squibb	NNI site 1/Thumb 1	III
Setrobuvir (ANA598)	Anadys/Roche	NNI site 4?/palm 1	II
NS5A inhibitors			
BMS-824393	Bristol-Myers Squibb	NS5A protein	II
PPI-461	Presidio	NS5A protein	II
PPI-668	Presidio	NS5A protein	II
Pibrentasvir (ABT-530)	Abbvie	NS5A protein	III
ACH-2928	Achillion	NS5A protein	I
Ruzasvir (MK-8408)	Merck	NS5A protein	II
Host targeting agents			
SCY-635	Scynexis	Cyclophilin inhibitor	II
Miravirsen	Santaris	miRNA122 antisense NA	II
RG-101-	Regulus	miRNA122 antisense NA	II
TT-0034	Tacere Therapeutics	RNA interference with HCV	II

Table 4 Selected directly acting antiviral agents and host targeting agents whose development has been stopped or temporarily halted^[63]

Drugs name	Company	Target/Active site
NS3 /4A protease inhibitors		
Ciluprevir BILN 2061	Boehringer Ingelheim	Active site/macrocylic
Narlaprevir (SCH900518)	Schering-Plough	Active site/linear
PHX1766	Pheromix	Active site
Danoprevir (R7227)	Roche/InterMune	Active site/macrocylic
Faldaprevir (BI201335)	Boehringer Ingelheim	Active site/linear
Telaprevir (VX-950)	Vertex	Withdrawn
Boceprevir (SCH503034)	Merck	Withdrawn
Nucleoside analogue NS5B polymerase inhibitors (NI)		
Valopicitabine (NM283)	Idenix/Novartis	Active site
R1626	Roche	Active site
Mericitabine (R7128)	Roche/Pharmasset	Active site
GS-938	Gilead	Active site
IDX184	Idenix	Active site
Non-nucleoside NS5B polymerase inhibitors (NNI)		
BILB 1941	Boehringer Ingelheim	NNI site 1/thumb
MK-3281	Merck	NNI site 1/thumb 1
VX-759	Vertex	NNI site 2/thumb 2
VX-222	Vertex	NNI site 2/thumb 2
VX-916	Vertex	NNI site 2/thumb 2
ABT-072	AbbVie	NNI site 3/palm 1
HCV-796	ViroPharma/Wyeth	NNI site 4/palm 2
Filibuvir (PF-00868554)	Pfizer	NNI site 2/thumb 2
IDX375	Idenix	NNI site 4/palm 2
Tegobuvir (GS-9190)	Gilead	NNI site 4/palm 2
GS-9669	Gilead	NNI site 3/palm 1
Deleobuvir	Böhringer	NNI site 3/palm 1
Host targeting agents		
NIM811	Novartis	Cyclophilin inhibitor
Alisporivir (Debio-025)	Novartis	Cyclophilin inhibitor

small groups of patients have been confirmed in the phase III studies and in “difficult-to-treat patients” [such as in check of previous regimens (including protease inhibitors), cirrhotic patients, liver or kidney

transplanted patients, HIV infected patients or multi-drug treated patients, at an increased risk of the drug interaction]^[63]. Nevertheless, viral variants resistant to DAAs can emerge during the antiviral therapy^[295-298]

(Table 4). The estimation of a putative virus resistance profile prior to an antiviral therapy can help to select the optimal treatment regimen for individual patients^[295,296].

CONCLUSION

A prominent genetic diversity of HCV in combination with non-trivial replication cycle poses serious problems in virus research and therapy of virus-associated diseases. However, during the last several years a significant progress in understanding of the HCV molecular biology and pathogenesis has been achieved. In many aspects that allows to making a great step forward towards antivirals design, that result in DAA therapy. However, even with the new drugs the HCV-associated problems, of both fundamental and applied origin, are not solved. In fact, the resolution of these problems is going hand in hand.

First, in the absence of direct cytopathic effect, the mechanism of lipid deregulation, that results in liver pathologies, is not completely understood. Second, the system of the HCV control during latency and putative triggers of virus active replication were poorly investigated. Third, design of prophylactic vaccine is still in its infancy. Finally, the access to drugs and therapies for the people leaving in the third world is very restricted. Even if SVR rates > 90% are reached, nearly 10 million people will be living without treatment options^[63]. Indeed, an easy access to diagnosis and treatment is still missing to drug users, people in difficult socio-economic situations, migrants, prisoners, because of themselves or health policy.

Therefore, fundamental studies on virus-cell interactions and studies directing towards development of the prophylactic vaccine should be intensified.

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