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# UP-REGULATION OF THE ATP-BINDING CASSETTE TRANSPORTER A1 INHIBITS HEPATITIS C VIRUS INFECTION

SSD (Settore Scientifico Disciplinare) MED/09

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# Index of contents

## Summary

Hepatitis C virus (HCV) establishes infection using host lipid metabolism pathways that are thus considered potential targets for indirect anti-HCV strategies. HCV enters the cell via clathrin-dependent endocytosis, interacting with several receptors, and virus-cell fusion, which depends on acidic pH and the integrity of cholesterol-rich domains of the hepatocyte membrane.

The ATP-binding Cassette Transporter A1 (ABCA1) mediates cholesterol efflux from hepatocytes to extracellular Apolipoprotein A1 and moves cholesterol within cell membranes. Furthermore, it generates high-density lipoprotein (HDL) particles. HDL protects against arteriosclerosis and cardiovascular disease. We show that the up-regulation of ABCA1 gene expression and its cholesterol efflux function in Huh7.5 hepatoma cells, using the liver X receptor (LXR) agonist GW3965, impairs HCV infection and decreases levels of virus produced. ABCA1-stimulation inhibited HCV cell entry, acting on virus-host cell fusion, but had no impact on virus attachment, replication, or assembly/secretion. It did not affect infectivity or properties of virus particles produced. Silencing of the ABCA1 gene and reduction of the specific cholesterol efflux function counteracted the inhibitory effect of the GW3965 on HCV infection, providing evidence for a key role of ABCA1 in this process.

Impaired virus-cell entry correlated with the reorganisation of cholesterol-rich membrane microdomains (lipid rafts). The inhibitory effect could be reversed by an exogenous cholesterol supply, indicating that restriction of HCV infection was induced by changes of cholesterol content/distribution in membrane regions essential for virus-cell fusion. Stimulation of ABCA1 expression by GW3965 inhibited HCV infection of both human primary hepatocytes and isolated human liver slices.

This study reveals that pharmacological stimulation of the ABCA1-dependent cholesterol efflux pathway disrupts membrane cholesterol homeostasis, leading to the inhibition of virus–cell fusion and thus HCV cell entry. Therefore besides other beneficial roles, ABCA1 might represent a potential target for HCV therapy.

# Introduction

## A brief history of viral hepatitis: from catarrhal jaundice to hepatitis C

Hepatitis has gone along with human history since its origins, being linked to jaundice, a very recognizable symptom. Catarrhal jaundice, as it was named in XIX century medicine, was deemed to be due to “a chill on the liver”, gastruoduodenal catarrh, or immoderate indulgence to the table, until the occurrence of jaundice in epidemic form prompted into consideration the hypothesis of a communicable disease [1]. In 1931, Findlay, Dunlop, and Brown presented a paper entitled “Observations on Epidemic Catarrhal Jaundice” at the Royal Society of Tropical Medicine and Hygiene, in which they concluded that catarrhal jaundice was likely due to infection by a “ultra-microscopic virus which is pathogenic only to man,” similar to varicella, herpes zoster, rubella, and dengue. The infectious hypothesis to explain jaundice received decisive support by epidemiological investigations of an outbreak occurring in the US army in 1942. At his press conference on July 24, 1942, Secretary of War Henry L. Stimson reported that 25,585 cases of jaundice had developed among army personnel between Jan. 1 and July 4, apparently from the use of vaccination against yellow fever. The modified strain of live virus on which the vaccine was based was exonerated on immunological grounds, leaving the possibility that the responsible agent was a contaminating virus which gained entrance to the vaccine and which was carried over a series of tissue-culture passages. The terms, hepatitis A and hepatitis B, were first introduced by MacCallum in 1947 in order to categorize infectious (epidemic) and serum hepatitis [2]. These terms were eventually adopted by the World Health Organization Committee on Viral Hepatitis (World Health Organization. 1973. Viral Hepatitis Repository of WHO Scientific Group. WHO Technical Report Series 512. Geneva: WHO). Evidence of the oral/faecal transmission route of the hepatitis A virus (HAV) eliminated this virus as causative agent of blood transmitted hepatitis (“see for review” [3]). In 1963, while searching for a diagnostic marker of leukemia, Baruch Blumberg discovered a previously unknown protein in the serum of an Australian aborigine, hence called the Australia antigen [4], that he thought - erroneously - to be of value for the diagnosis of early acute leukemia. It is ironic that proof of a link between lymphoproliferative disorders and hepatitis B required 45 years [5,6]. It was only in

1970 that carriage of the Australia antigen was linked to blood-transmitted hepatitis [7,8], and renamed hepatitis B virus surface antigen (HBsAg). Soon after these findings, screening of blood from volunteer donors became mandatory in the USA. Blood testing for HBsAg and exclusion of positive donors decreased the rate of viral hepatitis up to 97% [9]. Nevertheless, despite screening for HBsAg, cases of post-transfusion hepatitis still occurred, suggesting that these subjects were infected with other putative virus(es) [10-12]. The research for the responsible agent progressed slowly, mainly because of the absence of a cell culture or animal model for propagation of the unknown agent causing the entity known at that time as non-A, non-B hepatitis (NANBH). Identification of the pathogen responsible of this type of viral hepatitis occurred only in 1989. After 6 years of hard work, Michael Houghton, in collaboration with Dan Bradley, succeeded in the identification of the hepatitis C virus, approaching the problem with a blind immunoscreening strategy [13] ("see for review" [14]). The authors extracted and reverse transcribed the RNA from the plasma of chimpanzees infected with the NANBH agent and the cDNA fragments obtained were used to build an expression library in E. coli. Subsequently, expressed proteins were screened with antibodies derived from the plasma of a patient with NANBH. Finally, the sequence of an immunoreactive clone was determined. From the sequence analysis emerged that the NANBH agent contains a positive sense RNA genome of approximately ~9600 nucleotides showing analogy with members of the Flaviviridae family [15]. The new virus is currently classified as a member of the Flaviviridae family (other members: yellow fever, dengue, West Nile virus), Hepacivirus genus, and is named hepatitis C virus (HCV).

## The disease

### *Epidemiology and variability*

According to the World Health Organization, 170 million people are chronically infected by hepatitis C virus (HCV) and therefore are at risk of developing liver cirrhosis and/or liver cancer: more than 350,000 people die from hepatitis C-related liver diseases every year. Globally, the prevalence of people seropositive for HCV has increased from 2.3% to 2.8% and >122 million to >185 million between 1990 and 2005 [16].

HCV shows a high degree of variability, with different HCV genotypes diverging up to 30%-35% at the nucleotide level. To date, seven major genotypes and more than 120 subtypes of HCV have been identified and characterized [17]. HCV genotypes are not uniformly distributed, and significant geographic differences in risk group targeting and epidemiology exist [18] (Figure 1). In the US, the predominant genotype is 1a [19]. In Europe and Asia genotypes 1b, 2a and 2b are the most prevalent, having been linked to past blood transfusion, whereas genotype 3a has been associated with drugs injection [20]. In the Middle East the main genotype is the 4a, especially in Egypt, where the infection spread was due to the parenteral anti-schistosomal therapy carried out from the 1950s to the 1980s without the use of sterilized needles [21]. In sub-Saharan Africa and East Asia, despite large geographical and genetic variabilities, one genotype seems to predominate over the others (genotype 1 in Central Africa, genotype 2 in West Africa and genotype 6 in East Asia) [18].

Although several factors contribute to the high HCV genetic variability, the two most important are the immunologic pressure exerted by the host, that makes the virus evolve, and the absence of proofreading activity by the error-prone viral RNA polymerase. As a consequence, within an infected individual a large number of viral variants (“quasispecies”) is generated. The quasispecies consist of a pool of closely related viral variants whose relative frequency depends on their replication efficiency (“fitness”) in the host’s environment [22,23], “see for review” [24]. Importantly, the generation of viral targets for host cytotoxic T-lymphocytes that are continuously changing over time allows persistence of a chronic infection that escapes immune control, as elegantly shown in human immunodeficiency virus infection (HIV) [25].

The nucleotide sequence variability concerns the whole viral genome, but some regions show a higher degree of diversity than others. Regions encoding envelope proteins, especially in the hypervariable region 1 (HVR-1) are the most variable (hence its name), whereas the 5’ NTR is the most conserved region [26,27].

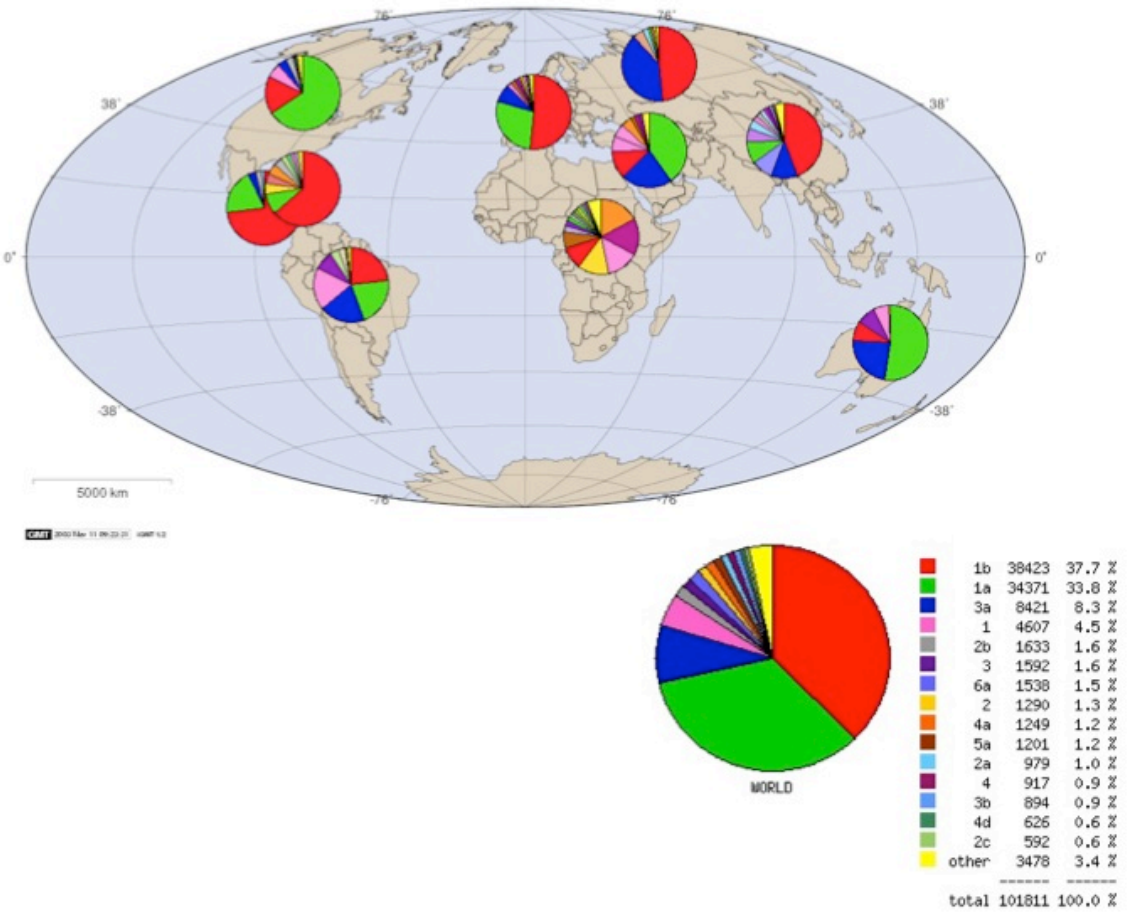


**Figure 1.** Worldwide HCV genotypes distribution and ratio.

## *Natural History*

Only a few reliable prospective long-term cohort studies on HCV infection are available to study its natural history. In fact: 1) both acute and chronic HCV infection are commonly either asymptomatic or characterized by nonspecific symptoms, making difficult to establish with certainty when infection occurred; 2) HCV patients are frequently co-infected by HIV and/or HBV, two viruses with which HCV shares risk factors, making difficult to assess the relative role played by each virus in the development of clinical endpoints; 3) correct estimates of alcohol consumption, a very important factor as far as liver fibrosis progression is concerned (see below), are notoriously difficult to obtain; 4) new natural history studies cannot be devised today, since effective therapies are available and cure of infection is possible, and performing such studies would be unethical [28].

### The Acute Phase of HCV infection

HCV RNA becomes detectable in the serum 7-21 days after infection, but the incubation period can be longer, depending on the inoculum size [29,30]. After 4-12 weeks from infection, an increase in the level of alanine aminotransferase (ALT) is observed, indicating liver injury [31]. ALT level reach concentrations ten times the upper normal level or higher, and may be followed by a rise in serum bilirubin concentration [32,33]. In addition, some patients develop symptoms 4-12 weeks after exposure, although the majority remain asymptomatic [29,34].

Symptoms commonly reported include nausea, fatigue, abdominal pain, loss of appetite, and mild fever. All these symptoms are nonspecific, but 16-33% of patients develop jaundice, in association with carriage of a single nucleotide polymorphism upstream the IL28B locus; patients with acute hepatitis C who become jaundiced are more likely to clear the infection [35].

Fulminant hepatitis C is a very rare event [36,37]. It is characterized by massive liver cells necrosis, and develops within 2-8 weeks post infection. In an anedoctal case of fulminant liver failure unequivocally caused by HCV [36], the peak of serum ALT concentration coincided with extremely high serum HCV RNA of a HCV strain with unusually low degree of diversity. These findings were interpreted by the Authors as reflecting a lack of selective pressure on the viral population because of insufficient time for the development of a specific immune response.

In a minority of cases, HCV infection can undergo spontaneous clearance, usually within 3-4 months from infection, but it is estimated that 54-86% of HCV infected patients establish a persistent infection [38]. Carriage of favourable alleles at the IL28B polymorphic locus is associated with spontaneous clearance [39].

### The chronic phase of HCV infection

HCV infection is defined chronic when HCV RNA is still detectable in the blood more than six month after transmission [40]. Persistent infection can cause liver damage, that leads sequentially to fibrosis deposition, cirrhosis and finally to development of hepatocellular carcinoma (HCC). The course of the disease is highly variable and patients report symptoms such as nausea, myalgia, loss of weight, right abdominal pain, and fatigue. All these symptoms are non-specific and often go unrecognized until advanced fibrosis/cirrhosis are established. Cirrhosis is defined histologically as a diffuse process characterized by replacement of the

normal liver parenchyma with fibrous tissue and the conversion of normal liver architecture into structurally abnormal regenerative nodules. This process ends in the loss of functional liver cells and the establishment of portal hypertension, to which most of liver-related mortality via liver decompensation and/or HCC development is associated. Data from a meta-analysis indicate that on average cirrhosis develops in 16% of patients within 20 years from infection [41]. This estimate is consistent with earlier findings suggesting that cirrhosis is associated with HCV infection in 27% of cases although ample (14-62%) variability exists, probably due to regional difference and other factors [42]. A key challenge for clinicians is to differentiate individuals with low risk of developing cirrhosis from those with high risk who necessitate immediate treatment. Several risk factors for fibrosis progression in hepatitis C have been identified, and include older age [43]. This makes sense, since the longer is the duration of infection the higher is the degree of the reparative processes leading to fibrosis. However, cirrhosis seems to be generated in a dynamic process that accelerates in parallel with age increase. Studies on cohorts of young subjects showed a rather low prevalence of cirrhosis, suggesting that in the young fibrosis deposition occurs slowly [43]. On the contrary, it has been demonstrated that people achieving a HCV infection at an age older than 40 years are at risk for fibrosis progression in a shorter period of time [44]. Indeed, it is known that infection during childhood lead to milder course, but on the other hand, it has been demonstrated that most of HCV patients develop fibrosis at about 65 years independently of the age of infection [45]. Gender seems to be another important predictive factor; in fact, fibrosis deposition is faster in men with respect to women, but, when menopause occurs, women are no longer protected [46], confirming earlier data suggesting the involvement of hormonal factors [47]. Viral factors have also been thoroughly investigated, and probably play a minor role. Exceptions are studies showing that genotype 2 and 3 are possibly linked to an accelerated and severe course of the disease [48,49]. Interestingly, viral load is not related to the extent of liver fibrosis or liver damage [47,50].

Finally, another recognized risk factor for disease progression among HCV infected patients is excessive alcohol consumption, obviously a major cause of liver cirrhosis on its own: in the context of HCV infection, chronic intake of more than 50 g per day increases dramatically the progression of the disease [51].

**Hepatocellular carcinoma**

HCC is the most common liver cancer and the 3rd leading cause of cancer-related death worldwide. Nearly always, HCC arises in patients affected by chronic hepatitis and cirrhosis [52]. Indeed, in the last 40 years, HCC incidence in industrialized countries has increased rapidly, to the extent that in the US HCC incidence is now three times that reported in the 1970s. Worldwide spread of HCV infection largely contributed to this phenomenon.

The various causes of HCC (including HBV, HCV, and aflatoxin B1, that together are responsible for more than 80% of HCCs) are perhaps better understood than those of any other major cancer in humans. Nevertheless, the molecular pathogenesis of HCC — that is, the specific genomic alterations that drive its development — is not understood, neither in HCV infected patients nor in other settings. Specifically, HCV does not have a reverse transcriptase (RT) and does not integrate into the host genome. In contrast to HBV-related carcinogenesis, where the higher is the viral load the higher is the risk of developing HCC [53], in HCV infection viral factors are thought to play a minor role. The most convincing data associate HCV infection with hepatocarcinogenesis through repeated cycles of inflammation and free radical production ("see for review" [54]). In fact, infection-driven activation of the inflammatory response cause the release of profibrogenic cytokines that lead to fibrosis deposition and liver cell proliferation ("see for review" [55]). On the other hand, the host of proinflammatory cytokines, chemokines, growth factors and inflammatory enzymes released during inflammation are thought to induce cellular DNA mutations through oxidative/nitrosative stress ("see for review" [56]). Moreover, HCV-infected patients have been shown to display increased lipid peroxidation levels [57]. Interestingly, microarray and proteomics studies demonstrated an increased oxidative stress gene response in patients with HCV-related fibrosis and cirrhosis [58]. In a recent study, the role of 50 different cytokines, chemokines, and growth factors has been investigated in 26 patients with HCV-related cirrhosis and HCC [59]. A number of proinflammatory molecules were found to be significantly increased in HCC patients, compared to healthy controls. Interestingly, serum IL-8 and IL-6 concentrations in HCC patients had a direct correlation with tumor burden [59].

## Immunobiology

### *Innate immunity*

Innate immunity is the first line of defence against infectious diseases. In particular, the interferon (IFN) system plays a pivotal role in the setting of viral infections. The interferon system consists of three type of interferons, namely type I (alfa and beta), type II (gamma) and type III (lambda). In addition, each type contains several subclasses. Type I IFNs are produced by infected cells as well as macrophage and dendritic cells (several IFN alpha and one IFN beta), whereas type III IFNs (lambda 1, 2 and 3 or IL-29, IL-28A and IL-28B) are produced by natural killer (NK) and NKT. T lymphocytes (both CD4+ and CD8+) also produce type III IFN.

To date, two mechanisms have been identified by which Type I and III IFNs are induced: one involves the toll like receptor (TLR) dependent pathway, and the other the RNA helicase retinoic acid inducible gene-I (RIG-I) pathway [60-62]. TLRs are a group of transmembrane receptors known for their role in the recognition of microbial pathogen associated molecular patterns (PAMPS) and in the activation of expression genes involved in the immune-inflammatory response [60]. TLRs are found on antigen presenting cells but also on fibroblast and epithelial cells. Almost all of them are expressed on the plasma membrane, but TLR3, TLR7 and TLR9 are localized intracellularly at the endosome level, and are involved in viral response. These three receptors respectively recognize double strand RNA (dsRNA), single strand RNA and non-methylated DNA with CpG motif [63-65]. Despite these differences, their pathways converge on the activation of the transcription factors NF-κB and interferon regulatory factor (IRF) 3. Once activated, these two transcription factors bind to responsive elements in the promoter of type I and III IFN genes. Virus escaping the above described pathways can be detected by RIG-I in the cytosol through interaction with viral ds-RNA and 5'-triphosphate RNA. Binding of RIG-I with viral nucleic acid determines conformational changes, interaction with mitochondrial antiviral signalling proteins (MAVS) and activation on IRF3 and NF-κB [66].

Type I, II and III IFNs bind to different receptors but they transmit the signal from the cell surface to the nucleus via the JAK-STAT pathways. All IFNs induce formation of STAT1 homodimers, which act as transcriptional factors for hundreds

of genes known as interferon-stimulated genes (ISG) [67]. Importantly, the set of ISG is different between IFN types and target cells [68,69].

Activation of the IFN system during the acute phase of infection is crucial to facilitate viral clearance, or at least to keep viral load under control. In the chimpanzee, a linear correlation between ISG stimulation and viral load exists [70,71]. HCV PAMPS are likely the most important regulators of ISG through TLR and RIG-I. In vitro experiments suggest the uncapped 5' phosphate dsRNA and the 3' NTR as PAMPS[72,73], but the evidence in support of this hypothesis is limited. It is also controversial which liver cells secrete IFNs, whether the hepatocytes or non-parenchymal cells, with the latter favoured by most [74,75]. Indeed, in the infected cells the protease NS3/4 is able to block both TLR and RIG-I pathways impeding IRF3 activation, cleaving MAVS and an adaptor molecule downstream TLR3 ("see for review" [76]).

### *Adaptive immunity*

One of the characteristics of chronic HCV infection is a defective adaptive immune response despite ISG induction and high HCV load. Nevertheless, HCV specific T cells and antibodies are detectable respectively within 5-9 and 8-20 weeks after the infection.

*Humoral response*. The role played by antibodies in HCV clearance is likely not paramount. Indeed, recovery from primary infection does not correlate with HCV-specific antibody titers, nor with the level of antibodies directed against the E1 and E2 glycoproteins [77,78]. In addition, immunocompromised patients without efficient humoral response can clear HCV infection [79]. Interestingly, absence of IgG subclasses other than IgG1 indicate no antiviral humoral response [80]. On the other side, data obtained from chimpanzees infected with antibodies neutralized–HCV give some weight to the role of humoral response [81].

The HVR-1 in the HCV envelope - thought to bind the viral entry factor complex - has been proposed to represent a target for neutralizing antibodies [36,82]. In the acute phase of infection, this region is subjected to a high mutation rate, that leads to quasispecies generation and escape from neutralization [83]. In this way, antibodies exert a selection pressure on viral variants contributing to the evolution of the HCV envelope sequences throughout the course of infection [84,85]. During

the chronic phase, serum IgG concentration and IgG secreting B cells increase, but almost all of them are not HCV specific [86].

*Cellular response*. Several pieces of evidence support the notion that T cells response is pivotal for clearance of HCV infection. In particular, the decrease of viral RNA coincides with the appearance of HCV-specific T cells and IFN-γ expression in the liver, suggesting that viral clearance is T cell mediated [87]. The role of IFN-γ is not clear, though, as it may simply be a marker of T cells function or of another antiviral factor. On the other hand, a direct antiviral effect exerted by IFN-γ is supported by the evidence that IFN-γ–mediated inhibition of subgenomic and genomic HCV RNAs is about 100-to 1,000-fold more effective than cytotoxicity [88].

HCV-specific, CD4+ T-cells are essential in the generation of a successful HCV-specific immune response. It has been demonstrated that patients showing vigorous proliferation of HCV-specific, CD4+ T-cells with concomitant IL-2 and IFN-γ expression recover and clear the infection [89-91]. In contrast, lack of an HCV-specific, CD4+ T-cell response or failure to maintain it for a sufficient time, especially in the case of viral mutations or quasi-species shifts, is associated with development of persistent infection and chronic hepatitis [91].

An early and strong multispecific CD8+ T-cell response is also important for viral clearance [92,93]. Chimpanzees able to develop a CD8+ T-cell response targeting multiple epitopes in HCV proteins resolve the infection, whereas others with limited response do not [94]. Interestingly, in vivo depletion of CD4+ T-cells from HCV-recovered chimpanzees abrogates protective CD8+ T-cell–mediated immunity upon reinfection, suggesting that CD4+ T-cell help is required for the generation and maintenance of protective CD8+ T-cells [95].

## Physico-chemical properties of HCV particles

Infectious, serum-derived HCV particles have diameters between 30 and 80 nm [96,97], while *in vitro* produced HCV particles (HCVcc; see below) show a diameter between 60 and 75 nm [98,99]. By electron microscopy (EM), HCVcc particles are pleomorphic, contain electron-dense cores, and lack discernible surface features [98-100].

Infectious HCV particles exhibit lower buoyant densities than other enveloped RNA viruses. Physical properties of HCV particles obtained from different sources have been analysed. Analysis of the infected patient serum in density gradients revealed that HCV RNA is distributed on a rage of densities from 1,03 g/ml to 1,20 g/ml and the peak of RNA is between 1,04 g/ml and 1,12 g/ml [101,102]. This heterogeneity is dependent on association of viral particles with different classes of lipoproteins (VLDL, LDL and HDL) and/or immunoglobulins [101,103-105].

HCV particles with higher buoyant densities are less infectious, underling the role of lipoproteins for HCV infectivity. Indeed, highly infectious virus particles present in chimpanzee serum were found to have densities between 1,03 and 1,10 g/ml [103]. Accordingly, whereas the peak of infectivity was found at lower density < 1.10 g/ml [106-108], most of HCVcc particles, had a higher density ~ 1,15 g/ml. In addition, both these densities are higher than the density HCV particles found in patient sera, since the hepatoma cell line currently used to produced HCV has deficient lipoprotein metabolism [109]. The low buoyant density of infectious HCV particles is likely the result of the association of the virion with serum lipoproteins [101,104,110,111]. Indeed, Apolipoproteins AI (ApoAI), ApoB, ApoC1, and ApoE have been shown to be associated with serum-derived HCV particles ("see for review" [112]). Several evidences support the presence of these apolipoproteins on HCV particles cultured *in vitro*, although the association with ApoB is controversial; likely due to inefficient VLDL production in cell culture, or methods used to its detection. [99,113-115]. In addition, HCVcc particles show lipid profile (cholesterol and lipid content) similar to low- and very low density lipoproteins (LDL and VLDL) [99,116]

The association of HCV particles with serum lipoproteins has led to the hypothesis that the virus is produced and secreted to the serum as a hybrid lipoviral particles (LVPs) following VLDL production and secretion pathway ("see for review" [117]).

The precise structure of the infectious lipoviral particles and the lipoprotein-HCV interactions are still unclear but two models have been proposed (Figure 2). Lipoprotein on LVPs may provide a shield, protecting virus from neutralizing antibodies [110,118].

In addition to infectious LVPs, other structures are present in the serum of HCV infected patients such as non-envelopped nucleocapsids [119] and more recently discovered non-infectious lipoviral particles [120].

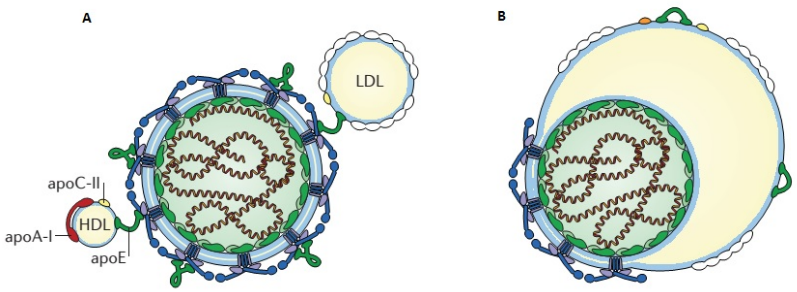


**Figure 2.** The two-particle model for lipoviral particle (LVP) structure, In the first HCV particles and serum lipoproteins transiently interact (A) In (B) the single-particle model for LVP structure, illustrating a low-density lipoprotein (LDL) particle integrated in an HCV particle). (Lindenbach, Nat rev. 2013)

## Molecular virology of HCV

HCV contains a 9.6 kb single stranded positive RNA genome composed of 5' non translated region (NTR), characterized by the presence of an Internal Ribosome Entry Site (IRES), one open reading frame (ORF) coding for structural and non structural proteins and the 3' NTR ("see for review" [121]). Structural proteins that form the viral particle, include glycoprotein E1 and E2 and the core protein. The non-structural proteins that are necessary for viral replication and assembly of infectious virus particle are the ion channel p7 protein, NS2 protease, the NS3-4A complex with protease and NTPase/RNA helicase activities, the NS4B, the NS5A phosphoprotein, and the NS5B RNA-dependent RNA polymerase (RdRp) [122].

IRES mediated translation leads to synthesis of a polyprotein residing on endoplasmic reticulum (ER) that is cleaved by cellular and viral proteases. Core protein is cleaved sequentially by the cellular signal peptidase (SP) and the cellular signal peptide peptidase (SPP) and p7 only by SP, whereas non-structural proteins are processed by NS2 and NS3/NS4 proteases [123,124] (Figure 3).

Intriguingly is the multifunctional aspect of HCV proteins, for example the replicase components that are involved in viral replication and assembly or the role of NS3-4A complex in the replication and pathogenesis of HCV [125-127]. In addition, functions of HCV proteins may be influenced by interaction with other viral or cellular molecules, which may induce different structural conformations [128].

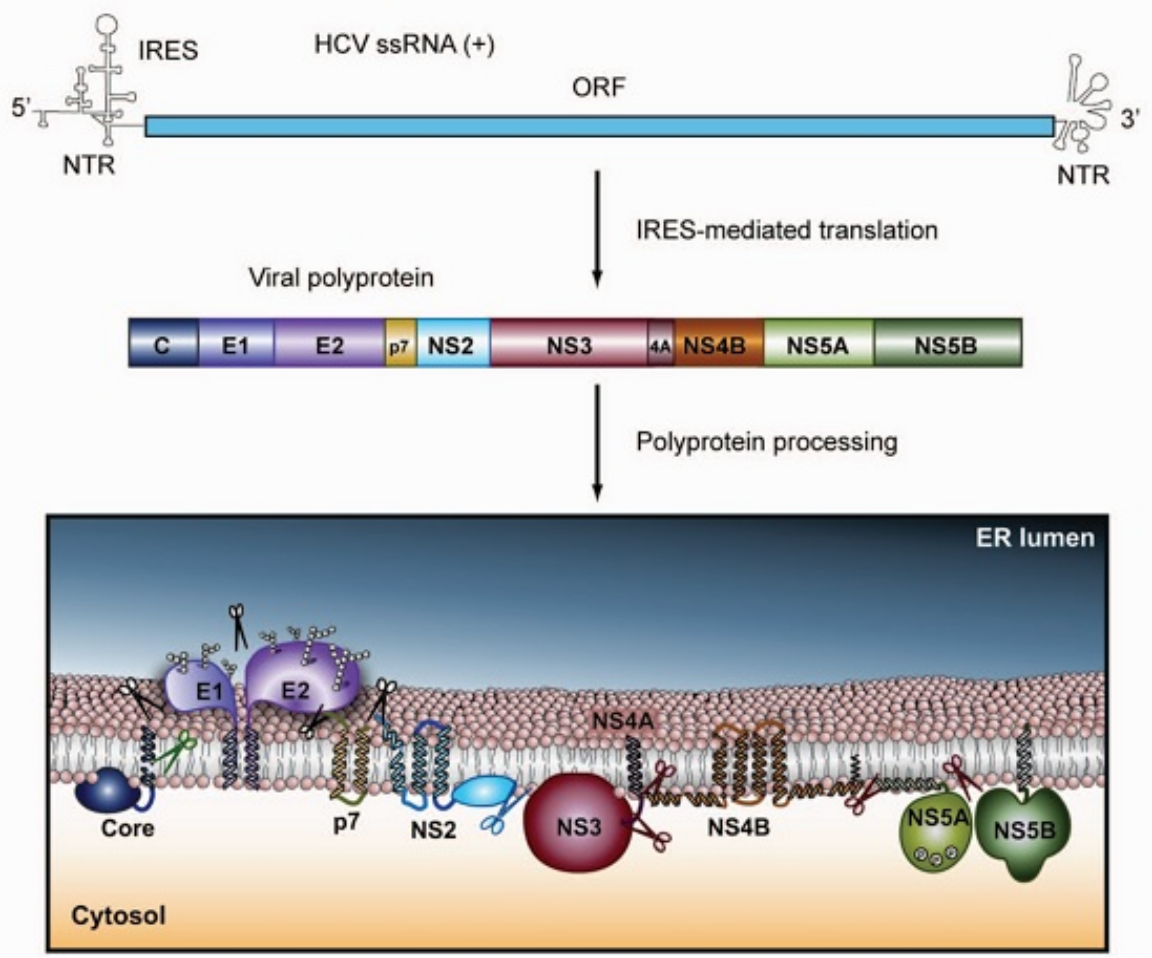


**Figure 3.** HCV genome contain only one ORF flanked by the 5' NTR and 3'NTR. The IRES in the 5' NTR drive the viral polyprotein synthesis that is co- and post- translationally processed by viral and cellular proteases. Polyprotein cleavages mediated by cellular SP are indicated by black scissor whereas cleavage mediated by NS2 or by NS3/NS4A complex are indicated respectively by blue and red scissor. The cleavage removing the carboxy-terminal region of the core protein, mediated by cellular SPP, is indicated by green scissor. The cleavage of the polyprotein, residing the ER, cause the release of structural protein (Core, E1-E2) and non-structural protein (p7, NS2, NS3/NS4A complex, NS4B, NS5A and NS5B). (Alvisi, G RNA Biology 2011)

### *5' NTR*

The 5′ NTR comprises the first 342 nucleotides (nts) and plays two key functions in the HCV replication cycle. First, in the positive strand it works as IRES driving RNA translation, and thus polyprotein synthesis. Secondly, in the negative strand it is thought to direct the synthesis of progeny positive-strand. The dual role of 5′-NTR is strengthened by the finding that in the positive and negative strands it adopts very different secondary structures. [129,130]. 5'-NTR in the positive strand contains four major stem loops (SL) or domain whereas in the negative strand seven predicted SL are present.

### *3' NTR*

The 3′NTR is pivotal for viral RNA replication [131], likely for the initiation and regulation of negative-strand synthesis [132]. It is constituted by three regions: a variable region, a polyU/UC tract of variable length (between 30 and 90 nts among HCV isolates) and a highly conserved element known as X-tail or 3′X (98 nts) [133].

The variable region deletion results in replication impairment but not its disruption, suggesting that it is not absolutely essential [131,134]. The poly U/UC function still needs to be clarified.

The X-tail is relatively conserved among HCV isolates and likely it contains the main regulatory elements required for the negative-strand synthesis [133]. Since mutations in this region are not well tolerated, both its conserved sequence and structure are critical. [134]

### *HCV Core protein*

The core protein is the first structural protein encoded by HCV genome and it forms the virus nucleocapsid. Between the core and E1 proteins is located a signal sequence that targets polypeptide to the translocon of the ER membrane, which mediates transferring of E1 ectodomain into the ER lumen [135]. In addition, the signal sequence is recognized and cleaved by the signal peptidase (SP) with production of an immature, 191 amino acid (aa) core (23 kDa). Further cleavage at the C terminus of immature core by the signal peptide peptidase (SPP) leads to the production of the mature ~177aa core protein (21 kDa) [135,136]. Although the C-terminal (aa177-191) domain D3 is cleaved soon after core is translated and thus is absent from the mature form of HCV core, it appears to be very important in terms of HCV core stability, targeting, and function [137]. The mature core is a dimeric membrane protein and may be stabilized by an intramolecular disulphide bonds [138,139].

The mature core protein is composed of two domains: the N-terminal hydrophilic D1 domain (aa 1-117) and the C-terminal hydrophobic domain D2 (aa 118-~177). The D1 plays a role in RNA binding and homo-oligomerization. Indeed, core D1 possess RNA chaperone activity probably required for the structural remodelling and packaging of the viral RNA [140]. D1 is also supposed to interact with several host factors and thus altering cellular functions upon HCV infection [128,141]. D2

is more hydrophobic and it is thought to interact with phospholipids on lipid droplets (LD) through amphipathic regions [142].
The HCV core protein, besides its role in formation of the virus nucleocapsid modulates many different host pathways by interacting with a variety of cellular factors. Indeed, the core protein is supposed to interact directly or indirectly with more than 200 cytoplasmic and nuclear molecules. This includes numerous transcription factors, such as heterogeneous nuclear ribonucleoprotein K, leucine zipper transcription factor (LZIP), 14-3-3 protein, RNA helicase CAP-Rf, p53, p21, NF-kB and x DDX3 protein ("see for review" [143])

It has been suggested to be involved in apoptosis and cell cycle regulation; thus contributing to the pathogenesis of HCC [128]
The role of core in the regulation of apoptosis is not completely clear. Indeed, it has been proposed a dual-role for the core protein.
*Pro-apoptotic effect*. Core protein can promote oxidative stress inducing production of reactive oxygen species (ROS). ROS cause DNA cleavage, increasing mutation rate, and promote the apoptosis *via* p53. Moreover, core interacting with the 14-3-3 epsilon protein causes release of Bcl-2-associated X protein (Bax), which provokes activation of mitochondrial apoptotic pathway [144].
*Anti-apoptotic effect.* during HCV infection, hepatocyte apoptosis could be induced by immune response. It has been demonstrated that the expression of HCV core inhibits Fas ligand mediated apoptosis and cell death as demonstrated in transgenic mice model [145,146]. Aside of the influence on the Fas ligand mediated pathways, HCV core protein has been found to suppresses the TNF-α mediated apoptosis [147]. As observed by Saito et al. in human hepatoma cells, HCV core may inhibit the TNF-α-mediated apoptosis inducing the expression of the cellular FLICE-like inhibitory protein (c-FLIP), an endogenous inhibitor of caspase-8 [148]. Finally, the HCV core protein may prevent the apoptotic effect of TNFα and Fas-ligand inhibiting the signal transducer and activation of transcription factor (STAT) 1 and activating STAT3, which protect the infected cells from cellular mediated immune response. [145].

HCV core protein is considered as a major viral factor inducing development of hepatocarcinoma during HCV infection. HCV core can modulate the expression of the cyclin-dependent inhibitor p21, a major target of p53 and regulate the activities

of cyclin/cyclin-dependent kinase complexes involved in cell-cycle control and tumor formation [149]. It has also been suggested that HCV core protein interferes with Wnt/β-catenin pathway playing a major role in the initiation of carcinogenesis [150].

It may equally contribute to the carcinogenesis *via* inducing insulin resistance up-regulating serine phosphorylation of insulin receptor substrate-1 (IRS-1), which in turn impairs the activity of protein kinase B (PKB), a pivotal signalling molecule of the insulin pathways [151]. In addition, HCV core protein expression induces development of hepatic steatosis, a risk factor for HCC in HCV patients [152]. Indeed, it has been proved that HCV core enhance the transcriptional activity of sterol regulatory element binding protein 1 (SREBP1) and peroxisome proliferators-activated receptor gamma (PPARγ) [153].

Although the pathogenic mechanisms leading to development of HCC in HCV patients have not yet been completely elucidated, several lines of evidence point to the core protein as a major factor contributing to these processes. Its particular role might be linked to its unusual subcellular localisation. Although the core protein predominantly resides in the cell cytoplasm, associated with lipid droplets, the site of virus morphogenesis, it has also been detected in the nucleus and mitochondria [154,155]. Indeed, whereas the presence of core on the LDs is mandatory for the virus assembly, the significance of its presence in the nucleus and mitochondria for the virus life cycle is still not clear. However, in tumor tissues from patients with HCC have been found to contain truncated forms of the HCV core protein within the nucleus [156-158].

Chang et al, demonstrated that core protein N- terminal contains three nuclear localisation signals (NLS), in the aa(5–13),aa(38–43), and aa(58–71) sequences. NLS are recognised and bound by importin-α that targets core to the nucleus [159]. This region is also necessary for core retention in the nucleus through its interaction with the proteasome activator 28γ (PA28γ) [160].

In addition to the tripartite NLS in the core protein, a functional nuclear export signal (NES) localised in the aa(109–133) sequence has been identified in HCV core, which is required for efficient virus production [154]. In particular, it has been shown that NLS and NES allow the core protein shuttling between the cytoplasm and the nucleus, enabling the virus to optimize its cell cycle and/or establish chronic infection [154].

**Alternative open reading frame protein (Core+1; ARFP)**

Normally correct placing of RNA template leads to synthesis of the HCV polyprotein of 3000 aa, however an overlapping ORF may generate an alternate reading frame product(s). Indeed, a novel HCV protein (named protein F, core +1 or alternative reading frame protein) encoded by an alternative open reading frame in the core region of HCV genome is produced in *in vitro* models [161]. The existence of the humoral and T-cell responses against core+1 in HCV infected individuals suggests that this protein is also produced in natural HCV infection [162]. Nevertheless ARFP has not yet been detected either in patients' sera or in infected tissues and its biological role remains unknown.

It has been reported that a +1 or -2 frameshifts can give rise to ARFP product *in vitro*, and thus result in production of corresponding immune responses detected *in vivo* [161,163]. In addition, high levels of anti-Core +1 antibodies have been found in patients with particular mutations in the core gene, infected with HCV-1a genotype [164]. Such mutations might induce conformational changes in the apical loop of SL-337 and thus favor the initiation of core+1 protein synthesis during natural HCV infection. These observations, in patients from South East Asia also suggested that *in vivo* similarly to studies *in vitro* [165]. HCV core+1 synthesis might negatively regulate production of the "normal" HCV core protein. The levels of anti-core+1 antibodies are elevated in HCC patients [166]. Core+1 protein seems to not be necessary for HCV life cycle [167], but the presence of the corresponding antibodies in HCV patients suggest its production *in vivo* and potential role in the pathogenesis.

### *E1E2 envelope glycoproteins*

Glycoprotein E1 and E2 are type I membrane proteins with a molecular weight of approximately 35 kDa and 70 kDa, respectively. Both contain a large N-terminal ectodomains (of 160 and 334 aminoacids in E1 and E2, respectively) and a short C-terminal transmembrane domain. The transmembrane domain (TMD) anchors E1 and E2 in the membrane and mediates their localization to the endoplasmic reticulum. The TMD is composed of two stretches of hydrophobic residues separated by a short segment containing at least one positively charged amino acid. These positively charged residues as well as the N-terminal part of E1 TMD have a key role in E1-E2 heterodimerization and retention in the ER whereas the entire TMD seems to be necessary for anchoring to the membrane. [168,169].

The ectodomains are characterized by the presence of six (E1) and eleven (E2) putative glycosylation sites, which are extensively N-glycosylated when they are translocated into the ER lumen. These sites are highly conserved among HCV genotypes and have been reported to play specific roles in HCV glycoprotein folding and entry [170]. The N-terminus of E2 contains the hyper variable region 1 (HVR1), a stretch of the HCV polyprotein rich in basic amino acids known to influence HCV pseudo particles (HCVpp; see below) infectivity [171,172]. HVR1 may be in part responsible for HCV escape from the immune response [85]. It has been shown that viruses lacking HVR1 are less infectious and exhibit impaired fusion, moreover they were more prone to neutralization and precipitation by E2 specific antibodies and soluble CD81 [173]. Nascent E1 and E2 are first assembled as a non-covalent heterodimer on the ER membrane but then the HCV glycoproteins undergo a dramatic change; indeed on the viral particle they have been found as large covalent complexes stabilized by disulfide bridges [174].

HCV glycoproteins E1 and E2 are present in tandem within the precursor polyprotein and this organization is similar to alphaviruses and flaviviruses that encode class II fusion proteins, although in the case of HCV the prediction of which glycoprotein acts as the fusion protein remain controversial. In fact, over the years many models have been proposed trying to explain fusogenic activity. Firstly, sequence analysis seemed to reveal that the E1 ectodomain contains a fusion peptide-like motif similar to the fusion peptide of paramyxoviruses and flaviviruses [175]. In addition has been demonstrated that specific residues but not the structure of this motif are required for mediating cell fusion and cell entry [176]. On the other hand, by analogy with other members of the Flaviviridae family, it has been proposed that E2 is a class II fusion protein [177,178]. Accordingly, Lavillette et al. have found at least three putative regions involved in virus-cell fusion by a mutagenesis approach, suggesting that these regions can contribute to the fusion of viral and cellular membranes, either by direct interaction with the lipid membrane or by induction of conformational change of E1E2 complex [179].

Very recently, Kong et al have succeeded in obtaining a crystal structure of E2 [180]. The crystallographic analysis shows a globular structure and contains many regions with no regular secondary structure despite the presence of eight disulfide bonds. Compared with class II fusion protein E2 shares with them only the Ig-fold beta sandwich and it does not adopt their extended three-domain fold. Finally,

data obtained by the authors and previously by others give further information on the putative CD81 binding site [180-182].

Despite these controversial data concerning fusion properties, E1 and E2 seem to have synergic roles in different HCV lifecycle steps. Indeed, it has been shown that the E1-E2 heterodimer binds CD81 stronger than E2 alone, so E1 may modulate E2 activity [183,184].

### *Protein p7*

p7 is a small integral membrane of 63 aa and is considered a NS protein although there is not a clear evidence if it is or not associated with viral particle. It contains two transmembrane α-helices (M1 and M2) connected by a positively charged cytosolic loop and the N and C termini that are oriented towards the ER lumen [185]. Early electron microscopy studies suggested that p7 monomers assemble into hexamers or heptamers [186,187] in artificial membranes. Recently, a study conducted by electron microscopy revealed a hexameric complex with a flower-shaped architecture and six protruding petals oriented toward the ER-lumen [188]. Furthermore a third α-helix upstream M1 has been found [189]. A more recent model, obtained using similar strategy suggests that hexameric and heptameric complexes may coexist, forming minimalist, yet robust functional ion channels [190].

p7 shares some analogies with viral protein of other viruses such as 6k of alphaviruses, M2 of influenza virus A, Very unique protein (Vpu) of HIV-1 which all belong to the viroporin family [190]. Generally, viroporins are not essential for viral RNA replication but they promote assembly and release of virus particles. Interestingly, some data indicate that p7 may be involved even in HCV replication [191].

*In vitro* and *in vivo* studies confirmed that p7 is essential for assembly and release of HCV particles [192-194]. p7 exerts its role in assembly through interaction with NS2. In viral release step, the role of p7 is based on its ability to form ion channels that equilibrate pH gradients within the secretory and endo-lysosomal compartments thus protecting HCV from uncoating during egress. [95,186,188,189].

### *NS2 protein*

NS2 is a transmembrane protein containing three putative transmembrane domains at its N-terminal and one cysteine protease domain at C-terminal. Homo-dimerisation of NS2 induces folding of the catalytic domain and formation of two active sites at dimer interface [195]. The unique known substrate for its activity is the junction NS2/NS3. NS2 might play a role in the HCV RNA replication. NS2 is necessary for polyprotein processing and full-length HCV genome replication but it is not required for RNA replication of the subgenomic replicon [196,197].

NS2 plays a key role in the assembly of infectious HCV particles and this function is independent of its protease activity [192,198]. NS2 exerts its roles at an early stage during virus assembly, acting in concert with E1-E2 glycoproteins, p7, and the NS3–4A enzyme complex [199-202]. Furthermore, Counihan and colleagues showed that recruitment of the core protein from LDs to the putative sites of virus assembly requires the NS2 and NS3–4A interaction [203]. As reported above, interaction between NS2 and p7 is also important for the assembly. In particular, this interaction is required to localize NS2 and LDs associated with core to putative sites of the virus assembly [199-201].

### *NS3-NS4A complex*

NS3-4A is a non-covalent complex composed of NS3 and the cofactor NS4A. NS3 is a 70 kDa protein with two well characterized enzymatic activities. Its structure has been solved by Yao et al. in 1999 [204](“see for review” [76]). The N-terminal contains the serine protease activity responsible for NS proteins cleaving from the HCV polyprotein, whereas in the C-terminus resides the NTPase/RNA helicase activity.

The N-terminal domain encoded protease belongs to the trypsin/chymotrypsin protease superfamily [205,206]. Protease activity relies on the NS3 catalytic triad and the NS4A cofactor facilitates the interaction between catalytic site and substrates, enhancing the efficiency and specificity [207]. NS3 substrates recognition is dependent on the presence of multiple amino acid residues; however, recent identification of its cellular substrates makes the story more complicated. Indeed, only few of a large number of cellular proteins containing the consensus sequence are cleaved by NS3-4A. On the other hand, several identified cellular substrates have less canonical sequence (“see for review” [76]).

NS3 belongs to the helicase super family 2 and shares six helicase motifs with related proteins [208]. NS3 exerts its nucleic acid unwinding activities with ATP consumption. When expressed alone NS3 helicase is less functional compared with the NS3-NS4A complex. The NS3 helicase is essential for HCV RNA replication and thought to play a role in the virus assembly [209,210].

Membrane association and structural organization of the NS3-4A complex are ensured by an in-plane amphipathic α-helix at the N terminus of NS3 and the transmembrane α-helix harboured in the NS4A N-terminal [211].

NS3-NS4A has been found also on the on mitochondrial or mitochondria-associated membranes. This finding might explain how it can inactivate the mitochondrial antiviral-signaling protein (MAVS) [212,213].

### *NS4B protein*

NS4B is a hydrophobic 27 kDa integral membrane protein. NS4B induces the formation of specific membrane alterations termed membranous webs, consisting of locally confined membranes in which HCV NSs accumulates and the viral replication takes place (Egger et al. 2002; Gosert et al. 2003). NS4B posses multiple functions: NTPase activity, interacts with other HCV NS proteins, binds RNA and plays a role in the assembly of viral particles [213-216]. As other HCV NS proteins, NS4B forms oligomers [217]. Furthermore, NS4B is essential for formation of functional replication complex: indeed mutation affecting its oligomerization prevented membranous web formation and consequently HCV replication. Probably NS4B exert its function inducing membrane curvature and formation of vesicles [218].

### *NS5A protein*

NS5A is a membrane-associated phosphoprotein of 447 aa that plays an important role in modulating HCV RNA replication and particle formation. It posses N-terminal membrane anchor and three domains separated by two low complexity sequence (LCS) [219]. The first two domains, D1 and D2 are mainly involved in RNA replication whereas D3 is required for virus assembly [125,127]. HCV NS5A was found in two states: basally phosphorylated (56 kDa) and hyper-phosphorylated (58 kDa). While basal phosphorylation concerns NS5A C-terminal and central residues the hyper-phosphorylation regards specific residues in the LCS. Observation that cell-culture adaptive mutations fall on these residues and

data obtained with kinase inhibitors led to the hypothesis that NS5A regulates HCV RNA replication, probably interacting with specific host factors [220]. NS5A is anchored to the ER membrane trough an N-terminal amphipathic alpha helix that is located in plane into the cytosolic face of the membrane. The NS5A ability to associate with phospholipid monolayer makes possible its interaction with the core protein located on LDs or on the LDs-ER interface. From the crystal structure of D1 emerged the presence of a dimer that extending form a channel, which can accommodate either ssRNA or dsRNA [221]. D1 domain of NS5A is known to bind RNA as a dimer [222]. Likely, NS5A dimers by attaching and gliding RNA may coordinate its fate during HCV replication [223]. D2 and D3 are natively unfolded and probably they reach stable conformation (mainly alpha helical) interacting with specific cellular or viral proteins. D2 and D3 have been found to interact and to be a substrate of Cyclophilin A (CypA) [224], CypA, is an isomerase required for HCV replication as demonstrated by its inhibition with cyclosporine A (CsA). ("see for review" [225]). In particular, D2 and D3 bind the active site of CypA and their proline residues are substrates for isomerase acivity [224,226]. Often mutations conferring CsA resistance were found in NS5A D2 [227].
Indeed, CypA plays crucial role in HCV RNA replication by interaction with NS5A and probably in virus assembly with the participation of NS2 [228,229].

### *NS5B protein*

Isolated more than two decades ago, the NS5B has been extensively studied and characterized [230,231]. NS5B is a viral enzyme of 68 kDa and is composed by a catalytic domain spanning from aa 1 to 530, a linker domain of 40 aa and a short C-terminal membrane anchor of 21 aa (CMA). The CMA is essential for replication *in vivo* but is dispensable for RNA synthesis *in vitro* [232]. *In vivo* the CMA is thought to be responsible for cytosolic orientation of the RdRp catalytic domain [233]. Similarly to many single-subunit polymerases it shows an organization similar to a right hand shape. In the "hand" is possible to recognize fingers, thumb and palm [234]. In addition, on the thumb is present a beta-flap that is characteristic of *Flaviviridae* RdRp [235]. Two conformations have been observed: closed (*de novo* initiation step) and open (elongation step). The close conformation is able to bind ssRNA template and the two initiating nucleotides [236]. In this step template and nucleotides are inside a completely encircled active site, which is closed from one side by fingers and on the other by interaction

between linker and beta-flap. After the *de novo* initiation step, the NS5B undergoes a transitional change during which the linker and the beta-flap are removed and the RdRp may begin the elongation. During this step the channel become enough wide to accommodate dsRNA.

## *In vitro* models to study HCV cell cycle

Since the discovery of HCV many unsuccessful attempts have been done to establish an efficient *in vitro* system to cultivate the virus. In early experiments, human and chimpanzee primary cells were infected with serum samples from HCV patients or transfected with cloned viral RNA. These trials failed to establish a robust replication model. In primary human foetal hepatocytes infected with HCV derived from patients' sera, the positive strand of virus RNA was detectable but the replication was very low. Cultures of primary hepatocytes were very difficult to establish. For these reasons several human hepatoma cell lines were tested. Human hepatoma cell lines 7721, PH5CH, HepG2 and Huh7 appeared susceptible to infection with HCV from patient serum but the system was still inefficient ("see for review" [237]).

Transfection of hepatoma cell lines with cloned viral RNA gave poor results due to non-functional sequences or mutations introduced by RT-PCR. This problem has been circumvented by the construction of a consensus sequence. The first construct generated with this strategy was derived from a patient defined H77, which was infected with HCV genotype 1a [238]. Despite these consensus genomes capable to establish infection *in vivo* all attempts to obtain robust replication and production of infectious virus particles *in vitro*, failed.

Hopefully, new molecular techniques allowed developing efficient *in vitro* cell culture systems to study HCV lifecycle. (Figure 4).

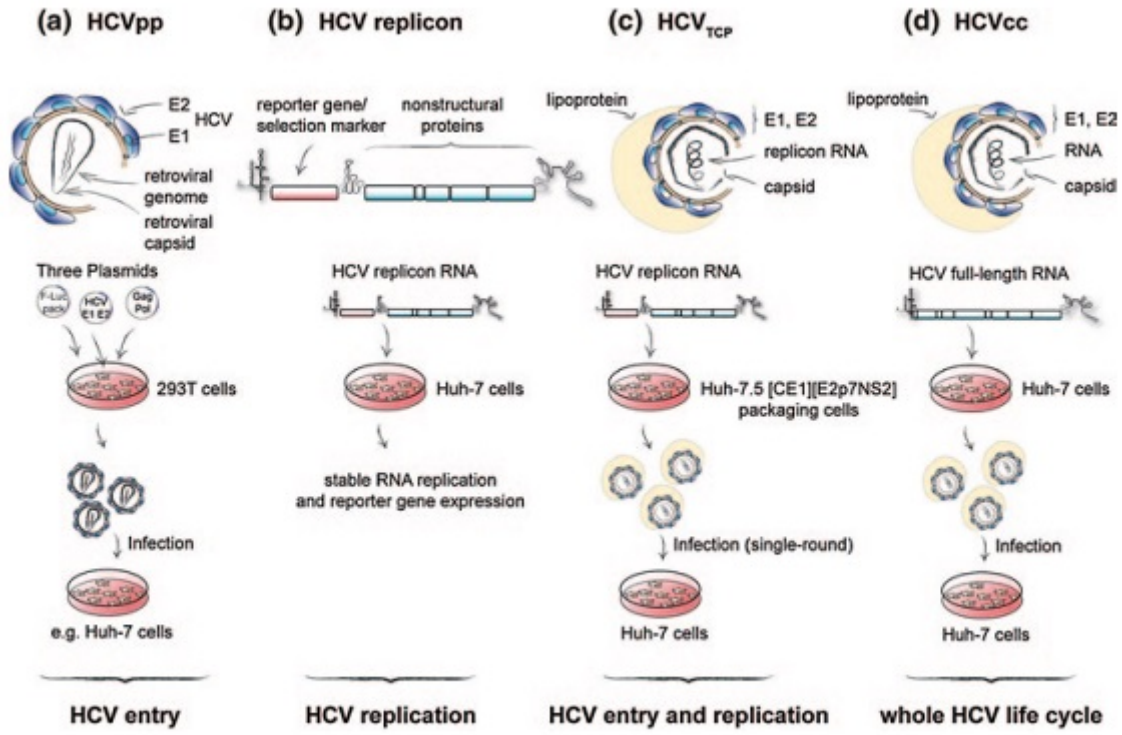


**Figure 4**. In vitro model systems developed to study HCV life cycle. Systems are listed accordingly to the HCV life cycle step(s) that they allow to study. (Steinmann E, Pietschmann T., Curr Top Microbiol Immunol.2013).

### *HCV replicons*.

Modified HCV genomes called replicons (genetic elements that can replicate autonomously) have been developed to study HCV replication [239]. At present, several systems are available ("see for review" [240]). The replicons may be sub-genomic e.g.; to contain only the non-structural HCV proteins required for RNA replication or full-length, so called genomic replicons that contain structural and non-structural proteins. As the first generation, most of them contain the HCV 5'-NTR, the neomycin phosphotransferase gene for selection, an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) driving translation of the HCV non-structural genes which are followed by the HCV 3'-NTR. The upstream T7 promoter drives transcription of all replicons types. Following transcription with T7 RNA polymerase, replicon RNA is transfected into human Huh7 hepatoma cell line.

Several studies showed that replicons might acquire adaptive mutations, which are generally located in the NS3, NS5A and NS5B proteins [241,242]. Importantly, adaptive mutations in NS5A region cause interferon resistance. In addition, a

replicon carrying three adaptive mutations (two in NS3 and one in NS5A) has shown strong increase of RNA replication ("see for review" [243]).
The replicon system allowed to study the host and viral signalling, adaptive mutations necessary for virus replication and most importantly screening of candidates for anti-viral molecules [244,245] ("see for review" [243]).
However, due to intrinsic limitations, this system could not be used to study viral entry and assembly process. Indeed, the replicon cells are not able to produce viral particles. ("see for review" [246]).

***HCV pseudo-typed particles (HCVpp).***

HCVpp was introduced as the first HCV *in vitro* infection system. Nevertheless it allowed only the investigation of the virus cell entry step and neutralization of HCV with anti-envelope antibodies. Indeed, HCVpp are composed of retro- or lentiviral nucleocapsid surrounded with HCV envelope glycoproteins E1 and E2 [247]. HCVpp are produced by co-transfection of the human embryo kidney cells (HEK293T) with three vectors: -the packaging vector carrying the gene encoding for retroviral structural proteins Gag and Pol, -the transfer vector containing a sequence required for encapsidation and a reporter gene (Luciferase or GFP) which are flanked at 5' and 3' by sequences required for its integration in the cellular DNA; - finally the vector which encodes unmodified HCV envelope glycoproteins E1 and E2, necessary for virus binding and fusion with the target cell membrane.
Virus pseudoparticles produced in 293T cells are used to transduce Huh7 cells and their infectivity is evaluated by quantifying expressed luciferase or GFP. It is also possible to transduce primary hepatocytes with HCVpp, yet the infection levels are usually lower than those in Huh7 cells [247].
HCVpp cell entry can be neutralized with sera of HCV infected patients and monoclonal antibodies targeting E1, or E2 proteins [248] ("see for reviews" [249,250]). In addition, they are often used to identify and characterise molecules able to block HCV entry and to investigate virus-cell-fusion mechanisms.
Importantly, because HCVpp originate from non-hepatic cells, which do not produce lipoproteins, these virus pseudotypes are not associated with lipoproteins (unlike infectious HCV virions, see below), and thus the entry mechanisms are not exactly same as for real, lipoprotein associated virus.

***HCV trans-complemented particles ($HCV_{tcp}$).***

$HCV_{tcp}$ are authentic viral particles that contain a JFH-1 (see below) based subgenomic replicon RNA instead of the full-length genome. These particles are generated in Huh7 packaging cell line after transfection with replicon RNA. The Huh7 packaging cell lines have been obtained through transduction with vector encoding HCV structural proteins and thus the sequences are stably integrated into the cellular genome [251]. This strategy permits to avoid the use of helper virus for providing structural proteins in *trans*, (which might result in contamination with HCV particles containing full genome). $HCV_{tcp}$ due to their origin show association with lipoproteins, and consequently resemble more to HCV than $HCV_{pp}$ [252]. Because $HCV_{tcp}$ contain only replicon genome, lacking of sequences encoding structural proteins, the infection of naïve cells with these particles results in a single round infection with only one viral entry and RNA replication.

***Cell culture derived HCV (HCVcc)*.**

In 2001 it has been reported that a sub-genomic replicon, obtained from a clone termed Japanese Fulminant Hepatitis 1 (JFH-1) replicated very efficiently in Huh7 cells without adaptive mutations [253-255]. Few years later three groups reported that the full JFH-1 genome was able to replicate and to produce virus particles in Huh7 cells, which were infectious for these cells as well as in animal models such as humanized mice and chimpanzees [100,256,257]. These virus particles were designated HCVcc and this system represented the turning point for HCV research. Indeed, it boosted the studies of all steps of the viral life cycle, besides early events like viral entry and replication. It enabled studying genome packaging, virion assembly, maturation, and release. Because the JFH-1 is a genotype 2a based system, many groups have worked to obtain clones from other genotypes able to produce infectious virus particles in cell culture [108,258]. However, these new systems seem not be able to establish a robust production of virus particles. To overcome this problem a wide panel of intra- and inter-genotypic chimeras has been developed to obtain infectious systems of different genotypes [259,260]. Most of the available chimeras have been produced by substituting the region spanning from core to NS2 protein in the JFH-1 backbone with the same region of other genotypes. Recently, the development of chimeras has been reported in which JFH-1 sequences encoding NS3/4A or NS5A proteins were replaced with

homologous sequences of other genotypes [261-263]. These new chimeras may permit the analysis of antiviral compounds (especially those targeting NS3/4A and NS5A) and studies of antiviral resistance developed by all HCV genotypes.

## Lifecycle

### *Attachment and entry*

The HCV entry is a multistep process that involves several factors and co-factors sequentially interacting with the virus that promotes initiation of productive infection. Below are described all factors and co-factors identified so far and a putative model of HCV cell entry process.

**Attachment factors:**

**Glycosaminoglycans (GAGs).** GAGs are long unbranched polysaccharides, consisting of repeated disaccharide units that have high degrees of heterogeneity with regard to molecular weight, disaccharide construction, and sulfation. Based on core disaccharide structures, GAGs are classified into four groups: heparin/heparan sulfate, chondroitin/dermatan sulfate, keratan sulfate and hyaluronic acid GAGs. They combine with cellular proteins to form a variety of molecules exposed on cell surfaces, known as proteoglycans (PG). Many viruses, like filoviruses, the HBV and the respiratory syncytial virus (RSV), use PG for their attachment to host cells [264-266]. Especially, a heparan sulfate proteoglycan (HSPG) seems to be crucial for the early steps of HCV infection.

The direct interaction between the HCV E2 glycoprotein and cell surface GAGs *via* the positively charged residues at the N terminus has been demonstrated and extensively studied using HCV-like particles produced in the insect cell line and recombinant E2 glycoprotein [267]. Nevertheless, it is generally accepted that the primary interaction of HCV with the liver heparan sulphate, especially Syndecan 1 is mediated by lipoproteins associated to LVPs and especially ApoE [268]. Indeed, the role of Apo E exposed on the HCV surface in virus attachment to heparin/heparin sulfate PG has been demonstrated by showing that antibodies targeting ApoE and synthetic peptides derived from ApoE were able to inhibit HCV cell binding [269]. These findings were further extended by Shi et al., who

investigated the role of different HSPGs core proteins in the HCV binding process, demonstrating elegantly that Syndecan 1 plays a major role in virus attachment compared to other member of the Syndecan family and others HSPG [268].

***DC-SIGN and L-SIGN.*** Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and the related protein liver/lymph node-specific (L-SIGN or DC-SIGNR) are calcium-dependent lectins expressed on dendritic and endothelial cells in the liver and lymph nodes, respectively. DC-SIGN and L-SIGN have been thoroughly investigated mainly because they were found to mediate human immunodeficiency virus (HIV) binding and internalisation [270]. Binding between these molecules and HIV is dependent on the presence of mannose N-linked chains in the HIV envelope protein (Env). Glycosylation of HCV E1-E2 glycoproteins is similar to that of HIV Env, and L-SIGN is expressed in the liver. Several interesting results emerged from early studies conducted with soluble E2 (sE2) and HCVpp, despite the intrinsic limitations of the two systems. In particular, sE2 and virus-associated E1 and E2 were able to bind DC-SIGN and L-SIGN [271]. These data have been confirmed with the HCVcc system [272]. All these studies supported the hypothesis that L-SIGN expressed on sinusoidal endothelium cells may serve as a docking site for circulating HCV within sinusoids, helping the virus transfer to hepatocytes. Absence of DC-SIGN and L-SIGN on hepatocytes suggests that they do not play a role as virus entry factors; rather, these molecules could enhance infection by promoting virus attachment. Finally, as suggested by Pöhlmann et al., sinusoidal endothelial cells may capture and concentrate circulating virions in the liver allowing their presentation to the hepatocyte [273].

**Entry factors:**

***Low-density lipoprotein receptor (LDLR).*** LDLR is a member of the low-density lipoprotein family and, since its identification [274], it has been extensively studied mainly due to its role in cholesterol homeostasis. LDLR, is a cell-surface receptor that recognises ApoB100 embedded in LDL and ApoE in intermediate lipoprotein (IDL), and mediates endocytosis of these lipoproteins [275]. The presence of lipoprotein components on the virus surface and the role of LDLR in HCV entry were first suggested by V. Agnello [276]. Several studies confirmed that indeed

ApoB100 and ApoE are present on the HCV surface [113,114,118]. This hypothesis was also strengthened by the finding that cell entry of patient-derived HCV strains required LDLR [277].
Although ,several studies clearly demonstrated the role of LDLR in HCV cell entry [276-279] in one study the role of LDL-R in HCV RNA replication has been suggested rather than in the virus entry process [280].

***Cluster of Differentiation 81 (CD81)***. CD81 is a 26 KDa transmembrane protein belonging to the *tetraspanin* family, ubiquitously expressed, and involved in regulation of cell morphology, motility and signalling [281]. CD81 is a type III membrane glycoprotein consisting of four transmembrane domains producing two extracellular loops and one short intracellular domain. The role of CD81 as an HCV entry factor has been suggested by Pileri et al, on the basis of its interaction with E2 protein [282] and subsequently confirmed using HCVpp model [247]. Further studies showed that disulphide bonds between cysteine residues in the large extracellular loop (LEL) are responsible for the stabilization and integrity of CD81 enabling its interaction with E2 [181,283]. Specifically, the LEL has been demonstrated as crucial for E2 binding [284-286] and residues on CD81 and E2, involved in the interaction have been identified [182,282,287]. HCV E1E2 heterodimers have a stronger interaction with CD81 than soluble E2 [183] and thus CD81 might induce a conformational change in E1E2 heterodimers to promote low pH-dependent fusion and endocytosis [288].
Using high-resolution fluorescence microscopy techniques, others have demonstrated the presence of the dot-like tetraspanin-enriched microdomains (TEMs), on the cell surface, in which tetraspanins are present at higher concentrations [289]. These areas, equally observed in experiments with other viruses [290], suggested that CD81 clustering on the cell membrane might be linked to susceptibility to HCV infection [291].
EWI-2wint (EWI-2 without its N-terminus) has been suggested as a regulatory ligand of CD81, which could inhibit HCV entry in cells non-susceptible to infection [292]. Moreover, silencing CD81 expression by small interfering RNA inhibited HCVpp and HCVcc entry into hepatoma cell lines [293], while conversely CD81 expression in non-hepatic cells conferred susceptibility to HCV infection. [247,294]. Furthermore, anti-CD81 antibodies inhibit HCV entry but not its binding,

confirming the role of CD81 as co-receptor required for the virus cell entry after attachment step [294,295].
Importantly, CD81 has been found in the cholesterol rich-microdomains (lipid rafts) and in the cell junctions, where it co-localises with SR-BI and CLDN1, respectively [296,297].

***Scavenger Receptor Class B type I (SR-BI)***. SR-BI is a 509 aa glycoprotein of 82 kDa found on the membrane of several cell types including hepatocytes. It is involved in the bidirectional transport of cholesterol and has been characterised as a receptor for various classes of lipoproteins [298]. It has a large extracellular loop anchored at the membrane at both N- and C- termini [299].
SR-BI was identified as a potential receptor for viral entry on the basis of its interaction with soluble E2 protein [300]. Furthermore, a possible interaction with the hypervariable region 1 (HVR1) has been proposed [301]. Further studies, have better defined the importance of SR-BI for HCV binding, and identified a domain (aa70-87) in SR-BI necessary for E2 recognition [302]. However, the experiments conducted with serum-derived (thus lipoprotein-associated) HCV have demonstrated that effective interaction between SR-BI and virus-associated ApoB-containing lipoproteins occurs *via* virus-associated VLDL [118].
The endogenous function of SR-BI as the lipid transporter may also play a role in susceptibility to HCV infection: in fact, using HCVpp and HCVcc, it has been shown that high density lipoproteins (HDL) enhanced HCV infection [301,303], while oxidized LDLs reduced HCV entry [304,305].
Moreover, Ploss et al. have demonstrated that whether human CD81 and Occludin (OCLN) are essential to render mouse cells susceptible to infection, murine homologues of SR-BI and CLDN1 may function similarly to the human proteins in promoting HCV entry [306]. This study provided important information concerning the possibility of development of the small animal models for studies of HCV infection.
Finally, inhibition experiments have shown that anti-SR-BI antibodies block HCV infection of hepatic cell lines [307] and of chimeric mice with transplanted human hepatocytes [308], confirming that SR-BI is an essential factor for HCV infection.

Recent studies have validated the hypothesis that SR-BI is involved in HCV cell entry at binding and post-binding steps [309] as well as in the cell-to-cell virus transmission [302].
SR-BI, to exert its role in HCV entry requires co-localization with CD81 on cholesterol-rich microdomains of plasma membrane and with CLDN1 (see below)

***Claudin 1 (CLDN1).*** CLDN1 is a 211 amino acid, transmembrane protein of 23 KDa. It is an important component of tight junctions involved in cellular permeability and polarity [310]. It is expressed in all epithelial tissues but especially in the liver and also at the basolateral membrane of hepatocytes as non-junctional CLDN1 [311]. This molecule was identified as a novel entry factor for HCV infection by screening a library of cellular proteins with HCVpp [312]. Although there is no homology between CLDN1 and tetraspanins [313], CLDN1 has been shown to interact directly with HCV virions [314]. Furthermore, CLDN1 forms entry complexes with CD81, playing a role in the post-binding step [315]. The critical region of CLDN1 for HCV entry is the extracellular loop 1 [312] and especially the domain containing the highly conserved motif $W_{30}$-GL$W_{51}$-$C_{54}$-$C_{64}$ [316]. Moreover, some studies have demonstrated that lateral diffusion of CD81-CLDN1 complexes is crucial for HCV entry *in vitro* [296,315]; however it has not been completely clarified if these complexes are pre-existing in the cells [317].
It has also been hypothesised that CLDN1 expression in the tight junctions might be related with HCV permissiveness [318], thus suggesting that tight junctions play a critical role for HCV entry. In fact, in infected cells CLDN1 is down-regulated to avoid superinfection [319]. Moreover, Mee et al. have investigated the role of the cell polarisation in the HCV cell entry: the polarisation affected tight junction formation altering the localisation of CLDN1 and other proteins [320]. This led to the formation of a non-junctional CLDN1 pool with altered capacity to bind CD81 and thus reduced HCV entry [321]. Equally, it has been proposed that other proteins belonging to the Claudin family, such as CLDN6 and CLDN9, might be involved in the mediation of the HCV entry although with different efficiency [322,323].

***Occludin (OCLN).*** OCLN is a 65 KDa transmembrane protein highly expressed in the tight junctions of polarized cells [324]. This protein plays an important role in cell-cell adhesion and in anchoring the junctional complex to the cytoskeleton [325,326]. After the identification of this protein as a new cellular factor for HCV entry [306] it has been clarified that OCLN is required for late, post-binding entry events [319,325].

Currently, OCLN and CD81 are considered the two critical cellular factors responsible for human HCV tropism. In fact, the expression of these human proteins confers susceptibility to HCV infection of mouse cells [306]. It has also been demonstrated that glucocorticosteroids may increase OCLN expression levels stimulating HCV entry, as already shown for SR-BI [327]. A direct interaction between OCLN and viral proteins remains controversial and further studies are advocated to fully understand these mechanisms [313].

**HCV entry co-factors**:

***EGFR* and *EphA2***. The epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane glycoprotein whose intracellular domain possesses tyrosine kinase activity. EGFR overexpression has been detected in a large proportion of hepatocellular carcinoma cases (40-70%) [328]. Recently EGFR has been identified as a co-factor for HCV entry by RNA interference kinase screening [329]. A second entry co-factor was the ephrin type A receptor 2 (EphA2), a transmembrane tyrosine kinase protein involved in cell positioning, cell morphology, polarity and motility [330].

Data obtained from experiments performed with HCVpp, HCVcc and replicon systems and several lines of hepatic origin and primary hepatocytes, showed that EGFR and EphA2 do not interact directly with HCV particles but they facilitate virus entry modulating CD81-claudin-1 association and viral glycoprotein–dependent membrane fusion [329]. This mechanism appears to be dependent on the expression and the activity of both EGFR and EphA2. Binding of HCV with CD81 but not with CLDN1 activates EGFR, triggering the internalisation of the HCV- CLDN1-CD81 complex.

HCV entry is apparently mediated by EGFR activation but not by its kinase activity [331]. The complex HCV-CD81-CLDN1 associates with two other proteins: CD81-

associated protein ITGB1 and Rap2B, which were identified as cofactors for HCV entry. This complex activates a GTPase protein, HRas that represents the link between the HCV entry complex and the signalling pathway of EGFR. EGFR trough HRas activation, mediate HCV entry *via* the MAPK pathway, promoting CD81-CLDN1 complex assembly. [332]. Furthermore, it is hypothesised that Rap2B, another GTPase protein, acts by regulating tetraspanin-enriched microdomains formation promoting CD81 and ITGB1 clustering [332]. These findings may have therapeutic implications for the treatment of HCV and other viral infections.

***Niemann Pick C1-like 1 receptor*** **(NPC1L1).** NPC1L1 is a glycoprotein with a molecular mass of 170 to 200 kDa, predicted to contain 13 transmembrane domains with three large extracellular loops (LEL) of which LEL1 has been shown to bind cholesterol. NPC1L1 is involved in cellular cholesterol absorption and in humans it is highly expressed in the liver and the gastrointestinal tract. In hepatocytes, NPC1L1 has been found to localize mainly in the endocytic recycling compartment during steady state but, in the case of cholesterol depletion, it is translocated to the canalicular membrane. Once on the plasma membrane, NPC1L1 exerts its function driving the influx of biliary cholesterol into the cells. Recently, a model of NPC1L1 function has been proposed [333], according to which NPC1L1 on the membrane binds to cholesterol present on bile micelles and transfers it to form a NPC1L1-flotillin-cholesterol microdomain that is subsequently endocytosed *via* clathtrin coated vesicles.

The mechanism of action of NPC1L1 and its role in cholesterol homeostasis suggest a possible link between this cell surface cholesterol-sensing receptor and HCV entry, due to the presence of cholesterol on virus particles [116,334] (“see for review” [335]). It has also been shown that knock down of NPC1L1 as well as pharmacological endocytosis inhibition or blockage of its LEL1 by antibodies dramatically reduced HCV entry [336]. Furthermore, NPC1L1 seems to be an entry cofactor specific for HCV since no effect on vesicular stomatitis G protein pseudotyped particles was observed. Finally, it seems that HCV entry *via* NPC1L1 is highly dependent on HCV particle cholesterol content.

It remains to be clarified if NPC1L1 interacts directly with HCV by removing cholesterol associated with virions, thus revealing a binding site on E1-E2, or

conferring required conformational changes for optimal fusion [336]. NCPC1L1 might play a role similar to that suggested for EGFR, i.e. the cholesterol triggered endocytosis of NPC1L1 and consequently entry of HCV particles.

***Transferrin receptor 1*** **(TfR1)**. TfR1 mediates cellular iron-transferrin complex uptake and is ubiquitously expressed in most human tissues. TfR1 is a 760 aa single pass type II membrane protein that upon complex binding undergoes endocytosis in a clathrin dependent way. Once iron is delivered inside the cells, TfR1 returns to the cell membrane to collect more iron [337]. TfR1 has been proposed as entry factor for several arenaviruses including Machupo virus, Guanarito virus and Sabiá virus [338,339].
Several pieces of evidence highlight a correlation between iron metabolism and HCV infection. First, a significant proportion of HCV patients have altered iron indexes suggesting iron overload [340]. In addition, microarray analysis revealed that changes in genes involved in iron metabolism occur during HCV infection. Recently, the role of TfR1 in HCV entry has been proposed. Preliminary evidence shows that TfR1 may interact with viral envelope glycoproteins. Probably such interaction may take place after virus binding to CD81 [341].

***Cluster of differentiation 63 (CD63)*****.** CD63 is a member of the tetraspanin superfamily, not belonging to the CD subfamily. In fact, CD63 constitutes its own subfamily and has a more ancient origin than other CD molecules [342]. CD63 is expressed ubiquitously and localizes both to the cell surface and to the endosomal system. This tetraspanin contains a lysosome-targeting motif that is recognised by AP-2 and AP-3, adaptor proteins responsible for the sorting of clathrin-coated vesicles, which respectively mediate endocytosis from the plasma membrane and redistribution from endosomes to lysosomes [343,344].
Recently Park et al [345] identified CD63 as a new entry factor by a novel approach based on computational prediction and data integration. The authors further analysed CD63 and discovered that it binds directly to HCV E2. In addition, HCV infection was inhibited by anti-CD63 antibody and by a polypeptide corresponding to the extracellular domain 2 of CD63.

### *HCV entry: a model*

As represented in Figure 5, HCV circulating in the blood might interacts with endothelial cells of the liver sinusoids, where molecules of the lectin family, in particular L-SIGN, might act as capture receptors for the transmission of the viral particles to the hepatocytes [346]. Subsequently virions are supposed to be trafficked to the basolateral membrane of the hepatocytes [294]. The first site of attachment is believed to be the HSPG [267,347], especially Syndecan-1 [268]. This might be due to the capacity of the LVPs to interact with both GAGs and LDLR through the virus associated lipoproteins allowing the attachment of HCV virions to the hepatocyte [276].

Post-binding events have not been completely determined, but it is supposed that, after the primary attachment, the viral particles interact with SR-BI that is able to bind HCV indirectly *via* VLDL associated to the virus [118] and then facilitate their uptake due to its cholesterol transfer function [348]. In sequence, the entry process probably involves the virus interaction with CD81 through E1E2 heterodimers [183]. Then, CD81 forms a complex with proteins of the CLDN family, [296], though it has not been completely understood if these interactions are pre-existing or induced by HCV binding.

After these early steps, the HCV entry may be altered by the presence of molecules circulating in the blood such as HDL and LDL [349], VLDL [118], or Lipoprotein Lipase (LPL) [350,351], facilitating (HDL) or inhibiting (VLDL, LDL, LPL) HCV infectivity,

The role of other factors is still unclear, although, according to the most recent data, HCV entry co-factors such EGFR and EphA2, once activated by their ligands, are responsible for HCV-CD81-CLDN1 complex modulation and transport into the tight junctions [322]. This step triggers viral glycoprotein–dependent membrane fusion and endocytosis by an actin-dependent mechanism [352,353]. The role of TfR1 is not completely clear; however it could play a role during endocytosis [341].

The tight junctions have been identified as necessary for protein localisation and virion internalization [306,320,321]; at this step of the entry process, complexes constituted by virion-CD81-CLDN most probably interact with other co-factors such as OCLN and NPC1L1 [336] leading to clathrin-mediated endocytosis [312,354] a common internalization mechanism for different viruses [355-358].

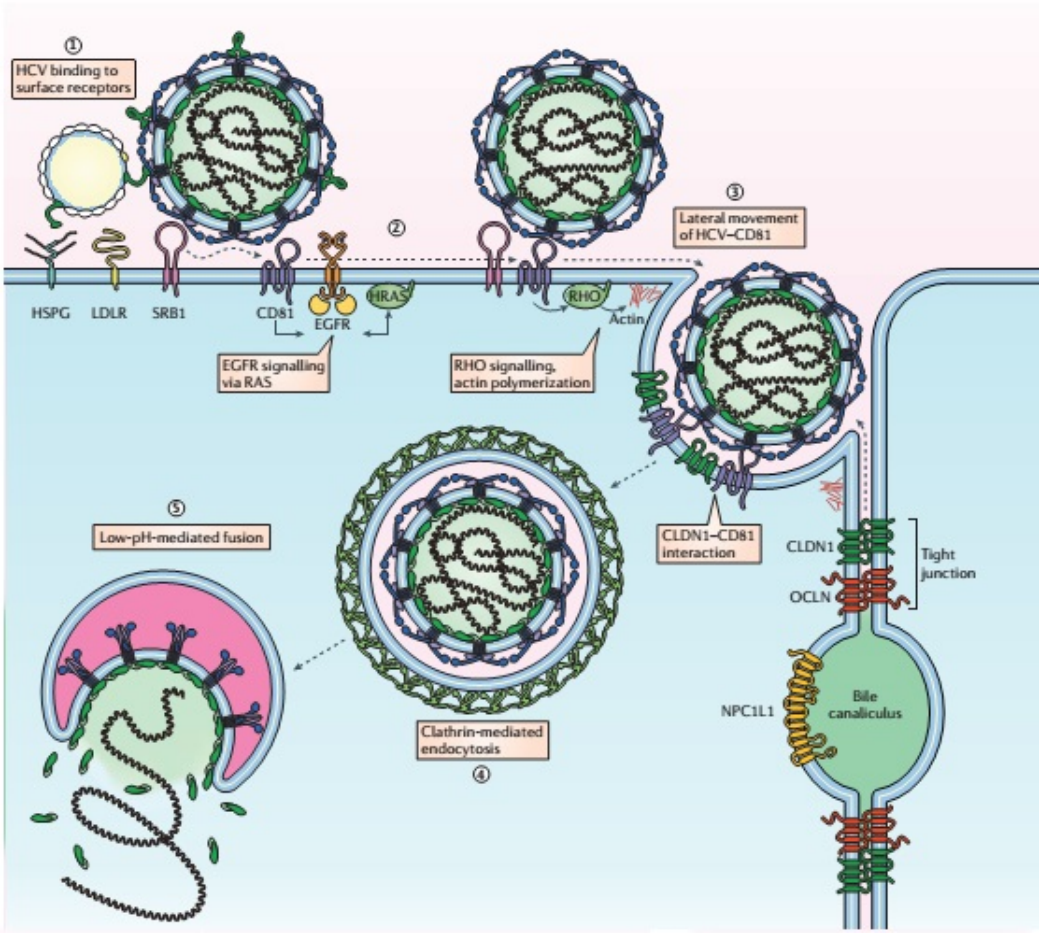


**Figure 5.** Putative mechanism of HCV entry illustrating interaction between the virus and entry factors (1-3). Step 4-5 show the fusion step that leads to HCV uncoating. (Lindenbach, Nat rev. 2013)

### *Fusion mechanism*

The mechanism of HCV fusion is not clear and for many years it was thought that it was mediated by the presence of a class II fusion protein, similar to those of other viruses possessing this class of protein [359,360]. Class II fusion proteins are known to induce membrane fusion within the endosome in a process highly dependent on an acidic environment [178,247,361], a process that has been demonstrated also for HCV entry [362] following a clathrin-mediated endocytosis [354,363]. In the case of *Flaviviridae* family it has been demonstrated that low pH induces conformational changes in the glycoproteins and heterodimer dissolution with the formation of a fusion-competent homo-trimer [283,364]

Also cholesterol facilitates HCV-mediated fusion dependent upon the presence of functional E1 and E2 proteins [179]. The fusion proteins act in common with lipid and cholesterol assemblies at the virus-cell fusion step. Level of virion-associated

cholesterol is likewise important, indeed the most fusion competent HCV show the same density as the predominant cholesterol rich lipoprotein [365,366]. Depletion of cholesterol from the virus almost completely abolished HCV infectivity, affecting internalization but not attachment [116].

Cellular lipids, mainly glycerophospholipids, sphingolipids and sterols, contribute through their physical, mechanical and/or chemical properties, whereas cholesterol can play a role through its preferential partitioning into rafts or its binding affinity for certain viral envelope proteins. Cholesterol-rich microdomains are implicated in the entry of many virus species such as Ebola and Marburg viruses, Vaccinia virus, murine Hepatitis virus, lymphocytic choriomeningitis virus and Herpes Simplex virus [367-371]. Indeed, when cholesterol dispersion or reorganisation of lipid microdomains were achieved using drugs such as filipin and nystatin, virus entry was largely reduced, although virion attachment was unaffected. Since all these viruses enter host cell *via* cholesterol-rich microdomains, these observations suggested drug action on a fusion step [372].

### *Translation*

The positive-strand RNA genome of the HCV is used directly as the template for translation in the cytosol of infected cells. The presence of an IRES element in the 5'-NTR of the viral RNA allows the virus to bypass the cellular mRNA processing events and permits the recruitment of the translation apparatus to the viral RNA to start translation of the viral proteins.

HCV IRES is characterized by the presence of seven stem-loops (IIIa-IV). Stem-loops IIId, IIIe,IIIf and IV constitute the core of IRES and they form a double pseudoknot structure, with a transfer RNA (tRNA) like structure [373]. This structure is known to bind strongly the small ribosomal 40S subunit due to multiple contacts. These multiple contacts make possible the IRES binding to 40S without contribution of any initiation factors [374,375]. The interaction between the structure and the 40S ribosomal subunit is believed to contribute to the positioning of the AUG codon in the mRNA binding cleft of the 40S ribosome [376]. Stem-loop II is required for translation but not stem-loop I [377,378]. The stem-loop II through the interaction with stem-loop IV may place the region with AUG into the 40S channel [379]

HCV IRES requires only three eukaryotic initiation factors (eIF) to form the 48S ribosomal complex and subsequently the 80S. The eIF3 interacts with 40S through

binding to the apical part of stem-loop III [380,381]. eIF2 acting in concert with initiator tRNA (tRNAi) and guanosine-5'-triphosphate (GTP) forms the eIF2-GTP-Methionine (Met)-tRNAi$^{Met}$ complex, which transfer the Met-tRNA$_i^{Met}$ to 40S in a GTP dependent way. eIF5 supports the start codon of the ORF recognition by aforementioned complex and induce eIF2 to hydrolyse GTP. Finally, joining of the 60S to the already established large complex leads to the formation of the translation-competent 80S [382].

Sequence and probably the secondary structure in the region coding for core may positively influence translation [383]. The 3' NTR also enhances the translation especially through elements mapped in its variable region, poly (U/C) tract and the 3'-X region [384].

eIF2 activity is suppressed In the case of the host cell-response to the virus and alternative eIF supporting the IRES mediate translation has been found. In particular, eIF5B can substitute both eIF2 and eIF5 or the eIF2a that promote delivery of tRNA in a GTP-independent way [385].

Several other elements, such as La protein and the microRNA 122 are suggested to contribute to the efficient translation but the mechanisms of their action need to be elucidated [386,387].

### *Replication*

Following translation and processing of the polyprotein, NS proteins comprised between NS3 and NS5B rapidly constitute the replication complex on the ER membrane. Establishment of the replication complex is thought to lead to membrane alterations [388]. Altered membranes are known since early studies on the human and chimpanzee liver tissue [389,390]. Expression of NSs, in particular NS4B induced vesicles accumulations, designated membranous web [391]. Several studies support the hypothesis that the HCV RNA replication sites are protected by membranes [392-394]. Others have suggested that these vesicles are membrane invagination with a pore that allow exchange of hydrophilic molecules like nucleotides [395]. It has also been assumed that each replication site contains only one copy of negative ssRNA that may be a part of double strand intermediate [393,394]. Aside single vesicles, more complex structures have been observed in HCV infected cells. These structures are defined as double-membrane vesicles (DMVs) and multiple-membrane vesicles (MMVs) but their functions as well their dynamic are not completely understood [396,397].

Morphology of membranous web is not dependent on RNA synthesis but it relies on the expression of NS3-N5B module that probably interacts with host factors (Romero-Brey et al. 2012; Reiss et al. 2011; Ferraris et al. 2010). NS4B has the major role in the formation of membranous web. Indeed, its expression generates membranous web resembling to the one generated by NS3 to NS5 proteins [391]. It has also been reported that sole expression of NS3/4A, NS5A or NS5B can induce membranous web morphogenesis, but only NS5A occasionally produces vesicles, which are morphologically identical to DMVs [398]. In addition, HCV impairs expression of genes involved in lipid metabolism, resulting in intracellular lipid accumulation that is crucial for optimal replication [58,399,400]. Lipids may be used in two ways: helping replication site formation through membrane proliferation and by protein modifications like geranyl-geranylation and palmitoylation [217,401].

Once membranous web are formed, RNA replication takes place. This step is a complex process and it has not been yet completely elucidated. Almost all data came from *in vitro* studies but the *in vivo* mechanism remains elusive due to lack of appropriate model systems. From studies with purified NS5B emerged that RNA synthesis may initiate in two ways: by the primer-dependent mechanism or by the *de novo* synthesis [230,402]. The *de novo* synthesis is thought to be the physiological mode of RNA synthesis in HCV infected cells.

The 3' end of the HCV negative strand is a template for RNA synthesis initiation; on the contrary, the 3' end of the positive strand is part of stable structure that cannot access to the closed NS5B conformation [132,403]. This suggests that the synthesis of the negative strand from the positive strand requires other factors like the NS3 helicase. RNA binding is a slow and inefficient process [404]. The NS5B enzymatic core binds with high affinity single strand RNAs with more than seven nucleotides [405]. Following the binding of the template, a dinucleotide primer is synthesized on the 3' end [406]. This step requires high concentration of nucleotides and is highly efficient, probably due to the closed conformation of NS5B [236]. Switch to the elongation step requires conformational change and high concentration of both GTP and the third base incorporated [406,407]. In conformational change, the C-terminal is removed to lodge the egressing dsRNA and "fingers" shift adapting contacts with the "thumb" [30,408]. During elongation,

NS5B incorporates 100-400 nucleotides per minute [409]. Termination step of RNA synthesis is almost unknown.

### *Viral assembly, maturation and release*

The exact mechanisms of the assembly of infectious virus particles remain elusive but behind these events there is a complex interplay between viral and cellular factors. HCV core protein due to presence of amphipathic regions behaves like the membrane protein and this allows its association to the surface of cytosolic LDs (cLD), which derive from the outer leaflet of the ER [142]. Mutations in core protein D2 may affect interaction with cLDs, impeding virus production, likely affecting the assembly [410,411]. In particular, mutation of the residue phenylalanyne 130 in D2 compromises protein stability and blocks targeting of core to LDs [410]. The trafficking of core to cLD requires also the cytosolic phospholipase A2 G4A (PLA2GA4) and is enhanced by the diacylglycerol acyltransferase 1 (DGAT-1), highlighting importance of the interaction of HCV with lipid metabolism [412,413].

The first evidence for the role of LDs in the mechanisms of HCV assembly was provided by Miyanari et al., who proposed the first HCV morphogenesis model [411]. In this study, core was proposed as a key element in the recruitment of E1E2, replication complexes and viral genome to LDs. According to this model LDs were the microenvironment in which all players come together to facilitate virus assembly.

More recently it has been shown that NS2 plays a crucial role in the early stage of assembly keeping together E1E2, p7 and NS3-4A [200,202]. In addition, interaction between NS2 and NS3-4A is recognised mandatory to recruit core-cLDs into the assembly site, as well as the interaction with p7, which is also required to localize NS2 to the site of the virus assembly [199,201,203].

Mutations in NS3 and NS5A impair the assembly, suggesting that they may contribute to the replication/assembly switch [414]. During the assembly of viral particles, NS5A is recruited on LDs to interact with core and probably also with ApoE, a fundamental element for this step [125,411,415]. In fact, interaction between ApoE and NS5a may contribute for to the building of the viral assembly platform [415].

Similar to other members of the *Flaviviridae* family HCV particles may be formed through budding into ER. Indeed pharmacological inhibition of ER-Golgi transport

induces accumulation of HCVcc inside the cell [416]. Based on these observations it might be hypothesised that HCV assembly originates in a site close to intracellular LD structures and the nucleocapsid formation may take place concomitantly with budding from ER.

After assembly, HCV particles undergo some complex modifications that may occur in the post-ER compartment. Several evidences support the hypothesis that maturation and release of HCV virions share features with the pathway of VLDLs production [114].

In hepatocytes, VLDL particles are assembled in a two-step process that requires several actors. In the first step, ApoB is co-translationally loaded with lipids by the microsomal triglyceride transfer protein (MTP) with formation of pre-beta VLDL in the ER. In the second step, pre-beta VLDL are further lipidated, but the mechanism is still unclear and two models have been proposed [417,418]. On one hand, VLDL precursor acquires lipids following the fusion with LDs facing the ER lumen, which are associated with ApoE and ApoC. On the other hand, VLDL may be lipidated in a post-ER compartment, likely in the Golgi. In circulation, triglyceride-rich core of VLDL is hydrolysed *via* LPL with formation of cholesterol-rich LDL [419].

Because ApoB, ApoE and MTP are all required for VLDL formation they were investigated to unveil their role in viral particle production. Blocking of VLDL synthesis through silencing of ApoB or ApoE as well as pharmacological inhibitions of MTP or the acyl-CoA-synthase 3, resulted in disruption of HCV production with no effect on viral replication [113,114,416,420]. On the contrary, others suggested that ApoB and MTP were not involved, whereas depletion of ApoE resulted in a strong negative effect on HCVcc production [113,421]. These authors, suggested that HCV maturation was dependent on fusion with ApoE associated LDs rather that VLDL pathways. However, these data might be affected by model system limitations. Indeed, most of results were obtained using Huh7 cells that are known to have incomplete lipoprotein synthesis pathways and thus produce low amounts of ApoB. Recently, ultrastructural analysis of HCV produced in primary human hepatocytes confirmed the association of ApoB with secreted viral particles [422].

Despite this controversial aspect, it is believed that HCV particles transiting through the secretory pathway associate with VLDL containing ApoE, reaching

their characteristic buoyant density. This hypothesis is supported by two essential findings: the patient-derived virus particles are associated with VLDL and extracellular HCVcc shows a lower buoyant density than nascent intracellular particles [416]. To date it is not clear if VLDL is attached to or integrated into the virion [113,114,423,424].

Furthermore, during egress the viral glycoproteins are heavily glycosylated, and their disulphide bonds are rearranged [174]. Finally, mature particles are secreted in vesicles protected from exposure to low pH by the presence of p7 on their membrane, likely preventing premature uncoating [194].

## Current and novel HCV therapies

### *Preventive and therapeutic Vaccination*

Despite all the efforts since the discovery of HCV, to date no preventive vaccine is available due to the high degree of virus variability. Indeed, recovery from infection does not provide protection to a second exposure to the virus. To date, two therapeutic DNA vaccines have entered clinical trials: CIGB-230 and chronVac-C. In addition, TG4040, a viral vector-based vaccine just ended a phase II clinical trial. The CIGB-230 vaccine is based on a plasmid that expresses HCV E1E2 glycoproteins and a recombinant core protein [425]. In an animal model, this vaccine induced a very strong production of antibody and T cell mediated response against HCV [426]. In a phase 1 clinical trial, it was shown to induce the production of antibodies able to target HCVpp [427]. In addition, one third of the patients developed cellular immunity against the infection. The risk of fibrosis was reduced, and 46% of cases cleared the infection [425]. In the phase 2 trial, the vaccine was used in combination with the standard therapy for HCV infection, interferon alpha plus ribavirin [428].

ChronVac-C consists of a plasmid coding for optimized NS3and NS4a genes [429]. One of major difficulties to use DNA vaccines is represented by the method of delivery. Indeed, the classical delivery through injection does not allow cells to capture the naked DNA. To overcome this problem, developers coupled the injection with *in vivo* electroporation, a short electrical pulse causing permeabilization of cellular membranes resulting in the induction of a local

inflammatory response. Reported data show that the vaccine gives rise to an evident T cell response after the second booster dose, without side effects. In the phase I/IIa clinical trial, 75% of the vaccinated patients infected by HCV genotype 1, were cured after treatment with the standard therapy.

TG4040 is a vaccine constituted by a recombinant vaccinia poxvirus containing sequences that encode the NS3, NS4, and NS5B proteins from the genotype 1b HCV [430]. In the phase I trial, this vaccine demonstrated to have acceptable safety profile and activity. In the phase II trial, it was used either in previously treated patients or in combination with standard therapy, showing efficacy respectively in 58% and 51% [431].

### *HCV therapy (with emphasys on novel drugs targeting HCV entry)*

Pegylated interferon alpha (pegIFNα) plus ribavirin has been the standard of care in HCV therapy for more than 10 years. The goal of HCV therapy is to achieve the so called sustained virological response (SVR), defined as undetectable HCV RNA (<15 IU/ml) after 24 weeks of treatment [432]. Success of therapy is highly dependent on several host factors, like gender, age, ethnicity, single nucleotide polymorphisms at the *IL28B* gene locus as well as on viral factors, like viral load and HCV genotype. In fact, pegIFNα plus ribavirin eradicate the infection in approximately 80% of patients infected by genotype 2 but the rate drops to approximately 40% in the case of genotype 1 [433,434]. Progress in the knowledge of HCV life cycle allowed the development of a host of novel anti-HCV compounds, many of which are undergoing clinical development. Recently, two NS3-4A protease inhibitors, telaprevir and boceprevir, have been approved for triple therapy (i.e., in association with pegIFNα and ribavirin) for the treatment of patients chronically infected with genotype 1 [434]. Although this therapy greatly improved response against genotype 1, they do not cure infection by other genotypes, and thus – at least in Italy - the standard of care for non-HCV1 genotypes is based on pegIFNα and ribavirin [433]. Importantly, relatively common genotypes like genotype 3 and 4 are quite resistant to the standard therapy and for this reason new drugs are urgently needed [435].

Novel treatments targeting host cell molecules involved in various steps of the HCV life cycle have been proposed, often to be used in combinations to prevent the development of antiviral resistance and to cover all HCV genotypes [436,437].

HCV entry is the first step in HCV life cycle and a potential therapeutic target for several drugs, either novel compounds, or molecules developed for other purposes and already available, but with different indication(s). Currently, one of the most interesting drugs targeting HCV entry is erlotinib, an epidermal growth factor receptor (EGFR) inhibitor used to treat lung cancer [438]. Through a previously described cell-to-cell transmission assay [439], Lupberger et al. demonstrated in a co-culture system that this molecule inhibits HCV infection and cell-to-cell transmission by blocking the EGFR kinase activity, necessary to regulate CD81-claudin1 association and membrane fusion in a post binding step [329]. Moreover, these authors showed that erlotinib treatment delayed the kinetics of HCV infection and decreased HCV-RNA level in a chimeric mouse model [329].

Another already marketed drug, ezetimibe, approved to treat hypercholesterolemia, showed an antiviral effect inhibiting HCV infection in vitro and in vivo in the transgenic mice model [336]. This molecule is a direct inhibitor of NPC1L1.

Another drug, ITX 5061, is a compound that causes reduction of SR-BI expression and thus inhibits the HCV infection, with a kinetic similar to anti SR-BI and anti-CD81 antibodies [440]. Considering the good safety profile showed by ITX 5061 in animals and humans, and its oral bioavailability, this molecule could be a promising HCV entry inhibitor and has indeed already entered a phase 2a study [441,442].

Other two well-known molecules have been shown to inhibit HCV entry: the green tea catechin epigallocatechin-3-gallate and silymarin. The first compound inhibits HCV entry *in vitro* in a dose dependent manner, blocking viral attachment to target cells; it is active against all HCV genotypes, cheap, innocuous in humans and very well tolerated in clinical studies involving healthy volunteers. [443]. Finally, silymarin, used in the past a “hepato-protective” drug, may come to a new life because it seems to limit HCV infection through the inhibition of virus entry and fusion. It probably acts by incorporating into lipid membranes of viruses and target cells, stabilizing them and making them less prone to fusion [444]. In a pilot study, short-term administration of high-dose intravenous silibinin rescued a few patients with ongoing minimal residual viremia while on interferon-based therapy [445]. Finally, ferroquine, an antimalarial ferrocenic analog of chloroquine has been

identified as a HCV entry inhibitor [446]. From this study emerged that ferroquine has an antiviral activity against all HCV genotypes, and inhibits HCV entry impairing the fusion process.

Host lipoproteins may also represent a "druggable" target because of their involvement in HCV lifecycle. It has been demonstrated that apoE peptides potently inhibit HCV infection suggesting the potential of developing of these molecules as novel HCV entry inhibitors by targeting HCV-host interactions [447].

Many other very promising drugs, targeting other steps of HCV life cycle, have undergone clinical development, and a few have already been approved by US and European regulatory agencies: their description is beyond the scope of this thesis.

## Role of lipoproteins and cholesterol in the HCV lifecycle

Lipids and lipoproteins are essential for the HCV life cycle [112,448,449]. HCV has developed lipoprotein-dependent mechanisms for cell entry [118,276,450], virus RNA replication regulated by fatty acids [400], virus morphogenesis is linked to lipid droplets [411] and the assembly and release of infectious virions follows the VLDL formation and secretion pathway [113,114]. Consequently, HCV circulates in the plasma of infected patients in association with VLDL and LDL forming LVPs [110,451].

The relationships between lipid metabolism and HCV are complex and intriguing. The expression of host genes involved in biosynthesis, degradation or transport of intracellular lipids is altered upon HCV infection [399,452]. Steatosis and insulin resistance associated with the metabolic syndrome increase fibrosis progression and reduce the response to the IFNα-ribavirin treatment. Moreover, a high baseline LDL level has been shown to be the best predictor of a sustained virologic response, whereas low lipid levels correlate with steatosis, progressing fibrosis and non-response to treatment [453].

Cholesterol is an important structural component of biological membranes and is essential for the uptake of many viruses. HCV cell entry requires cholesterol homeostasis and intact cholesterol-rich membrane microdomains [297]. Indeed perturbation of the alignment/packaging of cholesterol in lipid membranes

increases the energy barrier required for virus cell entry *via* fusion mechanisms [454].

Hepatocytes play a vital role in cholesterol homeostasis acquiring cholesterol by synthesis via the mevalonate pathway or LDL-R mediated endocytosis. Cholesterol is exported from hepatocytes together with triglycerides through the VLDL secretion pathway

Altogether, these observations reflect the role of lipids in the HCV life cycle. Therefore, host factors involved in cholesterol/lipid metabolism might represent potential targets for HCV strategies with only limited possibilities for escape mutations to develop [112,455] and improve regimens for patients infected with genotype 3 [437].

## Cholesterol homeostasis

Cholesterol is a vital component of cellular membranes and it is the precursor for the biosynthesis of bile acids, steroid hormones and oxysterol, which are important regulators of many metabolic pathways. Cholesterol modulates membrane fluidity, cell signalling, intracellular transport, in particular the caveola-dependent and clathrin-dependent endocytosis.

Cholesterol can be acquired through both diet (via LDLR mediated endocytosis) and *de novo* synthesis via 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) pathway. Cholesterol is a key factor for many cellular functions. Indeed, its over-accumulation results in cytotoxicity and progression of heart disease and cancer development [456]. Thus, several homeostatic mechanisms regulate its biosynthesis and export to keep the necessary balance. The ATP binding cassette A1 (ABCA1) acting in concert with ABCG1 plays a pivotal role in the cholesterol efflux.

## ATP Binding Cassette Transporter A1 (ABCA1)

ABCA1 belongs to a superfamily of proteins that uses energy from ATP hydrolysis to translocate substrate across the cellular membranes [457]. Within the superfamily there are eight (from ABCA to ABCH) sub-families based on their

amino acid homology. ABC transporters are widely present in all phyla from prokaryotes to humans and exert their functions as exporters and importers. In humans, some ABC transporters are involved in critical physiological functions and mutations in genes encoding ABCA1, ABCA4 and ABCC7 have been linked respectively to Tangier Disease (TD), retinal degeneration and cystic fibrosis [458-460].

Human ABCA1 gene is located on chromosome 9q31 and codes for 2261 aa protein with a molecular mass of ~250 kDa [461]. ABCA1 is highly expressed in the liver and tissue macrophages, nevertheless liver ABCA1 pathway appears to be responsible for generating most (70-80%) of plasma HDL [462].

Within the cells ABCA1 has been found to localize on the plasma membrane as much as on the early and late endosomes membrane [463,464]. In hepatocytes and other polarized cells, ABCA1 is expressed on the basolateral face of the plasma membrane [465].

ABCA1 a major regulator of cholesterol homeostasis, *via* mediation of cholesterol efflux and phospholipids to ApoA1 to form nascent pre beta-HDL particles (Figure 6) [466-468].

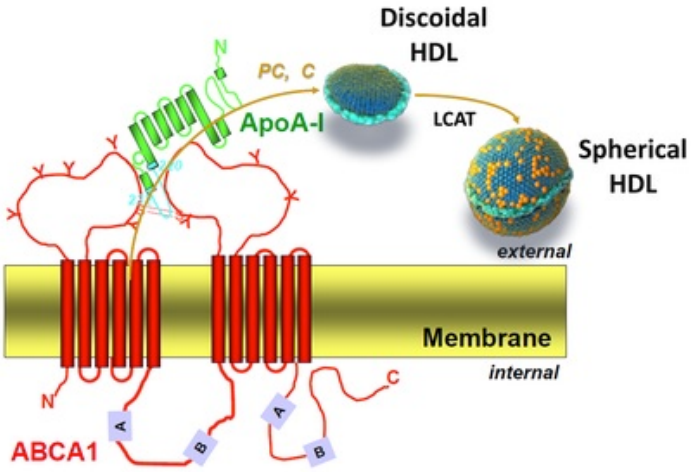


**Figure 6.** ABCA1 plays a pivotal role in HDL biogenesis. It transfers phosphorcholine (PC) and cholesterol (C) to the ApoA1 (in green) with formation of discoidal HDL (pre-beta HDL) in a mechanism dependent on ATP hydrolysis. Subsequently, pre-beta HDL are converted to mature HDL in a LCAT mediated mechanism.

Once loaded on ApoA1 cholesterol is processed by the lecithin-cholesterol acyltransferase (LCAT) to form cholesteryl esters (CE) in the core of nascent lipoprotein, promoting its conversion into the mature alfa-HDL. Circulating in the

blood HDL sequestrates lipids that LCAT converts into CE, leading to density decrease and size increase of the lipoprotein particle. This process gives rise to $HDL_2$ and finally $HDL_1$. CE contained in HDL can be delivered to the liver by SR-BI or to LDL by cholesteryl ester transfer protein (CEPT) [458].

ABCG1

ABCA1 works in concert with members of the ABCG subfamily of cholesterol transorters. In particular, with ABCG1 that like some other ABCG family members, is an “half transporter” that must homo- or heterodimerize with ABCG4 to become functional [469]. Function of ABCG1 may be complementary to that of ABCA1. Indeed, ABCG1 cholesterol efflux requires prior interaction of ApoA1 with ABCA1 and formation of nascent HDL [468,470].

### *Transcriptional regulation*

Liver X receptors (LXRs) belong to the nuclear hormone receptor subfamily of the ligand-activated transcription factors. LXR exist as two isoforms: LXR α and LXR β [471](“see for review” [472]). LXR α is mainly expressed in the liver, macrophages, kidney and adipose tissue while LXR β is expressed ubiquitously, suggesting difference in their physiological role [473,474]. Despite these receptors act as sensors for cholesterol levels, they do not interact with free cholesterol or CE [471]. Instead, their natural ligands are cholesterol derivate known as oxysterols or hydrocholesterols [471].

LXRs control cholesterol transport and lipid metabolism in the liver, intestine and macrophages through up-regulation of the cholesterol transporters: ABCA1, ABCG1, ABCG5/ABCG8 and the ApoE [475].

### *Post translational regulation*

ABCA1 has a relatively short intracellular half-life of 1-3 hours and like other proteins whose stability is tightly regulated possess a PEST motif [476]. PEST motifs are hydrophilic loop rich in proline (P), glutamic acid (E), serine (S) and threonine (T) [477]. PEST motif is reported to be a substrate for the thiol protease calpain [478]. ABCA1 turnover occurs at the cell surface following a non-well yet defined mechanisms, however it has been observed that the degradation process of ABCA1 is impaired by treatment with the calpain inhibitor calpeptin [476,479]. Phosphorylation of the PEST motif might also regulate cell surface expression of ABCA1 [479].

ApoA1 is known to stabilize ABCA1 and this suggests that this apolipoprotetin may act by reducing the phosphorylation and thus render the transporter insensitive to calpain-mediated degradation [480,481].

### *Mechanism of action of ABC family*

An inner and an outer leaflet compose mammalian cellular membrane, which are asymmetric in terms of lipid composition. The inner leaflet is mainly composed of phosphatidylserine (PS) and phosphatidylethanolamine (PE). Conversely, the outer leaflet is principally composed by phospholipids containing sphindomyelin (SM) and phosphatidylcholine (PC). This difference is fundamental for membrane functions and the asymmetry is maintained by three families of transporters: flippases, floppases (both are ATP-dependent) and scramblases (which is ATP-independent) [467,482,483]. Other ABC members may acts as a floppase and thus move lipid from the inner leaflet to the outer leaflet [467]. ABCA1, increasing levels of PS in the outer leaflet could promote ApoA1 binding to cell surface [484]. However, it is not clear if ApoA1 binds to ABCA1 or to phospholipids in the cell membrane.

### *ABCA1 and protection from Atherosclerosis*

Atherosclerosis is a chronic disease in which artery wall thickens due to progressive deposition of cholesterol and triglyceride. It may cause occlusion of the coronary arteries and lead to myocardial infarction. During the process, macrophages and white blood cells accumulate within the walls of arteries causing chronic inflammation and formation of multiple atheromatous plaques. LDL promotes inflammatory progression, without adequate removal of fats and cholesterol from the macrophages by HDL. However, to prevent atherosclerosis the body mobilises and excretes cholesterol and other lipids through the reverse cholesterol transport (RCT). Data concerning mechanism of RCT has been obtained by studying the rare syndrome caused by several mutations of ABCA1 gene. In Tangier Disease partial or total loss of ABCA1 function precludes cholesterol and phospholipid export from the cells and results in low levels of circulating HDL and cholesterol accumulation in the peripheral tissues. Liver ABCA1 expression and activity might influence hepatic VLDL secretion [485].

Currently, the therapy to lower cholesterol levels is based on the use of statins that decrease LDL cholesterol levels. Treatment with fibrates reduces triglyceride levels and increases HDL cholesterol levels.

Because HDL cholesterol levels are associated with a lower incidence of cardiovascular disease, several drugs are in development to increase HDL levels and thus reduce the risk of side effect of the current therapy.

Due to the crucial role of ABCA1 in HDL biogenesis, several approaches targeting cholesterol efflux are currently under study. LXR agonists inducing the transcription of ABCA1 and other molecules involved in the trafficking of lipids [475], might reduce atherosclerosis through raising HDL levels. Indeed, studies using the first generation of LXR agonists suggested they could reduce atherosclerosis in mice. However, due to their stimulation of fatty acid synthesis, these compounds also caused liver steatosis [486,487]. More recently, novel LXR agonists able to induce the expression of ABCA1 without side effects have been developed and tested [488,489]. Altogether, ABCA1 is considered a good target for atherosclerosis treatment.

### *ABCA1 as anti-cancer gene*

Aside the well-known role of ABCA1 in the protection from atherosclerosis development, it has been recently proposed as an anti-cancer molecule.

An important increase of the cholesterol levels have been observed In several tumour tissues as compared with normal ones, suggesting aberrant cholesterol homeostasis in cancer cells [490-493]. Indeed all the mechanisms involved in cholesterol homeostasis were impaired. The cholesterol uptake through LDLR was increased and the expression of ABCA1 decreased [494,495]. Although the role of cholesterol in oncogenic events has been established the mechanisms involved are not clear [496,497]. In cancer cells, down regulation of ABCA1 may induce cholesterol accumulation in mitochondrial membranes decreasing their fluidity and increasing retention of cell death-promoting molecules [498]. Accordingly, the stimulation of ABCA1 expression in cancer cells decreased mitochondrial cholesterol levels, facilitating the release of the cytochrome C, a pro-death molecule. Furthermore, LXR agonists known to induce ABCA1 expression, inhibited tumour growth in a model of prostate cancer [499]. In addition, the anti-

proliferative effects involved the activation of ABCA1 pathway, leading to reduction of cellular total cholesterol levels [500,501]. These data suggest the anti-tumour effect of the ABCA1-mediated cholesterol efflux.

## Aim of the study

Hepatitis C virus establishes persistent infection using host lipid metabolism pathways. HCV enters the cell *via* clathrin-dependent endocytosis, interacting with several receptors and virus-cell fusion mechanism, which depends on acidic pH and integrity of cholesterol-rich domains of the hepatocyte membrane. ABCA1 transporter mediates cholesterol efflux from hepatocytes to extracellular Apolipoprotein A1 and generates high-density lipoprotein (HDL) particles. Current therapies based on IFNα are successful in about 50% of cases and are characterised by wide range of side effects. Thus, other drugs either targeting virus encoded proteins (DAA) or cellular components essential for its life cycle (HTA) are under development. Due to the dependence of HCV life cycle on lipid and cholesterol metabolism, the molecules regulating these pathways might be considered as potential targets for indirect anti-HCV strategies. Therefore, the aim of the work presented in this thesis was to investigate the influence of the regulation of expression and cholesterol efflux function of the ABCA1 transporter – the main hepatic “controller” of cholesterol homeostasis - on HCV infection. To achieve this goal, we investigated the effects of the up or down regulation of ABCA1 gene expression and its specific function. For these studies we used the *in vitro* HCVcc multiplication system in Huh7.5 cell and primary human hepatocytes, the natural HCV target cell.

Several issues were addressed to better understand the role of ABCA1 in regulation of HCV infection:

- First we evaluated whether HCV infection by itself influences ABCA1 expression levels in Huh 7.5 cells.

- Secondly, we assessed whether up regulation of ABCA1 expression using synthetic LXR agonists, such as GW3965 and TO-901317 would influence the infection. In these studies we compared the expression of ABCA1 m-RNA, corresponding protein and its physiological activity of cholesterol transfer in drug-treated cells compared to non-treated cells.
- We also investigated the impact of silencing of ABCA1 gene on HCV infection and the capacity of ABCA1 knock-down cells to respond to

stimulation with LXR agonists. These studies were crucial to demonstrate the key role of ABCA1 in regulation of HCV infection levels.

- Since preliminary results showed an inhibitory effect of ABCA1 stimulating drugs on HCV infection, their influence on various steps of HCV cell cycle, was determined such as virus cell entry, RNA replication or assembly/secretion of virus particles.

- Finally, it was essential to evaluate the physico-chemical properties of the virus particles produced in ABCA1 over-expressing cells and their infectivity

Altogether this work was carried out to evaluate how manipulating cholesterol efflux pathway, by regulation of ABCA1 expression and its specific function, we can impair HCV cell entry and thus decrease the production of infectious virus.
We attempted to evaluate whether ABCA1 regulation might be proposed for future development of indirect acting anti-HCV drugs.

# Materials & Methods

**Cell infection with HCVcc**.

JFH1 HCV strain (2a genotype) was kindly provided by T. Wakita. Infectious HCV virions were generated as described [100]. Huh7.5 cells (kindly provided by C. Rice) were grown as previously described [351] and infected with the virus preparation containing $10^5$ ffU /ml (focus forming units per millilitre) at MOI=0.001-0.01 for 2h at 37°C, and grown further for the indicated time at 37°C.

Primary human hepatocytes (PHH) were isolated from two adult liver donors after resection for medical reasons and infected with HCVcc as previously described [502].

For the culture of liver tissue slices, human liver samples were obtained from adult HCV, HBV and HIV seronegative patients who underwent liver resection for metastasis in the absence of underlying liver disease and were infected with HCV as described [503].

**Stimulation of ABCA1 expression**.

Cell toxicity of GW3965 (Sigma) or TO901317 (Sigma) was determined using the CellTiter-Glo luminescent Cell Viability Assay (Promega). To stimulate ABCA1 expression, Huh7.5 cells or primary hepatocytes were treated with 1-10µM concentrations of the drugs diluted in DMSO for 24h before infection, or with a corresponding dilution of drug solvent (DMSO). Cells were grown for the indicated time and ABCA1 mRNA levels were analysed by qRT-PCR, ABCA1 protein determined by Western Blot followed by quantification using the Odyssey Infrared Imaging System (LI-COR Biosciences, NE, USA). ABCA1 function was measured using cholesterol efflux assay.

**ABCA1 silencing**.

Stealth siRNA targeting three different zones of human ABCA1 gene (Life Technologies Applied Biosystems) were used. The Huh7.5 cells were transfected with 25nM ABCA1 siRNA or with 25nM scrambled siRNA (Eurogentec), using RNAi Max reagent (Invitrogen). At 24h and 48h after transfection ABCA1 mRNA levels were determined by qRT-PCR, ABCA1 protein by Western Blot and ABCA1 function by cholesterol efflux assay.

**Quantitative RT-PCR (qRT-PCR).**

HCV RNA was determined as previously described [33]. For measurement of the ABCA1 mRNA, 5'-CCTGACCGGGTTGTTCCC-3' and 5'-TTCTGCCGGATGGTGCTC-3' primers were used for amplification and 5'-ACATCCTGGGAAAAGACATTCGCTCTGA-3' (Eurogentec) served as an internal probe. The results were normalized by quantification of the HPRT1 or GAPDH cellular genes using HPRT1 Taqman Gene Expression Assay (Life Technology Applied Biosystems) or GAPDH Control Kit (Eurogentec), respectively.

All assays were performed at least in quadruplicate. Error bars represent standard deviation of the mean values from at least quadruplicates.

**Cholesterol efflux assay.**

Control or drug-treated Huh7.5 cells were cholesterol-loaded using 1µCi/mL [3H] cholesterol-labelled SVF (10%) for 24h in DMEM medium. Cells were then incubated in serum-free medium containing 0.2% BSA with or without 1µM GW3965 for 16h. Cellular cholesterol efflux to 25 µg/mL lipid-free ApoA1 (Sigma) was assayed in serum-free medium containing 0.2% BSA for a 4h chase period, then culture media were harvested and cleared by centrifugation. Cell-associated radioactivity was determined by extraction in hexane-isopropanol (3:2), evaporation of the solvent and liquid scintillation counting (Wallac Trilux 1450 Microbeta). The percentage of cholesterol efflux was calculated according to the formula=100 x (medium cpm) / (medium cpm + cell cpm). ApoA1-specific cholesterol efflux was determined by subtracting the cholesterol efflux that occurred in apoA-I-free medium.

**Cell–to-cell fusion assay**.

The assay was performed as described previously [179]. HEK293T kidney cells (ATCC CRL-1573) were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids, 100U/ml penicillin and 100mg/ml streptomycin. Cells (2.5x105 cells/well seeded in 35 mm 6-well tissue culture dishes 24h before transfection) were co-transfected using the calcium phosphate reagent with a plasmid encoding HCV envelope proteins (H77 strain genotype 1a) encoding or Chikungunya envelope encoding-plasmid (ChickV) issued from ChickV E3E1E2 plasmid from the Réunion infectious clone and with an HIV-1 LTR (long terminal

repeat) luciferase reporter plasmid (a kind gift of Francoise Bex). After 12h, transfected HEK293T cells were detached with 0.53mM EDTA (Invitrogen) and co-cultured (5x104 cells/well) with Huh-7-Tat indicator cells (5x104 cells/well). Co-cultured cells were then incubated with 1µM of GW3965 or with DMSO. After 24h, the cells were washed with serum-free DMEM, incubated for 3 min in either pH 7 or pH 5 buffer (130mM NaCl, 15mM sodium citrate, 10mM MES and 5mM Hepes), then washed with serum-free DMEM. Cells were then incubated with 1µM of GW3965 or with DMSO for 48h. Luciferase activity was measured using a luciferase assay kit (Promega). The experiments were performed several times and results interpreted using student t-test.

**Cholesterol replenishment**.

Control or GW3965 treated Huh7.5 cells were incubated with 20µg/ml cholesterol:Methyl b Cyclodextrine D (MbCD) complexes (Sigma) for 1h at 37°C. Cells were extensively washed and either analysed for their lipoprotein content or infected with HCV.

**Analysis of GFP-FR distribution by fluorescence microscopy.**

Huh7.5 cells were transfected with 0,5µg of DNA encoding a fusion protein where GFP was fused to the Glycosylphosphatidyl-inositol-anchor attachment signal of folate receptor (GFP-FR) [504,505]. Four hours post-transfection cells were treated with 1µM GW3965 or with a drug solvent (DMSO) that was replenished at 24h post-transfection. Two days after transfection cells were fixed with 4% PFA containing 0.2% of glutaraldehyde. Fluorescence of GFP-FR was visualised with 488nm laser of a Zeiss Axioplan 2 microscope (x63 objective). Slices of 0.46µm were acquired. Images analysed were Z projections of 5 slices of the cell surface that faced the cell medium.

**Virus binding assay**.

GW3965-pre-treated or untreated Huh7.5 cells were washed with cold medium and incubated with 100µl of HCV preparation for 2h at 4°C. Cells were extensively washed to remove the unbound virus, total RNA was extracted and HCV RNA was determined by qRT-PCR.

**Quantification of mRNA of genes involved in lipid metabolism**.

Total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel). Reverse transcription and real time qPCR assays were performed as previously described [466] and mRNA levels were normalized compared to those of housekeeping genes (18S, human delta-aminolevulinate synthetase, human alpha-tubulin, human heat shock protein 90kDa alpha and human hypoxanthine phosphoribosyltransferase 1). Data were expressed as a fold change in mRNA expression relative to control values.

**Determination of intracellular lipids**.

Quantification of total cellular triglyceride mass was performed as previously described [506]. Free and esterified cholesterol mass was quantified using the Amplex Red cholesterol assay kit (Molecular Probes) [507].

**Determination of HCV infectivity**.

An infectivity assay was performed using Huh7.5 cells seeded in 96-well plates inoculated with serially (ten-fold) diluted HCVcc preparations. After 72h cells were fixed with 4% paraformaldehyde for 30 min at room temperature and the infected foci were visualized by In-Cell Western assay using human anti-HCV serum and DyLightTM800 labelled goat anti-Human IgG (KPL, Inc., MD, USA). Fluorescent foci were detected in infected cells using the Odyssey Infrared Imaging System (LI-COR Biosciences, NE, USA). Infectivity was expressed as focus forming units/ml (ffu/ml).

**HCV replicon**.

Huh7 cells harbouring the HCV JFH-1 sub-genomic replicon were kindly provided by J. McLauchlan and grown as described [508]. Replicon cells were treated with 1μM GW3965 for 72h. Every 24h the cell culture medium was replaced by fresh medium that contained the drug at the same concentration. As a control, replicon cells were treated with 0.66μM cyclosporine A (CsA, Sigma) an inhibitor of HCV replication. Total RNA was extracted every 24h and HCV RNA assessed by qRT-PCR.

**Flow cytometry**.

Drug- or solvent -treated Huh7.5 cells were grown in 6-well plates to obtain $1x10^6$ cells/well. Cells were harvested by incubation with Versene and centrifugation, and re-suspended in a FACS buffer containing 1% BSA and 0.01% Azide in phosphate-buffered saline (PBS). Cells were then incubated with primary antibodies: monoclonal antibody anti-CD81, rabbit anti-LDL-R (Abcam, Cambridge, UK), rabbit polyclonal anti SR-BI (kindly provided by T. Huby) for 30 min at 4°C, washed and incubated with APC-conjugated anti-mouse or FITC-conjugated anti-rabbit antibody (BD Pharmigen) for 30 min at 4°C. Cells stained with only secondary antibodies were used as negative controls. Cells were washed with FACS buffer, fixed with 1% paraformaldehyde and analysed by FACSCalibur (BD Bioscience) using FlowJo software (Three stars). A total of 25,000 to 50,000 events were collected per sample.

**Western Blot**

Cells were lysed with a buffer containing 20mM Tris-HCl pH 7.5, 150mM NaCl, 10% glycerol and 1% Triton X-100. After freeze-thaw steps and sonication, samples containing 50 µg of total protein were heated for 10 min at 95°C in a sample buffer containing SDS and reducing agent and subjected to electrophoresis in 3-8% Tris-Acetate PAGE and transferred to nitrocellulose membranes. After blocking with 5 % skim milk in PBS containing 0.1% Tween 20, blots were reacted with rabbit anti-CLD1, (Abcam, Cambridge, UK); rabbit anti-OCLN (Abcam, Cambridge, UK), rabbit anti-pan Cadherin (Abcam Cambridge, UK); rabbit anti-ABCA1 (Novus Bio) or mouse anti-NPC1L1 (Santa Cruz) as primary antibodies, followed by DyLight 680 conjugated anti-Mouse IgG or DyLight 800 conjugated anti-Rabbit IgG (WWR, France) and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, NE, USA).

**Duolink proximity ligation assay**.

Duolink assay (Sigma-Aldrich) based on proximity ligation technology (PLA) was used to determine the co-localisation of HCV receptors CLDN1 and SR-BI relative to CD-81 (<40nm) in drug-treated or solvent-treated cells. Monoclonal antibodies to CD-81 (Abcam, Cambridge, UK), and rabbit anti-CLDN1 (Abcam, Cambridge, UK) and rabbit polyclonal anti SR-BI (kindly provided by T. Huby) were used in the

assay. After signal amplification the slides were examined using fluorescence Zeiss axioplan 2 microscope (x63 objective) at 598nm. The obtained images were quantitatively analysed using Duolink Image Tool software.

**Determination of SR-BI function**.

To investigate cholesterol efflux to HDL via the SR-BI/Cla-1 pathway, SR-BI gene expression was silenced with 50nM siRNA. Alternatively a 50nM of a control siRNA was employed. After 24h incubation at 37°C, SR-BI knocked-down and control cells were labelled with [3H] cholesterol in the presence or absence of 1μM GW3965. Cholesterol efflux to 50μg/mL HDL isolated from normolipidemic plasma [509] was assayed during a 4h chase period in the presence or absence of 1μM GW3965.

**Ultracentrifugation in iodixanol gradient**.

Centrifugation was carried out as previously described [351]. A discontinuous 5-50% iodixanol density gradient (OptiPrep) was prepared in a buffer that contained 40 mM HEPES, 270 mM NaCl and 10 mM KCl. The supernatants from infected cells were concentrated using a Vivaspin concentrator (Vivascience) and centrifuged in the gradient for 24h at 38,000 rpm at 4°C in an SW41Ti rotor in a Beckman ultracentrifuge. Fractions (450μl) were collected and analysed for the presence of HCV RNA by qRT-PCR, HCV core protein, Apolipoprotein E and Apolipoprotein B by corresponding ELISA assays.

**Other assays.**

Apolipoprotein E and Apolipoprotein B were determined by ELISA (Mabtech AB, France), and HCV core was quantified by the Chemiluminescent Microparticle Immunoassay (Architect, HCVAg; Abbott Lbs, USA).

# Results

### *GW3965 up-regulates ABCA1 gene expression and cholesterol efflux function*

Liver X receptors (LXRα and LXRβ) are ligand-activated transcription factors, which act as cholesterol sensors and control hepatic cholesterol and fatty acid homeostasis [472,475]. GW3965 is a synthetic compound (LXR agonist) that up-regulates ABCA1 [510]. No appreciable cytotoxicity of the drug for Huh 7.5 cells was observed at a concentration range of 0.08-10μM (Figure 1A). Treatment of Huh7.5 cells with 1μM GW3965 for 24h increased ABCA1 mRNA levels up to 5-fold (Figure 1B). Up-regulation of the ABCA1 gene expression raised ABCA1 protein production (Figure 1C) and enhanced free cholesterol efflux to ApoA1 (Figure 1D). Studies of the kinetics of ABCA1 stimulation showed progressive increase of ABCA1 gene expression until a plateau was reached after 16-24h (Figure 1E), and gradually augmented cholesterol efflux to ApoA1 (Figure 1F). Thus, 24h of treatment of cells with the drug was used in further experiments to raise ABCA1 levels.

### *Stimulation of ABCA1 inhibits HCV infection*

Infection with HCV did not modify ABCA1 gene expression in Huh7.5 cells as shown by stable levels of ABCA1 mRNA over 72h (not shown). To assess whether stimulation of ABCA1 expression and physiological function would influence HCV life cycle, Huh7.5 cells were pre-treated with 1μM GW3965 for 24h and then infected with HCV. Up-regulation of ABCA1 (shown in Figure 1B) decreased intracellular HCV RNA levels (Figure 2A) and significantly reduced the virus production as evidenced by the decrease of HCV RNA in the cell supernatant (Figure 2B), which corroborated with a decrease of the concentration of HCV core antigen (not shown). Continuous GW3965 treatment for 7 days substantially reduced the amount of the HCV RNA in parallel with the increase of ABCA1 expression in virus-producing cells (Figure 2C).

TO901317, another synthetic LXR agonist known to be a potent inducer of ABCA1 [511,512], inhibited HCV infection to the same extent as GW3965 did. No further decrease of infection was obtained when the two drugs were used simultaneously or in higher concentrations (up to 50μM) (data not shown). These observations suggested that GW3965 and TO901317 inhibited HCV infection by similar mechanisms.

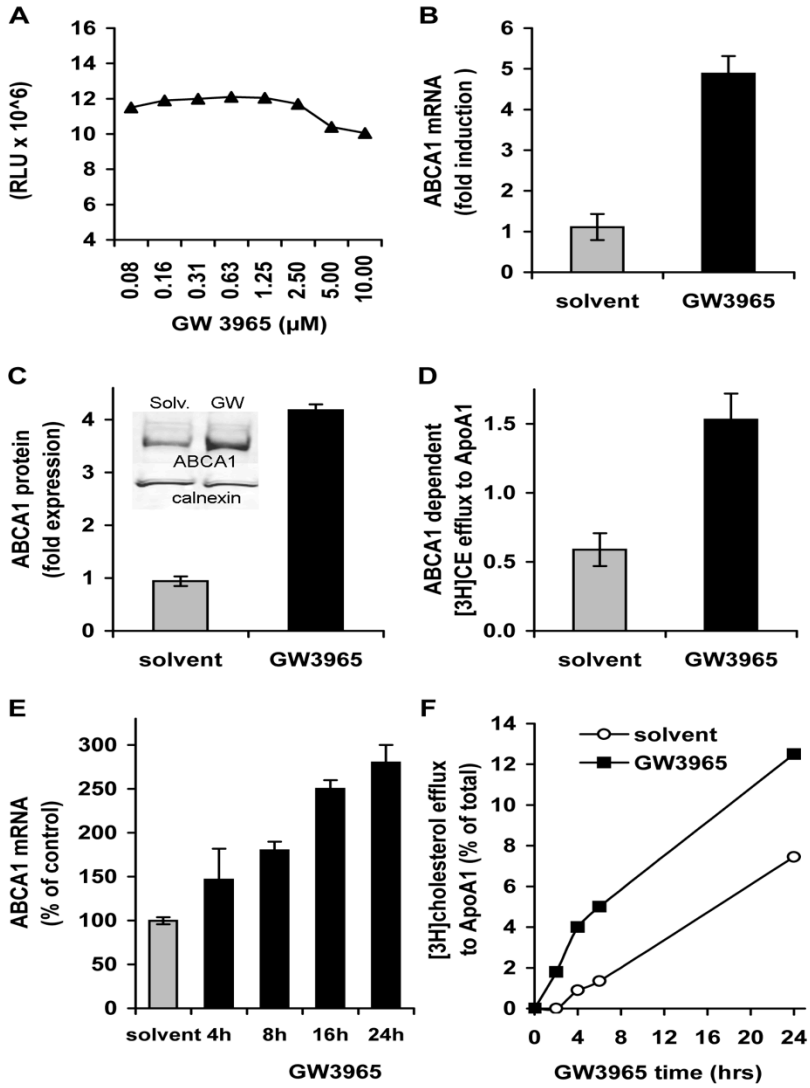


**Figure 1**. GW3965 treatment up-regulates ABCA1 expression and its cholesterol efflux function. (A) Cell toxicity of GW3965. Huh7.5 cells were cultured in the presence of indicated concentrations of the drug for 24h. The luminescent signal is expressed in luminescence units (RLU). (B) Up-regulation of ABCA1 mRNA expression by GW3695 treatment. Huh7.5 cells were treated for 24h with 1μM GW3695 or drug solvent (DMSO). Then ABCA1 mRNA was determined by qRT-PCR. (C) ABCA1 protein production in drug-stimulated Huh7.5 cells. Cells were treated for 24h with 1μM GW3965 and analysed by Western blot (shown in the insert). Protein content in the ABCA1 band (220kDA) in GW3965-(GW), and DMSO-(solv) treated cells was quantified relative to the calnexin band using the Odyssey Infrared Imaging System. (D) GW3965 stimulation promotes ABCA1-mediated cholesterol efflux to ApoA1. Huh7.5 cells were labelled with [3H] cholesterol then incubated with GW3965 or drug solvent. ABCA1-dependent [3H] cholesterol efflux was assayed by comparing cell-associated and free radioactivity. (E) Kinetics of ABCA1 gene expression following stimulation of cells with GW3965. Huh7.5 cells were treated with 1μM GW3965 for the indicated time and ABCA1 mRNA was determined by qRT-PCR. Results were expressed as relative values compared to ABCA1 expression in cells treated with drug solvent. (F) Kinetics of cholesterol efflux in cells stimulated with GW3965. Huh7.5 cells were labelled with [3H] cholesterol for 24h, and incubated for an additional 16h with 1μM GW3965 or drug solvent. ABCA1-dependent [3H] cholesterol efflux was assayed in the presence of ApoA1 and either GW3965 or solvent for the indicated period of time.

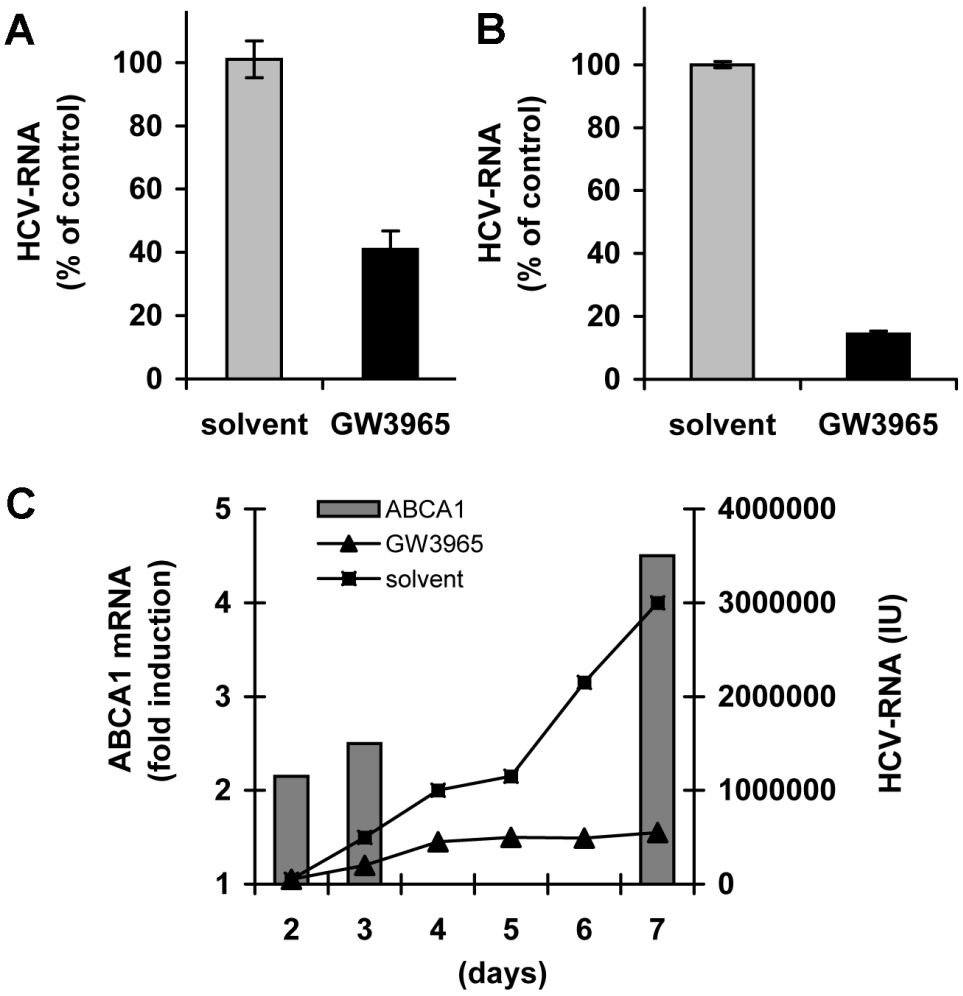


**Figure 2.** Stimulation of ABCA1 inhibits HCV infection. (A) Reduction of intracellular HCV RNA levels in cells that over-express ABCA1. Huh7.5 cells were pre-treated with 1μM GW3965 then infected with HCV. Cells were grown for a further 24h, total RNA was extracted and intracellular HCV RNA was determined by qRT-PCR. Results are expressed as the percentage of HCV RNA relative to that in cells treated with drug solvent prior to infection. (B) Decrease of HCV RNA levels in the supernatant collected from drug-stimulated cells. Huh7.5 cells were pre-treated with 1μM GW3965 then infected with HCV. After a further 72h, HCV-RNA in the culture medium was determined by qRT-PCR. Results are expressed as the percentage of HCV RNA secreted from drug-treated cells compared to solvent-treated cells. (C) Effect of GW3965 treatment on long-term HCV infection. Huh 7.5 cells were pre-treated with 1μM GW3965, infected with HCV and grown for up to 7 days in the presence of the drug. ABCA1 mRNA was determined by qRT-PCR every 24h and results are expressed as a fold-increase of ABCA1 mRNA compared to solvent-treated cells (grey bars). HCV RNA in the cell supernatant was measured at the same time points by qRT-PCR (line curves for GW3965 treated [filled triangles] or control [filled squares] cells) and is expressed in International Units (IU).

**ABCA1 plays a key role in the inhibition of HCV infection**.

Activation of LXRs by their agonists such as GW3965 or TO901317 enhances ABCA1 gene expression but also may affect the expression of other genes regulating lipid metabolic pathways [475]. Indeed, transcriptomic profiling of Huh7.5 cells treated with GW3965 revealed modified mRNA levels of several genes involved in hepatic lipid metabolism: increased mRNA levels of ABCA1 and ABCG1, nuclear LXRα (but not the LXRβ receptor), a sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase (FAS) and phospholipid transfer protein (PLTP). The treatment had no significant effect on mRNA levels of fatty acid transporter CD36 and ApoA1 mRNA (Figure 3).

We therefore investigated whether ABCA1 activation was responsible for the inhibition of HCV infection by knocking down ABCA1 gene. We used three siRNA targeting different regions of ABCA1 gene or their mixture to reduce ABCA1 expression. All approaches gave similar rate of ABCA1 reduction (60-80%) as compared to scrambled siRNA. Knocking down of ABCA1 gene in Huh 7.5 cells (Figure 4A) significantly reduced the production of ABCA1 protein (Figure 4B) and impaired cholesterol efflux to ApoA1 (Figure 4C) as compared with a control siRNA.

To assess whether ABCA1 stimulation was responsible for the inhibition of infection, Huh7.5 cells were transfected with ABCA1 siRNA or scrambled siRNA and then treated for 24 h with 1μM GW3965 or solvent. At 24h post-transfection, cells were infected with HCV and grown for further 24h. Infection levels were evaluated by measuring intracellular HCV RNA. Consistent with the requirement of ABCA1 stimulation to induce the inhibitory effect by GW3965 treatment, the drug did not decrease infection levels in ABCA1-silenced cells (Figure 4D).

These results provided evidence that the inhibition of infection was mediated by the over-expressed and fully functional ABCA1 in agreement with the hypothesis that ABCA1-mediated cholesterol efflux modulates HCV infection.

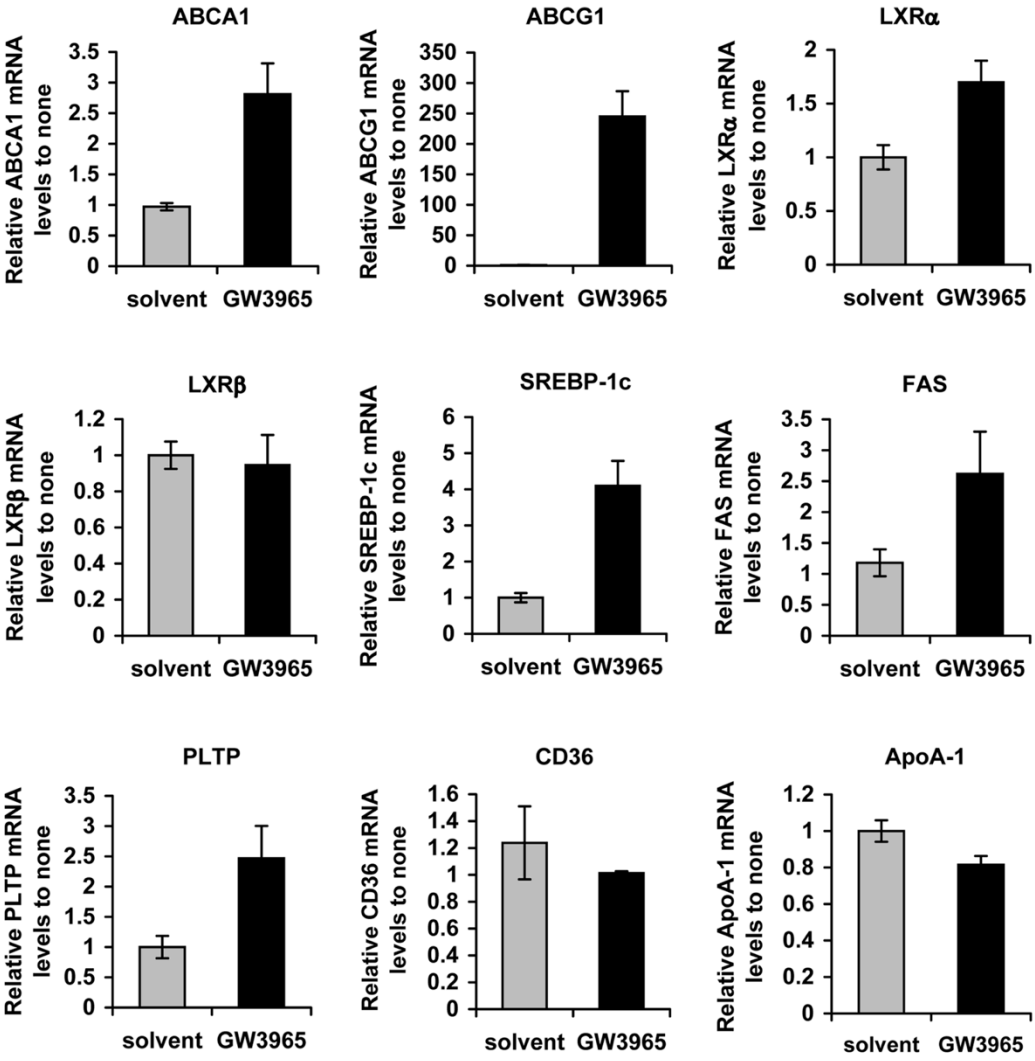


**Figure 3.** GW3695 treatment modulates expression of genes involved in lipid metabolism. Huh7.5 cells were treated with 1μM GW3695. Total RNA was extracted from cells and the mRNA levels corresponding to several genes regulating lipoprotein metabolism: ABCA1, ABCG1, nuclear LXRα and LXRβ receptors, a sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase (FAS) and phospholipid transfer protein (PLTP), CD36 and ApoA1 were determined by qRT-PCR. The results were normalized to housekeeping genes and compared to the levels of corresponding mRNAs in solvent-treated cells.

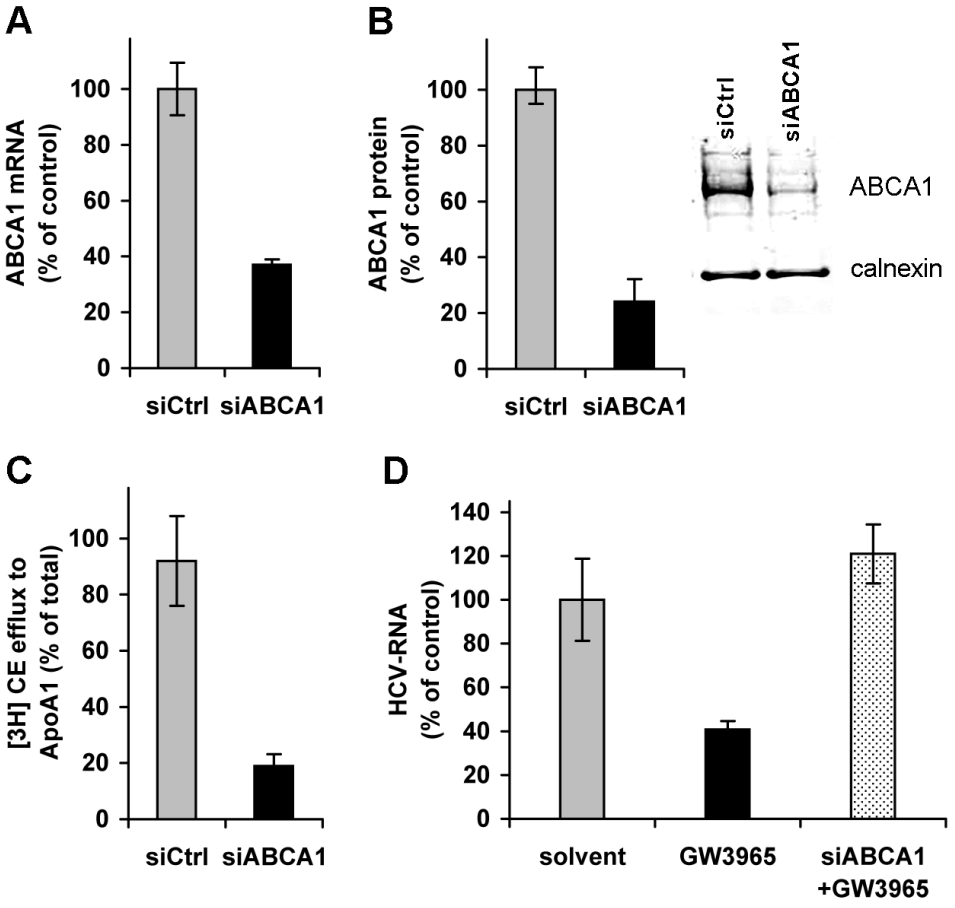


**Figure 4**. ABCA1 plays a key role in the inhibition of HCV infection. (A) Silencing of ABCA1. Huh7.5 cells were transfected with siRNA that targets ABCA1, or with a control siRNA. Total RNA was extracted after 48h and ABCA1 mRNA determined by qRT-PCR. Results are expressed as the percentage of ABCA1 mRNA in cells transfected with siRNA targeting ABCA1 relative to mRNA levels in control cells. (B) Decreased ABCA1 protein synthesis in ABCA1-knocked-down cells. Huh7.5 cells were transfected with ABCA1-specific siRNA as above. Cell lysates were subjected to Western blot analysis. Staining of the ABCA1 protein with specific antibodies is shown in the insert. Results are expressed as the ABCA1 protein content in ABCA1-siRNA transfected cells relative to that in control siRNA transfected cells, normalized to calnexin (quantification using the Odyssey Infrared Imaging System). (C) Loss of cholesterol efflux function in ABCA1-silenced cells. Huh7.5 cells were transfected with siRNA that targets ABCA1 or with control siRNA. ABCA1-dependent [3H] cholesterol efflux was assayed in the presence of ApoA1. Results are expressed as percentage of cholesterol efflux to ApoA1 in ABCA1 knocked-down cells relative to control si-RNA transfected cells. (D) Silencing of ABCA1 antagonizes GW3965-mediated inhibition of HCV infection. The expression of ABCA1 was reduced by transfection with ABCA1-specific siRNA (as in A) and cells were treated with 1µM GW3965, infected and grown for a further 24h. HCV RNA was determined by qRT-PCR. Results are expressed as the percentage of HCV RNA in drug-treated (GW3965) cells or ABCA1-silenced and subsequently GW3965-treated cells (siABCA1+GW3965), compared to solvent-treated cells.

### *GW3695 treatment inhibits HCV cell entry, but does not impair HCV RNA replication, or virus assembly/secretion mechanisms*

To determine which step of the HCV life cycle was affected by GW3965 treatment, we analysed the kinetics of activity of this compound using a “time-of-addition assay” e.g. by adding the drug at different time points (Figure 5). When cells were pre-treated with GW3695 before virus inoculation, and the drug was maintained during infection, intracellular HCV RNA levels decreased at 24h, 48h or 72h post-infection. No inhibition was observed when the drug was added concomitantly with the virus during infection, without cell pre-incubation. These observations provided evidence that, GW3965 had no direct detrimental effect on virus structure and infectivity, membrane composition, or fusion events and suggested that raising ABCA1 levels was required to affect infection.

Furthermore, no inhibitory effect was noted when the drug was applied at different time points post-infection implying that pre-treatment of cells with the drug stimulating ABCA1 impaired virus entry, but did not affect later steps of the virus life cycle. To assess whether the treatment influenced the initial virus attachment to the cell surface, or later steps in virus cell entry, Huh 7.5 cells were pre-treated with GW3965 for 24h to stimulate ABCA1 expression. The virus was then added and incubated with cells for 2h at 4°C. After washings, the cell-bound HCV was quantified by qRT-PCR. As shown in (Figure 6A and B), overexpression of ABCA1 after GW3965 treatment did not affect virus binding to the cell surface. Hence, the inhibitory effect induced by overexpressed ABCA1 concerned virus entry events after the binding step.

No inhibition of infection was observed when the drug was added at several time points (2h, 4h and 6h) after infection (Figure 5), suggesting that ABCA1 stimulation does not impair virus replication. This was further confirmed using the sub-genomic HCV replicon model, which permits studies of HCV replication without the expression of HCV structural proteins. HCV RNA replication was not affected by 72h of GW3965 treatment (Figure 6C), but was inhibited, in a time dependent manner by cyclosporine A known inhibitor of HCV replication, used here as a control (Figure 6D).

Increased ABCA1 expression did not affect mechanisms of assembly/secretion of virus particles. Indeed, when infected cells were continuously treated with GW3965 and the cell medium collected every 24h (to prevent the effect of raised

ABCA1 on virus cell entry) no difference in concentration of HCV core antigen was observed in supernatants collected from drug-treated as compared to non-treated cells (Figure 6E). The infectivity of virus particles in the same supernatants secreted from drug-treated and non-treated cells was also very similar (Figure 6F). Collectively, these data demonstrated that stimulation of ABCA1-mediated cholesterol efflux inhibits virus entry step, after virus attachment. However the treatment did not affect other steps of the HCV life cycle and the infectivity of virus particles was unchanged. Accordingly, a decrease of virus production in ABCA1 over-expressing cells was a consequence of reduced virus cell entry.

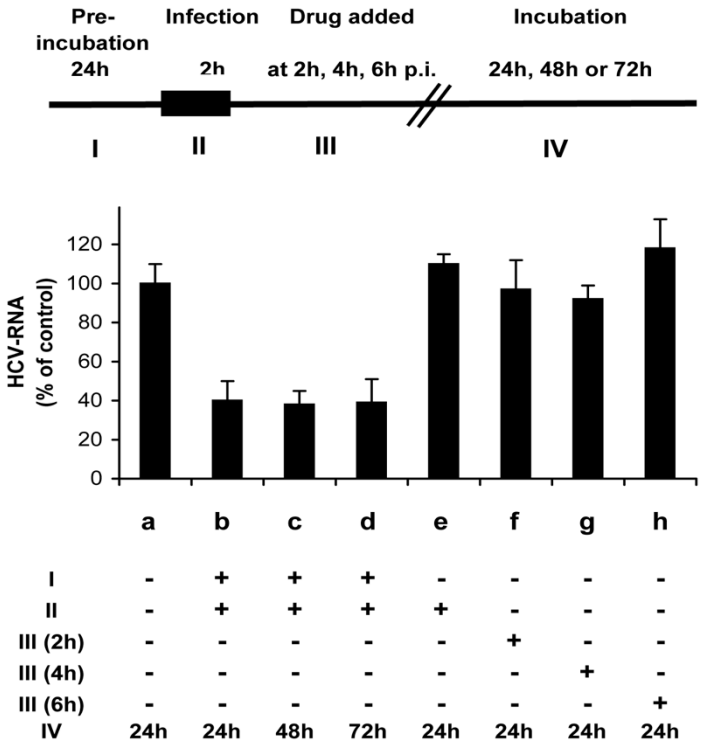


**Figure 5**. Up-regulation of ABCA1 inhibits HCV cell entry. The effect of GW3965 on the HCV cell cycle was analysed by adding the drug at different time points. A flow-chart is depicted in the upper panel of the graph. RNA in Huh7.5 cells infected in the presence of DMSO is shown in (a); that in cells pre-treated for 24h with 1μM GW3965 and infected in the presence of the drug are shown in (b), (c) and (d); results for cells treated with GW3965 during virus inoculation without pre-treatment are shown in (e); those of assays where the drug was added at 2h, 4h, or 6h post-infection are presented in (f), (g) and (h) respectively. For each experiment cells were incubated for the indicated time period after infection (IV). The efficiency of infection was expressed as intracellular HCV RNA measured by qRT-PCR as a per cent of the control (a).

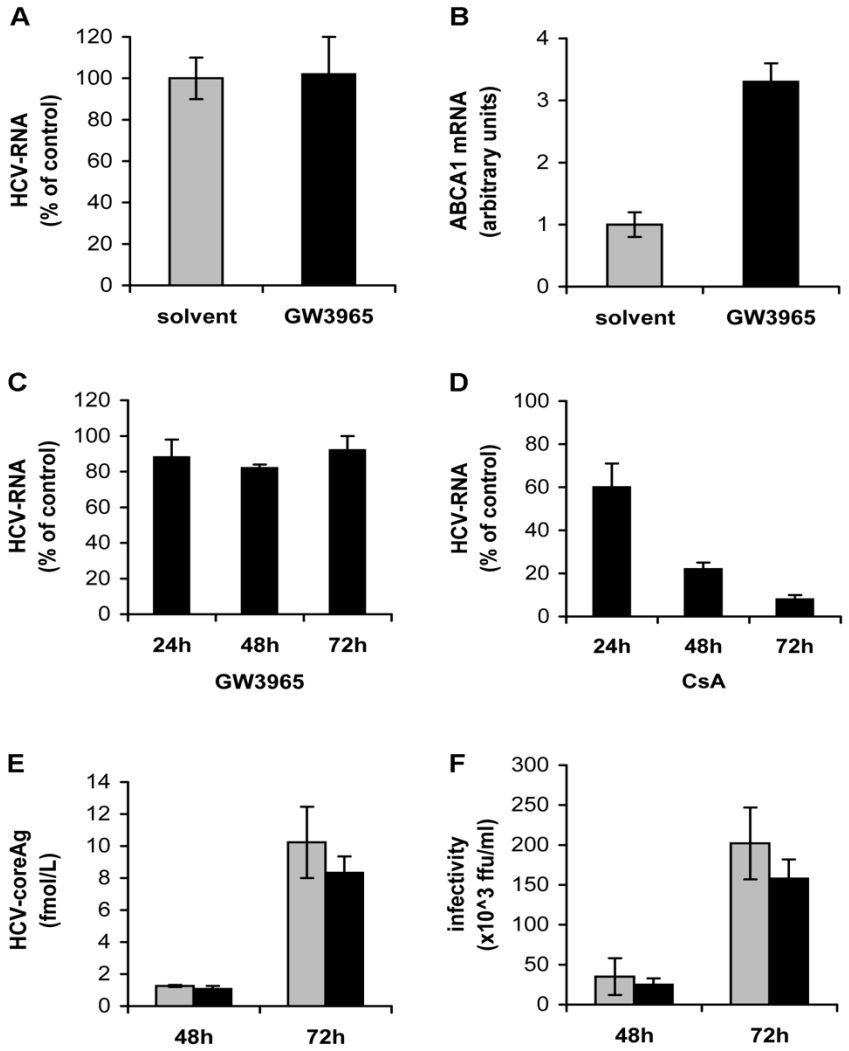


**Figure 6.** Stimulation of ABCA1 has no impact on HCV cell attachment, HCV RNA replication, assembly/secretion or infectivity of virus particles. (A-B) GW3965 treatment does not affect HCV cell attachment. Huh7.5 cells were pre-treated for 24h with 1μM GW3965, to raise ABCA1 levels and tested for their capacity to attach HCV using a "binding assay". (A) HCV RNA attached to drug pre-treated cells (GW3965) is expressed as per cent relative to HCV RNA attached to cells treated with DMSO (solvent). (B) ABCA1 mRNA levels were determined by corresponding qRT-PCR and expressed in arbitrary units. (C) Stimulation of ABCA1 does not affect HCV RNA replication. Huh7 cells that express the sub-genomic replicon were incubated for 72h with 1μM GW3965 or with the equivalent concentration of drug solvent. HCV RNA was quantified by qRT-PCR in drug-treated cells relative to HCV RNA in control replicon cells grown in the presence of drug solvent. (D) HCV RNA replication is inhibited by CsA (control for C). Replicon cells were grown in medium containing 0.67 μM CsA or drug solvent (EtOH) for 72h. HCV RNA was measured by qRT-PCR every 24h and results were expressed as the percentage of HCV RNA in drug treated cells relative to HCV RNA content in cells grown in the presence of drug solvent. (E) ABCA1 up-regulation does not affect virus particle assembly/secretion. Huh7.5 cells were infected with HCV and 1μM GW3965 was applied to cells 2h post-infection. Every 24h drug was replenished. HCV core antigen secreted to the cell supernatant was quantified at 48h and 72h post infection. Results were normalized with respect to the total protein content in the supernatant and are expressed in fmol/L. (F) Infectivity of virus particles secreted to the cell supernatant from cells treated according to the procedure described in (E) and determined using In Cell Western Blot assay. Results are expressed in ffu/ml.

### *Analysis of HCV particles produced in GW3965 treated cells*

The increased cellular cholesterol efflux did not affect levels of ApoB secreted to the cell supernatant (data not shown) and only slightly affected the total (Figure 7A) and free cholesterol (Figure 7B) contents in Huh7.5 cells. Instead, the treatment reduced the concentration of cholesteryl esters (Figure 7C) and increased cellular triglycerides (Figure 7D), as a result of well-known stimulation of lipogenesis *via* LXRs. These changes might alter physical characteristics of the virus produced in drug-treated cells.

We therefore investigated the properties of virus particles produced in cells continuously stimulated with GW3965 by centrifugation in a discontinuous 5-50% iodixanol gradient. Since significantly less virus was produced and secreted from drug-stimulated compared to non-stimulated cells (shown in Figure 2B) the cell culture medium was concentrated before ultracentrifugation. Nevertheless, in spite of quantitative differences, overall properties of viruses produced in the presence or absence of the drug were very similar (Figure 8 A and B). In both gradients HCV RNA was detected in two peaks at a density of 1.1 and 1.12g/ml. The low-density virus peak co-localised with ApoB and partially with HCV core antigen, which was more abundant in the high density RNA peak.

Thus, stimulation of ABCA1 expression upon GW3965 treatment did not significantly change either infectivity (shown in Figure 6F) or physicochemical properties of the virus particles produced (Figure 8) regardless of the influence on the levels of HCV infection and virus production.

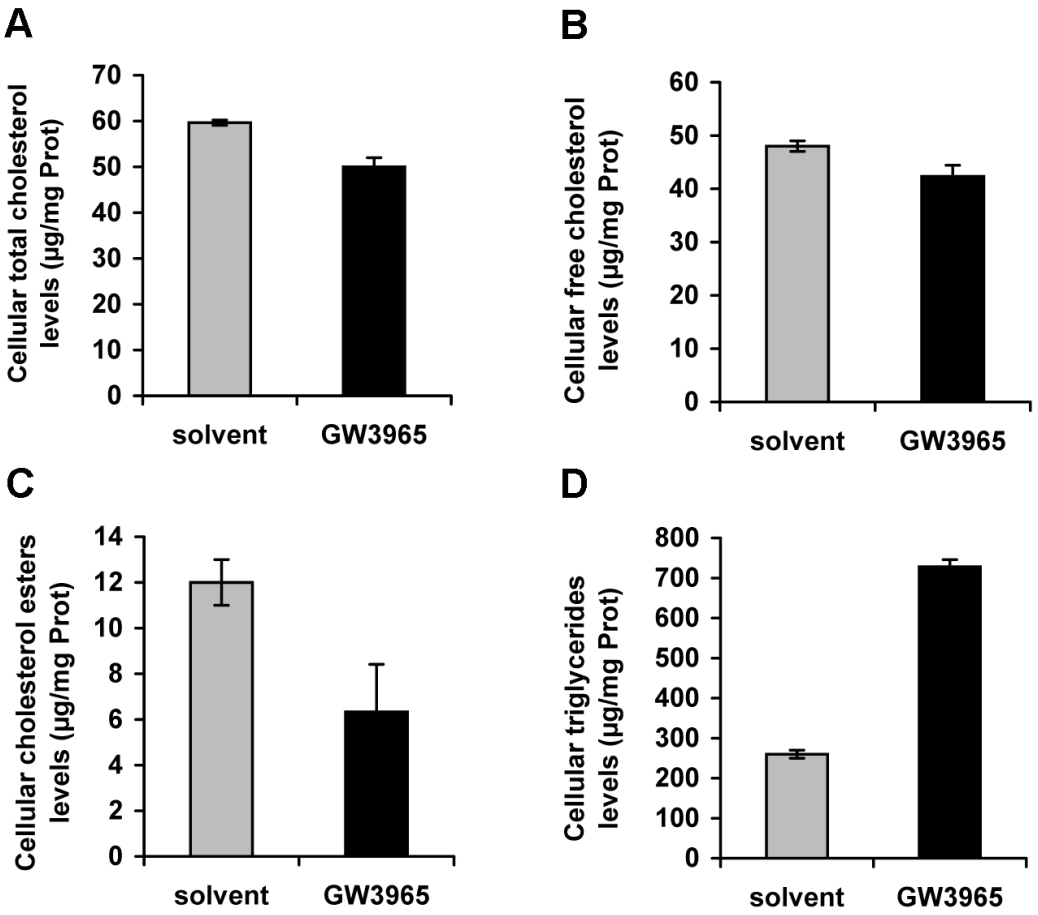


**Figure 7.** Analysis of cellular lipids in GW3965-treated cells. Huh7.5 cells were treated with 1µM GW3965 (GW3965) or drug solvent (solvent). Cellular lipids were extracted and (A) cellular total cholesterol, (B) cell-free cholesterol, (C) cholesterol esters and (D) triglyceride levels were quantified and expressed relative to total protein levels.

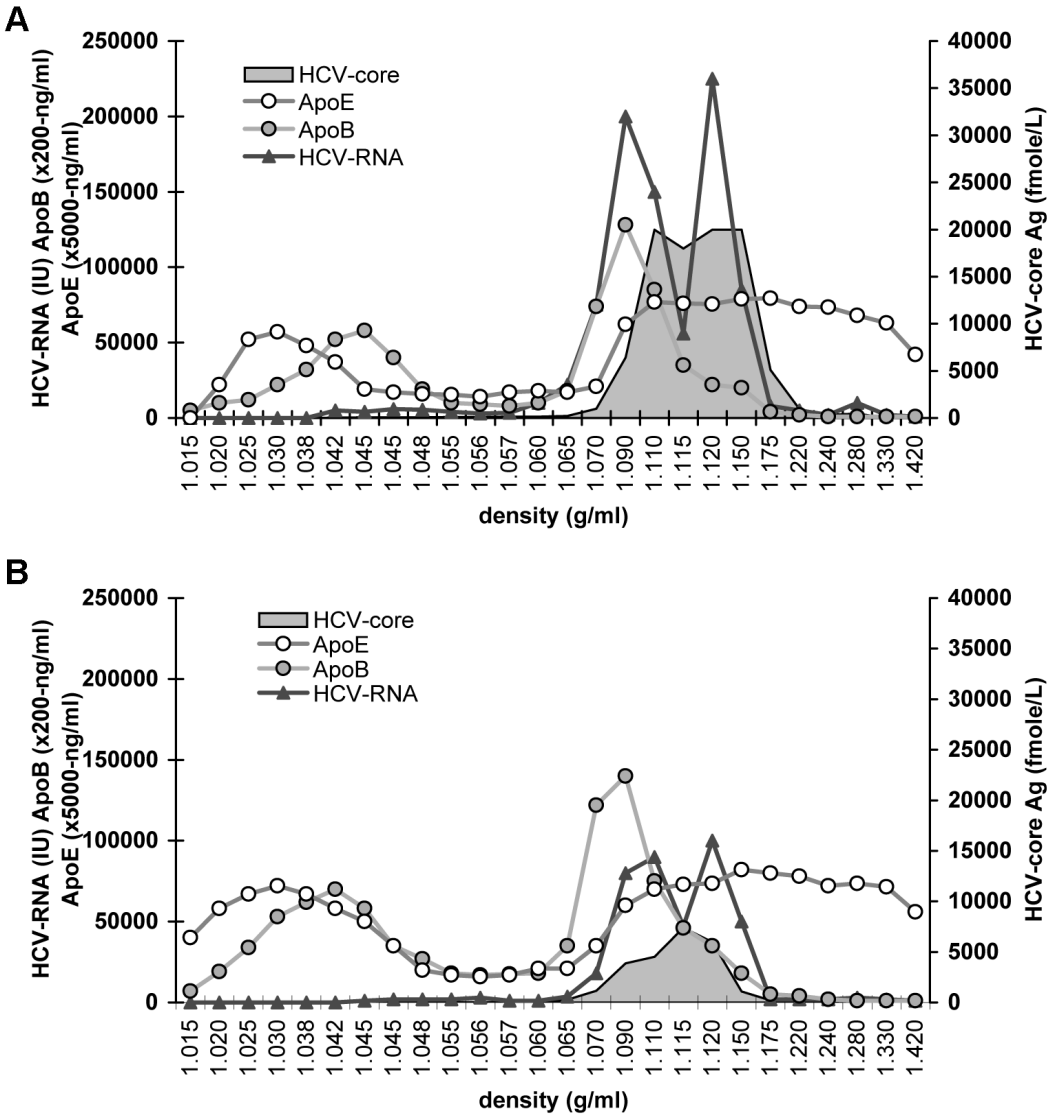


**Figure 8**. Analysis of HCV particles secreted from cells that over-express ABCA1. Physical properties of the nascent virus particles produced in cells stimulated or not with GW3965 were analysed by centrifugation in iodixanol gradient. Huh7.5 cells were pre-incubated with solvent (panel A) or 1μM GW3965 (panel B) and the drug was maintained until 72h post-infection when cell supernatants were collected, concentrated and subjected to gradient centrifugation. HCV RNA in gradient fractions was quantified by qRT-PCR and core antigen, ApoB and ApoE by ELISA assays.

**GW3695 does not change the expression of HCV receptors**.

We further assessed whether the inhibition of HCV cell entry by ABCA1 over-expression was associated with changes in expression of major HCV receptors. Flow cytometry analyses demonstrated that GW3965 stimulation did not change the global expression levels of CD81, SR-BI, and LDL-R in Huh 7.5 cells (Figure 9A). Equally, Western Blot analyses (Figure 9B) confirmed that the expression of NPC1, CLDN1, OCLN were not modified by the treatment. In addition, ABCA1 stimulation did not modify the cell surface distribution of CD-81 and SR-BI, or CD-81 and CLDN1, which interact at the plasma membrane forming HCV receptor complexes [296,297]. The distance between the pairs of the receptors was determined to be <40nm, by Duolink Proximity Ligation Assay (data not shown). Furthermore, ABCA1 over-expression did not reduce SR-BI specific cholesterol transfer activity to HDL that is required for HCV cell entry [348](data not shown).
Hence, impaired HCV entry following ABCA1 over-expression was not due to changes in the overall expression of major HCV receptor molecules, or distribution of receptors known to co-localise. It was not equally due to reduced SR-BI cholesterol transfer activity to HDL.

### *Stimulation of ABCA1 affects virus-cell fusion*

ABCA1 exerts an important influence on the plasma membrane structure, moving cholesterol within the membrane to disorganise cholesterol-rich raft domains [513-517]. These changes might impair virus-cell fusion mechanisms. Using a previously developed assay [179], we found that indeed increased ABCA1 expression upon GW3965 stimulation inhibited cell fusion induced by HCV envelope proteins (*$p<0.03$), whereas Chikungunya envelope-induced fusion (a control) was not significantly affected (Figure 9 C).
These observations underline the impact of the over-expression of the cholesterol transporter on specific membrane rearrangements required for HCV-induced fusion.

### *GW3965 treatment affects the organization of cholesterol enriched membrane microdomains*

ABCA1 disorganises cholesterol-rich raft microdomains and redistributes cholesterol/sphingolipids from raft to non-raft domains making it available for ApoA1 and facilitating HDL production [513-517]. Supplying cells over-expressing ABCA1 with exogenous cholesterol should redistribute membrane cholesterol between these domains and inverse the effect of ABCA1 [518].

To assess whether ABCA1 stimulated inhibition of HCV entry might be reversed by the cholesterol supply, we incubated cells that over-expressed ABCA1 with cholesterol/MbCD complexes. Cholesterol/MbCD–treatment increased total cellular cholesterol content (Figure 9D) and completely reversed the inhibitory effect of ABCA1 on HCV cell entry (Figure 9E).

These data suggested that the increased cholesterol efflux *via* ABCA1 induced remodelling of the cholesterol-rich lipid raft microdomains and accordingly affected virus-induced fusion. The inhibitory effect could be reversed by an exogenous cholesterol supply, providing evidence that restriction of virus infection was induced by changes of cholesterol content/distribution in membrane regions essential for virus cell entry.

To confirm that indeed GW3965 treatment affected cholesterol-enriched membrane microdomains we analysed the plasma membrane distribution of a raft-associated Glycosylphosphatidyl-inositol-anchor attachment signal of the folate receptor (GFP-FR). Glycosylphosphatidyl-inositol (GPI)-anchored proteins are cholesterol-dependent for their plasma membrane organization and thus can be used as lipid raft markers [504,519]. Fluorescence microscopy analysis demonstrated that, while in untreated cells GFP-FR was homogenously distributed at the plasma membrane in GW3965 treated cells GFP-FR expression was drastically different from the control (Figure 9 F). Thus, ABCA1 over-expression affected the organization of cholesterol enriched membrane microdomains.

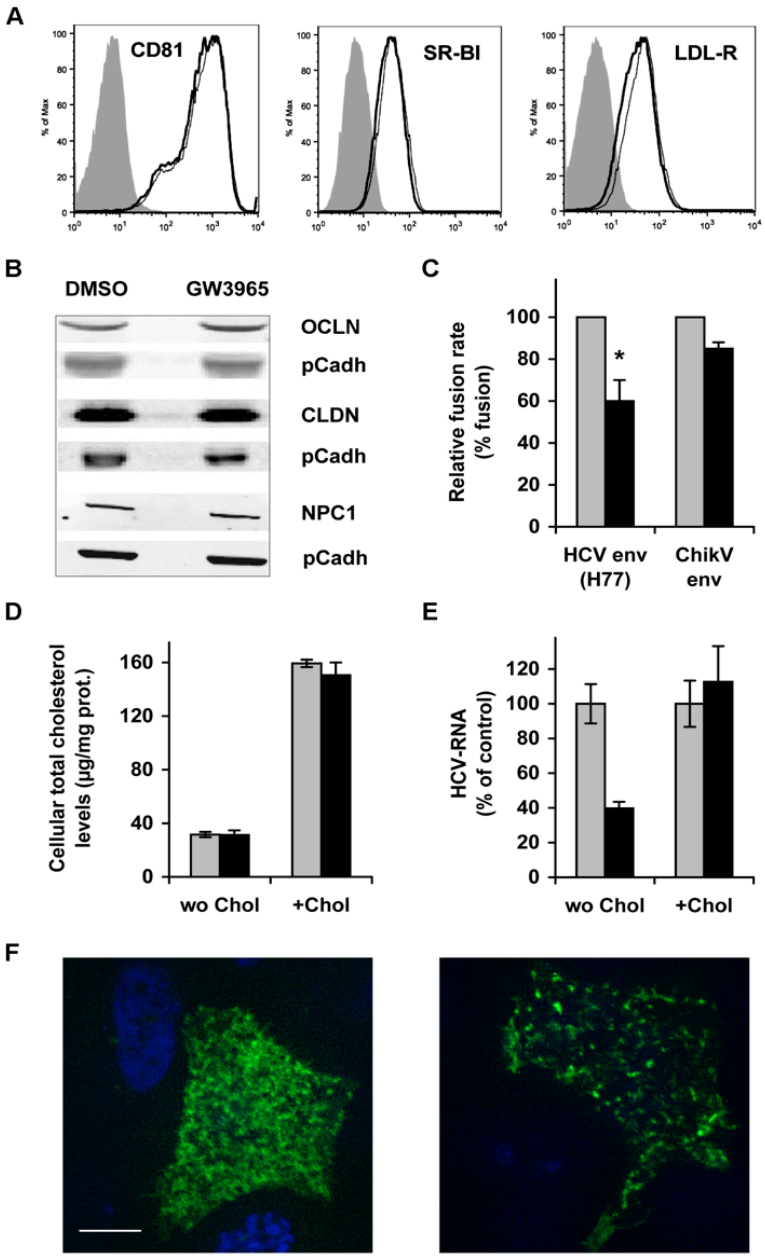


**Figure 9**. GW3965 treatment does not change the expression of HCV receptors, but affects HCV-cell fusion and modifies lipid raft structure. (A) Analysis of the expression of CD-81, SR-BI and LDL-R in Huh7.5 cells treated with 1µM GW3965 was analysed by Flow Cytometry using anti-receptor antibodies. GW3965-stimulated cells are shown by bold lines and non-treated cells by plain lines. Filled histograms represent cells stained with the secondary antibody only. (B) Western Blot assessment of the expression of OCLN, CLDN1 and NPC1 receptor in GW3965-treated cells, compared to solvent-treated cells. Pan Cadherin (pCadh) was used as a loading control. (C) GW3965 treatment inhibits HCV envelope-induced cell fusion. 293T cells that co-express a luciferase marker and the HCV E1-E2 envelope glycoproteins or the Chikungunya virus envelope glycoproteins were co-cultured with Huh7-Tat indicator cells. Co-cultured cells were incubated with 1 $\mu$M GW3965 or DMSO and exposed to pH 5. Luciferase activity was measured 72h later. Data are presented as the fusion rate in the presence of the drug (black bars) relative to fusion in the absence of drug (gray bars), which was considered as 100%. The graph represents the average of 3 independent experiments (*P < 0.03). (D-E). Cholesterol loading counteracts the inhibitory effect of ABCA1 over-expression on HCV infection. Huh7.5 cells were stimulated with 1 $\mu$M GW3965 to over-express ABCA1, and then incubated with 20 $\mu$M cholesterol/M $\beta$CD. In (D) the determination of total cellular cholesterol after replenishment of ABCA1 overexpressing cells with cholesterol/M $\beta$CD is shown. GW3965-treated cells (black bars) were compared to solvent-

treated (grey bars). The infection of Huh7.5 cells after cholesterol supply is shown in (E). Intracellular HCV RNA was determined by qRT-PCR at 24h post-infection, and is expressed as the percentage of HCV RNA in drug-treated (black bars) compared to solvent-treated (grey bars) cells, supplied (+Chol) or not (wo Chol) with cholesterol. (F) GW3965 treatment modifies plasma membrane organisation and thus the distribution of lipid raft-associated protein. Huh7.5 cells were transfected with DNA encoding the Glycosylphosphatidyl-inositol-anchor attachment signal of the folate receptor fused to GFP (GFP-FR). Cells were subsequently exposed to 1µM GW3965 or drug solvent (control). Two days post-transfection cells were fixed and GFP-FR fluorescence was visualised using a Zeiss axioplan 2 microscope (x63 objective). Slices of 0.46 $\mu$ m were acquired. The images shown are a Z projection of 5 slices of the cell surface that face the cell medium. The right panel represents GW3965-stimulated cells and the left control cells. The scale bar corresponds to 10 $\mu$ m.

**GW3965 inhibits HCV infection of primary human hepatocytes**.

Since Huh7.5 cells have several differences in lipid metabolism compared to primary human hepatocytes, we investigated the effect of ABCA1 up-regulation on HCV infection of human hepatocytes in primary culture [502] or HCV infection of human liver slices [503], the two available models that are permissive for a full HCV life cycle and display normal lipid/lipoprotein metabolic pathways.

First, hepatocytes obtained from two liver donors were pre-treated with various concentrations of GW3965 (2-10µM). Cells were then infected with HCV and grown for 24h and HCV RNA was determined by qRT-PCR. As shown in Figure 10 A-B ABCA1 up-regulation was associated with a dose dependent decrease of intracellular HCV RNA levels.

A substantial inhibition of HCV infection was also observed in the model of primary human liver slices in culture. Cultures of human liver slices were established with differentiation status. Human liver slices were treated with 5µM and 10µM concentrations of GW3965 (which were not toxic) or with drug solvent, for 24h before infection with HCVcc. These drug concentrations were selected on the basis of the results, which showed that 5µM concentration of GW3965 was required to raise ABCA1 levels.

HCV RNA levels decreased almost by two logs in drug-treated cells, while ABCA1 gene expression concomitantly increased 2.5 fold over the time course of the experiment (Figure 10 C and D).

Altogether these data provided evidence that pharmacological stimulation of ABCA1 efficiently inhibited HCV infection of primary hepatocytes.

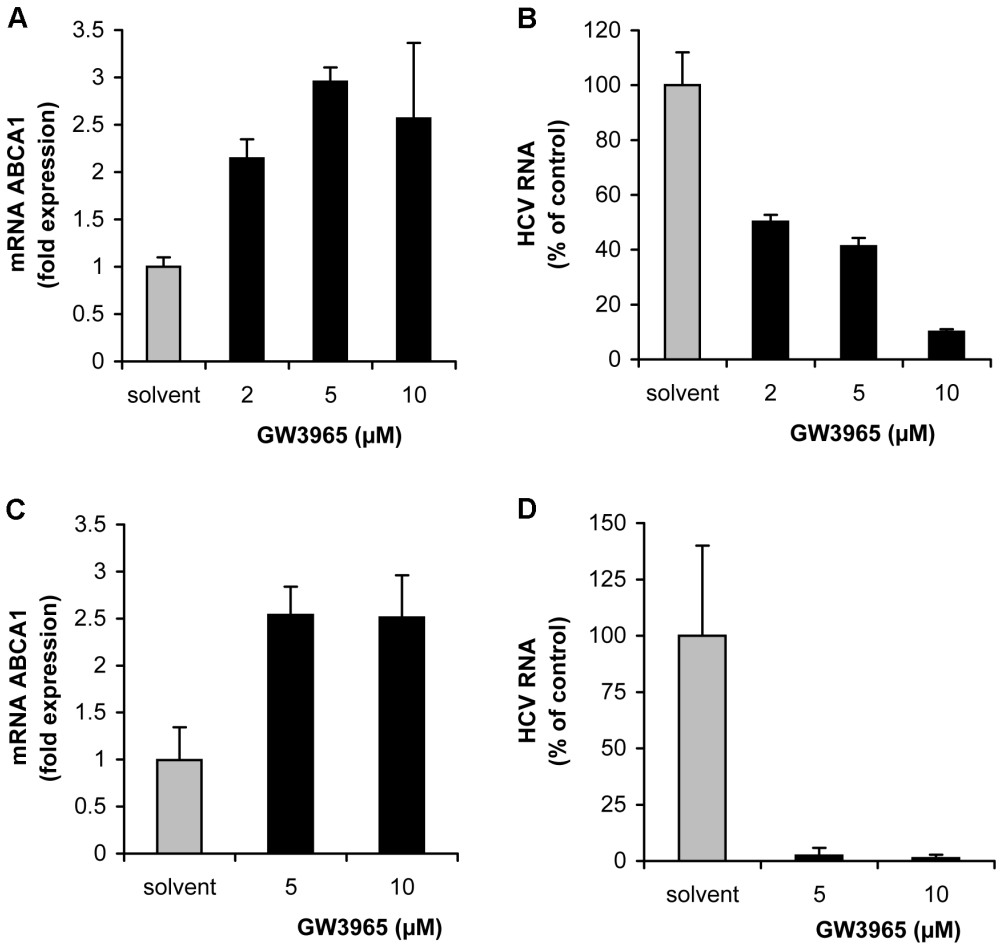


**Figure 10**. Over-expression of ABCA1 inhibits HCV infection of primary human hepatocytes and human liver slices. (A-B) Inhibition of HCV infection of primary human hepatocytes. (A) Primary human hepatocytes were treated with 2-10µM GW3965 (non-toxic concentrations for cells) or with drug solvent, prior to HCV infection. Twenty-four hours post-infection, ABCA1 mRNA was determined by qRT-PCR and expressed in arbitrary units, taking into account ABCA1 levels in liver cells pre-treated with the drug. (B) GW3965-treated and solvent-treated primary human hepatocytes were inoculated with HCV. After 24h, intracellular HCV RNA was quantified by qRT-qPCR. The efficiency of infection in drug pre-treated cells was expressed as the percentage of infection compared to solvent-treated cells. (C-D) ABCA1 over-expression inhibits HCV infection of human liver slices. Human liver slices were cultured for 24h, treated with 5 or 10µM GW3965 or with DMSO before infection with HCV. At 24h post-infection, total RNA was extracted and ABCA1 mRNA (C) and HCV RNA (D) were quantified by corresponding qRT-PCR assays and expressed as the percentage of RNA compared to the values obtained for solvent-treated cells.

# Discussion & Conclusions

Hepatitis C virus (HCV) infection affects 3% of the world population and is major cause of chronic liver disease with severe hepatic consequences such as steatosis, cirrhosis and hepatocarcinoma. Recently, numerous direct acting anti-viral drugs (DDA) have been introduced, targeting essential viral functions. These new treatments represent a significant step forward as compared to standard Pegylated IFN-α-ribavirin therapy. DDA are mainly inhibitors of NS3/NS4 HCV protease, and others drugs are under development that target NS5B polymerase or NS5A playing essential roles in HCV replication [437]. However, these DDA still have side effects and induce the development of drug-resistance [520].

HCV has developed lipoprotein-dependent mechanisms for cell entry [118,276,450], replication regulated by fatty acids [400], virus morphogenesis connected with lipid droplets [411] and the assembly and secretion of infectious virion *via* the VLDL pathway [113,114]. The relationships between lipid metabolism and HCV are complex and intriguing. The expression of host genes involved in biosynthesis, degradation or transport of intracellular lipids is altered upon HCV infection [399,452].

Altogether these observations reflect the role of lipids in the HCV life cycle. Therefore, host factors involved in cholesterol/lipid metabolism might represent potential targets for HCV strategies with only limited possibilities for escape mutations to develop [112,455] and improve regimens for patients infected with genotype 3 [437].

Cholesterol is an important structural component of biological membranes and is essential for the uptake of many viruses. HCV cell entry requires cholesterol homeostasis and intact cholesterol-rich membrane microdomains. The modulation of intracellular and membrane cholesterol homeostasis has dramatic effects on the early stages of several viral infections [521,522]. Thus, we hypothesised that the ABCA1-mediated cholesterol efflux may influence the course of HCV infection.

In our study presented in this thesis we provide first demonstration that pharmacological stimulation of ABCA1 gene expression and its cholesterol efflux function impairs HCV infection, acting on virus protein mediated-host cell fusion. The inhibitory mechanism was associated with re-organisation of cholesterol-rich membrane microdomains (lipid rafts). Stimulation of ABCA1 expression also efficiently inhibited infection of human hepatocytes, which represent the natural target cells for HCV, and in culture of isolated human liver slices,.

In our study overexpression of ABCA1 was achieved using LXR agonists, mainly GW3965. LXRs are ligand-activated transcription factors, which maintain cholesterol homeostasis. LXR agonists increase ABCA1 expression but also modulate several other genes that regulate lipid metabolic pathways [523,524]. Indeed, we found that GW3965 affected the expression of several genes involved in hepatic lipid metabolism. Nevertheless, silencing of the ABCA1 gene and reducing its protein expression and specific efflux function reversed the inhibitory effect of GW3965, supporting the key role of ABCA1 in the inhibition of HCV infection.

In hepatocytes, the specific cholesterol efflux function and nascent HDL formation only depends on ABCA1, whereas ABCG1, another cholesterol transporter acts in synergy with ABCA1 to promote cellular cholesterol efflux to preformed nascent HDL particles. SR-BI, one of the main HCV receptors, contributes neither to cholesterol efflux to ApoA1 nor to HDL formation [525]. Thus the specific cholesterol efflux function to Apo A1 activated by the treatment with GW3965 or TO90317 was due to the ABCA1 activity.

Stimulation of ABCA1 by these drugs impaired HCV cell entry by acting on virus protein-mediated cell fusion, presumably blocking virus nucleocapsid release into the cytosol. GW3965 did not directly affect the virus particle or cell membrane structure, since adding the drug during virus inoculation did not reduce its infectivity. Instead, inhibition of infection required ABCA1 over-expression and activation of its function during several hours. GW3965 did not either influence virus binding to the cell surface or HCV RNA replication.

Up-regulation of ABCA1-mediated hepatic cholesterol efflux might decrease hepatic VLDL secretion [526]. In our study GW3965 treatment did not affect either levels of ApoA1 in the cell supernatant, or virus assembly/secretion mechanisms. It did not change physicochemical properties and infectivity of the virus particles. Thus decrease in HCV production and secretion was due to the impaired virus entry.

The inhibition of HCV cell entry *via* stimulation of the ABCA1 pathway was not associated with changes in overall expression of HCV receptors: LDL-R [276], HSPG [347], CD-81 [282], SR-BI [300], NPC1 receptor [336] and tight junction molecules CLDN1 [312] and OCLN [306].

Nevertheless, besides its reliance on the expression of receptor molecules, initiation of a productive HCV infection depends on the cholesterol content of the target cell membrane [297]. Indeed, cholesterol constitutes an essential component of lipid-rich membrane microdomains, organised parts of the plasma membrane that compartmentalise/segregate proteins and lipids and thus play a key role in virus entry, either influencing clustering of receptors or acting on virus-cell fusion [454,527].

Depletion of the membrane cholesterol with MβCD inhibited HCV infection [297] showing for the first time that cholesterol-rich raft environments likely serve as portals for HCV entry. While MβCD disrupts lipid rafts by depleting cells of cholesterol with high cytotoxicity [528], ABCA1 stimulation leads to the activation of the physiological cholesterol efflux pathway that does not affect total and free cellular cholesterol content, and has low cytotoxicity. Whereas MβCD treatment resulted in important changes of cell surface expression of CD-81 and SR-BI [297] and OCLN [529] such changes were not observed in cells over-expressing ABCA1. Indeed, using the Duo Link assay we showed that the cell surface distribution of SR-BI and CLDN1 relative to CD-81, the HCV receptors supposed to co-localise [296,297]was not modified (distance <40nm) in GW3965-stimulated cells.

Up-regulation of ABCA1 leads to a perturbation of cholesterol packaging in cell membranes and changes of its distribution in the lipid rafts or between raft and non-raft microdomains [513,514,517,518]. Such modifications might impair HCV-host cell fusion as shown in our fusion assay either by influencing distribution of both lipids and proteins or by changing membrane fluidity [454].

Indeed, cholesterol facilitates HCV-mediated fusion dependent upon the presence of functional E1 and E2 proteins [179]. The fusion proteins act in common with lipid and cholesterol assemblies at the virus-cell fusion step. Lipids, mainly glycerophospholipids, sphingolipids and sterols, contribute through their physical, mechanical and/or chemical properties, whereas cholesterol can play a role through its preferential partitioning into rafts or its binding affinity for certain viral envelope proteins. Cholesterol-rich microdomains are implicated in the entry of many virus species such as Ebola and Marburg viruses, Vaccinia virus, murine Hepatitis virus, lymphocytic choriomeningitis virus and Herpes Simplex virus. Indeed, when cholesterol dispersion or reorganisation of lipid microdomains were

achieved using drugs such as filipin and nystatin, virus entry was largely reduced, although virion attachment was unaffected. Since all these viruses enter host cell via cholesterol-rich microdomains, these observations suggested drug action on a fusion step [372].

Several studies underline the fact that ABCA1 expression does not alter the overall cellular cholesterol content or its subcellular distribution [514]. The current model proposes that ABCA1 exerts an influence on plasma membrane structure, modifying organisation of cholesterol-rich microdomains by redistributing cholesterol/sphingolipids to non-raft domains to facilitate ApoA1 interaction and efflux [513,514,517,518].

In agreement with this notion, in our study activation of the ABCA1 pathway had only a minor effect on total and free cholesterol content in Huh7.5 cells. Instead, stimulation of ABCA1 modified the cholesterol distribution in cell membranes. Lipid raft-dependent localisation of GFP-FR in membranes was dramatically changed in GW3965 treated cells. These changes most probably disturbed virus cell entry, confirming that the integrity of cholesterol-rich membrane microdomains is essential for the initiation of a productive HCV infection.

Supplying cells with a surplus of exogenous cholesterol to GW3965-treated cells overturned the inhibitory effect on HCV cell entry in accordance with the notion that adding of exogenous cholesterol to cells, which over-express ABCA1, tightens lipid packaging in raft and non-raft microdomains, while decreasing ABCA1-dependent cholesterol efflux [518].

HCV (JFH-1 strain) infection of the Huh 7.5 cell line is currently used for studies of the HCV life cycle and the evaluation of HCV inhibitors. Nevertheless, this *in vitro* cell culture system, based on transformed and poorly differentiated hepatoma cells, has several limitations, especially concerning lipoprotein metabolism [109]. Thus, we confirmed our findings in primary culture of human hepatocytes and in human liver slices, which maintain the 3D structure and gene expression of the liver with normal lipid/lipoprotein metabolic pathways. We provide evidence that stimulation of ABCA1 expression with GW3965 inhibits HCV infection of primary hepatocytes even more efficiently than in Huh 7.5 cells.

## Conclusions and future perspectives

1. In our study Liver X receptor (LXR) agonist GW3965 was used to stimulate ABCA1 expression and cholesterol efflux. The antiviral effect of ABCA1 overexpression was evaluated using the HCVcc *in vitro* infection model and in primary human hepatocytes. Our studies showed ABCA1 stimulation inhibited HCV cell entry by acting on virus-cell fusion, consequently decreasing levels of produced and secreted virus. The inhibition mechanisms involved reorganisation of cholesterol-rich membrane microdomains (lipid rafts). Our findings highlight for the first time ABCA1 as a possible novel target for HCV treatment.

2. Several viruses, including Echovirus-1, and Dengue virus, have shown their dependence on plasma membrane cholesterol for optimal entry into host cells [530,531]. Recently it has been demonstrated that also HIV infection depends on membrane cholesterol for virus assembly, budding and cell entry, which also required lipid rafts [532]. Strikingly, *via* its protein Nef, HIV down-regulates ABCA1 expression and impairs cholesterol efflux activity to infect its target cell [533]. ABCA1 overexpression achieved using TO901317 impaired HIV infection: accordingly, pharmacological stimulation of ABCA1 has been proposed as an approach to inhibit HIV infection [534]. Since infection *in vitro* with both viruses HIV and HCV is impaired by stimulation of ABCA1-dependent cholesterol efflux, it would thus be interesting to investigate further the idea whether raising ABCA1 levels might improve treatment of HIV/HCV co-infected patients, who represent a special population particularly difficult to cure.

3. ABCA1-mediated cholesterol efflux leads to the formation of HDL, a lipoprotein important for prevention of arteriosclerosis and cardiovascular disease. Thus, in addition to this known beneficial role, ABCA1 emerges as a potential target to better control HCV infection.

4. New drugs are under development that should reduce the side effects found for LXR agonists such as an increase in triglyceride levels [535,536] also observed in our study.

5. Preliminary results obtained during preparation of this thesis suggested that knocking-down ABCA1 gene might stimulate infection in HCVcc model. These intriguing observations should be further investigated in terms of virus production, secretion and infectivity, including application of si-RNA approaches. If confirmed, ABCA1 would appear as a interesting regulator of HCV infection levels.

6. Since a chimeric mouse UpA/SCID model with transplanted human primary hepatocytes susceptible to HCV infection is currently available [537], it would be interesting to assess the effect of ABCA1 up regulation upon HCV infection in this *in vivo* model.

7. Recently, it has been demonstrated that ABCA1 expression is reduced in the presence of high levels of TNF-α, a pleiotropic cytokine playing key roles in the regulation of cellular immune response in HCV infection [538,539]. Serum TNF-α has been shown to bear a positive correlation with histological grade and a negative correlation with response to IFN in hepatitis C [540]. Moreover it has been claimed that TNF-α contributes to fibrosis progression and HCC development in HCV patients [541]. Finally, the cholesterol efflux function has been linked to anti-tumour activity, and thus ABCA1 is considered a putative tumour-suppressor molecule [498]. This particular property of ABCA1 might additionally help to prevent development of HCV-related HCC. Based on these premises it would be interesting to quantify ABCA1 expression in liver biopsies both in relationship with response to antiviral treatment and, most importantly, the development of HCC. Alternatively, down-regulation of ABCA1 expression mediated by TNF-α might be investigated in transgenic mice carrying the HCV core gene [542] to evaluate whether this mechanism could contribute to HCC development.

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# Other Projects

During my PhD studies I also participated to several projects focused on the characterization of pathophysiological mechanisms involved in the progression of liver diseases (in particular related to chronic viral infections) towards advanced fibrosis, cirrhosis and hepatocellular carcinoma. In particular, my efforts have been focused on:

1) The role of the polymorphism rs12979860 C/T on the IFNα and disease stage in HCV-HIV co-infected patients

2) A longitudinal study with the aim to asses if high repetition variant of dopamine receptor $D_4$ (DRD4) may serve as proxy measure of alcohol consumption, and to verify whether it may affect histologic outcome in HCV patients.

Specifically, I contributed to these studies carrying out the molecular biology part of the work. In particular, in the first project I genotyped the IL-28B rs12979860 C/T polymorphism that was performed by PCR-based restriction fragment at length polymorphism assay. In the second project, I participated in the genotyping of DDR4 through analysing PCR product on agarose gels to establish the number of tandem repeats in exon III.
From each project we obtained one publication (enclosed).
In addition, thanks to the knowledge gained during the period spent working on the project at the Pasteur Institute I had also the opportunity to take part in writing a review focused on HCV entry

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# IL28B Polymorphism, Blood Interferon-Alpha Concentration, and Disease Stage of HCV Mono-Infected and HCV-HIV Co-Infected Patients

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**Abstract:** Interferon (IFN) preactivation, interleukin-28B (IL28B) alleles, and liver fibrosis act as predictors of response to antiviral therapy against hepatitis C. We aimed to verify if blood IFN concentration, a putative biomarker of interferon preactivation, might depend on carriage of a given IL28B genotype and/or advanced hepatic fibrosis. The study population included 187 hepatitis C patients (75 of whom were HIV coinfected), who were genotyped for the rs12979860 polymorphism and staged non-invasively by transient elastography. Blood IFN, measured by an enzyme immunoassay, was detectable in 68/187 patients (36%). Seventy-three patients (39%) were C/C homozygotes, 25 (13%) were T/T homozygotes, and 89 (48%) were heterozygotes. The fibrosis stage was F0-F1 in 70 patients (37%), F2-F3 in 54 patients (29%), and F4 in 63 patients (34%). IFN levels were higher among patients with HIV coinfection (p=0.044) and patients with better renal function (p=0.041), without association with the IL28B genotype or the hepatitis C stage. From the multivariate analysis, the only independent predictor of higher level of IFN was the age of patients (p=0.019), whereas independent predictors of a fibrosis stage ≥F2 were age (p=0.007), belonging to the HIV/HCV group (p=0.048) and current alcohol consumption (p=0.008). In conclusion, a sizable proportion of HCV carriers have detectable IFN levels that do not indicate a greater severity of disease or display any relationships to specific rs12979860 variants.

**Keywords:** Fibroscan, HIV/HCV, IL28B genotype, IFN-alfa, liver fibrosis.

## INTRODUCTION

During the last 25 years, interferon (IFN)-alpha has been the backbone of antiviral treatment in hepatitis C, although it is not clear what mechanisms are responsible for why some people respond to IFN and clear hepatitis C virus (HCV) and others do not [1]. Originally, IFN treatment was considered as a replacement therapy because the persistence of viral replication in patients with chronic viral hepatitis was deemed to be due to defective endogenous IFN production [2]. Indeed, studies published in the 1980s reported that IFN-alpha is undetectable in sera from patients with acute and chronic viral hepatitis [3], and IFN-alpha production by peripheral blood mononuclear cells is impaired [4]. Currently, we know that hepatitis C patients can be divided into two broad categories with regard to the status of their endogenous IFN system: patients who have IFN in a pre-activated state in the liver, who are unlikely to respond to exogenous IFN, and patients who do not have IFN pre-activation, who are likely to respond to exogenous IFN [5]. However, this information requires a liver biopsy and a sophisticated array of molecular biology-based techniques, which are not suitable for implementation in clinical practice. On the other hand, IFN-alpha levels are not always undetectable in blood from patients with chronic hepatitis C, and sometimes they are exceedingly high. In fact, in the era of conventional IFN monotherapy of hepatitis C, the blood IFN-alpha level was suggested by our group as a putative inexpensive marker of IFN susceptibility [6]. The observation of high blood IFN-alpha concentrations was not limited to the HCV setting: indeed, patients with HIV infection have different blood IFN levels depending on the stage of disease [7], the speed of the progression to AIDS [8] and the presence of symptomatic manifestations of the disease [9].

Recently, the way clinicians approach treatment outcome prediction in hepatitis C patients has been revolutionized by three independent genome-wide association studies that established single nucleotide polymorphisms (SNP) upstream the interleukin-28B (IL28B) gene as strong, novel treatment outcome predictors [10-12]. IL28B is a cytokine belonging to the IFN family in a subgroup named type-III interferon, including three ligands (IFN lambda-1, lambda-2 and lambda-3, the latter also known as IL28B), which are generated by the immune system in response to viral infection. Further studies documented that hepatitis C virus (HCV) carriers with an unfavorable IL28B genotype have their hepatic IFN-induced genes predominantly in a pre-activated state [13]. The exact mechanisms linking IL28B polymorphisms to pre-activation of the IFN system in the liver are unknown.

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We wondered whether a paradox of maximal efficacy in patients less in need of a cure applies to IL28B polymorphisms and hepatitis C, *i.e.*, if carriers of the favorable IL28B genotypes have less severe forms of hepatitis C (and *vice versa*) and whether this could be reflected in serum IFN levels in untreated patients as a marker of IFN pre-activation. The present study was designed to answer these questions.

## MATERIALS AND METHODS

### Study Design

Retrospective cohort study.

### Patients

The study population originated from a database including 218 adults who were consecutive patients referred for evaluation and treatment of hepatitis C to either the human immunodeficiency virus (HIV) clinic or the liver clinic of a university hospital in northern Italy. All patients were genotyped for rs12979860, a SNP located in the genomic region upstream of the IL28B coding sequence. To be included in the study, patients needed to fulfill the following criteria: a) to have performed a valid liver stiffness measurement by transient elastography (Fibroscan, Echosens, Paris); b) to have donated a blood sample to our biobank within one week of when the Fibroscan was performed; c) to be HBsAg-negative; and d) to be IFN treatment-naïve. HBsAg-positive patients were excluded. Twenty-eight patients did not have a valid liver stiffness measurement. Therefore, the study population consists of 187 patients, 114 males and 73 females, of whom 112 were HCV-monoinfected and 75 were HCV-HIV-coinfected, none had concurrent infections at the time of blood donation. All patients gave an informed consent for their participation in the study, which was conducted in strict accordance to the principles of the declaration of Helsinki.

Sixty-eight out of 75 HCV-HIV-coinfected patients were on highly active antiretroviral therapy (HAART) for HIV at the time of their participation in the study; the blood HIV RNA was ≤50 IU/ml (51 of which were on active HAART) in 52/75 patients, and the median CD4 count was 331 cells/μl (95% confidence interval, 302-371). After being evaluated by transient hepatic elastography, 79 patients started antiviral treatment with pegylated IFN and ribavirin (77 patients in the HCV-monoinfected group and 2 in the HCV-HIV-coinfected group).

### Transient Elastography and Non-Invasive Assessment of Liver Fibrosis

Liver stiffness, a proxy measurement of liver fibrosis, was obtained by the shear elasticity probe, a device based on 1-D transient elastography, which is well adapted to the study of liver elasticity. For a more detailed description of this technique and of the examination procedure, see Sandrin *et al.* [14]. All tests were performed by external personnel who performed >100 examinations and were not involved in the planning of the study or in the analysis of data. Examinations were classified as follows: a) valid; b) failed, when no valid shots were obtained; c) unreliable, when fewer than 10 valid shots, an interquartile range (IQR)/liver stiffness measurement greater than 30%, or a success rate less than 60% were obtained [15]. For the purposes of the present study, only valid examinations were considered. Fibrosis stage was classified according to Castera *et al.* [16].

### rs12979860 Genotyping

DNA was obtained from 200 μl of whole blood using the Genomic DNA Blood Kit (Sigma, Italy) and amplified by polymerase chain reaction (PCR) in a thermal cycler (Applied Biosystems) using the forward primer 5'-GCTTATCGCATACGGCTAGG-3' and the reverse primer 5'-AGGCTCAGGGTCAATCACAG-3'. The amplicons were digested with 3 U of BstU-I restriction endonuclease (New England Biolabs, Hitchin, UK) at 37°C overnight (16 hours).

### Biohumoral and Viral Studies

Blood IFN-alpha concentrations were measured by a commercially available enzyme immunoassay (human IFN-alpha ELISA BMS216CE, Bender MedSystems, Burlingame, CA, USA). According to the manufacturer, the limit of detection for human IFN-alpha, defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (the mean plus 2 standard deviations) was determined to be 7.8 pg/ml.

HCV and HIV viral loads were quantified by reverse transcription-polymerase chain reaction using the Abbott RealTime HCV kit (Abbott Laboratories, Abbott Park, IL, USA), which has a detection limit of 12 IU/ml, and the Versant HIV-1 RNA 1.0 Assay (kPCR) (Siemens Healthcare Diagnostics, Deerfield, IL, USA), which has a detection limit of 50 copies/ml, respectively.

### Statistical Analysis

For the statistical analysis of data, the biomedical MedCalc statistical software package, version 11.6.1.0 (MedCalc Software, Broekstraat 52, 9030 Mariakerke, Belgium) was used, and the normal distribution of the data in continuous variables was preliminarily determined by the D'Agostino-Pearson test. The variability of the data around the central value was presented as the median (95% confidence interval of the median, CI 95%), while the comparisons between groups were made by the Mann-Whitney test. The correlation between two non-parametric variables was investigated by Spearman's coefficient of rank correlation. Categorical variables were presented as frequencies (percentage). The association between categorical variables was investigated by the Pearson Chi-square test, the Cochran-Armitage or the Fisher exact test when appropriate. The Hardy-Weinberg equation and allele frequencies were calculated using unrestricted software developed by Michael H. Court, which was downloaded from the website www.tufts.edu. Finally, to verify the relationship between a dichotomous dependent variable (detectable IFN-alpha in peripheral blood; liver stiffness ≥7.1 kPa, indicating significant fibrosis) and a set of independent predictor variables, logistic regression analysis with a stepwise forward approach was conducted. The level of statistical significance was chosen at $p<0.05$ (two-tailed).

## RESULTS

### Characteristics of Study Population

The main demographic and clinical characteristics of study participants are presented in Table **1**.

### Quantification of Blood IFN-Alpha

The analysis of the blood IFN-alpha values distribution in HCV-monoinfected and HCV-HIV-coinfected patients showed that in approximately two thirds of the cases, blood IFN-alpha was below the limit of detection. HCV-HIV-coinfected patients had a value of blood IFN-alpha that was significantly higher (median 7.8 pg/ml, 95% CI of the median 7.8-16.4) than in HCV-monoinfected patients (median 7.8 pg/ml, 95% CI of median 7.8-7.8) ($p = 0.04$). One-hundred nineteen patients had blood IFN-alpha ≤7.8 pg/ml (41 (34%) HCV-HIV-coinfected and 78 (66%) monoinfected) and sixty-eight patients had a blood IFN-alpha concentration greater than 7.8 pg/ml (34 (50%) HCV-HIV-coinfected and 34 (50%) HCV-monoinfected) ($p=0.053$).

There was no relationship between pre-treatment interferon levels and the achievement of sustained virological response, either considering dichotomized values ($p=0.761$), or considering continuous values ($p=0.415$).

Finally, to exclude the possibility that the stage of HIV infection might represent a significant confusing factor, we investigated the related blood IFN levels count to the CD4 count of HCV-HIV coinfected patients and we did not find any correlation (Spearman's rank correlation -0.061, $p=0.6$).

### Liver Stiffness

The distribution of liver stiffness values was similar in the two groups of patients ($p = 0.241$). In detail, a liver stiffness value <7.1 kPa (suggestive of absent or mild fibrosis, METAVIR stage F0-F1) was observed in 70 patients (23 (33%) HCV-HIV-coinfected *vs* 47 (67%) HCV-monoinfected), while among the 54 patients with a stiffness value between 7.1 and 12.5 kPa, 26 (48%) were HCV-HIV-coinfected and 28 (52%) were HCV-monoinfected. Among 63 patients with a stiffness value ≥12.5 kPa (suggestive of severe fibrosis, METAVIR stage F4), 26 (41%) were HCV-HIV-coinfected and 37 (59%) were HCV-monoinfected ($p = 0.221$).

### rs12979860 Genotyping

Seventy-three (39%) patients were C/C homozygotes (34 HCV-HIV-coinfected and 39 HCV-monoinfected), 25 patients (13%) were T/T mutant homozygotes (12 HCV-HIV-coinfected and 13 HCV-monoinfected), and 89 patients (48%) were C/T heterozygotes (29 HCV-HIV-coinfected and 60 HCV-monoinfected). The study population was in perfect agreement with the Hardy-Weinberg equation ($p = 0.795$), and the distribution of different genotypes was not significantly different between the two groups, HCV-monoinfected and HCV-HIV-coinfected ($p = 0.134$). The allele frequencies were 0.63 for allele C and 0.37 for allele

**Table 1. Main Demographic and Clinical Features of the Studied Population**

| | Total (N. = 187) | HCV Monoinfected (N. = 112) | HIV Coinfected (N. = 75) | P |
|---|---|---|---|---|
| Male : Female | 114 (60) : 73 (40) | 50 (45) : 62 (55) | 64 (85) : 11 (15) | <0.001 |
| Age (years) | 51 (50-54) | 58 (54-61) | 48 (46-49) | <0.001 |
| Transmission mechanism: | | | | |
| Unknown | 77 (41) | 73 (65) | 4 (5) | |
| Drug addiction | 77 (41) | 11 (10) | 66 (88) | |
| Transfusion | 24 (13) | 23 (20) | 1 (1) | |
| Other | 9 (5) | 5 (5) | 4 (5) | <0.001 |
| ALT, U/L | 62 (51-71) | 67.5 (50-76) | 55 (46-69) | 0.684 |
| INR, unit | 1.02 (1.00-1.08) | 1.00 (1.00-1.1) | 1.03 (1.01-1.09) | 0.684 |
| Bilirubin, mg/dl | 0.8 (0.7-0.8) | 0.8 (0.7-0.9) | 0.7 (0.6-0.9) | 0.369 |
| Creatinine, mg/dl | 0.90 (0.86-0.90) | 0.90 (0.90-0.90) | 0.86 (0.81-0.88) | 0.003 |
| HCV genotype | | | | |
| HCV1 | 95 (54) | 58 (57) | 37 (49) | |
| HCV2 | 30 (17) | 29 (28) | 1 (1) | |
| HCV3 | 36 (20) | 11 (11) | 25 (34) | |
| HCV4 | 16 (9) | 4 (4) | 12 (16) | <0.001 |
| Alcohol consumption | | | | |
| Teetotallers | 89 (50) | 71 (69) | 18 (24) | |
| Past drinkers | 39 (22) | 12 (12) | 27 (36) | |
| Current drinkers | 50 (28) | 20 (19) | 30 (40) | <0.001 |
| Serum HCV RNA IU/ml ($\times 10^3$) | 635 (426-807) | 510 (365-671) | 820 (530-1490) | 0.026 |

Data in continuous variables are presented as medians (95% confidence interval), and those in categorical variables as frequencies (%). The p values refer to the comparison between HCV monoinfected and HIV coinfected patients by Mann-Whitney test or Fisher exact test, as appropriate. Serum HCV RNA was available of 165/177 and HCV genotype of 177/187 patients. Information on alcohol consumption was available for 178/187 patients.

T. In the 60 patients who underwent pegylated IFN plus ribavirin treatment and completed the follow-up treatment necessary for the definition of sustained viral response (SVR; 24 weeks after completion of the treatment), SVR was achieved in 36 patients, 15/20 C/C homozygotes (75%), 19/33 C/T heterozygotes (57%) and 2/7 T/T homozygotes (28%) (p= 0.031).

### Determinants of Blood IFN-Alpha Concentration

IFN levels of HCV-HIV coinfected patients who underwent HAART (n.=68) were not statistically different from those observed among coinfected patients who did not undergo HAART (n.=7) (7.8 IC 7.8-72.47, and 7.8 IC 7.8-18.3, respectively) (p=0.74).

Univariate analysis of factors associated with detectable (>7.8 pg/ml) blood IFN is presented in Table **2**; patients with detectable blood IFN were significantly more likely to belong to the group of HCV-HIV-coinfected patients and had lower median serum creatinine concentrations. Following logistic regression analysis, after inclusion of each of the variables analyzed in the univariate analysis, the only independent predictor of detectable blood IFN was age (OR 0.97, 95%CI 0.94-0.99, p = 0.019).

### Relationship Between IL28B Genotype and Outcome

IL28B genotypes were distributed according to the liver stiffness measurement: liver stiffness ≤7.0 kPa (METAVIR F0-F1), C/C 24/73 (33%), and C/T or T/T 46/114 (40%); liver stiffness 7.1-12.4 kPa (METAVIR F2-F3), C/C 28/73 (38%), and C/T or T/T 26/114 (23%); and liver stiffness ≥12.5 kPa (METAVIR F4), C/C 21/73 (29%), and C/T or T/T 42/114 (37%) (p = 0.147). The univariate analysis of the factors associated with the presence of significant fibrosis (defined as liver stiffness >7.1 kPa) is presented in Table **3**. Following logistic regression analysis, after inclusion of each of the variables analyzed in the univariate analysis, the independent predictors of significant fibrosis were age (OR 1.04, 95%CI 1.02-1.08, p=0.007), current alcohol consumption (OR 2.91, 95%CI 1.33-6.39, p=0.008) and belonging to the group of HCV-HIV-coinfected patients (OR 2.02, 95%CI 1.01-4.04, p=0.048).

## DISCUSSION

In our study, the proportion of patients with chronic hepatitis C who showed detectable blood IFN levels was lower in HCV-monoinfected patients (34/112, 30%) than in patients coinfected with HCV and HIV (34/75, 45%), a result that would seem consistent with the hypothesis of a synergistic mechanism of the activation of the IFN system between the two viruses. However, it should be noted that the group of HCV-HIV-coinfected patients was composed of, on average, younger subjects. Aging has been shown to reduce the systemic production of IFN in an animal model of chronic viral infection, resulting in impaired viral clearance [17]. Moreover, denditric cell number and the amount of

**Table 2. Univariate Analysis of Factors Associated with Detectable Blood Interferon-Alpha Concentration**

| | Blood Interferon-Alpha | | |
|---|---|---|---|
| | Undectable ≤7.8 pg/ml (N. = 119) | Detectable >7.8 pg/ml (N. = 68) | P |
| Gender, M : F | 71 (60) : 48 (40) | 43 (63) : 25 (37) | 0.644 |
| Age (years) | 53 (51-56) | 50 (47-51) | 0.087 |
| Group, monoinfected : coinfected | 78 (65) : 41 (35) | 34 (50) : 34 (50) | 0.044 |
| Serum HCV RNA IU/ml ($\times 10^3$) | 678 (514-1,067) | 430 (287-1,081) | 0.291 |
| Liver stiffness | | | |
| kPa | 8.8 (7.8-10.7) | 8.3 (6.9-10.3) | 0.173 |
| METAVIR F0-1 : F2-F3-F4 | 42 (35) : 77 (65) | 28 (41) : 40 (59) | 0.436 |
| IL28B Genotype | | | |
| C/C | 44 (37) | 29 (43) | |
| C/T | 59 (50) | 30 (44) | |
| T/T | 16 (13) | 9 (13) | 0.567 |
| Creatinine, mg/dl | 0.90 (0.9-0.9) | 0.85 (0.8-0.9) | 0.041 |
| ALT, U/L | 66 (56-79) | 48.5 (39-70) | 0.070 |
| Alcol consumption | | | |
| Teetotallers | 59 (52) | 30 (47) | |
| Past drinkers | 27 (24) | 12 (19) | |
| Current drinkers | 28 (24) | 22 (34) | 0.358 |

Continuous variables are presented as medians (95% CI of median), categorical variables as frequencies (%). P values refer to Mann-Whitney test, Cochran-Armitage test, or Fisher exact test, as appropriate. Serum HCV RNA was available in 165/187 patients. Information on alcohol consumption was available for 178/187 patients.

**Table 3. Univariate Analysis of Factors Associated with Significant Fibrosis (Defined as Liver Stiffness 7.1 kPa or Higher)**

| | Liver Stiffness <7.1 kPa (F0-F1) (N. = 70) | Liver Stiffness ≥7.1 kPa (F2-F3-F4) (N. = 117) | p |
|---|---|---|---|
| Gender, Male : Female | 37 (53) : 33 (47) | 77 (66) : 40 (34) | 0.090 |
| Age (years) | 50 (47-52) | 53 (50-56) | 0.033 |
| Group, monoinfected:coinfected | 47 (67) : 23 (33) | 65 (56) : 52 (44) | 0.126 |
| Serum HCV RNA IU/ml ($\times 10^3$) | 636 (385-1281) | 625 (422-856) | 0.703 |
| Interferonemia | | | |
| ≤7.8 pg/ml : >7.8 pg/ml | 42 (60) : 28 (40) | 77 (66) : 40 (34) | 0.436 |
| IL28B genotype | | | |
| C/C | 24 (34) | 49 (42) | 0.365 |
| C/T | 38 (54) | 51 (44) | |
| T/T | 8 (12) | 17 (14) | |
| Creatinine, mg/dl | 0.90 (0.9-0.95) | 0.86 (0.8-0.90) | 0.019 |
| ALT, U/L | 50 (43-64) | 70 (55-82) | 0.022 |
| Alcohol consumption | | | |
| Teetotallers | 41 (60) | 48 (44) | 0.018 |
| Past drinkers | 16 (24) | 23 (21) | |
| Current drinkers | 11 (16) | 39 (35) | |

Interferonemia detectable >7.8 pg/ml. For categorical variables, p values refer to the Fisher exact test or, when appropriate, the Cochran-Armitage test. For continuous variables, p values refer to the Mann-Whitney test. Serum HCV RNA was available in 165/187 patients. Information on alcohol consumption was available for 178/187 patients.

IFN-alpha produced decline with aging [18]. Finally, some IFN-inducible genes, in particular IFI-16, are expressed differently according to age, as recently published by our group. Consistently with these data, in the present series the only independent predictor of detectable blood IFN was age.

A critical question to answer is to what extent blood IFN levels may reflect the state of activation of the IFN system in the liver. The release of IFN into the blood is not exclusive to infections by HCV and/or HIV; for example, other common infections, including those mediated by Epstein-Barr virus [19] and influenza virus [20], autoimmune disorders, such as systemic lupus erythematosus [21], and psychiatric syndromes [22] may present high IFN levels. Moreover, all of the 16 patients studied by Sarasin-Filipowicz *et al.* had blood IFN levels below the limit of detection, although six patients showed signs of intense preactivation of the IFN system in the liver [5]. In our series, among the 60 patients who completed treatment for hepatitis C infection, the pretreatment blood IFN level was not a significant predictor of sustained viral response, in contrast with the IL28B genotype. In fact, the patient with the highest IFN value was able to achieve viral clearance.

Scrutinizing the traditional predictors of response to antiviral therapy and the predictors of fibrosis progression, it appears that a large overlap exists between these two categories. For instance, coinfections (with hepatitis B and/or HIV), male gender, age, disease duration, obesity and insulin resistance, steatosis, and transplantation favor both the lack of response to antiviral treatment and disease progression, and fibrosis itself is a powerful predictor of non-response to antiviral treatment. Therefore, one may wonder whether the current standard of care we are using to treat hepatitis C patients is only successful in patients who have a reduced risk of developing severe complications. We have recently provided data suggesting that this paradox may apply to IL28B T/T carriers [23, 24], but these findings were not replicated here using an independent series of mixed HCV-monoinfected and HCV-HIV-coinfected patients. Moreover, two recent European studies did not confirm the existence of an association between carriage of an unfavorable IL28B genotype and fibrosis progression [25, 26]. One possible explanation for this discrepancy is the excess of T/T homozygotes that is expected to be represented in cross-sectional series of HCV-positive patients with advanced disease. In fact, these patients are prone to progress to severe fibrosis simply due to their inability to clear the virus either spontaneously or following antiviral treatment. Indeed, the excess of T/T homozygotes observed in HCV carriers who are liver transplant candidates can be viewed as a proxy demonstration of the efficacy of IFN-based regimens on definite endpoints, like death or liver transplantation.

The proportion of patients in the coinfected group who underwent antiviral treatment for HCV was strikingly low. It is well known that HIV/HCV coinfected patients are considerably undertreated [27] in comparison to HCV monoinfected patients. In our series, besides the usual factors pertaining to patients (including refuse of treatment), lack of confidence with antiviral treatment of HCV in the HIV clinic likely played a role. In any case, these data underline the need to overcome treatment barriers for these patients.

Finally, in our series, disease severity (judged by the results of transient elastography) was not different at univariate analysis between HCV-monoinfected and HCV-HIV-coinfected patients, a finding that may be considered at variance with what has been clearly established by previous

studies [28-30]. However, this discrepancy is more apparent than true, because HCV-HIV-coinfected patients were, on average, more than 10 years younger than their HCV-monoinfected counterparts, suggesting that the HCV-HIV-coinfected patients reach similar infection stages at an earlier age. Indeed, at multivariate analysis, belonging to the group of HIV-HCV-coinfected patients emerged as an independent predictor of the transient elastography stage, together with age and history of alcohol consumption.

In conclusion, the present study shows that a significant proportion of HCV carriers, especially if the patients are young, have signs of activation of the IFN system in the blood, although this is unlikely to reflect precisely what occurs in the liver. In fact, high blood IFN levels do not indicate a more severe disease course and are not related to the possession of specific allelic variants of the IL28B polymorphism.

## CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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Declared none.

## PATIENT CONSENT

Declared none.

## HUMAN/ANIMAL RIGHTS

Declared none.

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ALCOHOLISM CLINICAL & EXPERIMENTAL RESEARCH

# Fibrosis Progression in HCV Carriers with Mild Hepatitis Who Possess the High-Repetition Variant of the DRD4 Gene, a Genetic Marker for Binge-Drinking and Risk-Seeking Behavior: A Longitudinal Study

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**Background:** Alcohol is a major determinant of the outcome of chronic hepatitis C virus (HCV) infection, but self-reported drinking habits lack reliability. We hypothesized that carriage of high-repetition variants (HRV) of the variable number of tandem repeats (VNTR) in exon III of the dopamine receptor $D_4$ gene, linked to binge-drinking and risk-seeking behavior, might be a proxy measure of alcohol consumption, and aimed to verify whether it may affect histologic outcome.

**Methods:** A cohort of HCV patients with normal or near-normal aminotransferases ($N = 128$) underwent a liver biopsy as part of diagnostic work-up. None admitted to exceed low-risk alcohol consumption; most (90/128, 70%) described themselves as teetotalers. They received advice on abstaining from alcohol, but not antiviral treatment. After a median follow-up period of 10 years, all underwent a second liver biopsy. HRV allele frequencies were compared with those of a group of healthy blood donors ($N = 128$) and related to liver histology.

**Results:** HRV allele frequencies were 0.19 in patients and 0.16 in controls ($p = 0.182$). In the subgroup of patients who admittedly had consumed alcohol, 20/38 (53%) carried HRV, in comparison with 27/90 patients (30%) who had denied to consume alcohol ($p = 0.026$ by Fisher's exact test). Carriage of HRV was associated with higher histologic grade ($p = 0.002$) and stage ($p = 0.009$) at the final biopsy. At multivariate analysis, among a set of variables also including viral genotype, viral load, body mass index, gender, and history of alcohol consumption, only age (OR = 1.06, 95% CI 1.02 to 1.11) and HRV (OR = 3.13, 95% CI 1.28 to 7.68) were independent predictors of significant fibrosis at the end of follow-up.

**Conclusions:** The link between HRV carriage and histologic outcome in a subgroup of HCV patients at low risk of progression underlines the need for intense scrutiny of alcohol habits in hepatitis C.

**Key Words:** DRD4 Allele, Alcohol Consumption, HCV.

THE NATURAL HISTORY of hepatitis C virus (HCV) infection is highly variable, with only a minority of patients progressing to severe fibrosis and end-stage liver disease over a prolonged course that may require decades. This variability is influenced by genetic and environmental factors, among which chronic alcohol use is considered paramount. To acquire new, longitudinal, good-quality natural history data in this field is difficult, due to the availability of effective antiviral treatments and the scarce acceptance of multiple liver biopsies by patients. Moreover, the reliability and validity of self-reported alcohol-drinking habits, that is, the extent to which accurate information is provided by the respondent (Midanik, 1982; Rehm et al., 1999), have been questioned. However, the propensity to drink alcohol has a genetic component; for example, the dopamine receptor $D_4$ (DRD4) gene is a candidate gene for alcoholism. DRD4 is characterized by a highly polymorphic 48 base pair variable number of tandem repeats (48 bp VNTR) sequence in exon III, and the 7-repeat allele of 48 bp VNTR is associated with craving induced by alcohol cues, such as pictures or smell, as well as with an increased alcohol consumption per occasion in males (Du et al., 2010; Hutchison et al., 2002; Laucht et al., 2007; Muramatsu et al., 1996; Roman et al., 1999). Recently, this polymorphic variant has been related with risk-seeking behavior (Kuhnen and Chiao, 2009) and the

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binge-drinking phenomenon (Vaughn et al., 2009). Hypothetically, possessing this variant might make it more difficult for HCV carriers to abstain from alcohol and therefore make them more susceptible to progressive disease.

In the present study, our aim was to evaluate whether carriage of the 7-repeat DRD4 polymorphism might affect the histologic outcome in a cohort of untreated HCV patients with persistently normal or near-normal transaminases (PNALT), observed with serial biopsies along a median follow-up period of 10 years.

## MATERIALS AND METHODS

### *Study Population*

The study population consisted of 128 HCV-positive patients, fulfilling the EASL definition for PNALT, who underwent a liver biopsy as part of their diagnostic work-up. None of these patients admitted to exceed low risk and responsible alcohol consumption as defined in the International Drinking Guidelines (Anonymous, 1999); most of them (89/127, 70%) described themselves as teetotalers. Nevertheless, all received strong advice on avoiding alcohol consumption and other lifestyle issues, but not antiviral treatment. Following periodic clinical monitoring along a median follow-up period of 10 years (minimum, 62 months), they underwent a second (and final) liver biopsy. Baseline demographic and clinical features of these patients are presented in Table 1.

The allele frequencies of the VNTR were compared with those obtained from a control group, consisting of 128 individuals randomly chosen between a larger group of blood donors.

### *Molecular Biology*

Genomic DNA was extracted from whole blood samples by means of GeneElute Blood Genomic DNA kit (Sigma, Milan, Italy) according to the manufacturer's instruction. The VNTR in exon III in the DRD4 gene was analyzed as previously described by Lichter and colleagues (1993) with minor modification. In detail, the 48 bp repeat polymorphism was amplified by polymerase chain reaction (PCR) in a total volume of 10 $\mu$l in presence of 1.5 mM MgCl2, 0.5 U FastStart Taq DNA Polymerase, 1 × GC Reach Solution (for sequences characterized by high GC content; Roche, Milan, Italy), and 2 $\mu$M of each primer (Fwd 5′-GCGACTACGTGGTCTACTCG-3′; Rev 5′-AGGACCCTCATGGCCTTG-3′). The cycling condition for amplifications were 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, followed by 7 minutes at 72°C using the Applied BioSystem 2700 thermocycler (Monza, Italy). Whole PCRs were analyzed by electrophoresis in 2% agarose gel stained with GelRed (Biotium Inc., Hayward, CA). All the different size alleles, and some possible genotypes observed in our population survey, are displayed in Fig. 1. The high guanine and cytosine nucleotide content of the repetitive region promotes heteroduplex formation: strands of different size can anneal and cause a third band that migrates slower than the 2 homoduplex bands for each heterozygous individuals. All the different VNTR amplified were sequenced by Eurofins MWG Operon (Ebersberg, Germany) to confirm the number of repeats.

**Table 1.** Main Demographic and Clinical Characteristics of the Studied Population. Categorical Variables are Presented as Frequencies (%), Continuous Variables as Medians [95% CI of Median]

| Variable | ($N$ = 128) |
|---|---|
| Male/Female | 55/73 (43/57) |
| Age at initial biopsy, years | 41.5 [38.4 to 45.0] |
| Viral genotype, $N$[a] | |
| HCV-1 | 73 (57.5) |
| HCV-2 | 38 (29.9) |
| HCV-3 | 11 (8.7) |
| HCV-4 | 5 (3.9) |
| Circulating HCV RNA, IU/ml × $10^3$ | 1,162 [800 to 1,419] |
| ALT at first biopsy, U/l | 27 [22 to 29] |
| Regular alcohol consumption in the past, $N$ | 38 (30) |

HCV, hepatitis C virus.
[a]One patient missing.

### *Statistical Analysis*

Statistical analysis of data was performed by means of the biomedical statistical software MedCalc Version 12.3.0.0 (MedCalc Software, Mariakerke, Belgium). Continuous variables are presented as medians [95% CI]; categorical variables are presented as frequencies (%). The associations between categorical variables have been investigated by means of the Fisher's exact test, the Pearson chi-square test and, when appropriate, the chi-square test for linear trend (Cochran-Armitage test). Multivariate logistic regression analysis was employed to identify, among different variables, those independently associated with significant liver fibrosis (Ishak staging score 3 or higher) at the end of follow-up. A level of 0.05 (2-tailed) was chosen to indicate statistical significance.

## RESULTS

Table 2 presents DRD4 allelic frequencies in patients and controls. The allelic frequency of high-repetition variants (HRV; $\geq$7 repetitions) was <20% in both patients and controls, and the distribution was similar ($p$ = 0.182 by Fisher's exact test).

Among the 38 patients who admitted alcohol consumption, 20 (53%) carried HRV, in comparison with 27 of the 89 patients (30%) who did not report to consume any alcohol ($p$ = 0.027 by Fisher's exact test).

There was a strict correlation between grading at the initial and at the final biopsies (chi-square = 38.2, $p$ < 0.0001), and

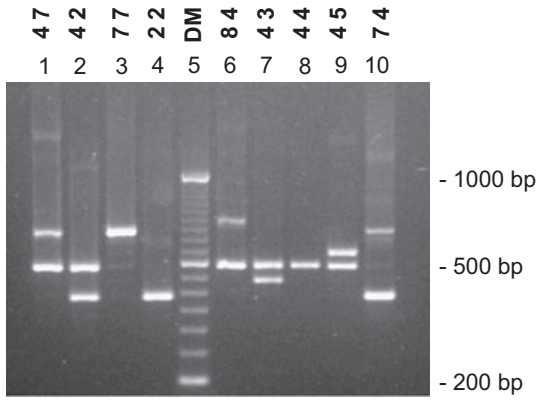


**Fig. 1.** Polymerase chain reaction analysis of the dopamine receptor $D_4$ (DRD4) polymorphism. DRD4 gene amplicons were electrophoresed on a 2% agarose gel. Lanes 1 to 4 and 6 to 10: patient samples, Lane 5: 50 bp DNA Markers (DM). The numbers on top of each lane indicate the DRD4 genotype.

**Table 2.** Dopamine Receptor $D_4$ Allelic Frequencies (AF) in Patients with Hepatitis C and Persistently Normal Aminotransferases and Controls

| | Repetitions, *N* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 10 | Total |
| Patients | | | | | | | | | |
| *N* | 28 | 14 | 161 | 0 | 4 | 48 | 1 | 0 | 256 |
| AF | 0.11 | 0.06 | 0.62 | 0 | 0.02 | 0.19 | 0.003 | 0 | |
| Controls | | | | | | | | | |
| *N* | 36 | 6 | 162 | 4 | 5 | 42 | 1 | 0 | 256 |
| AF | 0.14 | 0.02 | 0.63 | 0.02 | 0.02 | 0.16 | 0.003 | 0 | |
| Total | | | | | | | | | |
| *N* | 64 | 20 | 319 | 4 | 11 | 90 | 2 | 0 | 510 |
| AF | 0.125 | 0.039 | 0.625 | 0.007 | 0.021 | 0.176 | 0.003 | 0 | |

staging at the initial and the final biopsies (chi-square = 114.6, $p < 0.0001$). At each time point, grading and staging were also strictly associated each other (initial biopsy, chi-square = 35.9, $p = 0.0003$; final biopsy, chi-square = 39.3, $p = 0.0001$). In 70/128 cases (55%), staging at the final biopsy was higher than staging at the initial biopsy; in 26/128 cases (20%), the difference between the initial stage and the final stage was 2 points or more. In 8/128 cases (6%), the final stage was lower than the initial stage. Similarly, in 97/128 cases (76%), grading at the final biopsy was higher than grading at the initial biopsy; in 64/128 cases (50%), the difference between the initial grade and the final grade was 2 points or more. In 22/128 cases (17%), the final grade was lower than the initial grade.

Both grading and staging at the final biopsy were associated with carriage of HRV (Table 3). Categorizing patients according to stage change between the initial and the final biopsy as stable/improved (difference between initial and final stage $\leq 0$ points), mildly worsened (difference between initial and final stage: 1 point), and greatly worsened (difference between initial and final grade $\geq 1$ point), there was a progressively higher representation of patients with HRV, from 12/58 (21%), to 18/44 (41%), to 16/26 (62%) ($p = 0.0002$). Similarly, categorizing patients according to grade change between the initial and the final biopsy as stable/improved (difference between initial and final grade $\leq 1$ point), mildly worsened (difference between initial and final grade 2 to 3 points), and greatly worsened (difference between initial and final grade $\geq 4$ points), there was a progressively higher representation of patients with HRV, from 17/64 (27%), to 17/43 (40%), to 12/21 (57%) ($p = 0.009$).

When only patients with minimal fibrosis (Ishak stage $\leq 1$; $N = 93$) at the initial biopsy were considered, progression to stage 3 fibrosis or higher at the final biopsy was significantly associated with carriage of HRV (12/20, 60%, vs. 22/73, 30%, $p = 0.019$).

Finally, at multivariate analysis, among a set of variables that included viral genotype, viral load, body mass index, gender, history of regular alcohol consumption in the past, carriage of HRV, and age, the latter 2 were the only independent predictors of stage 3 fibrosis or higher at the end of follow-up (Table 4). When the same set of predictive variables was applied to the subset of patients with minimal or absent fibrosis (Ishak stage $\leq 1$), the only independent predictor of progression to stage 3 or higher was a history of alcohol consumption in the past (odds ratio 4.11, 95% CI 1.02 to 16.5, $p = 0.046$).

**Table 3.** Association Between Grading and Staging Scores According to Ishak and colleagues (1995) at the Final Biopsy and Dopamine Receptor $D_4$ (DRD4) Allelic Variants

| | Ishak grade | | | *p* |
|---|---|---|---|---|
| DRD4 allele | $\leq 4$ | 5 to 8 | $\geq 9$ | 0.002 |
| Low repetition | 29 | 40 | 13 | |
| High repetition | 6 | 25 | 15 | |
| | **Ishak stage** | | | |
| DRD4 allele | 0 to 1 | 2 to 3 | 4 to 6 | 0.009 |
| Low repetition | 42 | 33 | 7 | |
| High repetition | 15 | 20 | 11 | |

**Table 4.** Logistic Regression Analysis of Factor Associated with Stage 3 Fibrosis at the End of Follow-Up ($n = 127$ Patients with No Missing Data)

| Variable | Odds ratio | 95% CI | *p* |
|---|---|---|---|
| Age | 1.06 | 1.02 to 1.11 | 0.006 |
| DRD4 | | | |
| Low-repetition variant | Reference | | |
| High-repetition variant | 3.13 | 1.28 to 7.68 | 0.013 |
| HCV genotype | | | |
| HCV-1 | Reference | | |
| HCV-2 | 0.95 | 0.37 to 2.40 | |
| HCV-3 | 0.66 | 0.09 to 4.64 | 0.908 |
| HCV-4 | 0.39 | 0.03 to 5.13 | 0.675 |
| Circulating HCV RNA | 1.00 | 1.00 to 1.00 | 0.474 |
| Gender | | | |
| Female | Reference | | |
| Male | 1.34 | 0.55 to 3.23 | 0.514 |
| History of alcohol consumption in the past | | | |
| No | Reference | | |
| Yes | 1.53 | 0.60 to 3.91 | 0.370 |
| Body mass index | 1.00 | 0.79 to 1.27 | 0.962 |

Dopamine receptor $D_4$ (DRD4), hepatitis C virus (HCV) genotype, and gender were entered in the model as categorical variables, age, circulating HCV RNA and body mass index as continuous variables.

## DISCUSSION

In the present study, we report an association between carriage of HRV of the DRD4 gene, necroinflammatory indexes, and fibrosis progression in chronic hepatitis C. One major feature of the study consists in having being conducted entirely on patients with PNALT, a subgroup of hepatitis C patients considered at low risk of severe sequelae of HCV infection. In the past, the recommended management of these patients was no treatment and clinical and histologic periodic monitoring (EASL International Consensus Conference on Hepatitis C [Anonymous, 1999]), and although it has now been proven that they do respond to antiviral treatment similarly to their counterparts with abnormal transaminases (Zeuzem et al., 2004), the cost-effectiveness of treatment for this special HCV population is likely lower, albeit acceptable from a societal perspective (Hornberger et al., 2006). Furthermore, one might hypothesize that PNALT patients who do progress to advanced forms of liver disease may be more genetically predisposed to hepatic inflammation and fibrosis progression than their homologues with mild, nonprogressive disease. For all these reasons, we believe that PNALT patients give a unique opportunity to study the natural history of the disease and how it is influenced by genetic factors.

Two main strategies have been devised to allow the identification of genes linked to a specific trait or outcome: candidate gene association studies and genome-wide association studies. In the latter, the entire genome is scanned. They are an important advance in discovering genetic variants influencing disease and disease outcomes, but have been criticized because of their potential for false-positive results, lack of information on gene function, insensitivity to rare variants and structural variants, requirement for large sample sizes, and possible biases due to case and control selection and genotyping errors (Pearson and Manolio, 2008). On the other hand, candidate gene association studies, that is, those conducted with a hypothesis driven by the presumed function of a single gene, are limited by their reliance on existing knowledge about known or theoretical biology of disease, which is often incomplete. Moreover, besides DRD4, many other genes have been shown to be associated with alcoholism and alcohol-related liver damage, among which the most well established are those coding for the alcohol-metabolizing enzymes alcohol dehydrogenase-1B and aldehyde dehydrogenase-2 (Higuchi et al., 1996; Muramatsu et al., 1995). In principle, we cannot exclude that these genes, or others linked with drinking behavior, may play a role in determining a different outcome of HCV infection in the patients of our cohort; however, our study was not designed to prove or refute this hypothesis.

The DRD4 gene encodes the $D_4$ subtype of the dopamine receptor, a G protein-coupled receptor which, when activated upon binding of the neurotransmitter dopamine, inhibits adenylate cyclase, thereby decreasing intracellular cAMP. The main function of $D_4$ receptors is neuronal signaling in the mesolimbic system of the brain, an area that regulates emotion and complex behavior. DRD4 mutations have been associated with various behavioral phenotypes, including autonomic nervous system dysfunction, attention deficit/hyperactivity disorder, and the personality trait of novelty seeking. With specific reference to alcohol consumption, DRD4 polymorphism has been linked to alcoholism (overcoming the protective effect of carrying the ALDH2*2 allele in Asians) (Du et al., 2010; Hutchison et al., 2002; Laucht et al., 2007; Muramatsu et al., 1996) and binge drinking (Vaughn et al., 2009).

Most of the diversity in the DRD4 gene is the result of length and single-nucleotide polymorphism variation in the 48-bp VNTR in exon III, which encodes the third intracellular loop of the receptor. Variant alleles containing 2 (2R) to 11 (11R) repeats are found, with the resulting proteins having 32 to 176 amino acids at this position. Polymorphic repeat variants of DRD4 appear to have different relative affinities to form homo- and heterodimers, resulting in different exporting of the receptor from the endoplasmic reticulum and cell surface trafficking (Van Craenenbroeck et al., 2011). In a global survey of this polymorphism, the 4-repeat allele was the most prevalent (allele frequency = 64.3%) and appeared in every population with a frequency ranging from 0.16 to 0.96. The 7-repeat allele was the second most common (allele frequency = 20.6%), and the 2-repeat allele ranked third (allele frequency = 8.2%) (Chang et al., 1996). Our data, obtained in Caucasians with chronic hepatitis C and controls, are in almost perfect agreement with these reported frequencies, and the similarity between patients and controls means that the polymorphism does not influence the susceptibility to develop chronic hepatitis C.

The simplest way to explain the association between HRV and the histologic outcome of hepatitis C in patients with PNALT is that it reflects concealed alcohol consumption. The validity of self-reported alcohol consumption has long been questioned, especially as far as uncontrolled consumption is concerned (Watson et al., 1984). On the other hand, heavy drinking may be reported more accurately retrospectively than currently, in particular by females (Czarnecki et al., 1990): This may explain our observation that regular alcohol consumption in the past is an independent predictor of fibrosis progression among patients with initially mild hepatitis. It has been shown that consuming >50 g of alcohol per day has harmful effects on hepatitis C, but there is no defined threshold level below which alcohol consumption might be considered safe for HCV carriers (Westin et al., 2002). Indeed, HCV and alcohol share common pathogenic mechanisms, including the modulation of cytokine production, lipopolysaccharide-TLR4 signaling, and reactive oxygen species production (Szabo et al., 2010). Moreover, as excess alcohol intake may increase HCV RNA replication and interfere with response to treatment (Pessione et al., 1998; Romero-Gomez et al., 2001), complete abstinence

from alcohol is strongly recommended by current guidelines for patients with hepatitis C who undergo antiviral therapy (Anonymous, 2011). We speculate that in the cohort of HCV carriers that we studied, carriage of HRV was disproportionately represented among patients unable to abstain from alcohol, thereby damaging their liver with more inflammation and eventually more fibrosis.

An alternative, in our opinion, far less likely explanation for this association might call into question a direct influence of HRV on inflammation and fibrosis in the liver, not mediated by alcohol consumption. In fact, although catecholamines are able to induce an inflammatory response in human hepatocytes, this does not apply to dopamine (Aninat et al., 2008), and in any case, $D_4$ receptors in HRV carriers appear to have a blunted response to dopamine. Furthermore, we found no evidence in the literature that $D_4$ receptors (or any $D_2$-like receptor) are expressed in the liver (Gingrich and Caron, 1993).

HCV treatment has recently been revolutionized by the advent of direct-acting antiviral agents that offer high sustained viral response rates at the expense of increased adverse effects and costs. Patients with mild hepatitis and predictably slow progression rates such as HCV carriers with PNALT, if unfit for treatment or unwilling to be treated, are often counseled on lifestyle issues and observed without treatment, and this strategy is likely not to change in the near future. Our study suggests that, despite patient denial of consuming alcohol in excess, a genetic predisposition to risk-seeking behavior may be a factor of nonadherence to drinking guidelines and, thereby, may affect the histologic outcome of hepatitis C. The novel natural history data presented here indicate that, despite the apparent benign course of hepatitis C in PNALT patients, for those among them who undergo clinical monitoring without treatment ongoing alcohol consumption should be identified through intense scrutiny and the need for complete abstinence from alcohol reinforced.

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