PhD program in Translational and Molecular Medicine

DIMET

Virus-host interactions in hepatitis C virus infection: implications for pathogenesis and therapy

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CHAPTER 1

GENERAL INTRODUCTION
A. Clinical aspects of Hepatitis C Virus infection

1. The discovery of the Hepatitis C Virus

In the 1950s and ‘60s the field of viral hepatitis was initiated by the differentiation between “infectious” and “serum” hepatitis. Later on these two types of hepatitis were shown to be caused by hepatitis A virus and hepatitis B virus infection, respectively. Surprisingly, when serological tests became available in the mid-1970s, the majority of parentally-transmitted hepatitis infections could not be assigned to either virus. Despite international research efforts, the agent responsible for this so-called non-A non-B hepatitis (NANBH) remained unidentified for the next decade. In 1989, the causative agent of NANBH was identified by molecular cloning and was denominated hepatitis C virus (HCV). The virus was identified and characterized by molecular cloning techniques using serum from a NANB hepatitis virus infected chimpanzee and based on the similarity of the genome organization and hydropathy profiles of several precursor proteins classified as a member of the Flaviviridae family. However, the low sequence homology compared to other flaviviruses eventually lead to its classification into a new genus hepacivirus, distinct from the other flavivirus members.

The discovery of HCV was an important milestone in the field of viral hepatitis. It allowed screening of blood products and the installment of an antiviral treatment.
2. Epidemiology and geographic distribution of Hepatitis C

Since its discovery, HCV has been recognized as a major cause of chronic liver disease worldwide. Currently, the World Health Organization (WHO) estimates that 2.2-3% of the world population is chronically infected with HCV, representing 130 to 170 million people and more than one million new infection cases are reported annually. In the United States alone, nearly four million persons are infected and 30,000 acute new infections are estimated to occur each year. In Europe and Japan, the disease is already more important numerically than is either hepatitis B virus (HBV) or human immunodeficiency virus (HIV) infection and due to the availability of the HBV vaccine the impact of hepatitis C infections will increase further. HCV infection causes a substantial portion of chronic liver disease mortality due to the induction of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). About 4 to 20% of patients with chronic hepatitis C will develop liver cirrhosis within 20 years and HCC may develop after about 20 to 35 years. There is a significant rise in the incidence of HCC in many developed countries including Japan, Spain, France, and Italy, where the proportion of HCC attributable to HCV ranges from 50% to 70%. In Japan, HCV-related HCC incidence has more than tripled over the past four decades and accounts in the 60–70 year age group for as much as 90%. Apart from HCC, co-infections with other viruses, especially HIV-1 and other hepatitis viruses, have gained more attention. These are of clinical importance, since the course of HCV
infection is accelerated by co-infection with HIV-1, hepatitis A virus (HAV) or HBV.

HCV shows a large degree of geographic variability in its distribution. Countries with the highest reported prevalence rates are located in Africa and Asia; areas with lower prevalence include the developed nations in North America, Northern and Western Europe, and Australia (Fig. 1). The seroprevalence rate is about 1% in Western Europe and North America, 3-4% in some Mediterranean and Asian countries and up to 10-20% in parts of Central Africa and Egypt. Populous nations in the developed countries with relatively low rates of HCV seroprevalence include Germany (0.6%), Canada (0.8%), France (1.1%), and Australia (1.1%). Low, but slightly higher seroprevalence rates have been reported in the USA (1.8%), Japan (1.5–2.3%), and Italy (2.2%) (for a review see ).

Fig. 1. Prevalence of HCV in the world (Source, WHO 2007)
(http://www.nathnac.org/pro/factsheets/images/hep_c_epi.jpg)
There is a wide range of prevalence estimates from developing countries, and generally less data are available to validate assumptions about the burden of disease compared with the developed world. China, whose citizens account for one fifth of the world’s population, has a reported seroprevalence of 2.5-4.9%\textsuperscript{16}. In other countries like India, Indonesia and Pakistan, this may range from 0.9 % to 6.5%\textsuperscript{17-19}. Egypt, with an estimated population of 73 million, has the highest reported seroprevalence rate at 22% due to the use of parenteral antischistosomal therapy contaminated with HCV\textsuperscript{20}.

HCV genotypes have a geographic distribution with genotype 1 being more common in the Americas and Europe, followed by genotypes 2, 3 and 4. Subtype 1b accounts for most infections in China and Japan, genotype 4 is more prevalent in the Middle East and North Africa, genotype 5a is seen almost exclusively in South Africa, and genotype 6 is common in Hong Kong and South-East Asia\textsuperscript{21,22}.

Through a phylogenetic analysis of nucleotide sequences of the HCV genome, such as the NS5B region, it is possible to demonstrate clustering of HCV isolates that are more or less closely related, even within a single subtype. Using these techniques spread of HCV genotype 1b through infected blood products and subtype 1a and 3a in intravenous drug users, was demonstrated\textsuperscript{23,24}. Together with the coalescent theory of population genetics, phylogenetic analysis has been used to estimate the historical age and distribution rate of different HCV genotypes. As a result, these models indicate that genotype 1 originated within the last 100 years, whereas types 4 and 6 are several times older. Subtype diversity within genotypes 1, 2 and 4
from Western Africa and genotypes 3 and 6 in South-East Asia implicates long-term presence of HCV in these populations. Based on the genotype distributions, it was suggested that HCV has been endemic in sub-Saharan Africa and South-East Asia for a considerable time, and that the occurrence of infection in Western and other non-tropical countries represents a relatively recent emergence in new risk groups. In the 20th century, widespread use of blood transfusion, unsterilized needles for injections and vaccinations caused HCV to spread. These new transmission routes account for the epidemic spread of HCV over the past 50 years in Europe, Egypt and elsewhere.

3. Clinical characteristics of viral Hepatitis C and natural history of the pathology

The term natural history refers to the description of the course of the disease after infection, including clinical characteristics and factors that influence the progression of the disease. The accurate assessment of natural history of HCV infection has been very demanding, because generally the acute phase of the infection is silent, although the determination of the onset is critical to follow the full course of the disease. Furthermore, the disease progression is modified by factors and treatments that in principle may raise possible controversies on the definition of the long-term natural history of chronic HCV infection.

HCV is most efficiently transmitted by parental exposure to blood and blood products. The main causes of its spread are blood transfusions and the use of contaminated needles by intravenous drug
users. Nowadays, transfusion-related HCV infection is almost eliminated in countries where routine screening of blood is obligatory\textsuperscript{31}. At present, the most common risk factor in the development world is injection drug use. Also organ transplantations, inadequately or improperly sterilized medical or dental equipment, tattooing, body-piercing and acupuncture can be risk factors for HCV infection. Other routes of HCV transmission are less efficient and include perinatal, sexual and occupational transmission. Perinatal or “mother-to-child” transmission refers to the transmission of infection from an infected mother to her child during the birth process (respectively 2.7-8.4\%). A higher proportion of infants are born with HCV infection when the mother is co-infected with HIV\textsuperscript{32}. Sexual transmission of HCV has been observed, but is far less efficient than the transmission of other sexually-transmitted viruses\textsuperscript{33}. Occupational transmission is largely confined to health-care workers who become HCV infected by contaminated needle stick injuries\textsuperscript{34}.

Acute HCV infection is in most cases asymptomatic or associated with mild clinical illness. HCV RNA becomes detectable within 1 to 2 weeks after exposure and reaches high levels of up to 105 to 107 genome equivalents/ml in a few days\textsuperscript{35}. The acute phase is mainly characterized by mild and non-specific symptoms such as malaise, nausea, anorexia, fatigue and abdominal discomfort; 20-30\% of patients may have jaundice. Severe acute hepatitis however is rare and occurs in less than <1\% of patients. Therefore, HCV is not often diagnosed in the early stages of infection. The first indication of hepatic injury is an elevated alanine aminotransferase (ALT) level,
which can occur 4 to 12 weeks after viral exposure. Altogether the mild clinical manifestation of acute hepatitis generally lasts for 3 to 12 weeks. Fifteen to 20% of patients with acute hepatitis C spontaneously clear the virus, primarily during the first 3 months following clinical onset of infection\textsuperscript{36}. Spontaneous clearance occurs more often in the presence of symptomatic disease. Several viral and host factors appear to affect the clinical course of infection (HCV genotype, race, gender, HLA, co-infection with HIV, advanced age); however, none of these factors can accurately predict spontaneous clearance. The majority of HCV infected patients (80-85\%) will develop chronic disease, defined as the presence of HCV RNA in the blood for more than six months following infection\textsuperscript{37}. Chronic HCV infection usually remains asymptomatic for decades, until the patient develops to a more advanced stage of liver disease. Therefore, HCV is often designed as a “silent disease”, since the majority of infected patients is not aware of their disease for years after infection. Elevated ALT levels are only found in two-thirds of patients and do not correlate with disease severity\textsuperscript{35}. Overall, 25\% of chronically infected patients will develop progressive liver fibrosis and cirrhosis. Both virus and host factors may influence the severity and onset of liver fibrosis such as alcohol use, immune status, sex, race etc. Each year, 4 to 5\% of patients with chronic hepatitis C develop hepatocellular carcinoma (HCC)\textsuperscript{38}. The time between infection and HCC onset varies between 10 to 30 years.

To date, liver disease related to chronic HCV infection is the most common reason for liver transplantation in Western countries. Being
a silent disease, the contribution of chronic hepatitis C to global morbidity and mortality is generally underestimated.

However, the reported rates of cirrhosis development have been shown to vary among studies, from 2-8% in studies of young subjects to 20-30% in older patients\textsuperscript{39}. These differences account for the fact that many host factors modulate the risk of liver disease progression, as further explained below. The pathogenesis of the liver disease is mainly immune-mediated. Chronic infection is associated with portal inflammation, periportal necrosis, fibrosis and often steatosis\textsuperscript{40}. Destruction of hepatocytes by the chronic inflammation is accompanied by liver regeneration. For mechanisms not completely understood, in a sizable fraction of cases, liver destruction is followed by scar formation and deposition of fibrotic tissue instead of the normal tissue. Hepatic fibrogenesis represents a wound-healing response characterized by a net accumulation of extracellular matrix (ECM) resulting from increased synthesis and decreased degradation\textsuperscript{41}. Hepatic Stellate Cells (HSCs) represent the primary source of ECM\textsuperscript{42}. In normal liver, HSCs are described as being in a quiescent state. A distinguished feature of quiescent HSC is the presence in their cytoplasm of multiple lipid droplets containing high amounts of vitamin A. In response to inflammatory stimuli and Reactive Oxygen Species (ROS), HSCs become activated, proliferate and transform into myofibroblasts expressing α-smooth muscle actin (α-SMA), an actin isoform found in smooth muscle cells\textsuperscript{42}. An increased production of collagen type I is characteristic of this phase, a process regulated both transcriptionally and post-transcriptionally\textsuperscript{43, 44}. Hepatocytes, Kupffer
cells, platelets and endothelial cells contribute to the activation of HSC. Kupffer cells are an important source of TGF-β1, which is a potent stimulus for the production of ECM. TGF-β1 also acts in an autocrine loop, because HSCs are themselves a source of this cytokine. In addition, TGF-β1 inhibits cell proliferation and promotes differentiation or apoptosis. Nevertheless, a fraction of people with chronic HCV infection will never progress to cirrhosis, because the disease progression may be particularly slow, depending on both nonmodifiable and modifiable cofactors. The risk of progression is increased by many host factors, including older age at acquisition of the infection, male gender, alcohol consumption, coinfection with Hepatitis B Virus (HBV) and Human Immunodeficiency Virus (HIV), iron overload and other metabolic factors (insulin resistance, obesity).

4. Current management of Hepatitis C: diagnostic tools, clinical decisions and therapies

The optimal approach to detecting HCV infection is to screen persons for a history of risk of exposure to the virus, and to test selected individuals who have an identifiable risk factor. Currently, injection drug use is the primary mode of HCV transmission in the U.S; thus, all persons who use or have used illicit injection drugs in the present or past, even if only once, as well as intranasal drug users who share paraphernalia, should be tested for HCV infection. Individuals who have received a blood or blood component transfusion or an organ transplant before 1992 should also be tested. With the introduction of sensitive tests to screen blood donors for HCV...
antibodies in 1992, transfusion-transmission of HCV has become rare\textsuperscript{48}. Individuals with unexplained elevations of the aminotransferase levels (alanine and/or aspartate aminotransferase; ALT/AST), those ever on hemodialysis, children born to HCV-infected mothers, or those with human immunodeficiency virus (HIV) infection should be tested for the presence of HCV infection.

Other potential sources of HCV transmission include exposure to an infected sexual partner or multiple sexual partners, exposure among health care workers to HCV contaminated blood and blood products, and tattooing\textsuperscript{33, 49}. The prevalence of HCV infection is consistently higher among persons with multiple sexual partners, whereas sexual transmission of HCV between monogamous partners is uncommon\textsuperscript{33}. Nevertheless, between 1\% and 5\% of monogamous sexual partners of index HCV cases test positive for anti-HCV. The hepatitis C virus is not transmitted by hugging, kissing, sharing of eating utensils or breastfeeding.

Folk medicine practices, including acupuncture and ritual scarification, as well as body piercing, tattooing and commercial barbering are potential modes for transmission of HCV infection when performed without appropriate infection control measures\textsuperscript{50}. Transmission of HCV infection by body piercing is, however, rare. Because symptoms are generally absent in individuals with chronic HCV infection, recognition of infection requires risk factor screening, which should be done whenever it is possible to link with appropriate HCV testing and counseling\textsuperscript{47}. 
Laboratory Testing. Two classes of assays are used in the diagnosis and management of HCV infection: serologic assays that detect specific antibody to hepatitis C virus (anti-HCV) and molecular assays that detect viral nucleic acid.

Tests that detect anti-HCV are used both to screen for and to diagnose HCV infection. Anti-HCV can be detected in the serum or plasma using a number of immunoassays. Two enzyme immunoassays (EIAs) are approved by the U.S. Food and Drug Administration (FDA) for clinical use, Abbott HCV EIA 2.0 (Abbott Laboratories, Abbott Park, IL) and ORTHO® HCV Version 3.0 ELISA (Ortho-Clinical Diagnostics, Raritan, NJ), as well as one enhanced chemiluminescence immunoassay (CIA) VITROS® Anti-HCV assay, (Ortho-Clinical Diagnostics, Raritan, NJ). Many commercial assays for the detection (qualitative assays) or quantification (quantitative assays) of HCV RNA are available. Historically, qualitative assays have been more sensitive than quantitative assays. With the recent availability of real time polymerase chain reaction (PCR)-based assays and transcription-mediated amplification (TMA) assays, with sensitivities of 10-50 IU/mL, there is no longer need for qualitative assays. A highly sensitive assay with this lower limit of detection is considered appropriate for monitoring during therapy. All currently available assays have excellent specificity, in the range of 98% to 99%. In 1997, the World Health Organization established the first International standard for HCV RNA nucleic acid technology, and the IU rather than viral copies is now the preferred unit to report test results.
For monitoring purposes, it is important to use the same laboratory test before and during therapy.

**Genotyping Assays.** Genotyping is useful in epidemiological studies and in clinical management for predicting the likelihood of response and determining the optimal duration of therapy. The hepatitis C virus can be classified into at least 6 major genotypes (genotypes 1 to 6) based on a sequence divergence of 30% among isolates\(^2^2\). Genotype 1 (subtypes 1a and 1b) is the most common in the U.S., followed by genotypes 2 and 3. Less common genotypes (genotypes 4-6) are beginning to be observed more frequently because of the growing cultural diversity within the United States\(^5^3\). Several commercial assays are available to determine HCV genotypes using direct sequence analysis of the 5’ non-coding region, that include Trugene 5’NC HCV Genotyping kit (Siemens Healthcare Diagnostics Division, Tarrytown, NY), reverse hybridization analysis using genotype specific oligonucleotide probes located in the 5’ non-coding region, INNO-LiPa HCV II, (Innogenetics, Ghent, Belgium), and Versant HCV Genotyping Assay 2.0 (Siemens Healthcare Diagnostics Division, Tarrytown, NY). Incorrect typing among the major genotypes is rare (<3%) and mixed genotypes occur but are uncommon. Occasionally (<5%), tested samples cannot be genotyped. This usually results from low viral levels, issues with the PCR amplification step of the assay, or extreme nucleotide variability within the HCV genome\(^5^4\).

**Diagnosis of Acute and Chronic HCV Infection and Interpretation of Assays.** The diagnosis of acute or chronic HCV infection generally requires testing of serum for both antibody to HCV (anti-HCV) and for
HCV RNA. Patients suspected of having acute or chronic HCV infection should first be tested for anti-HCV.

HCV RNA testing should be also performed in patients with unexplained liver disease whose anti-HCV test is negative and who are immunocompromised or suspected of having acute HCV infection. A sensitive quantitative HCV RNA assay is recommended for diagnosis because it also provides information on the level of virus which is helpful in management.

HCV genotyping should be performed in all HCV-infected persons prior to interferon-based treatment in order to plan for the dose and duration of therapy and to estimate the likelihood of response.

One pattern is the identification of both anti-HCV and HCV RNA in a person with recent elevation of the ALT value. This scenario is consistent with either acute HCV infection when there is a recent known risk exposure, with exacerbation of chronic HCV infection, or with an acute hepatitis of another etiology in a patient with chronic HCV infection. Another pattern is the detection of anti-HCV but with a negative test for HCV RNA. This may represent acute HCV infection during a period of transient clearance of HCV RNA, a false positive or negative result or, more commonly, recovery from HCV infection.

Utility of the Liver Biopsy and Noninvasive Tests of Fibrosis. There are three primary reasons for performing a liver biopsy: it provides helpful information on the current status of the liver injury, it identifies features useful in the decision to embark on therapy, and it may reveal advanced fibrosis or cirrhosis that necessitates surveillance for hepatocellular carcinoma (HCC). The biopsy is assessed for grade and
stage of the liver injury, but also provides information on other histological features that might have a bearing on liver disease progression. The grade defines the extent of necroinflammatory activity, while the stage establishes the extent of fibrosis or the presence of cirrhosis. Several scoring systems have been conceived, the most common being the French METAVIR, the Batts-Ludwig, the International Association for the Study of the Liver (IASL) and the Ishak Scoring systems. The two more common non-HCV conditions that might affect disease progression and possibly impede treatment response are steatosis and excess of hepatocellular iron. Identifying either of these two features does not preclude initiating treatment, but their presence provides additional information regarding the likelihood of response to treatment.

The liver biopsy has been widely regarded as the “gold standard” for defining the liver disease status, but it has drawbacks that have prompted questions about its value. The procedure is not without risks (including pain, bleeding and perforation of other organs), it is subject to sampling error, it requires special expertise for interpreting the histopathology, it adds cost to medical care, and it is anxiety-provoking for the implicated person. Thus, efforts are underway to seek alternative means of establishing information on the extent of fibrosis by focusing on noninvasive blood marker panels. These markers are useful for establishing the two ends of the fibrosis spectrum (minimal fibrosis and cirrhosis) but are less helpful in assessing the mid-ranges of fibrosis or for tracking fibrosis progression.
A liver biopsy may be unnecessary in persons with genotypes 2 and 3 HCV infection, since more than 80% of them achieve a sustained virological response (SVR) to standard-of-care treatment. There is, however, an ongoing debate about whether a biopsy is warranted for persons infected with HCV, genotype 1, whose response to such treatment approximates 50% among Caucasians and 30% among African Americans\textsuperscript{67, 68}. Even more uncertain is whether there is need for a liver biopsy in persons infected with the other less common genotypes (4 through 6). Thus, although the liver biopsy was previously regarded as routine for defining the fibrosis stage in persons with genotype 1 infection\textsuperscript{65}, the issue is now in a state of flux and possible transition. Supporters of a biopsy cite the difficult nature and high cost of current antiviral therapy and are therefore willing to withhold or delay treatment if liver histology displays minimal to moderate fibrosis stage ≤2, especially if the infection is known to have been long-standing. However, treatment is advised for those with more advanced fibrosis stage ≥3. Therefore, the decision to perform a liver biopsy should be based on whether treatment is being considered, taking into account the estimated duration of infection and other indices of advancing liver disease (e.g., the platelet count), the viral genotype, and the patient’s willingness to undergo a liver biopsy and motivation to be treated.

In conclusion, a liver biopsy should be considered in patients with chronic hepatitis C infection if the patient and health care provider wish information regarding fibrosis stage for prognostic purposes or to make a decision regarding treatment.
Treatment of HCV Infection. Natural history studies indicate that 55% to 85% of individuals who develop acute hepatitis C will remain HCV-infected. Spontaneous resolution is more common among infected infants and young women than among persons who are older when they develop acute hepatitis. The risk of developing cirrhosis ranges from 5% to 25% over periods of 25 to 30 years. Progression to cirrhosis may be accelerated in persons who are of older age, who are obese, who are immunosuppressed (e.g., HIV co-infected), and who consume more than 50g of alcohol per day, although the precise quantity of alcohol associated with fibrosis progression is unknown. Persons with HCV-related cirrhosis are at risk for the development of hepatic decompensation (30% over 10 years) as well as hepatocellular carcinoma (1% to 3% per year). Infection with HCV can also cause extrahepatic diseases including mixed cryoglobulinemia, types II and III. Indeed, symptomatic cryoglobulinemia is an indication for HCV antiviral therapy regardless of the stage of liver disease.

The goal of the therapy is to prevent complications and death from HCV infection. Because of the slow evolution of chronic HCV infection over several decades, it has been difficult to demonstrate that therapy prevents complications of liver disease. Accordingly, treatment responses are defined by a surrogate virological parameter rather than a clinical endpoint.

Short-term outcomes can be measured biochemically (normalization of serum ALT levels), virologically (absence of HCV RNA from serum by a sensitive PCR-based assay), and histologically (>2
point improvement in necroinflammatory score with no worsening in fibrosis score\textsuperscript{67}.

Several types of virological responses may occur, labeled according to their timing relative to treatment. The most important is the sustained virological response (SVR), defined as the absence of HCV RNA from serum by a sensitive PCR assay 24 weeks following discontinuation of therapy (Fig. 2). This is generally regarded as a “virological cure”, although liver cancer has been identified years later, especially if cirrhosis existed at the time of achieving an SVR\textsuperscript{72}.

Undetectable virus at the end of either a 24-week or 48-week course of therapy is referred to as an end-of-treatment response (ETR). An ETR does not accurately predict that an SVR will be achieved but is necessary for it to occur.

A rapid virological response (RVR), defined as undetectable HCV RNA at week 4 of treatment, using a sensitive test with a lower limit of detection of 50 IU/mL, predicts a high likelihood of achieving an SVR\textsuperscript{73}. Only 15% to 20% of persons with HCV genotype 1 infection and 66% with HCV genotype 2 and 3 infections achieve an RVR\textsuperscript{74, 75}. Because of the rapid clearance of virus from serum, patients who achieve an RVR may be able to shorten the duration of treatment\textsuperscript{75}.

An early virological response (EVR) is defined as a $\geq 2$ log reduction or complete absence of serum HCV RNA at week 12 of therapy compared with the baseline level. Failure to achieve an EVR is the most accurate predictor of not achieving an SVR and identifying non-responders\textsuperscript{67, 75}. Ninety-seven to 100% of treatment-naive patients with HCV genotype 1 infection who did not reach an EVR failed to
achieve an SVR. Monitoring viral kinetics is thus useful for predicting whether or not an SVR is likely to develop.

Virological breakthrough refers to the reappearance of HCV RNA while still on therapy, while virological relapse is the reappearance of HCV RNA in serum after treatment is discontinued and an ETR was documented.

Persons who fail to suppress serum HCV RNA by at least 2 logs after 24 weeks of therapy are null responders, while those whose HCV RNA levels decrease by ≤2 logs IU/mL but never become undetectable are referred to as partial nonresponders.

Measuring the rate of viral clearance from serum is helpful in predicting the likelihood of a response to therapy, for determining the optimal duration of therapy and as a stopping rule for patients with chronic HCV infection. This approach may have the benefit of limiting exposure to PEG-IFNα and ribavirin, thus potentially leading to reduced toxicity and a cost savings.
Fig. 2. Graphic display of virological responses.
RVR, rapid virological response (clearance of HCV from serum by week 4 using a sensitive PCR-based assay); EVR, early virological response (≥2 log reduction in HCV RNA level compared to baseline HCV RNA level at treatment week 12); SVR, sustained virological response (HCV RNA negative 24 weeks after cessation of treatment); relapse, reappearance of HCV RNA in serum after therapy is discontinued; non responder, failure to clear HCV RNA from serum after 24 weeks of therapy; partial non responder, 2 log decrease in HCV RNA but still HCV RNA positive at week 24; null non responder, failure to decrease HCV RNA by <2 logs after 24 week of therapy.55

The Optimal Treatment of Chronic HCV: PEG-Interferon Alfa and Ribavirin. The currently recommended therapy of chronic HCV infection is the combination of a pegylated interferon alpha and ribavirin.

There are two licensed pegylated interferons in the United States, PEG-IFNα -2b (PEG-Intron, Schering Plough Corp., Kenilworth, NJ), with a 12-kd linear polyethylene glycol (PEG) covalently linked to the standard interferon alfa-2b molecule, and PEG-IFNα -2a (Pegasys, Hoffmann-La Roche, Nutley, NJ) with a 40-kd branched PEG covalently linked to the standard interferon alfa-2a molecule.76 The doses of these two forms of pegylated interferons differ.
The optimal dose of PEG-IFNα-2b, based on the original registration trial, is 1.5 µg/kg/week dosed according to body weight, together with ribavirin 800 to 1400 mg daily.

PEG-IFNα-2a is administered at a fixed dose of 180 µg/week given subcutaneously together with ribavirin 1000 to 1200 mg daily.

Ribavirin has two beneficial effects: an improvement in the ETR but, more importantly, a significant decrease in the relapse rate as compared to PEG-IFNα monotherapy treatment.

The optimal duration of treatment should be based on the viral genotype. The study established that patients with genotype 1 should be treated for 48 weeks with PEG-IFNα-2a plus standard weight-based ribavirin, whereas patients with genotypes 2 and 3 could be treated with PEG-IFNα-2a plus low dose ribavirin (800 mg) for 24 weeks.  

For patients with HCV genotype 4 infection, combination treatment with pegylated interferon plus weight-based ribavirin administered for 48 weeks appears to be the optimal regimen. Patients with genotypes 5 and 6 are underrepresented in trials of PEG-IFNα and ribavirin due to their limited worldwide frequency.

Currently, the major challenge with regard to therapy is what new approaches are needed to increase the SVR rates in (1) patients with genotype 1 infection and a high viral load; (2) persons who fail to achieve an SVR using the currently approved treatment regimens.

Multivariate analyses have identified two major predictors of an SVR among all populations studied: the viral genotype and pretreatment viral load. Sustained virological response rates were higher in patients infected with genotype non-1 infection (mostly
genotype 2 and 3) and in those with a viral load of less than 600,000 IU/mL\textsuperscript{68}. Other less consistently reported baseline characteristics associated with a favorable response include the doses of PEG-IFNα (1.5 µg/kg/week versus 0.5 µg/kg/week) and ribavirin (>10.6 mg/kg), female gender, age less than 40 years, non–African-American race, lower body weight (≤75 kg), the absence of insulin resistance, elevated ALT levels (three-fold higher than the upper limit of normal), and the absence of bridging fibrosis or cirrhosis on liver biopsy\textsuperscript{67}.

High dose interferon induction regimens have generally been unsuccessful. High dose ribavirin given together with standard dose PEG-IFNα was also evaluated. Ninety percent of patients achieved an SVR, but safety issues are the major concern for this approach since significant anemia developed in all patients, requiring the use of growth factors in all and blood transfusions in two patients.

**Adverse Events.** Almost all patients treated with PEG-IFNα and ribavirin experience one or more adverse events during the course of therapy. Adverse events are a major reason that patients decline or stop therapy altogether. In the registration trials of PEG-IFNα alfa-2a and 2b plus ribavirin, 10% to 14% of patients had to discontinue therapy due to an adverse event\textsuperscript{67}. The most common adverse events in these trials were influenza-like side effects such as fatigue, headache, fever and rigors, which occurred in more than half of the patients, and psychiatric side effects (depression, irritability, and insomnia), which occurred in 22% to 31% of patients.

Laboratory abnormalities are the most common reasons for dose reduction. Among these, neutropenia was a frequent laboratory
abnormality. Despite the decline in the neutrophil count, serious infections are uncommon and granulocyte colony stimulating factor is rarely necessary except in patients with advanced cirrhosis. Anemia was observed in approximately one-third of patients.

Neuropsychiatric side effects include depression, insomnia, emotional lability, mood disorders, frank psychosis, suicidal ideation, actual suicide, and homicide. Interferon-induced depression appears to be composed of two overlapping syndromes — a depression-specific syndrome characterized by anxiety and cognitive complaints, and neurovegetative symptoms, characterized by fatigue, anorexia, pain and psychomotor slowing.

The most consistent risk factors for developing depression are the presence of mood and anxiety symptoms prior to therapy. Mental or psychiatric disease represents a significant barrier to treatment in patients with chronic HCV infection. Significant depressive symptoms occur in 21% to 58% of interferon-treated patients. These patients may be successfully treated with a multidisciplinary approach to management of adherence and neuropsychiatric side effects. Using this approach, they can achieve SVR rates that are similar to patients without psychiatric disorders. Most psychotropic agents are thought to be safe for use in the management of patients with chronic HCV infection and psychiatric disease. However, consideration should be given to drug–drug interactions and dose modification in patients with advanced liver disease. A past history of depression and of receiving higher doses of interferon, as well as being female, have been
identified as risk factors for neuropsychiatric side effects of PEG-IFNα, but are less reliable ones\textsuperscript{79}.

Pegylated interferon may induce autoimmune disorders, such as autoimmune thyroiditis, or may worsen preexisting autoimmune disorders. Therefore, the presence of autoimmune conditions prior to treatment is a relative contraindication to therapy.

With regard to ribavirin, the most common side effect is hemolytic anemia. Since ribavirin is cleared by the kidney, the drug should be used with extreme caution in patients with renal disease and renal failure. Other side effects associated with ribavirin include mild lymphopenia, hyperuricemia, itching, rash, cough and nasal stuffiness. Ribavirin is reported to cause fetal death and fetal abnormalities in animals and thus it is imperative for persons who receive the drug to use strict contraceptive methods both during treatment and for a period of 6 months thereafter. The education of patients and caregivers about side effects and their management is an integral component of treatment and is important for a successful outcome.

\textbf{Retreatment of Persons Who Failed to Respond to Previous Treatment.} The approach to patients who fail therapy depends on the nature of the initial response, on the potency of initial treatment and on host–viral factors. Twenty to fifty percent of patients treated with pegylated interferon and ribavirin will not achieve an SVR. Failure to achieve an SVR with a course of pegylated interferon and ribavirin can be a consequence of non-response, virological breakthrough, or relapse. Poor adherence to the prescribed treatment and inappropriate dose reductions can contribute to poor response rates.
The induction of antibodies to PEG-IFNα accounts for only a minority of cases.

Non-responders to PEG-IFNα and ribavirin with advanced fibrosis should follow AASLD guidelines for screening for HCC and varices and be evaluated for liver transplantation if they are appropriate candidates. Patients with mild fibrosis should be monitored without treatment.

In the majority of instances, virological relapse occurs within the first 12 weeks and late relapse, beyond 24 weeks, is extremely uncommon. Patients with virological relapse are likely to respond to the same regimen given a second time but will still experience an unacceptable rate of relapse\(^{55}\).

**Diagnosis and Treatment of HCV-Infected Children.** The risk of perinatal HCV transmission is 4% to 6%, and is 2- to 3-fold higher for mothers with HIV/HCV co-infection. Although HCV has been identified in breast milk of infected mothers, there are no data to show that HCV is transmitted in breast milk; therefore breastfeeding is not prohibited in HCV-infected mothers\(^{80}\).

Children who are acutely infected with HCV, like adults, are generally asymptomatic, but they are more likely than infected adults to spontaneously clear the virus and are more likely to have normal ALT levels. Children with chronic HCV infection, irrespective of mode of acquisition (vertical versus transfusion), have been shown to have minimal progression of their disease over 5 to 20 years. Biopsy studies in children generally have demonstrated minimal fibrosis and rare cirrhosis 15 to 20 years after infection\(^{81}\).
Treatment of Persons with Compensated and Decompensated Cirrhosis. Patients with HCV-related compensated cirrhosis can be treated with the standard regimen of pegylated interferon and ribavirin but will require close monitoring for adverse events. Treatment of patients with decompensated cirrhosis, defined as one or more of the clinical complications of chronic liver disease — ascites, encephalopathy, variceal bleeding, and/or impaired hepatic synthetic function — is more problematic. They should be referred for consideration of liver transplantation. For those undergoing liver transplantation, reinfection of the allograft with HCV is the rule and progressive post-transplantation disease of the grafted liver is common. Accordingly, since eradication of HCV pre-transplantation is associated with a lower likelihood of post-transplantation infection, there is a strong incentive to treat the HCV infection before transplantation, provided the risks of treatment are acceptable. Graft re-infection is almost universal and graft loss due to recurrent HCV occurs in approximately 25% to 30% of patients.

Treatment of Persons with Acute Hepatitis C. The response rate to treatment is higher in persons with acute than with chronic HCV infection. There is consistent evidence that treatment reduces the risk that acute hepatitis C will evolve to chronic infection. Studies using high doses of interferon (5-10 million units per day) for at least 12 weeks, or until serum enzymes normalized, report sustained viral response rates of 83% to 100%, which are much higher than any estimates of spontaneous clearance, or of response rates in persons with chronic HCV infection. No recommendation can be made for or
against the addition of ribavirin and the decision will therefore need to be considered on a case-by-case basis. It is reasonable to start treatment within 8-12 weeks after identified acute hepatitis C, and thus patients should be monitored monthly for this purpose. Although HCV RNA in patients with acute infection generally is cleared from the blood by 8 to 16 weeks in most persons who recover spontaneously, viremia has been observed as late as 48 weeks after acute infection in injection drug users who ultimately clear35.

Other Management Issues. An important adjunct to the therapy of HCV is to advise chronically affected persons of measures that might be helpful in reducing or even preventing further fibrosis progression, independent of treatment. Most important is the issue of the potential deleterious effect of alcohol. Excess alcohol intake may increase HCV RNA replication and interfere with response to treatment83. Controversy exists, however, about the level of alcohol intake that is clearly harmful to the HCV-infected person. It is widely believed that the daily consumption of more than 50 grams of alcohol has a high likelihood of worsening the fibrosis, but there are reports of levels of alcohol intake of less than that amount having a deleterious effect on the liver disease84. It is reasonable to recommend either the complete suspension of alcohol intake while on treatment or restricting its use to an occasional drink during the course of the treatment.

Obesity and its associated nonalcoholic fatty liver disease are believed to play a role in the progression of fibrosis in HCV-infected individuals and response to treatment85. Weight reduction and
improvement in insulin resistance may improve the response to PEG-IFNα plus ribavirin therapy.

Furthermore, it is recommended that persons with chronic HCV infection who lack evidence of preexisting antibody to hepatitis A and/or B receive the hepatitis A and B vaccines\(^86\).

Other metabolic factors contribute to the modification of the natural course of HCV liver disease. These include: iron overload, steatosis, insulin resistance and obesity. All these factors have been shown to accelerate disease progression\(^60\). With regard to steatosis, a complex relationship exists with the virus and, additionally, the separation of the relative contributions of steatosis, obesity and insulin resistance is challenging, because of the underlying relationships between them. Moreover, accelerated rates of fibrosis progression associated with steatosis are observed in a genotype-specific way. Both in vitro and in vivo, HCV has been shown to modulate lipid metabolism, mainly through gene expression regulation or direct induction of intracellular lipid droplets accumulation\(^87, 88\). Moreover, HCV replicates within a compartment derived from intracellular membrane alterations induced by nonstructural proteins, thus providing another direct link between lipids and HCV life cycle.

5. Recently licensed HCV inhibitors

No direct acting antiviral drugs to treat infection with HCV have been licensed in the 20 years since its identification. Excitingly, recent publications herald several small revolutions in antiviral treatment of HCV that have considerable relevance for prospective HCV therapies.
After a decade in which PEG-IFNα–ribavirin therapy was the only available option, triple therapy with HCV nonstructural protein (NS) 3/4A protease inhibitors (HCV PI) in combination with PEG-IFNα–ribavirin will become the new standard of care. In the first half of 2010, important phase II studies of orally available inhibitors of HCV NS3/4A protease were published.

First, McHutchison and colleagues reported on the efficacy and safety of telaprevir in combination with PEG-IFNα–ribavirin in 465 patients with chronic hepatitis C who had not responded to at least one course of PEG-IFNα–ribavirin therapy. The addition of telaprevir to standard therapy markedly increased SVR rates in these difficult to treat patients (up to 53% compared with 14% in the control arm). Remarkably, in the group treated for 24 weeks with this triple therapy followed by another 24 weeks of PEG-IFNα-2b plus ribavirin the SVR rates for patients who had previously relapsed was 76%. Even for patients who had not responded to standard therapy the SVR rate was up to 39% with the novel triple therapy.

In the second publication of a phase II study, Kwo et al. reported the results of treatment with another HCV PI, boceprevir, showing that its addition to the standard treatment leads to a notably increased SVR (75% versus 38%) in treatment-naïve patients who are infected with HCV genotype 1.

Importantly, all of the published studies confirm that the combination of three agents (PEG-IFNα–ribavirin plus an HCV PI) is needed to substantially enhance SVR rates. The potency and safety of these two first generation HCV PIs have now been confirmed in large
phase III studies. In treatment-naive patients infected with HCV genotype 1, the SVR rates were 75% with the addition of telaprevir versus 44% with standard therapy, and 68% versus 40% for boceprevir.

Although the upcoming triple therapy protocols for telaprevir and boceprevir will be different, both will involve “response-guided therapy”. This strategy will mean that in treatment-naive patients, treatment duration will be reduced to 24 weeks or 28 weeks for patients with a rapid viral response—those with a negative result for serum HCV RNA after 4 weeks of exposure to an HCV PI.

Although the addition of HCV PIs to HCV therapy promises to markedly improve outcomes, IFN and ribavirin are still part of all the treatment regimens tested in the above mentioned trials; thus important adverse effects and several contraindications remain major problems in HCV therapy. An IFN-free method of achieving SVR is, therefore, the ultimate goal for HCV therapy.

In October 2010, the Lancet reported on 88 patients infected with HCV genotype 1 who were treated with two direct acting antiviral drugs (DAAs): a combination of the HCV PI, danoprevir, and a nucleoside NS5B polymerase inhibitor, RG7128. Treatment with a combination of the highest doses of both drugs led to an average 5.1 log reduction of HCV RNA plasma levels within 14 days. Overall, this treatment was well tolerated and some patients even had HCV RNA concentrations below the limit of detection. Notably, no viral breakthroughs were observed during 4 weeks of treatment, which
might reflect the high genetic barrier to resistance associated with nucleoside polymerase inhibitors.

The combination of two DAAs, as investigated in the INFORM-1 study, is an important step towards an IFN-free regimen that is administered orally—hopefully with an efficacy comparable to that of regimens that contain IFN. However, because of the protocol design of this study all patients received a full course of PEG-IFNα–ribavirin therapy following the 12 weeks of combination danoprevir and RG7128 therapy. Thus, proof of the concept that SVR can be achieved with a DAA approach administered orally is still missing.

At the moment, several companies are exploring different anti-HCV oral therapies in ongoing studies that incorporate different combinations of linear and cyclic HCV PIs, nucleoside as well as non-nucleoside NS5B polymerase inhibitors, NSSA inhibitors and cyclophilin A inhibitors.

Even in view of these imminent improvements in the standard of care of patients with hepatitis C, there is still an urgent need for other antiviral drugs that act via different molecular mechanisms, as the development of viral resistance and toxic effects will certainly be a major challenge to any new therapy. Given that HCV has a very high mutation rate, therapies that target the host factors essential for HCV replication might be a greater barrier to resistance than drugs that target viral enzymes.

In the search for novel targets, Lanford and colleagues have taken a highly original approach by antagonizing microRNA-122, the most abundant miRNA in the liver and an essential cofactor for viral RNA
replication that binds to the 5'-noncoding region of the HCV genome\textsuperscript{93}. They showed that using a locked nucleic-acid-modified phosphorothioate oligonucleotide (SPC3649) to inhibit miRNA-122 causes marked and prolonged reduction of HCV viremia in chronically infected chimpanzees\textsuperscript{94}. Except for a decrease in serum levels of cholesterol, they did not observe any important adverse effects in the treated animals. These data indicate the feasibility and safety of SPC3649 as a treatment for patients with hepatitis C. A drug that targets a specific human miRNA in a clinically common disease and demonstrates clinical efficacy represents an extremely innovate approach that might also be interesting for other disease areas as our understanding of the biological functions of miRNAs expands.

How useful the novel agents will be in the most difficult to treat patients, such as those with advanced or decompensated liver disease or after liver transplantation, is still unclear.

Furthermore, DAAs also have to be developed for the other HCV genotypes. Some progress has been made in meeting the next challenges, including the management of viral resistance, tailoring optimal treatment approaches for individual patients and eventually the replacement of treatments that contain IFN with regimens that do not contain IFN and are administered orally. Such therapies have to be developed to achieve an SVR and thus cure patients infected with HCV, even in so-called null responders to IFN-based therapies. Reported cases of an SVR in single patients who refused to continue standard treatment after a course of therapy with novel anti-HCV agents, such as cyclophilin inhibitors, are promising\textsuperscript{95}. Finally, with the expansion of
treatment options and an increasing understanding of how host factors (such as polymorphisms in the gene that encodes interleukin-28) affect treatment outcomes, regimens will increasingly have to be individualized to each patient’s characteristics and preferences.

6. IL28B genotype and response to therapy

An outstanding advance in understanding the interplay between host immunogenetics and viral clearance was the discovery of the association between the IL28B locus on chromosome 19 and the spontaneous or treatment-induced viral clearance. IL28B encodes for interferon-λ3 (IFN-λ3), a member of the type-3 interferon group. This cytokine is structurally related to the IL10 family, but is functionally related to type-1 IFN. Actually, it is an antiviral cytokine involved in innate immune responses that functions via the JAK/STAT signaling pathway in regulating the expression of IFN-stimulated genes (ISGs) and thus suppressing viral infections. Indeed, recent evidence showed that IFN-λ suppresses HCV replication and early clinical trials reported a successful antiviral effect of pegylated-IFN-λ1 plus ribavirin in treatment-naive patients. The identification of IL28B as a significant predictor of treatment-induced viral clearance resulted from various GWASs performed in population of different ethnicity. These findings were further validated and the association with spontaneous viral clearance was also demonstrated. The first GWAS performed by Ge et al. reported the association between rs12979860 a Sustained Virological Response (SVR) in patients with genotype 1 and treated with PEG-IFN-α2a or PEG-IFN-α2b. An
approximate twofold change in response to treatment was observed in patients with the SNP rs12979860 CC genotype compared with the TT genotype. The reported Odds Ratio (OR) was 7.3 in European-Americans, 7.1 in African-Americans and 5.6 in Hispanics. The SNP rs12979860 is located ~3 kbp upstream the IL28B gene and resulted the strongest predictor of SVR, compared to baseline fibrosis or baseline viral load.

Indeed, the actual causative variants have not been yet identified, although targeted sequencing of the IL28B region revealed two possible candidates: a non-synonymous substitution (rs8103142, K70A) and a variant in the promoter region of IL28B (rs28416813). Recently, gene expression has been linked to IL28B genotypes, thus providing a direct link between genotype and function. Indeed, lower pre-treatment intrahepatic expression levels of ISGs have been reported for the genotypes of rs8099917 and rs12979860 associated with SVR. Conversely, no association was reported between the IL28B mRNA expression and different genotypes. Hence, the emerging picture is the following: a) subjects with a IL28B genotype associated to SVR have a lower pre-treatment activation of ISGs; b) this reduced expression of ISGs in treatment-naive patients may account for the higher viral loads observed in those carrying the IL28 genotype associated with SVR; c) the IL28B variants associated with a poor response correspond to higher pre-treatment levels of ISGs, a condition that in principle might impair a strong response to exogenous IFN during therapy; d) the therapy outcome seems not to
be influenced by differential expression of IL28B gene in groups with different IL28B genotype.

Finally, the exact interplay between type-1 and type-3 IFNs remains to be fully elucidated and a mechanistic model to accurately explain this scenario is currently unavailable.

A short-term translational value of these findings could be the individualization of the therapy in accordance to the host genotype. A significant number of patients fail to respond to PEG-IFN plus ribavirin or have adverse effects, including influenza-like symptoms, haematologic abnormalities and neuropsychiatric symptoms. Therefore, it is of outstanding interest to predict the therapeutic outcome. A laboratory test for IL28B genotype is already available in the United States and this could in principle be used together with viral load and genotype plus other host factor to predict SVR, since a 100% predictive power is currently not achievable with the genotype data alone.

7. Still unmet clinical needs in Hepatitis C: diagnostic tools, biomarkers of disease progression and response to therapies, new therapies

Liver cirrhosis and its sequelae are the most unwanted consequences of chronic HCV infection. Apart from virus eradication, a very major clinical goal for HCV is to prevent or to follow closely the transition from fibrosis to cirrhosis. The progression rate from fibrosis to cirrhosis, varies widely among HCV patients going from few years to several decades and it is often unpredictable\cite{109}. While in some cases
the rate of HCV disease progression is clearly influenced by a number of external factors such as age at infection, gender, immune status and alcohol consumption, obesity, and liver co-infections, in most cases, there are no clinical markers available to assess the risk of developing progressive liver disease and liver cancer in individual subjects\textsuperscript{110}. Moreover, the liver is an organ that can often compensate very well for the lost of a sizeable fraction of hepatic tissue. Indeed, it is not infrequent that cirrhosis is diagnosed only at very advanced stages as patients live acceptably well with a liver that has only 20-30\% of its functionality. Understanding what are the patients at risk of developing a faster progressive liver disease would have great advantages. From the clinical point of view, antiviral therapy would be the most beneficial in patients at higher risk of developing progressive liver disease and would therefore be given as soon as possible. Furthermore, the gold standard for assessing hepatic fibrosis, that is liver biopsy, is invasive, subject to sampling errors, and has rare but occurring potentially life threatening complications, thus limiting its acceptability in patients with mild or moderate disease. There is therefore the medical need to develop non-invasive and reliable serum markers that accurately reflect hepatic fibrotic and cirrhotic disease. Moreover, effective surveillance of patients at high risk of developing HCC (i.e., patients with cirrhosis) could potentially decrease HCC-related mortality rate, and the availability of early and reliable serological markers of HCC remains a huge unmet medical need. Finally, from the basic research point of view, the study of how host and viral factors could affect disease progression may shed light on
fundamental pathogenic mechanisms in the path from fibrosis to cirrhosis to cancer, which still remain poorly defined.

Another important need in the Hepatitis C field regards the prediction of the rate of response to therapy for each patient. Antiviral therapy based on long-term administration of Pegylated Interferon alpha (PEG-IFNα) and ribavirin (RBV) permits to achieve viral clearance only in about 40-60% of cases. In addition, almost all patients treated with PEG-IFNα and RBV experience one or more adverse events during the course of therapy. Adverse events are a major reason that patients decline or stop therapy altogether. The most common adverse events in the registration trials of PEG-IFNα alfa-2a and 2b plus ribavirin were influenza-like side effects such as fatigue, headache, fever and rigors, which occurred in more than half of the patients, and psychiatric side effects (depression, irritability, and insomnia), which occurred in 22% to 31% of patients. Both viral and host genetic variability are thought to influence treatment success rates. Recently, whole-genome association studies identified host single nucleotide polymorphisms (SNPs) near the genomic region encoding IL28B as strongly associated with both spontaneous and treatment-induced viral clearance under PEG-IFNα/RBV therapy, HCV1 patients having the rs12979860 CT or TT genotype display reduced viral load decline and lower SVR rates. Regarding viral genetics, HCV genotypes 1 and 4 are considered more difficult to treat than genotypes 2 and 3. Although IL28B genotype is a significant pretreatment predictor of response to therapy, there are insufficient data to determine whether IL28B testing can be used to recommend addition of a direct antiviral agent, such as
one of the recently approved NS3/NS4A protease inhibitors, to the “standard” combination of pegylated interferon-alpha and ribavirin\textsuperscript{112}. Moreover, while the inclusion of a NS3/4A protease inhibitor in a triple combination therapy promises to markedly improve outcomes, IFN and ribavirin are still part of all the approved treatment regimens. There is therefore the medical need for additional predictors of response to IFN-based therapy.

In consideration of what discussed above, the ultimate goal for HCV therapy is the development of an IFN-free method of achieving SVR. At the moment, several companies are exploring different anti-HCV oral therapies in ongoing studies that incorporate different combinations of linear and cyclic protease inhibitors, nucleoside as well as non-nucleoside NS5B polymerase inhibitors, NS5A inhibitors and cyclophilin A inhibitors. Even in view of these imminent improvements in the standard of care of patients with hepatitis C, there is still an urgent need for other antiviral drugs that act via different molecular mechanisms, as the development of viral resistance and toxic effects will certainly be a major challenge to any new therapy. Given that HCV has a very high mutation rate, therapies that target the host factors essential for HCV replication might be a greater barrier to resistance than drugs that target viral enzymes\textsuperscript{113}. The identification and the functional characterization of host determinants essential for HCV lifecycle will likely allow the development of new antiviral therapies targeting host factors.
B. Molecular aspects of Hepatitis C Virus infection

1. HCV description

HCV is a positive single-stranded RNA virus and the only member of the *Hepacivirus* genus within the *Flaviviridae* family. The RNA genome of HCV is an uncapped linear molecule encoding a single open reading frame (ORF) of approximately 9.6 kb. The ORF is flanked by 5’ and 3’ untranslated regions (UTRs) which are highly conserved RNA structures important for genome replication and protein translation. The genetic organization of the HCV genome is depicted in Fig. 3.

![Fig. 3. Genome organization of hepatitis C virus.](image)

Solid diamonds denote cleavage sites of the HCV polyprotein precursor by the endoplasmic reticulum signal peptidase. The open diamond indicates further C-terminal processing of the Core protein by signal peptide peptidase. Arrows indicate cleavages by the HCV NS2/3 and NS3/4A proteases. Adapted from 114.

The 5’ UTR is highly conserved among different HCV isolates and is composed of four highly ordered domains (I-IV). It contains an internal ribosome entry site (IRES) which is required for cap-independent RNA translation. The IRES is formed by domains II, III and IV of the 5’ UTR and the first 24-40 nucleotides of the Core coding region. Recently it
was reported that an abundant liver-specific miRNA, miR-122, binds to the 5’ UTR and that this interaction is required for efficient HCV replication but not for translation. The 3’ UTR structure consists of three parts: (i) a short, highly variable region, (ii) a polypyrimidine (poly(U/UC)) tract and (iii) a highly conserved X-tail. The X-tail consists of three stem-loop structures which direct synthesis of the negative-strand RNA and also increase IRES-mediated translation. Besides the 5’ and 3’ UTRs, an additional cis-acting replication element (CRE) was identified in the C-terminal region of NS5B, namely 5BSL3.2. This stem-loop was found to interact with a stem-loop in the 3’ X-tail via a long-range RNA-RNA or “kissing” interaction.

Translation of the ORF leads to the synthesis of a polyprotein precursor of approximately 3000 amino acids. The HCV polyprotein is co- and post-translationally processed by viral and cellular proteases into mature HCV proteins and can be divided into a structural (Core, E1, E2) and non-structural region (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The structural region is processed by host cell signal peptidases at the endoplasmic reticulum (ER) membrane. Core protein is additionally cleaved by signal peptide peptidase to remove the C-terminal E1 signal sequence. All non-structural proteins, except p7, are cleaved by the NS2/3 autoprotease and the NS3/4A protease.
Fig. 4. Structures and membrane associations of the HCV proteins.
Scissors indicate cleavages by the endoplasmic reticulum (ER) signal peptidases, except on the cytosolic side where it indicates the processing of the Core protein by signal peptide peptidases. The cyclic arrow depicts cleavage by the NS2/3 protease. Straight arrows indicate processing by the NS3/4A protease complex. Known protein structures are shown as ribbon diagrams. Proteins or protein segment of unresolved structures are represented as spheres or cylinders with their approximate sizes.

The Core protein forms the nucleocapsid of the HCV virion. It consists of three domains, the N-terminal hydrophilic domain I (D1), the hydrophobic central domain II (D2) and the C-terminal domain III (D3). D1 is involved in RNA binding and homo-oligomerization. D2 supports the association with LDs and plays important role in virus assembly. D3 serves as a signal sequence for E1 and is removed by host signal peptide peptidase to yield mature Core protein. Core protein inhibits microsomal triglyceride transfer protein (MTP), which is essential for VLDL production. Inhibition of this enzyme might contribute to steatosis. As a result of a -2/+1 ribosomal frameshift in the N-terminal Core-encoding region of the polyprotein, an additional HCV protein, the “F” (frameshift) protein or “ARFP” (alternative reading frame protein) is produced. Anti-F/ARFP antibodies are detected in the serum of HCV infected patients, which indicates that this protein is expressed during HCV infection in vivo. However, the
biological role of this protein remains to be elucidated. The Core protein has been indicated to be involved in liver steatosis, fibrosis and carcinogenesis in various mouse model systems. HCV Core protein is normally localized on the surface of cytoplasmic lipid droplets, which are believed to represent sites of virus assembly and production of nascent virions. Overexpression of intracellular Core protein has been reported cause abnormal accumulation and redistribution of lipid droplets, as well to induce the production of free radicals and apoptotic cell death, all phenomena that could result in hepatocyte injury.

The structural proteins E1 and E2 are highly N-glycosylated transmembrane type I proteins located at the virus envelope and are involved in the receptor-binding and membrane fusion of the virion. E1 and E2 contain a large N-terminal ectodomain and a C-terminal hydrophobic region harboring a transmembrane domain. During synthesis, the N-terminal domain is targeted towards the ER lumen and modified by N-glycosylation, while the transmembrane domain anchors the glycoproteins to the ER membranes. E1 and E2 contain up to respectively 6 and 11 potential glycosylation sites. The glycans on the envelope proteins are involved in the proper folding of these proteins either directly or through interaction with calnexin, an ER chaperone, and some of these glycans have been shown to be essential for the entry function of E1 and E2. Besides membrane anchoring, the transmembrane domains also function as a signal sequence, an ER retention signal and as a major regulator of the assembly of the non-covalent E1-E2 heterodimer. This heterodimer is
the functional unit for productive HCV infection. E2 plays an important role in the interaction between HCV and its major cellular receptors CD81 and SR-BI. Since E1 and E2 are located at the surface of the HCV virion, they are targeted by neutralizing antibodies. These neutralizing antibodies often inhibit the interaction(s) between E2 and the CD81 receptor.

The HCV p7 protein belongs to the family of viroporins. Viroporins are small, highly hydrophobic, virally-encoded proteins that oligomerize to form channels or pores that increase the permeability of the cell membrane to ions and other small molecules. Other known viroporins include influenza A virus M2 protein and HIV-1 Vpu protein. P7 is a small transmembrane protein that forms hexameric channels. The N- and C-termini are oriented towards the ER lumen, while the connecting loop is facing the cytoplasm. It behaves as an ion channel when reconstituted into artificial lipid membranes but its function in infected cells is not well characterized. P7 is not required for HCV replication in vitro but is essential for productive infection in vitro and in vivo. It has been reported that p7 is involved in late steps of virion assembly. However, recent studies suggest that p7 increased the proton conductance in vesicles thereby preventing acidification in otherwise acidic intracellular compartments. This loss of acidification is required for productive HCV infection.

The NS2/3 protease is required for intramolecular cleavage between NS2 and NS3. The NS2 protein that is derived from the cleavage of NS2/3 is an integral membrane protein that is targeted to ER by its N-terminal hydrophobic domain. The C-terminal domain is a
cysteine protease domain and is capable to form dimers creating a composite active site\textsuperscript{131}. The function of mature NS2 is not yet fully understood. It is not required for HCV replication, but it is essential for the production of infectious virions\textsuperscript{132}. Interestingly, the protease activity is not necessary for virion assembly. Recently it was reported that the NS2 protein serves as a key organizer of virus assembly by connecting essential viral components\textsuperscript{133}. It recruits the envelope proteins to the assembly site and it crosstalks with non-structural proteins for virus assembly\textsuperscript{134}. The colocalization of NS2 with non-structural proteins is influenced by the phosphorylation state of NS5A. The HCV NS3 is a multifunctional protein\textsuperscript{135}. The N-terminal domain of NS3 forms, together with NS4A, a heterodimeric serine protease that cleaves the downstream region of the HCV polyprotein into four functional non-structural proteins (NS4A, NS4B, NS5A and NS5B). The C-terminal domain exerts a helicase/NTPase activity that unwinds the viral RNA. The NS3/4A protease is a serine protease and contains a catalytic triad composed of His-57, Asp-81 and Ser-139. The minimal NS3 domain required for full protease activity is mapped to 180 aminoacids at the N-terminus. In addition, the NS3/4A protease activity is also responsible for an escape mechanism of innate immune response. The intracellular presence of HCV RNA can be sensed by the toll-like receptor 3 (TLR3) and by the retinoic acid-inducible gene I (RIG-I). Activation of TLR3 of RIG-I leads to the activation of interferon regulatory factor 3 (IRF3) which will transactivate the IFN-β receptor in the nucleus. This signal pathway between activated TLR-3 or RIG-I and activated IRF3 requires adaptor proteins TRIF (also known as TICAM-1)
or MAVS (also known as Cardif, IPS-1 or VISA). Both TRIF and MAVS are substrates for the NS3/4A protease\textsuperscript{136}. Cleavage of TRIF or MAVS thereby blocks IRF3 activation. Furthermore, NS3 interacts directly with NS5B and also with NS4B and NS5A via NS4A within the replication complex. The NS3 helicase is a member of the superfamily 2 helicases and consists of three domains, of which domain 1 and 2 are RecA-like domains shared with other motor proteins\textsuperscript{137}. It can travel along RNA or ssDNA in a 3’-5’ direction and is essential for viral RNA replication. Various activities are attributed to NS3 helicase including unwinding of duplex substrates, displacement of proteins bound to nucleic acids, translocation along single-stranded nucleic acids, and packaging of the RNA genome to form infectious viral particles\textsuperscript{138}. The unwinding of double-stranded RNA or single-stranded RNA with secondary structures is coupled to ATP hydrolysis\textsuperscript{139}. ATP binds the helicase between domain 1 and 2, causing a conformational change that leads to a decreased affinity for nucleic acids. This conformational change allows the helicase to move like a motor. The NS3 helicase unwinds RNA in an “inchworm” or “ratchet-like” fashion\textsuperscript{140}. Genetic variations in the HCV helicase influence its activity, which is shown by the fact that adaptive mutations in HCV replicons frequently arise in the helicase region. Interestingly, the enzymatic activities of protease and helicase domains are functionally interdependent. The protease domain promotes the binding of RNA substrate and it improves the translocation efficiency of the helicase.

**NS4A** is an essential cofactor for the NS3 serine protease\textsuperscript{141}. NS4A activates the protease by inducing conformational changes and by
increasing binding affinity to the substrate. Furthermore, its hydrophobic N-terminal part forms a transmembrane region that is required for the targeting and anchoring of NS3 to the ER membrane. Recently, it was shown that the C-terminal acidic motif of NS4A is essential for regulating the ATPase activity of the NS3 helicase. Furthermore, at least for some HCV isolates, NS4A is required for hyperphosphorylation of NS5A.

**NS4B** is a highly hydrophobic protein consisting of an N-terminal part, a central part harboring four transmembrane segments and a C-terminal part. It is an integral membrane protein with the N- and C-terminal parts located on the cytosolic side of the ER membrane. The N-terminal region contains two amphipathic α-helices of which the second helix can traverse the membrane bilayer. Based on sequence homology, it was proposed that the N-terminal part contains a leucine zipper motif. Very recently, the role of this leucine zipper domain in the efficiency of HCV replication was demonstrated. The C-terminal part of NS4B contains two palmitoylation sites. The importance of C-terminal palmitoylation of NS4B remains to be clarified. NS4B plays a central role in HCV RNA replication and assembly. It induces the formation of the membranous web; this is the alteration of intracellular membranes which contains the HCV replication complexes and is essential for replication. However, the mechanisms by which NS4B induces web formation are unknown. NS4B also interacts with other non-structural HCV proteins and with negative-strand HCV RNA. Furthermore, NS4B was found to possess NTPase activity, suggested by the presence of a nucleotide-binding motif similar to the P-loop of a
number of NTPases. However, this motif is not absolutely conserved and a mutation within this motif was identified as a cell-culture adaptive change in Con1 HCV replicons. Therefore, the enzymatic activity of NS4B remains controversial\textsuperscript{145}.

The NSSA protein is a pleiotropic protein that plays essential role in the HCV viral lifecycle both by affecting the viral RNA replication as well as by modulating the physiology of the host cell to favor viral replication. It has no intrinsic enzymatic activity, but likely exerts its functions through interactions with viral and cellular factors. NSSA consists of three domains from which the structure of domain I has been resolved\textsuperscript{148}. Domain I contains an N-terminal amphipathic helix that anchors NSSA to the membranes and lipid droplets and a RNA binding domain. Crystal structure determination of domain I revealed that a “claw-like” dimer is formed\textsuperscript{149}. The groove generated by multiple dimers is believed to serve as a railway for viral RNA, tethering the RNA onto intracellular membranes. On the other hand, an alternative arrangement of the domain I homodimer was reported in which the proposed RNA binding cleft is absent\textsuperscript{150}. It is conceivable that the two dimer conformations observed thus far in crystallized domain I reflect two different functional states of membrane-tethered NSSA and that other configurations (even monomeric) could exist. Domain II is poorly conserved, but is required for RNA replication\textsuperscript{151}. It is able to bind NS5B and protein kinase R (PKR), which is an IFN-induced gene product. It also contains the so-called “interferon sensitivity determining region” (ISDR). The ISDR has been correlated with the efficacy of IFN treatment, but this finding remains
controversial\textsuperscript{152}. Domain III is dispensable for replication, but is a key factor for the assembly of HCV virions\textsuperscript{153}. NS5A occurs in a basally (p56) and hyperphosphorylated form (p58), with different putative functions during HCV replication. It is speculated that hyperphosphorylation serves as a molecular switch between replication and assembly\textsuperscript{154}. Mutations in NS5A that enhance viral replication reduce NS5A hyperphosphorylation\textsuperscript{155}. NS5A exerts multiple functions by interacting with viral and cellular proteins. It was shown that NS5A interacts with NS5B and that this interaction is essential for viral replication. Furthermore, NS5A was found to interact with Core protein onto lipid droplets\textsuperscript{156}. This interaction seems to be required for viral assembly. NS5A also interacts with several cellular signal cascades\textsuperscript{157}. The best studied interaction is that of NS5A and the IFN signaling pathway. This interaction includes the binding and inactivation of PKR. PKR is activated by binding to dsRNA, an intermediate formed during viral RNA replication. When PKR is activated, it shuts down protein translation. Furthermore, NS5A is also thought to interact with pathways of cell proliferation, apoptosis, stress-response and others.

Very recently, many groups has demonstrated the physical and functional interaction between NS5A and the phosphatidylinositol 4-kinase PI4KIII\textgreek{a}. The interaction between NS5A and PI4KIII\textgreek{a} was observed for the first time in a Yeast two-hybrid screening\textsuperscript{158}. Then, a number of groups have used small interfering RNA (siRNA) screens to identify cellular cofactors of HCV infection. Although there has been notable divergence in the array of host cofactors of HCV infection
identified in these screens, a remarkable constant has been the identification of phosphatidylinositol (PI) 4-kinase III alpha (PI4KIIIα) as an essential cofactor of HCV RNA replication\textsuperscript{159-162}. This finding is further supported by data showing that the kinase inhibitors wortmannin or LY294002, used at doses known to favor inhibition of type III PI4Ks, significantly decreased HCV replication\textsuperscript{159, 161}. PI kinases and the lipids they phosphorylate are essential regulators of membrane trafficking and protein sorting. PI4KIIIα is one of four cellular PI 4-kinases that all function to phosphorylate phosphatidylinositol at the 4 position of an inositol head group, which then serves as a beacon to recruit proteins containing lipid-binding motifs with affinity for PI4P (reviewed in references \textsuperscript{163}, \textsuperscript{164} and \textsuperscript{165}). The PI 4-kinases differ with respect to their subcellular localization, thus generating pools of PI4P at specific membrane compartments. PI4KIIIα is the predominant endoplasmic reticulum (ER) resident PI 4-kinase\textsuperscript{163}. In HCV-infected cells, PI4KIIIα colocalizes with HCV NS5A and viral double-stranded RNA, the HCV replication intermediate, suggesting a role in replication complex formation or function\textsuperscript{159}.

All positive-stranded RNA viruses reorganize cellular membranes to create their sites of replication. In the case of HCV, infection produces an accumulation of heterogeneous, ER-associated vesicular structures that has been termed the membranous web\textsuperscript{147, 166, 167}. This is thought to be the site of HCV replication, since de novo-synthesized viral RNA colocalizes with these structures\textsuperscript{166}. The expression of NS4B in the absence of other viral proteins produces structures reminiscent of the membranous web\textsuperscript{147}, suggesting that it is a primary mediator of
membrane reorganization. PI4KIIIα is also implicated in HCV replication complex formation. siRNAs that reduce PI4KIIIα accumulation appear to perturb membranous web formation. In cell lines expressing the HCV polyprotein, PI4KIIIα siRNAs alter the localization of the HCV NS5A protein, while membranous webs are difficult to detect in HCV-infected cells pretreated with PI4KIIIα siRNAs. This led to the hypothesis that HCV may hijack PI4KIIIα to generate PI4P, which would serve to nucleate viral and cellular proteins to ER membranes for HCV replication complex formation.

The NS5B protein is a RNA-dependent RNA polymerase (RdRp), which catalyzes the synthesis of a complementary minus-strand RNA, using the viral RNA genome as a template, and the subsequent synthesis of genomic plus-strand RNA form this minus-strand RNA template. The HCV RdRp can initiate the synthesis of viral RNA de novo, without the need of a primer, which is also thought to be the mode of action in vivo. It has the typical polymerase structure which is frequently compared with a right hand, where the palm domain contains the active site of the enzyme and where the fingers and the thumb are responsible for the interaction with the RNA. Specific characteristics of the viral RdRps include the fingertips; these are two loops that extend from the finger domain and that make contact with the thumb domain. These fingertips completely encircle the active site, ensuring a closed conformation. The catalytic site in the palm domain is the most conserved region in the RdRp. In contrast, the thumb domain is the most diverse domain among viral RdRps. The HCV thumb domain contains protruding extension that folds back into
the molecule. These features make primer-dependent initiation impossible. A conformational change to an “open” form is essential to switch between initiation and elongation. The C-terminal part of the RdRp anchors the protein to the membrane. This C-terminal part is dispensable for RdRp activity in vitro, but indispensable for RNA replication in cells.

2. Genomic heterogeneity and classification systems of HCV

The HCV genome shows remarkable sequence variation generated by the lack of proofreading activity by the RNA polymerase and a high in vivo productive rate of an estimated 10^12 virions per day. To encompass this diversity, HCV has been classified in genotypes and subtypes. Initially, genotypes were considered to differ more than 20% at the nucleotide level and more than 15% at the amino acid level. However, phylogenetic analyses indicated that genotype 10 is closely related to genotype 3 and genotype 7, 8, 9 and 11 to genotype 6. Therefore, a new consensus nomenclature system was proposed to be used for HCV classification. According to this system, HCV is classified into genotypes on the basis of < 70% similarity of nucleotide sequence and phylogenetic relationship. The more closely related HCV strains (75-80% sequence similarity) within some genotypes are designated subtypes. Genotypes are numbered in order of discovery and subtypes are assigned lowercase letters (Table 1). Nowadays, HCV is classified into 6 major genotypes and more than 90 subtypes.

Sequence variation between the HCV genomes was on average 0.9%, indicative of the presence of HCV quasispecies in hepatitis C.
patients. Furthermore, sequence heterogeneity was distributed throughout the genome except in the 5’UTR, while diversity was higher (1.6%) in the envelope genes than the others. Currently, intensive sequencing of HCV genomes is being conducted and nearly 50,000 HCV sequences, including 507 full-length genomes, have been deposited in generic data banks so far. To perform sequence classification, annotation, and analysis of such a large collection, several sequence databases are dedicated specifically to HCV, such as Los Alamos HCV database (http://hcv.lanl.gov/content/hcv-index), the hepatitis C virus database (http://s2as02.genes.nig.ac.jp/) and HCVDB (http://euhcvdb.ibcp.fr/euHCVdb/jsp/index.jsp).

Table 1. Genotypes/subtypes that are presently defined in the database

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<th>Genotype</th>
<th>Assigned subtypes</th>
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<tr>
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</tr>
<tr>
<td>3</td>
<td>a, b, c, d, e, f, g, h, i, k, l</td>
</tr>
<tr>
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<td>a, b, c, d, e, f, g, h, k, l, m, n, o, p, q, r, s, t</td>
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<tr>
<td>5</td>
<td>a</td>
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<td>6</td>
<td>a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t</td>
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3. HCV lifecycle

Insights in the HCV lifecycle, and thus potential antiviral targets, have long been hampered by the lack of efficient cell culture systems. However the generation of subgenomic HCV replicons, the HCV pseudoparticle model and more recently infectious HCV culture models were landmark of developments that helped the understanding of the lifecycle of HCV and drug development.
Fig. 5. Schematic representation of the lifecycle of hepatitis C virus.

Virus binding and internalization (a); cytoplasmic release and uncoating (b); IRES-mediated translation and polyprotein processing (c); RNA replication (d); packaging and assembly (e); virion maturation and release (f). The topology of HCV structural and non-structural proteins at the endoplasmic reticulum membrane is shown schematically. HCV RNA replication occurs in a specific membrane alteration, the membranous web. Note that IRES-mediated translation and polyprotein processing, as well as membranous web formation and RNA replication, which are illustrated here as separate steps for simplicity, might occur in a tightly coupled fashion. IRES, internal ribosome entry site. Adapted from\textsuperscript{114}.

3.1. HCV entry

HCV entry is a complex and multi-step process requiring a set of entry proteins\textsuperscript{176}. In the early stage of HCV entry, HCV virions are concentrated at the cell surface by non-specific attachment factors. Glycosaminoglycans seem to be the initial attachment sites for HCV. Also the LDL receptor is thought to be involved in early attachment of HCV, although this finding remains somehow controversial. Following initial attachment, HCV virions bind virus-specific receptors. Currently, a number of receptors essential for HCV entry are identified: the
scavenger receptor class B type I (SR-BI), the tetraspanin CD81, and tight junction proteins claudin-1 (CLDN1) and Occludin. Very recently, new players in HCV entry were uncovered: receptor tyrosine kinases (RTKs).

HCV associates with very-low-density and low-density lipoproteins (LPs) in the bloodstream. It binds the basolateral surface of hepatocytes through glycosaminoglycans (GAGs) and the low-density lipoprotein receptor (LDLR). Both SR-BI and CD81 bind HCV glycoprotein E2 and are necessary but not sufficient for HCV entry. SR-BI is a lipoprotein receptor that is responsible for the selective uptake of cholesteryl esters from HDL (high density lipoprotein) particles. In addition, SR-BI plays an important role in VLDL catabolism. SR-BI acts as a post-binding receptor, since SR-BI antibodies inhibit HCV infection when added 60 min following virus binding. CD81 belongs to the family of tetraspanins which are ubiquitously expressed. Similar to SR-BI, it acts as a post-binding entry factor, since anti-CD81 antibodies inhibit HCV infection only after virus attachment. Furthermore, CD81 actively promotes infection upon HCV binding by triggering signaling cascades that are important for virus entry. Very recently, RTKs were identified as cofactors required for HCV entry, and it was shown how pharmacological blockage of these molecules can prevent viral infection in vitro and in vivo. Clinically approved inhibitors exist for two of these kinases: epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2). EGFR activation promotes CD81–CLDN1 interactions and membrane fusion, thereby enhancing productive HCV uptake. RTKs, upon dimerization and autophosphorylation, provide
signals inducing proliferation, migration, morphogenesis and other fundamental cellular processes\textsuperscript{181}. CLDN1 is predominantly expressed in the liver, where it forms networks at tight junctions. Silencing of CLDN1 inhibits HCV infection in susceptible cells\textsuperscript{179}. Nevertheless, there is still no evidence for a direct interaction between the virus and CLDN1. CLDN1 plays a role in the post-binding phase subsequent to HCV binding to CD81 and SR-BI. Analogous to other viruses like coxsackievirus B, it is speculated that the interaction with CLDN1 takes place after migration of the virus-CD81/SR-BI complex to tight junctions\textsuperscript{182}. Occludin is expressed in tight junctions of polarized cells and is also involved in the post-binding phase of HCV entry\textsuperscript{180}.

Engagement by the virus of SR-BI, the tetraspanin CD81 and the tight junction proteins claudin-1 and occludin leads to clathrin-mediated endocytosis of virions\textsuperscript{183}, delivering HCV-receptor complexes to early endosomes. The low pH in the endosome triggers fusion of the virion envelope with the membrane of the early endosome particle. Membrane fusion is catalyzed by fusion peptides embedded in the viral glycoproteins. For related viruses like flaviviruses, the structural basis for low pH-induced membrane fusion has been elucidated\textsuperscript{184}. The envelope proteins of these viruses contain an internal fusion peptide, which is exposed during membrane reorganization mediated by low pH (class II fusion). The scaffolds of class II fusion proteins are remarkably similar. Therefore it has been suggested that the entry of all \textit{Flaviviridae}, including HCV, may include a class II fusion step. The nature of the fusion protein of HCV is not known yet. There is some evidence that E2 is the fusion protein; however, also E1 has been
suggested as possible fusion protein. Following membrane fusion, the viral RNA is released into the cytosol\textsuperscript{185}. How the viral RNA is exactly released from the nucleocapsid is not known. It was shown for other positive strand RNA viruses (alphaviruses) that binding of ribosomes to the viral genome supports RNA release\textsuperscript{186}.

3.2. Translation and replication

The released positive RNA genome is translated by a cap-independent mechanism at the rough ER by the host machinery into one polyprotein. This translation process is mediated by the IRES located in the 5’ UTR. The translated polyprotein is cleaved co- and post-translationally by cellular and viral proteases into ten different proteins. Similar to related viruses, expression of HCV proteins triggers intracellular membrane alterations\textsuperscript{166}. This specific virus-induced membrane structure is referred to as the membranous web. The formation of the membranous web can be induced by solely expressing HCV NS4B\textsuperscript{147}. The membranous web serves as a scaffold for the assembly of the viral replication machinery. Based on analogy of the replication of related viruses like Dengue virus, it is hypothesized that the viral replication complexes are located in invaginations of ER-derived membranes\textsuperscript{187, 188}. Viral RNA is thereby protected from degradation and recognition by intracellular sensors such as RIG-I is avoided. However, this hypothesis remains to be confirmed for HCV. Newly synthesized positive HCV RNA can either be used for translation, replication or encapsidation.
3.3. Assembly and release

Newly synthesized HCV RNA genomes can be assembled into new virions. Viral proteins involved in this assembly process are the core protein, the envelope proteins E1 and E2, viroporin p7, and non-structural proteins NS2 and NS5A. Lipid droplets and the VLDL assembly pathway are major contributors of the host cell to virion assembly. The mature HCV virion consists of a nucleocapsid, composed of core proteins and the viral genome, and an outer envelope, containing a lipid membrane and the envelope proteins. The early stage of assembly, i.e. nucleocapsid formation, involves oligomerization of the core and encapsidation of the viral genome. Currently it is not clear where the nucleocapsid formation initiates. In a first model, nucleocapsid formation is initiated at the surface of cytosolic LDs. Viral RNA is delivered to the core protein via NS5A that is also mobilized onto LDs or remains bound to the ER. In a second model, assembly initiates at the ER membrane. Core protein is initially transferred to LDs but is re-recruited to ER membranes at the assembly sites where it interacts with NS5A.

Very recently, it was demonstrated that a concerted action of HCV p7 and NS2 regulates Core localization at the ER and virus assembly. The JFH1 HCV strain replicates and assembles in association with LD-associated membranes, around which viral core protein is predominantly detected. In contrast, despite its intrinsic capacity to localize to LDs when expressed individually, they found that the Core protein of the high-titer Jc1 recombinant virus was hardly detected on
LDs of cell culture-grown HCV (HCVcc)-infected cells, but was mainly localized at ER membranes where it colocalized with the HCV envelope glycoproteins. Furthermore, high-titer cell culture-adapted JFH-1 virus, obtained after long-term culture in Huh7.5 cells, exhibited an ER-localized core in contrast to non-adapted JFH-1 virus, strengthening the hypothesis that ER localization of core is required for efficient HCV assembly. They also demonstrated that p7 and NS2 are HCV strain-specific factors that govern the recruitment of core protein from LDs to ER assembly sites. Indeed, using expression constructs and HCVcc recombinant genomes, they found that p7 is sufficient to induce Core localization at the ER, independently of its ion-channel activity. Importantly, the combined expression of JFH1 or Jc1 p7 and NS2 induced the same differential core subcellular localization detected in JFH1- vs. Jc1-infected cells. Finally, results obtained by expressing p7-NS2 chimeras between either virus type indicated that compatibilities between the p7 and the first NS2 trans-membrane domains is required to induce core-ER localization and assembly of extra- and intra-cellular infectious viral particles.

Another group studied the recruitment of core from LD into nascent virus particles. To investigate the kinetics of core trafficking, they developed methods to image functional core protein in live, virus-producing cells. During the peak of virus assembly, core formed polarized caps on large, immotile LDs, adjacent to putative sites of assembly. In addition, LD-independent, motile puncta of core were found to traffic along microtubules. Importantly, core was recruited
from LDs into these puncta, and interaction between the viral NS2 and NS3-4A proteins was essential for this recruitment process\textsuperscript{192}.

The late stage of assembly, i.e. the maturation and release of HCV virions, is closely associated with the VLDL pathway\textsuperscript{193, 194}. The nucleocapsid can be incorporated in the precursor of VLDL, the luminal LDs. How the envelope proteins are incorporated into virions is not clear. Recently it was reported that NS2 might “crosstalk” between envelope glycoproteins and non-structural proteins by forming multiple protein-protein interactions\textsuperscript{133}. Mature HCV virions contain apoB, apoE and eventually other apolipoproteins. After maturation, HCV virions are transported and secreted along the VLDL secretory pathway. Therefore, the structure of HCV virions resembles that of triglyceride-rich lipoproteins containing a core of neutral lipids surrounded by a phospholipid monolayer and stabilized by apolipoproteins\textsuperscript{195}.

4. The discovery of HCV defective particles

HCV subgenomes were firstly isolated from four patients among 23 chronic active hepatitis C patients, in two cases HCC, and in one case acute fulminant hepatitis following liver transplantation\textsuperscript{196}. The regions of deletions in individual subgenomes differed from each other; however, they all shared common structural features. They contained in-frame deletions from E1 to NS2, and retained complete sequences for both core protein and NS2 metal-dependent cysteine protease domain, encoding the entire NS3-NS5B proteins that are essential for RNA replication. The HCV polyprotein encoded by the subgenome was
processed as the standard HCV polyprotein generating core, a
glycosylated E1-NS2 fusion protein that was susceptible to
endonuclease H treatment, and full size NS3 in cells carrying the cDNA
of the subgenome$^{196}$. These data suggested that the cysteine protease
activity of NS2 should be involved in the processing of HCV
polyproteins from the HCV subgenome, and emphasized the
importance of domain preservation in the HCV subgenome.

The first question that arises after the discovery of HCV subgenome
was if they could work as DI (defective interfering) genomes. DI viruses
are subgenomic mutants derived from the parental virus that lack the
essential viral components for virus particle production; the DI virion is
therefore produced only under certain conditions. The replication of DI
virus is trans-complemented with the materials essential for virion
production from a helper virus, which is usually the parental virus. DI
viruses often interfere with parental virus replication by competing
with the materials essential for replication and/or by their advantages
in replication, they can also facilitate the establishment and
maintenance of persistent viral infection of cells$^{197}$.

The HCV subgenomes have a close resemblance with those of
naturally-arisen DI genomes identified in Flaviviridae. DI genomes have
been identified in pestiviruses, bovine viral diarrhea virus (BVDV)$^{198}$,
$^{199}$, classical swine fever virus (CSFV)$^{200}$ and a flavivirus Murray Valley
encephalitis virus (MVE)$^{201}$. The structures of MVE DI genomes were
most similar to those of the HCV subgenomes.

Synthetic subgenomic RNA replicons of HCV indicated that NS3-
NS5B proteins are both essential and sufficient viral components for
HCV RNA replication\textsuperscript{202} and, with the exception of NS5A, these elements cannot be complemented in trans due to a limited trans-complementation of viral components in RNA replication of other flaviviruses\textsuperscript{203}. In cell cultures it was possible to replicate artificial HCV subgenomic RNA with E1 and E2 sequence deletions\textsuperscript{204, 205}. It was demonstrated that they can replicate autonomously in hepatoma cells\textsuperscript{206}.

Long term (at least 2 years) persistence of the HCV subgenome in a patient’s blood with the standard HCV genome, and the approximate 120-day life of hepatocyte, indicate that the HCV subgenome circulates in blood and re-infects naïve hepatocytes. The fact that virus RNA and the core antigen in the serum of patients with an HCV subgenome were fractionated approximately 1.16 g/ml after buoyant density gradient centrifugation\textsuperscript{196} suggested that the HCV subgenome should be packaged within a virion. The HCV RNA replication lacking envelope proteins did not produce an infectious virus\textsuperscript{204}. All these data indicate that the standard HCV could function as a helper virus and trans-complement the envelope proteins, E1 and E2. This hypothesis was recently demonstrated in vitro by Pacini et al\textsuperscript{206}.

Data collected so far about the ratio of HCV subgenome titer to those of the standard genome indicate that they were approximately 5:1\textsuperscript{196}. It indicates that the competition of the envelope proteins and the advantage of RNA replication of the HCV subgenome would cause interference with standard HCV replication. The 20\% shortage in length of the HCV subgenome might give the subgenome an advantage
in virus replication, while the processing rate of HCV RNA polymerase is independent of the sequence.

The HCV subgenome offers clues to HCV packaging pathways. It was shown that core proteins assembled to form a capsid-like structure, and that core-E1-E2 did package HCV RNA to form virus-like particles (VLP) in many types of cells\textsuperscript{207}. The presence of HCV subgenome circulating in the blood also suggests that the HCV subgenome lacking an E1-E2 sequence should be packaged within a virion. Based on these observations, it should be reasonable to predict that one of the packaging signal sequences of HCV RNA should be localized in the core sequence.

According to the data collected so far, it should be noted that the ratio of HCV subgenome per HCV standard genome in liver is significantly higher than those in blood, suggesting that the subgenome packaging efficiency could be lower than that of the standard genome, or that the number of cells infected with both genomes might be limited. It is also possible that the coupling of RNA replication with packaging of the RNA genome affects HCV packaging efficiency\textsuperscript{208}.

The comparison between the HCV subgenomes and the MVE DI subgenomes illustrates another interesting feature of DI genomes: both subgenomes carry entire core sequences. The coincidence of these structural features suggests the importance of the core region in viral replication. Although one possible interpretation is the packaging signal, as discussed below, there remain other possible explanations for the core sequence preservation in the subgenomes: core is
essential for correct processing of the following proteins in the context of the HCV subgenome, or core might be required for RNA recombination to generate subgenomes.

The heterogeneous nature of HCV particles in patients suggested another pathway for HCV subgenome packaging. Nucleocapsids without envelopes were demonstrated in hepatitis patients, in addition to particles comprising HCV core protein, HCV RNA and lipoproteins in very low-density fractions of the patient’s serum. HCV core proteins also interact with HCV RNA and assemble into capsid-like particles. These findings suggest that the HCV subgenome might be released without the assistance of a helper virus.

The HCV subgenomes highlight an inconsistency with respect to the p7 gene product. Although p7 was thought to be indispensable for infection in a chimpanzee intrahepatic transfection system, the HCV subgenome is a replication entity. How might this apparent discrepancy be explained? In the VLP production system, using hepatoma cells infected with recombinant baculovirus, efficient expression and secretion of structural proteins from cells was achieved with p7 and NS2; however, this was not the case in the VLP production system using insect cells. Almost all the issues mentioned in this section should be addressed by investigations using another reliable model system to monitor viral production, such as an in vitro HCV replication system.

From the molecular point of view, many questions are still opened about how HCV subgenomes arise from the standard genome. Phylogenetic analysis and blast searches of quasispecies of HCV
subgenomes and the coexisting standard genomes suggest that they share common ancestral HCV quasispecies\textsuperscript{196}. Other types of HCV subgenomic mutations were recovered from five cases among 23 hepatocarcinoma patients. Almost all were minor populations of the HCV RNA quasispecies in patient specimen, and had out-of-frame deletions that were not conserved between isolates. However, in their putative parental HCV RNA, nearly identical sequences (one or two nucleotides in length) were found in the deletion donor and acceptor regions\textsuperscript{196}. These homologous sequences could serve the cross-over sites where strand switching occurs during RNA genome replication, which was one of the proposed models of the RNA virus recombination\textsuperscript{214}. Cross-over sites in short stretches of similar sequences were implicated to be involved in BVDV RNA recombination\textsuperscript{215}. In the case of the HCV subgenomes, there is no such identical sequence found at the deletion boundaries in the putative parental genome. It has been observed that RNA recombination often occurred around the regions with a higher ordered RNA secondary structure, which hindered the RNA replication\textsuperscript{214}. A computational analysis predicted the conserved RNA structures in both core and NS5B sequences\textsuperscript{216}. The functions of sequences with a conserved folding motif in the core are controversial; however, these sequences might also be involved in RNA recombination and packaging events. Intergenotypic HCV recombinants exhibiting cross-over near the NS2-NS3 junctions have been isolated\textsuperscript{217}, and the recombinants between DI and the standard genome were reported in BVDV\textsuperscript{215}. Recombination
between the HCV subgenome and the standard genome might occur since both genomes retain the region of intergenotypic recombination.

**Fig. 6. Schematic representation of the lifecycle of the HCV subgenome.**

Subgenomic mutations arise from HCV standard genome. These mutations will not persist while they lack the ability for autonomous RNA replication. Once generated, the HCV subgenomes start to replicate autonomously. They are packaged into virions with envelope proteins transcomplemented by the standard HCV genome (lower panel) and thereafter circulate in the patient’s blood. Although the HCV defective particle can infect naïve cells and replicate autonomously, it will not exit the hepatocytes (middle panel) without coinfection with the helper virus. WT: HCV complete virion; DI: defective interfering HCV particle.

The HCV subgenome life cycle is proposed in Fig. 6. The necessary features of an infectious HCV subgenome are the following: the ability for autonomous RNA replication and for being packaged into a virion. It is questionable whether the HCV RNA replicon, a synthetic subgenomic RNA lacking core, will be packaged into a virion by superinfection with HCV carrying the standard genome. Very recently, Pacini et al modified the infectious isolate JFH-1\textsuperscript{204, 205, 218} in order to
recapitulate in vitro the genetic structure of two of the most representative in-frame natural subgenomic deletion-containing RNAs found circulating in patients. Using this system, they analyzed natural subgenomic variants for their ability to replicate autonomously and demonstrated that the natural subgenomic deletion mutants are replication competent and are trans-packaged into infectious virions when coexpressed together with the wild-type virus. Furthermore, their data suggest that the presence of the NS2 protease domain is required in order to generate the correct NS3 N terminus, required for RNA replication. Unexpectedly, the presence of NS2 generates, in turn, a strict cis requirement for the core region in order to allow efficient trans-packaging of the subgenomic RNA, revealing a complex interplay between the NS2 and the core viral genes.

5. Host-pathogen interactions

Viral lifecycle as that of HCV completely relies on host cell infrastructure, presupposing that the virus has evolved mechanisms to utilize and control all cellular molecules or pathways required for viral lifecycle. Hence, HCV must have acquired the ability to gain access to key pathways controlling processes, such as cell growth, apoptosis and protein synthesis, which are all considered to also be crucial for liver regeneration. This occurs in a balanced way permitting persistent replication of viral genomes and production of infectious particles without endangering host cell viability and survival. In particular during the last decade, accumulating evidence indicates that HCV utilizes signaling pathways of the host with major impact on cellular growth,
viability, cell cycle or cellular metabolism, such as epidermal growth factor-receptor mediated signals, the PI3K/Akt cascade or the family of Src kinases. Furthermore, HCV specifically interacts with parts of the cellular machinery involved in protein translation, processing, maturation and transport, such as components of the translation complex, the heat shock protein family, the immunophilins or the vesicle-associated membrane protein-associated proteins A and B (VAMP-A and B)\(^{219}\).

5.1. Lipid metabolism

A particular hallmark of HCV is the intimate link with the host lipid metabolism. HCV virions were found to circulate in the blood in complex with lipoproteins\(^{220}\). These lipoviroparticles (LVP) are highly enriched in triglycerides and contain apoB and apoE\(^{210}\). The circulating LVPs are a heterogeneous population with buoyant densities ranging between 1.03 to 1.2 g/ml. The infectivity of HCV-positive inocula is inversely correlated with the buoyant density of LVPs\(^{221}\).

Since HCV virions are associated with host lipoproteins, it seems logical that HCV entry is connected to the host lipid metabolism. Lipoprotein receptors (LDL receptor, SR-BI receptor) have been reported to be involved in HCV entry. Furthermore, ligands of SR-BI can modulate HCV entry. For instance, HDL enhance HCV entry, whereas oxidized LDL inhibits HCV entry\(^{222}\). Also the lipid composition of the plasma membrane influences HCV entry. Cholesterol depletion from the plasma membrane decreases HCV entry by reducing the expression of CD81 receptors at the cell surface\(^{223}\).
HCV exploits the VLDL assembly and secretion pathway to be released from hepatocytes. Furthermore, it has been shown that lipid droplets (LDs) play an important role in HCV virion assembly. LDs are intracellular storage vesicles that contain triacylglycerols and cholesteryl esters surrounded by a phospholipid monolayer. HCV core protein accumulates around these LDs. This association is a peculiarity of the Hepacivirus genus in the Flaviviridae family and is essential for the recruitment of other viral proteins like NS5A and NS3 and for virus production\textsuperscript{224}.

HCV also requires elements of the cholesterol and fatty acids biosynthetic pathways for efficient replication. Fatty acids can stimulate or inhibit HCV replication depending on their degree of saturation. Polyunsaturated fatty acids inhibit HCV replication, whereas saturated and mono-unsaturated fatty acids stimulate it\textsuperscript{225}. Fatty acids can be used as prosthetic groups that may facilitate the anchoring of viral non-structural proteins to membranes. For example, NS4B undergoes palmitoylation at two cysteine residues in the C-terminal region. These lipid modifications were shown to be necessary for protein-protein interactions\textsuperscript{226}. Thus, palmitoylation can be a post-translational modification required for the life cycle of HCV.

Statins, which are widely used for the treatment of hypercholesterolemia, have also been reported to inhibit HCV replication. Statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis in the liver. The HMG-CoA reductase catalyzes the conversion of HMG-CoA into mevalonic acid. Besides their cholesterol-
lowering effect, statins were shown to inhibit the replication of HCV subgenomic genotype 1b replicons and to suppress RNA replication of infectious JFH-1 HCV. The precise mechanism of the anti-HCV activity of statins has not yet been unraveled. The anti-HCV activity of statins may result from inhibition of geranylgeranylation of cellular proteins rather than the inhibition of cholesterol synthesis. Geranylgeranylation is a post-translational modification that covalently attaches geranylgeranyl groups to various cellular proteins to facilitate their membrane association. These geranylgeranyl groups are isoprenoids synthesized in the cholesterol biosynthesis pathway. More recently, FBL2 has been reported to be a host target for geranylgeranylation. Geranylgeranylation of FBL2 appears to be critical for HCV replication because the association between FBL2 and NS5A, an interaction that is a prerequisite for HCV replication, depends on geranylgeranylation of FBL2. In vivo results of statin monotherapy for the treatment of HCV infected patients have yielded conflicting results so far. Neither atorvastatin (after conventional 12-week therapy) nor rosuvastatin resulted in a reduction in viral load. Nevertheless, fluvastatin was found to inhibit HCV RNA replication in HCV infected patients in a recently published study. The drug was well tolerated and resulted at relatively low doses (20-80 mg daily) in a transient reduction in viral load (-0.5 to -1.75 log10, 2-5 weeks). However, higher doses of fluvastatin did not reduce viral load. A recent retrospective analysis demonstrated that statin use in combination with PEG-IFN and ribavirin was associated with a significantly higher sustained virological response (SVR) and with a
higher viral response at week 4 and 12\textsuperscript{232}. A pilot study evaluating the combination of fluvastatin (20 mg daily) with PEG-IFN/ribavirin revealed that fluvastatin could be used safely to increase the response to PEG-IFN and ribavirin\textsuperscript{233}. There is today obviously no compelling evidence that statins, used in monotherapy, may result in a marked reduction in HCV viral load in chronically infected patients. However, statins may have the potential to increase the efficacy of current or future HCV therapy.

The importance of HCV and host lipid interactions has also been demonstrated clinically. Hepatitis C infection is associated with decreased cholesterol and LDL levels. When HCV is cleared by antiviral treatment, serum cholesterol levels are elevated again\textsuperscript{234}. It is hypothesized that HCV, by interrupting cholesterol synthesis and using host lipids for replication, decreases circulating lipids. Furthermore, the prevalence of steatosis in chronic hepatitis C is approximately 40\%\textsuperscript{235}.

5.2. Cellular stress, apoptosis, cell cycle

Apoptosis is central for the control and elimination of viral infections. In HCV-infected liver, however, despite enhanced hepatocyte apoptosis, viral persistence is observed.

Viral proteins interfere with the cellular apoptotic signaling pathway and block key cellular elements of the host cell. Until recently, the lack of an infectious HCV tissue culture system did not allow studying the impact of HCV infection on hepatocyte apoptosis. Overall, the data regarding the role of different HCV proteins are controversial and
Ascribe to a given viral protein pro- and anti-apoptotic effects, depending on the experimental system used. Since in most models viral proteins are overexpressed by non-viral promoters, for virtually all HCV proteins a pro-apoptotic effect has been described. Apart from the unphysiological expression of viral proteins, these models further lack the balance of intracellular viral expression of the different HCV proteins and their interactions. Especially in HCV infection, intracellular viral protein expression is very low\textsuperscript{236}.

Further, HCV is genetically highly variable and exists as quasispecies in a given patient. Different pro- and anti-apoptotic effects of the HCV core protein from an individual patient have been described\textsuperscript{237}, suggesting special properties of different quasispecies proteins. These protein differences may explain in part the different effects of viral proteins on apoptosis. Studies of the contribution of genotypes or quasispecies to the effects on apoptosis are largely missing. Further, experiments designed to study the impact of HCV infection on hepatocyte apoptosis must also consider the interactions between the different HCV proteins. Therefore, only models based on the complete and infectious virus may reflect to some extent the in vivo situation.

Very recently, gene expression profiling during multiple time-points of acute HCV infection of cultured Huh-7.5 cells was performed to gain insight into the cellular mechanism of HCV-associated cytopathic effect. Maximal induction of cell-death–related genes and appearance of activated caspase-3 in HCV-infected cells coincided with peak viral replication, suggesting a link between viral load and apoptosis. Gene ontology analysis revealed that many of the cell-death genes function
to induce apoptosis in response to cell cycle arrest. Labeling of dividing cells in culture followed by flow cytometry also demonstrated the presence of significantly fewer cells in S-phase in HCV-infected relative to mock cultures, suggesting HCV infection is associated with delayed cell cycle progression. Regulation of numerous genes involved in antioxidative stress response and TGF-b1 signaling suggest these as possible causes of delayed cell cycle progression. Significantly, a subset of cell-death genes regulated during in vitro HCV infection was similarly regulated specifically in liver tissue from a cohort of HCV-infected liver transplant patients with rapidly progressive fibrosis. Collectively, these data suggest that HCV mediates direct cytopathic effects through deregulation of the cell cycle and that this process may contribute to liver disease progression238.

5.3. Other host factors important for HCV lifecycle: results from siRNA screenings and other studies – The emerging role of PI4KIIα

Like all viruses, HCV relies heavily on the host cell to replicate. As such, there is significant interest in trying to identify cellular genes that are required for HCV infection, both to understand the basic biology of the HCV life cycle and to unearth potential new therapeutic targets.

Small or short interfering RNAs (siRNAs) mediate the degradation of complementary mRNA that results in a specific silencing of gene expression. siRNA mimics cellular miRNA that consists of small double stranded RNA strands and regulates gene expression by degrading the complementary mRNA. A key protein for the generation of these
molecules is DICER, an RNase enzyme that is responsible for the production of short interfering RNAs and microRNAs. Specific siRNAs targeting the expression of individual cellular cofactors are successfully applied as tools to study the impact of defined host factors for HCV entry and replication. Furthermore, functional RNA interference (RNAi) screens have been shown to represent a powerful tool to identify novel cellular cofactors for pathogen-host interactions.

Up to now, nine limited and two genome-wide siRNA screens designed to identify cellular cofactors required for HCV infection and based on different HCV replication models have been performed. However, the overlap of identified genes is very low, which may be due to different experimental conditions, reagents, or hit calling criteria.

There are numerous reasons for the varied results, including differences in RNAi libraries, cells, transfection protocols, HCV genotypes and HCV replication systems (sub-genomic replicons versus infectious HCV).

The most consistent hit identified by all siRNA screens as HCV dependency factor was phosphatidylinositol 4-kinase IIIa (PI4KIIIα). Encoded by the PIK4CA gene and localized primarily to the endoplasmic reticulum (ER), this enzyme is one of four kinases (PI4KIIα, PI4KIIβ, PI4KIIIα, and PI4KIIIβ) in mammalian cells that catalyze the synthesis of PI4P. All four enzymes have different subcellular localizations and regulation mechanisms of their activity state, thus producing distinct PI4P pools inside the cell.
It was proposed that PI4KIIIα plays a role in establishing HCV replication complexes, which consist of the replication machinery married to unique membranous structures induced by HCV infection\textsuperscript{159}. Membrane rearrangements are hallmarks of all positive-stranded RNA virus infections\textsuperscript{249}. In the case of HCV infection, these structures are clusters of intracellular, cytosolic membranes and are a major pathology associated with replication of the viral RNA genome in vivo\textsuperscript{147}. Similar membranous structures are observed using in vitro HCV replication and viral protein expression systems and have been termed “membranous webs”. Evidence that HCV replication proteins and de novo synthesized viral RNA localize to the membranous web, which forms in close proximity to the ER, implicates the webs as sites of viral replication and the ER as a potential membrane source\textsuperscript{166}. It is unclear whether membrane rearrangements serve primarily to provide a high local concentration of replication components, provide a scaffold for replication, or shield the viral RNAs from recognition by the innate immune system. The mechanism of HCV replication complex formation, including intracellular membrane rearrangements, remains to be determined.

The proposal that PI4KIIIα functions in HCV replication complex formation was based on the following data. Treatment with siRNAs and pharmacological inhibition of PI4KIIIα reduces HCV replication. PI4KIIIα is not required for HCV entry or initial translation of the viral genomic RNA. It co-localizes with markers of the HCV replicase, and most importantly, membranous webs fail to accumulate in cells that have been silenced for PI4KIIIα expression\textsuperscript{159}. A role for PI4KIIIα in
forming replication complexes is also supported by elegant studies from Tai et al. who show that the HCV replicase-associated NS5A protein has aberrant localization in cells that have inducible expression of the full HCV polyprotein and have been silenced for PI4KIIIα expression. On the basis of these results, it was proposed a model in which infecting RNA genomes are translated at the rough ER, giving rise to high amounts of polyprotein. NS5A generated by polyprotein cleavage binds to the kinase in a domain I-dependent manner and recruits the enzyme to ER-derived membranes. Binding of NS5A to the kinase stimulates its activity, resulting in high levels of PI4P at these membrane sites. Those membranes thus obtain a PIP signature, which might contribute directly to membrane properties or recruit viral or host factors required for proper architecture and functionality of the membranous web.

The main question remaining is the mechanism of HCV replication complex formation. RNAi analysis has identified a number of cellular candidates that may be involved in constructing membrane-associated sites of replication.

These include early endosomes (EEA1, RAB5 and PIK3C2G), late endosomes (RAB7A, RABEPK), vesicles associated with the Golgi apparatus (COPI components and RAB7L1), and the ER (PIK4CA, TBC1D20 and RAB1B). Components of vesicular trafficking and membrane fusion (VAP-A and VAPB) and regulators of actin reorganization (CDC42 and ROCK2) may also be involved. A role in replication complex formation for EEA1, RAB5, RAB7, COPI subunits, PIK4CA, TBC1D20, VAP-A, VAP-B and CDC42 is further
supported by microscopy, proteomic and/or protein biochemistry studies\textsuperscript{158, 159, 251}. These cofactors, in addition to others, may work in combination to establish the membrane-associated replication complexes in infected cells.

One hypothesis is that phosphorylation of PI molecules by PI4KIII\(\alpha\) and subsequent downstream modifications of PI4P attract cellular and/or viral proteins to phospholipids. This serves to nucleate replication proteins and membrane-bound vesicles, potentially establishing the membranous web, which appears to be a non-uniform, heterogeneous mix of vesicles. Additionally, PI4KIII\(\alpha\) itself may be directly required for establishing and maintaining an intimate interaction of the HCV replicase with cellular membranes. This is suggested from yeast two-hybrid analysis wherein PI4KIII\(\alpha\) interacted with HCV NS5A\textsuperscript{158}.

In addition to the interest in the biology of PI4KIII\(\alpha\) in HCV infection, it is possible that PI4KIII\(\alpha\) may be a legitimate drug target for treating HCV infection. Pharmacological inhibitors of PI kinase activity prevent HCV replication in vitro\textsuperscript{159, 161} and PI-3 kinase inhibitors have been successful therapies against certain cancers\textsuperscript{252}. We speculate that inhibitors specific to PI4KIII\(\alpha\) may be successful therapeutics for HCV with fewer issues of resistance than is observed for drugs targeting viral enzymes.

5.4. Inhibition of HCV by compounds that target host factors

The viral replication cycle has three stages: entry, replication, and assembly/release\textsuperscript{114}. All stages depend on various host encoded
factors\textsuperscript{219}. Targeting these host factors is an attractive option for future HCV therapies. The intention is to minimize development of HCV resistance and to enable efficient treatment of all HCV genotypes. This approach has been pursued successfully in the HIV field, where maraviroc (Selzentry, Celsentri), a small molecule compound that binds to the essential HIV co-receptor chemokine receptor type 5 (CCR5) and prevents its interaction with HIV, is now in clinical use\textsuperscript{253}.

**Cell entry.** It has been shown that neutralizing antibodies against CD81 can block HCV infection in vitro and in vivo and are currently in preclinical development\textsuperscript{254}. Prophylactic injection of monoclonal anti-CD81 antibodies prevented infection of human liver-uPA-SCID mice. However once infection had been established, no significant difference in viral load was observed between anti-CD81-treated and control animals (irrelevant antibody). Another approach to inhibiting HCV entry is to develop small molecules targeting one of the entry factors. ITX 5061 is an orally bioavailable small molecule compound that enhances HDL levels in animals and patients by targeting the SR-BI. ITX 5061 has a good safety profile in animal toxicology studies and in clinical studies involving over 250 subjects. This compound exhibits picomolar potency in inhibiting both genotype 1 and genotype 2 HCV presumably by blocking the interaction between the HCV glycoprotein E2 and SR-BI\textsuperscript{255}. Currently, the safety of ITX 5061 is being evaluated in HCV-treatment naive patients (phase 1b). In March 2011, the company iTherx announced that it has also commenced patient recruitment in an open-label, proof-of-concept phase 1b study of ITX 5061 in liver transplant patients with HCV. For CLDN1 and OCLN, efficient
neutralizing antibodies have not yet been developed but due to their essential function in HCV entry both are potential targets analogous to CD81 antibodies.

Such entry blockers are especially interesting in the context of liver transplant where an efficient means of blocking cell entry might help to prevent re-infection of the liver graft after transplantation.

Very recently, it has been reported that erlotinib can block HCV entry at post-binding steps at similar timepoints as anti-CD81 antibodies by inhibition of the activity of EGFR\textsuperscript{181}. Using FRET analyses and tagged entry factors expressed in polarized HepG2 cells it was suggested that EGFR activity is required for the formation of CD81-claudin-1 co-receptor associations in liver-derived cell lines. Furthermore, erlotinib also could control viral spread and dissemination in cell culture. Importantly, treatment of animals with the EGFR inhibitor erlotinib, which is in clinical use for the treatment of non-small cell lung cancer and pancreatic cancer, resulted in a somewhat delayed HCV infection and decreased viral load. However, whether erlotinib has a clinical benefit in the setting of HCV infection that outweighs its side effects has to be evaluated in the future.

**HCV RNA replication.** By the use of siRNA-based and other screening approaches, several dozens of host factors involved in or, in some cases, even essential for HCV RNA replication have been identified in recent years. A representative factor that generated a lot of interest is cyclophilin A (CypA), a cellular cis-trans-prolyl isomerase that is required for HCV RNA replication and probably also assembly\textsuperscript{256,257}. It has been shown in vitro that CypA binds to HCV NS5A and can
facilitate replication through an unknown mechanism. Moreover, it has been shown that HCV is much more sensitive to CypA inhibitors in the presence of an intact NS2/3 junction, indicating that important interactions between HCV and CypA may occur outside NS5A. Importantly, CypA seems to be important for all HCV genotypes. Interestingly, the first compound exerting an inhibitory effect on HCV replication by targeting CypA was the immunosuppressant cyclosporine A. Subsequently, cyclosporine A derivatives without immunosuppressive properties were developed as potential antivirals. The CypA inhibitor alisporivir, formerly known as Debio-025, is currently in a phase III trial for the treatment of treatment-naive HCV genotype 1 patients; patient recruitment for this study is ongoing. At the EASL International Liver Congress in April 2011, results of a phase II study were presented: a triple regimen consisting of PEG-IFN/RBV plus alisporivir for 48 weeks achieved SVR in 76% of the patients compared to 55% in the control arm treated with PEG-IFN/RBV alone. The study involved nearly 300 previously untreated patients infected with HCV genotype 1. Treatment with alisporivir resulted in a low incidence of adverse events, with discontinuation rates comparable between intervention and control groups. As all HCV genotypes seem to be similarly dependent on CypA, alisporivir may offer an effective treatment option for a broad range of HCV genotypes. In fact, alisporivir has also shown antiviral activity against other common HCV genotypes (2-4) in clinical studies. Interestingly, this drug might also have the potential to be important for an interferon-free regimen since cases of SVR after 29 days of alisporivir monotherapy were
reported in HCV genotype 3 infected individuals. Moreover, the genetic barrier for development of viral resistance seems to be very high in comparison to HCV polymerase or protease inhibitors both in vitro and in vivo. Thus alisporivir arguably represents the most advanced and most promising anti-HCV drug targeting a host factor at this point.

Another host factor critical for HCV genome replication has recently been described by several groups: the enzyme phosphatidylinositol-4-kinase III alpha (PI4KIIIα) binds to HCV NS5A and its enzymatic activity is required for efficient HCV RNA replication\textsuperscript{159-161, 243}. Recent data suggest that the direct activation of this lipid kinase by HCV NS5A contributes critically to the integrity of the membranous viral replication complex\textsuperscript{248}. Whether this kinase is also a suitable antiviral target for the treatment of HCV and if it is possible to design a potent inhibitor with tolerable side effects in vivo remain to be determined.

An unusual and scientifically intriguing host encoded antiviral target is the microRNA 122 (miR-122)\textsuperscript{93}. miR-122 is an abundant liver-specific miRNA which is crucial for efficient HCV RNA replication in cultured Huh7 cells stably expressing HCV replicons. It stimulates HCV RNA replication and translation through interaction with two adjacent sites downstream of stem loop I within the HCV 5′ untranslated region. Moreover, a recent study found that among chronically HCV-infected individuals pre-treatment intrahepatic miR-122 levels were significantly lower among patients who responded poorly to interferon therapy. Santaris Pharma has developed a locked nucleic acid-modified oligonucleotide (miravirsen or SPC3649) complementary to the 5′-end
of miR-122 that resulted in functional inactivation of miRNA-122. Miravirsen was shown to be active in HCV positive chimpanzees, markedly reducing HCV RNA replication and showing no significant side effects except for a profound decrease in serum cholesterol levels. Moreover, miravirsen-induced miR-122 antagonism had a potent antiviral effect against HCV genotypes 1-6 in vitro. Thus miravirsen holds promise as a new antiviral therapy with a high barrier to resistance and a tolerable side effect profile. Moreover, functional inactivation of a miRNA to treat an infectious disease represents a truly novel therapeutic paradigm far beyond the HCV or virology field. A phase II trial of miravirsen in chronically HCV infected individuals is currently recruiting patients.

Finally, HCV RNA replication seems to be intricately linked to the cholesterol and fatty acid biosynthesis pathways. Statins, a widely used group of drugs that target cholesterol metabolism by inhibition of 3-hydroxyl-3-methylglutary coenzyme A (HMG CoA) reductase, have been reported to inhibit HCV RNA replication in vitro, albeit the exact mechanism is still under investigation. Interestingly, the addition of fluvastatin to PEG-IFN/RBV has been reported to improve rapid viral response rates (RVR) but not SVR in HIV/HCV genotype 1 co-infected patients and SVR rates in diabetic patients with chronic hepatitis C. However, in another study atorvastatin showed no effect on HCV RNA and statins are currently not considered part of the standard of care.

Assembly and release. The final stages, i.e., assembly and release of progeny virions, are as yet the least understood part of the HCV replication cycle. Several laboratories worldwide are involved in the
hunt for the critical host factors required for HCV assembly and release. Any factor identified would then become a potential target for future antiviral strategies. An early example of a compound targeting the final replication cycle stages was celgosivir, an oral prodrug of the natural product castanospermine which is derived from the Australian chestnut. Celgosivir is a potent inhibitor of alpha-glucosidase I, a host enzyme required for viral assembly, release, and infectivity\textsuperscript{262}. Alpha-glucosidase I inhibitors have been shown to inhibit the replication of a broad range of enveloped viruses by preventing the correct folding of their envelope glycoproteins. The drug demonstrated broad antiviral activity in vitro and although the agent was not active as a monotherapy for HCV infection, it demonstrated a synergistic effect in combination with the current standard of care, both in vitro and in phase II clinical trials. However, development of celgosivir has recently been terminated due to safety concerns. Another cellular system emerging as central to HCV virion production is the LDL and VLDL pathways with apoE and microsomal triglyceride transfer protein being thought to have central roles\textsuperscript{193, 194}. However, this interesting and rapidly moving research field has yet to produce promising therapeutic footholds.

6. Experimental models to study HCV

The study of HCV replication and lifecycle has been hampered for a decade by the fact that HCV cannot be cultured in vitro and by the lack of appropriate HCV models. The discovery of subgenomic HCV replicons and the infectious virus JFH-1 clone were major
breakthroughs for the knowledge of the HCV lifecycle and drug discovery.

6.1. HCV subgenomic replicon

A major problem for the study of HCV was the ineffective cell culture replication of primary and molecular clones of HCV. To overcome this problem, the group of Bartenschlager created antibiotic-resistant HCV genomes, allowing the selection of replication-competent clones by antibiotic pressure. To this purpose, the sequence coding for the structural proteins was replaced by a neomycin resistance gene. Additionally, a second heterologous EMCV IRES was introduced to promote translation of the non-structural proteins, thereby creating a so-called bicistronic subgenomic replicon. A replicon is defined as a genetic element that can replicate under its own control. When transfected in the human hepatoma cell line Huh7, a low number of neomycin resistant colonies could be established that carried stably replicating HCV RNAs.

The efficiency of the replicon model depends on cell culture adaptive mutations and the selection of a highly permissive cell line. Cell culture adaptive mutations enhance RNA replication and emerge under neomycin selection. They cluster in three regions: the N-terminus of the NS3 helicase, two distinct positions in NS4B and in the centre of NS5A. Most adaptive mutations are found in NS5A, often affecting a serine residue that is involved in NS5A hyperphosphorylation.
An important drawback of the subgenomic replicon model is the inability to produce infectious viral particles. This may be the result of the presence of adaptive mutations, which seem to block viral assembly. In chimpanzees adaptive mutations were found to attenuate in vivo infectivity of HCV genomes. Moreover, these adaptive mutations were never observed in HCV infected patients or in experimentally infected chimpanzees. Furthermore, since Huh7 cells are immortalized tumor cells that differ from regular hepatocytes, caution may be required when studying processes that are dominated by the host cell (e.g. response to IFN). Despite these short-comings, HCV replicons remain a major tool to study the replication of HCV and to characterize new antiviral inhibitors that target the non-structural proteins of HCV.

Several variations on the initial bicistronic replicon were created including replicons of different genotypes and replicons containing reporter genes such as fluorescent proteins or the firefly luciferase, allowing their use in high-throughput screening.

6.2. HCV cell cultured infectious model (HCVcc)

In 2005 a cell culture model enabling efficient propagation of HCV was developed (HCV produced in cell culture; HCVcc). This model is based on the HCV isolate from a Japanese patient with fulminant hepatitis (JFH-1, genotype 2a). This isolate was used to construct subgenomic JFH-1 replicons. The JFH-1 subgenomic replicon replicated efficiently in cell culture without the need of cell culture adaptive mutations. Moreover, when an in vitro transcribed full length
genome was transfected in Huh cells, secretion of HCV virions was observed. These secreted virions proved to be infectious in cultured cells, chimeric uPA/SCID mice and chimpanzees. The infectivity of secreted virions was improved by using highly permissive cell lines such as Huh7.5 cells and by generating chimeric constructs with structural genes, p7 and NS2 of another genotype 2a clone, J6218. Furthermore, the HCVcc virions can be neutralized by antibody against E2 and the CD81 receptor as well as sera of HCV infected patients. Several variants of JFH-1 were constructed, including chimeric JFH-1 based genomes of all HCV genotypes. In these chimeric genomes, the structural genes, p7 and NS2 of JFH-1 were replaced by genotype-specific sequences132. Hence, the development of the HCVcc model proved a milestone in HCV research, since all steps in HCV lifecycle can be studied, including HCV assembly and secretion.

6.3. Additional models for HCV

A model that has proven to be very useful to study the early steps of HCV infection is the HCV pseudoparticle model. In this model, unmodified HCV envelope glycoproteins are incorporated onto lentiviral or retroviral cores such as MLV or HIV267, 268. HCVpp are produced by transiently transfecting human 293T cells with expression vectors encoding the HCV glycoproteins and an envelope-defective MLV or HIV proviral genome. Characterization of the HCV pseudoparticles revealed that they preferentially infect hepatocytes and hepatocarcinoma cells, mimicking the tropism of HCV infection268. Furthermore, HCVpp are neutralized by anti-E2 antibodies as well as
sera of HCV infected patients\textsuperscript{267}. Multiple entry receptors were identified using HCVpp, such as glycosaminoglycans, LDLR, claudin-1 etc.

The development of animal models to study HCV infection is challenging because HCV can only infect humans and chimpanzees. The chimpanzees model has been a cornerstone in HCV research and studies in this model yielded important insights in virus-host interactions. Many features of HCV infection are similar between human and chimpanzees; however, the clinical course of infection is milder compared to humans. The use of this model is limited because of the restricted availability of chimpanzees, the high costs and ethical concerns.

The construction of a small-rodent model for HCV has proven to be very complex. Because of the narrow host tropism of HCV, rodents have to be transplanted with human cells and therefore require a constitutive lack of immune rejection toward these xenografts. This goal can be achieved using the uPA/SCID chimeric mouse model. In this model, the overexpression of the urokinase plasminogen activator (uPA) gene results in severe hepatotoxicity, supporting the repopulation of diseased liver by transplanted donor hepatocytes of murine origin. By backcrossing the uPA transgenic mice with immunodeficient mice (SCID, severe combined immune deficient), it was possible to replace the diseased liver by human hepatocytes to mimic human liver. These transplanted uPA/SCID mice are fully permissive for HCV\textsuperscript{269}. The uPA/SCID chimeric model is physiologically closest to a natural human HCV infection and is therefore the most
successful small animal model for HCV infection at this moment. However, there are some major drawbacks: (I) this model is not suitable to study adaptive immune responses, immunopathology and vaccine strategies because of the immunodeficiency of the chimeric mice; (II) the transplantation technique is very challenging; (III) good quality of primary human hepatocytes is required; (IV) the success of repopulation of the liver after xenotransplantation is very variable.
C. SCOPE OF THE THESIS

Virus-host interactions are crucial for the pathogenesis of Hepatitis C. Disease progression and response to therapy depends from viral and host factors and from their mutual interactions. The study of host and viral factors is also of primary importance for the development of new antiviral therapies.

The goal of this work was to investigate some of the most relevant viral and host factors in order to improve their knowledge and the possibility to translate this knowledge to a useful clinical application.

CHAPTER 2: Metabolism of Phosphatidylinositol 4-Kinase IIIα-Dependent PI4P is Subverted by HCV and Is Targeted by a 4-Amino Quinazoline with Antiviral Activity

The enzymatic activity of PI4KIIIα is required for efficient HCV RNA replication and the direct activation of this lipid kinase by HCV is critical for the integrity of the viral replication complex. Since we demonstrated that the anti-HCV compound AL9 is an inhibitor of PI4KIIIα, this kinase is a suitable antiviral target for the treatment of HCV.
CHAPTERS 3-4: Unraveling host responses to the emergence of hepatitis C virus particles with defective RNA genomes

HCV particles with defective RNA genomes have been recently identified in the serum of some patients with chronic HCV infection and represent a significant proportion of viral load.

In order to investigate whether HCV defective genomes could play a role in any of the hepatic disease manifestations associated with chronic HCV infection, or affect response to antiviral therapy, we adopted a two-fold ex vivo/in vitro approach.

On one hand, we performed a retrospective screening campaign aiming at assessing the presence of defective genomes in the serum of HCV-infected individuals stratified according to disease severity as well as response to PEG-IFN/RBV combination therapy (CHAPTER 3).

On another hand, we studied the direct role of defective HCV genomes in hepatocyte injury using an infectivity model system in vitro. (CHAPTER 4).
Chapter 1 – References

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CHAPTER 2

Metabolism of Phosphatidylinositol 4-Kinase IIIα-Dependent PI4P is Subverted by HCV and Is Targeted by a 4-Anilino Quinazoline with Antiviral Activity

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A. Abstract

4-anilino quinazolines have been identified as inhibitors of HCV replication. The target of this class of compounds was proposed to be the viral protein NS5A, although unequivocal proof has never been presented. A 4-anilino quinazoline moiety is often found in kinase inhibitors, leading us to formulate the hypothesis that the anti-HCV activity displayed by these compounds might be due to inhibition of a cellular kinase. Type III phosphatidylinositol 4-kinase α (PI4KIIIα) has recently been identified as a host factor for HCV replication. We therefore evaluated AL-9, a compound prototypical of the 4-anilino quinazoline class, on selected phosphatidylinositol kinases. AL-9 inhibited purified PI4KIIIα and, to a lesser extent, PI4KIIIβ. In Huh7.5 cells, PI4KIIIα is responsible for the phosphatidylinositol-4 phosphate (PI4P) pool present in the plasma membrane. Accordingly, we observed a gradual decrease of PI4P in the plasma membrane upon incubation with AL-9, indicating that this agent inhibits PI4KIIIα also in living cells. Conversely, AL-9 did not affect the level of PI4P in the Golgi membrane, suggesting that the PI4KIIIβ isoform was not significantly inhibited under our experimental conditions. Incubation of cells expressing HCV proteins with AL9 induced abnormally large clusters of NS5A, a phenomenon previously observed upon silencing PI4KIIIα by RNA interference. In light of our findings, we propose that the antiviral effect of 4-anilino quinazoline compounds is mediated by the inhibition of PI4KIIIα and the consequent depletion of PI4P required for the HCV membranous web. In addition, we noted that HCV has a
profound effect on cellular PI4P distribution, causing significant enrichment of PI4P in the HCV-membranous web and a concomitant depletion of PI4P in the plasma membrane. This observation implies that HCV – by recruiting PI4KIIIα in the RNA replication complex – hijacks PI4P metabolism, ultimately resulting in a markedly altered subcellular distribution of the PI4KIIIα product.

B. Introduction

Hepatitis C virus (HCV) is an enveloped, single-stranded RNA virus classified as member of the Hepacivirus genus within the Flaviviridae family. The 9.6 kb positive-sense RNA genome contains a single open reading frame encoding a polyprotein of about 3,000 amino acids, flanked by highly structured 5’ and 3’ untranslated (UTR) regions. Following its release into the cytoplasm of the host cell, viral RNA is translated via an internal ribosome entry site (IRES), giving rise to a single polypeptide that is cleaved into 10 different mature protein products: Core, gpE1, gpE2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. HCV RNA replication takes place in the cytoplasm, in association with a virus-induced intracellular membrane structure termed “membranous web”, onto which NS proteins assemble to form the so-called RNA replication complexes.

It is estimated that 3% of the world’s population are chronically infected by the hepatitis C virus (HCV). Most infections become chronic and over time evolve into chronic hepatitis. The most unwanted complication of chronic hepatitis is cirrhosis, a massive liver fibrosis, which can lead to liver failure and hepatocellular carcinoma.
Since the discovery of hepatitis C virus (HCV) in the late 1980's much progress has been made in the understanding of the viral life cycle of HCV. Nonetheless, to date no vaccines are available and the current standard of care, involving lengthy treatment with a combination of ribavirin and pegylated interferon-α (PEG-IFN-α), eradicates the infection in half of treated patients. A large effort has been made in the past two decades in order to develop novel anti-HCV therapies with greater efficacy. Two oral direct-acting antiviral agents (DAA) targeting the HCV NS3/4 protease, boceprevir and telaprevir, have recently reached the market and more are being developed. While the initial efforts to the discovery of DAA focused almost exclusively on the best characterized HCV enzymes required for viral replication – the NS3/4A protease and the NS5B polymerase – in the past few years the NS5A viral protein has been attracting more and more attention as a target for drug development. NS5A possesses no known enzymatic activity. It is a multifunctional non-structural protein important for viral replication as well as viral assembly. It is a phosphoprotein consisting of three domains. Domain I is highly conserved and forms a dimeric structure, whereas domain II and III are believed to adopt a “natively unfolded” conformation.

In recent years, several anti-HCV compounds identified using cell-based replicon screens were indicated to target NS5A based on the analysis of the mutations associated with emergence of resistance in the replicon system. The most studied series of these “NS5A inhibitors” is represented by BMS-790052, an agent that is leading the field, having
demonstrated potent antiviral activity in clinical studies. Compounds in this class are characterized by a complex, dimeric or pseudo-dimeric structure and a high molecular weight, when compared with conventional “drug-like” small molecules. Resistance mutations against these compounds emerge readily in Domain I of NS5A, with the most recurrent of these changes corresponding to variant of tyrosine at position 93. Although direct interaction with purified NS5A has not been demonstrated, compelling reverse genetic experiments as well as molecular models strongly support the notion that NS5A is the direct target of these compounds.

A less characterized series of compounds, belonging to a different chemical class, was also initially indicated to target NS5A on the basis of the mutation pattern observed in resistant replicons. The common structural element of this latter class of inhibitors is a 4-anilinoquinazoline core. A representative member of this class of compounds is A-831/AZD-2836, an experimental antiviral agent that entered clinical trials but was later discontinued due to the lack of adequate exposure. For these agents, the mutations reported to be associated with resistance were found to be different from those expected for the NS5A inhibitor described above, pointing to a different mechanism of action: a few mutations were found at the C-terminal end of NS5A Domain I (E212D, L199F and T200P), whereas most mutations occurred in NS5A Domains II and III (P299L, S370P, V388D, V362A, S390G and S370P). Additional mutations were also found in NS4B (S258T) and NS5B (S76A). Reverse genetics studies in which these mutations were reintroduced in the replicon, however, did not recapitulate the
resistant phenotype observed in the original cellular clones, leaving thus the possibility open that these compounds act through a different viral or cellular target.

Interestingly, many kinase inhibitors, including some approved antitumoral drugs (gefitinib, lapatinib, erlotinib) are 4-anilino quinazoline derivatives\textsuperscript{19-21}. Altogether, these considerations led us to investigate whether the anti-HCV activity displayed by these compounds might be due to inhibition of a cellular kinase.

Recently, several small-interfering RNA (siRNA) screening campaigns have identified type III phosphatidylinositol 4-kinases (PI4K) as crucial host factors for HCV replication. In particular, PI4KIII\(\alpha\) was found to be required for HCV RNA replication in a cell line- and genotype-independent manner, whereas the requirement for the \(\beta\) isoform was observed to be less dramatic and limited to Con-1 (genotype 1b) replicons\textsuperscript{22-25}. It was shown that the catalytic activity of PI4KIII\(\alpha\) is required to rescue HCV replication in cells with a stable knock-down of PI4KIII\(\alpha\). In addition, it has been proposed that NS5A stimulates PI4KIII\(\alpha\) activity by direct interaction via Domain I\textsuperscript{26-28}. All these observations taken together made us consider the phosphatidylinositol 4-kinases a potential alternative candidate target for 4-anilino quinazoline inhibitors of HCV replication.

In this paper, we present evidence that AL-9, a member of this class of compounds previously reported to target NS5A, inhibits PI4P formation by direct inhibition of phosphatidylinositol 4-kinase III\(\alpha\) (PI4KIII\(\alpha\)). In addition, we provide evidence that pharmacological inhibition of PI4KIII\(\alpha\) with AL-9 results in altered subcellular
distribution of NS5A similar to that observed after RNAi knock-down of the PI4KIIIα mRNA, strongly supporting a mechanism of HCV inhibition mediated by the inhibition of PI4KIIIα. Moreover, we show that HCV subverts components of the phosphatidylinositol-4 phosphate (PI4P) pathway to function in favor of its own life cycle, thereby enriching the PI4P concentration in the membranous web while depleting the plasma membrane PI4P pool.

C. Results

1. Compound AL-9 inhibits HCV replication in vitro

AL-9 is a member of 4-anilino quinazoline-containing HCV replication inhibitors described previously (18; Fig. 1). In order to confirm its anti-HCV activity, we tested the effect of this compound on HCV replication in Huh7.5 cells stably expressing genotype 1b or 2a subgenomic replicons (Con1-SR and JFH-A4, respectively). The EC50 values, calculated by measuring viral RNA after incubation with AL-9 for three days, are reported in Table 1. Replicon EC50 values for AL-9 were found to be 0.29 µM and 0.75 µM for genotype 1b and 2a, respectively. In order to prove that AL-9 inhibits HCV replication not only in the context of a HCV subgenomic replicon, but also in the context of the complete viral life-cycle, we determined the inhibitory activity using the J6/JFH-1 HCV virus. In this case, the EC50 value was found to be 1.2 µM, a figure comparable with the result obtained with genotype 2a subgenomic replicon. CC50 values are shown for Con1-SR, JFH-A4 and Huh7.5 cells, respectively. In summary, AL-9 inhibits HCV
across different genotypes with activity in the sub-micromolar to low micromolar range in the absence of significant cytotoxic effects.

Fig. 1. Chemical structure of AL-9
For the synthetic pathway and procedure see Supporting Information.

Table 1. List of EC50 values of AL-9 for different HCV genotypes

<table>
<thead>
<tr>
<th>genotype</th>
<th>EC50 (µM)</th>
<th>CC50 (µM)</th>
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<tbody>
<tr>
<td>Con1-SR</td>
<td>1b 0.29 (+/- 0.09)</td>
<td>29.3 (+/- 2.8)</td>
</tr>
<tr>
<td>JFH-A4</td>
<td>2a 0.75 (+/- 0.15)</td>
<td>18.9 (+/- 3.2)</td>
</tr>
<tr>
<td>Huh7.5 + J6/JFH-1 HCV</td>
<td>2a 1.2 (+/- 0.37)</td>
<td>25.1 (+/- 4.6)*</td>
</tr>
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Table 1. List of EC50 values of AL-9 for different HCV genotypes

Huh7.5 cells replicating subgenomic replicons of genotype 1b or 2a (Con1-SR and JFH-A4, respectively) or Huh7.5 cells infected with the chimeric virus J6/JFH were treated with AL-9 for three days and intracellular viral RNA was measured by real time PCR. The data are representative of at least three independent experiments, and the standard deviations are shown. *CC50 measured in uninfected Huh7.5 cells.

2. AL-9 is an inhibitor of PI4KIIIα

In the following experiment, we investigated whether AL-9 inhibits the purified type III phosphatidylinositol 4-kinases PI4KIIIα and PI4KIIIß
Both enzymes were inhibited by AL-9 with a five-fold preference for PI4KIIIα (IC50 of 0.57 µM and 3.08 µM, respectively). This result demonstrates that AL-9 inhibits type III PI4 kinases in vitro at concentrations similar to those required for its anti-HCV activity, displaying a moderate selectivity for the α over the β isoform. We also tested the activity of AL-9 on two class I PI3-kinases (p110α and p110β). While PI3-kinase p110α was inhibited with an IC50 of 1.1 µM, the potency of AL-9 for PI3-kinase p110β was significantly lower (40% inhibition @10 µM, data not shown).

The enzymes were preincubated for 10 min with the indicated concentrations of AL-9 or DMSO and the reaction was started by addition of 100 µM ATP and 150 µM PI:PS substrate as described in Materials and Methods. Activity, measured as conversion of ATP to ADP, is expressed as percent of the DMSO control. Shown is a representative experiment of three independent experiments performed in duplicate. IC50 and SD of PI4KIIIα and PI4KIIIβ are indicated.

Fig. 2. Inhibitory dose-response curve of AL-9 for purified PI4KIIIα and PI4KIIIβ

The enzymes were preincubated for 10 min with the indicated concentrations of AL-9 or DMSO and the reaction was started by addition of 100 µM ATP and 150 µM PI:PS substrate as described in Materials and Methods. Activity, measured as conversion of ATP to ADP, is expressed as percent of the DMSO control. Shown is a representative experiment of three independent experiments performed in duplicate. IC50 and SD of PI4KIIIα and PI4KIIIβ are indicated.
Our hypothesis is that AL-9 inhibits HCV replication via inhibition of PI4KIIIα. Thus, we wanted to assess whether AL-9 also inhibited PI4KIIIα in living cells. To this aim, we needed to set up an assay that allowed us to monitor the activity of this kinase in intact cells. PI4KIIIα is primarily localized to the ER, whereas PI4KIIIβ is localized to the Golgi membranes\(^{29}\). It was shown that PI4KIIIβ contributes to the synthesis of PI4P at the Golgi membranes\(^{30, 31}\). Subcellular localization of the enzymes, however, does not always coincide with their function. Thus, PI4KIIIα, considered to be an ER-resident enzyme, has previously been shown to be critical for the generation and maintenance of the plasma membrane PI4P pool during phospholipase C activation and Ca\(_2^+\) signaling in HEK-293 or Cos-7 cells\(^{31, 32}\) as well as in resting Cos-7 cells\(^{33}\). Whether PI4KIIIα is responsible for the maintenance of the plasma membrane PI4P pool under normal cell culture conditions in hepatoma cells is currently not known. Hammond et al\(^{33}\) have developed immunocytochemical techniques that enable selective staining of the PI4P pool present in the plasma membrane (plasma membrane staining protocol) or in the intracellular membranes (Golgi staining protocol), respectively. We used this technique, in combination with RNA gene silencing or pharmacological inhibition, to decipher which of the type III enzymes participates in the synthesis of the Golgi- or plasma membrane PI4P pools in HuH7.5 hepatoma cells.

To address which type III PI4 kinase is responsible for the synthesis of the different cellular PI4P pools, HuH7.5 cells were treated with siRNAs targeting PI4KIIIα, PI4KIIIβ or an unrelated siRNA (mock-siRNA) as described in the Materials and Methods section. Immunoblots
assays show specific knockdown of PI4KIIIα or PI4KIIIβ by their corresponding siRNAs (Fig. 3C). Three days after siRNA treatment, PI4P was revealed either by the plasma membrane staining protocol (Fig 3A, upper panel) or by the Golgi membrane staining protocol (Fig. 3A, lower panel). In cells treated with the unrelated siRNA (mock-siRNA), PI4P was detected both in the plasma membrane and in intracellular membranes. Intracellular PI4P was localized primarily in the Golgi membranes, as judged by the colocalization with the Golgi marker giantin. Silencing of PI4KIIIα resulted in a significant decrease of the PI4P level in the plasma membrane. Concomitantly with the decrease in the plasma membrane PI4P levels, we consistently observed a pronounced increase of PI4P level in the Golgi membrane following PI4KIIIα knockdown. In the case of PI4KIIIβ knockdown, we observed a ~30% decrease of Golgi membrane PI4P level, whereas the PI4P levels of the plasma membrane remained substantially unaffected (Fig. 3B).

These results are in line with the previously reported role for PI4KIIIα in maintaining the PI4P plasma membrane pool\textsuperscript{31-33} and confirm the importance of PI4KIIIβ for the synthesis of at least part of the Golgi membrane PI4P\textsuperscript{30, 31}. We also observed that decreased expression of PI4KIIIα resulted in an unexpected increase in the level of the Golgi membrane pool (Fig. 3A), suggesting a complex level of cross-talk between the cellular type III PI4 kinases in maintaining the physiological PI4P levels at the Golgi membrane, at least in our experimental model.
Fig. 3. RNA interference analysis of PI4P production in Huh7.5 cells.

Huh7.5 cells were treated with irrelevant (mock) siRNA or siRNA targeting PI4KIIIα or PI4KIIIβ as detailed in Materials and Methods. The data were collected three days after initial siRNA transfection.

(A) Confocal microscopy images of Huh7.5 cells treated with PI4KIIIα siRNA, PI4KIIIβ siRNA or mock siRNA. Cells were fixed and stained as described in Materials and Methods. PI4P (green) localized to the plasma membrane (PM) was detected using the plasma membrane staining protocol (upper panel)\(^{33}\). Nuclei were stained by the Hoechst dye (blue). PI4P in the intracellular membranes (IM) was revealed using the Golgi staining protocol (lower panel). Together with PI4P, Golgi membranes were stained with the Golgi marker giantin (red). Colocalization of PI4P with Golgi membranes results in yellow color.
(B) Quantification of PI4P levels by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the control (mock siRNA) are shown. Four randomly picked fields were analyzed per each condition, as described in Materials and Methods. Data are presented as averages ± SEM. *, P < 0.05; **, P < 0.01.

(C) Immunoblot analysis of protein expression after RNAi silencing. Lysates prepared from Huh 7.5 cells transfected with irrelevant siRNA (mock), PI4KIIIα siRNA (PIKA) or PI4KIIIβ siRNA (PIKB) were analyzed by immunoblotting with PI4KIIIα, PI4KIIIβ or β-actin antibodies as indicated in the figure. Positions of the protein molecular weight markers are shown on the left side.

In order to confirm and extend the results described above, we utilized a known pharmacological inhibitor of the type III PI4 kinases. PIK93 was previously exploited to distinguish between the roles of the two PI4KIII isoforms\textsuperscript{34, 35}. In particular, a concentration of 0.5 µM PIK93 is expected to affect only PI4KIIIβ, whereas 30 µM PIK93 should inhibit both PI4KIIIβ and PI4KIIIα. Thus, Huh7.5 cells were treated with 0.5 µM or 30 µM PIK93 or with DMSO as control. After two hours of incubation, PI4P was revealed either by the plasma membrane staining protocol (Fig 4A, upper panel) or by the Golgi staining protocol (Fig. 4A, lower panel). PI4P levels associated with the Golgi membranes decreased by ~25% after incubation with 0.5 µM PIK93 (Fig. 4B). This is in line with PI4KIIIβ contributing to the production of PI4P present in the Golgi membranes (PI4KIIIα, another contributor of Golgi-localized PI4P is not inhibited by PIK93\textsuperscript{34, 35}). Increasing PIK93 concentration to 30 µM further increased the inhibition of the intracellular membrane PI4P pool, to ~65% (Fig. 4B). This could be due to a more complete inhibition of PI4KIIIβ; however, based on this experiment, we cannot rule out a contribution of PI4KIIIα activity to the maintenance of the Golgi membrane PI4P pool. In contrast to what observed in the Golgi-associated membranes, the plasma membrane PI4P level was not
significantly affected upon incubation with 0.5 µM PIK93, but decreased by nearly 50% after incubation with 30 µM of PIK93 (Fig. 4B). Combined with the RNAi experiments described above, these results support the notion that, in Huh7.5 cells, PI4KIIIα is involved in the maintenance of the plasma membrane PI4P pool, whereas PI4KIIIβ is at least partly responsible for the maintenance of the Golgi membrane PI4P pool.
Fig. 4. Effect of PIK93 on Golgi or plasma membrane PI4P in Huh7.5 cells

(A) Confocal microscopy images of Huh7.5 cells incubated with DMSO (left column), 0.5 µM PIK93 (central column) or with 30 µM PIK93 (right column) for 2 hours prior to fixation and staining as described in Materials and Methods. PI4P (green) localized to the plasma membrane (PM) was detected using the plasma membrane staining protocol (upper panel) 33. Nuclei were stained by the Hoechst dye (blue). PI4P in the intracellular membranes (IM) was revealed using the Golgi staining protocol (lower panel). Together with PI4P, Golgi membranes were stained with the Golgi marker giantin (red). Colocalization of PI4P with Golgi membranes results in yellow color (zoomed sections are indicated by a yellow square).

(B) Quantification of PI4P levels by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the control (DMSO) are shown. Four randomly picked fields were analyzed per each condition. Normalization was performed as detailed in Materials and Methods. Data are presented as averages ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

We then evaluated the PI4K inhibitory activity of AL-9 in Huh7.5 cells using the same methodology. Briefly, Huh7.5 cells were incubated either with DMSO or with increasing concentration of AL-9 (1, 2, 4 or 8 µM) for two hours (Fig. 5A). Treatment with AL-9 gradually reduced the amount of PI4P in the plasma membrane (Fig. 5B). Conversely, the concentration of PI4P in the Golgi-associated membranes remained substantially unaltered up to the highest AL-9 concentration used (Fig. 5B).
Fig. 5. AL-9 inhibits PI4KIIIα in Huh7.5 cells

(A) Confocal microscopy images of Huh7.5 cells treated for 2 hours with DMSO (left column) or with 1, 2, 4 or 8 µM of AL-9 (columns 2 to 5). PI4P (green) localized to the plasma membrane (PM) was detected using the plasma membrane staining protocol (upper panel). Nuclei were stained by the Hoechst dye (blue). PI4P in the intracellular membranes (IM) was revealed using the Golgi staining protocol (lower panel). Together with PI4P, Golgi membranes were stained with the Golgi marker giantin (red). Colocalization of PI4P with Golgi membranes results in yellow color (zoomed sections are indicated by a yellow square).

(B) Quantification of PI4P levels by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the control (DMSO) are shown. Four randomly picked fields were analyzed per each condition. Normalization was performed as detailed in the Materials and Methods. Data are presented as averages ± SEM. **, P < 0.01; ***, P < 0.001.
In all, the results described above suggest that AL-9 inhibits PI4KIIIα also in living cells, while not appreciably affecting the activity of PI4KIIIβ. This is in line with the selectivity for PI4KIIIα over PI4KIIIβ observed in the biochemical assays.

3. HCV alters the intracellular and plasma membrane distribution of PI4P

Viral infection induces modification of intracellular membrane structures\textsuperscript{36} and, for some RNA viruses including HCV, it has been shown that these induced membranous structures are highly enriched for PI4P\textsuperscript{28, 37}. Before testing the activity of AL-9 in HCV-infected cells, we wanted to know what the impact of HCV on cellular membrane structures was, with special regard to the subcellular membrane distribution of PI4P.

Naïve Huh7.5 cells or cells actively replicating the genotype 2a or 1b HCV subgenomic replicon were investigated for their PI4P concentration in internal membranes or plasma membranes, respectively (Fig. 6A). As previously shown, cells expressing the HCV replicon form a membranous web that is highly enriched for PI4P (Fig. 6A, lower panel). The level of PI4P in these virus-specific membrane structures is markedly higher in JFH-A4 cells, containing the very efficient genotype 2a JFH-1 replicon, compared to the Con1-SR cells, which are based on the genotype 1b Con1 replicon, possibly mirroring the different RNA replication efficiency. It is well established that the kinase responsible for the production of the PI4P pool present in these structures is PI4KIIIα. In the current model, PI4KIIIα interacts with the
viral protein NS5A, leading to up-regulation of the kinase activity and accumulation of PI4P in the virus-specific membranous web\textsuperscript{26-28}. Conversely, the results shown in Figures 3-5 suggest that – in absence of viral replication – a major function of PI4KIII\(\alpha\) is the synthesis of the PI4P pool in the plasma membrane. We therefore asked ourselves whether the presence of HCV could not only influence distribution and enrichment of PI4P in internal membranes, but also alter the PI4P plasma membrane pool. In Fig. 6A, we show that, concomitantly with the increase of PI4P in the internal membranes (lower panel), HCV replication promotes a marked decrease of PI4P concentration in the plasma membrane (upper panel). Relative quantification of the PI4P levels in the different experimental conditions is shown in Fig. 6B. This experiment demonstrates that the presence of HCV causes a dramatic change of PI4P localization in cellular membranes, whereby the increase of PI4P concentration in the virus-specific membranous structures appears to be accompanied by a depletion of the PI4P pool normally present in the plasma membrane.
Fig. 6. HCV impacts subcellular PI4P distribution

(A) Huh7.5 cells, JFH-A4 and Con1-SR cells were analyzed by confocal microscopy for the presence of PI4P (green) in the plasma membranes (PM, upper panel) or in the intracellular membrane (IM, lower panel) using the protocols described in Materials and Methods. Nuclei were stained by the Hoechst dye (blue). For internal membrane staining, giantin (red) was used as a specific marker for Golgi membranes.

(B) Quantification of PI4P levels by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the control (Huh7.5 cells) are shown. Four randomly picked fields were analyzed per each condition. Normalization was performed as detailed in Materials and Methods. Data are presented as averages ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
We next investigated whether HCV-associated changes in PI4P distribution could be reverted upon cure of the HCV replicon by specific inhibitors. We treated JFH-A4 cells for two weeks either with the HCV RdRp inhibitor HCV-796 or with the HCV NS3/4A protease inhibitor MK-5172 and followed PI4P localization in internal membranes and in the plasma membrane (Fig. 7). Independent of the type of inhibitor used, the result shows that the HCV-induced PI4P-enriched membranous web in JFH-A4 cells disappeared upon suppression of HCV replication and that the intracellular PI4P localization returned to the Golgi-localization as observed in the naïve Huh7.5 cells (left column). In parallel, the plasma membrane concentration of PI4P increases to the levels observed in naïve cells (middle column). NS5A staining (right column) as well as real-time RT-PCR (not shown) indicated that the prolonged treatment with HCV-inhibitor led to complete and stable suppression of viral protein expression and undetectable level of HCV RNA. Thus, removal of HCV RNA brings PI4P synthesis and distribution back to a level comparable to naïve Huh7.5 cells. It is worth of note, however, that the previous presence of HCV replicons in the cured cells induced some irreversible morphological changes of unknown nature, such as a smaller cell size.
Fig. 7. Reversibility of HCV-induced changes in PI4P subcellular distribution

JFH-A4 cells were incubated for 14 days with the HCV RdRp inhibitor HCV-796 (2 µM) or the HCV NS3/4A protease inhibitor MK-5172 (0.2 µM). Cure from HCV was controlled by detection of NS5A with a specific NS5A antibody (red, right column). As control, untreated Huh7.5 cells or JFH-A4 cells were used. Cells were fixed and PI4P (green) was detected in the internal membranes (IM, left column) or in the plasma membrane (PM, central column). For internal membrane staining giantin (red) was used as a specific marker for Golgi membranes. Nuclei were stained by the Hoechst dye (blue).
4. AL-9 inhibits PI4KIIIα in HCV-replicating cells

We have shown that PI4KIIIα is inhibited by AL-9 in naïve Huh7.5 cells. As discussed above, in HCV-replicating cells, the kinase activity of PI4KIIIα is up-regulated by a direct protein-protein interaction with the viral protein NS5A. In the following experiment (Fig. 8), we explored whether AL-9 is able to inhibit PI4KIIIα also in this context. JFH-A4 cells were incubated with increasing concentration of AL-9 for 4 hours and PI4P concentration in the HCV membranous web was followed by immunostaining (Golgi staining protocol). Treatment of cells with AL-9 lead to clear inhibition of PI4P accumulation in the HCV membranous web. Incubation with 8 µM AL-9 depleted as much as 70% of the PI4P present in the intracellular membranes of replicon-containing cells. This result confirms that AL-9 inhibits PI4KIIIα independent of its membranous localization and suggests that this inhibition could be responsible for the observed antiviral effect. Since AL-9 has anti-HCV activity in the concentration range used here, longer incubation of HCV replicons with AL-9 results in inhibition of HCV-RNA and -protein synthesis. As a consequence the PI4P–enriched HCV membranous web would disintegrate. In this case loss of PI4P in the internal membranes could be not a direct consequence of PI4KIIIα inhibition, but a consequence of disintegration of the HCV membranous web.

In order to rule out this possibility, we checked localization of NS5A, a presumed marker for HCV replication sites, after 4 hours of incubation with AL-9. Localization of NS5A does not change, suggesting that AL-9 does not significantly change the structure of the HCV membranous web upon 4 hours of treatment. Moreover, incubating
the same replicon cells for 4 hours with HCV-796, an HCV polymerase inhibitor, did not lead to appreciable depletion of the membranous web PI4P pool indicating that the loss of PI4P in the HCV-induced intracellular membranes is the direct consequence of inhibition of PI4KIIIα, and not the consequence of inhibition of HCV replication. Additional evidence is provided in the experiment below, in which expression of the HCV polyprotein, and consequently formation of a membranous web, was driven by cDNA plasmid rather than by autonomously replicating HCV RNA.
Fig. 8. AL-9 inhibits PI4KIIIα in HCV-replicating cells
(A) JFH-A4 cells were treated with DMSO or AL-9 for 4 hours and internal membranes were stained for PI4P (green) and the Golgi marker giantin (red) using the Golgi staining protocol, as described in Materials and Methods. DMSO or AL-9 concentrations are indicated within the image. Alternatively, cells were stained for NS5A as described in Materials and Methods (indicated as NS5A). Nuclear DNA was stained with Hoechst dye (blue). PI4KIIIα, associated with the HCV-associated membranous web is inhibited by AL-9. The decrease of PI4P is not due to inhibition of the HCV replication indicated by unchanged NS5A expression and localization (lower panel).

(B) Quantification of PI4P levels by immunofluorescence analysis. Changes in mean fluorescence intensity relative to the control (DMSO) are shown. Four randomly picked fields were analyzed per each condition. Normalization was performed as detailed in Materials and Methods. Data are presented as averages ± SEM. **, P < 0.01; ***, P < 0.001.

5. PI4KIIIα inhibition by AL-9 alters sub-cellular distribution of NS5A

It was previously shown that knock-down of PI4KIIIα expression by RNAi resulted in the production of large NS5A clusters. This was achieved in an experimental setting where the HCV polyprotein was expressed from DNA constructs, thus avoiding potential confounding effects due to inhibition of HCV RNA replication. We wanted to assess whether pharmacological inhibition of PI4KIIIα kinase activity would lead to similar effects on NS5A subcellular localization. Thus we followed the effect of AL-9 on NS5A localization after transient DNA transfection in Lunet-T7 cells with a plasmid expressing genotype 2a nonstructural proteins NS3-NS5B under the control of a T7 promoter. Cells were treated either with DMSO (upper panels) or with 8 µM AL-9 (lower panels) for 2, 8 or 16 hours and localization of NS5A as well as PI4P were followed by indirect fluorescence microscopy (Fig. 9). Cells successfully transfected with the HCV polyprotein expressed NS5A and induced the PI4P -enriched membranous web. After 8 hours of
treatment with AL-9, changes in NS5A localization in form of larger clusters become visible. At the same time, PI4P concentration in the membranous web started to decrease. After 16 hours of incubation with AL-9, NS5A was concentrated almost exclusively in large clusters. At this time-point, PI4P in the internal membranes had completely vanished.

In summary, this experiment shows that, in cells expressing the HCV polyprotein from cDNA, prolonged treatment with AL-9 results in a redistribution of NS5A into large clustered structures with high resemblance to the structures previously observed after silencing of the PI4KII\(\alpha\) gene by RNAi\(^{23,28}\). Concomitantly, we observed a depletion of the PI4P pool present in the HCV-induced membranous structures. These results indicate that the catalytic activity of PI4KII\(\alpha\) is directly or indirectly required for the proper localization of HCV NS5A protein into the membranous web. Furthermore, the experiment just described lends additional support to the notion that the antiviral effect of AL-9 is mediated by the inhibition of PI4KII\(\alpha\).
Fig. 9. Inhibition of PI4KIIIα by AL-9 induces the formation of large NS5A clusters

Huh7-Lunet/T7 cells were transiently transfected with the plasmid pTM-NS3-5B which expresses the HCV nonstructural proteins under the control of the T7 RNA polymerase promoter. Cells were treated with DMSO (upper panels) or 8 µM AL-9 (lower panels) for 2, 8 or 16 hours and were then stained for NS5A (red) and PI4P (green) using the Golgi staining protocol as described in Materials and Methods. Nuclear DNA was stained with the Hoechst dye (blue). Zoomed sections are indicated by a yellow square. Long incubation with AL-9 (8-16 hours) results in increased NS5A clustering and concomitantly a decrease of PI4P in the internal membranes.
D. Discussion

In the present paper, we show that a compound belonging to the class of 4-anilino quinazoline inhibitors of HCV replication is an inhibitor of PI4KIIIα, a cellular lipid kinase required for viral replication.

PI4KIIIα belongs to the family of type III phosphatidylinositol 4-kinases, enzymes that catalyze the conversion of phosphatidylinositol to phosphatidylinositol 4-phosphate (PI4P). PI4P is the most abundant monophosphorylated inositol phospholipid in mammalian cells and the importance of this phospholipid is just started to be unraveled. In addition to playing important roles in intracellular signaling and membrane trafficking, phosphatidylinositol lipids and their metabolizing enzymes are also exploited by many different viruses in order to transform cellular membranes in structures supporting their replication. PI4KIIIβ was shown to be a host factor required for enterovirus replication, whereas several reports have demonstrated that PI4KIIIα is crucial for HCV replication. Owing to the importance of this pathway, the need for specific inhibitors of PI4III kinases is increasing. Only recently, some enviroxime-like compounds with antiviral activity against enterovirus have been demonstrated to target PI4KIIIβ. One of these agents is a very specific inhibitor of the β-isoform of the type III PI4-kinases. So far, no such compound exists for the PI4KIII-α isoform. A commonly used inhibitor for type III phosphatidylinositol 4-kinases is PIK93, which has originally been designed to inhibit class I PI3-kinases. This compound allows differential inhibition of PI4KIIIβ alone or PI4KIIIα and PI4KIIIβ together depending on the concentration used. In this paper, we show that a 4-
anilino quinazoline derivative, termed AL-9 (Fig. 1 and S1), is able to inhibit PI4KIIIα in a test tube as well as in living cells. AL-9 inhibited purified PI4KIIIα, with a moderate (~5-fold) selectivity over the β isoform (Fig. 2). In cell culture, we observed that treatment with AL-9 efficiently inhibits the maintenance of the plasma membrane PI4P pool in Huh7.5 cells while not significantly affecting the Golgi membrane pool at the highest concentration used (Fig. 5). This finding is in line with the moderate selectivity observed in the biochemical assay. Thus, AL-9 represents a lead candidate for the development of more potent and more specific inhibitors of PI4KIIIα.

Anti-HCV compounds of the 4-anilino quinazoline class were previously assumed to exert their antiviral effect via inhibition of the viral protein NS5A. This conclusion rested on analysis of the mutations found in the HCV replicon in association with resistance to these agents. Mutations generated against 4-anilino quinazolines where localized mainly in NS5A, in triplets that occurred all in NS5A or appeared concomitant with changed in NS4B or NS5B (see also Introduction). Reverse genetic experiments, in which these mutations were reintroduced in the replicon (single, double and triple combinations), however, did not support a role for these mutations in conferring resistance to 4-anilino quinazolines. In order to assess whether the reported mutations conferred any level of resistance to AL-9, we independently performed reverse genetics studies in which selected mutations triplets, reported to be associated with the higher level of resistance, were reintroduced in a genotype 1b replicon with the same genetic background as the one reported in the original
resistance study (Fig. S2). These mutation triplets are: FAG: L199F+V362A+S390G (NS5A), DLD: E212D+P299L+V388D (NS5A), and PPA: T200P+S370P(NS5A)+S76A(NS5B). We observed that the replicon containing the first triplet lost the ability to replicate at significant level. For replicons containing the latter two combinations of mutations, RNA replication could be measured, although at a lower level compared to the parental construct (35% and 20%, respectively). These replicons, however, remained equally sensitive to AL-9 as the parental replicon (Fig. S2), opening the question as to which really is the target of this compound class. We are currently trying to select HCV replicons resistant to AL-9. So far we were unable to identify mutations that confer resistance to AL-9.

Our new data on AL-9 suggest that inhibition of HCV replication by 4-anilino quinazoline compounds is a consequence of PI4KIIIα inhibition. Our conclusion rests on a number of experimental findings. First of all, we showed that AL-9 is an inhibitor of purified type III PI4 kinases. Furthermore, we clearly demonstrated that AL-9 inhibits PI4KIIIα both in naïve Huh7.5 cells (Fig. 5, discussed above) as well in cells harboring actively replicating HCV RNA (Fig. 8). In cells where HCV replication occurs, PI4KIIIα interacts physically with HCV NS5A. This interaction, in turn, leads to the stimulation of PI4P synthesis at the HCV replication sites\(^{28}\). Treatment of replicon-harboring cells with AL-9 leads to efficient suppression of the PI4P pool at the HCV replication sites and does so independently of inhibition of HCV replication. This indicates that – although the enzymatic activity of PI4KIIIα is
modulated by the interaction with an HCV protein NS5A – it remains sensitive to the action of the 4-anilino quinazoline inhibitor.

We also investigated whether the dramatic changes observed in PI4P membrane levels by treatment with AL-9 could be associated with alteration in the subcellular distribution of type III PI4 kinases. To this aim, we analyzed the subcellular distribution of the type III PI4 kinases in Huh7.5 or Luc-A4 cells following incubation with AL-9 (Fig. S3). We observed no major effect of AL-9 on the localization of either PI4KIIIα or PI4KIIIβ, in line with the notion that the observed effects are primarily due to the inhibition of the kinase activity rather than to an altered protein subcellular distribution.

In cells that express the HCV polyprotein from a trans-gene, knockdown of PI4KIIIα by RNAi was previously shown to cause a dramatic change in NS5A subcellular distribution, from a pattern consistent with localization in the membranous web replication complexes to abnormally large cytoplasmic clusters. In Fig. 9, we show that AL-9 treatment of cells ectopically expressing the HCV nonstructural proteins results in a time-dependent depletion of PI4P and a concomitant change of NS5A localization to the large-clustered structures discussed above, reinforcing the notion that the anti-HCV effect of AL-9 and related compounds are likely to be mediated by the inhibition of PI4KIIIα.

We also found that PI3K p110α is inhibited by AL-9 in vitro at concentration similar to those needed to inhibit type III PI4-kinases. However, no Class I PI3-kinase has been shown to influence HCV replication thus inhibition of HCV replication by AL-9 is not due to
inhibition of Class I PI3-kinases. So far, the only PI3-kinase that resulted as positive hit for HCV replication inhibition in siRNA screens is PI3-kinase C2 gamma\(^{24}\). Future work will have to address whether AL-9 inhibits PI3KC2G in addition to Type III PI4-kinases.

During the characterization of AL-9 we focused our attention on various aspects of PI4P metabolism in Huh7.5 cells with and without replicating HCV. We observed a typical Golgi localization of PI4P in intracellular membranes of naïve Huh7.5 cells and confirmed a role for PI4KIIIβ in maintaining at least part of this pool. In order to get the complete picture we also investigated the PI4P pool present in the plasma membrane. In yeast, Stt4p, the ortholog to the mammalian PI4KIIIα, is localized at the plasma membrane and it is the major contributor for the synthesis of the plasma membrane-localized PI4P\(^{39}\). In mammalian cells, the role of PI4KIIIα for the maintenance of the plasma membrane PI4P pool has been demonstrated in HEK-293 and Cos-7 cells\(^{31-33}\). Here we demonstrate that liver-derived Huh7.5 cells are endowed with a rich PI4P pool in the plasma membrane and that the enzyme responsible for its maintenance is PI4KIIIα. In HCV-replicating cells, the subcellular PI4P distribution is profoundly altered. As already reported previously, the presence of HCV causes the induction of a membranous web highly enriched for PI4KIIIα-synthesized PI4P. In accordance, several reports demonstrate that NS5A recruits PI4KIIIα to the membranous web by direct protein-protein interaction, thereby stimulating its enzymatic activity\(^{26-28}\). Concomitantly with the induction of highly PI4P-enriched internal membranes, we observe a marked decrease of PI4P in the plasma
membrane. One possible explanation could be that – by hijacking PI4KIIIα - HCV might be able to enrich PI4P in the virus-induce membranous web not only by directly activating the enzymatic activity of PI4KIIIα recruited into the HCV RNA replication compartment, but also by preventing transport of the PI4KIIIα-synthesized PI4P from the synthesis site to the plasma membrane. How PI4KIIIα, localized at the ER, synthesizes the PI4P pool present in the plasma membrane it is still an enigma. This topological discrepancy can partially be resolved assuming that PI4KIIIα-dependent PI4P production occurs on ER-PM contact sites, that is, sites of close apposition between ER and PM. In yeast it has been demonstrated that a complex interplay between different proteins regulate the PI4P metabolism at the plasma membrane. Among these proteins are Osh, the yeast ortholog of the human OSBP and the ER membrane VAP proteins Scs2 and Scs22, the yeast orthologs of human VAP proteins. Interestingly, h-VAP-33 and OSBP have been shown to be important for HCV replication. It may be possible that recruitment of PI4KIIIα to the HCV membranous web through NS5A prevents interaction of PI4KIIIα with its cellular protein partners required to direct PI4P to the plasma membrane. Upon withdrawal of HCV from the cells (Fig. 7) PI4KIIIα is again free for interaction with the adequate partners. A possible role of PI4KIIIα in PI4P trafficking between the plasma- and intracellular membranes is suggested by our finding that RNAi silencing of this PI4 kinase results in decreased concentration of PI4P in the plasma membrane with a concomitant increase in the level of PI4P in the endomembranes (Fig. 3). Such a function of PI4KIIIα would have to be independent of the
kinase activity, since pharmacological inhibition (with PIK93 or AL-9) does not recapitulate this phenomenon observed by knocking down the protein expression.

In summary, the presence of HCV may change PI4P metabolism not only by activating the catalytic activity of PI4KIIIα by NS5A but also by modulating the PI4P distribution between different membrane compartments. The net result is an enrichment of the PI4P pool in the HCV-induced membranous web with a concomitant depletion of the plasma membrane PI4P pool.

Concluding, in this paper we demonstrate that a class of HCV inhibitors originally proposed to target NS5A does in fact target the host factor PI4KIIIα. Compounds targeting host factors may have the general advantage of imposing a higher genetic barrier to the development of resistance. AL-9, a member of this class of compounds, inhibits PI4KIIIα and to our knowledge, it is the first compound with a clear preference for PI4KIIIα over PI4KIIIβ. For this reason, AL-9 offers a good candidate as lead compound for the development of more potent and specific pharmacological inhibitors of PI4KIIIα to be used both as important research tools as well as leads for initial drug discovery.
E. Materials and Methods

1. Reagents and plasmids

The HCV RNA polymerase inhibitor HCV-796 and the PI kinase inhibitor PIK93 were a gift from Arrow Pharmaceuticals. The HCV protease inhibitor MK-5172 was purchased from Selleck Chemicals. Nucleic acids were manipulated according to standard protocols. Plasmid FBac-His-CD-PI4KA was constructed as follows: the catalytic domain of PI4KIIIα was amplified by PCR using the oligonucleotides 5'-cactgcggatccataatggggatgatgcagtgtgtgattg-3' (sense), 5'-cctgcgaattctcagtaggggatgtcattc-3' (antisense) and the plasmid pEF1A-PIK4CA untagged (a kind gift from G. Randall, Department of Microbiology, University of Chicago) as template. The resulting PCR fragment was subcloned into the vector pCR-Blunt II-Topo (Invitrogen) and finally cloned into the BamH1–XhoI cloning sites of the plasmid vector pFastBac THT-B. The resulting protein expressed from this plasmid contains an N-terminal hexa-histidine tag and starts at PI4KIIIα amino acid G873 (reference sequence NM_058004). pTM-NS3-5B expression vector expressing the HCV genotype 2a nonstructural proteins under the control of the T7 promoter was a generous gift from V. Lohmann (Department of Molecular Virology, University of Heidelberg). Synthesis of compound AL-9 is described in Supporting Information.

2. Cell lines and culture conditions

The human hepatoma-derived cell line Huh7.5 were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10%
fetal bovine serum, 100 U/ml penicillin, 100 μg/ml Streptomycin and 2 mM L-glutamine; G418 (0.8 mg/ml) was added to cell lines containing the HCV replicon. Stable cell lines expressing HCV genotype 1b or 2a subgenomic replicons were generated by electroporation of in vitro-transcribed RNA into Huh7.5 cells and following selection with G418 (0.8 mg/ml) for three weeks. Con1-SR: Huh7.5 cells replicating the Con1 subgenomic replicon with the adaptive mutations E1202G in NS3 and S2204R in NS5A. JFH-A4: Huh7.5 cells replicating the JFH-1 subgenomic replicon together with the luciferase reporter gene constructed as described previously. JFH-A4 cells were cured from the HCV replicon by two weeks of treatment with the protease inhibitor MK-5172 (0.2 μM) or the HCV RNA polymerase inhibitor HCV-796 (2 μM), respectively. Huh-LUNET/T7 cells were a kind gift from V. Lohmann (Department of Molecular Virology, University of Heidelberg, Germany).

3. Replication and infection assays

For replication assays, JFH-A4 or Con1-SR cells were plated at the density of 3x10^4 or 6x10^4 cells/well, respectively, in 24-well dishes the day before the experiment. Cells were treated with AL-9 resulting in a final concentration of 1 % DMSO in the cell medium. After three days of treatment, RNA was extracted using the RNeasy Mini Kit (Qiagen) and HCV RNA was quantified by real time PCR using the following oligonucleotide and probe set designed for the HCV IRES as described previously: sense (5’-GCAGAAAGCCTTGTGGTACT-3’), antisense (5’-CACGGTCTACGAGACCTCCC-3’), and probe (5’-
CCTGATAGGGTGGCTTGCGAGTGCC-3’, 5’ 6-carboxyfluorescein [FAM]/3’ 6-carboxytetramethylrhodamine [TAMRA]). GAPDH mRNA was used as internal control for data normalization.

Production of infectious virus was performed as follows: J6/JFH-1 chimeric RNA (1-846(J6CF)/847-3034(JFH1) was electroporated into Huh7.5 cells using the protocol described previously\(^\text{48}\). Briefly, 2x10\(^6\) cells were electroporated with 10 \(\mu\)g of RNA in a final volume of 200 \(\mu\)l and 4x10\(^6\) cells were plated in a T-75 flask. Three days post electroporation, medium was harvested and stored at -20°C in small aliquots. Calculation of EC50 of AL-9 using the infectious HCV virus was performed as follows: Huh7.5 cells were plated at 4x10\(^4\) cells/well in 24-well plates the day before infection. Infection was started by addition of 10 \(\mu\)l of cell medium containing infectious virus (see above) at an MOI of 50 in a final volume of 400 \(\mu\)l. After 6 hours of incubation, medium was removed and replaced with 400 \(\mu\)l of fresh medium containing serial dilutions of AL-9. RNA was collected after 72 hours of incubation and quantified by real time PCR. Cell cytotoxicity (CC50) of AL-9 was calculated using the cell viability assay CellTiter-Blue (Promega). Huh7.5, JFH-A4, or Con1-SR cells (5x10\(^3\) cells/well in 96-well dishes) were plated the day before treatment. AL-9 was added and cell viability was measured after four days of treatment.

4. Expression and purification of the catalytic domain of PI4KIII\(\alpha\)

Recombinant baculovirus was generated with the plasmid FBac-His-CD-PI4KA using the Bac-to-Bac system following the instructions of the manufacturer (Invitrogen). For protein expression, Sf9 cells were
infected with recombinant baculovirus at a density of 2\times10^6 Sf9 cells/ml for 3 days at 20°C. To prepare cell extract (1.5\times10^8 cells), cells were incubated in hypotonic buffer (10 mM HEPES (pH 7.5), 10 mM NaCl, 1 mM Tris(2-carboxyethyl)phosphine (TCEP) and EDTA-free protease inhibitor cocktail (Complete, Roche) for 30 min in ice and mechanically broken by 20 strokes of a Dounce homogenizer. After homogenizing, cells were incubated in lysis buffer (50 mM HEPES (pH 7.5), 500 mM NaCl, 10 % glycerol, 1 % Triton-X100, 1 mM TCEP and EDTA-free protease inhibitor cocktail (Complete, Roche) for further 30 min in ice and cell extract was cleared by centrifugation for 45 min at 20.000g. The cleared supernatant was incubated in batch with Ni-Sepharose High Performance (GE Healthcare) for 2 hours at 4°C with continuous shaking. The resin was first washed with 10 resin-volumes of wash buffer (50 mM HEPES (pH 7.5), 10 % glycerol, 0.4 % Triton X-100, 150 mM NaCl and 20 mM imidazol) followed by elution with wash buffer containing 250 mM imidazole. Active fraction (0.5 ml) were dialyzed against 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.4 % Triton X-100 and 10% glycerol and stored at -80°C in small aliquots.

5. In vitro kinase assay

PI4K kinase activity was assayed with the ADP-Glo Kinase Assay (Promega), according to the manufacturer’s instructions. Briefly, 0.5 µl of PI4KIIIα-CD or 0.05 µl PI4KIIIβ (32 ng, Invitrogen) were preincubated with DMSO or AL-9 in reaction buffer (20 mM Tris (pH 7.5), 5 mM MgCl2, 2 mM DTT, 0.5 mM EGTA, 0.4% Triton X-100) for 10 min at room temperature in a final volume of 8 µl. The reaction was started
by addition of 2 µl of ATP and PI:PS Lipid Kinase Substrate (Invitrogen) to give a final concentration of 100 µM and 150 µM, respectively. After 1 hour of incubation at room temperature the reaction was stopped and further processed as described by the manufacturer. In parallel the reaction was performed without PI:PS substrate in order to detect contaminating ATPase activity present in the protein fractions. This activity was subtracted from the measured kinase activity. Kinase activity of PI3Kα (p110α/p85α) and PI3Kβ (p110β/p85α) was assayed as above using 5 ng or 20 ng, respectively (Millipore). Reaction buffer was changed to 50 mM HEPES pH7.5, 10 mM MgCl2 and 1 mM DTT.

6. Indirect Immunofluorescence

Cells were plated one day before the experiment in 24-well plates (5x10^4 cells/well for Huh7.5 and JFH-A4 cells, 7x10^4 cells/well for Con1-SR and 1x10^5 cells/well for cured JFH-A4 cells). Cells were either untreated or treated with compounds for the time as indicated in the figure legend. PI4P staining of the plasma membrane or internal membranes was performed exactly as described previously. Primary antibodies used were: anti-PI4P IgM (Cat. No. Z-P004, 1:300, Echelon), anti-Giantin antibody (Cat. No. PRB-114C-200 1:1000, Covance), affinity-purified rabbit anti NS5A antibody (1:2000), anti-PI4KIIIα kinase (Cat. No. 4902, 1:50, Cell Signaling), anti-PI4KIIIβ kinase (Cat. No, 611817, 1:500, BD Transduction). Secondary antibodies used were goat anti-mouse IgM Alexa Fluor 488 (Cat. No. A-21042, 1:600, Invitrogen) and goat anti-rabbit Alexa Fluor 568 (Cat. No. A-11011, 1:600, Invitrogen). For type III PI4K kinases or NS5A staining, all
incubations were performed at room temperature. Cells were washed once with PBS and fixed with 300 μl of 4% PFA for 15 min. Cells were washed three times with PBS and permeabilized with 500 μl of 0.1% Triton X-100 (or 0.5% for PI4KIIIα kinase staining) in PBS for 10 min. Unspecific binding was blocked by incubation with 3% BSA in PBS (for PI4KIIIα staining no blocking was performed). After incubation with the primary antibody in blocking buffer, cell were washed with PBS and subsequently incubated with goat secondary antibodies conjugated to Alexa-Fluor 568, or Alexa-Fluor 488 at a dilution of 1:600. Nuclei were stained with Hoechst dye 33342 (Sigma; 1:4000). Slides were then mounted with 5 μl ProLong Gold Antifade (Invitrogen) and analyzed by using an inverted Leica TCS SP5 scanning laser confocal microscope. Digital images were taken using LAS AF software (Leica) and processed using Volocity software (Perkin Elmer). Quantification of fluorescence intensity was determined from multiple images using Volocity. Relative changes in fluorescence intensity mean values where obtained from four randomly picked fields for each condition (150 ~ 300 cells). For plasma membrane staining, total PI4P fluorescence intensity obtained in each condition was normalized to the number of cells present in each field. For the quantification of relative PI4P levels in internal membranes, PI4P fluorescence intensity was normalized using the fluorescence intensity of the Golgi marker giantin. Quantitative immunofluorescence data are presented as means ± the standard error of the mean (SEM). For the calculation of statistical significance, a two-tailed, unpaired t-test was performed.
7. siRNA silencing

3x10⁴ Huh7.5 cells/well were seeded in 24-well plates on microscope cover glasses and transfected with 50 nM of siRNAs in serum-free Opti-MEM (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer’s protocol. For western blot analysis, the transfection reaction was proportionally scaled up to 6-well plates. In order to maximize the silencing efficiency, 24 hours after the first transfection, the cells were subjected to a second round of siRNA transfection. siRNA sequences were the following (5’->3’ sense strand): mock siRNA, GUA UGA CCG ACU ACG CGU ATT (custom, Sigma-Aldrich); PI4KIIIα siRNA, CCG CCA UGU UCU CAG AUA ATT (custom, Sigma-Aldrich); and PI4KIIIβ siRNA, GCA CUG UGC CCA ACU AUG ATT (Silencer Validated siRNA s10543; Ambion). Three days after the initial transfection, cells were stained for PI4P as described previously³³, or subjected to western blot analysis. For immunoblot analysis of protein expression, cells were harvested with TEN buffer (10mM Tris/Cl pH 8.0, 1 mM EDTA, 100 mM NaCl), washed once with PBS and lysed with 2X protein sample buffer (125 mM Tris-HCl pH 6.8, 10 mM EDTA, 0.003 gr bromophenol blue, 20% glycerol, 4% SDS and 10% β-mercaptoethanol; 200µl). The samples were then sonicated, heated at 95°C and loaded onto 7.5% polyacrylamide-SDS page (Criterion, Biorad). After electrophoresis proteins were transferred to a nitrocellulose membrane and unspecific binding was blocked by PBS supplemented with 0.5% Tween (PBS-T) and 5% milk. Membranes were then incubated overnight at 4°C with primary antibodies (anti-PI4KIIIα, cat no. 4902, 1:250 Cell Signaling, anti-PI4KIIIβ, cat. No.
611817, 1:3000 BD Transduction Laboratories, mαβ-actin, cat. No. A1978, 1:5000, Sigma). HRP-conjugated secondary antibodies (donkey anti-rabbit, Cat. No. 9341 and sheep anti-mouse, Cat. No. 9311, GE Healthcare) were incubated for 1 hour at room temperature and detection was performed using SuperSignal-Femto chemiluminescent substrate (Pierce-Thermo Scientific).

8. T7-driven HCV polyprotein expression

1.5 x 10⁶ Huh7-Lunet/T7 cells/100 mm dish were transfected with 20 µg pTM-NS3-5B using the transfection reagent Lipofectamine 2000 (Invitrogen). Six hours after transfection, cells were seeded in 24-well plates on microscope cover glasses for indirect immunofluorescence. After 5 hours, cells were treated either with DMSO or with 8 µM AL-9 for 2, 8 or 16 hours and co-staining of NS5A and PI4P was performed using the Golgi staining protocol, as described previously.

F. Supporting Information

1. Figure S1 (see p. 155). Synthetic pathway for compound AL-9

2. Figure S2 (see p. 156). HCV replicons harboring putative 4-anilino quinazoline resistance mutations retain sensitivity to inhibition by AL-9

3. Figure S3 (see p. 157). Effect of AL-9 on subcellular distribution of type III PI kinases
Fig. S1. Synthetic pathway for compound AL-9
Reagents and conditions: (a) n-BuLi, dry THF, 278uC 1 h, 20uC 3 h, Bu3SnCl, 278uC 2 h, RT overnight; (b) Formamide, 155uC, 16 h; (c) SOCl2, dry DMF, reflux, 5 h, 4-morpholinoaniline, dry CH3CN, reflux, 16 h; (d) compound 1, bis(triphenylphosphine) palladium dichloride, dry THF, reflux; (e) HCl 2M, THF/H2O 1:1, RT; (f) NaBH(OAc)3, CH2Cl2/AcOH (15:1), RT.
Fig. S2. HCV replicons harboring putative 4-anilino quinazoline resistance mutations retain sensitivity to inhibition by AL-9.

Huh7.5 cells where transiently transfected with genotype 1b subgenomic replicons carrying mutation triplets reported to be associated to resistance to 4-anilino quinazolines (ET-FAG, ET-PPA or ET-DLD) or with the parental replicon (ET). The ET replicon is a derivative of the Con-1 replicon that contains adaptive mutations at positions E1202G, T1280I, and K1846T, i.e., the same genetic background used in the original resistance study. The putative resistance mutations triplets engineered in this replicon were as follows: ET-FAG (L199F, V362A, S390G in NS5A); ET-PPA (T200P, S370P in NS5A and S76A in NS5B); ET-DLD (E212D, P299L, V388D in NS5A). Transfected cells were treated with AL-9 for three days. Inhibitory dose-response curve of AL-9 are shown. Transient HCV replication was measured by luciferase activity and is expressed as % of the DMSO control. The data are averages from of three experimental replicates. EC50 values +/-1 SD are shown in the figure inset. Replicon ET-FAG did not replicate at appreciable levels.
Fig. S3. Effect of AL-9 on subcellular distribution of type III PI kinases

Cellular localization of PI4KIIIa (green), PI4KIIIb (green) or NS5A (red) was analyzed by immunofluorescence in Huh7.5 or JFH-4A cells incubated for 4 hrs with 8 mM AL-9 or DMSO (control). Zoomed sections are indicated by a white square. No major effect of AL-9 on the localization of either PI4KIIIa or PI4KIIIb was observed. Under our experimental conditions, we observe very limited colocalization of PI4KIIIa with NS5A (yellow) independent of the treatment with AL-9.

All the reagents were commercially available and used without further purification unless indicated otherwise. All solvents were anhydrous grade unless indicated otherwise. When dry conditions were required, the reactions were carried out in oven-dried glassware under a slight pressure of argon. Reaction were magnetically stirred and monitored by thin-layer chromatography (TLC) on silica gel. TLC was performed on Silica Gel 60 F254 plates (Merck) with UV detection, or using a developing solution of 0.5% orcinol in EtOH/H$_2$SO$_4$ (3%), followed by heating at 180°C. Flash column chromatography was performed on silica gel 230–400 mesh (Merck). The petroleum ether used as eluent in chromatography has boiling range of 40–60°C. $^1$H and $^{13}$C-NMR spectra were recorded on a Varian 400 MHz MERCURY instrument at 300 K. Chemical shifts are reported in ppm downfield from TMS as internal standard. Mass spectra were recorded on ESI-MS triple quadrupole (model API2000 QTrap™, Applied Biosystems).

*tributyl-(5-(diethoxymethyl)furan-2-yl)stannane (1)*

To a solution of 2-furaldehyde diethyl acetal (500μL, 2.9 mmol) in diethyl ether (3 mL) cooled to -78°C, n-butyllitium (3.9 mmol) was added under Ar atmosphere and the solution was stirred at -78°C for 1h and then for 3 h at 20°C. The solution was cooled again to -78°C and tributyltin chloride (1 mL, 3.9 mmol) was added dropwise; stirring for 2 h at -78°C and 12 h at room temperature.

The reaction was quenched by adding aqueous NH$_4$Cl and product extracted with diethyl ether. The organic layer was dried over Na$_2$SO$_4$
and concentrated in vacuo. The crude product was used without any further purification (brown oil, Rf = 0.74, AcOEt/hexane 0.2:9.8).

6-bromoquinazolin-4-ol (2)

To a solution of 2-ammino-5-bromobenzoic acid (5 g, 23.1 mmol), formamide (15 mL, 377.3 mmol) was added and the mixture was refluxed under Ar atmosphere for 16 h. Then 6 mL of water were carefully added (warning! boiling solution) and product immediately precipitated as a white solid. The mixture was then cooled at RT and 12 mL of water were added slowly. The mixture was stirred 30 min and the resulting precipitate was filtered and re-crystallized from cold ethanol obtaining product (white solid, yield: 53%, Rf = 0.51, AcOEt/petroleum ether 9.4:0.6)

1H-NMR (400 MHz; CDCl3/CD3OD 8:1) δ = 8.34 (d, 1H, J = 2 Hz), 7.97 (s, 1H), 7.83 (dd, 1H, J = 8.7 Hz, J = 2.3 Hz), 7.55 (d, 1H, J = 8.7 Hz).

6-bromo-N-(4-morpholinophenyl)quinazolin-4-amine (3)

To a solution of 6-bromoquinazolin-4-ol (2.84 g, 12.6 mmol) in DMF (0.84 mL) thionyl chloride (28 mL) was added and the mixture was refluxed for 5 h. The solvent was evaporated in vacuo by adding some toluene (3 x 28 mL) to completely remove thionyl chloride. The residue was dissolved in CH3CN (38 mL), 4-morphoaniline (2.48 mg, 13.9 mmol) was added and the mixture was refluxed for 72 h. Crude product precipitated upon cooling the solution and the solid precipitate was recrystallized from diethyl ether obtaining pure product (orange solid, yield: 79%, Rf = 0.37, AcOEt).

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\( ^1 \text{H-NMR} \) (400 MHz, DMSO-d\(_6\)) \( \delta = 11.30 \) (bs, 1H, NH), 9.04 (bs, 1H), 8.87 (s, 1H), 8.19 (bd, 1H, J = 8.8 Hz), 7.79 (d, 1H, J = 8.8 Hz), 7.57-7.04 (AA’XX’ system, 4H, J = 9.0 Hz), 3.74 (m, 4H), 3.14 (m, 4H).

6-(5-(diethoxymethyl)furan-2-yl)-N-(4-morpholinophenyl)quinazolin-4-amine (4)

To a solution of 6-bromo-N-(4-morpholinophenyl)quinazolin-4-amine (3) (300 mg, 0.78 mmol) in dry THF (7 mL), tributyl-(5-(diethoxymethyl)furan-2-yl)stannane (1) (1.4 g, 3.1 mmol) and bis(triphentilphosphine) palladium dichloride (44 mg, 0.06 mmol) were added. The reaction mixture was heated to reflux for 25 h under Ar atmosphere. The solvent was evaporated \textit{in vacuo}, the crude product was dissolved in AcOEt and washed with water and brine. The organic layer was dried over Na\(_2\)SO\(_4\) and concentrated \textit{in vacuo}. The crude product obtained was purified with flash chromatography on silica gel (AcOEt/exane 8:2) obtaining pure product (yellow solid, yield 74%, \( R_f = 0.24 \), AcOEt/petroleum ether 9.4:0.6).

\( ^1 \text{H-NMR} \) (400 MHz, CDCl\(_3\)) \( \delta = 8.65 \) (s, 1H), 8.20 (d, 1H, J = 1.5 Hz), 7.97 (dd, 1H, J = 8.8 Hz, J=1.5 Hz), 7.84 (d, 1H, J = 8.8 Hz), 7.56-6.93 (AA’XX’ system, 4H, J = 8.5 Hz), 6.70 (d, 1H, J = 2.7 Hz), 6.53 (d, 1H, J = 2.7 Hz), 5.57 (s, 1H), 3.87 (m, 4H), 3.60 (m,4H), 3.13 (m, 4H), 1.25 (m, 6H).

\( ^{13} \text{C-NMR} \) (400 MHz, CDCl\(_3\)) \( \delta = 158.02, 155.05, 152.70, 151.97, 149.30, 148.73, 130.24, 129.03, 128.89, 128.48, 124.21, 116.19, 115.31, 114.91, 110.71, 106.86, 96.27, 66.88, 61.54, 49.51, 15.15.

ESI-MS: calculated 474.34, found: 475.21 [M+H\(^+\)]; 497.21 [M+Na\(^+\)].

160
5-((4-morpholinophenyl)amino)quinazolin-6-yl)furan-2-carbaldehyde (5)

To a solution of 6-((5-diethoxymethyl)furan-2-yl)-N-(4-morpholinophenyl)quinazolin-4-amine (4) (87 mg, 0.18 mmol) in THF (8 mL), HCl 2M (1.6 mL) was added and the mixture was reacted for 2 h at room temperature. The reaction mixture was basified to pH 8 with NaOH 2M. Organic solvent was evaporated in vacuo and the aqueous residue was extracted with AcOEt. The organic layer was dried over Na$_2$SO$_4$ and concentrated in vacuo. Product (orange solid, yield 98%, $R_f = 0.61$, AcOEt/MeOH 9:1) was used without any further purification.

$^1$H-NMR (400 MHz, CDCl$_3$) $\delta = 9.60$ (s, 1H), 8.65 (1H, s), 8.41 (1H, d, J = 1.3 Hz), 8.01 (dd, 1H, J= 8.8 Hz, 1.5 Hz ), 7.85-7.57 (AA’XX’ system, 4H, J = 8.8 Hz), 7.32 (d, 1H, J = 3.7 Hz), 6.92 (m, 3H), 3.82 (t, 4H, J = 4.8 Hz), 3.11 (t, 4H, J = 4.8 Hz).

$^{13}$C-NMR (400 MHz, CDCl$_3$) $\delta = 177.21$, 158.30, 156.05, 152.12, 150.55, 148.81, 130.03, 129.35, 129.31, 126.35, 124.28, 117.92, 116.03, 115.47, 108.67, 66.84, 49.39.

ESI-MS: calculated 400.15, found: 401.13 [M+H$^+$].

(5-((4-morpholinophenyl)amino)quinazolin-6-yl)furan-2-yl)methanol (6)

To a solution of 5-((4-morpholinophenyl)amino)quinazolin-6-yl)furan-2-carbaldehyde (5) (140 mg, 0.35 mmol) in dry CH$_2$Cl$_2$ (5mL) and AcOH glacial (0.3 mL), sodium triacetoxyborohydride (148 mg, 0.70 mmol) was added. The solution was reacted for 3 h at room temperature. Fresh CH$_2$Cl$_2$ was added and washed with water and brine. Aqueous layer was washed again with fresh AcOEt. The
combined organic layers were dried over Na$_2$SO$_4$ and concentrated in vacuo. The crude product obtained was purified with flash chromatography on silica gel (AcOEt) obtaining pure product (yellow solid, yield 35%, $R_f$ = 0.53 AcOEt/MeOH 9:1).

$^1$H-NMR (400 MHz, DMSO-d$_6$) $\delta$ = 9.84 (s, 1H), 8.74 (d, 1H, $J$ = 1.4 Hz), 8.44 (s, 1H), 8.11 (dd, 1H, $J$ = 8.8 Hz, $J$ = 1.4 Hz), 7.74-7.61 (AA’XX’ system, 4H, $J$ = 8.8 Hz), 7.03 (d, 1H, $J$ = 3.2 Hz), 6.98 (d, 2H, $J$ = 8.8 Hz), 6.48 (d, 1H, $J$ = 3.2 Hz), 5.32 (t, 1H, $J$ = 5.7 Hz), 4.50 (d, 2H, $J$ = 5.7 Hz), 3.74 (t, 4H, $J$ = 4.7 Hz), 3.09 (t, 4H, $J$ = 4.7 Hz).

$^{13}$C-NMR (100 MHz, DMSO-d$_6$) $\delta$ = 157.80, 156.04, 154.53, 151.79, 148.88, 147.90, 130.79, 128.36, 128.09, 124.14, 116.59, 115.43, 115.07, 109.59, 107.65, 66.13, 55.84, 48.81.

ESI-MS: calculated, 402.17, found: 403.19 [M+H$^+$].

5. Protocol S2. Construction and assays of HCV replicons harboring putative resistance mutations

The mutants pFKi341-PiLuc-NS3-3’/ET-DLD (E212D, P299L, V388D in NS5A) and pFKi341-PiLuc-NS3-3’/ET-FAG (L199F, V362A, S390G in NS5A) were generated starting from pFKi341-PiLuc-NS3-3’/ET (provided by V. Lohman). Two synthetic fragments (737 or 767 bp, respectively) containing the indicated triple mutations were provided from MWG-Eurofins. Using Crossover PCR-based method SalI-BamHI fragments carrying mutations were obtained and transferred into pFKi341-PiLuc-NS3-3’-ET backbone. The mutant pFKi341-PiLuc-NS3-3’-ET-PPA (T200P, S370P in NS5A and S76A in NS5B) was obtained using Multi Site-Directed Mutagenesis Kit (Agilent Technologies). In
particular, the MluI-SpeI fragment of pFKi341-PiLuc-NS3-3’-ET was subcloned into pCR2.1 vector, mutated according to manufacturer’s protocol and transferred back into the pFKi341-PiLuc-NS3-3’-ET backbone. All numbers refer to aminoacid position of HCV proteins (HCV Con-1; EMBL database accession number AJ238799).

The pFKi341-PiLuc-NS3-3’/ET construct and the mutants pFKi341-PiLuc-NS3-3’/ET-DLD, pFKi341-PiLuc-NS3-3’/ET-FAG and pFKi341-PiLuc-NS3-3’/ET-PPA were linearized at the 3’ end of the viral genome by Scal digestion and purified by Sodium Acetate precipitation. RNA transcripts were generated and purified by MEGAscript T7 kit (Ambion) according to the manufacturer’s protocol. 2x10^6 Huh7.5 cells were electroporated with 10 μg of each RNA construct in a final volume of 200 μl as described previously. Electroporated cells were plated at the density of 7.5x10^3 cells/well in 96-well plates. 6 hours after electroporation, cells were treated with serial dilutions of AL-9. After three days of treatment, cell viability was measured by CellTiter-Blue® (Promega) and luciferase activity was measured by Bright-Glo™ Luciferase Assay System (Promega), according to the manufacturer’s protocols.

G. Acknowledgments

We wish to thank Glenn Randall, Volker Lohmann and Charles M. Rice for providing us with essential constructs and cells. We express our gratitude to Roberto Cighetti for his contribution to the synthesis of the compound AL-9 and to Manila Boca for her assistance and training in operating the confocal microscope. We also would like to
thank Richard Angell and Magdalena Laver from Arrow Therapeutics for helpful discussions during this research.

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H. Chapter 2 – References


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48. Pacini, L., Graziani, R., Bartholomew, L., De Francesco, R. & Paonessa, G. Naturally occurring hepatitis C virus subgenomic deletion mutants replicate efficiently in Huh-7 cells and are

CHAPTER 3

The presence of hepatitis C virus deletion mutants is associated with necro-inflammatory activity and pattern of response to therapy in HCV1 patients

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# These authors contributed equally to the manuscript

This work is going to be submitted for publication to Hepatology.
A. Abstract

Hepatitis C virus (HCV) variants characterized by genomic deletions in the region of structural proteins have been sporadically detected in the serum and liver of hepatitis C patients. These defective genomes are capable of autonomous RNA replication and are packaged into infectious viral particles in cells co-infected with the wild-type virus. The frequency of such forms in HCV infected population, as well as their impact on the progression of liver disease and antiviral therapy outcome, are currently unknown.

To investigate the incidence of HCV defective variants and their correlations with clinical characteristics, a screening campaign was performed on pre-therapy serum samples from a well-characterized cohort of HCV1 naïve patients who received treatment with Pegylated Interferon alpha (PEG-IFNα) and Ribavirin (RBV). 132 HCV1 subjects were successfully analyzed for the presence of defective species exploiting a long-distance nested PCR assay spanning from Core to NS3.

HCV forms with deletions affecting prevalently E1, E2 and p7 proteins were found in a high fraction (25/132, 19%) of the subjects. Their presence was associated with patient older age, higher viral load and increased inflammatory activity. While the presence of subgenomic HCV did not appear to influence significantly virological decline nor sustained virological response rates to PEG-IFNα and RBV (24% vs. 38%) it resulted to be an independent predictor of relapse in
the population reaching the viral clearance at the end of the treatment (OR:3.35).

In conclusion, in chronic HCV1 patients, the presence of HCV with defective genomes was associated with hepatic necro-inflammatory lesions. Furthermore, defective HCV RNA may represent a novel predictor of relapse after initial virological response following PEG-IFNα/RBV therapy.

B. Introduction

Hepatitis C virus (HCV) is an enveloped virus belonging to the family of Flaviviridae. Its genome consists of a positive-stranded RNA molecule of approximately 9.6 Kb that is structured in a 5’ non-coding region (5’UTR), a long open reading frame encoding a polyprotein precursor of about 3010 amino acids and a 3’UTR region. The polyprotein is processed by the action of both viral and cellular proteases to produce structural (Core and E1-E2 glycoproteins) and non-structural proteins (p7, NS2, NS3, NS4A-B and NS5A-B). The RNA-dependent RNA polymerase NS5B produces with a high frequency of incorrect nucleotide insertion; this characteristic, coupled with the lack of proofreading ability and the high in-vivo productive rate of new virions, is believed to be the driving force of HCV genetic diversity\textsuperscript{1,2}.

HCV infection affects an estimated 170 million people worldwide and is the major cause of chronic liver disease, liver cirrhosis and hepatocellular carcinoma\textsuperscript{3}. Standard antiviral therapy is based on long-term administration of Pegylated Interferon alpha (PEG-IFNα) and
ribavirin (RBV), but permits to achieve viral clearance only in about 40-60% of cases.

Both viral and host genetic variability are thought to influence treatment success rates. Recently, whole-genome association studies identified host single nucleotide polymorphisms (SNPs) near the genomic region encoding IL28B as strongly associated with both spontaneous and treatment-induced viral clearance\(^4\-^6\): under PEG-IFN\(\alpha\)/RBV therapy, HCV1 patients having the rs12979860 CT or TT genotype display reduced viral load decline and lower SVR rates. Regarding viral genetics, HCV genotypes 1 and 4 are considered more difficult to treat than genotypes 2 and 3\(^7\).

HCV variants characterized by genomic deletions have been highlighted in the serum and liver of chronic HCV patients\(^8\-^10\) as well as in the serum of immunosilent infections\(^11\) and liver transplant recipients with recurrent HCV infection\(^12\). These defective genomes contain large in-frame deletions affecting prevalently the Envelope (E1 and E2) region, but retain the Core region in addition to the genome portions that are essential for autonomous HCV replication (5'UTR and NS3-NS5-3'UTR)\(^8\). It has recently been demonstrated\(^9\,\,10\) in \textit{in vitro} infectivity models that subgenomic HCV RNAs with the architecture of the naturally occurring ones are capable of autonomous replication and are expressed at the protein levels, display a higher replication fitness compared to the full-length genome and are efficiently packaged into infectious viral particles when co-expressed together with full-length viral genome. Their structural peculiarities confer to HCV defective particles remarkable features: although they require the
complementation of structural proteins by a wild type virus to complete virion assembly and exit, upon infection of a naïve hepatocyte they will transduce self-replicating HCV subgenomes, resulting in the persistent expression of HCV proteins even in the absence of co-infection with the wild-type helper virus.

Deleted HCV species are reminiscent of defective interfering (DI) forms that are reported for other RNA viruses, including Flaviviridae family members pestivirus (Classical Swine Fever Virus – CSFV – and Bovine Viral Diarrhea Virus – BVDV – 13-15) and flavivirus (Murray Valley encephalitis virus16). DI variants are characterized by large genomic deletions, but retain the regions that are critically required for genome replication and encapsidation17; therefore, they are both dependent on and competitive with the parental virus.

While a key pathogenic role of DI forms is already well-established for flaviviruses such as CSFV and BVDV, to date the clinical relevance of HCV defective particles, as well as their incidence in general HCV infected population, have never been systematically evaluated. To address this point, we undertook a screening campaign for HCV defective genomes on pre-therapy serum samples of a well-characterized cohort of chronic HCV1 patients undergoing standard therapy treatment.
C. Results

1. HCV particles with defective genomes are found in the serum of a large fraction of chronic hepatitis C genotype 1 patients

Pre-therapy serum samples of 132 subjects fulfilling the selection criteria were screened for the presence of HCV defective species exploiting a long-distance nested PCR assay spanning from core to NS3.

Demographic and clinical characteristics of the patients are described in Tables 1A and 1B. Both genders were well represented (52.3% males) and the median age was 56 years (IQR 19.25). 30.3% of patients had moderate to severe histological activity (Grading ≥9) and 37.1% of them showed advanced fibrosis (Staging ≥4).

RVR (rapid virological response) and cEVR (complete early virological response) were obtained respectively in 34.1% and 58.3% of the cohort. At the end of treatment and follow-up period, 47 subjects (35.6%) achieved SVR, while 27 experienced a viral relapse and 58 were non-responders.

Large fragment PCR amplification, from nucleotide 127 (5'UTR region) to 3649 (NS3 protein), followed by agarose gel analysis revealed in a portion of the subjects the presence of amplicons consistently smaller than those expected from the amplification of full-length HCV genome (ranging from 1200 to 2000 bps, compared to 3522), thus suggesting the occurrence of large deletions (Fig. 1A). This screening strategy, being based on a very sensitive nested PCR technique that gives a preferential amplification of shorter amplicons, is able to detect the defective variant even if more rare than the full-
length variant. However, we cannot completely rule out the possibility to have classified as full-length patients in which the % of defective is very low.

Subsequent cloning and nucleotide sequencing investigation of the shorter amplicons was employed in each putative defective sample to confirm and further characterize the deletion events. Defective HCV genomes were confirmed in a high fraction of the cohort: we observed defective variants in 25/132 (18.9%) of the patients. Sequencing analysis highlighted the presence of in-frame deletions affecting prevalently envelope proteins and p7 (Figure 1C). In fact, in all the defective variants, E2 was completely deleted and E1 and p7 were at least partially missing; the initial portion of NS2 was lost in 21 (84%) of them. Two deletions in a single genome were observed in 3 patients, in which a small residual fragment ranging from 28 to 43 bps was localized between the two large deletions.
<table>
<thead>
<tr>
<th>Factor</th>
<th>All patients (n=132)</th>
<th>Stratified by HCV defective presence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Full-length (n=107)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>56 ± 19.25</td>
<td>54 ± 22</td>
</tr>
<tr>
<td>HCV RNA (IU/ml)</td>
<td>740820.5±1004706</td>
<td>703751 ± 859449</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>103 ± 97.5</td>
<td>101 ± 95</td>
</tr>
<tr>
<td>GGT (IU/l)</td>
<td>60 ± 63.25</td>
<td>60 ± 62</td>
</tr>
<tr>
<td>Male gender</td>
<td>69 (52.3%)</td>
<td>57 (53.3%)</td>
</tr>
<tr>
<td>BMI ≥ 25 kg/m²</td>
<td>43 (32.6%)</td>
<td>36 (33.6%)</td>
</tr>
<tr>
<td>Grading ≥ 9</td>
<td>40 (30.3%)</td>
<td>26 (24.3%)</td>
</tr>
<tr>
<td>Staging ≥ 4</td>
<td>49 (37.1%)</td>
<td>38 (35.5%)</td>
</tr>
<tr>
<td>IL28B CT/TT</td>
<td>76 (57.6%)</td>
<td>66 (61.7%)</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>35 (26.5%)</td>
<td>29 (27.1%)</td>
</tr>
<tr>
<td>RVR</td>
<td>45 (34.1%)</td>
<td>35 (32.7%)</td>
</tr>
<tr>
<td>cEVR</td>
<td>77 (58.3%)</td>
<td>60 (56.1%)</td>
</tr>
<tr>
<td>ETR</td>
<td>74 (56.1%)</td>
<td>59 (55.1%)</td>
</tr>
<tr>
<td>SVR</td>
<td>47 (35.6%)</td>
<td>41 (38.3%)</td>
</tr>
<tr>
<td>Relapse in ETR</td>
<td>27/74 (36.5%)</td>
<td>18/59 (30.5%)</td>
</tr>
</tbody>
</table>

Table 1A. Demographic and clinical features of the 132 HCV1 patients, stratified by presence of HCV defective particles
### Table 1.

<table>
<thead>
<tr>
<th>Factor</th>
<th>All patients (n=132)</th>
<th>Stratified by IL28B genotype</th>
<th>Pval</th>
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<tbody>
<tr>
<td></td>
<td>rs12979860 CC (n=56)</td>
<td>rs12979860 CT+TT (n=76)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>56 ± 19.25</td>
<td>55 ±19.75</td>
<td>56 ±19</td>
</tr>
<tr>
<td>HCV RNA (IU/ml)</td>
<td>740820.5±1004706</td>
<td>700032.5±858765</td>
<td>778654±1004706</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>103 ± 97.5</td>
<td>123 ± 155</td>
<td>91.5 ± 65.5</td>
</tr>
<tr>
<td>GGT (IU/l)</td>
<td>60 ± 63.25</td>
<td>45 ± 60</td>
<td>72.5 ± 81</td>
</tr>
<tr>
<td>Male gender</td>
<td>69 (52.3%)</td>
<td>27 (48.2%)</td>
<td>42 (55.3%)</td>
</tr>
<tr>
<td>BMI ≥ 25 kg/m²</td>
<td>43 (32.6%)</td>
<td>17 (30.4%)</td>
<td>26 (34.2%)</td>
</tr>
<tr>
<td>Grading ≥ 9</td>
<td>40 (30.3%)</td>
<td>22 (39.3%)</td>
<td>18 (23.7%)</td>
</tr>
<tr>
<td>Staging ≥ 4</td>
<td>49 (37.1%)</td>
<td>19 (33.9%)</td>
<td>30 (39.5%)</td>
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<tr>
<td>IL28B CT/TT</td>
<td>76 (57.6%)</td>
<td>----</td>
<td>47 (17.1%)</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>35 (26.5%)</td>
<td>13 (23.2%)</td>
<td>22 (28.9%)</td>
</tr>
<tr>
<td>RVR</td>
<td>45 (34.1%)</td>
<td>30 (53.6%)</td>
<td>15 (19.7%)</td>
</tr>
<tr>
<td>cEVR</td>
<td>77 (58.3%)</td>
<td>50 (89.3%)</td>
<td>27 (35.5%)</td>
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<tr>
<td>ETR</td>
<td>74 (56.1%)</td>
<td>48 (85.7%)</td>
<td>26 (34.2%)</td>
</tr>
<tr>
<td>SVR</td>
<td>47 (35.6%)</td>
<td>34 (60.7%)</td>
<td>13 (17.1%)</td>
</tr>
<tr>
<td>Relapse in ETR</td>
<td>27/74 (36.5%)</td>
<td>14/48 (29.2%)</td>
<td>13/26 (50.0%)</td>
</tr>
</tbody>
</table>

**Table 18. Demographic and clinical features of the 132 HCV1 patients, stratified by IL28B genotype**
Fig. 1. Viral genome architecture of the defective forms identified in the serum of HCV1 patients

Representative agarose-gel pictures showing full-length (lanes 1-2, 4-5 of the first gel and 1-5 of the second gel) and defective bands (lanes 3, 6 of the first gel and 6, 7 of the second gel) after the second round of nested PCR (A). Usually, in samples showing the shorter band, the full-length genome could not be detected, given the preferential amplification of the deletion mutant.

Representative agarose-gel picture showing amplification of a small core segment from the same RNA (B).

Schematic representation of the architecture of the 25 defective variants identified in the sera of HCV1 patients (C).
2. The presence of defective genomes is associated with patient older age, higher viral load and increased necro-inflammatory activity.

We then examined the correlations between the presence of defective HCV forms and clinical characteristics or viral kinetics on the 132 HCV1 patients of the cohort.

As visible in Table 1A, the presence of HCV defective genomes was found to be associated with older patient age (Wilcoxon test Pval 0.0367), higher viremic load (Wilcoxon test Pval 0.0046) and mild to severe hepatic necro-inflammatory activity (histological activity index ≥9; Fisher’s test Pval 0.003).

Analyzing viral kinetics during PEG-IFNα/RBV treatment, we could not detect substantial differences in the decline of viral load at early time-points (RVR and cEVR rates) in carriers of deleted variants; also ETR percentages were unchanged. SVR rates tended to be lower for patients with defective genomes (24% compared to 38.3%), but the difference was far from statistic significance (Fisher test Pval 0.247, Fig 2A).

Strikingly, however, among 74 patients who were HCV RNA-negative at the end of the treatment, virological relapse was observed in 60% (9/15) of the subjects carrying HCV defective genomes compared to 30% (18/59) of those infected with only the full-length virus (Fig. 2B). The association is characterized by a strong odds ratio (3.35) and a moderate statistic significance in univariable analysis (Chi-test Pval 0.034, Fisher’s test Pval 0.069).
RVR, cEVR, ETR and SVR rates in the overall population as well as relapse rates in ETR-positive subjects, according to the presence of HCV deletions (A-B) or IL28B genotype (C-D), are reported. The presence of HCV defective particles does not have a significant effect on RVR, cEVR, ETR or SVR rates, while it correlates with a higher probability of relapse in ETR-positive subjects (A, B). IL28B CT/TT genotypes are significantly associated with lower RVR, cEVR, ETR and SVR rates and correlate with a higher probability of relapse (*** Pval<0.0001).

3. IL28B genotype profoundly influences viral kinetics during treatment and is associated with SVR

IL28B genotype has recently emerged as a strong predictor for viral clearance in HCV1 patients undergoing PEG-IFNα/RBV therapy. Therefore, to better elucidate a possible role of HCV defective variants in influencing treatment outcome, it was essential to take into account also the well-established effect of IL28B genotype. For this reason,
rs12979860 polymorphism was determined in the patients of our cohort. SNP genotype frequencies in HCV1 population were 42.4% CC, 28% CT and 29.6% TT; therefore, 57.6% of the subjects were carriers of the “non-responder” T allele.

The relationships between IL28B genotype and clinical characteristics and viral kinetics are listed in Table 1B. IL28B unfavorable genotype resulted associated with lower ALT and higher GGT pre-therapy levels (Wilcoxon test Pval 0.0133 and 0.0316 respectively). Moreover, a weak association (Fisher’s test Pval 0.059) was noticeable between IL28B unfavorable genotype and lower grading scores.

Analyzing the therapy outcome, IL28B unfavorable genotype strongly correlated with treatment failure (OR 7.35, Pval 3.71e-7); the effect is noticeable already at early time points (RVR and cEVR rates, Fig2C-D).

4. The association between HCV defective particles and higher histological grading is independent from IL28B genotype

In order to confirm the correlation between increased necro-inflammatory activity and the presence of HCV defective particles by also taking in account the possible role of IL28B genotype and other confounding factors, a stepwise multivariable logistic regression model was exploited. The stepwise approach was used for the selection of the variables and a final model was established on the basis of the minimum Akaike Information Criterion (AIC). As shown in Table 2, the model confirmed the role of HCV deletions (Pval 0.028, OR 2.89), along
with patient age, as risk factors for higher grading index, while IL28B genotype had a modest effect (Pval 0.079, OR 0.48 for the CT/TT variants).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Multivariable Pval</th>
<th>OR</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Outcome: Grading</strong></td>
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<td></td>
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</tr>
<tr>
<td>HCV deletion</td>
<td>0.028</td>
<td>2.89</td>
<td>1.12-7.44</td>
</tr>
<tr>
<td>Age</td>
<td>0.043</td>
<td>1.46(^a)</td>
<td>1.02-2.15(^a)</td>
</tr>
<tr>
<td>IL28B CT/TT</td>
<td>0.079</td>
<td>0.48</td>
<td>0.23-1.02</td>
</tr>
<tr>
<td><strong>Outcome: ETR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL28B CT/TT</td>
<td>3.95 e-7</td>
<td>0.06</td>
<td>0.02-0.18</td>
</tr>
<tr>
<td>Age</td>
<td>0.004</td>
<td>0.51(^a)</td>
<td>0.32-0.79(^a)</td>
</tr>
<tr>
<td>Viremia</td>
<td>0.035</td>
<td>0.96(^b)</td>
<td>0.92-0.99(^b)</td>
</tr>
<tr>
<td>GGT</td>
<td>0.008</td>
<td>0.79(^c)</td>
<td>0.65-0.93(^c)</td>
</tr>
<tr>
<td><strong>Outcome: Relapse</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV deletion</td>
<td>0.049</td>
<td>3.73</td>
<td>1.01-13.77</td>
</tr>
<tr>
<td>Grading ≥ 9</td>
<td>0.017</td>
<td>4.11</td>
<td>1.29-13.07</td>
</tr>
<tr>
<td>IL28B CT/TT</td>
<td>0.021</td>
<td>3.90</td>
<td>1.23-12.33</td>
</tr>
</tbody>
</table>

Table 2. Multivariable logistic regression for Grading, ETR and Relapse
OR: odds ratio – CI: 95% confidence interval
a: odds ratio and confidence intervals have been calculated considering an interval of 10 years.
b: odds ratio and confidence intervals have been calculated considering an interval of $10^5$ units of viremia

c: odds ratio and confidence intervals have been calculated considering an interval of 20 units of GGT

5. Defective HCV particles and IL28B genotype are independent predictors of relapse in ETR-positive patients

Using the same approach, we exploited a multivariable logistic regression model to pinpoint the parameters associated with viral clearance at the end of treatment (ETR). In the final model, summarized in Table 2, IL28B CC genotype was the variable most strongly and positively associated with ETR (Pval 3.95e-7, OR 0.06 for CT/TT variants, corresponding to an OR of 16.6 for CC genotype). Younger patient age and lower pre-treatment viral load and GGT levels also resulted significantly associated with ETR. No impact on ETR obtainment was found for the presence of defective HCV particles, that therefore was not included as co-variate in the model.

Similarly, a model was constructed to evaluate the explanatory variables predicting virological relapse in the 74 subjects who where HCV-negative at the end of treatment. As described in Table 2, the presence of deletions in HCV genome represented a risk factor for relapse with an odds ratio of 3.73 (Pval 0.049), along with mild-to-severe histological grading (OR 4.11, Pval 0.017) and IL28B CT/TT genotypes (OR 3.90, Pval 0.021).
D. Discussion

In this study, we analyzed the presence of viral defective forms in a well-characterized cohort of HCV1 patients. The screening campaign was designed to detect deletion events occurring in the region of Core-NS3 and revealed the presence of large in-frame deletions affecting prevalently E1, E2 and p7 proteins. This finding is in line with already published results; in fact, the subgenomic HCV species reported in previous studies conserved the regions essential for RNA replication (5’UTR, 3’UTR and from NS3 to NS5B), intact Core and the protease domain of NS29,10,12,18.

Analyzing pre-therapy sera of 132 patients, defective genomes were found in about 20% of them; to our knowledge, this is the first systematic analysis done on such a large number of subjects, and it permits to infer that the emerging of deletion mutants, far from being a sporadic phenomenon, occurs in a relevant portion of HCV1-infected subjects.

Defective interfering forms are reported for a variety of viruses, and their clinical impact is variegated19. In fact, as a consequence of DI particles insurgence, a reduction in the titers of the parental virus20 and attenuation of disease severity21 have been reported; on the other hand, it has been proposed that DI viruses may play a role in the establishment and maintenance of persistent infection, as described for Murray Valley Encephalitis Virus and Japanese Encephalitis Virus16,22,23. Moreover, a crucial pathogenic role has been established for some DI viruses belonging to the Flaviviridae family. In the case of BVDV, a highly cytopathogenic viral isolate was shown to contain a DI
virus lacking the genes encoding the structural proteins\textsuperscript{14}. In fact, the pathogenesis of lethal mucosa disease in animals persistently infected with non-cytopathogenic BVDV has been ascribed to the insurgence of cytopathogenic variants containing genomic rearrangements, among which internal deletions\textsuperscript{24}. Similarly, cytopathogenicity of CSFV was demonstrated to be associated to a DI variant lacking the structural protein-coding region\textsuperscript{25}.

Regarding HCV, the ability of defective particles to influence the clinical course of the disease is largely unexplored. Focalizing on the clinical characteristics of our cohort, we observed that the presence of HCV defective variants correlates with higher viral load, older patient age and higher degree of hepatic inflammation. Since defective viruses are thought to be generated from the wild type through errors of the polymerase, the probability of their insurgence increases proportionally with the number of replication events. This may be at the basis of the association with older patient age (which probably could be considered as a proxy of the duration of infection) and higher viremia. From an etiopathological point of view, the association with the grading score appears more intriguing, raising the possibility that HCV defective particles exacerbate the liver inflammation processes. Given their high replication fitness and inability to autonomously exit from the cell, deleted variants are likely more prone to lead to the accumulation of viral proteins inside the cell; therefore, the persistent expression of HCV defective genomes in liver hepatocytes could have important pathological consequences. From this point of view, the pathogenic activity of defective forms could results from the balance
between the ability to accumulate in host cells (in absence of the helper virus) and, when complemented with the wild type structural proteins, to spread to other cells. Since the presence of an intact Core is one of the hallmarks of defective HCV genomes we found, it is conceivable that cells infected with subgenomic HCV will accumulate very high level of intracellular Core protein that, in contrast to the case of cells infected with the wild type virus, will not be counterbalanced by the formation of mature virions and virus exit. HCV Core protein is normally localized on the surface of cytoplasmic lipid droplets (LDs), which are believed to represent sites of virus assembly and production of nascent virions. Overexpression of intracellular Core has been reported to cause abnormal accumulation and redistribution of LDs, as well as to induce the production for free radicals and apoptotic cell death. Moreover, Core protein has been indicated to be involved in liver steatosis, fibrosis and carcinogenesis in various mouse model systems.

Altered expression of other viral proteins may also negatively impact the hepatocyte function. As already described, all the deleted variants found were characterized by the partial or total loss of E1, E2 and p7. While the involvement of E1 and E2 envelope glycoproteins in viral entry is well-established, the function of p7 in HCV life cycle is less understood, although it has been reported to be fundamental for virus infection but not necessary for RNA replication. More recently, it has been demonstrated that p7 plays a crucial role in re-directing Core from the LDs to the ER, probably favoring virus assembly; more precisely, the interactions between Core, p7 and the first
transmembrane domain of NS2 determine the degree of localization of core in ER or LD\textsuperscript{36}. In this context, the partial or complete lack of p7 may be another mechanism that favors the accumulation of Core on the lipid droplets. Finally, also the fusion protein produced by the deletion event may be able to exacerbate the pathological changes in the cell. Induction of ER stress has been reported in both \textit{in vitro} models of HCV and biopsies of HCV patients\textsuperscript{37}; since fusion proteins are probably more prone to be unfolded or misfolded, it could be speculated that ER stress is intensified in patients carriers of defective variants. A growing body of evidence suggests that ER stress and the inflammatory response are strictly interconnected\textsuperscript{38}.

We did not observe a correlation between the presence of defective forms and the staging score. However, it should be considered that, for this cohort, the lack of knowledge of the time of infection and of insurgence of the defective variants prevents us to correctly estimate the fibrosis progression rate and the impact of the subgenomic virus on it.

We then evaluated the viral kinetics during standard therapy treatment in HCV1 patients carrying the defective forms compared to those in which only the full-length variant was detected. No difference was found in RVR, cEVR and ETR rates, thus indicating that defective particles do not seem to play a key role in determining viral load decline during treatment. This result was confirmed also by logistic regression analysis, in which IL28B genotype, patient age and pre-treatment viremia, but not the presence of defective HCV, were identified as independent predictors of ETR. Refining the analysis on
the ETR-positive population, however, we observed that defective particle carriers displayed a higher probability to experience virological relapse during the follow-up period. This result is particularly interesting, since it has been proposed that defective virus belonging to the Flaviviridae family may play a role in the establishment and maintenance of chronic infections, although the majority of the data comes from in-vitro experiments\textsuperscript{16, 23}. A possible role in viral persistence has been suggested also for deleted variants of hepatitis B virus (HBV). Deletions in the surface antigen regions S1 and S2 of HBV genome have been reported in sera and liver of HBV patients\textsuperscript{39, 40} and, since pre-S region contains several epitopes for T or B cells\textsuperscript{41}, the emergence of deleted HBV variants may result in an impairment of viral clearance and therefore potentially be a mechanism of evasion of immune surveillance. Moreover, the incidence of these variants increases with the progression of the disease\textsuperscript{42}.

Multivariable analysis confirmed the role of subgenomic HCV as independent risk factor for relapse, with an effect magnitude (OR 3.73) comparable to that of IL28B genotype (OR 3.90). Interestingly in our cohort, 3 out of 4 (75\%) of ETR-positive subjects carriers of defective forms and IL28B unfavorable genotype experienced virological relapse, while only 22\% (8/37) of patients with the IL28B favorable genotype and lacking defective forms had a post-treatment relapse.

Although this might suggest a role for both IL28B genotype and HCV with defective genomes in determining the pattern of response to PEG-IFNα/RBV, we acknowledge that external validation of our data is necessary. Indeed, although our cohort is the largest one to date to be
analyzed for the presence of defective forms of HCV, it is still quite limited in size to provide conclusive evidence of their role in modifying treatment response. Further reinforcing the need for external validations is the fact that our treatment cohort might not reflect well the everyday clinical practice as all our patients were caucasian, with a relatively high prevalence of the IL28B CC genotype and in all cases received PEG-IFNα-2b.

In conclusion, our study indicated that, in pre-treatment sera of chronic HCV1 patients, HCV particles with large in frame-deletions in E1-NS2 region are detected in about 20% of the subjects and correlate with a higher degree of liver inflammation measured by the Ishak score. While RVR and ETR rates were not affected, the presence of defective particles was more frequent in subject experiencing a viral relapse after standard PEG-IFNα-2b/RBV therapy.

E. Materials and Methods

1. Patients

152 Patients infected by HCV genotype 1 who received PEG-IFNα-2b/RBV therapy in a previous study conducted at the Center for Liver Disease at Maggiore Hospital (Milan, Italy), were recalled and offered participation in the current study.

Treatment exclusion criteria were Hepatitis B Virus (HBV) or Human Immunodeficiency Virus (HIV) co-infection, decompensated liver disease, drug dependence or >40 g/day alcohol intake. Subjects affected by poorly controlled diabetes, severe depression,
autoimmune disease or concomitant malignant neoplastic diseases were also excluded.

A diagnostic liver biopsy performed prior to antiviral treatment was available for each subject. Liver biopsies were evaluated by a single expert pathologist and scored according to the Ishak system in separate reports for grading (from 0 to 18, ranging from 0 to 4 for piecemeal necrosis, focal necrosis and portal inflammation, and from 0 to 6 for confluent necrosis) and staging (from 0 -no fibrosis- to 6 - cirrhosis-).

Serum HCV RNA was assessed by in-house nested reverse transcriptase (RT)-PCR, using specific primers for 5’UTR and quantified by Versant HCV RNA 3.0 assays (bDNA 3.0; Bayer Corporation, Emeryville, CA, USA).

Excluded from the current analysis were 12 patients who discontinued anti-HCV treatment prematurely for non virological reasons, as well as 8 patients who were lost to follow-up. A total of 132 HCV-1 were enrolled and analyzed.

2. Treatment

Patients were treated for 48 weeks with PEG-IFNα-2b (PEGINTRON®; Schering–Plough now MSD, Kenilworth, NJ, USA) at doses of 1.5 μg/kg once per week subcutaneously. RBV (REBETOL®; Schering–Plough now MSD) was dosed according to baseline weight (800 mg for weight <65 kg, 1000 mg for 65–85 kg and 1200 mg for >85 kg).
Clearance of serum HCV RNA was assessed at week 4 (RVR: rapid virological response), at week 12 (cEVR: complete early virological response) and at week 48 of treatment (ETR: end of treatment response). Sustained virological response (SVR) was defined as undetectable HCV RNA at post-treatment week 24. ETR-positive patients who became HCV-RNA positive during the follow-up were classified as relapsers. Patients who had any other virologic response were considered as non-responders.

3. Viral RNA extraction and amplification of HCV genomes

Total RNA was extracted from 200μl of serum by High Pure Viral RNA kit (Roche Diagnostics GmbH) and eluted in 35μl of nuclease free water. Reverse transcription was performed with Transcriptor HiFi cDNA Synthesis kit (Roche Diagnostics GmbH) using degenerate HCV1-specific reverse primer 3636R (5’-TGGTCYACATTGGRTRCATYTG, nts 3636-3658) according to manufacturer’s instructions. cDNA fragments were amplified by nested PCR with Expand High Fidelity PCR System (Roche Diagnostics GmbH). The first round of amplification was performed with 4.5μl of template and primers 32F (sense, 5’-ATCACTCCCCCTGAGGAAAC, nts 36-55) and 3636R (antisense, 5’-TGGTCYACATTGGRTRCATYTG, nts 3636-3658) in 25 μl of reaction volume. For the second round of amplification, 1 μl of template and primers 66F (sense, TCCCGCGAGAGCCATAGT, nts 127-144) and 3627R (antisense, TTGGTRTACATYTGGTGGAYHGG, nts 3627-3649) were used. PCR cycling conditions consisted of an initial denaturation step of 94°C for 2 min followed by 35 cycles of 15 sec at 94°C, 90 sec at annealing
temperature (from 48°C to 55°C), 4 min at 68°C and final elongation 10 min at 68°C. To improve the yield, the first 10 PCR cycles were performed at a lower annealing temperature and the following 25 cycles by improving the elongation time for 5 sec for every cycle. As positive control for first round amplification, part of core sequence was amplified with AmpliTaq Gold DNA Polymerase (Applied Biosystems) using primers 953F (sense, 5′-AGGTCTCGTAGACCGTGCATCATG, nts 321-344) and 951R (antisense, 5′-CAYGTRAGGGTATCGATGAC, nts 705-724).

PCR products were examined on 1% agarose gel stained with ethidium bromide. Amplified products showing lower height compared to HCV full length genome were prepared for sequencing analysis: PCR amplicons were gel purified with Wizard® SV Gel and PCR Clean-Up System (Promega), cloned into pCR®-Blunt II-TOPO® (Zero Blunt® TOPO® PCR Cloning Kit; Invitrogen) following manufacturer’s instructions and sequenced. Sequences were provided by Primm s.r.l. and analyzed by BioEdit and Vector NTI softwares. HCV genome of H77 strain (accession NC_004102) was used as reference sequence for primers and deletion nucleotide positions.

4. Genomic DNA extraction and determination of IL28B genotype

Genomic DNA (gDNA) was extracted from 100 µl of serum using QIAamp DNA Micro kit (Qiagen) and pre-amplified with Genoplex Whole-genome Amplification kit (Sigma) following manufacturer’s instruction.
rs12979860 TaqMan SNP Genotyping assays were run starting from 30 ng of pre-amplified DNA on a 7900HT real time PCR instrument (Applied Biosystems, Carlsbad, CA), following manufacturer’s instructions.

5. Statistical analysis

Continuous variables were expressed as median ± interquartile range (IQR), while categorized variables were expressed as frequencies; statistical comparisons between groups were performed by the Wilcoxon test (also known as Mann-Whitney test) and Fisher’s test respectively. The threshold of significance was set at 0.05 for all the analyses.

Multivariable logistic regression models were established exploiting an automatic stepwise procedure; the final model was set up on the basis of the minimum AIC (Akaike Information Criterion). All statistical analyses were performed in R\textsuperscript{43}, using MASS library for the stepwise procedure\textsuperscript{44}.

F. Acknowledgments

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G. Chapter 3 – References


CHAPTER 4

The expression of HCV genomes resembling natural subgenomic deletions is cytopathic and alters the expression of cholesterol biosynthesis and stress responses genes

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The data presented in this chapter are part of an ongoing project.
A. Abstract

Chronic infection with hepatitis C virus (HCV) is the leading cause of chronic liver disease, including primary liver cancer. HCV particles with defective RNA genomes have been recently identified in the serum of some patients with chronic HCV infection. The HCV defective genomes are characterized by large in-frame deletions encompassing the region encoding the viral envelope proteins and retaining all the essential proteins for RNA replication. It was recently demonstrated that HCV defective genomes are capable of autonomous RNA replication, and they are efficiently packaged into infectious viral particles when co-expressed together with full-length viral genome. We found HCV defective genomes circulating in the serum of an unexpectedly high fraction of patients with chronic hepatitis C of different genotypes (about 20%).

In order to investigate whether HCV defective genomes could play a role in any of the hepatic disease manifestations associated with HCV infection, we studied whether defective HCV genomes may have a direct role in hepatocyte lesions using an infectivity model system in vitro.

We used the HCV infectivity system based on the HCV strain JFH-1, that is able to replicate and generate viral particles in a competent cell line, i.e. the hepatoma Huh7.5 cells. These cells were transfected with two defective genomes J6/JFH-Δ284-736 and J6/JFH-Δ212-886 and the cellular effects of their replication were compared to those elicited by the HCV2a full-length chimera J6/JFH-1. Notably, we observed a
significant decrease in cell viability after expression of the HCV genomes containing genomic deletions, compared to cells expressing the full-length chimera.

In addition, we observed by confocal microscopy that the expression of the HCV defective genomes – but not of the corresponding full-length genome – induced abnormal accumulation of peri-nuclear, Core-decorated LDs. This phenomenon could be recapitulated by a dicistronic replicon expressing Core separately from the HCV polyprotein (J6-Core-JFH-1-replicon), thus supporting a causative role for the unbalanced expression of Core protein.

In order to dissect further the cytopathic effect associated with expression of defective HCV genomes, we performed a whole-genome expression profiling of Huh7.5 cells transfected with either full-length or defective HCV RNA. Gene ontology analysis of the differentially expressed genes revealed significant functional enrichment of pathways related to Biosynthesis of Steroids.

In addition to the Cholesterol Biosynthesis genes, other pathways were found to be differentially represented in cells expressing HCV defective genome that are related to inflammatory/immune response (TNFR2, Acute Phase Response signaling) or carcinogenesis (p53 Signaling).

In summary, our data suggest that HCV particles with defective genomes have the potential to be implicated in the alterations of lipid/cholesterol metabolism and/or liver steatosis often observed in hepatitis C patients.
B. Introduction

Hepatitis C virus (HCV) is an enveloped virus belonging to the family Flaviviridae. The virus genome is a positive-stranded RNA of about 9,600 nucleotides, which contains a single open reading frame (ORF) encoding both structural (core, E1, E2, and p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. Two highly conserved untranslated regions (UTRs) are found at the 5’ and 3’ ends, which play critical roles in both viral translation and replication. HCV is estimated to infect 170 million people worldwide and in a high percentage of individuals causes a chronic liver infection that frequently evolves into an array of diseases, including cirrhosis and hepatocellular carcinoma.

The HCV RNA-dependent RNA polymerase (NS5B) has a high frequency of incorrect nucleotide insertions, in the range of 10^4 to 10^5 base substitutions per site, which can result in the rapid generation of HCV quasispecies. Because of this huge genetic diversity, HCV is currently classified into six major genotypes and more than 80 subtypes. Recombination may be another mechanism exploited by HCV to increase genetic diversity: naturally occurring intergenotypic recombinant viruses that often have their recombination points in the trans-membrane domains of NS2 were recently identified. Recent publications have reported the presence of natural HCV subgenomic RNAs in serum and liver of infected patients, mostly containing large in-frame deletions from E1 up to NS2, always found together with the full-length wild-type (wt) RNAs. These mutant viral genomes
persist for a long time, and sequence analysis suggests that subgenomic (the predominant species during this period) and full-length HCV evolve independently\(^\text{14}\). In the first screening campaign for HCV defective particles, we found HCV defective genomes circulating in the serum of an unexpectedly high fraction of patients with chronic hepatitis C (20\%) (see Chapter 3 of this thesis).

Analysis of the genetic structure of independent subgenomic deletion-containing RNAs\(^\text{11-14}\) strongly suggests that the possibility of recombination and/or deletion is restricted to specific regions. As expected, the 5’ UTR, the 3’ UTR, and the region coding from NS3 to NS5B are always conserved, in line with the notion that these regions are the minimal requirements for RNA replication. In addition to the regions required for RNA replication, however, naturally occurring subgenomic HCV RNA invariably contains an intact core region and the protease domain of NS2.

Very recently, Pacini et al modified the infectious isolate JFH-1\(^\text{15-17}\) in order to recapitulate in vitro the genetic structure of two of the most representative in-frame natural subgenomic deletion-containing RNAs found circulating in patients\(^\text{18}\). Using this system, they analyzed natural subgenomic variants for their ability to replicate autonomously and demonstrated that the natural subgenomic deletion mutants are replication competent and are trans-packaged into infectious virions when coexpressed together with the wild-type virus. Furthermore, their data suggest that the presence of the NS2 protease domain is required in order to generate the correct NS3 N terminus, required for RNA replication. Unexpectedly, the presence of NS2 generates, in turn,
a strict cis requirement for the core region in order to allow efficient trans-packaging of the subgenomic RNA, revealing a complex interplay between the NS2 and the core viral genes.

HCV deleted genomes are “exit-defective”: thus, it is conceivable that cells infected with defective HCV will accumulate very high level of intracellular Core protein. HCV Core is localized on the surface of cytoplasmic lipid droplets (LDs), believed to represent sites of virus assembly and production of nascent virions. Overexpression of intracellular Core protein is associated with abnormal accumulation and redistribution of LDs, a phenomenon that could be linked to hepatic steatosis and altered lipid metabolism.

In the present study, we observed a significant decrease in cell viability and a clear reorganization of lipid droplets after expression in hepatoma cells of HCV genomes containing genomic deletions similar to those observed in HCV-infected patients. In order to dissect further the cytopathic effect associated with the expression of defective HCV genomes, we performed whole-genome expression profiling of Huh7.5 cells transfected with either full-length or defective HCV RNA. Gene ontology analysis of the differentially expressed genes revealed significant functional enrichment of pathways related to Biosynthesis of Steroids, inflammatory/immune response (TNFR2, Acute Phase Response signaling) or carcinogenesis (p53 Signaling).
C. Results

1. Expression of HCV defective RNA genomes in Huh7.5 cells

In order to study the host response to the replication of HCV defective RNA genomes, Huh-7.5 cells were transfected with *in vitro* transcribed HCV RNA as described in Materials and Methods. The effect of HCV defective RNA genomes replication was investigated by comparing the cellular responses to the transfection of the HCV-2a full-length chimera J6/JFH-1 and the defective genomes J6/JFH-Δ284-736 and J6/JFH-Δ212-886 (Fig. 1A). These mutants recapitulate two representative natural subgenomic deletions\(^\text{13}\) in the context of the J6/JFH-1 chimera. The J6/JFH-1 chimera was originally generated to improve the exit efficiency and the titer of secreted virions by fusing the nonstructural genes of JFH-1 with structural genes, p7 and NS2 of another genotype 2a clone, J6\(^\text{15}\). The J6/JFH-1 construct was used as reference in our study.

The construct J6-core-JFH-rep is a dicistronic JFH-1 replicon expressing HCV NS3-5B and Core under the ECMV or HCV IRES, respectively (Fig. 1A). This construct was transfected in parallel to FL-J6/JFH-1 and its defective mutants in order to distinguish the cellular effects due to Core accumulation from those specifically raised by the presence of the fusion protein encoded across the deletion (Fig. 1A). The GND mutant of the J6/JFH-1 construct (J6/JFH-1-GND) was used as mock. Following transfection, HCV-positive cells were visualized by NS5A staining. As shown in Figure 1B, the majority of the cells expressed the viral antigen NS5A by 48 hrs post-transfection, thus indicating a transfection efficiency above 80%.
Fig. 1. Constructs used for cell culture experiments and transfection efficiency

(A) Huh7.5 cells were transfected with different HCV RNA constructs: the HCV-2a full-length chimera J6/JFH-1, the defective genomes J6/JFH-Δ284-736 and J6/JFH-Δ212-886, and the J6-core-JFH-rep construct. (B) Confocal microscopy analysis of cells transfected with the construct indicated in (A). Two days post transfection, cells were stained as described in Materials and Methods and analyzed by confocal microscopy (40X magnification). Transfection efficiency was determined counting NS5A positive cells (red) per total number of nuclei, stained by the Hoechst dye (blue), for at least 200 cells per sample.
2. Replication of HCV defective RNA genomes into Huh7.5 impairs cell viability

In order to measure the cell viability under expression of HCV mutants resembling natural subgenomic deletions, Huh7.5 cells were transfected with the constructs described in Fig. 1A and cell viability was measured 48, 72 and 96 hrs after transfection. As controls, the constructs lacking p7 (J6/JFH Δp7-Rluc2A) or lacking E1-E2 (J6/JFH ΔE1-E2-Rluc2A), in addition to their full-length control (J6/JFH Rluc2A), were transfected in parallel. These defective genomes differ from the two HCV mutants resembling natural subgenomic deletions because they do not express a fusion protein encoded across the deletion. The expression of HCV defective genomes resembling natural subgenomic deletions induced a strong decrease of cell viability, that was not observed with the other constructs considered. The cell viability is reduced to the 60% of mock already at 48hrs post transfection. The cytopathic effect became more prominent at 72 hrs and appeared to affect about the 70% of the cells by 96 hrs post-transfection (Fig. 2).

These findings support the hypothesis that the toxicity of HCV defective genomes resembling natural deletions could be due to the expression of a fusion protein across the deletion (presumably unfolded), rather than to the accumulation of Core: in fact, accumulation of Core can also occur in cells expressing the other exit-defective constructs (J6/JFH Δp7-Rluc2A, J6/JFH ΔE1-E2-Rluc2A and J6-core-JFH-rep), where cell viability is not significantly impaired.
Fig. 2. The replication of HCV mutants resembling natural subgenomic deletions decreases the viability of hepatoma cells

Cell viability was measured by CellTiterBlue at the indicated timepoints after transfection. The cells were incubated with the CellTiterBlue reagent for four hours at 37°C following the instructions of the manufacturer. Cell viability was calculated as percentage of FL-J6/JFH-1 GND. Shown is a representative experiment of three independent experiments performed in triplicates. The cell viability of cells transfected with J6/JFH-Δ284-736 and J6/JFH-Δ212-886 was the 60% of mock already at 48 hrs post transfection, went to 40% at 72 hrs and fell to 30% 96hrs after transfection. No significant toxicity was observed in cells expressing the other constructs considered.
3. The expression of HCV defective genomes induces a typical reorganization of lipid droplets that co-localize with intracellular Core.

The replication efficiency of HCV defective genomes was investigated by Pacini et al and compared to the parental construct J6/JFH-1. At 48hrs the HCV defective genomes replicate at four-fold higher levels than the FL genome, while after this timepoint the replication of the HCV defective genomes fall down dramatically \(^{18}\), presumably due to the inherent cytopathic effect. In order to clarify if replication of the HCV defective genomes could lead to Core accumulation into the hepatoma cells, a confocal microscopy analysis of Core and lipid droplets-associated Core was performed.

Lipid droplets (LD) are sites of HCV assembly. Soon after synthesis and processing, the HCV core protein is transferred to the lipid monolayer of the LD surface. The core protein also directs the transfer of the HCV replication complex to the LD surface where the assembly of virions is supposedly taking place \(^{19}\). It is well established that Core expression in hepatoma cells induces a typical reorganization of lipid droplets that cluster around the endoplasmic reticulum and co-localize with Core. This clustering was observed when Core is expressed alone or as part of a polyprotein encoding HCV structural proteins (Core-E1-E2) \(^{20}\), as well as for exit-defective HCV constructs, such as those variants lacking p7 or E1-E2 \(^{21}\) and personal observations). The same LD distribution is also observed in cells infected with the original JFH-1 strain: it can be considered an “exit-defective” virus since its exit
efficiency is very low if compared with the J6/JFH-1 chimera (21 and personal observations).

In our experimental conditions, a clear reorganization of lipid droplets was observed in Huh7.5 cells expressing either J6/JFH-Δ212-886 or J6/JFH-Δ284-736 (Fig. 3) and also with J6-core-JFH-rep (data not shown), concomitantly with the accumulation of Core in these cells.

Since Core was found to be cytotoxic in vitro and in animal models22, 23, we wondered if Core accumulation in cells expressing HCV defective genomes could change the host transcriptional pattern and lead to cytotoxicity.
Figure 3. The expression of the HCV defective genomes induces abnormal accumulation of peri-nuclear, Core-decorated lipid droplets
Huh7.5 cell transfected with J6/JFH-1, J6/JFH-Δ284-736 and J6/JFH-Δ212-886 were stained for lipid droplets (green) and Core (red), then analyzed by confocal microscopy (60X magnification). Nuclear DNA was stained with the Hoechst dye (blue). Colocalization of Core with lipid droplets results in yellow color.

The expression of the HCV defective genomes (central and lower panels), but not of the corresponding full-length genome (upper panel) triggered a clear reorganization of lipid droplets, that accumulate around the nucleus and strongly co-localize with Core.

4. Global transcriptional response to the replication of HCV defective RNA genomes in cultured hepatoma cells
In an attempt to gain an insight into the mechanism of HCV-defective particles-associated cytotoxicity, whole-genome expression
profiling of the cells expressing defective HCV RNA genomes was set up. The constructs FL-J6/JFH-1-GND (mock), FL-J6/JFH-1, J6/JFH-Δ284-736 and J6/JFH-Δ212-886 were electroporated as described in Materials and Methods. Cells were harvested at 48 hours post-transfection and total RNA was isolated for global gene expression profiling as described in Materials and Methods. At this timepoint cell sufferance started to be evident (Fig. 2). Genome-wide gene expression was analyzed by the Illumina microarray platform. Experiments were performed in quintuplicate and 9332 transcripts were found to be expressed in at least one of the four cellular conditions in all the biological replicates. 208 genes that showed at least 1.5 fold change with a Pval<0.01 (calculated as described in the method session) in both the defective genomes compared to the full-length were selected for further investigation (Fig. 4A).

The list of 208 genes generated from the differential expression analysis was analyzed for biological function and pathway enrichment by Ingenuity Pathway Analysis software (IPA). This analysis was used to identify the cellular processes represented by the changes in steady-state abundance of transcripts associated to the replication of HCV defective RNA genomes, compared to the expression patterns associated with the replication of FL-J6/JFH-1. Functional analysis by IPA reveals a significant functional enrichment of the canonical pathways indicated in Fig. 4B: the pathway of Steroid Biosynthesis was the most significantly altered in cells expressing either J6/JFH-Δ212-886 or J6/JFH-Δ284-736 (Fig. 4B).
Figure 4. Gene expression profiling of hepatoma cell line transfected with defective genomes vs. full length HCV RNA

(A) Total cellular RNA was collected 48hrs post transfection and genome-wide gene expression was analyzed by the Illumina platform, as described in Materials and Methods. Experiments were performed in quintuplicate and the signal average was used to calculate the fold-change between the full-length and the defective genomes. Statistical significance was calculated applying the Student t-test to each defective genome versus the full-length.
Genes showing at least 1.5 fold change and a Pval<0.01 in both the defective genomes were selected for further investigation.

(B) The analysis of pathways and biological function was performed using IPA software (Ingenuity Pathway Analysis, Ingenuity System) and the enrichment was determined taking into account the built-in Fisher exact test. This statistic test gave a p value of significance for each pathway included in the Gene Ontology database: low p values indicate that a significant number of genes belonging to a given pathway are differentially expressed in cells expressing the HCV defective genomes versus the cells expressing the FL genome. In this graph, p values are indicated as – Log: the higher –Log Pval, the stronger is the statistical significance of pathway enrichment.

Gene ontology analysis of genes that are differentially expressed in cells transfected with either defective genomes reveal the involvement of pathways related to synthesis of steroids and response to stress.

5. Genes of Cholesterol Biosynthesis pathway are downregulated in cells expressing either J6/JFH-Δ212-886 or J6/JFH-Δ284-736

Since the intimate connection between HCV lifecycle and cholesterol is well known\textsuperscript{19}, the expression of the enzymes involved in cholesterol biosynthesis in our experimental conditions was investigated more in detail (Fig. 5). Genes crucial for cholesterol biosynthesis, as SQLE (Squalene Epoxidase), HMGCR (Hydroxymethylglutaryl-CoA Reductase), EBP (Emopamil Binding Protein - Sterol Isomerase) and IDI1 (Isopentenyl-diphosphate Delta-Isomerase 1) are stable or upregulated in J6/JFH-1 expressing cells but are downregulated in cells expressing defective HCV genome. This expression pattern could be induced by a regulation system that acts upstream to the expression of many cholesterol biosynthesis genes, such as for example a regulation mediated by a transcription factor (i.e. SREBP-2).
Figure 5. Genes of Cholesterol Biosynthesis pathway are downregulated in both Δ212-886 and Δ284-736 expressing cells

Heat map of significant differentially expressed genes of cholesterol biosynthesis, generated by the TMev software. Genes are stable or upregulated in J6/JFH-1 expressing cells but are downregulated in cells expressing defective HCV genome (values are expressed in log2-fold change vs. Mock).

FDFT1: farnesyl-diphosphate farnesyltransferase 1; SQLE: squalene epoxidase; HMGCR: hydroxymethylglutaryl-CoA reductase; FDPS: farnesyl diphosphate synthase; DHCR7: delta-7-dehydrocholesterol reductase; ACAT2: acetyl-CoA acetyltransferase 2; LSS: lanosterol synthase; EBP: emopamil binding protein (sterol isomerase); SC4MOL: sterol-C4-methyl oxidase-like; DHCR24 and Name: 24-dehydrocholesterol reductase; IDI-1: isopentenyl-diphosphate Delta-isomerase 1.
6. The cellular stress sensor gene ATF3 is strongly induced by the HCV defective genomes

We wondered if the expression of markers of cell stress was linked to accumulation of Core protein, or to other features of HCV deleted genomes resembling naturally occurring HCV defective particles.

Among the 461 significant differentially expressed genes from whole-genome expression profiling, there were two members of the ATF family of transcription factors: ATF3 and ATF6. The ATF proteins are sensors of cellular stress signals, including oxidative stress and unfolded protein response (UPR)\textsuperscript{24, 25}.

The expression of Activating transcription factor 3 (ATF3) was investigated by Real-Time PCR as described in Materials and Methods in cells expressing the FL-J6/JFH-1, its dead mutant FL-J6/JFH-1 GND, the defective genomes J6/JFH-Δ284-736 and J6/JFH-Δ212-886, the minimal replicon JFH-neo-rep, the J6-core-JFH-rep construct described above, or both FL-J6/JFH-1 and J6/JFH-Δ284-736. ATF3 was previously found to be upregulated in cells infected by J6/JFH-1\textsuperscript{26}. We observed a much stronger induction in cells expressing defective genomes compared to cells expressing the JFH-1 replicon or the dicistronic replicon expressing Core separately from the HCV polyprotein (J6-Core-JFH-rep). The observed overexpression did not appear to be related to the block of HCV particles exit, since it is present also when the HCV defective genome is transfected together with the full-length genome (Fig. 6), that acts as helper virus and allows the exit of HCV defective particles harboring deleted genomes\textsuperscript{18}. These observations support the hypothesis that Core accumulation alone is not sufficient
to induce the 100-fold induction of ATF3 expression observed in cells expressing HCV defective RNA genomes. In conclusion, our data suggest that this huge ATF3 overexpression is rather triggered by the unfolded fusion protein encoded across the deletion junction.

Figure 6. Cell stress sensor genes ATF3 and ATF6 are strongly induced by the expression HCV defective genomes
Huh7.5 cell were transfected with the construct listed in Fig. 6. The ATF3 mRNA was measured by Real-Time PCR and the expression of ATF3 was compared between cells transfected with FL-J6/JFH-1 GND and the other constructs considered. Here the fold change of ATF3 expression vs. FL-J6/JFH-1 GND was shown for each construct. Every construct induced the expression of ATF3 more than 20 fold (blue and green bars), but the induction triggered by the expression of HCV defective genomes was at least 100 fold (red and orange bars). This huge induction is maintained when the construct J6/JFH-Δ284-736 was expressed together with the FL (purple bar), indicating a role of the fusion protein encoded across the deletion junction in inducing ATF3 expression. Shown is a representative experiment of three independent experiments.
D. Discussion

In this study, we have investigated the host responses to the *in vitro* replication of two representative in-frame subgenomic deletion mutants found circulating in patients. The infectious isolate J6/JFH-1 was modified in order to recapitulate *in vitro* the genetic structure of two of the most representative in-frame natural subgenomic deletion-containing RNAs found circulating in patients\textsuperscript{18}. We observed a clear sufferance of cells harboring either defective genomes J6/JFH-Δ284-736 and J6/JFH-Δ212-886, this was not observed in cells where the HCV 2a full-length chimera was replicating (Fig. 2). Similarly, no toxicity was detected in cells harboring other exit-defective constructs (J6/JFH Δp7-Rluc2A, J6/JFH ΔE1-E2-Rluc2A and J6-core-JFH-rep). The two defective constructs resembling naturally occurring defective HCV genomes share some peculiarities with the J6-core-JFH-rep construct and the Δp7 or ΔE1-E2 constructs: (I) they all replicate autonomously; (II) they produce complete HCV Core and non-structural proteins; (III) they cannot produce viral particles without the presence of an helper virus or without trans-complementation of the missing proteins\textsuperscript{18, 27}, so (IV) the viral proteins accumulates into the hepatocytes. Conversely, the HCV genomes resembling natural subgenomic deletions have the unique characteristic of expressing a fusion protein encoded across the deletion: the HCV polyprotein processing of J6/JFH-Δ284-736 generates an E1-E2 fusion protein, while the processing of J6/JFH-Δ212-886 generates an E1-NS2 fusion protein. These proteins are likely
to be unfolded, so they may trigger an unfolded protein response and/or an Endoplasmic Reticulum (ER) stress.

In an attempt to elucidate the pathogenic relevance of the HCV defective particles, we investigated the formation and distribution of cytoplasmic lipid droplets in cells transfected with defective HCV genomes. The relationship between the in vivo steatogenic properties of HCV and the ability of HCV Core protein to associate with intracellular lipid droplets and to induce lipid accumulation in vitro is well established for different HCV genotypes, especially for HCV genotype 3. The steatogenic potential of Core correlated with a specific distribution of lipid droplets. Core protein binding was associated with an accumulation of lipid droplets in the perinuclear area. Here we found that the expression of HCV defective RNAs resembling naturally occurring defective particles induces a typical reorganization of lipid droplets that co-localize with intracellular Core around the nucleus (Fig. 3). This clustering was also observed when Core is expressed alone or as part of a polyprotein encoding HCV structural proteins (Core-E1-E2), as well as for other exit-defective HCV constructs, such as those variants lacking p7 or E1-E2 (and personal observations). The same LD distribution was also observed in cells infected with the original JFH-1 strain, which can be considered an “exit-defective” virus since its exit efficiency is very low if compared with the J6/JFH-1 chimera (and personal observations). Morphological changes in intracellular LD are induced by different HCV genotype Core sequences; however, it has been reported that LD induced by genotype 3a Core are larger and contain more neutral
lipids than those induced by genotype 1b sequences\textsuperscript{28}. Since the infection with HCV genotype 3 is strongly correlated with steatosis in HCV-infected patients, the alterations of LD could be linked to altered lipid metabolism in hepatoma cells.

In summary, we demonstrated that naturally occurring HCV defective particles are exit-defective HCV particles that are able to induce the reorganization of lipid droplets around the nucleus \textit{in vitro}. This particular lipid droplets pattern has been correlated with the steatogenic potential of HCV \textit{in vivo}\textsuperscript{28}.

Further, we investigated the host transcriptional responses to the expression of defective HCV RNA genomes by a whole-genome expression profiling of the cells expressing defective HCV RNA genomes (Fig. 4A), in order to gain an insight into the mechanism of HCV-defective particles-associated cytotoxicity.

Gene ontology analysis of the differentially expressed genes revealed significant functional enrichment of pathways related to Biosynthesis of Steroids, Acute Phase Response Signaling, TNFR2 Signaling and p53 Signaling (Fig. 4B). In particular, we found that many genes of Cholesterol Biosynthesis pathway were downregulated in cells expressing defective HCV genomes. Conversely, these genes were stable or upregulated in cells expressing the full-length J6/JFH-1 genome (Fig. 5). This phenomenon could be related to a negative feedback regulation due to excessive intracellular lipid accumulation and/or impaired lipoprotein secretion. Downregulation of genes involved in the Cholesterol Biosynthesis pathway, concomitant with
the altered morphology of LDs, was related to the block of HCV particles exit.

In addition to the Cholesterol Biosynthesis, other pathways were found to be differentially represented in cells expressing HCV defective genome that may be related to inflammatory/immune response (Interferon, TNFR2, Acute Phase Response signaling) or carcinogenesis (p53 Signaling).

Among the 461 significant differentially expressed genes from whole-genome expression profiling, there were two members of the ATF family of transcription factors: ATF3 and ATF6. The ATF proteins are sensors of cellular stress signals, including oxidative stress and unfolded protein response (UPR). In our gene expression profiling, ATF6 and ATF3 showed similar expression pattern. ATF6 is a recognized ER-stress sensor in hepatocytes and recent studies demonstrated its activation in liver of patients with chronic hepatitis C\textsuperscript{29}.

Here we showed that HCV defective particles trigger substantial overexpression of Activating transcription factor 3 (ATF3). This gene was previously found to be upregulated in cells infected by J6/JFH-1\textsuperscript{26}. We observed a much stronger induction in cells expressing defective genomes compared to cells expressing the JFH-1 replicon or the dicistronic replicon expressing Core separately from the HCV polyprotein (J6-Core/JFH-1replicon). The observed overexpression did not appear to be related to the block of HCV particles exit, as demonstrated by the fact that co-transfection of a deleted genome with a full-length genome did not rescue the induction of ATF3.
overexpression. Our data suggest that ATF3 overexpression is triggered by the unfolded fusion protein encoded across the deletion junction.

Increased ATF3 expression accounts for suppression of p53-dependent senescence and enhanced tumorigenic potential\textsuperscript{30}. ATF3 was found to be also relevant in modulating innate immune response, inflammation and cell cycle regulation\textsuperscript{24, 31}, but its role in these mechanisms is still controversial and needs further investigation.

Taken together, our data suggest that HCV particles with defective genomes could represent important determinants of steatosis and cell sufferers in hepatocytes infected by HCV.
E. Materials and Methods

1. Reagents and plasmids

The generation of the HCV defective genomes J6/JFH-Δ284-736 and J6/JFH-Δ212-886 was described previously\textsuperscript{18}. The construct J6-core-JFH-rep was cloned as follows. HCV-IRES-Core was amplified by PCR from the FL-J6/JFH-1 with the primers DeltaF1new (CGACGCGCATGTAATTCTAATACGACTC, containing the EcoRI site) and Core-stop-Pmel-R (CCGCGCGCCGTTTAAACAAAGCTTCAAGCGGAGACCAGGGTGGTGATGC AGGACAGC, containing the flanking bases, the Pmel site and the stop codon). The PCR product and the pUC19-JFH-neo-rep were digested and cloned with EcoRI and Pmel (this digest excises the HCV-IRES-neo fragment from the pUC19-JFH-neo-rep plasmid). All nucleic acids manipulations were performed according to standard protocols.


2. Cell culture, transfection and measure of cell viability

The human hepatoma-derived cell line Huh7.5\textsuperscript{32} were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml Streptomycin and 2 mM L-glutamine. Electroporation of in vitro-transcribed HCV genomic RNA into Huh7.5 cells was performed as described previously\textsuperscript{18}. 2x10\textsuperscript{6} cells were electroporated with 10 μg of RNA in a final volume of 200 μl.
1.4x10^4 electroporated cells were plated in triplicates in 96-well plates and cell viability was measured at the indicated timepoints using the cell viability assay CellTiter-Blue (Promega), following the instructions of the manufacturer.

3. Indirect immunofluorescence

After electroporation, cells were plated in 24-well plates with glass coverslides (8x10^4 cells/well) and stained for HCV markers and/or lipid droplets at the timepoints indicated in the figure legends. Primary antibodies used were: mouse anti-NS5A antibody (Cat. No. HCM-131-5, 1:1000, Austral Biologicals) and human anti-Core antibody (1:1000, a generous gift by M. Mondelli, University of Pavia). Secondary antibodies used were goat anti-mouse IgG Alexa Fluor 568 (Cat. No. A11004, 1:600, Invitrogen) and goat anti-human IgG Alexa Fluor 568 (Cat. No. A21090, 1:600, Invitrogen). Lipid droplets were stained by 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503, Cat. No. D-3922, 1:300, Invitrogen).

Cells were washed once with PBS and fixed with 300 μl of 4% PFA for 10 min. Cells were washed three times with PBS and permeabilized with 500 μl of 0.1 % Triton X-100 in PBS for 10 min. Unspecific binding was blocked by incubation with 3 % BSA in PBS. After incubation with the primary antibody in blocking buffer, cell were washed with PBS and subsequently incubated with goat secondary antibodies conjugated to Alexa-Fluor 568 and BODIPY 493/503. Nuclei were stained with Hoechst dye 33342 (Sigma; 1:4000). Slides were then mounted with 5 μl ProLong Gold Antifade (Invitrogen) and analyzed by
using an inverted Leica TCS SP5 scanning laser confocal microscope. Digital images were taken using LAS AF software (Leica).

4. Isolation of cellular RNA, expression microarray format and data analysis

Total RNA was isolated from approximately $10^6$ cells using mirVana Isolation Kit (Ambion) following the instructions of the manufacturer. Extracted RNA was quantified by Nanodrop. All extracted RNA samples were quality controlled for integrity with 2100 Bioanalyzer (Agilent Technologies) and samples with RIN lower than 8 were discarded.

Illumina Direct Hybridization Assays were performed according to the standard protocol (Illumina Inc.). For each sample, 250 ng of total RNA were reverse transcribed according to the Illumina TotalPrep RNA Amplification kit (Ambion) and cRNA was generated after a 14 hours of in vitro transcription. Washing, staining and hybridization were performed according to the standard Illumina protocol: briefly, 750 ng of cRNA of each sample in a final volume of 15 µl were hybridized onto Illumina Expression BeadChip arrays (HumanHT-12 v3 Expression BeadChip). Hybridization and scanning were performed according to the manufacturer’s indications on an Illumina iScan System and data were processed with BeadStudio v.3; arrays were quantile normalized, with no background subtraction, and average signals were calculated on probe-level data for genes whose detection p-value was lower than 0.0001 in at least one of the experimental conditions examines (9332 probes). After logarithmic transformation, the expression ratio between full length or defective constructs and mock condition was
calculated in each experimental session and then used for the calculation of the mean among experimental sessions; the statistic significance was determined by applying an unpaired two-tailed $t$ test. Transcripts were defined as differentially expressed if there was a 1.5-fold or greater difference in the gene expression levels and a $P_{val}<0.05$ in both the defective constructs versus the full-length.

Pathway and biological function analyses were performed using IPA software (Ingenuity Pathway Analysis, Ingenuity System) and the enrichment was determined taking into account the built-in Fisher exact test.

Global visualization of differentially expressed genes by heat map was obtained using the TMev software.

5. Quantitative Real-Time PCR

Quantitative Real-Time PCR (qPCR) was used to validate gene expression changes. Total RNA was extracted by mirVana as described above. Quantitative PCR was performed by the QuantiTect Probe RT-PCR Kit (Qiagen), using a one-step strategy, according to the manufacturer’s instructions. 50 ng of total RNA extracted from cells were assayed and GAPDH expression was measured as internal reference (Applied Biosystems). Relative quantification was obtained by the $D_{DCt}$ method of qPCR data analysis. The probe used to measure the expression of ATF3 was the Hs00231069_m1* (Applied Biosystems).
F. Chapter 4 – References

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CHAPTER 5

SUMMARY, CONCLUSIONS AND
TRANSLATIONAL IMPACT OF THE PROJECTS
A. Discovery of HCV inhibitors targeting host factors

Most novel drugs directed against HCV, including the recently approved NS3/4A protease inhibitors boceprevir and telaprevir, are inhibitors of viral enzymes. Since HCV is an RNA virus with a short and highly variable genome, these direct-acting antivirals (DAAs) are prone to rapidly inducing the emergence of resistant HCV variants. This problem could be mitigated by the development of drugs that target host factors that the virus depends on during the various stages of its replication cycle. An increasing understanding of the molecular interactions between the virus and its host cell has allowed the identification of promising host targets for anti-HCV therapy and host-targeting agents (HTAs) are currently under development. The most advanced compound is an inhibitor of cyclophilin A, a host factor known to be critical for viral RNA replication and possible virion assembly or release. This compound, alisporivir, has demonstrated in vivo efficacy and is now in a phase 3 trial. Several other HTAs with very different host targets are further upstream in the development pipeline.

In CHAPTER 2 of this thesis we demonstrated that the 4-Amino Quinazoline compound named AL-9 inhibits HCV replication by targeting PI4KIIIα. The importance of this enzyme for HCV replication has recently been described by several groups: it binds to HCV NS5A and its enzymatic activity is required for efficient HCV RNA replication. PI4KIIIα belongs to the family of type III phosphatidylinositol 4-kinases, enzymes that catalyze the conversion of phosphatidylinositol
to phosphatidylinositol 4-phosphate (PI4P). PI4P is the most abundant monophosphorylated inositol phospholipid in mammalian cells and the importance of this phospholipid is just started to be unraveled\(^7\). In addition to playing important roles in intracellular signaling and membrane trafficking, phosphatidylinositol lipids and their metabolizing enzymes are also exploited by many different viruses in order to transform cellular membranes in structures supporting their replication\(^8,9\). PI4KIII\(\beta\) was shown to be a host factor required for enterovirus replication\(^10\), whereas several reports have demonstrated that PI4KIII\(\alpha\) is crucial for HCV replication\(^2,3,5,11\). Owing to the importance of this pathway, the need for specific inhibitors of PI4KIII kinases is increasing. Only recently, some enviroxime-like compounds with antiviral activity against enterovirus have been demonstrated to target PI4KIII\(\beta\). One of these agents is a very specific inhibitor of the \(\beta\)-isoform of the type III PI4-kinases\(^12\). So far, no such compound exists for the PI4KIII\(\alpha\) isoform.

We demonstrated that a class of HCV inhibitors originally proposed to target NS5A does in fact target the host factor PI4KIII\(\alpha\). Compounds targeting host factors may have the general advantage of imposing a higher genetic barrier to the development of resistance. AL-9, a member of this class of compounds, inhibits PI4KIII\(\alpha\) and to our knowledge, it is the first compound with a clear preference for PI4KIII\(\alpha\) over PI4KIII\(\beta\). For this reason, AL-9 is a good lead compound for the development of more potent and specific pharmacological inhibitors of PI4KIII\(\alpha\) to be used both as important research tools as well as leads for initial drug discovery\(^1\).
B. HCV-defective RNA genomes as biomarkers of disease severity and response to anti-HCV therapy

Among the unmet clinical needs in the field of Hepatitis C there is the necessity to find useful biomarkers of disease progression and response to therapies.

Understanding which are the patients at risk of developing a faster progressive liver disease would have great advantages. From the clinical point of view, antiviral therapy would be the most beneficial in patients at higher risk of developing progressive liver disease and would therefore be given as soon as possible. Furthermore, the gold standard for assessing hepatic fibrosis, that is liver biopsy, is invasive, subject to sampling errors, and has rare but occurring potentially life threatening complications, thus limiting its acceptability in patients with mild or moderate disease. There is therefore the medical need to develop non-invasive and reliable serum markers that accurately reflect hepatic fibrotic and cirrhotic disease. Moreover, effective surveillance of patients at high risk of developing HCC (i.e., patients with cirrhosis) could potentially decrease HCC-related mortality rate, and the availability of early and reliable serological markers of HCC remains a huge unmet medical need. Finally, from the basic research point of view, the study of how host and viral factors could affect disease progression may shed light on fundamental pathogenic mechanisms in the path from fibrosis to cirrhosis to cancer, which still remain poorly defined.
Another important need in the Hepatitis C field regards the prediction of the rate of response to therapy for each patient. To date, the standard antiviral therapy, based on long-term administration of PEG-IFNα/RBV, permits to achieve viral clearance only in about 40-60% of cases. Both viral and host genetic variability are thought to influence treatment success rates. SNPs near the IL28B gene are strongly associated with both spontaneous and treatment-induced viral clearance\textsuperscript{13-15}. Regarding viral genetics, HCV genotypes 1 and 4 are considered more difficult to treat than genotypes 2 and 3\textsuperscript{16}. Although IL28B genotype is a significant pretreatment predictor of response to therapy, consideration should be given to ordering the test when it is likely to influence either the physician’s or patient’s decision to initiate therapy\textsuperscript{17}. There is therefore the medical need for additional novel predictors of response to therapy.

In this study, we analyzed pre-therapy sera of 132 patients and we found defective genomes in about 20% of them; to our knowledge, this is the first systematic analysis done on such a large number of subjects, and it permits to infer that the emerging of deletion mutants, far from being a sporadic phenomenon, occurs in a relevant portion of HCV1-infected subjects. Defective interfering forms are reported for a variety of viruses, and their clinical impact is variegated\textsuperscript{18}. Regarding HCV, the ability of defective particles to influence the clinical course of the disease is largely unexplored. We observed that the presence of HCV defective variants correlates with higher viral load, older patient age and higher degree of hepatic inflammation. Since defective viruses are thought to be generated from the wild type through errors of the
polymerase, the probability of their insurgence increases proportionally with the number of replication events. This may be at the basis of the association with older patient age (which can be considered as a surrogate of the duration of infection) and higher viremia. From an etiopathological point of view, the association with the grading score appears more intriguing, raising the possibility that HCV defective particles exacerbate the liver inflammation processes.

We then evaluated the viral kinetics during standard therapy treatment in HCV1 patients carrying the defective forms compared to those in which only the full-length variant was detected. No difference was found in RVR, cEVR and ETR rates, thus indicating that defective particles do not seem to play a key role in determining viral load decline during treatment. This result was confirmed also by logistic regression analysis, in which IL28B genotype, patient age and pre-treatment viremia, but not the presence of defective HCV, were identified as independent predictors of ETR. Refining the analysis on the ETR-positive population, however, we observed that defective particle carriers displayed a higher probability to experience virological relapse during the follow-up period. This result is particularly interesting, since it has been proposed that defective virus belonging to the Flaviviridae family may play a role in the establishment and maintenance of chronic infections, although the majority of the data comes from in-vitro experiments\textsuperscript{19, 20}. In conclusion, our study indicated that, in pre-treatment sera of chronic HCV1 patients, HCV particles with large in frame-deletions in E1-NS2 region are detected in about 20% of the subjects and correlate with a higher degree of liver
inflammation measured by the Ishak score. While RVR and ETR rates were not affected, the presence of defective particles was more frequent in subject experiencing a viral relapse after standard PEG-IFNα-2b/RBV therapy. The finding that the presence of circulating HCV defective genomes determines a different pattern of treatment failure and potentially a higher risk for liver damage in patients with genotype 1 HCV should be taken into account in the design of new algorithms aimed at maximizing HCV therapy efficacy (CHAPTER 3).

From the molecular point of view, we demonstrated that the expression of HCV genomes resembling natural subgenomic deletions is cytopathic and alters the expression of genes related to carcinogenesis, inflammation and stress responses (CHAPTER 4). These properties could be mechanistically linked to the increased inflammatory activity found in the liver of patients harboring HCV defective particles (as described in CHAPTER 3).

Another pathway differentially regulated in cells harboring HCV defective genomes regards the biosynthesis of cholesterol. These genes are upregulated in cells infected with wild-type FL-J6/JFH-1 but downregulated in cells where HCV defective RNA genomes actively replicate. Concomitantly, perinuclear lipid droplets accumulate in cells which express the deleted HCV genomes. This phenotype supports the notion that the expression of HCV defective genomes profoundly alters lipid metabolism in infected cells. In the future, we will investigate whether the presence of HCV defective particles is correlated with steatosis and/or other markers of dysregulation of lipid metabolism in HCV-infected patients.
In conclusion, our study is the first to elucidate the host response elicited by the expression of defective RNA genomes in hepatocytes and to investigate the role of defective virus in the HCV-associated liver disease.
C. Chapter 5 – References


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Metabolism of Phosphatidylinositol 4-Kinase Illα-Dependent PI4P Is
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Antiviral Activity.