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Hepatitis C Virus II

Infection and Disease

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Part I
Chronic Hepatitis C

Natural History of Chronic Hepatitis C

Marc G. Ghany and T. Jake Liang

Abstract Globally it is estimated there are 200 million persons with chronic hepatitis C virus infection. The infection becomes persistent in 50–80 % of persons who are exposed to hepatitis C virus. Chronic infection with hepatitis C virus is a major cause of cirrhosis, end-stage liver disease and hepatocellular carcinoma. The prognosis of chronic hepatitis C is highly variable and many host, viral and environment factors influence outcome. Approximately 25 % of persons with chronic hepatitis C will progress to cirrhosis over a 25–30 year period and be at risk for complications of end-stage liver disease and hepatocellular carcinoma. Modeling data predicts that the number of individuals with cirrhosis is expected to double by 2030. Many of these individuals will be at risk for end-stage liver disease and hepatocellular carcinoma. Could substantially reduce risk of cirrhosis, decompensation, cancer, and liver-related deaths.

Keywords Hepatitis C virus • Chronic hepatitis C • Natural history • Outcome • Cirrhosis • Decompensated liver disease • Hepatocellular carcinoma

1 Introduction

The natural history of a disease refers to a description of the uninterrupted progression of a disease in an individual from the moment of exposure to causal agents until recovery or death (Bhopal 2002). Defining the natural history of chronic hepatitis C has been difficult for several reasons: the majority of cases of acute hepatitis C are asymptomatic, so identifying cases has proven challenging. By the time they present for medical attention, most subjects have usually progressed to chronic hepatitis. Establishing the duration of disease based on time of exposure is notoriously inaccurate because most individuals cannot recall the date of exposure unless it was due to receipt of a single blood transfusion or accidental needlestick.

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Another problem is the duration required to follow patients for the outcomes of the infection. This usually means decades of follow-up generally exceeding the careers of most researchers. Nevertheless, a fairly comprehensive understanding of the natural history of chronic hepatitis C has been pieced together from prospective and retrospective studies of untreated subjects. What has emerged from this data is the remarkably variable outcome of infection and the large number of factors that seem to influence its outcome. This review will provide a summary of over 30 years of work that has been carried out by many investigators to understand the natural history of chronic hepatitis C.

2 Outcome of Acute Hepatitis C

2.1 Clinical Course

Acute hepatitis C usually presents without symptoms and the majority of persons are unaware of the infection. Approximately 15–30 % are symptomatic and variably report fatigue, lethargy, myalgia and loss of appetite. Fewer than 1 % of cases present with jaundice. Hepatitis C virus (HCV) RNA is usually detectable within 2 weeks after infection and HCV specific antibody within 12 weeks of exposure. Serum alanine aminotransferase (ALT) levels usually rise within 8–10 weeks with the peak ALT ranging from 10 to 20 times the upper limit of normal. Interestingly, as the disease progresses, serum HCV RNA levels may be observed to fluctuate and even become negative only to reappear again. This finding is characteristic of the acute but not chronic phase of the infection and may be a clue to the diagnosis of acute infection.

Follow-up studies from cohorts with acute hepatitis C in which the time of exposure could be established with some precision, such as cases of post-transfusion hepatitis or injection drug users who were prospectively monitored with serial HCV RNA determinations, indicated that approximately 15–25 % of patients with acute hepatitis C spontaneously resolve their infection (Gerlach et al. 2003; Deterding et al. 2013; Villano et al. 1999; Tremolada et al. 1992). Rates of spontaneous resolution of acute hepatitis C may be higher, 45–50 %, in certain populations for example subjects who present with jaundice compared to asymptomatic individuals, (Gerlach et al. 2003) in persons who are infected at younger compared to older age (age >40 years) (Vogt et al. 1999) and among women compared to men (Kenny-Walsh 1999; Wiese et al. 2000). More recently, certain polymorphisms (the rs12979860-C, (Ge et al. 2009) rs8099917-T (Tanaka et al. 2009) and the ss469415590 TT (Prokunina-Olsson et al. 2013) variants), near to the IL28B gene that encodes for lambda interferon – a type III interferon – were shown to be associated with higher rates of spontaneous clearance of HCV infection. Viral factors also appear to affect resolution of infection and persons with

recovery from acute hepatitis C were found to have less genetic diversity of the virus compared to those who progressed to chronic infection (Farci et al. 2000).

3 Outcome of Chronic Infection

Persons who fail to clear virus after a period of 6 months are generally accepted to have chronic infection, but viral clearance may occur well beyond 6 months in many infected individuals. Some, but not all of individuals with chronic infection are at risk for progressive liver disease including cirrhosis, decompensated liver disease, hepatocellular carcinoma and death (Fig. 1). The ideal natural history study would follow a large cohort of both men and women with known date of infection and who remained untreated throughout their course until death. For obvious reasons, such a study would be very difficult to conduct. So investigators have had to resort to other approaches to define the natural history of HCV infection. Three approaches have been taken, retrospective, prospective and retrospective-prospective cohort studies.

3.1 Retrospective Studies

Retrospective studies identified subjects with chronic hepatitis C and compared the severity of liver disease based on liver histology or clinical features with the time of exposure based upon receipt of blood products or first use of injection drugs (Table 1). These studies were influenced by selection bias, in that most were conducted in tertiary medical centers where patients with presumably more advanced disease were disproportionately represented since they were more likely

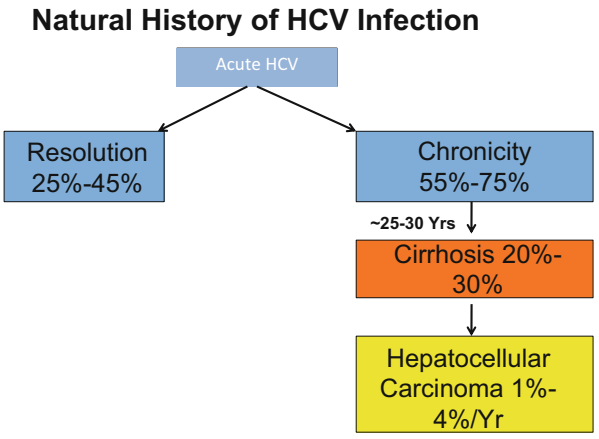

Fig. 1 Natural history of HCV infection

Table 1 Retrospective studies

Author	No.	Mean age	Mode of transmission	Duration of F/U (years)	Prevalence of cirrhosis	HCC	Liver related death/LT
Kiyosawa	231		PTH	10–29	35	23	
Tong	131	57	PTH	1–15	51	5	
Niederau	838		Mixed	9–16	17		
Gordon	627		Mixed	1–25	37	4	9
Gordon	140	58/38	PTH/IDU	13/16	66/33		
Ferenci	485	22[a]	PTH	31	34	5	13
Franchini	102	45	PTH	15–34	7	2	3
Forns	116		Multiple/ Unk	21–27	39	7	6
Posthouwer	212	1.8[a]/ 21		11–28	5[b]		

PTH post-transfusion hepatitis, *IDU* Injection drug use, *Unk* unknown
[a]Mean age at transfusion
[b]All cases HIV-HCV co-infected

to present for medical attention and are also affected by recall bias regarding the time of exposure. These retrospective studies presented a rather grim view of chronic hepatitis C. Cirrhosis was reported to be present in 14–55 % of subjects with chronic hepatitis C after average an disease duration of 1–34 years (Tong et al. 1995; Kiyosawa et al. 1990; Gordon et al. 1993, 1998; Niederau et al. 1998; Ferenci et al. 2007; Franchini et al. 2001; Forns et al. 2001; Posthouwer et al. 2006). Moreover, 2–23 % had developed hepatocellular carcinoma (HCC) and mortality from liver disease was 3–13 % (Tong et al. 1995; Kiyosawa et al. 1990; Gordon et al. 1993, 1998; Niederau et al. 1998; Ferenci et al. 2007; Franchini et al. 2001; Forns et al. 2001; Posthouwer et al. 2006). In one notable study, 131 subjects with transfusion acquired chronic hepatitis C were identified at a tertiary medical center in the Unites States. At initial evaluation 44 % had chronic hepatitis, 50 % had cirrhosis and 5 % had HCC, a mean of 22 years after transfusion. There was a clear trend for worse outcomes with longer duration of disease (Tong et al. 1995). The mean time from transfusion to diagnosis of cirrhosis was 21 years and for HCC, 28 years.

3.2 Prospective Studies

Prospective studies identified subjects with acute hepatitis C with known date of exposure and followed patients over time for development of outcomes. Several prospective studies of patients with transfusion and community acquired hepatitis C have been conducted (Table 2). The major limitations of these studies have been the relatively short period of follow-up ranging from 2 to 16 years and not all subjects

Table 2 Prospective studies

Author	No.	Mean age (years)	Mode of transmission	Duration of F/U (years)	Incidence of cirrhosis	HCC	Liver related death
Realdi	21	48	PTH	2–5	24		5
Tremolada	135	54	PTH	7.5	32	1	
DiBisceglie	65	52	PTH	9.7	20		6
Mattsson	61		PTH	13	8		1.6
Koretz	90	52	PTH	16	11		2
Thomas	1,667	34	IDU	14	1		2

underwent evaluation including liver biopsy. Another bias was that subjects were mostly middle-aged at time of exposure to HCV with an average age at transfusion that ranged between 48 and 54 years and were more likely to have underlying co-morbid conditions that could affect outcome of hepatitis C. Collectively, these studies reported incidence rates of cirrhosis of 8–32 % and liver-related death from 2 to 6 % over 2–16 years (Di Bisceglie et al. 1991; Tremolada et al. 1992; Mattsson et al. 1993; Koretz et al. 1993; Realdi et al. 1982; Thomas et al. 2000a). In studies that reported on the development of HCC, the rate was 0–1.3 %. In one study, subjects who contracted hepatitis C as a result of transfusions administered during cardiac surgery were prospectively followed for development of outcomes. Among 1,070 transfused patients, 65 (6.1 %) developed post-transfusion hepatitis, in whom 45 (69 %) the infection became chronic (Di Bisceglie et al. 1991). Thirty-nine subjects were followed for a mean of 9.7 years (range 1–24 years). During this period, cirrhosis developed in 20 % of subjects and 12 % developed decompensated liver disease (Di Bisceglie et al. 1991). In another study from Italy, outcomes were reported on 135 cases of post-transfusion hepatitis C, most cases following cardiac surgery (Tremolada et al. 1992). Spontaneous resolution was observed in 31 (23 %) cases while chronic hepatitis developed in 104 (77 %). After a mean follow-up of 90 months (range 13–180), cirrhosis was observed in 21 of 65 (32 %) of subjects who underwent one or more liver biopsies, 3 of 104 (3 %) developed end-stage liver disease and 1 (1 %) developed HCC (Tremolada et al. 1992). Thus, the prospective studies of subjects with post-transfusion hepatitis C also suggested high rates of development of cirrhosis but low rates of end-stage liver disease and HCC presumably due to the relatively short duration of follow-up.

3.3 Retrospective-Prospective Studies

Many studies have delineated the outcome of chronic hepatitis C based on identification of subjects who acquired hepatitis C in the past and were re-investigated and prospectively followed to determine the incidence of spontaneous clearance, cirrhosis, end stage liver disease, HCC and liver-related death. These studies have included a broad spectrum of populations infected with hepatitis C through multiple

Table 3 Retrospective-prospective studies

Author	No.	Mean age	Mode of transmission	Mean duration of disease	Incidence of cirrhosis	HCC	Liver related death
Bedogni	139	58		8	16	5	6
Harris	924	44[a]		16			1
Kenny-Walsh	376	28[a] 45[b]	PTH (anti-D immunoglobulin)	17	2		
Vogt	458	2.8[a] 20[b]	PTH (cardiac surgery)	20	1		
Wiese	1,018	24[a] 44[b]	PTH (anti-D immunoglobulin)	20	<1 %		<1
Just	230	58	PTH	22	19		9
Rodger	98	19	IDU	25	8		1
Seeff	222	49	PTH	25	17		4
Wiese	718		PTH (anti-D immunoglobulin)	35	9		4
Seeff	17	<25	Community acquired	45			6

[a]Mean age at transfusion
[b]Mean age at assessment

routes and include cohorts of young women who were infected after receipt of contaminated anti-rhesus factor, children and middle aged persons transfused during cardiac surgery, injection drug users, community acquired infection through uncertain exposure routes and general population-based cohorts, Table 3. These studies have provided a starkly different view of the natural history of chronic hepatitis C suggesting an entirely more benign course. As a group, the incidence of cirrhosis ranged from 0.4 to 19 %, HCC from 0 to 5 % and liver-related death from 0 to 9 % over a follow-up that ranged from 8 to 45 years (Bedogni et al. 2008; Harris et al. 2006; Kenny-Walsh 1999; Vogt et al. 1999; Wiese et al. 2000, 2014; Just et al. 2012; Rodger et al. 2000; Seeff et al. 2000, 2001).

3.3.1 Studies Among Young Women

Two of the studies reported on cohorts of young women, one from Ireland and one from Germany, who were infected from a single source infection of contaminated anti-D immune globulin. In the Irish cohort, screening for hepatitis C was advised for 62,667 women who had received contaminated lots of anti-D immune globulin. Among these women, 704 tested positive for HCV antibody, of whom 390 (55 %) were positive for HCV RNA indicating chronic infection (Kenny-Walsh 1999). Three hundred and seventy-six (96 %) of these women were followed for an average of 17 years after infection. Liver biopsies were performed in 356 of 363 women (98 %) and only 7 (2 %) had cirrhosis (Kenny-Walsh 1999). In two of the seven cases with cirrhosis, there was a history of excessive alcohol

consumption. Repeat liver biopsies were performed on a subset of 186 women without cirrhosis on the baseline biopsy a mean of 10 years later (mean 27 years from infection) (Levine et al. 2006). The incidence of cirrhosis was low at 2.1 %.

In the other cohort, 2867 German women received a batch of anti-D immune globulin contaminated with HCV. One thousand and eighteen women were evaluated after an average of 20 years following exposure (Wiese et al. 2000). Ten percent of these 1,018 women had spontaneously cleared the infection within 6 months after exposure to anti-D immune globulin. Twenty years later, only 55 % were HCV RNA positive. Among the women who were HCV RNA negative, the majority cleared the virus spontaneously, 91 %, and the remainder cleared the infection following treatment. Liver biopsies were performed on 220 (44 %) subjects with chronic infection. None of the biopsies showed cirrhosis. Clinical cirrhosis was evident in four (0.4 %) of the evaluated cohort and two subjects died, one from fulminant hepatitis following superinfection of hepatitis B and one from concomitant alcoholic liver disease (Wiese et al. 2000).

Seven hundred and eighteen (71 %) of the original cohort were reevaluated at an average of 35 years after initial exposure to anti-D immune globulin (Wiese et al. 2014). Five hundred and twenty-nine subjects who failed to clear HCV spontaneously, (including 197 untreated subjects, 183 who were treated and failed to clear the virus and 149 who responded to treatment), were compared to 189 subjects with spontaneous clearance. Overall, the cohort continued to have mild disease but significant progression to clinical cirrhosis was observed in 9.3 % of subjects (Wiese et al. 2014). The proportion of patients with clinical signs of liver cirrhosis increased from 0.4 % at 20 years to 0.5 % at 25 years and to 7.8 % at 35 years after infection. Progressive liver disease was strongly related to infection status. The highest rates of clinical cirrhosis were observed in the 183 subjects who were non-responders to therapy, 15.3 %. In contrast, clinical cirrhosis was present in 6 % of subjects who responded to treatment and only 1 % among those with spontaneous viral clearance. In total, 30 (4.2 %) subjects evaluated at 35 years after infection had died. Mortality among the HCV RNA negative subjects (including those with spontaneous and treatment-related clearance) was 10/338 (3.0 %) and only two deaths were directly attributed to liver disease; both were related to alcoholic cirrhosis (Wiese et al. 2014). Among the group who were still HCV RNA-positive, 20/380 (5.3 %) died, 9 (1.3 %) of whom death was liver related.

Together these studies suggest that hepatitis C in young women exposed in the second decade of life is associated with a high rate of spontaneously clearance, is generally mild but significant disease progression is observed in a small minority and usually associated with other mitigating circumstances.

3.3.2 Studies Among Young Children

Generally, the rate of chronicity and severity of hepatitis C tends to be milder in children compared to adults, but significant fibrosis and even cirrhosis can occur. The milder nature may be partly because of shorter duration of disease or

differences in the liver's reparative capabilities between children and adults. In one study, 458 children who underwent cardiac surgery as infants and were exposed to HCV through blood transfusion were evaluated for clinical outcomes and compared to a control group of subjects not undergoing surgery matched for age and sex (Vogt et al. 1999). Sixty-seven of 458 (14.6 %) who had undergone cardiac surgery had detectable HCV antibody compared to 0.7 % among the control subjects. Notably a high proportion of children had spontaneous resolution of hepatitis C, 30 of 67 (45 %) a mean of 20 years following cardiac surgery. Liver biopsies were performed on approximately half of subjects with chronic infection a mean of 21 years after surgery. Mild fibrosis was observed in two who had underlying congestive heart failure and cirrhosis in one who had been infected with hepatitis B (Vogt et al. 1999). In another study of 212 multi-transfused subjects with inherited bleeding disorders, 57 % tested HCV antibody positive but only two-thirds were HCV RNA positive (Posthouwer et al. 2006). After a mean duration of infection of 21 years, only 2 (5 %) of subjects had clinical and radiographical evidence of cirrhosis, and both of them were co-infected with HIV (Posthouwer et al. 2006).

Liver biopsy studies in children have demonstrated generally milder disease with less inflammation, steatosis and fibrosis compared to adults (Kage et al. 1997; Guido et al. 1998; Mohan et al. 2007; Hoshiyama et al. 2000; Goodman et al. 2008). An analysis of the pre-treatment liver biopsies for 121 children (age range 2–16 years) participating in the PEDS-C trial, a multicenter trial of peginterferon and ribavirin, showed that even among children deemed to warrant therapy, only 5 (4 %) had bridging fibrosis and 2 (2 %) had cirrhosis (Goodman et al. 2008). Overall, liver disease was mild but 5 (4 %) had bridging fibrosis, and 2 (2 %) had cirrhosis. However, one study from a single U.S. center reported cirrhosis in 3 of 40 (8 %) biopsies (Badizadegan et al. 1998). Progressive liver disease was demonstrated in a paired liver biopsy study of 44 children with a mean duration of 6 years between liver biopsies. The mean age at first and final biopsy was 8.6 and 14.5 years, respectively, and the mean duration of infection at time of biopsy was 7.7 and 13.5 years, respectively. Bridging fibrosis/cirrhosis was present on 11 % of biopsies at initial biopsy and increased to 20 % on the repeat biopsy. Differences in severity of liver disease among studies are likely related to referral bias.

3.3.3 Studies of Post-Transfusion Hepatitis C

Information on the outcome of hepatitis C has come from studies of subjects with transfusion acquired hepatitis C who have been retrospectively identified and followed for development of HCV-liver related outcomes. Collectively, these studies have revealed that all cause mortality among patients with transfusion-acquired HCV infection after 16–25 years of disease was not different from transfused, uninfected controls, however, there was a small but significantly higher rate of liver-related mortality indicating that the disease was progressive in some over 25 years.

In one study, each case of transfusion-acquired hepatitis from five transfusion associated hepatitis studies was compared with two transfused controls without hepatitis, matched for center, age, sex, race, use of hepatitis immunoglobulin, history of alcoholism, number of units transfused and date of transfusion. After 18 years of follow-up, there was no difference in all-cause mortality, 51 % and 51 % respectively, but a slightly increased liver-related mortality between in the HCV cohort, 3.3 % compared to the controls, average 1.6 % (Seeff et al. 1992). In a follow-up study 7 years later (25 years from infection), restricted to three studies that had archived sera, all cause mortality was 67 % among 222 HCV-related cases and 65 % among 377 controls. Liver-related mortality was approximately three times higher among cases compared to controls 4.1 % versus 1.3 %, respectively (Seeff et al. 2001). Among 129 subjects who were alive, 90 (70 %) were proven to have HCV-related hepatitis and the other 39 (30 %), non-A-G hepatitis. Follow-up of the 90 hepatitis C cases revealed that 77 % were still viremic, 23 % were anti-HCV positive/HCV RNA negative and 7 % had no markers of previous HCV exposure, thus 23 % spontaneously cleared the infection. Twenty subjects with raised ALT levels were biopsied and 7 (35 %) had cirrhosis.

A national look-back study of transfused individuals was performed in Denmark in 1996 and 1,018 subjects with transfusion-acquired HCV were identified (Just et al. 2012). At the time of the initial evaluation in 1996, 288 were alive and 230 were assessed for the status of their HCV infection. Among the 230 subjects, 124 (54 %) were viremic, 43 (19 %) had spontaneous clearance of HCV and 63 (27 %) had unknown infection status (Just et al. 2012). The 230 cases of transfusion-associated hepatitis C were matched 1:4 with 916 unexposed transfusion recipients. Subjects were reassessed in 2009, a median of 22 years after transfusion. The all-cause mortality rate of the 230 cases was 4.9 per 100 person years and was similar to that of the unexposed transfusion recipients, 4.6 per 100 person years. Among the HCV group, 16.5 % had cirrhosis at time of death. Liver-related mortality was significantly higher (tenfold) among the HCV-infected cohort compared to the non-HCV infected cohort.

In another study from England, 924 subjects with transfusion-acquired HCV were compared to 475 anti-HCV negative transfusion recipients to determine if there was any excess mortality from HCV infection. After the first decade of follow-up, there was some evidence for higher all-cause mortality among the HCV-infected cohort (hazard ratio 1.41) (Minola et al. 2002; Harris et al. 2006). Six years later (16 years from time of infection), all cause mortality was similar between the HCV-infected and non-infected cohorts; among 924 cases 255 (28 %) had died compared to 112 of 475 (24 %) of controls (hazard ratio 1.17, $p = 0.21$) (Harris et al. 2006). However, the risk of liver-related death was higher among the HCV cases 66/255 (26 %) compared to 6/112 (5 %) (controls hazard ratio 2.71, $p = 0.03$) (Harris et al. 2006).

3.3.4 Studies of Community Acquired HCV Infection

Studies of the natural history of HCV infection were also reported on "community-acquired" HCV infection where the source of infection was either unknown or possibly related to prior injection drug use. Stored serum samples from a cohort of patients admitted to an infectious diseases hospital in Australia with acute hepatitis were retrospectively tested 25 years later for HCV antibody (Pradat et al. 2007; Rodger et al. 2000). Among 1,511 patients, 238 (16 %) tested anti-HCV positive. The HCV antibody cohort was compared to a random sample of the HCV antibody negative cohort in a 2:1 ratio to determine if there was a difference in liver-related outcomes. One hundred and fifty of the HCV cohort and 292 of the HCV negative cohort were located. Complete follow-up was achieved on 98 HCV antibody positive subjects and 201 HCV antibody negative subjects (Rodger et al. 2000). Injection drug use was the presumed route of infection in the majority (87 %) of the HCV cases. After a mean of 25 years of follow-up, 54 % of the HCV cohort were viremic and 46 % had cleared the infection spontaneously. Among the HCV viremic cohort, 8 % had overt cirrhosis and there were no cases of HCC compared to only one case among the HCV uninfected cohort. The all cause mortality in the HCV cohort (12 %, 18 of 150) was similar to that of the uninfected cohort (8 %, 21 of 292). Liver mortality rates were also similar, 1 % (2 of 150) in the HCV-infected cohort and 0 % in the HCV uninfected cohort (Rodger et al. 2000). The two liver-related deaths in the HCV subjects were related to fulminant hepatitis B and complications of cirrhosis. The main cause of death in the HCV infected cohort was not liver disease but drug overdose 3 % (5 of 150) and suicide 2 % (3 of 150) (Rodger et al. 2000).

In another study, serum saved from 8,568 military recruits between 1948 and 1955 during an investigation of an outbreak of group A strepotoccal infection and acute rheumatic fever, were retrospectively tested almost 45 years later for HCV antibody (Seeff et al. 2000). Among the 8,568 persons, 17 (0.2 %) tested positive for HCV antibody. Follow-up was performed on the entire cohort. During the 45-year follow-up, liver disease occurred in 2 of the 17 (11.8 %) HCV-positive persons and 205 of the 8,551 (2.4 %) HCV-negative persons (Seeff et al. 2000). Seven of the 17 (41 %) HCV-positive persons and 2,226 of the 8,551 (26 %) HCV-negative persons had died by December 1996. Of persons who were HCV-positive, 1 (6 %) died of liver disease 42 years after the original phlebotomy, 5 (29 %) died of non-liver-related disease a median of 37 years after the original phlebotomy, and 1 (5.9 %) died of unknown causes (Seeff et al. 2000). In comparison, 119 (1.4 %) HCV-negative persons died of liver disease.

Another community-based prospective cohort study followed 1667 persons with a history of injection drug use and a positive test for HCV antibody for the development of decompensated liver disease (Thomas et al. 2000a). After a median follow-up of 8.8 years, a majority of subjects continued to have chronic hepatitis (79 %) but only 2 % had evidence of end-stage liver disease (Thomas et al. 2000a). Indeed, the most common causes of death in this cohort were from complications of

HIV infection, drug overdose or bacterial infections. Thus, community based studies suggest that only a minority of HCV-infected persons progress to end stage liver disease and the majority of HCV infected persons do not have overt liver disease.

From the data that has been reviewed, we can begin to piece together a better picture of the outcome of chronic HCV infection. Development of cirrhosis and end-stage liver disease definitely occurs but in a small percentage of cases and is usually associated with the presence of other co-factors that can accelerated liver fibrosis. The liver disease appears to be slowly progressive in the majority and most HCV infected persons will die with rather than from the infection. Still follow-up studies are necessary to identify whether disease will accelerate beyond a 25–30 years duration and what are the predictive factors associated with disease progression. Many factors have been identified and are discussed later in the review.

4 Fibrosis Progression

Another way of estimating the natural history of HCV is to study progression of liver fibrosis. Progression of fibrosis is the precursor of cirrhosis and rate of progression of fibrosis is a measure of the natural history of the disease. The initial stage of fibrosis was shown to be a good predictor of development of fibrosis progression and cirrhosis (Yano et al. 1996; Ghany et al. 2003). Ishak fibrosis stage was also shown to be predictive of development of clinical outcomes, need for liver transplantation, and liver-related death confirming the importance of fibrosis as a surrogate for development of clinical outcomes (Everhart et al. 2010). In an analysis of the Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) trial dataset, baseline laboratory markers of liver disease severity were worse and the frequency of esophageal varices higher with increasing Ishak stage. The HALT-C trial was one of the largest trials that prospectively observed a large cohort of patients with advanced chronic hepatitis C over time. The study was designed to show whether long-term, low dose pegylated interferon could prevent progression of liver disease as indicated by death, hepatocellular carcinoma, hepatic decompensation, or, for those with bridging fibrosis at baseline, an increase in the Ishak fibrosis score of 2 or more points as compared to observation only (Di Bisceglie et al. 2008). Among 1050 subjects, the 6-year cumulative incidence of first clinical outcome (defined as ascites, spontaneous bacterial peritonitis, variceal hemorrhage, hepatic encephalopathy, increase in Child-Turcotte-Pugh score by at least 2 points and liver-related death) was 5.6 % for stage 2, 16.1 % for stage 3, 19.3 % for stage 4, 37.8 % for stage 5, and 49.3 % for stage 6 (Everhart et al. 2010). This study demonstrated that most liver-related complications from hepatitis C do not occur until cirrhosis has developed and highlights the importance of preventing the development of cirrhosis in individuals with chronic HCV infection.

Since development of cirrhosis is one of the clinical outcomes of chronic hepatitis C, the stage of fibrosis from a liver biopsy and duration of infection have been used to estimate how rapidly chronic hepatitis C progresses. Interpretation of these cross-sectional studies has been limited by the assumption that progression of fibrosis is linear and proceeds in a staged manner. But the time required to progress from stage 1 to stage 2 might be much longer than the time to progress from stage 3 to 4. Also fibrosis may remain stable for long periods only to rapidly progress and then become quiescent again. Moreover, the scoring systems for staging of fibrosis are all semi-quantitative meaning that moving from stage 1 to stage 2 does not represent a doubling of fibrosis. There is also a bias to perform liver biopsies in patients suspected to have more advanced disease. Finally, this type of study relies on recall to identify the date of exposure, which is not always reliable. With these caveats in mind, a landmark study of 2235 subjects with chronic hepatitis C who had undergone liver biopsy scored using a five point scoring system for fibrosis (Metavir scale) estimated a median time of 30 years to progression to cirrhosis following infection (Poynard et al. 1997). One study addressed the issue of bias among liver biopsy studies by assessing fibrosis stage at autopsy on HCV antibody positive persons with injection drug with or without chronic infection. Two hundred and twenty out of a cohort of 523 anti-HCV positive injection drug users had died at the time of study (Kielland et al. 2014). The mean age at autopsy was 37 years and mean time from HCV exposure to autopsy was 17 years. Liver tissue was available from 102/220 subjects, 61 of whom were HCV RNA positive. Among HCV RNA positive subjects, 11 % (7/61) had cirrhosis compared to 2 % (1/41) who were HCV RNA negative after a mean period of 17 years (Kielland et al. 2014).

Performing repeated liver biopsies in subjects without intervening therapy should provide a more accurate determination of the rate of progression of fibrosis as the rate of fibrosis progression can be accurately determined between biopsies. Since the first therapies for chronic hepatitis C were approved, these studies are no longer ethical to perform. So most longitudinal biopsy studies have been retrospective and been biased by the fact that the indication for biopsy may have been the suspicion of more advanced disease (Table 4). Thus, these subjects may not be reflective of all patients with chronic hepatitis C. Interpretation of the results is also affected by sampling error of liver biopsy, which may over or underestimate true progression of fibrosis. With these caveats, a paired retrospective liver biopsy study was performed to evaluate the rate of fibrosis progression among subjects with CHC. Patients with all stages of fibrosis were included. After a mean period of 44 months, fibrosis progression (defined as a one point increase in fibrosis score between the first and second biopsy) was observed in 39 % of subjects, regression in 24 % and no change in 37 % (Ghany et al. 2003). Cirrhosis developed in 9 % of subjects all whom had bridging fibrosis on initial biopsy with the exception of a single patient indicating that rapid progression to cirrhosis from initially mild disease is uncommon. In this study, the rate of fibrosis progression was 0.12 fibrosis units per year, at which rate cirrhosis would require an average of 49 years to develop (Ghany et al. 2003). Several paired biopsy studies have been conducted in

Table 4 Paired biopsy studies

Author	No.	Duration between biopsies	Initial disease	Progression	No change	Regression
Ryder	214	2.5	Mild	33	57	10
Levine	167	3	Mild	27	49	24
Marcellin	110	3.2	Mild	32		
Collier	105	3.4	Mixed[a]	22	52	26
Ghany	123	3.7	Mixed 11 % cirrhosis	39	37	24
Hui	61	6.3	Mild	32	58	10
Boccato	106	7.8	Mild	60	40	0
Yano	70	8.8		50		

[a]39 subjects treated with interferon between biopsies; five achieved SVR

subjects with initially mild disease. These studies have reported fibrosis progression, defined as a one-point increase in fibrosis stage, in 32–59 % of subjects over a period of 2–8 years between biopsies (Boccato et al. 2006; Marcellin et al. 2002; Ryder et al. 2004). The frequency and severity of progression both increased with increasing duration between biopsies. These studies estimated a rate of fibrosis progression between 0.1 and 0.17 fibrosis units per year, predicting time to development of cirrhosis between 30 and 40 years. Paired biopsy studies among subjects with advanced fibrosis at baseline (bridging fibrosis) reported progression to cirrhosis in approximately one-third over a 4-year period (Di Bisceglie et al. 2008).

Thus, histological studies confirm that fibrosis is progressive in patients with chronic hepatitis C but the rate of progression is quite variable, worsens over time, related to the amount of fibrosis on the index biopsy and presence of co-factors in the population under study. The estimates derived from biopsy studies align fairly well with those from cohort studies suggesting a period of about 30–40 years for cirrhosis to develop in a subject without risk factors for cirrhosis and a shorter duration in a subject with one or more risk factors.

5 Natural History of HCV-Related Cirrhosis

Once cirrhosis develops, patients are at risk for decompensation events including the development of ascites, spontaneous bacterial peritonitis, variceal hemorrhage and hepatic encephalopathy. Once these complications develop there is an increased risk of death or need for liver transplant. Information on the natural history of hepatitis C after the progression to cirrhosis has been mostly derived from studies conducted at tertiary referral centers, which may not be representative of all persons with chronic hepatitis C. The duration of cirrhosis is unknown in most of these studies and patients followed at tertiary referral centers may be different from those in the community. These studies indicated that survival in the short and medium term is quite good for most patients with HCV-related cirrhosis. Five-year

survival ranges from 85 to 91 % and 10-year survival 60–79 %. The rate of clinical decompensation was approximately 2–5 %, per year and the development of HCC 1–4 % per year. In a notable European study, 384 European patients with biopsy proven HCV-related cirrhosis were enrolled at seven tertiary referral hospitals and followed up for a mean period of 5 years (Fattovich et al. 1997). The 5-year risk of decompensation was 18 % and that of hepatocellular carcinoma was 7 %. Death occurred in 51 patients (13 %), with 70 % dying of liver disease. The probability of survival was 91 % and 79 % at 5 and 10 years, respectively. In another study from the United States (U.S.), 112 patients with compensated HCV-cirrhosis were followed for an average of 4.5 (2–7.7) years. The cumulative probabilities for decompensation and development of HCC were 22 % and 10 % in 5 years, with an estimated yearly incidence of 4.4 % and 2.0 %, respectively. The cumulative survival probability was 83 % from entry and 51 % from decompensation in 5 years, with estimated yearly events of mortality and liver transplantation of 3.4 % and 9.8 %, respectively (Hu and Tong 1999). In another study with longer duration of follow-up, 214 patients with Child-Pugh class A cirrhosis who had no previous clinical decompensation were prospectively recruited and followed up with periodic clinical and abdominal ultrasound examinations (Sangiovanni et al. 2006). During 114 months (range 1–199), HCC developed in 68 (32 %), ascites in 50 (23 %), jaundice in 36 (17 %), upper gastrointestinal bleeding in 13 (6 %), and encephalopathy in 2 (1 %), with annual incidence rates of 3.9 %, 2.9 %, 2.0 %, 0.7 %, and 0.1 %, respectively (Sangiovanni et al. 2006). Clinical status remained unchanged in 154 (72 %) and progressed to Child-Pugh class B in 45 (21 %) and class C in 15 (7 %). Hepatocellular carcinoma was the main cause of death (44 %) and the first complication to develop in 58 (27 %) patients, followed by ascites in 29 (14 %), jaundice in 20 (9 %), and upper gastrointestinal bleeding in 3 (1 %). The annual mortality rate was 4.0 % per year and was higher in patients with other potential causes of liver disease than in those without them (5.7 % vs. 3.6 %; $P = 0.04$) (Sangiovanni et al. 2006). In another single center study of 103 patients with compensated cirrhosis, 59 received interferon monotherapy, but only 3 (5 %) achieved an SVR, complications occurred in 26 patients after an average follow-up of 40 months. Hepatocellular carcinoma developed in 11 patients, and decompensation unrelated to HCC in 19 patients. Sixteen patients died, 94 % of liver disease. Three patients were transplanted for liver failure. The 4-year risk of HCC was 11.5 % (annual incidence 3.3 %) and that of decompensation was 20 %. Survival probability was 96 % and 84 % at 2 and 4 years, respectively (Serfaty et al. 1998).

The HALT-C trial provided important data on the natural history of patients with advanced fibrosis and cirrhosis as treatment proved ineffective (Di Bisceglie et al. 2008). Outcomes occurred at a similar rate between the treated group, 34.1 % and the control group 33.8 %. The most common clinical outcome was an increase of 2 or more points in the Child–Turcotte–Pugh score (documented on two consecutive visits), which occurred in 109 patients (10.4 %). Ascites was the most common clinical decompensation event that occurred in 59 patients (5.6 %), followed by hepatic encephalopathy in 37 patients (3.5 %), variceal hemorrhage in 16 patients (1.5 %), and spontaneous bacterial peritonitis in 6 patients (0.6 %).

Hepatocellular carcinoma occurred in 29 patients (2.8 %); 13 (2.1 %) in the patients without cirrhosis and 16 (3.7 %) in patients with cirrhosis. Fifty-three patients (5.0 %) died, 31 in the treatment group (15 of liver-related causes) and 22 in the control group (12 of liver-related causes). At 3.8 years, the overall death rate was 6.6 % among patients who received peginterferon and 4.6 % among control patients.

Once decompensation develops, there is an increased risk of death or need for liver transplant. One study followed 200 patients with HCV-related cirrhosis without known hepatocellular carcinoma (HCC) after hospitalization for their first hepatic decompensation (Planas et al. 2004). During a mean follow-up of approximately 3 years HCC developed in 33 (16.5 %) patients, and death occurred in 85 patients (42.5 %). The probability of survival after diagnosis of decompensated cirrhosis was 82 % and 51 % at 1 and 5 years, respectively (Planas et al. 2004). Development of HE and/or ascites as the first hepatic decompensation event was associated with a lower survival rate.

6 Factors that Affect the Rate of Disease Progression and Outcome of Chronic Hepatitis C

Many factors broadly grouped as host, viral and environmental have been identified that affect outcome of chronic hepatitis C (Table 5). Most of the host and viral factors are not modifiable, so there is little than can be done to attenuate their affect on outcome. In contrast, all the environmental factors are modifiable and clinicians should counsel patients on avoidance of these factors (with the possible exception of caffeine). The evidence for each of the factors identified to influence rate of disease progression and outcome of CHC are discussed.

Table 5 Factors associated with disease progression

Host	Viral	Environmental
Age	HBV-coinfection	Alcohol
Gender	HIV-coinfection	Smoking
Race	Viral load	Cannabis
Metabolic	Genotype	Caffeine
Steatosis		
Insulin resistance/diabetes		
Obesity		
Genetics	Viral quasispecies	
Exercise		
ALT		

Factors in italics are potentially modifiable

6.1 Host Factors

6.1.1 Age at Infection

In nearly all medical conditions, outcome is usually worse with older age and chronic hepatitis C is no exception. Multiple studies have shown that older age at infection was associated with more rapid progression of fibrosis (Poynard et al. 1997; Minola et al. 2002; Wright et al. 2003; Strasser et al. 1995; Pradat et al. 2007; Matsumura et al. 2000; Serra et al. 2003). In a large study of 2,235 patients from France who had undergone a liver biopsy and had never received treatment, nine clinical factors believed to affect progression of fibrosis- age at biopsy, estimated duration of infection, sex, age at infection, alcohol consumption, hepatitis C virus genotype, HCV viral level, route of infection, and histological activity grade were correlated with progression of fibrosis (Poynard et al. 1997). Fibrosis progression was defined as the ratio of stage of fibrosis by estimated duration of infection. Older age at infection >40 years, daily alcohol consumption of 50 g or more and male sex were associated with a faster rate of fibrosis progression (Poynard et al. 1997). In another study of 247 untreated patients undergoing liver biopsy, a fibrosis progression rate was estimated, based on date of exposure and stage of liver biopsy, for different ages of infection (Pradat et al. 2007). The estimated rate of fibrosis progression increased from 0.13 U of fibrosis/year for persons infected at age ≤19 years to 0.36 U of fibrosis/year for persons infected ≥37 years (Pradat et al. 2007). In another study, 268 of 392 persons with transfusion-acquired HCV underwent liver biopsy to assess the frequency and rate of progression to cirrhosis. After a mean follow-up of 18 years from infection, 54 (20 %) persons had developed cirrhosis. On multivariate analysis, factors associated with the development of cirrhosis were the duration of follow-up, age at infection at the time of liver biopsy and serum alanine aminotransferase levels at biopsy (Minola et al. 2002). The estimated time necessary to have a 50 % probability of developing cirrhosis was 33 years in patients aged 21–30, 23 years in persons aged 31–40, and 16 years in persons more than 40 years, highlighting the importance of age of infection for development of cirrhosis (Minola et al. 2002). The prevalence of cirrhosis was also higher in subjects with post-transfusion HCV who were infected at middle age (mean age of infection 48–52 years), 25–55 % after 16–25 years of infection compared to persons who were infected in childhood or as young adults, <1–5 %, with similar duration of follow-up (Kenny-Walsh 1999; Wiese et al. 2000; Vogt et al. 1999).

The reasons for the age-related differences in outcome are not clear. Physiologic or immunologic alterations with increasing age may affect fibrogenic and fibrinolytic processes. Alternatively, older individuals may be more likely to have a higher prevalence of co-factors associated with faster fibrosis progression such as greater alcohol consumption, diabetes and hepatic steatosis. Whatever the explanation, these data suggest that persons who acquire HCV later in life should be monitored more closely for disease progression and considered for treatment earlier in the

course of their infection compared to persons who acquire the infection at a younger age.

6.1.2 Gender

Most but not all studies have reported that spontaneous clearance of HCV is higher among females compared to males (Bakr et al. 2006; Wang et al. 2007a; Inoue et al. 2000; Guadagnino et al. 1997; Cox et al. 2005; Alter et al. 1999). Almost half (45 %) of women who acquired HCV infection via receipt of contaminated Rh immune globulin, cleared infection spontaneously (Kenny-Walsh 1999; Wiese et al. 2000). Studies of injection drug users with acute hepatitis C with known dates of HCV seroconversion have consistently shown that female gender was associated with a higher rate of spontaneous clearance (van den Berg et al. 2011; Page et al. 2009; Grebely et al. 2014; Mosley et al. 2008). In one study of 632 persons with acute HCV, spontaneous clearance was observed in 25 % of subjects within the first year of infection. HCV clearance was associated with female gender, IL28B rs12979860 CC genotype and HCV genotype 1 (Grebely et al. 2014). Interestingly, the effect of IL28B genotype and HCV genotype on spontaneous clearance was greater among females, compared to males (Grebely et al. 2014). The reason for the higher rate of spontaneous HCV clearance among women compared to men is unknown but may be linked to sex-based differences in immunity.

Gender has also been shown to have an effect on outcome of chronic HCV infection. Male gender has been associated with a faster rate of progression of fibrosis (Poynard et al. 1997; Wright et al. 2003) and higher rates of development of HCC (Chiaramonte et al. 1999; Ganne-Carrie et al. 1996; Degos et al. 2000). These observations are independent of alcohol and tobacco consumption, iron overload and being overweight, factors that are generally more frequent in men and have a negative influence the course of chronic hepatitis C.

The explanation for the association of male gender with faster rates of fibrosis progression is unclear. It is possible that estrogen may have a protective effect against development of fibrosis. In support of this, estrogen has been shown to modify extracellular matrix ECM (collagen) production and perhaps ECM degradation. In a dimethylnitrosamine rat model of fibrosis, high estrogen was shown to be associated with less fibrogenesis (Yasuda et al. 1999). Furthermore, estradiol was shown to suppress hepatic fibrosis in animal models, and attenuate hepatic stellate cell activation by suppressing the generation of reactive oxygen species in primary cultures (Shimizu et al. 1999).

The in-vitro data suggesting a protective effect of estrogen on fibrosis development are also supported by clinical observations. In one study, 472 HCV-infected women who had undergone a liver biopsy, were surveyed regarding prior pregnancies, menopause, and the use of oral contraceptives and hormone replacement therapy to evaluate the impact of these variables on liver fibrosis and its progression (Di Martino et al. 2004). Among 201 women who completed the survey, the

estimated rate of fibrosis progression was higher among postmenopausal (0.12 METAVIR Units per year) compared to pre-menopausal women (0.09 Metavir Units per year) and among nulliparous (0.14 METAVIR Units per year) than women with one or more children (0.07 METAVIR Units per year) (Di Martino et al. 2004). Interestingly, among postmenopausal women, the estimated rate of fibrosis progression was lower in women who received hormone replacement therapy compared with untreated women (0.1 versus 0.13 METAVIR units per year) and was similar to that of premenopausal women (0.09 METAVIR units per year (Di Martino et al. 2004). These findings suggest that menopause may be associated with accelerated liver fibrosis progression in HCV-infected women, an effect that may be prevented by hormone replacement therapy.

Another study evaluated the histological severity of chronic hepatitis C in 250 women, in relation to the menopause, steatosis and hormone replacement therapy (Codes et al. 2007). Women with F2–F4 were more often menopausal (67 % v 47 %) and the probability of fibrosis stage F2–F4 was lower for menopausal women receiving hormone replacement therapy (Codes et al. 2007). Thus, post-menopausal women may be at risk for greater liver disease progression and should be monitored more closely than pre-menopausal women and considered for early antiviral treatment. Hormone replacement therapy may ameliorate this risk of accelerated fibrosis but its potential benefits need to be confirmed in clinical trials and must be balanced against other health risks.

6.1.3 Race/Ethnicity

The overall prevalence of anti-HCV has been declining in the United States from 1.8 %, among 21,241 persons 6 years old or older who participated in the third National Health and Nutrition Examination Survey (NHANES) conducted during 1988 through 1994,(Alter et al. 1999) to 1.0 % among 30, 074 persons 6 years old or older conducted during 2003–2010 (Denniston et al. 2014). In all surveys, the seroprevalance of anti-HCV was higher among non-Hispanic Blacks compared to non-Hispanic Whites, 2.7 % versus 1.1 %, respectively, in the most recent survey (Denniston et al. 2014). The prevalence of anti-HCV was similar among Latinos and non-Hispanic Whites (Denniston et al. 2014). Additional studies have reported that compared to Whites, African Americans were more likely to be infected with genotype 1, (Wiley et al. 2002; Sterling et al. 2004; Crosse et al. 2004) have lower baseline serum ALT levels, (Wiley et al. 2002; Sterling et al. 2004; Crosse et al. 2004) less piecemeal necrosis, (Sterling et al. 2004; Wiley et al. 2002; Crosse et al. 2004) less fibrosis, (Sterling et al. 2004; Wiley et al. 2002; Crosse et al. 2004) but higher rates of HCC (El-Serag 2002).

Latinos were also reported to demonstrate more necroinflammatory activity compared to non-Hispanic Whites and a higher prevalence of cirrhosis and HCC compared to African-Americans and non-Hispanic Whites (Bonacini et al. 2001; Rodriguez-Torres 2008; El-Serag et al. 2014). The causes for more advanced disease among Latinos are complex. A higher prevalence of the metabolic

syndrome, insulin resistance, and hepatic steatosis as well as genetic differences among Hispanics are likely important contributing factors (Rodriguez-Torres 2008).

Response rates to interferon-based treatment were associated with race. African Americans and Latinos have lower response rates compared to non-Hispanic whites (Conjeevaram et al. 2006; Muir et al. 2011; Rodriguez-Torres et al. 2009). This is partly due to a lower prevalence of the IL28b C allele amongst African Americans and Latinos compared to Caucasians and Asians (Ge et al. 2009).

6.1.4 Steatosis

Hepatic steatosis is a common finding among the general population ranging from 10 to 24 % depending on the geographical location of the study. In the NHANES-3 study, a survey of the non-institutionalized, U.S. population, the prevalence of hepatic steatosis was 21 % based on the presence of fat on ultrasonography and the absence of significant alcohol consumption (Lazo et al. 2013). Steatosis is also a frequent histologic finding among patients with chronic hepatitis C. The prevalence of hepatic steatosis is approximately two to three times higher among patients with chronic hepatitis C compared to the general population, ranging from 42 to 73 % (Hourigan et al. 1999; Adinolfi et al. 2001; Monto et al. 2002; Perumalswami et al. 2006; Leandro et al. 2006). Epidemiological studies have identified older age, presence of diabetes, obesity, higher body mass index, hyperlipidemia, alcohol use, higher ALT levels, hepatic inflammation, fibrosis and genotype 3 as factors associated with the presence of steatosis on biopsy in patients with chronic hepatitis C (Hourigan et al. 1999; Adinolfi et al. 2001; Monto et al. 2002; Perumalswami et al. 2006; Leandro et al. 2006). Certain polymorphisms of the patatin-like phospholipase domain containing three gene, which encodes for a triacylglycerol lipase that mediates triacylglycerol hydrolysis in adipocytes, may contribute to steatosis in persons with chronic hepatitis C particularly those infected with non-3 genotypes (Cai et al. 2011; Valenti et al. 2011). These data indicate that steatosis in persons with chronic hepatitis C is strongly related to features of the metabolic syndrome but also related to infection with the virus itself particularly, HCV genotype 3. The demonstration of resolution of steatosis in subjects who successfully eradicate the virus after therapy but not in subjects who fail treatment provides strong evidence that HCV genotype 3 infection is associated with the development of steatosis (Kumar et al. 2002; Reddy et al. 2008; Castera et al. 2004).

The etiology of steatosis in patients with hepatitis C is multifactorial involving multiple mechanisms that are not necessarily mutually exclusive. Overexpression of the HCV core protein in a transgenic mouse model was shown to interfere with the hepatic assembly and secretion of triglyceride-rich very low density lipoproteins (VLDL) through inhibition of microsomal triglyceride transfer protein (MTP) activity (Perlemuter et al. 2002). Several HCV proteins have been shown to upregulate the transcriptional factor sterol regulatory element-binding protein 1c (SREBP-1c), which in turn activates the transcription of lipogenic genes, including

fatty acid synthase to induce steatosis (Oem et al. 2008; Lerat et al. 2009; Xiang et al. 2010; Li et al. 2013). The HCV core protein has also been shown to reduce the expression of PPAR-α, a nuclear receptor regulating gene responsible for fatty acid degradation, an effect that may be mediated through upregulation of microRNA-27 (Tanaka et al. 2008; Singaravelu et al. 2014; Shirasaki et al. 2013). It is now recognized that in subverting the cellular machinery of the host for its replication, HCV induces a number of unique pathological processes that inadvertently affect lipid metabolism.

The accumulation of fat in the liver has been shown to adversely affect the outcome of chronic hepatitis C. Hepatic steatosis has been suggested to promote the development of fibrosis and hasten progression to cirrhosis, increase the risk for HCC and lower the response to interferon-based therapy. Many cross-sectional studies have demonstrated a correlation between hepatic steatosis and more severe fibrosis on liver biopsy, (Hourigan et al. 1999; Adinolfi et al. 2001; Hsieh et al. 2007; Cholet et al. 2004; Hu et al. 2004; Patton et al. 2004) but a few have not (Asselah et al. 2003). However, in paired biopsy studies, which should provide stronger evidence for whether steatosis worsens fibrosis, the results have been equivocal with one study suggesting an association (Castera et al. 2003) and another no association (Perumalswami et al. 2006). Steatosis was shown to be associated with fibrosis progression in meta-analysis of 3,068 patients with chronic hepatitis C, from diverse geographical regions (Leandro et al. 2006). Steatosis was present in 51 % and fibrosis in 88 % of the cohort. Although steatosis was shown to be an independent predictor of fibrosis along with higher inflammatory activity, male gender, and older age, the association between steatosis and fibrosis appeared to be dependent on co-existent hepatic inflammation (Leandro et al. 2006). This finding would suggest that steatosis per se may not be associated with fibrosis progression but rather may be an end result of injury and a marker of necroinflammation. In addition, most studies have not controlled for insulin resistance, which may be the primary mediator of fibrogenesis in chronic hepatitis C, particularly since steatosis is strongly associated with insulin resistance and other features of the metabolic syndrome. Interestingly, when cirrhosis develops, steatosis usually improves (Lok et al. 2007). To date, there has been no prospective study showing that steatosis is independently associated with fibrosis progression in patients with chronic hepatitis C and the current evidence suggests only a weak association.

Steatosis has also been reported to increase the risk of hepatocellular carcinoma among persons with chronic hepatitis C (Ohata et al. 2003; Pekow et al. 2007; Kurosaki et al. 2010). In one study, data from 161 subjects with chronic hepatitis C were retrospectively analyzed to identify factors associated with the development of HCC. The cumulative incidence of HCC was 24 %, 51 %, and 63 % at 5, 10 and 15 years, respectively. Multivariate analysis identified hepatic steatosis, older age, cirrhosis, and having never been treated with interferon as independent factors associated with development of HCC (Ohata et al. 2003). The associations between worse steatosis and higher risk of HCC seem to be discrepant with the findings reporting improvement of steatosis with the development of cirrhosis. The above

study also found that steatosis was correlated with BMI, serum ALT levels, and triglyceride levels. This raises the question of whether steatosis per se is truly associated with development of HCC or whether it is underlying obesity. Not all studies have demonstrated an association between steatosis and HCC. A small case control series compared 25 cases of HCV-related HCC with a similar number of controls without HCC and found no effect of steatosis on development of HCC (Kumar et al. 2005). How steatosis may influence the development of HCC is uncertain. Steatosis may induce oxidative stress, lead to free radical formation and DNA damage (Okuda et al. 2002). While these studies suggest a role for hepatic steatosis with HCC, they do not establish causality. There is insufficient data at present to recommend more intensive monitoring of patients with steatosis for development of HCC.

Steatosis may also affect response to therapy with interferon-based regimens (Poynard et al. 2003; Patton et al. 2004). Steatosis has also been associated with higher rates of virological relapse, independent of viral load in patients with HCV genotype 3 infection (Shiffman et al. 2007). This finding raises the important question whether steatosis contributes to the higher relapse rate observed in subjects with genotype 3 treated with direct acting antiviral agents (Lawitz et al. 2013).

6.1.5 Non-alcoholic Steatohepatitis

Non-alcoholic steatohepatitis (NASH) represents a more advanced form of steatosis and is itself a progressive condition that can result in cirrhosis. Therefore, co-existent NASH and chronic hepatitis C may result in more rapid progression of liver disease. The association between NASH and chronic hepatitis C was investigated in 296 subjects, 178 with CHC alone, 94 with CHC and steatosis and 24 with CHC and NASH (Bedossa et al. 2007). Patients with CHC and NASH had significantly higher AST and triglyceride levels and lower high-density lipoprotein HDL cholesterol or total cholesterol than patients with CHC and steatosis. They also had more steatosis and higher fibrosis stage than patients with CHC and steatosis suggesting that NASH may result in faster progression of fibrosis in patients with chronic hepatitis C (Bedossa et al. 2007). NASH was also more common among subjects infected with genotype 3. Another study demonstrated similar findings of more advanced fibrosis among patients with chronic hepatitis C with co-existent NASH (Ong et al. 2001). Since there is no specific treatment for NASH other than diet and exercise and NASH is associated with obesity, patients with chronic hepatitis C should try to maintain ideal body weight.

6.1.6 Insulin Resistance/Diabetes

The development of insulin resistance (IR) in patients with chronic hepatitis C is complex and appears to be related to presence of the metabolic syndrome as well as a result of the viral infection. Development of IR can lead to diabetes, independent

of the presence of cirrhosis, a recognized risk factor for diabetes (Megyesi et al. 1967; Conn et al. 1969, 1971; Gentile et al. 1993). The development of IR or diabetes can also promote the development of cirrhosis and HCC. Given this interplay among obesity, IR and steatosis, it has been difficult to understand the complex relationship of each of these factors with the pathogenesis and outcome of HCV infection. A number of epidemiological studies including two meta-analyses suggest a strong association between CHC and the development of insulin resistance (IR) and diabetes (Antonelli et al. 2005; Mason et al. 1999; Sangiorgio et al. 2000; Bigam et al. 2000; Mehta et al. 2000, 2003; Imazeki et al. 2008; Allison et al. 1994; Caronia et al. 1999; Wang et al. 2007b). Both meta-analyses reported that persons with HCV infection had a 1.7-fold higher risk for diabetes compared to persons without HCV infection (White et al. 2008; Naing et al. 2012). However, other studies have not found such an association (Papatheodoridis et al. 2006; Butt et al. 2006). Problems with the definition of IR and diabetes, selection of appropriate controls and ascertainment bias may be responsible for differences between studies. Findings were also inconsistent among population-based studies. An analysis of the NHANES 3 dataset, which included 9,841 persons of whom 8.4 % had type 2 diabetes and 2.1 % were anti-HCV positive, was performed to examine the association of CHC and development of diabetes. Persons aged 40 years or older with HCV infection were three times more likely than those without HCV infection to have type 2 diabetes (adjusted odds ratio, 3.77) (Mehta et al. 2000). In another study from Taiwan, 4,958 persons aged 40 years or older without diabetes from a community-wide cohort in southern Taiwan were followed for 7 years (1997–2003) to study the risk of diabetes associated with HCV infection. Three thousand four hundred and eighty-six had no serologic evidence of HCV or hepatitis B virus (HBV), 812 were anti-HCV+, 116 were co-infected with HBV and HCV, and 544 had chronic hepatitis B. A total of 474 participants developed diabetes. Anti-HCV positivity was associated with a 1.7-fold increased likelihood of diabetes (Wang et al. 2007b). However, two studies suggested that necroinflammation and not specifically CHC was associated with development of diabetes. A population-based cohort study from Italy reported no association between HCV and diabetes but HCV was associated with DM 2 only in subjects with elevated ALT (OR 1.5) (Montenegro et al. 2013). An analysis of the NHANES dataset from 1999 to 2010 using American Diabetes Association criteria for diabetes and prediabetes, found no association between either of these conditions with HCV status. However, elevated alanine aminotransferase and gamma glutamyltransferase levels were closely associated with diabetes regardless of HCV status (Ruhl et al. 2014). Studies have shown that eradication of HCV can lead to improvement and reversal of IR (Kim et al. 2009; Delgado-Borrego et al. 2010; Butt et al. 2012) and reduction in the incidence of diabetes (Arase et al. 2009; Romero-Gomez et al. 2008). Whether this is related to elimination of HCV, improvement in hepatic inflammation or both, remains to be clarified.

Several mechanisms have been reported for the development of IR and DM in patients with HCV infection. HCV has been shown to impair hepatic insulin signaling through the insulin receptor substrate 1 and 2 and phosphoinositide-3-

kinase/protein kinase B pathways (Aytug et al. 2003). The HCV core protein has been shown to suppress insulin receptor substrate 1 tyrosine phosphorylation through an interaction of SOCs3, through activation of proinflammatory c-Jun N-terminal kinase (JNK) pathways or through m-TOR activation (Shintani et al. 2004; Banerjee et al. 2008; Pazienza et al. 2007). The core protein of HCV genotype 3 has also been shown to promote IRS-1 degradation through an effect on PPAR-γ (Pazienza et al. 2007; Kawaguchi et al. 2007).

Insulin resistance and diabetes are associated with faster progression fibrosis, increased risk of HCC and lower response to therapy in patients with CHC. Diabetes has been shown to increase the risk of HCC in persons with CHC in data from cross-sectional, case control and prospective studies.

Several studies have reported that IR was associated with more severe fibrosis (Hui et al. 2003b; Moucari et al. 2008; D'Souza et al. 2005; Fartoux et al. 2005). In a cross-sectional study of 250 patients with CHC, homeostatic model assessment (HOMA)-IR was an independent predictor of degree of fibrosis and rate of fibrosis progression (Hui et al. 2003b). Another study of 600 subjects (500 with CHC and 100 with chronic hepatitis B) reported that significant fibrosis was associated with IR independent of steatosis among subjects with CHC but not chronic hepatitis B (Moucari et al. 2008). An analysis of the Taiwanese National Health Insurance Research Database, which is comprised of data from >99 % of the entire population reported that diabetes was associated with a higher risk of cirrhosis, and decompensation in persons with CHC. 6,251 adult CHC patients were identified from a random sampling of one million persons and followed prospectively for 12 years. Four hundred and twenty-four persons with CHC who developed diabetes were compared to 1708 persons with HCV who did not develop diabetes for outcomes of CHC. New onset diabetes shown to be an independent predictor for cirrhosis (HR = 2.5; 95 % CI = 1.609–3.897; $P < 0.001$) and decompensation, (HR = 3.6) (Huang et al. 2014).

The presence of diabetes has also been shown to promote the development of HCC among persons with CHC (Elkrief et al. 2014; N'Kontchou et al. 2006; Wang et al. 2009; Veldt et al. 2008; El-Serag et al. 2001). The influence of diabetes on HCC development in subjects with CHC was evaluated in a population-based study from Taiwan, involving 5,929 persons in southern Taiwan who were followed for 7 years. One hundred and thirty-two subjects with hepatitis C and diabetes were compared with 850 with hepatitis C without diabetes. Anti-HCV positive subjects with diabetes had a threefold higher rate of HCC compared to anti-HCV positive subjects without diabetes. The finding that treatment of diabetes in subjects with CHC was associated with a reduction in HCC also supports a role for diabetes in development of HCC (Lai et al. 2012).

IR also affects the response to therapy among HCV persons. A meta-analysis of 14 studies reported that HCV patients with IR have a 20 % lower SVR than patients without IR (Deltenre et al. 2011).

6.1.7 Obesity

Small studies report that obese patients have more fibrosis than lean patients but this may be due to other factors such as steatosis, insulin resistance or presence of diabetes. Given the close association between obesity and hepatic steatosis and insulin resistance, it is difficult to ascertain the relative contribution of obesity relative to these other factors and further studies are needed.

6.1.8 Genetics

Although several host, viral, and environmental factors have been linked with outcome of CHC, they do not completely explain the variable outcome of the disease. Epidemiological studies suggest that clinical factors explain only approximately 30 % of variability observed in fibrosis progression. Other studies have demonstrated that a strong host immune response against HCV favors viral clearance. Thus, variability in genes involved in the immune response may contribute to the ability to clear the virus. In a landmark study, a genetic polymorphism near the IL28B gene, encoding interferon-lambda-3 (IFN-lambda-3), was shown to be strongly associated response to treatment with interferon and spontaneous clearance of HCV (Ge et al. 2009). The C allele of rs 12979860 and G allele of rs8099917 were associated with an almost twofold change in treatment-related clearance of HCV compared to the T allele at both loci (Ge et al. 2009). This observation was true for individuals of both European and African ancestry. In another study of 1,015 subjects with chronic infection and 347 who spontaneously cleared the infection, the minor allele (G) of the SNP at position rs8099917 was associated with a greater than twofold progression to chronic HCV infection (Rauch et al. 2010). A study of patients with resolution of hepatitis C from a single source outbreak of HCV from contaminated anti-D from Germany, revealed that spontaneous clearance was more common in patients with the CC genotype (43/67; 64 %) compared with CT (22/90; 24 %) or TT (2/33; 6 %) (Tillmann et al. 2010). Jaundice during acute infection was twice as common among patients with CC genotype (33 %) than non-CC patients (with C/T or T/T) (16 %). This finding may explain the clinical observation of higher clearance of HCV in patients who present with jaundice. Among patients with the CC genotype, rates of clearance were high regardless of the presence of jaundice or not, 56 % with jaundice, and 61 % without jaundice, but in patients with non-CC genotype, those who presented with jaundice were three times more likely to clear the infection than those without jaundice (42 % vs 14 %, respectively) (Tillmann et al. 2010). The IL28B CC genotype was also shown to be associated with greater hepatic necroinflammation, higher ALT, and worse clinical outcomes in subjects with CHC (Noureddin et al. 2013).

The interplay between stimulatory and inhibitory natural killer cell receptors and their corresponding human leukocyte antigen ligands have been shown to influence the outcome of acute HCV infection (Khakoo et al. 2004; Romero et al. 2008).

Genes encoding the inhibitory NK cell receptor KIR2DL3 and its human leukocyte antigen C group 1 (HLA-C1) ligand were shown to affect resolution of HCV infection in a study of 685 subjects with persistent infection and 352 with resolved infection (Khakoo et al. 2004). The frequency of individuals with two copies of HLA-C1 alleles (HLA-C1C1) was higher in persons with resolved HCV infection (38 %) compared to those with persistent infection (30 %) (Khakoo et al. 2004). This appears to be weakly protective as it was only observed in patients with expected low infectious doses of HCV but not in those with high-dose exposure, in whom the innate immune response is likely to be overwhelmed. The data strongly suggest that inhibitory NK cell interactions are important in determining antiviral immunity and that diminished inhibitory responses confer protection against HCV. Other HLA genes have been identified that influence outcome of acute HCV infection. The frequency of DQB1*0301 and DRB1*1101 alleles was higher in patients with transient infection than in those with persistent infection (Alric et al. 1997).

Other specific immunoregulatory molecules, such as chemokines and chemokine receptors may also influence outcome of HCV infection. Chemokines are important mediators of hepatic inflammation and injury (Murai et al. 1999). Some studies have suggested that the homozygous 32-base pair deletion in the CCR5 gene (CCR5-Delta32) that protects against human immunodeficiency virus infection (HIV) was associated with a higher rate of chronicity after exposure to HCV, higher HCV RNA levels and lower response to interferon monotherapy (Ahlenstiel et al. 2003). However, other studies have found no such association with chronicity or response to antiviral therapy (Promrat et al. 2003; Tommasi et al. 2006). The role of this gene product and its polymorphisms on outcome of HCV infection is currently believed to be minor.

A combination of single nucleotide polymorphisms has been used to predict the risk of developing cirrhosis in patients with chronic hepatitis C. A 7 SNP variant signature consisting of AZIn1, TLR4, TRPM5, AQP2, rs2290351, rs4290351 and rs17740066 was shown to be better than clinical factors at predicting risk of cirrhosis in Caucasian patients with chronic hepatitis C (Huang et al. 2007). The usefulness of this risk score was also validated in a population with initially mild chronic hepatitis C (Marcolongo et al. 2009) and a population with more advanced disease (Curto et al. 2011).

6.1.9 Exercise

Metabolic abnormalities, including liver steatosis, obesity diabetes and insulin resistance have been associated with a worse clinical course of CHC. Exercise has been shown to improve these metabolic derangements and may therefore improve outcome of HCV infection particularly in individuals with the metabolic syndrome. In a small study of 16 obese patients with chronic hepatitis C, dietary intervention and increased exercise was associated with reduction in BMI, improved insulin sensitivity and serum ALT and AST levels suggesting that dietary

and exercise intervention may improve hepatic and metabolic status in obese insulin-resistant CHC (Pattullo et al. 2013). In another study from Japan, aerobic exercise was associated with significant improvement in BMI and ALT levels in subjects with chronic hepatitis C (Konishi et al. 2011). Aerobic exercise has also been shown to improve psychological well-being and quality of life in overweight and obese patients with CHC (Abd El-Kader et al. 2014; McKenna et al. 2013). Whether these improvements in metabolic parameters and serum ALT levels translate into histological improvement is currently unknown since liver biopsies were not performed in any of the reported studies. In addition, whether the short-term improvements from life style modification can influence long term clinical outcome of chronic hepatitis C remains to be determined. But given the other known health benefits of exercise, it should be recommended for both obese and lean patients with CHC.

6.1.10 ALT

Serum alanine aminotransferase level (ALT) is a sensitive indicator of liver cell injury and is useful in identifying cases of acute and chronic liver injury. Moreover, it has been shown to correlate with liver-related mortality. In population studies, an increase in ALT levels were shown to be associated with a progressive increase in death from all causes and in particular liver-related death (Kim et al. 2004). In addition, the increase in liver-related mortality seemed to become significant once the ALT level reached the range of 30–39 IU/L. There was an almost tenfold increase in liver-related mortality among individuals with an ALT of 30–39 IU/L compared to individuals with an ALT <20 U/L (Kim et al. 2004).

Among patients with chronic hepatitis C, ALT values are generally normal in approximately one-third of subjects, elevated $<2 \times$ upper limit of normal (ULN) in one third and elevated $>2 \times$ ULN in one third (McOmish et al. 1993; Conry-Cantilena et al. 1996). Serum ALT levels fluctuate over time and in one study ~20 % of subjects with normal ALT levels followed over 1–8 years, experienced an ALT elevation (Martinot-Peignoux et al. 2001). Subjects with persistently normal ALT levels are more likely to have mild liver fibrosis. Data from over 1100 subjects from 23 studies indicated that 80 % of subjects with a normal ALT level have mild fibrosis and approximately 20 % have advanced fibrosis (Alberti et al. 2004). Progression of fibrosis has been demonstrated in subjects with persistently normal ALT levels but is less frequent compared to patients with elevated ALT levels and usually mild (Hui et al. 2003a; Persico et al. 2000; Nunnari et al. 2013; Mathurin et al. 1998).

In paired biopsy studies baseline serum ALT was shown to be a predictor of fibrosis progression, with faster progression associated with higher ALT levels (Ghany et al. 2003; Boccato et al. 2006; Marcellin et al. 2002). Serum ALT was not shown to be associated with development of clinical outcomes but rather the ratio of AST/ALT was (Ghany et al. 2010). This highlights an important limitation

of ALT in that once the disease becomes advanced, it is not a very good predictor of clinical outcome.

6.2 Viral Factors

6.2.1 Co-infection with HBV

The prevalence of hepatitis B surface antigen (HBsAg) among persons with chronic hepatitis C is estimated to be 2–10 % (Crespo et al. 1994; Liu et al. 2005; Kaur et al. 1996; Liang et al. 1994; Reimer et al. 2007). There is significant geographical variation ranging from a low of 3 % in the U.S. to >15 % in India, Taiwan, Japan and Mediterranean (Caccamo et al. 2014; Tyson et al. 2013; Chakravarti et al. 2005; Dai et al. 2001; Sato et al. 1994; Di Marco et al. 1999; Fattovich et al. 1991). The prevalence is also higher among populations at higher risk for viral hepatitis such as injection drug users, patients receiving hemodialysis and those with hemoglobin disorders prior to blood screening (Reimer et al. 2007).

Studies of patients with HBV-HCV co-infection have reported more disease activity and consequently a high rate of clinical outcomes including a higher prevalence of cirrhosis, decompensated liver disease and HCC compared to subjects with mono-infection (Sagnelli et al. 2000, 2004; Mohamed Ael et al. 1997; Lee et al. 2007; Kaklamani et al. 1991; Kew et al. 1997; Kirk et al. 2004; Fong et al. 1991; Benvegnu et al. 1994). This was despite the fact that most studies of HBV-HCV co-infected subjects show that usually only a single virus predominates, though which virus does so was unpredictable (Zarski et al. 1998; Fong et al. 1991). In a large study of 648 subjects of whom 84 had HBV-HCV co-infection, 155 had occult HBV and HCV, 161 HBV monoinfection and 193 HCV monoinfection, persons with dual HBV and HCV infection were more likely to have severe activity and a higher prevalence of cirrhosis (13 %) compared to monoinfected patients with HBV (10 %) and HCV (6 %) (Sagnelli et al. 2000). Interestingly patients with occult hepatitis B and HCV (anti-HBc positive, anti-HCV positive) were also found to have more severe disease than their mono-infected counterparts (Sagnelli et al. 2000). In another study to assess HCC risk among patients with compensated cirrhosis, 66 patients with HBV monoinfection, 166 with HCV monoinfection and 27 with HBV/HCV co-infection were prospectively followed every 6 months with ultrasound and alfa fetoprotein testing (Chiaramonte et al. 1999). Overall, 20 % of the cohort developed HCC during a mean follow-up of approximately 5 years. However, the frequency of HCC development was twice as high in HBV-HCV co-infected subjects (40 %) compared to those with HCV mono-infection (21 %) and four times as high as subjects with HBV mono-infection (9 %). The incidence of HCC per 100 person-years of follow-up was 3.7 in HCV positive subjects, 2.0 in those HBsAg positive, and 6.4 in those with dual infection (Chiaramonte et al. 1999). A meta-analysis of 32 studies to assess the effect of HBV-HCV infection on HCC risk confirmed the higher HCC risk among subjects with

HBV-HCV co-infection OR 165 based on 191 cases and eight controls exposed compared to an OR of 22.5 for HBsAg positivity and anti-HCV/HCV RNA negativity and an OR of 17.3 for anti-HCV/HCV RNA positivity and HBsAg negativity (Donato et al. 1998). Thus, subjects with HBV-HCV co-infection should be monitored closely for the development of cirrhosis and end-stage liver disease and may warrant more intensive screening to detect HCC.

6.2.2 HIV

HCV and HIV share similar routes of transmission. As a result worldwide there are approximately four to five million persons with HIV-HCV co-infection (Alter 2006). In the US there are approximately 150,000–300,000 persons with HIV/HCV coinfection (Sherman et al. 2002). The prevalence of HIV-HCV co-infection varies geographically and by mode of transmission. The highest rates are seen among injection drug users and men who have sex with men; rates are lower among heterosexuals because HCV is not efficiently transmitted by sex. HCV infection is not associated with an increased rate of AIDS-defining events or deaths (Hernando et al. 2012). However, HIV has a number of adverse consequences on the outcome of HCV infection. HIV has been shown to increase the rate of chronic HCV infection, (Daar et al. 2001; Messick et al. 2001) to increase HCV RNA levels (Daar et al. 2001; Eyster et al. 1994; Ghany et al. 1996; Thomas et al. 2000b) and is associated with faster progression of fibrosis and development of cirrhosis. Response rates to interferon-based treatment are also lower among co-infected persons.

Prior to HAART therapy, most co-infected individuals died from complications of HIV infection. However, in the post-HAART era, HCV-related liver disease (primarily end-stage liver disease) is a major cause of death among co-infected persons (Rosenthal et al. 2007; Weber et al. 2006).

Two national retrospective multicenter cohort surveys were performed in France that included 17,487 HIV-infected patients during 1995 and 26,497 during 1997 to assess all cause and liver-related mortality and HIV related mortality. Comparative results between the 1995 and 1997 surveys showed a dramatic decline in AIDS-related mortality rates (7.47 % vs. 1.73 %) but not in HCV-related mortality rates (0.06 % vs. 0.07 %) (Cacoub et al. 2001). With extended follow-up, an increase in liver – related mortality was reported and duration of infection with HCV and alcohol consumption were identified as important contributors to disease progression (Rosenthal et al. 2003, 2007). A recent analysis from the VA reported similar findings in 4,280 co-infected patients who initiated ART and 6,079 HCV-monoinfected patients receiving care between 1997 and 2010 (Lo Re et al. 2014). The incidence of hepatic decompensation was greater among co-infected than monoinfected patients (7.4 % vs. 4.8 % at 10 years respectively; $P < 0.001$). Despite receiving ART, patients co-infected with HIV and HCV had higher rates of hepatic decompensation than HCV-monoinfected patients (Lo Re et al. 2014). It remains controversial whether HAART therapy is associated with

slowing of disease progression. Some studies showed no affect (Martinez-Sierra et al. 2003; Thein et al. 2008b; Smit et al. 2008) whilst others did (Brau et al. 2006; Macias et al. 2006). The use of protease inhibitors was shown to slow the progression of fibrosis but not NNRTIs (Benhamou et al. 2001).

HIV has been shown to accelerate progression of fibrosis among persons with chronic hepatitis C, including those with persistently normal ALT levels (Martin-Carbonero et al. 2009; Martinez-Sierra et al. 2003). Approximately, one-third of persons with HIV-HCV co-infection progress to cirrhosis over a 20 year period and about 50 % will progress to cirrhosis over a 30 year period compared to 25 % over 25–30 year period among mono-infected subjects (Thein et al. 2008b). Cross-sectional liver biopsy studies comparing HIV-HCV co-infected to mono-infected persons universally demonstrated more severe liver damage and higher prevalence of cirrhosis among co-infected persons (Fuster et al. 2004; Benhamou et al. 1999). A meta-analysis of 27 studies involving 4,970 with HCV monoinfection and 2,636 with HIV-HCV co-infection reported worse outcomes among co-infected persons in the HAART era. Disease progression was strongly linked to the duration of HCV infection and HAART therapy did not reduce the adverse effects of HIV on HCV outcome (Thein et al. 2008b). How HIV mediates faster progression of fibrosis is not well understood. Higher HCV levels may promote a more robust inflammatory response, alteration of the cytokine pattern to a more pro-fibrotic state, alternatively, HIV may indirectly affect fibrosis by increasing reactive oxygen species which in turn leads to upregulation of profibrotic proteins monocyte chemotactic protein-1 and tissue inhibitors of metalloproteinases-1 (Lin et al. 2011). HIV infection has been associated with enhanced microbial translocation, which may promote fibrogenesis through increased lipopolysaccharide levels or through immune dysfunction (Balagopal et al. 2008). HIV-HCV subjects should be monitored more closely for disease progression and consideration should be given to early therapy in this sub-population of subjects with HCV.

6.2.3 HCV Genotype

The HCV viral polymerase lacks proof reading capacity as a result many errors are introduced during replication. As a consequence, the virus circulates as a viral swarm or quasispecies. Viral quasispecies were shown to affect spontaneous viral clearance (Farci et al. 2000). Acute resolving hepatitis was associated with relative evolutionary stasis of the viral quasispecies, whereas chronic infection correlated with genetic evolution of HCV.

Six major genotypes have been identified based on a sequence divergence of 30 % among isolates. HCV genotypes have a geographical distribution with genotype 1 being the most common worldwide, accounting for 46 % of all HCV cases, approximately one-third of which are in East Asia. Genotype 3 is the next most prevalent globally, 30 %. Genotypes 2, 4, and 6 are responsible for a total 23 % of all cases and the remaining cases are comprised of genotype 5 (Messina et al. 2015).

HCV genotype is strongly related to the source infection, but not to the intrinsic pathogenicity of HCV, and is a strong predictor of sustained response to therapy.

Initial studies suggested that HCV genotype 1b was associated with a higher risk for cirrhosis, hepatic decompensation and HCC (Fattovich et al. 2001; Zein et al. 1996; Nousbaum et al. 1995; Bruno et al. 1997; Booth et al. 1995; Silini et al. 1996). However many of these earlier studies did not adequately control for other important factors of disease progression such as age at infection, duration of disease and alcohol use. Subsequent studies have shown no link between HCV genotype and outcome of infection (Serfaty et al. 1998; Bruno et al. 1997; Martinot-Peignoux et al. 1999; Freeman et al. 2003). A meta-analysis of 16 studies suggested that HCV genotype 3 was associated with accelerated fibrosis progression in single biopsy studies but not paired biopsy studies (Probst et al. 2011). The most important clinical utility of HCV genotype is as a predictor of response to therapy. Although the importance of genotype is diminishing in the era of direct acting antiviral agents. The development of pangenotypic regimens may obviate the need for genotyping for management of patients with CHC.

6.2.4 Viral Load

Unlike HIV infection, there is little evidence to support the notion that HCV viral load affects outcome of CHC. The viral load observed among persons with chronic hepatitis C ranges from 10^4 to 10^8 copies per ml with an average HCV RNA level of approximately 10^6 copies/ml (Thomas et al. 2000b). Typically, HCV RNA levels do not fluctuate more or less than a one log of the baseline viral load in a patient with chronic infection. HCV viral load does not differ among viral genotypes (Yamada et al. 1994). Most studies that have analyzed the role of HCV RNA with severity of disease have been cross-sectional in design and made comparisons with ALT or liver biopsy. Few have been longitudinal and focused on hard clinical outcomes. Although a few studies have shown a correlation between higher HCV viral load and higher serum ALT levels (McCormick et al. 1996) and hepatic inflammation (Lau et al. 1993; Naito et al. 1994; Fanning et al. 1999) most studies demonstrated no relationship between HCV viral load and disease severity (Puoti et al. 1999; Gervais et al. 2001; Lee et al. 2001; Freeman et al. 2003). The few longitudinal studies that examined the role of viral load and progression in fibrosis found no association (Lagging et al. 2002; Ghany et al. 2003; Poynard et al. 2001). A large study that involved 6,570 injection drug users with hepatitis C, some of whom were co-infected with HIV or HTLV-2 suggested that HCV RNA level was a predictor of death from end stage liver disease in patients with chronic hepatitis C (Hisada et al. 2005). The relative hazard of death from end stage liver disease increased with increasing viral load (HR 2.26) even after adjustment for alcohol (HR 2.56). However, the role of HIV co-infection and the effect on viral load was not carefully explored raising the question whether HCV viral load would still have been a predictor of death.

In summary the bulk of the evidence suggests that viral load does not affect outcome of chronic hepatitis C and there is no role for serially assessing viral load in a patient. Viral load is predictive of response to treatment with lower viral load being associated with higher response rates.

6.3 Environmental Factors

6.3.1 Alcohol

There are limited data on the prevalence of alcohol use among persons with chronic hepatitis C. The frequency and quantity of alcohol use was collected in the Dionysos Study, a prospective cohort study initiated to study the prevalence of liver disease among 6,917 persons from the general population of Northern Italy of whom, 3.2 % were anti-HCV positive and 2.3 % HCV RNA positive (Bellentani et al. 1999). Twenty-three percent of the anti-HCV cohort consumed a significant amount of alcohol (defined as >30 g/day) (Bellentani et al. 1999). Data on alcohol use was also available from a large meta-analysis conducted to examine progression of fibrosis. Among 111 studies which included 33,121 subjects, 19 % of subjects consumed alcohol ranging from 20 to 80 g/day (Thein et al. 2008a). Thus, approximately one-fifth to one-quarter of persons with chronic hepatitis C consume significant quantities of alcohol.

The prevalence of chronic hepatitis C appears to be five to tenfold higher among persons with a history of alcohol abuse either with or without known liver disease, compared to the general population. Among studies of drinkers, the proportion who test anti-HCV or RIBA positive ranges from 14 to 35 % and HCV RNA positive from 11 to 29 % (Bode et al. 1991; Befrits et al. 1995; Caldwell et al. 1991; Nalpas et al. 1992; Zarski et al. 1993; Oshita et al. 1994). However, there is substantial geographical variation in prevalence rates, for example the rate is ~10 % in the U.S., 14 % in Northern Europe and France and 45–80 % in Japan (Mendenhall et al. 1991; Befrits et al. 1995; Zarski et al. 1993; Caldwell et al. 1991; Nalpas et al. 1992; Oshita et al. 1994). The reason for the higher rate of hepatitis C among alcohol abusers is unclear. With first generation tests, cross-reacting antibodies in patients with cirrhosis may have led to false positive results. It is also possible that alcoholics were more likely to have risk factors for acquiring HCV infection (Brillanti et al. 1991). However, the prevalence remained higher among alcoholics compared to general population after controlling for blood transfusion and injection blood use. Alcoholic patients may be more susceptible to chronic infection with HCV because of impairments in cellular immunity (Caldwell et al. 1991; Oshita et al. 1994).

Alcohol adversely affects the natural history of chronic hepatitis C. The rate of chronicity appears to be higher, progression of fibrosis faster and the incidence of HCC higher among alcoholics. Indeed, alcohol consumption may be the single most important factor affecting disease progression in patients with chronic

hepatitis C. The mechanisms whereby alcohol exerts its deleterious affects on chronic HCV are unclear. Alcohol may increase HCV replication, may potentiate HCV-related cytotoxicity through hepatic oxidative stress or adversely modulate the immune response.

The importance of alcohol in progression of fibrosis was demonstrated in a landmark retrospective study from France. Among 1,157 patients with chronic hepatitis C who underwent a liver biopsy, the mean stage of fibrosis was significantly higher in patients whose daily alcohol consumption was 50 g or more compared to those who consumed less than 50 g, irrespective of age or duration of infection. Subjects who drank >50 g /day had a 34 % increased rate of fibrosis progression as compared to non-drinkers (Poynard et al. 1997). Many studies from tertiary care centers as well as population-based studies, have shown that persons with chronic hepatitis C who regularly consume 30–40 g/day of alcohol have a two to fourfold greater risk of cirrhosis and decompensated liver disease compared to non-drinkers (Wiley et al. 1998; Bellentani et al. 1999; Harris et al. 2001). Indeed, a large meta-analysis conducted to explore the relationship between advanced liver disease and alcohol use taking into account the different definitions of heavy alcohol consumption across many studies, including more than 15,000 patients with HCV infection, demonstrated that heavy intake between 210 and 560 g/week was associated with a 2.3-fold increase risk of cirrhosis (Hutchinson et al. 2005). Even moderate alcohol consumption, defined as 31–50 g/day, was shown to be associated with an increased risk of fibrosis (Hezode et al. 2003b). Moreover, the rate at which subjects developed cirrhosis was faster in persons with chronic hepatitis C with significant alcohol intake compared to non-drinkers. Although most studies indicate that alcohol use is associated with more severe disease, it is important to note that for a given quantity of alcohol use there is a broad spectrum of fibrosis seen and it is possible to see significant fibrosis including cirrhosis in non-drinkers and mild fibrosis in heavy drinkers (Monto et al. 2004). Moderate-large quantities of alcohol in patients with chronic hepatitis C have also been associated with an increase in liver-specific mortality and overall mortality (Niederau et al. 1998).

Currently, there is insufficient evidence to determine a “safe” amount of alcohol use. Therefore, despite the beneficial cardiovascular effects of light alcohol use (10–20 g/day), given the uncertainty on the effects of this amount of alcohol on liver disease progression, patients should be counseled of the adverse effects of alcohol on outcome of chronic hepatitis C and advised to refrain from alcohol use.

6.3.2 Smoking

Evidence that smoking may cause liver disease or worsen liver disease of any etiology is lacking. However, epidemiological studies have identified a link between smoking and an increased risk of cirrhosis in patients with alcoholic liver disease and hepatitis B (Klatsky and Armstrong 1992; Yu et al. 1997). Whether smoking has any effect on outcome of chronic hepatitis C is uncertain.

A community-based study showed that smoking was associated with higher serum ALT levels among anti-HCV positive persons (Wang et al. 2002). The effect of smoking on outcome of chronic hepatitis C was examined in 310 subjects who were undergoing first liver biopsy. Smoking was defined as never, ≤15 or >15 pack years; 57 % of the cohort were current smokers. Smoking >15 pack years was shown to be an independent predictor of liver fibrosis on multivariate analysis but this association was lost when the degree of disease activity was controlled for in the multivariate analysis (Pessione et al. 2001). Another study reported that smoking was associated with higher necro-inflammatory scores but not fibrosis in persons with hepatitis C undergoing liver biopsy (Hezode et al. 2003a). However, a small retrospective analysis reported that a greater proportion of smokers had stage F3/F4 compared to non-smokers and demonstrated higher VGEF levels among smokers suggesting that smoking may mediate its effects on fibrosis through a vascular mechanism (Dev et al. 2006). Other possible mechanisms by which smoking may promote fibrogenesis include induction of oxidative stress, enhanced production of proinflammatory cytokines or an effect on immune response but direct experimental evidence for this in the liver is lacking. In summary, the evidence demonstrating an association between smoking and outcome of chronic hepatitis C is weak. Despite this, given the known adverse effects of smoking on multiple human diseases, patients with chronic hepatitis C should be advised to not smoke.

6.3.3 Cannabis

There is both clinical and experiment evidence implicating daily cannabis use as co-factor modulating disease progression in patients with chronic hepatitis C. Two independent studies have reported a strong association between cannabis use and significant fibrosis (≥F3). In one study from France, 270 consecutive patients with chronic hepatitis C undergoing liver biopsy were studied. Cannabis use was categorized as none, occasional or daily. Daily cannabis use was associated with severe fibrosis on biopsy and a faster rate of fibrosis progression (Hezode et al. 2005). Another study from the U.S. performed a liver biopsy on 204 patients with chronic hepatitis C and compared the severity of fibrosis among daily and non-daily cannabis users. On multivariate analysis, daily cannabis use was strongly associated with more severe fibrosis (≥F3) (Ishida et al. 2008). Cannabis has also been shown to be associated with hepatic steatosis, another factor that contributes to more severe liver disease in subjects with CHC (Hezode et al. 2008).

There is very good experimental evidence supporting a role for cannabis as a mediator for fibrosis. Two receptors for delta 9-tetrahydrocannabinol the active compound mediating the psychoactive effects of cannabis have been identified, and designated CB1 and CB2. CB1 receptors were shown in several murine models to be potent enhancers of liver fibrogenesis and to prolong survival of hepatic myofibroblasts in culture (Teixeira-Clerc et al. 2006). Given the available clinical

and experimental data, patients with chronic hepatitis C should abstain from use of cannabis.

6.3.4 Caffeine

Population-based, epidemiological studies from different geographical regions have noted a link between daily caffeine consumption, particularly coffee, and lower risk of an elevated serum ALT among persons without known liver disease (Casiglia et al. 1993; Honjo et al. 2001) or who are at high risk for liver disease (Ruhl and Everhart 2005). Coffee consumption has also been associated with a lower risk of advanced liver disease, cirrhosis and hepatocellular carcinoma in patients with chronic liver disease. Among subjects with chronic hepatitis C, daily caffeine consumption was associated with lower ALT levels compared to no caffeine use. In one study from France, daily caffeine consumption was estimated and correlated with liver disease severity in 238 patients with chronic hepatitis C. A daily caffeine consumption greater than 408 mg/day (equivalent to >3 cups/day) was associated with a lower risk of disease activity greater than Metavir grade A2, but no relationship was found with fibrosis stage (Costentin et al. 2011). In another study from the U.S., 177 patients scheduled to undergo liver biopsy were asked to complete a detailed caffeine questionnaire on three occasions over a 6-month period. Caffeine intake was correlated with severity of liver disease. A daily caffeine consumption >308 mg (equivalent to 2.25 cups/day) was associated with less severe hepatic fibrosis (Modi et al. 2010). Interestingly, caffeine from other sources such as tea or caffeinated beverages was not associated with stage of liver fibrosis (Modi et al. 2010). A prospective study found that Japanese patients with chronic hepatitis C who consumed caffeine daily were three times more likely to maintain a normal ALT level compared to non-drinkers (Sasaki et al. 2013).

The role of coffee consumption with liver disease progression in individuals with advanced hepatitis C–related liver disease was examined in the HALT-C trial (Freedman et al. 2009). Higher coffee use was associated with a lower AST/ALT ratio, suggesting a lower prevalence of cirrhosis on cross-sectional analysis. On longitudinal analysis, higher, regular coffee consumption was associated with a markedly lower rate of disease progression. Rates of outcome were 11.1/100 person-years for subjects who drank none, 12.1 for less than 1 cup/day, 8.2 for 1 to fewer than 3 cups/day, and 6.3 for 3 or more cups/day (Freedman et al. 2009). It remains unclear whether it is coffee, caffeine or some other component of coffee that is responsible for the beneficial effects on liver disease. Experimental evidence suggests that coffee and some of its major components (caffeine, cafestol, and kahweol) alter expression and activity of enzymes involved in xenobiotic metabolism and may protect the hepatocyte from toxic metabolites (Cavin et al. 2008). Other work suggests that caffeine may inhibit the transforming growth factor beta pathway through an effect on PPAR-gamma and thus inhibit fibrogenesis (Gressner et al. 2008). Experimental data also suggests that caffeine at non-toxic

concentrations may inhibit HCV replication in a dose-dependent manner in an in-vitro model of HCV replication (Batista et al. 2015).

Although the evidence of the protective effects of coffee/caffeine on liver disease is growing, no prospective trials have been conducted on the use of caffeine/coffee to improve liver disease. In addition, since the amount of caffeine varies considerable from cup to cup of coffee, the amount required for a beneficial effect is unknown. Until more data is forthcoming we cannot recommend that patients with chronic hepatitis C use caffeine/coffee excessively.

7 Summary

The preponderance of evidence supports the notion that chronic hepatitis C is a progressive disease but the rate of progression is highly variable and influenced by a large number of factors, some of which are modifiable and others that are not. Based on the current evidence, the majority of persons with chronic hepatitis C are unlikely to die from their chronic liver disease. However, about a quarter of subjects will be at risk for cirrhosis, end-stage liver disease and HCC. In the era of direct acting antivirals these individuals should receive treatment to prevent these dire consequences. The advances in therapy make it unethical to continue to conduct natural history studies so it is unlikely that new outcome data will be forthcoming. Information on the outcome of treated patients with cirrhosis will be still needed in order to manage and council these patients of their post-treatment outcome. As simpler, safer and more affordable therapy becomes available, all chronically infected patients should be treated and the natural history of chronic hepatitis C will be relegated to history.

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The Multifaceted Features of HCV Infection Beyond the Liver

Kazuhiko Koike

Abstract A major problem in patients with hepatitis C virus (HCV) infections is progression from acute to persistent infection, resulting in development of serious liver diseases including hepatocellular carcinoma. However, HCV infection is a multifaceted disease. Extrahepatic manifestations include cryoglobulinemia, glomerulonephritis, porphyria cutanea tarda, Sjögren's syndrome, and lymphoma, most of which are evoked by the virus or the interaction thereof with the host immune system. Recently, partly extrahepatic features of HCV infection, including disturbance of lipid metabolism and insulin resistance, have been described. Such metabolic disturbances provoked by HCV are now considered to be essential features of the pathogenesis of liver disease induced by HCV infection. Some of these diseases/syndromes are cured by antiviral treatment. In the present review, the systemic manifestations of HCV infection will be examined and their clinical relevance discussed.

Keywords Hepatitis C virus • Extrahepatic manifestation • Metabolic disease • Sjögren's syndrome • All-cause mortality • Hepatocellular carcinoma

1 Introduction

Worldwide, approximately 170 million people are persistently infected with hepatitis C virus (HCV), which induces a spectrum of chronic liver diseases, ranging from chronic hepatitis, through cirrhosis, and, eventually, to hepatocellular carcinoma (HCC) (Saito et al. 1990). HCV has received increasing attention because of widespread viral dissemination across broad communities, linked to very high incidences of HCC in those with persistent infections. Once liver cirrhosis is diagnosed in patients persistently infected with HCV, the annual risk of HCC is approximately 7 % (Ikeda et al. 1998), resulting in development of HCC in almost 90 % of HCV-associated cirrhotic patients by 15 years later. It was recognized

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Table 1 Extrahepatic manifestations of HCV infection

Extrahepatic manifestations	Clinical manifestations	Pathogenesis	Prevalence of HCV antibody	References
Cryoglobulinemia	Purpura, arthralgia, and renal impairments	Apoptosis suppression of B lymphocytes. Persistent stimulation of lymphocytes induces the B-cell clonal expansion leading to the antibodies production. Intrahepatic growth of CD5- and CD81-positive B lymphocytes has been observed, suggesting monoclonal IgM induction as a possible cause	80–90 %	Mayo (2002), Johnson et al. (1993), Agnello et al. (1992), Saadoun et al. (2006, 2007), Okuse et al. (2003), Misiani et al. (1994), Zuckerman et al. (2000), Cacoub et al. (2002, 2008), Ubara et al. (2000)
Renal impairment and glomerulonephritis	Membranoproliferative glomerulonephritis (MPGN), membranous nephropathy, mesangial proliferative glomerulonephritis, Henoch-Schönlein purpura nephritis, and tubulointerstitial nephritis	Accumulation of an immune complex formed by monoclonal or polyclonal IgM-κ with rheumatoid factor activity produced by HCV-infected B lymphocytes in the glomerular vascular endothelium and mesangium	10–60 %	Kasuno et al. (2003), Johnson et al. (1993, 1994), Arase et al. (1998), D'Amico and Fornasieri (1995), Sinico et al. (2000), Misiani et al. (1999), Dammacco et al. (1994), Saadoun et al. (2010)
Porphyria cutanea tarda	Solar photosensitivity and hepatic damage	A reduced activity of uroporphybrinogen decarboxylase associated with an excessive deposition of iron in the liver induced by HCV infection	60–100 %	Sarkany et al. (2001), Fargion et al. (1992), Okano et al. (1997)
Sjögren's syndrome	An aggregate of symptoms characterized by insufficient tear production by the lacrimal glands and insufficient saliva production by the salivary glands, causing dryness of the eyes and mouth	The involvement of host immunologic responses to HCV. HCV envelope proteins may induce cytokines	14–57 %	Fox (2005), Nagao et al. (2003), Haddad et al. (1992), Pawlotsky et al. (1995), Koike et al. (1997), Takamatsu et al. (1992), Arrieta et al. (2001)

Diabetes and insulin resistance	High plasma glucose and serum insulin levels. Improvement after achieving SVR	The involvement of insulin resistance and insulin secretory deficiency. Disruption of tyrosine phosphorylation of the insulin receptor substrate (IRS-1). The involvement of TNF-α which level is increased in HCV infection	50 %	Allison et al. (1994), Mehta et al. (2000), Petit et al. (2001), Zylberberg et al. (1999), Pradhan et al. (2002), Shintani et al. (2004), Aytug et al. (2003), Hui et al. (2003)
Lipid metabolism disturbances and steatosis	Hepatic steatosis and hypobetalipoproteinemia. Improvement after achieving SVR	Induction of SREBP-1c by HCV. Suppression of MTP activity leading to a reduction in VLDL secretion from the liver. Insulin resistance, which increses the release from the perioheral and uptake into the liver of fatty acids	50 %	Powell et al. (2005), Adinolfi et al. (2001), Castera et al. (2003), Moriya et al. (1997, 1998, 2001, 2003), Perlemuter et al. (2002), Moriishi et al. (2007), Tsutsumi et al. (2002)
Lichen planus	An inflammatory disease associated with abnormal chronic dermal and intraoral keratinization	The involvement of HCV-specific T cells	0–65 %	Scully et al. (1998), Bellamn et al. (1995), Nagao et al. (1995a, b, 1996, 1999, 2000), Arrieta et al. (2000), Doutre et al. (1992), Baccia et al. (1993), Protzer et al. (1993)
Oral cancer		Unknown	70–100 % (HCV-RNA)	Nagao et al. (1995a, b, 2000)
Malignant lymphoma	Non-Hodgkin lymphoma (NHL) and specific B-NHL subtypes (diffuse large B-cell lymphoma, marginal zone lymphoma, and lymphoplasmacytic lymphoma)	The involvement of myc gene mutation in some cryoglobulinemia patients. Enhanced mutations of immunoglobulin and protooncogenes	0–33 %	Machida et al. (2004), Mizuochi et al. (2011), Ito et al. (2009, 2010a, b), Gisbert et al. (2003), de Sanjose et al. (2008), Perl et al. (1989), Ferri et al. (1995), De Vita et al. (1995),

(continued)

Table 1 (continued)

Extrahepatic manifestations	Clinical manifestations	Pathogenesis	Prevalence of HCV antibody	References
				de Sanjose et al. (2008), Hermine et al. (2002), Vallisa et al. (2005)
Autoimmune thyroid disease	Thyroid dysfunction (hypothyroidism)	The involvement of liver/kidney microsomal antibody type 1 (LKM1)	10 %	Montella et al. (2003), Testa et al. (2006), Antonelli et al. (2004), Muratori et al. (2005)
Idiopathic interstitial pneumonitis	Dry cough, short of breath, progressive course to respiratory failure	The involvement of activated T lymphocytes and eosinophils. Further studies are necessary but not have been achieved	28 %	Ueda et al. (1992), Kubo et al. (1996), Irving et al. (1993), Karim et al. (2001)
Myocardial impairment	Dilated cardiomyopathy, hypertrophic cardiomyopathy, arrhythmogenic right ventricular dysplasia cardiomyopathy and chronic myocarditis	The involvement of host immunologic responses to HCV, particularly that of the human major histocompatibility (MHC) class II antigen. Further studies are necessary but not have been achieved	6–10 %	Matsumori (2000, 2006), Maisch et al. (2003)
Mooren's ulcer	Progressive ulcer associated with congestion and pain around the cornea	Further studies are necessary but not have been achieved	Unknown	Wilson et al. (1994), Moazami et al. (1995), Zegans et al. (1999), Jain et al. (2004)
Cognitive disorders and mental disorders	Depression, cognitive impairment and fatigue, and a reduction in the health-related quality of life	Unknown	28 % of chronic hepatitis C patients had depression. 33 % had cognitive impairment	Perry et al. (2008), Jacobson et al. (2010)
All-cause mortality in HCV-positives	Compared with anti-HCV seronegatives, anti-HCV seropositives had higher mortality from both hepatic and extrahepatic diseases			Lee et al. (2012), Mahajan et al. (2014)

relatively soon after the discovery of HCV that infection with the virus involves not only the liver but also other organs and tissues. Several extrahepatic manifestations, which are complicated disorders of organs other than the liver, occur in association with HCV infection (Gumber et al. 1995). In addition, disease manifestations that are extrahepatic in part, including disturbances in lipid and glucose metabolism, add to the multifaceted nature of HCV infection (Koike and Moriya 2005). Of the latter conditions, most are definitely associated with HCV infection in both clinical and basic terms, but the links between some others await verification via conduct of more case studies and/or pathogenetic analyses (Table 1).

2 Manifestations Associated with HCV Infection

2.1 Cryoglobulinemia

In many patients infected with HCV, cryoglobulinemia is subclinical, thus not symptomatic, but the incidence of the extrahepatic complication, essential mixed cryoglobulinemia (EMC), is highest in HCV-infected patients. The clinical symptoms of EMC include purpura, arthralgia, and renal impairments (Mayo 2002). The latter include membranoproliferative glomerulonephritis (MPGN), which occasionally progresses to renal insufficiency (Johnson et al. 1993). Approximately 80–90 % of EMC patients are infected with HCV (Agnello et al. 1992; Saadoun et al. 2006). Cryoglobulins were detected, using a highly sensitive gel diffusion method, in 70 % of patients chronically infected with HCV (Okuse et al. 2003).

Cryoglobulins are abnormal immunoglobulins that form white deposits at 4 °C but are liquids at 37 °C. Cryoglobulins are classified into three types, namely, monoclonal cryoglobulins (type I), polyclonal cryoglobulins (type III), and mixed cryoglobulins (type II). The cryoglobulinemia associated with HCV infection is mainly of the mixed type. Specifically, such patients produce monoclonal IgM and polyclonal IgG antibodies exhibiting rheumatoid factor activity (Brouet et al. 1974; Wong et al. 1996).

The pathogenesis of cryoglobulinemia in HCV-infected patients remains poorly elucidated. However, persistent stimulation of lymphocytes induces B-cell clonal expansion, leading to the production of antibodies including rheumatoid factor, which become incorporated into cryoglobulins (Saadoun et al. 2007). Intrahepatic growth of CD5- and CD81-positive B lymphocytes has been observed, suggesting that induction of monoclonal IgM synthesis may be the cause of the disease (Curry et al. 2003). Tissue damage in patients with EMC-induced vasculitis would thus be T-cell mediated.

Antiviral agents including interferon (IFN) have been used to treat HCV-associated cryoglobulinemia (Lunel et al. 1994). Cryoglobulinemia symptoms improved in 15 of 25 patients with HCV-associated disease after IFN treatment commenced, but the symptoms recurred when treatment concluded (Misiani

et al. 1994). A combination of (PEG)-IFN and ribavirin has also been used to treat HCV-associated cryoglobulinemia. Administration of both drugs to nine EMC patients who had not responded to IFN monotherapy alleviated cryoglobulinemia in all nine, and improved clinical symptoms in seven of the nine (Zuckerman et al. 2000). Recently developed direct-acting antivirals (DAAs) for HCV are also effective, as these agents exhibit strong antiviral activities (Saadoun et al. 2014).

In addition, in patients with severe cryoglobulinemia, in whom antiviral agents may not adequately improve symptoms, combination therapy with IFN and steroids or immunosuppressants has been considered effective (Cacoub et al. 2002). Other treatment strategies, including plasma exchange therapy and splenectomy (Ubara et al. 2000), have also been attempted. Recently, rituximab was reported to improve vasculitis, including skin lesions, arthralgia, neuropathy, and glomerulonephritis (Cacoub et al. 2008).

2.2 Renal Impairments and Glomerulonephritis

Renal impairments associated with HCV infection include membranoproliferative glomerulonephritis (MPGN), membranous nephropathy, mesangial proliferative glomerulonephritis, Henoch-Schönlein purpura nephritis, and tubulointerstitial nephritis (Kasuno et al. 2003).

MPGN, in particular, is considered to be a typical example of the renal impairment associated with hepatic disease caused by HCV, and is also termed HCV-associated nephritis. In 1993, eight patients with HCV infections complicated by MPGN were reported, for the first time (Johnson et al. 1993). The incidence of HCV-associated nephritis has not yet been determined. In an autopsy study on 188 cases of chronic hepatitis C, 11.2 % exhibited MPGN, 2.7 % membranous nephropathy, and 17.6 % mesangial proliferative glomerulonephritis (Arase et al. 1998). The pathogenesis underlying HCV-associated nephritis is considered to be accumulation of an immune complex, formed by monoclonal or polyclonal IgM-κ, exhibiting rheumatoid factor activity. The antibody is produced by HCV-infected peripheral B lymphocytes, and is deposited in the glomerular vascular endothelium and mesangium (D'Amico and Fornasieri 1995).

The histopathological features of HCV-associated nephritis are similar to those of general MPGN of type I, but the former disease is occasionally associated with cryoglobulin deposition (Sinico et al. 2000). If EMC and nephrotic syndrome patients produce rheumatoid factor, HCV-associated nephritis should be suspected, and the presence/absence of HCV infection determined.

Antiviral therapies, including IFN, are effective when used to treat HCV-associated nephritis (Johnson et al. 1994). Administration of IFN to 14 patients with HCV-associated nephritis improved proteinuria, but relapse occurred after the end of therapy, in association with a relapse of HCV infection. IFN/ribavirin combination therapy, which is associated with a low relapse rate, has also been tested (Misiani et al. 1999). A combination of a steroid and

cyclophosphamide has been used as immunosuppressive therapy, but use of an immunosuppressant alone has not yet afforded good results (Dammacco et al. 1994). As patients with HCV-associated nephritis have been reported to have poor prognoses, the recent introduction of DAAs, as HCV treatments, may allow development of therapeutic procedures effective to treat the renal impairment associated with HCV infection. Also, addition of rituximab may be valuable in treatment of patients with HCV-related EMC exhibiting renal involvement (Saadoun et al. 2010).

2.3 Porphyria Cutanea Tarda

Porphyria cutanea tarda (PCT) is an acquired condition; patients exhibit photosensitivity to the sun, and hepatic damage caused by reduced activity of liver uroporphyrinogen decarboxylase (Sarkany 2001). The contributions of all of alcohol, excess iron, and medications to hepatic impairment were previously examined in the context of PCT development. However, as HCV infection is present in 60–100 % of patients with PCT, a linkage between such infection and the pathogenesis of PCT is suspected. The mechanism has not yet been clarified. It is assumed, however, that PCT is caused by a reduction in uroporphyrinogen decarboxylase activity associated with excessive deposition of iron in the liver, in turn triggered by HCV infection (Fargion et al. 1992).

IFN has been shown to be effective for treatment of PCT, in combination with avoidance of exposure to the sun, abstention from alcohol, and phlebotomy. IFN therapy normalized serum ALT levels, caused serum HCV-RNA to disappear, normalized porphyrin and ferritin levels, and improved clinical symptoms—including vesicle formation and hypertrichosis—in PCT patients infected with HCV (Okano et al. 1997).

2.4 Sjögren's Syndrome

Sjögren's syndrome is an aggregate of symptoms characterized by insufficient tear production by the lacrimal glands and insufficient saliva production by the salivary glands, caused by infiltration of exocrine lymphocytes, which in turn causes dryness of the eyes and mouth (Fox 2005).

An association of Sjögren's syndrome with certain viral infections has long been known, and reports indicating that 0–45 % of patients were positive for anti-HCV antibodies suggest that Sjögren's syndrome and HCV infection may be associated (Nagao et al. 2003). Differences in anti-HCV positivity rates may be attributable to regional differences in HCV infection rates. Also, lymphocytic sialadenitis, which resembles Sjögren's syndrome, was found in 16 of 28 patients (57 %) with HCV infection, but only 1 of 20 controls (5 %) (Haddad et al. 1992). Pawlotsky

et al. reported a 14 % prevalence of pathological abnormalities resembling Sjögren's syndrome in patients with HCV infections, compared to 0 % in controls without HCV infection. Of interest, the cited authors found that almost 50 % of patients with HCV infection had lymphocytic capillaritis of the salivary glands, which is found in all patients with sialadenitis. Lymphocytic capillaritis could therefore represent an early stage in the development of more severe lesions resembling the lymphocytic sialadenitis of Sjögren's syndrome (Pawlotsky et al. 1995).

Koike et al. found a direct association between HCV infection and sialadenitis in work with transgenic mice carrying genotype 1b HCV envelope genes, in which sialadenitis resembling Sjögren's syndrome develops spontaneously. In particular, it was noteworthy that lymphocytic capillaritis preceded sialadenitis in such animals (Koike et al. 1997), which may reflect the pathological sequence of development of Sjögren-like syndrome in humans chronically infected with HCV (Pawlotsky et al. 1995). In a clinical study, HCV-RNA was detected in salivary gland tissues from anti-HCV-positive patients with Sjögren's syndrome, via reverse transcriptase (RT)-PCR analysis (Takamatsu et al. 1992). Upon in situ hybridization of salivary gland tissue samples from 8 anti-HCV-positive and 11 anti-HCV-negative patients with chronic sialadenitis or Sjögren's syndrome, HCV-RNA was detected in all salivary gland samples from the anti-HCV-positive patients. Moreover, HCV-infected salivary gland epithelium supported viral multiplication (Arrieta et al. 2001). Such lines of evidence indicate that HCV plays some role in the development of the sialadenitis of Sjögren's syndrome, but it remains unclear whether HCV per se or an immunological response to HCV infection induces the condition.

No reports on the efficacy of IFN therapy for HCV-associated sialadenitis have yet appeared (Lunel and Cacoub 2000). Artificial lacrimal fluid and artificial saliva are used to alleviate dryness, and a non-steroidal anti-inflammatory drug or corticosteroid is administered to treat the fever and articular symptoms (Fox 2005).

2.5 Diabetes and Insulin Resistance

Allison et al. reported an epidemiological link between diabetes and HCV infection, but only in a cirrhotic cohort (Allison et al. 1994). This initial report made little impact, however, in view of the fact that glucose tolerance is well known to be impaired in patients with advanced chronic liver disease. Several other reports followed, both from the same group and others. The trend toward acceptance of a positive association between diabetes and HCV infection seems to have been triggered by a population-based study performed in the United States (Mehta et al. 2000), which found a solid association between the two conditions. In addition, it has been shown that comorbid diabetes is a risk factor for HCC (Hassan et al. 2002) in cirrhotic patients (Bianchi et al. 1994). These reports support the existence of an association between HCV infection and type 2 diabetes.

Petit et al. reported that insulin resistance was increased in chronic hepatitis C patients with even slight hepatic impairment, and that the index of impairment (HOMA-IR) correlated with the severity of liver fibrosis (Petit et al. 2001). Tumor necrosis factor (TNF)-alpha, the levels of which correlate closely with the extents of hepatic inflammation and fibrosis in chronic hepatitis C patients (Zylberberg et al. 1999), is considered to enhance glucose uptake in peripheral tissue and to promote gluconeogenesis in the liver, leading to induction of insulin resistance (Pradhan and Ridker 2002). However, any association between diabetes and HCV infection is difficult to establish, because cirrhosis, obesity, and old age are common in HCV patients. Hence, experimental systems have been employed. Shintani et al. used transgenic mice carrying the genotype 1b HCV core gene (Moriya et al. 1998), and found that (1) the HCV core protein induced insulin resistance in vivo; (2) tyrosine phosphorylation of the insulin receptor substrate (IRS)-1 in the insulin signal transduction pathway was disrupted; and, (3) the insulin resistance that developed was hepatic in nature, as shown by hyperinsulinemic euglycemic clamp analysis. The mice had high TNF-alpha liver levels, and administration of anti-TNF-alpha antibody improved insulin resistance. These results indicate that a direct link exists between HCV infection and type 2diabetes (Shintani et al. 2004).

At the time of publication of the above findings, Aytug et al. reported on insulin signaling in biopsied liver specimens from patients with chronic hepatitis C (Aytug et al. 2003). Specifically, the cited authors measured changes in IRS-1, IRS-2, and phosphatidylinositol (PI)3-kinase levels. Upon insulin stimulation of the biopsy samples, the levels of insulin-receptor proteins and IRS-1 increased, whereas phosphorylation of the tyrosines in IRS-1 decreased the levels thereof to 50 % of baseline values, and also reduced the activity of the P13-kinase associated with IRS-1. These data were in agreement with those of Shintani et al., who explored the mechanism of insulin resistance in mice. Both studies found that impaired tyrosine phosphorylation of IRS-1 induced insulin resistance in animals infected by HCV. It is, however, surprising, in a sense, that the mechanism of insulin resistance induced by HCV infection is identical in both clinical samples and experimental animals, because, earlier, hepatic IRS-2 was thought to be more important than IRS-1 in terms of development of insulin resistance (Suzuki et al. 2004).

Insulin resistance in patients with HCV infections may have an additional significant clinical implication. In 260 patients with chronic HCV infections, Hui et al. sought to establish a relationship between liver histology, and indicators of glucose metabolism and insulin resistance, using the homeostasis model assessment of such resistance (HOMA-IR). It was found that insulin resistance was already evident in patients with stage 0 or 1 liver fibrosis, indicating that such resistance in HCV-infected patients was not attributable to advanced liver disease (Hui et al. 2003). HOMA-IR was a significant and independent predictor of hepatic fibrosis stage, and the speed of development thereof. Others have reported similar results (Hickman et al. 2003). These data are important because they implicate a role for hyperinsulinemia, and (by inference) insulin resistance in promoting progression of hepatic fibrosis. Insulin has been shown to aggravate not only

atherosclerosis, but also systemic inflammation and fibrosis. The liver would not be an exception in this context.

2.6 Lipid Metabolism Disturbances and Steatosis

Hepatic steatosis is frequently observed in chronically HCV-infected patients (Bach et al. 1992; Lefkowitch et al. 1993; Czaja et al. 1998). The liver fatty acid composition in core gene transgenic mice (Moriya et al. 1997, 1998) differs from that in fatty livers caused simply by obesity. The levels of carbon 18 monounsaturated fatty acids (C18:1), such as oleic or vaccenic acid, which encourage proliferation of cancer cells (Kudo et al. 2011), were significantly increased in transgenic mice. This was also true when liver tissues from hepatitis C patients, and obese patients with simple "fatty livers", were compared (Moriya et al. 2001). Patients with HCV infections also exhibit dyslipidemia as part of the phenotype of hypo-beta-lipoproteinemia (Moriya et al. 2003). Chronic hepatitis C patients, in whom the liver fibrosis stages were comparable to those of control patients with chronic hepatitis B, showed reduced serum levels of total cholesterol and apolipoproteins B1, C2, and C3, whereas the levels of apolipoproteins A1, A2, and E were similar to those of controls.

The mechanism of steatogenesis in hepatitis C patients has been investigated using (principally) the mouse model described above. At least three pathways are involved in development of steatosis. One is the insulin resistance pathway that frequently develops in hepatitis C patients, as well as in core gene transgenic mice, as described above (Shintani et al. 2004). Insulin resistance increases the peripheral release and hepatic uptake of fatty acids, resulting in lipid accumulation in the liver. The second pathway involves suppression of the activity of microsomal triglyceride transfer protein (MTP) by HCV core protein (Perlemuter et al. 2002). This, in turn, reduces the secretion of very-low-density-lipoprotein (VLDL) from the liver, increasing liver triglyceride levels. The last pathway involves the sterol regulatory element-binding protein (SREBP)-1c, which regulates the production levels of triglycerides and phospholipids. In HCV core gene transgenic mice, SREBP-1c is upregulated, but this is not true of either SREBP-2 or SREBP-1a (Moriishi et al. 2007). Such results have been obtained in both in vitro studies (Kim et al. 2007; Waris et al. 2007) and work with chimpanzees (Su et al. 2002). Thus, all three pathways result in development of hepatic steatosis in hepatitis C patients (Fig. 1). Steatosis exacerbates the production of reactive oxygen species and accelerates progression of liver disease.

Recently, it has been reported that assembly and budding of HCV occur around lipid droplets accumulated within the endoplasmic reticulum (Miyanari et al. 2007). Furthermore, increases in saturated and monounsaturated fatty acid levels enhanced HCV-RNA replication. Such data suggest that regulation of lipid metabolism by the core protein plays a crucial role in the HCV life cycle. An inflammatory cytokine, TNF-alpha, is known to impair the insulin-signaling pathway via inhibition of

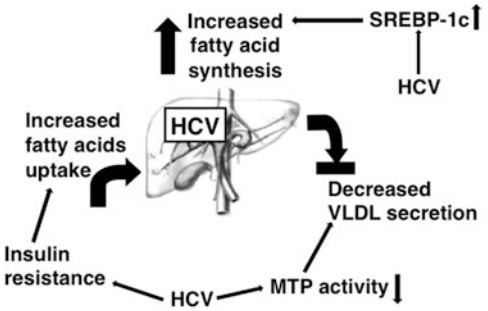

Fig. 1 HCV induces liver steatosis by affecting three lipid metabolism pathways. First, the HCV core protein induces insulin resistance, increasing the peripheral release and hepatic uptake of fatty acids. Second, the HCV core protein suppresses MTP activity, inhibiting VLDL secretion from the liver, thus increasing triglyceride levels in that organ. Lastly, a transcription factor, SREBP-1c, is upregulated by the HCV core protein, resulting in increased production of triglycerides. Thus, all three pathways are involved in the development of hepatic steatosis in hepatitis C patients. *MTP* microsomal triglyceride-transfer protein, *VLDL* very-low-density lipoprotein, *SREBP* sterol regulatory element-binding protein

tyrosine phosphorylation of IRS. Indeed, overproduction of TNF-alpha (Tsutsumi et al. 2002) has been reported to reduce phosphorylation of both IRS-1 and Akt in core gene-transgenic mice. Moreover, the hyperinsulinemia that developed was cured by depletion of TNF-alpha, suggesting that upregulation of TNF-alpha level contributes to core protein-induced insulin resistance (Shintani et al. 2004).

Hepatic steatosis is significantly associated with accelerated progression of liver fibrosis (Powell et al. 2005). A study on 180 hepatitis C patients for whom the dates of infection were known confirmed that steatosis of grades 3–4 was significantly associated with a higher annual rate of fibrosis progression, and that alcohol and steatosis acted together to increase fibrosis (Adinolfi et al. 2001). In another study on 96 untreated patients with CHC, who underwent serial liver biopsies, fibrosis progression was strongly associated with worsening of the steatosis, which was the only significant factor so associated upon multivariate analysis. (Castera et al. 2003).

2.7 Lichen Planus

Lichen planus is an inflammatory disease associated with chronic abnormal dermal and intraoral keratinization of unknown etiology. The assumed causes include viral or bacterial infection, immunological responses, circulatory disorders, allergy, mental stress, abnormal autonomic function, medication, and disorders of glucose metabolism (Scully et al. 1998).

A number of reports on relationships between lichen planus and HCV infection have appeared, but the anti-HCV antibody positivity rate in lichen planus patients

exhibited marked regional differences, ranging from 0 to 65 % (Bellamn et al. 1995; Nagao et al. 1995a). HCV reproduction in the skin and oral mucosal epithelium has been examined by in situ hybridization and RT-PCR (Nagao et al. 2000; Arrieta et al. 2000). HCV-specific T cells have been reported to be linked to the pathogenesis of the condition (Pilli et al. 2002), but none of HCV level, genotype, or pathological severity, was so associated (Nagao et al. 1996). Antiviral therapy (IFN) has been reported to be effective (Doutre et al. 1992). However, other investigators have reported that IFN rather induces or aggravates lichen planus (Baccia et al. 1993; Protzer et al. 1993). Thus, the effectiveness of IFN used to treat lichen planus remains debatable. Nagao et al. found no macroscopic changes in lesions 1 year after the end of IFN administration (to treat intraoral lichen planus lesions) in patients with chronic HCV infection, but macroscopic and histological improvements were evident 3 or more years after IFN administration ceased. It was assumed that not HCV infection, but rather the immunological response to such infection, was associated with development of oral lichen planus, as positive-strand HCVRNA was detected in the oral mucous membrane of some lichen planus patients although they had (histologically) recovered after IFN therapy (Nagao et al. 1999).

2.8 Oral Cancer

A relationship between HCV infection and oral cancer was first reported by Nagao et al., who found that the HCV infection rate was higher in oral cancer patients than in those with esophageal, gastric, or colorectal cancer (Nagao et al. 1995b). When HCV-RNA levels were examined in oral cancer tissues from 17 patients, via RT-PCR, positive-strand HCV RNA was detectable in all anti-HCV-positive patients, and negative-strand HCV-RNA in 71.4 % of such patients (Nagao et al. 2000). These findings indicate that HCV may possibly replicate in oral cancer tissues. No definite consensus has yet emerged on any relationship between oral lichen planus and oral cancer. However, as lichen planus is considered to be precancerous, oral examination may be necessary in patients with chronic HCV infections.

2.9 Malignant Lymphoma

HCV replicates in lymphocytes, although not robustly (Ito et al. 2010a), and short-term HCV culture systems using lymphocytes have been developed (Ito et al. 2001; Shimizu et al. 1992). Infected lymphocytes may undergo malignant transformation, leading to development of malignant lymphoma, after acquisition of certain genetic mutations (Machida et al. 2004; Mizuochi et al. 2011; Ito et al. 2009, 2010b). HCV infection is considered to be associated with development of malignant lymphoma,

particularly the pathogenesis of non-Hodgkin's B-cell lymphoma (Gisbert et al. 2003; de Sanjose et al. 2008). Some cryoglobulinemia patients have been assumed to develop non-Hodgkin's B-cell lymphoma in association with *myc* gene mutation (Perl et al. 1989). The anti-HCV positivity rate in patients with non-Hodgkin's B-cell lymphoma ranges from 0 to 33 % (Gisbert et al. 2003). Such variation may be associated with regional differences in HCV infection rates. Fourteen of 500 patients with chronic HCV infections had disease complicated with non-Hodgkin's B-cell lymphoma, and HCV-RNA was found in peripheral blood lymphocytes of all patients (Ferri et al. 1995). Positive- and negative-strand HCV-RNAs were detected in parotid glands of patients with parotid non-Hodgkin's B-cell lymphoma associated with HCV infection, and the presence of HCV in the parotid gland was confirmed by in situ hybridization (De Vita et al. 1995). Data from the International Lymphoma Epidemiology Consortium confirmed an association between HCV infection, and NHL and specific B-NHL subtypes (diffuse large B-cell lymphoma, marginal zone lymphoma, and lymphoplasmacytic lymphoma) (de Sanjose et al. 2008).

The treatment of HCV-associated malignant lymphoma is similar to that for non-HCV-associated non-Hodgkin B-cell lymphoma. However, recently, IFN monotherapy, or IFN and ribavirin combination therapy, has been reported to be effective. IFN was effective when used to treat certain specific lymphomas, including splenic lymphoma associated with production of villous lymphocytes (Hermine et al. 2002). Administration of pegylated IFN and ribavirin to 13 patients with HCV-associated non-Hodgkin's B-cell lymphoma afforded complete responses in 7 (Vallisa et al. 2005).

2.10 Autoimmune Thyroid Disease

The relationship between thyroid disease and HCV infection has been analyzed in a number of studies (Montella et al. 2003; Testa et al. 2006), and a causal linkage has been suggested. The incidence of thyroid dysfunction was assessed in 630 chronic HCV patients, without cirrhosis or HCC, who had not been treated with IFN. These patients had higher levels of thyroid-stimulating hormone, and lower levels of free thyroxine and triiodothyronine, than controls. In addition, the patients exhibited hypothyroidism and tended to have anti-thyroglobulin and anti-thyroid peroxidase antibodies. These findings suggest that a relationship exists between HCV infection and thyroid disorder (Antonelli et al. 2004). A possible relationship between such infection and thyroid cancer has attracted recent attention. The mechanism underlying the pathogenesis of thyroid disease associated with HCV infection has not yet been elucidated, but involvement of liver/kidney microsomal antibody type 1 has been suggested (Muratori et al. 2005). In general, patients with thyroid disorders caused by HCV infection are asymptomatic, and require no special treatment. Thyroid disorders are also known to develop as adverse reactions to IFN-alpha therapy for chronic HCV infection (Prummel and Laurburg 2003). Thyroid

dysfunction caused by administration of IFN-alpha is generally transient, resolving spontaneously after completion of treatment. Thus, discontinuation of IFN-alpha is not required in most cases.

2.11 Idiopathic Interstitial Pneumonitis

Viral infection has been suggested to be a cause of idiopathic interstitial pneumonitis. In terms of HCV infection, the anti-HCV positivity rate was 28.8 % in 66 patients with idiopathic interstitial pneumonitis (IIP), thus significantly higher than that in 9464 normal controls (Ueda et al. 1992). Any role for HCV infection, however, in the pathogenesis of idiopathic interstitial pneumonitis, remains unclear. Activation of T lymphocytes and eosinophils was suggested to be a feature of IIP pathogenesis associated with HCV infection, because increases in the counts of activated T-lymphocytes and eosinophils were noted in bronchoalveolar fluids from 13 chronic hepatitis C patients, although the total cell counts were identical to those of normal subjects (Kubo et al. 1996). Some studies, however, suggest that no relationship exists between HCV infection and IIP (Irving et al. 1993); further work is necessary. IIP has also been reported as an adverse reaction to IFN therapy in chronic HCV patients, who often exhibit high pretreatment KL-6 levels, suggesting that IIP might potentially develop. Recovery from IFN therapy-induced IIP is achieved by discontinuation of therapy, but steroid administration is required in some cases (Karim et al. 2001).

2.12 Myocardial Impairments

Causal relationships between HCV infection and certain myocardial impairments have been suspected; the impairments include dilated cardiomyopathy, hypertrophic cardiomyopathy, arrhythmogenic right ventricular dysplasia cardiomyopathy, and chronic myocarditis (Matsumori et al. 2000; Matsumori 2006). The rate of serum anti-HCV positivity was 6.3 % (42/663) in patients with hypertrophic cardiomyopathy and 10.6 % (74/697) in those with dilated cardiomyopathy, thus higher than the rate (2.4 %) observed among age-matched Japanese blood donors (Matsumori et al. 2000). Positive- and negative-strand HCV-RNAs were detected in cardiac muscle samples of these patients, indicating potential intramyocardial HCV multiplication (Matsumori 2006). HCV-RNA has also been detected in cardiac muscle samples from patients with arrhythmogenic right ventricular dysplasia cardiomyopathy and chronic myocarditis, suggesting that HCV plays a pivotal role in the genesis of myocardial impairments (Maisch et al. 2003).

In terms of the cause of myocardial impairments associated with HCV, the involvement of host immunological responses to HCV, particularly that of the human major histocompatibility (MHC) class II antigen, has been suggested

(Matsumori 2006). The suggested relationships between myocardial disorders and HCV infection require further investigation.

2.13 Mooren's Ulcer

Mooren's ulcer is a progressive ulcer associated with congestion and pain around the cornea. HCV infection has been suggested to contribute to development of this disease (Wilson et al. 1994; Moazami et al. 1995). IFN has been reported to be effective for treating HCV-associated Mooren's ulcers, but ocular pain became exacerbated following IFN discontinuation; hence, caution is required when treating such patients (Wilson et al. 1994). Systemic corticosteroid administration has also been reported to be effective. However, some investigators are of the view that no association exists between HCV infection and Mooren's ulcer (Zegans et al. 1999; Jain et al. 2004). Further detailed studies would clarify this issue.

2.14 Cognitive and Mental Disorders

Certain mental, psychiatric, and cognitive disorders have been associated with HCV infection in a number of studies. Those include depression, cognitive impairment, and fatigue; all reduce health-related quality of life. For further information, please refer to the comprehensive reviews that have appeared on this issue (Perry et al. 2008; Jacobson et al. 2010).

2.15 All-Cause Mortality in HCV-Seropositives

Recently, several studies on all-cause death levels in HCV patients have been performed. One was a community-based long-term prospective study in Taiwan. In this work, 23,820 adults aged 30–65 years were enrolled during 1991–1992, and vital status was ascertained via computerized linkage to national death certificate profiles in the years 1991–2008. Of all subjects, 1095 were anti-HCV positive and 69.4 % had detectable serum HCV-RNA levels. Compared to anti-HCV seronegative subjects, the seropositive subjects exhibited higher mortalities from both hepatic and extrahepatic diseases. The multivariate-adjusted hazard ratios (with 95 % confidence intervals) were 1.89 (1.66–2.15) for all causes of death; 12.48 for hepatic diseases, 1.35 for extrahepatic diseases; 1.50 for circulatory diseases; 2.77 for nephritis, nephrotic syndrome, and nephrosis; 4.08 for esophageal cancer; 4.19 for prostate cancer; and 8.22 for thyroid cancer. Thus, anti-HCV seropositives with detectable HCV-RNA exhibited significantly higher mortality from not only

hepatic, but also extrahepatic diseases, than did anti-HCV seronegatives (Lee et al. 2012).

Similarly, Mahajan et al. evaluated mortality among patients with HCV infection, who were in care, in the Chronic Hepatitis Cohort Study (CHeCS) conducted from 2006 to 2010 in the United States. Mortality was compared with national Multiple Cause of Death (MCOD) data from 12 million US death certificates issued in 2006–2010. It was found that the CHeCS cohort exhibited higher mortalities from both hepatic and extrahepatic diseases: non-alcoholic liver-related death levels were 24.4-fold higher in the CHeCS cohort; deaths from liver cancer 8.7-fold higher; deaths from cancers other than liver cancer 1.28-fold higher; deaths from circulatory disease 1.42-fold higher; deaths from diabetes 1.77-fold higher; and deaths from genitourinary conditions 3.75-fold higher (Mahajan et al. 2014). Thus, HCV-positive patients exhibit higher mortality not only from liver diseases but also from extrahepatic diseases, confirming that extrahepatic manifestations of HCV infection, such as EMC, diabetes, or disorders in lipid metabolism (as described above) increase the mortality rates of such patients.

3 Conclusion

HCV infection is a multifaceted disease exhibiting both hepatic and extrahepatic manifestations caused by viral replication. Although the risk of death from extrahepatic conditions is lower than that associated with hepatic disease, it is important to be aware of the extrahepatic manifestations and diseases associated with HCV infection so that appropriate antiviral or immunomodulating treatments are administered, in a timely manner, to cure patients thus affected.

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Hepatic Fibrosis in Hepatitis C

Melissa A. Sheiko and Hugo R. Rosen

Abstract Infection with the Hepatitis C virus (HCV) leads to liver inflammation and fibrosis, which progresses to cirrhosis. Cirrhosis leads to complications including hepatocellular carcinoma (HCC), end-stage liver disease, the necessity for liver transplantation, or death. In fact, HCV is the leading cause of liver transplantation, accounting for more than 40 % of liver transplants in the United States (Organ Procurement and Transplantation Network 2010). Because of the extended interval between infection and the emergence of complications, the proportion of HCV-infected patients with cirrhosis is expected to reach 45 % by 2030 (Davis et al. 2010). Fibrosis stage predicts morbidity, including liver-related deaths (Everhart et al. 2010). Patients with cirrhosis from HCV infection have an increased risk of developing HCC, estimated at 1–3 % per year (Fattovich et al. 1997), and the risk increases when comparing patients with cirrhosis relative to those with bridging fibrosis (Lok et al. 2009). With an estimated 180 million people infected by HCV worldwide, fibrosis and its progression to cirrhosis represent a major global problem (Rosen HR 2011).

Keywords Fibrosis • Stellate cells • Innate immunity • SVR (sustained virologic response)

1 Introduction

Infection with the Hepatitis C virus (HCV) leads to liver inflammation and fibrosis, which progresses to cirrhosis. Cirrhosis leads to complications including hepatocellular carcinoma (HCC), end-stage liver disease, the necessity for liver

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transplantation, or death. In fact, HCV is the leading cause of liver transplantation, accounting for more than 40 % of liver transplants in the United States (Organ Procurement and Transplantation Network 2010). Because of the extended interval between infection and the emergence of complications, the proportion of HCV-infected patients with cirrhosis is expected to reach 45 % by 2030 (Davis et al. 2010). Fibrosis stage predicts morbidity, including liver-related deaths (Everhart et al. 2010). Patients with cirrhosis from HCV infection have an increased risk of developing HCC, estimated at 1–3 % per year (Fattovich et al. 1997), and the risk increases when comparing patients with cirrhosis relative to those with bridging fibrosis (Lok et al. 2009). With an estimated 180 million people infected by HCV worldwide, fibrosis and its progression to cirrhosis represent a major global problem (Rosen HR 2011).

The rate of fibrosis in HCV varies greatly by patient characteristics (Table 1). Duration of infection is associated with increased rates of cirrhosis; the cumulative rates of cirrhosis are estimated to be 16 % at 20 years after infection and 41 % at 30 years (Thein et al. 2008). In a large French review of 2235 patients, mean duration to development of cirrhosis was 30 years (Poynard et al. 1997). Older age at infection also leads to increased rates of cirrhosis, and older individuals have faster rates of fibrosis progression (Tong et al. 1995; Poynard et al. 1997, 2001). Females have slower fibrosis progression than men (Freeman et al. 2001). An Irish cohort study followed 376 women who developed HCV from contaminated anti-D immunoglobulin, and after 17 years, only 1.9 % had developed cirrhosis (Kenny-Walsh 1999). Another study looked at 184 women with hepatitis C for 27 years, and only 2.1 % had cirrhosis (Levine et al. 2006). Other concurrent insults such as alcohol consumption of more than 50 g/day are also associated with progressive fibrosis (Freeman et al. 2001). Smokers have higher fibrosis rates (Dev et al. 2006). Moreover, co-infection with human immunodeficiency virus (HIV) or hepatitis B have also emerged as a risk factors for fibrosis (Macias et al. 2009). Co-infected patients with HIV and HCV with cirrhosis have increased mortality from liver-related complications as compared to HCV-monoinfected patients (Lopez-Dieguez et al. 2011). However, fibrosis rates do not vary by genotype or viral load. Some, but not all studies, have suggested that post-transfusion HCV infection is associated with a greater risk of cirrhotic decompensation (Gordon et al. 1998).

2 Pathogenesis of Fibrosis

Fibrosis is a complex process involving many pathways and cell types (macrophages, natural killer cells, hepatic stellate cells) (Pellicoro et al. 2014). Many pro-fibrogenic pathways are conserved across tissues (liver, lungs, heart) and represent protective responses to tissue injury (Pellicoro et al. 2014). HCV leads to hepatocyte injury and the initiation of inflammatory cascades that ultimately result in excessive extracellular matrix (ECM) deposition into the space of Disse. Mediators generated by both cellular damage and recruited immune cells [including

Table 1 Fibrosis progression rates by different risk factors

Risk factor	Median fibrosis progression rate per year	Number of patients	95 % CI
Overall	0.133	1,157	0.125–0.143
Females	0.111	517	0.100–0.125
Males	0.154	639	0.143–0.167
Age >50 at infection	0.333	136	0.272–0.375
Age <20 at infection	0.091	268	0.083–0.100
No daily alcohol	0.125	598	0.111–0.143
Alcohol >50 g/day	0.167	111	0.133–0.174

From Poynard et al. (1997)

platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and interleukin-13] activate mesenchymal precursors to trans-differentiate into myofibroblasts (Pellicoro et al. 2014). HSC can also become activated after phagocytosis of apoptotic bodies from hepatocytes infected by HCV (Jiang et al. 2008; Zhan et al. 2006). This process is initially reversible, particularly if the inciting insult is stopped. However, with the deposition of more scar tissue and replacement of the normal hepatic parenchyma by ECM, the patient progresses to cirrhosis, characterized by nodules of regenerative parenchyma with surrounding scar tissue. The ECM of the liver sinusoids normally contains mostly collagen IV and VI, but in fibrosis, these are replaced by collagen I, collagen III, and fibronectin (Hernandez-Gea and Friedman 2011). The collagen content in the liver can be upregulated tenfold in the setting of cirrhosis (Schuppan et al. 2001). In HCV, collagen I and IV increase the most with advancement of fibrosis (Chen et al. 2014). This change leads to impeded flow of plasma to the hepatocytes. In normal liver, the deposition of collagen is balanced by its removal by proteolytic enzymes; the most important of which are the metalloproteinases (MMPs). With fibrosis, the deposition of collagen and ECM overwhelms the removal by MMPs, leading to uncontrolled ECM increases. Tissue inhibitors of metalloproteinases (TIMPs) are inhibitors that bind MMPs and prevent degredation of the ECM, and TIMP-1 decreases apoptosis of HSC (Murphy et al. 2002). Polymorphisms of TIMP-1 and TIMP-2 correlate with faster fibrosis progression in HCV patients (Ikebuchi et al. 2013). ECM contains many other proteins, discussion of which is beyond the scope of this chapter, but a few specific components deserve mention. Decorin and biglycan are a part of the ECM and have the ability to bind TGF- β. Integrins, proteoglycans, laminin, and matricellular proteins are other important components of the ECM (Choi and Diehl 2009; Kalluri and Neilson 2003).

The cells most implicated in the deposition of ECM are hepatic stellate cells (HSC) that reside in the subendothelial space of Disse and represent about 15 % of the total resident cells in the liver (Berardis et al. 2014) (Fig. 1). In their quiescent state, HSC have important roles in vitamin A storage, ECM remodeling, contraction

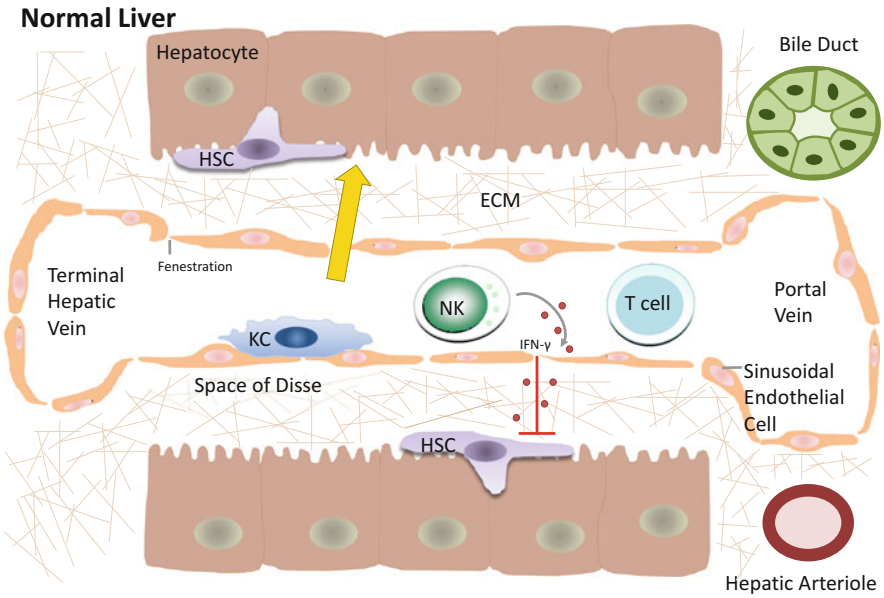

Fig. 1 Depiction of the anatomy of a normal liver. There is unrestricted flow of plasma to the hepatocytes. Interferon-gamma from Natural Killer (*NK*) cells inhibits hepatic stellate cells (*HSC*) (Partially adapted from Hernandez-Gea and Friedman 2011)

of the sinusoids, and interactions with hepatocytes via cytokines and chemokines (Sato et al. 2003). HSC become activated in the presence of pro-inflammatory cytokines or other cell signals (Fig. 2) and transform into myofibroblasts, the latter characterized by loss of lipid droplets and enhanced release of profibrogenic factors such as TGF-β (Pellicoro et al. 2014). The myofibroblasts increase the expression of cell adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) and alpha-smooth muscle actin (α-SMA) and increase the secretion of pro-inflammatory cytokines (Friedman 2008). Polymorphisms of ICAM-1 in HCV genotype four patients are associated with different rates of fibrosis (Rizk and Derbala 2013). Portal fibroblasts, bone marrow-derived cells, and circulating fibrocytes also play a role in the deposition of ECM but to a lesser extent (Hernandez-Gea and Friedman 2011). Epithelial cells may also lead to fibrosis through a process called epithelial to mesenchymal transition (EMT) where the epithelial cells migrate and change to a mesenchymal phenotype capable of producing ECM (Hernandez-Gea 2011). However, this process is controversial.

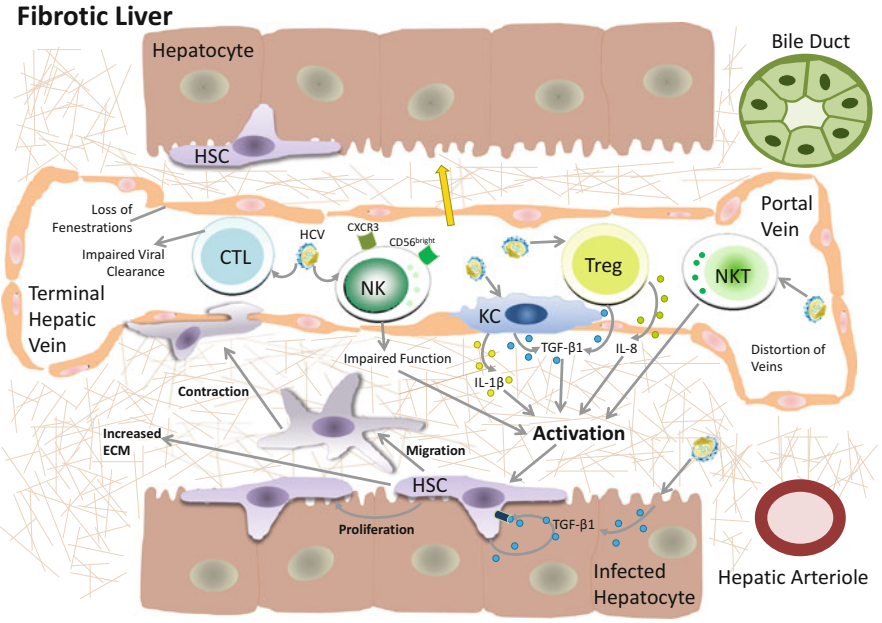

Fig. 2 Depiction of a fibrotic liver in the setting of HCV. Multiple pathways lead to activation of HSC including Natural Killer T cells (*NKT*), IL-8 and TGF-β1 secreted by Tregs, TGF-β1 and IL-1β secreted by Kupffer cells (*KC*), and impaired function of CXCR3 + $CD56^{bright}$ NK cells. TGF-β1 secreted by infected hepatocytes and the HSC themselves amplifies HSC activation. Activation of HSC leads to HSC proliferation, migration, and contraction. There is also increased deposition of ECM. The loss of fenestrations impedes the flow of plasma to the hepatocytes (Partially adapted from Hernandez-Gea and Friedman 2011)

3 Perpetuation of Liver Injury

Signaling from other cells is the main process by which HSC become activated; cytokines and chemokines are secreted by natural killer cells (NK cells), hepatocytes, lymphocytes, dendritic cells, and Kupffer cells (KC) to name a few. One of the key drivers of fibrosis is TGF-β since it has a pivotal role in HSC activation. TGF-β1 signals via Smad2 and Smad3 proteins and MAPK signaling pathways, which then increases procollagen I and III transcription (Inagaki and Okazaki 2007). In HCV, TGF-β is required for the production of α-SMA along with type 1 collagen, two of the main components of ECM (Presser et al. 2013). TGF-β has also been implicated in EMT (Zavadil and Bottinger 2005). Interestingly, production of TGF-β by HCV-specific CD4+ and CD8+ T lymphocytes decreases fibrosis (Li et al. 2012). Other important cytokines include interferons (IFNs) and interleukins (IL). The interferons activate signal transducer and activator of transcription 1 (STAT1), which inhibits HSC proliferation and TGF-β signaling (Jeong et al. 2006). IFN-γ also leads to activation of macrophages while recruiting T

cells, NK cells, and natural killer T cells (NKT), attentuating fibrosis (Bertoletti and Maini 2000). Polymorphisms in the IFN-γ 2 receptor affect fibrosis rates (Nalpas et al. 2010). Independently of productive infection, HCV enters intrahepatic macrophages to produce IL-1β via inflammasome activation, leading to further release of proinflammatory cytokines and chemokines (Negash et al. 2013). IL-1β serum levels correlate with HCV disease progression (Zampino et al. 2013). IL-6 and IL-22 activate STAT3, which controls liver regeneration and repair (Costa et al. 2003). IL-8 secreted from regulatory T cells (Tregs) activates primary HSC in vitro, and this activation is blocked by the addition of a neutralizing IL-8 antibody (Langhans et al. 2013). IL-8 is increased in patients with HCV, and its levels also correlate with fibrosis (Mahmood et al. 2002). In contrast, IL-10 is antifibrogenic. IL-28B polymorphisms are known to predict rates of response to HCV therapy and spontaneous clearance rates, but they are also related to fibrosis. In a meta-analysis of IL-28B polymorphisms, the rs12979860 CC and rs8099917 TT genotypes were associated with the progression of liver cirrhosis in HCV (Sato et al. 2014). In particular, in patients with non-genotype 1 infection, the presence of IL-28B alleles that reduce the chances of viral clearance may paradoxically slow the rate of fibrosis progression in the setting of viral persistence (Bochud et al. 2012). Chemokines also likely play pivotal roles in HCV fibrosis (Fahey et al. 2014). Elevated serum levels of monocyte chemotactic protein 1 (MCP-1), also known as chemokine (C-C motif) ligand 2, correlate with rapid progression of fibrosis in HCV (Farci et al. 2012).

The endocannabinoid system and its receptors also control fibrosis. Marijuana users with HCV have increased progression of fibrosis (Hezode et al. 2005); daily marijuana use compared to non-daily marijuana use had an odds ratio of 3.21 for moderate to severe fibrosis (Ishida et al. 2008). Cannabis compounds and endocannabinoids bind the cannabinoid type 1 receptor (CB1) and the cannabinoid type 2 receptor (CB2). CB1 is considered fibrogenic while CB2 is anti-fibrogenic (Julien et al. 2005). In cirrhotic livers, the expression of cannabinoid receptors is increased, and these receptors are mainly located on HSC and myofibroblasts. In HCV infection, CB1 messenger RNA (mRNA) is increased in liver samples compared to controls, and hepatocyte cell lines show increased CB1 mRNA after transfection with HCV (Van der Poorten et al. 2010). Antagonism of the CB1 receptor in three mouse models of liver injury inhibited the progression of fibrosis (Teixeira-Clerc et al. 2006). Cannabinoids also have other detrimental effects, namely they can contribute to steatosis, which can worsen fibrosis (Parfieniuk and Flisiak 2008).

4 Intrahepatic Blood Flow and Fibrosis

HSC can mediate sinusoidal contraction and decrease blood flow with various vasoactive mediators including endothelins, nitric oxide, the renin-angiotension system, prostaglandins, vasopressin, and thrombin known to play central roles

(Hernandez-Gea and Friedman 2011). As cirrhosis progresses, there is increased expression of the endothelin type A receptor in HCV liver stains (Kitamura and Hayashi 2008). Cyclooxygenase type 2 (COX-2) is an enzyme that converts arachidonic acid to prostaglandin and is involved in inflammatory responses. Patients with HCV have increased COX-2 expression in liver samples, and the expression correlates with progressive fibrosis (Nunez et al. 2004). The -1195GG polymorphism in the COX-2 promoter gene is also associated with progressive fibrosis and inflammation in Japanese HCV patients (Miyashita et al. 2012). Both endothelin receptor antagonists and angiotensin – converting enzyme inhibitors have been proposed as potential treatments for fibrosis (Cho et al. 2000; Jonsson et al. 2001). Other factors that lead to hypercoagulability and impaired microvascular flow such as Factor V Leidin mutation, protein C deficiency, and increased factor VIII are also associated with increased fibrosis in HCV (Anstee et al. 2011). There is considerable excitement in the identification of antifibrotic molecules such as relaxin (Poelstra 2014) that directly inhibit the contractility of HSCs and reduce portal hypertension, thus potentially serving as the basis of a new therapeutic strategy in liver fibrosis.

5 Effects of HCV Proteins on Fibrosis

Hepatitis C viral proteins have been found to stimulate fibrosis (Fig. 3). HCV core protein can stimulate HSC directly or indirectly through hepatocytes. However, HCV does not replicate in HSC. In the human HSC line LX-2, exposure to core protein increases the expression of α-SMA along with mRNA of α-SMA, procollagen a2, and TGF- β1, which are markers of activation of LX-2 cells (Wu et al. 2013). This activation of LX-2 cells occurs at least partially through the obese receptor (Wu et al. 2013). Adenoviral infection of HSC with HCV core protein leads to cell proliferation, accelerated cell activation, increased secretion of TGF- β1, expression of procollagen 1, and increased production of α-SMA (Bataller et al. 2004). Additionally, HCV core protein transfection causes TGF-β1 expression in hepatocyte cell lines, which then activates HSC (Benzoubir et al. 2013). HCV core protein can also stimulate hepatocytes to undergo EMT that can lead to further fibrosis (Battaglia et al. 2009). HCV core protein in murine and in vitro cell models has been shown to be pro-apoptotic, which can contribute to fibrosis (Schuppan et al. 2003). In cell culture, HCV core interacts with TNF-α1 and lymphotoxin β receptors, leading to increased apotosis (Zhu et al. 1998; You et al. 1999). However, in vivo, HCV causes variable amounts of apoptosis, depending on the patient (Pavio and Lai 2003). Reactive oxygen species can lead to liver injury, and HCV core has been shown to attach to the mitochondrial membrane and increase the production of these reactive oxygen species (Choi 2012).

Other HCV proteins have been implicated in HSC activation. Expression of non-structural HCV proteins increases TGF-β production in hepatocytes (Schulze-

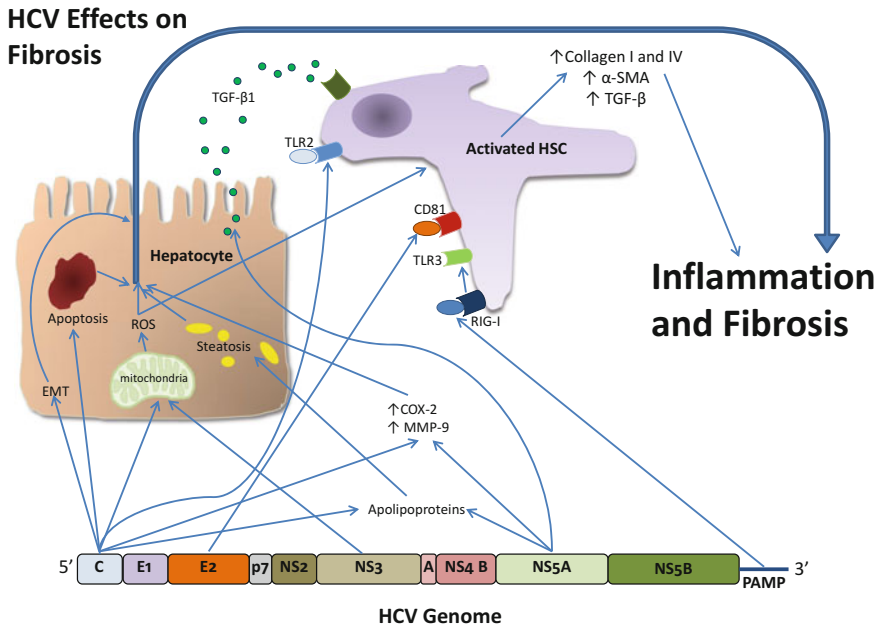

Fig. 3 Different HCV proteins can lead to inflammation and fibrosis either by activating HSC or through their effects on the hepatocyte (Partially adapted from Schuppan et al. 2003)

Krebs et al. 2005). Both non-structural protein NS5A and HCV core protein increase apolipoproteins, which lead to steatosis and further liver damage (Schuppan et al. 2003). HCV core and NS5A also upregulate COX-2 and MMP-9 gene expression in hepatocytes; intrahepatic expression of these proteins is associated with fibrosis (Nunez et al. 2004). NS5A can cause oxidative stress (Gong et al. 2001) and decrease the antifibrotic effect of interferon (Schuppan et al. 2003). Transduction of NS5A with an adenoviral vector into HSC induces chemokine secretion, expression of ICAM-1, secretion of TGF-β1, and the expression of procollagen 1 (Bataller et al. 2004). However, NS5A is not always inflammatory; it also interacts with the TGF-β receptor and inhibits TGF- β signaling (Choi and Hwang 2006). HCV protein E2 stimulates TGF-β production through degradation of AIMP1/p43 (Kim et al. 2014). It can also bind CD81 on HSC and lead to MMP-2 upregulation and the deposition of type I collagens (Mazzocca et al. 2005).

6 MicroRNAs and Fibrosis

MicroRNAs (miRNAs) are small non-coding RNA that binds mRNA and regulates its transcription. MicroRNAs are thought to regulate up to 60 % of the genome, and there is increasing evidence that HCV infection is affected by miRNAs (Griffiths-Jones S 2004; Friedman et al. 2009). There is evidence that miRNAs can also directly bind HCV (Singaravelu et al. 2014). An inhibitor of miRNA-122 was recently shown to decrease viral load after 29 days of treatment (Janssen et al. 2013). The role of miRNA in fibrosis is also becoming elucidated. Hepatic levels of miRNA-122 decrease with progression of fibrosis in HCV (Trebicka et al. 2013). Other miRNAs have important roles. MicroRNA-199a, 200a, and 200b correlate with progression of fibrosis in mouse models and with progression of fibrosis in HCV (Murakami et al. 2011). MicroRNA-199a, 199b, 200a and 200b are thought to be involved in the TGF-β signaling pathway and the expression of MMP-13, alpha 1 procollagen, and TIMP-1 (Murakami et al. 2011). MicroRNA-21 represses Smad 7, which is an inhibitor of TGF-B signaling (Marquez et al. 2010). MicroRNA-29a negatively regulates for the transcription of several collagen genes. HCV-induced TGF-β production downregulates miRNA-29a, leading to increased production of different collagens and ECM deposition (Bandyopadhyay et al. 2011). Other miRNAs that have been implicated in HCV liver fibrosis include 107, 200c, 221, 222, and 449a (Ramachandran et al. 2013; Sarma et al. 2012, 2014; Ogawa et al. 2012). A signature of nine miRNAs (155, 34a, 222, 23b, 361, 455, 30b, 30c, and 27b) has also been shown to be predictive of post-liver transplantation aggressive fibrosis in HCV patients (Gehrau et al. 2013).

7 Innate Immune Response and Fibrosis

The response of the innate immune system to HCV can be pro-fibrotic or anti-fibrotic. NK cells are considered the principal cells of the innate immune system that respond to viral infections (Golden-Mason and Rosen 2013). NK cells can also trigger the activation of the adaptive immune system. NK cells can sense pattern associated molecular patterns (PAMPs) and release cytokines to stimulate other cells, or they can kill virally infected cells or HSC themselves. NK cells tend to impede fibrosis through inhibition of HSC (Safadi et al. 2004). In HCV, NK cells have an enhanced ability to kill HSC, inhibiting fibrosis (Muhanna et al. 2011; Morishima et al. 2006). Intrahepatic NK cells produce IFN which inhibits HSC activation (Dong et al. 2004; Melhem et al. 2006) and can also induce apoptosis of HSC through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and natural killer group 2, member D (NKG2D) (Radaeva et al. 2006). The cytotoxicity of NK cells is increased by IFN-α treatment, while alcohol and TGF-β decrease the cytotoxicity of NK cells (Gao and Radaeva 2013). NK cells have multiple important activating and deactivating receptors. One important receptor is the NKp46

receptor, which is activating. NKp46^{high} NK cells accumulate in the liver of patients with HCV, and increased numbers of NKp46^{high} NK cells inversely correlate with fibrosis stage (Kramer et al. 2012). This effect is likely due to the fact that NKp46 ^{high} cells secrete more IFN-γ, which decreases HSC activation. Furthermore, the killing of HSC by NK cells is NKp46^{high} dependent (Gur et al. 2012), so the upregulation of this receptor leads to more efficient killing of HSC, limiting fibrosis. The chemokine receptor CXCR3 on NK cells and its chemokines are associated with fibrosis stage (Zeremski et al. 2008). CXCR3 + CD56^{bright} NK cells have an increased ability to inhibit HSC, but these cells have impaired function in HCV patients (Eisenhardt et al. 2012). Therefore, NK cells are critical in the control of fibrosis by regulation of HSC both by direct killing and release of inhibitory cytokines (Tian et al. 2013).

Other cells of the innate immune system also are also involved in the development of fibrosis and inflammation. Kupffer cells, tissue macrophages of the liver, are activated by HCV and release pro-inflammatory cytokines such as TGF-β, IL-1β, IL-6, and TNF (Boltjes et al. 2014). Kupffer cells also regulate ECM deposition and breakdown by other cells (Xidakis et al. 2005). Increased numbers of Kupffer cells are found in the areas of liver with increased inflammation (Marrogi et al. 1995). The role of neutrophils in HCV fibrosis is unclear. HSC can directly interact with NKT cells (Park et al. 2009). In contrast to NK cells, NKT cells are pro-fibrogenic. In mouse models of cirrhosis, depletion of NKT cells decreases the amount of fibrosis (Park et al. 2009). However, HCV patients have decreased percentage of NKT cells in the liver (Deignan et al. 2002).

Pattern recognition receptors that recognize PAMPs, such as toll-like receptors, are also involved in fibrosis. All types of toll-like receptors (TLR) are expressed on HSC (Yang and Seki 2012), and HCV infection increases expression of TLR2, 3, 4, 6, 7, and 8 in peripheral mononuclear cells (He et al. 2006; Shehata et al. 2006; Sato et al. 2007). HCV core protein activates TLR2 signaling on HSC, causing activation (Coenen et al. 2011). TLR2 and TLR4 mRNA is increased in cirrhosis and viral hepatitis (Soares et al. 2012). TLR3 activation leads to the production of inflammatory cytokines and chemokines. In patients with rapid fibrosis after HCV recurrence post-liver transplantation, TLR3 and TLR7/8 have impaired cytokine responses (Howell et al. 2013). TLR4 has been found to have the strongest connection to fibrosis. TLR4 affects TGF-β signaling in HSC (Guo and Friedman 2010; Seki et al. 2007) and blocks the effects of miRNA-29 (Roderburg et al. 2011). Several polymorphisms in TLR4 have been found to be protective of liver fibrosis because they decrease the TLR4-mediated inflammatory signaling and lower the threshold for apoptosis in HSCs (Guo et al. 2009; Huang et al. 2007).

Other pattern recognition receptors also contribute to liver fibrosis. HCV is recognized by retinoic acid-inducible gene 1 receptor (RIG-I), and this leads to induction of interferons and interferon stimulated genes (Saito et al. 2008). RIG-I is expressed on HSC, and its stimulation leads to production of IFN-β by HSC and increased expression of TLR3 (Wang et al. 2013). This RIG-I stimulation causes the HSC to further activate the innate immune response.

Lectins are also pattern recognition receptors involved in HCV fibrosis. Mannose-binding lectin (MBL) recognizes PAMPs from HCV and elicits the innate immune response (Brown et al. 2007b). HCV has increased MBL activity and complement activation (Brown et al. 2007a). Polymorphisms of the MBL gene that lead to decreased MBL activity are inversely correlated with liver fibrosis (Alves Pedroso et al. 2008). LecT-Hepa, a novel glycol-marker derived from three lectins, correlates with fibrosis stage in HCV adults (Ito et al. 2012). Lectins are also involved in activation of the complement system. The lectin pathway is one of the three pathways to activate complement. Complement activation has been shown to correlate with the degree of fibrosis and inflammation (Vasel et al. 2014). Patients with HCV have lower total plasma complement activity than controls, which suggests complement activation. Lower plasma complement activity correlated with inflammation and fibrosis scores (Vasel et al. 2014). In children, complement 4a levels decreased with progression of fibrosis (Behairy et al. 2013). Levels of mannose-binding lectin associated serine protease (MASP) 1, a component of the lectin complement pathway, do correlate with liver fibrosis (Brown et al. 2007a). Exposure of HSC to MASP-1 leads to HSC activation (Saeed et al. 2013). Therefore, lectins have a dual role in fibrosis; they activate the innate response and the complement pathway.

8 Contribution of Adaptive Immunity

The adaptive immune system also contributes to hepatic fibrosis in HCV, but there is less clear evidence than for the innate immune system. It is thought that an inefficient T-cell response to HCV leads to poor viral clearance and induction of secondary inflammation leading to fibrosis. MHC-class-I-restricted peptide-specific CD8+ T cells (CTL) can detect infected cells and kill them or release cytokines to stimulate other cells of the immune system. The number of CTLs has been correlated to liver injury in some studies (reviewed by Bowen and Walker 2005). However, in a study of 209 patients, intrahepatic CTLs did not correlate with fibrosis (Rothman et al. 2005). When the number of CTLs is decreased in a mouse model of fibrosis with carbon tetrachloride, this led to decreased fibrosis (Safadi et al. 2004). Consequently, it is likely that the inflammatory effects of CTLs promote fibrosis.

The significance of CD4+ T cells in fibrosis is also being elucidated. When HSC phagocytose CD4+ lymphocytes, this activates the HSC (Muhanna et al. 2008). CD4+ T cells that produce IL-17 (Th17) have recently been implicated in liver fibrosis in non-HCV models (LeibundGut-Landmann et al. 2007; Lemmers et al. 2009; Rong et al. 2009). HCV promotes pro-inflammatory Th17 responses (Lee et al. 2013). The livers of HCV patients have an increased number of Th17 CD4+ T cells, which correlates with the severity of liver inflammation (Foster et al. 2012).

Other T cell subsets can also contribute to fibrosis. Gamma delta T cells in the peripheral blood are increased in HCV, and increased amounts of gamma delta T cells in the liver correlate with hepatic injury (Nikolopoulou et al. 1995; Yonekura et al. 2000; Agrati et al. 2001). Tregs also are involved in fibrosis. Peripheral Tregs are increased in HCV infection (Ebinuma et al. 2008), and they are enriched in the liver near HSC (Langhans et al. 2013). Tregs have the ability to secrete TGF-β and IL-8, both of which active HSC (Stassen et al. 2004; Langhans et al. 2013). Exposure of CD4+ T cells to HCV-infected hepatocytes leads to increased differentiation toward Tregs (Ji et al. 2013). Since Tregs can also suppress the activity of other lymphocytes, their presence may also dampen the inflammatory effects of HCV in other pathways. Many highly activated Tregs localize to the liver in HCV and seem to decrease fibrosis (Claassen et al. 2010). One subclass of Tregs produces IL-10, which is antifibrogenic (MacDonald et al. 2002). Therefore, the exact role of Tregs in hepatic fibrosis is complicated and incompletely understood and seems to depend on the subtype of Treg.

9 Histologic Fibrosis

Liver biopsy has been the gold standard for measurement of fibrosis. The area under the receiver operating characteristic curve (AUROC) for liver biopsy is 0.97 (Ahmad et al. 2011). Obtaining the correct fibrosis stage is critical because it controls clinical decisions on HCV treatment, although this is likely to evolve in the setting of new direct-acting antiviral regimens with high efficacy and minimal side-effects, as well as prediction of HCV-related complications. There are three main scoring systems, Metavir, Ishak, and Knodell, which are used to score fibrosis. Attempts have been made to increase the accuracy of fibrosis staging in liver biopsies. Different staining techniques, other than the standard trichrome stain, are being utilized, and sirius red has been suggested as a better alternative (Huang et al. 2013). Different methods of digital image analysis have been developed to also try and objectively and accurately determine fibrosis. The ratio between the fibrosis area and the total sample area as determined by digital analysis correlates with Metavir and Ishak scores (Campos et al. 2014). In HCV, digital measurement of portal-bridging fibrosis area has been proposed as being more accurate than whole fibrosis or perisinusoidal fibrosis (Sandrini et al. 2014). Computer measurement of collagen proportionate area (CPA) is separate way to verify fibrosis stage, and CPA correlates with morbidity and HCC risk in HCV (Huang et al. 2014; Tsochatzis et al. 2014). In fact, Germani suggests that CPA correlates better with clinical outcomes than histologic stages (Germani et al. 2010). Liver biopsy is limited in its practicality due to its invasive nature, expense, variations in the histologic grading, and sampling variability. Much of the current HCV research is focused on efforts to find accurate non-invasive imaging techniques and serum markers that will stage fibrosis.

10 Fibrosis Imaging

Conventional ultrasound imaging of patients with cirrhosis has a sensitivity of 84 % and specificity of 100 % for cirrhosis, but poorly differentiates between levels of fibrosis (Ahmad et al. 2011). Proposed imaging techniques to measure fibrosis include transient hepatic elastography (TE), Acoustic radiation force impulse elastography (AFRI), shear wave elastography, microbubble ultrasound, molecular magnetic resonance imaging (MRI), and magnetic resonance (MR) elastography. Transient hepatic elastography by Fibroscan® (Echosens, Paris, France) is the most well-studied non-invasive imaging technique. It utilizes elastic waves and low frequency ultrasounds (50 Hz) to determine hepatic elasticity (Schiavon Lde et al. 2014). In 183 HCV patients, Fibroscan® was superior to serum testing for determination of advanced fibrosis, with an AUROC of 0.95 (Castéra et al. 2005). The utility of Fibroscan® has been verified in multiple other studies (Arena et al. 2008; Castéra et al. 2009; Nitta et al. 2009; Ziol et al. 2005) and in special populations including HIV and HCV co-infected patients (de Ledinghen et al. 2006; Vergara et al. 2007) and post-transplantation patients (Adebajo et al. 2012). In general TE correlates well with fibrosis stage. However, TE has not found to be as accurate for portal hypertension and risk of esophageal varices (Schiavon Lde et al. 2014). In about 5 % of cases, TE is not successful due to patient obesity, small inter-costal spaces, or the presence of ascites (Ahmad et al. 2011). There is also inter-observer variability in TE measurements with discordance of 35 % for at least one stage of fibrosis and 5 % for two or more stages (Perazzo et al. 2014).

Other imaging techniques are also being investigated. Microbubble ultrasound does not perform as well as TE in HCV with AUROC of 0.78 for cirrhosis and 0.76 for advanced fibrosis (Cobbold et al. 2012). AFRI is another ultrasound technique that measures liver stiffness by shear wave velocity. In a comparison to liver biopsy in 51 patients with HCV, the AUROC was 0.90 for ARFI in advanced fibrosis and was more effective than various serum markers (Silva Junior et al. 2014). Other studies have found similar results (Yamada et al. 2014; Nishikawa et al. 2014). Acoustic structure evaluation by computer software of ultrasound images has not been found to be equivalent to TE, with an AUROC of 0.71 for advanced fibrosis (Ricci et al. 2013). MR elastography does identify patients with advanced fibrosis who are at increased risk of hepatic decompensation, but further studies are needed to evaluate its usefulness (Asrani et al. 2014). A study in rat cirrhosis has suggested that molecular MRI with a collagen-specific probe can identify fibrosis, but this has not yet been validated in HCV or in humans (Polasek et al. 2012). So far, only AFRI has been found to be somewhat comparable to TE.

11 Serum Markers of Fibrosis

Similar to imaging, finding a useful serum marker of fibrosis has been an area of much research and interest. There are numerous blood indices using clinical characteristics and serum tests to assess for the presence of cirrhosis including FIB-4, APRI, Hepascore, and enhanced liver fibrosis (ELF) score to name a few (Chou and Wasson 2013). In a meta-analysis, the FIBROspect II® index had the highest AUROC at 0.86 for fibrosis (Chou and Wasson 2013). FIBROspect II® includes serum levels of TIMP-1, α_2-macroglobulin, and hyaluronic acid (Patel et al. 2004). Other indices with high AUROC include Fibrometer™ at 0.82 and ELF at 0.81 (Chou and Wasson 2013). Fibrometer™ includes sex, α_2-macroglobulin, prothrombin time (PT), γ-glutamyltransferase (GGT), and blood urea (Cales et al. 2005), while ELF measures hyaluronic acid, N-terminal propeptide of type II collagen, and TIMP-1 (Rosenberg et al. 2004). In comparison, platelet count alone has an AUROC of 0.71 and APRI (the aspartate aminotransferase (AST) – platelet ratio index) has an AUROC of 0.77 (Chou and Wasson 2013). A separate meta-analysis of APRI felt that it had a moderate degree of accuracy to determine fibrosis, and AUROC improved to 0.83 for cirrhosis (Lin et al. 2011b). For cirrhosis, Fibrometer™ had the best AUROC at 0.91, hyaluronic acid alone was 0.90, platelet count was 0.89 and the AST to alanine aminotransferase (ALT) ratio was 0.72 (Chou and Wasson 2013). A separate meta-review found that the Hepascore, which includes bilirubin, GGT, hyaluronic acid, α_2-macroglobulin, gender, and age, had the highest AUROC with 0.85 for significant fibrosis and 0.94 for cirrhosis (Ahmad et al. 2011).

Research is continuing for novel biomarkers that correlate with fibrosis. Adding angiotensin 2 to platelet count, PT, AST, age, and GGT leads to an AUROC of 0.89 (Hernandez-Bartolome et al. 2013). Protein C and retinol binding protein 4 could distinguish fibrosis in preliminary studies (Qin et al. 2012). Cytokine levels including TGF-β and PDGF are associated with fibrosis progression (Zhang et al. 2003; Kanzler et al. 2001). TIMP-1 levels are increased in HCV cirrhosis (Boeker et al. 2002). MMP-1 and apolipoprotein A1 are included in other indices that are recommended clinically (Saludes et al. 2014). A preliminary study of serum Fas receptor, a death receptor that is critical in controlling apoptosis in the liver, found that Fas levels differentiated HCV patients without cirrhosis from those with cirrhosis (Hammam et al. 2012). In summary, finding a serum marker of fibrosis is an ongoing area of research with the perfect marker, i.e., one that discerns the non-extreme stages of fibrosis, yet to be found.

12 HCV and HIV Co-infection

The risk factors that lead to HCV, namely intravenous drug use and transfusions, also lead to infection with HIV. Unfortunately, patients co-infected with HIV and HCV have accelerated fibrosis. In 135 patients infected with HIV and HCV who had two liver biopsies over a mean interval of 3.3 years, 28 % of patients progressed by one stage of fibrosis, 16 % progressed two or more stages, and 13 % developed cirrhosis (Macias et al. 2009). They also have increased rates and earlier incidence of HCC (Brau et al. 2007). It is estimated that there are five million people worldwide who are co-infected, and one million people in the US (Kim and Chung 2009; Rotman and Liang 2009; Sherman et al. 2011). Treatment of HIV with highly active antiretroviral therapy (HAART) has been shown to slow fibrosis progression and decrease cirrhosis-related mortality (Macias et al. 2009; Lopez-Dieguez et al. 2011). On the other hand, treatment of HCV in HIV-positive patients has lower rates of success (Kim and Chung 2009). Successful treatment of HCV does slow the progression of liver fibrosis in co-infected patients and increases the rate of fibrosis regression (Labarga et al. 2014).

HIV can directly interact with hepatocytes and HSC. Unlike HCV, HIV cannot replicate in hepatocytes, but HIV can induce cell signaling in hepatocytes, HSC, and immune cells (Lin et al. 2013). Specifically in HSC, HIV can infect the cells and leads to collagen I and MCP-1 expression (Tuyama et al. 2010). HIV proteins also increase TGF-β levels in hepatocytes and have an additive effect on TGF-β expression in HCV models (Lin et al. 2008; Presser et al. 2011). In addition, HIV proteins cause an increase in reactive oxygen species production in hepatocytes and HSC, as well as an increase the expression of procollagen 1α and TIMP-1 (Lin et al. 2011a). Co-infection with HCV and HIV also increases hepatocyte apoptosis markers such as caspase 3/7 and cell-membrane death receptors 4 and 5, more than either virus alone (Jang et al. 2011).

HIV also impairs CD4+ T cell function and decreases their numbers. This leads to decreased stimulation of NK cells by CD4+ cells (Glassner et al. 2013). Without the inhibitory effect of NK cells on fibrosis, co-infected patients can have more rapidly progressive fibrosis. HIV depletes the immune cells and lymphoid tissue of the intestines, allowing more entry of bacteria into the portal circulation (Lin et al. 2013). Exposure to lipopolysaccharide (LPS), a component of the walls of gram negative bacteria, can initiate hepatic fibrogenesis. LPS binds to TLR4 on HSC, which causes HSC activation (Seki et al. 2007). Patients with HCV have increased serum levels of LPS (Sandler et al. 2011), and in co-infected patients, LPS levels correlate with cirrhosis (Balagopal et al. 2008). Therefore, co-infected patients lack an intact gut immune barrier, leading to increased LPS, which causes HSC activation and promotes fibrosis.

13 Contribution of Other Factors to Fibrosis

Other factors, such as steatosis, fructose intake, cholesterol, and vitamin D, contribute to fibrosis. Patients with steatosis and hepatitis C have more advanced fibrosis (Hwang and Lee 2011). Genotype three disease has an increased risk of steatosis although it is not known if this is a direct viral cytopathic effect or indirect effect related to inflammatory pathways. Other factors that can cause steatosis include obesity, hyperlipidemia, and metabolic syndrome (Hwang and Lee 2011). Insulin resistance and metabolic syndrome are associated with advanced fibrosis as well, not just steatosis (El-Zayadi and Anis 2012; Yoon et al. 2013). Increased cholesterol intake also correlates with fibrosis progression (Yu et al. 2013). Moreover, high fructose intake can lead to fibrosis in animal models and has been suggested as a modifier of fibrosis in HCV (Rizkalla SW 2010). One study found a connection between industrial fructose intake and fibrosis score from liver biopsies in 147 genotype one patients (Petta et al. 2013b). However, in a study of 313 men, there was no association between fructose intake and serum fibrosis correlates (Tyson et al. 2013). Therefore, the connections between diet and other metabolic diseases and fibrosis are complicated and warrant further research.

A high percentage of HCV patients are vitamin D deficient. In one study, 90 % of the patients had 25-OH vitamin D levels of less than 32 ng/ml, which is considered deficient (Fisher and Fisher 2007). Multiple studies have found the association of more advanced liver disease with low vitamin D levels (Fisher and Fisher 2007; Petta et al. 2013a; Arteh et al. 2010; Lange et al. 2011). However, not all studies have found this correlation (Kitson et al. 2013). Vitamin D deficiency also correlates with liver fibrosis in HCV and HIV co-infected patients (Guzman-Fulgencio et al. 2014). Low vitamin D receptor expression in hepatocytes and lymphocytes is associated with increased portal inflammation (Barchetta et al. 2012). The cause of the vitamin D deficiency is likely multifactorial from a shortened half-life of 25-OH vitamin D in the setting of inflammation, decreased absorption in the setting of cholestasis, and decreased hepatic production of vitamin-D binding protein (Gutierrez et al. 2011). It is currently unclear if vitamin D deficiency is just a secondary marker of liver fibrosis, or if vitamin D deficiency contributes to the fibrosis itself, but increasing evidence suggests a more active role. In vitro, vitamin D inhibits HCV production in hepatocyte cell lines (Gal-Tanamy et al. 2011). The addition of vitamin D therapy improves sustained viral response (SVR) (Abu-Mouch et al. 2011). Vitamin D also affects the immune system by inducing Th2 cytokines, stimulating T-regulatory T cells, and suppressing the innate immune response (Han et al. 2013). Therefore, vitamin D is likely both a marker of cirrhosis and a contributor to fibrosis.

14 Post-Transplantation Fibrosis

In the immunocompetent setting, chronic HCV infection is a very slowly progressive disease, making prospective evaluation of its natural history very problematic (Feld and Liang 2006). Consequently, long periods of time are required to document any clear-cut evidence of progressive liver injury. In contrast, the proportion of HCV-positive liver transplant recipients who develop cirrhosis at 5 years ranges from 21 to 35 % (Gane et al. 1996; Prieto et al. 1999; Roche and Samuel 2007; Hughes and Rosen 2009). Accordingly, the median and mean rates of fibrosis development (which are non-linear) are significantly higher than that observed pre-transplantation ($p < 0.0001$) (Berenguer et al. 2000). As a result, annual protocol liver biopsies have been recommended in HCV-positive liver allograft recipients (Arjal et al. 2007). The telescoped natural history of HCV has allowed identification of specific factors associated with disease progression; donor age (Berenguer et al. 2002; Wali et al. 2002) and use of T cell depletion for treatment of rejection (e.g., OKT3) (Burton and Rosen 2006; Rosen et al. 1997) have consistently been associated with more rapid disease progression. A recent study of over 500 liver transplant recipients demonstrated that single nucleotide polymorphisms associated with enhanced production of RIG-I, the cytosolic receptor involved in recognition of HCV and induction of downstream IFN pathways, as well as IL-28B (*IFNL3*) within the donor liver, may increase the risk of allograft fibrosis/cirrhosis (Biggins et al. 2013). Specifically, patients who received a donor liver with the IL-28B CC genotype (rs12979860) but were themselves non-CC had a greater than sevenfold risk of advanced fibrosis.

15 Fibrosis Resolution

Fibrosis is thought to be at least a partially reversible process. After treatment of HCV, fibrosis can be reversible (Iredale 2007). Much of what is known about fibrosis resolution is based on rodent models of injury, not specifically on HCV. One of the processes that appear critical for fibrosis resolution is apoptosis of HSC (Herandez-Gea 2011). The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is integral to this process and prevents apoptosis of activated HSC. Adiponectin, elevated in advanced HCV fibrosis (Korah et al. 2013), can suppress HSC proliferation and promote apoptosis (Hernandez-Gea and Friedman 2011). NK cells are also apoptotic to HSC. Degradation of the ECM is controlled by MMPs, namely MMP-2, MMP-3, MMP-9, and MMP-13 can all break down ECM. TIMPs inhibit the activity of MMPs and prevent HSC apoptosis, and TIMP-1 levels decrease during fibrosis resolution (Ramachandran and Iredale 2012). The stimulation of fibrosis resolution could be a potential treatment option.

16 Effects of HCV Treatment

Recently, the advent of new direct-acting antiviral therapy for HCV has greatly improved SVR. For patients with cirrhosis, these new medications have greatly improved treatment since the interferon-based regimens were often difficult for these patients to tolerate (Everson et al. 2014). At any stage of liver disease, patients benefit from HCV clearance with decreased morbidity and mortality (Lee and Friedman 2014; Bernstein et al. 2002). The majority of patients have improvement in their fibrosis after SVR with IFN-based treatments, but a minority, approximately 7–12 %, have stability or progression of their fibrosis (Maylin et al. 2008; Poynard et al. 2002). There is some concern that patients with SVR with continued fibrosis progression have occult HCV infection, but this is not felt to be the case in most patients (Lee and Friedman 2014). Patients with cirrhosis seem to benefit the most from treatment, and many demonstrate decreases in their hepatic pressure venous gradient (Roberts et al. 2007).

For the small percentage of patients with fibrosis progression, the reasons for the lack of improvement are unclear, but there are numerous theories. Some of the progression of fibrosis might be due to other insults such as alcohol intake, but in studies that have controlled for these factors, there still remains a subset of patients that have progressive fibrosis. In animal models, fibrous septae that are older than 1 year are more resistant to degredation due to decreased cellularity and increased ECM crosslinking (Issa et al. 2004). Elastin, a component of ECM, accumulates more during advanced cirrhosis and is known to be resistant to breakdown (Lee and Friedman 2014). With withdrawal of the inflammatory agent, HSC can revert to a quiescent state but remain primed and can react more strongly to other irritants (Kisseleva et al. 2012). In a study of 38 patients with cirrhosis who had follow up biopsies at a mean of 67 months after SVR, staining for α-SMA did not differ before or after SVR, and in 11 patients, there was increased staining after SVR (D'Ambrosio et al. 2012). While SVR does decrease the rate of HCC development, from 3.3 % per person per year to 1.05 % (Morgan et al. 2013), cirrhotic patients still require HCC screening after SVR. Pre-treatment fibrosis stage and age at SVR remain the major risk factors for HCC development (Yamashita et al. 2011).

17 Anti-fibrotic Therapy

Clearly the first step in treating fibrosis in HCV is to clear the virus. There is no specific treatment of fibrosis in HCV, other than treatment of HCV infection. Many therapies have been proposed as being potentially useful. Suppressing TGF-β1, one of the most important cytokines in the activation of HSC, is a potential treatment option, but its usefulness is limited due to its immune suppression side effects (Friedman et al. 2013). There are currently two humanized antibodies that target TGF-β1 that are under investigation in patients with kidney disease and

scleroderma (Friedman et al. 2013). More specific inhibitors of other parts of the TGF-β pathway are also being developed, including largazole, a histone deacetylase inhibitor that inhibits TGF-β and vascular endothelial growth factor signaling (Liu et al. 2013). MicroRNA is another target for therapeutics. There are numerous treatment trials for idiopathic pulmonary fibrosis, which may have efficacy in the liver, because many fibrogenic pathways are conserved across tissues (Pellicoro et al. 2014). Some trials of treatment for HCV fibrosis have failed; a study of IL-10 for fibrosis caused increased viral loads (Nelson et al. 2003). Several trials of IFN-γ in HCV have failed to show a statistical change in the amount of fibrosis after a year of treatment (Pockros et al. 2007; Muir et al. 2003; Soza et al. 2005). Long-term IFN- α 2a treatment for 3.5 years has also been trialed in 1,050 patients, but no change was seen in fibrosis compared to controls (Di Bisceglie et al. 2008). Research using animal models is currently underway to identify new therapeutic targets for fibrosis treatment. Current potential targets include CB1 receptor antagonists and CB2 receptor agonists (Baldassarre et al. 2013). Much attention has also been placed on peroxisome proliferator activated receptor-α agonists, such as fenofibrate and curcurmin, because they inhibit the production of inflammatory cytokines and suppress HSC activation (Mohamed et al. 2013; Xu et al. 2003). Treatments targeting prevention of apoptosis of hepatocytes are also being developed. With the extensive amount of ongoing research, there is hope that useful anti-fibrotic agents will soon be discovered.

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Hepatocellular Carcinoma and Hepatitis C Virus

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Abstract Hepatocellular carcinoma (HCC) is an important health issue worldwide. It is a liver malignancy with heterogeneous etiologies. Among them, chronic hepatitis C virus (HCV) infection is one major cause. In chronically infected hepatocytes, HCV directly induces oxidative stress, impairs DNA damage response, manipulates cell cycle control and generates insulin resistance, possibly promoting DNA mutations accumulated in host genome from hepatitis stages in a long time. The predisposing polymorphisms or genetic mutations in critical proto-oncogenes and tumor suppressor genes, in synergy with epigenetic alterations after HCV invasion, would eventually transform hepatocytes to HCC tumor cells. Therefore, this chapter focuses on reviewing the virologic role of HCV in disease progression, as well as the host genetic changes for HCC development. Besides, the oncogenic mechanisms evoked by HCV at cellular level and the sex disparity of HCV-related HCC based on sex hormone effects are discussed individually. The comprehensive understanding of HCV-induced hepatocarcinogenesis paves the way to develop novel prophylactic or therapeutic strategies for HCC in future.

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1 Epidemiology of HCV-Related HCC

Hepatocellular carcinoma (HCC) is a global health issue, which is the sixth most common type of malignancy and the second leading cause of cancer death, accounting for approximately half a million deaths every year (Ferlay et al. 2010). Chronic viral hepatitis such as hepatitis B virus (HBV) infection or hepatitis C virus (HCV) infection is the major etiological factor of hepatocarcinogenesis, accounting for approximately 80 % of HCC patients. Behind chronic hepatitis B, chronic hepatitis C (CHC) is believed to be the second common cause for HCC development, which is associated with approximately 20-fold increase in HCC risk compared with negative subjects (El-Serag 2012; Saito et al. 1990).

Depending on the efforts and advances in universal vaccination and antiviral therapies for controlling HBV infection in endemic areas, the HBV-related HCC incidence rates are stable and even gradually declining in certain areas. Whereas the number of HCV-induced HCC cases remains substantial and even increasing, especially in Japan, southern Europe and North America where HCV infection is predominant (El-Serag 2012; McGivern and Lemon 2011). In these areas, HCV transmission is horizontally spread by contaminated blood transfer due to needle sharing of drug injection or unsurveyed blood donation system before the 1960s, therefore indirectly leading to the high prevalence of HCC in aged population nowadays (Shlomai et al. 2014). Under chronic infection, around 10–30 % of HCV carriers are estimated to develop HCC over 30 years (Grebely and Dore 2011). In this disease process, hepatic fibrosis and eventually cirrhosis caused by repeated inflammation and regeneration are commonly observed. People with HCV-induced cirrhosis are the highly perilous population for HCC development at an annual rate of 2–4 % with the 5-year cumulative risk up to 30 % (El-Serag 2012; Fattovich et al. 2004; El-Serag and Rudolph 2007). Other factors determining HCV-related hepatocarcinogenesis include male gender, HBV coinfection, alcoholism, diabetes and obesity (El-Serag 2012).

Despite therapeutic improvement for HCC patients such as sorafenib applied as first-line drugs, overall prognosis and survival rates are still low and unsatisfied in advanced stages (Cheng et al. 2009; Llovet et al. 2008). Application of effective anti-HCV regimens in patients, achieving sustained virologic response (SVR), has been proved in many studies to reduce risk for HCC (Morgan et al. 2013). In developed countries, the SVR rate is close to 45 % in genotype 1 CHC by using combinatorial treatment of pegylated interferon with ribavirin, and recently it is approved to be further elevated to 75 % by adding with antiviral protease inhibitors (Poordad et al. 2011; Jacobson et al. 2011). However, SVR is not perfectly

guaranteed and more likely to be achieved in the earlier infectious stages than in the later fibrotic and cirrhotic stages, suggesting the importance of in-time antiviral intervention for the risk reduction of HCV-induced HCC development (Everson et al. 2006). The recent advent of direct antiviral agents for CHC can achieve almost SVR among 90–95 % of CHC patients, a remarkable progress. However, CHC patients at advanced stage of fibrosis or cirrhosis can still develop HCC, despite of SVR. Therefore, a comprehensive understanding of host and virus factors leading to the progression of HCV-related HCC will be helpful to design novel strategies for disease prevention and cancer treatment.

In this chapter, the carcinogenic role of HCV together with susceptible genetic polymorphism and epigenetic alteration in hepatocarcinogenesis are reviewed. Besides, the HCV-induced oncogenic events in hepatocytes and the sex disparity of HCV-related HCC occurrence are discussed individually.

2 The Role of HCV in Hepatocarcinogenesis

HCV is a hepatotropic RNA virus with positive strands as genetic materials and replicates in cytoplasm through RNA-dependent RNA polymerization. Thus, in HCV life cycle, it is believed to be unlikely to integrate its genome into cellular DNA chromosomes for insertional mutagenesis. On the other hand, HCV-infected individuals without effective antiviral medication usually become persistent carriers and thus extensively suffer from chronic inflammation, progressive hepatic fibrosis and cirrhosis, which are undoubtedly preneoplastic environments for HCC development. The general consequences, such as the virus-induced hepatic injury, immune-mediated cytotoxicity and repeated hepatocyte regeneration, certainly contribute to HCV-associated hepatocarcinogenesis (Nakamoto et al. 1998), while the direct role of HCV in HCC formation is clearly supported by some observations. Besides, heavy alcohol intake which is the major cause of cirrhosis beyond viral hepatitis, has also been considered an important risk factor synergistically cooperated with CHC for HCV-induced hepatocarcinogenesis, especially.

2.1 Direct Role of HCV in HCC Development

First, although cirrhosis is inevitably diagnosed in HCV-associated HCC development, there is still some recognition of non-cirrhotic HCC observed in the minor population of HCV patients (Yeh et al. 2010; Lok et al. 2009; Bralet et al. 2000). In addition, the HCC incidence in HCV-related cirrhosis is significantly higher than that in autoimmune hepatitis-related cirrhosis, which occur at an annual rate of 1 % or even lower (Lok et al. 2009; Yeoman et al. 2008; Teufel et al. 2009; Degos et al. 2000). Besides, successful eradication of hepatic HCV infection is still benefit for the decrement of HCC risk in advanced fibrotic patients (Cardoso et al. 2010;

Morgan et al. 2010; Yu et al. 2006). Therefore, these evidences highly imply the virologic effect of HCV for HCC development other than promotion of preneoplastic lesions in liver tissues.

Second, the validation of HCC occurrence in several independent lineages of transgenic mice with expression of HCV proteins and lack of detectable inflammation convincingly approves the direct cancer-promoting impact exploited by HCV. The transgenic mice expressing core, NS5A or entire HCV polyprotein are reported to develop hepatic steatosis and finally HCC in old ages, albeit at a relative low but statistically significantly percentage (Wang et al. 2009a; Naas et al. 2005; Moriya et al. 1998; Lerat et al. 2002). These HCV oncoproteins may actively induce oxidative stress in liver tissues by stimulating reactive oxygen species (ROS) production and membrane lipid peroxidation, which are also certified in culture system (Gong et al. 2001; Moriya et al. 2001; Korenaga et al. 2005). In addition, they are also demonstrated to synergistically enhance the susceptibility of hepatocytes to carcinogen exposure, as displayed by the increased HCC incidence in these transgenic mice with treatment of alcohol, carbon tetrachloride or diethylnitrosamine (Machida et al. 2009a; Kato et al. 2003; Kamegaya et al. 2005). Accordingly, based on these epidemiological studies and mouse models, hepatic HCV infection is potential to directly induce hepatocarcinogenesis and coordinately leads to tumorigenic microenvironment with continuous live damage and hepatocyte replacement.

2.2 HCV Variants and HCC

Another clue for the concept that HCV itself acts as an independent risk factor for HCC development is the genotype difference in oncogenic potentials. The six HCV genotypes and related subtypes identified so far, which are mainly diverse in 30–35 % of nucleotides in the complete RNA genome, are distributed with geographic difference and possibly correlated with disease outcome (El-Serag 2012). In these HCV strains, genotype 1b which is the most common genotype diagnosed in the United States, Europe and Japan, is permanently correlated with higher HCC risk than any other genotypes, as statistically indicated by 1.8-fold of relative risk based on a meta-analysis of well-adjusted studies. This HCV strain-specific risk is more elevated to 2.5-fold in non-cirrhotic patients, further supporting the autonomous importance of HCV genotypes for HCC formation other than their promotive effects on cirrhosis (Raimondi et al. 2009).

Currently, the unique mechanism of HCV genotype 1b for rapid disease progression or hepatocarcinogenesis is still obscured and not directly defined, while some important point mutations in the core gene detected from this strain may provide one of possible molecular explanations. These missense mutations are observed in codon 71 and 90 of viral core protein translated and processed from HCV polyprotein, which are proved to be associated with failure of interferon treatment (Akuta et al. 2006, 2007a, 2009) and development of HCC (Kobayashi

et al. 2010; Nakamoto et al. 2010; Akuta et al. 2007b) in longitudinal studies from Japan. This could be theoretically caused by substitution of two amino acids (R70Q and L91M) for introducing an alternative translational system in addition to the traditional IRES, which would be helpful for viral replication and overcome cellular antiviral defenses for IRES shutdown.

On the other hand, the N-terminal deleted mini-core protein translated in this mutation-containing context may also be suspected to antagonize the interferon pathway, thus counteracting its antiviral and anti-proliferative activities (Ahmad et al. 2011). However, the causal relationship between HCV core mutation and HCC promotion remains uncertified and still warrants more investigations. Furthermore, the fact that majority of HCV-infected carriers do not proceed to cirrhosis or even HCC stages suggests host genetic factors and epigenetic responses are also prerequisite for disease control and progression. Therefore, with limitations in clinical follow-up studies, a more comprehensive and convenient HCV infection model is critical to determine candidate host factors and dissect these issues at molecular levels.

2.3 Alcohol and HCV-Related HCC

Alcoholism is another leading cause of HCC other than viral hepatitis (Morgan et al. 2004). In fact, chronic alcohol abuse (more than 80 g intake per day over 10 years) is an independent etiologic factor for HCC development, especially in areas where viral hepatitis is not endemic (Fassio et al. 2009, 2010; Hwang et al. 1996). It is believed for a long time that there is a pathological synergism between alcohol consumption and hepatitis C in hepatocarcinogenesis (Saito et al. 1990; Fattovich et al. 2004; Miyakawa et al. 1993; Takada et al. 1993). Actually, in HCV-infected population, the HCC risk is statistically elevated with approximately two to fourfold in heavy alcohol drinkers compared with teetotalers, which was fundamentally supported by many cross-sectional and case-control studies from different areas (Tsutsumi et al. 1996; Tanaka et al. 1991; Tagger et al. 1999; Donato et al. 2002; Yu et al. 1991; De Bac et al. 1994; El-Serag et al. 2001; Hassan et al. 2002). This synergy in HCC development was also confirmed in longitudinal studies (Aizawa et al. 2000; Ikeda et al. 1993). The alcohol use for the risk of HCV-related HCC is probably a dose-response effect (Tagger et al. 1999), as the odds ratio is not significant in light drinkers or populations without intake stratification (Mori et al. 2000; Sun et al. 2003). Besides, heavy alcohol consumption in CHC patients is associated with younger onset of HCC formation, higher likelihood of HCC progression and poorer survival rate after HCC resection (Tsutsumi et al. 1996; Kubo et al. 1997; Okada et al. 1996; Shimauchi et al. 2000; Miyakawa et al. 1994).

The abuse of alcohol drinking in HCV-infected patients not only influences HCC development at end stage, but also exacerbates liver diseases such as cirrhosis and even hepatitis C at early stage (Peters and Terrault 2002). For example, excessive

alcohol intake is significantly correlated with the two to fivefold increased risk of cirrhosis in HCV carriers (Khan and Yatsuhashi 2000; Harris et al. 2001; Corrao and Arico 1998), and its abuse indeed worsens the course of hepatitis C and accelerates liver disease progression (Poynard et al. 1997; Wiley et al. 1998; Bellentani et al. 1999; Cromie et al. 1996). In addition, heavy consumption of alcohol has been suggested to directly interfere the efficacy of interferon-based therapy for anti-HCV treatment and indirectly enhance HCV replication through immunosuppression, although these mechanisms remain unclear (Peters and Terrault 2002; McCartney and Beard 2010).

The promotive effect of alcohol on HCV-induced hepatocarcinogenesis has been further validated in animal models, by using transgenic mice which express HCV NS5A protein (Mercer 2011). In this study, HCC was only observed in the group receiving ethanol-containing diet after 1 year, but not in the group feeding with control diet. The synergistic effect between alcohol and HCV protein was approved to be mediated by toll-like receptor 4 (TLR4), as HCC tumors cannot be detected in alcohol-fed mice when TLR4 was knockout (Machida et al. 2009a). This TLR4-mediated carcinogenic event, stimulated by alcohol and HCV NS5A, may evoke the proliferation of hepatic progenitor cells. On the other hand, in HCV core-expressing transgenic mice, chronic alcohol application additively increased hepatic lipid peroxidation and synergistically enhanced cytokine secretion, such as tumor necrosis factor α (TNFα) and transforming growth factor β (TGFβ) (Perlemuter et al. 2003). Thus, the microenvironment with repeated inflammation and regeneration might be more easily established in the livers of HCV patients with alcohol abuse, which could be an oncogenic niche as one of possible mechanisms explaining their synergism in HCC development.

However, transgenic mouse models expressing single HCV proteins rather than complete HCV genomes may simplify the interactive effect of alcohol with natural HCV infection on HCC development. A more comprehensive experimental design like human liver-chimeric mice would be a expectable model to address the detailed mechanisms. Moreover, the controversy of alcohol intake at light level for HCV-related HCC risk, as well as the therapeutic consideration in CHC patients with alcoholism, are still open questions and warrant further investigations.

3 Genetics of HCV-Related HCC

In hepatocarcinogenesis, multiple genetic changes and epigenetic alterations are pathogenically involved and occurred in HCV-infected hepatocytes. Upon systemic approaches, a better understanding of the interaction between HCV and host genetics is helpful to dissect the critical points in HCC development.

3.1 Genetic Mutation and Polymorphism for HCC Susceptibility

In HCV-infected population, only few persons progress to cirrhosis and even HCC in end stages. On the contrary, family history of viral hepatitis-induced liver cancer is significantly associated with increased HCC risk, especially in first degree relatives (Hassan et al. 2009). These observations emphasize the regulatory involvement of host genetic backgrounds in HCV-associated disease progression and HCC formation. For example, the somatic mutation of *p53* gene, as the dominant master of tumor suppressors, is one of the most frequent event observed in HCC tumors (Hussain et al. 2007). In CHC, long-term oxidative stress and ROS production may induce cellular DNA damage, thus accumulating hotspot mutations in the *P53* gene and coordinating with other transforming potentials in persistent inflammatory hepatocytes for hepatocarcinogenesis. On the other hand, female sex, reactive immune responses, serum neutralizing antibodies and host genetics, have been confirmed in prospective studies to be associated with successful and spontaneous clearance of acute HCV infection (Hajarizadeh et al. 2013). One of well-characterized factors is the *IL28B* gene, whose genetic polymorphism located upstream of open reading frame is strongly correlated with spontaneous clearance of HCV in an interracial manner (Grebely et al. 2010; Tillmann et al. 2010; Thomas et al. 2009). *IL28B* encodes a type III interferon-λ3 and its polymorphic variant susceptible to viral clearance has already been proved to secret higher serum levels of interferon than other genotypes, possibly leading to more active antiviral responses for HCV eradication (Shi et al. 2012).

Other genetic factors such as TNFα and glutathione S-transferase (GST) are also reported as the risk predictors of HCV-related HCC development based on their single nucleotide polymorphisms (SNP) (El-Serag 2012). The promoter heterogeneity of TNFα gene at G-308A is recently believed to be an independent polymorphism correlated with HCC progression in meta-analysis (Qin et al. 2010; Guo et al. 2010; Yang et al. 2011). This association between the SNP of TNFα and hepatocarcinogenesis is also confirmed specifically in HCV-related cirrhosis patients but not alcohol-induced cirrhosis cases (Tarhuni et al. 2014), suggesting the impact of TNFα as a pro-inflammatory cytokine for HCC tumorigenesis in the HCV-induced inflammatory environment. Another example as a host predisposing factor for HCV-related HCC is the gene family of GST, which expresses lots of phase II isoenzymes responsible for hepatocellular detoxification by neutralizing oxidative byproducts and catalyzing carcinogenic xenobiotics. The genetic variants of GST family, especially for their enzymatic-dead genotypes found in class μ1 (GSTM1) and class θ1 (GSTT1), are modestly associated with the increased risk of HCC in HCV cirrhotic patients in several case-control studies reported from India and Egypt (Asim et al. 2010; Abd El-Moneim et al. 2008; Sarma et al. 2012). This mild but significant correlation with 1.3-fold of odds ratio is further verified by a meta-analysis (Wang et al. 2010), indicating the participation of metabolic stress and catalytic capacity in HCV-infected liver tissues for HCC development.

Recently, there are some novel genetic polymorphism candidates identified in genome-wide association studies (GWAS) which are specifically associated with HCV-induced HCC, including the loci mapped in epidermal growth factor (*EGF*) gene on chromosome 4, MHC class I polypeptide-related sequence A (*MICA*) gene on chromosome 6 and *DEPDC5* gene on chromosome 22 (Abu Dayyeh et al. 2011; Miki et al. 2011; Kumar et al. 2011). Their biological functions are either characterized or completely unknown, but their specific oncogenic potentials in HCV-infected hepatocytes, as well as TNFα and GST described above, remains unclear and requires more detailed investigations.

3.2 Profiles of Epigenetic Alterations in HCV-Related HCC

In addition to genetic mutations and polymorphisms, epigenetic modulation of host gene expression has been systemically considered as a fundamental field of HCV-related cancer development and progression. Specific expression profile of cellular genes in clinical HCV-infected liver tissues is observed in a cDNA microarray study, which are differential from HBV-infected or normal cases, especially for those genes involved in anti-apoptotic effects, cell cycle acceleration, and extracellular matrix maintenance (Honda et al. 2001). These epigenetic alternations specifically evoked by HCV infection are further determined in isolated hepatocytes, excluding the confounding effects from infiltrating lymphocytes and thus highlighting the role of HCV as an epigenetic modulator for hepatocarcinogenesis (Honda et al. 2006). Indeed, this virus-induced epigenetic change such as the gradually increased promoter methylation status of some tumor suppressor genes from hepatitis to HCC, is reported in Egyptian and Japan case-control studies (Zekri Ael et al. 2013; Nishida et al. 2008). It supports the epigenetic imprinting concept that the DNA methylation signatures with oncogenic potentials could be early events accumulated continuously from hepatitis stages for HCV-associated disease progression. From the virologic view for HCV evolving to adapt in hepatocytes thoroughly, manipulation of host gene expression profile which drives cell cycle and resist to apoptosis is believed to establish a cellular environment favored for viral gene expression, replication or even against defensive immune surveillance (McGivern and Lemon 2011). However, this selfish situation would be oncogenically malicious for hepatocyte transformation in chronic infection, as well as persistent hepatic inflammation.

In general, cellular epigenetic modifications include DNA methylation, post-translational modifications of histone proteins, chromatin remodeling, and noncoding RNAs at post-transcriptional level, which are four major types at molecular layers (Rongrui et al. 2014). Infection of HCV is evidentially associated with hypermethylation status in preferential cellular genes responsible for DNA mismatch repair, cell cycle arrest and cytokine signaling control, which is well characterized in clinical case-control specimens, experimentally reproducible in culture system and clearly confirmed in human hepatocyte chimeric mouse models

with HCV infection (Zekri Ael et al. 2013; Nishida et al. 2008; Rongrui et al. 2014; Hinrichsen et al. 2014; Okamoto et al. 2014; Quan et al. 2014; Formeister et al. 2010). Actually, HCV core protein is proved in cultured cells to enhance DNA methyltransferases expression at transcriptional level (Benegiamo et al. 2012). It would be reflected to the alteration of gene expression such as *p16*, *RB*, and *E2F*, providing one of possible explanations for dysregulation of cell cycle in HCV-infected cells (Machida et al. 2009b; Lim et al. 2012). Besides, protein phosphatase 2A induced by HCV proteins, as confirmed in cell culture and HCV transgenic mice, probably inhibits acetylation and phosphorylation of histone proteins which may epigenetically contribute to HCC development (Duong et al. 2010, 2014). Lately, the inactivating mutations of somatic *ARID2* gene, which encodes a modifier of chromatin remodeling, are specifically identified in HCV-related HCC patients but not HCC cases with other etiologies (Li et al. 2011). Although the reason why this gene selected to be nullified and how it achieved are still unknown, this study still supports the inevitable role of epigenetic changes in HCV-associated HCC development.

On the other hand, cellular gene expression can also be post-transcriptionally regulated by RNA interference, thus implying the decisive role of microRNAs in epigenetic profiles. One of well-recognized microRNAs involved in HCV pathogenesis is miR-122, which is uniquely and abundantly expressed in adult hepatocytes for control of numerous hepatocyte-specific genes and promotion of hepatocellular differentiation (Elmen et al. 2008; Krutzfeldt et al. 2005; Chang et al. 2004). It is believed that miR-122 functions as a tumor suppressor, because knockout mice lacking its expression are highly susceptible to HCC development (Tsai et al. 2012; Hsu et al. 2012), as well as its amount significantly reduced in majority of HCC patients, including HCV-related cases (Ura et al. 2009; Gramantieri et al. 2007; Kutay et al. 2006). However, it is an interesting dilemma that miR-122 is also an essential factor for HCV replication, as comprehensively approved in nonpermissive cell lines with its overexpression for HCV replication (Jangra et al. 2010; Jopling et al. 2005; Fukuhara et al. 2012), in HCV infection chimpanzee models with its antagomir for suppression of viral replication (Lanford et al. 2010) and in phase II clinical trial with its sequestration for decreased viremia (Janssen et al. 2013). Apparently, the decrement of miR-122 in HCV-induced HCC specimens broadly influences the epigenetic balance in HCC cells, possibly as one of molecular events coordinated in hepatocarcinogenesis. While it would impair its life cycle under this circumstance as well, since HCV replication is barely detected in HCC sections compared with adjacent tissues (Arzumanyan et al. 2013). Little is known about why HCV initially forces infected hepatocytes into its favored environment but eventually leads to host cell transformation which is gradually unsuitable for its replication. This puzzle remains unsolved and will be asked whether and how HCV infection directly leads to the downregulation of miR-122 expression. One of possibility is the enforced recruitment of miR-122 to HCV replication complex may passively interfere its regulatory effect on cellular genes (Shlomai et al. 2014), probably disrupting the epigenetic network for feedback maintenance of its expression amount.

4 Identified Mechanisms of HCV Proteins in Hepatocarcinogenesis

So far, in addition to genetic mutations and epigenetic alterations predisposed in CHC phase, malignant transformation of hepatocyte could be also stimulated by HCV viral proteins such as core and NS5A, as it has been experimentally confirmed in transgenic mouse models which are absolutely devoid of hepatic inflammation as discussed above. The oncogenic potentials of these viral proteins have been attributed to generation of oxidative stress, dysregulation of hepatocyte turnover and induction of metabolic abnormality (Jeong et al. 2012). These HCV-induced diverse events in liver tissues for hepatocarcinogenesis are reviewed in the following paragraphs.

4.1 HCV and Oxidative Stress

Unusual augmentation of cellular oxidative stress in liver tissues is one of the notable features in CHC (Shlomai et al. 2014), which seems to be more easily occurred in HCV patients than in other hepatitis cases with different etiologies (Farinati et al. 1995). Not just via infection-induced inflammation, HCV itself is capable of promoting oxidative stress aberrantly through its core protein and leading to the elevated ROS level in hepatocytes, as comprehensively verified in different cell lines, independent HCV transgenic mouse lineages and clinical case-control studies (Moriya et al. 2001; Korenaga et al. 2005; Farinati et al. 1995; Okuda et al. 2002). The overproduced ROS is thought to be deleterious to cause host cell DNA damage and genetic mutations, thus fundamentally contributing to the multistep of hepatocarcinogenesis. In this pathogenic process, oxidative stress is initiated by HCV core protein targeting to cytoplasmic mitochondria, resulting in the disturbance of mitochondrial membrane function and generating reactive free radicals subsequently. At molecular level, one of characterized mechanisms is that HCV core interacts with prohibitin, and thus interferes its chaperone activity which is required for maintaining the redox potentials of protein complexes in mitochondrial electron transfer system (Fujinaga et al. 2011).

Besides, in addition to the forced generation of oxidative stress by HCV, the cellular antioxidant system responded to this abnormality is simultaneously reported to be compromised by viral core protein, especially for some key antioxidant such as heme oxygenase 1 (HO-1) and NADH dehydrogenase quinone 1 (NDQ-1) (Moriya et al. 2010). They are very central to take charge of ROS management in iron-overloaded liver tissues, since they are inducible cytoprotective enzymes to specifically ameliorate oxidative stress aggravated by heavy storage of hepatic ion (Liao et al. 2013). Intriguingly, the iron accumulation is clearly observed in HCV transgenic mice and CHC patients (Farinati et al. 1995; Moriya et al. 2010), while HCV core protein evilly decreases their protein

expressions through unidentified mechanisms. Hence, hepatic HCV infection not only stimulates ROS production but also antagonizes specific antioxidant expression, synergistically exacerbating oxidative stress that would facilitate HCC development. One of the reasons why HCV represses the expression of HO-1 antioxidant is probably to overcome its well-studied inhibitory effect on viral replication (Zhu et al. 2008; Shan et al. 2007). This issue highlights the protective role of HO-1 in HCV-infected hepatocytes and warrants in-depth investigations for such viral adaption-related carcinogenesis.

4.2 HCV and Hepatocyte Cell Growth

After HCV infection, virus-induced dysregulation of cell cycle and interference of immune defense are believed to compel cellular machinery to efficiently support viral RNA replication and trickily evade host surveillance in infected hepatocytes at present. Multiple HCV encoded proteins are observed to evolutionally fulfill such virologic effects through manipulating host signaling pathways, which often inactivate tumor suppressors and accelerate cell growth, coincidentally initiating transforming potentials in chronic inflammatory stages.

For example, HCV NS5B protein recruits RB in its cytoplasmic complex, leading to its ubiquitination and then protein degradation (Munakata et al. 2005, 2007). On the other hand, expression of core protein decreases the RB gene expression at transcriptional level (Machida et al. 2009b). These RB-targeting events would relieve E2F from RB restriction for G1 to S phase transition of cell cycle, as well as impairing RB-mediated DNA damage responses. In the meanwhile, NS5A is able to interact with the protein SH3 domain that are found in many signaling molecules like the adaptor protein Grb2, the p85 subunit of phosphoinositide 3-kinase (PI3K) and some members of the Src kinase family (Street et al. 2004; Macdonald et al. 2004; Tan et al. 1999). These signaling pathways responsible for cell growth and anti-apoptosis are accordingly dysregulated by HCV infection, which may substantially favor viral replication but potentially stimulate cell transformation at long term. In addition, a cellular RNA helicase DDX3 is proved to be hijacked by HCV proteins, possibly core or NS3/4A, for essentially serving viral RNA replication (Owsianka and Patel 1999; Ariumi et al. 2007; Chatel-Chaix et al. 2013). This molecular kidnapping redistributes the cellular localization of DDX3 and is considered to obstruct its original functions such as upregulation of *p21* gene expression for cell cycle control and induction of interferon secretion for defensive responses (Oshiumi et al. 2010; Schroder et al. 2008; Chao et al. 2006).

To maintain viral persistence, HCV NS3/4A also targets host alert system for sensing double stranded RNA, including Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I) pathways. The viral protease is reported to hamper the activation of these signaling cascades by cleavage of their adaptor molecules, Toll-IL-1 receptor domain-containing adaptor inducing IFNβ (TRIF) and IFNβ

promoter stimulator 1 (IPS-1), respectively. It would compromise the activation of interferon regulatory factor 3 (IRF-3) and thus reduce the production of IFNβ (Wang et al. 2009b; Meylan et al. 2005; Li et al. 2005). Besides, NS3/4A may sustain viral replication by targeting Ataxia telangiectasia mutated kinase (ATM) and checkpoint kinase 2 (Chk2) to blunt their sensing activity for DNA damage (Ariumi et al. 2008). The aberrant activation of Wnt/β-catenin pathway and counteraction of p53 tumor suppressor function are proved in HCV-infected hepatocytes as well, suggesting the transforming potentials initiated and promoted in CHC (Park et al. 2009; Milward et al. 2010; Kao et al. 2004).

Take together, to thoroughly facilitate HCV replication in hepatocytes and sustain its infection, viral proteins notoriously accelerate cell cycle but impair DNA damage response and host innate immunity. These viral events are usually accompanied by dysregulated cell growth, leading to a long-term risk of hepatocarcinogenesis. However, the current knowledge based on these molecular studies is often derived from the overexpression system established in hepatoma cell lines, and therefore it should be interpreted exclusively and carefully. A more authentic model supporting HCV infection and even HCC development is required to clarify these molecular issues conclusively.

4.3 HCV and Insulin Resistance

Another characteristic of CHC is accompanied by metabolic liver disorders, especially type II diabetes (T2D). As well as cirrhosis, T2D is also evident to be an independent risk factor for HCC development, with approximate 2.5-fold risk ratio reported in several meta-analysis studies (Wang et al. 2012a, b; El-Serag et al. 2006). Recently, non-cirrhotic HCV-infected individuals have been approved to be a risky population predisposed to T2DM, as the adjusted odds ratio closed to twofold compared with HBV-infected persons in community-based study and meta-analysis study (Wang et al. 2007; White et al. 2008). This correlation is also confirmed in transplanted liver tissues with HCV positivity (Delgado-Borrego et al. 2004).

HCV-induced insulin resistance in hepatocytes is lately believed to be a basic virologic effect for explaining the direct role of HCV in T2DM development in liver tissues (Bose and Ray 2014). This viral infection-related glucose intolerance has been attributed to the impairment of hepatic insulin pathway, but not the deficiency of pancreatic insulin secretion (Lecube et al. 2004). The detailed mechanism targeting the insulin signaling pathway is not completely understood, possibly elicited by HCV core or other NS proteins that interferes phosphorylation status of signaling molecules or degrade adaptor proteins to shutdown the signal delivery (Lonardo et al. 2009). Because of insensitive glucose uptake and then dysregulated lipid metabolism, the pathological outcome of insulin resistance evoked by HCV somewhat resembles steatosis. This close and mutual link between steatosis and insulin resistance prompts hepatitis C to belong to a kind of virus-associated

steatohepatitis, based on the metabolic aspect. While HCV also disturbs expression of cellular genes and interferes transduction of signaling pathways, it may explain why the incidence of HCC is higher in CHC compared with that in nonalcoholic steatohepatitis (Koike and Moriya 2005). Similarly, together with other well-known predisposing factors for diabetes development, including obesity, hypertension, family history, and other metabolic syndromes, HCV-infected carriers are thus more susceptible to develop diabetes than other hepatitis patients, which could be a predictable disease progression coordinated with cirrhosis for HCV-related hepatocarcinogenesis.

Given that HCV is an RNA virus with quasispecies and various genotypes, the severity of insulin resistance induced by different viral strains and diabetes thereafter is not comparably analyzed and needed to be addressed, which could be one of genotype-dependent mechanisms for the disparity of HCC risk. In addition, synergistically with HCV infection, T2DM is observed to promote HCC formation, although the detailed crosstalk mechanisms are not fully confirmed (El-Serag et al. 2001; Hassan et al. 2002). Therefore, according to the metabolism-related essence of HCV as previously determined, therapeutic approaches targeting the abnormality of insulin resistance could be a novel concept to develop newly antiviral drugs, albeit possible side effects and the beneficial controversy of attaining SVR for prevention of T2DM (Arase et al. 2009; Giordanino et al. 2008; Simo et al. 2006).

5 Role of Sex Hormones in HCV-Induced HCC Development

The male dominance of HCC incidence is certainly an important issue observed for a long time, which is commonly reported worldwide, especially in virus hepatitis endemic areas (El-Serag 2012). This sex disparity is definitely noticeable in HCV-related HCC population, although the male to female ratio is lower than that in HBV-related cases (Lee et al. 1999). The male gender as a risk factor is faithfully reflected to disease progression, from early hepatitis to cirrhosis and then HCC development (Poynard et al. 2001; Chu et al. 1983). In recent years, HBV is well-identified as a sex hormone responsive virus at transcriptional level, thereby providing one of probable explanations based on basic virology for the disparity of HCC development in HBV patients (Tong 2012). Compared with HBV, a DNA virus which could be regulated by sex hormone pathways at viral mRNA level (Wang et al. 2009c, 2012c), some clues also point out that HCV infection is actually correlated with male and female sex hormone pathways for regulation of viral replication and host gene expression, as discussed in following sections.

5.1 Estrogen Axis and HCV-Related HCC

Based on the epidemiological study, female sex hormone estrogen is believed to play a protective role for decreasing HCC risk, either in HCV or in HBV-infected women (Yu et al. 2003). Exposure of estrogen is proved to be associated with a beneficial impact on hepatic fibrosis in female HCV patients, as the disease progression is accelerated after menopause but prevented by estrogen supplementation or delayed by pregnancy experiences (Campbell et al. 2005; Di Martino et al. 2004). One of possible mechanisms is that estrogen inhibits interleukin-6 secretion from Kupffer cells which could ameliorate cytokine-induced hepatic inflammation, hence indirectly reducing the risk of HCC development at long term (Naugler et al. 2007). On the other hand, the antiviral response to IFNα treatment for HCV eradication is also better and easier to achieve SVR level in pre-menopausal women than in men, as well as applied by combinatorial therapy with ribavirin (Sezaki et al. 2009; Hayashi et al. 1998). It is supported by clinical studies that the HCV clearance rate is undoubtedly higher in females than in males (Bakr et al. 2006; Seeff 2002). Besides, the polymorphism of *ESR1* which encodes estrogen receptor α (ERα) is analyzed to be associated with the outcomes of HCV infection in a Chinese population study (Tang et al. 2014), as well as previously proved in HBV patients (Yan et al. 2011; Zhai et al. 2006; Deng et al. 2004). Moreover, a microRNA called miR-18a targeting ERα transcript is significantly elevated in female HCC tissues compared with adjacent non-tumor sections, which is occurred in both HCV and HBV-related patients but not in focal nodule hyperplasia or adenoma cases, thereby highlighting hepatic ERα as a protector from virus-induced HCC formation (Liu et al. 2009). Therefore, these studies provide evidences indicating the involvement of estrogen-ERα axis in virus-induced liver diseases, although the molecular events are probably distinct in HCV and HBV.

In a culture-based study, 17β-estradiol, the most potent estrogen, effectively inhibits production of HCV infectious particles but not viral RNA amount, depending on a high concentration equivalent to pregnant level. This repressive effect seems to be mediated by ERα due to the failure treatment of ER antagonists (Hayashida et al. 2010). However, another in vitro study reported that ERα is crucial to promote the participation of HCV RNA polymerase NS5B in viral replication complex, while it is blocked by anti-estrogens (Watashi et al. 2007). While phytoestrogens such as coumestan derivatives are identified to be a novel series of NS5B-targeting inhibitors, with developing potential as a kind of non-nucleoside drugs (Kaushik-Basu et al. 2008). Based on these studies, the role of estrogen in HCV replication is still an controversial issue, which could be caused by impure agonists and antagonists with confused and undefined profiles of estrogen activity (Journe et al. 2008; Grilli 2006), whereas the druggable potential of estrogen-ERα axis is definitely attractive for development of novel antiviral therapies. This proof of concept is tested by selective estrogen receptor modulators and confirmed that multistep of HCV life cycle is actually influenced (Murakami et al. 2013). So, in addition to immune modulation, the regulatory effect of hepatic

estrogen pathway on HCV replication could be another instinctive mechanism for explaining the sex disparity of HCC development, while it should be further verified in a more realistic model system rather than cell culture.

5.2 Androgen Axis and HCV-Related HCC

Fewer studies have addressed the role of androgen pathway in HCV-related hepatocarcinogenesis. So far, a cross-sectional study indicates that total testosterone concentration in serum is correlated with increased risks of advanced fibrosis and active inflammation in hepatic tissues of males with CHC, which is defined as a dose-response association and thus suggesting the importance of androgen for the male dominance in the severity and progression of HCV-induced liver diseases (White et al. 2012). However, the representation of total testosterone level as the risk indicator may be somewhat inaccurate, possibly due to the variant availability of free form or the secondary effect derived from estrogen which is just biosynthesized behind androgen. Moreover, the association of serum androgen level with HCC occurrence is not systemically evaluated in this study, so the direct link between androgen and HCV-related HCC is not clearly defined and it should be given close attention as next issues.

In 2008, a culture-based research provides one possible mechanism of androgen pathway as a stimulatory role in HCV-associated hepatocarcinogenesis. In this study, HCV core protein alone or in genome context is proved to enhance androgen receptor (AR) activity in an androgen-dependent manner. This aberrant activation of AR leads to the gene expression of vascular endothelial growth factor, which is potential for hepatocyte transformation (Kanda et al. 2008). Notably, hepatic AR is recently identified to be an HCC promoter, as experimentally verified in chemically induced HCC mouse models (Ma et al. 2008). As well as the oncogenic effect of HBV X protein on AR activation (Yang et al. 2009; Chiu et al. 2007), the positive role of HCV core protein in androgen axis supports that male sex hormone pathway is central to virus-induced hepatocarcinogenesis. In vivo evidences such as transgenic mice is required to further confirm the regulatory effect of HCV core on AR pathway.

6 Conclusion and Prospective

HCV-related HCC is a complex malignancy progressed from preneoplastic lesions with heterogeneous etiologies in persistent inflammatory liver tissues. Other than chronic hepatitis, HCV itself is considered to be a virologic risk factor for HCC and sufficient to induce hepatocarcinogenesis even in the absence of inflammation, as confidentially evident in HCV transgenic mouse models. Synergistically with chronic HCV infection, alcohol abuse is a well-known costimulatory factor for

HCC development. The oncogenic potential of HCV is achieved by its core and other NS proteins, which could maliciously lead to induction of oxidative stress, impairment of DNA damage response, takeover of cell cycle control, and generation of insulin resistance at cellular level. Started from CHC, these long-term exposures are believed to cause DNA mutations accumulated in the genome of hepatocytes which continuously suffer from inflammatory injury and regeneration repair. Coordinated with epigenetic changes evoked by HCV infection, the predisposing polymorphisms or genetic mutations in critical proto-oncogenes and tumor suppressor genes would potentially transform hepatocyte for HCC tumor formation. Together with other independent risk factors such as diabetes and cirrhosis, which can be derived from HCV infection as well, HCV-induced HCC development is etiologically complicated with many undissected problems.

The in-depth delineation of HCV-induced hepatocarcinogenesis is helpful to develop newly strategies for HCC prevention, diagnosis and treatment. So a more convenient and comprehensive model for HCV infection, rather than hepatoma culture system with overexpression of viral proteins far above virologic levels, is an urgent issue to further investigate many HCV topics. For example, the chimeric mice transplanted with humanized hepatocytes is well-adaptable to temporarily study HCV infection, host immune responses and cellular stresses, although their life-span may be limited for thoroughly studying virus-induced hepatocarcinogenesis. Other issues like the disparity of HCV genotypes in disease progression and the definite roles of androgen and estrogen pathways in clinical contribution are still uncertain and warrants more detailed researches. Besides, HCV infection is evident to probably induce metabolic abnormality in addition to hepatitis, directly implying a non-traditional concept for antiviral therapies. Therefore, to completely achieve SVR in HCV-infected population, which is by far a more effective and economic strategy for HCC prevention, searching for novel therapeutic targets such as insulin pathway or ERα to alternatively inhibit HCV life cycle is important for future development of antiviral drugs in next generation.

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HCV, Alcohol, and the Liver

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Abstract Alcoholic liver disease (ALD) and hepatitis C virus (HCV) infection together account for the greatest causes of liver disease in patients worldwide. It is estimated that about 140 million and 170 million individuals worldwide suffer from alcohol dependence or chronic HCV infection respectively. ALD and HCV infection, independently, if not treated properly can progress to advanced liver disease including fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC). Their synergism, however, has been shown to dramatically exacerbate the rapid progression of liver disease to HCC. Several molecular mechanisms representing synergistic interactions of HCV and alcohol that accelerate the progression of liver disease have been identified including immune, metabolic, oxidative stress, proteomic and epigenetic modulations of target host systems. In this review, we summarize current advances in understanding of the various molecular cellular mechanisms by which HCV synergizes with alcohol to advance liver disease. Given the limited treatments available for HCV-infected patients who use alcohol, we also highlight new therapeutic targets and areas where more research would enhance our understanding of the pathophysiology of this disease. At this point in time, we recommend that HCV-infected patients should abstain from drinking alcohol and that those who abuse alcohol should be encouraged to stop alcohol use and be tested for HCV infection.

Keywords Chronic hepatitis C • Alcoholic liver disease • Steatohepatitis • Fibrosis • Reactive oxygen species • Exosomes • MicroRNA • Hepatocellular carcinoma • Endotoxins • Stress

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1 Introduction

Alcoholic liver disease (ALD) and hepatitis C virus (HCV) infection together account for the most frequent causes of liver disease in both developed and less developed countries. It is estimated that over 170 million people worldwide are currently infected with HCV which establishes chronic infection in about 50–80 % of infected individuals (Bartenschlager et al. 2011; Levrero 2006). The World Health Organization (WHO) estimates that there are over 140 million people who use excessive alcohol despite its negative consequences for predisposition to alcoholic liver disease (ALD), alcoholic hepatitis, liver fibrosis and HCC (Frazier et al. 2011; O'Shea et al. 2010; Szabo and Lippai 2012). While HCV and alcohol are independent risk factors for the development of liver disease, their dual occurrence in patients dramatically increases the likelihood of HCC development to 47.8 % from 8.6 % in patients with either HCV infection or alcohol abuse alone (Koike et al. 2008; Machida 2010). It is estimated that about 30 % of alcoholics[1] are currently infected with HCV (Rosman et al. 1996) and about 70 % of HCV infected patients have a history of alcohol abuse (Schiff 1999). The molecular mechanisms of how HCV and alcohol individually modulate liver disease have multiple overlaps and their synergistic interactions have been shown to enhance adverse molecular signaling mechanisms that accelerate liver damage, fibrosis and HCC development.

HCV and alcohol can modulate molecular signaling and host defenses through immune, proteomic, cell proliferation and regeneration, metabolic and epigenetic pathways (Stickel et al. 2002). Chronic alcohol consumption has been shown to change the composition of the gut microbiome and induce increased gut permeability that allows the translocation of gut-derived bacterial components to the liver (Enomoto et al. 2001; Garcia-Tsao and Wiest 2004; Wigg et al. 2001) leading to increased immune cell activation and inflammatory cytokine production (Wigg et al. 2001; Petrasek et al. 2010; Purohit et al. 2008; Szabo and Bala 2010). Additionally, chronic alcohol use and its metabolites induce hepatic oxidative stress (Petrasek et al. 2010; Purohit et al. 2008; Szabo and Bala 2010). HCV chronic infection can independently induce liver injury and inflammation by the direct effect of HCV proteins or its modulatory role on host cell signaling. Additionally, HCV infection modulates both host innate and adaptive immunity to establish chronic infection, induces direct hepatocyte damage, and promotes deregulated release of inflammatory cytokines. Together, HCV and alcohol use can create a sustained hepatic inflammatory condition leading to irreversible fibrosis with an

[1] Alcoholics can be defined loosely as heavy drinkers. The Substance Abuse and Mental Health Services Administration (SAMHSA) of the U.S. Department of Health and Human Services defines heavy drinking as drinking five or more drinks on the same occasion on each of 5 or more days in the past 30 days. 'Alcoholics' in this study were those who were admitted for detoxification.

increased risk of cirrhosis and in some cases liver cancer (Koike et al. 2008; Szabo et al. 2006).

In this review, we will describe recent advances in understanding the synergistic impact of combined alcohol abuse and HCV infection on the progression of liver disease, viral replication, host immune response, and response to anti-HCV treatments. We will also discuss new research findings that could serve as treatment targets and highlight areas where more research is needed to enhance our understanding of the molecular mechanisms by which HCV infection and alcohol use cause liver disease.

2 HCV and Alcohol Effects on HCV Replication and Hepatocytes

2.1 HCV and Alcohol Synergize to Induce Hepatocyte Death

A prominent feature of both chronic HCV infection and chronic alcoholic liver disease is the induction of sustained hepatic injury leading to programmed cell death of both parenchymal and non-parenchymal cells. Programmed cell death (apoptosis) can be mediated by two distinct pathways; the mitochondrial (intrinsic) and death receptor (extrinsic) mediated pathways, both of which occur during HCV infection and alcoholic liver disease. Apoptosis is an energy-dependent process that is deregulated during HCV infection or chronic alcohol use independently and dramatically exacerbated when both exist together in patients. In a chronic HCV-infected patient with associated alcohol abuse, hepatic apoptosis mediated by multiple deregulated signaling pathways is characterized by sustained and high levels of pro-inflammatory cytokine production, oxidative stress, mitochondrial dysfunction, steatohepatitis, and alterations in iron metabolism.

While the inflammatory and metabolic mechanisms by which alcohol can enhance HCV disease progression have received extensive research interest, it is now becoming evident that up-regulated programmed cell death pathways (apoptosis) also play a crucial role in HCV-infected hepatocytes in the presence of alcohol. Generally, it is widely believed that physiologic levels of alcohol or HCV infection per se do not have a direct effect in inducing hepatocyte cell death. Reports have shown that hepatocyte cell death during HCV infection independently or synergistically with alcohol is due to the host's immune response required for viral clearance or the exacerbated immune activation due to increased gut-derived LPS as a consequence of alcohol abuse. Through sustained immune response geared towards clearing HCV infection or alcohol-induced microbial products in the liver, activated cytotoxic T lymphocytes and natural killer (NK) cells can induce hepatocyte cell death (Izumi et al. 1983; Poralla et al. 1984; Laso et al. 2010; Maini et al. 2000; Cerny and Chisari 1999; Wang and Weinman 2013). Additionally, HCV infection or chronic alcohol use decreases expression of Bcl-2 protein, a proto-

oncogene located on chromosome 18 which can mediate the suppression of apoptosis by preventing activation of caspases (Nakamoto et al. 2002; Kountouras et al. 2003; Tsamandas et al. 2003). Clinical studies have demonstrated that heavy alcohol use is usually associated with high rates of hepatocyte apoptosis and very low levels of Bcl-2 protein expression (Pianko et al. 2000). Given that both alcohol and HCV independently induce hepatocyte cell death in a Bcl-2 dependent fashion it would not be surprising if HCV-infected alcoholics show higher hepatocyte cell death compared with alcoholics or patients having just HCV infection.

2.2 Hepatic Oxidative Stress Induced by HCV and Alcohol in Liver Disease

Oxidative stress has been shown to be a key mechanism by which alcohol and HCV infection either independently or together can enhance or exacerbate the progression of liver disease. HCV infection or alcohol abuse can independently cause oxidative stress characterized by excess production of free radicals and reactive oxygen species (Moriya et al. 2001; Dey and Cederbaum 2006; Szabo et al. 2010; Loguercio and Federico 2003). Numerous studies have advanced diverse mechanisms by which alcohol and HCV either independently or in concert can increase oxidative stress. Most importantly, the breakdown of alcohol in the liver involves chemical oxidation reactions defined by the addition of oxygen, the removal of hydrogen, or both, mainly by cytochrome P450 2E1 (CYP2E1) (Lieber 2005). Chronic alcohol consumption significantly increases the activity of the alcohol-oxidizing enzyme cytochrome P-450 (Nanji et al. 1994; Takahashi et al. 1993). Ethanol is first converted by alcohol dehydrogenase to acetaldehyde by removing hydrogen. Then, a second enzyme, acetaldehyde dehydrogenase converts acetaldehyde to acetate by removing additional hydrogen and adding oxygen. The second enzyme system is mainly activated following heavy alcohol consumption. This second enzymatic reaction sometimes generates not only stable, non-toxic molecules but also highly unstable and potentially harmful molecules, called free radicals. Many of these molecules contain oxygen and are called reactive oxygen species (ROS) or oxygen radicals. These include superoxide ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$). The presence of excess levels of oxygen free radicals leads to oxidative stress given that natural host antioxidants including vitamin E, vitamin C, and glutathione (GSH) cannot readily prevent or neutralize free radical formation induced during high and chronic alcohol use. High levels of oxygen radicals associated with chronic alcohol use can cause significant hepatocyte damage, which in turn, drives and contributes to the development of hepatic inflammation and fibrosis. Independent of alcohol, chronic HCV infection has been shown to induce endoplasmic and mitochondrial cellular stress leading to the production of ROS and nitric oxide (NO) (Bailey 2003).

While numerous studies have tried to decipher the molecular mechanisms responsible for induction of cellular stress during HCV infection with and without associated alcohol abuse, we are still far from understanding the entire molecular process. The development of new mouse models expressing some HCV proteins have been key in understanding the mechanism of hepatic stress induction during HCV infection with and without associated alcohol abuse (Moriya et al. 1998, 2001; Korenaga et al. 2005a, b; Mas et al. 2010; Okuda et al. 2002). When HCV core protein was expressed in mice, it was observed that the HCV core protein could induce significant reactive oxygen species (ROS) leading to substantial mitochondrial DNA damage when combined with alcohol (Moriya et al. 2001; Okuda et al. 2002; Dionisio et al. 2009; Otani et al. 2005). Studies have shown that HCV core protein associates with cellular organelles including the endoplasmic reticulum (ER) and mitochondria. In HCV core expression systems this viral protein induces mitochondrial stress and ER stress leading to the release of Ca2+ from these organelles (Benali-Furet et al. 2005; Li et al. 2007; Tardif et al. 2005; Piccoli et al. 2007). In addition to HCV core protein, HCV NS3/NS4a proteins have also been shown to induce ROS production via a mitochondria-dependent binding process (Machida et al. 2006).

In response to increased access of pathogen-associated molecular patterns (PAMPs) to the liver and the effect of HCV, hepatic immune cells are activated in an attempt to mount an effective immune response to maintain hepatic homeostasis. This process also results in the generation of ROS in the liver by Kupffer cells and hepatic neutrophils. High levels of ROS can have significant modulatory effects on cellular proteasome functional activities which are crucial for mounting an effective anti-viral immune response or mediating clearance of infected/damaged hepatocytes associated with HCV infection combined with alcohol use (Osna et al. 2014).

2.3 Host Protein and miRNA Modulation by HCV and Alcohol During Liver Disease

Numerous studies including those from our group have shown that alcohol synergizes with HCV infection to accelerate the development or progression of liver disease by diverse mechanisms (Szabo et al. 2006, 2015; Osna et al. 2014; Seronello et al. 2010).

HCV infection and alcohol abuse can modulate the development of liver disease by differential regulation of host protein expression as well as host proteasome activity. Modulation of host protein expression or proteasome degradation by HCV and alcohol can lead to increases or decreases in protein expression that can potentially enhance the progression of liver disease. As obligate intracellular pathogens, viruses like HCV use cellular proteins to their advantage to sustain replication and maintain their presence in a target host. We and others have shown

that alcohol can modulate HCV replication by multiple mechanisms including modulation of expression of host proteins (Osna et al. 2014; Zhang et al. 2013; McCartney et al. 2008; Bukong et al. 2013; Hou et al. 2013). While there are conflicting findings as to direct role of alcohol in modulating HCV replication in-vitro, we and others have recently showed that alcohol can increase HCV replication through alcohol metabolites (McCartney et al. 2008), increased expression of GW812 (Bukong et al. 2013) or HSP90 (Bukong et al. 2013), or decreased cyclinG1 expression (Hou et al. 2013). Thus, in vitro studies show that alcohol and alcohol metabolites increase HCV replication (Seronello et al. 2010; McCartney et al. 2008; Bukong et al. 2013; Hou et al. 2013). In the absence of alcohol, we recently showed that HCV infection can also modulate cellular expression of cytoskeletal proteins Ezrin, Moesin and Radixin during infection (Bukong et al. 2013). We found that HCV engagement of CD81 induced Ezrin phosphorylation by way of spleen tyrosine kinase (Syk), which was crucial for virus entry and infection of hepatocytes. Additionally, we found that chronic HCV infection of hepatocytes significantly decreased Moesin and Radixin expression leading to increased microtubule aggregate formation. Formation of these microtubule aggregates suggests that regulation of these cytoskeletal proteins may be a mechanism utilized by HCV to create rail tracts facilitating its cellular trafficking for effective infection and replication in hepatocytes (Bukong et al. 2013). The regulation of these cytoskeletal proteins during alcohol abuse, independently and in association with HCV infection, is currently being investigated by our group.

While the role of alcohol in modulating HCV replication remains controversial, our findings and those by others showing this positive correlation are somehow consistent with clinical observations demonstrating adverse and rapid liver disease progression in HCV infected patients who drink alcohol.

2.3.1 Modulation of HCV Replication by Alcohol via HSP90

Heat-shock protein 90 (HSP90) is an important mediator of HCV disease progression associated with alcohol abuse (Bukong et al. 2013). HSP90 is a 90KD protein that is an evolutionarily-conserved chaperone protein critical for modulating proper folding of newly synthesized protein in concert with other co-chaperone proteins. HSP90 is significantly unregulated at both mRNA and protein levels during hepatic stress conditions associated with alcohol liver disease or HCV infection, either independently or in association (Bukong et al. 2013; Ambade et al. 2014). HSP90 is significantly increased in both immune cells and hepatocytes to functionally maintain protein stability within complexes and modulate cellular response/adaptation during stress conditions associated with HCV infection and alcohol liver disease (Johnston et al. 2010; Makhnevych and Houry 2012; Mandrekar et al. 2008). In the liver, while HSP90 is mostly induced during diverse cellular stress conditions, including viral infections, numerous studies have demonstrated other roles for HSP90 in modulating gene transcription mediating neo-vessel development which is usually associated with hepatic neoplasia development and metastasis

(Bagatell and Whitesell 2004; Bohonowych et al. 2010; de Martel et al. 2012; Eustace et al. 2004; Freeman and Yamamoto 2002; Parkin 2006; Schlesinger 1990; Solit and Chiosis 2008; Tariq et al. 2009; Trepel et al. 2010; Zhao and Houry 2005). Ambade et al. showed that mice fed an alcohol diet had significant HSP90 protein upregulation in the liver, which positively correlated with the extent of liver inflammation and injury. Additionally, they showed that functional inhibition of HSP90 activity using 17DMAG could significantly alleviate hepatic steatohepatitis and hepatic injury in mouse model studies suggesting that HSP90 was critical in modulating the molecular pathophysiology of ALD (Ambade et al. 2014; Mandrekar et al. 2008). During HCV infection associated with alcohol use, in-vitro studies found that Hsp90, which is increased during HCV infection associated with alcohol, can increase HCV replication (Bukong et al. 2013; Nakagawa et al. 2007; Okamoto et al. 2006; Taguwa et al. 2009; Ujino et al. 2009, 2012). In addition to its role in HCV replication, HSP90 also plays an important role in the processing and activation of HCV NS3 and NS2 proteins (Varmus 1984). HSP90 also has been shown to directly interact with the HCV NS5A protein as well as other host proteins promoting HCV replication (Okamoto et al. 2006, 2008). Given the role of HSP90 in the pathophysiology of both HCV infection and ALD, it is to be expected that patients with both of these conditions will most likely progress to advanced liver disease including HCC. This is because HSP90 has modulatory functions that can enhance the proliferative potential of malignant cells as well as protect neoplastic cells from apoptosis thereby driving tumor development and persistence (Lanneau et al. 2008; Takayama et al. 2003). Because of the critical role played by HSP90 in the pathophysiology of both HCV infection and ALD, HSP90 has been highlighted as an important drug target for HCV infection and/or alcoholic liver disease (Bukong et al. 2013, 2014; Mandrekar et al. 2008; Nakagawa et al. 2007; Ambade et al. 2012; Lachenmayer et al. 2012; Lang et al. 2009).

2.3.2 MicroRNA Regulation of Liver Disease During HCV Infection Associated with Alcohol Use

MicroRNAs (miRNAs) are short non-coding RNAs of about 22 nucleotides that mainly function by suppressing gene expression by binding complementarily to the 3′UTR of target genes. Given the recent association of epigenetic modifications by miRNAs in disease processes, increasing reports show that these modifications occur during progressive HCV infection and progressive ALD independently and together (French 2013; Mandrekar 2011; Szabo and Bala 2013). In liver disease numerous studies have focused on miR-122 and miR-155. miR-122 is highly expressed in hepatocytes compared to all other cell types (Chang et al. 2004; Lagos-Quintana et al. 2002). Additionally, while most micro-RNAs suppress gene transcription by binding to the 3′ UTR, miR-122 has been shown to bind to the 5′UTR of HCV promoting HCV replication (Jopling 2008). In a recent study, we demonstrated that alcohol increases HCV replication in vitro and identified a critical role for miRNA-122, GW182, HSP90 and Cyclin G1 in the process

(Bukong et al. 2013; Hou et al. 2013), although some studies have shown that alcohol use does not increase HCV replication (Anand and Thornby 2005). Given the important roles played by miR-122 in the pathogenesis of liver disease due to HCV and or alcohol, functional inhibition of miR-122 has received extensive research as a therapeutic target. Administration of a miR-122 'antagomir' in human HCV infection was well tolerated and was effective in reducing HCV serum levels (Janssen et al. 2013). While this might prove beneficial for treating HCV infection, caution must be exercised and results from clinical trials fully evaluated (Janssen et al. 2013; Hsu et al. 2012; Lanford et al. 2010; Tsai et al. 2012).

Both HCV and alcohol contribute to inflammation in the liver. miRNA-155, a master regulator of inflammation, is increased in monocytes, macrophages, and Kupffer cells by HCV proteins and chronic alcohol, respectively (Sidorkiewicz et al. 2010; Bala et al. 2011, 2012; Bala and Szabo 2012). In a recent report we found that serum miR-155 increases during alcoholic liver disease or in HCV infection and positively correlates with disease severity (Bala et al. 2011, 2012; Bala and Szabo 2012; Shrivastava et al. 2013). In an animal model of alcoholic liver disease, serum miR-155 was also increased (Bala et al. 2011).

2.4 Exosomes

Recent discoveries indicate that in addition to direct infection of hepatocytes by HCV that involves entry via specific cell surface receptors (CD81, SB-RI and ApoE), HCV infection also occurs via small cell-derived vesicles (exosomes) both in cell culture systems and possibly HCV-infected patients (Bukong et al. 2014; Cosset and Dreux 2014; Ramakrishnaiah et al. 2013). Exosomes are 20–150 nm diameter membranous vesicles derived from hepatocytes (as relevant in HCV infection) that contain both proteins and nucleic acids (Bukong et al. 2014; Hosseini et al. 2013). It has been shown that HCV RNA-containing exosomes can activate dendritic cells to produce type I interferon (Dreux et al. 2012). In a recent study, we found that exosomes isolated from patients with chronic HCV infection contain single-stranded and, in some patients with history of no response to IFN-based therapy, double-stranded HCV RNA (Bukong et al. 2014). Furthermore, the HCV RNA was in complex with miR-122 and HSP90 in exosomes and these exosomes could transfer HCV infection to naïve human primary hepatocytes, suggesting that HCV infection could be mediated by exosomes derived from HCV infected hepatocytes (Bukong et al. 2014).

The effect of alcohol on exosomes and the role of exosomes in alcoholic liver disease are yet to be explored. However, we have recently shown that exosomes can functionally mediate the transfer of miRNA to hepatocytes and macrophages modulating proinflammatory cytokine induction both in-vitro and in-vivo (Momen-Heravi et al. 2014).

3 The Gut-Liver Axis in HCV Infection and Alcohol Use

The gut and the liver are closely linked anatomically and functionally. There is a continuous bidirectional process between these organs including the transfer of digested products and microbial components from the gut to the liver and the transfer of liver products including bile, hormones and inflammatory molecules to the gut. Products from the gut are metabolized, detoxified, transformed, broken down and removed as a natural functional process of the liver. In alcoholic liver disease in both human and animal disease models, numerous reports have shown that this bidirectional transfer and interaction through diverse mediators increases as a result of increased gut permeability. Similarly, HCV infected patients independent of alcohol use have been shown to have high serum gut-derived endotoxins which also gain access to the liver (Dolganiuc et al. 2007; Sozinov 2002). To functionally metabolize alcohol, and clear the gut endotoxins and HCV from the liver, cellular detoxification and immune mechanisms are activated. These processes during chronic alcohol abuse, chronic HCV infection, or both, are associated with high levels of pro-inflammatory cytokine and ROS production. Combined, chronic ALD and HCV infection at the gut-liver-axis creates a precarious health condition for patients characterized by increased gut permeability, impaired hepatic detoxification, and hyperactive immune responses aimed at maintaining gut-liver homeostasis. To prove these associations experimentally, numerous reports have used several disease models employing gut sterilization or treatments to suppress HCV infection/replication in hepatocytes. These approaches have proved successful in mitigating liver disease progression as well as in identifying the role of key molecular pathophysiologic processes in alcoholics (Adachi et al. 1995; Son et al. 2010) and potentially in HCV-infected patients who consume alcohol.

Chronic alcohol use has been shown by numerous reports, including our own, to compromise gut barrier integrity leading to increased gut endotoxin translocation to the liver (Lippai et al. 2014, In Press, Keshavarzian et al. 2009). Similarly, published reports have shown that patients with chronic viral infections including HCV or HIV have elevated blood lipopolysaccharide (LPS) levels (Dolganiuc et al. 2007; Sozinov 2002; Sandler et al. 2011; Caradonna et al. 2002). Additionally, chronic alcohol use and HCV infection have independently been shown to increase Toll-like receptor (TLR)2 and TLR4 expression in hepatocytes, Kupffer cells, and peripheral monocytes, both in-vitro and in-vivo (Szabo et al. 2005, 2006; Dolganiuc et al. 2006a; Pang et al. 2011; Testro et al. 2010). The synergism between HCV and alcohol increases TLR expression with induced TLR expression associated with increased serum endotoxin and significant proinflammatory cytokines in the liver (Szabo et al. 2006; Testro et al. 2010).

4 Immune Modulation by HCV and Alcohol in Liver Disease

ALD and HCV infection alone or in combination represents more than two thirds of the liver disease patients in the western world (Mueller et al. 2009). Studies have shown that HCV and alcohol use synergistically accelerate the progression of the liver diseases, cirrhosis and HCC (Szabo et al. 2010; Hassan et al. 2002; Yuan et al. 2004; Hutchinson et al. 2005; Siu et al. 2009). HCV and alcohol cause both immune impairment and tissue damage separately and in combination (Szabo et al. 2006, 2010; Singal et al. 2014; Lieber 1997; Nelson and Kolls 2002).

4.1 Innate Immune Modulation

A large body of evidence indicates that innate immune functions are direct targets of alcohol and HCV infection, both individually and additively. In human monocytes, chronic alcohol use or in vitro chronic alcohol exposure results in sensitization to TLR-mediated activation of pro-inflammatory cytokines (Mandrekar et al. 2009; Szabo and Mandrekar 2009). Upregulation of the pro-inflammatory cascade, and monocyte and liver macrophage activation are induced by HCV, its RNA and HCV proteins (Heydtmann 2009). There is recruitment of activated macrophages in the liver both in HCV infection and in ALD (Lalor et al. 2002; Tacke 2012). Several studies found that HCV core and NS3 or NS5 proteins activate blood monocytes to produce TNFα and IL-1ß (Dolganiuc et al. 2003a; Hosomura et al. 2011). Blood monocytes from patients with chronic HCV infection show in vivo sensitization to TLR4 or TLR2 activation by LPS or HCV proteins, respectively, resulting in an overall pro-inflammatory state (Bala et al. 2012). Recent studies found TLR7/8 activation in macrophages by single-stranded HCV RNA results in IL-1ß production via activation of the multiprotein NLRP3 inflammasome (Negash et al. 2013).

Dendritic cells of the innate immune system have the capacity to produce interferons, immunomodulatory cytokines and interact with T cells in antigen presentation or fine-tune T cell functions. Both HCV and alcohol were shown to modulate the functions of the different subtypes of dendritic cells. Plasmacytoid DCs, characterized by surface expression of BDCA-2 and BDCA-4 are the most potent cell type in production of IFN-α (Mathan et al. 2013; Tang et al. 2010; Dzionek et al. 2000). In chronic HCV infection decreased numbers of pDCs were found in the circulation and some studies detected an increase in pDC surface marker expression in the liver suggesting redistribution of this cell population (Szabo and Dolganiuc 2005; Dolganiuc et al. 2006b; Velazquez et al. 2012; Cicinnati et al. 2008; Brass and Brenndorfer 2014). Importantly, the IFN producing capacity of pDCs is decreased in chronic HCV infection (Dolganiuc et al. 2006b;

Ulsenheimer et al. 2005). Independently, alcohol exposure was also shown to decrease IFN production by human blood mononuclear cells in response to TLR7/8 stimulation (Pang et al. 2011).

Myeloid dendritic cells are represented by different populations including circulating mDC (myeloid DC1) and myeloid DC type 2 characterized by expression of BDCA-3 and monocyte-derived DCs that are generated in vitro from monocytes by IL-4+GM-CSF stimulation (Liu et al. 2001; Dauer et al. 2003). Most studies, but not all, found that monocyte-derived DCs have decreased capacity to activate naive T lymphocytes in an antigen-independent or antigen-dependent fashion (Woltman and van Kooten 2003; Lipscomb and Masten 2002). Some of the decreased T cell activating capacity of mDCs was attributed to their impaired capacity to reach the fully mature DC phenotype with high expression of CD80 and CD86 after in vitro stimulation with LPS or other triggers of maturation (Mandrekar et al. 2004; Szabo et al. 2001). In alcohol exposed human monocyte-derived DCs as well as in liver mDCs from alcohol-fed animals, studies consistently found an impaired capacity of accessory cell function and T cell activation (Szabo et al. 2004; Eken et al. 2011; Guo and Friedman 2010). This was associated with decreased production of IL-12 and increased production of IL-10 by mDCs (Mandrekar et al. 2004). There are striking similarities in the mDC phenotype and function between mDCs isolated from chronic HCV infected individuals and normal mDCs differentiated in the presence of in vitro alcohol (Dolganiuc et al. 2003b; Szabo et al. 2004). Both have impaired accessory cell function in a mixed lymphocyte reaction, attenuated CD80 and CD86 surface expression, increased IL-10 and decreased IL-12 production compared to controls (Dolganiuc et al. 2003b; Szabo et al. 2004; Lau et al. 2009). Strikingly, alcohol treatment of monocyte-derived DCs from HCV infected patients has a synergistic inhibitory effect with HCV to attenuate DC accessory cell function, suggesting that alcohol consumption in a chronic HCV infected individual dually undermines antiviral immunity (Szabo et al. 2010; Dolganiuc et al. 2004). BDCA-3^+ myeloid DC2 cells are a rare subset of mDCs. mDC2s are a major source of IFN-λ production by PBMCs in response to HCV-infected hepatocytes. A study showed that mDC2s are activated through the TLR3 pathway (Zhang et al. 2013).

Fine-tuning of the innate and adaptive immune responses relies on multiple subtle events and communication between various cell types of the immune system (Szabo et al. 2015). Many of these fine tuning events appear to be the target of either HCV, alcohol or both (Szabo et al. 2015). For example, we found that full NK cell activation required IL-13 production by monocytes (Szabo et al. 2001). Interestingly, in an earlier study it was shown that alcohol also increases IL-13 (Szabo et al. 2001) suggesting potentially common targets in immunomodulation by alcohol and HCV (Heydtmann 2009; Szabo et al. 2001; Weng et al. 2009).

4.2 Adaptive Immune Modulation

Cell-mediated adaptive immune response is driven by T cells which are critical for HCV clearance. Alcohol also inhibits cell-mediated immune response, perhaps contributing to a higher HCV prevalence in alcoholics (Szabo et al. 2006; Singal et al. 2014). It has been observed that the cells infiltrating the liver of alcoholic hepatitis and cirrhosis patients contain both $CD8^+$ and $CD4^+$ T cells that are activated and respond to T cell receptor stimulation by Th1 cytokines such as IFN-γ and TNFα (Chedid et al. 1993; Song et al. 2001; Albano 2012). Liver injury during chronic HCV infection is associated with increased Th1 cytokines and chronic alcohol consumption is also associated with increased T cell activation (McCaughan et al. 2000; Napoli et al. 1996; Cook 1998). Chronic alcohol leads to inhibitory effects on generation of antigen specific $CD4^+$ and $CD8^+$ T cell activity using HCV core as the immunogen (Geissler et al. 1997c). Experiments have also revealed that antigen-presenting cells may be cellular targets of the effects of chronic alcohol consumption (Encke et al. 1998, 1999; Encke and Wands 2000). Both acute and chronic alcohol reduces T cell proliferation (Szabo and Mandrekar 2009). It has been shown that some ALD patients have detectable autoantibodies and anti-hepatocyte cytotoxic T cells (Lieber 1997; Nelson and Kolls 2002). Anti-phospholipid antibodies are also increased in 80 % of patients with alcoholic hepatitis or cirrhosis (Chedid et al. 1994; Biron et al. 1995). In the liver of alcoholic hepatitis patients, IL-17-producing T Helper (Th-17) lymphocytes are present in the inflammatory infiltrates and there is also an increase in the IL-17 plasma levels (Lemmers et al. 2009). Th17 cells are important in the pathogenesis of HCV, thus the role of Th17 T cells in ALD is of particular importance (Hammerich et al. 2011). The process of apoptosis mediated by cytotoxic T cells and NK cells is synergistically enhanced in the presence of alcohol (Nakamoto et al. 2002; Kountouras et al. 2003).

Human studies have shown that $CD4^+$ and $CD8^+$ T cell activity directed against epitopes in nonstructural proteins (NS5) are important for viral clearance during the natural resolution of HCV (Rehermann and Nascimbeni 2005). Chronic alcohol consumption leads to substantial inhibition of NS5 specific $CD4^+$ and $CD8^+$ T cell activity, and is only partially restored followed cessation of chronic alcohol consumption (Encke et al. 1999). Studies have indicated that the suppressive effect of alcohol on CTL activity was reversed following co-immunization with a GM-CSF encoding plasmid, and this indicates that the major effect of alcohol on antiviral immune responses might be at the level of antigen presentation. As mentioned in the innate immune section above, DCs differentiated in vivo in the presence of chronic alcohol feeding have intrinsic functional defects, which may be responsible for the attenuated CTL activity observed after DNA-based immunization with HCV core and NS5 as the immunogens (Siu et al. 2009; Encke et al. 1998, 1999; Aloman et al. 2007; Geissler et al. 1997a, b).

Not much is known regarding the alteration in cellular immune responses that may predispose to persistent HCV infections in alcoholics. Previous clinical studies

of the adaptive host response to acute HCV infection help us in understanding the effect of alcohol on the immune response to HCV. HCV clearance is associated with a strong, multi-specific, $CD8^+$ T cell response along with a sustained $CD4^+$ T cell proliferation in response to HCV structural and non-structural proteins (Diepolder et al. 1995; Gerlach et al. 1999; Thimme et al. 2001). These activated T cells also secrete proinflammatory cytokines including IFN-γ and lead to a decrease in the viral load during acute HCV infection (Thimme et al. 2001). Heavy alcohol consumption is associated with reduced $CD4^+$ T cell proliferative responses and also alters delayed hypersensitivity reactions (Nelson and Kolls 2002; Jayasinghe et al. 1992; Waltenbaugh and Peterson 1997). These conditions are also associated with an increased incidence of viral and bacterial infections (Siu et al. 2009; Cook 1998). Further studies of antiviral immune responses in alcohol-consuming animal models will be necessary to expand our knowledge of the effects of alcohol on HCV infection.

5 HCV and Alcohol Synergize to Promote Liver Fibrosis and Hepatocellular Cancer

Clinical strudies identified HCV and chronic alcohol use as independent risk factors for liver cirrhosis and progressive liver damage leading to cirrhosis (Poynard et al. 2003; Massard et al. 2006). The exact pathogenic mechanisms underlying the synergistic effects of alcohol and HCV infection on the progression of liver fibrosis are not fully understood; however, they may have additive and synergistic effects at multiple levels. Both chronic HCV infection and chronic alcohol use lead to activation of innate immune cells in the liver resulting in a pro-inflamamtory cytokine environment that promotes stellate cell activation (Vera and Nieto 2006; Friedman 2008). In addition, HCV as well as alcohol can activate sinusoidal stellate cells and or promote their activation by LPS that acts on TLR4 expressed on stellate cells (Szabo and Bala 2010; McGettrick and O'Neill 2010; Kumagai and Akira 2010; Soares et al. 2010). Human data and animal models suggest that chronic alcohol use and even chronic HCV infection are independently associated with increased serum levels of LPS, a component of gram negative bacteria (Pinzone et al. 2012). It has been proposed that the increased serum LPS levels are likely a result of gut microbial translocation (Pinzone et al. 2012; Schnabl and Brenner 2014). Stellate cell activation leads to the loss of the "quiescent" phenotype, characterized by loss of retinoic acid droplets and induction of alph-smooth muscle action and collagen expression (Friedman 2008). In the setting of chronic HCV infection and/or chonic alcohol use, the triggers of HSC activation are sustained, leading to chronic HSC activation and increase liver fibrosis.

Clinical studies indicate that the elimination of HCV infection with succesful therapy leads to attenuation and/or resolution of liver fibrosis and clinical stabilization of patients with advanced cirrhosis (Arthur 2002). Reversal of cirrhosis still

remains controversial after HCV elimination and/or cessation of alcohol in cirrhotic patients (Arthur 2002).

The risk of HCC is increased by excessive alcohol use and/or chronic HCV infection, respectively (Machida 2010; Mueller et al. 2009). Furthermore, HCV and alcohol are synergistic in increasing the risk of HCC development (Machida 2010; Mueller et al. 2009). The mechanism(s) responsible for the synergistic effects of alcohol and HCV are not fully understood, however, TLR4-mediated intracellular signaling leading to activation of the transcription factor NANOG has been identified as a critical pathway in a mouse model (Machida et al. 2009).

6 Treatment of HCV Infection: Impact of Alcohol Use

In the era of IFN-based HCV therapies, the generally accepted practice was not to treat individuals with chronic HCV infection who were actively drinking, and the recommendation was to refrain from alcohol during HCV therapy even in moderate drinkers. These recommendations were based on the observations that alcohol use during IFN-based HCV treatments was associated with decreased compliance and decreased response rates compared to those who had no active alcohol use (Anand et al. 2006). In vitro studies support this practice, considering that it has been shown that downstream signaling via the type I interferon receptor is inhibited by alcohol (Plumlee et al. 2005; Morishima et al. 2006). Less-defined additional negative effects of alcohol may also include the inhibition of cross-talk between types I and III interferons, with a reduction in the augmenting effect of type I interferon on the induction of type III interferon and vice versa (Zhang et al. 2013).

In the new era of HCV treatment with combinations of direct acting antivirals, sustained virological response rates reach almost 95–100 % in most patient populations (Nookathota and Mukherjee 2014; Conteduca et al. 2014). To date there have been no studies to assess the efficacy of these new treatments in patients with ongoing chronic alcohol use. However, it is possible that the presence of ongoing alcohol use may decrease the otherwise high virological response rates to these new regimens considering that the biological action of these new therapies relies, at least partially, on unleashing the host immune system (Nookathota and Mukherjee 2014; Welsch et al. 2012). One can speculate that the immunosuppressive effects of chronic alcohol use and its negative direct effects on signaling induced by endogenous interferons could adversely affect HCV elimination even with drug regimens that are IFN-α-free. The production of endogenous interferons in response to HCV RNA can be attenuated even after one occasion of binge drinking in vivo or its equivalent based on in vitro studies (Pang et al. 2009). Further studies are needed to fully evaluate the effects of alcohol use on HCV elimination with the new oral regimens.

7 Future Perspectives and Unmet Needs; Summary

In conclusion, chronic HCV infection and chronic alcohol use can be devastating for the liver. Alcohol and HCV can mutually potentiate multiple factors involved in the progression of liver disease caused by either alcohol or HCV alone (Figs. 1, 2, and 3). The common targets of HCV and alcohol in the pathogenesis of chronic liver disease include hepatocyte damage, increased pro-inflammatory mediator production, increased serum levels of endotoxin (LPS), recruitment of activated macrophages to the liver, activation of liver resident macrophages, Kupffer cells, leading to increased liver inflammation, stellate cell activation and liver fibrosis (Figs. 1, 2, and 3). In addition, intracellular mechanisms and host factors required for HCV replication in hepatocytes may be augmented by alcohol including increases in miR-122, expression of HSP90 and GW182, all of which may promote the HCV replication complex (Fig. 3). Finally, synergistic interactions between alcohol and HCV in promoting HCC remains a major health concern because the

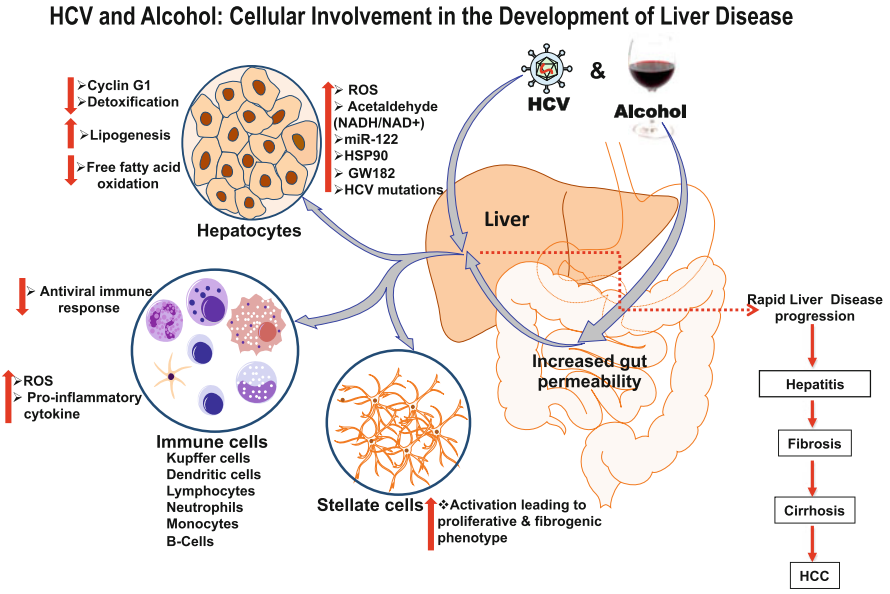

Fig. 1 HCV and alcohol induce sustained hepatic inflammation through induction of hepatocellular stress, modulation of hepatic proteins and miRNAs, as well as activation of immune cells and stellate cells. HCV infection associated with alcohol abuse compromises intestinal mucosal barrier integrity causing bacterial translocation and elevated serum endotoxin levels. This leads to liver immune cell activation driving pro-inflammatory cytokine production. Additionally, hepatocyte injury due to HCV and alcohol leads to stellate cell activation and fibrosis. Together, the complex mix of hepatocyte injury and increased proinflammatory cytokines in the liver lead to increased lipogenesis and decreased fatty oxidation, promoting the development of hepatic steatosis. HCV infection associated with alcohol use creates a vicious cycle that drives the rapid progression of liver inflammation to fibrosis, cirrhosis and ultimately hepatocellular cancer (HCC)

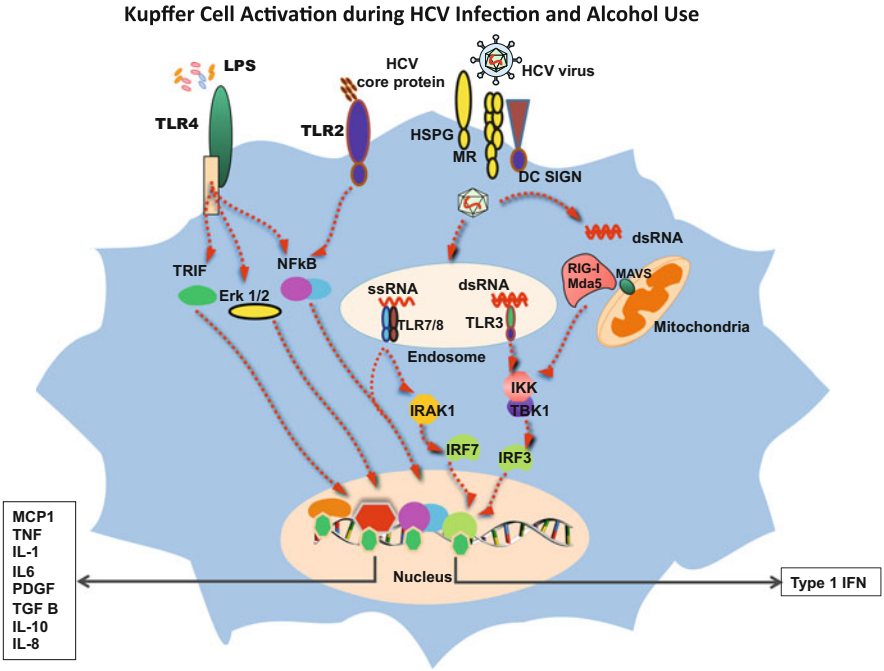

Fig. 2 HCV infection and alcohol synergize in modulating Kupffer cell activation. HCV virus or HCV protein engagement of pathogen recognition receptors in Kupffer cells induces their activation and triggers the release of type 1 interferon. Similarly, an alcohol-induced increase in serum endotoxins (LPS), can activate Kupffer cells via TLR4 driving pro-inflammatory cytokine production. *TLR4* Toll like receptor 4, *LPS* Lipopolysaccharide, *HSPG* Heparan sulphate proteoglycan, *MS* Mannose Receptor, *DC SIGN* Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin

high risk of HCC remains even after elimination of HCV and/or cessation of excessive alcohol use in cirrhotic patients, suggesting that insults sustained in the earlier phase of disease have a major effect on progression and development of HCC. With new therapies that have the potential to effectively eliminate HCV of all genotypes, the goal should be to treat early in the course of chronic HCV infection, before the development of cirrhosis to avoid the increased risk of HCC in this vulnerable patient population.

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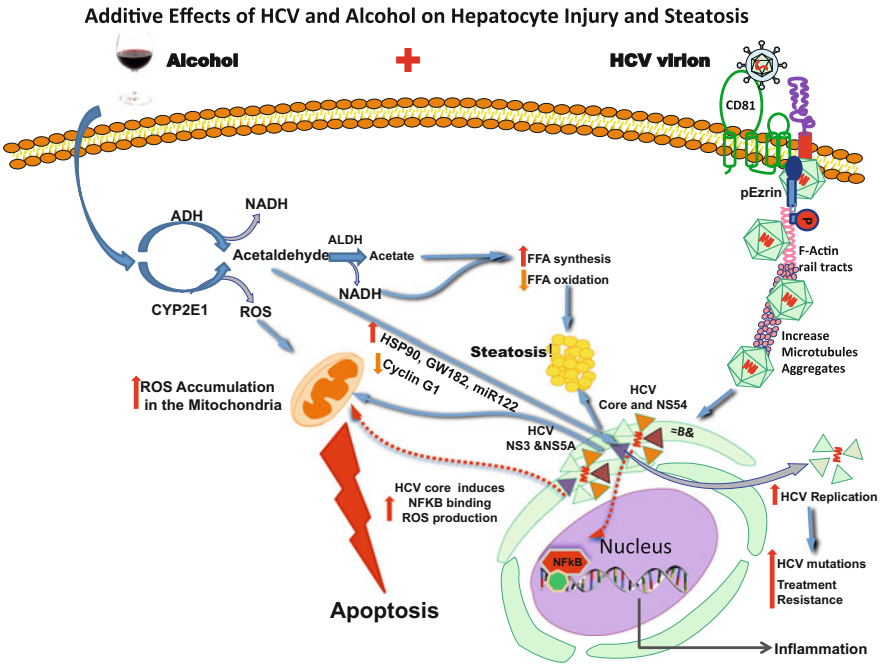

Fig. 3 The liver is the main site for alcohol metabolism and it is also the main organ targeted by HCV for infection. During excessive alcohol use, metabolism in the liver creates large amounts of ROS and acetate, which can drive increases in free fatty acid (FFA) synthesis and decreased FFA oxidation. This leads to hepatic steatosis, while high levels of ROS can lead to hepatocyte death. HCV usurps the host proteins CD81, Ezrin and cellular actin/microtubules, to infect hepatocytes. Following infection, HCV core protein and NS3 protein can induce hepatic steatosis by increasing free fatty acid synthesis. Additionally, NS3, core and NS5a proteins trigger ROS accumulation causing hepatic and mitochondrial stress leading to cellular apoptosis. Together, HCV and alcohol can modulate the expression of hepatocyte proteins, increasing HSP90 and GW182 and decreasing Cyclin G1 leading to increased HCV replication and hepatocyte injury. Additionally, HCV and alcohol can increase miR-122 expression, which supports HCV replication. Increased HCV replication during alcohol abuse could also lead to increased HCV mutations and treatment resistance

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Extrahepatic Replication of HCV

Wen-Chi Su, Keigo Machida, and Michael M.C. Lai

Abstract Cirrhosis and hepatocellular carcinoma are the prototypic complications of chronic hepatitis C virus (HCV) infection in the liver. However, HCV also causes extrahepatic manifestations, suggesting that HCV may infect other organs. In this chapter, we will first show the correlation between HCV infection and a variety of extrahepatic diseases by epidemiological investigations. Furthermore, to support the possible extrahepatic replication of HCV, the detection of HCV RNA in a number of organs and the accompanying genetic changes of viral sequences will be discussed. Finally, isolation of a lymphotropic HCV strain from B cell lymphoma and the consequence of HCV infection of lymphoid cells, providing unequivocal evidence for extrahepatic replication of HCV, will be described. We endeavor to summarize the published works and our own findings to highlight the evidence of the extrahepatic replication of HCV, which reveals an insight into HCV persistence.

Keywords Extrahepatic replication • Mixed cryoglobulinemia • Lymphotropic HCV • HCV persistence • SB virus • B cells • Co-receptors

1 Introduction

Hepatitis C virus (HCV) represents a growing public health burden with more than 170 million people being infected worldwide (Wasley and Alter 2000). A striking feature of HCV infection is its tendency toward chronicity, with at least 70 % of

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acute infections progressing to persistence, which is often associated with significant liver disease (Wasley and Alter 2000). Though the primary target organ of HCV is the liver, the pathogenesis of HCV infection has been linked to other organs. Extrahepatic manifestations of HCV infection was first reported in the early 1990s (Pascual et al. 1990), including a number of disease processes affecting the small blood vessels, skin, kidneys, thyroid, immune system, brain, etc (Ko et al. 2012). The clinical manifestation and pathogenesis of extrahepatic infection of HCV will be presented elsewhere in this book by Koike et al. The current chapter will summarize the evidence of HCV infection and replication in these extrahepatic tissues.

The primary entity of extrahepatic manifestations of HCV is mixed cryoglobulinemia (MC), also known as type II cryoglobulinemia. More than 90 % of patients with mixed cryoglobulinemia are infected with HCV, and about half of patients with HCV have cryoglobulins (Ferri et al. 1993; Lunel et al. 1994). In MC, the cryoglobulins are composed of two or more classes of immunoglobulins including a monoclonal IgM component with rheumatoid factor-like activity (Brouet et al. 1974). Immune complex depositions can be identified in small vessels and glomerular capillary walls, leading to palpable purpura in the skin and membranoproliferative glomerulonephritis in the kidney. About 8–10 % of patients with MC ultimately develop a lymphoproliferative disorder (Bachy et al. 2010). Other HCV-associated extrahepatic diseases, such as lichen planus, glomerulonephritis and neurocognitive disorders, have also been documented (Forton et al. 2004b; Ko et al. 2012). Forty to 75 % of patients with chronic HCV infection exhibit at least one clinical extrahepatic manifestation (Cacoub et al. 2000).

In this article, we will first review the association between HCV infection and extrahepatic diseases based on epidemiological data, clinical observations and biological studies. Furthermore, we will discuss the evidence of HCV replication in those organs and tissues, albeit controversial. In addition, in vitro infection of non-hepatic cells with HCV, indirectly supporting extrahepatic replication of HCV, will be described. We will present characterization of a lymphotropic HCV strain from B-cell lymphoma, providing evidence for HCV infection of lymphoid tissues in vivo. Finally, since the majority of extrahepatic manifestations are recognized as the consequences of the host immune response to HCV infection, the influence of extrahepatic replication, particularly in lymphoid cells, will be addressed. The studies regarding extrahepatic replication of HCV are the clues to understanding how HCV escapes immunity, persists in infection and causes extrahepatic manifestations.

2 Review of Epidemiological Evidence for Extrahepatic Involvement of HCV

A diverse catalogue of diseases has been linked to HCV infection by epidemiological studies. Here we review the most common extrahepatic manifestations of HCV.

2.1 B-Cell Lymphoma

Several studies have investigated a potential association between HCV and a variety of lymphoproliferative disorders (Bachy et al. 2010). Silvestri et al. have found that in 537 patients with lymphoproliferative disorders, the prevalence and the relative risk (RR) of being infected by HCV were increased only among B-cell non-Hodgkin lymphoma (B-NHL) (9 %; RR 3.24; $p < 0.0001$), indicating that HCV infection was prevalent in patients with B-NHL as compared to other lymphoid malignant neoplasms (Silvestri et al. 1996). Nevertheless, the prevalence of HCV in patients with B-NHL was variable in different countries (Negri et al. 2004). HCV is associated with some B-NHL, particularly in HCV endemic areas, such as Italy and Japan, where HCV prevalence rates hover around 20 % (Mele et al. 2003; Mizorogi et al. 2000). In contrast, in nonendemic areas, like North America, Northern Europe and United Kingdom, the prevalence rate of HCV infection in B-NHL is far lower than 5 % (Sy and Jamal 2006). This discrepancy might be caused by unknown environmental and genetic factors or the difference in HCV prevalence. Additionally, studies in the low-HCV-prevalence countries might not have included enough patients to detect a significant association between HCV and B-NHL. Actually, further cohort studies have indicated an increased risk of NHL in patients with HCV as compared to HCV-negative controls (Giordano et al. 2007; Nieters et al. 2006). Taken together, it appears that there is a greater tendency toward development of NHL upon HCV infection and the risk is particularly evident in populations with high HCV prevalence.

2.2 Renal Disease

Johnson et al. first revealed that HCV is associated with glomerulonephritis (GN) by investigating eight HCV-infected patients who presented with membranoproliferative glomerulonephritis (MPGN) (Johnson et al. 1993). Among HCV-related glomerulonephritis, MPGN associated with type II cryoglobulinemia is most common (D'Amico 1998). The prevalence of HCV infection in MPGN patients is 10–20 % in the United States (Johnson et al. 1993). Less common glomerulonephritis diseases have also been reported in HCV-infected patients, including MPGN without cryoglobulinemia (Johnson et al. 1993), membranous glomerulonephritis (MGN) (Uchiyama-Tanaka et al. 2004) and proliferative glomerulonephritis (Horikoshi et al. 1993).

2.3 Dermatologic Disease

Thus far, cutaneous vasculitis, porphyria cutanea tarda (PCT) and lichen planus (LP) are recognized as the major skin diseases frequently associated with HCV infection. In HCV-infected patients, cutaneous vasculitis, leading to palpable purpura, is reported in 24–30 % of MC-positive patients (Cacoub et al. 2000). The severity of vasculitis correlates with the level of HCV viremia. As to PCT, the prevalence of PCT patients and HCV infection varies according to geographical region. The prevalence is higher in southern Europe (65–91 %) and the United States (50–75 %), while it is lower in northern Europe (8–17 %) and Australia and New Zealand (~20 %) (Chuang et al. 1999). As to LP, a common inflammatory skin disease, the relationship between HCV infection and LP is more questionable; nevertheless, in most studies, the proportion of HCV-positive patients is higher in the LP group as compared to the control group without LP disease (Lodi et al. 2004).

2.4 Central Nervous System Disorders

About 50 % of patients with HCV infection suffer from neuropsychiatric symptoms, "brain fog", weakness, fatigue, along with some degree of quality of life impairment (Weissenborn et al. 2009). Impressively, by using magnetic resonance spectroscopy some in vivo evidence for a neurotropic role for HCV has revealed the alterations in the cerebral metabolite spectrum in the basal ganglia (Forton et al. 2001), the white matter and the frontal cortex (Weissenborn et al. 2004) in HCV patients. A recent study has demonstrated evidence of microglial activation, which positively correlated with HCV viremia and altered cerebral metabolism in the brains of patients with mild hepatitis C (Grover et al. 2012). It is worth noting that viral sequences in the brain are often different from those in the liver, but are closely related to those found in lymphoid tissue (Forton et al. 2004a). Therefore, it has been proposed that HCV-infected mononuclear blood cells cross blood-brain barrier to enter the brain, enabling the virus to reside within the brain (probably in microglia) and to infect brain cells, especially astrocytes (Weissenborn et al. 2009). HCV has been demonstrated to infect also the endothelial cells of the blood-brain barrier in biological specimens and infects neuroepithelioma cell lines in tissue culture (Fletcher et al. 2010, 2012).

2.5 Autoimmune Diseases

HCV infection has been reported to be associated with autoimmune diseases, such as the aforementioned MC, Sjogren's syndrome (SS) and autoimmune thyroid

disease. The prevalence of SS in the patients with HCV infection ranges from 4 % to 57 % (Haddad et al. 1992; Jacobson et al. 2010). The variable prevalence might have been caused by the difference in diagnostic criteria (Jacobson et al. 2010). The pathogenesis of HCV-associated SS is not clear. Thus far, HCV has not been definitely identified in glandular tissue (Ohoka et al. 2003), suggesting that the virus is unlikely to have a direct effect on progression of SS and that the HCV-associated SS might be caused by a host-immune-mediated mechanism. Thyroid disorders, such as thyroid dysfunctions and hypothyroidism, are common in patients with chronic HCV infection. Approximately 10–25 % of patients with persistent HCV infection have thyroid autoantibodies, including thyroglobulin autoantibodies (TGAs), thyroid microsome autoantibodies (TMAs), and antibodies to thyroid peroxidase autoantibodies (anti-TPO) (Antonelli et al. 2004; Ganne-Carrie et al. 2000). To date, the pathogenesis of HCV-related autoimmune thyroid disease is not clear.

2.6 Diabetes Mellitus

Besides immune-related disorders, metabolic syndrome is also reported to be associated with extrahepatic manifestations of HCV infection. The prevalence of type 2 diabetes in patients with chronic HCV infection is 13–24 % (Antonelli et al. 2005; Grimbert et al. 1996). HCV-related diabetes is usually insulin-resistant and might not be associated with antibodies targeting the beta cells (Petit et al. 2001).

3 Causal Association of Lymphoma with HCV: Clinical Evidence

HCV has been epidemiologically shown to correlate with HCV-associated extrahepatic diseases. This raises an intriguing question: Is HCV therapy also effective in the treatment of these diseases? Hermine O. et al demonstrated that in HCV-infected patients with splenic lymphoma with villous lymphocytes, treatment with interferon can lead to regression of the lymphoma (Hermine et al. 2002). The similar effect was also observed by Kelaidi et al. who showed that after treatment with interferon alpha and ribavirin, the evident virologic clearance is shown and accompanied with hematologic remission of lymphoma in some HCV-associated marginal zone lymphomas patients (Kelaidi et al. 2004). We have also observed similar effects in HCV-infected B-cell lymphoma patients (Levine et al. 2003). Zuckerman et al also demonstrated the efficacy of antiviral treatment on immunoglobulin heavy-chain gene (IgH) rearrangement and t(14;18) translocation in HCV-infected patients (Zuckerman et al. 2001). In fact, antiviral therapy with

pegylated interferon and ribavirin is also effective in other HCV-associated extrahepatic diseases, such as cutaneous vasculitis (Cacoub et al. 2005) and cryoglobulinemic glomerulonephritis (Alric et al. 2004). In sum, these clinical studies firmly support an etiologic relationship between HCV infection and HCV-associated extrahepatic diseases.

4 Consideration of Methods for Detecting Viral Replication: PCR Specificity?

Serological identification of infection by HCV is based on detecting anti-HCV IgG using well-developed immunoassays, including enzyme-linked immunosorbent assay (ELISA) and chemiluminescence. Theoretically, recombinant protein or synthetic peptide related to HCV proteins is used as antigen in immunediagnostic assay for detecting anti-HCV IgG. The diagnostic specificity of these assays can achieve around 99 % (Colin et al. 2001). Nevertheless, the immunoassays cannot determine whether an antibody-positive person has active HCV infection since anti-HCV IgG may still be detectable in persons who have resolved infection and are no longer viremic.

The use of reverse transcriptase polymerase chain reaction (RT-PCR) to detect negative-strand HCV RNA is recognized, as the strongest evidence for viral replication. Because HCV virions carry positive-sense HCV RNA, the detection of positive-sense HCV RNA in any tissue does not authentically represent viral replication. Instead, production of negative-strand HCV RNA, the replicative intermediate molecule, is regarded as a more convincing indicator of HCV genomic replication (Sangar and Carroll 1998). Negative-strand HCV RNA has been identified in peripheral blood mononuclear cells (PBMC), including B cells, T cells, and cells of monocyte/macrophage lineage from HCV patients (Bertolini et al. 1993; Bouffard et al. 1992; Shimizu et al. 1992; Wang et al. 1992). Besides, negative-strand HCV RNA has also been detected in other non-hepatic tissues, such as pancreas, thyroid, adrenal gland, spleen (Laskus et al. 1998b), brain (Radkowski et al. 2002) and kidney (Sansonno et al. 2005).

The extrahepatic replication of HCV can be enhanced by co-infection of HCV and human immunodeficiency virus (HIV). It is notable that viral clearance of HCV occurred more often in HCV mono-infected patients than the coinfected individuals (Thomas et al. 2000). A number of studies suggest that extrahepatic replication of HCV occurs frequently in patients with HIV infection (Laskus et al. 1998a, 2000). In addition, Laskus et al. has demonstrated that HIV infection facilitates HCV replication in naive human macrophages and Daudi B-cell line with engineered CD4 expression in vitro (Laskus et al. 2004). Moreover, patients coinfected with HIV and HCV have higher levels of HCV RNA and viral load and poorer sustained response to interferon therapy than HCV mono-infected patients (Di Martino et al. 2001). Although the mechanisms underlying these effects remain unclear,

they do suggest that HCV can infect and replicate in extrahepatic cells under some circumstances.

In spite of a considerable number of studies showing the possible extrahepatic replication of HCV, many of these reports remain controversial. Lanford et al. demonstrated that only positive-strand HCV RNA, but not negative-strand HCV RNA, could be detected in extrahepatic tissues of HCV patients (Lanford et al. 1995). In this study, reverse transcription was performed at higher temperature by using rTth, a thermostable reverse transcriptase, to avoid false priming of the incorrect strand (Lanford et al. 1995). The authors proposed that detection of positive-strand HCV RNA in extrahepatic tissues may be caused by contamination with circulating virus (Lanford et al. 1995). Nonetheless, other studies using highly strand-specific RT-PCR assays were able to detect negative-strand HCV RNA in PBMC (Hu et al. 2003; Lerat et al. 1996).

5 In Vitro Infection of Cultured Cells (Particularly B and T Cells) with HCV

Due to the uncertainty of the strand specificity and limitation of sensitivity for RT-PCR assays, whether HCV undergoes replication in cells outside of the liver remains questionable. Nevertheless, in vitro studies showing that several lymphoblastoid cell lines were permissive for HCV replication have indirectly supported extrahepatic replication of HCV.

5.1 Consideration of Viral Receptors and Host Factors for Extrahepatic Infection and Replication of HCV

In the human blood, HCV is physically associated with lipoproteins (Thomssen et al. 1992). On entering the liver through the circulating blood, HCV may be captured by liver sinusoidal cells, thus facilitating the viral infection of neighboring hepatocytes, which are not in direct contact with circulating blood. HCV may utilize different receptors to enter different cells. For example, both dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and Liver/lymph node-specific ICAM-3-grabbing nonintegrin (L-SIGN) have been shown to be the HCV receptor for liver sinusoidal endothelial cells (LSEC) (Cormier et al. 2004a; Gardner et al. 2003). Using various model systems, several cell surface molecules have been identified to be crucial for the HCV infection of various cells. These include the low density lipoprotein receptor (Agnello et al. 1999), highly sulfated heparin sulfate (HS) (Barth et al. 2003), glycosaminoglycans (Germi et al. 2002), scavenger receptor class B type 1 (SR-B1) (Bartosch et al. 2003; Scarselli et al. 2002), tetraspanin CD81 (Bartosch et al. 2003; Cormier

Table 1 HCV receptor and co-receptor candidates

Receptor candidate	Putative function	Tissue expression and character	Reference
CD5	T lymphocyte co-receptor	T lymphocytes	Sarhan et al. (2012, 2013)
CD81	HCV early stage entry, Tetraspanin protein	Ubiquitous (except erythrocytes and platelets)	Bartosch et al. (2003), Cormier et al. (2004b), (1998), Zhang et al. (2004)
Claudin-1 (CLDN1)	HCV late stage entry, Tight junction component	Highly expressed in liver and epithelial tissues	Evans et al. (2007)
SR-BI	HCV binding/entry, ligand: high-density lipoprotein (HDL)	Hepatocyte, steroldogenic tissue, dendritic cell	Bartosch et al. (2003), Scarselli et al. (2002)
Occludin	HCV late stage entry, Tight junction component	Epithelial cells	Ploss et al. (2009)
LDLR	HCV binding/entry	Ubiquitous	Agnello et al. (1999)
L-SIGN and DC-SIGN	HCV capture/binding, C-type lectins	LSEC, Kupfer cell	Cormier et al. (2004a), Lozach et al. (2004)
Highly sulfated HS	HCV capture and binding	Hepatocyte	Barth et al. (2003), Basu et al. (2004, 2007)
Asialoglycoprotein receptor	HCV binding, c-type lectin	Liver (HBV receptor)	Saunier et al. (2003)

Abbreviations: Scavenger receptor B type I (*SR-BI*), Low-density lipoprotein receptor (LDLR), heparan sulfate (HS), liver sinusoidal endothelial cells (LSEC)

et al. 2004b; Pileri et al. 1998; Zhang et al. 2004), claudin-1 (CLDN1) (Evans et al. 2007), and occludin (OCLN) (Ploss et al. 2009). The function and tissue expression of these putative receptors or co-receptors for HCV are summarized in Table 1. Among these receptors, some are expressed only in particular cells while others are ubiquitously expressed, consistent with the idea that the target of HCV infection is not restricted to hepatocyte. Among these candidate receptors, it is generally believed that HCV infects human cells through the interaction of E2 with a tetraspanin molecule CD81 (Pileri et al. 1998). Wakita et al. demonstrated that CD81-specific antibodies neutralized the infectivity of HCV to Huh7 cells (Wakita et al. 2005), consistent with the finding that CD81 is required for pseudotype HCV infection (Zhang et al. 2004).

However, CD81 is ubiquitously expressed in many tissues. There are likely other factors that confer tissue specificity. Thus far, it is not clear whether CD81 is the receptor for HCV infection of lymphocytes. A lymphocyte-specific glycoprotein, CD5, has recently been identified to be a lymphotropic receptor for HCV entry into lymphocytes (Sarhan et al. 2012, 2013). Blocking of CD5 with antibody or

silencing with specific short hairpin RNA (shRNA) decreased T cell susceptibility to HCV, while increasing CD5 expression had the opposite effect. Moreover, transfection of naturally CD5-deficient HEK-293 fibroblasts with CD5 facilitated viral infection (Sarhan et al. 2012). Further study demonstrated that SR-B1, CLDN-1 and CLDN-6 are not associated with the susceptibility to HCV, but they serve as the necessary co-receptors for HCV infection. In contrast, CD5 and CD81 expression coincided with the HCV lymphotropism (Sarhan et al. 2013), suggesting that lymphotropic HCV strain may not use the same receptors as the hepatotropic HCV strain. It should be noted that HCV can also be produced and spread through exosomes; the pathway is partially resistant to antibody neutralization (Ramakrishnaiah et al. 2013). The presence of multiple HCV-interacting receptors and multiple entry mechanisms suggests that HCV has capacity to infect multiple cell types other than hepatocytes.

The extrahepatic replication of HCV may be limited not only by the deficiency of a specific surface receptor, but also by possible deficiency of other host factors required for HCV infection or replication. These host factors may be expressed only in hepatic cells. For example, the introduction of micro-RNA 122 or apolipoprotein Apo E into kidney cells allowed the recipient cells to support complete replication of HCV (Da Costa et al. 2012). It is possible that some extrahepatic cells do possess these factors, or that some virus strains do not depend on these factors, thus becoming more efficient at replicating in these cells.

5.2 Surface Binding vs. Real Replication of HCV in PBMC

As previously described, HCV RNA can be detected in PBMCs of infected individuals. However, in vitro studies showed that retroviral and lentiviral pseudoparticles bearing HCV envelope glycoproteins (HCVpp) could not infect lymphoid cells (McKeating et al. 2004). This raised a question whether HCV indeed infects and replicates in PBMCs or just associates with PBMCs without undergoing replication. Marukian et al. in an in vitro culture study, showed that approximately 0.1 % of the input viral RNA remained associated with PBMC cultures after the input virus was extensively washed away (Marukian et al. 2008). The authors demonstrated that level of HCV RNA did not increase in cultures of PBMCs at any time, and that the low level of HCV RNA detected in PBMCs was not affected by 2′CMA, an inhibitor of HCV RNA-dependent RNA polymerase (Marukian et al. 2008). Thus, they proposed that viral RNA may be bound or taken up by cells without undergoing a complete infectious cycle and suggested that detection of HCV RNA associated with blood cells does not prove that the virus replicates in these cells. Nevertheless, accumulating evidence supports the idea that HCV authentically infects PBMCs, replicates in these cells and alters the properties of cells. In an in vitro culture study, we also showed that HCV could infect T cell lines (Molt-4 and Jurkat cells) as well as human primary CD4+ T cells in vitro (Kondo et al. 2007). After infection for several weeks, both positive-

and negative-strand HCV RNA were still detectable in the infected cells. Moreover, infectious HCV particles were produced from Molt-4 cell cultures and could be used to infect naïve T cell lines (Kondo et al. 2007). Shimizu and colleagues (Nakajima et al. 1996; Shimizu et al. 1992) also extensively characterized the replication of the virus in both B-cell (Daudi) and T-cell (HPBMa10-2 and Molt4) lines. In addition to B and T lymphocytes, HCV can infect monocytes, especially $CD14^{+}CD16^{+}$ monocytes (Coquillard and Patterson 2009) and dendritic cells (Navas et al. 2002). In vitro studies demonstrated that lymphoblastoid cell lines are permissive for HCV replication.

5.3 *Evolution of Viral Genome 5′-UTR During In Vitro Passages Through T Cell Lines and In Vivo Replication in Extrahepatic Organs*

Nakajima et al. have characterized long-term cultures of HCV in T cell lines by sequencing several parts of HCV genome and found that the virus with a mutation in the hypervariable region of E2 protein could replicate in both Daudi cells and HPBMa10-2 cells (Nakajima et al. 1996). In addition, the sequence in the HCV recovered from PBMC of the infected chimpanzee was identical to this mutant (Nakajima et al. 1996). These pieces of evidence suggested that HCV indeed replicates in lymphocyte and selects a particular sequence(s) that may facilitate viral replication in lymphocytes. Further studies indicated that certain sequence variations in the 5′ untranslated region (5′UTR) of the viral genome (Lerat et al. 2000) may be associated with replication in B and T lymphocyte (Lerat et al. 2000; Nakajima et al. 1996; Shimizu et al. 1992). These 5′UTR substitutions were required for maximally increased translational activity in the lymphoblastoid cells but not in hepatocytes, suggesting that HCV adapts itself to replicate in lymphoid cells (Lerat et al. 2000). The viral RNA sequence of the predominant viral isolates in different extrahepatic cells, such as lymphoid cells, brain and others from HCV patients have been compared with those of hepatic isolates in the same patient (Forton et al. 2004a). The HCV quasispecies derived from different tissues generally showed different quasispecies patterns, further indicating that HCV replicates in the extrahepatic tissues and undergoes genetic selection.

5.4 *Isolation of Lymphotrophic Virus SB Virus from B Cell Lymphoma*

We have previously isolated an HCV SB strain from an HCV-infected B-cell lymphoma cell line (Sung et al. 2003). This splenic B cell line, called SB cell line, was established from the splenic tumor of an HCV-infected patient who has

mixed cryoglobulinemia and B-cell lymphoma. It is an EBV-negative and HCV-producing cell line, yielding approximately 28,000 copies/ml of viral RNA in the culture media. The HCV SB virus belongs to genotype 2b, and its quasispecies pattern is similar to that in the spleen but different from that in the serum of the same patient. The isolated SB virus could infect primary B cells, the established B cell lines and primary hepatocytes (Sung et al. 2003). The isolation of this virus from a lymphoma tumor and subsequently from the cell line gives undisputable evidence that HCV infects and replicates in B cells in vivo. The culture supernatant, which contains virus particles, infects naïve Raji cells (a B-cell lymphoma cell line) but does not replicate or infect Huh7 hepatoma cells. In contrast, JFH1 strain of HCV, isolated from a case of fulminant hepatitis (Wakita et al. 2005), productively infects Huh7 cells but does not infect or replicate in Raji cells. Recently, the molecular nature of the SB strain of HCV has been established by molecular cDNA cloning of the full-length HCV genome. The transcript of the full-length SB virus clone could replicate robustly in Raji but poorly in Huh7 cells. In contrast, the JFH1 RNA replicated in Huh7 cells, but poorly in Raji cells (Makino, unpublished). The phylogenetic analyses have shown that these two strains differ in more than 40 % of their amino acids, particularly in the E1, E2 and p7 regions. The mix-match experiments between SB and JFH1 RNA have preliminarily identified the E2 sequence and 5′-UTR sequence as crucial for determination of the lymphotropism of SB virus. The isolation of SB virus from B-cell tumor unequivocally established that certain strains of HCV can infect and replicate in B cells in vivo. The SB virus will be useful for further characterization of the genetic determinants of extrahepatic infection of HCV. Very recently, another lymphotropic HCV of genotype 1b was identified by deep sequencing (Kondo et al. 2014). The viral replication in lymphocytes could be stimulated with various cytokines.

6 Consequences of HCV Infection of B and T Cells

Among the extrahepatic diseases with HCV infection, mixed cryoglobulinemia-related diseases, autoimmune-related diseases and lymphoma are related to lymphoid cells, suggesting an association between HCV and lymphoid cells. Though the biological significance of lymphotropic HCV remains elusive, lymphocytes are viewed as the HCV reservoir and may contribute to the recurrence of HCV infection.

Additionally, lymphotropic infection of HCV disturbs the humoral and cellular immunity and contributes to carcinogenesis of the lymphoid cells. Therefore, understanding of the effects of HCV on the lymphoid cells is important for clarification of the immunopathogenesis of HCV persistent infection and B-cell lymphoproliferative diseases. Here, we summarize a number of studies showing the effect of HCV on lymphoid cells and discuss the biological significance of lymphotropic HCV.

6.1 Consequences of Viral Infection of T Cells

To cause persistent infection, HCV must have effective ways to escape from humoral and cellular immunity. The failure of HCV-specific $CD4^+$ and $CD8^+$ T cell response, including type 1 helper T cells (Th1) hyporesponsiveness, cytotoxic T lymphocytes (CTL) exhaustion, excessive function of $CD4^+CD25^+FOXP3^+$ regulatory T cells, has been demonstrated in HCV-infected patients (Boettler et al. 2005; Manigold and Racanelli 2007). Moreover, in vitro studies with infection of T cells support the findings regarding the failure of T cell response. In T cell line infected with HCV in vitro, the expression of STAT-1 was reduced and its activation was inhibited, resulting in suppression of IFN-γ/STAT-1/T-bet signaling (Kondo et al. 2007). Besides, HCV suppressed proliferation of T cells and enhanced Fas-mediated apoptosis by inhibiting the expression of CD44 splicing variant 6 (Kondo et al. 2009). The suppression of T cell functions may contribute to T cell anergy and immune escape of HCV. In contrast to suppression of the immune response, HCV can activate partial immune response. For example, HCV core protein activates transcription of interleukin-2 gene, which is required for T-cell growth and differentiation (Bergqvist and Rice 2001). HCV infection has also been shown to enhance the development of Th17 cells (Kondo et al. 2014).

6.2 Consequences of Viral Infection of B Cells

The predominant effects of HCV infection on B cells are increase of B cell proliferation and mutation frequency. As stated above, CD81 is recognized as a receptor for HCV infection and is part of a complex with CD21, CD19, and Leu13 in B cells (Levy et al. 1998). The complex, composed of CD81, CD21, CD19 and Leu13, lowers the threshold for B cell activation via the B cell receptor by bridging antigen-specific recognition and CD21-mediated complement recognition (Levy et al. 1998). Lymphocytes in mice lacking CD81 develop normally but have altered their proliferation in distinct manners, dependent on the context of stimulation (Miyazaki et al. 1997). The external stimulation of lymphocyte receptors by HCV antigen might induce a proliferation signal. Rosa et al. have reported that engagement of CD81 on human B cells by a combination of HCV-E2 and an anti-CD81 mAb triggered the JNK pathway and led to preferential proliferation of the naïve ($CD27^-$) B cell subset (Rosa et al. 2005) . Moreover, Chen et al have demonstrated that both E2 and HCVcc activated IκBα and subsequently increased expression of NF-kB and NF-kB target genes, such as antiapoptotic Bcl-2 family proteins, and protected Raji lymphoma cells and primary human B lymphocytes from Fas-mediated death (Chen et al. 2011). Besides, the HCV-core protein induces the production of interleukin 6 in $CD14^+$ cells via Toll like receptor 2 and leads to increased B cell proliferation (Feldmann et al. 2006).

In addition to the proliferation signal, HCV-E2-CD81 interaction on B cells enhances mutation frequency of cellular genes. We have documented that HCV infection of B cells triggers double-strand DNA breaks (DSBs) in many cellular genes (Machida et al. 2004b). Interestingly, HCV-E2 alone, without viral replication, can induce DSBs specifically in the variable region of immunoglobulin (Ig) gene locus, leading to hypermutation in Ig of B cells (Machida et al. 2005); this effect can be blocked by pre-incubation with the anti-CD81 antibody. In addition, HCV-E2-CD81 interaction on the surface of B cells elevates the expression of activation-induced cytidine deaminase (AID), which causes deamination of deoxycytidine to deoxyuracil in the template DNA strand (Petersen-Mahrt et al. 2002). AID is involved in both somatic hypermutation and class-switching recombination of Ig gene in normal B cell development, leading to enhancement of the mutation frequency (Machida et al. 2004b). Silencing AID blocked the E2-induced DSBs and hypermutation of Ig gene (Machida et al. 2005). Therefore, HCV infection, through E2-CD81 interaction, may modulate host's innate or adaptive immune response by activation of AID and hypermutation of Ig gene in B cells. Notably, although HCV-E2 can bind to hepatocytes, which also express CD81, the expression of AID or DSBs was not induced in this non-B cell line (Machida et al. 2005). These findings suggest that the other components of the CD81 complex, such as CD21 and CD19, may be important for the signal transduction involved in the induction of AID. Moreover, HCV infection also induced hypermutation of many other cellular genes, including protooncogenes, and *p53* (Machida et al. 2004b). The nucleotide-substitution patterns of p53 and β-catenin were different from those of Ig heavy chain in HCV-infected cells, suggesting different mechanisms of mutation. Furthermore, we demonstrated that HCV infection stimulated the production of nitric oxide (NO) through activation of the gene for inducible NO synthase (iNOS) by the viral core and NS3 proteins, leading to DNA breaks and enhanced DNA mutation (Machida et al. 2004a).

Pestka et al. have shown that rapid induction of neutralizing antibody contributes to viral clearance during the early phase of infection (Pestka et al. 2007), supporting the important role of neutralizing antibody in HCV-specific adaptive immunity. We have previously demonstrated that the E2-CD81 interaction enhances mutation frequencies in the variable region of immunoglobulin (V_H) gene locus (Machida et al. 2005). Furthermore, we demonstrated that the V_H sequences in the HCV-infected hybridomas had a significantly higher mutation frequency as compared with uninfected hybridomas (Machida et al. 2008). These mutations lowered the antibody affinity against the targeting protein and also decreased the virus-neutralizing activity of anti-E2 antibodies. Moreover, antibody-mediated, complement-dependent cytotoxicity with the antibodies secreted from the HCV-infected hybridomas was impaired (Machida et al. 2008). These results suggest that HCV infection could cause some anti-HCV-antibody-producing hybridoma B cells to make less-protective antibodies. Zeisel et al has indicated that during chronic HCV infection, delayed and inefficient neutralizing antibody response induces HCV escape mutations at Ab recognition sites, resulting in the selection of viral mutants (Zeisel et al. 2008). Therefore, we hypothesize that HCV may infect and cause B

cells mutation, thereby losing the avidity and specificity of HCV-specific antibodies within a short period of time. As a consequence, HCV is able to escape from humoral immunity owing to the reduction of the neutralization activity or the antibody-mediated cell lysing activity. It is worth noting that Kohara's group have established HCV transgenic mice that expressed the full HCV genome on B cells and observed a 25 % incidence of diffuse large B-cell non-Hodgkin lymphomas (Kasama et al. 2010), supporting the idea that HCV infects B cells and results in B-cell lymphoproliferative disorders.

7 Perspectives

Extrahepatic infection by HCV contributes to the pathogenesis of HCV. This chapter summarizes the evidence for the prevalence and roles of such occurrence in HCV biology and pathogenesis. In particular, B cell abnormality is a common feature of HCV infection. Clinically, Rituximab, which is a chimeric antibody against CD20 and used for the treatment of autoimmune and lymphoproliferative disorders, has been shown to be advantageous when incorporated into the anti-HCV regimen (interferon and ribavirin), suggesting the importance of lymphoid cells in HCV-induced diseases (Dammacco et al. 2010). HCV infection also involves multiple organs other than the liver. The preponderance of evidence indicates that HCV does not merely attach to the target cells in these tissues, but also replicates in them. The viral genetic elements and the tissue-specific factors that determine the tissue tropism of HCV for various cell types will be an interesting topic. So far, in the literature, there is only one HCV isolate (SB virus) derived from nonhepatic tissues. How prevalent is such a virus and how important it is in HCV pathogenesis will be very interesting topics. Such knowledge will contribute to the understanding of HCV biology and pathogenesis.

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Part II
HCV Infections in Special Populations

Hepatitis C Virus Infection in Pregnancy and Childhood

Jessica Wen, Samantha Ohmer, and Jonathan Honegger

Abstract Both pregnancy and childhood uniquely affect the natural history of hepatitis C virus (HCV) infection. Chronically infected women frequently experience a rise in plasma viral levels and decline in hepatic transaminases in the latter half of pregnancy, likely due in part to suppression of HCV-specific cellular immunity by maternofetal tolerance mechanisms. These changes reverse following delivery, with viremia sometimes declining sharply due to an apparent postpartum resurgence of HCV-specific immunity. While pregnancy influences the natural history of HCV, recent studies suggest that HCV infection may also affect pregnancy, predisposing to premature delivery and other adverse outcomes. About 4–6 % of pregnancies to viremic mothers result in utero or peripartum vertical transmission, which is now the primary route of childhood HCV infection. Currently no antiviral therapies or prophylactic interventions are available to prevent the vertical transmission of HCV. Hepatitis C acquired in childhood appears to follow a distinct course from infection acquired in adulthood. In the acute phase of infection, hepatic transaminases usually rise less dramatically in young children than in adults. Children may spontaneously resolve infection at least as often as adults, and some studies suggest they retain capacity to resolve infection over a more prolonged period of time. Persistently infected children demonstrate a slower progression of liver fibrosis than adults, though more studies are needed to understand the long-term outcome of pediatric HCV infection. Immunological mechanisms governing vertical transmission and the natural history of HCV in children remain poorly understood. Standard therapy for children remains interferon and ribavirin while clinical trials are underway to test direct acting antiviral regimens in this population.

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Keywords Hepatitis C virus • Pregnancy • Pediatrics • Vertical transmission • Cellular immunity • Childhood infection

1 Introduction

Prior to 1989, clinicians had described cases of parenterally-transmitted chronic non-A non-B hepatitis (NANBH) in pregnant women and children along with potential mother-to-child transmission events (Tabor 1989; Infeld et al. 1979; Wejstal and Norkrans 1989). Upon discovery of the hepatitis C virus (HCV) (Choo et al. 1989), it was quickly established that HCV was the chief etiologic agent of disease in these cases and that vertical transmission was indeed possible (Wejstal et al. 1990; Bortolotti et al. 1992). Recent estimates suggest that one million or more women with HCV deliver annually (Yeung et al. 2001; Le Campion et al. 2012), that 4–6 % of pregnancies to viremic women result in vertical transmission (Yeung et al. 2001; Benova et al. 2014), and that roughly ten million children are infected with HCV as of 2013 (Gower et al. 2014). While these cases constitute less than one tenth of the existing global HCV burden, new infections in children and adolescents account for a disproportionate number of new cases of HCV infection, creating a reservoir for HCV that may last for decades. Here we review unique features of the epidemiology, natural history, pathogenesis, and clinical management of HCV infection in pregnancy and childhood.

2 Epidemiology of HCV in Pregnant Women and Children

Regional surveys conducted among pregnant women over the past two decades have reported anti-HCV seroprevalence rates of 0.15–2.4 % in high-income nations (Arshad et al. 2011; Conte et al. 2000; Mast et al. 2005; Ohto et al. 2010; Blasig et al. 2011; Hutchinson et al. 2004; Seisdedos et al. 2011) and 0.15–8 % or greater in parts of certain low- and middle-income nations (Gasim et al. 2013; Costa et al. 2009; Bosan et al. 2010; Benova et al. 2015), reflecting the marked geographical variation in overall HCV prevalence. Egypt is believed to have the highest national prevalence of HCV in the world (Miller and Abu-Raddad 2010). Anti-HCV seroprevalence among Egyptians aged 15–59 years was estimated to be 14.7 % in 2008, including 11.7 % among women aged 15–49 years, and over 5 % in women aged 20–30 who have the highest fertility rates (Benova et al. 2015). As in Egypt, pregnant women across the globe tend to have intermediate HCV prevalence for their geographical region, between the lower rates found in children and the peak rates usually found among individuals over 55 years of age (Mohd Hanafiah et al. 2013).

Iatrogenic transmission of HCV accounts for most maternal infections in many low- and middle-income nations, including highly endemic countries such as Egypt

(Gasim et al. 2013; Miller and Abu-Raddad 2010; Hauri et al. 2004; Pepin et al. 2014). Though HCV transmission associated with blood transfusion has markedly decreased worldwide since discovery of the virus, it continues on a significant scale in some resource-constrained nations due to lack of screening or use of insensitive HCV screening assays (Thursz and Fontanet 2014). Routine injections, hemodialysis, and dental and surgical procedures pose ongoing risk for HCV transmission in some nations due to reuse of needles and syringes, inadequate sterilization, and other gaps in infection control practices (Thursz and Fontanet 2014). Health care employment was also found to be a risk factor for maternal infection in Egypt (El-Kamary et al. 2015). Despite these continued challenges, it should be acknowledged that public health efforts to improve infection control practices have substantially reduced iatrogenic HCV transmission in numerous low- and middle-income nations since 2000 (Pepin et al. 2014; Apata et al. 2014).

Injection drug use is the other predominant route of maternal HCV acquisition, particularly in high-income nations where iatrogenic transmission is now uncommon (Ohto et al. 2010). Unfortunately, rates of HCV transmission by injection drug use are on the rise in some of these nations, including in the United States, where a recently worsening epidemic of heroin use is thought to account for the doubling of acute HCV incidence in young women in the US between 2006 and 2012 (Suryaprasad et al. 2014).

Other routes of maternal HCV acquisition include sexual transmission, vertical transmission, and other less well-understood mechanisms. HCV is not efficiently transmitted sexually between monogamous HCV-discordant heterosexual couples (Terrault et al. 2013; Vandelli et al. 2004), but the risk rises among individuals with multiple sexual partners and in those co-infected with HIV or other sexually transmitted infections (Gorgos 2013). Some infected mothers have acquired HCV in childhood by vertical transmission or possibly by horizontal household transmission. Infection acquired via these routes likely reaches a significant scale in highly endemic regions (Benova et al. 2015; Said et al. 2013; Akhtar and Carpenter 2013). Finally, in most studies a subset of infected pregnant women report no identifiable risk factor for HCV, indicating an unrecognized or undisclosed mode of transmission (Giles et al. 2003; Diab-Elschahawi et al. 2013; Lambert et al. 2013).

Children tend to have lower rates of HCV seroprevalence than other age groups (Mohd Hanafiah et al. 2013), ranging from 0.05 % to 0.36 % in certain high-income nations to as high as 1.8–5.8 % in Egypt (Gower et al. 2014; El-Shabrawi and Kamal 2013). Vertical transmission is the chief route of HCV infection in young children across the globe (Benova et al. 2015; El-Shabrawi and Kamal 2013; Jhaveri and Swamy 2014), though iatrogenic exposures including dental procedures, surgeries, injections, and blood transfusions are also important risk factors for childhood HCV infection in high prevalence countries with suboptimal infection control (Esmat et al. 2012). An intra-household non-sexual horizontal route of HCV transmission to children is also suspected in some endemic sites, though the mechanisms are not understood (Said et al. 2013; Akhtar and Carpenter 2013).

Finally, as children age into adolescence, injection drug use increasingly contributes to HCV acquisition in some locales (Suryaprasad et al. 2014).

3 Natural History of HCV Infection in Pregnancy

3.1 Acute HCV Infection in Pregnancy

While most women with HCV have uneventful pregnancies, an accumulating body of evidence indicates that pregnancy has unique effects on the control of HCV infection, and that HCV may affect pregnancy outcomes. In non-pregnant adults the acute phase of HCV infection (conventionally the first 6 months) begins with a prolonged viremic incubation period (7–8 weeks on average, range 2–26 weeks) usually followed by onset of hepatitis with alanine aminotransferase (ALT) elevation (Lauer and Walker 2001). The hepatitis is thought to be immune-mediated, as the leakage of hepatic enzymes into circulation coincides with the appearance of functional HCV-specific CD8+ and CD4+ T-cells in the liver and declining levels of viremia (Fig. 1) (Rehermann 2009; Bowen and Walker 2005). Overt clinical hepatitis with jaundice may occur, but most acute HCV infections are asymptomatic and go unrecognized (Lauer and Walker 2001). Approximately 20–40 % of acute infections spontaneously resolve, usually within 6–12 months of onset, while the remainder establish persistent infection associated with chronic viremia and progressive exhaustion of cellular immunity (Bowen and Walker 2005; Grebely et al. 2014). Clinical predictors of spontaneous resolution of acute infection include development of jaundice, female sex, and in some studies, young age (Grebely et al. 2014; Tillmann et al. 2010; Beinhardt et al. 2013). Immune predictors of viral clearance include enduring HCV-specific cellular immune responses, favorable polymorphisms in the promoter region of the IL-28B gene, and inheritance of natural killer (NK) cell receptor/ligand combinations that favor NK cell activation (Bowen and Walker 2005; Thomas et al. 2009; Khakoo et al. 2004; Schulze Zur Wiesch et al. 2012; Fitzmaurice et al. 2015).

Whether pregnancy alters the clinical presentation or outcome of acute HCV infection is not clear from available data, which are scarce. Of nine case reports of acute HCV infection during pregnancy, seven (77 %) were recognized due to jaundice (Gonzalez et al. 2006; Flichman et al. 1999; Honegger et al. 2013). While this high rate of jaundice could suggest that pregnancy increases the odds of an icteric presentation of acute HCV, it almost certainly also reflects some degree of diagnostic and publication bias. Despite the presence of jaundice, none of five cases with follow-up experienced spontaneous resolution, possibly suggesting that infection acquired in pregnancy may be predisposed to chronicity. Approximately half of the reported cases of acute HCV in pregnancy resulted in premature delivery, but again, this finding may reflect selective reporting of more severe cases (Gonzalez et al. 2006; Honegger et al. 2013).

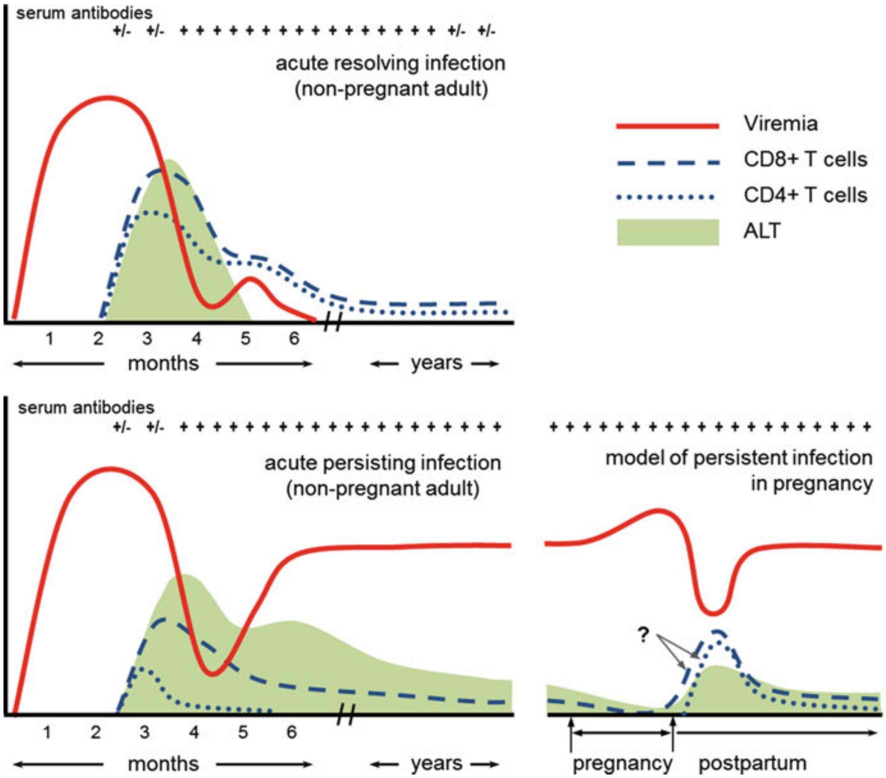

Fig. 1 Course of viremia, alanine aminotransferase (ALT) levels, and cellular immunity in acute hepatitis C virus (HCV) infections and influence of pregnancy on the natural history of chronic HCV infections. *Top left* in acute resolving infections, the onset of enduring functional HCV-specific CD8+ cytotoxic and CD4+ helper T-cell responses coincides with control of viremia and elevation of ALT levels from immune-mediated hepatotoxicity. *Bottom left* in acute persisting infections, HCV-specific T-cells transiently control viremia but CD8+ T-cells develop functional exhaustion and CD4+ T-cells are lost, resulting in viral rebound. *Bottom right* with pregnancy, HCV viral loads often climb during gestation and drop after delivery, sometimes sharply. Conversely, ALT levels often decline during pregnancy and peak in the first months postpartum. Some evidence suggests that these reciprocal changes in viral load and ATL reflect suppression of HCV-specific CD8+ T-cell activity by maternofetal tolerance mechanisms during pregnancy, followed by a rebound in CD8+ T-cell function after delivery. Whether functional HCV-specific CD4+ T-cell activity also sometimes emerges after delivery is unknown

3.2 Chronic HCV Infection in Pregnancy

Compared to the scant literature on acute HCV infection during pregnancy, the course of chronic HCV infection in pregnancy is better described. Even before the discovery of the hepatitis C virus, women with chronic NANBH were noted to have a drop in ALT levels in late pregnancy (Wejstal and Norkrans 1989). These findings were later replicated in studies of pregnant women with chronic HCV infection

(Wejstal et al. 1998; Gervais et al. 2000; Paternoster et al. 2001). These ALT declines are greater than would be expected from hemodilution alone and are sometimes associated with elevations in HCV viremia (Honegger et al. 2013; Wejstal et al. 1998; Gervais et al. 2000). ALT and virus levels tend to return to baseline pre-pregnancy levels after delivery, but some women experience sharp flares in ALT or marked declines in viremia 3–6 months after delivery (Fig. 1) (Honegger et al. 2013; Ruiz-Extremera et al. 2013; Lin and Kao 2000). Spontaneous resolution of chronic HCV in the postpartum period has also been rarely reported (Honegger et al. 2013; Hattori et al. 2003).

Given that HCV viral levels are normally stable in chronic infection (McGovern et al. 2009), changes in viremia and ALT during and after pregnancy are of interest because they may offer insight into mechanisms of immune control of chronic HCV. Placental interferon production was hypothesized to explain ALT declines during pregnancy, but the later discovery of a concomitant rise in HCV viremia during pregnancy was difficult to reconcile with this theory (Gervais et al. 2000; Paternoster et al. 2001; Floreani et al. 1996; Conte et al. 2001). Maternofetal tolerance mechanisms of pregnancy that dampen cellular immunity have also been proposed to explain the reciprocal changes in viremia and ALT that occur during and after pregnancy (Wejstal et al. 1998; Gervais et al. 2000). Recent work has supported this hypothesis. One study of postpartum ALT flares, viral load, and serum cytokines associated postpartum ALT flares with increased serum levels of Th1/Tc1 cytokines and the counter-regulatory cytokine IL-10 (Ruiz-Extremera et al. 2013). A study of viral evolution in women with circulating viral genomes that had accumulated escape mutations in HLA class I epitopes prior to pregnancy provided evidence for fluctuating HCV-specific CD8+ T-cell activity during and after pregnancy (Honegger et al. 2013). By the third trimester of pregnancy, viruses emerged that had lost escape mutations in several of these class I epitopes, thereby reverting to "wild-type" sequences. Viruses bearing these "wild-type" versions of class I epitopes replicated better in vitro than those bearing escape mutations and were transmitted to offspring. Following delivery viral loads dipped sharply, "wild-type" variants were lost, and viruses bearing escape mutations reemerged. This study supports the hypothesis that maternofetal tolerance mechanisms of pregnancy directly inhibit HCV-specific CD8+ T-cells during gestation and that these T-cell responses rebound after delivery (Fig. 1). Further work is needed to better understand which hormonal, cytokine, or cellular components of the maternofetal tolerance process dictate the suppression and rebound of HCV-specific cellular immunity (Price et al. 2014).

While pregnancy may significantly affect HCV-specific cellular immunity, the long-term effects of altered immunity during and after pregnancy on the course of chronic HCV infection is less clear. A case-control study of 12 HCV-infected women who underwent liver biopsy before and after pregnancy reported significantly greater progression of necroinflammatory scores and a trend towards increased fibrosis following pregnancy compared to 12 non-pregnant women biopsied twice over a similar timeframe (Fontaine et al. 2000). However, some of the postpartum histologic changes reversed years later, suggesting that they may

have represented a transient postpartum inflammatory state. A retrospective study of 157 HCV-infected women found that women with past pregnancies had lower fibrosis scores than nulliparous women, suggesting that the long-term net effect of pregnancy on liver fibrosis was benign, and possibly protective (Di Martino et al. 2004). Estrogen signaling is thought to protect hepatocytes from oxidative stress and inflammatory injury, which could possibly explain the beneficial effects of pregnancy and the overall slower progression of liver fibrosis in pre-menopausal women compared to men or post-menopausal women (Baden et al. 2014). Whether pregnancy affects the future risk of hepatocellular carcinoma in chronic HCV infections is unclear due to contradictory study results (Amr et al. 2014; McGlynn et al. 2015).

3.3 Pregnancy Outcomes for HCV-Infected Women

The earliest work examining pregnant women with chronic NANBH or chronic HCV did not identify significant adverse pregnancy or birth outcomes, particularly when contrasted with the overt risk of maternal and fetal mortality posed by other hepatic conditions, such as liver cirrhosis or acute hepatitis E virus infection (Infeld et al. 1979; Wejstal and Norkrans 1989; Shaheen and Myers 2010; Dienstag and Alter 1986; Bohman et al. 1992). Later studies however have identified a number of adverse pregnancy outcomes in HCV-infected mothers, though distinguishing the specific effects of HCV has been challenging in cohorts from high-income nations where injection drug use is the primary risk factor for maternal infection. Recent large studies attempting to test for the independent effects of HCV on pregnancy by controlling for alcohol, tobacco, and drug exposures have identified possible increased risk of preterm delivery (Connell et al. 2011), low birth weight (Connell et al. 2011; Pergam et al. 2008), small size for gestational age (SGA) (Pergam et al. 2008), neonatal abstinence syndrome (Berkley et al. 2008), adverse neonatal neurologic outcomes including feeding problems and seizures (Salemi et al. 2014), gestational diabetes (Pergam et al. 2008; Reddick et al. 2011), and intrahepatic cholestasis of pregnancy (ICP) (Berkley et al. 2008), though specific associations were not necessarily reproduced across cohorts (Table 1).

Interestingly, studies of women infected by means other than injection drug use (eg. contaminated IgG anti-D, blood transfusions, or surgery) did not identify effects of maternal HCV infection on risks for adverse pregnancy outcomes such as prematurity, low birth weight, or SGA, although these studies were significantly smaller in size (Jaffery et al. 2005; Jabeen et al. 2000). Larger studies from such cohorts may be useful adjuncts to existing studies for gauging the independent effects of HCV on pregnancy outcomes. Mechanisms by which HCV would predispose to preterm delivery or reduced fetal growth are not well studied, but could potentially relate to the finding of enhanced placental NKT cell cytotoxicity in HCV-infected women (Hurtado et al. 2010).

Table 1 Adverse pregnancy outcomes associated with maternal hepatitis C infection following adjustment for demographic factors including tobacco and drug use. North American cohorts

Authors	Source	# HCV ab +	Comparator group	Outcomes				
				Gestational DM	Prematurity	LBW	SGA	Unique findings
Connell et al. (2011)	Florida discharge data 1998–2007	999	1,669,370	NS	**OR 1.40 (1.15–1.72)**	**OR 1.39 (1.11–1.74)**	NS OR 1.19 (0.97–1.46)	Congenital anomalies **OR 1.55 (1.14–2.11)**
Reddick et al. (2011)	NIS 1995–2005 discharge data	555	296,218	**OR 1.61 (1–2.59)**	NS OR 1.22 (0.94–1.52)			
Pergam et al. (2008)	Washington state birth certificate 2003–2005	506	2,024 random selected	If excess wt gain **OR 2.51 (1.04–6.03)**	NS OR 1.54 (0.97–2.43)	**OR 2.17 (1.24–3.80)**	**OR 1.46 (1–2.13)**	NICU **OR 2.91 (1.86–4.55)** Neonatal assisted ventilation **OR 2.37 (1.46–3.85)**
		124 drug using	1,439 drug using	QNS	NS OR 1.03 (0.66–1.61)	NS OR 1.19 (0.74–1.91)	NS OR 0.97 (0.57–1.64)	NICU **OR 2.80 (1.83–4.29)** Neonatal assisted ventilation **OR 1.82 (1.03–3.22)**
Berkley et al. (2008)	Univ. New Mexico pregnancy drug treatment program	159 drug using	141 drug using HCV neg.		NS	33 % vs 17 %, adj $P = 0.06$		Cholestasis of pregnancy (**6.3 % vs 0 %, $P = 0.002$**) Neonatal abstinence syndrome if mother on methadone (**88.4 % vs 36.4 %, $P < 0.001$**)
Salemi et al. (2014)	Florida discharge data 1998–2009	2,457	2,214,778					Feeding difficulty **OR 1.32 (1.06–1.64)** Neonatal seizure OR 1.74 (0.98, 3.10) Any neurological outcome **1.22 (1.03–1.44)**

While there is some discordance across studies regarding the contribution of HCV to several adverse pregnancy outcomes, the association of maternal HCV infection with intrahepatic cholestasis of pregnancy (ICP) has been replicated in multiple cohorts (Locatelli et al. 1999; Paternoster et al. 2002; Ropponen et al. 2006; Marschall et al. 2013). In two Scandinavian registry studies, women with ICP had anti-HCV prevalence three to four times that of pregnant women without cholestasis, such that some authors have advocated HCV screening in all women diagnosed with ICP (Marschall et al. 2013). It is not certain why HCV-infected women have increased susceptibility to ICP, but this may be linked to reduced expression of bile acid exporter pumps such as MRP2 in HCV-infected livers (Hinoshita et al. 2001).

Finally, while questions remain about the overall effects of HCV on pregnancy outcomes, it is important to note that HCV-infected women who have advanced liver disease such as cirrhosis with portal hypertension do face significant risks in pregnancy (Shaheen and Myers 2010). An Egyptian cohort of 129 pregnant women with cirrhosis due to viral hepatitis (more than half with HCV mono-infection) experienced increased rates of pregnancy complications including placental abruption, post-partum hemorrhage, preeclampsia, intrauterine growth restriction, maternal mortality (7.8 % versus 0.2 %), and neonatal mortality (4.7 % versus 0.8 %) compared to non-cirrhotic controls (Rasheed et al. 2013). Mortality among cirrhotic pregnant women also exceeded that of cirrhotic non-pregnant controls observed over a similar timeframe (7.8 % versus 2.5 %). Variceal hemorrhage at time of vaginal delivery was the major cause of maternal mortality. While multidisciplinary care by hepatology, maternal-fetal medicine, and neonatology specialists along with endoscopic monitoring and management of varices may improve pregnancy outcomes, these resources may not be readily available in some highly endemic nations (Rasheed et al. 2013; Russell and Craigo 1998).

4 Vertical Transmission of HCV

4.1 Rates of Vertical Transmission

Vertical (mother-to-child) transmission of HCV is now the chief route of HCV acquisition in children across the globe, and substantial effort has been given to defining the rates of such transmission. A 2014 meta-analysis of more than 2,000 pregnancies to HCV-RNA positive, HIV negative mothers living primarily in high-income nations derived a HCV vertical transmission rate of 5.8 % (95 % CI 4.2–7.8 %) (Benova et al. 2014), similar to the rate of 4.3 % that was found in a 2001 meta-analysis (Yeung et al. 2001). In the same meta-analysis from 2014, an increased HCV vertical transmission rate of 10.8 % was found for mothers co-infected with HCV and HIV (Benova et al. 2014). In contrast, vertical transmission from an HCV-RNA negative, anti-HCV seropositive mother appears to be

exceedingly rare (Granovsky et al. 1998; Resti et al. 2002a). In such cases, maternal "aviremia" is generally thought to represent a single snapshot of a fluctuating viral load or, alternatively, to be attributable to insensitive PCR testing (Benova et al. 2014).

Despite reasonable concordance between the two largest meta-analyses of vertical transmission rates (Yeung et al. 2001; Benova et al. 2014), results from individual studies vary significantly, likely reflecting their heterogeneous study populations, HCV assay types, and case definitions. Some controversy exists regarding whether a significantly greater number of HCV transmission events occur than are accounted for by the stringent definitions of vertical transmission used in many studies (Jhaveri and Swamy 2014; Shebl et al. 2009; Wen and Haber 2014). Specifically, many studies of vertical transmission require persistence of HCV-antibody through 18 or 24 months or repeatedly positive HCV-RNA PCR to qualify children as vertically infected. However several studies have documented the presence of HCV-RNA in 12, 14, or even 20 % of vertically exposed infants, with more than half of the infected infants resolving viremia by 12 months of age and losing antibody by 18 months of age (Shebl et al. 2009; Ceci et al. 2001; Ruiz-Extremera et al. 2011). Though these cases would not be counted by some definitions, they may represent true cases of vertically transmitted infection. It is important to note, however, that some of these studies had other irregularities such as very late appearance of transiently detectable HCV-RNA (8 months) (Ceci et al. 2001) or unexpectedly high rates of transient viremia detected in children born to HCV-RNA negative women (Shebl et al. 2009). Another study supported the concept of a high rate of vertical transmission and viral clearance within infants, finding that a large proportion of children born to mothers with HCV infection have HCV-specific T-cell activation despite persistently negative HCV PCR (Della Bella et al. 2005). Resolution of this uncertainty is more than just a matter of semantics; it has significant implications for the pathogenesis of vertical transmission and immunity in infected infants. Efforts to mainstream HCV viral load testing for clinical use in the past decade have led to an expanded availability of highly sensitive quantitative real-time PCR with reduced false positive rates, such that the true rate of vertical transmission and the frequency and viral dynamics of "transient infections" in vertically exposed infants may be defined more precisely in the coming years.

4.2 Clinical Risk Factors for Vertical Transmission

In addition to maternal HIV co-infection and detectable maternal viremia, other clinical risk factors for perinatal transmission include high maternal viral load, maternal injection drug use, fetal scalp electrode monitoring, and rupture of amniotic membranes for more than 6 h (Table 2) (Yeung et al. 2001; Benova et al. 2014; Mast et al. 2005; Resti et al. 2002a; Lin et al. 1994; Okamoto et al. 2000; Indolfi et al. 2013a; European Paediatric Hepatitis, C.V.N 2005a). Despite the apparent

Table 2 Factors affecting risk of HCV vertical transmission (Prasad and Honegger 2013)

Increased risk of transmission	Inconsistent effect on transmission
High maternal viral load (esp. $>10^6$ IU/mL)	Fetal inheritance of certain HLA class I or class II alleles
HIV co-infection (untreated)	**No effect seen**
Internal fetal monitoring	Breastfeeding
Maternal lacerations during birthing process	Mode of delivery
Prolonged rupture of membranes (esp. >6 h)	HCV genotype
Maternal IV drug use	Maternal IL28B genotype
Maternal-fetal HLA class II concordance	Fetal IL28B genotype
Female sex of fetus	Maternal antibody neutralization of heterologous HCV strains
HCV detection in maternal PBMC	Maternal-fetal HLA class I concordance

influence of peripartum factors on risk of vertical transmission, studies conflict on whether elective cesarean section reduces the risk virus transmission. Thus, elective cesarean delivery is not routinely recommended in mothers who are mono-infected with HCV (Cottrell et al. 2013; Ghamar Chehreh et al. 2011). HCV RNA has been found in colostrum and breast milk, but there is no evidence to suggest that transmission occurs via breastfeeding (Conte et al. 2000; Mast et al. 2005; Lin et al. 1994; Gibb et al. 2000; Mast 2004). Therefore, mothers who are mono-infected with HCV are encouraged to breastfeed unless they have bleeding or cracked nipples. Post-partum household exposure is another possible route by which mothers may transmit HCV to their children and housemates, but the frequency of non-sexual horizontal transmission remains unclear (Indolfi et al. 2013b; Meisel et al. 1995).

4.3 Pathogenesis of Vertical Transmission

4.3.1 Timing and Route of Vertical Transmission

As vertical transmission is the leading route of HCV infection in young children (Jhaveri and Swamy 2014) and no prophylactic measures exist, there is significant impetus to understand when and how vertical infection occurs. Both intrauterine and peripartum transmission of HCV are possible. Studies using PCR to test for HCV in very young infants (<3 days old) suggest that intrauterine transmission occurs in approximately one-third to one-half of cases (Mok et al. 2005; Resti et al. 1998), while about half of vertically-infected children seem to acquire virus either late in utero or peripartum (Tosone et al. 2014). The occurrence of intrapartum transmission is further supported by the finding that rates of

transmission may be increased in cases of fetal exposure to infected maternal blood, including with prolonged rupture of membranes, maternal lacerations during the birthing process, and fetal monitoring via scalp electrodes (Mast et al. 2005; Indolfi et al. 2013a). While maternofetal blood mixing through placental or cutaneous injury may explain some cases of intrapartum transmission (Kwiek et al. 2006), the route by which in utero transmission occurs is less clear. Proposed mechanisms include transplacental passage of free virus or cell-associated virus, direct infection of placental trophoblasts, and transcytosis through placental trophoblasts (Le Campion et al. 2012).

It has been found that maternal immune cells may pass through the placenta and into umbilical cord blood, creating maternal microchimerism in the fetus (Jonsson et al. 2008). The passage of infected maternal cells into the fetus could therefore provide a potential route for HCV vertical transmission. Although there has been a longstanding debate on whether HCV generates productive infection in lymphocytes and other peripheral blood mononuclear cells (PBMC) (Kronenberger and Zeuzem 2009; Marukian et al. 2008), many groups have demonstrated HCV RNA associated with PBMC (Azzari et al. 2008; Resti et al. 2002b; Di Lello et al. 2014; Jablonska et al. 2013; Kao et al. 1997; Lerat et al. 1996, 1998). In several instances, both the positive and negative strands of HCV have been detected in PBMC (Azzari et al. 2000; Cribier et al. 1995a; Li et al. 2005; Manzin et al. 1994; Pawelczyk et al. 2013; Sansonno et al. 1998; Zignego et al. 1995), while others have demonstrated possible viral evolution in PBMC cultures (Di Lello et al. 2014), suggesting that PBMC can serve as sites of extra-hepatic viral replication. At least one group has presented data that the detection of HCV in maternal PBMC was a strong predictor of vertical transmission, even better than maternal plasma viral loads (Azzari et al. 2008, 2000), and argued that maternal PBMC may be the vector for HCV vertical transmission. PBMC infection is suggested to occur more frequently in HCV infection acquired by use of injection drugs, which could help explain why injection drug use has been has been linked to an increased risk HCV vertical transmission (Yeung et al. 2001; Granovsky et al. 1998; Resti et al. 2002a; Azzari et al. 2008). Further, HLA class II mismatch between mother and child was associated with a reduced risk of HCV vertical transmission in one study (Bevilacqua et al. 2009), supporting a hypothesis that fetal T cells exerting an allo-reactive attack on infected maternal PBMC that cross the placenta may protect against vertical transmission (Indolfi et al. 2013a).

Placental trophoblast infection has also been proposed as a possible route of in utero HCV vertical transmission. Expression of HCV receptors including the LDL receptor, DC-SIGN, CD81, SR-B1, claudin-1 and occludin has been demonstrated on primary trophoblasts or trophoblastic cell lines, at least at low level (Le Campion et al. 2012; Mostafavi et al. 2012). It has been suggested that HCV can infect cultured first trimester primary placental trophoblasts in vitro (Nie et al. 2012). Others have found that early trophoblastic cell lines are permissive for HCV entry (Mostafavi et al. 2012), though whether they are also competent for replication of HCV is still unclear. To date there has been no in vivo evidence of HCV replication in trophoblasts, and emerging evidence suggests that the placenta is uniquely

equipped to resist viral infections including HCV (Delorme-Axford et al. 2013; Ouyang et al. 2014). Thus the role of trophoblast infection in HCV vertical transmission remains speculative.

The increased rate of HCV vertical transmission in HCV/HIV co-infected mothers (Mast et al. 2005; Gibb et al. 2000; Tosone et al. 2014; Pappalardo 2003; Marine-Barjoan et al. 2007; Roberts and Yeung 2002; Airoldi and Berghella 2006) could potentially yield insight into the route of HCV infection, and multiple mechanisms for this effect of HIV have been proposed. HIV not only induces immunosuppression and allows HCV viral load to climb (European Paediatric Hepatitis, C.V.N 2005a; Matthews-Greer et al. 2001; Thomas et al. 1996; Cribier et al. 1995b), but may also damage the placental architecture (as in the case of HIV-induced chorioamnionitis), and thereby break down the barrier that prevents passage of HCV from mother to child (Tosone et al. 2014). HIV co-infection has also been linked to an increased rate of HCV detection in PBMC, potentially increasing the odds of PBMC-associated virus transmission (Blackard et al. 2005). Highly active antiretroviral therapy appears to reduce the risk of HCV vertical transmission, but the mechanism by which this occurs remains to be determined (European Paediatric Hepatitis, C.V.N 2005a; Checa Cabot et al. 2013).

4.3.2 Viral Quasispecies Selection in Vertical Transmission

Examination of maternal and infant viral quasispecies has the potential to provide additional insight into the mechanisms of vertical transmission. In several small case studies of HCV envelope gene sequences, vertically transmitted viruses appeared to have traversed a significant population bottleneck because most (Kudo et al. 1997; Manzin et al. 2000; Murakami et al. 2000; Sitia et al. 2001; Weiner et al. 1993) but not all (Murakami et al. 2000) cases involved transfer of just one or two founder variants. Interestingly, transmitted founder variants did not necessarily reflect the dominant quasispecies present in the maternal plasma at time of delivery (Kudo et al. 1997). These findings have yet to be verified by single-template sequencing, the current standard approach for tracing the lineage of transmitted viral variants (Li et al. 2012). Further, it remains to be demonstrated whether sequences of viruses compartmentalized within maternal PBMC more closely match infant viral quasispecies than those from maternal serum (Indolfi et al. 2013a).

4.3.3 Immune Factors Affecting Vertical Transmission

By one estimate, 10^{13}–10^{14} HCV particles may circulate through the placenta during a term gestation (Babik et al. 2011), which begs the question of why only 5 % of infants vertically acquire the infection (Benova et al. 2014). Although some groups have proposed that the actual vertical transmission rate is two to three times

higher than current estimates when cases with rapid clearance of transient viremia are considered (Jhaveri and Swamy 2014; Wen and Haber 2014), the vast majority of pregnancies to viremic women do not result in detectable transmission to their offspring. These rates of HCV vertical transmission are significantly lower than those for other viremic illnesses such as HIV or hepatitis B. Given the down-regulation of maternal cellular immunity necessary to achieve maternofetal tolerance, the rise in maternal HCV viral load that often occurs during pregnancy, and in vitro evidence that fetal hepatocytes are readily susceptible to HCV infection (Andrus et al. 2011), the low rate of vertical transmission suggests the existence of other potent immune mechanisms that prevent establishment of fetal infection.

Transplacental passage of maternal anti-HCV antibodies has been hypothesized as one mechanism limiting vertical transmission. Although the overall role of humoral immunity in controlling HCV infection has been uncertain, broadly reactive neutralizing antibody responses are detectable in most individuals and their rapid appearance has been associated with spontaneous resolution of acute infection (Osburn et al. 2014; von Hahn et al. 2007). Active transport of these antibodies across the placenta in the third trimester of pregnancy may therefore prevent infection in the fetus. One study compared neutralizing activity of serum from HCV-transmitting and non-transmitting mothers co-infected with HIV (Dowd et al. 2008). Using a genotype 1 strain H77 pseudoparticle system to measure neutralization, they found no difference in HCV-neutralizing antibody titers between transmitting and non-transmitting mothers, suggesting that there was no protective effect of these heterologous neutralizing antibodies. Nevertheless, it is appreciated that HCV neutralization is strain-specific and neutralization of the H77 strain may not reflect the capacity to neutralize autologous virus. Indeed, in chronic infection the HCV envelope proteins appear to continuously evolve to escape neutralizing antibody responses (von Hahn et al. 2007). Studies are warranted to determine whether non-transmitting women can better neutralize autologous circulating virus than transmitting women, but ongoing maternal viremia in the face of these antibodies may indicate that there are at least some viral variants in all viremic women that can escape neutralization (Dowd et al. 2008).

Antiviral immunity at the placental interface is not well characterized, but emerging data suggest that the placenta contains unique cellular and molecular mechanisms of virus resistance. On the one hand, the placenta is known to be replete with tolerogenic mechanisms that suppress adaptive cellular immunity, potentially increasing susceptibility to infection. Thus growing attention has been given to cells of the innate immune system. One study enumerated and characterized immune cells in the decidua, placenta, and umbilical cord blood of term pregnancies to HCV-infected and uninfected women (Hurtado et al. 2010). The study found enhanced frequencies and cytotoxicity of certain innate immune effector cells in the maternal decidua and fetal placenta relative to cord blood, comprising a potential gradient of antiviral defense. The placental compartment was notable for enrichment of NKT and gamma-delta (γδ) T cells and greater cytotoxicity of NKT and NK cells relative to cord blood. In mothers with HCV infection placental γδ T cell frequency, NKT cell frequency, and NKT cytotoxicity

were further increased while NK cells actually had reduced expression of activation markers. It is possible that these innate immune cells help limit HCV transmission to the fetus in utero. Interestingly, it has also been conjectured that enhanced placental NKT- and γδ T cell-mediated immunity may contribute to increased rate of pre-term labor in HCV-infected women, as placental innate immune hyperactivity has previously been shown to predispose normal pregnancy to pre-term labor in animal models (Hurtado et al. 2010; Gomez-Lopez et al. 2014).

Beyond the enrichment of certain innate immune cells populations, additional placental antiviral mechanisms may limit virus transmission. Placental trophoblasts, fetal cells in direct contact with maternal circulation, constitute the primary physical barrier separating maternal and fetal circulations and form the first line of defense against infectious agents in the maternal circulation. Primary trophoblast cultures are resistant to numerous virus infections (Delorme-Axford et al. 2013). Further, transfer of medium from these trophoblast cultures confers resistance to viral infection to many normally permissive cell lines, including resistance of Huh7.5 cells to cell-culture adapted HCV strains. This was recently found to relate in part to production of a placenta-specific microRNA cluster encoded on chromosome 19, which when transferred in exosomes appears to confer resistance by induction of autophagy. Further work is needed to understand the in vivo relevance of these findings and whether placental expression of this chromosome 19 microRNA cluster limits vertical transmission of HCV or other viruses (Delorme-Axford et al. 2013; Ouyang et al. 2014).

It is likely that at least some HCV particles traverse the placenta in most pregnancies, and fetal immunity may thus also play a role in limiting vertical transmission (Babik et al. 2011). Fetal/neonatal T-cell responses to HCV are of interest in this regard because data from adults show that virus-specific T-cells not only play a critical role in controlling acute HCV infection (Bowen and Walker 2005), but are also often found in HCV-exposed seronegative, aviremic individuals (Heller et al. 2013). Cord blood T-cell responses to HCV were not detected by intracellular cytokine stain in one study of seven vertically exposed infants (Babik et al. 2011), suggesting that in utero exposure does not usually prime functional HCV-specific T-cell responses. However, this study did note reduced expression of activation markers on global T-cell populations and exaggerated IFN-γ production by T-cells following polyclonal stimulation (Babik et al. 2011), suggesting an immunological effect of in utero exposure to HCV. Studies of older vertically exposed, aviremic children have yielded conflicting results. In one study, more than 70 % of 1–4 year old aviremic vertically exposed children demonstrated HCV peptide induced T-cell expression of activation markers, which was interpreted as a memory response to prior HCV exposure (Della Bella et al. 2005). However a study of 3–8 year old vertically exposed children found HCV-specific T-cell responses by IFN-γ enzyme-linked immunosorbent spot (ELISpot) assay in just 14 % of aviremic children, a proportion similar to that found in children born to uninfected mothers in this highly endemic region (El-Kamary et al. 2013). Of note, HCV-specific ELISpot responses were detected in 80 % of children with a history of transient viremia, suggesting that T-cells may play an important role in the spontaneous clearance of

established infection in children (El-Kamary et al. 2013). In support of a role of T-cell immunity affecting vertical transmission, several HLA class I and class II alleles have been associated with increased or decreased risk of vertical transmission, but specific associations have not been replicated across cohorts (Bevilacqua et al. 2009; Bosi et al. 2002; Martinetti et al. 2006). Despite the existence of several studies on HCV-specific T-cells in vertically infected children, their role in preventing vertical transmission remains unclear.

Whether fetal or neonatal NK cells contribute to viral clearance is not known. One study identified an association of HLA-C alleles with vertical transmission, possibly indicating a role for NK KIR-HLA-C interactions (Martinetti et al. 2006). Another study examined the influence of an IL28B polymorphism that is strongly associated with spontaneous and treatment-induced resolution of HCV infection in adults (Thomas et al. 2009; Ge et al. 2009). Interestingly, neither maternal nor infant IL28B genotype affected risk of vertical transmission of HCV, but infant IL28B genotype was associated with spontaneous resolution of infection in those infants who had acquired infection from their mothers (Ruiz-Extremera et al. 2011).

A final unexplained factor affecting fetal immunity to HCV is the increased susceptibility of female children to vertical transmission, at a ratio approaching 2:1 in some studies (Granovsky et al. 1998; European Paediatric Hepatitis, C.V.N 2005a; Bortolotti et al. 2007; England et al. 2011). It has been conjectured that sex-specific differences in the fetal immune response in utero and in the peri-partum period put females at higher risk (Indolfi et al. 2013a). This is an intriguing finding that bears further investigation, as it is in contrast with the comparatively favorable outcome of HCV infection reported in adult females, who are not only more likely to spontaneously clear HCV than males, but who also suffer less severe consequences of chronic infection (Hattori et al. 2003; Baden et al. 2014; Chen and Morgan 2006; Inoue et al. 2000; Li et al. 2014; Micallef et al. 2006; Nishida et al. 2012). Similarly, among young children parenterally exposed to HCV, female sex is protective against the establishment of infection (England et al. 2011). Aside from sex-based differences in estrogen and other hormones that may directly affect immune function, several immune-related genes are encoded on the X chromosome, such as the Toll-like receptors 7 and 8, as well as the IL-2 receptor gamma, CD132 (van Lunzen and Altfeld 2014). It remains to be determined what role, if any, these factors play in the increased risk for vertical transmission of HCV to female neonates.

5 Natural History of HCV Infection in Children

5.1 Clinical Course of HCV in Children

Though not extensively studied, the clinical presentation of acute HCV infection in infants and young children may be even more subtle than it is in adults. In adults, a

1–3 month incubation period characterized by high-level viremia and normal ALT is followed by a peak in ALT that often exceeds ten times the upper limit of normal (Lauer and Walker 2001; Alter et al. 1992; Hoofnagle 2002). In vertically acquired infection, high-level viremia is also usually detected in the first months of life (Farci et al. 2006), but ALT typically does not reach peak levels until 3–24 months of age, exceeds five times the upper limit of normal in less than half of children, and in some cases remains persistently normal (Tovo et al. 2000; European Paediatric Hepatitis, C.V.N 2005b; Resti et al. 2003). Whereas up to one-third of acutely infected adults experience symptomatic hepatitis, often with jaundice (Tillmann et al. 2010; Hoofnagle 2002), icteric hepatitis has not been described in several major series of vertically infected infants (England et al. 2011; Tovo et al. 2000; European Paediatric Hepatitis, C.V.N 2005b; Abdel-Hady et al. 2011; Yeung et al. 2007). Young children infected by means other than vertical transmission also usually lack recognizable symptoms and are identified only by testing following known or potential exposures or after incidental detection of elevated transaminases (England et al. 2011; Abdel-Hady et al. 2011; Vogt et al. 1999; Mohamed et al. 2005). In adolescents the clinical manifestations of acute HCV more closely resemble those of adults.

Symptomatic presentation of acute HCV infection, particularly icteric hepatitis, has been linked to increased likelihood of spontaneous viral clearance in adults (Tillmann et al. 2010). Despite their paucity of symptoms, young children may be at least as likely to resolve infection as adults. Whether acquired by vertical or parenteral transmission, HCV infection in young children spontaneously resolves in 20–40 % of cases (average about 25 %) (Grebely et al. 2014; Ceci et al. 2001; Tovo et al. 2000; European Paediatric Hepatitis, C.V.N 2005b; Yeung et al. 2007; Vogt et al. 1999; Garazzino et al. 2014). Interestingly, young children seem to retain the capacity to resolve infection several years beyond the 6–12 month timeframe in which most adult infections resolve (Grebely et al. 2014; European Paediatric Hepatitis, C.V.N 2005b; Yeung et al. 2007).

Among children in whom HCV infection persists, liver disease is typically milder than in adults (Murray et al. 2005; Barshes et al. 2006). Liver biopsies taken years to several decades after infection reveal mild inflammation and low fibrosis scores in most children (Vogt et al. 1999; Mohan et al. 2007; Jara et al. 2003). ALT levels are often normal or minimally elevated in chronically infected children, though some have persistently elevated ALT, which may be associated with hepatomegaly (European Paediatric Hepatitis, C.V.N 2005b) and higher grade inflammation on liver biopsy (Mohan et al. 2007). As in adults, normal ALT in children does not exclude advanced fibrosis (Mohan et al. 2007). Age at acquisition appears to be a key determinate of the pace of progression towards cirrhosis. In a retrospective study of transfusion acquired cases, the probability of cirrhosis after 30 years of infection increased from 5 % to 13 % to over 50 % for individuals infected at ages 0–20, 21–30, and greater than 30 years respectively (Minola et al. 2002).

HCV rarely progresses to end-stage liver disease in children. Indeed, though hepatitis C occurs in 0.2–0.4 % of the pediatric population in the U.S., only

67 pediatric patients received liver transplants due to HCV infection from 1988 to 2005 (Barshes et al. 2006). By extension, HCV-related hepatocellular carcinoma is extremely uncommon in the pediatric population, with only three case reports in the literature (Gonzalez-Peralta et al. 2009; Malik et al. 2014). One patient was successfully transplanted and subsequently cleared of hepatitis C infection with post-transplant treatment; one patient died while waiting for transplant; and the other died shortly after liver transplantation due to intraoperative complications.

While the progression of HCV-related liver disease is generally slow in childhood, whether it will accelerate in adulthood remains to be determined. There is some thought that exposure to other co-factors such as hepatotoxic medications, alcohol, and other injuries could accelerate disease progression in adulthood (Mohan et al. 2007). Indeed, in several pediatric observational studies, more advanced liver fibrosis does occur in older children, suggesting insidious chronic progression of liver disease despite a low incidence of the most severe sequelae of infection (Abdel-Hady et al. 2011; Jara et al. 2003)

Finally, it is important to mention extra-hepatic manifestations of HCV in children. One study has linked childhood HCV infection with cognitive problems even in children who display normal liver function tests, in association with elevation of certain immune cytokines such as plasma IFN-α and IL-6 (Abu Faddan et al. 2015). Autoantibodies or cryoglobulins are detected in up to 50 % of untreated vertically infected children, but their clinical significance is unclear as manifestations of autoimmune phenomena appear much less commonly in children than in adults (European Paediatric Hepatitis, C.V.N 2005b; Garazzino et al. 2014; Indolfi et al. 2012). Some studies however have linked detection of antibodies to LKM-1 to more advanced liver disease in children (Bortolotti et al. 2003).

5.2 Immune Response to Pediatric HCV Infection

Certain features of the natural history of HCV infection in young children, including their blunted and delayed rise in ALT, slow disease progression, and prolonged capacity to resolve infection, point to unique effects of young age on the host immune response to HCV. In acutely infected adults, the rise in ALT levels 1–3 months after infection is temporally associated with onset of functional cellular immunity to HCV and is thought to reflect immune-mediated hepatocyte injury rather than viral cytotoxicity. Thus, the subdued rise in ALT in children may indicate a prolonged delay in the onset of functional cellular immunity to HCV. This is not surprising in one sense, as the adaptive cellular immune response is both immature and tightly regulated in utero to afford tolerance of maternal antigens. Equally interesting however, is that despite their presumably restrained onset of adaptive cellular immunity, young children are as likely, or perhaps more likely, to resolve infection than adults. This paradox is not seen with other persistent viral infections such as hepatitis B, in which a lack of immune mediated symptoms in

infancy is accompanied by a higher rate of persistence than for infections acquired in adulthood.

T-cell responses to HCV are central mediators of the outcome of acute infection in adults, and several lines of evidence suggest that T-cell immunity may play a role in control of HCV infection in children. Two studies have shown that ALT elevation over five times the upper limits of normal in the first 2 years of life—a possible indicator of T-cell mediated immune activity—predicts resolution of vertically acquired infection (Resti et al. 2003; Garazzino et al. 2014). HCV-specific T-cell responses were detected by IFN-γ ELISpot in four of five vertically exposed children aged 3–8 years who had resolved transient viremia in infancy, versus 0 of 6 seropositive children with persistent viremia (El-Kamary et al. 2013; Hashem et al. 2011). Interestingly, three viremic children who were seronegative for anti-HCV and had household contacts to HCV did have moderate HCV-specific ELISpot responses, possibly reflecting T-cell responses during an acute phase of infection (Hashem et al. 2011). A seronegative 10-year-old with known vertically acquired infection had weak IFN-γ ELISpot responses to HCV peptides below the typical threshold for positivity (Larouche et al. 2012). Another study that included three vertically infected children ages 1–4 years found that none had detectable proliferative HCV-specific CD4+ T-cell responses (Della Bella et al. 2005), suggesting that like adults, children with persistent HCV infection demonstrate weak or absent HCV-specific cellular immunity.

Important gaps in the understanding of T-cell responses to HCV in young children arise from a lack of longitudinal studies, particularly in the first year of infection when the outcome of infection is often determined. The kinetics, frequency, cytotoxicity, and cytokine secretion profile of these cells in the acute phase of pediatric infection are unknown, both for children who go on to resolve infection and for those with persistent infection. Further, tolerogenic mechanisms that might explain the delayed onset of ALT elevation have not been explored in the context of HCV vertical transmission. Recent evidence suggests that children born to HCV-infected mothers may be exposed to significant concentrations of HCV protein antigens in utero, potentially predisposing T-cells that target these antigens to a regulatory phenotype (Attallah et al. 2015). Finally, studies of cellular immunity have not been performed in children infected through routes other than vertical transmission, though similar profiles of viremia and ALT between vertically and parenterally infected children suggest that their immune responses may share some common features (England et al. 2011).

Essentially all infants born to HCV seropositive mothers will be seropositive for anti-HCV antibodies in the first months of life due to transplacental passage of maternal antibody. The proportion of uninfected (aviremic) infants with residual maternal antibody (as detected by enzyme-linked immunoassay) falls to about 90 % by 3–4 months, 80 % by 5–6 months, 15 % by 12 months, and <1 % by 18 months of age (Mast et al. 2005). Determining the time of onset of humoral immune responses in vertically infected infants is somewhat difficult due to persistence of maternal antibody, but in some infected infants a distinct decline of maternal anti-HCV titers precedes a later rise in infant anti-HCV titers (Mast et al. 2005;

Murakami et al. 2000), sometimes separated by a 3–12 month gap of seronegativity (Mast et al. 2005). Using the more stringent recombinant immunoblot assay (RIBA) test to determine anti-HCV seropositivity, the onset of the infant response was estimated to range from 5 to 15 months of age in one study (Farci et al. 2006), suggesting that the onset of humoral immunity in infants may be more delayed than it is in acutely infected adults. Rarely, viremic children may remain anti-HCV seronegative for years (Larouche et al. 2012), particularly in the presence of HIV co-infection (Granovsky et al. 1998).

Whether maternal or infant antibodies affect the course of HCV infection in infants is not clear. Certainly, high-level viremia can occur regardless of the presence of maternal antibodies (Farci et al. 2006; Meunier et al. 2011), including those that broadly neutralize heterologous viruses (Meunier et al. 2011). It is conceivable that transmitted viral variants are pre-selected to escape maternal antibodies (Meunier et al. 2011). Infants also appear to have the capacity to generate humoral responses that broadly neutralize heterologous viruses (Meunier et al. 2011) and exert selection pressure on viral envelop protein targets (Farci et al. 2006), but onset of these responses has not been linked to changes in viremia. Some toddlers do clear virus after seroconversion (European Paediatric Hepatitis, C.V.N 2005b; Resti et al. 2003), but to date, no studies have compared neutralizing capacity of antibody responses of seropositive young children who clear infection to those with persistent infection (Meunier et al. 2011). Numerous studies have documented cases of spontaneous resolution of confirmed viremia in infants who tested negative for anti-HCV at age 12 months or earlier (Ruiz-Extremera et al. 2011; El-Kamary et al. 2013; European Paediatric Hepatitis, C.V.N 2005b). Whether these infants failed to prime a humoral response or instead mounted a very transient response, their cases suggest that robust humoral immunity is not an absolute requirement for control of viremia in infancy.

Even fewer studies have explored innate immunity in children. As noted earlier, one study found that a polymorphism in the promoter region of IL28B known to be associated with resolution of HCV infection in adults was also associated with resolution of viremia in vertically infected infants (Ruiz-Extremera et al. 2011). Mechanisms of this polymorphism are not completely understood and its association with adaptive responses is not clearly determined. No studies have examined the effects of cellular components of the innate immune system on the course of HCV infection in children.

Together, these studies suggest that immune responses to HCV in children share some features with adults, including the capacity to mount HCV-specific T-cell responses that are detectable in much greater frequency in those with resolved infection than those with persistent infection, the capacity to mount broadly neutralizing antibody responses, and a protective role of IL28B polymorphisms. Nevertheless, children seem to lack the same destructive cell-mediated immune attack on hepatocytes that contributes to much of the liver pathology in adults (Garcia-Monzon et al. 1998). Whether this is a result of tolerance due to exposure to antigen from a young age or from exhaustion and deletion, as in adults, remains to be seen.

6 Clinical Management of HCV in Pregnancy and Childhood

6.1 Diagnosis of HCV in Pregnant Women

While some medical institutions have incorporated universal testing for hepatitis C in pregnancy, most rely on a risk-based screening approach to identify mothers who are HCV-infected (Blasig et al. 2011; Giles et al. 2003; Diab-Elschahawi et al. 2013; ACOG 2007; Pembrey et al. 1999; Selvapatt et al. 2015; McDermott et al. 2010). Pregnant women with risk factors for HCV such as those listed in Table 3 are tested for anti-HCV antibody, and if positive, by HCV PCR to confirm viremia and inform risk of vertical transmission. Beyond traditional risk factors, some recent studies also suggest that women with intrahepatic cholestasis of pregnancy should be screened for HCV (Marschall et al. 2013). While targeted risk-based screening should in theory capture the vast majority of women with HCV (Martyn et al. 2011), in practice this approach may miss a large proportion of infected women, due in part to the practical difficulties of relying on providers to assess risk factors, hesitancy of pregnant women to disclose risk factors, and cases of HCV among women without common risk factors (Blasig et al. 2011; Giles et al. 2003). In highly endemic nations, certain HCV risk factors such as prior surgical and dental procedures are highly prevalent and render development of a targeted screening approach for pregnant women challenging (El-Kamary

Table 3 Indications for HCV testing in pregnant women and children

CDC: HCV testing recommended (Centers for Disease Control (CDC) 1998)
Persons who have ever injected illegal drugs, including those who injected only once many years ago
Recipients of clotting factor concentrates made before 1987
Recipients of blood transfusions or solid organ transplants before July 1992
Patients who have ever received long-term hemodialysis treatment
Persons with known exposures to HCV, such as health care workers after needlesticks involving HCV-positive blood or recipients of blood or organs from a donor who later tested HCV-positive
All persons with HIV infection
Patients with signs or symptoms of liver disease
Children born to HCV-positive mothers
CDC: HCV testing of uncertain need (Centers for Disease Control (CDC) 1998)
Recipients of transplanted tissue (e.g., corneal, musculoskeletal, skin, ova, sperm)
Intranasal cocaine and other noninjecting illegal drug users
Persons with a history of tattooing or body piercing
Persons with a history of multiple sex partners or sexually transmitted diseases
Long-term steady sex partners of HCV-positive persons
Pregnancy specific (Marschall et al. 2013)
Consider in women with intrahepatic cholestasis of pregnancy

et al. 2015). Cost-effectiveness studies in high-income nations have favored targeted screening over universal screening. This is due in large part to low prevalence of HCV in many locales, the lack of methods to prevent vertical transmission, the slow progression of vertically acquired infection, and limitations of interferon therapies for HCV which render the costs of quality-adjusted life years gained through universal screening unacceptably high (Plunkett and Grobman 2005). On the other hand, recent data indicate that availability of highly effective direct-acting-antiviral therapies for HCV may render universal HCV screening in pregnancy cost-effective, even in locales with relatively low prevalence (Selvapatt et al. 2015).

6.2 Management of HCV in Pregnant Women

Antiviral therapy for HCV is not currently recommended in pregnancy. Combined pegylated interferon-α (PEG-IFN-α) and ribavirin was until recently the standard therapy for HCV, but this regimen is contraindicated in pregnancy due to fetal safety concerns. Ribavirin in particular is a pregnancy category X drug because of teratogenicity at low doses in animal studies and is also to be avoided in women and their male partners 6 months prior to conception (Roberts et al. 2010). PEG-IFN-α is considered a category C agent in pregnancy, though no major safety problems have emerged in case series (Yazdani Brojeni et al. 2012). Several new direct acting antiviral therapies appear safer in animal pregnancy models and open the possibility that treatment of HCV in pregnancy may be a viable option in the future for resolving maternal infection and preventing vertical transmission (Arshad et al. 2011). Studies will be needed to examine the safety, efficacy, and risk/benefit of this approach versus early postpartum treatment of mothers and the 5 % of children who acquire the infection.

While HCV-infected women are not yet given antiviral therapy during pregnancy, several aspects of prenatal and postpartum care do warrant attention. Testing for viremia late in pregnancy can be useful in counseling about risk of vertical transmission, as transmission rarely occurs in mothers who have undetectable viral loads. Comorbid conditions such as drug abuse need to be addressed if present. At delivery, HCV-infected women generally receive routine peripartum care, though avoidance of fetal scalp electrode monitoring is advised if feasible. Postpartum breastfeeding should be encouraged except in cases of cracked or bleeding nipples, in which case pumping and discarding of milk is advised until the affected breast heals (Mack et al. 2012; Lin et al. 1995). Finally, pregnancy provides an opportune time to link infected women to care for postpartum management of their HCV.

While most women with HCV have uneventful pregnancies, those who have advanced liver disease do need to be managed in consultation with a hepatologist during pregnancy as they may be at risk of complications including variceal hemorrhage, hepatic failure, encephalopathy, and maternal or fetal death (Rasheed et al. 2013; Russell and Craigo 1998).

6.3 Diagnosis of HCV Infection in Children

After infancy the approach to diagnosis of HCV in children is the same as for adults. Children with risk factors for HCV (Table 3) should be screened by anti-HCV antibody testing, followed by HCV-RNA PCR if antibody positive (Smith et al. 2012). Children with recent exposures to HCV or immunocompromised children may be seronegative and require direct screening with HCV-RNA PCR.

In vertically exposed infants, HCV antibody testing is not recommended before age 18 months to avoid detection of persistent circulating maternal antibodies (Mast et al. 2005; Mack et al. 2012). If earlier testing is desired, either to address parental anxiety or to improve rates of follow-up, HCV-RNA PCR after 2 months of age is the recommended diagnostic test. PCR testing before 1 month of age appears to have significantly less sensitivity, likely owing to low viral load (Gibb et al. 2000; Polywka et al. 2006; Davison et al. 2006). If initial PCR testing is positive in young infants, it should be repeated after 12 months of age to look for viral persistence as some infants may spontaneously clear the infection in infancy (Mack et al. 2012).

By one estimate, only 5–15 % of HCV infected children in the United States have been diagnosed (Delgado-Borrego et al. 2012). Because HCV is usually clinically silent, vigilance is needed to identify infected children. Comprehensive efforts may be needed to improve ability to identify, track, and screen at risk children, including those who are vertically-exposed (Abughali et al. 2014).

6.4 Monitoring HCV Infection in Children

Since young children with HCV infection are generally asymptomatic, the goals of clinical care primarily focus on ongoing education about the infection as well as assessing the clinical progression of disease while making decisions regarding choice and timing of therapy. Families often feel anxiety about the diagnosis and care of their child, since many of these children are either too young to be treated at the time of diagnosis or may benefit from waiting for better treatment options. Anticipatory guidance should be provided, including the importance of receiving immunizations against other hepatotropic virus such hepatitis A and B; the prevention of viral transmission by avoid sharing of personal items that can be contaminated by blood, such as toothbrushes and nail clippers; and techniques for cleaning up blood spills, such as wearing gloves and using diluted bleach or disinfectants. Conversely, families should be educated that hepatitis C is not spread by casual contacts such as kissing or hugging, and children should not be isolated due to their infection status. There is often apprehension about disclosing the child's infection status to school, daycare, other care providers or family and friends. Although there may be some variation between regulations geographically, there is generally no legal requirement to disclose infection in the United States to schools and daycare.

Therefore, the decision for disclosure of infection status should be individualized and carefully weighed by the family.

In general, infected children are monitored with annual blood work, including aminotransferases, bilirubin, albumin, complete blood count with platelets, and prothrombin time (if cirrhosis is suspected or present). Additionally HCV RNA monitoring is important, particularly in very young children, as spontaneous clearance of infection can occur. HCV genotype is identified so that treatment options may be anticipated. Presence of co-infections such as hepatitis B and HIV infection should also be screened, as these co-infections impact progression of liver disease and treatment options. Liver biopsy is generally not utilized unless there is concern for development of cirrhosis or if it will impact clinical decision-making, particularly since the new treatments now in development have high cure rates. In adults, there are now available non-invasive markers of liver fibrosis, both laboratory based (fibrotest/Fibrosure) as well as imaging based techniques such as Fibroscan (Echosens, Paris, France) and acoustic radiation force impulse imaging (ARFI), thus decreasing the need for liver biopsy in patients with hepatitis C infection (Rockey and Bissell 2006; Gonzalez et al. 2013; Bota et al. 2013). However, these techniques have not yet been widely utilized in children since these techniques are often disease- and population- specific and validations are still needed in the pediatric population. Once these non-invasive liver fibrosis markers are validated in children, they may potentially be useful for monitoring disease progression, particularly in older, untreated children.

There have only been few cases of reported hepatocellular carcinoma in children with hepatitis C infection (Gonzalez-Peralta et al. 2009; Malik et al. 2014), and therefore, routine surveillance ultrasound is generally not indicated in all children with hepatitis C infection. However, in those with advanced liver disease and cirrhosis, surveillance ultrasound and serum alpha-fetoprotein should be considered annually or biannually (Mack et al. 2012).

6.5 Treatment of HCV in Children

The goal of treating HCV in children includes eradicating the HCV virus, halting the progression or preventing the development of cirrhosis, reducing the occurrence of hepatocellular carcinoma, and relieving the social stigmatization associated with HCV infection. Given the relatively slow progression of disease during the first decade of infection, the optimal timing for treatment is often dictated by family preference and social situation rather than clinical necessity. Early childhood may be an opportune time, as young children generally tolerate treatment well, experience fewer side effects, and have motivated caregivers to ensure compliance (Mack et al. 2012). Teenagers may have demanding school and activity schedules, which in turn may impact their willingness to undergo treatment. However, treatment during this time may still be preferred, as older adolescents may move away from home to attend college, at which point effective treatment may be even more

difficult to achieve. Some argue that because of the overall slow disease progression, it is reasonable to delay treatment until adulthood. Providing treatment before patients become sexually active may provide additional benefits, however, including prevention of further spread of infection either through sexual contact or via vertical transmission. In reality, considerations regarding the timing of treatment may soon become less important with the introduction of treatment regimens that are shorter in duration and have minimal side effects, as these treatments should have few barriers to prevent early treatment.

Until recently the standard therapy for HCV infection was combination PEG-IFN-α and ribavirin. Interferon-α is a cytokine naturally produced by host immune cells to modulate the innate immune system and to impede viral replication. Ribavirin is an oral nucleoside analogue that, when used in combination with interferon, has synergistic anti-viral effects by inhibiting viral RNA synthesis and also by modulating the immune system, although its exact mechanism of action is not known. Injections of recombinant interferon-α three-times weekly was first approved as a treatment of chronic hepatitis C in the U.S. in the early 1990s, initially as monotherapy, and later on in combination with ribavirin for improved efficacy. In 2001, a pegylated formulation of interferon-α, wherein the interferon molecule is covalently linked to polyethylene glycol, became available. The pegylated form of interferon-α has a longer circulating half-life and a more favorable pharmacokinetic profile, with the subsequent benefit of needing only to be injected once weekly and as well as providing better rates of sustained virologic response.

The use of interferon in combination with ribavirin for the treatment of chronic hepatitis C in children was first published in several studies during early 2000s (Christensson et al. 2000; Lackner et al. 2000; Wirth et al. 2002; Gonzalez-Peralta et al. 2005). These trials demonstrated a comparable or superior efficacy of the regimen in children as that seen in adults. The use of PEG-IFN-α in combination with ribavirin was also soon studied in children (Wirth et al. 2005, 2010; Schwarz et al. 2011; Abdel-Aziz et al. 2011), which subsequently led to the licensing of combination PEG-IFN-α and ribavirin in the U.S for the treatment of chronically infected children over the age of 3 years. Similar to the adult regimen, the length of treatment recommended by the North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) practice guidelines is 48 weeks for genotype 1 and 4 infection, and 24 weeks for genotype 2 and 3 infection. However, if the viral load has not dropped by at least 2-$\log_{10}$ at 12 weeks of therapy or viral clearance is not achieved by 24 weeks of therapy, prolonged treatment is not recommended.

6.5.1 Side Effects

Almost all patients experience side effects with PEG-IFN-α/ribavirin treatment, and most of the hesitancy regarding treatment is due to the side effect profile related to this regimen. PEG-IFN-α has been associated with a variety of side effects such

as flu-like symptoms, fatigue, fever, headaches, anorexia, weight loss, depression, and irritability. Additionally, laboratory abnormalities such as leukopenia, neutropenia, anemia, and thrombocytopenia require the practitioner to closely monitor lab parameters and reduce doses when necessary. Furthermore, PEG-IFN-α can induce or worsen existing autoimmune diseases, including autoimmune thyroid disorders, and its use may be contraindicated in such situations. The main side effect of ribavirin is dose-dependent hemolytic anemia. Ribavirin is also a teratogen and strict birth control must be practiced during treatment and for a period of 6 months after treatment in both males and females.

In general, however, children tolerate therapy with PEG-IFN-α/ribavirin relatively well, as they are generally healthy and lack co-morbidities that may exacerbate drug side effects. Furthermore, most children undergoing treatment do not have cirrhosis, which also tends to cause worsened side effects. In two large pediatric studies conducted in the U.S. (Schwarz et al. 2011) and Europe (Wirth et al. 2010), almost all children experienced constitutional symptoms with PEG-IFN-α injection, though these symptoms usually resolved within 24 h after the injection. Neutropenia was the next most common side effect, requiring PEG-IFN-α dose reduction in 38 % of children in the U.S. study and 12 % in the European study. Nevertheless, there was no discontinuation of treatment, nor serious infection associated with neutropenia in either study. Stimulating growth factors such as G-CSF were not used. Anemia was another common side effect, requiring ribavirin dose reduction in 25 % of children in the U.S. study (Schwarz et al. 2011) and 7 % in the European study (Wirth et al. 2010). There was no early discontinuation of treatment due to anemia, however.

The use of PEG-IFN-α is withheld until at least 3 years of age in children with chronic hepatitis C infection due to the fact that the clinical course is relatively mild early in life and because of concerns regarding interferon's impact on growth during such a critical period of childhood development. This concern may be warranted, as a majority of children treated with PEG-IFN-α experience weight loss while on treatment, and there is also a decrease in the velocity of height change during treatment. However, the weight is regained after discontinuing treatment and there is catch up growth during long-term follow up of children who completed treatment (Wirth et al. 2010; Jonas et al. 2012).

6.6 Applications of Emerging Therapies for Children

In recent years there has been a burst of drug development for HCV. Direct-acting antiviral (DAA) agents such as telaprevir (Vertex, Boston, MA) and boceprevir (Merck, Whitehouse Station, NJ) were incorporated into the treatment of genotype 1 chronic hepatitis C in 2011, significantly improving the virologic response rate compared to the prior clinical standard of combination PEG-IFN-α and ribavirin. In 2013 and 2014, the FDA approved second generation DAAs including sofosbuvir (Gilead, Foster City, CA), fixed dose sofosbuvir/ledipasvir (Gilead, Foster City,

CA), simeprevir (Janssen, Titusville, NJ)) and fixed-dose ombitasvir/paritaprevir/ritonavir in combination with dasabuvir (Abbvie, North Chicago, IL). These agents further improved virologic response rates, shortened duration of treatment, and led to better tolerance by patients. The emergence of so many effective DAAs has provided a clear path towards all-oral, interferon-free regimens for all genotypes, such that interferon therapy for HCV may soon be obsolete. In fact, the current American Association for the Study of Liver Disease and Infectious Disease Society of America recommendations (AASLO-IDSA 2015) for adults with chronic hepatitis C infection no longer include interferon for most genotypes. Interferon may continue to have a role in more resource constrained countries until affordable DAA regimens are widely available.

It should be acknowledged that use of DAAs in children is not yet established. At the time of this publication, the recommended and FDA-approved treatment of chronic pediatric hepatitis C infection remains the combination of PEG-IFN-α with ribavirin. The first pediatric studies of all-oral DAA regimens are ongoing (www.clinicaltrials.gov). If proven safe and effective for children, DAA-based therapies will almost assuredly replace interferon-based regimens and significantly alter the approach to treatment of chronic hepatitis C in children.

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Part III
Approaches to Therapy

Host Genetics and Responses to Antiviral Therapy in Chronic Hepatitis C

Yasuhito Tanaka and Masashi Mizokami

Abstract Genome-wide association studies (GWAS) have revealed recently that certain interleukin-28B (*IL28B*) polymorphisms are strongly associated with responses to pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy in patients chronically infected with hepatitis C virus (HCV) genotype 1. Subsequent reports revealed that these *IL28B* polymorphisms also affect treatment efficacy in chronic infection with other HCV genotypes. Therapy of chronic hepatitis C (CHC) recently has entered a new era with the availability of direct-acting antiviral agents (DAAs). These include non-structural 3/4A protease inhibitors which have shown promise in combination with PEG-IFN/RBV in several clinical trials, as well as in clinical practice. IFN-free therapy is expected to be useful especially for IFN-resistant patients and may become the standard of care in the future. Several clinical trials have revealed an association between *IL28B* genotype and treatment efficacy in triple therapy and in some interferon-free regimens. Recently, it was shown that a polymorphism of *IFN-lambda 4* (*IFNL4*) is in high linkage disequilibrium with that of near *IL28B* and more strongly associated with spontaneous or treatment-induced HCV clearance than the *IL28B* polymorphisms, especially in individuals of African ancestry. This finding provides new insights into the genetic regulation of HCV clearance and its clinical management. *IL28B* genotyping will be useful for personalized treatment of CHC in the forthcoming era of DAAs.

Keywords Hepatitis C virus (HCV) • Single nucleotide polymorphism (SNP) • Genome-wide association study (GWAS) • Interleukin 28B (IL28B) • Favorable *IL28B* genotype (CC at rs12979860 or TT at rs8099917) • Direct-acting antivirals (DAAs)

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1 Introduction

Chronic hepatitis C virus (HCV) infection is a significant health problem worldwide with approximately 170 million people infected (Lavanchy 2009). Over 70 % of individuals acutely infected with HCV go on to develop chronic infection and are at significant risk of progressive liver fibrosis and subsequent liver cirrhosis and hepatocellular carcinoma (HCC). Antiviral treatment has been shown to improve liver histology and decrease the incidence of HCC in chronic hepatitis C (CHC) (Yoshida et al. 2004; George et al. 2009). Up to 2011, the standard treatment for chronic HCV infection was weekly pegylated interferon (PEG-IFN) in combination with daily doses of ribavirin (RBV); however, less than 50 % of patients infected with HCV genotype 1 treated in this way achieve a sustained virological response (SVR) (Fried et al. 2002; Hadziyannis et al. 2004). In 2009, genome-wide association studies (GWAS), including our study of HCV infection (Tanaka et al. 2009), showed that a single nucleotide polymorphism (SNP) near the interleukin-28B (*IL28B*) gene is strongly associated with response to PEG-IFN/RBV therapy for chronic HCV genotype 1 infection (Tanaka et al. 2009; Ge et al. 2009; Suppiah et al. 2009; Rauch et al. 2010). As a result, prediction of treatment outcome, especially non-responsiveness to PEG-IFN/RBV, has been greatly improved by genotyping for the *IL28B* SNP, enabling personalized medicine to be developed for chronic hepatitis C (CHC). Newly developed treatments involving direct-acting antiviral agents (DAAs), including nonstructural (NS) 3/4A protease inhibitors, have shown promising outcomes in combination with PEG-IFN/RBV in several clinical trials as well as clinical practice, wherein >70 % of patients infected with HCV genotype 1 achieved SVR (Zeuzem et al. 2011; Poordad et al. 2012; Jacobson et al. 2011a). Several clinical trials have revealed an association between *IL28B* polymorphism and treatment efficacy in triple therapy and in some interferon-free regimens.

1.1 Predictors of Response to IFN-Based Therapy

Various viral and host factors have been identified as significant determinants of the outcome of IFN-based treatments. Viral genotype and baseline viral load are well known predictors of response to therapy. Other viral factors in patients infected with HCV genotype 1 include amino acid substitutions at positions 70 and 91 in the HCV core region (Akuta et al. 2005) and in the interferon sensitivity-determining region (ISDR) in NS5A (Enomoto et al. 1996). Several host factors related to failure of treatment-induced viral clearance include older age, insulin resistance, advanced fibrosis and hepatic steatosis (McHutchison et al. 2002, 2009). Ethnicity is also a factor in treatment outcome. The proportion of African-American patients achieving SVR on treatment with PEG-IFN/RBV is lower than of Caucasian patients

(Conjeevaram et al. 2006; Jeffers et al. 2004; Muir et al. 2004), indicating that host genetic factors can be an important determinant of treatment outcome.

1.2 *The Association Between* IL28B *and Response to PEG-IFN/RBV*

The success of the Human Genome Project accelerated studies of genetic factors involved in different outcomes of HCV infection. Significant breakthroughs in identifying phenotype-associated SNPs followed when the GWAS approach was established. Unlike the traditional candidate gene approach, GWAS can identify functionally important polymorphisms in genes that have no predicted role in disease pathogenesis. In 2009, four independent groups simultaneously published the results of GWAS to assess the role of genetic variation in response to PEG-IFN/RBV for CHC patients (Tanaka et al. 2009; Ge et al. 2009; Suppiah et al. 2009; Rauch et al. 2010). These studies revealed a strong association between genetic polymorphisms near the *IL28B* locus on chromosome 19 and treatment-induced HCV clearance (Table 1). Ge et al. studied patients from the IDEAL trial (Ge et al. 2009), a large randomized controlled trial involving Caucasians, African-Americans and Hispanics in North America (n = 1137). The CC genotype at rs12979860 showed a twofold greater rate of achievement of SVR in Europeans and Hispanics, and a threefold higher rate of SVR in African-Americans, relative to the non-CC genotype (Ge et al. 2009). Suppiah et al. also analyzed Caucasians, comprising 293 Australian individuals infected with HCV genotype 1, and validated their findings in an independent replication cohort consisting of 555 - Europeans from the UK, Germany, Italy, and Australia. They showed that

Table 1 GWAS on association between the favorable *IL28B* genotype (rs8099917 TT or rs12979860 CC) and SVR in PEG-IFN/RBV therapy

Study	Population	GWAS/ replication size	Significant SNP	Odds ratio	P value	HCV genotype
Ge et al.	European American, African American, Hispanic	1137/none	rs12979860	3.10	1.37×10^{-28}	1
Suppiah et al.	Australian, European	293/555	rs8099917	1.98	7.06×10^{-8}	1
Tanaka et al.	Japanese	142/172	rs8099917	12.10	1.18×10^{-18}	1
Rauch et al.	European	465/none	rs8099917	5.19	3.11×10^{-8}	1–4

GWAS genome-wide association study, *SVR* sustained virological response, *PEG-IFN* pegylated interferon, *RBV* ribavirin

rs8099917 was the polymorphism most strongly associated with SVR (Suppiah et al. 2009). On the other hand, we reported host factors associated with a null virological response (NVR) to treatment with PEG-IFN/RBV in 142 Japanese CHC patients infected with HCV genotype 1, and an independent replication cohort of 172 Japanese patients. In that study, rs8099917 showed the most significant associations ($P = 2.68 \times 10^{-32}$, OR = 27.1) (Tanaka et al. 2009). Rauch et al. investigated 465 Caucasians infected with HCV genotypes 1, 2, 3, or 4 and a strong predictive value of the *IL28B* polymorphism was observed in genotype 1 and 4 patients, but not in genotype 2 and 3 infection (Rauch et al. 2010). The above studies document that rs12979860 and rs8099917 are the polymorphisms most significantly associated with response to therapy. These SNPs are in strong linkage disequilibrium, except for patients of African ancestry; they are in partial linkage disequilibrium in Caucasians (Ge et al. 2009; Rauch et al. 2010), but in near-complete linkage disequilibrium in East Asians.

Moreover, significant differences have been reported between ethnicities in response to PEG-IFN/RBV therapy: the SVR rate was approximately 20–28 % in African Americans and 40–52 % in Caucasian patients with HCV genotype 1 (Conjeevaram et al. 2006; Jeffers et al. 2004; Muir et al. 2004), and 57 % vs. 82 % in those with genotype 2/3 (Shiffman et al. 2007). The frequency of the favorable *IL28B* genotype (CC at rs12979860 or TT at rs8099917) for treatment varies with ethnicity, being >80 % in certain Asian populations, 35–55 % in Caucasians and <20 % in patients of African ancestry. This variation explains, in part, the lower response rates of African-Americans, compared to Caucasians, and the higher response rates of Asians compared to Caucasians (Ge et al. 2009; Thomas et al. 2009). However, it was reported that the *IL28B* polymorphism and ethnic background were independent pretreatment predictors for SVR in the IDEAL study (Thompson et al. 2010): *IL28B* polymorphism (CC vs. non-CC at rs12979860: OR = 5.2, $P < 0.0001$) and ethnic background (Caucasian vs. African American: OR = 2.8, $P < 0.0001$; Hispanic vs. African-American: OR = 2.1, $P = 0.0041$), suggesting that *IL28B* polymorphisms did not account for all of the ethnic differences in responses to treatment.

Following the above-mentioned GWAS, many studies have confirmed the impact of *IL28B* on response to treatment. Thompson et al. reported that the *IL28B* polymorphism also affected the early viral kinetics during PEG-IFN/RBV therapy for patients infected with HCV genotype 1. Patients with a favorable *IL28B* genotype achieved a higher rate of rapid virological response (RVR). Even when an RVR was not achieved, a favorable *IL28B* genotype was also strongly associated with SVR. In contrast, the *IL28B* polymorphism was not associated with SVR in patients who experienced an RVR (Thompson et al. 2010). These findings indicate that the *IL28B* polymorphism is useful as an on-treatment predictor of SVR in patients not experiencing RVR. In the IDEAL study cohort, SVR rates in patients with advanced liver fibrosis (METAVIR F3-4) were considerably lower, namely 41 % for patients with CC, 22 % for CT, and only 11 % for TT at rs12979860 (Thompson et al. 2010). Thus, liver fibrosis is an important predictive factor of treatment efficacy, in addition to the *IL28B* polymorphism. The *IL28B*

polymorphism is also associated with the outcome of PEG-IFN/RBV therapy in patients co-infected with HCV genotype 1 and human immunodeficiency virus (HIV), as well as in HCV mono-infected patients (Rallon et al. 2010). In patients who underwent liver transplantation, the *IL28B* polymorphisms of both the donor and recipient were associated with treatment efficacy (Fukuhara et al. 2010; Charlton et al. 2011).

1.3 IL28B *and Treatment Efficacy in Patients with HCV Genotype Non-1*

We have summarized previous reports on the effect of *IL28B* polymorphism on treatment efficacy in patients infected with HCV genotype non-1 (Table 2). Rauch et al. reported that there was no significant association between *IL28B* polymorphism and response to PEG-IFN/RBV in patients infected with HCV genotype 2 or 3 in their GWAS study (OR = 1.58; $P = 0.18$) (Rauch et al. 2010). Mangia et al. noted that the *IL28B* polymorphism was associated with SVR in patients with genotype 2 or 3, especially those who did not experience RVR in PEG-IFN/RBV for 24 weeks: the SVR rates were 87 %, 67 %, and 29 % in patients with CC, CT, and TT at rs12979860, respectively ($P = 0.0002$) (Mangia et al. 2010). Sakamoto et al. examined the relationship between *IL28B* polymorphism and response to therapy in Japanese patients infected with HCV genotype 2 who were treated with PEG-IFN/RBV, also for 24 weeks. They showed that patients infected

Table 2 The association between a favorable *IL28B* genotype (rs8099917 TT or rs12979860 CC) and SVR in patients with HCV non-1 genotype who were treated with PEG-IFN/RBV

Study	Population	SNP	Number of patients	Odds ratio	P value	HCV genotype
Rauch et al.	European	rs8099917	230	1.58	0.18	2, 3
Mangia et al.	European	rs12979860	268	1.80	0.0046	2, 3
Sakamoto et al.	Japanese	rs8099917	129	3.01	0.013	2
Yu et al.	Taiwan	rs8099917	482	1.37	0.50	2
Moghaddam et al.	North European	rs8099917	281	0.91	N.S.	3
Asselah et al.	Egyptian, European, Sub-Saharan African	rs12979860	82	3.32	0.0008	4
Antaki et al.	Syria	rs8099917	182	4.17	<0.0001	4
Abdo et al.	Saudi Arabia	rs12979860	129	1.5	0.008	4
Antaki et al.	Syria	rs8099917	49	1.04	0.9	5
Seto et al.	Hong Kong	rs8099917	60	N.A.	0.014	6

SVR sustained virological response, *PEG-IFN* pegylated interferon, *RBV* ribavirin, *N.S.* not significant, *N.A.* not available

with genotype 2b had significantly lower RVR rates than those infected with genotype 2a. Moreover, both RVR and SVR were significantly associated with a favorable *IL28B* genotype in patients infected with genotype HCV 2b (Sakamoto et al. 2011). Other investigators showed that a favorable *IL28B* genotype was associated with RVR, but not SVR, in patients infected with HCV genotype 2 or 3 (Yu et al. 2011; Moghaddam et al. 2011). Taken together, these data suggest that the effect of *IL28B* polymorphism on SVR is weaker in patients infected with genotype 2 or 3 than in those with genotype 1. With regard to HCV genotype 4, the *IL28B* polymorphism correlates with response to PEG-IFN/RBV therapy as well as it does for genotype 1 (Rauch et al. 2010; Asselah et al. 2012; Antaki et al. 2012a; Abdo et al. 2013). In CHC genotype 4 patients, favorable genotypes of both SNPs of *IL28B* are valuable for predicting SVR. Additional genotyping of rs8099917 in carriers of the heterozygous C allele of rs12979860 can improve the prediction of SVR (Ragheb et al. 2014). There are very few reports of these associations in patients infected with HCV genotype 5 or 6. Antaki reported that the *IL28B* polymorphism did not predict response to treatment in a small study of patients infected with HCV genotype 5 ($n = 49$) (Antaki et al. 2012b). Seto et al. noted that the SVR rate was higher in patients with a favorable *IL28B* genotype than in those with an unfavorable genotype (96.2 % vs. 62.5 %, $P = 0.014$) in their analysis of a total of 60 patients infected with HCV genotype 6 (Seto et al. 2013).

1.4 IL28B *Polymorphism and Responses to Regimens Including DAA*

The HCV genome is translated into one polyprotein that is subsequently cleaved by viral and cellular proteases, being processed into ten structural and non-structural proteins. DAA therapies directly inhibit specific steps in the HCV replication cycle, with targets including the NS3/4 protease, NS5B polymerase, and NS5A phosphoprotein which are essential for replication. To date, the first-generation protease inhibitors, telaprevir and boceprevir have been approved and various clinical trials of new DAAs are ongoing.

The SPRINT-2 (Poordad et al. 2011) and ADVANCE trials (Jacobson et al. 2011a) of boceprevir and telaprevir, respectively, showed that, in treatment-naive patients, the *IL28B* SNP: rs12979860 affected treatment outcome. The SVR rates in SPRINT-2 and ADVANCE were higher in patients with CC (80 %, 90 %) than CT (71 %, 71 %) or TT (59 %, 73 %) (Table 3) (Poordad et al. 2012; Jacobson et al. 2011b). On the other hand, in treatment-experienced patients, the RESPOND-2 (Bacon et al. 2011) and REALIZE (Zeuzem et al. 2011) trials of boceprevir and telaprevir, respectively, showed that the previous response to PEG-IFN/RBV strongly affected SVR; thus the SVR rate increased from null-response, to partial response and then relapse to previous therapy (Poordad et al. 2012; Pol et al. 2011).

Table 3 Associations between *IL28B* genotype and SVR in DAA plus PEG-IFN/RBV therapy

Drug	Study population	Treatment arm	rs12979860 genotype		
			CC	CT	TT
Boceprevir	Sprint-2 (treatment-naïve)	BOC PR48	44/55 (80 %)	82/115 (71 %)	26/44 (59 %)
	Respond-2 (treatment-experienced)	BOC PR48	17/22 (77 %)	48/66 (73 %)	13/18 (72 %)
Telaprevir	Advance (treatment-naïve)	TVR12	45/50 (90 %)	48/68 (71 %)	16/22 (73 %)
	Realize (treatment-experienced): overall	All TVR	60/76 (79 %)	160/266 (60 %)	49/80 (61 %)
	Prior relapse	All TVR	51/58 (88 %)	100/117 (85 %)	29/34 (85 %)
	Prior partial response	All TVR	5/ 8 (63 %)	33/57 (58 %)	10/14 (71 %)
	Prior null response	All TVR	4/10 (40 %)	27/92 (29 %)	10/32 (31 %)
Simeprevir (TMC-435)	Quest-1 (treatment-naïve)	SMV 150 mg	72/77 (94 %)	114/150 (76 %)	24/37 (65 %)
	Aspire (treatment-experienced)	SMV 150 mg	21/24 (88 %)	67/90 (74 %)	17/28 (61 %)
Faldaprevir (BI-201335)	Silen-C1 (treatment-naïve)	FDV 240 mg	22/22 (100 %)	CT + TT 34/48 (71 %)	
Vaniprevir (MK-7009)	Study 009 (treatment-naïve)	All VNV	22/25 (88 %)	12/19 (63 %)	2/5 (40 %)
Daclatasvir	Command-1 (treatment-naïve)	All DCV	76/95 (80 %)	83/158 (53 %)	16/35 (46 %)
Sofosbuvir	Neutrino study (treatment-naïve)	SOF 400 mg	93/95 (98 %)	CT + TT 202/232 (87 %)	

SVR sustained virological response, *DAA* direct-acting antiviral, *PEG-IFN* pegylated interferon, *RBV* ribavirin, *Ref.* reference number

The *IL28B* polymorphism was not significantly associated with SVR in those who relapsed or in partial responders, whereas the SVR rate tended to be higher in prior null-responders with the favorable *IL28B* genotype than in those with unfavorable genotypes (McHutchison et al. 2010). Bota et al. performed a meta-analysis and discerned a role for *IL28B* polymorphisms as predictors of SVR in patients treated with triple therapy. They selected five studies (1641 cases) in which the regimens of four were telaprevir/PEG-IFN/RBV and the fifth was boceprevir/PEG-IFN/RBV. The SVR rate was significantly higher in patients with CC at rs12979860 than in those with non-CC (OR = 3.92, $P < 0.0001$). Moreover, higher SVR rates were seen in patients with CC, regardless of therapeutic status (treatment-naïve patients: OR = 3.99, $P < 0.0001$; treatment-experienced patients: OR = 2.15, $P = 0.001$) (Bota et al. 2013).

In addition to the *IL28B* polymorphism, several factors influencing responses to triple therapy have been identified. The REALIZE study showed that the severity of

liver fibrosis was a predictive factor for SVR in telaprevir/PEG-IFN/RBV therapy: the SVR rate was 74 % in those with F0-F2 fibrosis, 66 % in those with F3, and 47 % in those with F4 (Zeuzem et al. 2011). Akuta et al. showed that the SVR rate was 84 %, irrespective of substitution of core aa70 in patients with TT at rs8099917, whereas in those with non-TT, the SVR rate was 50 % for patients with the wild type core aa70 and 12 % in those with non-wild type (Akuta et al. 2010). Combining these factors with *IL28B* genotyping might improve the prediction of responsiveness to triple therapy.

To date, there have been several reports on the effects of the *IL28B* polymorphism on the efficacy of treatment with next-generation DAA plus PEG-IFN/RBV (Table 3). The QUEST-1 trial investigated the efficacy of two doses of simeprevir, together with PEG-IFN/RBV, in treatment-naïve patients infected with HCV genotype 1. The SVR rate with simeprevir at 150 mg was 94 %, 76 %, and 65 % in patients with CC, CT, and TT at rs12979860, respectively. Viral breakthrough was seen exclusively in the non-CC genotype (Jacobson et al. 2014). The ASPIRE trial in treatment-experienced patients infected with HCV genotype 1 showed that the SVR rate with simeprevir at 150 mg was 88 %, 74 %, and 61 % in patients with CC, CT, and TT at rs12979860, respectively (Zeuzem et al. 2014). The SILEN-C1 trial investigated the efficacy of faldaprevir combined with PEG-IFN/RBV in treatment-naïve patients infected with HCV genotype 1. In the subgroup treated once-daily with faldaprevir at 240 mg and PEG-IFN/RBV, the SVR rate was 100 % (22/22) in patients with CC at rs12979860 and 71 % (34/48) in non-CC (Sulkowski et al. 2013). A phase 2 study of vaniprevir in treatment-naïve patients with HCV genotype 1 also showed that the SVR rate was 88 %, 63 %, and 40 % in patients with CC, CT, and TT at rs12979860, respectively (Manns et al. 2012). As shown in Table 3, daclatasvir (Hezode et al. 2015) or sofosbuvir (Lawitz et al. 2013) combined with PEG-IFN/RBV in treatment-naïve patients infected with HCV genotype 1 showed similar results to the regimens including the protease inhibitor. Thus, next-generation DAA plus PEG-IFN/RBV therapy will likely weaken the restriction of *IL28B* polymorphism. However, the *IL28B* polymorphism will remain relevant to treatment efficacy, especially in treatment-naïve patients.

Furthermore, Lok et al. demonstrated that the combination of daclatasvir (NS5A inhibitor) and asunaprevir (NS3 protease inhibitor) with PEG-IFN/RBV was effective for patients infected with HCV genotype 1 who had had a null-response to prior PEG-IFN/RBV therapy: the SVR rate was 90 % (Lok et al. 2012). In robust treatments such as this quadruple therapy, the *IL28B* polymorphism might not be associated with treatment outcome.

IFN-free therapy is expected to become the standard of care in the future and is clearly required, especially for IFN-resistant patients. Chayama et al. demonstrated that nine of ten patients infected with HCV genotype 1b and who had failed to respond to prior PEG-IFN/RBV therapy experienced SVR on an IFN-free regimen containing daclatasvir (NA5A inhibitor) and asunaprevir (NS3 protease inhibitor) (Chayama et al. 2012). This suggests that combination therapy with potent DAAs might obscure the influence of *IL28B* polymorphisms on treatment efficacy, as supported by large-scale clinical trials (Manns et al. 2014; Kumada et al. 2014).

However, it has been reported that *IL28B* polymorphisms may affect viral kinetics even in the context of interferon-free regimens, in the case of a combination of mericitabine (NS5B polymerase inhibitor) and danoprevir (NS3 protease inhibitor) (Chu et al. 2012). Moreover, in a phase 2b, randomized, open-label trial of faldaprevir (NS3 protease inhibitor) and deleobuvir (NS5B polymerase inhibitor), the SVR rates tended to be higher in patients with CC at rs12979860 than in those with non-CC (Zeuzem et al. 2013). This suggests that innate immunity may still be important and *IL28B* polymorphism may affect treatment efficacy in certain interferon-free regimens. Larger cohort sizes will be required to confirm such associations. On the other hand, recent IFN-free regimens such as ABT450/r + ABT267 + ABT333 +/− RBV (Kowdley et al. 2014) and Ledipasvir + Sofosubvir (Afdhal et al. 2014a, b) showed very high SVR rates for any *IL28B* polymorphism, suggesting no association of *IL28B* polymorphism with SVR achievement by these IFN-free DAAs combination therapies (Table 4).

1.5 Association Between IFN-λ and HCV Infection

IL28B encodes IFN-λ3, which belongs to the type III IFN-λ family consisting of *IL29*/IFN-λ1, *IL28A*/IFN-λ2, and *IL28B*. Signalling by IFN-λ is initiated through a membrane receptor distinct from the receptors for type I IFNs and which is composed of heterodimers of an IL28RA/IFN-λR subunit and an IL10R2 subunit (Kotenko et al. 2003; Sheppard et al. 2003). Type I and III IFNs induce the transcription of IFN-stimulated genes (ISGs) by activating the Janus kinase-signal transducer and activator of transcription (Jak-STAT) pathway through various cell surface receptors (Kotenko et al. 2003; Sheppard et al. 2003), in order to mediate their potent antiviral effects. There are several reports regarding the profile of expression of ISGs in liver and peripheral blood mononuclear cells (PBMCs). It has been reported that high level expression of intrahepatic ISGs was associated with a poor response to PEG-IFN/RBV therapy (Sarasin-Filipowicz et al. 2008; Feld et al. 2007). Moreover, recent studies have revealed an association between the *IL28B* polymorphism and expression levels of intrahepatic ISGs (Honda et al. 2010; Urban et al. 2010). In the innate immune system, induction of the Toll-like receptor 3 (TLR3) and Retinoic acid-inducible gene I (RIG-I) signalling pathways of IFN-β has an essential role in host antiviral defense against HCV infection. Asahina et al. showed that intrahepatic gene expression involving innate immunity was strongly associated with the *IL28B* polymorphism and response to PEG-IFN/RBV (Asahina et al. 2008, 2012). Suppiah et al. and we have shown *IL28* expression in PBMCs to be higher in patients with a favorable *IL28B* genotype (Tanaka et al. 2009; Suppiah et al. 2009). Asahina et al. showed that the induction of several ISGs in PBMCs after the initial administration of PEG-IFN/RBV tended to be stronger in patients with SVR than NVR, but the difference was not statistically significant (Asahina et al. 2008). Similarly, most other investigators have indicated a less marked association between the expression of ISGs in PBMCs and treatment

Table 4 Associations between *IL28B* genotype and SVR in IFN-free therapy

Drug	Study population	Treatment arm	rs12979860 genotype		
			CC	CT	TT
Asunaprevir + Daclatasvir	Hallmark-dual study (treatment-naïve)	ASV100mg + DCV60mg 24 W	68/76 (89 %)	87/99 (88 %)	27/28 (96 %)
ABT450/r + ABT267 + ABT333 + RBV	Aviator study treatment-naïve	8 W	20/22 (91 %)	37/41 (90 %)	13/17 (76 %)
		12 W	22/22 (100 %)	37/39 (95 %)	17/18 (94 %)
		24 W	17/22 (77 %)	44/46 (96 %)	12/12 (100 %)
	Prior null response	12 W	2/2 (100 %)	25/28 (89 %)	15/15 (100 %)
		24 W	1/1 (100 %)	24/25 (96 %)	16/17 (94 %)
Ledipasvir + Sofosbuvir	ION-1 study (treatment-naïve)	12 W	51/58 (88 %)	100/117 (85 %)	29/34 (85 %)
	ION-2 study (treatment-experienced)	12 W	10/10 (100 %)	CT + TT 92/99 (93 %)	
	ION-3 study (treatment-naïve)	8 W	54/56 (96 %)	CT + TT 148/159 (93 %)	

SVR sustained virological response, *DAA* direct-acting antiviral, *PEG-IFN* pegylated interferon, *RBV* ribavirin, *Ref*. reference number

outcomes or *IL28B* polymorphism, in comparison with their expression in the livers of the same patients (Sarasin-Filipowicz et al. 2008; Abe et al. 2011). Thus, though there are several reports about the association between ISGs in liver or PBMCs and *IL28B* polymorphism or response to IFN therapy, the biological pathways linking *IL28B* genetic variants to spontaneous and/or treatment-induced HCV clearance remain unknown. However, recent reports suggest some possible scenarios. Using primary human hepatocytes and chimpanzees, Thomas et al. found that type III, but not type I, IFNs are primarily induced after HCV infection and that the degree of induction is closely correlated with the levels of ISGs (Thomas et al. 2012). These results strongly suggest that hepatic IFN-λ production may have important roles and could be a principal driver of ISG induction in response to HCV infection. On the other hand, in chronically HCV-infected chimeric mice that have the characteristic of immunodeficiency, larger amounts of IFN-λs on HCV-infected human hepatocytes were produced in liver with a favorable *IL28B* genotype on treatment with IFN-α (Watanabe et al. 2013). However, no significant differences in HCV RNA reduction related to *IL28B* variants were observed because of the lack of intrinsic immune cells in the model. In contrast, Zhang et al. and Yoshio et al. reported that a certain subset of dendritic cells (DCs) within human PBMCs could recognize HCV and produce large amounts of IFN-λs (Zhang et al. 2013; Yoshio et al. 2013) and the ability to produce IFN-λ3 was superior in subjects with a favorable *IL28B* genotype (Yoshio et al. 2013). Moreover, IFN-α directly affected DC function and significantly increased IFN-λ production (Zhang et al. 2013). Based on these findings, it is tempting to speculate that exogenous IFN-α would increase IFN-λ production by DCs and/or HCV-infected hepatocytes during IFN-α therapy, and this could provide a potential explanation as to why *IL28B* genetic variants predict the outcome of IFN-α therapy (Fig. 1).

Recently, Olsson et al. performed RNA sequencing in primary human hepatocytes activated with synthetic double-stranded RNA to mimic HCV infection. They discovered that a new, transiently induced region that harbors a dinucleotide variant ss469415590 (TT or ΔG) was strongly associated with HCV clearance. The ss469415590 polymorphism is located upstream of *IL28B* and is in high linkage disequilibrium with rs12979860. The ss469415590 ΔG allele is a frameshift variant that creates a novel gene, designated *IFNL4*, encoding the type III IFN-λ4 protein, which is somewhat similar to IFN-λ3. Interestingly, compared to rs12979860, ss469415590 is more strongly associated with spontaneous and treatment-induced HCV clearance in individuals of African ancestry, whereas it did not improve prediction among Caucasians and Asians. This can be explained by a lower level of linkage disequilibrium between the two polymorphisms in African-Americans ($r^2 = 0.71$) than Caucasians ($r^2 = 0.92$) and Asians ($r^2 = 1.00$) (Prokunina-Olsson et al. 2013). Bibert et al. also noted that this polymorphism improved prediction of treatment-induced HCV clearance in patients infected with HCV genotype 1/4 or 2/3. In addition, they determined that induction of *IL28B* and IFN-γ-inducible protein 10 (IP-10) mRNA in PBMCs relies on ss469415590 but not rs12979860 (Bibert et al. 2013). Their findings provide new insights into the genetic regulation of HCV clearance and have implications for its clinical management.

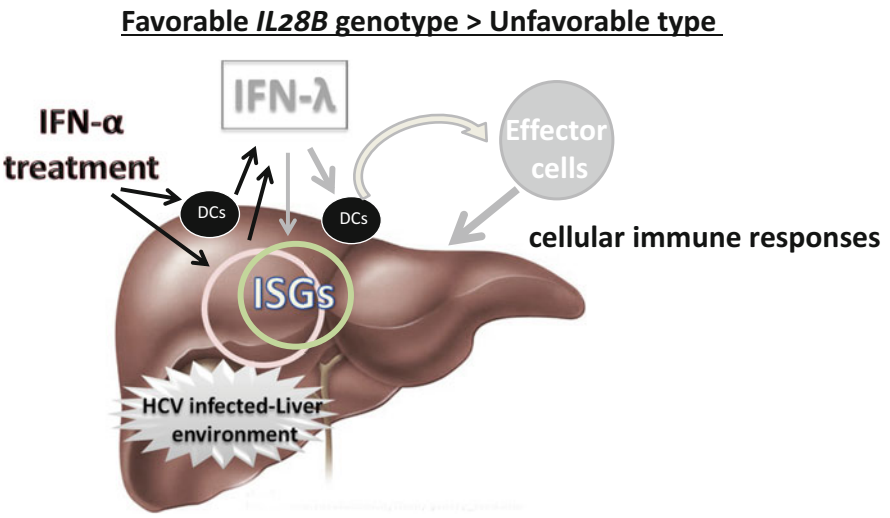

Fig. 1 Potential role of the *IL28B* SNP in the responses to interferon (IFN)-α therapy. IFN-α upregulates hepatic interferon-stimulated genes (ISGs). According to in vivo models of chronic HCV infection (Watanabe et al. 2013), exogenous IFN-α would increase IFN-λ production by HCV-infected hepatocytes during IFN-α therapy. The amounts of IFN-λs produced by HCV-infected human hepatocytes were larger in livers with a favorable *IL28B* genotype. Dendritic cells (DCs) also produce large amounts of IFN-λ, following an immune response against HCV infection in the liver environment. The ability of DCs to produce IFN-λ3 was superior in subjects with a favorable *IL28B* genotype (Yoshio et al. 2013)

2 Conclusions

Application of GWAS technology has revealed an unexpected role of *IL28B* in HCV infection. This finding could provide a strong rationale for developing novel therapeutic strategies for HCV infection as well as furthering basic studies of IFN-λs. The *IL28B* polymorphism could assist clinical decision-making for the treatment of acute HCV infection. In the context of PEG-IFN/RBV therapy for CHC, *IL28B* polymorphisms are strongly associated with treatment efficacy in patients infected with HCV genotype 1 or 4, with some effects on other HCV genotypes. *IL28B* genotyping is also useful for pretreatment prediction of the outcome of DAA plus PEG-IFN/RBV therapy, especially in treatment-naïve patients. In the future, more aggressive treatments, such as quadruple therapy or potent DAA combinations, might obscure the influence of *IL28B*, but *IL28B* genotyping will remain useful for making decisions on suitable regimens and treatment duration in patients in the forthcoming era of DAAs. The mechanisms by which IFN-λs are active against HCV infection must be elucidated through the functional analyses of IFN-λs.

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Viral Variation and Response to Therapy

Shinya Maekawa and Nobuyuki Enomoto

Abstract Till recently, interferon (IFN)-based non-specific antiviral therapy was only the choice for the eradication of hepatitis C virus (HCV) in patients with chronic hepatitis C. However, antiviral activity of IFN was quite limited, and the eradication rate did not exceed 50 % in the treatment of the most refractory genotype-1 HCV. In the treatment of chronic hepatitis C with IFN, it was gradually disclosed that sequences of certain viral genomic regions significantly affect viral response.

With this background, various novel small compounds with potent antiviral effects called direct antiviral agents (DAAs) which directly inhibit enzymatic activities of HCV have been recently developed, and their significant antiviral activity is literally changing anti-HCV therapy. In DAA-based therapy, rare treatment failure is associated with the development of DAA-resistant mutations, and it is an important issue if pretreatment prediction of the appearance of DAA-resistant mutations is possible in the DAA-based therapy.

In this chapter, the association between viral sequence variations and the response to IFN-based as well as DAA-based therapy is described.

Keywords Hepatitis C virus • Viral predictive factor • Drug-resistance • Peg-interferon plus ribavirin therapy • DAA • NS3/4A protease inhibitor • NS5A replication complex inhibitor • NS5B polymerase inhibitor

1 Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases worldwide, and 170 million people are infected with HCV. Seventy percent of acute infections become persistent, and 50–75 % of patients with chronic HCV infection progress to hepatocellular carcinoma.

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Till recently, interferon (IFN)-based non-specific antiviral therapy was only the choice for the eradication of HCV in patients with chronic hepatitis C. However, antiviral activity of IFN was quite limited, and the eradication rate did not exceed 50 % in the treatment of the refractory genotype-1 HCV even with the advanced regimen of pegylated-interferon (PEG-IFN) plus ribavirin (RBV) (Fried et al. 2002; Manns et al. 2001). Under the circumstance, various novel small compounds with potent antiviral effects called direct antiviral agents (DAAs) which directly inhibit enzymatic activities of HCV have been developed, and some DAAs have been already approved for the clinical use. Significant antiviral activity of DAAs is literally changing anti-HCV therapy.

HCV is known to have high mutation rate because of its rapid turnover rate in host and of its error-prone RNA-dependent RNA polymerase lacking proofreading activity. As a result, HCV exists in host as genetically-related but distinct viral subpopulations called as quasispecies. In cases with the treatment failure, viral sequence variations as well as the quasispecies state are considered to be important factors determining the treatment response both in IFN-based therapy and in DAA-based therapy.

2 Interferon-Based Therapy

IFN was the first approved drug for clinical use in the therapy of HCV. Historically, IFN was first approved as the IFN mono-therapy, but IFN-based therapy was advanced with the development of long-acting PEG-IFNs and the introduction of RBV, resulting in that the combination PEG-IFN plus RBV therapy has long been the standard of care (SOC) for almost a decade till the introduction of DAA at 2011. IFN exerts its antiviral activity through activating IFN signal pathway resulting in activation of multiple interferon-stimulated antiviral genes with multiple mechanisms of action. Because of the suspected multiple antiviral mechanisms, it is considered that genetic barrier preventing the development of IFN-resistant variants to be high.

However, through the analyses of patients with treatment failure, it has been gradually disclosed that variations of viral genomic structures affect the response to IFN in various previous studies. Namely, that viral genotypes and certain viral sequences, Core and NS5A, are highly associated with the response in the treatment of IFN-based therapy.

2.1 HCV Genotypes

By phylogenetic analysis, HCV is classified into seven major genotypes, and these major genotypes are further classified into subtypes in each genotype determined

by their genetic distances. Among all the viral factors investigated, viral genotypes are the most influential, and well-established predictive factor determining the treatment outcome in IFN-based therapy. Geographically, genotypes 1–3 are associated with worldwide epidemic, while genotypes 4–6 are endemic. In comparison among major genotypes 1–3, a high SVR rate (~84 %) was observed in patients with genotype 2 or 3, while a low SVR rate (~42 %) was observed in genotype 1 (Fried et al. 2002; Manns et al. 2001; Hadziyannis et al. 2004) in the PEG-IFN/RBV therapy. When compared between genotype 2 and 3, genotype 2 could have more favorable outcome (Dalgard and Mangia 2006; von Wagner et al. 2005). The studies of genotype 4 were mainly from Egypt, and the SVR rate was reported to be intermediate (55–69 %) (El-Zayadi et al. 2005; Kamal et al. 2005). In genotype 5 or 6, the SVR rate has been considered as intermediate between the SVR of genotype 1 and genotype 2–3, but studies focusing the response of genotype 5 and 6 are limited because of their minor distribution (Nguyen and Keeffe 2005).

2.2 Core

The core protein forms the viral nucleocapsid of HCV, and its mature form consists of a secondary structure made of a large folded multimer of ~24 monomers. It is 21 kDa in size, and is separated into two domains, an N-terminal two-thirds hydrophilic domain (D1, residues 1–117) and a C-terminal one-third hydrophobic domain (D2, residues 118–170), respectively. The D1 domain contains many positively charged amino acids, and is implicated to bind RNA. The D2 domain is required for proper folding of domain D1. The mature core protein shares high homology among HCV genotypes. The core protein has been reported to interact with a variety of cellular proteins and to influence numerous host cell functions, such as its proapoptotic or antiapoptotic actions (Kountouras et al. 2003), immunomodulatory roles (Hahn 2003), or oxidative stress (Wang and Weinman 2006). Recently, much attention has been paid for its relation with liver steatosis, insulin resistance and hepatocellular carcinoma (Mori et al. 2008; Serfaty and Capeau 2009). HCV core proteins of genotype 3a and 1b were reported to interfere with the insulin signaling pathway differently depending on genotype-specific mechanisms (Pazienza et al. 2007).

As its contribution for the clinical treatment response, Akuta et al. first reported that substitutions of the amino acid (aa) 70 and 91 in the core protein were significantly related to the final outcome in the 48 weeks of interferon plus ribavirin combination therapy in 50 Japanese patients infected with genotype 1b HCV (Akuta et al. 2005). In successive studies, they reported that substitutions in those core regions were related to the final outcome, viral kinetics, early viral response, and these viral responses in extended 72 weeks of therapy (Akuta et al. 2007a, b, c, 2009). The importance of core aa 70 was further confirmed through the full HCV open reading frame (ORF) study. Namely, through the analysis of HCV full ORF in the PEG-IFN/RBV therapy, core aa 70 was extracted as the single host spot

significantly associated with the null response among all 3010 HCV amino acids (Maekawa et al. 2012): glutamine (Q) or histidine (H) ("mutant-types") compared to arginine (R) ("wild-type") at core aa 70 was significantly observed in the patients with null response. Core aa 70 was associated with elevated alpha-fetoprotein, and hepatocarcinogenesis (Akuta et al. 2007c, 2008; Miura et al. 2011).

Interestingly, significant link between core aa 70 and IL28B single nucleotide polymorphism (SNP), the most important host factor determining viral response to the PEG-IFN/RBV therapy, was disclosed. Namely, IL28B minor types (IL28B TG or GG at rs8099917) were found with significant high frequency in cases with core aa 70 mutants (core 70Q/H), suggesting significant interaction between host and virus. Since the IL28B typing is genetically determined in host (human) from birth, it is speculated that high mutant frequency of core aa 70 would have been the result of the host-virus interaction. Of note, the role of IL28B in hepatocarcinogenesis seems to be weak whereas core aa 70 is significantly associated with hepatocarcinogenesis, making the correlation between IL28B and core aa 70 to be rather mysterious (Miura et al. 2011).

Through the recent advancement of deep sequencing technique and of relative quantification of the variants, it was disclosed that core aa 70 often exists as mixed status of wild-type (R) and mutant-types (Q/H): deep sequencing of the core region in the clinical samples showed a mixture of Q/H and R at core aa 70 position in most cases (71/79 [89.9 %]), and the ratio of mutant residues to wild-type residue (R) increased as liver disease advanced to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (Miura et al. 2013). Phylogenetic analysis of the core region revealed that the HCV isolates differed genetically depending on the mutation status at core aa 70 (Miura et al. 2013).

In the treatment of genotype-2a HCV, association of core aa 110 and the response to the PEG-IFN/RBV therapy was also reported (Kadokura et al. 2011a).

2.3 NS5A

NS5A is phosphorylated on multiple serine and threonine residues, and forms two distinct molecules of basal phosphorylated form (p56) and hyperphospyorylated form (p58), being 56 kDa and 58 kDa in size, respectively. The protein has three distinct domains (domain I, II, and III), and the separated by low complexity sequences (LCS I and II). The study of the X-ray crystal structure analysis of domain I suggested that the NS5A is a dimer, and it forms a large putative RNA binding groove (Tellinghuisen et al. 2005). Recent genetic study has shown many residues in domain II are essential for RNA replication, while domain III is less conserved and might be dispensable. Though the true function of NS5A is still not fully understood, the protein is considered as a component of HCV replication complex, and modulates HCV replication through interaction other viral proteins.

Among all HCV proteins, NS5A has been most extensively explored for its relation with the response in the interferon-based therapy.

2.3.1 ISDR and PKR-BD

Interferon Sensitivity Determining Region (ISDR), located in the C-terminal half of NS5A, was originally identified as the 40 amino acid region (aa2209–2248) significantly related to the treatment outcome in the monotherapy of IFN-alpha in Japanese patients infected with genotype-1b HCV (Enomoto et al. 1995, 1996). "Mutant-type" meant for four or more mutations in the region was associated with high SVR rate (16/16: 100 %), while SVR rate was low in the "intermediate-type" (one to three mutations: SVR rate 5/38 (13 %)), or "wild-type" (no mutation: SVR rate 30/30 (0 %)). Successive studies from Japan were also concordant with the initial study (Chayama et al. 1997; Komatsu et al. 1997; Kurosaki et al. 1997). However, there occurred controversy as to the predictive value of ISDR since studies from Europe and North America did not always show evident correlation between ISDR and the treatment outcome (Chung et al. 1999; Hofgartner et al. 1997; Khorsi et al. 1997; Odeberg et al. 1998; Squadrito et al. 1997; Zeuzem et al. 1997). However, a later meta-analysis study clearly confirmed its value even in the western countries (Pascu et al. 2004). Different results observed in North America and Europe at first might have been caused partly by smaller rates of mutant-type patients in western countries, and partly by the different treat regimen in Japan and in western countries (Pawlotsky et al. 1998; Saiz et al. 1998; Sarrazin et al. 1999, 2000; Witherell and Beineke 2001). Though ISDR was found in the era of IFN monotherapy, its predictive value in the treatment outcome of PEG-IFN/RBV therapy was also reported (Akuta et al. 2009; Okanoue et al. 2009; Shirakawa et al. 2008; Veillon et al. 2007; Yen et al. 2008). Especially, through the detailed study with full ORF analysis, it was disclosed that amino acid mutations in ISDR was associated with rapid viral response (defined as undetectable HCV-RNA 4 weeks) in PEG-IFN/RBV therapy (Maekawa et al. 2012).

In searching for the biological ISDR function, Gale et al. found that PKR-binding domain (PKR-BD, aa2209-2274) in NS5A protein consisting of ISDR(aa2209-2248) and its adjacent region (aa2249-2274) could directly bind to PKR, an important antiviral protein, and could suppress antiviral PKR activity in an ISDR-dependent manner (Gale et al. 1997). Thus, they insisted that inactivation of PKR may be one mechanism by which HCV avoids the antiviral effects of IFN. Kurosaki et al. also found that ISDR was related to IL28B SNPs (Kurosaki et al. 2011).

In the treatment of genotype-2a/2b HCV, association of NS5A heterogeneity and the response to the PEG-IFN/RBV therapy was also reported (Kadokura et al. 2011a, b).

2.3.2 V3 Domain and IRRDR

V3 domain located in the C-terminal region of NS5A was originally identified as a genomic region of genotype-1b HCV showing a marked heterogeneity between Japanese and American isolates (Inchauspe et al. 1991). A correlation of its mutations and the response to IFN-based therapy was first reported by Duverlie et al. and they reported that sequences of V3 domain were highly conserved in resistant strains, but were highly variable in sensitive strains (Duverlie et al. 1998). Most following studies also reported the concordant results (Veillon et al. 2007; Murphy et al. 2002; Puig-Basagoiti et al. 2005; Sarrazin et al. 2002; Vuillermoz et al. 2004). El-Shamy et al. reported a high degree of sequence variations in the V3 and the flanking pre-V3 regions (aa2334–2355) of NS5A, and they designated the region as interferon/ribavirin resistance-determining region (IRRDR) (aa2334–2379). They reported substitution number in the IRRDR was closely correlated with early virological response (EVR) by week 16 in 47 HCV-1b – infected patients treated with peg-interferon plus ribavirin (El-Shamy et al. 2007). In their follow up study for the same group of patients, sequence variation in the IRRDR was also significantly correlated with the final outcome. The positive predictive values of IRRDR of six or more for SVR was 89 % (16/18), whereas negative predictive values of IRRDR of five or less for non-SVR was 81 % (22/27) (El-Shamy et al. 2008). The importance of IRRDR was confirmed by the ORF study and it was disclosed that mutations in IRRDR was related to the treatment relapse in PEG-IFN/RBV therapy and unrelated to IL28B SNPs (Maekawa et al. 2012).

3 DAA-Based Therapy

DAAs under development or in clinical use mostly target enzymatic activities of viral nonstructural proteins resulting in inhibition of the intracellular viral RNA replication in the life cycle of HCV (Hayes and Chayama 2014). Among those NS proteins, NS3-4A protease, NS5A replication complex and NS5B polymerase are the target enzymatic proteins at the current status. Since DAAs are specifically designed for HCV, their antiviral activity is significant and adverse events are few compared to the conventional IFN.

On the other hand, since each DAA targets single viral enzymatic function, HCV could escape from the antiviral activities of DAA through developing resistant mutants by introduction of mutations in their viral genome and changing their protein structures. In this sense, DAAs generally have low genetic barrier, and since those resistance soon appears with the DAA monotherapy, regimens of DAA-based therapy should be based on the combination with IFN or other DAA (s) though DAA resistant variants gradually disappear and are replaced by wild-type normally after discontinuation of DAAs due to the low replication capacity (or replication fitness). However, it was gradually disclosed that certain types of

DAA resistant variants persists even after discontinuation of DAAs. Moreover, it was also reported that even DAA treatment-naïve patients might naturally have substantial proportion of DAA-resistant HCV variants. And of course, treatment failure for multiple classes of DAAs finally induces DAA super-resistant HCVs. With the background, much attention has been given to the issues of DAA resistance because of the clinical significance.

3.1 HCV Genotypes

Since most HCV-specific DAAs have been primarily designed to inhibit genotype-1 HCV isolates, some DAAs have weak antiviral activity for other genotypes (Sarrazin and Zeuzem 2010). In protease inhibitors (PIs), first-generation PIs such as telaprevir or boceprevir have the strongest antiviral activity in genotype-1b HCV, though second-generation PIs have broader antiviral activities and are effective in other genotypes. Of note, concerned to the genetic barrier for DAA resistance, viral subgenotypes are also important determinants for the development of viral resistance: R155K substitution conferring resistance to first-generation PIs occurs with one nucleotide substitution in genotype-1a HCV while it occur only after two nucleotide substitutions in genotype-1b HCV. In NS5A replication complex inhibitors, differences in the antiviral activity are also observed, and their antiviral activity is strongest in genotype-1b HCV. It is speculated that the difference results from the high heterogeneity of the NS5A. In NS5B polymerase inhibitors, non-nucleoside polymerase inhibitors (NNI) show different antiviral activities in different genotypes while nucleoside inhibitors (NI) are effective in all genotypes.

3.2 NS3

HCV-specific protease inhibitors (PIs) target an essential step in HCV replication by blocking the nonstructural 3/4A (NS3/4A) protease-dependent cleavage of the HCV polyprotein. PIs are classified into two groups according to the difference in structure, first-generation PIs with the linear structures (telaprevir, boceprevir) and second-generation PIs with the macrocyclic structures (simeprevir, vaniprevir, faldaprevir, asunaprevir, sovaprevir and grazoprevir), and drug-resistance profile is somewhat different between these two groups. Because of the low replication fitness in PI-resistant variants, these PI-resistant variants were considered to emerge only when wild-type HCV is suppressed significantly with the introduction of PIs, but soon become undetectable after discontinuation of PIs.

However, recent studies have reported the presence of these PI-resistant variants in high frequencies ~10 % by direct sequencing in patients untreated with the PIs (Susser et al. 2009; Kuntzen et al. 2008; Shindo et al. 2011). Moreover, with the use of recently-developed deep sequencing technique enabling the detection of minor

variants, it was reported that most patients are infected with resistant variants to some degree (Jabara et al. 2014). As a mechanism of the appearance of these naturally-occurring resistance, Gaudieri et al. suggested that regions of NS3 protease are likely to be under HLA immune pressure, and that drug-resistant variants may occur naturally to escape the immune system (Gaudieri et al. 2009). Especially, much concern has been raised as to how those resistant variants influence on the outcome of PI-based therapy. Actually it was reported that those resistant variants are effective to IFN equally as wild-type (Shindo et al. 2011), and did not affect the treatment outcome if the PIs are used in combination with IFN (plus RBV) (Akuta et al. 2013, 2014).

As a special problem in the pretreatment resistant variants, it is known that NS3 Q80K exists with high frequency (19–48 %) as a dominant isolate in genotype-1a HCV infection, and the presence of Q80K affects and lowers the outcome in simeprevir, asunaprevir or sovaprevir-included therapy even IFN is included in the regimen. In phase III clinical studies of QUEST-1 and QUEST-2 evaluating the combination of simeprevir with PEG-IFN/RBV in patients infected with genotype-1, SVR rate was low among patients with genotype-1a with Q80K at baseline (58 %) compared to patients without Q80K (84 %) (Poveda et al. 2014). On the other hand, in IFN-free DAA therapies, it is gradually disclosed that the pretreatment existence of PI-resistant RAVs decreases SVR rate, and might induce the development of multiple DAA resistance.

Another problem in PI-resistant mutation is the post-treatment persistence of PI-resistant HCVs. In general, PI-resistant mutations have low replication fitness, and those variants gradually become undetectable and replaced by the wild-type after discontinuation of PIs. However, it was reported that the speed of wild-type reversion is different between genotype-1a and 1b, and it is slower in genotype-1a HCV compared to genotype-1b HCV: genotype 1b-resistant variants persisted for a median of approximately 1–2 months compared with approximately 8–11 months for genotype 1a in phase 3 telaprevir trials (Wyles and Gutierrez 2014). In the IFN-free DAA combination therapy, the post-treatment persistence of the PI-RAVs lowers the SVR rate, and even induce the development of multi DAA resistant HCVs.

3.3 NS5A

NS5A inhibitors (daclatasvir, ledipasvir, ombitasvir and elbasvir) are considered to act as an inhibitor of NS5A protein to form replication complex with other viral components as well as host factors in hepatocytes. As described before, NS5A inhibitors have the strongest antiviral activity for genotype-1b HCV, and antiviral activity of these NS5A inhibitors are significant among various DAAs. Due to the strong antiviral activity, NS5A inhibitors are one of the key drugs to be used in combination with other DAAs.

On the other hand, it was gradually disclosed that a substantial proportion of patients (8–30 %) with genotype-1b patients have NS5A-resistant mutations in its natural state (Miura et al. 2014; Suzuki et al. 2012), and these variants affect and lower the treatment outcome in the DAA-free regimen of daclatasvir (NS5A

inhibitor) plus asunaprevir (PI). In the treatment of daclatasvir/asunaprevir therapy, patients with one of the NS5A mutations (L31M/V or Y93H) showed resistance in half of patients, and moreover, those patients with the treatment failure finally resulted in the acquisition of triple mutations (L31M/V and Y93H in NS5A and D168A/V/E in NS3) (Suzuki et al. 2013). In addition, it was reported that NS5A mutations do not significantly lower replication fitness of the virus, and therefore, those NS5A mutations were reported to persist even after discontinuation of these NS5A inhibitors (Wang et al. 2013). Importantly, since NS5A inhibitors are most frequently used in the DAA combination therapies with DAAs of other classes, and the failure of DAA combination therapy would be likely to result in the development of "monster" HCV showing resistance to all used DAAs, clinicians should pay extra attention to the pretreatment existence of NS5A mutations. In particular, since Y93 is the most frequent mutant site among NS5A mutations, it is important to clarify the cut-off values as to the mixture ratio of Y93H to Y93 wild type in establishing clinical resistance. Interestingly, Y93H was significantly correlated with the IL28B major (TT) genotype of the host indicating the existence of host-virus interaction also in this site (Miura et al. 2014).

3.4 NS5B

Two different classes of polymerase inhibitors are undergoing clinical trials or in clinical use: nucleoside inhibitors (NIs) and non-nucleoside inhibitors (NNIs) (Soriano et al. 2013). NIs mimic natural nucleotides and are incorporated into the elongated RNA, and finally causes chain termination (sofosbuvir). Since the active site of NS5B polymerase is highly conserved across different genotypes, it is pangenotypically potent. Due to the poor replication fitness in resistant variants (S282T), genetic barrier of NIs is high, and pretreatment existence as well as treatment-induced development of NI-resistant variants is rare event clinically.

On the other hand, NNIs (dasabuvir and beclabuvir) suppress NS5B polymerase activity through binding to NS5B allosteric sites distant from the active site. NS5B polymerase consists of finger, palm and thumb domain, and has at least 5 different allosteric sites that could be targeted by NNIs: Therefore, the profiles of resistant variants are different dependent on each NNI-targeted site. Since those allosteric sites are not strictly conserved across the genotypes, antiviral potency is most significant in genotype-1 HCV, but weaker in other genotypes. Moreover, genetic barriers of NNIs are low. Because of the low genetic barrier, NNIs are scheduled to be used in multi-combination with other classes of DAA.

4 Conclusions

In this section, potential influences of viral genomic variations on the treatment response in the IFN-based therapy as well as in the recent DAA-based therapy in chronic HCV infection were briefly described. Though anti-HCV therapy has been greatly advanced with these new DAAs, it is still important for each clinician to understand the relationship between biological features of those drugs and the appearance of drug-resistant mutants to achieve and obtain the best treatment outcome in all patients.

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HCV NS3/4A Protease Inhibitors and the Road to Effective Direct-Acting Antiviral Therapies

Nicolas Tremblay, Alex Young Park, and Daniel Lamarre

Abstract Hepatitis C virus (HCV) currently infects over 185 million individuals around the world. Its persistence causes infected individuals to enter a chronic phase where their condition can lead to several different outcomes of liver disease. HCV therapies are becoming highly efficient and well tolerated due to improved understanding of the HCV biology and to the discovery of antivirals targeting essential viral functions. The NS3 protein with its co-factor NS4A forms a serine protease (NS3/4A protease) that is indispensable for the maturation of HCV polyprotein. The NS3/4A protease was identified as a prime antiviral drug target. Its exploration led to the development of peptidomimetic inhibitors based on substrates and N-terminal products and to the rapid selection of drug candidates. Ciluprevir (BILN 2061) was the first NS3/4A protease inhibitors in clinical trials and it established the proof-of-concept in humans for a novel class of selective anti-HCV agents specifically designed to inhibit an essential viral enzyme. The inclusion of protease inhibitors with the standard of care therapy established the first major therapeutic advancement against HCV infection. In conjunction with other classes of anti-HCV agents, these inhibitors contributed to the revolution of all-oral interferon-free combination therapies. This chapter provides a historical perspective of the past 25 years in the validation of NS3/4A protease as an important drug target and in the rationale behind inhibitor design that led to the development of promising HCV antiviral drugs for the treatment of HCV infection.

Keywords HCV • NS3 • Serine protease • Antivirals • Protease inhibitors • Drug discovery • N-terminal product inhibition

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1 Introduction

1.1 Epidemiology and Disease

Hepatitis C virus (HCV) infection currently affects over 185 million individuals worldwide with genotype 1 infection being the most common (Messina et al. 2015). The virus primarily infects hepatocytes, and only a minority of infected individuals are able to clear the virus during an acute infection. Persistence of the virus causes infected individuals to enter a chronic phase where their condition can lead to several outcomes of liver disease such as cirrhosis and hepatocellular carcinoma.

1.2 Therapeutic Advance Strategy

The treatment of HCV infection has evolved over the last two decades. In the 1990s, low cure rates, often less than 10 %, were achieved with type I interferon (IFN) regimens administered for 24–78 weeks. These low cure rates were reflected in low frequencies of a sustained virologic response (SVR) (Manns and von Hahn 2013). A substantial breakthrough was achieved with the replacement of standard IFN with pegylated-IFN (Peg-IFN) (once-weekly injections and improved pharmacokinetics). In combination with ribavirin (RBV), Peg-IFN, in 2001, became the most effective available treatment and the standard of care (SOC) for patients with chronic hepatitis C (Pol and Corouge 2014). Despite the promise of such a therapy, the suboptimal efficacy and adverse effects, as well as the increasing number of patients with advanced liver disease and no therapeutic option had emphasized a major need for improved therapeutic regimens. With the discovery of HCV, significant efforts were made, mainly focused on decoding the functions of virally encoded enzymes, to rapidly initiate the search for antiviral approaches. The absence of a clear understanding of HCV biology in humans led to a long-term clinical hypothesis that inhibition of key viral enzymes would block viral replication and would be therapeutically beneficial for infected patients. The viral enzyme-targeted drug discovery approach was also predicting significant therapeutic advance in patients not responding to the standard IFN therapy given the differing mechanism of action of potential anti-HCV agents. Thus potent and specific small molecules targeting essential HCV functions were needed to fight this devastating liver disease. It was felt, however, that an intense commitment to antiviral drug discovery would be required to discover such a breakthrough therapy.

1.3 Targeting Essential Functions of HCV

The discovery of HCV in 1989 (Choo et al. 1989) had provided fundamental molecular insights for initiating antiviral drug discovery. The HCV genome encodes a linear single-stranded RNA of positive polarity with approximately 10,000 nucleotides. The analysis of the amino acid sequences extrapolated from the nucleotide sequences initially revealed a gene organization similar to those of flaviviruses, which was later assigned to a new genus (the genus *Hepacivirus*) within the *Flaviviridae* family. The research on HCV and related viruses immediately focused on annotating functional elements. This rapidly led to major key advances associated with the identification of viral enzymes and *cis*-acting RNA elements of the genome that could serve as targets for intervention. The HCV genome was shown to be translated into a single polyprotein via an internal ribosome entry site (IRES) located at the 5′ end of the virus coding sequence (Tsukiyama-Kohara et al. 1992). The concept of using RNA molecules as therapeutic agents had aroused increasing interest and led to the identification of *trans*-cleaving ribozymes and antisense RNAs as potential inhibitors of HCV translation (Welch et al. 1996; Wu and Wu 1998). This culminated in the identification of the clinical candidate, ISIS 14803, a 20-base antisense oligodeoxynucleotide inhibitor of HCV expression that functioned via binding to the IRES region. Unfortunately, during testing in phase 1b clinical trials, it generated inconclusive clinical results (Soler et al. 2004; McHutchison et al. 2006).

Viral enzymes constitute the most promising targets for the development of novel antiviral compounds. The discovery of HCV inhibitors targeting the best characterized viral enzymes has therefore been the focus of intense research in both academia and in the biopharmaceutical industry. In the early 1990s, a growing body of information was generated on polyprotein maturation by host and viral proteases. This robustly demonstrated a major role of the virally encoded serine protease associated with the amino-terminal domain of NS3 (Tomei et al. 1993; Grakoui et al. 1993a; Eckart et al. 1993; Hirowatari et al. 1993), and a second protease (Grakoui et al. 1993b) that is responsible for the cleavage between NS2 and NS3. All other mature HCV proteins were shown to be processed from the polyprotein by host signal peptidases resulting in the mature C, E1, E2, p7 and NS2 proteins. Proteolytic processing of the HCV polyprotein by the two virally encoded proteases produced all mature non-structural (NS) proteins, but most importantly yielded the NS3 and NS5B proteins that contain characteristic sequence motifs for RNA helicases (found next to the serine protease domain in NS3) and RNA polymerases (NS5B) respectively. As similar sequence motifs were described in all flaviviruses and pestiviruses in their homologous proteins, this suggested that these enzymes would play an important role in the respective virus replication cycle (Miller and Purcell 1990).

The NS3 helicase domain contains seven highly conserved motifs (Gorbalenya and Koonin 1993), which based on mutational and crystallographic analyses, have been implicated in nucleotide triphosphate (NTP) binding, NTP hydrolysis, and

nucleic acid binding. The NS3 helicase was classified into the superfamily II (SF-II) and as part of the NS3/NPH-II family of DExH proteins based on the conserved helicase motif II (Jankowsky and Fairman-William 2010). The unwinding reaction by HCV NS3 is perhaps the best understood of any RNA helicase to date and many crystal structures are available for HCV NS3 and related flavivirus NS3 proteins. The biological role of these NS3 proteins however is less clear, although it is known that these enzymes are essential for viral replication and that they interact with the viral RNA polymerase and other viral cofactors (Chatel-Chaix et al. 2012). While many high-throughput screening assays involving NS3 helicase/ATPase were established and while crystallographic structures were available (Kim et al. 1998), few NS3 helicase inhibitors have been reported. In light of the lack of helicase inhibitors being approved for clinical use, this confirmed that targeting the helicase domain was traditionally difficult. Even today, no selective HCV NS3 helicase inhibitors are being investigated nor are any in early clinical trials as an antiviral agent.

The NS5B-encoded protein contains the classic palm, fingers and thumb structural sub-domains of a polymerase and its activity has been studied intensively by a number of groups (Behrens et al. 1996; Lohmann et al. 1997; Yuan et al. 1997). The interaction of the different sub-domains is thought to ensure coordinated movement and to assist in modulating initiation, elongation and termination of RNA synthesis by promoting high processivity of viral replication. With the marketable success of polymerase inhibitors in other viruses, the high potential of a polymerase-based target was rapidly established for an anti-HCV strategy. Both nucleoside and non-nucleoside inhibitors of HCV polymerase were rapidly identified through HTS and rational drug design using available crystallographic structures (Bressanelli et al. 1999).

Even with the rapid identification of specific HCV inhibitors through biochemical and surrogate cell-based assays (Walker and Hong 2002), practical models for HCV replication were necessary. Such models would allow pharmacological validation and assessment of antiviral potency that are often required for compounds to qualify as pre-development candidates. A key discovery was a sub-genomic replicon (Lohmann et al. 1999; Blight et al. 2000) that provided an in vitro HCV RNA replication model to evaluate antiviral activity of lead compounds and to better characterize virally encoded enzyme targets in cell culture.

The HCV-infected chimpanzee was initially the only animal model supporting HCV replication, it was, however, an impractical model for routine studies of HCV biology. This model was used to demonstrate that in vitro transcribed RNAs from HCV clones are infectious in chimpanzees upon intrahepatic inoculation (Kolykhalov et al. 1997; Yanagi et al. 1997; Hong et al. 1999). The limited availability and the high cost of the chimpanzee model led to the development of a small animal model which supported HCV infection (Mercer et al. 2001) and which proved to be predictive of the clinical outcomes when using antiviral compounds (Kneteman et al. 2006).

2 NS3/4A Serine Protease: A Role in Virus Life Cycle and Pathogenesis

2.1 NS3/4A Cleavage in HCV Polyprotein Processing

HCV polyprotein cleavage at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions is catalyzed by the NS3/4A serine protease (Fig. 1). The viral NS2/3 protease autocleaves itself from the NS3 protein and allows the NS3 protease to perform its cleavage activity on all other sites of the NS portion of the polyprotein. Based on expression, purification and in vitro enzymatic reconstitution, the NS3 serine protease was one of the most thoroughly characterized HCV enzymes. The amino-terminal 180 amino acids of the NS3 protein encodes a chymotrypsin-like serine protease that, in conjunction with the NS4A cofactor, mediates efficient processing. The structures of the NS3 protease domain have been solved by X-ray crystallography with and without an NS4A peptide partner (Love et al. 1996; Kim et al. 1996). A comparison of the NS3 protease crystal structure

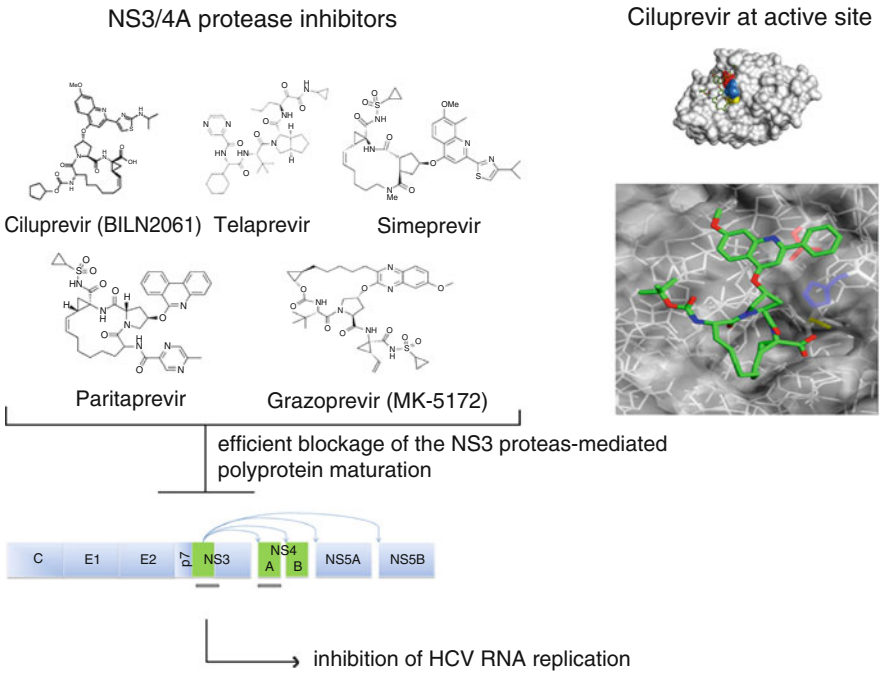

Fig. 1 Mechanism of action of NS3/4A protease inhibitors. Selected chemical structures are shown for BILN 2061, for approved drugs and for molecules in clinical development. NS3/4A protease inhibitors inhibit the cleavage of the HCV polyprotein at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions, which in turn prevents HCV RNA replication. Binding of BILN2061 at the active site of NS3/4A, a highly conserved enzymatic pocket, is shown

showed that the presence of NS4A re-orients the position of the catalytic residues within the serine protease (Yao et al. 1999).

Substrate recognition by the NS3/4A protease is mainly characterized by a strong preference at the P1 position (nomenclature according to Schechter and Berger 1967) and requires an extended series of interactions distributed over the surface of the substrate. More precisely, the NS3/4A protease requires at least decamer peptide substrates spanning P6-P4′, with a preference for an acidic residue in P6, cysteine in P1, serine or alanine in P1′, and a hydrophobic residue in P4′ (Landro et al. 1997; Urbani et al. 1997; Zhang et al. 1997). The requirement for a minimal substrate peptide is believed to be dictated by the shallow, solvent-exposed substrate-binding cleft of the enzyme. These studies have been complemented with solution NMR and with biochemical analyses to elucidate the structure of a target substrate (Barbato et al. 1999) and of product analogues (Cicero et al. 1999; LaPlante et al. 1999). Structural observations had raised concerns in the identification of specific and potent inhibitors, which would be suitable for clinical trials. Although the NS3 protease was considered a valuable target for drug discovery, it represented a formidable challenge for the design of small-molecule inhibitors.

2.2 *Subversion of Antiviral Innate Immune Response*

HCV has evolved mechanisms to interfere with the induction of type I IFN and innate immune response via its NS3/4A protease activity (Foy et al. 2003). The central role of the enzyme was firmly established following the elucidation of mechanisms that counteract TLR3- and RIG-I/MDA5-mediated signaling pathways. Through the cleavage of the host cell adaptors TRIF and MAVS (Meylan et al. 2005; Li et al. 2005), HCV can prevent the transcriptional activation of type I IFN genes and IFN stimulated genes (Dustin and Rice 2007). The protease activity of NS3 that targets these pathways was also reported in dendritic cells of HCV-infected individuals. The loss of pro-inflammatory cytokine expression that results from the disruption of these pathways could be restored ex-vivo with treatment with NS3/4A protease inhibitors (Rodrigue-Gervais et al. 2010). Since the NS3/4A protease plays a critical role in evading host immune responses, it represents a dual therapeutic target; its inhibition may block viral replication and protect from a loss of antiviral IFN response. Based on these findings, the NS3/4A protease was considered one of the most attractive targets for drug discovery.

2.3 *Abrogation of Cellular Functions*

A great variety of animal viruses encode proteases for the proteolytic processing of polypeptide precursors. Other than the production of mature proteins involved in the virus life cycle, viral proteases are often responsible for the proteolysis of host

cellular substrates therefore contributing significantly to viral pathogenesis. The NS3/4A protease has been found to target several cellular proteins for which cleavages seem to be required for efficient HCV replication. In addition to TRIF and MAVS from the IFN pathway, the NS3/4A protease has been reported to cleave the T-cell protein tyrosine phosphatase (TC-PTP) thereby modulating growth factor signaling (Brenndorfer et al. 2009). Similarly, there are some data indicating that it cleaves the UV-damaged DNA binding protein 1 (DDB1), a core component of the E3 ubiquitin ligase complex (Kang et al. 2013), and membrane-associated peroxidase GPx8, cleavage of the latter being observed in liver biopsies from patients with chronic HCV (Morikawa et al. 2014).

We recently identified the main karyopherin β nuclear import carrier protein KPNB1 as a host interactor of the NS3/4A protein (Germain et al. 2014) that also appears to be cleaved by the NS3/4A protease (B Gagné, A Young Park and D Lamarre, personal communication). We further showed that KPNB1 is a key mediator of the nuclear translocation of transcriptional factors IRF3 and p65 that are both required for IFN production. This data support a more generalized mechanism of viral immune evasion by NS3/4A than expected from a novel mechanism targeting nucleocytoplasmic transporter host proteins. Thus, the NS3/4A protease is not only an essential component of the viral replication complex and a prime target for antiviral intervention, but is also a key player for the persistence and pathogenesis of HCV. Therapeutic targeting of NS3/4A may not only disturb viral replication by blocking viral polyprotein processing but may also exert unanticipated beneficial effects to further decrease viral replication.

3 NS3/4A Serine Protease as a Drug Target

3.1 Relevance and Validation of Viral Targets for the Disease

The identification of a novel drug target is obtained through descriptive studies on protein expression (and/or RNA) in target tissues and through comparing features from diseased and healthy tissues. As such, the NS3/4A protease is expressed only in virally-infected hepatocytes (in other words, it is expressed selectively in diseased tissues) and its expression is consistent with viral propagation and liver disease. However, its initial selection as a drug target was done prior to having a deep understanding of the disease and of its causative molecular mechanisms. To make matters worse, there were no predictive models to validate the serine protease target in accordance with a proposed mode of action outside a cell-based model that expressed the NS3 to NS5B portion of the HCV polyprotein. Indeed, in vitro cell-based mechanistic studies were used to reveal regulatory characteristics of this target such as the kinetics, the cleavage order within the polyprotein, and the importance/necessity of each protease-mediated cleavage site to the virus life cycle. Experiments directly demonstrating the indispensable role of NS3/4A

protease activity for HCV replication and infectivity were performed much later, in vitro, using the HCV replicon cell culture system and, in vivo, with HCV-infected chimpanzees. Introduction of an active site mutation (Ser to Ala) within the NS3 protease gene locus of the subgenomic HCV replicon blocked RNA replication (Lohmann et al. 1999), while injection of an infectious HCV RNA mutated clone into chimpanzee livers abolished virus production (Kolykhalov et al. 2000). These functional studies thus demonstrated that NS3/4A serine protease was essential for HCV replication and viral propagation. They also proved that this enzyme was a suitable target with sufficient validation for full-fledged antiviral drug discovery program by the reference standards used in pharmaceutical industries.

Another important criterion was drug tolerability. Many cellular serine proteases exist in mammals (examples include the four human serine proteases trypsin, chymotrypsin, plasmin and elastase). This can raise concerns if designed inhibitors are non-selectively acting against host serine proteases. In humans, these proteases are responsible for coordinating various physiological functions, including those involved in digestion, in the immune response, in blood coagulation and in reproduction. Remarkably, inhibition of the NS3/4A protease target with a class of substrate-based inhibitors demonstrated excellent protease selectivity (Llinas-Brunet et al. 1998a) and was not predicted to lead to mechanistic toxicity. In contrast, electrophilic carbonyl-containing derivatives from the same class were shown to inhibit host serine proteases very effectively (Llinas-Brunet et al. 1998a). One of the concerns with the introduction of electrophilic carbonyls in the peptide sequence was the specificity of these inhibitors against other proteases (Hedstrom 2002). Overall, many factors that normally contribute to successful target identification were lacking with the initial selection of NS3/4A protease as a target for HCV treatment. These factors included, but were not limited to, the lack of in vitro infectious systems and of an appropriate animal disease model. The relevance of the target to the disease was mainly based on the genetic validation within HCV replication that was directly linked to the probability of clinical efficacy.

3.2 Catalytic Mechanism of Serine Protease

Proteases encompass a wide array of enzymes that catalyze the cleavage of a peptide bond. There are four main classes: cysteine proteases, aspartate proteases, metalloproteases and serine proteases, all of which are classified according to the chemical species involved in catalysis. NS3/4A and other serine proteases are enzymes for which a serine serves as the nucleophilic amino acid at the active site during the cleavage of the peptide bonds (Hedstrom 2002). The nucleophilic properties of the serine are strengthened by a charge relay system known as the catalytic triad, which consists of histidine (His1083), aspartic acid (Asp1107), and serine (Ser1165) in the NS3/4A protease (based on HCV polyprotein numbering). The mechanism implicating the triad involves the formation of an acyl-enzyme

intermediate. The acyl-enzyme complex, when attacked by a water molecule, generates a new tetrahedral intermediate that collapses. This, in turn, results in the formation of an N-terminal product acid and a leaving group. Serine proteases fall into two broad categories based on their structure; they are either chymotrypsin-like (trypsin-like) or subtilisin-like. In addition, their specificity is mainly driven by the shape and properties of their binding pockets (Madala et al. 2010).

3.3 3D Structure of NS3/4A Serine Protease

The NS3 serine protease domain folds as a chymotrypsin/trypsin-like protease with two β barrels and reveals a zinc binding site distal to the active site. The catalytic triad is located in a cleft between the two β barrels, with His1083 and Asp1107 in the N-terminal barrel and Ser1165 in its C-terminal counterpart. The C-terminal sub-domain contains the conventional six-stranded β barrel common to most members of the chymotrypsin family. On the other hand, the N-terminal sub-domain, in complex with an NS4A segment, consists of eight β-strands, seven from the NS3 protein and one from the NS4A peptide. The extensive interaction between NS3 and NS4A results in a tightly packed β-barrel that buries an additional 2,400 Å of the surface area of the NS3 protease. The mechanism by which the NS4A cofactor induces the NS3 serine protease activity was illustrated by a direct comparison of the various X-ray structures of NS3 protease domain. This showed a striking effect of NS4A binding on the orientation of the catalytic triad in the characteristic position expected from a chymotrypsin-like serine protease (Kim et al. 1996; Yan et al. 1998). The structure of NS3 protease has a substrate-binding site which exhibits a shallow nonpolar S1 binding pocket. The phenylalanine (Phe1180) found within this binding pocket is believed to be crucial for sequence specificity. NS3/4A protease binds its substrates or other peptide ligands in an extended bioactive conformation. Thus, an antiparallel β-sheet is formed via a number of important points of contact and such throughout a large active site (LaPlante et al. 2014). The peptide ligands exhibit rather complex conformational behavior providing some insight as to why this target class is not very successful in identifying non-peptidic active site inhibitors when screening a large collection of compounds. Overall, the solvent exposable surface of the NS3/4A protease active site illustrated the challenge in the design of small-molecule inhibitors, especially given the rather shallow pocket of the active site when compared to other chymotrypsin-like proteases.

4 Discovery of NS3/4A Protease Inhibitors

The combination of specific and potent anti-HCV agents achieving a high genetic barrier offered a strong rationale to eradicate infection in HCV-infected patients by the rapid and significant reduction of plasma HCV RNA. Major efforts were concentrated on identifying and designing compounds that specifically inhibit essential viral functions for HCV replication. The NS3/4A protease was among the best characterized viral enzymes and was the first to have its mode of action validated in polyprotein processing. Convenient homogenous ultra high-throughput screening (uHTS) assays were developed using the NS3/4A protease domain or the full-length protein for testing large collections of compounds in search of selective inhibitors. In parallel, early discovery of N-terminal product inhibitors for the NS3/4A protease (Steinkuhler et al. 1998; Llinas-Brunet et al. 1998b) linked to the insights gained from the design of human immunodeficiency virus (HIV) protease inhibitors paved the way for rational design of active site peptidomimetic inhibitors. As of today, a search within the scientific and patent literature of inhibitors of this enzyme mainly reveals peptidic species that are nearly all derived from the N-terminal product sequence.

4.1 HTS Approach

Significant efforts of several biopharmaceutical groups have been devoted to the identification of nonpeptidic small-molecule inhibitors of the NS3/4A protease (see review by Steinkuhler et al. 2001; Beaulieu and Llinas-Brunet 2002). Nonpeptidic molecules have emerged through random screening and mainly displayed a noncompetitive mechanism of action. The identification of several classes of protease inhibitors were reported including derivatives of 2,4,6-trihydroxy-3-nitro-benzamides (Sudo et al. 1997a), thiazolidines (Sudo et al. 1997b) and benzanilides (Kakiuchi et al. 1998), as well as compounds isolated from natural products (Chu et al. 1999). These compounds were mostly non-selective and inhibited the activity of host serine proteases in addition to that of the desired NS3/4A protease target. Overall, screening efforts of large compound libraries were mostly unsuccessful in identifying selective hits that would serve as lead molecules for optimization.

4.2 Rational Drug Design Approach

In light of the enzyme features, various alternative strategies for developing NS3/4A protease inhibitors were initially envisioned in a rational approach such as interfering with NS4A interaction, zinc binding and substrate binding. While the

first two strategies have been viewed as extremely difficult (De Francesco et al. 1999), the design of active site inhibitors for the NS3/4A protease was considered the most promising approach. A number of peptidomimetic inhibitors were described falling mainly into two mechanistic classes of NS3/4A protease inhibitors so-called N-terminal product-like inhibitors and covalent inhibitors (De Francesco et al. 2003).

4.2.1 N-Terminal Product-Like Inhibitors

An important class of peptide-based inhibitors was identified from the finding that the NS3/4A protease is susceptible to feedback inhibition by the N-terminal products released from the substrate after enzymatic cleavage (Llinas-Brunet et al. 1998a; Steinkuhler et al. 1998). The relevance of such product inhibition for the enzyme was nicely underlined by the three-dimensional structure of the full-length NS3-NS4A heterodimer protein complex (Yao et al. 1999), in which the C-terminal threonine of the NS3 helicase domain (P-side product of the cleavage between NS3 and NS4A) occupies the active site of the protease domain. This discovery was exploited mainly by two groups and resulted in the synthesis of hexapeptide-like inhibitors of the NS3/4A protease (Ingallinella et al. 1998; Llinas-Brunet et al. 1998b, 2000). By NMR, the hexapeptide Asp-Asp-Ile-Val-Pro-Cys (DDIVPC) was shown to bind its target in a well-defined way (LaPlante et al. 1999) and, by enzymatic studies, it was shown to competitively inhibit the enzyme ($Ki = 19\ \mu M$) thus fulfilling the requirement of a lead molecule. This peptide-based inhibitor series was particularly attractive in view of the C-terminal carboxylate that imparts good solubility, as well as a dramatically improved specificity (Llinas-Brunet et al. 1998b) given by the absence of an activated warhead commonly incorporated for serine protease inhibition. It is well known for peptide inhibitors of serine proteases that the P1 residue contributes considerably to the potency and specificity of the ligand. For these NS3/4A protease inhibitors, both the P1 side chain and its terminal carboxylic acid contributed to the binding energy (Steinkuhler et al. 1998). A major challenge was designing suitable and chemically stable replacements for the P1 sulfhydryl group. The continuing efforts of research culminated in the successful replacement of the unstable P1 residue with aminocyclopropane carboxylic acid (ACCA) derivatives (Rancourt et al. 2004). The incorporation of a vinyl-ACCA, as a carboxylic acid containing P1 residue, produced NS3 protease inhibitors that were significantly more active than inhibitors containing a cysteine at the same position (Rancourt et al. 2004).

Attempts to rationally improve inhibitor potency were successful, yielding compounds effective at low picomolar range with enzymatic characteristics of slow, tight-binding inhibitors of a full-length genotype 1 enzyme (Pause et al. 2003). These compounds were highly selective for the viral NS3/4A protease and inactive ($IC50 > 30\ \mu M$) against representative human serine proteases (Pause et al. 2003). Efforts were progressively devoted to reducing the size and peptidic

nature of the peptidyl carboxylic acid inhibitors (LaPlante et al. 2000). The NMR-derived conformation of a substrate-based enzyme-bound tetrapeptide provided a model that led to the design of a very potent macrocyclic inhibitor series. This inhibitor series was obtained by the addition of a macrocyclic ring that connected the side chain of the P1 and the P3 residues (Tsantrizos et al. 2003). The macrocyclization design from P1 to P3 resulted in the breakthrough compound BILN 2061 (Lamarre et al. 2003; Llinas-Brunet et al. 2004). This inspired others to employ macrocyclization by using an alternative macrocyclic connection between the P2 and the P4 moieties as a strategy for conformational restriction (rigidification). This led to the creation of very potent inhibitors many of which have been recently approved and are in development. In these inhibitors the P1 to P3 and the P2 to P4 macrocycles bind to the NS3/4A protease active site (Fig. 1). Various C-terminal moieties have been used to increase non-covalent inhibitor potency and its drug-like properties. These include the carboxylate moiety and the acylsulfonamide moiety, which establish prominent stabilizing interactions with the enzyme oxyanion hole and its surrounding subsites. Despite the fact that recently approved drugs and inhibitors currently in clinical trials vary substantially with regard to their C-termini and their side chain appendages, their docking into the enzyme active site demonstrated the same core structure derived from DDIVPC and a similar bioactive conformation (LaPlante et al. 2014). Attachments to the peptidic structures often consist of P1 ACCA derivatives, modified P2 proline and P3 residues with a bulky tert-butyl side chain tethered to P1 (Fig. 1). The variety of appendages discovered for these compounds that properly satisfy the same bioactive conformation requirements indicate that a large variety of NS3/4 protease drug candidates can be designed thus ensuring the long-term efficacy of this drug class against the rapid emergence of resistance.

4.2.2 Covalent Inhibitors

Many covalent small-molecule and/or irreversible inhibitors that rely on the fundamental mechanism of using enzyme activity to trap and inactivate a protease have been developed. These peptide molecules use a reactive warhead that binds to the catalytic machinery of the enzyme, and include specificity elements that are linked to the warhead moiety effectively gaining selectivity for one target. With serine protease, inhibitors are developed by derivation of the known substrate through the replacement of the scissile amide bond with an electrophilic warhead that forms a covalent adduct with the catalytic serine residue. Inhibitors of this mechanistic class are often referred to as transition state analogues, serine-trap inhibitors or covalent serine protease inhibitors. Several groups have reported electrophile-based peptidic NS3/4A protease inhibitor series harboring unique modifications and functionalities such as a-ketoamides, boronic acids, phosphonates, hydrazinourea, ketoacids and pyrrolidine-5,5-trans lactams (Steinkuhler et al. 2001; Fischmann and Weber 2002). Electrophile-based covalent inhibitors are believed to be of limited use in a clinical setting due to their irreversible binding to mechanistically unrelated

biomolecules and thus to their potential for idiosyncratic toxicity (Sanderson 1999). Despite these concerns, NS3/4A protease inhibitors of this class were the first to be approved by the FDA, in combination with conventional therapeutic approaches, to treat patients infected with HCV genotype 1. Boceprevir and telaprevir (Fig. 1) are both linear α-ketoamide derivative inhibitors which formed a covalent bond with the active site of the enzyme in a reversible manner. Given the reactive nature of the warhead and the mechanistic and structural similarities displayed by protease families, the continued development of drug classes that have the ability to selectively inhibit these enzymes is necessary.

4.3 Pharmacological Validation of NS3/4A Protease Inhibitors In Vitro

4.3.1 NS3/4A Protease Inhibitors Abrogate HCV RNA Replication

HCV RNA replication in the replicon cell system described by Lohmann et al. (1999) is dependent on the various NS proteins (Lohmann et al. 2001), and therefore replicon-containing cells are useful for testing specific inhibitors of viral RNA replication. With the identification of sub-micromolar NS3 protease inhibitors using in vitro enzymatic assays, few reports emerged to demonstrate inhibitory efficacy of this compound class in decreasing HCV RNA replication in replicon-containing cells. Results of in vitro studies using the subgenomic HCV replicon cell culture system with a potent and specific peptide-based inhibitor of the NS3/4A protease were reported by Pause et al. (2003). The results demonstrated a reduction of HCV RNA levels and a blockage of NS3-mediated polyprotein maturation (see below). In view of the cell-based efficacy at reducing intracellular HCV RNA to undetectable levels through a confirmed mechanism of this NS3/4A protease inhibitor, this class of peptidomimetic inhibitors was considered a promising series for HCV replication inhibitors. The findings provided additional support for targeting the NS3 protease and the continued development of potent and specific HCV inhibitors with therapeutic potential for the treatment of HCV infection.

4.3.2 NS3/4A Protease Inhibitors Block HCV Polyprotein Processing

In order to confirm the intracellular mode of action of protease inhibitors that lead to reduction in subgenomic HCV RNA, the HCV NS protein processing was assessed by Western blot analysis of cell extracts (Pause et al. 2003). Dose-dependent decrease in NS3 protein was observed in NS3/4A protease inhibitor-treated cells resulting in the complete disappearance of mature NS3 protein and the detection of an HCV polyprotein precursor. Intermediate proteins were not detected under these conditions possibly because of a short polyprotein half-life. In order to detect HCV NS protein precursors, replicon-containing cells were also labeled with

35S-methionine/cysteine and treated with NS3/4A protease inhibitors. Cell extracts were immunoprecipitated with specific anti-NS3 protein antibodies and products were analyzed by SDS-PAGE. Treatment of cells with NS3/4A protease inhibitors resulted in complete disappearance of NS3 protein and the concomitant appearance of a product of larger molecular weight consistent with the size of the polyprotein precursor. The effective inhibition of cellular HCV RNA replication by NS3/4A protease inhibitor was evidenced by efficient blockage of the NS3 protease-mediated polyprotein maturation that is essential for HCV replication in human liver cells.

5 Clinical Development of NS3/4A Protease Inhibitors

5.1 Proof-of-Concept Studies

Ciluprevir (BILN2061) was the first NS3/4A protease inhibitor to enter clinical trials and to establish the proof-of-concept (Fig. 1) (Lamarre et al. 2003). The purpose of a short trial design in humans was to assess the in vivo antiviral efficacy of BILN 2061. The latter had been selected solely on the basis of its in vitro potency in surrogate enzymatic and cell culture assays as well as its oral pharmacokinetic profile in animals. Following a phase I study in healthy individuals to assess its safety and pharmacokinetics parameters, the antiviral activity was investigated in patients infected with HCV genotype 1 in a randomized, double-blind, placebo controlled study. The plasma HCV RNA virus load (VL) was measured up to 10 days post-administration in patients treated orally and twice daily with 200 mg of BILN 2061 or placebo. BILN 2061 was highly effective, inducing a rapid VL decline to undetectable levels within 24–28 h of administration. This substantial and impressive effect corresponds to a 2–3 log10 or greater reduction in VL. Although positive at the 50 HCV RNA copies/mL detection limit using the qualitative transcription mediated assay (Bayer), the VL remained undetectable 48–72 h post-initiation of treatment. The VL decline was followed by a virus rebound that returned to pre-treatment levels within 6–12 days after initiation of BILN 2061 treatment. No significant reduction in VL was observed in plasma samples of placebo-treated patients. Similarly, a considerable decline in VL was observed in BILN 2061-treated patients that were either treatment-naive or previously treated with IFN with either minimal or advanced liver disease (Benhamou et al. 2002; Hinrichsen et al. 2002, 2004). The efficacy of BILN 2061 in humans established the first proof-of-concept for an NS3 protease inhibitor and represented a novel class of a selective anti-HCV agents specifically designed to inhibit an essential viral enzyme. While the data for BILN 2061 in a human trial clearly demonstrated the great potential of selective and potent anti-HCV agents, BILN 2061 and other compounds targeting HCV specific functions had to be assessed for sustained antiviral activity and therapeutic benefit in prolonged trials.

5.2 NS3/4A Protease Inhibitors in Combination with Pegylated-IFN and Ribavirin Therapy

From 2001 to 2011, pegylated-IFN and ribavirin (Peg-IFN/RBV) were the standard of care (SOC) for HCV infection. Despite its widespread usage, its therapeutic efficacy is relatively modest by today's standards with a sustained virological response (SVR) of about 40–50 % for genotype 1 patients and 70–80 % in genotype 2 and 3 patients (Fried et al. 2002; Manns et al. 2001). Moreover, Peg-IFN/RBV treatment comes with a myriad of side effects leading to treatment discontinuation (Ward and Kugelmas 2005). Furthermore, the therapy is often contraindicated because of its higher risk-to-benefit ratio in many patients with HCV-associated comorbidities such as decompensated liver cirrhosis (Annicchiarico et al. 2008). Thus, while Peg-IFN/RBV was the SOC for more than 10 years, its low efficacy and its poor tolerability profile prompted a redirection of research towards new therapeutic agents against HCV. By 2010, more than 15 NS3/4A protease inhibitors, five NS5B polymerase inhibitors and many other direct-acting antivirals (DAA) against HCV were assessed in clinical trials (AASLD 2010). In 2011, protease inhibitors telaprevir (Vertex Pharmaceuticals) and boceprevir (Merck & Co.) received approval in the US, Canada and Europe (telaprevir was also approved in Japan) for the treatment of HCV chronic infection. This represented a major advance in the availability and diversity of HCV treatment (Table 1). Overall, these two drugs, used in combination with Peg-IFN/RBV, were the first to show a significantly improved SVR rate with genotype 1 patients (from 40 % to 70 %) and to further shorten the treatment regimen (from 48 weeks down to 24 weeks) (Jacobson et al. 2011; Poordad et al. 2011). However, both of these drugs failed to improve the tolerability profile of an HCV treatment regimen causing severe adverse event in almost 50 % of patients with decompensated liver cirrhosis (Hezode et al. 2014). Thus, despite the first therapeutic improvement in over a decade, there was still a need to develop a pan-genotypic Peg-IFN/RBV-free treatment regimen that would simultaneously increase the viral resistance barrier, be more tolerable and facilitate regimen adherence.

In 2013, a transition toward a protease inhibitor combination therapy was initiated with the approval of simeprevir (Janssen Research & Development) in Canada, Japan and US in 2014. The QUEST-1 and QUEST-2 clinical trials showed that daily doses of simeprevir taken with Peg-IFN/RBV increased the SVR rate to 80 % in genotype 1 patients while not worsening adverse effects (Jacobson et al. 2014; Manns et al. 2014a, 2014b). However, a major pitfall of this first combination therapy was a low genetic barrier for resistance as demonstrated by the lower SVR rate of 58 % in a subgroup of patients infected with a genotype 1a virus bearing a Q80K mutation. Based on these results and the necessity to screen a patient's HCV subtype, clinicians may find themselves reluctant to use this therapy. The approval of sofosbuvir (Gilead Sciences Inc.), a NS5B polymerase inhibitor, for usage in combination with Peg-IFN/RBV confirmed that DAA would lead to a breakthrough in the treatment of HCV. This was evidenced by SVR rates in clinical

Table 1 Overview of the trial phase of selected NS3/4A protease inhibitors

NS3/4A PI	Trial phase	Combination	SVR rate
Telaprevir	Approved	Peg-IFN/RBV	70 %; SVR24
Boceprevir	Approved	Peg-IFN/RBV	70 %; SVR24
Simeprevir	Approved	Peg-IFN/RBV	80 %; SVR24
		Sofosbuvir	92 %;
		(NS5B inh.)	SVR12
Paritaprevir (ABT-450/r)	Approved	Ombitasvir	95–100 %; SVR12
		(NS5A inh.)	
		Dasabuvir	
		(NS5B inh.)	
		Ritonavir	
		(PI enhancer)	
Grazoprevir (MK-5172)	Phase III	Elbasvir	Near 100 %; SVR12
		(NS5A inh.)	
Asunaprevir (BMS-650032)	Phase III	Daclatasvir	95 %; SVR12
	Approved in Japan	(NS5A inh.)	
Vaniprevir (MK-7009)	Phase II	Peg-IFN/RBV	95–100 %; SVR24
Vedroprevir (GS-9451)	Phase II	Ledipasvir	63 %; SVR12
		(NS5A inh.)	
		Tegobuvir	
		(NS5B inh.)	
		Ribavirin	

Trial phase according to North American standards. Combination regimen SVR rates are shown followed by SVR12 or SVR24 to denote the number of weeks after which SVR rates were measured

trials which ranged from 82 % to 100 % with genotypes 1, 4, 5 and 6 (Kowdley et al. 2013). Using the momentum of its success, sofosbuvir would eventually lead the way into an era of IFN-free therapy by boasting SVR rates of 90–100 % when used in combination with the protease inhibitor simeprevir or daclatasvir (Bristol-Myers Squibb), an NS5A inhibitor, with or without ribavirin. These impressive SVR rates were also observed in naïve and hard-to-treat patients (EASL 2013; Sulkowski et al. 2014; Zeuzem et al. 2014). From this time on, HCV therapy would transition definitively from an IFN-based therapy to a combination-based therapy using the best assortment available of NS3/4A protease, NS5A and NS5B polymerase inhibitors (nucleoside or non-nucleoside) with or without the addition of ribavirin.

5.3 All Oral Protease Inhibitor-Containing Combination Therapy

HCV treatment has been revolutionized by the approval of the first IFN-free DAA-based therapy. It is now possible for many patients around the world to

benefit from an HCV cure that boasts a high level of success, that is easy to take (i.e., one pill per day) and that has a very good tolerability profile. As of now, a number of combination therapies (one of which does not contain a protease inhibitor) have been approved for treatment of HCV genotype 1: Harvoni® (Gilead), Viekira Pak™ (AbbVie) and Sovaldi® (Gilead) + Olysio® (Janssen) (Table 1).

Ledipasvir and sofosbuvir combination therapy (Harvoni®, Gilead) was approved in October 2014 by the FDA in the US for the treatment of genotype 1 HCV-infection. This all-oral IFN-free therapy is composed of a first-generation NS5A inhibitor (ledipasvir) and first-generation nucleoside analogue NS5B inhibitor (sofosbuvir). Its use in an open-label phase III clinical trial showed an SVR12 rate of 100 % in naïve patients as well as patients who have undergone prior treatment while demonstrating an excellent adherence to treatment regimen and a good tolerability profile (Mizokami et al. 2015).

In November 2014, the combination therapy comprised of simeprevir (Olysio®, Janssen) and sofosbuvir (Sovaldi®, Gilead) was approved for the treatment of genotype 1 HCV infections. This all-oral IFN-free therapy is composed of a first-generation protease inhibitor and first-generation nucleoside analogue NS5B inhibitor (sofosbuvir). In a phase IIa clinical study, an SVR12 rate of 92 % was observed in naïve and treatment-experienced patients with a favorable tolerability profile and a good adherence to the treatment regimen (Lawitz et al. 2014a, 2014b).

Finally, the ombitasvir/paritaprevir/ritonavir and dasabuvir combination therapy (Viekira Pak™, AbbVie) was approved in December 2014 by the FDA in the US for treating genotype 1 HCV infections. This all-oral IFN-free therapy is composed of a first-generation NS5A inhibitor (ombitasvir), a second generation protease inhibitor (paritaprevir) coupled to a pharmacological enhancer (ritonavir) and a non-nucleoside NS5B inhibitor (dasabuvir) (Klibanov et al. 2015). The phase III clinical trial data demonstrated a very high SVR12 rate (95–100 %) among patients infected with HCV genotype 1 as well as demonstrating a good adherence to the treatment regimen and a favorable tolerability profile (Ferenci et al. 2014; Poordad et al. 2014).

These three approved therapies highlight how quickly the transition away from an IFN/RBV era is taking place. In less than 5 years, HCV treatment has changed from a complicated and painful process to a clever and easily manageable regimen exemplifying the finesse of combining different DAAs which each independently interfere with different vital steps of an HCV infection. Moreover, this transition marks only the beginning as many combination therapies are either still in clinical development or approved in small, restricted markets. An example of an upcoming combination therapy is one which incorporates two second-generation molecules, namely the protease inhibitor grazoprevir (MK-5172, Merck) and the NS5A inhibitor elbasvir (MK-8742), both of which are equally effective against multiple HCV genotypes. Early data show spectacular results with SVR12 rates of almost 100 % across genotypes 1a, 1b, 4 and 6 and with an SVR12 rate of 87 % in HCV genotype 1/HIV co-infected individuals (Lawitz et al. 2015; Sulkowski et al. 2015; Zeuzem et al. 2015). This therapy was also linked to good compliance and to an excellent

tolerability profile for cirrhotic treatment-naive patients. On the scale of smaller more localized markets, the protease inhibitor asunaprevir (BMS) and the NS5A inhibitor daclatasvir (BMS) combination therapy was approved in Japan in July 2014. This therapy proved to be highly effective (SVR12 rates of 95 %) against HCV genotype 1b infection which is responsible for approximately 70 % of all HCV infections in this area (Lok et al. 2014).

6 Resistance, Eradication and Perspective

6.1 Resistance Pattern for NS3/4A Protease Inhibitors

NS3/4A protease inhibitors have been associated with the selection of HCV genotype-specific resistance strains or quasi-species in vivo that can lead to virologic failure in certain individuals. This is because HCV mutations that occur at or near the binding site of an NS3/4A protease inhibitor can hinder the pharmacological activity of said inhibitors by decreasing its affinity and thus can confer resistance. Many mutations observed under selective pressure in vitro (V36M, T54A, Q80K/R, R155K, A156S/T, V170A, D168A/E/V/Y, V/I 170 A/T, V36M + R155K) conferred a low- to high-level resistance to boceprevir, telaprevir, asunaprevir, faldaprevir, paritaprevir and grazoprevir (Lenz et al. 2010, 2013; Summa et al. 2012; Tong et al. 2006). There is no definitive pattern of shared resistant mutations between protease inhibitors, but a tendency for similar mutations between protease inhibitors with similar structure (covalent linear, non-covalent linear or non-covalent macrocyclic) can be observed. The baseline prevalence of these resistant quasi-species can vary but is generally low during an HCV infection (Bartels et al. 2008; Palanisamy et al. 2013). Thus, it is not all baseline variants that are of clinical significance. However, retrospective and prospective studies should be performed in individuals failing therapy to determine whether a resistance mutation is responsible for a viral relapse. The importance of identifying resistance patterns is exemplified with the instance of the Q80K mutation, a relatively common polymorphism (30 %) in genotype 1 HCV associated with a much lower SVR24 rate (57.1 % vs. 81.8 %) and with a higher chance of viral breakthrough (14.3 % vs. 3.0 %) in patients treated with the combination therapy of simeprevir and Peg-IFN/RBV (Fried et al. 2013). These major differences in SVR rates and the recurrence of this mutation led the FDA to recommend the screening of baseline quasi-species before initiating this particular protease inhibitor-containing therapy. These regulatory measures rendered the therapy impractical in many clinical settings. With the release of novel classes of DAAs and their combinatory use, one may wonder whether resistance is still relevant in cases of proper regimen adherence. This is because, in theory, cure should occur earlier than the simultaneous appearance of multiple resistance mutations and compensatory mutations. Future clinical research will definitely focus on patients who have failed

a first combination therapy to assess whether resistant strains are equally resistant to a second combination of DAAs different from the first.

6.2 Contribution to Eradication

It is clear that improved pharmacological management of HCV can contribute to the control of the current HCV epidemic, to the elimination and possibly eventually to the eradication of this virus. Protease inhibitor-containing combination therapies should be able to confer a pan-genomic coverage and an SVR rate of 100 % in the near future. However, the availability of an effective and extremely potent therapy is but one piece of a multi-dimensional puzzle that our society needs to solve to eradicate HCV. At the research level, there is a need to develop an effective vaccine against HCV as current costs and availability of IFN-free HCV therapy make it quite difficult for underprivileged countries to contribute to a decrease in the global disease burden. At the political level, policy makers, politicians, public health officials and stakeholders with industry ties to health care will need to work together to improve and sustain prevention programs, to promote the availability of diagnostic tools, and to ensure accessibility to proper care following HCV diagnosis. Thus, only the combination of a strong-willed society and proper political engagement may, one day, overcome the HCV epidemic.

6.3 Perspectives for NS3/4A Protease Inhibitors and Conclusion

From the discovery of the HCV genome in 1989 to the pending approval of DAA combination therapies that promise a pan-genomic SVR rate of almost 100 %, it is safe to say that the last 25 years have been full of fruitful discoveries in the field of HCV biology (Fig. 2). As we have seen thus far, NS3/4A protease inhibitors have played a major role in the initiation of the therapeutic revolution that is currently taking place. The identification of BILN 2061 was pivotal in the current design of HCV therapy as it established the first proof-of-concept in humans and demonstrated that a potent and selective therapy against HCV was feasible. The challenges of the upcoming IFN-free treatments will be to maintain a high SVR rate, to further improve their tolerability and adherence profile and to become available for all infected individuals (naïve, treatment-experienced, co-infected, etc.).

Firstly, "real-world" data are needed to assess the SVR rate outside of a controlled clinical trial environment. These data will be useful to implement tailored treatment recommendations that best fit with patient history, HCV genotype, and other variables. It will be interesting to see whether a therapy developed to

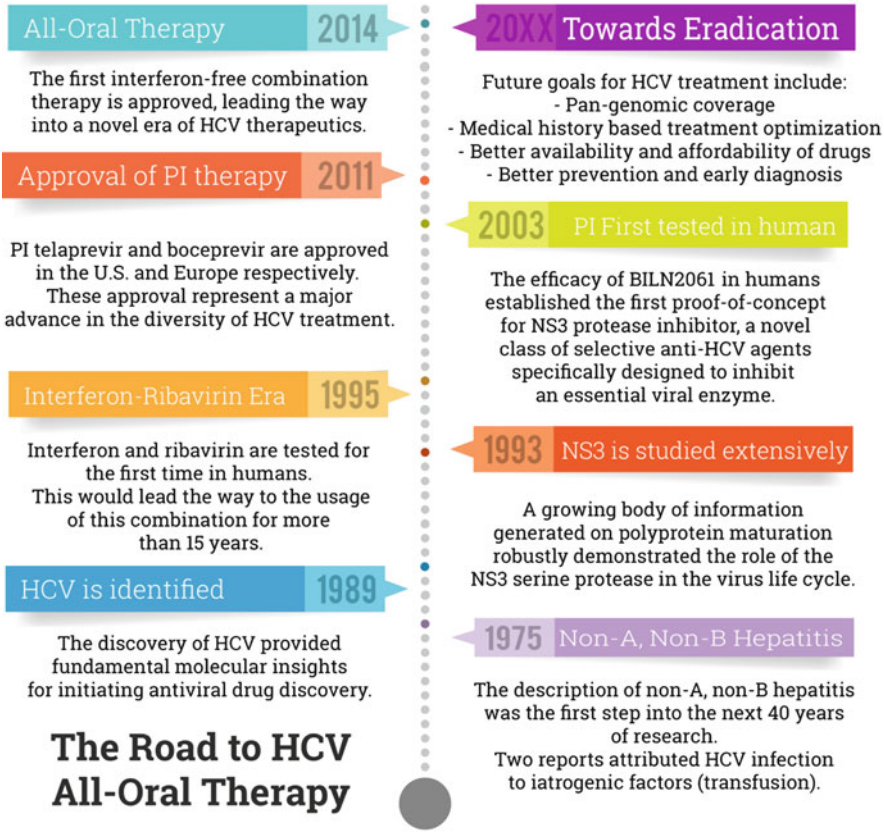

Fig. 2 The road to HCV all-oral therapy. This timeline highlights selected milestones of HCV research with an emphasis on the impact and future challenges of protease inhibitor-containing all-oral therapy

cure genotype 1 infection will maintain its therapeutic promise when applied in a large population affected by many other HCV genotypes.

Secondly, additional studies will be required to establish the optimal treatment duration. At the beginning of the protease inhibitor era, the common treatment course lasted 48 weeks and gradually shortened to 24 weeks eventually lasting as little as 12 weeks (Kohli et al. 2012; Nelson et al. 2015). Now, some studies are investigating the possibility of using an 8-week therapy regimen and perhaps an even shorter one. Ideally, treatment should be as short as possible, but how short can it really be? Future research addressing the impact of novel therapy (including immune-based therapy) in conjunction with clinical studies will be able to better provide an answer to this question.

Lastly, now that very potent combination therapies are available, the focus should be put on strategies to promote its widespread access and its incorporation into common practice. The cost of treatment regimens is putting enormous

economic pressure on the healthcare infrastructure of wealthy countries all the while not being remotely viable for many others. It will be interesting to see how treatment accessibility will be managed whether it be by prioritizing care, by providing special access programs for low-income individuals, or simply by dramatically decreasing the cost. It will depend on whether the industry is motivated more by its social responsibility or by its business imperative. In conclusion, protease inhibitors have and are still playing a major role in HCV therapy. When used in combination with equally potent molecules targeting different viral enzymes, it is quite clear that they will continue their contribution to HCV elimination.

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Mechanism of Action of Direct-Acting Antivirals: New Insights into the HCV Life Cycle

Sara E. Williford and David R. McGivern

Abstract The urgent need for novel therapeutics to address the burden of disease associated with chronic hepatitis C has driven the development of direct-acting antivirals (DAA) that target hepatitis C virus (HCV) replication. Combination therapies targeting multiple viral proteins can increase the genetic barrier to resistance, and all-oral, interferon-free regimens combining different classes of DAA are proving highly effective in treating chronic hepatitis C. Most if not all HCV nonstructural proteins are multifunctional and act in concert with viral and cellular interaction partners in macromolecular complexes. As a consequence, inhibitors that target a single HCV protein can impact several different stages in the HCV life cycle. Studies designed to understand how DAAs act on the functions of individual HCV proteins at the molecular level have provided novel insights into the viral replicase complex, viral RNA synthesis, and virion assembly. DAAs currently used in the clinic include inhibitors that target the NS3/4A protease, the NS5A protein, and the NS5B RNA-dependent RNA polymerase. This review will discuss the mode of action of each of the major classes of antiviral drugs and how they interrupt the HCV life cycle at the molecular level.

Keywords NS5A inhibitor • Protease inhibitor • Helicase • RNA-dependent RNA polymerase

1 A New Era of Antiviral Therapy for Chronic Hepatitis C

Until recently, the standard of care for treatment of chronic hepatitis C was dual antiviral therapy with pegylated interferon-α (Peg-IFN) and ribavirin (RBV), which was lengthy, not well tolerated, and typically achieved sustained virological response (SVR) in only ~50 % of patients infected with the most common HCV genotypes (Fried et al. 2002). Over the past 10–15 years, intense efforts to identify new therapies for chronic hepatitis C have resulted in the development of several

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direct acting antivirals (DAAs) that target viral proteins essential for HCV replication. In 2011, two NS3/4A protease inhibitors (PIs), boceprevir (marketed as Victrelis by Merck) and telaprevir (marketed as Incivek by Vertex), were the first DAAs to be approved by the U.S. Food and Drug Administration for treatment of chronic infection with genotype 1 HCV. These drugs were only approved for use in combination with PEG/IFN and RBV since they have a low genetic barrier to resistance. In clinical trials, PI monotherapy resulted in the rapid emergence of resistant variants (Rong et al. 2010). Triple therapy with PEG-IFN/RBV and either of these PIs increased SVR rates to 70–80 %.

Since then, the discovery of newer, more potent DAAs has resulted in ever increasing rates of SVR. The treatment landscape has changed rapidly and the availability of newer, more effective drugs with fewer side effects has led to the discontinuation of boceprevir and telaprevir use in the U.S. The use of combinations of DAAs that target different viral proteins has resulted in lower rates of virologic breakthrough by increasing the genetic barrier to resistance. Additionally, the high SVR rates achieved with combinations of DAAs have reduced or eliminated the need for PEG-IFN, resulting in much greater tolerability and fewer side effects for patients.

DAAs currently in use in the clinic include small molecule inhibitors that target the NS3/4A protease, the NS5B RNA-dependent RNA polymerase (RdRP), and the NS5A protein (Fig. 1). For patients living with chronic hepatitis C, these DAAs have vastly improved outcomes. For molecular virologists studying HCV they have also provided fascinating insights into the virus life cycle and represent tools with which to dissect virus protein function.

2 NS3/4A Protease Inhibitors

2.1 Targeting NS3/4A to Block Polyprotein Processing

The HCV serine protease NS3, and its cofactor NS4A, play an essential role in the HCV life cycle as the complex they form directs cleavage of the polyprotein

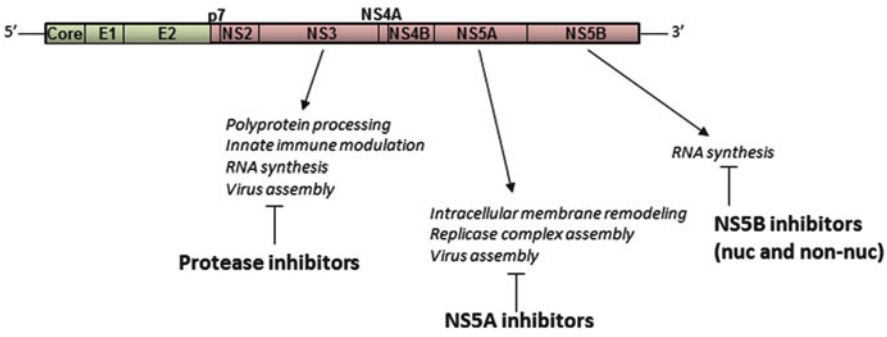

Fig. 1 Schematic of the HCV genome highlighting the proteins targeted by the major classes of DAA currently used for therapy of chronic hepatitis C and their functions within the viral life cycle

at the NS3-NS4A, NS4A-NS4B, NS4B-NS5A and NS5A-NS5B junctions (Grakoui et al. 1993a, b), thus generating the mature non-structural proteins that make up the HCV replicase complex. NS3/4A has long been recognized as an attractive target for the development of antiviral therapies (see the chapter "HCV NS3/4A Protease Inhibitors and the Road to Effective Direct-Acting Antiviral Therapies" by Tremblay and colleagues in this volume). The N-terminal protease domain of NS3 was the first of the HCV protein domains for which a high resolution X-ray crystal structure was determined (Kim et al. 1996; Love et al. 1996). Small molecule inhibitors designed to mimic the natural substrate of the NS3/4A protease and block polyprotein processing were the first class of antivirals demonstrated to be effective against HCV (Lamarre et al. 2003) and the first DAAs to be approved for therapy of chronic hepatitis C.

In addition to the peptidomimetic PIs that bind to the protease active site, a class of NS3 inhibitors has been developed using a fragment-based screening approach that bind to an allosteric site at the interface between the protease and helicase domains of NS3 (Saalau-Bethell et al. 2012). These allosteric NS3 inhibitors (no longer in clinical development) block polyprotein processing by locking the full length NS3 in a closed, auto-inhibited conformation.

2.2 PI Effects on HCV RNA Synthesis

PIs have long been assumed to target RNA synthesis by inhibiting polyprotein cleavage and thus cutting off the supply of new non-structural proteins required to generate new replicase complexes. However, peptidomimetic PIs such as boceprevir and telaprevir are capable of almost completely blocking new viral RNA synthesis at very early time points (<12 h) after addition of drug to HCV-infected cell cultures, prior to any detectable decline in intracellular abundance of non-structural proteins (McGivern et al. 2015). NS3 is an essential component of a macromolecular RNA replicase complex formed by multiple nonstructural (NS) proteins, and the early kinetics of RNA synthesis inhibition suggest that PIs block an essential function of NS3 within this complex that is required for RNA synthesis (McGivern et al. 2014). How might a PI act to rapidly block RNA synthesis? Since NS3 is a bifunctional enzyme consisting of distinct protease and helicase domains connected by a flexible linker, the binding of PIs at the protease active site may constrain the helicase activities of NS3 within the replicase complex. Helicase activity is essential for HCV RNA replication (Lam and Frick 2006). The protease domain has been shown to regulate the helicase activity in vitro and to be required for efficient binding and unwinding of dsRNA by full length NS3 (Frick et al. 2004; Beran et al. 2007). A model for NS3 helicase activity during RNA synthesis has been proposed in which the protease domain of NS3 acts as clamp, holding

dsRNA while the helicase unwinds the dsRNA template to allow NS5B, the catalytic core of the replicase complex, to access the negative strand and synthesize new genomes (Aydin et al. 2013). Binding of a PI to the protease domain may prevent interactions of NS3 with the RNA template or with other NS proteins within the complex that are required for RNA synthesis.

2.3 PI Effects on Virus Assembly

Modeling analyses of the decline in patient serum viral load following monotherapy with telaprevir suggest that this PI can block HCV assembly or its egress from infected cells (Guedj et al. 2013), although the block to virus secretion appears less efficient than that mediated by the NS5A inhibitor daclatasvir (discussed below). Importantly, genetic and biochemical studies have implicated the NS3 helicase domain in virus assembly (Ma et al. 2008; Jones et al. 2011).

In HCV-infected cell cultures, treatment with a PI such as telaprevir or boceprevir blocks the production of infectious virus within 12 h of the addition of drug (McGivern et al. 2014, 2015). Again, this effect precedes any significant reduction in the abundance of processed mature polyprotein products. A similar blockade of virus production was observed with an allosteric NS3 inhibitor, AT23708 (Saalau-Bethell et al. 2012) (no longer in clinical development) that binds the inter-domain interface between the protease and helicase domains. Rate-zonal centrifugation analyses of lysates from HCV-infected cell cultures showed that treatment with either telaprevir or AT23708 did not affect the assembly of HCV RNA-containing particles but did abolish the infectivity of those particles (McGivern et al. 2015). These data indicate that the PI and allosteric NS3 inhibitor can block a very late stage in assembly or infectious virus maturation prior to egress. Collectively, the effect of PIs on both RNA synthesis and virion maturation point to the multifunctional nature of HCV nonstructural proteins, a common theme among positive-strand RNA viruses that because of restrictions on the length of their genomes express only small numbers of proteins.

2.4 Restoration of Innate Immune Signaling by PIs

HCV infection is sensed in infected hepatocytes by the pattern recognition receptors RIG-I (retinoic acid-inducible gene I) and TLR3 (Toll-like receptor 3). Binding of double-stranded RNA replication intermediates to RIG-I or TLR3 initiates a signaling cascade that results in activation of several transcription factors including interferon regulatory factor (IRF) 3, IRF7, and nuclear factor-kB (NF-kB), thereby resulting in subsequent transcription of antiviral genes including type I IFNs. RIG-I and TLR3 signaling are mediated by the adaptor proteins MAVS (mitochondrial

antiviral-signaling protein) and TRIF (Toll-interleukin1 receptor domain-containing adaptor inducing IFN-β, or TICAM1), respectively. The HCV NS3/4A has evolved the capacity to cleave both MAVS (Meylan et al. 2005) and TRIF (Li et al. 2005) to evade these host innate immune responses.

Early studies showed that high concentrations of prototype NS3/4A inhibitors restored IRF3 activation in HCV replicon cells that were infected with Sendai virus (SenV), a potent inducer of IFN-β (Foy et al. 2003). These studies suggested that blocking NS3/4A activity might have a "double whammy" effect: not only would PIs directly inhibit viral replication but they might also restore innate immune signaling pathways in infected hepatocytes. Treatment of HCV infected cells with the potent macrocyclic PI simeprevir increased the abundance of full length, uncleaved MAVS in HCV-infected cells, but higher concentrations of simeprevir (>100-fold the antiviral EC_{50}) were required to restore SenV-induced IFN-signaling compared to that needed to block HCV replication (Liang et al. 2008). In comparison, an NS5B inhibitor had no effect on restoring IFN-signaling through the IRF3-dependent IFN-β promoter and, despite suppressing NS3 abundance, only the cleaved form of MAVS was detected at the time points assayed.

While all classes of DAA targeting HCV can ultimately restore MAVS-mediated RIG-I signaling as an indirect consequence of eradicating HCV from the infected cell, other cell culture studies have confirmed that the restoration of antiviral signaling occurs more rapidly with PI treatment. In HCV replicon-bearing cells, the PIs boceprevir, telaprevir and simeprevir were shown to restore levels of uncleaved, mitochondrially localized MAVS and SenV-induced IFN-β promoter activation faster than inhibitors targeting NS5A and NS5B (Kalkeri et al. 2013). It is likely that PIs can also restore TLR3-dependent signaling, but whether PIs also block cleavage of TRIF has yet to be determined. As important, it remains unknown whether this 'double-whammy' action results in any clinical benefit in the context of combination DAA therapies.

3 NS5A Inhibitors

3.1 Discovery of NS5A Inhibitors and Identification of NS5A as an Antiviral Target

For NS3 (protease) and NS5B (RdRP), a detailed knowledge of structure-activity relationships allowed design of specific inhibitors that inhibit enzyme activity. An alternative strategy, involving high throughput screening of small molecule libraries in an HCV replicon cell-based assay, was used to identify a novel class of iminothiazolidinone compounds that could inhibit HCV RNA replication (Gao et al. 2010). Two distinct lines of evidence identify NS5A as the target of this class of compounds. First, sequencing of replicons that were able to grow under

selection with the compounds identified mutations in domain I of NS5A that confer resistance (Gao et al. 2010; Fridell et al. 2010). In addition, biotinylated versions of these compounds were able to pull down NS5A from lysates of HCV replicon-bearing cells. Initial lead compounds were further refined to improve antiviral activity and symmetry was identified as an important contributor to potency, resulting in the development of daclatasvir (formerly BMS-790052) (Gao et al. 2010).

Following the identification of the first-in-class NS5A inhibitor, daclatasvir (Bristol Myers Squibb), many other groups identified similar compounds that were also able to inhibit NS5A including ledipasvir (Gilead) and ombitasvir (Abbvie). In cell culture models, NS5A inhibitors were able to inhibit HCV replication at concentrations in the low picomolar range, placing them among the most potent antiviral drugs ever discovered.

However, as with the PIs, resistance is a problem for most NS5A inhibitors identified so far and this prevents their use as a monotherapy. Importantly, the resistance profiles of NS5A inhibitors do not overlap with other DAAs so they can be used effectively in interferon-free combinations with other classes of antiviral such as PIs or polymerase inhibitors. Combination therapies currently approved for use in the clinic include combinations of NS5A inhibitors with nucleotide analog NS5B inhibitor (e.g. ledipasvir or daclatasvir with sofosbuvir), or in triple therapy with a ritonavir-boosted protease inhibitor and a non-nucleoside NS5B inhibitor (e.g. ombitasvir with pariteprevir and dasabuvir).

As a protein with no known enzymatic activity, NS5A is an unusual and fascinating antiviral target. NS5A is a phosphoprotein that associates with intracellular membranes through an amphipathic alpha helix at its N-terminus (Brass et al. 2002). Like many other viral proteins encoded by small viruses, NS5A is multifunctional and is required for viral RNA replication (Tellinghuisen et al. 2008b), virus assembly (Tellinghuisen et al. 2008a; Masaki et al. 2008), and modulation of cellular signaling pathways (Pflugheber et al. 2002).

NS5A has no cellular or viral homologs and its structure is only partially characterized (Tellinghuisen et al. 2005; Love et al. 2009). Of the three distinct domains currently considered present within the NS5A protein, domains 2 and 3 are intrinsically disordered; structural information is only available for domain 1, which includes the N-terminal amphipathic helix and a zinc binding motif. Crystal structures available for domain 1 (minus the N-terminal amphipathic helix) reveal alternative dimerization interfaces (Tellinghuisen et al. 2005; Love et al. 2009; Lambert et al. 2014), possibly resulting from the slightly different truncations of domain 1 used in the three studies. While NS5A dimer formation may be an artifact of crystallization, the dimeric symmetry of highly potent NS5A inhibitors suggests that these compounds may interact with NS5A in a dimeric form. NS5A dimerization may be important for HCV replication, but NS5A inhibitors do not block dimer formation (Lim et al. 2012). NS5A inhibitors bind to domain 1 and resistance-associated variants (RAVs) that arise both in vitro and during treatment in patients contain amino acid substitutions within domain 1 (Fridell et al. 2010, 2011b).

Differential phosphorylation of NS5A has been proposed as a mechanism by which its involvement in several diverse functions in the virus life cycle is regulated (Cordek et al. 2014). Two forms of NS5A, basal and hyperphosphorylated, are observed by SDS-PAGE analysis of lysates of infected cells, and phosphorylation at specific residues or groups of residues have been suggested to regulate NS5A functions in RNA replication and virus assembly (for a review see (Ross-Thriepland and Harris 2015)). Early studies of NS5A inhibitors using the genotype 2a strain of HCV, JFH-1, or the genotype 1b strain, Con1, have been interpreted to suggest that NS5A inhibitors act by blocking hyper- but not basal phosphorylation of NS5A (Qiu et al. 2011; Fridell et al. 2011a). However, NS5A phosphorylation is influenced by different cellular conditions, and it is possible that changes in phosphorylation status represent an epiphenomenon rather than the primary mode of action of NS5A inhibitors. A detailed understanding of the mode of action of inhibitors that target NS5A is likely to shed additional light on the functions of this protein in the HCV life cycle.

3.2 Membranous Web and Replicase Complex Assembly

Accumulating evidence suggests that one mode of action for NS5A inhibitors is to block establishment of the intracellular "factories" where HCV RNA genomes are replicated. Kinetic analyses of the inhibition of RNA synthesis (Fig. 2) following addition of drug to infected cell cultures suggest a model where NS5A inhibitors do not block RNA synthesis from pre-existing replicase complexes, but rather prevent formation of new replicase complexes (McGivern et al. 2014; Targett-Adams et al. 2011). The replicase complexes responsible for RNA synthesis form in association with remodeled intracellular membranes, collectively referred to as the membranous web (Egger et al. 2002; Gosert et al. 2003). The membranous web acts as a platform for virus replication, concentrating the viral proteins that form the replicase complex and potentially protecting viral RNA from detection by intracellular pattern recognition receptors such as RIG-I. Biochemical studies (Romero-Brey et al. 2012) have demonstrated that the membranous web is primarily derived from endoplasmic reticulum and is composed of single and double membrane vesicles (DMVs). The DMVs are currently considered to be the sites of active HCV RNA synthesis, although there is no direct evidence for this. The induction of DMVs by HCV is not fully understood, although the concerted action of multiple HCV non-structural proteins is required. Deletion studies have identified the N-terminal alpha helix and domain 1 of NS5A as key determinants of DMV induction (Romero-Brey et al. 2015). Treatment of HCV-infected Huh7 cells with NS5A inhibitors blocks the formation of the membranous web independently of inhibiting RNA replication and without affecting the stability of NS5A (Berger et al. 2014).

How might NS5A inhibitors block formation of the membranous web? NS5A has been shown to interact with many cellular proteins (Ross-Thriepland and Harris

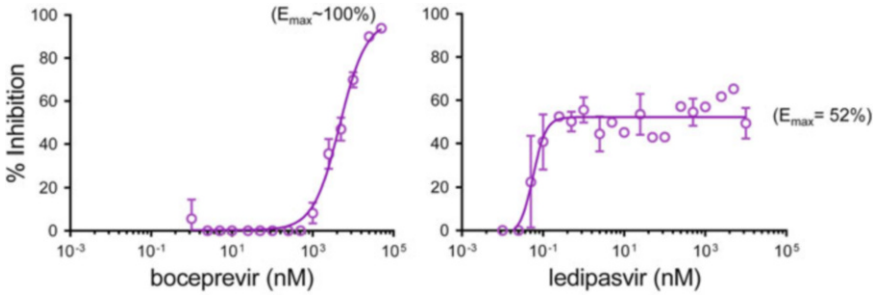

Fig. 2 Inhibition of HCV RNA synthesis in H77S.3-infected cells by a PI (boceprevir) or an NS5A inhibitor (ledipasvir). RNA synthesis was measured by incorporation of 5-EU into nascent HCV RNA between 2 and 12 h after addition of inhibitor to culture medium. During this early time interval following addition of ledipasvir, the inhibition of RNA synthesis is potent but partial. These data suggest a model where NS5A inhibitors block new replicase complex formation but have limited ability to inhibit previously assembled complexes. In contrast, the PI boceprevir can almost completely block new RNA synthesis at high concentrations. No reduction in residual abundance of non-structural proteins was detected after 12 h PI treatment (McGivern et al. 2015), suggesting disruption of an activity of NS3 within the replicase complex (Figure adapted from McGivern et al. (2014))

2015). Among these, its interaction with the cellular lipid kinase, phosphatidylinositol 4-kinase IIIα (PI4KIIIα) is critical for virus replication (Lim et al. 2012) and establishment of the membranous web (Reiss et al. 2011). NS5A interacts with and stimulates PI4KIIIα activity (Reiss et al. 2011; Berger et al. 2011) resulting in locally increased levels of the product phosphatidylinositol-4-phosphate (PI4P) in the intracellular membranes of infected cells (Bianco et al. 2012). The accumulation of PI4P is not by itself sufficient for maintenance of the integrity of the membranous web, but it results in recruitment of an effector, oxysterol-binding protein (OSBP) to NS5A-bearing membranes (Wang et al. 2014). The activity of PI4KIIIα and its effector OSBP were shown to regulate cholesterol trafficking to the membranous web, possibly contributing to its formation and integrity.

Changes in the membranous web following NS5A inhibitor treatment are similar to effects observed following treatment with PI4KIIIα inhibitors (Bianco et al. 2012; Reghellin et al. 2014). However, NS5A inhibitors such as daclatasvir do not directly inhibit PI4KIIIα. NS5A inhibitors impair but do not completely abolish the interaction of NS5A with PI4KIIIα (Reghellin et al. 2014), and daclatasvir has been shown to alter NS5A localization, suggesting a model in which NS5A inhibitors bind to NS5A and block conformational changes required to stimulate the activity of PI4KIIIα (Chukkapalli et al. 2015). The inhibitor-mediated blockade of NS5A stimulation of PI4KIIIα activity prevents high-level accumulation of PI4P, and subsequent trafficking of cholesterol, essential for formation and integrity of the membranous web (Reghellin et al. 2014; Chukkapalli et al. 2015).

3.3 Virus Assembly

In human subjects with chronic hepatitis C, a single dose of 10 or 100 mg daclatasvir results in a very rapid initial decline in serum viral load (3 $\log_{10}$ reduction by 8 h after initiation of therapy), followed by a subsequent slower decline over the next 40 h (Guedj et al. 2013). Mathematical modeling of the decline in viral load suggested that daclatasvir has two modes of action: the initial rapid decline in serum viral load being explained by the drug blocking virus assembly or release from infected hepatocytes, while the second phase likely reflects the capacity of the drug to block RNA production (as a consequence of its inhibition of replicase complex assembly described above).

Detailed kinetic analyses of infectious virus production from cultured Huh7 cells infected with the genotype 1a HCV strain H77S.3 showed that NS5A inhibitors can efficiently block virus release within 6–9 h following addition of the drug (McGivern et al. 2014). At these very early time points there is negligible effect on intracellular viral RNA levels as measured by RT-PCR. Following treatment with NS5A inhibitors, persistently-infected Huh7 cells were rapidly depleted of intracellular infectious virus and RNA-containing HCV particles (McGivern et al. 2014), indicating that the inhibitors block virus assembly. In contrast to NS5A inhibitors such as daclatasvir and ledipasvir, inhibitors of the lipid kinase PI4KIIIα (Leivers et al. 2013) that block HCV replication by preventing membranous web formation do not inhibit virus assembly at early time points (McGivern et al. 2014). This suggests that NS5A inhibitors target a PI4KIIIα-independent function of NS5A to block virus assembly. Taken together, these data provide direct evidence for disruption of virus assembly by NS5A inhibitors, in addition to their PI4KIIIα-dependent action in blocking the establishment of the membranous web: truly dual mechanisms of action. Unfortunately, RAVs associated with resistance to the antiviral effects of NS5A inhibitors provide for escape from both antiviral mechanisms (McGivern et al. 2014).

4 NS5B RNA-Dependent RNA Polymerase Inhibitors

4.1 NS5B as an Antiviral Target

The RdRp, NS5B, is the enzyme that catalyzes synthesis of the HCV genome and thus a major target for the development of antiviral compounds. Crystal structures of the HCV polymerase were solved in 1999, and since that time biochemical enzymatic assays and cell based replicon systems have provided the means to explore molecules targeting various sites of the RNA polymerase (Lesburg et al. 1999). The structure of NS5B is that of a typical RNA polymerase, resembling a right hand with thumb, finger, and palm domains, similar to the architectures of the RNA polymerases of picornaviruses and bovine viral diarrhea virus. The active

site is located within the palm domain and has a highly conserved GDD motif (Miller and Purcell 1990). NS5B inhibitors are classified into two general classes; nucleoside/nucleotide inhibitors (NIs) and non-nucleoside inhibitors (NNIs).

4.2 Nucleoside/Nucleotide Inhibitors (NIs)

When properly phosphorylated, these inhibitors interact with the well-conserved catalytic active site of the enzyme, causing chain termination and/or errors when incorporated into a growing RNA chain (Ma et al. 2005). Since the active site is well conserved, these inhibitors show relatively similar efficacy across HCV genotypes and demonstrate a relatively high barrier to resistance. Like all DAAs, monotherapy with NIs results in unacceptably poor treatment outcomes, but such antiviral compounds provide an ideal "backbone" upon which to build combination DAA therapies.

Sofosbuvir, a potent NI with pangenotypic activity was discovered in 2007 by Pharmasset, Inc. and approved by the U.S. Food and Drug Administration in 2013 for treatment of genotypes 1–4. It was the first NS5B nucleoside inhibitor to be introduced into clinical use because of its potency, pan-genotypic activity, once daily dosing, low incidence of adverse effects, and high barrier to resistance. Similar compounds are now in advanced stages of clinical development by other pharmaceutical companies. The most common resistant variant is a substitution of serine to threonine at position 282 (S282T) in the active site. This variant is unfit and the replication rate is 3–15 % of that seen with wild-type virus in vitro assays (Migliaccio et al. 2003). While it can pre-exist and may be selected during therapy, its poor fitness generally does not allow this variant to expand. Other variants that confer in-vitro resistance to nucleoside inhibitors (S96T and N142T) have not been detected in patients with HCV genotype 1 infections (Kuntzen et al. 2008).

4.3 Non-nucleoside Inhibitors (NNIs)

The NNIs bind one of several different allosteric sites on the surface of the polymerase, creating a change in the conformation of the polymerase that reduces its activity. There are four main allosteric sites that have been targeted in the NS5B polymerase; thumb domains 1 and 2 and palm domains 1 and 2. Since different sites are targeted these inhibitors could act synergistically or in a non-antagonistic way (Gentile et al. 2015).

In contrast to NIs, the NNIs have shown a restricted spectrum of activity against the various HCV genotypes. They have a lower genetic barrier to resistance because the allosteric sites may undergo a series of mutations without affecting the overall function of the NS5B polymerase (Sarrazin and Zeuzem 2010). Also, variants with

low sensitivity to these inhibitors are pre-existing at relatively high frequencies at baseline in many patients.

Dasabuvir (also known as ABT-333) is a relatively potent NS5B NNI that was developed by modification of an aryl dihydrouracil fragment identified in high-throughput screening of libraries for compounds that inhibit activity of recombinant polymerase in vitro (Liu et al. 2012). Since dasabuvir is an allosteric inhibitor, it has a relatively low barrier to resistance and is approved for use in combination with an inhibitor targeting HCV NS3/4A protease (paritеprevir boosted with ritonavir) and NS5A (ombitasvir) for the treatment of HCV genotype 1 infection. Ribavirin is added if there is cirrhosis (which worsens the outcome for all DAA therapies), or if the patient is infected with genotype 1a. Predominant dasabuvir-variants selected in the genotype 1a replicon studies were S556G and C316Y, while C316Y and M414T were predominant in the genotype 1b replicon studies (Kati et al. 2015). Resistance profiles, genotype specificity and structure activity relationships indicate that dasabuvir binds to the palm I allosteric inhibitory site of the NS5B polymerase (Liu et al. 2012).

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Modeling HCV Dynamics in Clinical Practice to Personalize Antiviral Therapy

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Abstract Modeling by mathematical equations the dynamics of hepatitis C virus (HCV) infection has been helpful to uncover the mechanisms of viral persistence and to study the mode of action of different antiviral therapies. Standard biphasic models well describe the viral load decline in the first 2–4 weeks of therapy, however the hypothesis of a constant clearance rate of the infected cells during longer treatment periods provides unrealistic estimates of the chance to reach a sustained virological response (SVR).

For this reason, we have developed a multiphasic model in which the analysis of alanine aminotransferase (ALT) kinetics during the first 4 weeks of therapy allows for better assessment of the infected-cell clearance rate, which is feedback regulated to the reduction of the infected cell number during the following treatment. By this approach, we found a very high correlation between the residual number of infected cells at the end of therapy and its outcome, regardless to HCV genotype, IL28B polymorphism and characteristics of the liver disease. This modeling approach was successfully applied in a pilot study to tailor the duration of peg-interferon and ribavirin therapy, as well as to make accurate predictions of SVR at the individual level in HCV genotype 1 patients receiving P/R lead-in before adding first generation protease inhibitors.

The unique experience generated by the application of our bio-mathematical model in clinical practice, suggests that deterministic models of viral dynamics can be used as simulators of treatment response to help physicians in personalizing treatment management. This is especially valuable when we have to take into account the costs of the different schedules and their actual efficacy in patients with clinical features more complex than those of the drug registration trials.

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Keywords Viral hepatitis • HCV • Bio-mathematical model • Viral dynamics • DAAs • Cost-efficacy

1 Introduction

The availability in the 1990s of quantitative assays to measure with accuracy changes of viral load in the blood during antiviral therapies, prompted the growth of a new field in HCV biology: the study of viral kinetics by mathematical models.

Since HCV-RNA levels represents the equilibrium between virus production and clearance, the kinetics of their changes under given conditions allows for studying the underlying biological mechanisms responsible for the antiviral effects. Bio-mathematical models, indeed, can simulate complex biological phenomena starting from logical assumptions, hypotheses and experimental evidences to provide a simplified description of the process, which retains sufficient complexity to reveal the major determinants of the process itself.

This approach has provided valuable insights in understanding the modes of action of Peginterferon (Peg-IFN) and Ribavirin (RBV) and estimating key parameters of the HCV life cycle, thus offering an efficient tool for the prediction of treatment outcome (Colombatto et al. 2003; Dixit et al. 2004; Snoeck et al. 2010). Here, we review the development and clinical application of the modeling approach in the context of treatment personalization, discussing its potential role in the rapidly evolving scenario started with the availability of Direct Acting Antivirals (DAAs).

2 Standard Biphasic Models

In chronic viral infections, standard models describe viral dynamics based on the division in two distinct but connected compartments: the cellular one, where viruses replicate inside the infected cells, and the extracellular one, where free viruses circulate to reach non-infected cells. This modeling approach, which proved useful in understanding pathogenesis and guiding therapy of human immunodeficiency virus (HIV), was firstly applied to study the kinetics of HCV-RNA under IFN-α therapy by Zeuzem et al. (1996). Their model described with ordinary differential equations productively infected hepatocytes and viral load dynamics. In their fits to experimental data, they were able to produce only single exponential viral decay in response to therapy (assuming alternatively 100 % block of viral production or 100 % block of de novo infections), whereas most patient showed a biphasic viral decline. However, with the assumption of completely blocking of de novo infection, a good fit of the biphasic decline was observed, supporting the conclusion that this was the main mechanism of IFN therapy that lead to cure hepatitis C patients.

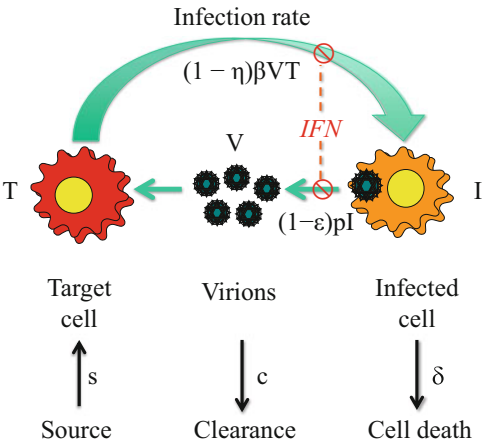

Fig. 1 Biphasic model of viral dynamics

Neumann et al. (1998), further extended the model by including a separate compartment for susceptible healthy hepatocytes (Fig. 1). Dynamics and relations among uninfected target cells (T), infected cells (I) and viral load (V) are described by three different equations:

- $dT/dt = s - dT - (1 - \eta)\beta VT$
- $dI/dt = (1 - \eta)\beta VT - \delta I$
- $dV/dt = (1 - \varepsilon)pI - cV$

Briefly, this model assumes that target liver cells (T) replicate at rate s, die at rate d, and become infected (I) at a certain rate (βTV) when they encounter free virions circulating in the blood. The infected cells are eliminated at the rate of δI and free virions, which are produced at the rate of pI, and cleared at the rate of cV. Antiviral therapy can reduce either target-cell sensitivity to infection or the production rate of free virions that are released in the blood from the already infected cells. In this model, the impact of antiviral drugs on the dynamics of viral infection is considered by the coefficients (1-η) and (1-ε) of the equations that describe the rate of target cell infection and free virion production, where η and ε represent the reduction in cell susceptibility to the infection and the reduction in the viral production, respectively. With a very frequent sampling of viral load in the first 2 days of therapy from 23 patients, Neumann et al. observed that daily IFN doses of 5, 10 and 15 MIU were correlated to viral release and production blocking efficacy of 81 %, 95 % and 96 %, respectively. In presence of a strong direct antiviral activity that blocks virus production not completely, the model predicts a biphasic pattern of viral load decline if the half-life of the circulating virions and that of the infected cells are different. The short HCV-RNA half-life of the first phase (day 0–2) was reasonably associated with the clearance of circulating virions rather than infected cells, and provided estimated HCV virion half-life of 2.7 h. The second phase viral load decline was slower, leading to estimated infected hepatocytes half-life of 1.7–70 days. Interestingly, the infected cell half live was directly correlated with

viral load at baseline, but inversely correlated with alanine aminotransferase (ALT) levels.

The need to further improve this model to better describe viral kinetics in patients treated with the combination of peg-IFN and RBV, was pointed out by the study of Herrmann et al. (2003). In fact, they observed a more rapid viral load decline (day 7–28) in these patients, as compared to those treated with peg-IFN alone. The faster second phase decline was associated with undetectable viral load at the end of therapy and higher rates of sustained virological response (SVR). They explained this phenomenon by assuming that RBV increases the death rate of infected cells. However, Pawlotsky et al. (2004) noticed a transient effect in viral decline upon addition of RBV that could be attributed to a direct antiviral effect of RBV. In their mathematical model, they assumed that RBV rendered a proportion of newly infected hepatocytes unproductive, in agreement with the evidence in a poliovirus model of RBV mutagenic effects that made part of the newly produced virions non-infectious (Crotty et al. 2001). By considering the additional efficacy of RBV as a separate term, Dixit et al. (2004) were able to analyze its dose-dependent effect in several situations. They could explain why RBV was ineffective in monotherapy and not necessarily required for high efficacy of IFN.

Altogether, the results of these studies brought to the conclusions that model analysis of the antiviral effects during the first month of peg-IFN/RBV therapy could predict treatment outcome, thus helping to tailor individual schedules. However, the use of a biphasic model to decide treatment duration was not able to improve therapy outcomes in a large clinical trial (Zeuzem 2005). For this purpose, a major limit of the biphasic models was the assumption of a constant clearance rate of the infected cells during the whole treatment. Although reasonable in the first month of therapy, such assumption in the long run would invariably lead all patients to clear HCV, provided the presence of a minimal direct antiviral effect and a long enough treatment.

3 Multiphasic Viral Dynamics Models

To overcome some of the limits of the biphasic model, a multiphasic one was developed in which the immune system activity has the possibility to be modulated with the progression of the treatment (Colombatto et al. 2003). There are, in fact, several reasons to hypothesize that the efficacy of the immune mediated clearance of the infected cells may change after the first month of therapy: (i) infected cells trigger the immune response, and their progressive reduction is expected to determine a reduction of specific cytotoxic T cells infiltrating the liver, (ii) HCV replication and antigen production is likely to be lower in infected cells during antiviral therapy, making more difficult CTL recognition of the residual infected hepatocytes, (iii) IFN alpha can have immune-suppressive effects by limiting T cell proliferation (Kaser et al. 1999).

In addition, the kinetics of the infected cells can be investigated during the second phase not only by the viral load decline but also by the ALT decline. Serum levels of ALT are valid surrogate parameters of hepatocyte turnover (Zeuzem et al. 1998) and the normalization of ALT levels during antiviral treatment is considered a major parameter to assess the remission of liver damage. Dahari et al. (2005), who analyzed data from ten Chimpanzees to better understand the virus-host dynamics during primary HCV infection, set up a model where ALT dynamics were described in a separate differential equation. In addition, the introduction of the homeostatic proliferation of hepatocytes helped to analyze complex and long-term viral dynamics. By this approach they concluded that both cytolytic and noncytolytic mechanisms were responsible in varying half-life of infected hepatocytes. Thus, the combined analysis of ALT and HCV-RNA decline appears very useful to discriminate between the cytolitic clearance of the infected cells and the additional direct antiviral effects that can yet occur in the second phase, i.e. due to RBV and to the increase of the IFN concentration (Bruno et al. 2004), which may eventually lead to extinguish the infection in a subset of hepatocytes.

By fitting HCV-RNA and ALT declines during the first month of therapy, the model developed at the Hepatology Unit of the University Hospital of Pisa (Colombatto et al. 2003) can simulate long term viral and infected cell dynamics to predict individual (peg)-IFN/RBV therapy outcome. In this model the dynamics of target (T) and infected cells (I), and that of the viral load (V) are described by the following equations:

$$dT(t)/d(t) = \xi(T,I) - \Theta \cdot T(t) - (1-\eta) \cdot \beta \cdot T(t) \cdot V(t) \quad (1)$$

$$dI(t)/d(t) = (1-\eta) \cdot \beta \cdot T(t) \cdot V(t) - [\delta 0 \cdot (I(t-\sigma)/I0)k + \Theta + \alpha] \cdot I(t) \quad (2)$$

$$dV(t)/d(t) = (1-\varepsilon) \cdot \Psi(t) \cdot I(t) - \lambda \cdot V(t) \quad (3)$$

In Eq. 1, the term $\xi(T,I)$ yields the number of target cells that are produced daily: at variance with the standard biphasic model its value depends on the overall number of target cells (T) plus the number of infected cells (I) and increases (due to liver regeneration) if the overall hepatocyte number (T + I) tends to decrease; $\Theta \cdot T(t)$ describes the number of target cells dying daily by natural death (Θ = 1/300 days) and $(1-\eta) \cdot \beta \cdot T(t) \cdot V(t)$ the number of target cells infected daily.

In Eq. 2, the term $\delta_0 \cdot (I(t-\sigma)/I_0)k + \Theta + \alpha] \cdot I(t)$ describes the number of infected hepatocytes lost every day. The rate constant of the immune mediated clearance of the infected cells δ_0 is computed the least square fit of ALT values measured during the first 28 days of therapy using the equation ln[(ALT(t)–ALTn)/(ALT_0 – ALTn)]/t, where ALTn = ALT normal value (assumed 20 U/L for the initial computation, then adjusted to obtain best fitting of the experimental data). During therapy, after a delay time (σ = 4 weeks), the infected cell clearance rate does not remain constant but it is feedback regulated to the reduction of the infected cells $I(t-\sigma)/I_0)^k$ by the exponent k (value: 0.2); the hepatocyte natural turnover Θ and the term α, that represents a minimal natural immune system clearance activity,

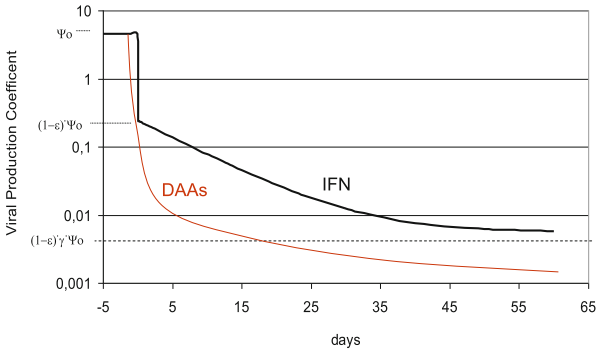

Fig. 2 Kinetics of inhibition of viral production

become relevant for I(t) approaching zero and warrants the maintenance of the response after the end of therapy.

In Eq. 3, the term that describes the number of virions produced and removed daily is $(1-\varepsilon)\Psi(t)\cdot I(t)$. The effectiveness of IFN in blocking viral production, responsible for the first phase rapid viral load decline, is computed at day 2 as: $(1-\varepsilon) = V(d2)/V(d0)$. Thereafter, the viral production coefficient $\Psi(t)$ does not remain constant like in Neumann's model, but it continues to decrease down to its asymptotic value $\gamma\cdot\Psi_0$ in patients whose second phase decay of HCV-RNA is faster than that of the infected cells, as computed by ALT decline. Comparing the kinetics of viral inhibition predicted by this model in patients treated with peg-IFN/RBV with those observed with direct acting antivirals (DAAs), like protease inhibitors (Fig. 2), it is interesting to note how the faster and deeper inhibition of viral replication induced by Telaprevir (TVR) (Levin et al. EASL Conference, 2006, Vienna, Austria) brings the viral production coefficient close to its asymptotic levels after few day of therapy only, making the second phase viral load decline really reflecting the slow clearance rate of the cells maintaining residual viral production.

4 Prediction of SVR by Viral Dynamics Models

Using the multiphasic modeling approach developed in Pisa, the good correlation observed between model parameters and long term virological response allowed for studying the application of this model to tailor peg-IFN/RBV treatment at the single patient level in clinical practice. In particular, the end of therapy infected cell number (Ieot), which is computed after 4 weeks of treatment, represents a virtual parameter summarizing the major drug-virus-host interactions occurring during therapy and showed, by logistic regression analysis, the best correlation with the outcome in the 28 patients treated with IFN/RBV of the original study (Colombatto et al. 2003).

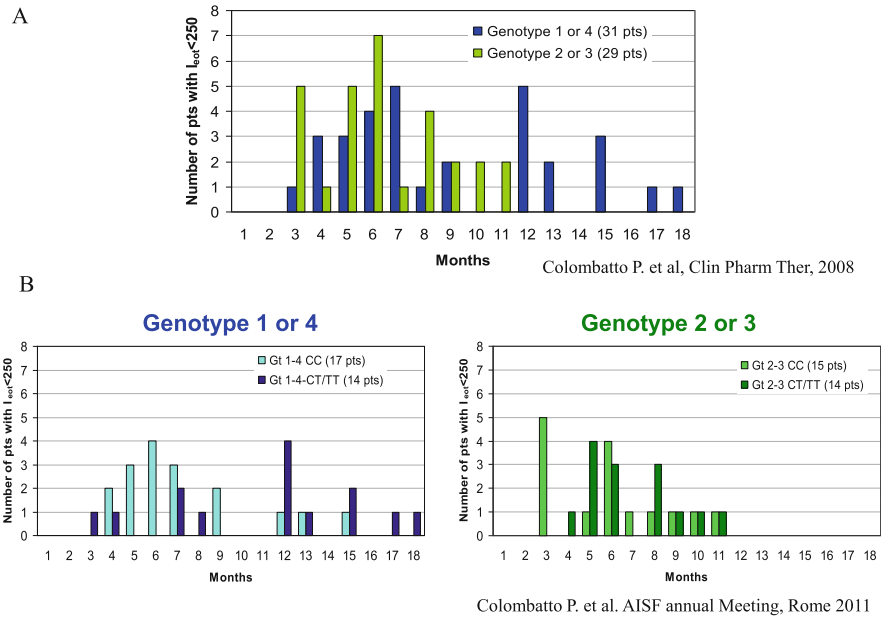

Fig. 3 Treatment duration according to model computed Ieot and IL28B status

Therefore, the predictive value of the Ieot index was subsequently assessed in a larger cohort of 78 patients (16 Non Responders, 12 Relapsers and 50 SVR, 48 G1/4 and 30 G2/3) treated according guideline protocols with peg-IFNs/RBV. This study confirmed that the Ieot numbers were lower in SVR (50) than in Relapser (Rel: 12) and in Non Responder (NR: 16) patients (median value: 31 vs 2190 vs 1,090,000; $p < 0.001$) (Colombatto et al. 2008). The achievement of Ieot <250 was associated with 93 % Positive Predictive Value of SVR and showed higher Diagnostic Accuracy (81 %), as compared to Rapid (74 %) and Early Virological Response (77 %), in prediction of SVR.

Using the time to achieve Ieot <250 thresholds to individually define treatment duration, 60 (3/16 NR, 8/12 Rel and 49/50 SVR) of the 78 patients analyzed would have reached this target in a period of 3–18 months (Fig. 3a). Interestingly, in genotype 1 and 4 patients treatment durations required to achieve I_{eot} <250 showed a bimodal profile: the first peak of frequency occurred at month 5, paralleling the one observed for genotype 2 and 3 patients; the second peak occurred at month 12, due to patients who behaved as slow responder. This predictions were in agreement with the high rates of SVR obtained with 24 weeks of peg-IFNs/RBV treatments in a subgroup of genotype 1 patients, thus considered eligible for shorter schedules (EASL Clinical Practice Guidelines 2011). The biological explanation for the existence of two distinct populations in terms of IFN sensitivity among genotype 1 patients, came from the discovery of the IL28B polymorphism (Ge et al. 2009). Noteworthy, the large majority (76 %) of the patients for whom

the multiphase model predicted short treatment durations (3–9 months) were indeed carriers of the favorable CC allele at the IL28B rs12979860 SNP (Fig. 3b).

In the meantime, other Authors (Snoeck et al. 2010) attempted early predictions of SVR extending the original Neumann's model to include infected and non infected hepatocytes proliferation (Dahari et al. 2007), and considering the effect of ribavirin rendering a fraction of newly produced virions non-infectious (Dixit et al. 2004). This non linear model was used to simultaneously describe individual long-term HCV kinetic profiles of 2100 CHC patients treated with peginterferon α-2a alone or in combination with ribavirin. At variance with the multiphasic model, their basic principle was the stochastic extinction of the virus when the viral load is reduced below one virus in the entire extracellular fluid in a patient. By this approach the authors were able to successfully simulate realistic viral pattern of HCV infection relapse. Their simulations showed that individual treatments could be optimized by increasing the direct antiviral effects with higher peg-IFN doses or DAAs combinations in patients with on treatment viral breakthrough and/or prolonging duration in those with relapse. However, the interplay between treatment duration, doses and schedules, remained to be elucidated in this model and never tested in clinical trials.

On the contrary, at the end of the last decade, the multiphase model developed in Pisa was tested in a prospective randomized trial (EudraCT 2006–002483–26) to compare the cost-effectiveness of Peg-IFN/RBV treatments tailored according to the model (MT) or defined by Guide Lines (GL) (Colombatto et al. 2010). Overall 117 consecutive patients, stratified by therapy exposure (74 naïve, 43 experienced), HCV genotype (66 G1–G4 and 51G2–G3) and Peg-IFN type (72 2a and 45 2b), were randomized 1:1 to receive MT or GL schedules (24 weeks if G2–G3 and 48 weeks if G1–G4). The week 12 stopping rule was applied in G1 to identify NR in the GL arm. In MT patients ALT and HCV-RNA were measured at day 0–2–4–7–14–21–28 to compute their infected cells number at the end of therapy. Treatment was stopped at week 4 in patients with Ieot >5000 (at the end of therapy defined by GL), otherwise tailored to achieve Ieot <250 (Fig. 4).

The multiphase model was able to fit ALT and HCV-RNA kinetics in 50 (84.7 %) patients of the MT arm, the remaining nine patients, in whom Ieot could not be computed, were treated according to GL schedules but not included in the comparative analysis. Eventually, 83 patients (36MT and 47 GL) completed treatment according to the study protocol, and their outcome among MT and GL, respectively, were: SVR 58 % vs 66 %, Rel 8 % vs 19 %, NR 34 % vs 15 %, ($p = 0.088$). The 8 % higher SVR rate observed in the GL arm parallels the 10 % higher prevalence of patients with the favorable IL28B CC alleles found in a post hoc study, suggesting that a selection bias might account for the slightly better outcome observed in GL treated patients. On the contrary, the lower relapse rate observed in the MT arm (8 % vs 19 %), counterbalanced by the higher rate of NR (34 % vs 15 %), supports the better accuracy of the model in defining patients at higher risk of relapse, whose treatment in the MT arm was withdrawn at week 4 and patients counted in the NR group.

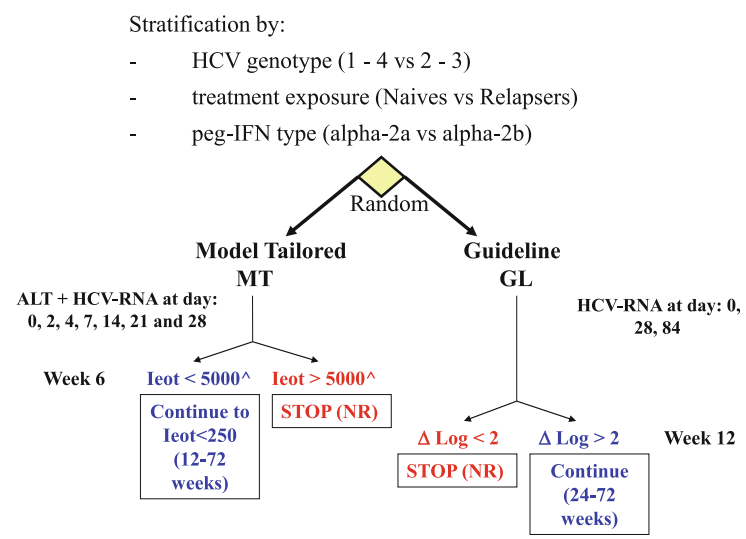

Fig. 4 Prospective randomized trial design comparing cost-efficacy of model tailored (*MT*) vs Guide Line (*GL*) treatments (EudraCT: 2006-002483-26)

Interestingly, despite the five additional ALT and HCV-RNA assays required for viral kinetics, cost-efficacy analysis in patients who completed treatment protocol, showed a lower cost for SVR in the MT (18.612 Euro) than in the GL (20.401 Euro) arm. As predicted by model simulations in the retrospective cohort (Fig. 3a), treatment duration in MT patients who achieved an SVR was significantly shorter in G2 than in G1–G3 patients (mean 21 vs 36 weeks, $p = 0.012$) and showed a wide range of distribution: 17–55 weeks in 8 G1, 17–28 weeks in 5 G2 and 19–56 weeks in 8 G3 patients.

The application in a prospective trial of the multiphase model to tailor individual treatment, provided the first clinical validation of a model computed index that appears able to predict when peg-IFN/RBV therapy can be safely stopped in responder patients. In fact, 21 of the 24 patients (87.5 %) at the time when they achieved Ieot <250 were SVR (Fig. 5). In addition, this study documented the wide individual variability in the time required to achieve an SVR by peg-IFN/RBV therapy, confirming the simulations made by the model in the retrospective cohort of patients (Fig. 3a).

In conclusions, these studies showed that, although the chance of SVR are greatly influenced by baseline predictors of response like HCV and IL28B genotypes, once peg-IFN/RBV therapy is started, tailoring treatment to Ieot <250 in responder patients is the most accurate tool for treatment personalization and allows for cost savings (Colombatto et al. 2012). In the era of the new DAAs, the Ieot index computed by the multiphasic model after 4 weeks of "lead-in" Peg-IFN/RBV therapy, qualifies as a decision tool that can help to optimize DAAs indication and tailor the overall treatment duration.

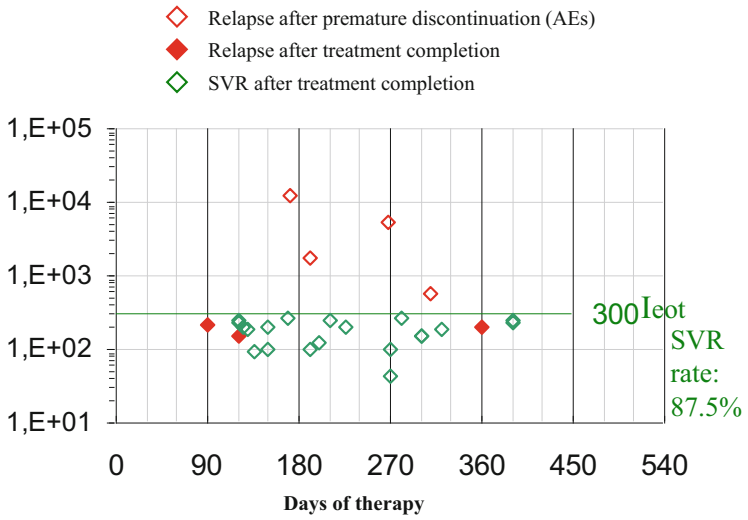

Fig. 5 Therapy outcome after individualized treatment duration by model computed Ieot

5 Analysis of Individual Differences in Response to Antivirals

The use of viral dynamics models proved useful to better investigate the reasons for the different antiviral responses observed in HCV patients. It has been reported above that IL28B polymorphisms influences SVR to pegIFN/RBV, although its biological mechanism did not appear immediately clear. Early viral kinetics analyzed according to Neumann's biphasic model pointed out that IL28B polymorphisms were independently associated with the first phase viral decline, irrespective of HCV genotype (Bochud et al. 2011). However, using the same modeling approach, Scott J et al. (2011) came at the opposite conclusion that its antiviral effect is more likely based on an immune-mediated mechanism, which increases the infected hepatocyte death rate. Besides the unclear effects on viral dynamics, the biological mechanisms by which IL28B polymorphisms impact on the innate immunity remain to be elucidated. Experimental studies showed that the IL28B polymorphism was associated with differential intrahepatic gene expression profiles, where the IFN sensitive IL28B variants correlate with lower levels of hepatic IFN Stimulated Genes expression (ISG) (Urban et al. 2012). More recently it has been reported (Prokunina-Olsson et al. 2013) that a dinucleotide variant upstream of IL28B in ss469415590 (TT or ΔG) is in strong linkage disequilibrium with rs12979860, and accounts for the production of a mutated form of IFN-λ3, designated as interferon-λ4 (IFNL4). Nevertheless, protein sequence variants of IFN-λ3 do not explain the differences in ISG expression or anti-HCV response by IL28B genotype (Urban et al. 2012).

The study of the IL28B polymorphisms together with the ALT and HCV-RNA kinetics (days: 0–2–4–7–14–21–28) by the multiphase model in the 78 patients of the retrospective study (Colombatto et al. 2008), showed that IL28B-CC patients had a higher block of viral replication, as compared to CT/TT (0.9264 vs 0.8142, $p = 0.006$) but also a faster second phase of HCV-RNA decline (0.2829 vs 0.1626 $days^{-1}$; $p < 0.001$) associated with a faster infected cell decline computed by ALT (0.1238 vs 0.0816 $days^{-1}$; $p = 0.002$). Interestingly, both model predicted end of treatment HCV Infected Cells (Ieot) and circulating Virions (Veot) were lower in CC than in CT/TT pts (Log-I_{eot}: 1.87 vs 3.27; $p = 0.001$ and Log-V_{eot}: −0.95 vs 1.69; $p < 0.001$); at the multivariate analysis only Log-V_{eot} remained significant ($p = 0.002$). Such results support the view that the IL28B favorable alleles are primarily associated with a stronger direct inhibition of viral replication/production by IFN. However, their impact is not limited to the first phase but contributes to the decay of the infected hepatocytes in the following phases, more likely through non-cytolytic rather than cytotoxic immune-mediated clearance (Colombatto et al. AISF Annual Meeting, Rome 2011).

Modeling the dynamics of HCV infection was also used to investigate the effects of humoral and cell-mediated immune responses that may be elicited by a therapeutic vaccine to improve viral clearance. In a recently published study (Colombatto et al. 2014), a prototype HCV-E1/E2 vaccine in combination with pegIFNα2a/RBV therapy was tested in a phase Ib pilot trial for experienced Genotype 1 HCV patients. Viral load decline during therapy was mainly influenced by the effectiveness of peg-IFN/RBV therapy and by the CTL mediated clearance of the infected cells. However, among patients with higher and homogeneous block of virus production (ε above the median value of 0.800), the combination of the Vaccine (V) with PegIFNα2a/RBV brought viral load to significantly lower levels at week 16 (Mann-Whitney *U* test, $P = 0.026$), just 4 weeks after the end of the first course of vaccine injections (Fig. 6). Interestingly, the neutralizing antibody titers (NOB) against HCV envelope proteins, whose baseline values were positively correlated with therapy outcome, decreased during therapy in patients treated with peg-IFN/RBV alone and increased significantly at week 12 ($P = 0.023$) in those who received also the vaccine. A significant increase of the anti-E1E2 HCV specific CD4 responses (20-fold of the mean LPA index at week 12; $P = 0.019$) was also observed in peg-IFN/RBV + V treated patients, with larger magnitude in those with SVR.

More recently, viral kinetics and immune responses against the HCV non-structural region (NS), were studied using the same model in a phase 1b trial where nine relapser patients received sequential injections of Adenovirus 6 (Ad6NSmut) and Modified Vaccinia Ankara orthopox (MVA-NSmut) vectors, engineered to carry the HCV NS coding sequence with a mutation (mut) inactivating the polymerase gene, before and 2 weeks after the beginning of PegIFN-RBV re-treatment (EudraCT Number: 2010-022700-49).

Altogether, different models and different experimental contexts may increase the understanding of innate and adaptive immune responses to HCV infection,

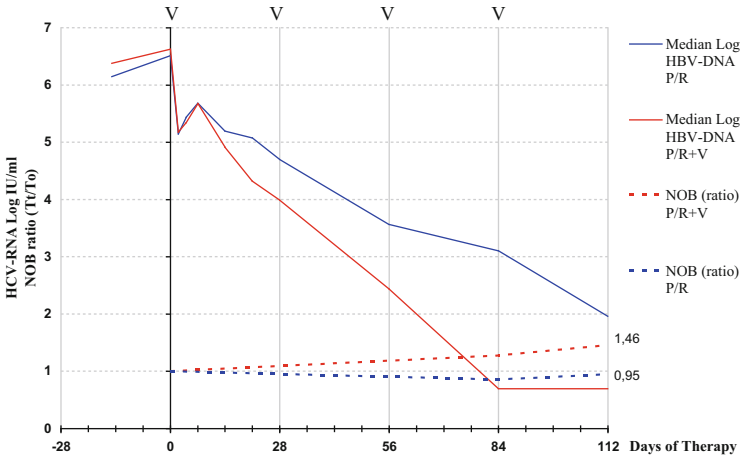

Fig. 6 HCV-RNA and NOB Titer (*T*) in patients with higher effectiveness of PegIFN/RBV (P/*R*) + Vaccine (*V*)

which maintain, despite direct antiviral agents that block viral replication more efficiently, a role not yet fully understood in the clearance of HCV.

6 Modeling Viral Dynamics in the Era of DAAs

Triple therapy (TT) with peg-IFN/RBV and boceprevir (BOC) or telaprevir (TVR) is more effective than dual therapy (DT) in genotype 1 HCV patients, however the increase of costs and side effects of TT supports the adoption of clinical approaches aimed to personalize treatment choices. Provided that patients with a rapid virological response (RVR: undetectable HCV-RNA at week 4) have more than 90 % chance of SVR after 24–48 weeks of peg-IFN/RBV (EASL Clinical Practice Guidelines 2011), naive patients with low fibrosis (Metavir F0-2) are advised to start treatment with DT to assess week 4 viral load decline before adding a DAA. The evidence of RVR, which qualifies the patient for DT, occurs only in a part of those who achieve SVR after 48 weeks of peg-IFN/RBV, as patients with weaker block of viral replication display slower kinetics of viral clearance. To identify no-RVR patients who have high chance of SVR with DT, the multiphase model (Colombatto et al. 2003, 2008, 2011) could be used. Accordingly, the prediction accuracy of Ieot, Veot and their product (V*Ieot), was assessed in a large cohort of 97 genotype 1 (20 % 1a) patients (77 % males, 63 % naïve, 31 % F3–F4) who completed protocol assigned peg-IFN/RBV therapy with <20 % reduction of the total dose (Colombatto et al. 2013). The thresholds that better discriminated SVR (45.4 %) from NO SVR (Relapser: 18.6 %, Partial Responder: 12.3 % and Non Responder: 23.7 %) patients were Ieot <250 (AUROC: 0.953), Veot <1.5 (AUROC: 0.964) and their combination I*Veot <800 (AUROC: 0.975).

Interestingly, the combination of the two most important model computed variables in their product I*Veot appears to have superior accuracy than the single ones, allowing for the identification of genotype 1 patients who achieve SVR without DAAs with the remarkable sensitivity and positive predictive value of 95.5 %. Thus, the use of a decisional algorithm based on the model computed I*Veot index has the potential to significantly improve cost/efficacy of the antiviral therapy in HCV patients.

One of the major limit for a model tailored treatment approach that hampers its widespread clinical application, is the need for frequent measures of ALT and HCV-RNA during the first 4 weeks of treatment. To overcome it, we recently developed a simplified algorithm to run the model with less experimental data (Colombatto et al. 2014, unpublished observations). This approach was applied to all the 150 consecutive genotype 1 patients (median age 49 years, 76 % males, 57 % naïve, 47 % F0–F2, 17 % infected with subtype 1a), who received, at the Hepatology Unit of the University Hospital of Pisa, peg-IFN/RBV standard (101) or model tailored (49) treatments. The multiphase model was applied using either the full data set (ALT and HCV-RNA at day 0–2–4–7–14–21–28) or a simplified data set (ALT at day 0–7–14–28 and HCV-RNA at day 0–28). Successful fittings were obtained with both methods in 135 (90 %) patients and the V*Ieot index computed with the different data sets showed a very high correlation (Fig. 7). The best prediction of SVR using the simplified data set was achieved using the threshold level of V*Ieot = 400 (Fig. 8). Such index was able to identify SVR patients with a sensitivity of 87.5 %, more than twice better than RVR (40.6 %), and

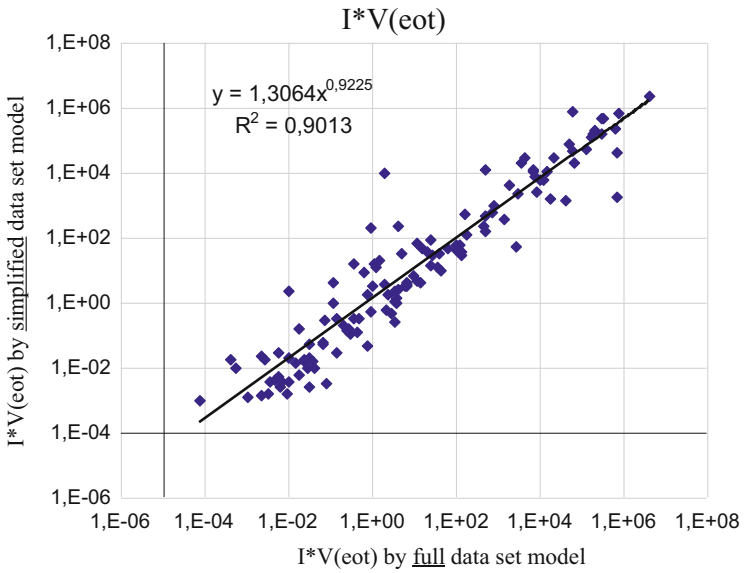

Fig. 7 Correlation between the I*Veot index computed by full or simplified data set model in 135 genotype 1 patients

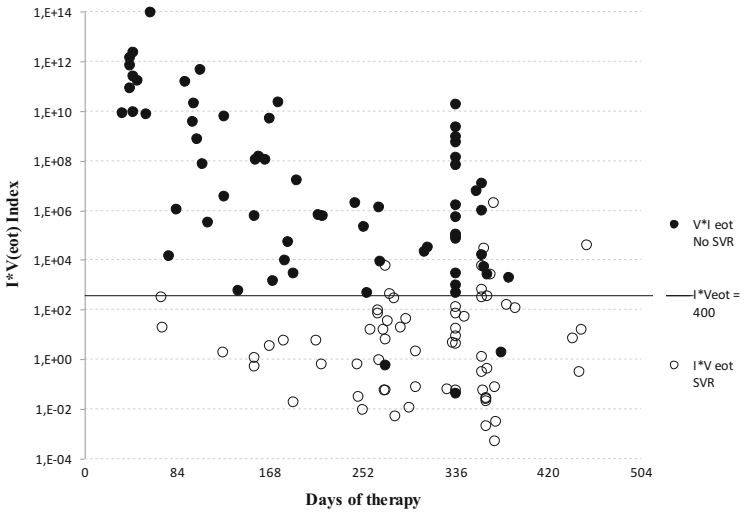

Fig. 8 Correlation between SVR and I*Veot index computed by simplified data set model in 135 genotype 1 patients

a positive predictive value of 94.9 %, still higher than that of RVR (92.9 %). Therefore, the adoption of a more sophisticated bio-mathematical predictive criterion, requiring only two additional measures of a cheap and universally available assay (ALT at week 1 and 2) plus those of standard management (ALT and HCV-RNA at baseline and at week 4), can identify patients who are effectively treated with DT, reserving high-cost BOC- and TVR-based TT only to those who really need it. The next task would be to develop a comprehensive model that could simulate virus-host interactions and predict the exact time of eradication during DAAs treatment.

It has already been shown that a biphasic viral decline is present also during Protease Inhibitors (PIs) administration, whether in monotherapy (Guedj and Perelson 2011) or in combination with peg-IFN/RBV. Surprisingly, PIs showed not only higher antiviral effectiveness ($\varepsilon > 0.999$) in the first phase, but also a second phase viral decline four to tenfold more rapid than with IFN-RBV therapy (Guedj and Perelson 2011). According to the standard model, the faster second phase viral decline during PI therapy must be due to a more rapid elimination of infected cells. However, ALT kinetics analyzed by the multiphase model in paradigmatic relapser patients subjected to re-treatment with TVR + peg-IFN/RBV at the Hepatology Unit of the University Hospital of Pisa (Fig. 9), showed only moderate increase (1.25-fold) of the infected cell clearance rate. Moreover, there is a direct correlation between the extent of viral load decline in the first phase and the slope of the second phase, either with TVR (Guedj and Perelson 2011) or pegIFN/RBV treatment (Colombatto et al. 2008, 2014). To explain this feature Guedj et al. (2010) proposed a model that hypothesizes the existence of a critical

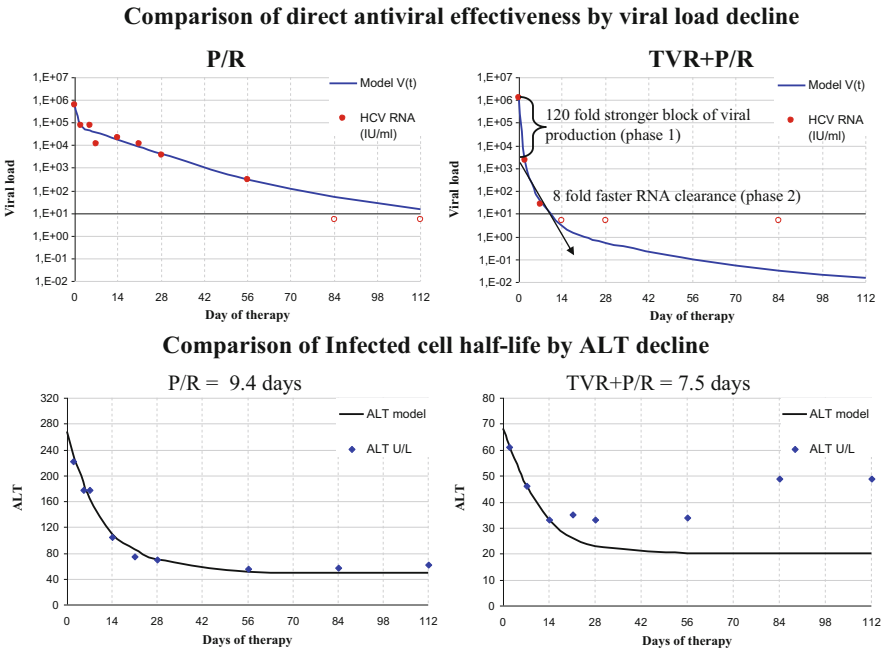

Fig. 9 Viral kinetics in a cirrhotic genotype 1b patient during P/R therapy (relpase) and TVR + P/R (SVR)

intracellular drug effectiveness that limits the possibility of degrading viral replicative intermediates inside an infected cell. If treatment is highly potent, intracellular viral RNAs are predicted to be progressively eliminated with the result that the second slope of viral decline is more rapid because reflects the combined effect of loss of infected cells and concomitant continuing decrease in viral production. This explanation is in good agreement with the two-step decline of the viral production coefficient (Fig. 2) already pointed out by the multiphase model (Colombatto et al. 2003) by comparing HCV-RNA and ALT Log decline in the second phase. In addition, in vitro experiments using the replicon system support the assumptions of these models, showing that viral replication not only initially declines by the factor (1- ε), but also continues to decline under PI or IFN treatment (Lin et al. 2004; Dahari et al. 2009). Hence, potent antiviral treatments with DAAs could cause the progressive elimination of intracellular replication complexes; assuming that this event occurs at least in a proportion of infected cells, this would explain the shorter time required to reach an SVR with these new drugs and its higher rate of success.

Although both biphasic and multiphase models brought valuable insights into the origin of viral declines observed during IFN-based treatment, extensions of these model are needed to deeper investigate the patterns of viral decline during DAA therapy. For instance, to simulate the effects of the NS5A inhibitor daclatsvir

(DCT) required a multiscale model that incorporates drug effects on the HCV intracellular lifecycle (Guedj et al. 2013). Patients treated with DCT showed a very rapid two Log decrease in serum HCV RNA levels within 6 h of administration (Chatterjee et al. 2012), in the absence of any known enzymatic functions of NS5A, making it difficult to understand its mode of action. Thus, the model developed by Guedj et al. (2013) simulated such very early biphasic HCV-RNA decline assuming that DCT efficiently blocks two distinct stages of the viral lifecycle, namely viral RNA synthesis and virion assembly/secretion with mean effectiveness of 99 % and 99.8 %, respectively. If this model holds true, the first phase viral decline may reflect two phases, firstly the decay due to virion clearance, then the decay due to loss of intracellular viral RNA, and, finally, serum HCV half-life would be much shorter than previously estimated: 45 min, rather than 2–3 h.

7 Viral Dynamics and Pharmaaeconomic Models

The major limit for large scale treatment of HCV patients with the new direct antivirals is the high cost of these drugs. Cost-efficacy of the antiviral therapy in resource constraint settings could take advantage from the bio-mathematical models that help to better manage treatment at the individual patient level, especially in those with low stage fibrosis (Metavir F0-2) who have no urgency to be treated with DAAs and higher rates of response to peg-IFN/RBV therapy. For this scenario, we simulated the simplified application of the Pisa's multiphase model described above in the context of a cost-effectiveness analysis. As previously shown, viral (V) and infected cell (I) loads at the end of therapy (eot) were computed in 135 genotype-1 CHC-patients treated with DT using a simplified data set containing baseline plus week 4 HCV-RNA and week 1–2–4 ALT (Fig. 8). The correlation of the V*Ieot index with SVR was assessed in the subgroup of 64 patients with fibrosis stage F0-2, showing that the index I*Veot <400 identified SVR patients with 93.8 % PPV and 88.2 % sensitivity. A decision-analytic economic model was developed (Iannazzo et al. 2015) to compare the long-term costs and outcomes achieved by the Model-Guided strategy (MG) rather than the RVR criterion (Guideline-Guided strategy: GG) to identify the patients suitable to continue DT rather than adding BOC or TVR. The analysis, carried out for F0–F2 naïve patients, provided outcomes with MG- and GG-strategy of 19.1–19.4 and 18.9–19.3 quality-adjusted-life-years (QALY). Total per-patient lifetime costs were €25,200–26,000 with MG-strategy and €28,800–29,900 with GG-strategy. When comparing MG- with GG-strategy the former resulted more effective and less costly, being defined as dominant according to pharmacoeconomic definition (Fig. 10).

In conclusion, the cost-effectiveness analysis of the adoption of a model guided strategy in clinical practice demonstrated that this strategy would provide benefits both in terms of clinical outcomes and cost containment allowing a more accurate

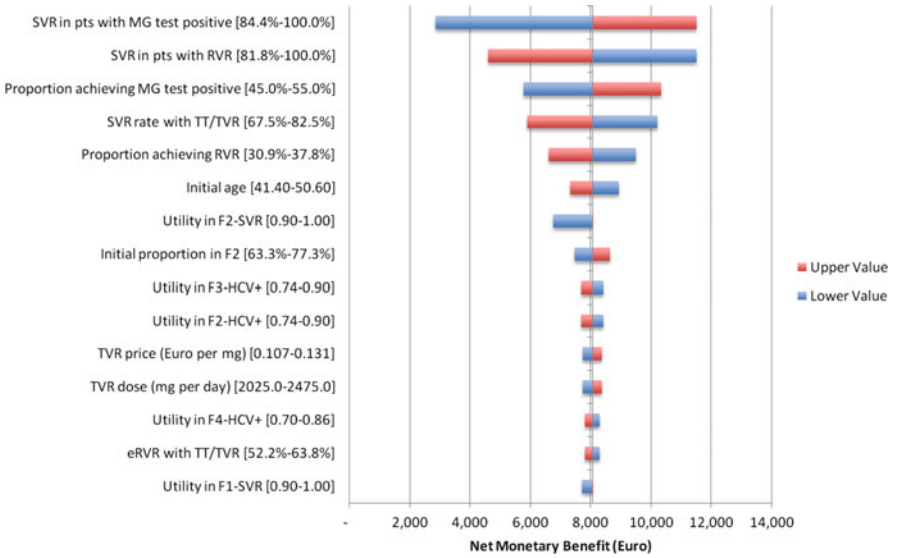

Fig. 10 One-way sensitivity analysis on the MG-TVR versus GG-TVR comparison. In the base case analysis, MG-TVR was dominant. The net monetary benefit, calculated assuming a willingness-to-pay of €30,000/QALY, was €8502

identification of patients who can be effectively treated with DT and reserving new high-cost DAA only to those who really need them.

8 Conclusions

The relevant amount of biological, clinical and epidemiological evidences accumulated in the past 25 years from the discovery of HCV combined with the recent availability of long waited potent antiviral agents, strongly support the adoption in clinical practice of a systems medicine approach to individualize antiviral therapy in HCV patients, adapted to their whole clinical and socio-economical context. The deterministic bio-mathematical modeling of both virus and infected cell dynamics, substituting the standard probabilistic criterion used to compare in RCT the efficacy of different compounds or schedules, can improve the cost-effectiveness of anti-HCV therapy in the individual patient in many different scenarios.

In the future, we expect to see even more sophisticated models that take into consideration both intracellular and extracellular HCV dynamics. Moreover, a better understanding of the liver cell turnover and of their biochemical functions according to the different stages of the disease, would probably help to improve treatment outcomes in patients with more advanced liver disease, who are still less prone to benefit from the new potent antiviral therapies. Models of the future will

hopefully provide insights on how HCV spreads in the liver spatially within the hepatocytes, and the interplay between these phenomena and the effectors of the immune response, such as neutralizing antibodies, or the host factors required to vehicle viral particles through the blood stream.

Finally, wherever these models will succeed in capturing accurately the effects of the new generation DAAs, they will be able to identify individual characteristics that allow for selecting the best therapeutic options and optimize treatment duration at the single patient level. If this will be the case, the personalized therapeutic approach would provide benefits both in terms of clinical outcomes and also cost containment for the whole community.

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Part IV
Prevention and Control of HCV Infection

Prophylactic Vaccines for the Hepatitis C Virus

Andrea L. Cox

Abstract With the advent of oral, interferon-sparing HCV regimens, it has become much easier to safely and effectively treat HCV infection. However, HCV eradication is not likely to be achieved with treatment alone. Identification of those with HCV infection is challenging, therapies are too costly for countries with the highest incidence, reinfection can occur following treatment, transmission of drug-resistant HCV is possible, and treatment does not fully reverse severe liver damage even when cure is achieved. There is a rising epidemic of acute HCV infection in adolescents and young adults that gives new urgency to prophylactic vaccine development efforts. However, numerous challenges for vaccine development remain, including limited populations in which candidate vaccines can be tested, the enormous sequence diversity of HCV, and incomplete understanding of what mediates protective immunity. The study of immune responses to HCV has provided important insight into protective immunity. However, more research is needed to identify clear correlates of immunity to assess before candidate vaccines are tested in the limited at-risk populations available. Multiple candidate HCV vaccines that induce robust immune responses have been tested in rodents. Fewer have been tested in chimpanzees, with minimal ability to generate sterilizing immunity and variable capacity to prevent persistent infection. A vaccine that was highly immunogenic in healthy volunteers is now being tested in at-risk subjects. The combination of effective antiviral agents to treat HCV infection and a vaccine to prevent persistent infection provide our best chance of substantially reducing morbidity and mortality from HCV on a global scale.

Keywords Hepatitis C • HCV • Viral hepatitis • Vaccines • Prophylactic vaccination • Viral vaccines • HCV prevention • HCV eradication

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1 Do We Need a Vaccine in the New Era of HCV Therapy?

Effective therapies exist for a wide variety of pathogens. For example, safe, effective, and inexpensive treatment for syphilis has been available since the widespread use of penicillin in the 1940s. Despite that, syphilis remains a significant problem in most parts of the world, including high as well as low and middle income countries. Historically, vaccination has been a central part of most effective infectious disease control programs. The relatively low cost and high efficacy rates have made vaccines the foundation of modern public health programs. Two pathogens have been eradicated globally as agents of disease; small pox in humans and rinderpest in cattle. The eradication of both pathogens occurred following global vaccination campaigns. Even without complete control of infection, the impact in other infections of vaccination has been great. For example, for hepatitis B virus (HBV) infection, a universal vaccine campaign has been highly successful in reducing both HBV infection rates and the rates of HBV complications such as hepatocellular carcinoma (Chang et al. 1997).

Unfortunately, substantial control or eradication of HCV with treatment alone is even less likely than with other pathogens given several aspects of HCV disease (Table 1). First, both acute and chronic infection are largely asymptomatic. Infected individuals often don't know they are infected, making identification of those as risk of transmitting the infection to others difficult. Second, comprehensive screening programs are not in place in some of the most highly endemic regions of the world. As a result of the asymptomatic nature of infection and the lack of widespread testing, it is estimated that less than 5 % of the world's HCV infected population is aware they are infected (Gravitz 2011). Even in developed countries such as the United States, it is estimated that half of all infected people are unaware they have the infection (Holmberg et al. 2013). As long as those who are infected remain unaware, they will not receive treatment. Even if everyone with HCV infection were identified, treatment to eradicate the disease would be a challenging strategy.

Interferon-based regimens were the mainstay of HCV therapy for decades. Therapy for HCV has improved dramatically with the advent of potent directly acting viral agents (DAAs). These agents decrease treatment duration, increase treatment response rates, and have far fewer side effects than interferon-based regimens. Although it has become much easier to safely and effectively treat HCV infection, as with other infections, HCV eradication is not likely to be achieved with treatment alone. DAAs have a number of limitations that make their use less appealing than vaccination as a means to eradicate HCV infection. First, the cost of the DAA's is currently prohibitive for most of the nations with the highest prevalence of infection. Even in high income countries, the prospect of treating the millions of infected individuals is daunting. HCV transmission occurs through poor blood product and needle hygiene in many parts of the world. Nations lacking the financial resources and health care infrastructure to assure safety of the blood supply or to use disposable medical supplies as needed are unlikely to be able

Table 1 Challenges to HCV eradication with treatment alone

HCV infection is often asymptotic making identification difficult
Awareness of HCV infection status is limited world-wide
Drugs to treat HCV remain expensive
Reinfection is possible following HCV cure
Transmission of drug-resistant HCV variants occurs
Treatment does not fully reverse liver damage

to afford DAAs for treatment. Even with DAAs provided to developing nations at dramatically reduced costs, vaccination will likely be a less expensive path to reducing the prevalence of chronic HCV and the combination of DAAs and vaccination may prove the most cost effective strategy for global control. Second, the DAAs do not provide protection against reinfection. Those who remain at risk of infection may be reinfected following a course of successful therapy. Health care workers, people who inject drugs (PWID), men who have sex with men, and other groups documented to be at increased risk of HCV infection compared to the general population are at ongoing risk of infection as long as they engage in the activities that place them at risk. Reinfection following successful therapy with DAAs has been documented since use of these agents began, starting from their first use in clinical trials (Sulkowski et al. 2014). If a single round of therapy for those infected exceeds the resources of a nation, obviously multiple rounds of therapy will not be an affordable option. In contrast, vaccination after successful therapy might be a highly cost effective means to prevent reinfection. Third, the DAAs can select for resistance variants that may limit HCV treatment efficacy using DAAs in the same class. Transmission of a resistant HCV variant has already been documented between a person who failed DAA therapy and his sexual partner, who previously had been treated successfully for HCV with a DAA regimen (Franco et al. 2014). The impact of variants of HCV that render DAAs ineffective is unclear because the mutations don't always persist, but transmission of resistant variants is almost certainly more likely to occur following more widespread use of DAA therapy. Lastly, chronic HCV can result in severe liver disease and is the leading cause of liver cancer and end-stage liver disease requiring liver transplantation in many countries, including most of Europe, the United States and Japan. While there are not sufficient data to know how much liver damage will be reversed by HCV cure using DAAs, HCV cure using interferon and early data with DAAs demonstrate that cure does not completely reverse severe liver damage. Although liver disease may stabilize or decrease following cure, those with cirrhosis can further progress and generally continue to have significant disease following successful therapy (Veldt et al. 2007). Indeed, those with advanced fibrosis or cirrhosis require ongoing monitoring for liver failure and hepatocellular carcinoma following successful therapy. Because therapy cannot fully reverse the damage done by HCV, and that damage can be very severe, HCV prevention is more likely than treatment to maximally reduce HCV morbidity. Given these characteristics of HCV disease and limitations of available therapies, a vaccine will likely remain

important in the quest for HCV control and in eradication efforts. There have been multiple studies addressing the potential impact of a vaccine that support this concept.

2 Predicted Impact and Practical Aspects in Testing of an HCV Vaccine

The impact of a prophylactic HCV vaccine has been modeled, varying delivery strategy and levels of vaccine efficacy (Hahn et al. 2009). Using these models, high vaccination rates of high-risk seronegative PWID using a vaccine with 50–80 % efficacy had significant impact. A best-case scenario with 80 % vaccine efficacy and 1 % of high-risk PWID vaccinated per month was predicted to reduce HCV incidence from 13.5 to 3.2 % over 10 years. However, a reduction in incidence may not be necessary to decrease HCV morbidity and mortality. There are essentially no long term sequelae of acute HCV infection that resolves spontaneously. Thus, vaccine strategies that decrease rates of chronic infection rather than incidence may be highly effective in preventing disease from HCV, if not infection altogether.

The feasibility of testing vaccines that could reduce rates of chronic HCV infection rather than HCV incidence has been studied. Identification of populations with sufficiently high HCV infection rates to demonstrate vaccine efficacy will be important. Power calculations using data from PWID in Australia suggest a chronic HCV infection rate of at least 12/100 person years (py) (versus primary HCV infection rate 16/100 py) will be required for trials of even highly effective candidate vaccines to prevent chronic infection (White et al. 2014). Identifiable populations with infection rates that high are essentially limited to PWID and effective engagement and recruitment of large numbers of exposed but uninfected PWID will be required for successful vaccine trials. The incidence of HCV infection is 5–25 % per year in PWID, demonstrating both their suitability as a test population for an HCV vaccine trial and the continued need for HCV infection prevention in this population (Cox and Thomas 2013). Infection rates remain high in other settings, such as in people undergoing medical interventions in high-endemicity countries. However, prediction of risk far enough in advance to administer a vaccine and induce immune responses prior to the exposure is very difficult in those settings. That candidate HCV vaccine trials will likely be limited to PWID is one of the challenges in HCV vaccine development (Table 2). These trials will require overcoming the substantial and distinct challenges in enrolling and retaining PWID in trials. Developing appropriate protocols to assess vaccine safety, immunogenicity, and efficacy will also be a challenge given the increased risk of death due to trauma and drug overdose, high rates of incarceration, frequent changes in contact information, and social instability in this population. However, multiple cohorts have proven success in longitudinal monitoring of injection drug users for

Table 2 Challenges in HCV vaccine design and testing

Limited populations in which candidate vaccines can be tested
Correlates of protective immunity to HCV are not fully understood
HCV is a highly diverse virus, necessitating induction of broadly cross reactive immunity

incident HCV infection and that expertise will be valuable in future trials of candidate vaccines (Cox et al. 2009; Grebely et al. 2013; Edlin et al. 2009).

3 Who Could Benefit from a Preventative HCV Vaccine?

Health care workers, men who have sex with men, those living in countries with high rates of endemicity, and PWID are all at increased risk of acquiring HCV infection relative to the general population. Iatrogenic exposure is not a major mode of acquisition in developed regions of the world. Risk is greatest where the prevalence of HCV infection is high. Targeting some at-risk populations would be relatively easy, such as providing vaccination to nursing or medical students prior to substantial patient, and therefore blood, contact. However, identification of those in other risk groups and vaccinating them fully prior to exposure will be challenging. It will be particularly challenging to identify and vaccinate PWID who become infected shortly after initiation of injection drug use. Despite years of prevention effort, acute HCV infection rates are rising. The transition from recreational opiate use to injection drug use has fueled a rise in HCV infection among adolescents and young adults over the past decade, particularly in suburban and rural Caucasian adolescents (Klevens et al. 2012; Rolls et al. 2013; McNamara et al. 2014). A study examining HCV infection from 2006 to 2012 in 34 reporting States in the United States showed that 88 % of the States surveyed observed higher incidence in 2012 than in 2006 (Suryaprasad et al. 2014). In that time, the reported incidence of acute HCV increased significantly in young persons with a 13 % annual increase in non-urban counties and 5 % annual increase in urban counties. Eighty-five percent of the newly reported HCV-infected young persons were Caucasian. Modeling studies of urban IDU cohorts suggest that aggressive HCV screening and DAA treatment of active injectors could substantially reduce HCV prevalence and transmission (Martin et al. 2013; Rolls et al. 2013). However, the increasing proportion of HCV infection through injection drug use in rural parts of the United States, where fewer public health resources exist for intensive case finding and treatment, make those interventions less likely to be effective (Klevens et al. 2012). Even when such resources exist, young and nonurban injectors are some of the most difficult groups to engage in prevention activities(Hepatitis C Virus Infection in Young Persons Who Inject Drugs Office of HIV/AIDS and Infectious Disease Policy 2013). There is very little culturally appropriate drug treatment programming that appeals to young PWID, especially in the suburban and

rural areas most affected. HCV treatment options may be limited in these areas as well, due in part to small numbers of providers with experience treating HCV infection. When available, interest in HCV treatment among young PWID is often limited given that they generally don't feel ill as a result of infection and medical sequelae often occur decades later. Given the limited access to those most highly at risk before infection occurs combined with continued iatrogenic spread of HCV infection, universal vaccination rather than vaccinating only those at risk may be required to have the largest impact. In addition to the implementation issues surrounding efforts to reduce infection rates through vaccination, there are a number of scientific questions that remain in HCV vaccine development.

4 Is There Evidence of Protective Immunity Against HCV?

Although spontaneous clearance of HCV infection occurs in about 25 % of acutely infected individuals, whether it is possible to develop protective immunity against HCV remains controversial (Micallef et al. 2006). The existence of protective immunity against HCV infection has been questioned, primarily because chimpanzees and humans who spontaneously control an initial HCV infection can develop recurrent HCV viremia following additional HCV exposures (Farci et al. 1992; Prince 1994; Bassett et al. 2001; Major et al. 2002; Nascimbeni et al. 2003; Shoukry et al. 2003; Mehta et al. 2002; Osburn et al. 2010; Prince et al. 2005; Micallef et al. 2007). Reinfection following spontaneous HCV clearance in humans has been observed in PWID and men who have sex with men, suggesting that it does not generate sterilizing immunity in humans whether introduced via percutaneous or mucosal routes (Micallef et al. 2007; Osburn et al. 2010; van de Laar et al. 2009). However, following detectable viremia, clearance of multiple infections with homologous and heterologous virus has been observed in chimpanzees and humans (Bassett et al. 2001; Prince et al. 2005; Osburn et al. 2010; Page et al. 2009; Micallef et al. 2007) Clearance occurs far more often in reinfection, with people controlling secondary HCV infections about 80 % of the time; nearly the reverse of clearance versus persistence rates in primary infection (Osburn et al. 2010). This could be the result of fixed genetic traits that make individuals more likely to control infection every time they are exposed, generation of an adaptive immune response that controls subsequent infections, or both. Fixed genetic traits would allow viral control, but would not be predicted to substantially change the nature of the response to HCV in subsequent exposures since fixed genetic traits would not differ between first and subsequent exposures to HCV. Supporting the hypothesis that adaptive immunity is induced, Lanford and colleagues reported that clearance of both homologous and heterologous viral rechallenge was associated with decreased duration and magnitude of viremia in chimpanzees compared to primary infection (Lanford et al. 2004). Similarly, HCV reinfection in humans is characterized by a reduced peak and duration of viremia compared to initial infection of the same person (Osburn et al. 2010). Reinfection was associated with broadened

cellular immune responses compared to primary infection and the presence of broadly cross-reactive neutralizing antibodies (nAbs). More rapid and effective control of viral replication with subsequent exposures compared to initial exposure suggests that adaptive immunity is induced and, while not sterilizing, protects against chronic disease. These studies raise the question of what adaptive immune responses are required for protection against persistent HCV infection.

5 What Are the Correlates of Protective Immunity to HCV?

Through decades of research, the correlates of protective immunity to HCV are partially understood. There is substantial data from human and chimpanzee studies that HCV-specific $CD4^+$ helper and $CD8^+$ cytotoxic T cells are crucial in control of primary and secondary HCV infections. In acute primary infection, T cell responses are generated against HCV antigens in those who progress to chronic infection as well as in those who spontaneously control the infection. However, differences in the T cell response based on outcome have been identified. The presence of a vigorous and multispecific proliferative $CD4^+$ T cell response against HCV proteins is a strong immunological correlate of spontaneous control of acute HCV infection (Schulze zur Wiesch et al. 2012; Diepolder et al. 1996; Chang et al. 2001; Rehermann 2009) More recently, Schulze zur Wiesch and colleagues demonstrated that broadly directed HCV-specific $CD4^+$ T cells are universally detectable during early stages of infection, regardless of outcome (Schulze zur Wiesch et al. 2012). However, $CD4^+$ T cells show early functional defects in chronically evolving HCV infection and the $CD4^+$ T cell response rapidly collapses to undetectable with progression to chronicity. This absence of $CD4^+$ T cell help is thought to impair $CD8^+$ T cell immunity, resulting in functional exhaustion and selection of HCV variants that carry escape mutations in class I epitopes (Rehermann 2009). Consistent with this, selection of HCV variants with mutations that impair $CD8^+$ T cell recognition has been demonstrated in acutely infected PWID who progress to chronic infection while variants carrying escape mutations were not detected in PWID who spontaneously controlled infection (Cox et al. 2005).

Chimpanzees studies have also identified phenotypic differences that might explain maintenance of T-cell responses in spontaneous control of HCV. $CD8^+$ T cells from animals that controlled HCV replication were more likely during the early phase of infection to express CD127, the interleukin-7 receptor α subunit that is a marker of memory precursors and is expressed at low levels on effector and exhausted $CD8^+$ T cells (Shin et al. 2013). In another study, $CD8^+$ T cells from animals that control infection were more likely to have a phenotype associated with proliferation (Ki67 positive and Bcl-2 low) and to be activated (HLA-DR positive) (Zubkova et al. 2014). Some human studies have linked $CD8^+$ T cell surface expression of the inhibitory molecules programmed cell death-1 (PD-1) or T cell

immunoglobulin and mucin domain-containing molecule 3 (Tim-3) with viral persistence, but others don't support differences in expression related to infection outcome (McMahan et al. 2010; Rutebemberwa et al. 2008; Kasprowicz et al. 2008).

As discussed, HCV-specific T-cell memory generated after resolution of primary infection is thought to contribute to rapid control of subsequent infections in humans and in chimpanzees. Antibody-mediated depletion of $CD4^+$ T cells before reinfection of two immune chimpanzees resulted in persistence of HCV despite functional intra-hepatic memory $CD8^+$ T cell responses (Grakoui et al. 2003). Antibody-mediated depletion of $CD8^+$ T cells immediately before the third infection of two chimpanzees that had previously cleared infection resulted in prolonged viremia that was controlled only upon detection of $CD8^+$ T cells in the liver (Shoukry et al. 2003). The T cell response in PWID was analyzed in recurrent HCV infection as well and responses compared between five PWID who controlled the second infection and four who failed to control the second infection (Abdel-Hakeem et al. 2014). Protection against viral persistence was associated with broadening of the T-cell response and the expansion of effector memory T cells at the peak of the response. While all subjects had $CD8^+$ T cells with high expression of CD127 at the time of reinfection, the peak of infection was characterized in those who controlled infection by expansion of $CD127^{neg}$, $PD1^{lo}$ effector memory T cells. The four individuals who failed to clear their subsequent infection had limited expansion of HCV-specific $CD4^+$ and $CD8^+$ memory T cells that expressed variable levels of the exhaustion marker PD1 on HCV-specific $CD8^+$ T cells. Dominant epitope regions of HCV strains isolated from patients with persistent reinfection had sequence variations that were not recognized by the pre-existing memory T cells, consistent with escape seen in association with persistence of primary infection. These studies demonstrate that memory $CD4^+$ and $CD8^+$ T cells play a crucial role in protection of immune individuals who are re-exposed to HCV.

Although the importance of nAbs in spontaneous resolution of acute hepatitis C is less well understood than antiviral cellular immune responses, there has been significant progress in understanding the humoral response to HCV as well. The domains of the HCV envelope glycoproteins targeted by nAbs are highly variable and antibody responses can be broadly neutralizing or have very limited neutralizing capacity. Human data on the importance of HCV-specific antibodies in controlling HCV infection provide conflicting results. HCV infections resolve in some people with primary hypogammaglobulinemia, arguing against antibodies being required to mediate protection (Semmo et al. 2006). In a randomized, controlled, clinical trial, administration of human hepatitis C antibody enriched immune globulin product (HCIG) to patients undergoing liver transplantation for chronic hepatitis C did not prevent HCV recurrence after liver transplantation (Davis et al. 2005). This suggests that antibodies are also not sufficient to prevent infection, at least as administered in the setting of liver transplantation. However, a role for nAb in infection control became clearer with the development of assays to assess hepatocyte entry using pseudoparticles that display HCV envelope glycoproteins on

a vesicular stomatitis virus (VSV) or retroviral capsid (HCVpp). Two common source outbreaks furthered understanding of nAb responses since both allowed generation of envelope glycoprotein HCVpp derived from the same virus transmitted to multiple patients. In the first, nAb responses to HCVpp made from the infection donor's virus were assessed in 17 recipient patients infected via hemodialysis (Lavillette et al. 2005). Patients with the highest serum Ab titers against the transmitted virus had the lowest levels of viremia. In a second, Pestka and colleagues studied a cohort of women accidentally exposed to the same HCV strain in another single-source outbreak and found that viral clearance was associated with rapid induction of nAbs in the early phase of infection (Pestka et al. 2007). In contrast, women who progressed to chronic HCV infection had low-titer or no nAbs in the early phase of infection. Persistence occurred despite development of cross-neutralizing antibodies in the late phase of infection. While generation of nAb late in infection is generally associated with persistence, there is a report of spontaneous control of HCV in a patient after more than a year of infection following development of nAb (Raghuraman et al. 2012). In addition to development of nAbs early in infection, the development of broadly cross reactive nAbs has been associated with primary infection control (Osburn et al. 2014). Thus, the presence of broadly neutralizing antibodies early in infection, as would be expected following successful vaccination, may aid in control of HCV infection.

Although the early development of broadly nAbs directed against envelope protein antigens can play a role in clearance of infection and possibly in reinfection, specific envelope sequences or conformations that preferentially drive early, broadly neutralizing Ab production and sterilizing immunity remain unknown (Dowd et al. 2009; Osburn et al. 2010; Pestka et al. 2007). One study demonstrated that the breadth of nAb responses generated in the acute phase was not dependent on infecting genotype since some but not all subjects infected with genotype 1, 2, or 3 HCV were capable of mounting broad nAb responses against a genotype 1 HCVpp library (Osburn et al. 2014). This suggests that the HCV envelope sequence selected for inclusion in a vaccine may not be have to match the genotype of virus to which the person is subsequently exposed to provide protection, but what induces broadly neutralizing antibodies remains unknown. Given that the majority of those infected fail to mount early, broadly neutralizing antibody responses to HCV infection, more understanding may be required to design a vaccine that induces antibodies that facilitate HCV control.

HCV infection induces innate immune responses in the human host, including Type I interferon and inflammasome cytokine production (Chattergoon et al. 2011, 2014; Negash et al. 2013; Horner and Gale 2013; Burdette et al. 2012; Shrivastava et al. 2013). However, HCV has multiple mechanisms to regulate and evade innate immunity, including suppressing Type I IFN production in infected hepatocytes (Horner and Gale 2013). Whether the innate immune response differs between those who control infection and those in whom HCV persists remains unclear, as does whether different vaccine strategies induce innate responses that modulate downstream adaptive responses critical in preventing infection upon HCV exposure.

Given the absence of clear correlates of protective immunity, there is no way to reliably assess in vitro, in animal models, on in healthy volunteers whether immune responses induced through vaccination will provide protective immunity to those at risk. This requires that candidate vaccines be tested in at-risk individuals, which will be very challenging since those populations are rare. More rapid screening of potential vaccine candidates would be facilitated by an understanding of protective immune correlates so that only those vaccines with significant potential to be protective advance to clinical trials in at-risk subjects. Thus, additional research on effective immune control of HCV, including those who spontaneous control infection multiple times, remains important in the quest for an HCV vaccine.

6 HCV Sequence Diversity- An Additional Challenge to HCV Vaccine Design

There are at least seven HCV genotypes described, varying in nucleotide sequence by approximately 30 % (Smith et al. 2014). In addition to diverse genotypes, HCV circulates within and between individuals as a quasispecies of genetically distinct yet related sequences (Martell et al. 1992). The highly error-prone HCV NS5B polymerase yields a mutational rate of 10^{-5}–10^{-4} nucleotides per replication cycle, which , combined with the large number (10^{12}) of virions produced daily, contributes to quasispecies diversity. Quasispecies diversity is also driven by immune selection and constrained by the need to maintain replicative fitness. Although mutations are generated randomly, both antibody and T cell responses to select viral variants that thrive in the presence of immune pressure (von Hahn et al. 2007; Dowd et al. 2009; Cox et al. 2005; Weiner et al. 1992; Timm et al. 2004). This creates a swarm of viruses of remarkable diversity that present an immense challenge for vaccine development. Vaccine strategies meant to overcome this viral diversity must generate a broad immune response, capable of responding to abundant variations.

At least three methods for selecting HCV vaccine antigens have been proposed to maximize induction of robust and cross-protective T cell responses; (1) selection of specific known T cell epitopes (2) use of a single circulating HCV variant and (3) use of computational methods to minimize the degree of sequence dissimilarity between a vaccine strain and contemporary circulating viruses. One study demonstrated enhanced potential for computer-generated sequences to elicit cross-reactive T cell responses versus the other two methods and supports the use of a synthetically generated sequence to elicit robust $CD8^+$ T cell responses, but this has not yet been tested as a vaccine strategy in HCV (Burke et al. 2012).

7 HCV Vaccine Trials

Generation of live attenuated and inactivated whole virus vaccines has been effective against other viruses, but neither strategy is feasible for generating HCV vaccines. The inability to culture HCV until relatively recently and ongoing limitations of HCV culture systems makes production of a live-attenuated or inactivated whole HCV vaccine technically very challenging. The potential risk of causing HCV infection and disease with a live attenuated vaccine limits the utility of that strategy as well. Several strategies to induce immune responses to HCV have been tested in rodents, with a much smaller number progressing to nonhuman primate trials (Fig. 1). Most candidate vaccines have produced humoral and cell mediated immune responses in the animals in which they were tested. A much smaller subset of vaccine candidates have moved to trials in healthy human volunteers not at risk for infection (Fig. 1). As of 2016, only one candidate vaccine has moved to trials in an at-risk human population. Thus, this is the first trial with the potential to evaluate a candidate vaccine's ability to prevent chronic HCV infection in humans. However, several trials have demonstrated some encouraging results in the chimpanzee model of HCV using a variety of strategies.

7.1 Trials of Vaccines Designed to Elicit Neutralizing Antibody Responses

Most vaccines, including those against HAV and HBV infection, protect through induction of an antibody that mimics the protective antibody responses of natural infection. Targeting the HCV envelope glycoproteins to induce neutralizing antibodies was the initial strategy employed against HCV infection as well. Vaccine studies with recombinant HCV envelope (E1E2) glycoproteins have been completed in chimpanzees with the goal of inducing neutralizing antibody that prevents infection. Chiron (now Novartis) created a subunit vaccine based on E1 and E2 glycoproteins derived from a single genotype 1a HCV strain in oil-water emulsion

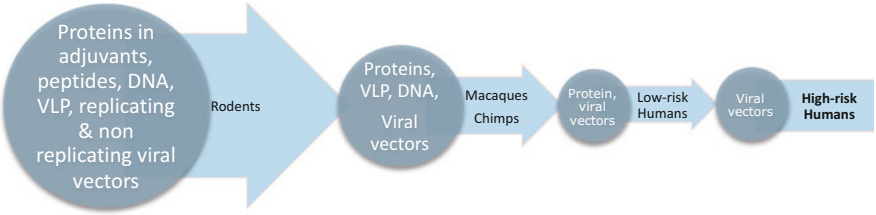

Fig. 1 Efforts to develop a prophylactic HCV vaccine with high risk populations as the target. Despite testing of several strategies in rodents, relatively few candidate vaccines have been tested in non-human primates and even fewer have progressed to testing in healthy volunteers. Only one candidate vaccine has been tested in at-risk individuals as of 2016

adjuvants, including MF59. In one of the earliest published studies on vaccination of multiple chimpanzees using an E1E2 vaccine, the chimpanzees that developed high titer antibodies were protected against apparent infection after low dose homologous challenge. (Choo et al. 1994) However, two of the seven vaccinees became infected after viral challenge. Those two animals failed to respond well to the vaccine and had the lowest antibody titers at the time of viral challenge. In another chimpanzee study, recombinant E1E2 administered with MF59 reduced the rate of chronicity following both homologous and heterologous 1a virus challenge. In some chimpanzees, it also completely prevented infection with the homologous challenge virus (Houghton 2011). Efficacy was linked to $CD4^+$ T cell responses as well as humoral immune responses in the chimpanzees (Puig et al. 2004). The safety and immunogenicity of this recombinant glycoprotein-based vaccine was then tested in human volunteers not at risk for infection. The vaccine induced strong humoral and $CD4^+$ T cell responses with minimal side effects in volunteers (Frey et al. 2010). In addition, adjuvanted E1E2 vaccines have been shown to induce broadly neutralizing antibodies against multiple HCV genotypes as assessed in the HCVpp cell culture assay (Meunier et al. 2011; Ray et al. 2010; Law et al. 2013). No further progress in clinical development of this vaccine has been reported and so its fate is uncertain.

Other approaches to elicit anti-HCV antibodies have been evaluated in animal models. Recombinant virus vectors and plasmid DNA encoding HCV envelope glycoproteins as well as virus-like particles (VLPs) pseudotyped for HCV proteins have been used to induce antibodies in macaques and chimpanzees (Forns et al. 1999; 2000; Garrone et al. 2011) Recombinant viral vectors and synthetic VLPs incorporating HCV E1E2 elicited broadly neutralizing antibodies in macaques (Garrone et al. 2011). The recent success in determining the crystal structure of the E2 core bound to a broadly neutralizing antibody may advance design of synthetic immunogens that induce broadly neutralizing monoclonal antibodies against the HCV glycoproteins (Kong et al. 2013).

The incomplete control of HCV with vaccines designed to generate neutralizing antibody responses, concern about the ability to induce broadly cross-reactive neutralizing antibody responses with E1E2 given the high degree of envelope protein sequence diversity, and data supporting a role for T cells in control of HCV prompted interest in vaccines to induce protective T-cell immunity.

7.2 Trials of Vaccines Designed to Elicit Cytotoxic T Cell Responses

Evidence supporting the necessity of T-cell–mediated immunity in control of HCV infection prompted development of candidate HCV vaccines designed to induce T-cell responses. Thus, the second major approach to a prophylactic vaccine for HCV is targeting the relatively conserved viral proteins within the non-structural

region of the genome to induce a broad T-cell response. Nonstructural (NS) proteins like NS3, NS4, and NS5 are more conserved across HCV genotypes than the envelope glycoproteins and are the dominant targets of $CD8^+$ T cells (Ward et al. 2002). A variety of strategies have been employed to introduce NS proteins in an immunogenic way, including (1) DNA-based immunization, (2) DNA priming followed by recombinant virus vector or HCV protein boosting, (3) recombinant adenovirus priming and DNA boosting, (4) combinations of replicating and nonreplicating recombinant viruses for prime and boost, (5) recombinant baculovirus-derived VLP, (6) hepatitis B virus surface antigen–HCV recombinants, and (7) pooled synthetic class I peptide epitopes or peptides incorporated in lysosomes (Cox and Thomas 2013; Youn et al. 2008) Genetic vaccines, including plasmid DNA or recombinant virus vectors, have been most commonly used. While most of these vaccines used NS proteins as antigens, VLP comprised of the HCV envelope glycoproteins and core protein have also been employed in vaccine strategies designed to induce T cell responses (Elmowalid et al. 2007). Most candidate vaccines have produced humoral and cell mediated immune responses in rodents with a small subset of candidate vaccines shown to elicit robust $CD8^+$ T cell immunity in macaques (Fig. 1) (Garrone et al. 2011; Colloca et al. 2012; Jeong et al. 2004; Fattori et al. 2006, Lang Kuhs et al. 2012; Polakos et al. 2001; Rollier et al. 2005)

Immunogenicity and protection of chimpanzees from HCV challenge have been assessed for even fewer candidate vaccines. These vaccines include VLP comprised of the HCV E1, E2, and core proteins, recombinant nonstructural proteins formulated with the ISCOMATRIX adjuvant, and genetic vaccines that encoded nonstructural proteins (Rollier et al. 2004; Folgori et al. 2006; Youn et al. 2008; Elmowalid et al. 2007; Zubkova et al. 2014). The genetic vaccines that encoded envelope glycoproteins induced antibodies as well as T cell responses, but none provided sterilizing immunity (Rollier et al. 2004; Youn et al. 2008). The outcome of infection in vaccinated chimpanzees was highly variable in these studies, which included fewer than six vaccinated animals per study (Dahari et al. 2010; Elmowalid et al. 2007; Youn et al. 2008; Rollier et al. 2004; Zubkova et al. 2014). While these vaccines did not uniformly provide sterilizing immunity, they reduced primary viremia after challenge with HCV by as much as several orders of magnitude (Dahari et al. 2010; Youn et al. 2008; Folgori et al. 2006; Rollier et al. 2004; Elmowalid et al. 2007; Zubkova et al. 2014). A meta-analysis of all the data from chimpanzee vaccine trials showed that suppression of acute-phase virus replication was associated with recall of vaccine-primed T cells (Dahari et al. 2010). The approaches to analysis of cellular immunity varied in these studies, but the frequency of circulating HCV-specific T cells that produced IFN-γ after vaccination and virus challenge was measured in all. The levels of induced T cell responses did not correlate with vaccine success in the meta-analysis of chimpanzee vaccine studies (Dahari et al. 2010). The analysis also noted that vaccines that contained only structural proteins had clearance rates that were significantly higher than vaccines that contained nonstructural components. However, the vectors and antigens used, the timing of vaccination prime and boost, and the timing and

identity of challenge HCV viruses used were highly varied, limiting the ability to pool data from or to compare these studies.

While the majority of chimpanzee vaccine studies showed reduced HCV persistence rates versus controls, that is not true of all HCV vaccine studies. Vaccination of five chimpanzees with recombinant NS3, NS4, and NS5 proteins formulated with the ISCOMATRIX adjuvant resulted in persistent HCV infection upon rechallenge in all animals. Acute-phase HCV replication was substantially suppressed in the five vaccinated chimpanzees compared to replication in mock-vaccinated controls. Vaccination also resulted in the appearance of vigorous HCV multi-specific $CD4^+$ and $CD8^+$ T-cell responses in the liver prior to experimental challenge and infection, but these failed to protect against chronic infection. A second study showed that immunization of naïve chimpanzees with core-E1-E2 and NS3 of a genotype 1b HCV induced robust HCV-specific immune responses. Vaccinated animals had significantly reduced viral RNA in plasma and in liver versus controls animals after challenge with heterologous HCV. However, despite control of HCV in plasma and liver in the acute period, three of the four vaccinated animals developed persistent infection. One of two mock-vaccinated animals controlled infection, demonstrating a higher rate of persistence in vaccinated than in control animals. Given the small numbers of animals in these studies, it is not clear that these vaccines actually reduced the chances of HCV control. If the vaccines did inhibit HCV control, how prior exposure to HCV antigens and induction of T cell responses made HCV control less likely is not clear. However, these studies demonstrate that vaccination with HCV proteins doesn't uniformly enhance HCV control and vaccination must proceed with caution. In general, the small number of chimpanzees tested and the diversity of the vaccines tested and methods to assess induced immune responses employed make it exceedingly difficult to draw conclusions about the immune responses that provide protection. More detailed phenotypic and functional analyses will be needed to gain insight into factors that determine if a vaccine will reduce or perhaps increase the rate of persistent infection in humans.

Two vaccines designed to prevent infection by eliciting T cell immunity have been tested in Phase I safety and immunogenicity trials in human volunteers not at risk for HCV infection. A prototype vaccine with the HCV Core protein and ISCOMATRIX adjuvant was assessed for its ability to induce T cell responses in healthy individuals not at risk for HCV infection (Drane et al. 2009). Although the vaccine was generally well tolerated, $CD8^+$ T cell responses were only detected in two of the eight participants receiving the highest dose. The second vaccine designed to elicit T cell responses that was tested in healthy volunteers is the only one to have advanced to a trial in at-risk human subjects. This vaccine is composed of a replication-defective adenovirus vector encoding NS proteins.

Replication-defective adenovirus vectors have long been studied as means to introduce antigens from other pathogens to generate potent, protective immune responses. Human adenovirus serotype 5 (Ad5) induces protective immune responses against diverse pathogens and cancer in animal models and elicit robust and sustained cellular immunity in humans. However, most humans have

neutralizing antibodies to Ad5, which can reduce the immunogenicity of Ad5-based vaccines. Replication-defective adenoviral vectors based on serotypes 6 (subgroup C) and 24 (subgroup D) were chosen to create a genetic vaccine because of their low seroprevalence in humans and lack of immunological cross-reactivity. A segment of DNA coding for the nonstructural region of the HCV genotype 1b was delivered by Ad6 followed by Ad24 and finally by electroporated plasmid DNA in chimpanzees (Folgori et al. 2006). Following HCV rechallenge with HCV genotype 1a virus, all vaccinated chimpanzees showed a significantly blunted peak of viremia with the average peak more than 100 times lower in the HCV vaccinated versus control groups. These viral kinetics are similar to those seen in PWID who successfully control HCV with repeated exposure (Osburn et al. 2010). All five animals that received the vaccine had minimal alanine amino transferase (ALT) increases relative to mock-vaccinated control chimpanzees challenged with HCV. Four of the five vaccinated chimpanzees had a significantly shorter duration of viremia versus the control group and eventually cleared the virus. However, one vaccinated chimpanzee maintained low levels of HCV RNA for the duration of the study. A follow-up study revealed that after challenge, vaccinated chimpanzees showed early expansion of $CD8^+$ T cells with higher expression of the memory precursor molecule CD127, lower levels of the inhibitory molecule PD-1, and enhanced effector functions when compared with primary T cells from the mock-vaccinated controls that developed persistent infections (Park et al. 2012). Analogous to this, early expansion of $CD127^+$ HCV-specific T cells with high functionality was previously demonstrated in chimpanzees that spontaneously control acute HCV infection (Shin et al. 2013).

Serotypes of human adenoviruses to which humans are less likely to have been exposed prior to vaccination are less likely to be neutralized, but have been shown to be less potent as vaccine vectors than Ad5 in mice and nonhuman primates (Colloca et al. 2012). Novel adenovirus strains from chimpanzees (ChAd) have been identified for potential use as vaccine vectors. Replication-defective vectors generated from a subset of novel ChAd serotypes were screened to determine whether they were neutralized by human sera and able to grow in human cell lines. Of these, ChAd3 expressing the NS region from HCV was found to induce long-lasting T and B cell memory responses in mice and macaques. Based on these data, vectors expressing NS proteins from HCV genotype 1b were constructed based on rare serotypes Ad6 and ChAd3. The Ad6-NS and ChAd3-NS vaccines were tested in heterologous prime-boost regimens in a safety and immunogenicity phase 1 clinical trial in healthy volunteers not at risk for HCV infection (Barnes et al. 2012) (see ClinicalTrials.gov NCT01070407) ChAd3-NS was highly immunogenic and showed a clear dose-response effect with 100 % of participants responding at a dose of 2.5×10^{10} viral particles (vp) that was well tolerated. Intracellular cytokine staining demonstrated that ChAd3-NSmut primed a large number of polyfunctional $CD8^+$ T cells. Antigen-specific polyfunctional $CD4^+$ T cells were detected at a lower frequency. Memory $CD8^+$ T cells that expressed CD127, but not PD-1, were sustained in circulation. Although more robust recognition of HCV genotype 1b peptides (matching the vaccine) was observed, there

was lower level recognition of genotype 1a and 3a peptide pools. Thus, the vaccine might provide cross-genotypic protection. Responses were sustained for at least a year after boosting with the heterologous adenoviral vector, as demonstrated by persistence of central and effector memory pools that retained polyfunctionality and proliferative capacity. This response was similar to sustained T cell responses of a magnitude and quality associated with protective immunity in other vaccine trials with other pathogens. However, boosting was not as robust with heterologous adenovirus as it was later found to be with modified vaccinia virus Ankara (MVA) boosting so the vaccine was advanced to HCV at-risk subjects with ChAd3-NS prime and MVA-NS boost (Swadling et al. 2014).

The ChAd3-NS prime and MVA-NS boost vaccine is now being evaluated in a staged phase I/II study in Baltimore and San Francisco (see ClinicalTrials.gov NCT01436357). Vaccine responses are primed with ChAd3-NS at 2.5×10^{10} vp (previously found to be well tolerated and immunogenic in healthy volunteers) followed by boosting 8 weeks later with MVA-NS at 1.8×10^{8} pfu. The goal of this study is to prevent HCV persistence in HCV-naïve populations of PWID at high risk for infection. Phase 1 of the trial was completed and enrollment in phase 2 of the trial began in 2013. If the initial results appear promising, confirmation that the vaccine reduces the rate of chronic HCV infection in PWID will likely require larger trials. However, this vaccine trial will at minimum demonstrate the feasibility of conducting trials in PWID.

In conclusion, successful control of HCV infection will most likely require a combination of mass global screening to identify those with infection, treatment of those infected, and prevention and harm reduction strategies for those who are uninfected and at risk. A prophylactic HCV vaccine remains an important part of this strategy because successful identification and treatment of those infected with HCV before irreversible liver damage occurs will remain a challenge. In sum, 25 years after the discovery of HCV, an ounce of prevention is still worth a pound of cure.

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Global Control of Hepatitis C Virus Infection

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Abstract Hepatitis C is among the most serious infectious diseases currently faced by human beings. With the discovery and identification of the causative agent, the hepatitis C virus (HCV), and extensive research on HCV virology, we have revealed the unique nature of HCV. Research findings were directly and promptly applied to medical and public health interventions. In particular, strict screening of blood transfusion drastically reduced the number of new hepatitis C cases worldwide. New and mechanistically diverse anti-HCV therapies has been developed one after another. HCV can be eradicated from individual patients, but hepatitis C cannot be eradicated globally. HCV is heterogeneous in nature. There are quasispecies of HCV in infected persons. The global distribution of genotypes continues to change and diversify. The persistence of HCV is closely related to the heterogeneity of human society. Notably, risk factors and epidemiological parameters are very dynamic. To control HCV infection globally, we must establish a robust international network capable of monitoring the real time epidemiology of HCV and accounting for its increasing disease burden.

Keywords Global epidemiology • Prevalence • Transmission routes • Blood transfusion • Community acquired infection • Intravenous drug injection

Abbreviations

CDC	Centers for Disease Control and Prevention
DAAs	Direct-Acting Antivirals
GBD	Global Burden of Diseases
GDBS	Global Database on Blood Safety
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus

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IDU(s)	Injection Drug Use(rs)
NAT	Nucleic Acid Technology
NBTS	National Blood Transfusion Service(s)
PCR	Polymerase Chain Reaction
STD	Sexually Transmitted Disease
SVR	Sustained Virologic Response
TTIs	Transfusion Transmissible Infection

1 Introduction

Hepatitis C is a major global health issue. Accurate and up-to-date epidemiological data is fundamental to global efforts to control C virus (HCV) infection. However, epidemiological parameters (prevalence, incidence, transmission routes, co-factors, genotype distribution, among others) have changed substantially during the last 25 years and since HCV was discovered. HCV infection via blood transfusion, which was the major route of transmission in the past, is now almost completely controlled in developed countries, where illicit injection drug use (IDU) is now the major route of HCV infection. In contrast, transfusion of unscreened blood, along with unsafe therapeutic injections, is still the major route of HCV transmission in developing countries. Many epidemics that were once considered community-acquired HCV epidemics are now known to have resulted from inadequate local medicinal remedies and practices. Studies on such epidemics directly contributed to the control of the disease and advances in HCV knowledge.

HCV infection is global, but its distribution is uneven. HCV genotype distribution around the globe has changed and diversified. Moreover, the hepatitis C disease burden has also been changing. Given the heterogeneity of HCV and the persistence of hepatitis C, global control of hepatitis C depends on the vigor of global public health efforts.

2 Global Epidemiology

To prevent and control HCV infection, it is essential to have accurate and up-to-date epidemiological data. Because of the nature of acute hepatitis C, disease- based surveillance is rather difficult even in developed countries. Most descriptions of global epidemiology rely on HCV seroprevalence studies. However, the design of such studies is typically cross-sectional, and these studies are done in selected populations that are not necessarily representative of the community or region (Shepard et al. 2005). Nonetheless, for several years, the WHO summarized data on worldwide prevalence of HCV infection based on published studies (WHO 1997, 1999, 2000). Based on a 1999 WHO report, approximately 3 % of the world's population (170 million persons) was infected with HCV (Report of a WHO

consultation 1999). Based on a 2011 estimate, 2.35 % of the world population (160 million persons) were infected with HCV (Lavanchy 2011). Although HCV infection is worldwide, there is consistently a large degree of geographic variability in its distribution; countries in Africa and Asia have the highest reported anti-HCV prevalence, while industrialized countries in North America, Western Europe, and Australia have lower prevalence. Among countries in the developed world, low seroprevalence has been reported from Germany (0.6 %), Canada (0.8 %), France (1.1 %), and Australia (1.1 %), while low but slightly higher seroprevalence has been reported in the USA (1.8 %), Japan (1.5–2.3 %), and Italy (2.2 %) (Shepard et al. 2005). These higher rates may indicate more sensitive detection systems in these three countries.

Recently, a comprehensive study based on the Global Burden of Diseases, Injuries, and Risk Factors 2010 (GBD 2010) operations guidelines resulted in new estimates of age-specific antibody and HCV seroprevalence (Hanafiah et al. 2013). In this study, seroprevalence in each of the 21 GBD regions was systematically reviewed and subject to meta-analysis based on the primary national data sources and 232 articles published after peer review. Age-specific prevalence curves constructed with 1990 versus 2005 datasets were compared. This review stated that global prevalence and the number of people with anti-HCV antibody had increased from 2.3 % (>122 million) to 2.8 % (>185 million) between 1990 and 2005, respectively. High prevalence (>3.5 %) was found in Central and East Asia and in North Africa and the Middle East; moderate prevalence (1.5–3.5 %) was seen in South and Southeast Asia; sub-Saharan Africa; Andean, Central, and Southern Latin America; the Caribbean; Oceania; Australia; and Central, Eastern, and Western Europe; the lowest prevalence (<1.5 %) was evident in Asia Pacific, Tropical Latin America, and North America. This study was an international collaborative effort to estimate the burden of disease using available parameters. Documentation of anti-HCV seroprevalence in this systematic review is the first step towards modeling the global burden of disease for HCV infection and controlling it worldwide.

Most recent estimates of the global prevalence of HCV were reported together with descriptions of HCV genotype distribution (Gower et al. 2014; Messina et al. 2015). Gower et al. further indicated the global prevalence (reports from 89 countries) of anti-HCV estimation as 1.6 %, corresponding to 115 million past infections, while 1.1 %, corresponding to 75 million viraemic infection. These estimates were lower than previous estimates because of the difficulty in generating accurate national estimates in some countries, like India and China. Globally, genotype 1 was most common; it accounted for 46 % of all HCV infections. Genotype 1 was followed by genotype 3 (22 %) and genotypes 2 and 4 (13 % each) (Gower et al. 2014). The report by Messina et al. (representing 117 countries and 90 % of the global population) summarized the data from 1989 to 2013. It reported prevalence of genotype 1 (46 %), genotype 3 (30 %), genotypes 2 (9 %), genotype 4 (8 %), genotype 6 (2 %), and genotype 5 (1 %); undefined or combination genotypes accounted for 3 % of the total HCV infections (Messina et al. 2015).

Since the first report of a recombinant HCV strain (Kalinina et al. 2002), there have been many reports describing the presence of recombination at all levels: between genotypes, subtypes of the same genotype, and strains of the same subtypes (reviewed: Gonzalez-Candelas et al. 2011). Recombination may be a rare event, and the number of well-documented cases is still very low. Simultaneous infection of the same cell by different strains may occur; however, detection of such events is rather difficult with the current assay system, and the actual incidence of such events may be underestimated. Research on viral recombination will impact the evaluation of new therapeutics and of HCV's persistence in human communities. Furthermore, it is noteworthy that many of the recombinant HCV isolates were found by researchers in HCV-endemic countries (Kurbanov et al. 2008). Serendipitous discoveries of recombinant HCVs may result from careful researcher in HCV-endemic countries where the virus changes rapidly for survival.

3 Routes of Transmission

The original reports on HCV state that the virus is transmitted via blood, and they simultaneously describe assay systems for the detection of infectious blood (Choo et al. 1989; Kuo et al. 1989). HCV is definitely a blood-borne virus, and it is transmitted via various other routes.

3.1 Blood Transfusion

The main route of transmission for HCV is blood transfusion. With nationally controlled and strict blood screening, the blood supply in developed countries became as safe as it has ever been (Bush et al. 2003). However, in many of the poorest countries of the world, less than 50 % of the blood supply comes from voluntary, unpaid donors that were adequately screened for transfusion-transmitted infections (TTIs)—including HIV, HBV, and HCV. According to the WHO Global Database on Blood Safety (GDBS) summary report in 2011, among the 164 countries monitored, only 40 collected less than 25 % of the their respective blood supply from voluntary blood donors (WHO, GDBS 2013). In 39 countries, blood donations were not routinely tested for TTIs. In low-income countries, 47 % of the donated blood was tested in laboratories without quality assurance processes (Layden et al. 2014). Another study highlights the fact that the median-risk TTIs in sub-Saharan Africa are high; 1, 4.3, and 2.5 HIV, HBV and HCV infections, respectively, occur with each 1000 units of blood (Jayaraman et al. 2010). Furthermore, nucleic acid testing (NAT) is not yet used systematically in many African blood banks because of high costs and the lack of infrastructural support. It is necessary to adopt more sensitive and inexpensive methods for screening the blood supply and identifying infectious blood (Tagny et al. 2014).

GDBS of WHO plays an important role in the global control of HCV infection (http://www.who.int/bloodsafety/global_database/en/). The Blood Safety Indicator tool is prepared using key elements of the questionnaire for the GDBS, and it is sent to all WHO member states for completion by the senior staff of the National Blood Transfusion Service (NBTS). By analyzing these annual responses, the WHO provides recommendations to establish national blood policy (Tapko et al. 2014). There are some limitations to this analysis, for example; (i) the data are self-reported by each country and cannot be independently verified, (ii) the quality of laboratory screening methods is variable among countries, (iii) the data do not represent all health facilities that collect blood outside each national blood transfusion service network, and (iv) there are some countries for which data is unavailable. Nevertheless, the annual analysis of collected database information is very useful. From 2000 to 2011, the number of countries in sub-Saharan Africa screening at least 95 % of donated blood units for HBV and HCV increased from 76 to 95 % and from 34 to 86 %, respectively (Apata et al. 2014; Tapko et al. 2014). Compared to screenings for other TTIs like HBV and HIV, screenings for HCV are not yet sufficient in developing countries. This lack of vigilance may result from insufficient concern about HCV infection in the region. The development of new, simpler, more sensitive assay systems that can be used to screen donated blood for HCV is urgently needed.

3.2 Mother-to-Child Transmission

Just after the discovery of HCV, evidence emerged that HCV is vertically transmitted from infected mothers to their children (Thaler et al. 1991). However, subsequent studies showed that mother-to-child HCV transmission was less common than mother-to-child HBV transmission (Reinus et al. 1992; Wejstal et al. 1992; Lam et al. 1993; Roudot-Thoraval et al. 1993). Findings from prospective studies with larger numbers of mother-child pairs showed that HCV can be transmitted from mother to child when the HCV RNA titer is high in the mothers (Novati et al. 1992; Ohto et al. 1994; Zanetti et al. 1995). Based on systematic reviews of previously published papers, mother-to-child transmission of HCV is estimated to occur with ~5 % of HCV-positive mothers (Conte et al. 2000; Yeung et al. 2001; Benova et al. 2015). High viral load in the mother is the key factor in such transmission. Furthermore, intravenous drug use (IDU), co- infection with HIV, and fetal exposure in the birth canal are each a risk factor for vertical transmission (Ohto et al. 1994; Zanetti et al. 1995; Mast et al. 2005; Murakami et al. 2012; Benova et al. 2014). Influences of genetic factors, especially HLA genotypes, have been examined, but because of small cohorts and variable genetic backgrounds of patients, a clear relationship between human genetics and vertical HCV transmission has not yet been established (Bevilacqua et al. 2009).

Heterogeneity of HCV RNA in mothers may play a role in mother-to-child transmission (Aizaki et al. 1996). HCV genotypes may be also important, but so

far no clear correlation has been identified (Yeung et al. 2001). Research on viral risk factors will provide information important to the control of vertical HCV transmission. In this sense, recent reports from Egypt are noteworthy. Egypt has the highest HCV prevalence in the world, and mother-to child transmission is among the most important routes of infection. However, the frequency of vertical transmission is higher than generally reported. Actual numbers of children infected with HCV from carrier mothers in 2008 were estimated to be between 3080 and 5167 (Benova et al. 2015). In Egypt, most of the HCV carriers were infected with only genotype 4 HCV. Studies in Egypt—which has a high HCV prevalence, high mother-to-child transmission rates, and a unique distribution of genotype 4—will provide information on the mechanisms responsible for vertical transmission and on potential interventions for prevention of mother-to-child transmission. The natural history of infected children should be evaluated carefully. Vertical transmission and perinatal transmission are two different types of mother- to-child transmission. Future studies in countries like Egypt with high HCV prevalence will provide clues to elucidate the mechanism of mother-to-child transmission.

Research on the immunological mechanisms of mother- to child transmission is particularly important. HCV-specific $CD8^+$ cytotoxic lymphocytes (CTLs) clearly play a pivotal role in the recovery of acute infections; nevertheless, in the 60–80 % of infections that persist, these cells become functionally exhausted, or select mutant viruses escape T-cell recognition (Bowen and Walker 2005; Cox et al. 2005; Rehermann 2009). Serum ALT levels in pregnant women with chronic hepatitis C decrease; moreover, serum HCV RNA levels increase during the later stages of pregnancy (Wejstal et al. 1998; Conte et al. 2000; Gervais et al. 2000; Paternoster et al. 2001). These changes may be the consequence of the complex phenomena responsible for maternal tolerance of the fetus (Guleria and Sayegh 2007). For example, the activity of HCV-specific CTLs may be impaired during pregnancy, and therefore, CTLs may exert less selection pressure on existing persistent viruses. Eventually, Honegger et al. characterized the circulating viral quasispecies in two obstetrics patients during and after consecutive pregnancies. They found that CTL-mediated selection pressure on persistent HCV was reimposed after childbirth, and escape mutations again predominated in the quasispecies and observed sharp decrease of viral load (Honegger et al. 2013). Further research into this unique virus-versus-host interaction may inform and improve clinical and public health strategies for prevention of mother-to-child transmission.

3.3 Injection Drug Users

Soon after the discovery of HCV, HCV infection was found to be common among injection drug users (IDUs) in developed countries (Bell et al. 1990; Girardi et al. 1990; van den Hoek et al. 1990). Since worldwide adoption of strict blood-screening protocols, acute hepatitis C has been drastically reduced globally. In

developed countries, contaminated blood products have been virtually eliminated as sources of HCV transmission. In the USA, 68 % of all newly acquired cases of hepatitis C are related to IDU (Alter 2002). The epidemic of HCV infection continues to escalate in Australia, predominantly through transmission related to IDU; 80 % of the epidemic-related HCV infections were determined to be associated with IDU (Dore et al. 2003).

In several European countries, nowadays, Currently, IDU is the most common HCV transmission route particularly among younger people. This conclusion is based on data from Italy (Mele et al. 2000), France (Elghouzzi et al. 2000), Norway (Dalgard et al. 2003), and England and Wales (Balogun et al. 2003). In Japan, the total number of HCV infection is decreasing; nevertheless, sporadic HCV transmission is mainly seen among young IDUs (Chung et al. 2010). A systematic analysis of data on HCV infection among IDUs indicated that approximately 10.0 million IDUs worldwide might be anti-HCV positive. China (1.6 million), USA (1.5 million), and Russia (1.3 million) were the three nations with the largest number of anti-HCV-positive IDUs. More IDUs had anti-HCV antibodies than HIV infection, although sensitivities of each assay must be taken into consideration when assessing these findings (Nelson et al. 2011). In some settings, HCV incidence has clearly declined among younger IDUs; nevertheless, new infections still occur IDUs aged >39 years in USA (Mehta et al. 2011).

Notably, heroin use, HCV infection, or both alter circulating miRNAs; these effects on miRNAs could be a novel mechanism that impairs innate anti-HCV immunity among IDUs (Zhou et al. 2015). HCV could be transmitted through sharing of unsterilized needles among IDUs, and at the same time, an injected drug could enhance the replication of HCV. The behavior of IDUs is usually illicit; consequently, it is difficult to manage HCV infection in this population (Robaeys et al. 2013). HCV targets very weak human populations for its survival and persistence.

3.4 Community-Acquired Infections

Several endemic outbreaks of non-A, non-B hepatitis were reported in isolated areas in Japan (Yamauchi et al. 1983). Most of these cases progressed to chronic hepatitis, but each such individual had no history of blood transfusion. Clinical data were collected; even serum samples were collected and kept. The sera of such patients were infectious to chimpanzees, and the inoculated animals developed hepatitis (Abe et al. 1986).

Immediately after the discovery of HCV, HCV antibody and HCV RNA detection revealed that most such outbreaks were eventually HCV outbreaks (Aramaki et al. 1991a, b; Fujisawa et al. 1991; Ito et al. 1991; Kuboki et al. 1991; Setoguchi et al. 1991; Wakayama et al. 1991; Fukuda et al. 1992; Kiyosawa et al. 1994; Murata et al. 1995). Examination of independently collected patient sera played a significant role in identification of HCV as the causative agent for such endemic

outbreaks in Japan. The Kiyosawa's report is important because it suggested risk factors for transmission. Folk remedies that involve unsterile materials for acupuncture or "Suidama" therapy seemed to be possible routes of transmission. "Suidama" therapy is no longer performed in Japan; it is an old, traditional folk remedy believed to relieve muscle stiffness by drawing blood from an artificial cut, which was often made with a unsterile knife (Kiyosawa et al. 1994). Definitive identification of the origin of outbreak is difficult for each case mentioned above; however, the lesson from Japan is important and instructive. HCV transmission by unsafe injections used for local remedies was reported in Egypt (described later), a province of Taiwan, Pakistan, and many other parts of the world (Wu et al. 1992; Ho et al. 1997; Luby et al. 1997). Most of these infectious injection-based remedies involved shared needles and syringes and were unsafe and even unnecessary; consequently, many of these practices are now abolished in industrialized countries. However, such practices remain a serious problem in developing countries, and the WHO estimates that unsafe injections results in 2.3–4.7 million new HCV infections worldwide every year (Simonsen et al. 1999; Drucker et al. 2001). People in poverty and those lacking adequate recognition are more vulnerable to HCV, which seems to survive and persistence in these populations.

Just after the discovery of HCV in 1989, a remarkably high prevalence of HCV was observed in Egypt (10–20 % of general population) (Saeed et al. 1991; Kamel et al. 1992; Darwish et al. 1993; Hibbs et al. 1993; Abdel-Wahab et al. 1994; Bassily et al. 1995; El-Gohary et al. 1995; Arthur et al. 1997). A high prevalence of HCV was also detected in Egyptian patients with chronic liver diseases (Waked et al. 1995). Unscreened blood transfusion seemed to be the main risk factor for infection (Khalifa et al. 1993; Abdel-Wahab et al. 1994). Several measures involving percutaneous procedures— like intravenous injection, and circumcision—were considered as possible routes of transmission, but this hypothesis did not explain the high prevalence among healthy blood donors. However, a history of schistosomiasis among anti-HCV-positive donors was significant (Darwish et al. 1993). Meanwhile, a high HCV prevalence was noticed among patients with schistosomiasis (Strickland 1994; Angelico et al. 1997).

In Egypt, schistosomiasis was traditionally the most serious public health problem (Abdel-Wahab et al. 1980). From 1950s until the 1980s, intravenous injection with an antimony compound was used in large public health campaigns to control schistosomiasis; in the 1980s, a novel oral anti-schistosomal drug replaced the intravenous injections. El-Zayadi et al. noticed high HCV seroprevalence was more pronounced among anti-schistosomial-positive sera. They speculated that the high HCV prevalence could be due to HCV transmission during anti-schistosomal parenteral therapy or due to depressed cell-mediated immunity associated with schistosomal infection (el-Zayadi et al. 1997). Notably, parenteral anti-schistosomial therapy was already considered as a risk factor for HBV transmission (Hyams et al. 1987; Madwar et al. 1989). Frank et al. conducted careful retrospective studies and found a direct relationship between extensive intravenous injection for the treatment of schistosomiasis and high, countrywide prevalence of HCV (Frank et al. 2000). They concluded that the nationwide campaign in Egypt may

represent the world's largest iatrogenic transmission of blood-borne pathogens. Genetic epidemiological analysis of HCV genotype 4 in Egypt indirectly evaluated the hypothesis that simultaneous dissemination of multiple HCV was transmitted extensively throughout Egypt before 1980 (Ray et al. 2000). A Bayesian coalescent analysis further indicated widespread iatrogenic transmission of HCV; this analysis was designed to estimate the transmission dynamics of HCV in Egypt (Pybus et al. 2003). Tracing of the evolution of genotype 4 HCV in Egypt demonstrated that the spread of this unique genotype increased exponentially during the1940s and up to 1980 (Tanaka et al. 2004; Mizokami and Tanaka 2005). As a consequence, HCV-related mortality is expected at least to double in the next 20 years in Egypt (Deuffic-Burban et al. 2006).

The originally commendable effort to control a major health problem, ultimately established a very large reservoir of HCV in Egypt (Strickland 2006). This large reservoir of HCV infection in the general population of Egypt (~6 million) provides an opportunity to investigate risk factors for transmission (Magder et al. 2005; Mohamed et al. 2005; Benova et al. 2015), mechanism of concomitant infections (El-Awady et al. 2006; Abdel-Rahman et al. 2013), the natural history of infection, and the effectiveness of new therapeutics. Research in Egypt will thus provide precious lessons for the global control of HCV infection (Arab Republic of Egypt, Ministry of Health and Population 2008). Furthermore, it is notable that Egyptian government succeeded in establishing an exclusive agreement to obtain DAA drugs at lower price from Gilead.

The following is the most recent information whose report is now in preparation (personal information from Dr. El-Gohary). *Sofosbuvir* (*Sovaldi*) *entered Egypt on 16 October 2014. It is being sold in medical centers affiliated to the health ministry. Ministry of Health in Egypt reached agreement on 15 July 2015 with Gilead Sciences* (*biopharmaceutical company*) *to import the drug at a heavily discounted cost. Egypt received the drug from Gilead for 1 % of Sovaldi's price. Cairo subsidized the remaining costs to provide the treatment to the Egyptian citizens. Patients will be treated for free as part of a government health program.*

This is less than a year after this drug, was cleared by the FDA in the US market.

In September 2014, Egypt imported 225,000 Sovaldi bottles from the U.S., manufactured by the Gilead Science Company and received a similar amount in March 2015.

On the website of the National Committee for the Control of Viral Hepatitis in Egypt, 996,642 patients applied to be treated by Sovaldi until last June, according to the past-Minister of Health in Egypt Adel El-Adawy. After medical check-ups patients receive Sovaldi (*Sofosbuvir*). *He added that 85,536 patients were provided with the drug until the end of June. According to the treatment protocols of hepatitis C treatment centers, response rate of Sovaldi is 92.7 %, said El-Adawy. The eventual aim is to treat 300,000 people a year. There are 31 specialized centers for liver in Egypt* (*where 350, 000 people have been treated in the past 6 years*). *El-Adawy announced that two new hepatitis C drugs, Harvoni and Viekira, are to be available at the hepatitis treatment centers by next November.*

The national hepatitis plan for 2013–2018, drawn up by the National Viral Hepatitis Committee, the Ministry of Health and supported by health partners, places a strong emphasis on prevention. Public awareness is also vitally important and the authorities are developing a communications campaign, with UNICEF and WHO support, to educate people about the need to avoid unnecessary injections and to insist that health workers use sterile syringes and needles.

The production of a "similar drug" using the same API is an alternative to compulsory licensing. Twelve companies registered to produce similar drugs to Sovaldi.

In Japan, the incidence of hepatocellular carcinoma was significantly elevated among patients with *shistosomiasis japonicum*, but the cause for this phenomenon was not clearly identified (Nakashima et al. 1975). After HCV was discovered and an anti-HCV antibody assay was developed in 1989, sera of patients with chronic schistosomiasis from Yamanashi prefecture, an area with high rates of endemic shistosomiasis in Japan, had significantly elevated prevalence of HCV antibody (36.5 %). However, the route of HCV transmission was not determined at that time (Iida et al. 1999). Tanaka et al. used molecular tracing to demonstrate that the exponential growth of HCV epidemics in Japan occurred in the 1920s (Tanaka et al. 2005). This time period coincided with the use of parenteral anti-schistosomal therapy. Similarly, in India, HCV transmission accelerated through the re-use of needles for administrating anti-kala-azar drugs (Singh et al. 2000); in this study, it was also found that HCV was somehow more prevalent than HBV.

3.5 Sexual Transmission

Epidemiological evidence suggests that HCV can be transmitted via sexual interaction, though less efficiently than HBV or HIV. The initial study using the first-generation anti-HCV assay demonstrates the efficiency of heterosexual transmission of HCV is poor (Brettler et al. 1991). However, data from more sensitive PCR methods indicate that there is a high frequency of intra-spouse transmission of HCV in Japan (Chayama et al. 1995); such transmission is plausible because HCV RNA is sometimes detected in the semen of infected men (Luruez-Ville et al. 2000). A cluster of acute HCV infection was even reported among men who have extensive sex with men (Goetz et al. 2005). However, such cases have other high risk factors that may influence on HCV transmission. These factors include multiple sex partners, a history of other STDs, and IDUs. Occult HCV infection has recently been reported among healthy spouses of patients with HCV infection (Shazly et al. 2015). These studies totally depend on how the cohort was selected and the sensitivity of detection. Sexual transmission of HCV does exist, but its significance or magnitude as a transmission route is difficult to assess.

3.6 Other Routes of Transmission

Since the main route of HCV transmission is through blood or blood products, it is possible that HCV is transmitted via minor procedures that involve blood or body fluid transfer. These procedures include tattooing, body piercing, acupuncture, circumcision, scarification, and other folk medical procedures (Karaca et al. 2006; Barakat and El-Bashir 2011; Tohme and Holmberg 2012). Education of people regarding HCV and HCV transmission is the only effective method for preventing such transmission. Infectivity (i.e. infectious units contained in blood) varies tremendously, and notably, Pybus et al. have hypothesized that HCV can be mechanically transmitted by arthropods bite is (Pybus et al. 2007). HCV cannot replicate in arthropods like other pestiviruses; consequently, it is impossible to passively transfer infectious HCV from humans to humans. However, the amount of infectious HCV in patients varies tremendously; this possibility cannot be completely ruled out, though it may be difficult to prove.

4 Non A–E Hepatitis Viruses

4.1 GB Viruses (Hepatitis G Virus)

The isolation of a fragment of the HCV genome was a landmark discovery in terms of the identification of a theretofore-unknown causative agent for a blood-borne non-A, non-B hepatitis virus (Choo et al. 1989; Kuo et al. 1989, reviewed; Levine and Enquist 2007). That work also clearly demonstrated the robustness of a newly developed cDNA library immunoscreening method that could detect a target gene with a specific antibody (Young and Davis 1983). The key issue for the success of this methodology was that the cDNA library being screen actually contained cDNA representing infectious RNA or DNA viruses. Eventually, the discovery presented that antibody is the most sensitive reagent whatsoever available.

However, HCV may not be the one and only causative agent of non-A, non-B hepatitis. Many researchers naturally considered the possibility that agents other than HCV could cause blood-borne non-A, non-B hepatitis. Intense research efforts to identify agents other than HCV involved their own blood materials that may contain the causative agents. However, in most studies, the recovered DNA by this method was cell derived not virus derived (Mishiro et al. 1999, many others. unpublished). Most HCVs isolated so far reported were derived from PCR products using sequences derived from the HCV reported.

An early study, before the discovery of HBV or HAV, on the transmission of human viral hepatitis to marmoset monkeys was important in the history of hepatitis research (Deinhardt et al. 1967). At that time, there was no robust replication system for growing hepatitis viruses in tissue culture cells. By reformulating Koch's postulates, Deinhardt et al. used a suitably cautious approach to prove

that their clinical materials contained hepatitis virus that could be successfully transmitted to monkeys. A representational difference hybridization method was used to clone GBV-A and GBV-B genes from sera that had been used to successfully infect marmoset monkeys (Simonds et al. 1995b). The letters "GB" represent the initials of a surgeon with acute hepatitis whose sera was used to transmit hepatitis to the marmoset monkeys (Deinhardt et al. 1967). GBV-A and GBV-B are similar to HCV, but still genetically distinct from HCV. They were originally derived from human sera of a patient with hepatitis, but subsequent studies showed that GBV-A and GBV-B are indigenous monkey viruses that seemed to be acquired during passages in tamarins. Although some human sera from patients with or without hepatitis showed antibody activity against GBV-A or GBV-B, sequences from these viruses were never detected in human sera by PCR. Instead, a third related virus, GBV-C, whose sequence had limited identity with those of GBV-A, GBV-B, or HCV, was detected in humans by the same research group (Simonds et al. 1995a). GBV-C was originally isolated from individuals including patients with hepatitis; therefore, it was naturally considered to be a candidate non-A-E hepatitis virus. Intriguingly, another group totally independently, and almost simultaneously, isolated a clone from a patient with non-A, non-B hepatitis (Linnen et al. 1996). The clone was isolated and identified by way of immunoscreening of a cDNA library constructed from serum of this patient with transfusion-associated, non-A, non-B hepatitis. The genome analysis indicates that the virus, designated hepatitis G virus (HGV), is closely related to GBV-C, and yet distantly related to HCV, GBV-A, and GBV-B. The patient was eventually co-infected with HCV, but the HGV genome was successfully isolated from the library constructed with this patient's sera. Based on sequence relatedness and overall genome structure, GBVs have been classified in the family *Flaviviridae* (Lindenbach et al. 2013). Among them, GBV-B, together with HCV, belongs to the genus *Hepacivirus* (Stapleton et al. 2011). It has been proposed that the remaining members—GBV-A, GBV-C (HGV), and the newly identified GBV-D (Epstein et al. 2010)—be classified to the genus *Pegivirus* within the family *Flaviviridae* (Stapleton et al. 2010).

Although GBV-C/HGV can easily cause persistent infection in humans, it does not appear to cause hepatitis (Tan et al. 1999). Nor does GBV-C/HGV worsen the course of HCV infection, with which it is frequently coexistent. It has been suggested that GBV-C/HGV might be a clinically favorable prognostic factor in co-existing HIV infections (Helinglake et al. 1997; Toyoda et al. 1998; Yao et al. 2000). Prospective and retrospective clinical studies indicated that coinfection with GBV-C is associated with a reduced mortality rate in HIV-infected patients (Tollman et al. 2001; Xiang et al. 2001). This observation was significant when GBV-C co-infection occurred in later stages of HIV disease, but finding was not significant when co-infection occurred in early-stage HIV (Williams et al. 2004; Zhang et al. 2006). In contrast, no effect of GBV-C co-infection on HIV disease progression has been observed in a cohort of African women infected with HIV (Kaye et al. 2005). The "beneficial" effect of GBV-C co-infection among HIV-infected individuals may be related a reduction in host immune activation (Chivero and Stapleton 2015).

4.2 Non-Primate Hepaciviruses

In an effort to identify additional hepatitis viruses other than A–E, sera from non-primate species were screened for HCV-like viruses. The potential existence of HCV-like viruses in other animals is a concern in the context of global HCV infection control. Non-primate hepaciviruses have been identified in various mammals, including dogs, horses, bats, rodents, and cattle (Epstein et al. 2010; Kapoor et al. 2011, 2013 Burbelo et al. 2012; Lyons et al. 2012; Drexler et al. 2013; Baechlein et al. 2015). Among these hepaciviruses, the equine hepacivirus shares the highest amino acid homology with HCV (Burbelo et al. 2012; Lyons et al. 2012); moreover, it exhibits liver-tropism with inflammation, and it causes acute and chronic infection in horses (Pfaender et al. 2015). Tanaka et al. also suggest that equine hepacivirus exhibits similar genetic and biological properties to HCV (Tanaka et al. 2014). The life cycle of non-primate hepaciviruses may be similar to that of HCV, and the characterization of these viruses will be useful for research on HCV, in particular for anti-viral research. However, all of the currently known non-primate hepaciviruses have strict host ranges, and there is no indication that humans are permissive hosts that can develop hepatitis from any of these viruses. These novel viruses, nevertheless, may provide promising animal models for the study of HCV infections in humans. They also have potential to establish zoonotic infections.

4.3 Non-(A–E) Hepatitis Virus

Even in developed countries like Japan, the etiology of 10–20 % of cases of acute hepatitis remains undetermined. In developing countries in Africa, many hepatitis cases are not clearly diagnosed. Considering recent isolation of *pegiviruses* from non-primate animals and their hepatotropic nature, it is still probable that there are some as-yet unidentified hepatotropic, non-A–E viruses with or without clear manifestation of hepatitis.

5 Global Control of Hepatitis C

In the context of global control of HCV infection, it is essential to have accurate epidemiological data and due regard to the disease burden of hepatitis C. Various epidemiological parameters have changed substantially since HCV was discovered. Recent systematic reviews of published reports indicated high and increasing prevalence of global HCV infection (Hanafiah et al. 2013; Gower et al. 2014; Messina et al. 2015). It is challenging to establish: (i) countrywide seroprevalence and molecular surveys, (ii) nationwide sentinel studies of acute HCV infection, (iii)

periodic and accurate notification of prevalence and incidence data to National Public Health Services with accessible databases, and (iv) continuous coordination of National Public Health Services with WHO (Esteban et al. 2008). It is quite obvious that there is a large gap between developed and developing countries with regard to public health infrastructure. The Global Hepatitis Programme of WHO is extremely important in the effort to set up a global network for HCV surveillance (www.who.int/hepatitis/en/). Development of new, simple, and sensitive technology to detect HCV carriers is also expected. Standardized methodology, collection of updated information, and meticulous data analysis are each key to establishment of a global network for the global control of HCV infection.

Because most HCV infections do not cause manifest symptoms, most HCV-infected persons are unaware of their infection. In the United States, it is estimated that less than half of HCV-infected individuals are aware of their infection. No more than 15 % of the >185 million HCV-antibody-positive persons worldwide are aware of their infection (Thomas 2013). From a public health perspective, the groundwork necessary for control of hepatitis C is active interventions directed toward those unknowing carriers of HCV and toward preventing disease development in these carriers.

In Japan, a national project started in 2002; specifically, any individual who has recently turned 40, 45, 50, 55, 60, 65, or 70 is offered tests for hepatitis B and C at routine health check-ups (Tanaka et al. 2011). This project was preceded by studies done in Hiroshima-prefecture showing that HCV check-ups every 5 years are very useful for gauging the real incidence of HCV infection in a population (Tanaka et al. 2008). However, only 30 % of the eligible persons have taken the tests. Furthermore, the follow-up to individuals diagnosed as HCV carriers is not complete. The government of Japan is strengthening this nationwide testing and follow-up system. Correct recognition of HCV infection and its disease burden are both important factors in the control of HCV infection in an advanced country such as Japan.

6 Conclusion

Considering its global prevalence and serious disease burden, hepatitis C is one of the most serious infectious diseases that human beings are now confronted with. After discovery and identification of the causative agent and subsequent extensive research on HCV virology, we learned many unique features of HCV. Furthermore, mainly because of gene expression experiments and development of robust viral replication in tissue cultured cells, our knowledge about virus-cell, virus-tissue, and virus-host interaction has expanded tremendously. Molecular biology-based studies on cellular viral pathogenesis are expanding. Strict blood screening before transfusion could drastically reduce the number of new HCV cases. New anti-HCV therapeutics with varying mechanisms have been developed one after another. Consequently, people have begun to believe that hepatitis C is a curable disease.

Hepatitis C is definitely controllable. HCV can be eradicated from individual patients, but hepatitis C cannot be globally eradicated. HCV, as an entity, uses a delicate mechanism to survive. Adequately selected and adapted mutants survive in varying circumstances in cells, tissues, host (humans), and human societies. Furthermore, HCV can cause deterioration of downstream cellular signaling cascades in not only infected cells, but also the whole innate immune response of an infected host to establish a persistent infection. Human beings, on the contrary, cannot abandon people because they are difficult to survive in varying circumstances. Diseased and weakened individuals should be indeed cared for the most. Nevertheless, accurate HCV carrier rates are yet determined, and many new drugs are too expensive to use in many developing countries. Meanwhile in developed countries, illicit IDUs are still the most vulnerable to HCV infection. HCV can survive in such weak, innocent, poor human populations, and HCV circulation can continue unnoticed. Global control of hepatitis C is, after all, a consequence of the interactions between the HCV population and the human community. Step by step, science could contribute to control HCV infection. The one and only strategy to fight against this formidable virus is to share correct and serious understanding of the virus globally.

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