From THE DEPARTMENT OF MEDICINE, HUDDINGE
Karolinska Institutet, Stockholm, Sweden

LONG-TERM CLINICAL, HISTOLOGICAL AND VIROLOGICAL OUTCOMES AFTER SUSTAINED VIROLOGIC RESPONSE IN PATIENTS WITH CHRONIC HEPATITIS C

Magnus Hedenstierna

Stockholm 2017

Long-term Clinical, Histological and Virological Outcomes after Sustained Virologic Response in Patients with Chronic Hepatitis C

THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i föreläsningssal 4Z, Alfred Nobels Allé 8, Huddinge

Onsdagen den 31 maj, 2017, kl 09.00

By
Magnus Hedenstierna

Principal Supervisor:
Associate Professor Soo Aleman
Karolinska Institutet
Department of Medicine, Huddinge
Units of Infectious Diseases and
Gastroenterology

Co-supervisor:
Professor Ola Weiland
Karolinska Institutet
Department of Medicine, Huddinge
Unit of Infectious Diseases

Opponent:
Professor Kristian Bjøro
Universitetet i Oslo
Department of Transplantation

Examination Board:
Professor Johan Giesecke
Karolinska Institutet
Department of Medical Epidemiology and
Biostatistics

Associate Professor Staffan Wahlin
Karolinska Institutet
Department of Medicine, Huddinge
Unit of Gastroenterology

Associate Professor Karlis Pauksens
Uppsala Universitet
Department of Medical Sciences
Unit of Infectious Diseases

ABSTRACT

Successful antiviral treatment of chronic hepatitis C (HCV), resulting in Sustained Virologic Response (SVR) has been shown to reduce the risk for liver related complications, hepatocellular carcinoma (HCC) and death. Several studies have also shown that liver histology improves after achieved SVR. In a small sub-set of patients, however, liver fibrosis will persist and sometimes even progress to cirrhosis after SVR. Furthermore, a risk to develop HCC seems to remain many years after SVR has been achieved. The aim of this thesis was to study the long-term effect of sustained virologic response on clinical, histological and virological outcomes, and to identify risk factors associated with persisting advanced fibrosis and a continued risk to develop HCC after SVR.

In **study I** we investigated the effect of antiviral treatment on liver-related complications, HCC and death. We included 351 patients with HCV related cirrhosis in a prospective cohort study. Mean follow-up time was 5.3 years. The risk to develop liver-related complications, HCC, and liver-related and all-cause mortality was 0.9, 1.0, 0.7 and 1.9 per 100 person years for patients with achieved SVR, compared to 4.9, 4.0, 4.5 and 5.1 per 100 person years for patients never treated for HCV. In **study II** we investigated the prevalence and clinical implications of occult hepatitis C. In a cross-sectional study, 54 patients with all stages of pre-treatment liver fibrosis and SVR 5-20 years prior to inclusion were tested for HCV RNA using a highly sensitive method. Three patients (6%) tested positive for HCV RNA in peripheral blood mononuclear cells (PBMCs), but this did not correlate to clinical, histological or immunological evidence of persisting liver disease. In **study III** we investigated the long-term risk and risk factors for the development of HCC after achieved SVR. In this cohort study we included 399 patients with SVR and pre-treatment advanced fibrosis or cirrhosis. Median follow-up time was 7.8 years. The incidence rate of HCC was 0.15 and 0.95 per 100 person years for patients with advanced fibrosis and cirrhosis respectively. The main risk factor for the development of HCC was pre-treatment cirrhosis, low serum albumin and diabetes mellitus. The risk to develop HCC diminished with longer follow-up time. In **study IV** we investigated fibrosis regression and risk factors for persisting advanced fibrosis after achieved SVR. In a cross-sectional study 269 patients were examined with transient elastography. Median follow-up time was 7.7 years. A majority of patients regressed to a lower fibrosis stage at follow-up, but 24% had persisting advanced fibrosis. This proportion, however, diminished over time. Risk factors associated with persisting advanced fibrosis were pre-treatment cirrhosis, higher age and high body mass index.

Our studies have contributed to the growing evidence of the positive effects of SVR in chronic HCV. With long follow-up time, we were also able to show that disease regression continues over time. We identified established cirrhosis and co-morbidity with metabolic disease as important risk factors for persisting advanced fibrosis and a continued risk to develop HCC after SVR has been achieved.

LIST OF SCIENTIFIC PAPERS

I. Aleman S, Rahbin N, Weiland O, Davidsdottir L, Hedenstierna M, Rose N, Verbaan H, Stal P, Carlsson T, Norrgren H, Ekbom A, Granath F, Hultcrantz R. **A risk for hepatocellular carcinoma persists long-term after sustained virologic response in patients with hepatitis C-associated liver cirrhosis**. Clin Infect Dis. 2013 Jul;57(2):230-6

II. Hedenstierna M, Weiland O, Brass A, Bankwitz D, Behrendt P, Uhnoo I, Aleman S, Cardell K, Fryden A, Norkrans G, Eilard A, Glaumann H, Pietschmann T, Sallberg M, Brenndorfer ED. **Long-term follow-up of successful hepatitis C virus therapy: waning immune responses and disappearance of liver disease are consistent with cure**. Aliment Pharmacol Ther. 2015 Mar; 41(6):532-43.

III. Hedenstierna M, Nangarhari A, Weiland O, Aleman S. **Diabetes and Cirrhosis Are Risk Factors for Hepatocellular Carcinoma After Successful Treatment of Chronic Hepatitis C**. Clin Infect Dis. 2016 Sep 15;63(6):723-9.

IV. Hedenstierna M, Nangarhari A, El-Sabini A, Weiland O, Aleman S. **Cirrhosis, high age and body mass index are risk factors for persisting advanced fibrosis after sustained virologic response in chronic hepatitis C.** Submitted.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AASLD	American association for the study of liver diseases
BMI	Body mass index
CI	Confidence interval
DAA	Direct-acting antiviral
EASL	The European Association for the Study of the Liver
ELISpot	Enzyme-Linked ImmunoSpot
ESLD	End-stage liver disease
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HR	Hazard ratio
IFN	Interferon
IR	Incidence rate
IRR	Incidence rate ratios
OR	Odds ratio
LSM	Liver stiffness measurement
NS	Non-structural
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SVR	Sustained virologic response

1 INTRODUCTION

Chronic infection with hepatitis C virus (HCV) is a global health problem. It is estimated that 70 million people are chronically infected with HCV and that 750 000 people die every year from HCV related causes [1, 2]. Chronic HCV infection is a major cause of hepatocellular carcinoma (HCC) in the world and has been the leading cause for liver transplantation in North America and Europe [3, 4]. Treatment with interferon-based therapy was introduced in 1986, even before the virus was identified, but was ineffective and burdened by side effects [5]. As a consequence, for many years the main focus of HCV research was the development of better treatment strategies. Twenty years later combination therapy with pegylated interferon and ribavirin cured 50% of the treated patients but often failed to cure patients with more advanced liver disease and a high risk of liver-related and other complications [6]. With the development of direct-acting antivirals (DAAs) this has changed, and we can now achieve virologic cure (sustained virologic response – SVR) in almost all patients regardless of the stage of liver disease [7].

This raises other questions. What is the effect of SVR on clinical outcomes? Can liver fibrosis and cirrhosis regress after SVR has been achieved? Does SVR really mean that HCV has been eradicated, or can small amounts of HCV persist in liver tissue or immune cells?

The aim of this thesis was to investigate the long term effect of SVR on clinical, histological and virological outcomes. Is successful treatment of chronic hepatitis C really a cure?

2 HEPATITIS C INFECTION

2.1 HEPATITS C VIRUS

The clinical picture of hepatitis C infection was first described as transfusion-associated non-A non-B hepatitis in 1975 [8]. Hepatitis C virus (HCV) was isolated in 1989, and was found to be a positive sense, single-stranded RNA virus belonging to the Flaviviridae family [9]. The HCV particle consists of a lipid membrane envelope and a nucleocapsid, containing the HCV genome. The HCV genome codes for a single polyprotein that is cleaved by viral and cellular proteases into three structural (core and envelope 1+2) and seven non-structural proteins (P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). HCV replication is dependent on the combined functions of the NS3/4A protease complex, the NS5B RNA polymerase and the NS4B and NS5A proteins [10]. Increased knowledge of these viral proteins has led to the development of new direct-acting antiviral drugs [11]. There are seven genotypes (GT 1-7) and several sub-genotypes of HCV, and the genetic diversity of HCV is enormous [12]. This is due to the high error rate of the NS5B RNA polymerase, but also to the long evolutionary relationship between HCV and humans [13, 14]. HCV genotypes have epidemiologic implications, but also affect the outcome of hepatitis C treatment [15].
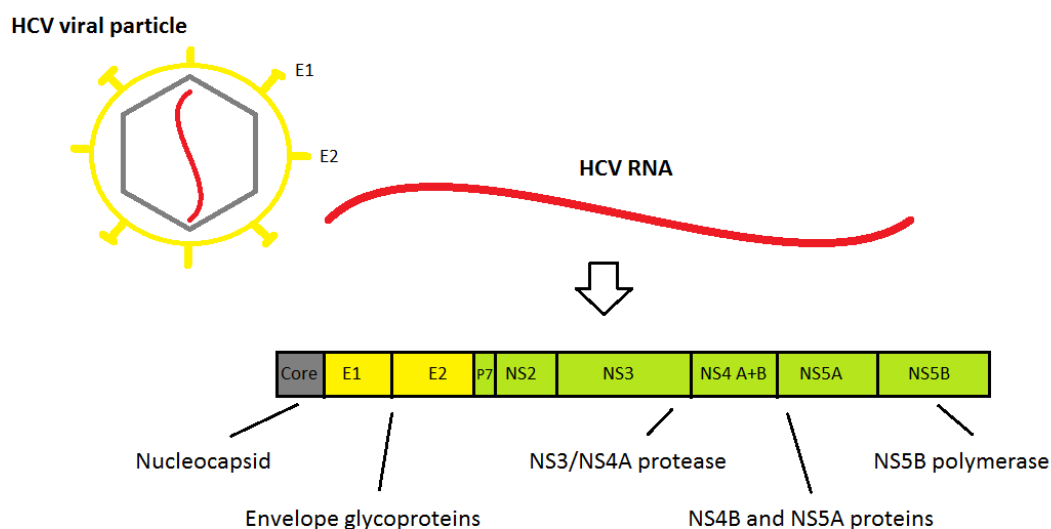

Figure 1: *Hepatitis C virus particle and viral proteins*

2.2 EPIDEMIOLOGY OF HCV INFECTION

Chronic infection with HCV is a global health problem and a growing epidemic with a seroprevalence that has increased from 2.3% to 2.8% between 1990 and 2005 [16]. Based on serologic evidence of infection it has been estimated that130-170 million people are

chronically infected with HCV [16, 17]. These estimations have been challenged, and recent studies using updated data on viraemic infections have estimated that 70-80 million have chronic HCV infection [1, 18]. The burden of disease is not evenly distributed globally, with the highest viraemic HCV prevalence in Eastern Europe and Central Asia [1]. There are several explanations for the new lower estimates for HCV prevalence. Older statistics have been incomplete and outdated, and new lower estimates for populous countries like China and India influenced the total prevalence significantly [1]. Another reason is that patients with chronic HCV are an ageing population which have already developed advanced liver disease and consequently have a high mortality rate [19, 20]. In many countries the rate of all-cause and liver related mortality among HCV infected people is higher than the rate of new infections, decreasing the HCV prevalence [1]. As a consequence the global morbidity and mortality caused by chronic HCV infection has increased by more than 50% between 1990-2013 despite a lower HCV prevalence, and 500 000-750 000 people now die from HCV related causes every year [2, 21].
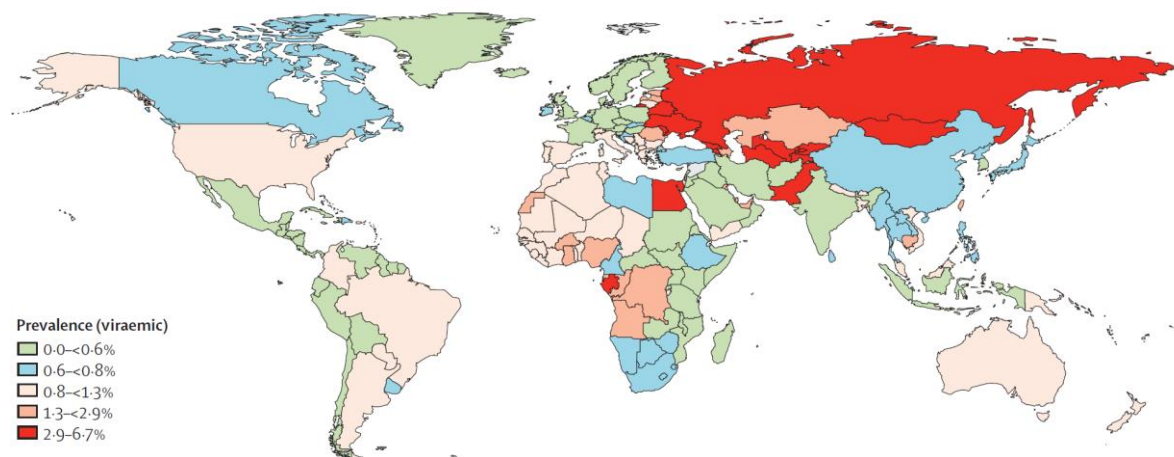
Figure 2: *Estimated viraemic HCV prevalence in 2015. From Bach S et al, The Lancet Gastroenterology 2017, http://dx.doi.org/10.1016/S2468-1253(16)30181-9, with permission: http://creativecommons.org/licenses/by-nc-nd/4.0/.*

Hepatitis C virus infection is an almost exclusively blood-borne disease, and the main routes of transmission are intravenous drug use and nosocomial transmission through transfusions or contaminated health care equipment [22]. Vertical transmission from mother to child is rare and occurs in 4-7% of pregnancies [23]. Sexual transmission is very rare, but higher incidence rates have been reported mainly among human immunodeficiency virus (HIV) co-infected men who have sex with men [24, 25].

In Sweden the spread of HCV increased during the 1960s to 1980s, as a result of injecting drug use. HCV infection is widespread among people who inject drugs in Sweden, and the seroprevalence of HCV in this group has been estimated to be over 80% [26]. Over 50 000

people have been diagnosed with HCV infection in Sweden since 1990, correlating to a seroprevalence of 0.5%, compared to over 2% in other Western European countries [16, 27]. It has been estimated that approximately 20% of HCV infected persons in Sweden have an undiagnosed infection [28].

2.3   NATURAL HISTORY OF HCV INFECTION

The incubation period for HCV infection is 2-6 months, but only 20% of HCV infected patients develop a clinical acute hepatitis with jaundice. The majority of acute HCV infections are instead subclinical and anicteric. Approximately 15-25% of HCV infected patients will clear the infection spontaneously and the rest develop chronic hepatitis C, defined as persisting HCV RNA after more than six months [29]. Young patients, women and patients with symptomatic acute hepatitis have a much higher rate of spontaneous clearance and the risk for chronic infection for these groups is only 55-60% [30].
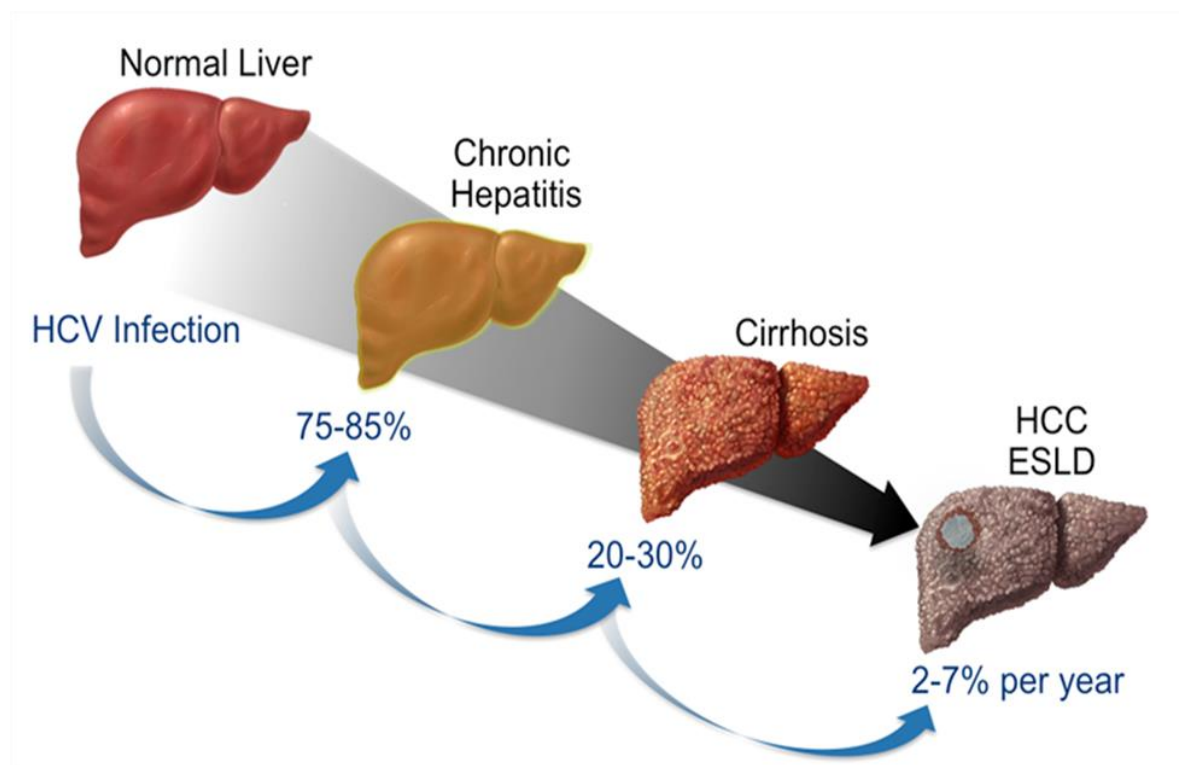
Figure 3: *The natural history of HCV infection. HCC=hepatocellular carcinoma, ESLD=end stage liver disease. From Hepatitis C Online: http://www.hepatitisc.uw.edu, with permission.*

Patients who develop chronic HCV infection are at risk for continued hepatic inflammation and fibrosis that will progress slowly over decades. After 20-40 years of chronic HCV infection, 20% of patients will have developed advanced liver disease with cirrhosis or hepatocellular carcinoma(HCC) [30]. Disease progression is not linear and the risk for

advanced liver disease increases with older age [31]. The risk for liver-related complications, HCC and liver-related death is low for patients with mild fibrosis, but increases with the stage of fibrosis. The annual risk to develop HCC for patients with chronic hepatitis C and cirrhosis is approximately 4% in the Western world, while higher estimates have been reported in Eastern Asia [32, 33]. Patients with advanced fibrosis, who have not yet developed cirrhosis, also seem to have a risk to develop HCC, with reported annual risks of 0.8-3.4% [34, 35]. Current guidelines from The European Association for the Study of the Liver (EASL) therefore recommend HCC surveillance with ultrasound every 6 months for patients with HCV infection and advanced fibrosis or cirrhosis [36]. Because of this, accurate measurement of hepatic fibrosis is important for the clinical management of patients with chronic HCV infection.

2.4 MEASUREMENT OF LIVER FIBROSIS

The gold standard for measuring the stage of liver fibrosis is by liver biopsy. A liver biopsy also provides information on hepatic necroinflammatory activity. It is, however, an invasive method, associated with pain and potentially serious complications. The most widely accepted scoring systems for assessment of liver fibrosis and necroinflammation are the Ishak and METAVIR scores [37, 38]. Both scoring systems measure fibrosis and inflammation separately, but use different scales. The METAVIR system describes five stages of fibrosis, with stage F0 correlating to absence of fibrosis and stage F4 to cirrhosis. The grade of inflammation is similarly divided into four steps, A0-3.
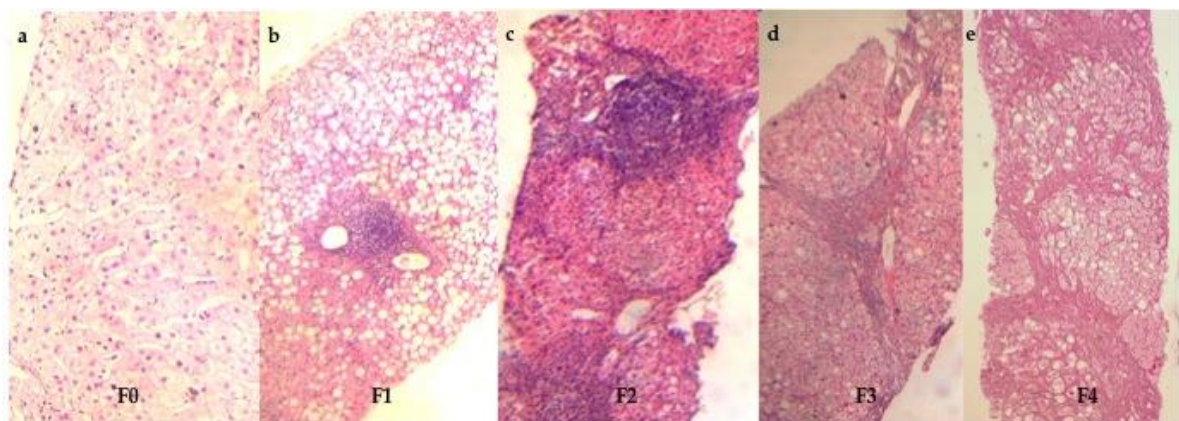

Figure 4. *Fibrosis stages F0-F4 according to the METAVIR system: (a) No fibrosis, F0. (b) Portal and periportal fibrosis, F1. (c) Periportal fibrosis with few septa, F2. (d) Septal fibrosis and bridging without cirrhosis, F3. (e) Cirrhosis, F4.*

A non-invasive alternative to liver biopsy is transient elastography. This method measures liver stiffness by ultrasound and the result is a composite variable measuring both liver fibrosis and inflammation. The method is validated for the diagnosis of significant fibrosis and cirrhosis in chronic hepatitis C [39]. An advantage of transient elastography is that since the outcome is a continuous variable, it is possible to stratify the degree of cirrhosis within the cirrhotic group and to correlate this to clinical outcome [40]. A drawback of transient elastography is that it will also measure increased liver stiffness due to other causes, such as recent food-intake, congestive heart disease and acute hepatitis. The method is also less validated for the assessment of residual liver fibrosis after successful treatment of chronic hepatitis C [41].
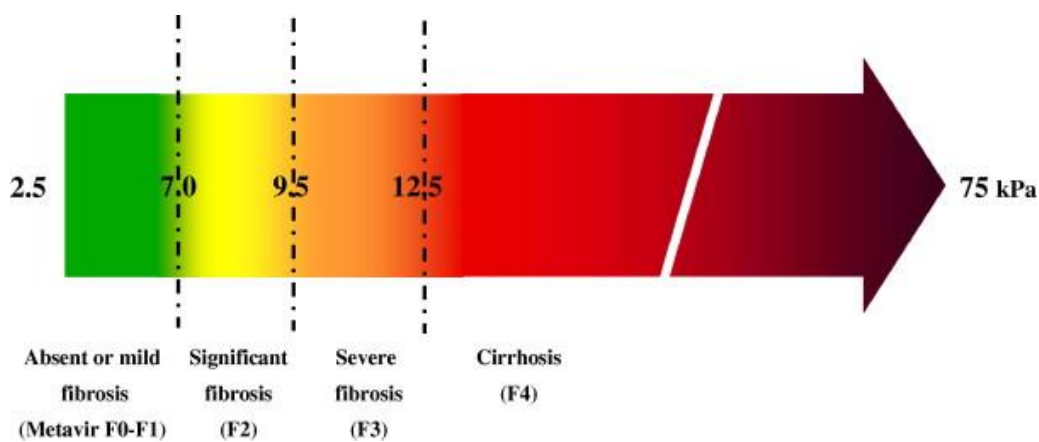
Figure 5: *Clinical cut-offs for liver stiffness measurements and corresponding fibrosis stage according to the METAVIR scoring system. From Castera L et al, Journal of Hepatology 2008: http://dx.doi.org /10.1016/j.jhep.2008.02.008, with permission.*

Another method to describe the severity of liver cirrhosis is by the Child-Pugh score, which is also used to assess the prognosis for patients with liver cirrhosis. This score combines biochemical markers (serum albumin, bilirubin and INR) and clinical complications (ascites and hepatic encephalopathy) to calculate a score of 5-15 points. The results are then grouped as Child-Pugh class A, B and C with increasing severity and reduction of life-expectancy [42].

2.5   HCV IMMUNOLOGY

Within 1-2 weeks after infection with HCV the patient becomes viraemic and the innate immune system reacts with an interferon (IFN) driven response to control viral replication [43, 44]. This response is not able to clear the infection and after a delay of 1-2 months the

adaptive immune system responds by recruiting HCV specific T-cells to the liver [43, 45]. The adaptive immune response consists of neutralizing antibodies and HCV specific cytolytic and non-cytolytic T-cell responses [46]. A dominating IFN-γ mediated non-cytolytic response correlates strongly with viral clearance and normalized liver blood tests, but this occurs in less than a third of acute HCV infections [43].

In patients that develop chronic infection, HCV somehow evades the adaptive immune response, and in many cases instead stimulate an ineffective IFN-mediated innate immune response in the liver [47]. This response has been associated with a genetic polymorphism in the IL28B gene, which is also associated with a poor response to IFN based antiviral therapy [48, 49].

2.6   TREATMENT OF HCV INFECTION

The goal of HCV treatment is virological cure in order to prevent HCV related complications such as fibrosis, cirrhosis, decompensated liver disease, hepatocellular carcinoma and death [7]. Virological cure is defined as undetectable HCV RNA 12-24 weeks after end of antiviral treatment, and is usually described as sustained virologic response or SVR [50]. The first attempts to treat HCV infection with recombinant human alpha-interferon were performed before the virus could be isolated and the clinical endpoint at this time was normalized serum aminotransferase levels [5]. Early attempts to treat HCV infection with ribavirin in monotherapy, led to high rates of clinical relapse [51, 52]. The combination of alpha-interferon and ribavirin, and later the development of pegylated interferon improved treatment results substantially with SVR rates of 40-80%, depending on HCV genotype and the stage of liver fibrosis [53-55]. However, IFN-based treatments were also associated with substantial side-effects, such as flu-like symptoms, depression, anemia and autoimmune hepatitis. Several patient groups with co-morbidities were therefore excluded from treatment. Patients with cirrhosis had a much lower chance of SVR, and patients with advanced cirrhosis tolerated IFN-based treatment poorly due to a risk for hepatic decompensation and infection [6]. As a consequence, patients with cirrhosis had both a high risk of liver-related complications and a low rate of SVR.

Since 2011, new highly effective antivirals, directly targeting viral proteins involved in replication such as NS3/4A protease inhibitors, NS5A inhibitors and NS5B polymerase inhibitors, have further improved treatment results. These direct-acting antivirals (DAAs) allow interferon-free treatment with SVR rates > 95% for all patients, regardless of genotype and stage of liver fibrosis [56-59]. The side-effects are generally mild and not frequent. As a result more patients with more advanced liver disease will achieve SVR in the coming years, and questions about how this will affect their clinical outcome becomes more important.

3 EFFECT OF SVR

3.1 LIVER-RELATED COMPLICATIONS, HCC AND MORTALITY

Sustained virologic response (SVR) is only a surrogate marker for the clinical end-point of successful antiviral treatment of chronic HCV. The ultimate goal of HCV treatment is to reduce the risk for liver-related complications, HCC and death [7]. Several studies have shown that the risk for liver-related complications and HCC decreases after SVR has been achieved [60-64]. A meta-analysis in 2010 estimated that achieved SVR reduces the risk for HCC in patients with HCV associated advanced liver disease by 68% (relative risk (RR) 0.32, 95% confidence interval (CI) 0.23-0.44), and the risk for decompensated liver disease by 87% (RR 0.13, 95% CI 0.06-0.27) [65]. Later studies have confirmed that the risk for liver-related complications, such as esophageal varicosal bleeding and hepatic decompensation after SVR is very low, but also that a risk for HCC remains [35, 66-68]. With longer follow-up time and growing cohorts of patients with SVR we now also know that SVR is associated with reduced liver-related mortality and longer over-all survival [68, 69].

The risk to develop HCC after SVR and risk factors associated with HCC development has been investigated in several studies on different populations. The largest studies have been performed in Asian cohorts, where the risk to develop HCC is generally higher and not as strongly associated with cirrhosis [70]. As a consequence these studies have included patients with all stages of liver fibrosis and only a small proportion of patients with pre-treatment cirrhosis. Almost all of these studies have associated the risk to develop HCC with more advanced fibrosis and higher age before treatment [60-62, 71-75]. Other risk factors, such as male gender, alcohol use, diabetes mellitus, liver steatosis and different biochemical markers of liver disease have been associated with a risk to develop HCC in some studies, but not in others [71, 73, 75-77]. The results from these studies are not automatically applicable on patients from Western cohorts with other environmental and genetic backgrounds.

In Western patients there is a strong association between advanced liver disease and the risk to develop HCC [70]. Studies on the risk to develop HCC after SVR in Western cohorts have therefore included a high proportion of patients with cirrhosis before treatment. As a consequence the number of patients with SVR included has been lower than in Asian studies, and it has been difficult to identify risk factors associated with the risk to develop HCC after SVR.

It is also not known if the risk to develop HCC after SVR decreases over time. Case reports of patients that develop HCC more than 17 years after SVR showed that the risk can persist for many years [78]. Current guidelines therefore recommend continued HCC surveillance with ultrasound every six months in high risk groups also after SVR has been achieved [36].

Author	Country	N= (total/SVR) (F4)	Follow-up time (years)	Cumulative incidence or IR of HCC $_{SVR}$	Cumulative incidence or IR of HCC $_{non-SVR}$	Risk factors for HCC in patients with SVR
Yoshida [60]	Japan	2392/836 (9%)	Mean 6.7-7.4	IR 0.57	IR 2.16	↑fibrosis, ↑age
Makiyama [61]	Japan	3626/1197 (2%)	Mean 5.9	2.3	8.8	↑fibrosis, ↑age, male
Iwasaki [71]	Japan	792/792 (2%)	Median 5.1	2.9	-	↑fibrosis, ↑age, alcohol
Kobayashi [62]	Japan	1124/373 (?)	Median 5.5	3.5	8.1	↑fibrosis, ↑age, male
Hirakawa [72]	Japan	1193/1193(3%)	Median 8.3	1.9	-	↑fibrosis, ↑age, male
Tanaka [73]	Japan	266/266(3%)	Mean 9.9	2.6	-	↑fibrosis, ↑age, ↑steatosis
Chang [74]	Taiwan	871/871 (28%)	Median 3.4	4.2	-	↑fibrosis, ↓platelets, ↑age, ↑
Asahina [76]	Japan	1818/913 (?)	Mean 6.1	2.3	6.9	↑alanine transaminase, ↑α-fetoprotein
Hung [77]	Taiwan	1470/1027 (28%)	Median 4.4	3.2	12.2	Diabetes in patients without cirrhosis
Huang [75]	Taiwan	642/642 (13%)	Median 4.4	IR 0.68	IR 4.54	F4, ↑age, ↑γ-glutamyl transferase
Veldt [63]	Europe/Canada	479/142 (75%)	Median 2.1	IR 1.07	IR 2.77	Not assessed
Bruno [64]	Italy	883/124 (100%)	Mean 8.0	IR 0.66	IR 2.10	Not assessed
Cardoso [35]	France	307/103 (59%)	Median 3.5	IR 1.24	IR 5.85	Not assessed
Morgan [67]	USA	526/140 (35%)	Median 6.6-7.2	1.4	8.5	Not assessed
Van der Meer [68]	Europe/Canada	530/192 (73%)	Median 8.4	IR 0.55	IR 2.63	Not assessed

Table 1: *Summary of studies investigating the risk for HCC after achievement of SVR in patients with HCV. The risk is described either as a cumulative incidence or an incidence rate (IR). The table only includes risk factors for HCC in patients with SVR.*

3.2 LIVER FIBROSIS

Another important question is whether fibrosis and cirrhosis can regress after successful antiviral treatment of chronic HCV infection or not. To answer this, several studies with varying follow-up times have compared liver fibrosis by different methods before and after achievement of SVR [41, 79-87].

Studies based on liver biopsies have all shown that fibrosis and cirrhosis can improve after SVR has been achieved in a majority of patients, but also that fibrosis will persist or progress after SVR in a subset of 1-14% [79-83]. The studies with the shortest mean follow-up times reported the highest rate of fibrosis progression, whereas studies with longer mean follow-up times reported lower rates of 1-7%. In the largest study, with 3010 patients of which 1094 achieved SVR, cirrhosis regressed in 75 of 153 patients (49%) and fibrosis progressed in 7% of patients with SVR. Mean follow-up time was 20 months, and factors strongly associated

with absence of significant fibrosis after treatment was achieved SVR, lower baseline fibrosis stage, age below 40 and a BMI below 27 [79].

Due to the risks associated with liver biopsies, transient elastography and biochemical markers have been used to evaluate liver fibrosis after SVR in more recent studies [84, 88]. In a cohort study with 10 years follow-up time, 993 patients were evaluated with paired Fibrotest (a score based on biochemical markers) and transient elastography measurements. Of 171 patients with SVR, 43 had pre-treatment cirrhosis, and of these 56% regressed to lower stages of fibrosis. Among patients without pre-treatment cirrhosis 12% progressed to cirrhosis during follow-up [88].

The diagnostic accuracy of these non-invasive methods to detect persisting cirrhosis after SVR has been questioned [41, 84]. In a study comparing liver stiffness measurements with follow-up biopsies 61 months after SVR had been achieved, the sensitivity of transient elastography to detect cirrhosis was only 61% when standard pre-treatment cut-offs were used [41]. The combination of transient elastography and biochemical markers did not improve the sensitivity and biochemical markers alone failed to predict persisting fibrosis [84].

Recent published studies with repeated liver stiffness measurements up to two years after SVR have shown a rapid initial improvement of the liver stiffness followed by a slower or plateauing phase of fibrosis improvement [85-87]. Since liver elastography measures both fibrosis and inflammation, early liver elasticity improvement after SVR could be explained by a rapid reduction of the liver inflammation and less pronounced fibrosis regression. Liver stiffness, however, continued to improve after treatment cessation in patients who achieved SVR but not in non-responder patients. This would correlate better to true fibrosis regression [87].

3.3   OCCULT HEPATITIS C

Negative HCV RNA 12-24 weeks after the end of antiviral treatment for chronic HCV infection is considered to be equivalent with virologic cure. However, several groups have described the presence of HCV RNA traces in serum, peripheral blood mononuclear cells (PBMCs) or liver tissue in patients who have achieved SVR, suggesting that these patients may not have completely eradicated the virus [89-91]. Furthermore, residual HCV RNA has been reported to increase the risk for necroinflammatory activity and fibrosis in the liver, indicating that residual RNA may be involved in liver disease development [92]. This has raised the concept of occult HCV infection, which could contribute to progressive liver disease after SVR and viral reactivation in immunosuppressed patients. Viral reactivation is, however a rare event, and there is so far no evidence of progressive fibrosis due to residual HCV RNA [82].

4 AIMS

The overall aim was to study the long-term clinical, histological and virological outcome after sustained virologic response (SVR) in patients with chronic hepatitis C.

4.1 SPECIFIC AIMS

1   To study if HCV RNA can persist in liver tissue or immune cells after successful treatment of chronic HCV infection, and if this correlates to clinical, immunological or histological outcomes.

2   To study the long-term effect of antiviral therapy on the risk to develop HCC, liver complications, and liver-related death in patients with HCV related liver cirrhosis.

3   To study the long-term risk to develop HCC after SVR has been achieved in patients with pre-treatment advanced fibrosis or cirrhosis and to identify risk factors associated with an increased risk for HCC.

4   To study fibrosis regression and risk factors for persisting fibrosis after SVR has been achieved in patients with pre-treatment advanced fibrosis or cirrhosis.

5 MATERIALS AND METHODS

5.1 PATIENTS AND STUDY DESIGN

The four studies of this thesis all included patients with chronic hepatitis C cared for at university hospitals in Sweden. Almost 700 individual patients from seven university hospitals in Stockholm, Gothenburg, Linkoping, Lund, Malmo and Uppsala were included. The vast majority of the patients were from Karolinska University Hospital, either in Solna or Huddinge. There was a substantial overlap of patients between studies. The included patients in the different studies are described in detail below.
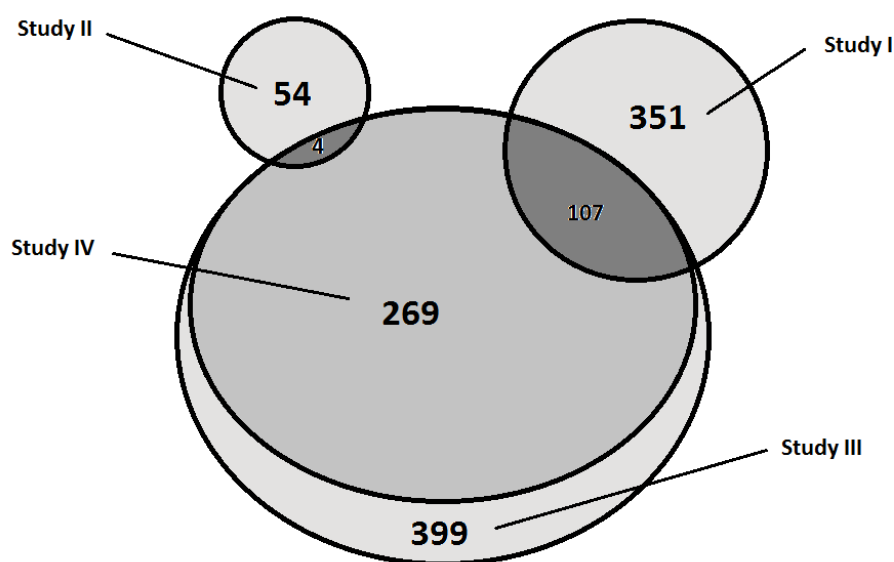

Figure 6: *Overview of the included patients of the different studies.*

5.1.1 Study I

In this cohort study, patients with HCV-related cirrhosis were followed prospectively to assess the effect of antiviral treatment on clinical outcomes. The risk for HCC, liver-related complications, and liver-related and overall mortality was compared for patients with or without sustained virologic response to antiviral treatment and patients never treated for HCV infection. A total of 351 patients were included, and of these 303 were treated for HCV infection during the study period. In total 110 patients achieved SVR. Patients were recruited from the HCV Cirrhosis Registry, a research data base that consecutively included patients with a diagnosis of HCV-related liver cirrhosis at six university hospitals between January 2001 and July 2009. Patients with decompensated cirrhosis, hepatocellular carcinoma, liver transplantation or hepatitis B or HIV co-infection at baseline were excluded. The majority of patients were recruited at the Department of Infectious Diseases and the Department of

Gastroenterology and Hepatology at Karolinska University Hospital. Lund University Hospital, Malmö University Hospital, Sahlgrenska University Hospital in Gothenburg and Uppsala University Hospital also contributed to the study. The baseline characteristics of the included patients are described in Table 1 of paper I.

5.1.2 Study II

In this cross-sectional study we investigated the possibility and clinical significance of residual HCV RNA after successful treatment of HCV (occult HCV infection). Patients with chronic HCV infection with achieved SVR after treatment with peg-interferon alpha-2b +/- ribavirin at least five years prior to inclusion were offered a clinical follow-up visit with a new liver biopsy. The liver biopsy, PBMC and serum were analyzed for residual HCV RNA with a highly sensitive method. The presence of residual HCV RNA was correlated to clinical, histological, immunological and biochemical outcomes. Between 2008 and 2010 we included 54 patients with all stages of pre-treatment liver fibrosis from five centers at Karolinska University Hospital and the University Hospitals in Linköping, Gothenburg and Uppsala. The baseline and follow-up characteristics of the included patients are described in Table 1 in paper II.

5.1.3 Study III

In this cohort study we investigated the long-term risk to develop HCC after SVR had been achieved in patients with pre-treatment advanced fibrosis (METAVIR F3) or cirrhosis (F4). We also aimed to identify risk factors associated with an elevated risk to develop HCC. The study included all patients with fibrosis stage F3 or F4 successfully treated for chronic HCV infection at Karolinska University Hospital between 1992 and 2013. Patients were identified from treatment records, the InfCare Hepatitis research database and the Hepatitis C Cirrhosis registry, as mentioned previously. Patients with HCC or liver transplantation before treatment or co-infection with hepatitis B virus (HBV) or human immunodeficiency virus (HIV) were excluded. The study ended in September 2015 and included a total of 399 patients. There was a significant overlap (107 patients) with the cohort from study I and some overlap (4 patients) with study II. The baseline characteristics of the included patients are described in Table 1 in paper III.

5.1.4 Study IV

In this cross-sectional study we investigated fibrosis regression and risk factors for persisting fibrosis after SVR had been achieved in the same cohort as in study III. Patients were offered a clinical follow-up visit with a liver stiffness measurement (transient elastography). At this visit potential risk factors for persisting fibrosis, such as age, body mass index (BMI), alcohol

consumption and presence of diabetes mellitus were recorded. Inclusion ended in October 2015 and a total of 269 patients were successfully examined at a follow-up visit. Patients who had developed HCC, died or undergone liver transplantation before inclusion were excluded (n=36), and the remaining patients were lost to follow-up (n=82) or had unreliable liver stiffness measurements (n=15). The baseline characteristics of the included patients are described in Table 1 in paper IV.

5.1.5 HCV treatment

The standard of care therapy for chronic HCV infection has changed over the years covered by these studies and ranges from interferon monotherapy to combination therapy with pegylated interferon, ribavirin and direct acting antivirals (DAAs) [5, 93]. However, during the whole period interferon or pegylated interferon has been the back-bone of HCV treatment, and the last patients included in these studies achieved SVR in 2013 and were still treated with peg-interferon based therapies. Only a small sub-set of patients (< 10%) received combination therapy with DAAs. In all studies SVR was defined as a negative HCV RNA 24 weeks after end of treatment.

5.2 METHODS

5.2.1 InfCare Hepatitis

The InfCare Hepatitis database is a national quality registry and research database that includes information on demographics, histology, virology, biochemistry and treatment history and outcomes for patients with chronic hepatitis B and C. The database has excellent coverage of patients treated at Karolinska University Hospital, and was used to identify patients, eligible for inclusion in the studies, and also to retrieve relevant data. Other registries such as the HCV Cirrhosis registry described previously and treatment records were also used to minimize loss to follow-up.

5.2.2 Virological methods

Routine HCV RNA analysis was performed using COBAS AmpliPrep/COBAS TaqMan HCV (Roche Diagnostics, Mannheim, Germany) with a sensitivity of at least 15 IU / ml. The HCV genotype was determined with a line probe assay (Inno-LiPA HCV II, Immunogenetics NV, Gent, Belgium).

In study II we used a highly sensitive method to detect minute amounts of HCV RNA in liver biopsies, PBMC and plasma. Liver biopsies and PBMCs (10^6 cells per patient) were homogenized and plasma was concentrated 40-fold by ultracentrifugation. The extracted RNA was then amplified in a nested polymerase chain reaction (PCR) using two sets of

primers in two successive reactions. The specificity of the end product was confirmed by restriction digest (ThermoFisher Scientific) and sequencing of the final PCR products (MWG, Ebersberg, Germany). The method is described in greater detail in paper II.

5.2.3 Immunological methods

Serologies for HBV, HCV and HIV were performed at local laboratories using routine methods.

In study II we measured NS3 specific antibody titers by an immunosorbent assay using serially 6-fold titrated plasma starting with a dilution of 1:60. We also measured the cross-neutralizing capacity of antibodies purified from patient plasma to neutralize HCV of a non-matching genotype (5a) [94].

Hepatitis C specific T-cell responses were measured by enzyme-linked ImmunoSpot (ELISpot) and pentamer staining. We incubated PBMC with recombinant viral proteins (NS3, NS4, NS5a, NS5b and core), and detected IFN-γ producing cells by ELISpot (MabTech, Nacka Strand, Sweden and Autoimmun Diagnostica, Strassberg, Germany). NS3-specific CD8$^+$ T-cells were detected by pentamer staining and flow cytometry. The methods are described in detail in paper II.

5.2.4 Biochemical methods

Biochemistry was performed at local laboratories using routine methods.

Fibrosis indices were calculated using the AST to platelet ratio index (APRI) score: AST(μkat/l) / AST$_{ULN}$ (upper limit of normal for AST(μkat/l)) x 100 / platelet count (10^9 /l) and Gothenburg University cirrhosis index (GUCI) score: AST(μkat/l) / AST$_{ULN}$ (μkat/l) x 100 / platelet count (10^9 /l) x PT-INR [95, 96].

5.2.5 Histological methods and assessment of liver fibrosis

Pre-treatment liver fibrosis stage was determined by the original assessment of a pre-treatment liver biopsy by a pathologist according to the METAVIR staging system [38]. From 2008 most patients were instead evaluated with transient elastography and pre-treatment fibrosis stage for these patients was estimated using established clinical cut-offs for METAVIR stages [39]. A clinical diagnosis of liver cirrhosis was also accepted for inclusion.

Liver stiffness measurements by transient elastography were performed with FibroScan (Echosense, Paris, France), using the M and XL probes, as appropriate. Only examinations with 10 valid measurements, a success rate of at least 60% and an inter-quartile range of less than 30% of the median result were considered valid.

In study II we performed new liver biopsies on the included patients at the follow-up visit. Liver biopsies were performed by the Menghini technique [97]. The pre-treatment and follow-up biopsies were re-assessed according to the METAVIR staging system by Magnus Hedenstierna and an experienced pathologist. We were blinded as to whether the biopsies were and performed before or after successful treatment.

5.2.6 Diagnosis of hepatocellular carcinoma

The diagnosis of HCC was based on a verified focal liver lesion by imaging techniques in accordance with the EASL and AASLD (American Association for the Study of Liver Diseases) guidelines [36, 98].

In study III we decided to use an unorthodox method to estimate the true date of HCC development. Many of the patients in this study were diagnosed with HCC late due to symptoms, instead of by routine HCC surveillance with ultrasound. Because of this, the true date of HCC development was arguably months to years before the date of diagnosis. We back-dated the time point of HCC diagnosis for all patients not diagnosed by routine surveillance, based on the assumption that a liver tumor will double its size in 6 months and that the smallest detectable tumor size is 1 cm [99].

5.2.7 National registries

The Swedish national health registries include the National Causes of Death Register, the National Cancer Register and the National Patient Register. These registries are continually updated and contain information on causes of death, cancer diagnosis and inpatient and specialized outpatient care. The registries have an estimated coverage of 95-99% [100-102].

We extracted data from the Swedish national health registries to obtain complete data on co-morbidities and clinical outcomes for all patients included in our studies, regardless of if they were still followed-up at the clinics participating in the studies. In this way we tried to minimize loss to follow-up and misclassification.

5.2.8 Statistical methods

In the two cohort studies (I and III) we calculated hazard ratios (HRs) using univariate and multivariate Cox regression analysis to determine the association between baseline factors (treatment, age, sex, BMI, diabetes, alcohol consumption, genotype etc.) and the risk to develop HCC. Kaplan-Meier curves and incidence rates and ratios (IRs and IRRs) were calculated.

In study II we used the Mann-Whitney U-test to compare baseline and follow-up biochemistry and histology. The Spearman's rank test was used to compare the distribution of antibody titers and IFN-γ spots for different follow-up periods.

In study IV we used quantile regression to determine the impact of different risk factors on liver stiffness at follow-up and logistic regression to determine risk factors for persisting advanced fibrosis. The Kruskal-Wallis rank test for equality of populations was used to compare the distribution of fibrosis stages between different follow-up periods.

All tests were 2-sided and a p-value of < 0.05 was considered significant. Statistical analysis was done with SAS 9.2 (SAS Institute, Cary, North Carolina), InStat 3 and GraphPad Prism (GraphPad Software Inc, La Jolla, California) and STATA 13.1 (StataCorp, College Station, Texas).

5.2.9 Ethics

All studies were performed in accordance with the Helsinki declaration and were approved by the Regional Ethics Committee.

Dnr. 01-232 with amendments 2008/2054-32 and 2012/979-32 for studies I, III and IV.

Dnr. 2008/157-31/1 for study II.

6 RESULTS AND DISCUSSION

6.1 THE CLINICAL RELEVANCE OF OCCULT HEPATITIS C (PAPER II)

To investigate the possible presence and clinical relevance of occult HCV infection, 54 patients with sustained virologic response at least 5 years prior to inclusion were examined at a clinical follow-up visit with a new liver biopsy. The included patients were evaluated for virological, histological, immunological and biochemical outcomes. Median follow-up time was 9.8 years (range 5-20 years).

6.1.1 The presence of residual HCV RNA

All included patients tested negative for HCV RNA by routine methods. Liver biopsies, plasma and PBMC were analyzed for minute amounts of HCV RNA using a highly sensitive method as described previously. All patients were negative for HCV RNA in liver biopsies and plasma, but three patients (6%) were positive for HCV RNA in PBMC after 8, 5 and 9 years of follow-up. These three patients had different HCV genotypes (3a, 1a and 1b) excluding the possibility of contamination. One patient had different genotypes before and after treatment (2b and 3a), suggesting a double infection or possibly a reinfection with spontaneous clearance. To investigate if the detected HCV RNA persists over time the three patients were re-tested in PBMC after another 4-5 years, and at this time-point all three were negative.

6.1.2 Signs of persisting liver disease

None of the included patients had clinical signs of liver disease at follow-up, and biochemical liver tests and fibrosis indices had improved significantly or normalized after successful treatment. Two of the three patients with detectable HCV RNA had normal liver tests at follow-up and one had slightly elevated liver enzymes (ALT 1.06 µkat/L and AST 1.18 µkat/L).

Paired liver biopsies (baseline and follow-up) were available for 39/54 patients (72%). Liver histology (both liver fibrosis and inflammation) had improved significantly ($p < 0.0001$) at follow-up and none of the patients had progressed to a higher stage of liver fibrosis. Mean liver stiffness measured with FibroScan was 4.9 kPa (range 2.4-8.8 kPa) confirming the histological evaluation. For two of the patients with detectable HCV RNA, follow-up liver biopsies were available and showed no signs of inflammation or fibrosis (F0 and A0). For the third patient no follow-up biopsy was available, but median liver stiffness measured with FibroScan was 4.7 kPa corresponding to a fibrosis stage of F0-1.
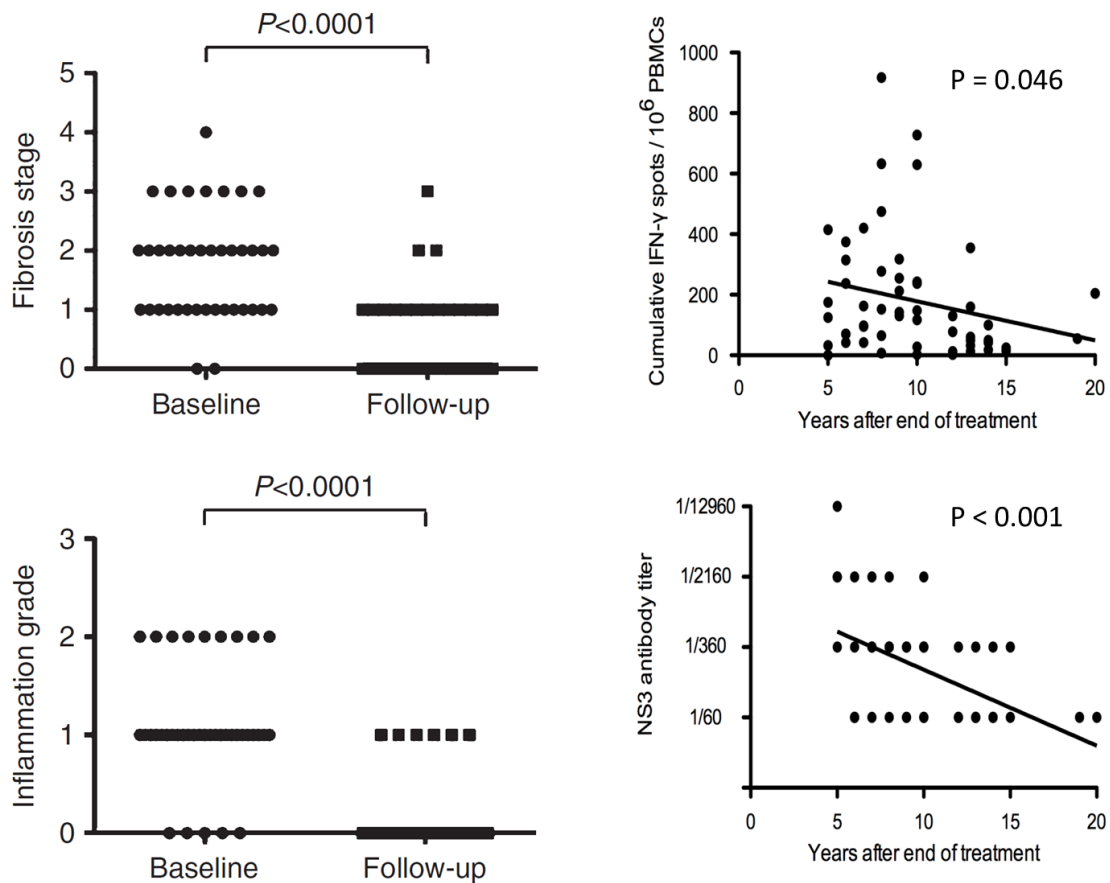
Figure 7: *Histological and immunological outcomes. Paired liver biopsies were available for 39/54 patients. Significance was tested with the Mann-Whitney U-test and the Spearman rank-test.*

6.1.3 HCV specific immune responses

HCV specific NS3 specific antibody titers of at least 1/360 could be detected up to 15 years after SVR. There was an inverse correlation between titer levels and follow-up time after SVR ($p < 0.001$), indicating waning immune response after viral clearance. The three patients with detectable HCV RNA at follow-up had differing NS3 antibody titers of 1/60, 1/2160 and 1/2160, suggesting that there is no clear correlation between residual HCV RNA and persisting humoral immune response. The follow-up times of these three patients were below median in the study which could contribute to the high titers measured in two patients, but continued antigenic stimulation by residual HCV RNA is also possible.

HCV specific T-cell immune responses were measured by IFN-γ ELISpot. The majority of patients 39/54 (72%) had at least 50 cumulative IFN-γ spots per 10^6 cells up to 20 years after SVR. As with humoral immune responses there was an inverse correlation between time after SVR and the level of immune response ($p < 0.046$). The patients with detectable HCV RNA had 130, 153 and 415 cumulative IFN-γ spots per 10^6 cells, which was comparable to other patients with similar follow-up times.

6.1.4 Discussion

This study was unique in its broad approach to the effect of SVR on histological, virological and immunological outcomes, making it possible to evaluate the clinical relevance of occult HCV infection (residual HCV RNA in PBMC after SVR).

Three patients (6%) tested positive for minute amounts of HCV RNA in PBMC, and this corresponds well to results from previous studies that have analyzed the prevalence of residual HCV RNA at one time point [103, 104]. However, in a longitudinal study, in which PBMCs were repeatedly tested 2-3 times with 3-6 month intervals, all patients (n=11) tested positive for residual HCV RNA at least once [90]. In another large study of 150 patients, residual HCV RNA could be sporadically detected up to 8 years after SVR but not thereafter [105]. The three patients in our study all had follow-up times below median in the study (5, 8 and 9 years), and were all negative when re-tested after a further 4-5 years, suggesting that occult HCV is a transient state.

Some studies have reported that it is possible to detect negative sense HCV RNA in PBMCs, indicating continued low-level replication in PBMCs after SVR has been achieved [91, 106]. In our study only positive sense HCV RNA was detected, and other studies have shown that HCV RNA can form immune-complexes with immunoglobulins and bind to the surface of PBMCs [107, 108]. This suggests that residual HCV RNA associated with PBMCs does not necessarily replicate in PBMCs, but could merely be bound to the surface of some immune cells.

Chronic HCV infection has been associated with depletion of HCV specific T-cell immune responses [109]. This has also been observed in patients with occult hepatitis C [110]. In our study there was no such correlation and instead we found an inverse correlation between immune responses and follow-up time after SVR, indicating that successful treatment equals cure. This conclusion was further supported by the lack of clinical, histological and biochemical signs of persisting liver disease. Since it is quite possible to become re-infected with HCV, it seems unlikely that persistent replicating HCV infection is possible without any signs of active hepatitis.

6.2 TREATMENT AND CLINICAL OUTCOMES (PAPER I & III)

In studies I and III we investigated the effect of HCV treatment on clinical outcomes, such as liver-related complications, HCC and death. Study I compared treated and untreated patients with cirrhosis, whereas study III focused on patients with achieved SVR, but included both patients with advanced fibrosis (METAVIR F3) and cirrhosis (F4). A total of 643 patients were included in these two cohort studies with some overlap of patients as described previously. Mean follow-up time was 5.3 years for study I and 8.4 years for study III, with follow-up times ranging from less than one year up to 8.6 and 23 years respectively.

6.2.1 Liver related complications, hepatocellular carcinoma and death

We retrieved data on clinical outcomes from patient records and the Swedish national health registries as described previously. In this way the risk for misclassification and loss to follow-up was minimized. In study I incidence rates for liver-related complications (ascites, esophageal varicosal bleeding and hepatic encephalopathy), HCC and all-cause and liver-related mortality were calculated. Study III focused on the risk to develop HCC but also included data on all-cause mortality.

The incidence rates for liver related complications and liver-related death was significantly lower for patients who achieved SVR with 0.9 and 0.7 events per 100 person-years, compared to 3.2 and 3.0 for non-responder patients and 4.9 and 4.5 for untreated patients.

All-cause mortality was highest among untreated patients with cirrhosis, with a mortality rate of 5.1 per 100 person years. This risk decreased successively for patients that received treatment, achieved SVR and was lowest (0.5 per 100 person years) for patients with SVR and pre-treatment F3 fibrosis.

The risk to develop HCC followed the same pattern with a high risk of 5.1 per 100 person-years for untreated patients with cirrhosis, 2.3 for treated non-responder cirrhotic patients and 1.0 for patients with cirrhosis and SVR. The lowest risk (0.16 per 100 person years) was again observed in patients with pre-treatment F3 fibrosis and SVR.

Group	N	Follow-up time for HCC	HCC	All-cause mortality	Liver-related mortality	Liver-related complications
F4 (untreated)	48	347 py	5.1	5.1	4.5	4.9
F4 (non-SVR)	193	1129 py	2.3	4.1	3.0	3.2
F4 (SVR), (study I)	110	589 py	1.0	1.8	0.7	0.9
F4 (SVR), (study III)	180	1467 py	0.95	1.7	nd	nd
F3 (SVR)	219	1899 py	0.16	0.5	nd	nd

Table 2: *Clinical outcomes for the different patient groups included in studies I and III. Follow-up time differed for different outcomes and is presented as person-years (py) for hepatocellular carcinoma (HCC). Incidence rates are presented as events per 100 py.*

In both studies the risk to develop HCC over time was analyzed. We divided the follow-up time to achieve similar numbers of clinical events in each time period. This resulted in a cut-off at 3 years in study I and at 2 years in study III. In study I none of the studied outcomes decreased significantly after 3 years of SVR, but there was a trend for a decreased risk of any event after 3 years (p=0.05). Study III had a larger cohort and longer follow-up time. In this study the risk to develop HCC decreased significantly from 2 per 100 person years to 0.6 per 100 person years after 2 years follow-up (p=0.039).

6.2.2 Risk factors for the development of HCC

We investigated the impact of potential risk factors at baseline on the risk to develop HCC after achieved SVR by Cox-regression analysis. Only six cases of HCC occurred in study I and the results from this study should be interpreted with caution. However, age over 50 years at SVR and male gender were significantly associated with a higher risk to develop HCC, with hazard ratios (HRs) of 2.45 (95% CI 1.16-5.61, p=0.02) and 2.09 (95% CI 1.06-4.62, p=0.047). Oher risk factors included in the analysis were alcohol consumption (<50 or >50 g/day), diabetes mellitus, and HCV genotype.

In the larger study (III) of patients with SVR, 17 cases of HCC were diagnosed during follow-up. Only three cases occurred among patients with pre-treatment F3 fibrosis and we decided to exclude all patients without cirrhosis from the final regression model. We included age at SVR, gender, BMI, diabetes mellitus, alcohol abuse, liver steatosis, HCV genotype and baseline platelet count and serum albumin in the analysis. Only the presence of diabetes mellitus and serum albumin <35 g/dL remained significant in the multivariate analysis with HRs of 6.26 (95% CI 1.7-23, p=0.006) and 6.23 (95% CI 1.6-25, p=0.01). As indicated by the broad confidence intervals, there was a degree of statistical uncertainty due to few HCC cases also in this study.

We constructed a Kaplan-Meier graph for the cumulative incidence of HCC among patients with pre-treatment cirrhosis, stratified for the main risk factor diabetes mellitus (DM). The graph shows that the high incidence rate of HCC during the first two years after SVR is most pronounced in patients with diabetes mellitus, and that patients without DM have a lower and more stable incidence rate of HCC.
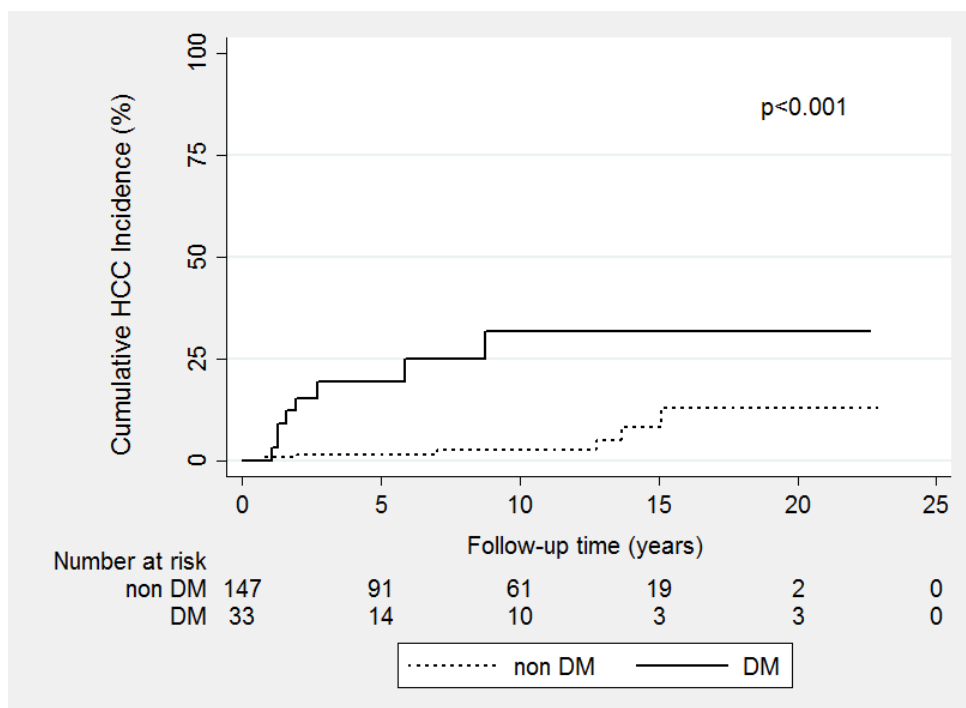

Figure 8: *Kaplan-Meier graph for the cumulative incidence of hepatocellular carcinoma for patients with pre-treatment cirrhosis and sustained virologic response, stratified for the presence of diabetes mellitus. The log-rank test was used to test for equality of survivor functions.*

6.2.3 Discussion

These two studies showed that the risk to die or to develop decompensated liver disease or HCC is significantly reduced after treatment for chronic HCV infection. The main risk factor for the development of HCC was liver cirrhosis before treatment, and the association between low serum albumin and the risk to develop HCC indicates that this risk continues to increase with more advanced cirrhosis. Several studies support this conclusion, and have associated cirrhosis, high liver stiffness and biochemical markers of more advanced liver disease with the risk to develop HCC [40, 74, 111-113]. This underlines the importance of early antiviral treatment of chronic HCV infection before cirrhosis is established.

Diabetes mellitus is an established independent risk factor for the development of HCC in patients with chronic HCV, but also in patients without chronic viral hepatitis [114-116]. The

mechanism for this is not fully understood, but these diseases share common risk factors such as obesity and liver steatosis [117]. There is also evidence of direct carcinogenic effects of hyperinsulinemia and insulin signaling [118]. Liver cirrhosis increases insulin resistance and causes hyperinsulinemia, whereas successful treatment of chronic HCV has been shown to decrease or even prevent the development of insulin resistance [119-121]. Previous studies on Asian cohorts have not identified DM as a risk factor for the development of HCC after SVR has been achieved, but there is growing evidence from recent studies on Western cohorts that support our finding [74, 112, 113, 122, 123]. This could be explained by different genetic and environmental backgrounds of the studied patients.

With the access to better treatment options, more patients with advanced liver disease are being cured and a growing question is how to manage these patients after successful treatment. Continued HCC surveillance after SVR has been associated with improved HCC survival and current guidelines recommend continued HCC surveillance by ultrasound every 6 months for patients with a high pre-treatment risk to develop HCC [36, 98, 124]. In our study only three patients with pre-treatment F3 fibrosis developed HCC during almost 2000 person years of follow-up time. The incidence rate for this patient group was well below the 1.5% incidence threshold usually suggested for HCC surveillance to be cost effective in patients with cirrhosis [36]. Patients without cirrhosis and patients successfully treated for HCV will, as these studies show, have a longer life expectancy and more life-years to gain from continued surveillance. As a comparison a HCC incidence threshold of 0.2% has been suggested for surveillance in non-cirrhotic patients with chronic HBV infection [36]. However, the risk to develop HCC after SVR for patients with F3 fibrosis was only 0.16 per 100 person years in our study and it is questionable if continued surveillance for this group is cost-effective. We also showed that for patients with cirrhosis, the risk to develop HCC decreases with longer follow-up time after SVR. This indicates that there might be a time point after SVR when it is no longer reasonable to continue HCC surveillance, especially in patients that lack other important risk factors such as diabetes mellitus. However, in our studies the incidence rate of HCC for cirrhotic patients was still at levels that warrant continued surveillance, and further studies and cost-effectiveness analysis are needed to decide this question.

All patients in our studies were treated with INF-containing regimens, and another important question is if we can apply the results on patients treated with DAAs. Early reports from cohorts treated with IFN-free regimens have shown unexpected high incidence rates of HCC, mainly among patients previously treated for HCC [125, 126]. This has been attributed to different immunological responses to IFN-containing and IFN-free regimens and the loss of IFN-mediated anti-proliferative effects on HCC [126, 127]. However, other studies have not identified an increased risk for HCC with DAA treatment, and it is possible that the observed high incidence is a consequence of selection bias [128, 129]. Patients that previously did not tolerate or responded poorly to IFN-based therapy had the highest risk to develop HCC, and are now effectively treated with DAAs [130]. The cohort in study III included all patients with F3 or F4 fibrosis successfully treated at Karolinska University Hospital between 1991

and 2013 (n=399). These patients were highly selected before treatment and only a small proportion achieved SVR and could be included in the cohort. Between 2014 and 2016 another 701 patients with F3 and F4 fibrosis have achieved SVR at Karolinska University Hospital after treatment with DAA, clearly illustrating that these populations are not comparable. As a consequence we need to be careful when we apply results from IFN-era studies on DAA-era populations.

6.3 FIBROSIS REGRESSION AFTER SVR (PAPER IV)

To investigate fibrosis regression after SVR, we performed a cross-sectional study on the cohort from study III. Of the 399 patients in the cohort, 269 (67%) were successfully examined with transient elastography (FibroScan) at a follow-up visit.

6.3.1 Liver stiffness after SVR

A majority of the included patients had a low liver stiffness at follow-up corresponding to METAVIR F0-1 fibrosis according to pre-treatment cut-offs for METAVIR fibrosis stages. The median liver stiffness at follow-up was 6.6 kPa (range 2-57), with significantly higher median liver stiffness for patients with pre-treatment cirrhosis (8.5 kPa, 95% CI 7-9.1) than for patients with F3 fibrosis (6 kPa, 95% CI 5.5-6.4), (p<0.0001). Only one measurement of liver stiffness was available for each patient and the follow-up time from SVR varied greatly between patients. To investigate a possible effect of longer follow-up time on fibrosis regression, we divided the include patients into groups based on 5 year follow-up periods.

This could introduce bias, since patients with longer follow-up times were treated with older and less effective treatment regimens, and might not be comparable to more recently treated patients. However, when baseline characteristics were compared the groups did not differ greatly. The included patients are described in detail in paper IV.

Liver stiffness improved early for patients with pre-treatment F3 fibrosis and the distribution of liver stiffness and corresponding METAVIR fibrosis stages then remained stable over time. Liver stiffness improved more slowly in cirrhotic patients but continued to improve with longer follow-up time. After more than 10 years of follow-up the difference in liver stiffness between patients with pre-treatment F4 and F3 fibrosis had decreased and was no longer significant with 7.0 kPa (95% CI 5.6-83) and 5.6 kPa (95% CI 4.5-6.7) respectively (p=0.13).

6.3.2 Risk factors for persisting advanced fibrosis

Most patients improved their fibrosis stage after SVR, but 24% had a liver stiffness ≥ 9.5 kPa indicating persisting advanced fibrosis according to pre-treatment cut-offs for METAVIR stages. Among patients with pre-treatment cirrhosis this proportion decreased with longer

follow-up time from 48% for patients with < 5 years of follow-up to 36 % for patients with 5-10 years of follow-up, and 21% when follow-up time was > 10 years (p=0.02).
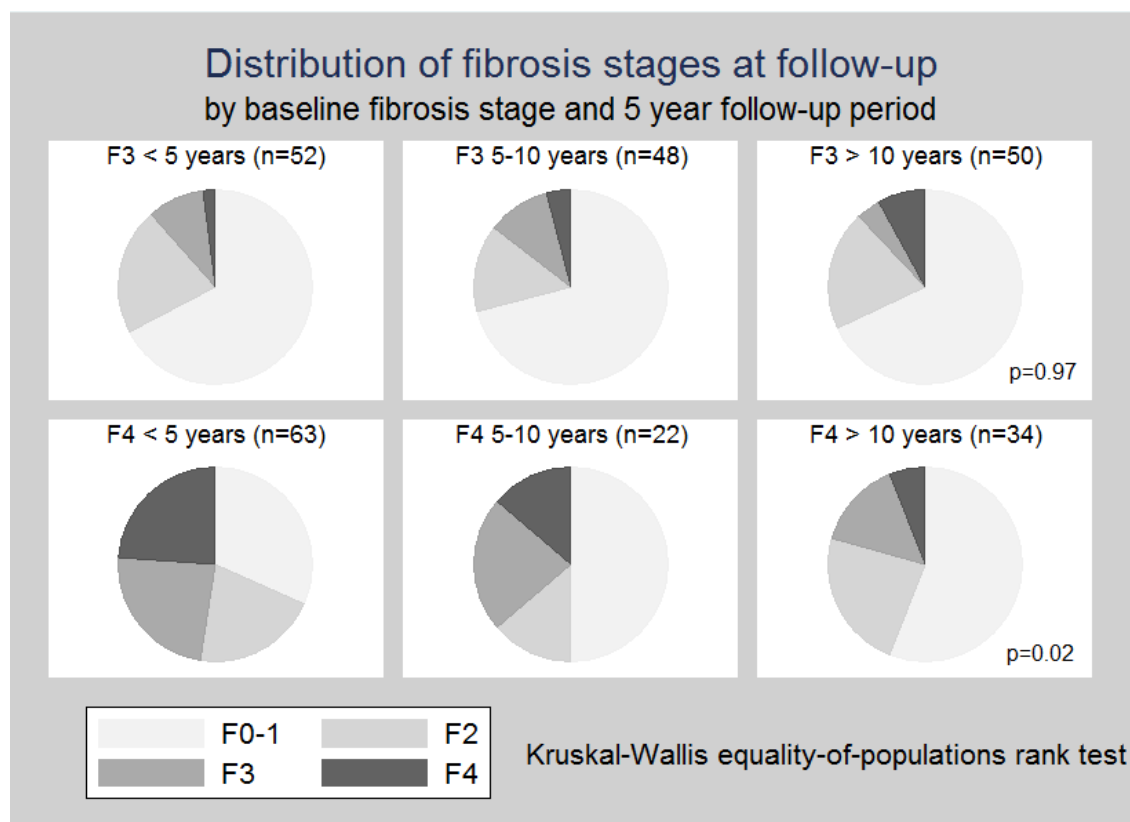

Figure 9: *The distribution of fibrosis stages at follow-up by METAVIR fibrosis stage at baseline and 5-year follow-up period.*

We estimated the risk for persisting advanced fibrosis at follow-up, defined as a liver stiffness ≥ 9.5 kPa, by calculating odds ratios (ORs) with logistic regression for patients with different risk factors. The analysis included age, gender, baseline fibrosis stage, BMI, the presence of diabetes mellitus and alcohol consumption. In the multivariate analysis, pre-treatment cirrhosis, age ≥ 55 years at SVR and BMI ≥ 25 kg/m^2 at follow-up remained significant risk factors for persisting advanced fibrosis with ORs of 3.9 (95 % CI 2.0-7.2), 2.3 (95 % CI 1.2-4.3) and 2.3 (95 % CI 1.1-4.6), respectively.

6.3.3 Discussion

This study confirmed that fibrosis and even cirrhosis can regress after SVR has been achieved in a vast majority of patients. Older patients who had already developed cirrhosis before treatment had higher liver stiffness after successful treatment, stressing the importance of early HCV treatment before cirrhosis is established. High BMI was also identified as an

important risk factor for high liver stiffness after SVR, again identifying the metabolic syndrome as an important cause of liver disease [117].

A third of the included patients had long follow-up times of 10-20 years after SVR. This group had significantly lower liver-stiffness than patients with shorter follow-up times after SVR, indicating that fibrosis regression is a process that continues for many years. This trend was mainly seen in patients with pre-treatment cirrhosis and the difference in liver-stiffness between patients with pre-treatment F3 and F4 fibrosis was no longer significant >10 years after SVR. This suggests that more profound changes of liver histology, such as the nodular architecture typical for cirrhotic livers take many years to improve. Studies on liver histology after SVR has shown that early improvement of liver-stiffness after SVR was associated with reduced necroinflammatory activity and reduced collagen content of the liver [41, 81]. This was seen both in patients with cirrhosis regression and in patients who maintained a cirrhotic nodular liver architecture [41]. With the long follow-up time of this study, it is unlikely that our results only reflect early changes in inflammation and collagen content, and we conclude that even cirrhosis will regress over time after successful treatment of chronic HCV.

The major weakness of this study was the cross-sectional design and the fact that we used transient elastography instead of liver biopsies to evaluate persisting fibrosis. There are no established liver stiffness cut-offs for corresponding METAVIR fibrosis stages after SVR has been achieved and the sensitivity of transient elastography to detect persisting cirrhosis has been questioned [41]. Recent studies with repeated liver stiffness measurements up to two years after SVR show that liver stiffness decreases rapidly after antiviral treatment probably due to decreased inflammation [85-87]. However, these studies also show that liver stiffness continues to improve with longer follow-up time indicating true fibrosis regression.

7 CONCLUSIONS

The four studies of this thesis provide encouraging evidence of the positive effects of successful treatment of chronic HCV infection.

We have shown that even though minute amounts of HCV RNA can be detected up to 9 years after SVR has been achieved, this does not correlate to continued liver disease. On the contrary, the persistence of HCV RNA in PBMC seems to be transient and HCV specific immune responses wane with time after SVR indicating complete virologic clearance.

Several studies have shown that successful treatment reduces the risk for liver-related complications and hepatocellular carcinoma. Our studies added to that knowledge by investigating the effect on all cause- and liver-related mortality and by identifying risk factors for the development of HCC. With long follow-up times it was also possible to study how the risk for HCC changes over time.

Our studies have shown that treatment reduces the risk for all clinical outcomes, but that a risk for HCC remains at least 15 years after SVR has been achieved. The risk to develop HCC for patients with advanced fibrosis without cirrhosis was low after achieved SVR, and the need for continued HCC surveillance for this group is questionable. Patients with cirrhosis before treatment had a higher risk to develop HCC after SVR had achieved, but our studies showed that this risk diminishes over time. This suggests that there could be a time-point even for this group when the risk for HCC is reduced below the threshold that warrants continued HCC surveillance.

Furthermore, we showed that liver fibrosis and even cirrhosis will improve after successful treatment of HCV infection, and that this process continues with time. The main risk factors for persisting advanced fibrosis after SVR had been achieved was established cirrhosis before treatment, older age and high body mass index. Metabolic disease is strongly and independently associated with liver disease, and these studies show that metabolic disease is also an important risk factor for persisting fibrosis and the risk to develop HCC in patients with SVR after treatment of chronic HCV infection.

A remaining question is how persisting cirrhosis after SVR correlates to the risk to develop HCC. We have shown that both cirrhosis and the risk to develop HCC diminishes with longer follow-up time after SVR, but our studies were not designed to correlated fibrosis regression with HCC risk. Six of the patients included in our studies developed HCC after they were examined with transient elastography, and two of these had a liver stiffness corresponding to a fibrosis stage F0-1 at follow-up. This indicates that liver stiffness after SVR is a poor prognostic marker for the risk to develop HCC, but larger studies designed to investigate this are needed.

All patients in these four studies were treated with IFN-based therapy, and our results should be interpreted with caution when applied to patients treated with DAAs. The long-term effect of SVR is likely to be independent of the type of therapy, but with IFN-free treatment we are

able to cure patients with more advanced liver disease and a higher baseline risk for liver related complications and HCC.

To conclude, virologic cure (SVR) dramatically improves the outcome for patients with chronic HCV infection, but patients with risk factors such as established cirrhosis and metabolic disease should be followed closely after SVR has been achieved.

8 ACKNOWLEDGEMENTS

9 REFERENCES

1. Blach S, Zeuzem S, Manns M, Altraif I, Duberg A. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. Lancet Gastroenterology and Hepatology **2017**; 2(3): 161–76.

2. Stanaway JD, Flaxman AD, Naghavi M, et al. The global burden of viral hepatitis from 1990 to 2013: findings from the Global Burden of Disease Study 2013. Lancet **2016**; 388(10049): 1081-8.

3. Parkin DM. The global health burden of infection-associated cancers in the year 2002. Int J Cancer **2006**; 118(12): 3030-44.

4. Durand F, Francoz C. The future of liver transplantation for viral hepatitis. Liver international : official journal of the International Association for the Study of the Liver **2017**; 37 Suppl 1: 130-5.

5. Hoofnagle JH, Mullen KD, Jones DB, et al. Treatment of chronic non-A,non-B hepatitis with recombinant human alpha interferon. A preliminary report. N Engl J Med **1986**; 315(25): 1575-8.

6. Hoofnagle JH, Seeff LB. Peginterferon and ribavirin for chronic hepatitis C. N Engl J Med **2006**; 355(23): 2444-51.

7. EASL Recommendations on Treatment of Hepatitis C 2016. J Hepatol **2017**; 66(1): 153-94.

8. Feinstone SM, Kapikian AZ, Purcell RH, Alter HJ, Holland PV. Transfusion-associated hepatitis not due to viral hepatitis type A or B. N Engl J Med **1975**; 292(15): 767-70.

9. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **1989**; 244(4902): 359-62.

10. Dubuisson J, Cosset FL. Virology and cell biology of the hepatitis C virus life cycle: an update. J Hepatol **2014**; 61(1 Suppl): S3-S13.

11. Sarrazin C, Hezode C, Zeuzem S, Pawlotsky JM. Antiviral strategies in hepatitis C virus infection. J Hepatol **2012**; 56 Suppl 1: S88-100.

12. Smith DB, Bukh J, Kuiken C, et al. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology **2014**; 59(1): 318-27.

13. Gray RR, Salemi M, Klenerman P, Pybus OG. A new evolutionary model for hepatitis C virus chronic infection. PLoS Pathog **2012**; 8(5): e1002656.

14. Simmonds P. Genetic diversity and evolution of hepatitis C virus--15 years on. J Gen Virol **2004**; 85(Pt 11): 3173-88.

15. Messina JP, Humphreys I, Flaxman A, et al. Global distribution and prevalence of hepatitis C virus genotypes. Hepatology **2015**; 61(1): 77-87.

16. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. Hepatology **2013**; 57(4): 1333-42.

17. Lavanchy D. Evolving epidemiology of hepatitis C virus. Clin Microbiol Infect **2011**; 17(2): 107-15.

18. Gower E, Estes C, Blach S, Razavi-Shearer K, Razavi H. Global epidemiology and genotype distribution of the hepatitis C virus infection. Journal of hepatology **2014**; 61(1 Suppl): S45-57.

19. Chhatwal J, Wang X, Ayer T, et al. Hepatitis C Disease Burden in the United States in the era of oral direct-acting antivirals. Hepatology **2016**; 64(5): 1442-50.

20. Gane E, Kershenobich D, Seguin-Devaux C, et al. Strategies to manage hepatitis C virus (HCV) infection disease burden - volume 2. Journal of viral hepatitis **2015**; 22 Suppl 1: 46-73.

21. Lozano R, Naghavi M, Foreman K, et al. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet **2012**; 380(9859): 2095-128.

22. Webster DP, Klenerman P, Dusheiko GM. Hepatitis C. Lancet **2015**; 385(9973): 1124-35.

23. Roberts EA, Yeung L. Maternal-infant transmission of hepatitis C virus infection. Hepatology **2002**; 36(5 Suppl 1): S106-13.

24. Terrault NA, Dodge JL, Murphy EL, et al. Sexual transmission of hepatitis C virus among monogamous heterosexual couples: the HCV partners study. Hepatology **2013**; 57(3): 881-9.

25. Hagan H, Jordan AE, Neurer J, Cleland CM. Incidence of sexually transmitted hepatitis C virus infection in HIV-positive men who have sex with men. AIDS **2015**; 29(17): 2335-45.

26. Lidman C, Norden L, Kaberg M, et al. Hepatitis C infection among injection drug users in Stockholm Sweden: prevalence and gender. Scandinavian journal of infectious diseases **2009**; 41(9): 679-84.

27. Duberg A, Janzon R, Back E, Ekdahl K, Blaxhult A. The epidemiology of hepatitis C virus infection in Sweden. Euro Surveill **2008**; 13(21).

28. Duberg AS, Blach S, Falconer K, Kaberg M, Razavi H, Aleman S. The future disease burden of hepatitis C virus infection in Sweden and the impact of different treatment strategies. Scand J Gastroenterol **2015**; 50(2): 233-44.

29. Thomas DL, Seeff LB. Natural history of hepatitis C. Clin Liver Dis **2005**; 9(3): 383-98, vi.

30. Seeff LB. The history of the "natural history" of hepatitis C (1968-2009). Liver Int **2009**; 29 Suppl 1: 89-99.

31. Thein HH, Yi Q, Dore GJ, Krahn MD. Estimation of stage-specific fibrosis progression rates in chronic hepatitis C virus infection: a meta-analysis and meta-regression. Hepatology **2008**; 48(2): 418-31.

32. Sangiovanni A, Prati GM, Fasani P, et al. The natural history of compensated cirrhosis due to hepatitis C virus: A 17-year cohort study of 214 patients. Hepatology **2006**; 43(6): 1303-10.

33. Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic

patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. Ann Intern Med **1999**; 131(3): 174-81.

34.   Lok AS, Seeff LB, Morgan TR, et al. Incidence of hepatocellular carcinoma and associated risk factors in hepatitis C-related advanced liver disease. Gastroenterology **2009**; 136(1): 138-48.

35.   Cardoso AC, Moucari R, Figueiredo-Mendes C, et al. Impact of peginterferon and ribavirin therapy on hepatocellular carcinoma: incidence and survival in hepatitis C patients with advanced fibrosis. J Hepatol **2010**; 52(5): 652-7.

36.   EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol **2012**; 56(4): 908-43.

37.   Ishak K, Baptista A, Bianchi L, et al. Histological grading and staging of chronic hepatitis. J Hepatol **1995**; 22(6): 696-9.

38.   Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology **1996**; 24(2): 289-93.

39.   Castera L, Forns X, Alberti A. Non-invasive evaluation of liver fibrosis using transient elastography. J Hepatol **2008**; 48(5): 835-47.

40.   Masuzaki R, Tateishi R, Yoshida H, et al. Prospective risk assessment for hepatocellular carcinoma development in patients with chronic hepatitis C by transient elastography. Hepatology **2009**; 49(6): 1954-61.

41.   D'Ambrosio R, Aghemo A, Fraquelli M, et al. The diagnostic accuracy of Fibroscan for cirrhosis is influenced by liver morphometry in HCV patients with a sustained virological response. J Hepatol **2013**; 59(2): 251-6.

42.   Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC, Williams R. Transection of the oesophagus for bleeding oesophageal varices. Br J Surg **1973**; 60(8): 646-9.

43.   Thimme R, Oldach D, Chang KM, Steiger C, Ray SC, Chisari FV. Determinants of viral clearance and persistence during acute hepatitis C virus infection. The Journal of experimental medicine **2001**; 194(10): 1395-406.

44.   Werner JM, Heller T, Gordon AM, et al. Innate immune responses in hepatitis C virus-exposed healthcare workers who do not develop acute infection. Hepatology **2013**; 58(5): 1621-31.

45.   Shin EC, Park SH, Demino M, et al. Delayed induction, not impaired recruitment, of specific CD8(+) T cells causes the late onset of acute hepatitis C. Gastroenterology **2011**; 141(2): 686-95, 95 e1.

46.   Klenerman P, Thimme R. T cell responses in hepatitis C: the good, the bad and the unconventional. Gut **2012**; 61(8): 1226-34.

47.   Wieland S, Makowska Z, Campana B, et al. Simultaneous detection of hepatitis C virus and interferon stimulated gene expression in infected human liver. Hepatology **2014**; 59(6): 2121-30.

48.   Thomas DL, Thio CL, Martin MP, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature **2009**; 461(7265): 798-801.

49.   Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature **2009**; 461(7262): 399-401.

50. Martinot-Peignoux M, Stern C, Maylin S, et al. Twelve weeks posttreatment follow-up is as relevant as 24 weeks to determine the sustained virologic response in patients with hepatitis C virus receiving pegylated interferon and ribavirin. Hepatology **2010**; 51(4): 1122-6.

51. Reichard O, Andersson J, Schvarcz R, Weiland O. Ribavirin treatment for chronic hepatitis C. Lancet **1991**; 337(8749): 1058-61.

52. Dusheiko G, Main J, Thomas H, et al. Ribavirin treatment for patients with chronic hepatitis C: results of a placebo-controlled study. Journal of hepatology **1996**; 25(5): 591-8.

53. Reichard O, Norkrans G, Fryden A, Braconier JH, Sonnerborg A, Weiland O. Randomised, double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. The Swedish Study Group. Lancet **1998**; 351(9096): 83-7.

54. Poynard T, Marcellin P, Lee SS, et al. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet **1998**; 352(9138): 1426-32.

55. Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet **2001**; 358(9286): 958-65.

56. Poordad F, McCone J, Jr., Bacon BR, et al. Boceprevir for untreated chronic HCV genotype 1 infection. N Engl J Med **2011**; 364(13): 1195-206.

57. Jacobson IM, McHutchison JG, Dusheiko G, et al. Telaprevir for previously untreated chronic hepatitis C virus infection. N Engl J Med **2011**; 364(25): 2405-16.

58. Gane EJ, Stedman CA, Hyland RH, et al. Nucleotide polymerase inhibitor sofosbuvir plus ribavirin for hepatitis C. N Engl J Med **2013**; 368(1): 34-44.

59. Sulkowski MS, Gardiner DF, Rodriguez-Torres M, et al. Daclatasvir plus sofosbuvir for previously treated or untreated chronic HCV infection. N Engl J Med **2014**; 370(3): 211-21.

60. Yoshida H, Tateishi R, Arakawa Y, et al. Benefit of interferon therapy in hepatocellular carcinoma prevention for individual patients with chronic hepatitis C. Gut **2004**; 53(3): 425-30.

61. Makiyama A, Itoh Y, Kasahara A, et al. Characteristics of patients with chronic hepatitis C who develop hepatocellular carcinoma after a sustained response to interferon therapy. Cancer **2004**; 101(7): 1616-22.

62. Kobayashi S, Takeda T, Enomoto M, et al. Development of hepatocellular carcinoma in patients with chronic hepatitis C who had a sustained virological response to interferon therapy: a multicenter, retrospective cohort study of 1124 patients. Liver international : official journal of the International Association for the Study of the Liver **2007**; 27(2): 186-91.

63. Veldt BJ, Heathcote EJ, Wedemeyer H, et al. Sustained virologic response and clinical outcomes in patients with chronic hepatitis C and advanced fibrosis. Ann Intern Med **2007**; 147(10): 677-84.

64. Bruno S, Stroffolini T, Colombo M, et al. Sustained virological response to interferon-alpha is associated with improved outcome in HCV-related cirrhosis: a retrospective study. Hepatology **2007**; 45(3): 579-87.

65. Singal AK, Singh A, Jaganmohan S, et al. Antiviral therapy reduces risk of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis. Clin Gastroenterol Hepatol **2010**; 8(2): 192-9.

66. Bruno S, Crosignani A, Facciotto C, et al. Sustained virologic response prevents the development of esophageal varices in compensated, Child-Pugh class A hepatitis C virus-induced cirrhosis. A 12-year prospective follow-up study. Hepatology **2010**; 51(6): 2069-76.

67. Morgan TR, Ghany MG, Kim HY, et al. Outcome of sustained virological responders with histologically advanced chronic hepatitis C. Hepatology **2010**; 52(3): 833-44.

68. van der Meer AJ, Veldt BJ, Feld JJ, et al. Association between sustained virological response and all-cause mortality among patients with chronic hepatitis C and advanced hepatic fibrosis. JAMA **2012**; 308(24): 2584-93.

69. Tada T, Kumada T, Toyoda H, et al. Viral eradication reduces all-cause mortality in patients with chronic hepatitis C virus infection: a propensity score analysis. Liver international : official journal of the International Association for the Study of the Liver **2016**; 36(6): 817-26.

70. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology **2004**; 127(5 Suppl 1): S35-50.

71. Iwasaki Y, Takaguchi K, Ikeda H, et al. Risk factors for hepatocellular carcinoma in Hepatitis C patients with sustained virologic response to interferon therapy. Liver Int **2004**; 24(6): 603-10.

72. Hirakawa M, Ikeda K, Arase Y, et al. Hepatocarcinogenesis following HCV RNA eradication by interferon in chronic hepatitis patients. Intern Med **2008**; 47(19): 1637-43.

73. Tanaka A, Uegaki S, Kurihara H, et al. Hepatic steatosis as a possible risk factor for the development of hepatocellular carcinoma after eradication of hepatitis C virus with antiviral therapy in patients with chronic hepatitis C. World J Gastroenterol **2007**; 13(39): 5180-7.

74. Chang KC, Hung CH, Lu SN, et al. A novel predictive score for hepatocellular carcinoma development in patients with chronic hepatitis C after sustained response to pegylated interferon and ribavirin combination therapy. J Antimicrob Chemother **2012**; 67(11): 2766-72.

75. Huang CF, Yeh ML, Tsai PC, et al. Baseline gamma-glutamyl transferase levels strongly correlate with hepatocellular carcinoma development in non-cirrhotic patients with successful hepatitis C virus eradication. Journal of hepatology **2014**; 61(1): 67-74.

76. Asahina Y, Tsuchiya K, Nishimura T, et al. alpha-fetoprotein levels after interferon therapy and risk of hepatocarcinogenesis in chronic hepatitis C. Hepatology **2013**; 58(4): 1253-62.

77. Hung CH, Lee CM, Wang JH, et al. Impact of diabetes mellitus on incidence of hepatocellular carcinoma in chronic hepatitis C patients treated with interferon-based antiviral therapy. Int J Cancer **2011**; 128(10): 2344-52.

78. Yamashita N, Ohho A, Yamasaki A, Kurokawa M, Kotoh K, Kajiwara E. Hepatocarcinogenesis in chronic hepatitis C patients achieving a sustained virological response to interferon: significance of lifelong periodic cancer screening for improving outcomes. J Gastroenterol **2014**; 49(11): 1504-13.

79. Poynard T, McHutchison J, Manns M, et al. Impact of pegylated interferon alfa-2b and ribavirin on liver fibrosis in patients with chronic hepatitis C. Gastroenterology **2002**; 122(5): 1303-13.

80. Shiratori Y, Imazeki F, Moriyama M, et al. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. Ann Intern Med **2000**; 132(7): 517-24.

81. D'Ambrosio R, Aghemo A, Rumi MG, et al. A morphometric and immunohistochemical study to assess the benefit of a sustained virological response in hepatitis C virus patients with cirrhosis. Hepatology **2012**; 56(2): 532-43.

82. Maylin S, Martinot-Peignoux M, Moucari R, et al. Eradication of hepatitis C virus in patients successfully treated for chronic hepatitis C. Gastroenterology **2008**; 135(3): 821-9.

83. Balart LA, Lisker-Melman M, Hamzeh FM, Kwok A, Lentz E, Rodriguez-Torres M. Peginterferon alpha-2a plus ribavirin in Latino and Non-Latino Whites with HCV genotype 1: Histologic outcomes and tolerability from the LATINO Study. Am J Gastroenterol **2010**; 105(10): 2177-85.

84. D'Ambrosio R, Degasperi E, Aghemo A, et al. Serological Tests Do Not Predict Residual Fibrosis in Hepatitis C Cirrhotics with a Sustained Virological Response to Interferon. PLoS One **2016**; 11(6): e0155967.

85. Chekuri S, Nickerson J, Bichoupan K, et al. Liver Stiffness Decreases Rapidly in Response to Successful Hepatitis C Treatment and Then Plateaus. PLoS One **2016**; 11(7): e0159413.

86. Bachofner JA, Valli PV, Kroger A, et al. Direct antiviral agent treatment of chronic hepatitis C results in rapid regression of transient elastography and fibrosis markers fibrosis-4 score and aspartate aminotransferase-platelet ratio index. Liver Int **2016**.

87. Hezode C, Castera L, Roudot-Thoraval F, et al. Liver stiffness diminishes with antiviral response in chronic hepatitis C. Aliment Pharmacol Ther **2011**; 34(6): 656-63.

88. Poynard T, Moussalli J, Munteanu M, et al. Slow regression of liver fibrosis presumed by repeated biomarkers after virological cure in patients with chronic hepatitis C. J Hepatol **2013**; 59(4): 675-83.

89. Pham TN, MacParland SA, Mulrooney PM, Cooksley H, Naoumov NV, Michalak TI. Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. J Virol **2004**; 78(11): 5867-74.

90. Radkowski M, Gallegos-Orozco JF, Jablonska J, et al. Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. Hepatology **2005**; 41(1): 106-14.

91. Castillo I, Rodriguez-Inigo E, Lopez-Alcorocho JM, Pardo M, Bartolome J, Carreno V. Hepatitis C virus replicates in the liver of patients who have a sustained response to antiviral treatment. Clin Infect Dis **2006**; 43(10): 1277-83.

92.   Castillo I, Pardo M, Bartolome J, et al. Occult hepatitis C virus infection in patients in whom the etiology of persistently abnormal results of liver-function tests is unknown. J Infect Dis **2004**; 189(1): 7-14.

93.   2011 European Association of the Study of the Liver hepatitis C virus clinical practice guidelines. Liver international : official journal of the International Association for the Study of the Liver **2012**; 32 Suppl 1: 2-8.

94.   Jensen TB, Gottwein JM, Scheel TK, Hoegh AM, Eugen-Olsen J, Bukh J. Highly efficient JFH1-based cell-culture system for hepatitis C virus genotype 5a: failure of homologous neutralizing-antibody treatment to control infection. The Journal of infectious diseases **2008**; 198(12): 1756-65.

95.   Wai CT, Greenson JK, Fontana RJ, et al. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology **2003**; 38(2): 518-26.

96.   Westin J, Ydreborg M, Islam S, et al. A non-invasive fibrosis score predicts treatment outcome in chronic hepatitis C virus infection. Scandinavian journal of gastroenterology **2008**; 43(1): 73-80.

97.   Menghini G. One-second needle biopsy of the liver. Gastroenterology **1958**; 35(2): 190-9.

98.   Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. Hepatology **2011**; 53(3): 1020-2.

99.   Colombo M. Natural history and pathogenesis of hepatitis C virus related hepatocellular carcinoma. Journal of hepatology **1999**; 31 Suppl 1: 25-30.

100.   Ludvigsson JF, Andersson E, Ekbom A, et al. External review and validation of the Swedish national inpatient register. BMC Public Health **2011**; 11: 450.

101.   Johansson LA, Bjorkenstam C, Westerling R. Unexplained differences between hospital and mortality data indicated mistakes in death certification: an investigation of 1,094 deaths in Sweden during 1995. J Clin Epidemiol **2009**; 62(11): 1202-9.

102.   Barlow L, Westergren K, Holmberg L, Talback M. The completeness of the Swedish Cancer Register: a sample survey for year 1998. Acta Oncol **2009**; 48(1): 27-33.

103.   George SL, Bacon BR, Brunt EM, Mihindukulasuriya KL, Hoffmann J, Di Bisceglie AM. Clinical, virologic, histologic, and biochemical outcomes after successful HCV therapy: a 5-year follow-up of 150 patients. Hepatology **2009**; 49(3): 729-38.

104.   Morishima C, Morgan TR, Everhart JE, et al. Interpretation of positive transcription-mediated amplification test results from polymerase chain reaction-negative samples obtained after treatment of chronic hepatitis C. Hepatology **2008**; 48(5): 1412-9.

105.   Veerapu NS, Raghuraman S, Liang TJ, Heller T, Rehermann B. Sporadic reappearance of minute amounts of hepatitis C virus RNA after successful therapy stimulates cellular immune responses. Gastroenterology **2011**; 140(2): 676-85 e1.

106.   Pawelczyk A, Kubisa N, Jablonska J, et al. Detection of hepatitis C virus (HCV) negative strand RNA and NS3 protein in peripheral blood mononuclear cells (PBMC): CD3+, CD14+ and CD19+. Virology journal **2013**; 10: 346.

107.   Riva E, Maggi F, Abbruzzese F, et al. Immune complexed (IC) hepatitis C virus (HCV) in chronically and acutely HCV-infected patients. Med Microbiol Immunol **2009**; 198(1): 13-8.

108. Fujiwara K, Allison RD, Wang RY, et al. Investigation of residual hepatitis C virus in presumed recovered subjects. Hepatology **2013**; 57(2): 483-91.

109. Heim MH, Thimme R. Innate and adaptive immune responses in HCV infections. Journal of hepatology **2014**; 61(1 Suppl): S14-25.

110. Roque-Cuellar MC, Sanchez B, Garcia-Lozano JR, et al. Hepatitis C virus-specific cellular immune responses in sustained virological responders with viral persistence in peripheral blood mononuclear cells. Liver international : official journal of the International Association for the Study of the Liver **2014**; 34(6): e80-8.

111. Ikeda M, Fujiyama S, Tanaka M, et al. Risk factors for development of hepatocellular carcinoma in patients with chronic hepatitis C after sustained response to interferon. J Gastroenterol **2005**; 40(2): 148-56.

112. El-Serag HB, Kanwal F, Richardson P, Kramer J. Risk of hepatocellular carcinoma after sustained virological response in Veterans with hepatitis C virus infection. Hepatology **2016**; 64(1): 130-7.

113. van der Meer AJ, Feld JJ, Hofer H, et al. Risk of cirrhosis-related complications in patients with advanced fibrosis following hepatitis C virus eradication. Journal of hepatology **2017**; 66(3): 485-93.

114. Davila JA, Morgan RO, Shaib Y, McGlynn KA, El-Serag HB. Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. Gut **2005**; 54(4): 533-9.

115. Veldt BJ, Chen W, Heathcote EJ, et al. Increased risk of hepatocellular carcinoma among patients with hepatitis C cirrhosis and diabetes mellitus. Hepatology **2008**; 47(6): 1856-62.

116. Arase Y, Kobayashi M, Suzuki F, et al. Effect of type 2 diabetes on risk for malignancies includes hepatocellular carcinoma in chronic hepatitis C. Hepatology **2013**; 57(3): 964-73.

117. Bellentani S. The epidemiology of non-alcoholic fatty liver disease. Liver international : official journal of the International Association for the Study of the Liver **2017**; 37 Suppl 1: 81-4.

118. Chettouh H, Lequoy M, Fartoux L, Vigouroux C, Desbois-Mouthon C. Hyperinsulinaemia and insulin signalling in the pathogenesis and the clinical course of hepatocellular carcinoma. Liver Int **2015**; 35(10): 2203-17.

119. Garcia-Compean D, Jaquez-Quintana JO, Gonzalez-Gonzalez JA, Maldonado-Garza H. Liver cirrhosis and diabetes: risk factors, pathophysiology, clinical implications and management. World J Gastroenterol **2009**; 15(3): 280-8.

120. Aghemo A, Prati GM, Rumi MG, et al. Sustained virological response prevents the development of insulin resistance in patients with chronic hepatitis C. Hepatology **2012**; 56(5): 1681-7.

121. Jung HJ, Kim YS, Kim SG, et al. The impact of pegylated interferon and ribavirin combination treatment on lipid metabolism and insulin resistance in chronic hepatitis C patients. Clin Mol Hepatol **2014**; 20(1): 38-46.

122. Sato A, Sata M, Ikeda K, et al. Clinical characteristics of patients who developed hepatocellular carcinoma after hepatitis C virus eradication with interferon therapy: current status in Japan. Intern Med **2013**; 52(24): 2701-6.

123. Huang CF, Yeh ML, Tsai PC, et al. Baseline gamma-glutamyl transferase levels strongly correlate with hepatocellular carcinoma development in non-cirrhotic patients with successful hepatitis C virus eradication. J Hepatol **2014**; 61(1): 67-74.

124. Toyoda H, Tada T, Tsuji K, et al. Characteristics and prognosis of hepatocellular carcinoma detected in patients with chronic hepatitis C after the eradication of hepatitis C virus: A multicenter study from Japan. Hepatology research : the official journal of the Japan Society of Hepatology **2016**; 46(8): 734-42.

125. Conti F, Buonfiglioli F, Scuteri A, et al. Early occurrence and recurrence of hepatocellular carcinoma in HCV-related cirrhosis treated with direct-acting antivirals. Journal of hepatology **2016**; 65(4): 727-33.

126. Reig M, Marino Z, Perello C, et al. Unexpected high rate of early tumor recurrence in patients with HCV-related HCC undergoing interferon-free therapy. Journal of hepatology **2016**; 65(4): 719-26.

127. Cardoso H, Vale AM, Rodrigues S, et al. High incidence of hepatocellular carcinoma following successful interferon-free antiviral therapy for hepatitis C associated cirrhosis. Journal of hepatology **2016**; 65(5): 1070-1.

128. Lack of evidence of an effect of direct-acting antivirals on the recurrence of hepatocellular carcinoma: Data from three ANRS cohorts. Journal of hepatology **2016**; 65(4): 734-40.

129. Kobayashi M, Suzuki F, Fujiyama S, et al. Sustained virologic response by direct antiviral agents reduces the incidence of hepatocellular carcinoma in patients with HCV infection. Journal of medical virology **2017**; 89(3): 476-83.

130. Foster GR, Irving WL, Cheung MC, et al. Impact of direct acting antiviral therapy in patients with chronic hepatitis C and decompensated cirrhosis. Journal of hepatology **2016**; 64(6): 1224-31.