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# **CLINICAL AND MOLECULAR FEATURES OF CHRONIC HEPATITIS C INFECTION AND ADVANCED LIVER DISEASE**

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Institutet**

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*Image on front page:* Progressive liver disease caused by hepatitis C virus infection.  
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“Scientific knowledge is the highest attainment upon the human plane, for science is the discovery of realities.”

- ‘Abdu’l-Bahá (1844-1921)

*To all those young Bahá’ís in Iran thirsting  
for knowledge, but deprived of access to  
higher education*



## GENERAL SUMMARY

The aim of this thesis was to study molecular and clinical aspects of hepatitis C infection (HCV), especially in patients with advanced liver disease.

In the first study we investigated whether the use of a second generation contrast agent in ultrasound (US) examinations can improve detection of hepatocellular carcinoma (HCC) and characterisation of focal liver lesions in 49 HCV-infected patients with liver cirrhosis. In total 96 examinations with conventional US followed by a contrast-enhanced ultrasound (CEUS) examination were analysed retrospectively. The number of diagnosed malignant liver lesions increased from one to ten after CEUS.

In the second study we analysed the efficacy and tolerability of combination therapy with pegylated interferon (peg-IFN) and ribavirin in 104 patients with HCV-associated Child-Pugh class A liver cirrhosis at a Swedish university clinic. Sustained virological response (SVR) was achieved in 13% of genotype 1-, 60% of genotype 2-, and 31% of genotype 3-infected patients. In treatment-naïve patients, the corresponding rates were 13%, 82% and 38% respectively. In 46% of patients, treatment was discontinued prematurely owing to a lack of virological response in the majority. SVR rates found in our study, in particular for genotype 1 patients, were lower than those generally found in randomised controlled studies.

In the third study we evaluated the long-term impact of SVR to antiviral therapy on the risks of developing HCC, liver complications and death in 351 HCV-infected patients with compensated Child-Pugh class A liver cirrhosis. They were followed prospectively for a mean of 5.3 years, up to 8.6 years. Among patients with SVR (n=110), 5.0% developed HCC, 3.6% ascites, 0.9% liver encephalopathy and none variceal bleeding. The incidences of HCC, any liver complication, liver-related and overall death per 100 person-years were 1.0, 0.9, 0.7 and 1.8% among patients with SVR versus 1.9, 2.5, 2.4 and 3.1% respectively among patients without SVR (n=241). Risks of HCC, liver decompensation and death were markedly reduced in patients with SVR, but the risk of developing HCC was remaining at 1% per year.

In the fourth study we investigated whether there is an association between levels of the HCV NS3 protein in liver biopsies, T cell protein tyrosine phosphatase (TCPTP) cleavage and clinical parameters in patients with chronic HCV infection. Hepatic NS3 and TCPTP protein levels were determined in liver biopsies from 69 HCV RNA-positive patients and 16 control patients. Levels were correlated to viral load or clinical parameters for the severity of liver disease. We found that intrahepatic NS3 expression and the viral load were inversely correlated with intrahepatic TCPTP protein levels. Detection of NS3 did not associate with any other clinical parameters. The clear link demonstrated suggests that TCPTP cleavage may have important consequences for the HCV life-cycle and HCV-induced liver diseases.

**Conclusions:** In HCV-infected patients, TCPTP cleavage may play an important role for the viral life-cycle and progress of HCV-induced liver disease. Patients with HCV-induced liver cirrhosis who receive standard of care therapy in clinical settings achieve SVR at lower rates than those generally found in randomised controlled studies, in particular genotype 1 patients. If SVR is achieved, risks of HCC, liver decompensation and death are markedly reduced in these patients, but the risk of HCC remains at a non-negligible level, warranting a continued surveillance for HCC. Diagnostic confidence may be improved with CEUS in surveillance for HCC. Patients with HCV-induced liver cirrhosis constitute a clinically challenging group of patients. Additional studies are needed to further understand the pathogenesis of HCV and how it establishes a chronic infection, in order to improve the rate of eradication by treatment and to identify prognostic factors for liver complications after achieving SVR, along with optimising surveillance in patients with chronic HCV infection, so that survival may be increased.



## LIST OF PUBLICATIONS

- I. **Nogol Rahbin**, Anna-Karin Siösteen, Anders Elvin, Lennart Blomqvist, Karin Hagen, Rolf Hultcrantz, Soo Aleman  
**Detection and characterization of focal liver lesions with contrast-enhanced ultrasonography in patients with hepatitis C-induced liver cirrhosis**  
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- II. Eliya Syed, **Nogol Rahbin**, Ola Weiland, Tony Carlsson, Antti Oksanen, Markus Birk, Loa Davidsdottir, Karin Hagen, Rolf Hultcrantz, Soo Aleman  
**Pegylated interferon and ribavirin combination therapy for chronic hepatitis C virus infection in patients with Child-Pugh Class A liver cirrhosis**  
*Scandinavian Journal of Gastroenterology*, 2008, 43, 1378-1386
- III. Soo Aleman, **Nogol Rahbin**, Loa Davidsdottir, Ola Weiland, Nina Rose, Hans Verbaan, Per Stål, Tony Carlsson, Hans Norrgren, Anders Ekbom, Fredrik Granath, Rolf Hultcrantz  
**Long-term impact of treatment induced sustained virological response in 351 Swedish patients with hepatitis C cirrhosis**  
*Manuscript*
- IV. **Nogol Rahbin**, Lars Frelin, Soo Aleman, Rolf Hultcrantz, Matti Sällberg, Erwin Daniel Brenndörfer  
**Non-structural 3 protein expression is associated with T cell protein tyrosine phosphatase and viral RNA levels in chronic hepatitis C patients**  
*Submitted*

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## LIST OF ABBREVIATIONS

aa	Amino acids
AASLD	American Association for the Study of Liver Diseases
AFP	Alpha fetoprotein
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BCLC	Barcelona Clinic Liver Cancer
CEUS	Contrast enhanced ultrasound
CHC	Chronic hepatitis C
CT	Computed tomography
DAA	Direct acting antiviral agents
EASL	European Association for the Study of the Liver
ELISA	Enzyme-linked immunosorbent assay
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
IDU	Injection drug use
IFN	Interferon
MAVS	Mitochondrial antiviral signaling protein
MELD	Model for end-stage liver disease
MRI	Magnetic resonance imaging
NANB hepatitis	Non-A, non-B hepatitis
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NLR	NOD-like receptor
NS protein	Non-structural protein
ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
Peg-IFN	Pegylated interferon
PRR	Pathogen recognition receptor
RCT	Randomised controlled trial
RIG-1	retinoic acid inducible gene I
RLR	RIG-I like receptor
RNA	Ribonucleic acid
SOC	Standard of care
TACE	Transarterial chemoembolization
TCPTP	T-cell protein tyrosine phosphatase
TE	Transient elastography
TLR	Toll-like receptor
TRIF	TIR domain-containing adaptor inducing IFN $\beta$
US	Ultrasound

# **1 INTRODUCTION: HEPATITIS C VIRUS**

## **1.1 HISTORY**

The field of viral hepatitis was initiated in the 1950s and 60s with the distinction of so-called “infectious” and “serum” hepatitis (1), later proven to be due to infection by the hepatitis A virus (HAV) in 1973 (2) and the hepatitis B virus (HBV) in 1968 (3). In the mid-1970s, serological tests were introduced to detect infection by HAV and HBV and soon it became obvious that neither virus caused a large portion of cases of parenterally transmitted hepatitis (4). This gave rise to the term non-A, non-B (NANB) hepatitis (5). The disease was transmissible to chimpanzees and its insidious development was demonstrated in humans with up to ~ 20% of infected patients slowly progressing to liver cirrhosis, typically over the course of many years (6, 7).

The genome of the infectious agent was first cloned and characterised in 1989, serological tests were developed and the cause for NANB hepatitis was named the hepatitis C virus (HCV) (8).

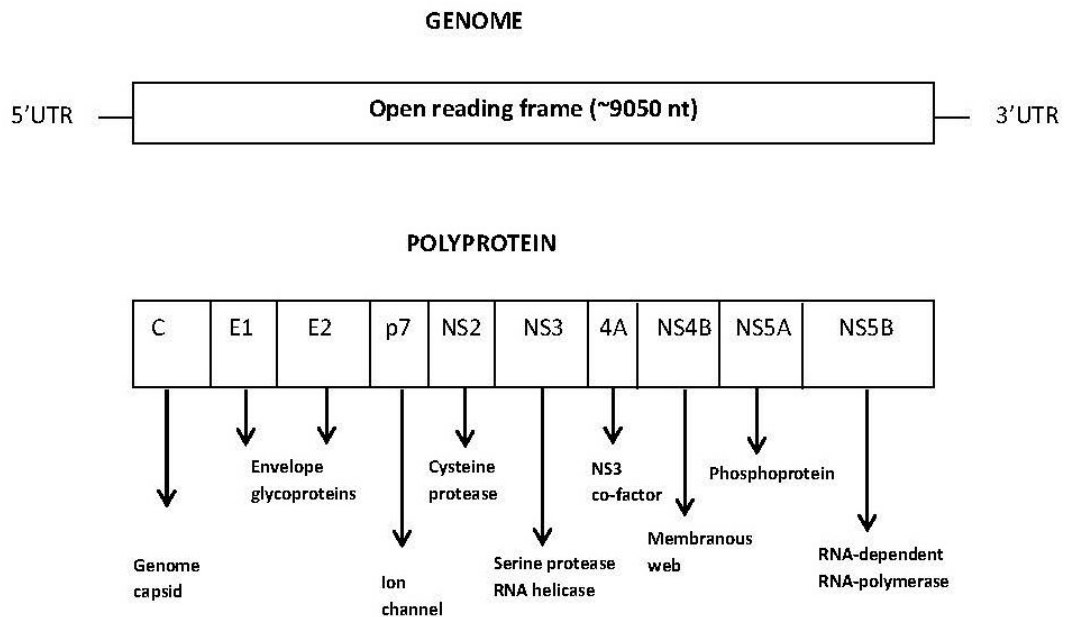
## **1.2 EPIDEMIOLOGY**

It is estimated that 130-170 million persons, or 2-3% of the world's population, are infected with HCV (9, 10), the majority living in Central/Southeast Asia and the Western Pacific regions (11). Transmission of HCV infection mainly include blood transfusion from unscreened donors, injection drug use (IDU), unsafe therapeutic injections and other health-care related procedures. IDU has been considered to be the predominant mode of HCV transmission in developed countries. Unsafe therapeutic injections have been of great importance in the spread of HCV in many developing countries where supplies of sterile syringes may be inadequate or non-existent (10, 12). In Egypt, the country with the highest reported seroprevalence of HCV in the world, a nationwide schistosomiasis treatment campaign was carried out from 1960 to 1987 using contaminated glass syringes, representing the largest outbreak of iatrogenic transmission of a bloodborne pathogen ever recorded (13). Other modes of transmission include occupational, perinatal and sexual exposures, but with much less efficiency compared to large or repeated percutaneous exposures.

In Sweden (population 9 million), the prevalence has been estimated to be about 0.5% (14). The spread of HCV started during the mid-1960s and culminated during the 70s with the rise of IDU (15), which is the predominant mode of transmission today. However, around 6% of registered cases in the country have been attributed to transfusions of blood or blood products. Since 1992, it is mandatory to test for HCV in donated blood and blood products and all newly diagnosed cases of HCV infection are to be reported by Swedish law. It has been estimated that about 60% of patients diagnosed with HCV infection were most likely infected in the 1970s and early 1980s and now, having been infected for 25-30 years, run an increased risk of developing HCV-related liver disease and hepatocellular carcinoma (HCC) (16).

### **1.3 MOLECULAR STRUCTURE**

The HCV was found to have a ribonucleic acid (RNA) genome with similar characteristics to the flaviviruses and pestiviruses. HCV was therefore classified as a third separate genus, hepacivirus, in the Flaviviridae virus family (17). The hepatitis C virion is a spherical particle of approximately 55-65 nm (8, 18). The genome consists of a single stranded positive sense RNA of approximately 9600 nucleotides, containing a single open reading frame (ORF). The ORF encodes a precursor poly-protein of 3010-3033 amino acids (aa) encoding the 10 viral proteins. The precursor protein is cleaved into the structural proteins core (C), envelope (E) 1, E2 and p7 and the non-structural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. (19, 20). The NS proteins are responsible for replication and packaging of the viral genome into capsids formed out of structural proteins (21). The genetic organisation of the HCV is summarised in a simplified manner in Figure 1.



**Figure 1.** The organisation of the HCV genome and the function of its proteins are shown.

### 1.3.1 Genetic heterogeneity: Genotypes

The genetic heterogeneity of HCV was revealed in the early 1990s (22, 23). The classification of HCV was put forward by the publication of a consensus paper in 1994 (24), dividing the HCV into 6 genotypes, using phylogenetic methods. Each genotype contains a number of subtypes, indicated by a letter (a, b, c, etc.). Genotypes differ from each other by ~32% at the nucleotide level, compared to ~22% between subtypes.

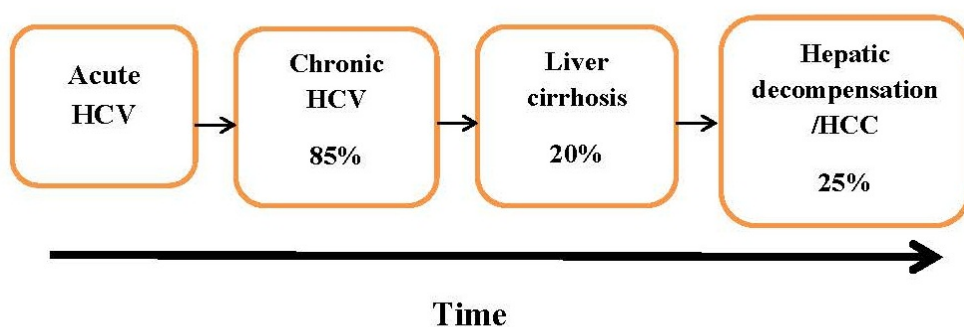
The HCV genotypes are clustered differently in the world. Genotypes 1, 2 and 3 and their subtypes are distributed worldwide. Genotype 4 appears to dominate in Africa, mainly in Zaire and Egypt, while genotype 5 is mainly found in South Africa and genotype 6 in Asia (25). Genotype 1 is the most common genotype in Sweden and has been estimated to account for 41-70% of HCV-infected patients (26, 27).

The realisation of the diversity of HCV has important clinical implications. A majority of patients infected with genotypes 2 and 3 are curable with standard of care (SOC) therapy, which consists of pegylated interferon (peg-IFN) in combination with ribavirin, while only 42–46% of genotype 1-patients are cured by such treatment (28, 29). However, with the introduction of first generation NS3/4A protease inhibitors in

2011 as part of treatment for genotype 1-patients, the cure rate in this group has increased substantially (30, 31).

#### 1.4 MILESTONES TOWARDS ADVANCED LIVER DISEASE

Several studies have explored the natural history of HCV infection. About 85% of patients infected will develop a chronic infection. Of these, approximately 20-30% will progress to develop liver cirrhosis during the following 20 to 40 years. Approximately 25% will go on to develop hepatic decompensation/HCC (32). Section 1.7 discusses this in more depth. Figure 2 describes the typical milestones towards HCV-induced advanced liver disease.



**Figure 2.** Steps of liver disease caused by HCV infection. Values shown as percentage of patients from each group.

#### 1.5 ACUTE HEPATITIS C INFECTION

Acute HCV infection is followed by viral clearance, which is defined as undetectable HCV RNA in the blood, and can develop 2-12 weeks after being exposed to the virus, lasting less than 3 months (33, 34). This occurs in about 25% of individuals infected (35). However, if viremia persists for more than 6 months, it is accepted as a chronic infection (36).

In the initial phase of infection, the virus appears in the blood within 2-15 days of exposure. Levels of liver-associated serum enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), increase and HCV-specific antibodies appear gradually within 20-150 days of exposure (37-39). The mean incubation period is 7 weeks and 10-15% of patients report symptoms. Primary HCV infection often has mild and non-specific symptoms, such as lethargy and myalgia. However, jaundice may be

present (40). Some patients clear the virus spontaneously and remain anti-HCV positive for decades with no HCV RNA detectable in serum or liver tissue (41, 42).

The following factors have been identified with spontaneous clearance: an effective immunity shown as jaundice or other signs and symptoms of hepatitis (33, 43-45), age less than 40 years (35, 46), female gender (35, 47, 48), a disease presented with lower viral load (43) and being of non-black ethnic origin (43, 49).

## **1.6 CHRONIC HEPATITIS C INFECTION**

In the majority of newly infected individuals, viremia persists beyond 6 months and leads to chronic hepatitis C (CHC) infection. CHC infection is diagnosed based on the presence of anti-HCV antibodies and HCV RNA. Serum is used to detect the presence of HCV RNA by testing for the quantity of viral particles by the reverse transcriptase-polymerase chain reaction. The detection of HCV antibody is usually carried out through commercially available enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay. A positive ELISA result may be confirmed with a more specific supplementary test called recombinant immunoblot assay (50).

Liver disease, manifested in the form of progressive fibrosis and the development of cirrhosis, determines the morbidity and mortality of CHC infection (51). Several factors affect the natural course of HCV infection. The most consistent environmental risk factor for accelerated disease course is alcohol abuse (52, 53). It has been established that HCV infection and heavy alcohol consumption synergistically accelerate liver injury and progression to cirrhosis (54) and HCC (55, 56). Even moderate alcohol intake seems to increase fibrosis progression (57). The mechanisms by which alcohol and HCV interact to synergistically accelerate liver damage are not yet fully understood.

Patients with CHC tend to have more severe liver disease if they are obese or diabetic (58, 59).

Table 1 shows factors associated with disease progression in CHC, divided into non-modifiable and potentially modifiable factors.

**Table 1.** Factors associated with disease progression in chronic hepatitis C.

Non-modifiable factors	Potentially modifiable factors
Age at acquisition of infection Duration of infection Male sex Race Host genetic factors Viral genotype	ALT level Activity on liver biopsy Alcohol consumption Co-infection with HBV or HIV Co-infection with schistosomiasis Metabolic factors (steatosis, insulin resistance) Cigarette smoking Daily cannabis use Iron overload

Reprinted from Missiha et al, 2008 (60), with permission from Elsevier.

HCV infection has also been associated with extrahepatic manifestations, such as cryoglobulinemia, porphyria cutanea tarda, membranous glomerulonephritis and with increased risk of non-Hodgkin lymphoma (61).

### 1.6.1 Viral evasion strategies by hepatitis C virus

As described further on, HCV establishes a chronic infection in the majority of cases. In order to survive the host immune responses, HCV has developed multifactorial mechanisms to evade immune elimination and can thereby achieve a persistent infection.

The first defence of the human body against viral infection is the innate immune response, which in the liver is constituted by natural killer (NK) cells, natural killer T (NKT) cells, Kupffer cells (liver macrophages), and a rapid interferon (IFN) or cytokine response exerted by the infected hepatocytes.

Human cells/hepatocytes recognize HCV or other microbial pathogens through a wide variety of pathogen recognition receptor (PRR) molecules, including the retinoic acid inducible gene I (RIG-I) like receptors (RLRs), Toll-like receptors (TLRs) and NOD-like receptors (NLRs). PRRs serve to distinguish self from non-self by their recognition and interaction with pathogen specific molecules, termed pathogen associated molecular patterns (PAMPs) (62). IFNs are a major product of PRR signaling and are produced and secreted from HCV-infected cells. IFNs inhibit viral replication, cell proliferation and apoptosis via different signal pathways and play a role in both the innate and adaptive immune responses.



RIG-I is the major PRR that recognizes HCV and triggers the antiviral immune response (63, 64). The role of TLRs in sensing the HCV and promoting antiviral immunity is not fully understood as that of RIG-1. TLR responses may play a role in sensing HCV within infected cells, possibly by increasing IFN production and responses, with promotion of inflammatory signaling within the infected liver.

A major strategy employed by HCV to subvert the host innate immune response is to undermine IFN antiviral activity (65). In this context, the NS3/4A protease has been proposed as the key viral protein (further described in 1.6.1.1), but also HCV core (66, 67), NS5A and the glycoprotein E2 have been shown to interact with IFN activity (68, 69).

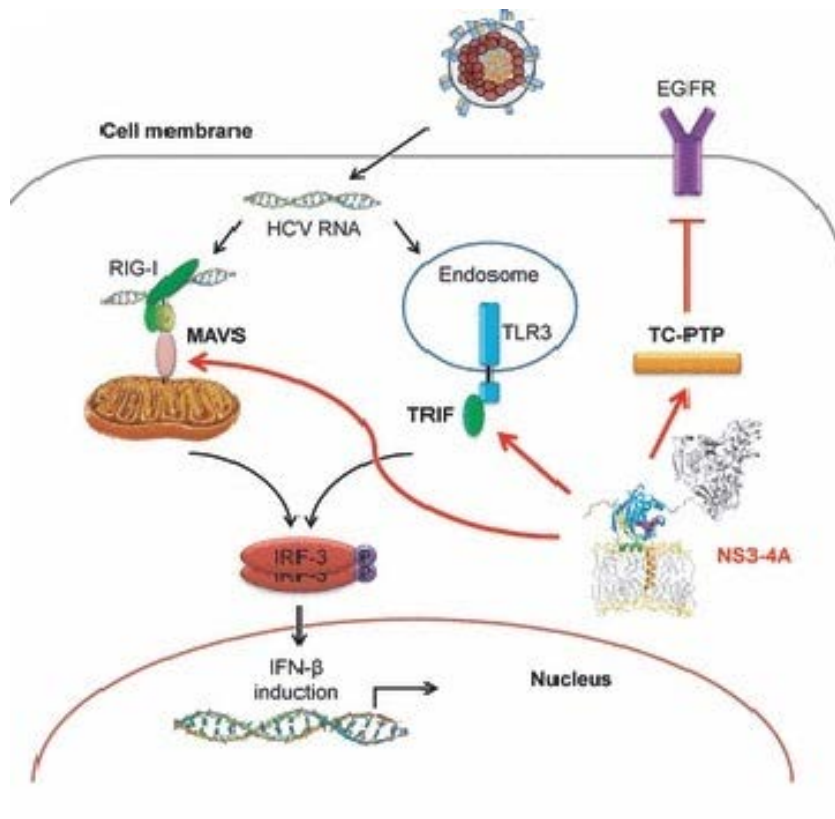
The innate immune responses are followed by activation of adaptive immune responses including CD4<sup>+</sup>, CD8<sup>+</sup> T cells and B cells. Various mechanisms have been suggested in which the HCV impairs the adaptive immune responses. Evidence suggests that CD4<sup>+</sup> and CD8<sup>+</sup> T cells have crucial but distinct roles in determining the outcome of HCV infection (70).

One of the most potent immune evasion strategies that the HCV employs is escape by mutations. Its error-prone RNA polymerase lacks proof-reading ability and together with the high viral replication rate a tremendous amount of mutations are generated (71) and hence immune escape is promoted.

#### *1.6.1.1 The NS3/4A complex and its cellular targets for establishing persistent infection*

The NS3 is a multifunctional protein with an N-terminal serine-protease domain of around 180 aa, and a C-terminal 442 aa domain with helicase/NTPase activities. The complete protease encompasses both the NS3 and the co-factor NS4A, comprising 54 aa. The NS3/4A complex combines the enzymatic activities of a protease and helicase, the first one needed for polyprotein cleavage and the latter responsible for unwinding and separation of the replicating double-stranded RNA. Due to its essential role for viral replication, the NS3/4A protease has been one of the most attractive targets for developing specific antiviral drugs against HCV. The first generation of NS3/4A protease inhibitors were introduced as part of therapy in the fall of 2011 (further described in section 1.8.4) and several other agents are under development (30, 31).

Apart from being essential for the viral life-cycle, the NS3/4A protease/helicase has been proposed as a key complex in modulating the infected hepatocyte by blocking innate immune pathways and thereby contributing to the persistence and pathogenesis of HCV. The cellular targets of the NS3/4A are illustrated in a simplified manner in Figure 3. By cleaving and inactivating cellular proteins which induce innate immune responses, being the first line of defence, HCV may establish persistent infection. The targets of the NS3/4A complex identified until now are mitochondrial antiviral signaling protein (MAVS), TIR domain-containing adaptor inducing IFN $\beta$  (TRIF) and T cell protein tyrosine phosphatase (TCPTP) (72).



**Figure 3.** Cellular targets of the HCV NS3/4A protease. By Morikawa et al, 2011 (72).

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#### 1.6.1.1.1 MAVS and TRIF

Hepatocytes are thus believed to sense HCV RNA through RIG-I and TLR3. By cleaving the adaptor molecules MAVS and TRIF, interferon regulatory factor 3 (IRF3) activation and IFN production are blocked. (73-75). This prevents the establishment of an antiviral state in infected and neighbouring cells.

#### 1.6.1.1.2 TCPTP

TCPTP, also known as PTPN2 (protein tyrosine phosphatase nonreceptor type 2), is an ubiquitously expressed phosphatase first described in T cells (76). For cellular homeostasis, the maintenance of proper protein tyrosine phosphorylation levels is critical (72). The fact that TCPTP-deficient mice die 3-5 weeks after birth because of systemic inflammation (77) shows the fundamental role of TCPTP as a negative regulator of diverse signal transduction pathways. Epidermal growth factor (EGF) stimulation causes TCPTP to exit from the nucleus, resulting in dephosphorylation of the EGF receptor. This leads to decreased downstream activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (78).

A rather recent finding has shown that the third cellular target of NS3/4A identified until now is TCPTP. The cleavage of TCPTP by NS3/4A causes the important negative feedback regulation of epidermal growth factor receptor (EGFR) signaling and Akt activation to be disrupted, resulting in an enhancement of EGF-induced signal transduction and an increase basal activity of Akt. These are both essential for the maintenance of sufficient viral replication (79). The increase in EGFR activity also enhances HCV cell entry (80). In addition, the inactivation of TCPTP has been found to have a possible implication for the development of HCC (81-84).

In short, cleavage of TCPTP may have important consequences for the HCV life-cycle and signal transduction as well as HCV-induced liver diseases.

### **1.7 HCV-INDUCED LIVER CIRRHOSIS**

The main histopathological feature of liver cirrhosis is the extensive deposition of extracellular matrix responsible for the increased resistance to portal blood flow, development of vascular shunting and regenerative nodules.

Certain risk factors leading to cirrhosis are known. The single most important factor known to contribute to the progression of liver damage in CHC infection is persistent inflammation. Acquired infection after age 40 years, male sex, excessive alcohol-consumption, HBV or HIV co-infection, steatosis and immunosuppressed state have been identified as co-factors associated with progression of fibrosis and development of cirrhosis (60, 85-89).

The exact prevalence of HCV-induced cirrhosis is unknown due to the high number of undiagnosed HCV-infected individuals and since compensated cirrhosis often goes undetected for an extended period of time. The prevalence of HCV-induced liver cirrhosis is estimated to be rising, with increased cases of HCC in the Western world.

### 1.7.1 Diagnosis of liver cirrhosis

Liver biopsy has been considered as the gold standard for fibrosis evaluation and treatment indication in patients with CHC (90). However, in recent years it has been gradually replaced with transient elastography (TE), which is a non-invasive method measuring liver stiffness (FibroScan®, Echosens, Paris, France) (91, 92). The disadvantages of liver biopsy are sampling errors and intra- and inter-observer variability that may lead to understaging (93-95). Also, liver biopsy is a painful procedure with rare, but potentially life-threatening complications. The limitation of TE is mainly failure (around 5% of cases), mostly in obese patients. Studies of TE have demonstrated very high accuracy for determining the presence or absence of advanced fibrosis (92).

The extent of liver fibrosis is of major importance in CHC. For patients with HCV, one of the few validated scoring systems designed is called METAVIR (96, 97). It uses two separate scores, one for the stage of fibrosis (F from F0 to F4 cirrhosis) and another for necro-inflammatory grade (A for activity, from A0 to A3). Activity scores are: A0 = no activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity. In Table 2, METAVIR score fibrosis staging and corresponding figures of TE are shown (98).

**Table 2.** METAVIR score and corresponding liver stiffness measurement with transient elastography are shown.

<b>Fibrosis stage according to METAVIR score</b>	<b>Transient elastography (kPa)</b>
F0 = no fibrosis	F0-F1: 2.5- 7
F1 = portal fibrosis without septa	
F2 = portal fibrosis with few septa	7–9.5
F3 = numerous septa without cirrhosis	9.5–12.5
F4 = cirrhosis	>12.5

Cirrhosis can also be diagnosed based on the clinical presentation of the patient. Patients with cirrhosis may present stigmata of chronic liver disease at physical examination, e.g. spider angiomas, palmar erythema, flapping tremor (asterixis) and gynecomastia. If signs of decompensation, such as ascites, variceal bleeding or hepatic encephalopathy and/or presence of varices or portal hypertensive gastropathy during gastroscopy are present, combined with changes in liver function test values (albumin, prothrombin index, bilirubin) and radiological imagery indicating cirrhosis, the diagnosis can be made without performing biopsy. Low platelet count and AST/ALT ratio  $> 1$  also indicate cirrhosis (95).

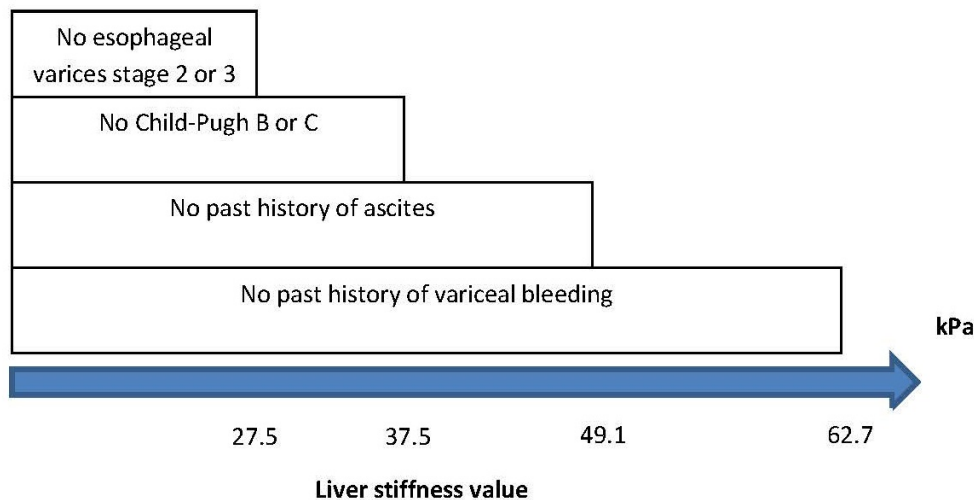
### **1.7.2 Clinical features of liver cirrhosis**

Cirrhosis is often asymptomatic and unsuspected until the occurrence of liver complications. When severe portal hypertension occurs with a caval-to-portal pressure gradient above the threshold value of 12 mmHg, the critical step is reached that eventually gives rise to liver decompensation (99). Chronic liver disease is said to be decompensated when one or the other complication of the disease has developed: ascites, variceal bleeding, impaired hepatic synthetic function, jaundice, or hepatic encephalopathy (Table 3).

Studies report that the first most common and most frequent complication to arise is HCC and then ascending in hierarchical order: ascites, jaundice, bleeding and encephalopathy (100, 101). TE has shown to be useful not only in diagnosing the presence of cirrhosis, but also assessing its severity. A study established cut-off values for complications of cirrhosis with a negative predictive value  $>90\%$  according to Figure 4 (102).

**Table 3.** Features of decompensated liver disease

	<b>Description</b>	<b>Cause</b>
<b>Ascites</b>	Abnormal accumulation of serous fluid in the abdominal cavity	Portal hypertension
<b>Variceal haemorrhage</b>	Bleeding from dilated vessels, usually in esophagus or stomach	Portal hypertension
<b>Impaired hepatic synthetic function</b>	Decreased level of albumin	Decreased hepatic production, sequestration into ascites and interstitium
<b>Jaundice</b>	Yellow discoloration of skin, sclerae and mucous membranes	Impaired hepatocyte excretory function, occurs when serum bilirubin >20 mg/L
<b>Hepatic encephalopathy</b>	Confusion, altered level of consciousness or coma	Accumulation of toxic substances

**Figure 4.** Usefulness of liver stiffness measurement with FibroScan® in clinical practice. By Foucher et al, 2006 (102). Adapted by permission from BMJ Publishing Group Limited.

The Child-Pugh score and the model for end-stage liver disease (MELD) are also frequently used to assess the risk of decompensation (103). The Child-Pugh score is by far the most widely used both in clinical practice and in clinical research. The score lacks variceal bleeding as a variable, since it was originally developed to predict the operative risk of mortality associated with surgical portosystemic shunt surgery in

patients with cirrhosis and variceal bleeding (104). MELD is primarily used to predict the 90-day mortality of patients awaiting liver transplantation (105). There is no evidence to support the superiority of one model over the other in terms of accuracy (106, 107).

Child-Pugh score is dependent on the following variables: bilirubin, albumin, ascites, encephalopathy and prothrombin time. The score corresponds to the total of points for each item (Table 4). According to the sum of these points, patients can be categorized into Child-Pugh grade A (5 to 6 points), B (7 to 9 points) or C (10 to 15 points).

**Table 4. Child-Pugh classification**

	<b>1 point</b>	<b>2 points</b>	<b>3 points</b>
Encephalopathy	Absent	Medically controlled	Poorly controlled
Ascites	Absent	Medically controlled	Poorly controlled
Bilirubin (mg/L)	<20	20–30	>30
Albumin (g/L)	<35	28–35	<28
INR	<1.7	1.7–2.2	>2.2

Child-Pugh (5–6 points), Child-Pugh B (7–9 points) and Child-Pugh C (10–15 points) predict a life expectancy of 15–20, 4–14, and 1–3 years respectively. INR=international normalised ratio.

The annual incidence rate of hepatic decompensation in cirrhotic patients has been found to be 2.9-4.4% and the 5-year cumulative incidence of developing decompensation after diagnosis of HCV-induced cirrhosis has been reported to be 28% (101, 108-110). Approximately 80% of patients with stable cirrhosis and no previous episodes of decompensation will survive the next 10 years (32). Once decompensation has occurred, the survival declines significantly; the 5-year survival rate in decompensated cirrhotics is 50% (111) and barely 30% during the next 10 years (112, 113). Liver transplantation (LT) is the treatment of choice in all such cases. If HCV is not eradicated before going to LT, re-infection with HCV occurs in all transplant recipients as a rule. This in turn leads to cirrhosis in around 30% of patients in 5 years (114).

Independent factors associated with hepatic decompensation (excluding HCC) in studies involving patients with HCV-related cirrhosis are older age at infection, higher baseline bilirubin level, lower albumin level, lower platelet count, stigmata of chronic liver disease on physical examination, the presence of esophageal varices, absence of interferon therapy and high baseline AST/ALT ratio (100, 101, 103, 109, 110, 112, 115).

There is evidence that the severity of portal hypertension, assessed by detection of esophageal varices, is the main independent predicting factor of decompensation, HCC, and mortality (116). A recent study found TE to be the single best individual predictor of clinical outcome (117).

## **1.8 TREATMENT OF HEPATITIS C INFECTION**

The goal of therapy is to eradicate HCV infection in order to prevent development of cirrhosis and the consequence of cirrhosis, with occurrence of decompensation, HCC, and liver-related death (118). Patients with liver cirrhosis are therefore the group of patients with the most urgent need to achieve eradication of HCV. The endpoint of therapy is sustained virological response, defined as undetectable HCV-RNA at the end of treatment and at follow-up 6 months after cessation of treatment. In clinical practice, a further HCV RNA test, at 6-12 months after SVR, is performed to rule out late relapse and to diagnose an eradicated infection. The incidence of late relapse has however been reported to be extremely low (<1%) (119). Patients having achieved SVR are informed that although negative in HCV RNA tests, they can be HCV antibody positive for many years and they are not allowed to be blood or organ donors. Neither are they immune to re-infection with HCV.

All patients who want to be treated without contraindications to therapy should be considered for therapy according to the EASL guidelines (118). However, not all patients are in need of therapy, due to the natural course of HCV infection. Some patients will never develop any complications of liver disease during their lifetime and the infection will affect neither their quality of life nor psychosocial well-being. In the Swedish treatment guidelines, it is therefore stated that it is important to determine whether the patient will benefit from treatment or not through assessing the extent of liver damage and take in account other factors as age, general health and personal considerations regarding therapy (120).



### 1.8.1 History of treating hepatitis C virus infection

Even before HCV was identified as the cause of the disease, studies began to evaluate treatment of patients with NANB hepatitis. Acyclovir was one of the first antiviral agents to be evaluated in the 1980s, but failed to show a positive effect (121). The first encouraging results were shown in trials with IFN- $\alpha$ , confirming its partial effectiveness with SVR rates of about 6%. When ribavirin was added to IFN- $\alpha$ , SVR rates were raised up to 42%. The next step was to enhance the half-life of IFN via pegylation, which further improved the virological response (122). Peg-IFN in combination with ribavirin have constituted standard of care (SOC) therapy for CHC infection during the past decade. In the fall of 2011, two first generation protease inhibitors were approved for use in combination with SOC therapy for genotype 1 patients with further improved SVR rates to around 70% (30, 31).

### 1.8.2 Standard of care treatment

One of the two peg-IFN- $\alpha$  molecules, peg-IFN- $\alpha$ -2a (Pegasys<sup>®</sup>) or peg-IFN- $\alpha$ -2b (Pegintron<sup>®</sup>), can be used in combination with ribavirin, together constituting the SOC therapy. During treatment, patients are regularly monitored with blood tests, HCV RNA measurements and clinical examinations. The virological terms connected to SOC are shown in Table 5. HCV RNA levels at 4, 12 and 24 weeks of therapy can be used to assess the likelihood of achieving SVR.

SOC therapy is often lengthy and associated with considerable side effects. Flu-like symptoms such as fever, headache, myalgia, fatigue, anemia (mainly ribavirin-associated), depression, skin rash and gastrointestinal symptoms are the most common side-effects. Full adherence to both peg-IFN and ribavirin is associated with improved SVR rates (123). Before starting treatment, the following has been recommended:

- Assessment of fibrosis
- Assessment of HCV genotype
- If cirrhosis, check for esophageal varices and HCC (gastroscopy, US)
- Exclude contraindications (psychiatric, severe cardiac disease etc.)
- IL28B genotype (introduced in recent years)

Genotype CC in genotype 1 patients has been associated with a higher probability of attaining SVR with SOC treatment (124).

**Table 5.** Virological Responses during SOC therapy and definitions. By Ghany et al, 2009 (125).

<b>Virological Response</b>	<b>Definition</b>	<b>Clinical Utility</b>
Rapid virological response (RVR)	HCV RNA negative at treatment week 4 by a sensitive PCR-based quantitative assay	May allow shortening of course for genotypes 2&3
Early virological response (EVR)	$\geq 2$ log reduction in HCV RNA level compared to baseline HCV RNA level (partial EVR) or HCV RNA negative at treatment week 12 (complete EVR)	Predicts lack of SVR
End-of-treatment response (ETR)	HCV RNA negative by a sensitive test at the end of 24 or 48 weeks of treatment	
Sustained virological response (SVR)	HCV RNA negative 24 weeks after cessation of treatment	Best predictor of a long-term response to treatment
Breakthrough	Reappearance of HCV RNA in serum while still on therapy	
Relapse	Reappearance of HCV RNA in serum after therapy is discontinued	
Nonresponder	Failure to clear HCV RNA from serum after 24 weeks of therapy	
Null responder	Failure to decrease HCV RNA by $< 2$ logs after 24 week of therapy	
Partial responder	Two log decrease in HCV RNA but still HCV RNA positive at week 24	

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Among factors associated with SVR, the IL28B genotype and rapid virological response (RVR), the latter regardless of genotype or treatment regimen, have the strongest predictive value (125, 126). With the SOC regimen in genotype 1 patients, 15-20% of patients were estimated to achieve RVR. For genotype 2 and 3 infections the percentage was shown to be 66% (127, 128) and these may be able to shorten duration

of treatment in case of low viral load at baseline. For genotype 1 non-cirrhotic patients who achieved RVR, a 24 week course therapy was recommended until 2011. The current recommendation for genotype 2- and 3-patients is 12-16 weeks of therapy in the presence of RVR and low viral load at baseline (125). Hence RVR is crucial to response-guided therapy.

In pivotal clinical trials for SOC therapy, SVR rates of 42-46% have been reported in patients with genotype 1 infection and 76-82% in patients with genotype 2 or 3 infections (29, 123, 129).

### **1.8.3 Treatment in patients with cirrhosis**

Patients with liver cirrhosis constitute a group of patients with the most urgent need for eradication of HCV, but have at the same time been found to be the most difficult to treat. For previously untreated patients with fibrosis stage F3/F4, large randomised controlled trials (RCTs) have shown SVR rates of 10-16% lower than patients with less progressed fibrosis (29, 129, 130), the difference being largest in patients with genotype 1 infection where 16% was seen (129). In cirrhotic patients with genotype 3, SVR was achieved in less than 50% and the risk of relapse was ten times higher than those with milder fibrosis (131). In previously treated patients, re-treatment with peg-IFN- $\alpha$ -2a and ribavirin comprising patients with >90% genotype 1 infection, SVR was achieved in only 9-10% (132).

No studies so far have evaluated the optimal length of treatment in cirrhotic patients. The recommended length of treatment, regardless of genotype or previous treatment, is 48 weeks. RVR was achieved only among 6% of F4 patients with genotype 1 after SOC treatment in a meta-analysis (133). However, RVR is not very predictive for SVR in these patients with a positive predictive value of 50%, so shortened therapy is not recommended, even if RVR is achieved. A shorter duration of treatment of 24 weeks may be considered for patients with genotype 3 or especially 2, who attain RVR and present favorable demographic features (low BMI, young age, genotype CC on IL28-B). Child-Pugh score class A (in genotype 1 cases only) and lower pre-transplantation viral loads are other positive predictors.

Therapy-associated side effects with peg-IFN and ribavirin are more common in cirrhotics than those with milder fibrosis. Cytopenia with neutropenia was seen in 38%

in cirrhotics, compared to 6% among non-cirrhotics. The corresponding figures for anemia and thrombocytopenia were 35% versus 15% and 24% versus 17% (134). Due to the higher risk of cytopenias, patients with cirrhosis have to be monitored more frequently during treatment. Risk of infection was seen in approximately 1% of cirrhotics compared to <1% in non-cirrhotics.

#### *1.8.3.1 Treatment in patients with decompensated disease*

Child-Pugh class B patients with a score  $\leq 9$  and history of a decompensated event that has vanished after treatment are recommended to be offered antiviral therapy in specialised units with close collaboration with transplantation clinics (135). Child-Pugh class B and C patients have a lower chance of achieving SVR than those with Child-Pugh class A. SVR rates of only 16% and 28% were observed in Child-Pugh class B patients with and without genotype 1 respectively (136). Generally, Child-Pugh class C patients are not considered for treatment because of the low probability of SVR and high risk of severe or fatal side effects. In Child-Pugh class B patients, the risk/benefit ratio should be assessed on a case by case basis (137) and treatment should especially be considered before transplantation, which in itself is the best treatment option in this population. Ribavirin-induced hemolytic anemia and interferon-induced neutropenia are reported to be one of the most common causes of antiviral dose reductions/withdrawal in patients with decompensated disease (135).

#### *1.8.3.2 The impact of SVR in patients with HCV-induced liver cirrhosis*

In patients with HCV-induced cirrhosis it has been found that achieving SVR reduces the risk of developing HCC, the annual risk being between 0.7-1.2% (138-141), although somewhat diverging results exist. Most studies have been of retrospective design, including relatively few patients, or have had short follow-up periods (142, 143). However, it has been found that SVR improves overall survival and reduces the incidence of other components of liver-related morbidity and mortality (i.e., hepatic decompensation, and liver-related death or liver transplantation) when compared to nonresponders (138, 142, 144).

It has also been shown that SOC therapy reduces incidence and progression of cirrhosis, especially if SVR is achieved and in these cases even cirrhosis reversal seems possible (99, 145, 146).

#### **1.8.4 Introduction of first generation protease inhibitors**

During the past decade efforts have been made to develop different compounds with antiviral activity against HCV genotype 1, called direct acting antiviral agents (DAAs) (147). Two HCV NS3/4A protease inhibitors called boceprevir and telaprevir have completed a clinical development programme and have been introduced as part of a standard triple therapy with peg-IFN- $\alpha$  and ribavirin in Europe, USA and other countries in the fall of 2011 (126), i.e. after inclusion of patients in the studies of this thesis. So far, these two agents have not been compared in any randomised study. For previously untreated patients, studies have shown SVR rates of 75% for telaprevir and 63-66% for boceprevir (30, 31, 148). The principal side-effect of boceprevir is anemia, while treatment with telaprevir has been associated with mild to severe skin rash (149).

At the moment there are limited data on using DAAs in patients with non-genotype 1 and these agents are not recommended in these groups of patients.

The addition of DAAs has resulted in a higher achievement of SVR and made possible a shorter duration of treatment in non-cirrhotic patients. The duration of treatment of patients with cirrhosis is still 48 weeks of therapy. Furthermore, DAAs also have to be developed for the other HCV genotypes (150).

There is currently somewhat limited data on using first generation protease inhibitors as part of triple regimen in cirrhotic patients with genotype 1 infection from registration trials, due to the rather small proportion of included patients with cirrhosis. However, it has been seen that the addition of boceprevir or telaprevir to standard treatment with peg-IFN and ribavirin increased SVR rates in patients with severe fibrosis or cirrhosis. In naïve patients with fibrosis stage F3/F4, SVR rates of 52-63% were seen. In treatment-experienced patients, SVR rates were 83-84% for relapsers, 34-46% for partial responders and 14% for null responders (30, 31, 148, 151, 152). Hence, the benefits of using protease inhibitors versus standard treatment were maintained also in cirrhotic patients. However, a higher relapse rate and more frequent side effects, mainly anemia, were seen in patients with cirrhosis compared with those without. There are no data on the efficacy of the triple regimen in patients with decompensated cirrhosis (137). The current recommendation as from 2011 concerning cirrhotic genotype 1 patients when using telaprevir is 12 weeks of triple treatment followed by 36 weeks with peg IFN-2a and ribavirin. When using boceprevir, a 4 week lead-in (using only

peg-IFN and ribavirin) is recommended, followed by 44 weeks of triple treatment. No response-guided therapy is available for cirrhotic patients and 48 weeks of therapy is thus needed.

## **1.9 HEPATOCELLULAR CARCINOMA**

Hepatocellular carcinoma (HCC) is a major cause of morbidity and mortality: HCC is the seventh most common cancer worldwide and the third leading cause of cancer-related deaths (153). Together, chronic HBV and HCV infections account for around 75% of all HCCs worldwide. Over 80% of HCCs occur in developing countries. High incidence rates are found in sub-Saharan Africa, Southeast Asia, and East Asia (including Mongolia), where the prevalence of HBV and HCV infections usually are high (153). HCV has been the dominant viral cause of HCC in North America, some Western countries and Japan (154). The risk of HCC in patients with HCV mainly depends on the degree of fibrosis. It has been estimated that of all HCCs, 80–90% develop in a cirrhotic liver (155). The strongest and the most common known risk factor for HCC is cirrhosis (156). The annual risk of HCC in untreated patients with HCV-induced liver cirrhosis is estimated to be 0.5-5% in the Western world (157) and in Japan as high as 7.9% (158).

To the present day, molecular mechanisms underlying HCC development are not clear (159, 160). It has not been possible to correlate specific changes in gene expression patterns with HCC development. Hepatocarcinogenesis can be described as an interaction between chronic inflammation, steatosis, fibrosis and oxidative stress and the damages they cause. Also, HCV proteins have been associated with causing direct oncogenic effects (161). In cirrhosis, the decreased liver reserve may increase accumulation of toxic metabolites, which could possibly increase the risk of HCC (162). Also, the cirrhotic liver is associated with telomere shortening, which in turn may lead to chromosomal instability and deletion of checkpoints (163). The development of HCC in a cirrhotic liver is believed to occur either as *de novo* hepatocarcinogenesis or through a multistep pathway that starts from a dysplastic focus arising within a regenerative nodule to early HCC and then finally progressed HCC (164).

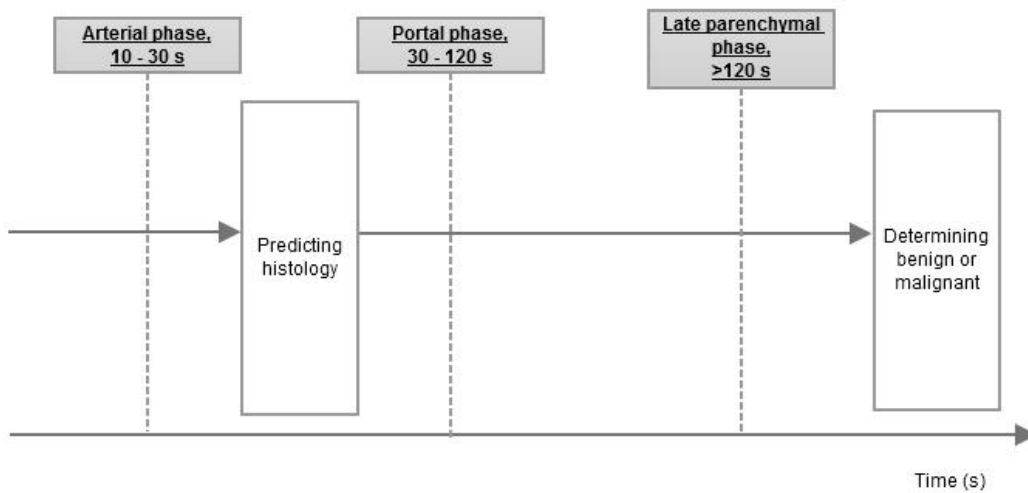
### **1.9.1 Risk factors of hepatocellular carcinoma**

It has been shown that host and environmental factors appear to be more important than viral factors in determining the progression of liver disease to cirrhosis and HCC in HCV-infected patients. These factors include: older age at diagnosis (>55 years: 2- to 4-fold increased risk) (165, 166), duration of infection (167), male sex (2- to 3-fold increased risk) (115), severity of liver disease at presentation, co-morbidities such as porphyria cutanea tarda (168), heavy alcohol intake (168-171), diabetes mellitus (172, 173), steatosis (174, 175), obesity (173, 176) and co-infections, especially with HBV (177, 178). Also, slightly elevated serum bilirubin levels, decreased platelet counts and manifestations of liver disease in the skin, such as vascular spiders and/or palmar erythema, have been associated with the HCC risk (177, 178). In addition, specific human leukocyte antigen (HLA) class II alleles have been correlated with the progression of chronic hepatitis C to decompensated cirrhosis or HCC (165).

### **1.9.2 Surveillance for hepatocellular carcinoma with ultrasound**

As previously described, the risk of HCC in patients with chronic hepatitis C is highest. It has been best studied in patients who have established cirrhosis (112, 167, 178, 179), in whom the incidence of HCC is between 0.5-8% per year (157, 180). The current understanding is that all patients with CHC and cirrhosis should undergo surveillance for HCC. The radiological test most widely used for screening and surveillance is ultrasonography (US) with a proposed interval of 6-12 months. In the 1990s, contrast enhanced (CE) US technologies were introduced. CEUS can accurately differentiate between benign and malignant liver tumours and is recognised as a screening or surveillance technique for HCC. Contrast agents are well tolerated after being intravenously injected in patients and carry very few contraindications. They consist of gas-filled microbubbles which remain within the vascular compartment and cannot move through the vascular endothelium into the interstitium, wherefore they are true blood pool agents. The microbubbles will remain in the circulation for some minutes, after which they will dissolve with the gas being exhaled and the shell metabolised by the liver to a large extent. An US wave will cause the microbubbles to respond by oscillating and produce a returning signal, called echo (181). The ability to bounce an echo is defined as echogenicity. The first-generation contrast agent (e.g. Levovist®) is useful for high mechanical index imaging and the second-generation agent (e.g. SonoVue®, Sonazoid® and Definity®) for low mechanical index imaging and vascularity assessment (182). The phases of contrast enhancement include: arterial (10-

35 s after injection), portal (30-120 s) and late parenchymal (>120 s) (181, 183). In a simplified manner it could be said that the arterial phase is useful for predicting the histology of a lesion, while the late phase helps distinguishing between its benign or malignant character (181, 184), (Figure 5). This is especially valid for larger lesions. The enhancement pattern of the lesion (hypo-, hyper- or iso-echoic) is compared to the adjacent liver parenchyma during these phases and evaluated in CEUS characterisation (185).



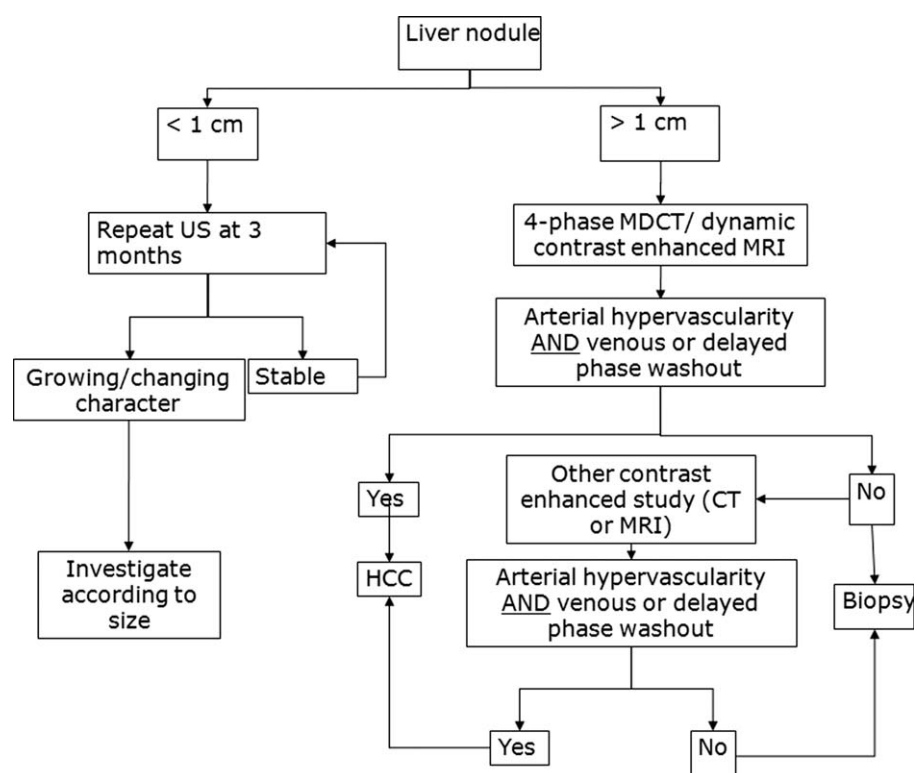
**Figure 5.** The three vascular phases of contrast enhancement in the liver

The radiological hallmark of hepatocellular carcinoma (HCC) is the phenomenon of arterial vascularity and venous washout (186, 187). It has been reported that >90% of HCCs enhance during the arterial phase and the majority, 83-97%, wash out the contrast in the late phase (181). Hence, a majority of HCCs will be appearing as hyperenhancing nodules in the arterial phase and hypoechoic in the late phase.

Serum AFP concentration has been the most commonly used marker for HCC, but due to its low sensitivity it is no longer generally recommended for surveillance (180, 188).

A diagnostic algorithm for suspected HCC has been elaborated by The American Association for the Study of Liver Diseases (AASLD) in 2010 (189), (Figure 6).

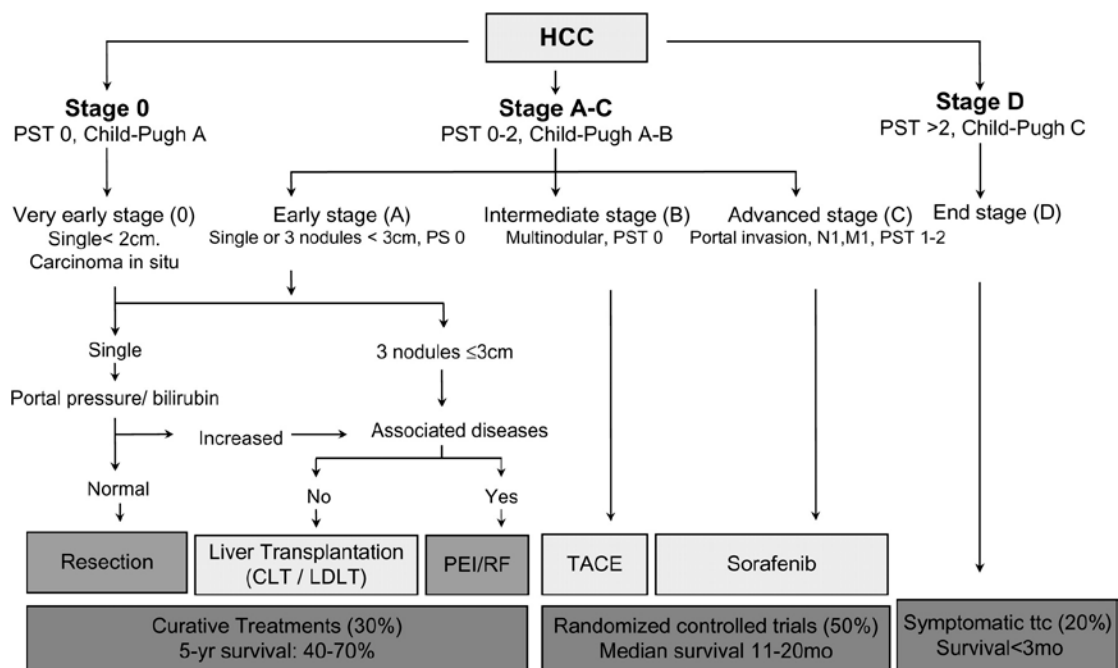




**Figure 6.** Diagnostic algorithm for suspected HCC. CT, computed tomography; MDCT, multidetector CT; MRI, magnetic resonance imaging; US, ultrasound (189). Copyright© 2011 American Association for the Study of Liver Diseases

### 1.9.3 Treatment of hepatocellular carcinoma

The Barcelona Clinic Liver Cancer (BCLC) has elaborated a staging and treatment strategy for HCC which is widely recognised and endorsed (Figure 7) (180, 190-192). The prognostic factors it includes are related to tumour status, liver function and general health status. This staging system suggests that curative treatment (resection, liver transplantation or percutaneous local ablative treatment) is appropriate for patients with very early (stage 0) and early (stage A) HCCs. Transarterial chemoembolization (TACE) is chosen for patients with intermediate (stage B) HCC, whereas chemotherapy with sorafenib is selected for patients with advanced (stage C) HCC. Best supportive care is recommended for patients with terminal (stage D) HCC.



**Figure 7.** Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy for HCC (190). By permission of Journal of the National Cancer Institute.

PST=performance status test, CLT =cadaveric liver transplantation, LDLT=living donor liver transplantation, PEI=percutaneous ethanol injection, RF=radio frequency, TACE=transarterial chemoembolization.

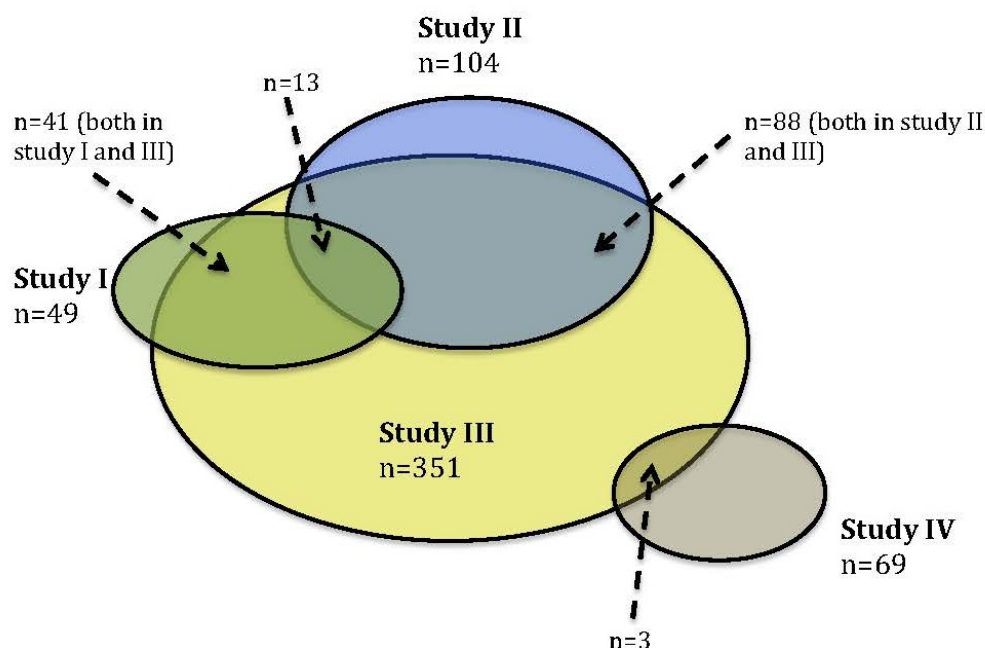
## **2 AIMS OF THE STUDY**

1. To investigate whether diagnostic confidence is improved by using a second-generation US contrast agent in a surveillance programme for HCC in patients with HCV-induced liver cirrhosis (Paper I)
2. To analyse the efficacy and tolerability of combination therapy in patients with Child-Pugh class A liver cirrhosis (Paper II)
3. To evaluate the long-term impact of SVR on the risk to develop HCC, liver complications and death in patients with HCV-induced liver cirrhosis (Paper III)
4. To investigate whether there is an association between TCPTP cleavage, levels of the HCV NS3 protein in liver biopsies and clinical parameters in patients with chronic HCV infection (Paper IV)

### 3 MATERIAL AND METHODS

#### 3.1 PATIENTS

Patients included in the studies were recruited from the Department of Gastroenterology and Hepatology (study I-IV) and the Department of Infectious Diseases at Karolinska University Hospital (study II, III), Malmö and Lund University Hospitals, Sahlgrenska and Uppsala University hospitals (study III). Data of medical history, physical examination, biochemical tests and virological data were retrieved from patient journals and stored in a central database at the Department of Gastroenterology and Hepatology, Karolinska University Hospital, Sweden. All patients tested positive for anti-HCV antibodies and HCV RNA. For study I-III, the patients had a diagnosis of HCV-related cirrhosis based on liver biopsies from 1984 and onwards or a clinical evaluation involving biochemical parameters, clinical signs of portal hypertension and/or radiological findings consistent with cirrhosis. Alcohol consumption was categorized as  $<50$  g/day or  $> 50$  g/day (study I, III). In study IV, high alcohol consumption was defined as  $\geq 50$  g/day for males and  $\geq 40$ g/day for females. Figure 8 shows the concurrently included patients in studies I-IV and Table 6 summarises the number of patients included in each study and the inclusion and exclusion criteria applied.



**Figure 8.** The concurrently included patients in study I-IV in this thesis.

**Table 6.** Inclusion and exclusion criteria applied to the patients in study I-IV.

<b>Study</b>	<b>N</b>	<b>Inclusion</b>	<b>Exclusion</b>
I	49	1. HCV induced liver cirrhosis 2. At least one combined ultrasound with contrast at surveillance 3. Years 2002-2004	1. Prior or present HCC at ultrasound investigation
II	104	1. HCV-induced liver cirrhosis 2. Child-Pugh class A 3. At least one dose of peg-IFN and RBV treatment 3. Years 1999-2005	1. Prior ascites or encephalopathy 2. Transplanted patients 3. Renal disease
III	351	1. HCV-induced liver cirrhosis 2. Child-Pugh class A 3. Years 2001-2009	1. Prior ascites, variceal bleeding or encephalopathy 2. Patients with lack of Child-Pugh class at inclusion 3. Patients with co-morbidities (HBV, HIV, hemochromatosis or AIH) 4. Patients with HCC or liver transplant at baseline/6 months after diagnosis of cirrhosis
IV	69	1. Patients with HCV-infection undergoing liver biopsies 2. Years 2006-2007	

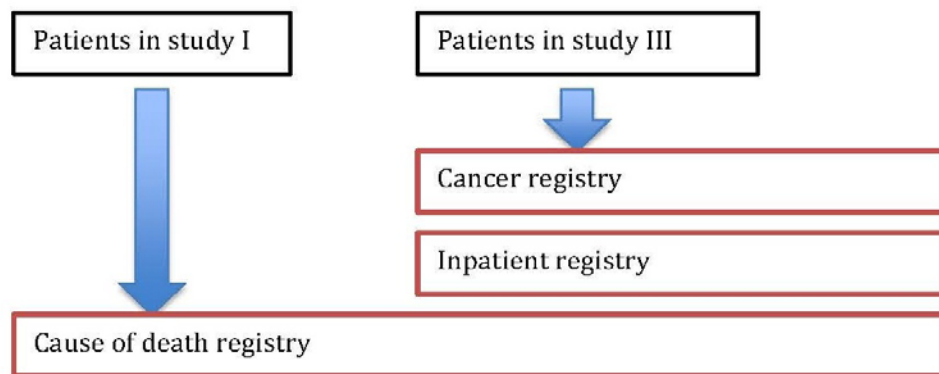
HCV: hepatitis C, HCC: hepatocellular carcinoma, peg-IFN: pegylated interferon, RBV: ribavirin, HBV: hepatitis B, HIV: human immunodeficiency virus, AIH: autoimmune hepatitis.

For study IV, liver biopsies were used from 69 HCV-infected patients attending the Department of Gastroenterology, Karolinska University Hospital Solna. The patients underwent the liver biopsy as part of the clinical follow-up for their HCV infection between the years 2006-7. Four patients had liver cirrhosis. As negative controls for NS3 detection, biopsies were used from non-HCV-infected patients who had undergone liver biopsy for other diagnostic purposes during the same time period. The patients' consent for use of the biopsy for research purposes was retrieved after both oral and written information. Demographic data of the HCV-infected patients were retrieved from patient journals around the time of the performed liver biopsy.

### **3.2 OBTAINING DATA FROM NATIONAL REGISTRIES (STUDY I, III)**

For studies I and III, data on the patients were retrieved from various National Swedish Registries. All Swedish residents are assigned a 10-digit personal identification number that is used in all contacts with the health care system. Reporting all newly diagnosed

malignant tumours to the *Cancer Registry* became mandatory in 1958 for both clinicians and pathologists and the Registry contains more than 95% of all detected tumours along with the date of diagnosis by two caregivers (study III). The *Swedish Registry of Causes of Death* contains information of  $\geq 99,5\%$  of all deceased persons in the country since 1997, including the date and cause of death (study I, III). The *Swedish National Patient (Inpatient Registry)* contains information about all residents who are hospitalized, including dates of admission and discharge, surgical procedures performed and diagnosis at discharge for as many as 8 medical conditions according to the International Statistical Classification of Diseases and Related Health Problems (ICD) (study III). The completeness since 1987 is estimated to be 98-99%. Figure 9 summarises how the national registries were used in the studies.



**Figure 9.** National registries used in study I and III for patients who were no longer followed up at their respective clinic.

### 3.3 IMAGING STUDIES (STUDY I)

The conventional US examinations in this study were all followed by a contrast-enhanced examination. The patients were examined with an Acuson Sequoia platform (Siemens Acuson, Mountain View, Calif., USA) at the Department of Diagnostic Radiology, Karolinska University Hospital Solna. The mechanical index (MI) ranged between 0.14 and 0.30. A convex array probe (4C1) at 3 MHz was used with Sequoia CPS software for contrast-enhanced examinations. Each examination with US immediately followed by contrast enhancement was performed by the same radiologist. Grayscale US scanning was performed using tissue harmonic and compound US imaging examinations. By enhancing examinations, the use of contrast medium made possible the evaluation of vascularity of focal lesions, whether being detected or not on

baseline examinations. When detecting a focal liver lesion, size, echogenicity and localization were determined and a diagnosis was made. After the baseline US imaging, 2.4 ml SonoVue was injected in a peripheral or central vein as a bolus, followed by a flush of 5 ml saline solution. This was immediately followed by a new investigation. The two pre- and post-contrast examinations of the same patient were then compared.

### **3.4 IMMUNOPRECIPITATION (IP) AND WESTERN BLOT (WB)**

#### **ANALYSES (STUDY IV)**

The obtained liver biopsies from chronically HCV-infected or control patients (as described in section 3.1) were homogenised and analysed by immunoprecipitation and then western blot (NS3) or only by western blot (TCPTP and GAPDH). In brief, 5 mg of each biopsy was lysed in 1 ml buffer (150 mM NaCl, 50 mM Tris/HCl pH 7.4, 1% Triton-X 100, 1% Na-deoxycholate, 1% sodium dodecyl sulphate (SDS), 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and 1 mM  $\text{Na}_3\text{VO}_4$ ), homogenised and sonicated twice for 30 seconds. For NS3 detection, protein A sepharose and anti-NS3 mouse polyclonal antibody (HCV genotype 1, in-house produced) were added and incubated overnight at 4°C. The washed pellets were re-suspended in SDS sample buffer, heated at 98°C for 5 minutes prior to SDS-PAGE on 4-12% Bis-Tris gels (Invitrogen, Paisley, UK) and transferred to Nitrocellulose membranes. Non-specific binding was blocked with 5% (w/v) non-fat dry milk powder in phosphate buffered saline (PBS)-T (20 mM Tris/HCl pH 7.4, 137 mM NaCl, and 0.05% Tween) or 5% bovine serum albumin (BSA) for 1 hour at room temperature. The blots were incubated overnight in PBS-T supplemented with primary antibodies. After extensive rinsing with PBS-T, blots were incubated with secondary antibody (goat anti-mouse IgG) conjugated to horseradish peroxidase for 1 hour (Dako, Glostrup, Denmark). After further rinsing in PBS-T, the immunoblots were developed with the enhanced chemiluminescence system (ECL; PerkinElmer, Shelton, CT, USA) following the manufacturer's instructions. NS3 was detected by using the anti-NS3 mouse polyclonal antibody. As positive controls, we used lysates from HepG2 cells transfected with plasmids coding for NS3/4A. As negative controls, homogenates of liver biopsies from non-HCV-infected patients were used, as described in section 3.1. For TCPTP and GAPDH detection, 30-40 µg of protein/lane were used for SDS-PAGE analysis. Protein concentration was estimated by using the BioRad protein assay. Antibodies against TCPTP and GAPDH were obtained from R&D Systems (Minneapolis, MN, USA) and Biodesign (Saco, ME, USA), respectively.

## **3.5 STATISTICS**

### **3.5.1 Study II**

For non-parametric group analyses, the  $\chi^2$  test was used. By using  $\chi^2$  and Student's T-test, predictive factors of SVR were assessed. The variable was further analysed by multivariate logistic regression analysis, if the p-value was less than 0.1 in univariate analysis. A p-value was considered significant if less than 0.05. The odds ratios (ORs) as well as the associated 95% confidence intervals (CIs) were calculated. The statistical package SPSS (SPSS Inc., Chicago, Ill., USA) was used for performing all calculations.

### **3.5.2 Study III**

Student's T-test and Chi-square test were used. The incidence of HCC, liver-related complications (ascites, variceal bleeding or encephalopathy) and disease-free survival in relation to SVR-status was estimated as the number of events occurring during non-SVR time and SVR-time divided by the corresponding person time at risk in the two groups. Cox regression was used to analyse the effect of SVR. Calendar time since January 1, 2001 was used as the time scale and SVR was considered as a time-dependent covariate. Models were also adjusted for alcohol consumption, age and diabetes. The hazard ratios together with 95% CIs presented were estimated by using the profile likelihood method. Survival curves with respect to SVR-status were estimated from the cumulative hazard functions for SVR and non-SVR follow-up time obtained from the Cox regression models and significant differences were assessed by testing  $HR=1$  using Wald's tests. All tests were two-sided and considered statistically significant if the p-value was less than 0.05. The software programme SAS 9.2 (Cary, NC, USA) was used to perform data analysis.

### **3.5.3 Study IV**

A densitometrical analysis of the bands obtained through western blot analysis was performed by using Image J. By using the Mann-Whitney U test by aid of the InStat 3 software, the values for each group were compared. The correlations after the Spearman's approach were determined by using the GraphPad Prism software.

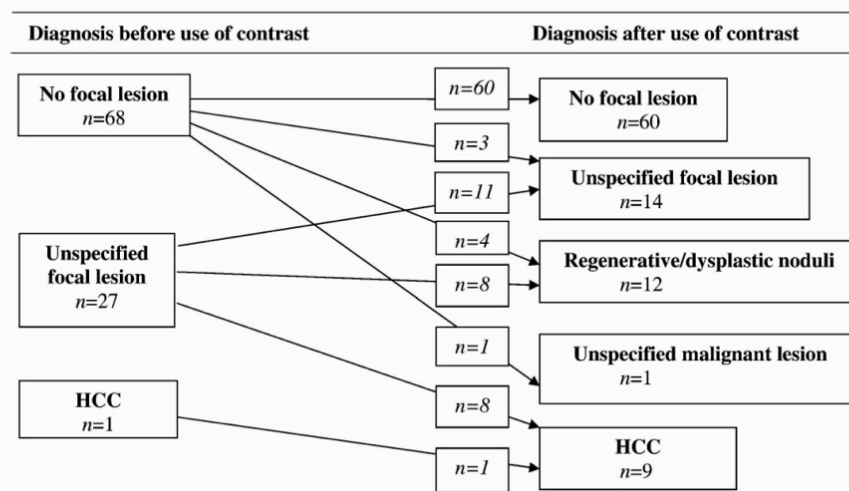


## 4 STUDY RESULTS

### 4.1 CEUS IMPROVES DIAGNOSTIC CONFIDENCE (STUDY I)

In total, 96 combined US and CEUS examinations of 49 patients with HCV-associated liver cirrhosis were analysed using second generation contrast agent SonoVue®. Diagnoses before and after use of contrast and the number of changed diagnoses are shown in Figure 10. It was shown that the number of patients with diagnosed malignant liver lesions increased from 1 to 10 cases after use of contrast medium.

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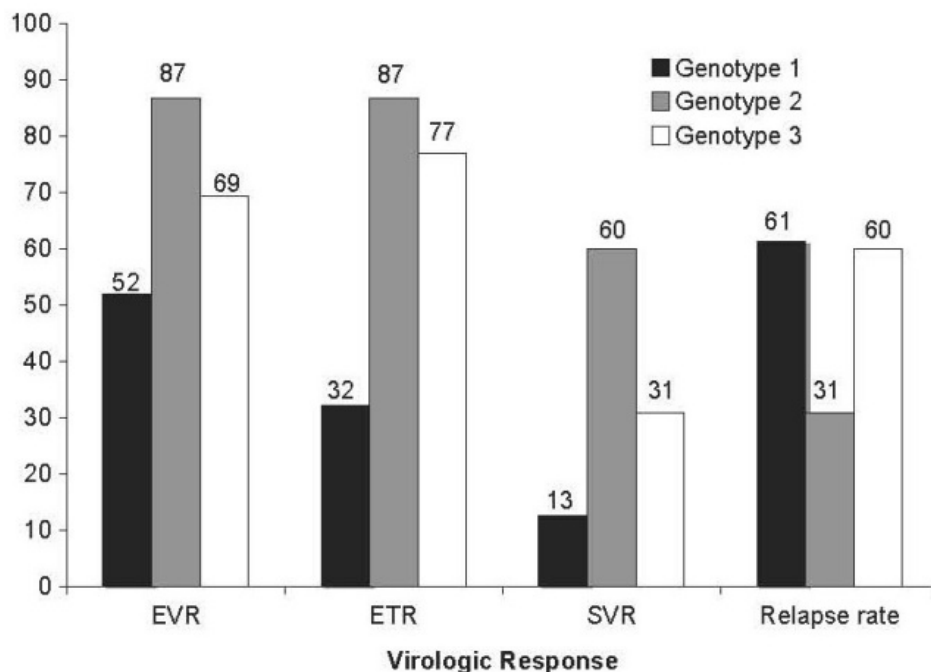
**Figure 10.** Ninety-six ultrasonography examinations before and after use of contrast agent in patients with HCV-associated liver cirrhosis. The diagnoses are shown with the number of findings. Boxes containing italicized numbers show the number of changed diagnoses.

For patients diagnosed with HCC, diagnoses were confirmed by either MRI (n=4) or CT (n=5) in all cases but one. This patient died within 1 month after diagnosis due to liver failure. The mean number of lesions detected was  $2 \pm 2.3$  (median 1, range 1-8) and the mean size of the lesions was  $3.2 \pm 1.4$  (median 2.4, range 1.5-5.5) cm. The mean alpha-fetoprotein level at time of diagnosis of malignancy was 64 (range 1.8-1384, reference limit <10 ng/ml). Eight patients with detected malignant lesions died within a mean of  $8.1 \pm 6.7$  months after they received their diagnosis. In the great majority of patients the causes of death were liver failure/malignancy (n=7). One patient died of sudden cardiac arrest. In the group without diagnosed malignant lesions (n=39) at CEUS, seven patients died. The causes of death were liver failure (n=5) and cerebral

hemorrhage (n=2). These patients had not presented any clinical or laboratorial signs of HCC during the follow-up period which was at least 12 months for each patient.

#### 4.2 LOWER SVR RATES IN A CLINICAL SETTING (STUDY II)

The virological response and adverse events were retrospectively analysed in 104 patients with HCV-associated Child-Pugh Class A liver cirrhosis, who had been treated with peg-IFN and ribavirin. Four patients were lost to follow up and excluded from the response analyses. Overall, sustained virological response (SVR) was achieved in 24% of all patients. The virological response rates according to genotype are summarised in Figure 11, with the SVR rates being 13% for genotype 1-, 60% for genotype 2-, and 31% for genotype-3 infected patients. IFN-experienced patients achieved SVR to a lesser extent than IFN-naïve patients, with rates of 14% versus 31%, but the difference was not statistically significant. The only variable at baseline significantly associated with SVR was genotype, where genotype 1 indicated a lower SVR rate ( $p=0.001$ , OR: 9.1, 95% CI: 2.5-33) and genotype 2 infection a higher rate ( $p=0.04$ , OR: 3.6, 95% CI: 1.1-11.8).



**Figure 11.** Virological responses after combination therapy with pegylated interferon and ribavirin in patients with chronic hepatitis C and Child-Pugh A class liver cirrhosis. All patients with genotype 1, 2, or 3 (n=97). Abbreviations: IFN=interferon; EVR=early virological response; ETR=end of treatment response; SVR=sustained virological response.

In patients where baseline HCV-RNA levels were below 800,000 IU/ml, 31% achieved SVR versus 17% in patients exhibiting levels above that limit. Table 7 states the SVR rates according to treatment adherence. In IFN-naïve patients with complete treatment course, the SVR was only 33% for genotype 1 patients, while the corresponding SVR rate was 80% for genotype 2/3 patients. In 46% of patients, treatment was discontinued prematurely owing to a lack of virological response in the majority. There was no significant difference between IFN-naïve and IFN-experienced patients in this regard. The most frequent reason for early cessation of treatment was reported to be lack of virological response, which was noted in 21%. In 15%, the reasons were other, classified as mild to moderately severe adverse events. These included fatigue, myalgia, headache, pyrexia, weight loss, nausea, diarrhoea, vertigo, irritability, abdominal pain, light sensitivity, pruritus, exacerbation of psoriasis, insomnia and depression. In 6%, discontinuation of treatment was caused by cytopenia. Seven patients (7%) developed infections during treatment. However, none of them had infection related to leukopenia or neutropenia, except for one patient with lower urinary tract infection at a neutrophil count of  $0.7\text{--}1.1 \times 10^9/\text{l}$ . Significant bleedings occurred in 3 patients, in which one had a peptic ulcer and variceal bleeding at a thrombocyte level of  $43 \times 10^9/\text{l}$ . The two other cases were not related to thrombocytopenia. The number of patients treated with reduced drug dose/s as a result of anemia, leukopenia, neutropenia and thrombocytopenia was, 16%, 3%, 10% and 15% respectively. HCC was detected in 2% and diagnosed at treatment week 26 and 39, respectively. Regarding hepatic decompensations during treatment, 4% developed ascites, one patient had variceal bleeding but none developed hepatic encephalopathy. During treatment, 1.9% of the patients died, the causes of death being HCC and heart disease.

**Table 7.** Sustained virological response (SVR) rates according to treatment adherence. The number and percentage of patients with SVR (%) are shown.

	Complete treatment course <sup>a</sup>	Full treatment duration <sup>b</sup>	Incomplete treatment <sup>c</sup>
All			
SVR gt 1	3/11 (27%)	2/10 (20%)	2/35 (3%)
SVR gt 2/3	9/16 (56%)	3/11 (27%)	5/14 (36%)
INF-naïve			
SVR gt 1	3/9 (33%)	1/5 (20%)	1/24 (4%)
SVR gt2/3	8/10 (80%)	2/7 (29%)	4/7 (57%)
INF-experienced			
SVR gt 1	0/2 (0%)	1/5 (20%)	1/11 (9%)
SVR gt2/3	1/6 (17%)	1/4 (20%)	1/7 (14%)

<sup>a</sup> Full treatment duration and maintained drug doses. <sup>b</sup> Full duration, but reduced drug doses.

<sup>c</sup> Truncated duration and reduced drug doses.

### 4.3 SVR REDUCES, BUT DOES NOT ELIMINATE THE RISK OF HCC (STUDY III)

351 patients with compensated Child-Pugh class A liver cirrhosis were followed for long-term outcomes up to 8.6 years to evaluate the effect of SVR on the risks of HCC, liver-related complications and death. The patients were divided into SVR (n=110) and non-SVR (n=241) groups. The results are summarised in Table 8. Six (5%) of the patients who had achieved SVR developed HCC during the follow-up period, corresponding to an incidence of 1.0/100 person-years, compared to 40 (17%) in the non-SVR group, corresponding to an incidence of 1.9/100 person-years. The incidence of HCC was found to be significantly lower in patients with SVR than those with non-SVR with a hazard ratio of 0.41 (CI 0.16-0.91). The cumulative risk of developing HCC was also significantly lower in the first group (p=0.04).

In patients achieving SVR, one patient was diagnosed with HCC within a period of one month after SVR. The other five patients who developed HCC were diagnosed at 1.6, 2.4, 4.3, 7.4, 7.4 and 7.6 years after achievement of SVR. Five were males, one had a history of heavy alcohol consumption and three had diabetes mellitus.

The risk of developing liver-related complications was significantly lower in patients with SVR than non-SVR (p=0.002). Four (3.6%) patients developed ascites after achievement of SVR. The diagnosis of ascites was made at 2, 13, 13, 48 months after achieving SVR. None of the patients developed variceal bleeding. Only one patient with SVR developed hepatic encephalopathy and this was seen at 4.1 years after SVR.

This patient had not developed ascites, variceal bleeding or HCC during the follow up period.

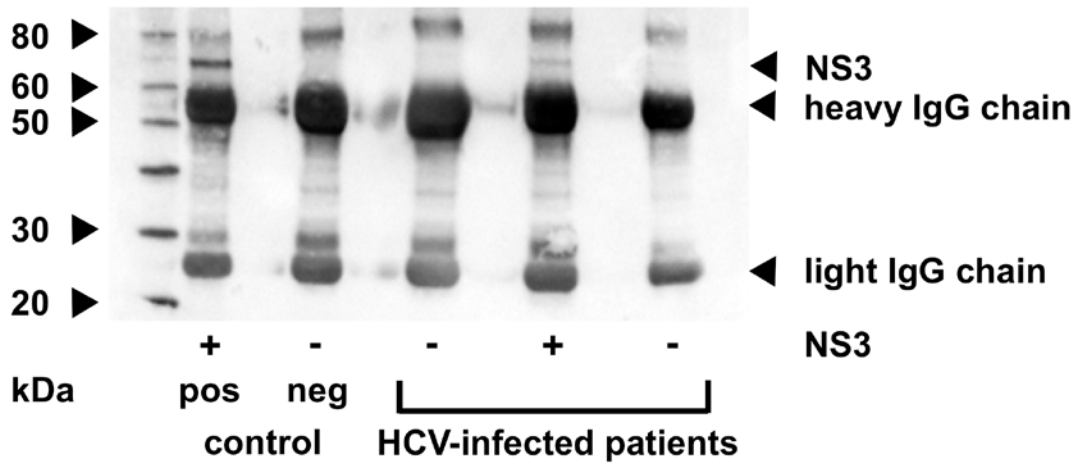
Finally, it was seen that the relative risks for liver-related death and death of any cause were lower in patients with SVR than non-SVR with a hazard ratio of 0.21 (CI 0.06-0.51) and 0.46 (CI 0.23-0.82) respectively. The cumulative risks for liver-related death and overall death were significantly lower in patients with SVR versus non-SVR ( $p=0.03$  and  $p=0.01$ , respectively). Eleven (10%) of patients having achieved SVR died during the follow-up period, among which four of liver-related causes. One patient with SVR underwent liver transplantation due to HCC.

**Table 8.** The incidences of HCC, liver complication, liver-related and all death per 100 person-years are shown in patients with and without sustained virological response (SVR). Hazard ratio (HR) between these groups and 95% confidence interval (CI) are shown.

Endpoint	<u>SVR</u> n=110			<u>Non-SVR</u> n= 241			HR (95% CI)	
	Events (n)	Person- years	Rate	Events (n)	Person- years	Rate		
HCC	6	589	1.0	40	2139	1.9	0.41	(0.16-0.91)
Any complication	5	583	0.9	52	2089	2.5	0.25	(0.09-0.57)
Ascites	4	583	0.7	42	2076	2.0	0.25	(0.08-0.63)
Variceal bleeding	0	595	0.0	14	2135	0.7	0.00	(0.00-0.37)
Encephalopathy	1	594	0.2	9	2146	0.4	0.26	(0.01-1.41)
Liver-related death	4	595	0.7	52	2157	2.4	0.21	(0.06-0.51)
All death	11	595	1.8	67	2157	3.1	0.46	(0.23-0.83)

#### 4.4 NS3 EXPRESSION AND HCV RNA LEVELS ARE INVERSELY CORRELATED WITH TCPTP LEVELS (STUDY IV)

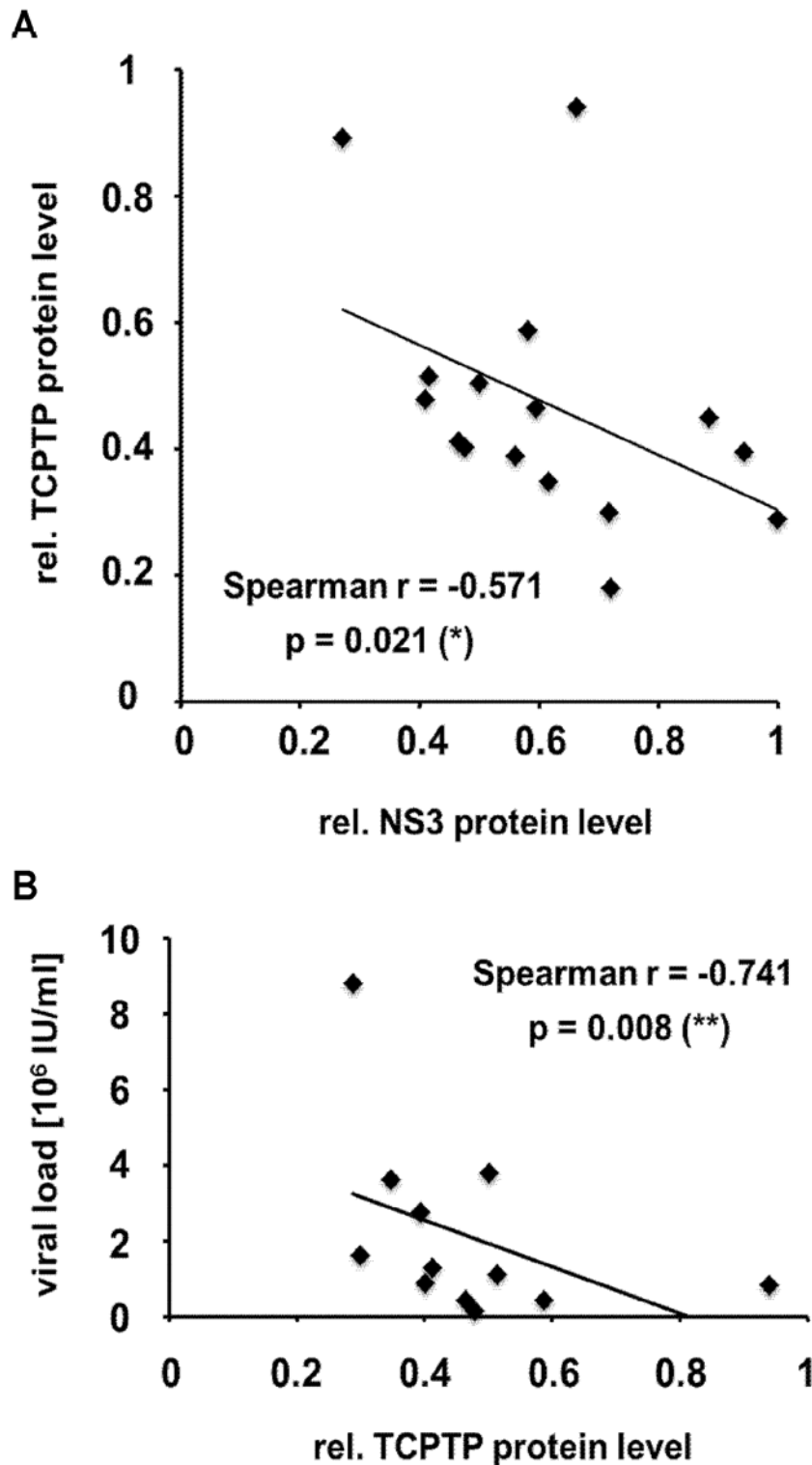
Liver biopsies from 69 patients with chronic HCV and 16 control patients were analysed to determine hepatic NS3 (Figure 12) and TCPTP protein levels, which were then correlated to viral load or clinical parameters for severity of liver disease.



**Figure 12. Detection of intrahepatic NS3 protein in liver biopsies from HCV-infected patients.** Liver lysates from HCV-infected patients, as well as a positive (lysates from HepG2 cells transfected with plasmids coding for NS3/4A) and a negative (homogenates of liver biopsies from non-HCV-infected patients) control sample, were analysed for NS3 by immunoprecipitation followed by western blot. A representative example is shown.

NS3 was detected in 31 of 69 liver samples (44.9%), and was more commonly detected in patients infected with the HCV genotype 1 than in those with non-1 genotypes (56.8% versus 28.6%). Detection of NS3 was not significantly associated to liver injury (measured as mean serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) at the time of biopsy), liver inflammation or fibrosis stage. Patients with detectable NS3 had mean HCV RNA levels of  $3.81 \times 10^6$  IU/ml ( $\pm 4.94 \times 10^6$  IU/ml) as compared to  $1.38 \times 10^6$  IU/ml ( $\pm 2.92 \times 10^6$  IU/ml) in patients without detectable NS3, showing a trend towards higher viral load in patients with detectable NS3 ( $p=0.0508$ ; Mann Whitney U test). NS3 expression levels analysed with densitometry showed a significant correlation to the viral load of the respective patients (Spearman  $r$  of 0.638 and  $p = 0.0008$ ).

TCPTP levels were determined in liver biopsies from 16 NS3-positive HCV patients and 16 controls. TCPTP was found to be significantly lower in HCV patients compared to control patients. Further, a significant inverse correlation was found between TCPTP and NS3 protein levels (Spearman  $r$  of  $-0.571$  and  $p = 0.021$ ) (Figure 13 a). Also, an inverse correlation was found between intrahepatic TCPTP levels and viral load (Spearman  $r$  of  $-0.741$  and  $p = 0.008$ ) (Figure 13 b). A trend of NS3-positivity was seen in patients with a history of high alcohol consumption ( $p=0.070$ ; Mann Whitney U test).



**Figure 13. Correlation of intrahepatic TCPTP levels with intrahepatic NS3 levels and viral load.** (A) Correlation of TCPTP and NS3 levels in liver biopsies from chronically HCV-infected patients. Total protein extracts were prepared from liver biopsies and analysed by western blot for TCPTP and GAPDH (loading control). The relative TCPTP protein levels represent the ratio of the net intensity of the TCPTP band and the GAPDH band. NS3 protein expression was analysed by immunoprecipitation followed by western blot. The relative NS3 protein levels represent the ratio of the net intensity of the NS3 band and the light IgG chain band. (B) Correlation of HCV viral load and the relative intrahepatic TCPTP protein levels in chronically HCV-infected patients.

## 5 DISCUSSION

Understanding how the HCV establishes a chronic infection and its mechanisms in achieving a progressive advanced liver disease have been of major importance. With the discovery of the HCV just over two decades ago, CHC infection is now the leading cause of chronic liver disease worldwide and of liver transplantation in the Western world. The aim of this thesis was to study the clinical and molecular features associated with CHC infection, especially in those with advanced liver disease.

### 5.1 THE ROLE OF CEUS IN DETECTING MALIGNANT LESIONS AT SURVEILLANCE

In study I we found that the use of CEUS improves detection of HCC and diagnostic confidence in characterising focal liver lesions in patients with HCV-induced liver cirrhosis. This study is of retrospective design with relatively few patients included. However, to our knowledge, it presents the first data to be published on the role of CEUS in surveillance of patients with HCV-associated liver cirrhosis. The differences between US and CEUS for detection and characterisation of liver lesions shown in our study are supported by results from other published studies with different populations and study settings (193, 194).

Due to the approximate 100 times increased risk of developing HCC in patients with HCV-induced cirrhosis (181) and curative treatment being appropriate for early stages of HCC only, it is of critical importance to detect HCC early on. The 5-year survival rate of cirrhotic patients with a small HCC lesion (<2 cm) is about 80% after transplantation. This is in stark contrast with the 5-year survival rate of untreated symptomatic HCC which has been reported to be <5% (195). The objective of surveillance is to identify HCC at a stage when cure is highly likely, i.e. early lesions of HCC which are identified as small lesions in the liver. Our study showed that the mean size of the detected lesions was  $3.2 \pm 1.4$  (median 2.4, range 1.5-5.5) cm. It has previously been shown that CEUS has shown a higher capability of characterising focal liver lesions sized 2.1-3.0 cm and less capability in lesions <2 cm (194). This could be explained by the finding that 17% of HCCs sized between 1.0-2.0 cm were hypoenhancing during the arterial phase compared to 100% of HCCs sized 2.1-3.0 cm



being hyperenhancing (196). As also concluded in our study, the higher sensitivity of CEUS seems to be dependent on vascularization.

The current understanding is that HCC can develop from regenerative noduli (benign lesions frequently detected in cirrhosis), over dysplastic nodules (pre-malignant dysplasia), to early well-differentiated HCC and ultimately to undifferentiated HCC. When using CEUS, small lesions, apart from being small HCCs, could also be regenerative nodules or dysplastic nodules and the challenge is to distinguish these between possibilities. Early HCCs, also known as vaguely nodular HCCs (197, 198), are the most difficult to diagnose as they lack typical radiological appearances and are hypovascular unlike classical HCCs (199). Also, they may not show washout very reliably and be isoechoic in the parenchymal phase. A study reported washout in only 50% in well-differentiated hypervascular tumours (200). A patient illustrative of this was noted in our study; after use of contrast, a 1.5-cm hyperenhancing lesion with no washout was seen. The patient underwent a liver transplant and the diagnosis of HCC was verified.

US carries many advantages as a screening tool. Firstly, it is non-invasive and secondly it is not associated with any radiation hazard. In addition, examinations can be done repeatedly, rapidly and at a low cost. The performance of US depends on the experience of the examiner, the technology used, the body habitus, the presence of cirrhosis and the size of the tumour (188). Introduction of second-generation US contrast media in the 1990s has transformed the characterisation of focal liver lesions. With its use, it is possible to visualise the enhancement characteristics in real time over a period of 5 minutes. Sensitivities and specificities have been reported to approach those of computed tomography (CT) and magnetic resonance imaging (MRI) (181). Contrast media are well tolerated and there are very few contraindications to their use. However, the use of these media carries a higher cost. Limitations associated with CEUS are those with conventional US, e.g. large body habitus, bowel gas and movement artifacts (201). Also, it has been shown that CEUS offers false-positive HCC diagnosis in patients with cholangiocarcinoma (202) and hence the technique is no longer used for diagnosing HCC. The current recommendations are that surveillance for HCC should be based on US examination, while diagnosis is based on one dynamic radiological procedure, either contrast-enhanced CT or MRI, including the detection of arterial hypervascularity and portal or delayed venous washout. When these features are

not present, either the contrast-enhanced examination or biopsy is recommended according to the AASLD (189).

After a more extended analysis of patients with HCV-associated liver cirrhosis at Karolinska University Hospital, it was found that CEUS didn't improve detection rate of HCC (*personal communication, Dr Anna-Karin Siösteen*). However, our study along with other subsequent studies, confirm the understanding that CEUS plays an important role in characterising focal liver lesions in patients with liver cirrhosis and increases diagnostic confidence (164, 203), also for small nodules between 1-2 cm (204). A prospective study performed in 2011 reported a sensitivity of 86-91% and specificity of 96-100% in identifying HCC with CEUS in cirrhotic livers (164). Until the present day, further published data on using CEUS in surveillance programmes for patients with HCV-induced cirrhosis are lacking. To our knowledge, the systematic use of CEUS in surveillance of patients with HCV-induced cirrhosis has been applied at Karolinska University Hospital and some other clinics in Sweden.

To conclude, patients with HCV-induced liver cirrhosis are at a large risk of developing HCC and surveillance programmes to detect HCC early on is of major importance. CEUS improves characterisation of focal liver lesions, although characterising early HCCs remains a challenge. Additional studies on the role of CEUS, as well as cost-benefit analyses, in the surveillance programme of this group of patients are needed to further help elucidate its role in this context.

## **5.2 SVR RATES AFTER STANDARD THERAPY IN A "REAL-LIFE" CLINICAL SETTING**

Once HCV has succeeded in establishing a persistent infection, chronic inflammation can lead to progressive liver fibrosis with liver cirrhosis as the end-stage liver disease. Due to the high risk of decompensation and HCC, patients with compensated cirrhosis (Child Pugh class A) are considered being prime candidates for treatment. However, it's a known fact that SOC-treatment with peg-IFN and ribavirin is less effective in patients with liver cirrhosis than in non-cirrhotic patients (29). Also, side-effects during therapy are more frequent in cirrhotics. Anemia has been seen in 35% of patients with F4, compared to 15% in those with a milder fibrosis grade. Corresponding rates of neutropenia were 38% versus 6% and thrombocytopenia 24% versus 17% (134). Also, it should be mentioned that in the clinical reality, anemia due to bone marrow

suppression or hypersplenism often already exists in patients with Child-Pugh class A cirrhosis before start of treatment.

In study II, the efficacy and tolerability of peg-IFN and ribavirin treatment during 1999-2005 were analysed in patients with HCV-associated Child-Pugh class A liver cirrhosis in a "real-life" clinical setting. In RCTs, patients only with certain criteria are included with possible selection bias. In IFN-naïve patients, we found that SVR was achieved in only 13% of genotype 1-patients and 58% of genotype 2- or 3-patients. These rates were considerably lower than those of 41-44% for genotype 1 and 74% for non-genotype 1 reported in RCTs (29, 129, 130). We believe that there are several factors which can explain this discrepancy. Firstly, only a limited number of cirrhotic patients have been included in RCTs and these patients have often been grouped together with patients with advanced fibrosis, but not yet fully developed cirrhosis. Furthermore, the strict inclusion criteria employed in RCTs has led to the exclusion of a considerable number of patients with compensated liver cirrhosis. In addition, in trials only including patients with advanced fibrosis and cirrhosis, exclusion criteria owing to laboratory abnormalities have been applied.

In our study, nearly half of the patients withdrew from treatment prematurely compared to 20% seen in RCTs (29, 129). The most common cause of withdrawal was lack of virological response, namely 21%, as compared to 6-9% seen in RCTs (29, 129, 130). This was probably caused by the frequent dose reductions of peg-IFN and/or ribavirin owing to fear of adverse events, which are known to be associated with lower SVR rates (205). The higher SVR rates seen for genotype 1-patients having completed the full treatment course or who received full treatment, being quite similar at 27% and 20% respectively, support this. Fear of neutropenias and thrombocytopenias have previously been a common cause of dose reduction. There have been rules of how to reduce doses of peg-IFN or ribavirin, or end both drugs totally, at certain levels of cytopenia. In the beginning of the treatment era, these rules were strictly followed. Since our study included SOC treatments between years 1999-2005, clinicians have mainly followed these rules of dose reduction. However, data has emerged describing how neutropenia and thrombocytopenia during SOC treatment do not increase the risk of infection and bleeding respectively (206, 207). Consequently, in clinical practice, clinicians no longer strictly follow these dose reduction rules, but rather judge on a case-to-case basis. In our study, 7% of patients developed infections during treatment,

but none were related to leukopenia or neutropenia, except in one patient at a neutrophil level of  $0.7-1.1 \times 10^9/l$ . No deaths were seen related to infections. It is interesting to correlate these findings to the ones of a recent French study, which included patients with HCV-induced liver cirrhosis who had early access to treatment with first generation protease inhibitors in a real-life clinical setting. In an interim analysis with data after 16 weeks of therapy, four deaths (0.8%) due to infection were reported. Also, frequent adverse events were seen, ranging from 38 to 49% (208).

Considering the abandonment of previous dose reduction routines, carrying out a similar study of SOC treatment today at 2012 would probably yield higher SVR rates. However, the rates would still not be very high, considering that genotype 1 cirrhotic patients with no reduction of doses and with full length of therapy achieved SVR at only 27% in our study. On the other hand, the improved SVR rates in genotype 1 cirrhotics associated with the addition of first generation protease inhibitors would lead to substantially higher SVR rates today. Nevertheless, the rates would probably be lower than those in RCTs, even with these new drugs, according to the pattern seen in this study which reflects patients with more advanced cirrhosis receiving treatment in a real-life clinical situation without selection of adherent patients and with regular follow-ups only.

### **5.3 RISKS FOR HCC, LIVER DECOMPENSATION AND DEATH AFTER SVR**

A 17-year cohort study of 214 patients with CHC revealed that HCC was the main cause of death (44%) and the first complication to develop in 27% of patients (101). Diverging results exist whether successful SOC treatment with achievement of SVR reduces the risk of developing HCC or not (138, 139, 142-144, 209, 210). These results have emerged from Western studies, which are further described in our manuscript. The reports have primarily been retrospective, consisting of relatively few cirrhotic patients and have often suffered from short follow-up periods. However, in meta-analyses with pooled data from both Asian and Western studies, a reduced risk of HCC, liver-related morbidity and mortality was seen in patients with SVR (140, 211).

In study III, we followed 351 HCV-infected patients with compensated Child-Pugh class A liver cirrhosis up to 8.6 years and analysed the long-term outcomes. This study carries several strengths. Firstly, the study was of prospective design and, in

comparison to previous studies, it comprised a rather large number of patients (n=351), who were followed up long-term. The frequent problem of loss of patients in previous studies, being reported between 10-22% (139, 144), could be avoided due to the use of Swedish national registries together with routine clinical follow-ups. Dropouts in our study were reported to be as low as 0.8%.

Evaluating the impact of SVR in cirrhotic patients has been challenging. Firstly, pivotal HCV treatment studies have included a low frequency of such patients. Secondly, in RCTs, patients with advanced fibrosis (F3) have often been grouped together with cirrhotic patients (F4). It has been shown that the risk of HCC and liver-complications differ in these groups and chances of achieving SVR are generally 10-15% lower in cirrhotic patients and probably, as we showed in study II, even lower in a clinical reality compared to rates reported in randomised studies. In this study, these problems could be avoided considering the nature of the cohort which was comparatively large (n=351) and comprised a rather homogenous group of patients (HCV-induced Child-Pugh class A cirrhosis).

We showed that the risk of developing HCC remained in patients with SVR at a rate of 1% per year. Based on this finding, terminating surveillance for HCC in cirrhotic patients after achievement of SVR can be seriously questioned. The recent AASLD guideline for screening of HCC states that an incidence of 1.5% per year is the cut-off which indicates cost-effective surveillance for HCC (189). However, EASL Clinical Practice Guidelines for management of HCV recommend surveillance in patients with HCV-related cirrhosis who have achieved SVR (118).

Even the rates of decompensation were markedly reduced after SVR in our study. No variceal bleeding was seen during the long-term follow-up in patients with SVR, which together with another long-term follow-up study (212), indicate that endoscopy surveillance may not be necessary in patients with no varices at the time of SVR.

Studies have shown that SOC therapy resulting in SVR also reduces incidence and progression of cirrhosis. Even reversal of cirrhosis seems to be possible (99, 145, 146). This could be of major importance concerning the continued clinical outcome after achieving SVR and play an important role in determining which patients are in need of surveillance. In this study, we did not carry out sequential measurements of liver

fibrosis after achievement of SVR to be able to analyse the correlation between remaining fibrosis and long-term risk of HCC. Future studies of prognostic factors for remained risk of HCC are needed to distinguish the subpopulation of patients with need of long-term surveillance.

#### **5.4 ASSOCIATION BETWEEN NS3, TCPTP AND HCV RNA LEVELS**

A major limitation in understanding the pathogenesis of HCV infection has been the challenge of identifying viral proteins in the liver. It is a known fact that HCV replication occurs at the hepatic level and to date no replication at extrahepatic sites has been reported (213). Identification of viral proteins in the liver by immunohistochemistry or fluorescence microscopy has been shown to be possible only at or near the limits of sensitivity (214-217). For example it has been reported that only 1.7 to 21.6% of hepatocytes in chronic HCV patients are infected by HCV (218). These findings have led to the conclusion that HCV proteins seem to be expressed by a limited number of hepatocytes with low levels of expression. One of the key viral proteins proposed for viral evasion and thus establishment of persistent infection by the HCV is the NS3/4A complex. In modulating the infected hepatocyte by blocking immune pathways, the NS3/4A cleaves or inactivates cellular proteins.

In study IV, we established a new method of detecting the HCV NS3 protein in liver tissue from HCV-infected patients. Hence, for the first time to our knowledge, we were able to show successful detection of HCV NS3 through a combination of immunoprecipitation and western blot analysis. This allowed us to study the association between intrahepatic NS3 levels with clinical parameters, virological markers and signaling pathways modulated by HCV and/or NS3. Detection of NS3 was more common in patients with the HCV genotype 1 than non-genotype 1, which may be explained by the usage of a NS3 antibody raised against a NS3 genotype 1 immunogen. The fact that NS3 was also detected in genotype 2- and 3-patients suggests that there is some degree of cross-reactivity towards these genotypes.

The correlation found between intrahepatic NS3-protein levels and the HCV viral load and their inverse correlation to intrahepatic TCPTP protein levels has led us to conclude that a high viral load results in an increased number of HCV-infected hepatocytes producing HCV NS3/4A in which TCPTP is cleaved, causing an overall decrease in intrahepatic TCPTP protein levels. The cleavage of TCPTP may have

important implications for the HCV life-cycle and development of HCV-induced liver diseases.

There was a lack of association between NS3 detection and grade of liver inflammation, fibrosis stage and serum levels of liver damage markers. From this we conclude that neither viral load nor the expression level of HCV proteins seem to be directly proportional to HCV-induced liver injury. Other studies also support the lack of correlation between serum titers of HCV and severity of liver disease (219-222).

The interesting finding that alcohol may have a positive effect on HCV replication we have no explanation for. The possible influence of alcohol on HCV viral replication has been analysed through a number of studies, in which some indicate increased HCV RNA levels in alcoholics (223-225) and others don't (226-228). This association must be investigated further.

To summarise, the results of this thesis confirm that patients with CHC constitute a clinically complex and challenging group. Clinical tools and interventions currently used for diagnosis and therapy improve clinical outcome. However, further studies are needed to clarify the pathogenesis of HCV infection and identify prognostic factors to enable individual optimisation of the medical care for each patient and thereby increase survival.





## **6 GENERAL CONCLUSIONS**

### **Study I**

CEUS improves characterisation of focal liver lesions, although characterising early HCCs remains a challenge. In low endemic countries, the use of CEUS in screening for HCC may be considered. However, further studies are needed to elucidate its role in surveillance of patients with HCV-induced liver cirrhosis.

### **Study II**

SVR rates found in our study, in particular for genotype 1-patients, were lower than those generally found in randomised controlled studies, which may reflect that these studies include a more select group of patients compared to the ones treated in a “real-life” clinical setting. For cirrhotic patients, new treatment alternatives are urgently needed to improve treatment outcome.

### **Study III**

The risks of HCC, liver decompensation and death were markedly reduced in cirrhotic patients having achieved SVR. However, a HCC incidence of 1% per year still remained after SVR, which indicates that continued surveillance for HCC is warranted. Endoscopic surveillance for varices in patients who have achieved SVR may not be necessary.

### **Study IV**

Intrahepatic HCV NS3 can be detected by immunoprecipitation and western blot analyses. In HCV-infected patients, both intrahepatic NS3 expression and viral load were inversely correlated with intrahepatic TCPTP protein levels. A high viral load may result in an increased number of HCV-infected hepatocytes producing HCV NS3/4A in which TCPTP is cleaved, followed by a decrease in intrahepatic TCPTP protein levels. This may have important consequences for the HCV life-cycle and HCV-induced liver diseases.

## 7 CONCLUDING REMARKS

Patients with advanced liver disease/liver cirrhosis constitute a clinically challenging group of patients, with difficulties of achieving eradication through therapy and with increased risk of decompensation, HCC and liver-related deaths. In this thesis, different aspects of chronic HCV infection have been studied, with especial focus on patients with liver cirrhosis.

One of the mechanisms by which the HCV manages to establish a chronic infection was studied, through analyzing levels of the NS3-protein and TCPTP-protein in liver biopsies from patients with CHC infection. We found an association between levels of intrahepatic NS3 and the HCV viral load and an inverse correlation to levels of intrahepatic TCPTP. In HCV-infected patients, TCPTP cleavage may play an important role for the viral life-cycle and progress of HCV-induced liver disease.

In patients with chronic infection, some develop liver cirrhosis over time. In our study we could see the difficulty of eradicating the virus with SOC therapy once the stage of liver cirrhosis has been reached. Therefore, in patients with proven progressive disease, it is preferable that HCV infection be treated before reaching end stage liver disease. Patients with HCV-induced liver cirrhosis who receive standard of care therapy in clinical settings achieve SVR at lower rates than those generally found in randomised controlled studies, in particular genotype 1 patients. New therapies are urgently needed for this patient group. Novel treatment regimens are being developed (some of which are interferon-free) and are expected to be available in the coming years, anticipated to significantly raise SVR rates for patients with liver cirrhosis.

If SVR is achieved, risks for HCC, liver decompensation and death are markedly reduced in patients with liver cirrhosis. Still, the risk of HCC does not remain at a negligible level, which warrants continued surveillance for HCC. However, it is not known for how long this surveillance should be continued. Identifying prognostic markers for sustained risk of HCC after SVR are needed. Diagnostic confidence may be improved with CEUS in surveillance for HCC, but larger studies are needed to confirm our findings. Variceal bleeding was not seen after SVR, so gastroscopy surveillance may be abolished after achievement of SVR.

There are still many aspects of patients with HCV-induced liver cirrhosis which need to be studied, such as further understanding the pathogenesis of HCV, increasing the rate of eradication by treatment and identifying prognostic factors for developing decompensation or HCC in patients having achieved SVR. Additionally, studies are needed to further evaluate the role of CEUS in surveillance for this group of patients, in which detection of especially small HCCs is of major importance to increase survival.

## 8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Syftet med avhandlingen var att studera kliniska och molekylära aspekter av hepatit C-virusinfektion och på vilka sätt infektionen kan leda till en kronisk och avancerad leversjukdom.

I den första studien utvärderade vi om användandet av kontrast vid ultraljudsundersökningar av levern förbättrade diagnostiken av levercancer inom ramen av ett uppföljningsprogram för hepatit C-infekterade patienter med skrumplever (cirros), vilka löper ökad risk för att utveckla levercancer. Totalt 49 patienter ingick i studien och 96 ultraljudsundersökningar – före och efter kontrasttillförsel - analyserades. Vi fann att kontrastförstärkt ultraljud ökar den diagnostiska säkerheten när det gäller att skilja på godartade och elakartade förändringar i levern.

Standardbehandlingen för många patienter med hepatit C är en kombinationsbehandling med peg-interferon och ribavirin, vilka är behäftade med kraftiga biverkningar. I den andra studien undersökte vi effektiviteten och toleransen av denna behandling hos patienter med levercirros vid en svensk universitetsklirik. Utläkning och biverkningar/komplikationer analyserades hos 104 patienter. Vi kunde se att andelen patienter som uppnådde utläkning var lägre än de siffror som generellt anges i randomiserade kontrollerade studier. Detta kan bero på att patienter som har sämre förutsättningar att klara behandlingen ofta utesluts i dessa studier. Vi fann även att behandlingen avbröts i förtid hos nästan hälften av patienterna, där den dominerande orsaken var påvisad brist på önskad läkemedelseffekt vid rutinkontroll under behandling. Detta kan förklaras av att läkemedelsdoser fick sänkas eller att en av medicinerna togs bort under behandlingstiden pga. biverkningar.

I den tredje studien ville vi utvärdera hur uppnådd utläkning av hepatit C-infektion påverkade risken att utveckla levercancer, leverkomplikationer och dödlighet. Totalt följdes 351 patienter med levercirros i genomsnitt under 5,3 år. Vi kunde visa att riskerna reducerades påtagligt hos patienter som hade läkt ut sin infektion. Risken att utveckla levercancer kvarstod dock och beräknades vara 1% per år, jämfört med 1,9% i gruppen med utebliven utläkning. Hepatit C-infekterade patienter med levercirros som läker ut sin infektion bör därför fortsätta följas upp avseende levercancer.

Det icke-strukturella proteinet NS3 ingår i hepatit C-virusets kärna och TCPTP är ett s.k. fosfatas som naturligt finns i våra celler och som deltar i det komplexa molekylära signaleringssystemet. Det har nyligen visats att NS3 inaktiverar TCPTP och man har framlagt att detta stänger av ett viktigt steg i kroppens immunförsvar mot hepatit C och möjliggör etablerandet av en kronisk infektion. I vår fjärde studie etablerades en ny metod för detektion av NS3 för att se hur proteinmängden förhöll sig till andra parametrar hos patienter med kronisk hepatit C-infektion. NS3- och TCPTP-nivåer bestämdes i leverbiopsier från 69 infekterade patienter. Vi kunde visa att det fanns ett omvänt förhållande mellan dessa nivåer, dvs. att patienter med höga nivåer av NS3 uppvisade låga TCPTP-nivåer. Det fanns också ett samband mellan höga NS3-nivåer och hög virusmängd i blod. Ingen koppling sågs mellan detektion av NS3 och kliniska parametrar. Detta visar att NS3:s interaktion med TCPTP kan ha en betydande roll i hepatit C-virusets livscykel och för den kroniska leversjukdom som viruset ger upphov till.

Sammanfattningsvis har vi kunnat visa att det finns ett omvänt samband mellan NS3- och TCPTP-nivåer i leverbiopsier från kroniskt hepatit C-infekterade patienter. Det påvisade sambandet, tillsammans med tidigare forskningsdata, stödjer att detta är en konsekvens av en av hepatit C-virusets mekanismer att etablera en kronisk infektion i levern. Vidare fann vi att andelen patienter med kronisk infektion och levercirros som behandlades framgångsrikt vid en svensk universitetsklirik var lägre än de siffror som generellt anges i randomiserade kontrollerade studier, vilket bl.a. kan förklaras av att patienter med sämre förutsättningar att klara behandlingen ofta utesluts i dessa studier. Vi visade även att risken för levercancer, leverkomplikationer och dödlighet minskade markant hos hepatit C-infekterade patienter med levercirros som uppnådde utläkning med behandling, men den kvarstående risken att utveckla levercancer föranleder att patienterna bör fortsätta följas upp. Slutligen konstaterade vi att kontrastförstärkt ultraljud ökar säkerheten när det gäller att skilja mellan godartade och elakartade förändringar i levern och kan övervägas i uppföljningsprogram för att upptäcka levercancer hos hepatit C-infekterade patienter med levercirros.

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