On the assessment and impact of liver fibrosis in patients with chronic Hepatitis C

Magdalena Ydreborg

Department of Infectious Diseases
Institute of Biomedicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2013

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magdalena.ydreborg@gu.se

ISBN 978-91-628-8827-5 (printed)
ISBN 978-91-628-8839-8 (pdf) http://hdl.handle.net/2077/33121

Printed in Gothenburg, Sweden 2013
Ineko AB

ABSTRACT

Hepatitis C virus (HCV) infection is associated with increased risk of severe liver damage, cirrhosis, and hepatocellular carcinoma (HCC). Despite pending highly efficacious HCV treatment, assessment of liver damage will remain important for prognostication, treatment decisions, and indication for HCC surveillance. The aims of this thesis was to evaluate (i) which patients benefit from look-back screening for HCV, (ii) factors impacting on survival in the HCV-associated liver transplant setting, (iii) non-invasive diagnostic markers of HCV-associated cirrhosis and (iv) host genetic factors impacting on HCV-associated fibrosis.

In **paper I**, we identified chronic HCV infection in 113 out of 13,573 subjects (0.8%) screened for HCV following blood transfusion prior to 1992. The majority of those individuals were eligible for therapeutic intervention. Additionally, 73% of the identified subjects were women, often infected following transfusions during childbirth. Thus, screening for HCV among recipients of blood transfusions prior to 1992 is meaningful.

In **paper II** we evaluated survival among 84 patients who underwent liver transplantation for HCV- related liver disease from 1992 to 2006. We found that portal inflammation and fibrosis in the donor liver may deleteriously affect both patient and graft survival. Thus, pre-transplant evaluation of donor histopathology may be of value in the selection of donors for transplantation of HCV positive individuals, especially among older donors.

In **paper III**, we created a new model for prediction of liver cirrhosis in a cohort of 278 patients comprising age, body mass index (BMI), platelet count, prothrombin-INR and D7-lathosterol. The model was validated in an independent set of 83 patients and could confidently predict cirrhosis using the novel index, referred to as the Nordic Liver Index (NoLI).

In **paper IV**, we noted an association between CC carriage at *rs12979860* and more pronounced liver damage among HCV genotype 3 infected patients in a cohort of 771 patients with HCV infection which suggest that *IL28B* may differentially regulate the course of HCV infection across genotypes.

Keywords: Hepatitis C virus; blood transfusion; liver fibrosis; cirrhosis; histopathology; liver transplantation; survival; Index; Biochemical markers; Non-invasive; AUROC; Genotype 1; Genotype 3; IL28B; Liver stiffness measurement; Transient Elastography; Liver Histology

ISBN: 978-91-628-8827-5 (printed)

SAMMANFATTNING PÅ SVENSKA

Kronisk Hepatit C virus (HCV)- infektion orsakar inflammation i levern med leverskada och ärrbildning (fibros) som följd. På lång sikt finns risk för skrumplever (cirrhos), leversvikt och levercancer. Vid framgångsrik behandling stannar fibrosbildningen i regel av och kan även gå tillbaka.

Hepatit C viruset smittar via kontaminerat blod. Sedan 1992 testas alla blodprodukter avseende Hepatit C, men innan dess var blodtransfusion en vanlig smittkälla även i Sverige. 2007-2008 genomfördes en screening av tidigare blodtransfunderade i Västra Götaland. Denna utvärderades i delarbete I. Vi fann att screeningen identifierade 113 patienter (0.08% av 13,573 provtagna) med kronisk Hepatit C infektion. Majoriteten (73 %) var kvinnor som många infekterats i samband med förlossning. De allra flesta kunde fortfarande behandlas, vilket gör screening av tidigare blodtransfunderade meningsfull.

Mängden fibros i levern har stor betydelse för behandlingssvar och prognos, och mikroskopisk (histopatologisk) bedömning av en leverbiopsi, d.v.s. ett vävnadsprov från levern, har länge varit den metod som använts för att bedöma fibrosmängd. För patient och läkare enklare, icke-invasiva metoder har utvecklats under senare år, bl. a olika blodprover (fibrosmarkörer) samt transient elastografi där ultraljudsteknik används för att mäta leverns elasticitet vilket anses relaterat till mängden ärrvävnad.

Delarbete III syftade till att skapa en ny metod för fibrosbedömning. Vi mätte ett stort antal fibrosmarkörer hos 278 patienter med kronisk Hepatit C. På statistisk väg tog vi därefter fram en modell, för prediktion av levercirrhos i leverbiopsi, innehållande ålder, BMI (vikt relaterat till längd) samt tre olika blodprover. Modellen utvärderades därefter i en grupp av 83 patienter med kronisk Hepatit C och visade sig kunna förutsäga cirrhos även där.

Graden av leverskada och mängden ärrvävnad varierar mycket mellan olika individer beroende av bl.a. ålder, alkoholintag och kön. Singel-nukleotidpolymorfism (mycket liten skillnad i arvsmassan) i ett baspar i närheten av den interferonkodande genen IL28B har relativt nyligen visat sig vara relaterad till mängden fibros i levern. I delarbete IV undersökte vi sambandet mellan denna polymorfism och fibrosmängd i levern mätt med transient elastografi hos 771 patienter med kronisk Hepatit C-infektion. Vi fann en association mellan Il28B och fibrosmängd hos patienter infekterade med genotyp 3 av viruset, men inte vid infektion med genotyp 1 eller 2.

dessutom fann vi att fördelningen av IL28B genotyp skiljer sig åt vid infektion med olika virusgenotyper. Sammantaget tyder detta på att effekten av IL28B genotyp skiljer sig åt mellan virusgenotyper.

Vid leversvikt är levertransplantation enda behandlingsmöjligheten. Viruset infekterar dock den nya levern relativt omgående (recurrent Hepatit C), och leder till en snabbare fibrosutveckling jämfört med före transplantation. En mängd olika faktorer har visat sig påverka detta förlopp. I delarbete II undersökte vi vilka faktorer som påverkade överlevnaden hos 84 patienter som levertransplanterats pga Hepatit C-relaterad leversvikt på Sahlgrenska sjukhuset mellan 1992-2006. Vi fann att portal inflammation och fibros i donatorlevern påverkar såväl patientens som den transplanterade leverns överlevnad i negativ riktning. Histopatologiska bedömning av donatorns lever kan därför vara av värde inför transplantation till en HCV-positiv patient.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Ydreborg M, Söderström A, Håkansson A, Alsiö Å, Arnholm B, Malmström P, Hellstrand K, Westin J and Lagging M. Look-back screening for the identification of transfusion-induced hepatitis C virus infection in Sweden. *Scand J Infect Dis. 2011 Jul;43(6-7):522-7.*

II. Ydreborg M, Westin J, Lagging M, Castedal M, Friman S. Impact of donor histology on survival following liver transplantation for chronic hepatitis C virus infection: a Scandinavian single-center experience. *Scand J Gastroenterol. 2012 Jun;47(6):710-7.*

III. Ydreborg M, Lisovskaja V, Lagging M, Brehm Christensen P, Langeland N, Rauning Buhl M, Pedersen C, Mørch K, Wejstål R, Norkrans G, Lindh M, Färkkilä M, Westin J. A novel fibrosis index comprising a non-cholesterol sterol accurately predicts HCV-related liver cirrhosis. *Submitted*

IV. Ydreborg M, Westin J, Rembeck K, Lindh M, Norrgren H, Holmberg A, Wejstål R, Norkrans G, Cardell K, Weiland O, Lagging M. Impact of IL28B-related single nucleotide polymorphisms on liver transient elastography in chronic hepatitis C infection. *PLoS One, 2013. In press*

CONTENT

ABBREVIATIONS

Acetyl CoA	Acetyl coenzyme A
ALT	Alanine aminotransferase
APRI	AST (see AST) to platelet ratio index
ARFI	Acoustic Radiation Force Impulse
AST	Aspartate aminotransferase
AUROC	Area under ROC, see ROC
BCLC	Barcelona Clinic Liver Cancer
BMI	Body mass index
C_{high}	Higher cutoff
C_{low}	Lower cutoff
CAP	Controlled attenuation parameter
CHC	Chronic hepatitis C
CI	Confidence interval
CMV	Cytomegalovirus
CPA	Collagen proportionate area
DAA	Direct-acting antiviral agent
ECM	Extra cellular matrix
ELF	Enhanced liver fibrosis (score)
FIB-4	A non-invasive fibrosis index
GGT	Gamma-glutamyltransferase
GLC	Gas–liquid chromatography
GUCI	Gothenburg university cirrhosis index
GWAS	Genome wide association screen
HA	Hyaluronic acid
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma

HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HMG CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HSC	Hepatic stellate cell
HVPG	Hepatic venous pressure gradient
I_{odds}	Log odds (predicting cirrhosis)
I_{prob}	Predicted probability (for cirrhosis)
ICTP	Carboxy-terminal telopeptide of type I collagen
IL28A	Interleukin 28A
IL28B	Interleukin 28B
IL29	Interleukin 29
INR	International normalized ratio
IQR	Interquartile range (i.e. the range between the 25th and the 75th percentile)
IR	Insulin resistance
ISG	Interferon stimulating gene
+LR	Positive likelihood ratio
-LR	Negative likelihood ratio
LSM	Liver stiffness measurement
MELD	Model for End-Stage Liver Disease
MGB	Minor groove binding
MMP	Matrix metalloproteinase
MR	Magnetic resonance
MTP	Microsomal triglyceride transfer protein
NFLD	Non-alcoholic fatty liver diease
NLTR	Nordic Liver transplant Registry
NoLI	Nordic liver index
NPV	Negative predictive value

NS3/4A	Non-structural protein 3/4A
OLT	Orthotopic liver transplant
PCR	Polymerase chain reaction
Peg-IFN	Pegylated interferon
PIIINP	Procollagen type III amino-terminal peptide
PNPLA3	Patatin-like phospholipase domain-containing protein 3
PPV	Positive predictive value
PTEN	Phosphatase and tensin homolog
RNA	Ribonucleic acid
ROC	Receiver operator characteristics
RT-PCR	Reverse transcription polymerase chain reaction
RVR	Rapid virological response
SNP	Single nucleotide polymorphism
SVR	Sustained virological response
TE	Transient elastography
TIMP	Tissue inhibitor metalloproteinases
ULN	Upper limit of normal
VLDL	Very low-density lipoprotein

1 INTRODUCTION

1.1 Hepatitis C virus (HCV) infection

Epidemiology and routes of transmission

Worldwide, an estimated 130-170 million people are infected with Hepatitis C (1). The geographic distribution of HCV infection is highly variable between and within countries. The highest prevalence has been reported in Africa and the Middle East with a reported prevalence of more than 10% in Egypt and Cameroon and 5% in Pakistan (1). The majority of developed countries in North America, Northern and Western Europe, and Australia have a low-prevalence below 2% (1). In Sweden, 54,289 cases were reported from 1990 until 2012. One fifth of them are deceased, which gives a prevalence of 0.4% (2).

Hepatitis C is a blood-borne virus transmitted through exposure to contaminated blood from an infected individual. Historically, blood transfusion was a major route of infection. Since the implementation of routine blood donor screening, the major route of transmission in developed countries has been intravenous drug use (3-5), while in developing countries, unsafe medical procedures and iatrogenic exposure remains a risk factor for HCV infection (6, 7). The risk of transmission from mother to child during pregnancy and delivery is estimated to be approximately 5% (8, 9). Sexual transmission seems to be rare between couples in long-lasting relationships, while high-risk sexual behavior and high prevalence of other sexually transmitted diseases is associated with a higher prevalence of hepatitis C infection (10). Of the 1981 cases reported in Sweden in 2012, the route of transmission was as follows: intravenous drug use (46%), sexual transmission (5%) and previous blood transfusion (4%), whereas for 40% of patients, no route of transmission was reported (2). In Sweden, no national HCV look-back screening of blood transfusion recipients was performed following the discovery of the virus. With improved treatment outcome (11, 12), the National Board of Health and Welfare revised the Swedish national guidelines with a final update reported in 2007. In these amended guidelines, look-back strategies targeting former pediatric patients were recommended, given that these patients may not be aware of having received a blood

transfusion and could be assumed to benefit the most from HCV combination therapy (13).

The Hepatitis C virus

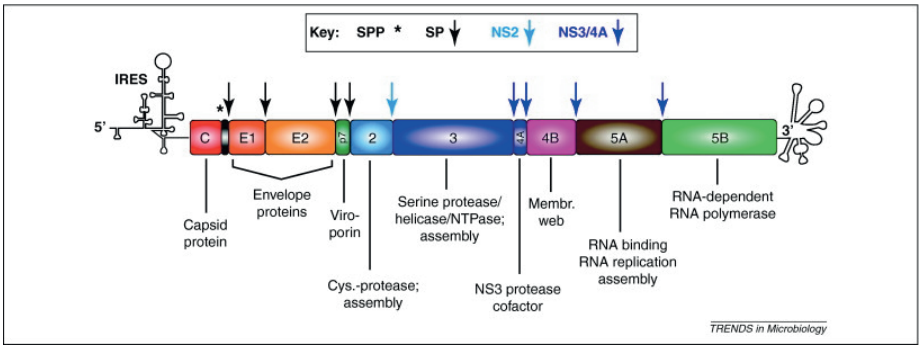

Figure 1. The genome of the Hepatitis C virus. From ***Bartenschlager*** *R, Penin F, Lohmann V, André P.Trends Microbiol. 2011 Feb; 19(2): 95-103. doi: 10.1016/j.tim.2010.11.005. Epub 2010 Dec 14. Reprinted with permission from Elsevier*

The Hepatitis C virus, formerly known as non-A, non-B hepatitis, was cloned and characterized in 1989 (14). The first serological tests became available in Sweden in 1990, with second-generation tests being introduced two years later, and on January 1st 1992, screening of blood donors became mandatory. Initially, the lack of viral culture systems hampered detailed analyses of the HCV genome. In 2005, Lindenbach et al were able to reproduce a replicative full-length genome that was infectious in cell culture (15), and the same year Wakita et al reported that cell-cultured HCV produced infectious HCV particles that were transmissible to chimpanzees (16). This enabled detailed studies of the viral genome and the development of direct acting antivirals (DAAs).

The HCV virus is a positive single stranded RNA virus and the genome consists of approximately 9 500 nucleotides (17). The genome contains a single open reading frame encoding a polyprotein flanked by two highly conservative, untranslated regions at the 5'and the 3' terminals. The polyprotein is cleaved by viral and host proteases into at least ten known proteins (figure 1). The virus replicates rapidly producing up to 10^{12} new

virions per day in chronic infection (18). As the HCV RNA-polymerase lacks proofreading this results in multiple quasispecies.

HCV has six major genotypes and each genotype has several subtypes. Genotype 1,2 and 3 have a broad geographic distribution (19), while genotype 4, 5 and 6 are more restricted to certain areas (genotype 4 in Africa and the Middle East, Genotype 5 in South Africa and genotype 6 in southeast Asia) (20, 21). In Sweden, genotype 1a (35%) and 3 (31%) has been the most common genotypes, followed by genotype 2 (17%) and genotype 1b (6%) (22).

1.2 Diagnostic methods

Detection of HCV specific antibodies is used for screening purposes and do not discriminate between persistent and resolved infection. In enzyme immune assays (EIAs), recombinant antigens based on HCV core, NS3, NS4 and NS5 proteins are used to capture circulating antibodies (23). Recombinant Immunoblot Assay (RIBA), a method for antibody detection, is used to confirm HCV-reactivity (24). EIAs have a high sensitivity and specificity but can be false negative in immunocompromised individuals.

Viremia can be detected and quantified by nucleic acid amplification with real time reverse transcriptase polymerase chain reaction (PCR). One commonly used, commercially available PCR (Roche Cobas TaqMan) method has a broad range of quantification; from 15 up to 7-8 log10 IU/ml, as well as a high sensitivity and specificity (25). The quantification process involves RNA capture, reversed transcription and amplification of the target sequence in a cyclic manner. The amount of virus in the sample corresponds to the number of cycles needed to reach a threshold value. HCV-RNA detection and quantification are used to confirm persistent infection and to evaluate response to treatment.

Genotype determination is essential for choice of antiviral therapy. The reference method for determination of genotype is direct sequencing of part of the genome and phylogenetic analysis (17). An alternative method is the use of PCR with genotype specific probes (26). This method is quicker and less expensive but still identify viral genotypes with a high accuracy.

1.3 Natural history

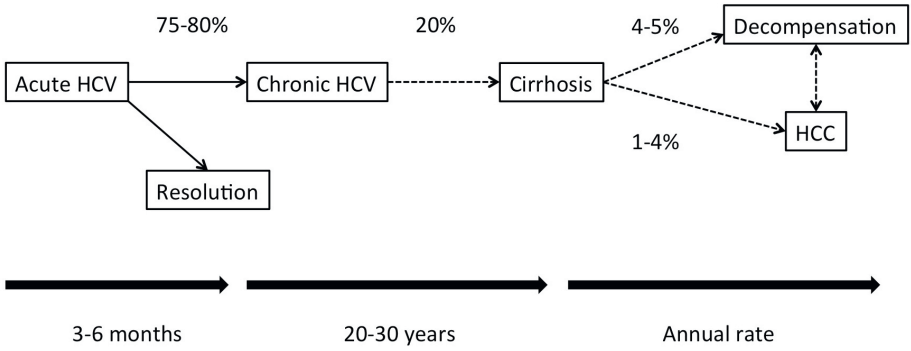

Figure 2. Natural history of Hepatitis C infection.

Acute HCV infection

After exposure, HCV RNA can be detected in serum within a week, while there is usually a 6-8 weeks "window-phase" period before HCV-specific antibodies can be detected (27, 28). The acute infection is usually asymptomatic, although some patients (≈10%) develop classical symptoms of acute hepatitis including jaundice. Progress to fulminant hepatitis with acute hepatic failure is rare. In a minority of patients, the acute infection is followed by viral clearance with normalization of ALT levels and undetectable HCV RNA although HCV antibodies may persist for years. The estimated rate of spontaneous clearance of infection varies depending on study criteria although, in a systematic review by Micallef et al., the estimated proportion of viral clearance was 26% (29). Thus, the majority of patients develop a chronic infection.

Factors associated with spontaneous viral clearance include symptomatic acute infection (30), high initial HCV RNA levels (31) and rapid decline in HCV RNA levels (32) as well as a strong, broadly directed and sustained CD4+ T cell response (33). In addition, certain human leucocyte antigen (HLA) genotypes (34) and single nucleotide polymorphism (SNP) on chromosome 19 in proximity to interferon coding gene IL28B are related to spontaneous clearance, (35, 36).

Chronic HCV infection

Chronic hepatitis C (CHC) is a slowly progressive disease, characterized by the development of fibrosis in the liver with cirrhosis as the end stage of the disease. The time to cirrhosis varies between different settings. A meta-analysis by Thein et al. estimated the prevalence of cirrhosis after 20 and 30 years of infection to 16% and 41% respectively, with a higher prevalence of cirrhosis at 20 years found in studies performed in clinical settings (18%) compared to non-clinical settings (7%) (37). In a large cohort study by Poynard et al., 33% of patients had an expected median time to cirrhosis of less than 20 years, while 31% of patients were not expected to develop cirrhosis after at least 50 years of infection (38). In studies evaluating outcome of chronic hepatitis C in females and children infected through blood transfusion, few subjects progressed to cirrhosis (39, 40).

Thus, the fibrosis progression rate is highly variable and prognosis correlates with fibrosis stage (41). Accordingly, potential risk factors for disease progression have been evaluated in numerous studies. Factors that have been consistently associated with a faster fibrosis progression include age at infection, duration of infection, male gender and alcohol consumption (37, 38, 42), as well as co-infection with HIV (43) or HBV (44), insulin resistance (45), in addition to obesity and steatosis (46, 47). The role of viral load and HCV genotype is more unsettled with diverging results in different studies (38, 48, 49) although genotype 3 may possibly be related to more rapid fibrosis progression (50, 51).

Although well established, these risk factors account for only a limited portion of the inter-individual variation in fibrosis progression, and it is probable that host genetic factors are involved. In recent years, genome wide association studies (GWAS) have sought to identify single nucleotide polymorphisms (SNPs) related to fibrosis progression. SNPs in the adiponutrin/patatin-like phospholipase-3 (PNPLA3) gene, a genetic determinant of liver fat content (52) has also been associated with steatosis, fibrosis and fibrosis progression in chronic HCV infection (53, 54). Additionally the presence of the otherwise favorable *IL28B* genetic variants has been associated to more pronounced steatosis and fibrosis especially in non-genotype 1 patients (55-57).

Development of fibrosis, progression to cirrhosis and HCC

Fibrosis progression in Hepatitis C is mainly driven by chronic inflammation in a complex interplay involving both innate and adaptive immune responses (58). The key cell in liver fibrosis formation is believed to be the Hepatic Stellate Cell (HSC)(59). Following persistent inflammation, the HSC is activated and transforms into a contractile myofibroblast capable of increased proliferation, migration, and contraction as well as the release of inflammatory cytokines and chemokines. In the normal state, deposition of extracellular matrix (ECM) is balanced by degradation and removal. Following prolonged inflammation the balance is skewed resulting in quantitative and qualitative changes in the ECM, the net result being an excessive deposition of ECM, fibrosis. The excess deposition of ECM impairs the flow of plasma between sinusoidal lumen and hepatocytes eventually leading to altered hepatic function (reviewed in (60)). Further progression of fibrosis eventually leads to cirrhosis which is characterized by distortion of the liver parenchyma with formation of nodules of regenerative parenchyma surrounded by fibrotic tissue accompanied by extensive vascular changes leading to portal hypertension (61)

Cirrhosis is characterized by initial, often asymptomatic phase, compensated cirrhosis. Development of complications due to portal hypertension or liver dysfunction marks the transition to decompensated cirrhosis, defined by the appearance of ascites, variceal bleeding, hepatic encalopathy or jaundice. The rate of decompensation is estimated to 4-5 % per year and while the 5-year survival in HCV related compensated cirrhosis is estimated to 80-90% it drops to around 50 % after decompensation (62-64). The risk of decompensation is related to portal hypertension (65) and the risk of decompensation differs depending on the existence of esophageal varices (62).

In patients with cirrhosis, assessment of the prognosis can be made by calculation of the Child-Pugh (66) score which is based on levels of bilirubin, albumin, PK-INR and the presence or absence of ascites and encelopathy. Based on the result, cirrhosis is classified as either A, B or C representing increased severity. The MELD-score (Model of End-stage Liver Disease)(67) includes levels of creatinine, bilirubin and PK-INR and, in a modified version, need for dialyses during the last week, predicts short-term survival and is used in the transplant setting. (Both are reviewed in (68)).

HCV related liver cirrhosis is a major risk factor for hepatocellular carcinoma (HCC) and several studies report HCC to be the first complication of liver

cirrhosis to occur in CHC patients as well as accounting for the majority of liver related death in compensated cirrhosis (62, 63, 69, 70). Once cirrhosis is established, the annual rate of HCC i estimated to 1-4 % in Europe and USA and 7% in Japan with a higher risk in patients with older age at acquisition of infection, heavy alcohol intake, co-infection with HBV or HIV, obesity and male gender (71). Staging of HCC with the Barcelona Clinic Liver Cancer (BCLC) staging system stratifies patients by tumor size and number, liver function and health status into categories with different prognosis and specific treatment proposals. Simplified, curative treatments for HCC are surgical resection and ablation for small, single nodules and liver transplantation for patients with portal hypertension and/or multifocal HCC meeting the Milan criteria (solitary nodule $\leq$ 5 cm or up to 3 nodules $\leq$ 3 cm)(72). These treatment options can result in complete remission or long-term disease free survival. Untreated, survival is poor (73, 74).

Most cases of HCV-related HCC occurs in patients with cirrhosis, thus HCC surveillance by ultrasound is recommended for these patients, and improved survival with screening every six months compared to once a year has been reported (75).

In the event of successful antiviral treatment, liver fibrosis may be reversed (76). A 5-year follow-up of patients with sustained virological response and paired liver biopsies pre- and post treatment showed a decrease in fibrosis stage in 80% of patients. The more severe fibrosis stages tend not to disappear altogether, although ten of twelve patients with bridging fibrosis and cirrhosis had decreased fibrosis stages in follow-up biopsies (77). However, although decreased, the risk of HCC remains, and thus patients with cirrhosis need to continue HCC surveillance in spite of eradication of HCV (78).

1.4 Liver steatosis

Hepatic steatosis, defined by the accumulation of lipids in the cytoplasm of hepatocytes, is a common feature of chronic HCV infection, more so in patients infected with HCV genotype 3 than in non-genotype 3 patients (70-80% and 45-50% respectively) (79). Additionally, steatosis in genotype 3 patients seems to be more severe (80). Two main types of steatosis have been defined in CHC although they might overlap to some extent. One is associated with metabolic factors such as obesity, insulin resistance and type 2 diabetes, and is present predominately among non-genotype 3 patients. In

genotype 3 patients, however, steatosis is independent of BMI and related to viral load (81), associated with hypocholesterolemia (82), and diminishes after successful antiviral treatment (83, 84), accompanied by normalization of serum cholesterol levels, thus suggesting a direct inhibitory effect of genotype 3 on lipid export (85, 86).

In cell culture, HCV core protein induces lipid droplet accumulation within the cell (87, 88), an effect that is more pronounced with genotype 3 core protein compared to other genotypes (89). The genotype 3 core protein down regulates PTEN phosphatase activity in vitro (a mechanism that has been proposed as contributing to development of steatosis in NAFLD) thereby affecting cholesterol metabolism and inducing accumulation of large lipid droplets (90, 91). Furthermore, the HCV core protein inhibits microsomal triglyceride transfer protein (MTP), an enzyme that is directly responsible for the assembly of VLDL-particles from triglyceride and apolipoproteins, resulting in the accumulation of intracellular lipids (92). When evaluated in multivariate analysis, MTP mRNA levels were independently associated with fasting insulin for genotype 1 and 2 patients, while in genotype 3 patients, only HCV RNA levels remained predictive (93), again suggesting a direct viral effect. Other proposed mechanisms for virus-induced steatosis include up regulation of fatty acid synthesis (94, 95) and the ability of the HCV-core protein to increase the production of reactive oxygen species (96), thereby affecting membrane lipids and VLDL secretion.

The role of steatosis for fibrosis progression is not fully understood. While steatosis has been significantly associated to fibrosis progression in several studies (97, 98), especially among HCV genotype 3 infected patients (99, 100), some studies have failed to show an association (101). Insulin resistance (IR) is known to promote fibrosis progression (102). Because obesity, insulin resistance and steatosis are closely interrelated, it is difficult to ascertain which one of the components that contributes the most to disease progression. Studies including the assessment of IR as well as steatosis, have found IR to be an independent predictor of severe fibrosis in both genotype 1 and genotype 3 infected patients (103, 104).

1.5 Assessment of liver fibrosis

Assessment of liver fibrosis is essential in order to determine the prognosis of HCV infection and aid treatment decisions and to diagnose cirrhosis, when

surveillance for complications should be initiated. Below are listed various methods for the evaluation of fibrosis.

Liver histopathology

Traditionally, liver fibrosis is assessed by histological staging of liver biopsy specimens. Liver biopsy can be obtained by the percutaneous Meninghi technique. Histochemical staining of formalin-fixed tissue samples is used to demonstrate various features of liver damage. Histopathologic assessment includes grading, which gives an estimate of the intensity of inflammation, and staging, which measures the degree of fibrosis and architectural alterations. Portal tract inflammation, with lymphoid aggregates or follicles, bile duct lesions and presence of steatosis are considered characteristic of Hepatitis C infection. Other histological features includes interface hepatitis and bridging necrosis resulting in porto-central bridging fibrosis (105).

Several pseudo numerical methods are currently used to express the grade and stage of viral hepatitis. The use of pseudo numeric scores allows comparison between patients and populations as well as statistical evaluation. The Ishak score grades inflammation 0-4 in four separate variables and stage of fibrosis from 0-6 (106). The METAVIR score is similar, but less complex and is evaluated in large cohorts of chronic HCV infected patients (107). Both scores show a low grade of inter-observer variability (108, 109). In the score proposed by Batts and Ludwig (110) which is commonly used in Sweden, stage of fibrosis is given from 0-4 using the same definitions as METAVIR.

Liver biopsy is still recommended in official guidelines for evaluation of liver fibrosis in chronic Hepatitis C patients (111), although transient elastography and serum markers are recommended as alternatives. An advantage with liver biopsy is that it provides information on other aspects of liver lesions that might impact on disease progression, such as steatosis and iron-load (112). In addition, non-invasive methods are evaluated using stage of fibrosis in liver biopsy as a reference. An alternative method for evaluation of fibrosis in liver biopsies is the Collagen Proportionate Area (CPA), *i.e.* the relative proportion of collagen to tissue measured by computer-assisted digital image analysis, and is correlated to Ishak fibrosis stage (113). Liver biopsy is associated with a potential risk of complications (114), demands a full-day visit at the clinic, and is limited by the risk of sampling error and inter-observer variability (115). Thus, different non-invasive methods for evaluation of liver fibrosis have been proposed.

Transient elastography

Liver stiffness measurement (LSM) by transient elastography (Fibroscan®), a completely non-invasive procedure, was first described by Sandrin *et al.* in 2003 (116), and has rapidly gained widespread usage. Briefly, a probe including an ultrasonic transducer mounted on the axis of a vibrator is placed at the skin surface in an intercostal space above the right liver lobe. The probe induces a vibration that propagates through the liver tissue as an elastic shear wave. A pulse-echo ultrasound is used to measure the velocity of the wave. The harder the tissue (i.e. the more fibrotic) the faster the shear wave propagates. The result is expressed in kilopascal (kPa) as the median value and interquartile range (IQR) of all valid measurements (range 2.5-75 kPa). A valid result generally is hard to obtain in obese patients and in patients with narrow intercostal space and impossible in patients with ascites (116). Definition of a reliable result, as proposed by the manufacturer, is ten valid measurements with a success rate >60% and an IQR to median ratio (IQR/M) of <30% (117, 118). However, more recent evaluations of reliability criteria have demonstrated that the most important measurement of the accuracy of an examination is the IQR/M and that the success rate is of less importance (119, 120). Boursier *et al.* propose a definition of very reliable, reliable and poorly reliable measurement based on IQR/M of <0.1, 0.1-0.3 and >0.3 respectively (121). The intra- and interobserver agreement is generally high, but reported to be reduced significantly in patients with steatosis, increased BMI, and lower fibrosis stages (117). In an evaluation of more than 13.000 examinations, liver stiffness measurements were not interpretable in nearly 20% of cases, mainly due to obesity and limited operator experience (122). At the same time, Boursier *et al.* reported excellent correlation in LSM results between novice and expert except for the number of valid measurements (123).

Transient elastography correlates well with fibrosis stage in liver biopsy as demonstrated in several independent studies (118, 124-127), although there is considerable overlap between adjacent stages in all studies. Proposed cut-offs in these studies range from 5.2 to 8.6 kPa for significant fibrosis and from 11.9 to 14.8 kPa for cirrhosis. A meta-analysis (not including individual patient data) proposed 7.6 kPa and 13.0 kPa as cut-offs for significant fibrosis and cirrhosis respectively (128). The corresponding AUROCS were 0.84 (95% CI 0.82-0.86) for significant fibrosis and 0.94 (95% CI 0.93-0.95) for cirrhosis (128).

In a recent meta-analysis evaluating TE for the assessment of fibrosis due to recurrent HCV after liver transplantation, the sensitivity and specificity for

detection of cirrhosis was 98% and 84% respectively. For detection of significant fibrosis the pooled estimate for sensitivity and specificity was 83%(129). Transient elastography has also been evaluated in relation to Hepatic Venous Pressure Gradient (HVPG) measurement for detection of portal hypertension. A good correlation was reported by Carrion (130) *et al.* in a cohort of patients with recurrent Hepatitis C after liver transplantation, and has since been confirmed in an additional cohort of 61 patients with CHC- related liver cirrhosis (131).

ALT-flares, especially ALT-levels 2-3 times above the ULN (132, 133) and ingestion of food within 3 hours prior to the examination (134, 135) may lead to an overestimation of liver stiffness. As to the impact of steatosis on liver stiffness measurement results are conflicting. Arena *et al.* observed no influence (136), whereas Sanchez-Conde *et al.(137)* and Boursier *et al. (138)* reported significant associations. However, in the latter two studies the influence of steatosis was noted predominately among patients with high-grade steatosis. This potential source of error can be avoided by use of a diagnostic tool called controlled attenuation parameter (CAP) that specifically measure liver steatosis using a process based on transient elastography (139).

Other non-invasive methods for assessment of hepatic fibrosis include Acoustic radiation force impulse (ARFI) elastography (140) MR-elastography (141) and two-dimensional shear-wave elastography (142). ARFI is implemented in an ultrasound-imaging device, with the advantage that the examiner can choose which part of the liver to investigate. A meta-analysis of pooled patient data reported accurate diagnostic performance for staging of liver fibrosis (143)

Serum fibrosis markers

Liver fibrosis can be estimated using biochemical markers measured in serum. The perfect serum marker for fibrosis would be liver-specific, not influenced by other concomitant diseases, easy to perform and sensitive enough to discriminate between different fibrosis stages. The search for this perfect biomarker started decades ago and is still ongoing. Two principally separate groups of serum fibrosis markers can be identified: direct and indirect markers. Direct markers are substances that directly reflect changes in the extracellular matrix, like tissue inhibitor metalloproteinases (TIMP-1), Matrixmetalloproteinases (MMPs), hyaluronic acid (HA), and procollagen

type III amino-terminal peptide (PIIINP). Indirect markers, on the other hand, reflect liver function like platelet count, prothrombin-complex INR, AST to ALT ratio, etc. When evaluated as a single marker, HA seems to be a good direct marker of liver fibrosis associated with fibrosis in patients with HCV (144). Although single fibrosis markers may be useful, they are often combined in order to enhance their diagnostic utility.

Combination of serum fibrosis markers

The first more complex score including several variables and achieved through statistical modeling was the FibroTest®, described by Imbert-Bismut *et al.* in 2001 (145). Since then, several more or less complex scores have been proposed (146-155), listed in table 1.

Table 1. The different parameters included in some of the available fibrosis indices.

	Age	Platelet count	AST	ALT	gender	Apolipoprotein A1	Bilirubin	Prothrombin-INR	Haptoglobin	γGT	α-2-macroglobulin	MMP-3	TIMP-1	Hyaluronate	Cholesterol	γ-globulin
FibroTest®	x				x	x	x		x	x	x					
Fibrometer®	x	x	x		x			x			x					
FibroSpect®											x		x	x		
ELF®	x											x	x	x		
Hepascore®	x				x		x			x	x			x		
Lok-index		x	x	x				x								
APRI		x	x													
GUCI		x	x					x								
FIB-4		x	x	x												
Fibroindex		x	x													x
Forns Index	x	x								x					x	

Validation, comparison and combination of different non-invasive methods

In a study by Björklund et al evaluating the clinical use of APRI, GUCI and FIB-4, GUCI was the most accurate in predicting severe fibrosis with a positive predictive value (PPV) of 85% and a negative predictive value of 78% for identification of severe fibrosis (156). When validated and compared externally, the different patented scores have similar accuracy for the diagnosis of significant fibrosis (157-159). In a multicenter study including 913 CHC-patients, Degos *et al.* reported that the diagnostic accuracy for significant fibrosis was moderate (AUROC 0.72-0.78) for all non-invasive methods evaluated (including FibroTest, FibroMeter, APRI and Transient Elastography), and that liver biopsy remains of use to diagnose intermediate stages of fibrosis (127). A study by Castera *et al.* comparing APRI, FibroTest and Transient Elastography to liver biopsy, reported no statistically significant differences between the three tests based on AUROC-values although there was a trend towards better performance of FibroTest and Transient Elastography as compared to APRI (126). Different combinations of the three tests were also evaluated. When combining FibroTest and Transient Elastography, agreement between the two tests correlated with liver biopsy in 84% and 94% for the detection of significant fibrosis and cirrhosis respectively. A recent multicenter study reported by Zarski *et al.* (160) evaluated different combination of serum markers and transient elastography. They noted that for significant fibrosis, a combination of two different diagnostic methods increased the percentage of well-classified patients from 70-73% to 80-83%. For cirrhosis, however, a combination did not entail improvement. APRI and FIB-4 are based on routine laboratory tests, making them inexpensive and simple to use in clinical practice. When combined with transient elastography or FibroMeter the diagnostic accuracy for significant fibrosis improves significantly (160, 161). Sebastiani *et al.* evaluated a stepwise combination of APRI, followed by FibroTest and then liver biopsy if necessary, called SAFE biopsy. This stepwise application of different methods allowed a 36% reduction of the need for liver biopsies (162). Finally, a combination of either FibroMeter®, FibroTest® or Hepascore® with ELF® reduced the need for liver biopsy with 50-55% for detection of significant fibrosis (METAVIR F≥2)(163).

Apart from predicting fibrosis and cirrhosis, the use of non-invasive markers to predict outcome has been evaluated. Vergniol *et al.* evaluated the prognostic value of TE, FibroTest, and APRI, FIB-4 and liver biopsy in a cohort of 1477 CHC-patients. At 5-year follow up, all tests were predictive

for shorter survival although FibroTest and TE had higher predictive values (164). Similar results were found when evaluating the predictive ability of ELF over a median of 7-years (165), and TE may help to identify patients at low risk for clinical decompensation within a 2-year period (166).

Both direct and indirect serum markers can be affected by concomitant disease, especially inflammation or medication. On the other hand, they require a minimal invasive procedure, are reproducible and a result will be obtained in almost all patients. Additionally, some of the indirect serum markers are based on readily available laboratory tests making them inexpensive and easy to use in every day clinical practice. Although the diagnostic performance of LSM is considered to be very reliable, the use of TE is hampered by the high percentage of unreliable results (122, 127). This had a negative impact on the performance of liver stiffness measurement by Fibroscan in a recent "intention–to-diagnose" evaluation (167).

Sterols as fibrosis markers

Endogenous cholesterol is synthesized predominantly by the liver (168) and the liver is responsible for the metabolism of cholesterol and non-cholesterol sterols. Some of the key metabolites of the cholesterol metabolic pathway are displayed in figure 3. D7-lathosterol and desmosterol are intermediates in the cholesterol synthesis pathway and their serum concentrations reflect cholesterol synthesis. D7-lathosterol is the most sensitive marker of cholesterol synthesis and correlates to 5-alfa-HMG-CoA-reductase activity, the rate-limiting enzyme of cholesterol synthesis (169, 170). Sitosterol, avenasterol and campesterol are plant sterols (phytosterols) derived from ingested food reflecting intestinal absorption, while cholestanol is produced by enzymatic cleavage of endogenous cholesterol. Cholestanol as well as plant sterols reflects biliary secretion, and cholestanol is a marker of chronic cholestasis (171, 172). In line with this, plasma levels of these non-cholesterol sterols have been associated with either chronic cholestasis or hepatocyte function, particularly in the setting of primary biliary cirrhosis (PBC) (173).

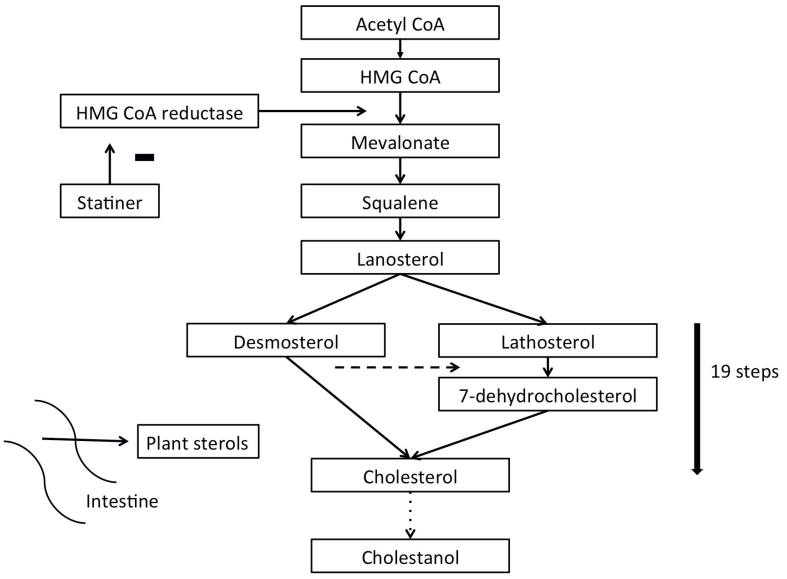

Figure 3. The cholesterol synthesis pathway and the uptake of plant sterols.

1.6 HCV Treatment

Unlike some other chronic viral diseases, HCV infection can be cured by antiviral treatment. The goal is to achieve a sustained virological response (SVR), *i.e.* viral eradication, in order to avoid fibrosis progression and reduce the risk of cirrhosis and HCC. SVR is defined by undetectable HCV RNA 24 weeks after cessation of treatment. In 1986 the first report on the use of interferon treatment for what was then referred to as ”non-A non-B hepatitis” was reported (174), with a minority of patients achieving persistent normalization of transaminases. Treatment efficacy was enhanced by the addition of ribavirin in the late 1990s (175-177) and further improved by the introduction of pegylated interferon in 2001 (peg-INF) (11).

Until a few years ago, the combination of peg-INF and ribavirin for 48 weeks (genotype 1) or 24 weeks (genotype 2 and 3) was the standard treatment regimen with an SVR rate of 40-50% in genotype 1-infected patients and

80% for genotypes 2 and 3 (178). This remains the standard treatment regimen in genotype 2 and 3 infected patients, with the option of a shortened treatment duration (12 -16 weeks) for patients with favorable baseline factors (no significant fibrosis), no dose reductions and undetectable HCV RNA at day 7 or at week 4 (age < 40 years) (179). The last years have seen an intense development of new direct acting anti-virals (DAAs). The first two such DAAs, telaprevir (180, 181) and boceprevir (182, 183), introduced in 2011, are first generation inhibitors of the viral NS3/4A protease. They are both used in combination with peg-INF/Ribavirin to treat genotype 1 infection, resulting in improved response rates.

Predictors of therapeutic response to interferon-based treatment include HCV genotype, fibrosis stage, baseline HCV RNA level, age, BMI, insulin resistance, levels of ALT and GGT, and coinfection with HBV and HIV (reviewed in (184, 185)), in addition to pretreatment activation of interferon stimulated genes (ISGs) (186, 187), including IP-10, and *IL28B* single-nucleotide polymorphisms, with the latter being the most important predictor for treatment outcome in genotype 1 infected patients (188)

New, highly efficaous treatment regimens also for more advanced fibrosis stages and including genotypes 2-6, are likely to be introduced shortly, and pending interferon-free treatment in the not-too-distant future.

1.7 *IL28B* single gene nucleotide polymorphism

Several genome-wide association studies have revealed that single nucleotide polymorphisms (SNPs) in the *19q13* region, in close proximity to three genes (*IL28A*, *IL28B*, and *IL29*) encoding cytokines of the interferon-λ (*i.e.* type III interferon) family, predict spontaneous clearance of HCV infection (35, 189) as well as sustained virological response (SVR) following peg-IFN and ribavirin therapy among patients infected with HCV genotype 1 (188-191).

Regarding genotype 2 and 3, however, reports on treatment response according to IL28B allele carriage have given conflicting results (192-196) and for these genotypes uncertainty prevails regarding the benefit of favorable *IL28B* genotype carriage. The C allele at rs*12979860* is associated with higher viral load (188, 197, 198), which otherwise is an established negative predictor of response to IFN-α and ribavirin therapy (11, 12, 178). These polymorphisms are strongly associated with the first phase viral

decline (*i.e.* the reduction of HCV RNA during the first days of treatment, which is assumed to result from the blocking of the production or release of hepatitis C virions (18, 199)), irrespective of HCV genotype (192, 198, 200). Among HCV genotype 1 infected patients this translates into higher frequencies of achieving both rapid virological response (RVR) and SVR among carriers of the favorable SNP alleles (192, 198).

IL28B polymorphisms have also been evaluated in relation to liver histopathology damage. In HCV genotype 3 patients, $CC_{rs12979860}$ indicated more pronounced inflammation than T allele carriage based on APRI and ALT levels (56), and in liver biopsies from HCV genotype 3 infected patients, carriage of the otherwise favorable allele was associated with more pronounced inflammation, steatosis and fibrosis (55, 57, 201). For non-genotype 3 patients, however, the results have been more conflicting. A study enrolling Japanese patients infected with HCV genotype 1 or 2 reported significantly more severe inflammatory activity and a higher proportion of more advanced fibrosis among those homozygous for the *Il28B* allele more favorable for treatment outcome (202). On the other hand, a rather recent report including 1483 predominately HCV genotype 1-infected patients, of whom 276 had paired liver biopsies, CC carriers at *rs12979860* had more severe hepatic necroinflammation, higher ALT and worse clinical outcome, but not more aggressive fibrosis progression (203), although this latter finding may have been secondary to the relatively short time that elapsed between biopsies (median 4 years).

1.8 HCV and liver transplantation

HCV associated end-stage liver disease is a leading indication for elective liver transplantation in the United States and Europe (204, 205). In Sweden, the number of HCV-associated liver transplants has increased in recent years (206). Reduced survival has been reported for patients transplanted due to HCV cirrhosis compared to other indications (207).

Reinfection of the transplanted liver graft is universal after liver transplantation for Hepatitis C virus infection. In a viral kinetics study by Garcia-Retortillo *et al*., HCV RNA was detectable in the blood stream during the an hepatic phase in most patients, and reached pre-transplant levels within four days in a significant proportion of patients (208). The reinfection of the

allograft is followed by an acute hepatitis in 2-6 months post transplantation and subsequent chronic hepatitis with decreasing viral load and immune-mediated injury (209, 210). A variant form of recurrence is cholestatic hepatitis, which occurs in <10% of transplant recipients, is associated with high viral load, frequent rejection episodes and HIV co-infection and can result in rapid graft loss within a year (211). The fibrosis progression rate is highly accelerated post transplantation with development of bridging fibrosis and cirrhosis in 20-54% in five years and 32-51% in seven years (212-216).

The mechanisms underlying the accelerated fibrosis progression remain unclear. As discussed earlier, development of liver fibrosis and fibrosis progression is the result of a complex interplay between factors promoting the formation or degradation of fibrotic tissue. The character of the immune response is thought to be of consequence for the pathogenesis of HCV after transplantation. For example, a broad, specific T-cell response post liver transplantation is correlated with improved histological and clinical outcome (217, 218).

Due to immune suppression, HCV RNA levels are significantly higher post-transplant as compared to pre-transplant (210). This may have both direct consequences as studies indicate that high HCV replication alone might induce fibrosis (219-221), and indirect consequences through an enhanced immune response in the liver although the overall immune response is attenuated (figure 4).

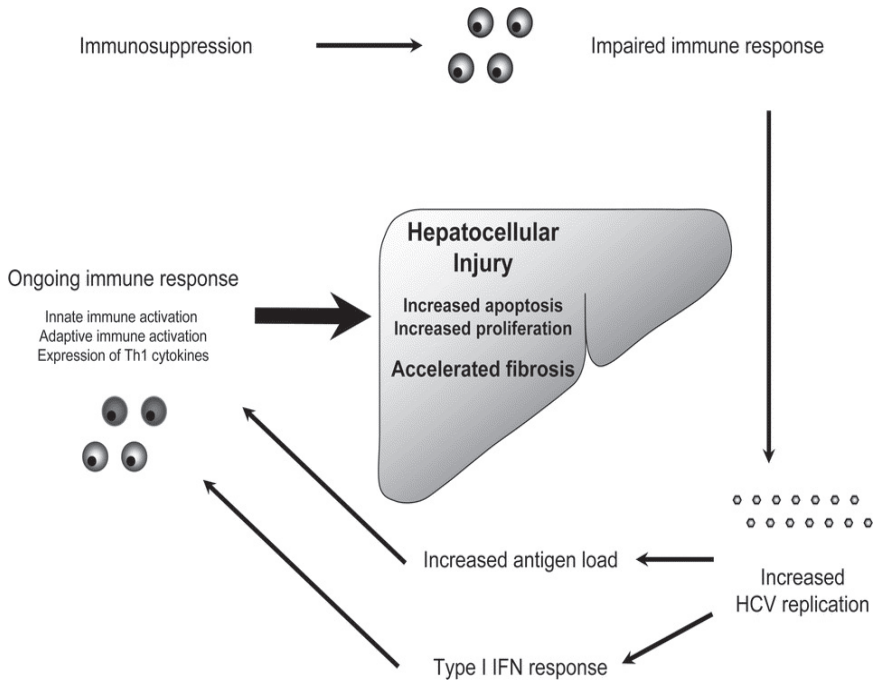

Figure 4. Proposed mechanisms underlying rapid progression of Hepatitis C virus (HCV)-related liver disease post transplantation. Immunosuppression leads to increased HCV replication in the face of an attenuated immune response. Increased viral replication is associated with activation of type I interferon responses within the infected liver, and with the presence of an increased antigen load. As the immune response is blunted rather than abrogated, this in turn likely results in activation of both innate and adaptive immune pathways, with the generation of Th1 cytokines and the recruitment of innate immune cells including macrophages, which may contribute to liver injury. Overall, there is increased hepatocyte apoptosis and proliferation, and accelerated fibrosis occurs. Adapted from McCaughan GW, Zekry A. Mechanisms of HCV reinfection and allograft damage after liver transplantation. J Hepatol 2004; 40: 368. McCaughan GW et al. Transplantation 2009;87: 1105–1111. Reprinted with permission from Wolters Kluwer Health and Elsevier

The course of fibrosis progression can be predicted at an early stage. Previous studies have shown that grade of inflammation as well as stage of fibrosis in 1-year protocol biopsies of the liver graft to be predictive of fibrosis progression as well as graft and patient survival (222, 223) (figure 5), while presence of steatosis at the same time point is associated with enhanced progression to significant fibrosis (224). Similarly, donor histology, especially steatosis and the presence of portal inflammation, has been reported to adversely influence outcome in terms of fibrosis progression post-transplant (225-227). Other factors adversely affecting outcome following HCV-associated liver transplantation include higher recipient age (228),

female gender (214), higher donor age (214, 228, 229), viral load (230, 231), reactivation of cytomegalovirus (CMV) infection and use of steroid boluses to treat rejection episodes (223, 232), while reduction of overall immunosuppression and avoidance of abrupt variations in immunosuppression seems to improve outcome (233). SVR following treatment is associated with fibrosis stabilization/improvement as well as increased graft- and patient survival (234). Additionally, *IL28B* (*rs12979860*) genotype in donor as well as recipient seem to affect outcome with donor CC genotype favoring development of fibrosis and a higher rate of progression to cirrhosis, liver related death and re-transplantation while the opposite was observed in recipient CC genotype (235, 236). Following HCV recurrence among 54 liver transplant recipients, a non-significant trend towards milder fibrosis was noted among CC *rs12979860* carriers possibly secondary to better therapeutic response (237). Similarly, a recent study found that donor, but not recipient PNPLA3 genotype affected post transplant outcome in terms of progression to ≥ Ishak stage 3 fibrosis or HCV-related mortality/graft loss (238).

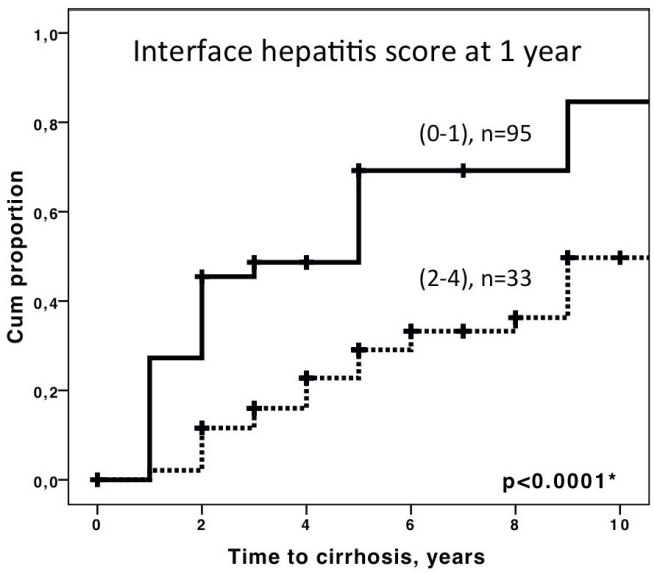

Figure 5. Time to cirrhosis according to grade of interface hepatitis at 1 year post transplant. Ydreborg et al, (2009), Abstracts 504. Hepatology, 50: 502A–599A. doi: 10.1002/hep.23302. Reprinted with permission from John Wiley and Sons.

2 AIM

The overall aim of this thesis was to explore the implications and consequences of liver fibrosis for patients with HCV infection in different settings.

Additional specific aims were:

- To analyze the prevalence of HCV infection among recipients of blood transfusions prior to 1992 in a regional observational study, and to evaluate whether a look-back screening effort would be beneficial for the patients identified considering degree of liver damage and their chance of receiving effective HCV treatment.

- To evaluate patient and graft survival following liver transplantation from 1992 to 2006 in HCV-infected liver transplant recipients in a single center study, and to identify factors influencing survival, with particular focus on donor liver histopathology.

- To create and validate a new model for accurate prediction of biopsy-verified HCV-related liver cirrhosis, based on patient characteristics and biomarkers of liver fibrosis, including a panel of non-cholesterol sterols reflecting cholesterol synthesis and absorption within the framework of a phase III treatment trial.

- To evaluate the impact of *IL28B* SNP variability on liver damage, evaluated by liver stiffness measurement in the context of a real-life trial for sequential patients with HCV infection undergoing routine evaluation.

3 PATIENTS AND METHODS

3.1 Study participants

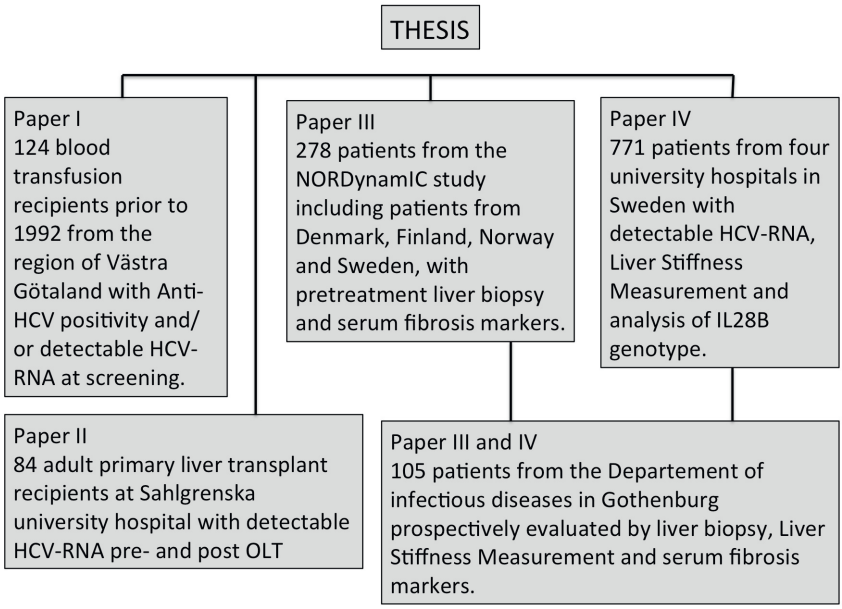

Figure 6. Overview of study participants and inclusion criteria in paper I-IV.

Paper I

From May 15, 2007, screening serologies for HCV among recipients of blood transfusion prior to 1992 were carried out at the Department of Virology, Sahlgrenska University Hospital, Gothenburg. During 2008, this routine was expanded to include all microbiology laboratories throughout the Västra Götaland Region (population 1.6 million). In total, 13 573 individuals were tested. For sera analyzed at the Department of Virology in Gothenburg, age and gender were available for all individuals tested. A confirmed HCV antibody analysis, along with a report to the local Department of Communicable Disease Control that blood transfusion prior to 1992 was the most likely cause of infection, was used as inclusion criterion. Clinical

evaluation was performed at the local Infectious Diseases outpatient clinic, and therapy was initiated at the discretion of the treating physician. The time and reason for the transfusion along with biomarkers of fibrosis, liver biopsy reports, and information regarding treatment for HCV were gathered retrospectively and recorded in an anonymous fashion.

Paper II

All adult recipients (n=84) of primary liver transplantation due to HCV-related end stage liver disease at Sahlgrenska University Hospital (Gothenburg, Sweden) between January 1992 and December 2006 were included (patient characteristics are detailed in Table 1 of paper II). Patients were followed until re-transplantation, death or, if alive, until April 2010. Median follow-up time was 57 months (range 28-87). Demographic and clinical data were collected from patient files, and donor data as well as cause of death from the Nordic Liver Transplant Registry (NLTR), comprising recipient data at acceptance to the waiting list and at transplantation as well as donor data, recorded prospectively since 1990. Graft survival was defined as absence of death or re-transplantation. HCV recurrence was determined on the basis of liver histopathology as reported by the local pathologist in combination with clinical, biochemical and virological findings. All patients were seronegative for antibodies to human immunodeficiency virus (HIV). One patient was HBsAg seropositive indicative of active hepatitis B virus infection. HCC was diagnosed pre- or post OLT in 33 patients (40%) and of these, 48% were within the Milan criteria (72).

In order to evaluate outcome over time, the patient cohort was divided in two time periods based on year of OLT: period 1 (1992-1998, n=16) and period 2 (1999-2006, n=69). Since the number of patients transplanted due to HCV-related liver disease increased substantially over time, the latter time period was further subdivided into period 2a (1999-2002, n=25) and period 2b (2003-2006, n=44). For details regarding immunosuppressive regimen and prophylaxis in order to avoid cytomegalovirus (CMV) and *Pneumocystis jiroveci* infections, I refer to the Method section of paper II.

Paper III

The study participants in this paper are derived from two different study cohorts that constitute the exploration and validation set respectively. The exploration set cohort was derived from a phase III multicenter treatment trial

for treatment-naïve HCV genotype 2 or 3 infected patients (the NORDynamIC study, n=382) conducted at 31 centers in Denmark, Finland, Norway and Sweden. Details regarding trial design and outcome have been published previously (239). A liver biopsy consistent with chronic hepatitis C within 24 months of entry was required. Of the 382 patients enrolled, 298 patients had a liver biopsy that fulfilled the criteria for staging and grading along with serum samples analyzed for potential fibrosis markers in accordance with the study protocol. When all patients with missing data in any of the parameters were excluded, 278 patients remained. These patients constituted the exploratory set. The validation set was derived from a cohort of 105 CHC patients enrolled in a study evaluating the use of liver biopsy, serum fibrosis markers and liver stiffness measurement performed at the Department of Infectious Diseases at Sahlgrenska University Hospital in Gothenburg in 2008-2010. All consecutive patients referred for a liver biopsy during an eighteen months period were asked to participate. Patients with missing data for any of the relevant parameters were excluded from the present study, leaving 83 patients in the validation set.

Paper IV

Eight hundred and two sequential HCV infected patients undergoing routine clinical liver stiffness measurement were recruited at four University Hospitals in Sweden from 2008 to 2012, and genotyped for *IL28B (rs12979860)*. Forty-one of the evaluated patients did not fulfill the inclusion criteria (HCV RNA was not detectable at time of liver stiffness measurement, or unavailable samples for *IL28B* or HCV genotyping) and thus were excluded. One patient with genotype 6, 21 patients with genotype 4, and two patients co-infected with more than one HCV-genotype were also excluded leaving a study cohort of 737 patients, of whom 614 had valid liver stiffness measurements (enrollment and disposition of patients detailed in figure 7). Demographic and clinical data were gathered from medical charts and anonymously registered in a joint database. Information regarding previous episodes of antiviral treatment was available for 708 patients (of whom 590 had an interpretable liver stiffness measurement) where 22% (n=150) of the patients were treatment experienced, without having achieved SVR. No patient was on treatment at the time of evaluation. Data regarding alcohol consumption or race was not available, although the overwhelming majority of patients are likely to be Caucasians of Scandinavian origin.

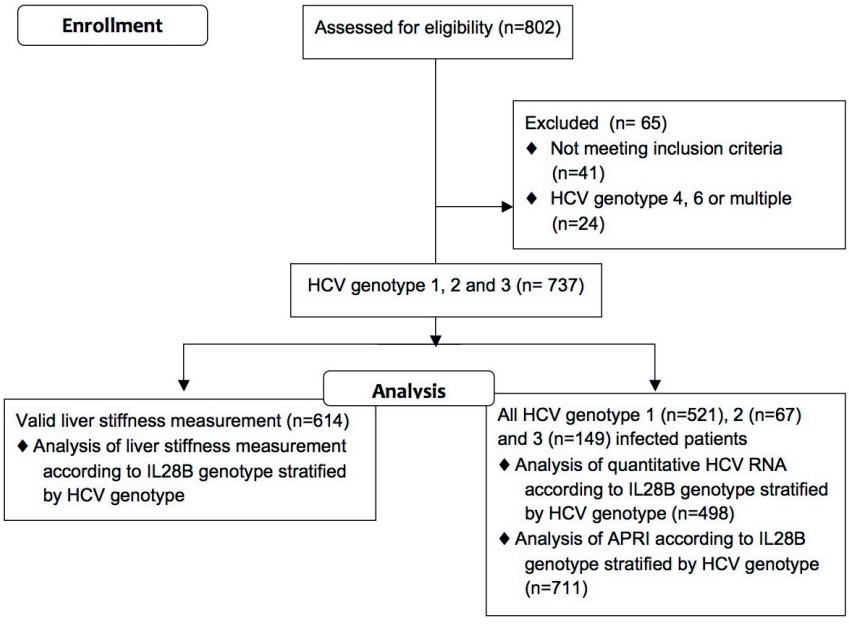

Figure 7. Study enrollment and disposition of participants in paper IV

3.2 Methods

Histopathologic assessment (papers I-III)

Paper I

Liver biopsies were obtained as part of the clinical evaluation at the discretion of the treating physician and assessed by the local pathologist according to the Ludwig and Batts protocol (110).

Paper II-III

In paper II, initial perioperative biopsies from the transplanted liver were available for 68 donor livers. In paper III pre-treatment liver biopsies were obtained from all 278 patients in the exploratory set and from the 83 patients constituting the validation set. All biopsies were retrieved and reassessed

centrally according to the Ishak protocol (including fibrosis stages 0-6) (106) by two experienced observers with a documented acceptable interobserver variability (108), in a dual observer consensus fashion as previously described (239). A consensus score was agreed upon, which then was used in the analysis. Steatosis was graded as absent (score of 0), mild (score of 1; <30% of the hepatocytes involved), moderate (score of 2; 30-70%) or severe (score of 3; >70%) (99). Cirrhosis was defined as Ishak fibrosis stage 5-6. Only biopsies containing at least four portal tracts or with a total length of ≥10 mm (paper II) or six portal tracts or with total length of ≥15 mm were evaluated (paper III).

HCV serology, HCV RNA quantification and HCV genotyping (papers I-IV)

For detailed description of diagnostic procedures, see the Method section of each paper, respectively.

Serum markers for liver fibrosis (paper I, III and IV)

The following indices, validated for the detection of fibrosis and/ or cirrhosis in chronic HCV infected patients, are used in this thesis:

APRI: (AST level (/ULN) /Platelet counts ($10^{9/L}$)) x 100 (148)

GUCI: (AST level (/ULN) x Prothrombin-INR x 100) / platelet count (10^9/L) (151)

Lok-index: log-odds (predicting cirrhosis) = -5.56-(0.0089 x platelet (x10^3/mm^3)) + (1.26 x AST/ALT ratio) + (5.27 x INR). Predicted probability = exp (log-odds) / (1 + exp (log-odds)). (153)

FIB-4: Age (years) x AST (U/L)/ ((platelets (10^9/L) x (ALT (U/L)) (155)

Evaluation of serum fibrosis markers (paper III)

In the exploratory cohort, baseline serum samples were drawn within 30 days prior to study entrance. Platelet count (x 10^9 /L) and Prothrombin complex-

INR were analyzed at each center. All other serum samples were stored at -70 °C and subsequently analyzed at a central laboratory at Helsinki University Hospital, Finland. Liver function tests and serum fibrosis markers analyzed by standard laboratory methods included normalized AST and ALT, Gammaglutamyl-transferase (GGT) (U/L), Bilirubin (µmol/L), Haptoglobin (g/L), alfa2-macroglobulin (g/L), Hyaluronic acid (HA) (ng/ml), amino-terminal propeptide of type III procollagen (PIIINP) (ug/L), Apolipoprotein A1 (g/L) and carboxy-terminal telopeptide of type I collagen (ICTP) and serum cholesterol (mg/100ml). The non-cholesterol sterols (cholestanol, D8-lathosterol, desmosterol, D7-lathosterol, campesterol, sitosterol, sitostanol, avenasterol and squalene) were analyzed by gas–liquid chromatography (GLC) as described in the Method section of paper III. To correct for differences in serum levels of sterols as a consequence of varying concentrations of lipoprotein particles, the non-cholesterol sterol values are expressed as proportions of serum cholesterol (µg/100 mg of cholesterol) as well as absolute concentrations (µg/100 ml).

In the validation set all serum samples were drawn the same day as the liver biopsy and immediately stored at -70 °C. Serum sterols were subsequently analyzed as stated above, all other analyzes were performed according to routine laboratory procedures at the Sahlgrenska University Hospital.

Liver stiffness measurement (papers III-IV)

Liver stiffness measurement was performed using the Fibroscan® device (EchoSens, Paris, France). This method, introduced in 2003 (116), is described in the introduction section of this thesis.

IL28B genotyping (paper IV)

SNP *rs12979860* was determined in plasma by allelic discrimination using Taqman MGB (minor groove binding) probes. The primers used and other details are described in the Method section of paper IV. All SNPs were in Hardy-Weinberg equilibrium. SNP *rs12979860* has previously been reported to have a stronger association with both first phase decline and SVR than *rs8099917* and *rs12980275* among Caucasian HCV infected patients, and was thus analyzed in the present study (198).

Statistical analysis

Statistical analyses were performed using either the IBM SPSS Statistics version 16.0 or 19.0 software package (IBM Corporation, Somers, NY) or the R software package version 2.15.0 (paper III). The package pROC was used to calculate ROC curves and the corresponding CI in paper III. All reported p-values are two-sided, and p-values < 0.05 were considered significant. Univariate analyses of patient characteristics were performed using either the Mann-Whitney U-test, the Chi squared test or Fischer´s exact test where appropriate. Correlations were examined using the Spearman's rank correlation coefficient r_s test.

Analysis of survival after liver transplantation (paper II)

Re-transplantation or death was considered major end-points and only primary liver transplants were included in the study. Patient and graft survival were evaluated by Kaplan-Meier analysis and the log rank test was used for comparison of survival distribution. In addition, Kaplan-Meier analysis and Cox regression analysis were used to perform univariate analysis of predictors of post-OLT patient and graft survival. Predictors of potential importance ($p<0.10$) were included in a stepwise Cox regression multivariate analysis. The potential explanatory variables included in the analyses were recipient and donor age, MELD-score, cold ischemia time, HCC in explant, repeated steroid boluses or steroid resistant rejection, sustained viral response (treatment post transplantation) and presence of inflammation, steatosis or Ishak fibrosis stage ≥2 in donor liver. Life Tables were used to estimate overall patient and graft survival.

Logistic regression and model selection for prediction of cirrhosis (paper III)

The following variables were analyzed in the exploratory set: age, sex, weight, BMI, genotype, platelets, PK-INR, normalized AST and ALT, GGT, Bilirubin, Haptoglobin, alfa2-macroglobulin, HA, PIIINP, Apolipoprotein A1, ICTP, total cholesterol, cholestanol, D8-lathosterol, desmosterol, D7-lathosterol, campesterol, sitosterol, sitostanol, avenasterol and squalene. Classification was made in two groups; group 1 (n=242), which encompassed fibrosis stages 0-4 and group 2 (n=36) which consisted of fibrosis stages 5-6.

The suggested classification procedure consisted of two steps: (i) an index that resulted from fitting of a model to the data and (ii) cut-off values for this index that can be used to divide the predictions into two groups.

The data was modeled through logistic regression; details regarding this procedure are described in the Method section in paper IV. The new index based on the model can be formulated either in terms of probabilities (I_{prob}) or log-odds (I_{odds}), which are equivalent. I_{odds} uses a more natural scale while I_{prob} may be perceived as more intuitive since it reflects the probability that a certain patient belongs to group 2 (i.e. has cirrhosis). I_{prob} was chosen for further calculations. Cutoff values were chosen in order to minimize the misclassification error. Rather than choosing one cut-off, we suggest applying two, C_{low} and C_{high}. If the index is below C_{low}, the observation will be classified into group 1. If it is above C_{high} it will be classified as group 2. If between C_{low} and C_{high} it will not be classified as either group and further investigations may have to be undertaken to make a correct diagnosis.

Diagnostic performance was analyzed by constructing receiver operator characteristics curve (ROC) for specificity and sensitivity, with calculation of the area under the ROC curve (AUROC) and the corresponding confidence interval (CI).

Ethical Aspects

The studies in paper I, II and IV was approved by the Regional Ethical Review Board in Gothenburg. In paper III, the ethical committee in each participating country approved the NORDynamIC trial that constituted the exploratory set and the Regional Ethical Review Board in Gothenburg approved the procedures involving the patients in the validation set. All studies were conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Calculation of costs for identification of patients with HCV infection (paper I)

The cost for identification and curing of one patient was calculated based on the sum costs for the screening process (i.e laboratory analysis and newspaper advertisements etc), the number of patients identified, the cost for

treatment in Sweden according to Lidgren *et al.* (240) for genotype 1 and 2/3 respectively, and by estimating the sustained viral response rate to be 50% for genotype 1 and 80% for genotype 2 and 3.

4 RESULTS AND DISCUSSION

4.1 HCV look-back screening (paper I)

HCV prevalence and patients demographics

From May 2007 through December 2008, sera from 13,573 patients who had received a blood transfusion prior to 1992 were screened for HCV antibodies at four microbiology laboratories in the Region Västra Götaland. Among tested subjects, age and gender were available in 5,022 cases; the median age was 58 years (IQR 47-66) and 80% were women.

One hundred and twenty-four out of 13,573 screened blood transfusion recipients (0.9%) had serum antibodies against HCV and 113 (0.8%) had detectable HCV RNA in serum (Figure 8). Ninety-one (73%) of these chronically infected patients were females and the median age was 57 years (IQR 48-65;). Fifty percent of patients with detectable HCV RNA were infected with genotype 1, 35% with genotype 2, and 14% with genotype 3. The earliest blood transfusion took place in 1963, and the highest frequency of HCV infected transfusions was observed between 1975 and 1989 (n=88, 78%). Cause of transfusion is demonstrated in figure 9. Among women, blood loss in association with childbirth (including caesarean section) accounted for 38% of HCV infections. The median age at the time of transfusion was 28 years IQR 21-38), and the median and mean follow-up time from transfusion until evaluation was 27 years (IQR 22-32).

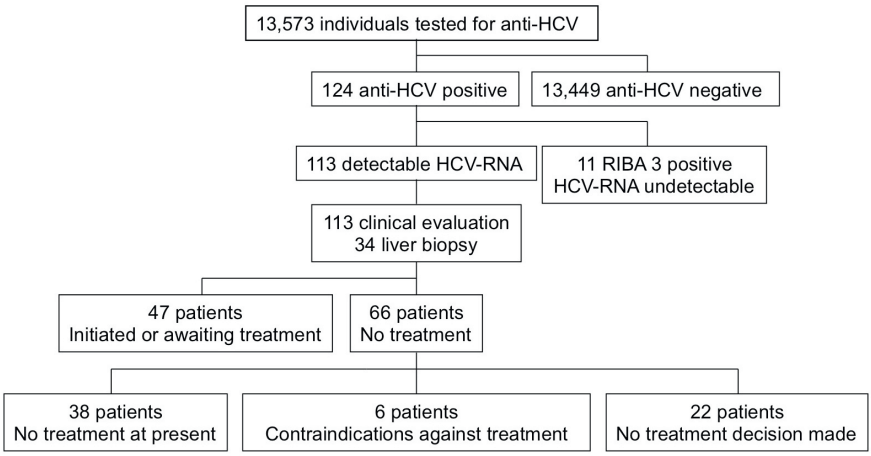

Figure 8. Outline of screening procedure for HCV transmission following blood transfusion prior to 1992. Published in Scand J Infect Dis. 2011; 43: 522–527 (paper I).

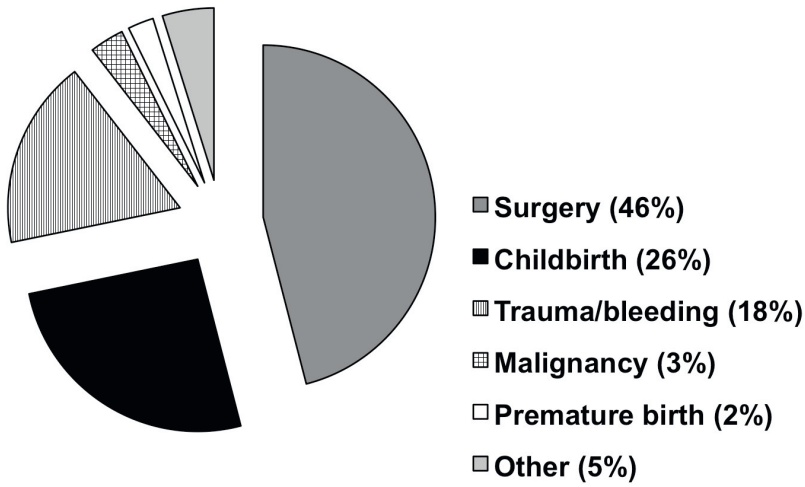

Figure 9. Reasons for blood transfusion among the 124 Anti-HCV positive blood transfusion recipients.

Previously reported prevalence of serological evidence of HCV infection from the same region of Sweden was 0.8% among pregnant women and 0.15% among female blood donors (241). Considering that 73% of the subjects identified were women, our findings indicate a slightly higher prevalence of HCV among those having received transfusions prior to 1992 than in the general population. Our study, however, likely underestimates the prevalence of transfusion-associated HCV infection, because many HCV infected transfusion recipients may have been diagnosed prior to this look-back study or may have expired from HCV-associated liver disease or other HCV-associated morbidity (242), or from other causes. Indeed only a minority of the identified patients were transfused because of malignancy, likely owing to a high mortality in this cohort.

The predominance of women in this population is surprising considering that approximately 50% of transfusion recipients (243) and almost 70% of patients with HCV in the Swedish national register (2) are male. A similar optional HCV screening program conducted in Ireland in the late 1990's, however, reported a similar gender disparity (244). This may indicate that women are more inclined to respond to public health information. Additionally, women reportedly have a higher rate of survival after blood transfusion (243). Similarly, because HCV infected men have a more rapid progression of fibrosis than women (38), a larger proportion of the male transfusion recipients may have been identified prior to this screening campaign or may have succumbed to HCV-associated disease. Thirty-eight percent of the women in the study cohort were infected after transfusion in conjunction with childbirth, thus indicating that childbirth might be an overlooked risk factor for HCV transmission.

Prevalence of fibrosis

A GUCI score could be calculated for 104 of the 113 subjects with detectable HCV RNA. No score was calculated for patients receiving warfarin (n=4) or herbal medication (n=1) likely to affect the GUCI score, nor if the necessary analyses were lacking (n=4). As shown in Figure 10 A, the majority of patients had mild fibrosis as indicated by low GUCI scores (median 0.42; IQR 0.29–0.66). A liver biopsy assessment as part of the pre-treatment evaluation was recorded for 32 patients (Figure 10 B). No fibrosis or portal fibrosis (stage 0–1) was noted for the majority of patients. Nine of 32 patients had periportal or septal fibrosis (stage 2–3), and one patient had cirrhosis. For one additional patient, clinical findings were consistent with

cirrhosis. Age at transfusion, age at diagnosis or duration of infection did not differ significantly between patients with stage 0–1 as compared to stage 2–4 (Table 2). The one patient with cirrhosis in the liver biopsy had a follow-up time of 39.5 y. An association was noted between GUCI and age at diagnosis (Spearman's rank correlation r_s=0.22, P =0.026). Similarly, a non-significant trend between GUCI and age at transfusion was observed, while no association was found between GUCI or fibrosis stage and the calculated duration of infection.

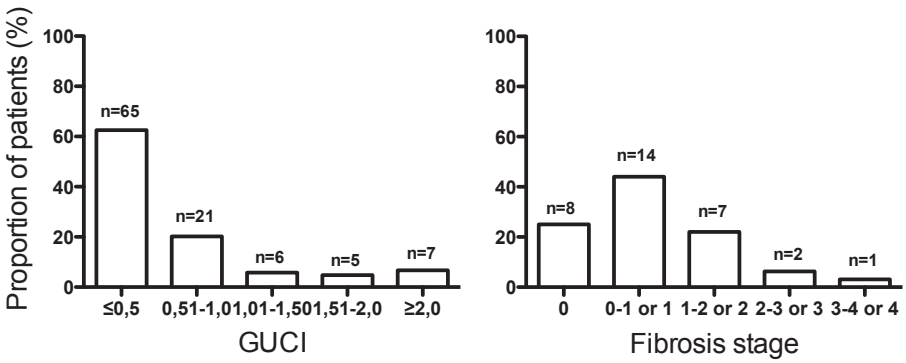

Figure 10. Assessment of liver fibrosis. For 104 patients a fibrosis index, the GUCI-score (Gothenburg University Cirrhosis Index), was calculated. A GUCI-score below 1.0 indicates a low risk of cirrhosis (A). Thirty-two liver biopsy evaluations using the Ludwig and Batts protocol were available. Published in Scand J Infect Dis. 2011; 43: 522–527 (paper I)

Table 2. Characteristics of patients according to stage of fibrosis

	Stage 0-1 (n=21)	Stage 2 (n=7)	Stage 3-4 (n=3)
Females (%)	71	71	67
Median age at transfusion (IQR)	27 (22-37)[1]	27 (17-47)	24 (0-41)
Median age at diagnosis (IQR)	57 (50-66)	51 (40-69)	57 (39-69)
Duration of infection (IQR)	28 (24-32)[2]	22 (19-35)	33 (28-39)
Genotype 1/ 2/ 3 (n)	17 / 2 / 2	4 / 3 / 0	2 / 1 / 0

IQR, Interquartile range.

[1] n=19
[2] n=19

Discordant outcomes have previously been reported for patients identified with transfusion-associated HCV infection. Whereas some report an unfavorable prognosis (245, 246), others observed a predominance of mild to moderate liver disease (39, 40, 247) in concordance with our study. These discrepancies may result from differences in gender, age, duration of follow-up, co-morbidity etc. While the majority of patients in the present study had mild disease, a subset with advanced liver disease was identified. These patients, along with two additional fatal cases of HCV infection identified outside our study within the Region Västra Götaland, reported in local press (248, 249), likely would have benefited from earlier diagnosis of HCV infection. Interestingly, we found no association between liver fibrosis and duration of infection, possibly secondary to the limited number of liver biopsies as well as sampling error in conjunction with this invasive procedure, inadequate approximations of fibrosis using GUCI, or incorrect recall of the time of transfusion. Additionally an underlying bias towards milder liver disease may have been present because individuals with advanced fibrosis are more likely to have been identified or expired prior to initiation of the screening campaign.

Antiviral treatment

Of the 113 patients with chronic HCV infection, a decision regarding anti-viral treatment was available for 86 subjects at the time of evaluation 3-18 months post testing. Forty-seven patients had started or were awaiting the initiation of treatment, while six patients were considered to have contraindications against HCV therapy (cirrhosis with hepatocellular carcinoma (n=1), psychiatric disorder (n=2), cardiovascular disease (n=2), and renal insufficiency (n=1)). For three patients, an age ≥70 years was the primary motive for abstaining from therapy. In the remaining 29 non-treated cases, treatment was not initiated due to either mild fibrosis or the patient's desire to deter therapy.

The main differences between treated and non-treated patients were a higher proportion of genotype 2 or 3 and more advanced fibrosis among treated patients. In order to evaluate the benefit of diagnosis and anti-viral treatment in the subgroup of patients with significant fibrosis, we identified sixteen patients in the treatment group with either a GUCI score greater than 1 or a fibrosis stage of ≥2. Fourteen of these patients had initiated anti-viral treatment. Three patients had discontinued treatment, two (both genotype 1)

due to lack of virological response, and one (genotype 2) due to development of vasculitis while on therapy. Eight patients (one genotype 1, four genotype 2 and three genotype 3) had completed the intended treatment course and all had undetectable HCV RNA at the end of treatment. Five of eight had completed six months of follow-up and had achieved a sustained viral response (one genotype 1, two genotype 2 and two genotype 3).

General aspects of screening including screening costs and benefit for the patient

During 2008 the total cost for the screening campaign, initiated by the local Department of Communicable Disease Control, amounted to 180,000 euros (145,000 euros for laboratory analyses and 35,000 euros for newspaper advertisements etc.). Thus the cost per identified chronically infected HCV patient amounted to approximately 1,800 euros.

Given that at the time of the study expected sustained viral response (SVR) rates of 80% and 50%, and treatment costs of approximately 12,000 euros and 23,000 euros for genotype 2/3 and genotype 1 infected patients (using pegylated interferon-α and ribavirin for 24 and 48 weeks respectively)(240), the total cost of identifying and curing an infected patient would be approximately 16,600 euros and 47,400 euros for genotypes 2/3 and 1, respectively. However, these calculations are limited by not including the continued costs for patients not achieving a sustained viral response following therapy as well as for patients abstaining from therapy. A proper cost-benefit analysis would have pre-requisites that were beyond the scope of this study.

We attempted to determine whether or not this screening process was beneficial for the patients identified. Criteria of an effective screening includes that the screening should target diseases with serious consequences such as mortality or prolonged morbidity, that the disease has a preclinical phase, that there is a treatment available and that treatment is more effective before the onset of symptoms (250). All this applies in general to chronic HCV infection although a subset of patients will never develop any symptoms of liver disease. The aspects of risk of transmission set aside, it could be argued that these patients would have been better off without the knowledge of a potentially life-threatening disease. On the other hand, awareness makes it possible for the patient to influence the disease progression to some extent, and disease progression cannot be fully determined without follow-up. In this study we limited our interpretation of

the word beneficial to whether patients identified could be offered treatment or not. In that aspect, the screening was beneficial since most patients identified were eligible for treatment.

4.2 Outcome following liver transplantation for HCV-related end stage liver disease (paper II)

Survival

The overall patient survival at 1, 3, and 5 years post-OLT were 90%, 77%, and 73% respectively. In total, 29 patients (35%) died during the study period. Three of these deaths occurred within 30 days post transplantation. The cause of death among these patients was often multifactorial, with the most common primary cause being relapse of HCC (n=10), followed by liver failure (n=4) which included 3 patients with recurrent HCV-cirrhosis and one patient with acute rejection. Three patients died from septic infections, 3 patients from cardiovascular disease and another 5 patients died from other causes (suicide (n=2), gastrointestinal bleeding (n= 1), lymphoma (n=1), multi-organ failure and CMV-pneumonitis (n=1)). In 4 cases the cause of death could not be identified.

Re-transplantation

The overall 1-, 3-, and 5-year graft survival rate throughout the study period was 80%, 77%, and 60% respectively. Twelve patients (14%) received a second allograft, one of which also received a third allograft. The median time to re-transplantation was 3 months. The indications for re-transplantation were recurrence of hepatitis C (n=4), primary graft non-function (n=3), acute arterial thrombosis (n=3), late arterial thrombosis with ischemic bile duct problems (n=2), and acute portal thrombosis (n=1).

Survival according to donor histology

Biopsies of the donor liver could be retrieved for 68 of the 84 primary liver transplant recipients. Some of the oldest biopsies were not available and thus, patients with no available donor biopsy had undergone transplantation earlier than patients with retrievable biopsies (median year of transplant 1999 (IQR 1998-2004) vs. 2003 (IQR 2000-2005), P= 0.024). There were, however, no significant differences regarding graft or patient survival, recipient or donor age, presence of HCC in the explant, cold ischemia time, or MELD-score between these two groups of patients. Histopathologic features of the donor livers are detailed in Table 3. For statistical comparison, stratification was made between stage F0-1 vs. F2-4 for fibrosis and absence vs. presence of steatosis and necroinflammatory activity for each inflammatory component separately.

Table 3. Characteristics of liver donors

Median donor age (range)	54 (11-77)
Donor sex female / male, n	35 / 46
Donor histology	
Grade of steatosis (n) 0 / 1 / 2 / 3	23/ 22/ 21 / 2
Stage of fibrosis (n) 0/ 1/ 2 / 3 / 4 / 5 / 6	25/ 26/ 11 / 1 / 3 / 0 / 0
Grade of portal inflammation (n) 0/ 1/ 2/ 3/ 4	56/ 8/ 1/ 0/ 0
Grade of interface hepatitis (n) 0/ 1/ 2/ 3/ 4	60/ 4/ 1/ 0/ 0
Grade of focal inflammation (n) 0/ 1/ 2/ 3/ 4	13/ 41/ 9/ 1/ 1

Figure 11 A displays an association between patient survival and the stage of donor liver fibrosis. This association remained statistically significant also after exclusion of patients with HCC. A similar, although not statistically significant, trend was noted for graft survival (data not shown). Portal inflammation in the donor liver was significantly related to graft survival (Figure 11 B). When analyzing donors older than 60 years (n=19), the presence of portal inflammation in the donor liver was strongly associated with both impaired graft and patient survival (P=0.004 and P<0.0001 respectively). Similarly a non-significant trend towards impaired graft survival due to interface hepatitis in the donor liver was noted (P=0.066). No such trend was observed between focal inflammation and graft or patient survival. We further observed a non-significant trend for impaired graft survival due to presence of steatosis in donor liver (P=0.061, data not shown). Three patients died and another 4 patients were re-transplanted due

to HCV recurrence. No associations between death and re-transplantation due to HCV recurrence and donor histopathology were noted.

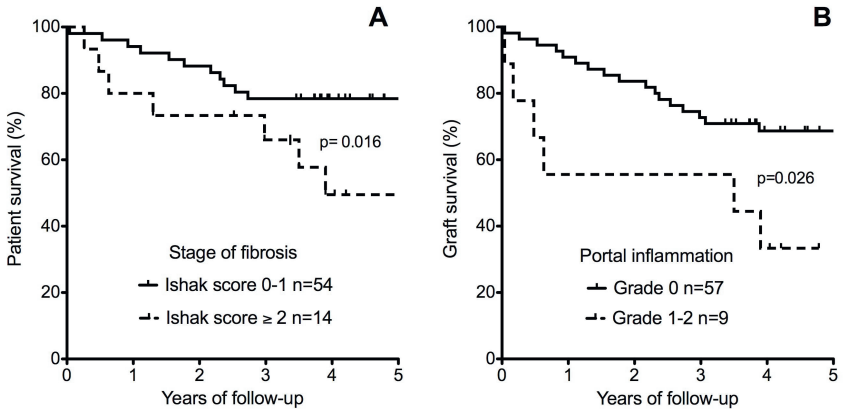

Figure 11.
A: Association between patient survival and fibrosis stage in the donor liver. Patient survival was significantly reduced in recipients receiving a graft with Ishak fibrosis stage 2-4 compared to recipients receiving a graft with Ishak fibrosis stage 0-1 (P= 0.016, Log rank test). Mean survival time was 4.5 years (95% CI 3.0-6.0) and 9.6 years (95% CI 8.0-11.2) respectively.
B: Association between graft survival and portal inflammation in donor liver biopsy. Graft survival was significantly impaired in patients receiving a graft with portal inflammation as compared to patients receiving a graft with no portal inflammation (P=0.026, Log-rank test). Mean survival time was 2.6 years (CI 1.3-3.9) and 8,2 years (CI 6.6-9.8) respectively. Published in Scand J Gastroenterol. 2012; 47: 710–717 (paper II).

Conceivable predictive factors for patient and graft survival evaluated in univariate analysis are detailed in Table 4 a and b respectively. Recipient age over sixty and stage of fibrosis in donor liver remained independently predictive of patient survival. Repeated steroid boluses or steroid resistant rejection and portal inflammation in the donor liver remained independently predictive of graft survival.

Table 3. Univariate and multivariate Cox-regression evaluating A) patient survival and B) graft survival among HCV patients following liver transplantation

A	**Univariate analysis P-value**	**Multivariate analysis P-value**	**Odds ratio (95% CI)**
Recipient age ≥ 60 years	0.088	0.007	3.98 (1.46-10.86)
Donor age ≥ 60 years	0.932		
Ishak fibrosis stage 2-4 in donor liver	0.033	0.007	3.56 (1.40- 9.04)
Steatosis in donor liver	0.570		
Portal inflammation in donor liver	0.381		
MELD-score	0.301		
Cold ischemia time	0.098	0.362	
Repeated steroid boluses or steroid resistant rejection	0.359		
HCC in explant	0.041	0.302	
Sustained Viral Response (treatment post transplantation)	0.138		
B			
Recipient age ≥ 60 years	0.119		
Donor age ≥ 60 years	0.391		
Ishak fibrosis stage 2-4 in donor liver	0.272		
Steatosis in donor liver	0.049	0.213	
Portal inflammation in donor liver	0.033	0.017	3.17 (1.23-8.16)
Interface hepatitis in donor liver	0.066	0.147	
MELD-score	0.402		
Cold ischemia time	0.142		
HCC in explant	0.398		
Repeated steroid boluses or steroid resistant rejection	0.017	0.004	3.72 (1.51-9.19)
Sustained Viral Response (treatment post transplantation)	0.047	0.066	

The main finding in paper II was that donor histology may be of utmost importance for graft and patient survival. We observed associations between fibrosis in the donor liver and patient survival as well as between portal inflammation in the donor liver and graft survival. To our knowledge, this is the first report on these findings, although previous studies have shown that grade of inflammation as well as stage of fibrosis in 1-year protocol biopsies of the liver graft to be predictive of fibrosis progression (222, 223).

Bahra *et al.* have previously reported that the presence of portal inflammation in the donor liver deleteriously impacted on fibrosis progression post-OLT but not long-term survival (225). The occurrence of intrahepatic inflammation was in the same study significantly increased in donors older than 33 years. In the present study, however, no association between donor age and portal inflammation was noted, although most donors were older than 33 years, and fibrosis progression post transplant could not be properly evaluated because of the lack of protocol biopsies from the earlier time period.

The underlying cause of fibrosis and inflammation in donor livers remains obscure and could not be properly investigated in the present study due to limited information regarding donor medical history, and because histological assessment does not permit an etiologic diagnosis. It must also be noted that the Ishak scoring method was originally designed for the assessment of chronic viral hepatitis and has not been validated for examination of donor livers without viral hepatitis. Thus, we believe that this warrants further investigation with systematic reporting of extended donor information.

The impact of steatosis on graft survival is subject to ongoing debate. In the present study a non-significant trend was noted towards impaired graft survival due to presence of steatosis in the donor graft. Previous studies have concluded that mild steatosis (<30%) is not of importance for survival (251, 252), while severe steatosis (>60%) is recognized as an important prognostic factor for graft dysfunction (226, 253). Furthermore, moderate/severe steatosis has been suggested to be of importance for the severity of recurrent hepatitis C as well as for graft survival (227). In the present study the degree of steatosis did not alter the observed influence on survival. This might in part be attributed to the scarceness of severely steatotic donor livers in this study cohort, but it is in line with some earlier studies (252).

Survival according to time of transplantation

A trend towards relatively poor patient as well as graft survival was noted during the period 1999-2002 (patient survival is displayed in figure 12), with an improvement in recent years. This development was not seen in patients transplanted at the same center for reasons other than HCV infection (data not shown), was not altered by the exclusion of HCC patients, and is in line with other reports (214, 254). The improved outcome in recent years noted in the present study occurred despite increasing age of both patients and donors. Fewer late CMV infections and better selection of patients with concurrent hepatocellular carcinomas (254), as well as optimized immunosuppression with fewer episodes of acute rejection and less need for steroid boluses have been suggested as contributing to this improvement (214, 233). Yet another topic has been the switch from ciclosporin to other calcineurin-inhibitors such as tacrolimus. Patients taking ciclosporin seems to have a significantly better chance of SVR following anti-viral treatment with peg-IFN and ribavirin, compared to tacrolimus (234), but no differences in post-liver transplantation clinical events have been reported (255).

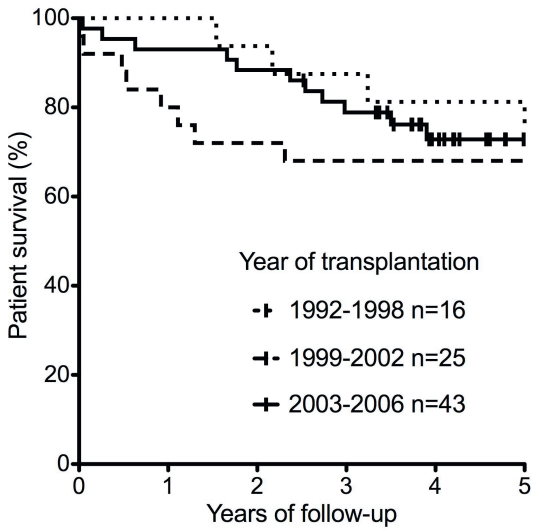

Figure 12. Association between patient survival and year of transplantation. Impaired survival during the period 1999-2002 was noted with a significantly reduced one and two-year survival as compared to the other time periods (p=0.05). Published in Scand J Gastroenterol. 2012; 47: 710–717 (paper II).

Table 5. Patient and donor characteristics displayed by time period of transplantation

	Period 1 1992-1998 (n=16)	Period 2a 1999-2002 (n=25)	Period 2b 2003-2006 (n=43)	p
Median age (range)	49.5 (39-61)	52 (38-68)	54 (46-68)	0.04[1]
Female sex %	25	28	21	
Median BMI (range)	24.7 (15.3-37.8)	26.1 (21.1-43.4)	26.5 (18.8-41.2)	
Preexisting alcohol abuse, n (%)	4 (25)	11 (44)	20 (45)	
HCC in explant, n (%)	5 (31)[2]	8 (32)	20 (45)	
Genotype 1 (%)	56	52	51	
Median MELD-score (range)	16.5 (10-23)	14 (7-48)	12 (6-42)	
Median cold ischemia time, hours (range)	11.0 (4.8-14.0)	10.0 (5.5-23.0)	8.3 (5.0-15.0)	0.009[3] 0.05[4]
Patients with steroid bolus treatment, n (%)	9 (56)	11 (44)	12 (28)	0.045[5]
Patients with repeated steroid bolus treatment or steroid resistant rejection, n (%)	3 (19)	6 (24)	6 (14)	
Patients receiving antiviral treatment post transplantation n (%)	7 (44)	13 (52)	27 (63)[6]	
Patients achieving sustained viral response, n (%)	1 (6)	3 (12)	7 (16)	
Median donor age (range)	43 (11-67)	54 (18-69)	56 (13-77)	0.002[7] 0.005[8]
Steatosis in donor liver, n (%)[9]	8 (73)	10 (50)	26 (70)	
Ishak fibrosis score ≥2 in donor liver, n (%)[10]	0 (0)	4 (16)	11 (26)	

[1] Period 2a vs 2b
[2] Missing cases n=2
[3] Period 1 vs 2b
[4] Period 2a vs 2b
[5] Period 1 vs 2b
[6] Missing cases n=1
[7] Period 1 vs 2b
[8] Period 1 vs 2a, 2b
[9] Missing cases, period 1 n=5, period 2 n=5, period 3 n=6
[10] Missing cases, period 1 n=5, period 2 n=5, period 3 n=6

In the present study, the effect of late CMV infections on graft- and patient survival could not be properly evaluated. However, it should be pointed out, that only 4 patients had a CMV D+/R- mismatch and that all patients received CMV-prophylaxis. In our study, fewer steroid treated rejection episodes and shortening of cold ischemia time appear to have contributed to the recent improvement. The negative impact of steroid boluses on the prognosis of recurrent HCV is well documented (232, 256, 257) and prolonged cold ischemia time has been related to severe HCV recurrence (251, 258).

Effect of donor age

Most previous studies have established donor age as a significant predictor of survival and fibrosis progression post-OLT (229, 259, 260), although others report no such association (216, 257). No effect of donor age on patient and graft survival could be detected in our present cohort. However, the overall donor age was relatively high throughout the entire study period (median 54 years range 11-77), compared to other centers (225, 261, 262) and the low number of young donors may have led to an underestimation of the effect of donor age. In a recent study Selzner *et al.* concluded that the effect of donor age on graft survival was most profound in older recipients (≥50 years) and in HCV-positive recipients and that the combination of older donor and older recipient should likely be avoided in HCV-positive patients (228). In order to assess the importance of donor histology in older donors, we evaluated the impact of stage of fibrosis, steatosis, and necroinflammatory activity on graft and patient survival in recipients receiving a liver from a donor aged 60 years or more. Particularly portal inflammation appeared to have great impact on graft and patient survival in this subgroup analysis. However, because of small sample size, these findings must be interpreted with caution.

The findings in paper II suggest that if possible, it may be worthwhile to undertake a histologic examination of the donor liver before transplantation. Although it can prove a logistic challenge, analysis of frozen sections may be both feasible and accurate for rapid assessment of liver histology (263, 264).

4.3 Prediction of cirrhosis by a non-invasive index (paper III)

Baseline characteristics

Baseline characteristics according to presence of liver cirrhosis in the exploratory set and the validation set are displayed in table 1 of paper III. The prevalence of cirrhosis was 13% (36/278) and 10% (8/83) in the exploratory and validation set respectively. There were no patients with clinically decompensated liver cirrhosis in either exploratory or validation set. Patients in the validation set were older than patients in the exploratory set; median age was 49 (IQR 44-55) vs. 42 years (IQR 34-50) ($P<0.001$), and had a longer duration of infection; 29 (IQR 24-34) vs. 13.5 years (IQR 6,8 – 25) ($P<0.001$) in the validation and exploratory set respectively. In the exploratory set, 69% of the patients were infected with HCV genotype 3, whereas in the validation set HCV genotype 1 was most frequent, present in 72% of patients.

Model selection

Application of the model selection methodology described in the subject and methods section lead to a final model comprising the following variables: Age, BMI, D_7-lathosterol, platelet count, and Prothrombin-INR. The model characteristics are summarized in table 5.

Table 5. Summary of the final model.

Coefficients	Estimate	Standard Error	P-value
(Intercept)	-12.174	3.63	0.0008
Age	0.110	0.03	0.0002
BMI	0.232	0.06	8.0×10^{-5}
D7-lathosterol	-0.013	0.006	0.02
Platelets	-0.018	0.004	7.0×10^{-5}
Prothrombin-INR	3.687	2.382	0.122

The regression formula for this final model is: Log-odds; I_{odds} (predicting cirrhosis) = -12.17 + (age x 0.11) + (BMI (kg/m^2) x 0.23) + (D_7-lathosterol (µg/ 100 mg cholesterol) x (-0.013)) + (Platelet count ($x10^9/L$) x (-0.018)) + (Prothrombin-INR x 3.69).

Predicted probability; I_{prob} = exp (log-odds)/ (1+exp (log-odds)).

Predicted probability (I_{prob}) was chosen for further calculations, referred to as the Nordic Liver Index (NoLI). Figure 13 displays the relation to Ishak fibrosis stage for each component separately as well as combined in the index.

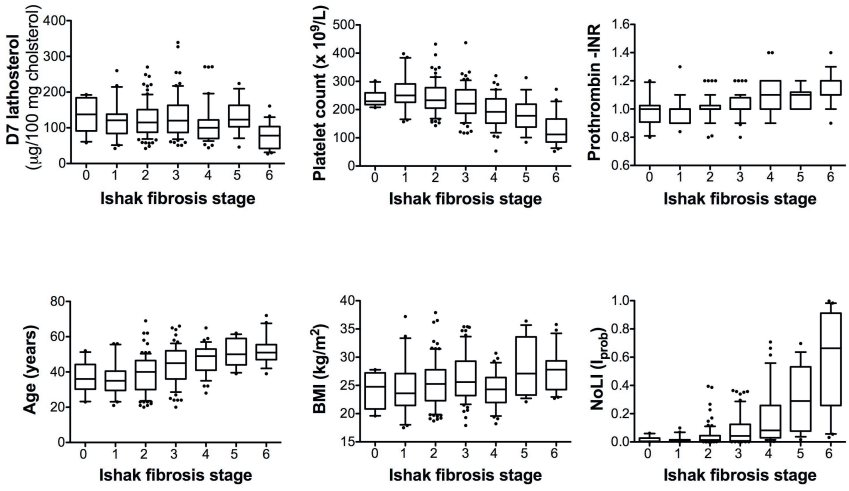

Figure 13. Box-plots displaying the different components of the NoLI index (D_7 lathosterol, platelet count, Prothrombin complex-INR, age and BMI) and the NoLI index in relation to Ishak fibrosis stage in the exploratory set (n=278). Fibrosis stages were distributed as follows: F 0=10, F1=41,F 2=85, F3=67, F4=39, F5=15, F6=21.

Area under ROC (AUROC)

The ROC curve formed by plotting sensitivity against specificity for the new index (NoLI) in prediction of cirrhosis in the exploratory set is shown in Figure 14. The area under the ROC curve (AUROC) was 0.91 (95% CI 0.86-

0.96). Figure 14 also display ROC curves for APRI (148), Lok index (153), GUCI (151) and FIB-4 (155). Theses indices were chosen for comparison since they are all freely available non-invasive scores, evaluated and validated for the detection of cirrhosis.

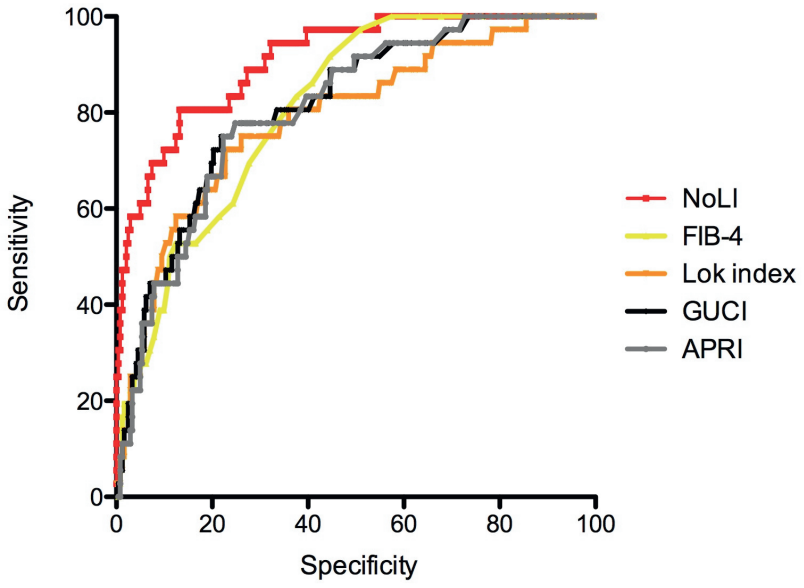

Figure 14. Receiver operator characteristics curves plotting sensitivity against specificity for prediction of cirrhosis in the exploratory set (n=278) for the new index (NoLI) in comparison with FIB-4, Lok index, GUCI and APRI. The AUROC for NoLI was 0.91 (95% CI 0.86-0.96). The corresponding AUROC for the other indices in the exploratory set were for FIB4 0.81 (95% CI 0.75-0.87), Lok 0.79 (95% CI 0.71-0.87), APRI 0.81 (95% CI 0.74-0.88), and for GUCI 0.81 (95% CI 0.74-0.88).

Cut-off values

Two cut-off values were chosen that would produce a minimal misclassification error of approximately 5% for each group. Using a predicted cut-off value of <0.053 for the exclusion of cirrhosis and >0.37 for the identification of cirrhosis would misclassify 5.6% (2/36) of patients with cirrhosis as non-cirrhotic and 5.0% (12/ 242) of non-cirrhotic as having cirrhosis (with a cirrhosis prevalence of 13%, a misclassification rate of 5.6%

is the closest to 5% we can get). Figure 15 shows the proportion of correct classifications for cirrhosis and non-cirrhosis according to different cut-offs.

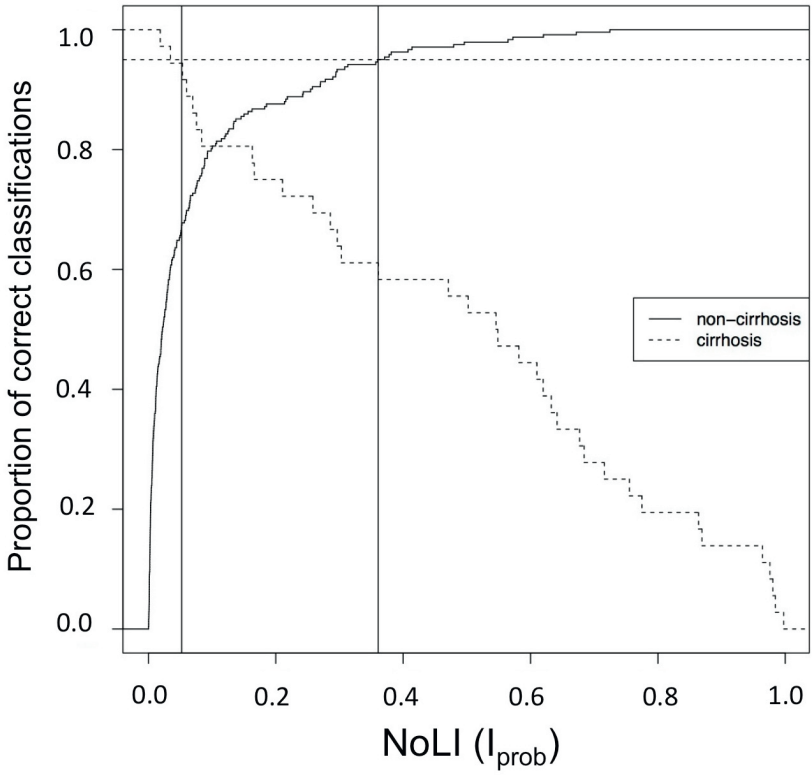

Figure 15. Proportion of correct classifications (i.e. non-cirrhotic classified as non-cirrhotic and cirrhotic classified as cirrhotic) according to different cut-off levels for NoLI. The solid vertical lines represent the chosen cut-offs 0.054 and 0.37. Using these cut-offs, the misclassification error would be approximately 5% for cirrhotic as well as non-cirrhotic patients, respectively (dotted horizontal line).

The sensitivity, specificity, likelihood ratios and negative and positive predictive values for the proposed cut-offs (0.053 and 0.37) are displayed in table 6. Among the 12 patients misclassified as having cirrhosis, the Ishak fibrosis stages were distributed as follows; F4 n=7 (58%), F3 n=3 (25%) and F2 n=2 (17%). HCV genotypes 2 and 3 were evenly distributed.

Table 6. Diagnostic accuracy for the proposed cut-off levels regarding the NoLI score for prediction of cirrhosis in 278 HCV-infected patients.

Cut-off	Sensitivity (%)	Specificity (%)	+LR	-LR	NPV (%)	PPV (%)
0.054	94(81-99)	68(62-74)	2.9(2.4-3.6)	0.08(0.02-0.32)	99(96-100)	30(22-40
0.37	58(41-75)	95(92-97)	10.9(6.0-19.7)	0.44(0.30-0.65)	94(90-97)	62(44-78

Abbreviations: +LR, positive likelihood ration; -LR, negative likelihood ratio; NPV, negative predictive value; PPV, positive predictive value. All values are presented with 95% confidence interval.

Prediction according to HCV genotype

Since the exploratory set included only patients infected with HCV genotypes 2 and 3 and the validation set mainly genotype 1 infected patients, we combined the exploratory and validation set to evaluate potential genotypic differences. No significant differences were detected and the AUROC for specificity vs. sensitivity for each genotype was 0.91 (95% CI 0.82-1.0), 0.86 (95% CI 0.73-0.98) and 0.93 (95% CI 0.88-0.97) for genotypes 1, 2, and 3 respectively.

Correlation with transient elastography

In the validation set, patients were also examined by transient elastography. The Spearman correlation coefficient for the new index and liver stiffness values was 0.54 ($p<0.001$; Figure 16). One patient (a 66 year-old genotype-1-infected male with compensated cirrhosis and mild steatosis) with a liver stiffness value of 50 kPa was included in the calculation but not in the figure. To evaluate the diagnostic capability of the new index using transient elastography as reference, we used a cut-off of 12.5 kPa for cirrhosis (118). The resulting ROC-curve had an AUROC for prediction of cirrhosis of 0.95 (95% CI 0.89-1.0).

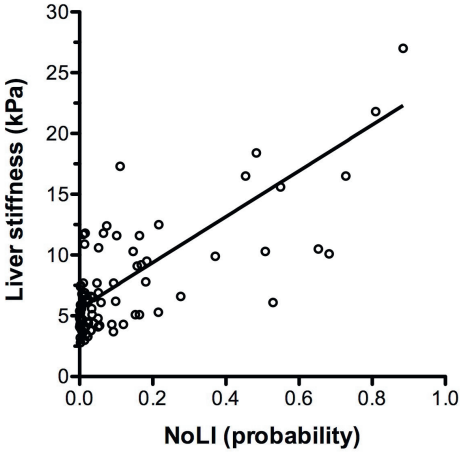

Figure 16. Scatter plot comparing the New index (NoLI) with liver stiffness measured by transient elastography. The Spearman correlation coefficient was 0.542 ($p<0.001$).

Exclusion of D7 lathosterol

BMI, age, prothrombin and platelet count are standard laboratory variables while lathosterol is not. Hence, we evaluated a simplified index including only BMI, age, prothrombin and platelet count using the same coefficients. Applying this simplified index, the AUROC for prediction of cirrhosis was 0.90 (95% CI 0.85-0.95) in the exploratory set and 0.86 (95% CI 0.75-0.98) in the validation set (figure 17 A). In order to optimize the index without lathosterol, the coefficients were recalculated which resulted in the following slightly different formula:

Log-odds; I_{odds} (predicting cirrhosis) = -12.29 + (age x 0.10) + (BMI (kg/m^2) x 0.16) + (Platelet count ($x10^9/L$) x (-0.018)) + (Prothrombin-INR x 4.55).

Predicted probability; I_{prob} = exp (log-odds)/ (1+exp (log-odds)).

The resulting AUROC for the modified formula for prediction of cirrhosis was 0.91 (95% CI 0.86-0.95) in the exploratory set and 0.88 (95% CI 0.79-0.98) in the validation set (figure 17 B).

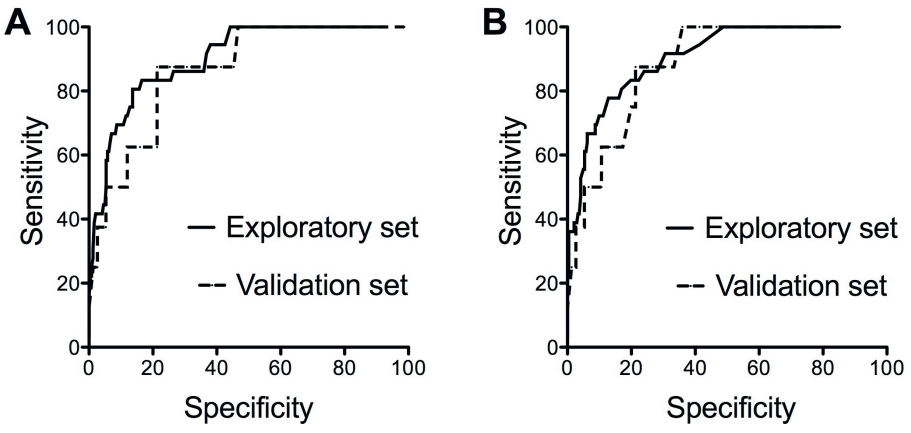

Figure 17. Receiver operator characteristics curves plotting sensitivity against specificity for prediction of cirrhosis in the exploratory and validation set respectively using the simplified index without lathosterol (A) and the index with modified formula (B). The corresponding AUROCS in were 0.90 (95% CI 0.85-0.95) in the exploratory set and 0.86 (95% CI 0.75-0.98) in the validation set (A) and 0.91 (95% CI 0.86-0.95) in the exploratory set and 0.88 (95% CI 0.79-0.98) in the validation set (B)

In paper III we aimed to create a cirrhosis-index based on serological markers. For the creation of the index, we investigated a range of potential fibrosis markers in order to find the combination of markers that would best predict liver cirrhosis in our study cohort. The result was the Nordic Liver Index, NoLI, which is an index based on age, BMI, platelet count and prothrombin index, i.e. factors that previously have been consistently associated with fibrosis (148, 153, 265-267), along with D7-lathosterol which, to our knowledge, has not previously been evaluated in this setting. With the development of liver cirrhosis, the synthetic function in the liver, including cholesterol synthesis, decreases (268). Thus, it is plausible that the concentration of D7-lathosterol in serum would reflect liver function and that decreased levels of lathosterol could be an early sign of advanced liver fibrosis. The sterols primarily reflecting cholestasis did not predict cirrhosis in this setting, which is not surprising since cholestasis is not a common feature in HCV-related cirrhosis.

The hepatitis C virus is known to interfere with host lipid metabolism resulting in reduced levels of s-cholesterol and hepatic steatosis (269). However, the evaluation of NoLI index according to HCV genotype in the

present study showed no statistically significant differences regarding cirrhosis prediction between genotypes and the level of serum lathosterol does not seem to be genotype-dependent in this setting.

Several studies on the prediction of cirrhosis have been reported during the past 10 years (145, 148-151, 153-155, 270, 271). In a large multicenter study by Degos *et al.* (127) evaluating the diagnostic accuracy of Fibroscan and FibroMeter, FibroTest, APRI and Hepascore for the prediction of cirrhosis, AUROC ranged from 0.77 to 0.86. FibroMeter (150), FibroTest (127) and Hepascore (149) are all validated and frequently used for the prediction of cirrhosis but they are all protected by patented formula making them less accessible. The AST-to-platelet ratio index (APRI) (148) as well as the Lok-index (153) and FIB-4 (155) are non-commercial, free-of-cost indexes based on routine biochemical markers making them easy to use in routine clinical practice. When compared in the exploratory set, our model performed slightly superior to these other non-patented scores. Unfortunately, the size of and cirrhosis prevalence in the validation set did not allow for in-depth comparison.

The prevalence of cirrhosis in the exploratory and validation sets was 13% and 10% respectively, which is low compared with some other studies on non-invasive fibrosis markers using liver biopsy as reference (153, 272). This lessens the ability to create a solid model for prediction. However, a cirrhosis prevalence of around 15% seems to be common in unselected HCV infected populations (38, 127). The limited sample size and the low prevalence of cirrhosis in the validation set is a major limitation and does not permit accurate comparison between different indices, and the diagnostic performance of the new index need to be tested also in other larger cohorts. Moreover, other studies of fibrosis markers have been validated in cohorts of similar size, *e.g.* APRI, a very reliable and widely used fibrosis index, was created in an exploratory set of 192 patients (15 (16%) cirrhotics) and validated in a cohort of only 78 subjects (13 (17%) cirrhotics)(148).

The patients in the exploratory and validation sets differed regarding HCV genotypes as well as country of residency, although none of these factors were independently associated with cirrhosis when patients in the two sets were analyzed together (cirrhosis was more common in Sweden and Denmark which was due to an age effect, data not shown). Thus they would not have influenced the statistical model. In the exploratory set, liver biopsies and serum samples were not taken at the same time point, although both were sampled prior to initiation of therapy. The index, however, performed well in the validation set where blood samples were drawn on the day of the liver

biopsy. Measurement of serum lathosterol is not a standard biochemical test. Although it is measured using a standard patent-free laboratory technique, commonly used for analysis of other compounds, it may be slightly cumbersome. Despite this, the presence of lathosterol improves the diagnostic performance of the NoLI index why we choose to retain it in the model.

As with all non-invasive tests, the use of liver biopsy as the "gold standard" comparison poses problems as liver biopsies are prone to sampling error and thus can both over- and underestimate the true liver fibrosis stage (107). As shown by Bedossa *et al.* only 65% of biopsies relying on 15-mm samples led to correct diagnosis using METAVIR scoring system (115). Hence, the absolute correctness of a non-invasive fibrosis marker would need to be estimated by use of other methods. Although not the primary aim of this study, it is interesting to note that the concordance of this index with Transient Elastography might be greater than with liver biopsy (AUC 0.95 (95% CI 0.89-1.0) vs. 0.90 (95% CI 0.83-0.98)). Another perhaps more appropriate endpoint would be the risk of liver related complications or death over time. A liver biopsy can provide much more pertinent information than serologic fibrosis markers on liver histology and may remain of importance in some cases in the future. However, we believe that serologic markers should be considered an important complement to liver biopsy and Transient Elastography.

4.4 Impact of *IL28B*-related single nucleotide polymorphisms on liver elastography in chronic hepatitis C infection (paper IV)

Baseline characteristics of patients with a valid liver stiffness measurement are displayed in table 1 of paper IV. The majority of patients were male (62%) and infected with HCV genotype 1 (69%). When comparing HCV genotype 1 and 3 infected patients, they differed significantly. The HCV genotype 1 patients were older (median age 53 vs. 47 years for genotype 1 and 3 respectively, P<0.0001), had a longer duration of infection (median 30 vs. 25 years for genotype 1 and 3 respectively, P<0,0001), had higher BMI (26 vs. 25 kg/m^2 for genotype 1 and 3 respectively, P=0.04), and were less likely to have been infected through intravenous drug use (50% vs. 64% genotype 1 and 3 respectively, P=0.03).

A strong association was noted between the distribution of HCV genotypes and *IL28B* SNP variants (P<0.0001; Figure 18), with CC at *rs12979860* being significantly more common in treatment-naïve patients with HCV genotype 2 or 3 infection than genotype 1. Treatment-experienced patients were excluded from this latter analysis in order to avoid potential bias resulting from differing treatment response.

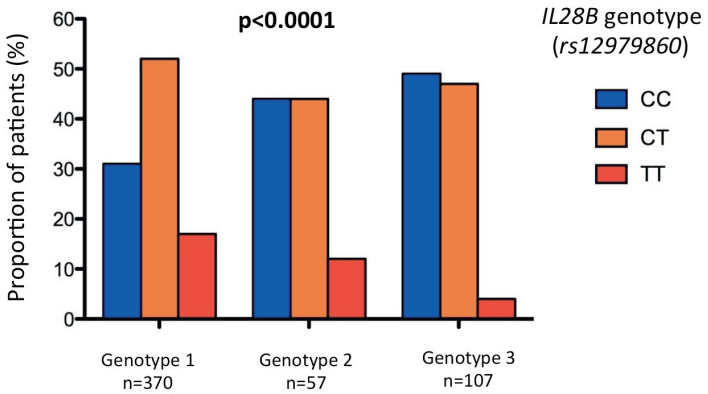

Figure 18. Frequency distribution of IL28B variants in relation to HCV genotypes 1-3 among treatment-naïve patients. Chi-squared (χ^2)-test was used to compare differences in distribution.

A valid liver stiffness measurement was obtained in 614 patients (83%). In general patients with invalid examinations were older than those with valid (median age 54 and 52 years, P=0.004), and had significantly higher body-mass index (BMI) (28 and 25 kg/m^2, P<0.0001). These findings were in line with a previous reported 5-year prospective study of 13,369 liver stiffness measurements, where unreliable results were noted in nearly one of five examinations, with obesity and old age being main causes (122).

Among HCV genotype 3 infected patients with CC at *rs12979860*, significantly higher liver stiffness values (median 8.2 vs. 6.4 kPa for CC and CT/TT respectively, P=0.004; Figure 19) as well as APRI (median 1.0 vs. 0.6 for CC and CT/TT respectively, P=0.02; Figure 20), were noted as compared to $T_{rs12979860}$ allele carriers. Conversely, among HCV genotype 1 infected patients with CC genotype, a non-significant trend towards lower liver stiffness values and APRI were noted. There were no significant differences in age, gender, BMI or duration of infection between CC and CT/TT carriers in neither genotype 1 nor genotype 3 infected patients (Table 7). These

results were unchanged when patients with unreliable liver stiffness measurements were excluded. No significant associations were observed among the 67 HCV genotype 2 infected patients, although a trend was noted towards slightly more pronounced liver pathology among CC carriers (median liver stiffness 9.2 vs. 7.0 kPa for CC and CT/TT respectively, P=0.13), (median APRI 0.8 vs. 0.5 for CC and CT/TT respectively, P=0.19). All of the abovementioned results remained unchanged if treatment-experienced patients were excluded from the analyses. No association was noted between alanine aminotransferase (ALT) and *IL28B* genetic variants.

The HCV genotype 1 infected homozygous CC carriers had significantly higher viral load (median 6.6 and 6.2 $\log_{10}$ IU/mL for CC and CT/TT respectively, P=0.001; Figure 21) with similar non-significant trend noted among HCV genotype 2 and 3 infected patients.

The following variables remained independently predictive in multivariate analysis of greater liver stiffness in HCV genotype 1 infected patients: older age (P<0.001), higher ALT (P<0.0001), and male gender (P=0.011). For HCV genotype 3 infected patients, older age (P<0.0001), higher ALT (P=0.001), CC at *rs12979860* (P=0.017), and male gender (P=0.029) were independently predictive of more pronounced liver stiffness. HCV genotype 2 infected patients could not be evaluated in multivariate analysis due to the small sample size.

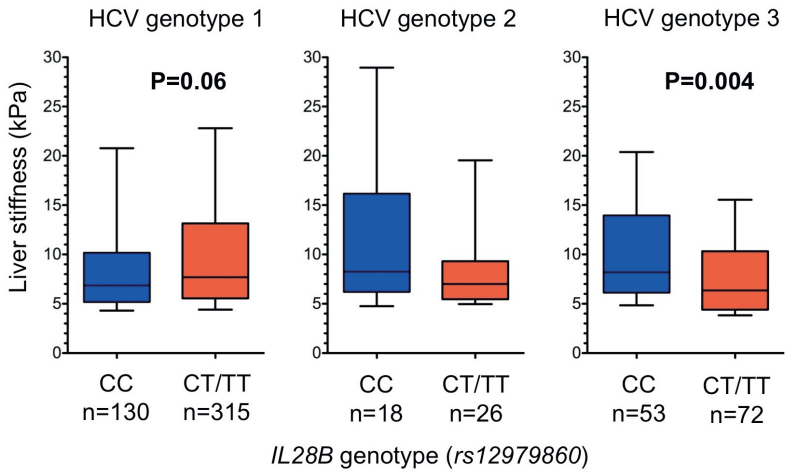

Figure 19. Tenth, 25th, 50th, 75th, and 90th percentiles of liver stiffness measurement level in relation to IL28B variants for genotypes 1, 2 and 3

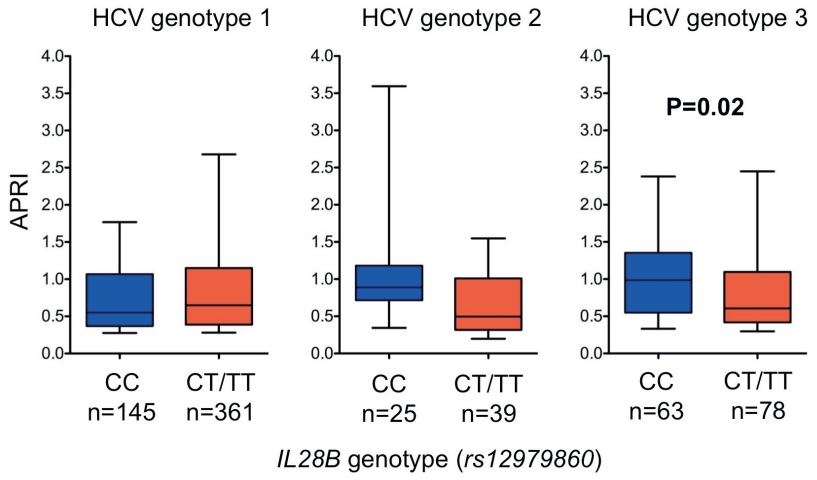

Figure 20. Tenth, 25th, 50th, 75th, and 90th percentiles of APRI score in relation to IL28B variants for genotypes 1, 2 and 3.

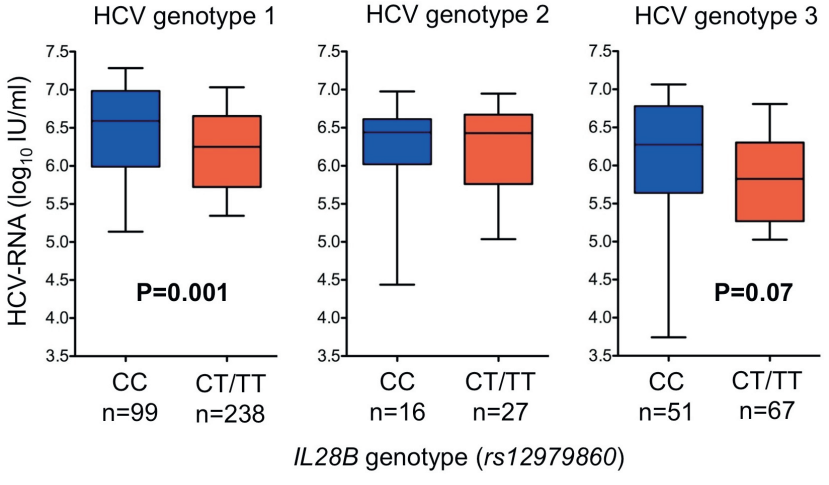

Figure 21. Tenth, 25th, 50th, 75th, and 90th percentiles of HCV RNA level in relation to IL28B

Table 2. Baseline characteristics according to HCV genotype and IL28 B genetic variations (*rs12979860*)

	Genotype 1			Genotype 3		
	CC n=151	CT/TT n=370		CC n=67	CT/TT n=82	
Age (years)[a]	52 (43-58)	53 (46-58)	n.s	48 (41-54)	47 (42-55)	n.s
Gender (male/female)[b]	96 / 55	240 / 130	n.s	38 / 29	45 / 37	n.s
BMI (kg/m^2)[c]	26 (23-28)	26 (24-29)	n.s	26 (23-27)	24 (23-26)	n.s
Duration of infection (years)[d]	30 (21-36)	31 (22-36)	n.s	26 (15-31)	24 (11-31)	n.s

Values are expressed as median (IQR)[a] or n[b]

[c] n=173 genotype 1, n=58 genotype 3
[d] n=373 genotype 1, n=109 genotype 3

In paper IV, we confirmed previous reports that $CC_{rs12979860}$ carriage is associated with more pronounced liver pathology in patients chronically infected with HCV genotype 3 as compared to genotype 1. Bochud *et al.* analyzed the association between *IL28B* polymorphisms and liver histology among 1527 chronically HCV genotype 1 and 2 infected Caucasian patients and noted that the *IL28B* $G_{rs8099917}$ allele, which has been associated with poor response to therapy, entailed less hepatic inflammation and fibrosis (55). When stratifying for HCV genotype the findings were statistically significant for genotype 3 only. Concordantly, Rembeck *et al.* found the *IL28B* $G_{rs8099917}$ allele (*i.e.* the unfavorable genotype for this SNP) to be significantly associated with milder fibrosis in genotype 3 but not genotype 2 infected patients with a similar trend observed for *IL28B* $T_{rs12979860.}$ Similarly, *IL28B* $T_{rs12979860}$ carriage in HCV genotype 3 infected patients was associated with less steatosis, whereas *IL28B* $G_{rs8099917}$ carriage in HCV genotype 2 was associated with less steatosis (57).

Liver pathology in the present study was evaluated by means of liver stiffness measurement rather than liver biopsy. Thus it was not possible to ascertain which histopathologic components contributed to the elevated measurements among HCV genotype 3 infected CC carriers, although the concomitantly elevated APRI suggests that more pronounced fibrosis weighed in. ALT reportedly confounds the use of transient elastography among HCV infected patients (273). However, in the present study ALT was not associated with

IL28B genetic variants, and in the multivariate analysis among HCV genotype 3 infected patients, both higher ALT and *IL28B* $CC_{rs12979860}$ carriage were independently predictive of the elevated liver stiffness measurement. Additionally, there are conflicting results as to the impact of steatosis on liver stiffness measurements (136), although influence of steatosis has been noted predominately among patients with high-grade steatosis (121, 137).

It is unclear how CC genotype carriage, in the context of HCV genotype 3 infection, may induce more pronounced liver pathology. Rembeck *et al.* previously suggested that the underlying mechanism of action might be secondary to higher baseline viral loads (55, 57, 274), although in the present study no significant association was noted between viral load and liver stiffness. Elevated HCV RNA levels in HCV genotype 3 infection have previously been reported to be associated with the increased presence and severity of steatosis (81, 275), which in turn entails accelerated fibrosis progression (99), suggestive of a cytopathic effect of HCV genotype 3 virus. How the HCV genotype 3 virus exerts this cytopathic effect remains to be determined, though previously it has been hypothesized that the greater propensity for development of steatosis, than observed for other HCV genotypes (81, 99), may be secondary to a greater impairment of lipid export from infected hepatocytes (85, 86) possibly mediated by inhibition of microsomal triglyceride transfer protein (MTP) (89, 93) or due to increased availability of free fatty acids by reduced oxidation or by increased *de novo* synthesis (94, 95, 276, 277) mediated by the HCV genotype 3 core protein.

Our finding that homozygous CC at *rs12979860* was significantly more common in the setting of treatment-naïve HCV genotype 2 or 3 infection than genotype 1 corroborates previous reports (197, 278). Indeed, the proportion of CC at *rs12979860* among HCV genotype 2 and 3 infected patients (40% and 45%, respectively) in our study is similar to the reported prevalence in HCV uninfected Caucasians (~40%), suggesting that this SNP genotype may be less beneficial following exposure to HCV genotype 2 or 3 as compared to genotype 1. This, and the finding that *IL28B* variability did not significantly impact on liver stiffness measurement among HCV genotype 1 and 2 infected patients, suggest that *IL28B* may differentially regulate the course of HCV infection across genotypes.

5 CONCLUSIONS

A look-back screening study identified 113 HCV-infected subjects previously unaware of their diagnosis, the majority of whom were eligible for therapeutic intervention. Additionally, a majority of identified subjects were women, often infected following transfusions during childbirth. Thus, screening for HCV among recipients of blood transfusions prior to 1992 is meaningful and should include women transfused during childbirth.

Histopathologic features, especially portal inflammation and stage of fibrosis in the donor liver may deleteriously affect graft and patient survival following HCV-related liver transplantation. Thus, pre-transplant evaluation of donor histopathology may be of value in the selection of donors for transplantation of HCV positive individuals, especially among donors older than 60 years.

HCV-related liver cirrhosis can be confidently predicted or excluded by use of an index combining well-known predictors of liver fibrosis with measurement of a non-cholesterol sterol. The index could be of value as a supplement to already existing methods and aid clinical decision-making, *e.g.* when deciding which patients require continued HCC surveillance monitoring in spite of successful antiviral treatment.

An association between CC carriage at *rs12979860* and more pronounced liver damage was detected among HCV genotype 3 infected patients. In this light, analysis of *IL28B* genotype may be beneficial among these patients so as to encourage homozygous CC carriers to initiate therapy. Additionally, our findings suggest that *IL28B* may differentially regulate the course of HCV infection across genotypes.

6 FUTURE PERSPECTIVES

The last years have seen a rapid development of new treatment options for HCV infection that will likely change the basis for Hepatitis C care. Several all-oral, interferon-free treatment options may become available in the not too distant future. Those regimens are expected to be more effective, with fewer side-effects and shorter treatment durations and will likely be effective even in more advanced stages of fibrosis. Although this will lessen the need for fibrosis staging for therapeutic decision-making and prognostication, fibrosis assessment will remain central for tailoring the duration and choice of therapy. Additionally, the diagnosis of cirrhosis will continue to be vital to establish the need for evaluation of portal hypertension and for HCC surveillance, irrespective of therapeutic outcome. Accordingly, confident exclusion of cirrhosis will be the most important characteristic in future fibrosis staging. Thus, the use of liver biopsy will likely be reduced on this indication in favor of non-invasive methods. Algorithms combining different non-invasive methods for prediction of fibrosis have been proposed. A consensus recommendation on how to use these methods in clinical practice is much needed. Additionally, a simultaneous validation and an international standard for interpretation and comparison of the different liver stiffness measurement options available today, would be of use for both clinicians and researchers.

HCV positive liver transplant recipients will likely benefit from improved antiviral treatment as well. Still, the quality of the donor liver seems to be of importance for clinical outcome including survival, possibly even more so in older donors. A systematic evaluation of donor livers, possibly including histopathologic evaluation, could be important in order to assess which liver grafts may be safely used in HCV positive recipients.

Careful evaluation of host genetics has revealed that nucleotide polymorphisms in close proximity of the IL28B gene affect spontaneous clearance of HCV infection, therapeutic outcome of interferon-based treatment as well as the natural course of HCV infection. The exact mechanism behind this effect, however, remains unknown. This warrants further investigation.

Application of a well tolerated and effective all-oral treatment for HCV infection raises for the first time the possibility of actually eliminating Hepatitis C disease and stop transmission. In light of this, screening of populations at risk of disease, e.g. recipients of blood transfusion prior to

1992 and former as well as present intravenous drug users, becomes even more important. Finally, since developing countries carry a large proportion of the Hepatitis C burden, the pricing and availability of new treatment options will largely determine if eradication will be a reality.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to everyone who has contributed to this work in some way and especially to:

Johan Westin, my supervisor, for guiding me through my PhD studies in an inspiring manner, always generous with your time, knowledge, constructive feed-back, encouragement and support, helping me to focus and see past the obstacles.

Martin Lagging, my co-supervisor, for your broad competence and your willingness to share it; always coming up with new research ideas. For your encouragement and for setting the good example of focusing on one thing at a time.

Lars Hagberg, Professor of the Department of Infectious Diseases, for providing a generous environment for research and education.

Rune Wejstål, head of the Department of Infectious Diseases, all my colleagues, friends and highly valued roommates. I really enjoy working with you!

Lars-Magnus Andersson, my clinical supervisor for your sound advice.

Anders Eilard, my hepatitis companion, Gunnar Norkrans, for everything you taught me about hepatitis, management of study patients and valuable comments on my work and Magnus Lindh for creating the IL28B assay.

All the “hepatitis-nurses” at the Department of Infectious Diseases. Lena Johansson, Mats Ahlqvist, Pia Paghold, for performing transient elastography measurements, and Helen Christiansen, Anna Rosnäs and Eva Karlsson for expert technical assistance.

Thomas Bergström, for creating a stimulating research environment at the Department of Clinical Virology.

Sigvard Olofsson, Bo Svennerholm, Jan-Åke Liljeqvist, Staffan Görander, Kristoffer Hellstrand, Peter Horal, Helen Norder and other collegues at the Virology section for pleasant lunches and interesting discussions.

Colleagues, PhD students, researchers and other friends at the third floor of the Virology section for creating such a friendly and stimulating atmosphere.

The entire staff at the Hepatitis Unit and the Unit of viral quantification for all your help with viral analysis.

All the patients who participated in this project.

All my co-authors for providing patients and for fruitful discussions and improvement of manuscripts.

Agneta Håkansson at the Virology section, Ann Söderström, Britta Wåhlander and everyone else at the Department of Communicable Disease control involved in the Hepatitis C screening project.

Styrbjörn Friman and Maria Castedal for your good-humored generosity and for sharing your knowledge in liver transplantations.

Christina Wibeck and Ingela Broman at the Transplant Institute, Sahlgrenska University Hospital for your all your assistance.

Martti Färkillä, at Helsinki University Hospital, for sharing your knowledge on lipids and cholesterol metabolism.

Olle Nerman and Vera Lisovskaja for excellent statistical modeling and Vera for answering my questions on statistics over and over again.

Ann-Sofi Tylö and Leena Kaipiainen for expert technical assistance.

Inger Gyllensten, Ann Ljungblom and Gaby Halbok, university secretaries, and Lotta Wikström, Yvonne Ballard, Kristina Weilöv and all the administrative staff at the Department of Infectious Diseases for much needed practical help and administrative assistance.

Åsa Alsiö and Karolina Rembeck, my fellow PhD students, for friendship and support.

All my friends, especially Karin and Magdalena, for your continuous encouragement.

My parents, Elisabet and Sven-Olof, for your love and encouragement, and for all your support and help while I´ve been working on this thesis.

Johanna, Andreas and Tobias with families, the best sister and brothers there are, Johan & Fia and Kalle & Anna for just being there, helping us out.

Martin, for your love and support and for struggling through life with me, and to our wonderful children Alfred, Kajsa and Ellen for bringing lots of joy, inspiration and constant challenges into our life, reminding me of what is really important. I love you.

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