Impact of Host Genetic Variants on Natural History and Treatment of Hepatitis C Virus Infection

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Abstract

Chronic hepatitis C Virus (HCV) infection causes liver disease and may progress to severe fibrosis, cirrhosis, and hepatocellular carcinoma. This thesis aimed to evaluate the impact of host genetics, i.e. genetic variants of *PNPLA3*, *IL28B* and *ITPA*, on liver disease severity and treatment outcome in HCV genotype 2 and 3 infected patients treated with pegylated interferon and ribavirin for either 12 or 24 weeks.

In paper I, 359 patients were evaluated retrospectively with regards to the impact of the *PNPLA3* genetic variants. No significant impact was observed on liver disease severity nor on treatment outcome, and the clinical need to screen Nordic HCV genotype 2 or 3 infected patients for these genetic variants seems low.

In papers II and III, in post-hoc evaluation encompassing 339 Nordic HCV genotype 2 or 3 infected patients, genetic variants of the *rs12979860* in proximity to *IL28B* were not associated with treatment outcome but the $CC_{rs12979860}$ and the $TT_{rs8099917}$ genetic variants (n=314) were found to be associated with more pronounced liver histopathology among HCV genotype 3 infected patients. Thus, these patients may benefit from early initiation of therapy.

In paper IV, in a real life trial (n=737) enrolling HCV genotype 1-3 infected patients evaluated by means of transient elastography, $CC_{rs12979860}$ was significantly associated with higher liver stiffness values among HCV genotype 3 infected patients; thus confirming the results of papers II and III in an independent cohort of patients.

In paper V, in a post-hoc analysis of Nordic HCV genotype 2 or 3 infected patients treated with 800 mg ribavirin daily and interferon reduced ITPase (n=354) activity was significantly associated with increased likelihood of achieving sustained virological response. Thus the majority of patients having normal ITPase activity may benefit more from a higher weight-based dosing of ribavirin.

SAMMANFATTNING PÅ SVENSKA

Kronisk hepatit C-virus (HCV)-infektion är associerad med progredierande leverskada som kan utvecklas till skrumplever (cirrhos), leversvikt eller primär levercancer. Hur fort leversjukdomen framskrider varierar kraftigt mellan individer. Tidigare studier visar att en tredjedel utvecklar skrumplever inom ca 20 års tid, en tredjedel under ca 50 års tid medan den sista tredjedelen löper liten risk att drabbas av leverskada under sin livstid. Värdfaktorer såsom manligt kön, konsumtion av stora mängder alkohol samt virala faktorer såsom infektion med hepatit C genotyp 3 och samtidig infektion med hepatit B har tidigare visat sig vara associerat med progressiv leversjukdom. Framgångsrik behandling av sjukdomen eradikerar hepatit C virus och kan i de flesta fall stoppa vidare utveckling av leverskada och/eller göra att en del av befintlig skada går tillbaka. Syftet med denna avhandling är att utvärdera hur genetisk variation av värdfaktorerna Patatin- like phospholipase domain containing protein 3 (*PNPLA3*), Interleukin 28B (*IL28B*) samt Inosine triphosphate pyrophosphatase (*ITPA*) påverkar naturalförlopp samt behandlingssvar hos nordiska HCV genotyp 2 och 3 infekterade patienter.

I södra Europa har *PNPLA3* 148M (en genetisk variant av genen som kodar för PNPLA3) visat sig vara associerad med mera fettinlagring i levern (steatos), fibros och cirrhos hos HCV infekterade patienter. I **delarbete I** utvärderade vi om *PNPLA3* 148M varianten var associerad med mera steatos, fibros eller cirrhos hos nordiska HCV genotyp 2 eller 3 infekterade patienter. Vi kunde inte påvisa någon association med ökad grad av steatos eller cirrhos eller påvisa någon påverkan på behandlingsutfallet efter behandling med pegylerat interferon och ribavirin.

I **delarbete II och III** visar vi genom att undersöka blodprover och leverbiopsier att CC varianten av *rs12979860* (i förhållande till CT eller TT) och TT varianten av *rs8099917* (i förhållande till TG eller GG) i närheten av *IL28B* genen var associerade med mer leverpåverkan hos HCV genotyp 3-, men inte HCV genotyp 2-infekterade patienter. Detta är viktigt då patienter infekterade med HCV genotyp 3 med $CC_{rs12979860}$ därför kanske bör rekommenderas behandling i ett tidigt skede.

I **delarbete IV** bekräftas resultaten från delarbete II och III att HCV genotyp 3-infekterade patienter med CC varianten av *rs12979860* (i förhållande till CT och TT) har högre leverelasticitetsvärden (undersökt med Fibroscan) samt högre APRI score (ett biokemiskt index som kan identifiera patienter med levercirrhos), fynd som kan sammanfattas som mera allvarlig leverpåverkan.

I **delarbete V** visar vi att reducerad Inosine triphosphate pyrophosphatase (ITPAse) aktivitet är associerat med bättre behandlingsresultat efter kombinationsbehandling med pegylerat interferon och 800 mg daglig dos av ribavirin hos HCV genotyp 2- eller 3-infekterade patienter. I likhet med tidigare studier visar vi också att reducerad ITPase ativitet är associerad med skydd mot ribavirininducerad blodbrist i behandlingsvecka 4.

LIST OF PAPERS

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

I. Rembeck K, Maglio C, Lagging M, Christensen PB, Färkkilä M, Langeland N, Buhl MR, Pedersen C, Mørch K, Norkrans G, Hellstrand K, Lindh M, Pirazzi C, Burza MA, Romeo S, Westin J. *PNPLA 3* I148M genetic variant associates with insulin resistance and baseline viral load in HCV genotype 2 but not in genotype 3 infection. BMC Medical Genetics. 2012,13:82.

II. Rembeck K, Alsiö Å, Christensen PB, Färkkilä M, Langeland N, Buhl MR, Pedersen C, Mørch K, Westin J, Lindh M, Hellstrand K, Norkrans G, Lagging M. Impact of *IL28B*-Related Single Nucleotide Polymorphisms on Liver Histopathology in Chronic Hepatitis C Genotype 2 and 3. PLoS One. 2012; 7(1):e29370.

III. Rembeck K, Westin J, Lindh M, Hellstrand K, Norkrans G, Lagging M. Association Between Interleukin-28B-Related Genetic Variants and Liver Histopathology Differs Between Hepatitis C Virus Genotypes. Hepatology. 2012; 56(1):394.

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 *IL28*-Related Single Nucleotide Polymorphisms on Liver Transient Elastography in Chronic Hepatitis C Infection. PLoS One. 2013; 8(11):e80172.

V. Rembeck K, Waldenström J, Hellstrand K, Nilsson S, Nyström K, Martner A, Lindh M, Norkrans G, Westin J, Pedersen C, Färkkilä M, Langeland N, Buhl MR, Mørch K, Christensen PB, Lagging M. Variants of the Inosine Triphosphate Pyrophosphatase Gene Are Associated With Reduced Relapse Risk Following Treatment for HCV Genotype 2/3. Hepatology. 2014; 59(6):2131-9

CONTENT

ABBREVIATIONS

Anti-HCV	Antibodies against HCV
ALT	Alanine aminotransferase
APRI	AST to platelet ration index
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BMI	Body mass index
DAA	Direct acting antiviral
dITP	Deoxyinosine triphosphate
GTP	Guanosine triphosphate
GUCI	Gothenburg university cirrhosis index
GWAS	Genome wide association study
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HOMA-IR	The homeostatic model assessment-insulin resistance
IFN-α	Interferon-α

IP-10	Plasma interferon-gamma-inducible protein
ISG	Interferon stimulated gene
IL28A	Interleukin 28A
IL28B	Interleukin 28B
IMP	Inosine monophosphate
IMPDH	Inosine monophosphate dehydrogenase
ISG	Interferon stimulating gene
ITP	Inosine triphosphate
ITPA	Inosine triphosphatase
ITPase	Inosine triphosphate pyrophosphatase
ITT	Intention-to-treat
JAK-STAT	Janus kinase- signal transducer and activator of transcription
MTP	Microsomal triglyceride transport protein
NAFLD	Non alcoholic liver disease
NASH	Non-Alcoholic Steato-Hepatitis
NS	Non structural protein
OR	Odds ratio
PCR	Polymerase chain reaction
PegIFN-α	Pegylated interferon- α
PNPLA3	Patatin-like phospholipase domain-containing 3

PP   Per-protocol

RT-PCR   Reverse transcription polymerase chain reaction

RNA   Ribonucleic acid

 RTP   Ribavirin triphosphate

RVR   Rapid virological response

SNP   Single nucleotide polymorphism

SVR   Sustained virological response

TE   Transient elastography

ULN   Upper limit of normal

VRVR   Very rapid virological response

XTP   Xanthosine triphosphate

1 INTRODUCTION

1.1 The Hepatitis C Virus

The hepatitis C virus (HCV) was cloned in 1989, following an intensive search for the major etiologic agent associated with non-A, non-B hepatitis (1). The HCV virus is a positive stranded RNA virus and belongs to the family of flaviviridae, classified in the genus hepacivirus (2). The virus replicates in the hepatocytes, and possibly also in B-lymphocytes, with an approximate production and clearance rate of 10^{12} virions per day and a virion half-life of approximately 2.7 hours (3, 4). The HCV viral genome consists of a 9,600 nucleotides, single open reading frame, shielded by a nucleocapside and a spherical envelope (5-7). Two highly conserved untranslated regions, the 5′ and 3′ regions, essential for translation and genome replication, flank the genome coding for HCV polyprotein. The polyprotein is cleaved by host and viral proteases into structural (the core, E1 and E2 envelope proteins, and p7 ion channel) and non-structural proteins (NS2 (transmembrane protease), NS3 (serine protease), NS4A (cofactor of NS3), NS4B (hydrophobic integral membrane protein), NS5A (hydrophilic phosphoprotein), and NS5B (RNA dependent RNA polymerase)) (8). Within each infected individual, HCV exists as multiple "quasispecies" (9, 10), resulting from the error-prone NS5B HCV RNA polymerase, the high viral replication rate, as well as host immune selective pressure (11). Recombination between HCV strains of different HCV genotypes are rare events (12). Through evolution, 6 major genotypes have arisen with differing global geographic distributions (fig 1) (13, 14). A sequence coding for a seventh genotype has been reported, but thus far only from one single isolate (15).

1.2 Epidemiology

According to the World Health Organization, approximately 170 million people are infected with hepatitis C worldwide (16), corresponding to 3% of all people. However, a recent update possibly indicates a lower global prevalence of 80 (range 64-103) million infected people, i.e. 1.6% (range 1.3-2.1%). The wide span of insecurity is secondary to poor estimates of HCV prevalence for large parts of the world, e.g. Nigeria, China, Pakistan, Ethiopia, Russia, Egypt and Congo. A probable decrease in HCV prevalence has been suggested due to increased mortality of a rapidly ageing HCV

infected population, paired with a decreased incidence resulting from improved implementation of blood supply screening and reduction in high risk behavior (17).
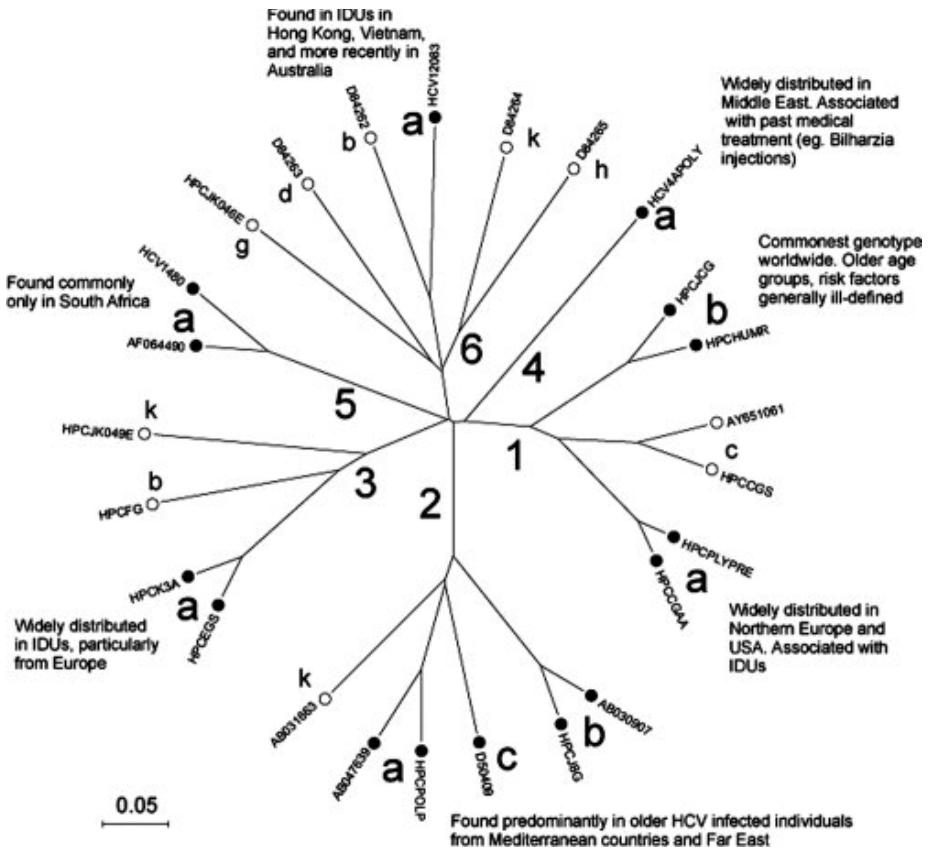

Figure 1. Evolutionary tree of available complete open-reading frame sequences for each HCV genotype, Simmonds et al, Hepatology 2005 (13), reprinted with permission.

In Sweden approximately 2000 new HCV cases are reported annually and the estimated HCV prevalence corresponds to 0.5% of the general population (18, 19). HCV genotype 1 is the most common genotype globally, with a prevalence of 83.4 million (46% of HCV strains genotyped), with more than 30% of infected individuals living in East Asia. HCV genotype 3 accounts for 54.3 million infected people (30%), with more than 75% living in South Asia. The third most common is HCV genotype 2 with an estimated 16.5 million cases (9%), closely followed by HCV genotypes 4 and 6 with an estimated prevalence of 15 million (8%) and 9.8 (5%) respectively. HCV genotype 2 and 6 are most common in East Asia, whereas HCV genotype 4 is most commonly found in the Middle East and North Africa (19). The 7[Th] genotype thus far identified has only been reported in one patient, originating from the Congo (15).

1.3 Natural History of HCV Infection

The course of the hepatitis C virus infection can vary extensively (fig 2). Acute HCV infection, defined as viral replication during the first 6 months after acquisition, may resolve spontaneously in 15% to 45% of cases (20, 21). HCV RNA is generally detectable within 3 weeks after exposure (22, 23). Throughout the first 6 months and up to a year after exposure, HCV RNA levels fluctuate significantly, and at times may temporarily, or persistently, become undetectable (20, 23). Alanine aminotransferase (ALT) levels commonly rise as HCV RNA becomes measurable, and antibodies against HCV (anti-HCV) become detectable within 4 to 13 weeks following exposure, but are not universally evident after spontaneous clearance (23, 24). The majority of acute infections are non-symptomatic, making this state rather difficult to evaluate as patients seldom seek care. However, if symptomatic, symptoms such as jaundice and nausea often arise within 7 weeks (range of 3 to 12 weeks) after exposure. Symptomatic infection favors spontaneous HCV resolution (20, 25). Other favorable prognostic factors for spontaneous resolution include female gender, plasma interferon-gamma-inducible protein 10 (IP-10, also known as CXCL10) below 380 pg/ml, HCV genotype 1, and *IL28B* CC genotype (26, 27). The impact of female gender on spontaneous clearance was studied extensively in two cohorts of young woman receiving HCV contaminated anti-D immunoglobulin, where 45% of the exposed women had detectable anti-HCV, but undetectable HCV RNA (21, 28). Fulminant hepatitis after acute HCV infection is fortunately very rare (29).

The majority of HCV infections do not spontaneously resolve, but rather
evolve into chronic infections, characterized by a low-grade, smoldering
inflammatory process, if successful therapeutic intervention is not initiated.
Spontaneous clearance of chronic infection is very rare, exemplified by the
report of undectable HCV RNA approximately 3 years after follow up in only
6 of 310 chronically HCV infected Japanese patients. Unfortunately, all 6
patients died from end-stage liver disease shortly after the event (30). The
rate of progression of liver fibrosis in the setting of chronic HCV infection is
outmost variable, with approximate one third progressing to cirrhosis within
20 years, one third progressing to cirrhosis between 20 and 50 years after
acquisition, and the last third probably never progresses to cirrhosis (31).
Among cirrhotic patients, there is an annual risk of approximate 4% of
developing hepatocellular carcinoma (HCC) (32, 33). In the absence of
decompensated cirrhosis, fatigue is the most frequently reported symptom
(34). Individual predictions of fibrosis progression are difficult as a range of
host genetic, environmental, and viral factors have considerable impact.

Studies of patients having undergone a liver biopsy established that male
gender, alcohol consumption in excess of 50 g/day, and age at infection
above 40 years are factors associated with accelerated fibrosis progression
(31). A subsequent study of paired liver biopsies has confirmed extensive
alcohol consumption as well as higher age at the first biopsy as unfavorable
predictors of fibrosis progression (35). Additionally in the latter study,
fibrosis progression increased as the time interval between the two liver
biopsies increased, and if interface hepatitis was present in both liver
biopsies. Female gender is a beneficial prognostic factor leading to slower
progression of liver disease as exemplified by the cohort of young woman in
Germany receiving anti-D immunoglobulin, where only 4% of those
developing chronic hepatitis C infection had progressed to cirrhosis after 20
years (28). Other prognostic factors that predict enhanced fibrosis
progression include HIV and HBV co-infection, steatosis, as well as insulin
resistance (36-39). Although HCV genotype previously was not considered to
be associated with fibrosis progression (31), recent studies have reported that
HCV genotype 3 appears to be associated with more severe liver disease as
well as increased morbidity and mortality (40, 41).

Figure 2. The natural course of the hepatitis C virus associated liver disease.
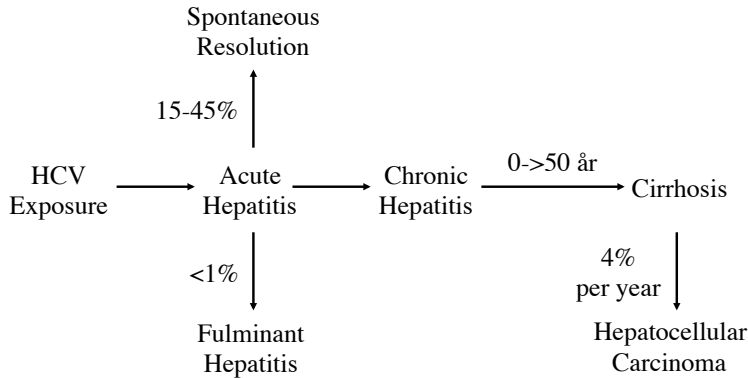
1.4 Histological Changes Throughout The Natural Course of Chronic HCV Infection

Initiation of HCV infection in hepatocytes is accompanied by histological changes in the liver. Debut of infection is associated with infiltration of inflammatory cells around portal areas as well as in the liver parenchyma. Interface hepatitis, also known as piece-meal necrosis, occurs when inflammatory cells extend beyond the margins of the portal tracts into the adjacent limiting plate of liver cells. Erosion of the limiting plate makes the margin of the portal tract irregular with an accompanying loss of periportal hepatocytes. In the liver acini, primarily in zone III, hepatocyte ballooning, apoptosis and lytic necrosis commonly are observed. The lytic necrosis of hepatocytes is often focal, but may confluence, thus creating bridging necrosis that interconnects the portal rooms and the central veins. Over time, the active hepatitis inflammatory process may be followed by fibrosis development with deposition of collagen tissue, primarily caused by activated hepatic stellate cells and portal myofibroblasts. If this hepatic fibrogenic process continues, hyperplasia/expansion of the remaining liver parenchyma may occur, compressing the fibrotic tissue concentrically around the

parenchyma, creating regenerative nodules, which characterize cirrhosis (42, 43). These histological changes leading to cirrhosis can be evaluated in biopsies from the liver by various scoring systems that grade the severity of necroinflammation and stage the extent of fibrosis (44). The Ishak scoring system (table 1) and Metavir are two examples of such pseudonumeric scoring systems, commonly used in the setting of HCV infection (45-47).

1.5 Assessment of Liver Fibrosis

The liver biopsy remains the gold standard for evaluation of the grade of necroinflammation and steatosis as well as the stage of fibrosis (43, 48), in spite the risk of sampling error, potential side effects, as well as the subjective categorical nature of its evaluation using pseudonumeric scoring systems.

Transient elastography (TE,) is a novel non-invasive method to assess liver fibrosis (49) by measuring liver stiffness. An ultrasound transducer is set in the intercostal space at the level of the right lobe of the liver and emits an elastic shear wave that propagates through the underlying liver tissue. The faster the shear wave propagates, the stiffer is the liver tissue. The Fibroscan method is rapid and reproducibly yields objective, continuous pressure measurements in the SI unit Pascal (Pa). A valid TE measurement requires an interquartile range of maximum 30% of the median value (the variability of validated measures) and a success rate of at least 60% (the ratio of successful measurements to the total number of attainments). According to the study by Castera et al., the clinical cut-off values for mild or absent fibrosis is <7.0 kPa (Metavir F0-1), 7.0 to 9.5 kPa for significant fibrosis (Metavir F2), 9.5 to 12.5 kPa for severe fibrosis (Metavir F3) and >12.5 kPa for cirrhosis (Metavir F4) (50). However, it is important to bear in mind that several factors may influence TE measurements. Steatosis may have an impact, although contradictive results have been reported (51, 52). Other factors including liver inflammation as indicated by ALT flares may increase the liver stiffness values 1.3 to 3-fold. Also, food intake within 3 hours before the measurement may result in transiently increased liver stiffness values (52, 53).

Other non-invasive models for predicting significant fibrosis and cirrhosis utilize biomarkers, such as the Fibrotest, AST-to-Platelet Ratio Index (APRI) and Gothenburg University Cirrhosis Index (GUCI) (54-56). Although they

are relatively good at identifying cirrhosis, the disadvantage of these biomarkers of liver damage is that they generally perform less optimal regarding differentiation of the intermediate stages of fibrosis. Thus, a combination of TE and biomarkers may be the best, validated non-invasive method of assessing the severity of liver fibrosis (57).

Table 1. Activity grade and fibrosis stage according to the Ishak score. Ishak. K et al. Journal of Hepatology 2005 (45), reprinted with permission.

Modified HAI grading: necroinflammatory scores

	Score
A. Periportal or periseptal interface hepatitis (piecemeal necrosis)	
Absent	0
Mild (focal, few portal areas)	1
Mild/moderate (focal, most portal areas)	2
Moderate (continuous around <50% of tracts or septa)	3
Severe (continuous around >50% of tracts or septa)	4
B. Confluent necrosis	
Absent	0
Focal confluent necrosis	1
Zone 3 necrosis in some areas	2
Zone 3 necrosis in most areas	3
Zone 3 necrosis+occasional portal-central (P-C) bridging	4
Zone 3 necrosis+multiple P-C bridging	5
Panacinar or multiacinar necrosis	6
C. Focal (spotty) lytic necrosis, apoptosis and focal inflammation*	
Absent	0
One focus or less per 10×objective	1
Two to four foci per 10×objective	2
Five to ten foci per 10×objective	3
More than ten foci per 10×objective	4
D. Portal inflammation	
None	0
Mild, some or all portal areas	1
Moderate, some or all portal areas	2
Moderate/marked, all portal areas	3
Marked, all portal areas	4

Modified staging: architectural changes, fibrosis and cirrhosis

Change	Score
No fibrosis	0
Fibrous expansion of some portal areas, with or without short fibrous septa	1
Fibrous expansion of most portal areas, with or without short fibrous septa	2
Fibrous expansion of most portal areas with occasional portal to portal (P-P) bridging	3
Fibrous expansion of portal areas with marked bridging (portal to portal (P-P) as well as portal to central (P-C))	4
Marked bridging (P-P and/or P-C) with occasional nodules (incomplete cirrhosis)	5
Cirrhosis, probable or definite	6

1.6 Liver Steatosis

Hepatic steatosis is caused by the accumulation of triglycerides in the form of lipid droplets in the cytoplasm of hepatocytes. Steatosis, defined as the presence of lipid droplets in >5% of hepatocytes (58), is surprisingly common in the general population, with a prevalence of 16 to 31%, with increased frequency observed among obese individuals as well as heavy drinkers (59, 60). The gold standard for assessment of liver steatosis is a liver biopsy, but magnetic resonance imaging, ultrasound and computed tomography can also be used (61, 62). The degree of hepatic steatosis may be influenced by several factors, including insulin resistance, excessive alcohol intake, or viral infections such as HCV (42, 63, 64).

Non-Alcoholic Liver Disease (NAFLD) is the common appellation of liver steatosis if alcohol, viral hepatitis and other serious liver diseases have been excluded. NAFLD is strongly associated with insulin resistance and the metabolic syndrome (63, 65), but mild NAFLD (without fibrosis or inflammation) is associated with a relatively favorable prognosis (66). However, NAFLD-associated steatosis may progress into Non-Alcoholic Steato-Hepatitis (NASH), which is defined by the presence of hepatocyte ballooning, lobular inflammation, and fibrosis (62). Among patients with NASH, approximately one-third progress with regards to fibrosis (67, 68), and NASH induced cirrhosis subsequently can result in liver associated morbidity and mortality including hepatocellular carcinoma (69, 70).

The prevalence of steatosis in HCV patients exceeds that in the general population, ranging from 41 to 65% (59, 60, 64, 71-73). Steatosis is more pronounced in HCV genotype 3 as compared with genotype non-3, with prevalence rates of 61 to 91% (64, 71-73). The mechanism of steatosis differs between HCV genotype 3 and non-3, where HCV genotype 3 induced steatosis appears to be strongly associated with the plasma viral load (64, 71, 72), and diminishes significantly if sustained virological response (SVR) is achieved following successful therapy (71, 72). In contrast, steatosis in HCV genotype non-3 is commonly associated with higher body mass index (BMI) and/or alcohol consumption (38, 64). The HCV genotype 3-induced steatosis has been hypothesized to be mediated by a direct inhibitive effect of the microsomal triglyceride transport protein (MTP). The MTP protein assemblies very low density lipoprotein (apoB in addition to triglycerides) and reduced MTP activity, as observed in HCV genotype 3 infected hepatocytes results in reduced lipoprotein secretion and a subsequent increase of intrahepatic triglycerides (74). An alternative suggested mechanism of action is up-regulation of fatty acid synthase promoter by hepatitis C

genotype 3 virus core protein, leading to intrahepatic triglyceride
accumulation by means of increased de novo synthesis (75, 76).

In terms of the natural course of the liver disease, HCV genotype 3 induced
steatosis has been associated with more advanced fibrosis stage as well as
accelerated rates of fibrosis progression (64, 77). Similarly, severe steatosis
in the presence of HCV genotype 1 infection may also be associated with
more pronounced fibrosis (38, 71).

Steatosis also impacts the likelihood of achieving SVR following antiviral
therapy with peg-interferon and ribavirin among patients infected with HCV
genotype non-3, but surprisingly not for patients infected with HCV genotype
3 (71, 72, 78), possible confounded by the underlying metabolic syndrome
associated with steatosis in HCV genotype non-3.

1.7 Treatment

HCV treatment has improved considerably throughout the past few years following the introduction of direct acting antivirals (DAA), allowing for currently available interferon-free treatment options, with or without the addition of ribavirin. Initially, interferon-α (IFN-α) mono-therapy was introduced for treatment of non-A non-B hepatitis, prior to the identification of the hepatitis C virus. In the first reported clinical trial, IFN-α treatment was suggested to control disease activity (79). During the 1990s ribavirin was added to this therapy and SVR rates were markedly improved secondary to reduced relapse rates (80, 81). Outcome of HCV treatment was further improved by chemically adding polyethylene glycol (PEG) to IFN-α, which prolonged the half-life and therapeutic activity (82, 83). For more than two decades, this combination of pegIFN-α and ribavirin was considered the standard-of-care for treating HCV. This regimen had SVR rates of approximately 80% in HCV genotype 2/3 infected patients, and 40-50% in HCV genotype 1 infected patients. For HCV genotype 2/3 infected patients, 800 mg ribavirin fixed daily dosing resulted in similar SVR rates as weight base dosing (1000 mg if <75 kg and 1200 mg if >75 kg) (84), and thus often was used. For patients younger than 40 years with HCV genotype 2 or 3 infection, and with HCV-RNA undetectable at treatment week 4 (i.e. rapid virological response (RVR)) as well as patients older than 40 years with HCV RNA below 1000 IU/mL at day 7 (very rapid virological response (VRVR)), pegIFN-α and ribavirin combination therapy could be reduced to as little as 12 weeks duration, thus sparing considerable side effects and cost (85). Favorable prognostic factors for treatment outcome included baseline HCV RNA below 600,000 IU/ml, female gender, age <40 years, lower body weight, as well as absence of insulin resistance and of bridging fibrosis or cirrhosis (83, 84, 86, 87). Beneficial baseline factors for treatment of HCV genotype 1 infected patients included genetic favorable variants of the *IL28B* as well as IP-10 levels below 150 pg/ml (88, 89). On-treatment factors such as greater first phase decline (decline of HCV RNA during the first days of treatment) and achieving RVR also favored SVR (85, 90-92).

Interferon has potent antiviral activity against HCV and acts indirectly through the stimulation of interferon stimulated genes (ISGs) (reviewed in (93)). A more detailed explanation hereof is provided below. Combination therapy with pegIFN-α and ribavirin is associated with considerable side effects. Interferon-induced depression is a common cause of premature discontinuation (94), and other common side effects include influenza-like symptoms, neutropenia, thrombocytopenia and hypothyroidism (5%). Hemolytic anemia is the most common side effect secondary to ribavirin,

with a mean hemoglobin decline of 20 g/L (95). Recently, a phase 2b study
comparing the traditional interferon-α therapy with interferon-lambda (λ),
reported similar SVR rates although fewer side effects, especially
hematological, were noted when using interferon-λ (96).

Ribavirin is considered a broad acting antiviral with potential effect against
several RNA-viruses including HCV. It is a guanosine analogue, which upon
HCV entry into cells is converted into ribavirin triphosphate (RTP) that
possibly may be miss-incorporated into HCV RNA by the less stringent HCV
polymerase. It also acts as an inhibitor of inosine monophosphate
dehydrogenase (IMPDH), resulting in intracellular guanosine triphosphate
(GTP) depletion.

Two first-generation protease inhibitors, Telaprevir and Boceprevir, were the
first direct acting antivirals (DAA) to be introduced in 2011, and were added
to pegIFN-α and ribavirin combination therapy for the treatment of HCV
genotype 1 infected patients, thus significantly improving SVR rates (97, 98),
but at the cost of augmented side effects. Throughout the past year, several
new DAAs have been licensed within the European Union, and others are
awaiting approval. These DAAs include sofosbuvir (uridine analogue, NS5B
polymerase inhibitor), simeprivir (a second generation NS3/4A protease
inhibitors), daclatasvir (NS5A inhibitor), AbbVie 3D (paritaprevir, a protease
inhibitor boosted with ritonavir, ombitasvir, a NS5A inhibitor, and dasabuvir,
a nonnucleoside analogue that inhibits the NS5B polymerase), faldaprevir
(NS3/4A protease inhibitor), and deleobuvir (nonnucleosid inhibitor of non-
structural protein 5B polymerase) (99-103). Interestingly, with the currently
available interferon-free treatment options, HCV genotype 3 has become the
most difficult-to-cure genotype, with lower SVR rates achieved than for other
genotypes (99, 101). New treatment guidelines for the different HCV
genotypes are under continuous revision as new therapeutic options are
introduced, with cost becoming a major impetus. Currently the recommended
interferon-free option for HCV genotype 2 is sofosbuvir in combination with
ribavirin for 12 weeks, and sofosbuvir, daclatasvir with or without ribavirin
for 12 weeks for HCV genotype 3 (104). For HCV genotype 1 infection, high
SVR rates are achieved following therapy with several regimens including
simeprevir and sofosbuvir for 12 weeks, ledipasvir and sofosbuvir (and
ribavirin) for 12 weeks, daclatasvir and sofosbuvir for 12-24 weeks, and
AbbVie 3D (and ribavirin for genotype 1a) for 12-24 weeks (99-102).

1.8 Genome Wide Association Studies

Genome wide association studies (GWAS) are used to identify associations between human genetic variations and a disease or trait. A GWAS evaluates hundreds of thousands of single nucleotide polymorphisms (SNPs) in large, well characterized population samples (105). In the context of this thesis, GWAS studies have revealed close associations between the variations in the interleukin 28B (*IL28B)* gene and treatment outcome as well as spontaneous resolution of HCV infection (88). Such studies also led to the reported association between reduced inosine triphosphate pyrophosphatase (ITPase) activity and protection against hemoglobin decline during HCV treatment with pegIFN and ribavirin (106, 107), as well as the association between pronounced steatosis and a genetic variant of the patatin-like phospholipase domain-containing 3 (*PNPLA3)* gene (106, 107).

GWAS evaluations became possible as a result of the human genome database on common sequence variations generated by the Human Hap Map project (108). The human genome contains 3 billion nucleotide bases, and a SNP is a site in the human genome where individuals differ at a single base position**.** One SNP often has only 2 allelic variants, one on each autosomal chromosome. For example, one person has a cytosine (C allele) at this locus and another person may have a thymidine (T allele), as is the case with the *rs12979860* SNP in proximity to *IL28B*. There are approximately one SNP per 300 bases (at 110 million sites) where both alleles have a frequency of >1%. The variations of these SNPs are often inherited together. This is defined as a haplotype. This linkage disequilibrium of SNPs (in one haplotype) in different populations (European, Asian and Africans) is dependent on the number of nucleotides between the SNPs. SNPs within a defined region of a chromosome are often inherited together. This means that there are only a few haplotypes that differ within a given population (109). Looking into certain SNPs ("tag" SNPs) thereby provides information of regional haplotypes, and negates the need to evaluate all potential SNPs, which otherwise would be rather cumbersome and costly.

A GWAS is often based on a three step analysis (105). It utilizes the "tag" SNPs from the Human Hap map project, usually approximately 300,000 evenly spread across the genome. Since there is no predefined hypothesis regarding a particular gene or locus associated with a given disease or trait, GWAS is considered to be a "hypothesis-free" analysis (110). The first step is a case control study where SNPs across the human genome are genotyped. The next step is to calculate the strength of the association as the difference of the prevalence of the genetic variants of the SNPs (common homozygote,

heterozygote or variant homozygote) between the cases with a particular
disease or trait, and controls. In the third step, a quality control screen is
performed, where identified associations hopefully can be replicated. This
process helps to rule out false associations. Interestingly, only 12% of the
depicted SNPs associated with a trait or a disease are located within protein-
coding genes. Approximately 40% of identified SNPs are located in introns
(the portion of the transcript that is removed before translation to a protein),
and another 40% are located in non-coding, intergenic regions (105). The
latter, as is the case of the SNP *rs12979860*, which is located in a non-coding
region in close proximity to the *IL28B* gene, coding for interferon-λ3 (88).

It is important to bear in mind that GWASs only can reveal a potential
genetic susceptibility for developing or having a certain disease or trait.
Developing a disease often also requires additional environmental factors,
and these are not taken into account in a GWAS.

1.8 PNPLA3, *IL28B* and *ITPA*

1.8.1 Patatin-like Phospholipase Domain-Containing 3

The patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene is located on chromosome 22 and encodes a 481 amino acid long protein that belongs to the patatin–like phospholipase family, involved in lipid metabolism (106, 111). In humans, PNPLA3 predominately is expressed in the liver, presumably in the hepatocytes, but is also expressed in skin and adipose tissue (112).

In 2008, a GWAS revealed that a genetic variant of *PNPLA3*, a cytosine to guanine substitution entailing an amino acid change from isoleucine to methionine at residue 148 (*PNPLA3* 148M), was associated with pronounced hepatic steatosis in a study enrolling more than 2000 individuals of different ethnologies, primarily African-Americans, European-Americans and Hispanics (106). Subsequent reports have confirmed this association between the *PNPLA3* 148M homozygotes, i.e. *rs738409* GG, and increased incidence of NAFLD and more rapid progression to advanced steato-hepatitis and hepatic fibrosis (113, 114). Furthermore, this association has also been corroborated in the setting of alcoholic liver disease and HCC (115, 116). In patients with HCV infection, homozygotes for the *PNPLA3* 148M similarly has been reported to be associated with more steatosis, fibrosis, cirrhosis, and HCC predominantly in Mediterranean populations (117-120).

The mechanism of action through which *PNPLA3* 148M results in steatosis remains unclear. The critical amino acid change of isoleucine to methionine at residue 148 has been proposed to result in reduced enzymatic hydrolyses of glycerol lipids, which subsequently leads to induction of steatosis (111, 121). An alternative hypothesized mechanism of action is that the substitution entails acyl-transferase activity leading to increased triglyceride synthesis (122).

The frequency of the *PNPLA3* 148M allele ($G_{rs738409}$) varies across ethnicities, with the highest prevalence noted among Hispanics (49%), with an approximate homozygote frequency of 25% (106). The prevalence of *PNPLA3* 148M homozygotes in the Italian population has been reported to be approximately 10% (8-14%) (117, 120, 123). In contrast, the homozygote frequency in Germany appears to be lower (5.5%) (124).

1.8.2 Interleukin 28B

In 2009, a GWAS revealed that genetic variants in close proximity to the *IL28B* (also known as interferon-λ3) gene predicted greater likelihood of achieving SVR following treatment with pegINF-α and ribavirin among adherent HCV genotype 1 infected patients. The strongest association, among a predominantly Caucasian population, was noted for the *rs12979860* SNP where the CC$_{rs12979860}$, had an almost 2-fold increased likelihood of achieving SVR as compared to the TT variant (88). The differences in C allele frequencies, with a greater frequency in Asian and European populations as compared to populations of African origin, to a great extent could explain the previously recognized racial differences in treatment response (88, 125). Furthermore the *rs12979860* C allele also was associated with a greater first phase decline (i.e. the reduction in HCV RNA during the first days of interferon therapy) as well as spontaneous clearance of the viral infection in HCV genotype 1, but somewhat counterintuitive, also with a higher baseline viral load (88, 126, 127).

Regarding patients infected with HCV genotype 2 or 3, the *IL28B* C allele also reportedly is associated with greater first phase decline as well as higher baseline viral load (127, 128). However, there is discordance regarding the potential benefit of the C allele regarding treatment outcome for these patients when treated with INF-α and ribavirin. Some studies have reported that CC$_{rs12979860}$, as compared to the TT variant, is a positive predictor of SVR among Caucasian patients (129, 130), while others have failed to demonstrate such an association (128, 131).

In the setting of DAA regimens, the *rs12979860* CC variant was a positive predictor of SVR in triple therapy with the first first-generation protease inhibitors, Telaprevir and Boceprevir (132, 133), but with the introduction of interferon-free regimens the importance of the *IL28B* genetic variants is of less importance (134). Though, in an interferon-sparing trial evaluating Faldaprevir and Deleobuvir, with and without ribavirin, among HCV genotype 1 infected patients, the CC$_{rs12979860}$ was associated with higher SVR rates in comparison with non-CC variants (103).

Another SNP, *rs8099917,* located in proximity to and in strong linkage disequilibrium (a non-random association of two alleles at two loci) with *rs12979860* (127, 135), also has been reported to be of significance regarding treatment outcome, especially among populations of Asian origin, where CC$_{rs12979860}$ is almost universal. For HCV genotype 1 infected patients, the *rs8099917* TT variant is associated with favorable treatment outcome,

spontaneous virus clearance, and greater first phase decline, but also with a higher baseline viral load as compared to the TG and GG variants (135-137). As described for the *rs12979860* CC variant, the association between $TT_{rs8099917}$ and a greater first phase decline was also observed among HCV genotypes 2 and 3 infected patients (127).

Additionally a third SNP, *rs12980275*, located downstream from *IL28B*, has also been associated with treatment response in HCV genotype 1 infected patients. However, this is the least explored SNP in proximity to *IL28B* and it will not be discussed further in the context of this thesis (137).

In the setting of liver disease progression prior to therapeutic intervention, there are reports of significant associations between $GG_{rs8099917}$ and milder fibrosis, less necroinflammatory activity and slower fibrosis progression rate in European HCV genotype non-1 infected patients (138). The CC variant of *rs12979860* was in the setting of Scandinavian HCV genotype 3 infected patients, significantly associated with higher ALT and APRI score, as compared to the CT and TT variants, indicative of a higher degree of inflammation and fibrosis (131). Similarly in Japanese patients infected with HCV genotype 1 or 2, the otherwise favorable T allele of *rs8099917* was associated with more severe inflammation activity and fibrosis (139). In a study of primarily HCV genotype 1 infected patients, 276 had paired liver biopsies, with a median of 4 years between the first and second biopsy. In this study, the CC *rs12979860* was associated with greater hepatic inflammation grade and higher ALT, but not with fibrosis progression (140). Thus regarding the natural course of HCV-associated liver disease, the less favorable genetic variants with regards to spontaneous resolution of HCV infection and therapeutic outcome, i.e. $GG_{rs8099917}$ and $TT_{rs12979860}$, appears to be protective of liver disease progression.

The type III or lambda (λ) interferons are cytokines that are secreted by cells, particularly leukocytes, in response to viral infections. They bind to receptors on uninfected cells and induce proteins that increase resistance to viral infection. The stimulation of the transcription of these genes is modulated by the Janus Kinase- Signal Transducer and Activator of Transcription (JAK-STAT) signaling pathway, which is a direct intracellular signaling pathway from the cell surface receptor to the nucleus (141). There are more than 300 ISGs that can be up regulated by interferons. There are three major classes of interferons, i.e. type I, type II and type III. The interferons in proximity to *rs8099917* and *rs12979860* are known as the type III or interferon-λ family, coded by the *IL28A* (*IFN-λ2*), *IL28B* (*IFN-λ3*) and *IL29* (*IFN-λ1*) genes. The type III interferons primarily bind to epithelial cells and hepatocytes, and are

comprised of the abovementioned interferon-λ1-3 (IFN-λ1-3) as well as the recently discovered interferon-λ4 (reviewed in (142)).

The $CC_{rs12979860}$ variant has been reported to be associated with lower intrahepatic expression of ISGs, e.g. the expression of IFI27, ISG15, RSAD2, HTATIP2, etc., in a study of 109 patients with chronic hepatitis C genotype 1-4 infection. In this same study, higher ISG expression also was noted among non-responders to interferon and ribavirin therapy irrespective of *IL28B* genotype. However, the finding of significantly lower ISG expression did not correlate causally with the expression of IL28B/IFN-λ3, where $TT_{rs12979860}$ was associated with lower IL28B/IFN-λ3 expression (143). The latter finding is consistent with the results from two other, independent studies where the *IL28B* mRNA expression levels in peripheral blood mononuclear cells were measured in HCV genotype 1 patients (n=20), as well as in whole blood a healthy cohort (n=49). These studies reported significantly lower mRNA expression levels of IL28B/IFN-λ3 in subjects with the *rs8099917* GG genotype (136, 137). In contrast, another larger study enrolling HCV genotype 1 infected patients (n=80) found no association between IL28/IFN-λ expression levels and *rs12979860* genotype (88). Also, a study measuring the intrahepatic levels of ISGs among Japanese HCV genotype 1 infected patients found an association between the $G_{rs8099917}$ allele and higher ISG levels, but this was not significant in the setting of the *rs12979860* variants. This illustrates the complexity and difficulty regarding possible causal coherences of *IL28B/IFN-λ3* variants and induction of endogenous interferons.

The most recently discovered member of the type III family, IFN-λ4, exists as a dinucleotide variant (*rs368234815* TT/ΔG) with the ΔG variant coding for the *interferon-λ4* (*IFN-λ4*) gene, whereas the TT variant results in a disruption of the *IFN-λ4* reading frame and thus no synthesize of IFN-λ4 (144). The *rs368234815* ΔG variant (also known as ss469415590) is in linkage disequilibrium with the unfavorable *rs12979860* T allele, and compared to *rs12979860*, is more strongly associated with HCV clearance in individuals of African ancestry, whereas it provides comparable information in Europeans and Asians (144). Similarly it recently has been reported that an amino-acid substitution in the IFNλ4 protein changing a proline at position 70 to a serine (P70S; i.e. G to A at *rs117648444*), with a minor A allele frequency of 0.11 among Caucasians, substantially alters the antiviral activity of IFN-λ4, and that both $ΔG_{rs368234815}$ and $G_{rs117648444}$ are independent predictors of impaired response to interferon-α based therapy for HCV (145). Thus patients expressing the IFNλ4-S70 variant display lower expression levels of ISGs, improved treatment response and better spontaneous

clearance rates, as compared with patients coding for the fully active IFNλ4-P70 variant (145). In a haplotype analysis, it was observed that 95% of chromosomes are composed of three haplotypes: (i) $TT_{rs368234815}$ and $G_{rs117648444}$, which produces no IFNλ4, (ii) $\Delta G_{rs368234815}$ and $A_{rs117648444}$, which produces the less active IFNλ4-S70 and (iii) $\Delta G_{rs368234815}$ and $G_{rs117648444}$, which produces the fully active IFNλ4-P70. Interesting among patients: (i) unable to produce IFNλ4, 29% spontaneous cleared acute HCV infection and 81% achieved SVR following pegIFN-α and ribavirin therapy), (ii) able only to produce the less active IFNλ4-S70, 15% of patients spontaneous cleared acute HCV infection and 69% achieved SVR following pegIFN-α and ribavirin therapy, and (iii) producing only the fully active IFNλ4-P70 or both P70/S70 variants, 7% of patients spontaneous cleared acute HCV infection and 47% achieved SVR following pegIFN-α and ribavirin therapy (145). It has been suggested that fully active IFNλ4-P70 binds to the IFN-λ receptor and strongly stimulates JAK-STAT signaling and thus up-regulates ISG induction (146). This higher ISG expression secondary to the production of the fully active IFNλ4-P70, subsequently leads to lower levels of viral replication, hampering adaptive immune responses such as intrahepatic lymphocyte degranulation activity (147), which otherwise aid in the resolution of infection.

1.8.3 Inosine Triphosphate Pyrophosphatase

The inosine triphosphate pyrophospatase (*ITPA*) gene is located on the chromosome 20 and encodes the enzyme inosine triphosphate pyrophosphatase (ITPase) that is involved in the purine metabolism. ITPase converts inosine triphosphate (ITP) to inosine monophosphate (IMP) (148, 149). Other potential substrates that can be utilized by ITPase include deoxyinosine triphosphate (dITP) and xanthosine triphosphate (XTP). Thus the presence of ITPase is essential in order to prevent intracellular accumulation of rogue nucleotides such as ITP, dITP and XTP, which otherwise might be falsely incorporated into RNA and DNA producing mistranslation, enzyme inhibition and genetic instability (150-152).

Two allelic variants of the *ITPA* gene have been associated with reduced ITPase activity, subsequently resulting in increased intracellular concentrations of ITP. The first is a proline to threonine substitution where homozygosis for the C variant of *rs1127354* entails normal ITPase activity. The second allelic variant is a splicing altering SNP in the second intron where the A variant of *rs7270101* has normal ITPase activity (148, 149, 153, 154). Table 3 presents the compound predicted ITPase activity based on homozygosis resp. heterozygosis of these two SNPs.

The first reports of reduced ITPase activity leading to accumulation of ITP in human erythrocytes were published in the 1960s (155). The general consequences of this altered metabolic activity remain to be clarified, but reduced ITPase activity has been associated with increased risk of adverse drug reactions, such as more frequent and severe episodes of febrile neutropenia in patients treated with purine analogues, e.g. mercaptopurine (156). Other studies using human cell lines, normal ITPase activity has been reported to protect against DNA damage, probably through cleansing of ITP and dITP from the nucleotide pool forms (157). ITP is generated continuously in all cells through nucleotide recycling, and ITPase mRNA expression has been reported from all human tissues tested (liver, erythrocytes, spleen, placenta, brain etc.) with highest mRNA expression levels obtained in the heart, thyroid gland, and skeletal muscle (150).

In 2010 a GWAS of HCV genotype 1 infected patients revealed that genetic variants associated with reduced ITPase activity, $A_{rs1127354}$ and $C_{rs7270101}$, were associated with reduced hemoglobin decline at week 4 after initiation of treatment with pegIFN-α and ribavirin. The cut-off values for reduced hemoglobin decline used were those when ribavirin dose reduction is recommended, that is a decline in hemoglobin of >3 g/dL or hemoglobin

levels <10g/dL (107). Further subsequent reports have confirmed this association (158-160), also in an interferon-free regimen containing faldaprevir, deleobuvir, and ribavirin (161). Additionally, reduced ITPase activity has been associated with a greater platelet reduction at week 4, possibly secondary to reduced erythropoietin stimulation (162). The mechanism by which reduced ITPase activity prevents ribavirin induced anemia has been hypothesized to be secondary to less adenosine triphosphate (ATP) depletion, which in turn prevents erythrocyte membrane oxidative damage that mediates premature erythrocyte removal (163, 164).

The reports of the impact of reduced ITPase activity on treatment outcome in HCV patients have been conflicting. In the setting of HCV genotypes 1, 2 or 3 infected patients treated with pegIFN-α and weight-based dosing of ribavirin, reduced ITPase has not been reported to influence treatment outcome (158, 160, 165). In contrast, other studies have reported significant association between *ITPA* variants entailing reduced ITPase activity and improved SVR rates. For example an Italian study enrolling HCV genotype 1-4 infected patients, treated with pegIFN-α and ribavirin, reported increased SVR rates in patients with reduced ITPase activity when all patients were included in the analysis (159). However, this association was no longer significant after subdivision by infecting HCV genotype (159). Another study of Japanese HCV genotype 1 patients treated with peg-interferon and ribavirin, reported an association between increased likelihood of achieving SVR and *ITPA* $A_{rs1127354}$ carriage among a subset of patients with the favorable *IL28B rs8099917* TT variant (166).

2 AIMS

The overall aim of this thesis was to investigate the influence of host genetic variants on treatment outcome and on liver histopathology in patients with chronic HCV infection.

The specific aims were:

1.   To evaluate the impact of the *PNPLA3* 148 M genetic variant on steatosis, fibrosis, cirrhosis, and sustained virological response (paper I).

2.   To evaluate the impact of genetic variants of *IL28B* (*rs12979860* and *rs8099917)* on fibrosis, cirrhosis, and sustained virological response (*rs12979860*) (papers II and III).

3.   To evaluate the impact of genetic variants of *IL28B* (*rs12979860*) on liver damage as measured by liver stiffness values in the setting of a real life trial (paper IV).

4.   To evaluate the impact of genetic variants of *ITPA* on treatment induced anemia, thrombocytopenia, and sustained virological response (paper V).
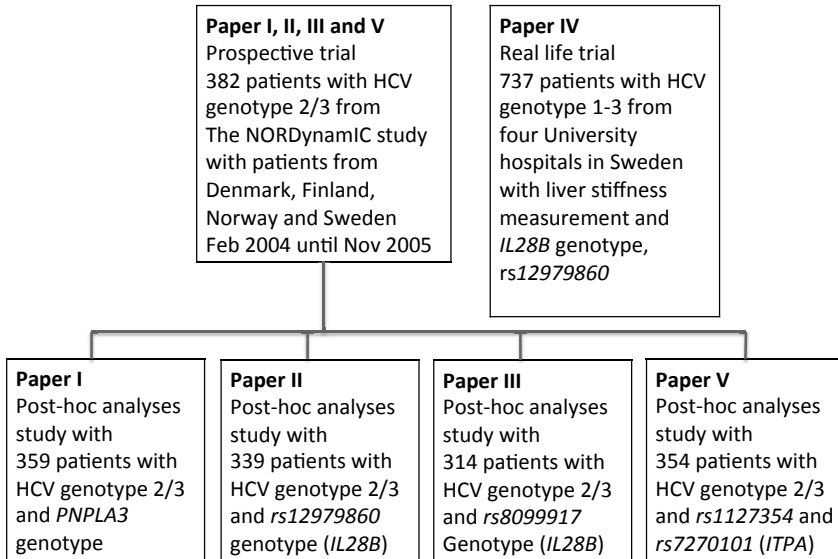
3 PATIENTS AND METHODS

| Paper I, II, III and V
Prospective trial
382 patients with HCV
genotype 2/3 from
The NORDynamIC study
with patients from
Denmark, Finland,
Norway and Sweden
Feb 2004 until Nov 2005 | Paper IV
Real life trial
737 patients with HCV
genotype 1-3 from
four University
hospitals in Sweden
with liver stiffness
measurement and
IL28B genotype,
rs*12979860* |

| Paper I
Post-hoc analyses
study with
359 patients with
HCV genotype 2/3
and *PNPLA3*
genotype | Paper II
Post-hoc analyses
study with
339 patients with
HCV genotype 2/3
and *rs12979860*
genotype (*IL28B*) | Paper III
Post-hoc analyses
study with
314 patients with
HCV genotype 2/3
and *rs8099917*
Genotype (*IL28B*) | Paper V
Post-hoc analyses
study with
354 patients with
HCV genotype 2/3
and *rs1127354* and
rs7270101 (*ITPA*) |

Figure 3. Summary of patients in paper I through V.

3.1.1 Patients (papers I, II, III and IV)

Papers I, II, III and IV are retrospective, post-hoc studies based on the NORDynamIC study. The NORDynamIC study is a phase three, open-label, randomized, multicenter, investigator-initiated trial where patients at 31 centers in Denmark, Finland, Norway and Sweden were randomized to either 12 or 24 weeks of treatment with 180 μg of peginterferon α-2a once weekly and 800 mg of ribavirin daily (85). Three hundred and ninety-two patients were enrolled between February 2004 and November 2005, 10 failed due to inclusion criteria or not being able to participate. Three hundred and eighty two patients were included in the intention-to-treat (ITT) analysis. One hundred and ninety four patients were randomized to 12 weeks and 188 patients to 24 weeks of treatment. Fifty-eight patients prematurely terminated treatment and premature termination was mainly due to adverse events.

Three hundred and three patients were included in the per-protocol analysis (PP), i.e. they received at least 80% of the target dose of ribavirin and peginterferon for at least 80% of the planned treatment duration. Twenty-six patients failed to have a plasma sample at follow up (24 weeks after completion of therapy), and these patients were regarded as treatment failures. Patients included were adults (≥ 18years), had compensated liver disease, were treatment naïve for hepatitis C, were seronegative for HBV and HIV, had a positive anti-HCV antibody test as well as HCV RNA of >15 IU/ml (quantified with the Roche Amplicor HCV monitor 2.0) within 6 months of treatment initiation. All patients had HCV genotype 2 or 3, and had a liver biopsy within 24 months of entry. Baseline characteristics stratified per HCV genotype in the NORDynamIC cohort is presented in table 2 (85).

In paper I, non-Caucasians as well as two patients with both HCV genotype 2 and 3 infection were excluded (n=23), and a total of 359 patients were genotyped for *PNPLA3* genetic variants and evaluated.

In paper II, 339 Caucasians could be analyzed for the *rs12979860* (43 patients excluded) and a total of 314 could be evaluated both by liver biopsy and *rs12979860* genotype.

In paper III, 314 patients were evaluated for both liver biopsy and the *rs8099917* genotype (68 patients excluded).

In paper V, 354 Caucasian patients were available for *rs1127354* and *rs7270101* genotyping (28 patients excluded).

3.1.2 Patients (paper IV)

Eight hundred and two HCV infected patients undergoing routine, clinical evaluation including liver stiffness measurement by means of Fibroscan were recruited at four University hospitals in Sweden between 2008 and 2012. Sixty-five were excluded, due to not meeting inclusion criteria or having HCV genotype 4, 6 or being infected with multiple HCV genotypes. Seven hundred and thirty-seven were finally included for further analysis of whom 711 had analysis of APRI and 614 had valid liver stiffness measurements. All patients had detectable HCV RNA at time of liver stiffness measurement, and had undergone *IL28B* (*rs12979860*) as well as for HCV genotyping as part of their routine, clinical evaluation. All patients were negative for HBsAg and

anti-HIV antibodies. Demographic and clinical data were gathered from medical charts. Seven hundred and eight patients had information of previous HCV treatment (590 had interpretable liver stiffness measurement) of which 21% (n=150) were treatment experienced without having reached SVR. No patient was on treatment at the time of evaluation. The majority of patients were likely to be Caucasians of Scandinavian origin, although no data on race was available, nor was data on alcohol consumption. *IL28B rs8099917* genotyping was not available.

Table 2. Baseline characteristics in the NORDynamIC study, no significant differences were noted in the treatment arms. The HCV genotype 2 patients were though significantly older than the HCV genotype 3 patients, no other significant difference were noted. Lagging et al, Hepatology 2008, reprinted with permission.

Characteristic	12 Weeks Treatment (n=194)	24 Weeks Treatment (n=188)
Gender		
Male[a]	123 (63%)	105 (56%)
Female[a]	71 (37%)	83 (44%)
Age (years)[b]	41.5 (10.87)	42.0 (10.8)
Body-mass index (kg/m^2)[b]	26.0 (4.4)	25.5 (4.3)
Body weight (kg)[b]	79.8 (16.4)	76.5 (16.1)
Route of transmission		
Intravenous drug use[a]	147 (77%)	131 (71%)
Transfusion[a]	12 (6%)	15 (8%)
Health care worker[a]	5 (3%)	4 (2%)
Sexual[a]	10 (5%)	7 (4%)
Unkown[a]	17 (9%)	28 (15%)
Genotype[a]		
2	55 (28%)	49 (26%)
3	137 (71%)	139 (74%)
No. of drinks per week[c]	1 (0-30)	1 (0-22)
Log$_{10}$ HCV-RNA (IU/mL)[b]	6.1 (0.8)	6.0 (1.1)
ALT (x ULN)[d]	1.5 (1.9)	1.3 (2.4)
Bridging fibrosis[a] (Ishak stage 3-4)	70 (39%)	70 (40%)
Cirrhosis[a] (Ishak stage 5-6)	23 (13%)	23 (13%)
Steatosis present[a] (grade 1-3)	113 (64%)	122 (69%)
Moderate or severe steatosis[a] (garde 2-3)	51 (29%)	48 (27%)

[a]No. (%); [b]Mean (SD); [c]Median (Range); [d]Median (Mean)

3.2 Methods

3.2.1 Study Design and Ethical Considerations (papers I-V)

Papers I, II, III, and V are retrospective studies based on post-hoc analyses of data from the NORDynamIC study. Written informed consent was obtained for each participating patient and the ethics Committees in each participating country approved the study. The study has been registered at the NIH trial registry (ClinicalTrials.gov Identifier: NCT00143000).

Paper IV was a real-life trial, where demographic and routine clinical data were retrospectively collected from medical charts and anonymously registered in a joint database. The study was conformed according to the guidelines of the 1975 Helsinki declaration, and the trial was approved by the Regional Review Board in Gothenburg (Regionala Etikprövningsnämnden i Göteborg).

3.2.2 Histological Assessment (papers I, II, and III)

Liver biopsies were obtained from all patients within 24 months prior to study entry. Biopsies with a length exceeding 1.5 cm and containing more than 6 portal tracts were evaluated. The fibrosis stage and necroinflammatory activity grade was evaluated according to the Ishak protocol (table 1) (45). Steatosis was graded as absent (grad 0), mild (grade 1, less than 30 % of hepatocytes involved) moderate (grade 2, 30-70 % of hepatocytes involved) or severe (>70 % of hepatocytes involved) (77). Two independent observers evaluated the liver biopsies and equivocal issues after the independent scores were debated and a consensus score was obtained. In paper I, fibrosis was defined as Ishak fibrosis stage ≥ 1 and cirrhosis as Ishak fibrosis stages 5-6. Steatosis was reported as absent or present (grade 1-3).

3.2.3 Fibrosis Index (papers I, II, III, and IV)

APRI was calculated as the ratio of normalized aspartate aminotransferase (AST), *i.e.* value divided by the upper limit of normal, to the platelet count multiplied by 100 (55).

3.2.4 Liver Stiffness Measurements (paper IV)

TE was performed for liver stiffness measurements. This non-invasive method to assess liver fibrosis is described in the Introduction of this thesis.

3.2.5 HCV RNA Quantification and HCV Genotyping (papers I, II, III, IV, and V)

In papers I to V, plasma was obtained using PPT-tubes and, HCV RNA was determined by reverse transcription polymerase chain reaction (RT-PCR) of plasma using Cobas AmpliPrep/COBAS TaqMan HCV Test (Roche Diagnostics, Branchburg, NJ), which quantifies HCV RNA with a limit of detection of ≤15 IU/mL.

In papers I, II, III and V HCV RNA was furthermore quantified on days 0, 3, 7, 8, 29, week 8, week 12, week 24 (for those receiving 24 weeks of therapy), and 24 weeks after completion of therapy. All samples were frozen (−70°C) and subsequently analyzed at the department of Virology in Gothenburg.

HCV genotyping in papers I, II, III and IV initially was performed at the local centers and later confirmed at the central laboratory (Dept. of Virology, Gothenburg) by RT-PCR and Taqman probes targeting the 5´non-coding region (167). HCV genotyping in paper IV was performed at the local center.

3.2.6 PCR *PNPLA3*, *IL28B* and *ITPA* genotyping

For the specific probes, forward and reverse primers used to determine the genetic variants of *PNPLA3* (*rs738409), IL28B (rs12979869 and rs8099917)* and *ITPA (rs1127354* and *rs7270101)* as referred to the method section of each paper. All SNPs were in Hardy-Weinberg equilibrium.

3.2.7 Homeostatic Model Assessment-Insulin Resistance (papers I and V)

Insulin resistance was assessed by using the Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) index. Baseline fasting glucose (mmol/L) was measured locally, whereas fasting serum insulin (mU/L, Architect Insulin, Abbott Park, IL) was analyzed at the department of Virology, Gothenburg. HOMA-IR was calculated as (Glucose x Insulin) / 22.5 (168).

3.2.8 Statistical methods (papers I, II, III, IV, and V)

All statistical analyses were performed using the IBM SPSS statistics version 19 (IBM Corporation, Somers, NY) software package and a two-sided P-value of <0.05 was considered statistically significant.

In paper 1, Continuous variables were presented as median and the range between the 25th and 75th percentile (interquartile range). Categorical variable distributions were compared using either the χ^2 test or the Fisher's exact test. Continuous variables were analyzed with linear regression, when necessary after logarithmic transformation in order to create a normal distribution. Both additive (II vs.IM vs. MM) and recessive (II+IM vs. MM) inheritance models were tested.

In papers II, III and IV Wilcoxon-Mann-Whitney U-test, Kruskal-Wallis test, as well as Chi squared ($\chi2$) test or Fisher´s exact test were utilized to evaluate relationships between groups. In paper IV, multivariate analysis of potential fibrosis predictors was made by General Linear Model and as in paper I, logarithmic transformation was used for quantitative data with skewed distribution and subjects were stratified according to genotype.

In paper V, Spearman's rank-order correlation test was used to analyze univariate relationships with ITPase activity. Logistic regression was performed on the PP patients to evaluate the relationship between SVR and ITPase activity (considered as a numerical value) both with and without baseline covariates: age, BMI, liver fibrosis stage, HCV RNA level, IP-10 level, and *IL28B* genotype, as well as change in hemoglobin on days 0-29, ribavirin concentrations day 29, and treatment duration.

4. RESULTS

4.1 The Impact of *PNPLA3* 148M Homozygosity on Liver Histology and Treatment Outcome (paper I)

In paper I, we aimed to investigate the importance of *PNPLA3* genetic variants on liver histology and treatment outcome in a Nordic cohort of hepatitis C patients with genotype 2 or 3. In total, 359 patients were genotyped and the frequencies of the *PNPLA3* variants were 60% (n=215), 37% (n=134) and 3% (n=10) for the II, IM and MM genotype respectively, stratified per HCV genotype, homozygosity for the *PNPLA3* 148M variant had a frequency of 4% (n=4) in the HCV genotype 2 cohort and 2% (n=6) in the HCV genotype 3 cohort.

There were more patients infected with HCV genotype 3 (n=256) compared with HCV genotype 2 (n=103). Patients with HCV genotype 2 were on average nine years older than the patients with HCV genotype 3 (p<0.001). Those infected with HCV genotype 3 had more steatosis (n=49 [50%] and n=171 [73%] in HCV genotype 2 and 3 respectively; p<0.001) and higher ALT values (median of 78 compared with 108 U/l in HCV genotype 2 and 3 respectively; p<0.001). The latter two observations were most probably secondary to the viral characteristics of HCV genotype 3 previously described. Homozygosity for the *PNPLA3* 148M genetic variant in HCV genotype 2 was also associated with higher HOMA-IR, compared to the IM and II variants (1.9, 2.9, 9.0 in the II, IM and MM group respectively, P *additive* = 0.023 and P *recessive*= 0.005).

No significant association were noted, for either HCV genotype 2 or 3, between homozygosity for the *PNPLA3* 148 M genetic variant and increased grade of steatosis, more advanced fibrosis or cirrhosis.

SVR rates were not affected by the *PNPLA3* genotype (66, 70 and 100% in HCV genotype 2 and 67, 74 and 67% in the HCV genotype 3 for *PNPLA3* II, IM, and MM respectively). Regarding viral kinetics during therapy, there was an initial significant association with lower early HCV RNA in HCV genotype 2 infected patients with homozygosity for the *PNPLA3* 148M noted at baseline, day 3 and day 7 as compared to the IM and II genotypes (p= 0.005, p=0.014 and p=0.003 at day 0, 3 and 7 respectively). Surprisingly, in contrast HCV RNA at baseline was significantly higher at baseline in HCV

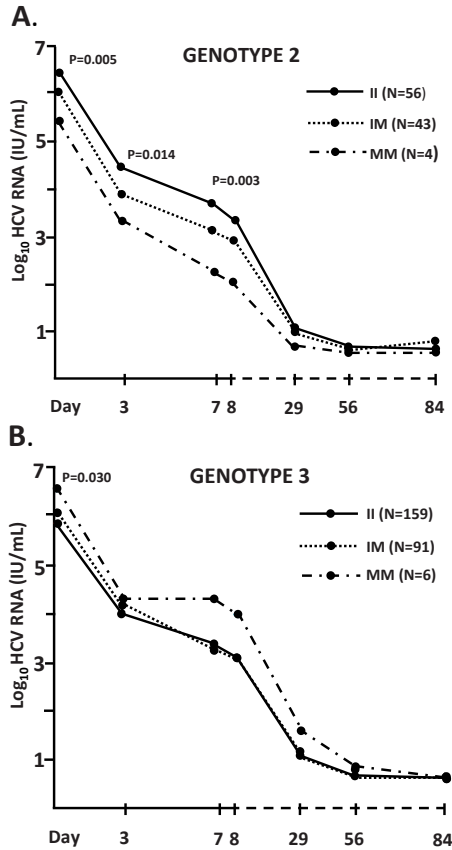genotype 3 infected patients with homozygosity for the *PNPLA3* 148M variant (P *additive*= 0.03) (fig 4).

Figure 4. HCV viral load according to the PNPLA3 148M genotype in HCV genotype 2 (A) and HCV genotype 3 (B) from start of treatment (day 0) through week 12 (day 84). P values were calculated for the difference in viral load at each time point using linear regression. Rembeck et al, BMC medical genetics 2012, reprinted with permission.

4.2 The Impact of *Interleukin 28B* Genetic Variants on Liver Histology and Treatment Outcome (papers II and III)

In total, 339 patients could be genotyped for the *rs12979860,* of whom 314 could be evaluated for liver histology (paper II). Three hundred and fourteen patients were also available for *rs8099917* genotyping and liver biopsy evaluation (paper III). Patients with HCV genotype 2 were significantly older than those with HCV genotype 3 (mean age 47.2 vs. 39.8, p<0.0001), and fewer patients were infected with HCV genotype 2 than genotype 3 (98 vs. 241). All patients were Caucasians, with the majority being of Scandinavian origin, and were treatment naive. Among patients with chronic HCV genotype 3 infection, the *rs12979860* CC genotype was associated with higher APRI score (p=0.001), greater normalized ALT (p< 0.0001), more severe portal inflammation (p=0.02), and higher steatosis grade (p=0.03) as compared to the CT and TT variants (fig 5 and 6).

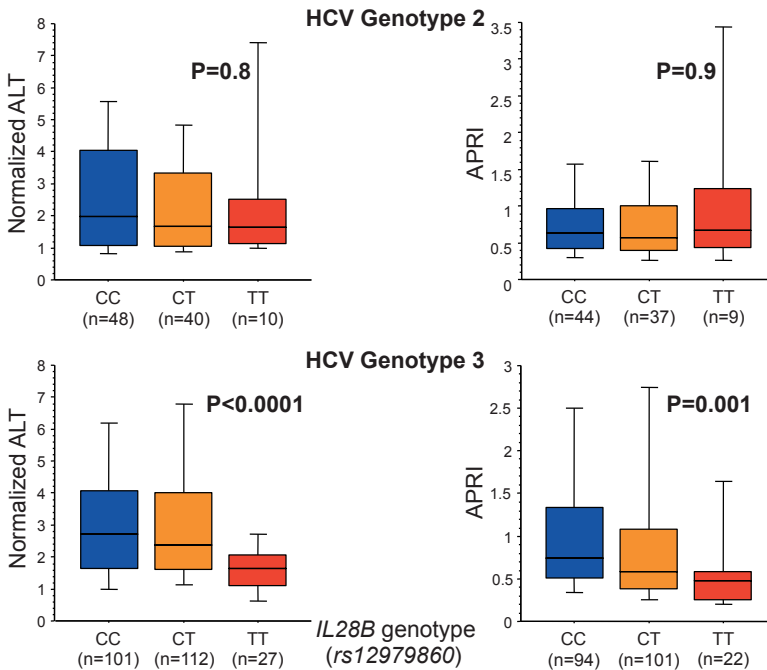*Figure 5. Impact of IL28B (rs12979860) on normalized ALT and AST to platelet ratio index (APRI) among HCV genotype 2 and 3 infected patients. Box plots displaying the 10th, 25th, 50th, 75th and 90th percentiles and P values obtained using Kruskall-Wallis test. Rembeck et al, PLOS one 2012, reprinted with permission.*
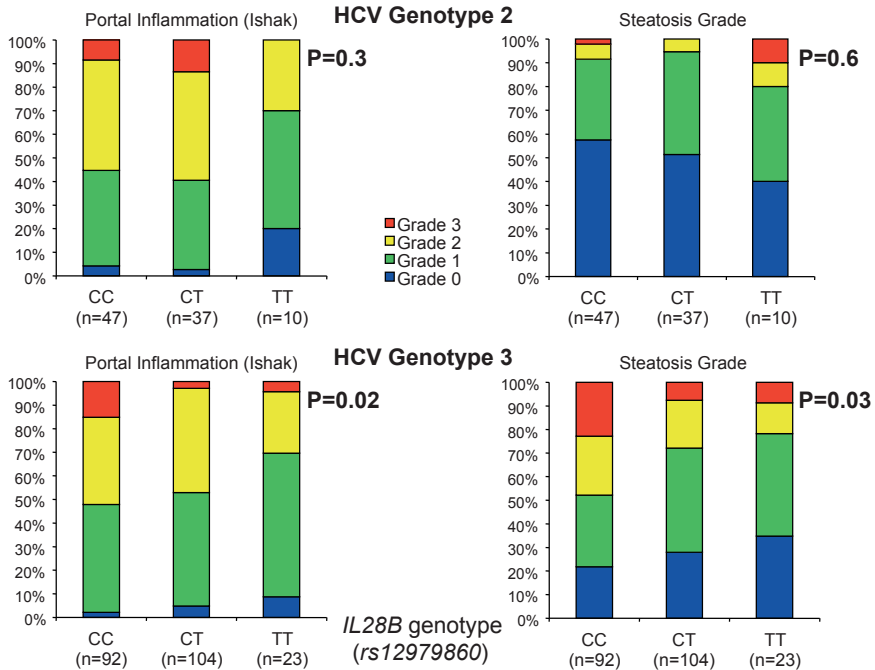
Figure 6. Impact of IL28B (rs12979860) on portal inflammation grade (Ishak protocol) and steatosis grade among HCV genotype 2 and 3. Histogram showing the proportion of patients and p-value obtained using Chi-squared test. Rembeck et al, PLoS ONE 2012, reprinted with permission.

Similarly, among HCV genotype 3 infected, the *rs8099917* TT variant also was associated with greater normalized ALT values (p=0.001), and higher APRI score (p=0.02) as compared to the GT and TT genotype. Additionally, the *rs8099917* TT variant was also significantly associated with more pronounced interface hepatitis (p=0.007) and fibrosis (p=0.01; fig 7). For neither *IL28B* SNP were any such associations observed among HCV genotype 2 infected patients. Thus, the *IL28B* variants that generally are regarded as beneficial regarding interferon-based treatment outcome among HCV genotype 1 infected patients, seems to be associated with more severe liver disease in the setting of HCV genotype 3 infection.
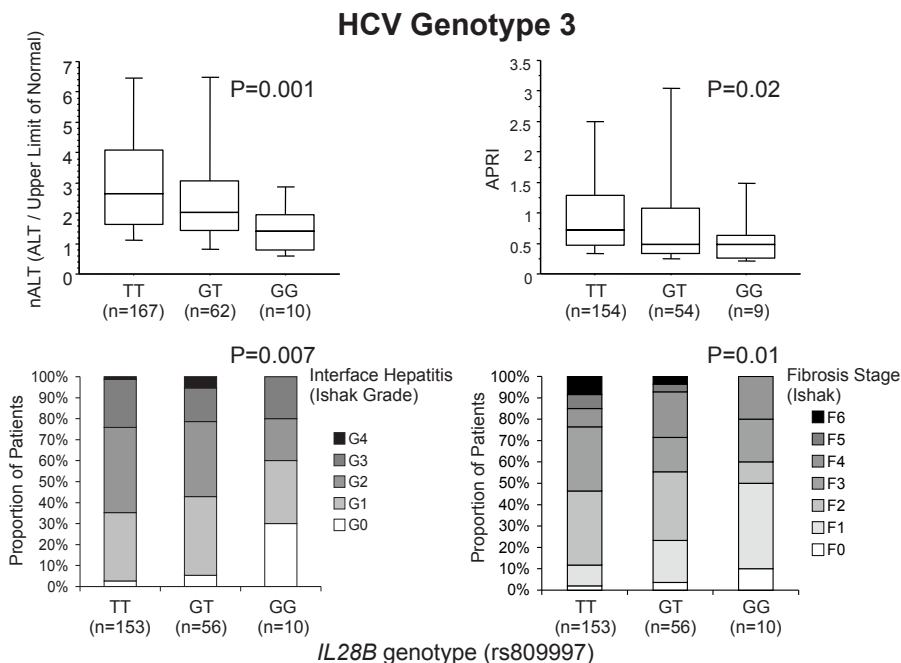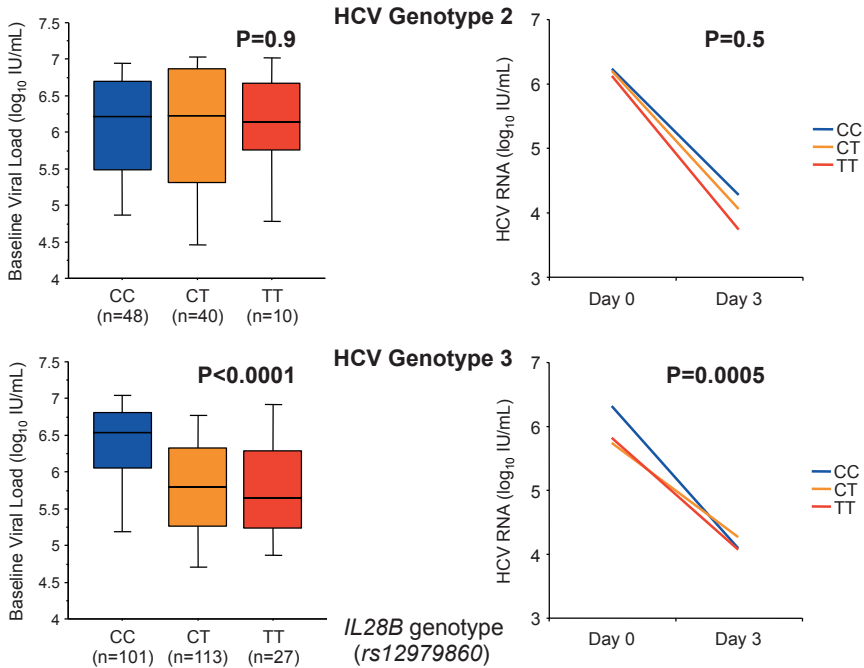
HCV Genotype 3

Figure 7. Box plots displaying the 10th, 25th, 50th, 75th and 90th percentiles of normalized ALT and APRI (P values obtained using Kruskal-Wallis test). Histograms displaying Ishak interface hepatitis grade and fibrosis stages (P values obtained using chi-squared and Fisher´s exact test) among HCV genotype 3 infected patients grouped by IL28B (rs8099917). Rembeck et al, Hepatology 2012, reprinted with permission.

Additionally, among HCV genotype 3 infected patients, the *rs12979860* CC variant was associated with higher baseline viral load (mean 6.3, 5.9, and 5.9 $\log_{10}$ IU/ml for CC, CT, and TT genotype respectively, p< 0.0001), as well as with a greater first phase decline in HCV RNA (2.1, 1.7, and 1.9 $\log_{10}$ IU/ml for CC, CT, and TT genotype respectively, p=0.0005; fig 8). In spite of this, no significant differences in SVR rates with regards to *IL28B* variants (*rs12979860)* were noted for either HCV genotype.

Figure 8. . *Impact of IL28B (rs12979860) on baseline viral load and decline in mean HCV RNA day 0-3 among HCV genotype 2 and 3 infected patients. Box plots displaying the 10th, 25th, 50th, 75th and 90th percentiles and P values obtained using Kruskall-Wallis test. Rembeck et al, PLoS ONE 2012, reprinted with permission.*

4.3 Impact of *IL28B*-related Single Nucleotide Polymorphism on Transient Liver Elastography in Chronic HCV Infection (paper IV)

The majority of patients were Caucasians of Scandinavian origin. Most patients were male (62%) as well as infected with HCV genotype 1 (69%). This proportion of HCV genotype 1 is greater than is commonly noted in Sweden (169), and possibly reflects a previous tendency to preferentially evaluate fibrosis stage among HCV genotype 1 infected patients, although such an evaluation is recommended regardless of HCV genotype. Patients with HCV genotype 2 infection (n= 44) were older in comparison to those infected with HCV genotype 1 (n=445) or 3 (n=125) (median of 56, 52, and 47 years for HCV genotype 2, 1, and 3 respectively, p <0.0001). HCV genotype 2 infected patients also had a longer duration of infection (median of 34, 30 and 24 years in HCV genotype 2, 1, and 3 respectively, <0.0001) and a higher BMI (29, 26, and 24 in HCV genotype 2, 1, and 3 respectively, p<0.02) in comparison to those infected with HCV genotype 1 or 3. Additionally the genotype 3 infected patients were most prone to have acquired the infection through intravenous drug use (64% versus 49% and 52% in HCV genotype 3, 1, and 2 respectively).

HCV genotype 3 infected patients with the *rs12979860* CC variant had significantly higher liver stiffness values (median 8.2 kPa in CC vs. 6.4 kPa in CT/TT, p=0.004) and higher APRI score (median 1.0 vs. 0.6 for CC and CT/TT respectively, p=0.02) indicating a more pronounced liver histopathology (fig 9). Also a significantly higher baseline viral load was noted among HCV genotype 1 infected CC patients as compared to CT/TT patients (6.6 versus 6.2 $\log_{10}$ IU/ml, p=0.001) (fig 10). No such significant association was noted HCV genotype 3 patients in this study, in contrast to what was previously mentioned for papers II and III above. Neither of the abovementioned associations were found among HCV genotype 2 infected patients, and there was no association regarding ALT levels and *rs12979860* for any HCV genotype. All associations remained significant after the exclusion of treatment experienced patients.

Figure 9. Liver Stiffness values as well as APRI score according to IL28B variants and HCV genotypes 1, 2 and 3. Boxplots displaying the 10th, 25th, 50th, and 90th percentiles. Ydreborg et al. PLoS ONE 2013, reprinted with permission.
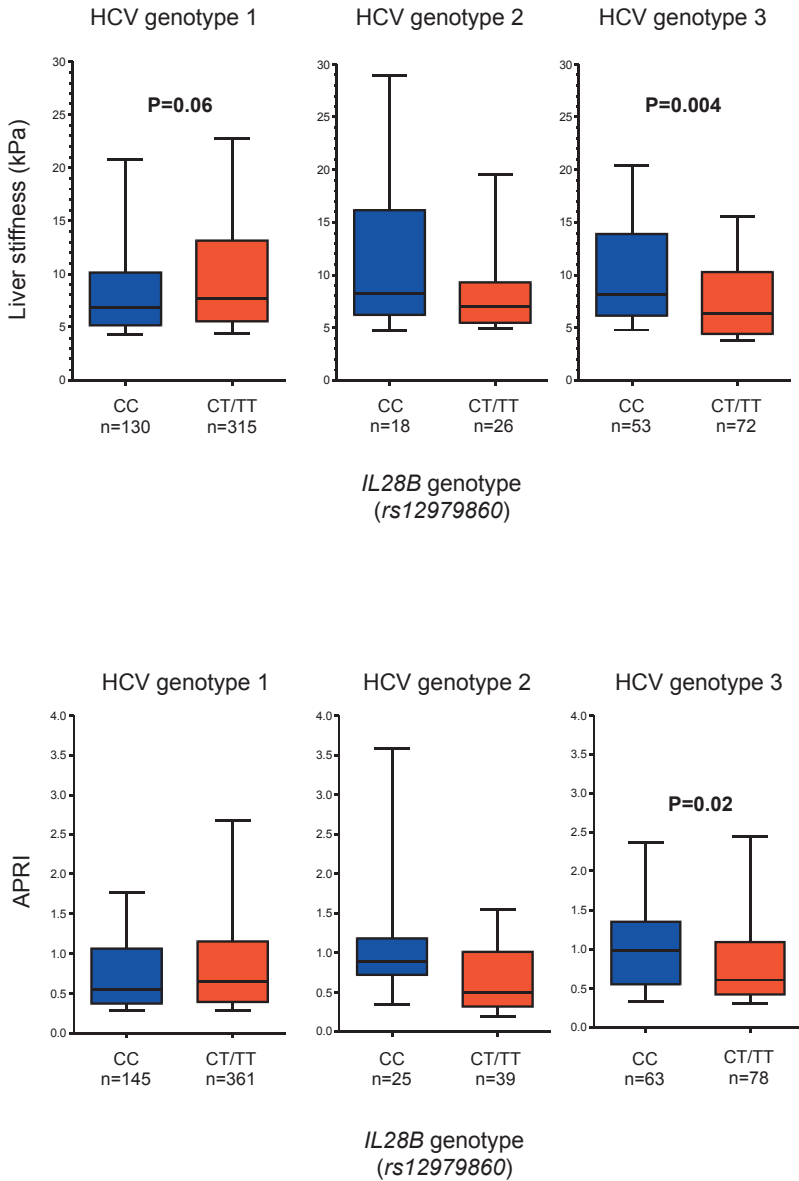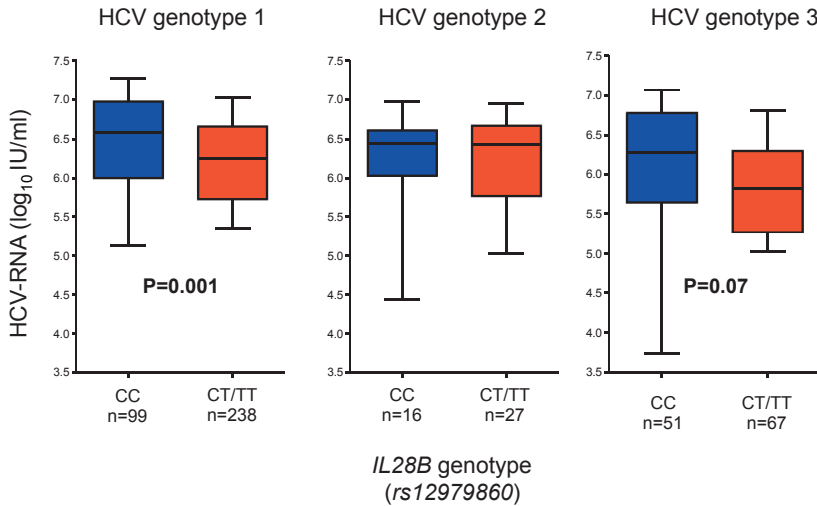
Figure 10. HCV RNA levels in relation to IL28B variants and HCV genotype 1, 2 and 3. Boxplots displaying the 10th, 25th, 50th, and 90th percentiles. Ydreborg et al. PLoS ONE 2013, reprinted with permission.

Interestingly, the frequency distribution of the genetic variants of the *rs12979860* among treatment naïve patients demonstrated a significantly lower proportion of the CC variant among the HCV genotype 1 infected patients as compared to HCV genotype 2 or 3 infected patients (p<0.0001) (fig 11). This observation supports previous reports that the CC variant in the setting of HCV genotype 1 is associated with improved spontaneous viral clearance (125), but may be less beneficial after exposure to HCV genotype 2 or 3.

Fig 11. Frequency distribution of IL28B variants in relation to HCV genotypes 1, 2 and 3 among treatment-naive patients. P values were obtained by Chi squared test. . Ydreborg et al. PLoS ONE 2013, reprinted with permission.
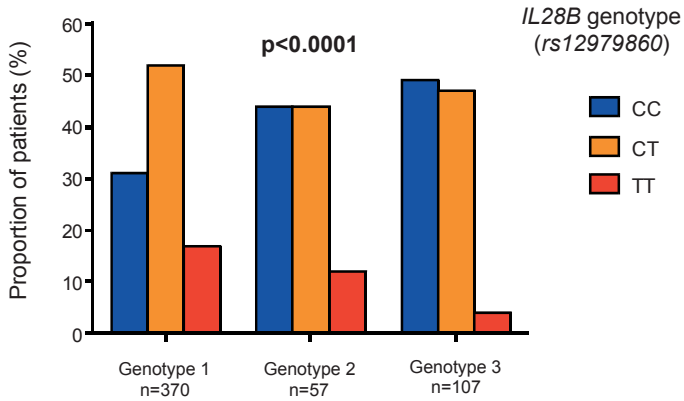
# 4.4 The Impact of *ITPA* Genetic Variants on Hemoglobin Decline During Therapy and Treatment Outcome.

Three hundred and fifty four patients out of the 382 patients in the NORDynamIC trial were available for *ITPA* related SNP genotyping, that is the genetic variants of *rs1127354* (CC, CA and AA) and *rs7270101* (AA, AC and AA). The majority of patients (63%) had normal ITPase activity with the CC variant of *rs1127354* and the AA variant of the *rs7270101*. The residual frequency distribution of ITPase genotypes within the study population and its compound ITPase activity is reported in table 3.

rs1127354	*rs7270101*	Predicted ITPase Activity (%)	Distribution in the study population % (n)
Wild type (CC)	Wild type (AA)	100	63 (219)
Wild type (CC)	Heterozygote (AC)	60	21 (73)
Wild type (CC)	Homozygote (CC)	30	2 (7)
Heterozygote (CA)	Wild type (AA)	25	12 (43)
Heterozygote (CA)	Heterozygote (AC)	10	1.7 (6)
Homozygote (AA)	Wild type (AA)	<5	0.3 (1)

Table 3. Predicted ITPase activity according to compound genotype of rs1127354 and rs7270101. Rembeck et al. Hepatology 2014, reprinted with permission.

As to be expected, compound reduced ITPase activity was significantly associated with less hemoglobin decline after 4 weeks of treatment with peg-interferon and ribavirin ($p<0.0001$), as well as a significantly greater platelet reduction ($p=0.007$), (fig 11). At treatment week 4, reduced ITPase activity was also significantly associated with lower ribavirin concentration ($p=0.02$) (fig 11). There was no significant association between predicted reduced ITPase activity and ribavirin dose reductions, likely due to the lower and thus also better-tolerated 800 mg ribavirin daily used in this study.

Figure 11. Impact of ITPase activity on change in hemoglobin (A) and platelet count days 0-29 (B), and on ribavirin concentration day 29 (C). Boxplots displaying the 10th, 25th, 50th, 75th, and 90th percentiles among patients grouped according to predicted ITPase activity based on compound ITPA genotype. P-values obtained for Spearman's rank correlation coefficient (r_s). Rembeck et al. Hepatology 2014, reprinted with permission.
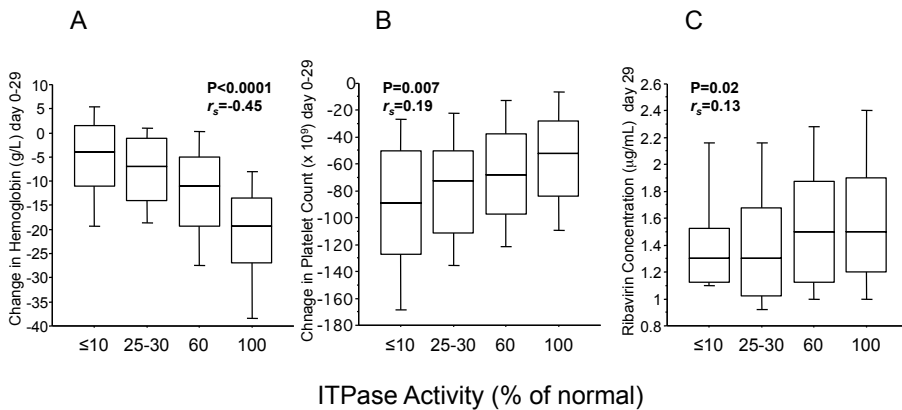
Reduced ITPase activity was not significantly associated with first phase decline, VRVR, RVR (43%, 65%, 61% and 63% for ITPase activity ≤10%, 30%, 60% and 100%, respectively) or HCV RNA level at the end of treatment (fig 12).

In the PP population, reduced predicted ITPase activity was significantly associated with increased likelihood of achieving SVR (odds ratio [OR] =6.4 for completely reduced activity, p=0.0003) (table 4). Reduced ITPase activity remained significant for SVR also in the ITT cohort (p=0.031 univariate and p=0.007 multivariate analysis). In the PP population predicted reduced ITPase activity was significantly associated with improved SVR rates also when the population sample was subdivided into treatment duration (12 versus 24 weeks), HCV genotype, fibrosis stage and IL28B variants (*rs12979860*) (table 4). Interestingly, SVR rates were continuously improved as ITPase activity decreased (OR 2.3 for 100% versus ≤60% and OR 4.4 100% versus 30%), and the association of reduced predicted ITPase with

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improved SVR rates was explained by lower rates of relapse (relapse rate 20%, 21%, 19% and 39% for ITPase activity ≤10%, 30%, 60% and 100%, respectively, in the 12 week treatment duration arm, and 0%, 0%, 13% and 14% for corresponding ITPase activity in patients treated for 24 weeks).

Apart from reduced ITPase activity, in multivariate analysis, among others, the TT variant of *rs12979860* (*IL28B*) was a negative predictor of SVR (OR=0.46, p=0.004).

By means of logistic regression analysis, protection against anemia as well as ribavirin adherence were ruled out as potential explanations for the improved likelihood of achieving SVR among patients with reduced ITPase.
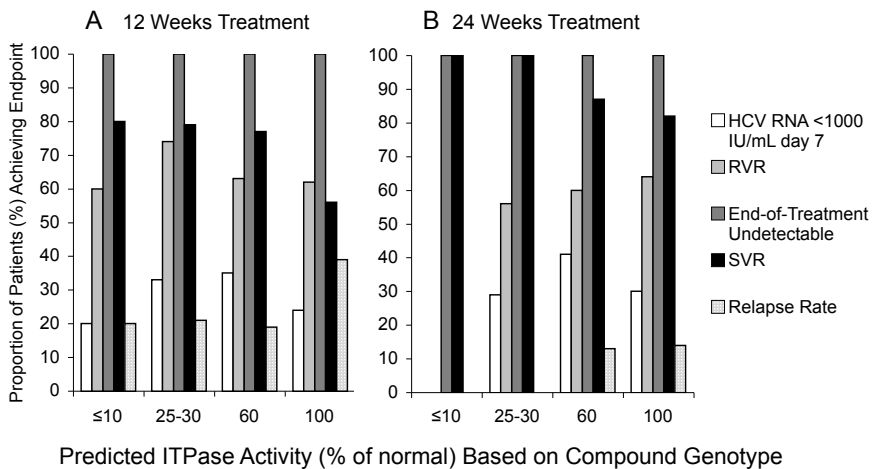

Figure 12. Proportion of patients (%) achieving treatment responses grouped according to predicted ITPase activity based on compound ITPA genotype and treatment duration 12 (A) or 24 (B) weeks. Rembeck et al. Hepatology 2014, reprinted with permission.

Table 4. Proportion of patients achieving SVR (Per-Protocol analysis). Odds ratio and P values obtained using logistic regression. Rembeck et al. Hepatology 2014, reprinted with permission.

	Predicted ITPase Activity (% of normal)				Odds Ratio	P
	≤10%	25-30%	60%	100%		
All Patients	6/7 (86%)	35/39 (90%)	50/61 (82%)	115/173 (66%)	6.4 (2.1-19.6)	0.0003
12-week Treatment Duration	4/5 (80%)	15/19 (79%)	23/30 (77%)	57/102 (56%)	4.9 (1.4-17.6)	0.01
24-week Treatment Duration	2/2 (100%)	20/20 (100%)	27/31 (87%)	58/71 (82%)	11.9 (1.03-138)	0.02
HCV genotype 2[a]	2/2 (100%)	8/9 (89%)	19/20 (95%)	29/51 (57%)	71.0 (3.5-1454)	0.0004
HCV genotype 3[a]	4/5 (80%)	27/30 (90%)	31/41 (76%)	84/120 (70%)	3.5 (1.04-11.7)	0.03
Non-significant Fibrosis (Ishak 0-2)	1/2 (50%)	18/19 (95%)	25/27 (93%)	59/77 (77%)	5.3 (0.76-37.0)	0.07
Bridging Fibrosis (Ishak 3-4)	4/4 (100%)	10/12 (83%)	18/23 (78%)	39/64 (61%)	7.5 (1.3-43.7)	0.01
Cirrhosis (Ishak 5-6)	1/1 (100%)	5/6 (83%)	5/9 (56%)	5/17 (29%)	27.4 (1.8-420)	0.009
IL28B CC	4/4 (100%)	14/17 (82%)	27/32 (84%)	48/73 (66%)	6.3 (1.2-31.8)	0.02
IL28B CT	1/2 (50%)	14/14 (100%)	17/21 (81%)	58/84 (69%)	6.6 (0.99-44.8)	0.03
IL28B TT	1/1 (100%)	4/5 (80%)	5/7 (71%)	9/16 (56%)	5.9 (0.35-99)	0.19

5 DISCUSSION

Chronic HCV comprises a broad spectrum of liver disease, ranging from no or minimal activity to active hepatitis that over the coarse of time may progress to severe fibrosis, cirrhosis, and hepatocellular carcinoma. HCV infection often is associated with considerable morbidity and mortality, if successful therapeutic intervention is not initiated. Previously, host (e.g. male gender, higher alcohol consumption and steatosis) as well as viral factors (e.g. HCV genotype 3 and co-infection with HIV or HBV) reportedly have been associated with more rapid liver disease progression. This thesis aimed to evaluate the impact of host genetics, i.e. genetic variants of *PNPLA3*, *IL28B* and *ITPA* on liver disease severity and treatment outcome.

5.1 Impact of host genetics (*PNPLA3* and *IL28B*) on liver disease severity

The *PNPLA3* 148M genetic variant, entailing a cytosine to guanine substitution resulting in a change of isoleucine to methionine at residue 148, previously has been reported to increase the risk of hepatic steatosis as well as fibrosis (106, 113). In the context of this thesis, the impact of the *PNPLA3* 148M genetic variant on liver disease severity was evaluated in a Nordic cohort of HCV genotype 2 and 3 infected patients (paper I).

In our study, homozygosity for the *PNPLA3* 148M was not significantly associated with hepatic steatosis, fibrosis or cirrhosis. This is discordant with previous reports from southern Europe, where homozygosity for the *PNPLA3* 148M genetic variant was associated with more severe liver histopathology (117-120). However, the low prevalence of homozygosity for the *PNPLA3* 148M genetic variant in our Nordic patient cohort (4% (n=4) among HCV genotype 2 infected and 2% (n=6) in genotype 3 infected) hampers the evaluation of any possible association, and thus caution must be exercised when interpreting these results. This low frequency of homozygosity for the *PNPLA3* 148M genotype is in line with a previous report from Germany (124), and is substantially lower than the frequencies of approximately 10% reported from Italy (117, 120, 123) and 25% reported among Hispanics (106). Interestingly, congruent with our results, previous studies with established associations between homozygosis for the *PNPLA3* 148M genetic

variant and steatosis, fibrosis and cirrhosis in HCV infected patients, the association with steatosis was no longer significant for genotype 3 upon stratification for HCV genotype (117-119). A possible explanation for this observation is that the proposed molecular mechanism through which *PNPLA3* 148M generates steatosis, i.e. either reduced hydrolyses of glycerol lipids or increased triglyceride synthesis (111, 121, 122), may be overshadowed by the steatosis induced by the HCV genotype 3 core protein (74-76). Thus in the setting of Nordic HCV genotype 2 or 3 infected patients, it seems to be of minor clinical relevance to screen for *PNPLA3* genetic variants.

In contrast to *PNPLA3* genetic variants, the otherwise favorable SNPs in proximity to *IL28B* were associated with more severe liver histopathology. The CC variant of *rs12979860* in HCV genotype 3 infected patients was significantly associated with higher normalized ALT, higher APRI score (fig 5), more severe portal inflammation and higher grade of steatosis as compared with the CT or TT variants (fig 6) (paper II). Similarly, the TT variant of *rs8099917* was significantly associated with higher normalized ALT and APRI score as well more pronounced interface hepatitis grade and higher fibrosis stage (fig 7) (paper III). These findings subsequently were confirmed in the independent, real-life trial, where liver stiffness values were measured in HCV genotype 1-3 by means of Fibroscan (paper IV). In the latter trial the HCV genotype 3 infected patients with the CC variant of r*s12979860* had significantly higher APRI score and liver stiffness values (fig 9). Thus it appears that the "protective" alleles in terms of treatment response for HCV genotype 1, negatively impact the natural course of progression of HCV associated liver disease, especially for patients infected with HCV genotype 3.

These results are consistent with previous reports, where HCV genotype 3 infected patients of Scandinavian origin having the CC variant of *rs12979860,* had significantly higher ALT levels and APRI score as compared with the CT or TT variant (131). Similarly another study among European patients reported significant associations between the otherwise less favorable *rs8099917* GG variant in HCV genotype non-1 and less fibrosis, less necro-inflammatory activity and slower fibrosis progression rate (fibrosis stage/duration of HCV infection) (138). Correspondingly, more severe activity and inflammation activity in Japanese HCV genotype 1 or 2 infected patients and the T allele of *rs8099917* (139).

The $CC_{rs12979860}$, among HCV genotype 3 infected patients, was in our study (paper II) also associated with higher baseline viral load (fig 8) indicating

that the changes in the liver histopathology observed may have been secondary to a direct viral cytopathic effect. HCV genotype 3 induced steatosis, which is also associated with higher HCV RNA levels, has previously been described to entail enhanced fibrosis progression (74-77). Although, in the real-life trial (paper IV), higher APRI score and higher Fibroscan values in HCV genotype 3 infected patients were not associated with higher baseline viral load. Higher baseline viral load was on the contrary found in patients infected with HCV genotype 1 (fig 10). These results underline the complexity and difficulty in interpreting the function and mechanism of the genetic variation in SNPs in proximity to the *IL28B* gene.

Thus many studies implicate a poorer prognosis characterized by more severe liver histopathology for patients with the otherwise therapeutically favorable CC variant of *rs12979860* and the TT variant of *rs8099917*, especially among HCV genotype 3 infected patients. HCV genotype 3 infection in general also appears to be associated with more severe liver disease as well as increased morbidity and mortality (40, 41). Interestingly, the CC variant of *rs12979860* appears more common among HCV genotype 3 than genotype 1 infected patients (89, 170). Unfortunately HCV genotype 3 currently is the most difficult to cure genotype in the new era of direct acting antivirals with higher relapse rates and fewer efficient treatment options available (101), but hopefully this will be remedied in coming years. Fortunately patients infected with this genotype generally have a relatively high likelihood of achieving SVR following interferon-based therapy. Thus HCV genotype 3 infected patients, especially those with the CC variant of *rs12979860* and the TT variant of *rs8099917*, may benefit from early initiation of therapy, and perhaps should consider interferon-based therapy, in spite of the considerable side effects, if they are unlikely to obtain interferon-free treatment within the foreseeable future.

The underlying mechanism of action of genetic variants in proximity to *IL28B* remains unclear. More evidence suggests that these variants are linked with the production and regulation of the endogenous interferon response. Indeed it has been hypothesized that the CC variant of *rs12979860* is associated with lower baseline intrahepatic expression of ISGs and that this could explain the improved response upon exogenous administration of interferon during HCV interferon-based combination treatment. Somewhat counterintuitive to this thought is the observation that lower ISG expression does not causally correlate with the levels of IL28B/IFN-λ3 expression (143), highlighting the complexity of this issue. The recent identification of the 2 SNPs, *rs368234815* and *rs117648444*, generating three major haplotypes where production of fully active fully active IFNλ4-P70 or the less active

IFNλ4-S70 is associated with significantly lower likelihood of spontaneous clearance as well as SVR after peg-interferon and ribavirin treatment in comparison to variants that negate the production of IFNλ4 (145), is a very compelling molecular mechanism that warrants further evaluation.

In conclusion, the genetic variants in close proximity of *IL28B* seems to regulate HCV genotype 2 and 3 differently where the CC variant of *rs12979860* and the TT variant of *rs8099917* is associated with more severe liver histopathology as well as higher Fibroscan measurement values in HCV genotype 3 infected patients. Thus it may be clinical relevant to screen HCV genotype 3 patients regarding *IL28B* genotype, and to encourage those with either the CC variant of *rs12979860* and the TT variant of *rs8099917* to consider earlier initiation of therapeutic intervention.

5.2 Impact of host genetic factors (*PNPLA3*, *ITPA* and *IL28B*) on HCV treatment outcome

Previous diverging reports have suggested that homozygosity for the *PNPLA3* 148M variant, aside from being associated with more pronounced steatosis, also either negatively impacts therapeutic outcome (120), or is not independently associated with treatment failure (117, 118). In the NORDynamIC that enrolled an HCV genotype 2 or 3 infected cohort of Nordic patients, SVR rates after 12 or 24 week with peg-interferon and ribavirin treatment were not associated with *PNPLA3* genotype, but as previously noted, the low prevalence of homozygosity for the *PNPLA3* 148M genetic variant may have obscured any possible association. However, interesting findings regarding viral kinetics during treatment were noted. Among patients infected with HCV genotype 2 with homozygosity for the *PNPLA3* 148 M variant, significantly lower baseline viral loads were observed as well as at treatment days 3 and 7 (fig 4) (118). HCV virions circulate in sera as lipoviral complexes, i.e. HCV particles in association with host lipoproteins, and the *PNPLA3* 148M genotype influences hepatic lipid accumulation (121, 171). Thus it has been hypothesized that the *PNPLA3* 148M genotype may impair both viral and lipoprotein release from infected hepatocytes, thus resulting in lower HCV RNA levels. However, this was only observed among HCV genotype 2 infected patients, but not in HCV genotype 3 infected patients, where baseline HCV RNA in homozygosity for the *PNPLA3* 148M on the contrary was higher, and again this may likely be a

direct viral effect of HCV genotype 3 (74-76). A novel finding observed among the NORDynamIC cohort was higher HOMA-IR among HCV genotype 2 infected patients having homozygosity for the *PNPLA3* 148M genotype (118, 172). Insulin resistance and steatosis are closely associated, although the interpretation and clinical implications of these findings are somewhat difficult to interpret. Previous studies noting an association between homozygosity for the *PNPLA3* 148M and increased steatosis, have not reported any such association with insulin resistance (106).

Regarding SNPs in proximity to *IL28B* and their impact on treatment outcome, the CC variant of *rs12979860* and the TT variant of *rs8099917,* previously have been reported to be strongly predictive of treatment outcome in HCV genotype 1 (88, 137). In the setting of the NORDynamIC trial, the CC variant of *rs12979860* was not found to be associated with SVR for HCV genotype 2 or 3. This is consistent with some previous reports that also failed to demonstrate any such association (128, 131). In contrast, other studies have reported that CC$_{rs12979860}$, as compared to the TT variant, is a positive predictor of SVR among Caucasian HCV genotype 2/3 infected patients (129, 130), especially among patients not achieving RVR.

Reduced ITPase activity, on the other hand, was predictive of SVR in addition to being protective of ribavirin induced anemia in the NORDynamIC study. Previous reports also have shown that reduced ITPase activity is protective of ribavirin induced anemia at week 4 although the majority of these reports have not found an association with SVR (158, 160, 165), possibly due to the use of higher, weight-based dosing of ribavirin in addition to less stringent monitoring of treatment adherence, thus not allowing for per-protocol analyses.

In the multivariate analyses using the NORDynamIC data, it was possible to demonstrate that SVR rates continuously improved as the predicted ITPase activity diminished, and that this improvement was linked to a reduced rate of relapse. Additionally, the increased rates of SVR was not secondary to improved adherence to ribavirin or protection against anemia, and remained significant after stratification for *IL28B* genotype, treatment duration and fibrosis grade. The reduced relapse rate in this study resembles that observed upon the addition ribavirin to peg-interferon monotherapy (80). Interestingly in the multivariate analysis, other independent, significant predictors, aside from ITPase activity, of the likelihood of achieving SVR in the NORDynamIC study included liver fibrosis stage, *IL28B* variant, baseline HCV RNA level, treatment duration (*i.e.* 12 or 24 weeks), and ribavirin concentrations day 29. Thus the CC variant of *rs12979860* did appear to

impact on treatment outcome in the NORDynamIC study in the multivariate analysis when predicted ITPase activity was included in the analysis.

Surprisingly, reduced ITPase activity was also associated with lower ribavirin concentrations at week 4 (fig 11), which otherwise is a predictor of treatment failure (173). This may have been secondary to a larger volume of distribution secondary to less anemia allowing for increased entrapment of ribavirin in erythrocytes, and thus leading to lower extra-cellular concentrations in plasma.

The discrepancy between our and previous studies regarding the impact of ITPase activity on SVR partially may be explained by ribavirin dosing, where other studies commonly have used weight-based ribavirin dosage of 800 to 1400 mg ribavirin per day in contrast to the standard, fixed 800 mg daily dose, as was used in the NORDynamIC study (158, 160, 165). Two studies enrolling Japanese patients that were genotyped only for the *rs1127354* SNP demonstrated that reduced ITPase activity was predictive of improved SVR (166, 174). In the case of the study by Kurosaki et al, the increased likelihood of achieving SVR was observed in the group of patients with the favorable *IL28B* TT variant of *rs8099917* and was mediated by a reduced relapse rate, but improved ribavirin adherence could not be ruled out as a possible confounder (166).

The protective effect of reduced ITPase activity on ribavirin induced anemia is hypothesized to be secondary to avoidance of ATP depletion, which prevents erythrocyte membrane oxidative damage (163, 164). However, this is unlikely to explain the improved likelihood of achieving SVR observed in the NORDynamIC study. In contrast, we hypothesize that the observed reduced relapse rate, associated with reduced ITPase activity, may be mediated through a ribavirin-like mechanism of action. Among other proposed molecular mechanisms, ribavirin reportedly is a competitive inhibitor of the enzyme IMPDH (175), and ribavirin induced inhibition of IMPDH results in increased intracellular concentrations of ITP as well as reduced levels of GTP. Similarly reduced ITPase activity results in increased levels of ITP and dITP, which falsely may be incorporated into RNA and DNA producing mistranslation, enzyme inhibition and genetic instability (150-152). Ribavirin also is proposed to be a relative inefficient inhibitor of the HCV RNA-dependent RNA polymerase (NS5B) polymerases by the incorporation of RTP into RNA (176), also leading to increased mutagenesis in HCV as demonstrated in the full-length replicon system (177), presumably resulting in the production of defective virus. Thus reduced ITPase activity and ribavirin may act synergistically by depleting GTP, and subsequently

increasing the likelihood of incorporation of ITP and RTP into the HCV
genome leading to increased frequency of random mutagenesis.

The reduced ITPase activity in our study is predicted by the compound
genotypic analysis of both *rs1127354* and *rs7270101* (table 3). These
compound estimates of activity are based on previous biochemical analyses,
where the ITPase activity was determined in lysated erythrocytes as
micromoles IMP formed per hour per gram hemoglobin (148, 149, 154). It is
important to bear in mind that erythrocytes may not be the optimal cell type
to determine ITPase activity, and that marked variations in ITPase activity
have been noted depending on the origin and age of the cells studied.
Additionally, technical difficulties such as the potential presence of inhibitory
Inosine diphosphate (IDP) in the ITP used as substrate in addition to the
relative high costs of the direct ITPase assay, make estimates of ITPase
activity based on the compound genotype preferential, especially for
population based screening of ITPase activity.

So in conclusion, reduced ITPase is associated with protection against
ribavirin induced anemia and increased likelihood of achieving SVR in HCV
genotype 2 and 3 infected patients when treated with standard 800 mg daily
ribavirin dosing, and patients with normal ITPase activity may benefit more
from the use of ribavirin.

6 CONCLUSION

- In our study of Nordic HCV genotype 2 and 3 infected patients, homozygosis for the *PNPLA3* 148M genetic variant was not associated with steatosis, fibrosis or cirrhosis. Nor was any association noted with SVR. The low frequency of the *PNPLA3* 148M genetic variant does not exclude the possibility of existing associations; although it allows us to conclude that it is of limited clinical relevance to screen Nordic HCV patients for variants of this gene (paper I).

- The genetic variants in close proximity of *IL28B* appear to regulate HCV genotype 2 and 3 differently, where the otherwise favorable CC variant of *rs12979860* and the TT variant of *rs8099917* is associated with more severe liver histopathology in HCV genotype 3 infected patients, and these patients may benefit from earlier initiation of therapy. However, the genetic variants of *rs12979860* were not associated with the likelihood of achieving SVR (paper II and III).

- The abovementioned finding that the CC variant of *rs12979860* among HCV genotype 3 infected patients was associated with more severe liver damage was confirmed by an independent, real-life trial evaluating liver stiffness by means of Fibroscan (paper IV).

- Reduced ITPase activity is associated with protection against ribavirin-induced anemia and increased likelihood of achieving SVR by means of reduced relapse risk in both HCV genotype 2 and 3 infected patients treated with standard 800 mg/day dose of ribavirin. Patients with normal ITPase activity may benefit more from ribavirin, possibly at a higher weight-based dosing (paper V).

7 FUTURE PERSPECTIVES

In HCV genotype 2 and 3 infected patients enrolled in the NORDynamIC study, *IL28B* genotyping was not associated with treatment outcome following peg-interferon and ribavirin treatment, in contrast to what has been reported for HCV genotype 1 (88). The clinical relevance of *IL28B* genotyping in the setting of the new interferon-free regimens likely will diminish, also for HCV genotype 1 (134). However, one study enrolling HCV genotype 1 patients treated with faldaprevir and deleobuvir with or without ribavirin reported of higher SVR rates in the $CC_{rs12979860}$ as compared to the non-CC variants, suggesting that the endogenous interferon response somehow affects treatment outcome in the absence of exogenous interferon. In addition, the recent discovery of the relation between IFN-λ4 and genetic variants in proximity to *IL28B* (145) remains to be elucidated in the setting of interferon-free regimens. The future clinical importance of *IL28B* genotyping and other host genetic analyses largely depends on the forthcoming cost of HCV interferon-free therapies, as they potentially may play a roll in tailoring the choice of treatment regimen and duration.

In the setting of the NORDynamIC trial, reduced ITPase activity was associated with increased likelihood of achieving SVR by means of reduced risk of relapse for both HCV genotype 2 and 3 infected patients, although the clinical relevance of *ITPA* genetic testing remains to be elucidated in the setting of interferon-free regimens.

The clinical utility of *IL28B* for prediction of the natural course of HCV-related liver disease may likely remain of clinically importance, particularly for HCV genotype 3 infected patients. The $CC_{rs12979860}$ and the $TT_{rs8099917}$ variants in HCV genotype 3 infected patients enrolled in the NORDynamIC study was associated with more pronounced liver histopathology, and thus these patients may benefit from initiating treatment in an early stage, also with the currently available interferon-free regimens.

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