Impact of Genetic Variants in Inosine Triphosphate Pyrophosphatase and Interferon-λ4 on Natural History, Treatment Response and Ribavirin Pharmacology in Hepatitis C Virus Infection

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"Science makes people reach selflessly for truth and objectivity; it teaches people to accept reality, with wonder and admiration, not to mention the deep awe and joy that the natural order of things brings to the true scientist" -Lisa Meitner
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ABSTRACT
Hepatitis C virus (HCV) impacts on global health with around 70 million chronically infected worldwide. The infection increases the risk of cirrhosis and primary liver cancer. The treatment until 2013 has been based on interferon-α and ribavirin, but is now replaced by direct acting antivirals. Ribavirin is still used in the most difficult-to-cure patients. This thesis evaluates host genetic variations in inosine triphosphate pyrophosphatase (ITPA) and interferon-λ4 (IFNL4) in relation to cure rates in patients treated with interferon-α and ribavirin as well as ribavirin pharmacology in the setting of chronic HCV infection, and spontaneous resolution of acute HCV infection. In a post-hoc analysis of 354 HCV genotype 2/3 infected patients receiving interferon-α and ribavirin, genetic variation in ITPA entailing reduced ITPase activity was associated with increased cure rates (paper I). Small inhibiting RNA aimed at ITPA reduced ITPase levels and increased the antiviral effect of ribavirin, ribavirin-associated viral mutations and concentrations of ribavirin triphosphate intracellularly, in vitro. ITPase was also shown to be able to dephosphorylate ribavirin triphosphate (paper III). In a randomized trial, standard interferon-α and ribavirin treatment was compared to four weeks ribavirin monotherapy prior to combination treatment and to two weeks of ribavirin double dosage alongside with interferon-α. Both experimental strategies succeeded in reaching high ribavirin concentrations at earlier timepoints in dual therapy. Ribavirin monotherapy resulted in a viral decline associated with IFNL4 genotype (paper II). IFNL4 genotype was associated with clearance in acute HCV genotype 1 as well as in genotype 2/3 infection. ITPA genotype showed significant associations with age at seroconversion and spontaneous resolution in males with favorable IFNL4 genotype (paper IV).

Keywords: Hepatitis C virus, HCV, Inosine triphosphate pyrophosphatase, ITPA, interferon-λ4, IFNL4, ribavirin, interferon, PWID, IP-10.
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Hepatit C virus (HCV) orsakar en kronisk leverinfektion hos ungefär 2/3 av alla som infekteras. Infektionen leder till en gradvis leverskada med ökad bindvävsinlagring som hos vissa orsakar levercirrhos med efterföljande decompenserad leversjukdom eller levercancer. Behandlingen bestod länge av interferon tillsammans med guanosinanalogen ribavirin (RBV), men har de senaste åren ersatts av mer effektiva läkemedel riktade direkt mot specifika virusproteiner. RBV används nu med viss framgång bl.a. vid svåra infektioner med respiratoriskt syncytievirus samt vid blödarfebrarna Lassa feber och Krim-Kongo feber, men även till de svårast sjuka patienterna med hepatit C och decellerad levercirrhos.

Syftet med denna avhandling var att undersöka hur vanliga värdgenetiska variationer i generna för inosintrifosfatpyrofosfatas (ITPA) och interferon-λ4 (IFNL4) påverkar frekvensen av spontanläkning av akut HCV infektion, utläkning vid kombinationsbehandling med pegylerat IFN (pegIFN) och RBV, virusnedgång under RBV monoterapi hos patienter med kronisk HCV-infektion, samt av hur dessa polymorfismer påverkar farmakokinetiken av RBV.

ITPA kodar för enzymet ITPas vars funktion är att bryta ner potentiellt skadliga nukleotider i våra celler som annars felaktigt skulle kunna inkorporeras i DNA, RNA eller påverka olika enzymer. Interferon-λ4 är en nypptäckt typ III interferon med antiviral effekt. En betydande del av befolkningen har en defekt gen för detta interferon.

Hos 354 patienter infekterade med HCV genotyp 2 eller 3 behandlade med pegIFN och RBV var genetiska variationer som orsakar försämrad funktion av ITPaset associerade med högre utläkningsfrekvens (odds ratio 6,4, p=0,003), lägre hämoglobinnedgång samt lägre plasmakoncentrationer av RBV (delarbete I). I en uppföljande in vitro-studie transfekterades en levertrumörcellinje med small inhibitng RNA (siRNA) mot ITPA. Cellerna behandlades sedan med RBV i olika koncentrationer och infekterades med HCV. siRNA-behandlingen minskade som förväntat mängden ITPase och detta resulterade i förbättrad antiviral effekt av RBV, fler RBV-inducerade HCV-mutationer och högre intracellulära RBV-trifosfat-nivåer. Vi kunde också i en separat analys visa att ITPaset defosforylerar RBV-trifosfat ungefär lika effektivt som dess naturliga substrat inosintrifosfat (delarbete III).

I en randomiserad nordisk multicenterstudie (n=61) med patienter infekterade med HCV genotyp 1, utvärderades effekten av två veckors behandling med...
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Genvarianterna i ITPA och IFNL4 analyserades även hos patienter med akut HCV från sprututbytesprogrammen i Malmö (n= 139) och Stockholm (n=115) (delarbete IV). Vid akut HCV var IFNL4, kopplat till markant förbättrad utläkningsfrekvens hos både HCV genotyp 1 och genotyp 2/3 infekterade patienter. Hos män med defekt gen för IFNL4 var nedsatt ITPas-aktivitet kopplat till både utläckning och ålder vid insjuknande av akut HCV.
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ABBREVIATIONS

ATP  Adenosine triphosphate
DAA  Direct acting antiviral
EOT  End of treatment
GTP  Guanosine triphosphate
HBV  Hepatitis B virus
HCC  Hepatocellular carcinoma
HCV  Hepatitis C virus
HCVcc  Hepatitis C cell culture system
IFN  Interferon
IFNL  Interferon lamda
IMP  Inosine monophosphate
IMPDH  Inosine monophosphate dehydrogenase
Indel  Insertion/deletion
IP-10  IFN-γ-inducible protein 10 kDa
ISG  Interferon stimulated gene
ITP  Inosine triphosphate
ITPA  Inosine triphosphate pyrophosphatase (gene)
ITPase  Inosine triphosphate pyrophosphatase (enzyme)
ITT  Intention-to-treat
JFH-1  Japanese fulminant hepatitis virus 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>LD</td>
<td>Linkage disequilibrium</td>
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<tr>
<td>NEP</td>
<td>Needle exchange program</td>
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<tr>
<td>PegIFN</td>
<td>Pegylated interferon</td>
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<tr>
<td>(rt)PCR</td>
<td>(real time) Polymerase chain reaction</td>
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<tr>
<td>PWID</td>
<td>People who inject drugs</td>
</tr>
<tr>
<td>RAS</td>
<td>Resistant associated substitution</td>
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<tr>
<td>RAV</td>
<td>Resistant associated variant</td>
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<tr>
<td>RBV</td>
<td>Ribavirin</td>
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<tr>
<td>RMP</td>
<td>Ribavirin monophosphate</td>
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<tr>
<td>RDP</td>
<td>Ribavirin diphosphate</td>
</tr>
<tr>
<td>RTP</td>
<td>Ribavirin triphosphate</td>
</tr>
<tr>
<td>RVR</td>
<td>Rapid viral response</td>
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<tr>
<td>siRNA</td>
<td>Small inhibiting RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOC</td>
<td>Standard-of-care</td>
</tr>
<tr>
<td>SVR</td>
<td>Sustained virological response</td>
</tr>
<tr>
<td>VRVR</td>
<td>Very rapid virological response</td>
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1 INTRODUCTION

1.1 HEPATITIS C VIRUS

Hepatitis C virus (HCV) infection is a blood-borne disease responsible for approximately 500,000 deaths annually, and it is estimated that 69 million people, i.e. ≈1% of the world’s population, are chronically infected (1). The disease was prior to the identification of HCV referred to as non-A non-B hepatitis, but in 1989 a research group lead by Michael Houghton was able to isolate, clone, and sequence the viral genome as well as also develop an antibody assay for detection of the virus (2). The virus belongs to the family Flaviviridae, which also include other well known viruses such as yellow fever virus, West Nile virus and dengue virus. It is, as the only known human pathogen, grouped in the genera of Hepacivirus. Until recently, HCV was the only described species in this genus, but recently several additional species have been discovered including a canine Hepacivirus (3). Interestingly, the most closely related virus is found in horses, the equine hepacivirus, suggesting that this may have been the zoonotic source of human HCV (4). Aside from humans, HCV is only able to infect chimpanzees (5). HCV exists in six major genotypes, although a total of eight different genotypes and several subtypes have been identified thus far (6, 7). Genotypes differ at around 30-35% of the nucleotide sites and relative prevalence differ based on geographic region. Generally genotype 1 is the most common, followed by genotype 3, and all remaining genotypes account for about one fifth of infections globally (8).

1.1.1 STRUCTURE AND GENOME

HCV is heterogeneous in size but typically range from 40-100 nm in diameter, and the shape is roughly spherical. It is surrounded by a thick shell of different forms of host apolipoproteins. The precise nature of the association between the virus and apolipoproteins remains unclear, but apolipoproteins seem to interact with the envelope lipids or proteins. HCV is sometimes referred to as a “lipoviral particle” or “lipovirion” (9, 10). Apolipoproteins likely shield the envelope proteins from immune detection, and seem to be important in HCV entry into hepatocytes. The viral RNA genome interacts with the viral core protein which is also the capsomere that forms the nucleocapsid. The capsid is surrounded by a lipid membrane envelope in which the viral glycoproteins E1 and E2 is anchored.

The viral genome is around 9.6 kb in length and is a positive-sense single-stranded RNA, thus it can be directly translated without any preceding
replication or transcription by the host polymerases. It codes for ten different proteins situated in one single open reading frame (ORF) that is translated into one polyprotein later processed into individual proteins by host and viral proteases. The genome is flanked with highly conserved untranslated regions (UTRs) in both the 5´ and the 3´ ends.

The 5´UTR is 341 bases in length and essential for viral replication and translation. The RNA in this region forms important secondary and tertiary structures. The outermost 125 nucleotides in the 5´ region binds the viral polymerase, whereas a 300 nucleotide sequence, partly overlapping the former, comprises the internal ribosomal entry site (IRES) (11). The IRES together with the host liver specific microRNA (miRNA) miR-122 facilitates the binding to the ribosome (12). HCV lacks a 5´-cap of methylated guanosine that is otherwise utilized by many other viruses to promote translation. Interestingly, a drug targeting miR-122 has been developed and was efficacious as HCV treatment in clinical trials, but the development was halted because of the rapid introduction of more effective DAAs (13), as well as fear of increased risk of hepatocellular carcinoma as loss of miR-122 is associated with gain of metastatic properties in liver cancer (14).

At the 3´ end, there is a highly variable poly U/UC tract followed by a 98 nucleotide long highly conserved region called the 3´X region and both these areas are needed for replication (15-17).

The ORFs ten proteins consist of the structural proteins core, E1 and E2, as well as the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The core protein binds RNA and is likely able to self-assemble into the viral nucleocapsid (18). The E1 and E2 are envelope proteins important for viral attachment and cell entry (19). They are heavily glycosylated and associate in heterodimeric complexes. The E2 protein contains two hypervariable regions (HVR), HVR1 and HVR2 which both show extensive nucleotide diversity (20). The p7 is a small protein that assembles to hexamers and seems to work as a channel for positively charged ions. It is not necessary for replication, but is required for the production of infectious virions (21). NS2 is a serine protease that cleaves between NS2 and NS3, but it is also involved in organizing the viral assembly (22, 23). Like p7, it is needed for productions of infectious virions, but not for replication. The NS3-NS5B are all needed for viral replication and together build a membrane associated replicase complex. The NS3 protein works partially as a helicase that likely unwinds the RNA duplex formed during replication (24). The other major function of NS3 is its protease activity, where it together with NS4A, cleaves the HCV polyprotein between NS3/4A, NS4A/NS4B, NS4B/NS5A, and
NS5A/NS5B. NS4B induces alternations of intracellular membranes needed to form the membranous web that has been shown to function as a scaffold for viral replication (25). The NS5A protein is together with NS5B and NS3/4, a major DAA target in HCV therapy. NS5A has no enzymatic function but seems to have different functions that are essential for both replication and virion assembly. Today is NS5A inhibitors the cornerstone of HCV treatment. NS5B is the RNA dependent RNA polymerase, and the drug target for nucleotide/nucleoside analogues (26).

1.1.2 REPLICATION CYCLE

Much of the knowledge on HCV replication cycle was gained by experiments using subgenomic replicons and later from the HCV cell culture (HCVcc) system. Entry of HCV is not fully understood, but it seems to involve complex procedures including many interacting cell surface molecules and intracellular signaling pathways. HCV in the form of an lipoviral particle (LVP) migrates from the blood into the hepatic perisinusoidal space (or space of Disse) through the fenestrated sinusoidal endothelium. The first attachment is mediated by the LDL-receptor and glycosaminoglycans (9, 10). The LVP is then able to interact with scavenger receptor B1 (SR-B1) allowing for E2 binding to CD81 (11, 12). The latter binding activates intracellular signaling pathways leading to migration of CD81 to the tight junction between hepatocytes where they form a complex able to interact with claudin-1 (CLDN1) and occludin (OCLD) (13, 14). Many more receptors are reportedly involved in viral entry and new interactions are continuously discovered (15). Entry into the cell is through clathrin mediated endocytosis. As pH decreases in the endosome, fusion between the viral envelope and the endosome membrane occurs leading to uncoating and release of viral RNA (16). As mentioned previously is the HCV genome positive-stranded RNA with an IRES, and thus ready to bind to the ribosome, which translates the RNA to the polyprotein. Cell and viral proteases subsequently cleave the polyprotein. The E1 and E2 proteins are secreted into the ER lumen and are glycosylated, whereas the core protein remains in the cytoplasm. The replicase complex consisting of NS3, NS4A, NS4B, NS5A, and NS5B is formed on the NS4A induced membranous web. The replicase complex initially makes a negative strand intermediate from the positive-strand HCV genome forming an RNA duplex, which is used as a template for further replications. The positive-sense RNA is then packed linked to the core protein which is likely released into ER and the newly synthesized virions leaves the cell via the secretory pathway (17).
1.1.3 QUASISPECIES AND ERROR CATASTROPHE

Hepatitis C virus is extremely efficient in creating new viruses with around $10^{12}$ new viruses being made daily in an infected human. They are short-lived with half-lives of around 45 minutes, and as the RNA polymerase is often inaccurate and lacks proof reading capability, considerable mutagenesis occurs (18). The high frequency of mutations leads to a swarm of genetically distinct but closely related viruses in each infected individual, referred to as quasispecies. Although the mutations caused during replication are almost random, evolutionary pressure rapidly shapes the quasispecies cloud, which reflects a balance between the need to preserve vital functions, selective forces in the environment from especially the immune system and the ability to replicate new variant viruses. As the error frequency of NS5B is approximately $10^{-3}$/per site, the viral genome is $10^4$ bases and around $10^{12}$ viruses are produced daily, theoretically $10^{13}$ mutations occur each day (19). However, the consensus sequence in an infected patient as measured by Sanger sequencing varies only around $1-3 \times 10^{-3}$/site annually (20, 21). Similarly, the genetic variants of HCV quasispecies within the same patient have around 91-99% sequence similarity in conserved regions such as NS5B and core-regions, but less so in HVRs, for example in E1 (22). This high mutation frequency makes the virus a difficult and evasive target for the immune system. There are of course limits to the mutation rate, and when surpassed, the virus loses its genetic integrity and viral replication is disrupted. This phenomena is sometimes referred to as lethal mutagenesis or error catastrophe (23). Drugs that increase mutation rates seems to be able to direct viruses into such a process, as has been reported for HIV, HCV, Hantaan virus, and Polio virus in vitro (24-27).

1.1.4 HCV CELL CULTURE SYSTEM

HCV has been notoriously difficult to propagate in cell culture systems. To understand the viral life cycle and to find suitable drug targets much effort has been spent to try to replicate the virus in vitro. The first major breakthrough was the development of the subgenomic replicons. These replicons are based on bicistronic RNA constructs carrying two different genes in the same vector. One gene is an antibiotic resistance gene directed for translation by the HCV IRES sequence and the other harbors the genetic code for the HCV non-structural genes necessary for replication (NS3-NS5B) driven by the IRES from encephalomyocarditis virus (EMCV). These replicons are propagated in Huh-7 cells (28), an immortalized liver tumor cell line, and have been developed for HCV genotypes 1-6. They are not infectious as they lack the HCV structural proteins, but have been invaluable for studying replication and protein function, as well as for anti-viral drug development (29).
To study the entry of HCV into cells, another system called HCV pseudo particles (HCVpp) was developed. This system is based on plasmids containing the HCV envelope proteins E1 and E2 (30, 31).

The second major breakthrough occurred in 2005 when an HCV genotype 2A virus from a Japanese patient with fulminant hepatitis (JFH-1) was demonstrated to replicate in Huh 7 cells, making it possible to study the whole HCV life cycle in vitro (32). The JFH-1 virus has later been improved to replicate more efficiently when the core-NS2 region was substituted for that of another HCV genotype 2a strain and used together with the original NS3-5B region of JFH-1. This virus is called J6/JFH-1 virus and is used in paper III. Further alterations to the virus to create different variants have been made. The cell line most permissive for infection with JFH-1 is an immortalized human hepatoma cell line called Huh-7 cells, originally derived from a 57-year old Japanese patient in 1982. One derivative especially permissive for viral replication is Huh-7.5. The Huh-7.5 cells have a defective gene for Retinoic Acid-Inducible Gene (RIG-1), one of the major pattern recognition receptors for detecting HCV that is needed to elicit a strong innate antiviral defense (33). In paper III we used this liver cancer cells with defective RIG I signaling (Huh-7.5).

1.2 ASSESSING HOST GENETIC VARIATION

The human genome consists of 3x10^9 base pairs and harbors approximately 20,000 genes, and interestingly 98% of DNA does not code for any protein. Differences in the DNA sequence between two individuals is found at between 4.2-5 million sites, affecting approximately 20 million base pairs. Geographically or ancestrally remote individuals differ more (34). Only around 60 new mutations are seen in each person as compared to those existing in his or her parents (35). The most common and simplest form of genetic variation is a single nucleotide polymorphisms (SNPs) which exists in 1 in 100-300 bases in the genome. By definition, a SNP is base position that differ in more than 1% of the population. Around 90% of all genetic variation identified is SNPs and in the latest update from the “1000 genomes project” roughly 85 million SNPs have been identified in 2,500 sequenced individuals (36). SNPs are situated throughout the genome, both in coding as well as non-coding regions. In the coding region, a SNP does not necessarily entail a change in the amino acid sequence of a protein as different triplets of RNA bases can code for the same amino acid, also known as codon degeneracy. A SNP is called synonymous if it does not change the amino acid sequence of protein, and nonsynonymous if it does. A nonsynonymous SNP can either be a missense mutation causing a change in the amino acid sequence, or a nonsense mutation.
resulting in a premature stop codon. A SNP, in a non-coding region, may also have a major impact if it is situated in a position affecting gene splicing, transcription factor binding or non-coding RNA. A SNP can be referred to as an allele, and often alleles in proximity on the same chromosome are inherited together making up a haplotype. This linkage is based on the fact that DNA sequences close to each other in the genome are more likely to be inherited together and not be subjected to recombination during meiosis. Coinherited alleles or alleles inherited together more often than random are said to be in linkage disequilibrium (LD). The information on a certain SNP can with varying degree of certainty be representative of a whole haplotype. A big collaborative project called the HapMap project has developed a haplotype map to describe common patterns of genomic variation. This is exploited in genome wide association studies (GWAS), where SNPs representatives for different haplotypes are identified at several hundred thousand or millions of positions throughout the genome, thus reducing the number of SNPs required to be evaluated. Consequently, SNPs identified in GWAS studies are not necessarily causal variants, but instead may be in LD with another causal variant within the same haplotype. A GWAS can offer information on genomic haplotypes in patients that are associated with a certain trait or outcome. An odds ratio and a p-value can be calculated using for example $\chi^2$-test. The p-value must, however, be corrected for the immense multiple testing which generally requires that the p-value for a significant result must be lower than $5\times10^{-8}$. To reach genome wide significance, a prerequisite is often a large sample size, as exemplified by a study on insomnia with 1.3 million participants (37). However, some small studies have found interesting results with as few as 150 participants, but then only assessing certain parts of the genome (38). The result can be displayed in a Manhattan plot, where the p-value is plotted on the Y axis and SNP position on the X axis, see figure 1. GWAS have been particularly successful in the field of HCV infection, and many of the results in this thesis are follow-up studies based on GWAS results from patients with acute or chronic HCV infections.
The second most common category of genetic variation is simple insertion and deletion (indels), and together with SNPs they make up 99.9% of all genetic variation (39). In a deletion, one or more bases are absent in the genome, whereas in an insertion one or more bases are inserted in the genome. Often it is difficult to determine whether an indel is an insertion or deletion, hence the use of the term indel. If an indel is situated within a gene, it causes a frameshift, i.e. a shift of the reading frame, unless the indel is a multiple of three nucleotides, which however is the most common configuration (39).

1.3 IMMUNE RESPONSES AGAINST HCV

HCV is able to cause a chronic infection in most, but importantly not all patients. Once a chronic infection is established, the virus often produces $10^{12}$ progenies daily. Only a few viruses, e.g. HIV and hepatitis B virus (HBV), have similar properties. The tropism for the liver is likely a key factor as liver immunology differs from that of other organs with its higher degree of tolerability. The majority of blood passing through the liver comes from the gut via vena portae, and contains many proteins that have foreign, non-self origins, coming from diet as well as from the resident microbiota, which contains numerous species of microorganisms. Indeed, there is a constant flow...
of bacterial endotoxins passing the liver without triggering an innate immune response, the so-called endotoxin tolerance. The fenestrated sinusoids allow direct contact with the blood which is thoroughly sampled by resident reticuloendothelial cells (macrophages and dendritic cells) in the liver. These cells need to confer immunotolerance in most cases to maintain homeostasis in the liver, but also be able to switch to inflammation when needed. Immunotolerance is partly explained by these cells ability to create an immunosuppressive cytokine milieu locally within the liver with secretion of among other IL-10 and TGF-β, but also systemically by cell to cell signaling via MHC molecules with protein antigens to T cells or unconventional antigens via CD1d to NKT cells and γδ T-cells together with PD-1 and CTLA-4 surface markers (40). Illustrative of hepatic immunotolerance is that liver allograft transplantation is associated with less T-cell dependent rejection than other transplants, and some patients may even be able to stop immunosuppressive therapy completely over time (41). Exactly how the T cell tolerance is created in the liver is unclear, but it seems to be a complex network of many different cell types, cytokines and innate immunity components working together (42, 43). The switch from homeostasis towards inflammation in the liver seems to be a question of reaching a threshold of pattern recognition receptor (PRR) signaling from both pathogen-associated molecular patterns (PAMPs) from e.g. HCV RNA, but also from damage-associated molecular patterns (DAMPs) signaling where sufficient activation from several receptor types favors an inflammatory reaction (44).

1.3.1 INNATE IMMUNE RESPONSES

HCV virus may be detected already at the cell membrane or in the endosome by extra-cytoplasmic PRRs, such as toll like receptor 3 (TLR3) or by PRRs in the cytoplasm such as RIG-I and MDA5 that sense double-stranded RNA. RIG-I and MDA5 activates the mitochondria associates membrane adaptor protein (MAVS) and TLR3 activation trigger an adaptor protein called TRIF. The downstream signaling pathway of these two adaptor proteins are similar with nuclear translocation of interferon (IFN) response factor 3 (IRF3), IRF7 and NF-κB leading to transcription of many inflammatory genes including IFN-β. IFN-β will then bind to receptors on neighboring cells as well as the parental cell resulting in transcription of IFN-α as well as many other antiviral proteins. The IFNs are the main cytokine in the antiviral defense, directing the transcription in the cell towards an antiviral state as well as activate other cells of the innate immune system such as NK cells. There are three different types of IFNs, type I with IFN-α and IFN-β, type II with IFN-γ, and type III with IFN-λ1-4. Type I and III IFNs can be produced by the infected cell, but also by macrophages and dendritic cells, whereas type II IFN is produced by NK
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cells and T cells. Type I and III IFNs signal through different receptors, where type I IFNs bind to the ubiquitious IFN-α receptor, whereas type III IFNs signal via the IL10R2 chain together with the IFN-λ1 receptor 1 chain.

\textit{IL10R2} is most commonly expressed on epithelial cells in the respiratory and intestinal tracts, but infection with HCV increases the receptor density in the liver. All IFNs activate signal transducer and activator of transcription 1 (STAT1) one to form homodimers that act as a transcription factor and bind IFN-gamma activated elements (GAS) in IFN-stimulated genes. Type I and III IFNs additionally signal through a different pathway, and activate a heterotrimeric transcription factor consisting of STAT1, STAT2 and IRF9 called IFN stimulated gene factor 3, which binds to IFN stimulated response elements (ISRE), causing induction of IFN stimulated genes (ISGs), \textbf{figure 2}. The gene expression profile of type III IFNs seems to dominate, and differ compared to type I IFNs in HCV infected chimpanzees and primary liver cells (45). The IFN signaling pathways are subjected to negative feedback loops with inhibitors such as suppressors of cytokine signaling (SOCs) and ubiquitin-specific peptidase 18 (USP18). Importantly, USP18 does not exert a negative feedback on IFN-λ or IFN-γ (46). The ISGs codes for several hundred different

\textbf{Figure 2. Type I and type III interferon signaling pathway. IFN, interferon; IL-10R2, interleukin 10 receptor 2; Jak-1, janus kinase 1; Tyk 2, tyrosine kinase 2; STAT, signal transducer and activator of transcription; IRF-9, interferon regulatory factor 9; interferon stimulated gene factor 3; ISRE, interferon-sensitive response element; ISG, interferon stimulated gene.}
proteins with antiviral, immunomodulatory and antiproliferative functions (47). Several of these have antiviral activity against HCV (48).

During the first weeks of infection, HCV RNA rapidly increases and so does the activation of ISGs, but the endogenous IFN response often is unable to clear the infection without aid from the adaptive immune response (49). However, treatment with pegylated (peg) IFN-α2a in monotherapy is extremely efficient during the acute infection, if initiated during the first months of infection, with around 80% achieving a cure (50). In the setting of chronic infection, some patients continue to have a highly active immune response with hundreds of ISGs highly expressed, whereas others do not. It has been demonstrated that patients with a continuously high expression of ISGs do not respond to IFN-based treatment, secondary to preexisting hepatic up-regulation of ISGs, to the same extent as patients not having a continuous expression of ISGs. Since IFN-λ1-4 are not affected by the negative feedback of USP18, they seem to be good candidates for being responsible for the continues high expression of ISGs observed in some patients (46).

HCV has several virulence factors interfering with IFN responses. The NS3/4 is able to cleave and at least to some extent block the signaling from the PRRs TLR3, MDA5, and RIG-I by cleaving the adaptor proteins MAVS and TRIF (51, 52). This is, however, not a complete blockage as ISGs are heavily transcribed during the acute infection shown in experiments on chimpanzees (53). The HCV core protein also seems to block IFN signaling pathways by influencing STAT1. Several viral proteins also affect PKR thereby suppressing ISGs and eIF-2 translation factor and protein synthesis (54, 55).

NK cells use activating, as well as inhibitory, receptors for human leukocyte antigen (HLA) A-C to determine their threshold for activity. They are activated in HCV infected patients with up-regulation of the NKG2D activating receptor, as well as increased IFN-γ production (56). Activated NK cells secrete cytokines including IFN-γ, IFN-α and TNF, which in turn up-regulate ISG expression, inhibit viral replication, promote maturation of dendritic cells, and promote release of chemokines. NK cells may also cause a direct lysis of infected hepatocytes (54). Certain NK cell genes have been linked to resolution of acute infection, for example the combination of homozygosity for NK cell receptor KIR2DL3 (weak inhibitor of activation) in combination with HLA-C1 (57). If the HCV infection becomes chronic the NK cells are impaired in their ability to exert effector functions, especially IFN-γ production (58). HCV E2 protein has been reported to have an inhibitory effect on NK cells, but this has been disputed by others (59, 60). HCV might also have an inhibitory effect
of NK cell activation of the professional antigen presenting dendritic cells and in part inhibit the activation of adaptive immune system (61).

### 1.3.2 **IFNL4 POLYMORPHISMS**

Genetic determinants of spontaneous resolution of HCV infection was assessed in a big GWAS study looking at SNPs throughout the genome. The most significant association was found in genetic polymorphisms in chromosome 19 around the *IFNL* gene area, [figure 3](#).

The highest significance was seen for a SNP, rs12979860, in proximity of the IFN-λ3 gene (*IFNL3* aka *IL28B*). Homozygous carriage of the C allele in rs12979860 increased the likelihood of clearance markedly (62, 63). A thorough investigation of this area revealed a novel, overlooked gene, IFN-λ4 (*IFNL4*). rs12979860 was found to be situated in intron 1 in *IFNL4* and was in strong LD with an indel polymorphism, rs368234815, in *IFNL4* exon 1. The ancestral allele sequence is gccGctg (ΔG), and the alternate allele variant has the sequence gccTTcctg (TT) with a substitution mutation from G to T and more importantly an inserted T, resulting in a disruption of the open reading frame and a non-functional gene. The C allele in rs12979860 and the TT allele in

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**Figure 3. The interferon-λ locus with the SNPs/indel rs12979860, rs368234815.**
rs368234815 are very strongly associated with each other (64). $\Delta G_{rs368234815}$ carriers, harboring the functional $IFNL4$ gene, are able to produce IFN-$\lambda$ and have higher continues expression of ISGs. IFN-$\lambda$ is highly antiviral, but also blocks IFN-$\alpha$ signaling by affecting the negative feedback loop of USP18 in vitro (46). This might in part explain the counterintuitive association between a functional $IFNL4$ and impaired spontaneous clearance as well as diminished response to IFN-$\alpha$ based treatment in patients with HCV infection (46, 64, 65). Recently rs368234815 has been reported to be slightly superior to rs12979860 in persons of African ancestry with regards to prognostication of spontaneous HCV clearance, while the results are very similar in people of European and Asian descent, consistent with a weaker LD between these polymorphisms in the African population (64).

### 1.3.3 ADAPTIVE IMMUNITY

A strong and broad T cell response, with an increase in HCV specific CD4+ T helper (Th) cells and CD8+ cytotoxic T-cells (CTL), appears after approximately 4-8 weeks in acute hepatitis C infection, and is essential for spontaneous clearance (66). The onset of adaptive cellular immune response coincides with increased liver inflammation (67). Spontaneous clearance of infection is associated with the T cells’ ability to produce IFN-$\gamma$, and with T cell responses targeting multiple HCV epitopes (66-68). Loss of CD4+ T cell responses during acute infection seems to be associated with increased viremia and risk of chronicity (49). In chronic infections HCV specific Th cells are rare or even absent, whereas HCV specific CTLs commonly comprise around 1-2% of all CTLs (49, 69). Th cells, in chronic infection, also become limited in the amount of HCV epitopes they are able to recognize (70). The CTLs subsequently lose much of their ability to produce IFN-$\gamma$, proliferative, and eliminate infected cells that display their cognate antigen. These “stunned” CTLs are typically seen in chronic HCV infection (54). In chronic infection no new CTL receptor specificities are identified even though the virus has mutated its antigen to escape recognition (71). One possible explanation for this is the inhibition of functional Th cells (72, 73).

Antibodies usually become detectable within 8-12 weeks, but might be significantly delayed or absent. Seronegative HCV infection is very uncommon in healthy blood donors, but more common in HIV/HCV coinfection, hemodialysis, and other immunocompromised patients (74). Additionally, antibodies are not always detectable despite spontaneous resolution. This was demonstrated in a cohort of East German HCV-infected patients, who had spontaneously cleared their HCV infection after being infected with HCV contaminated immunoglobulins, where 18/43 (42%) had
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undetectable antibodies 18-20 years after infection (75). In most chronically infected patients, antibodies are directed toward both structural and non-structural viral proteins (76). The importance of antibodies in HCV infection seems to be less than that of the cell mediated adaptive immune response (76). The humoral response is inefficient to protect from re-infection, although re-infected patients generally clear their infection to a greater extent (77). Furthermore, two boys with congenital agammaglobulinemia have been reported to have had a mild disease and clear an acute infection, but contrasting reports have been published on hypogammaglobulinemic patients with more severe and rapidly progressing HCV (78-80). Interestingly, immunoglobulin prophylaxis containing anti-HCV antibodies has been shown to likely have had some effect in preventing sexually transmitted HCV, and also immunoglobulin against hepatitis B (HBIG), which before 1990 likely contained anti-HCV antibodies, may have prevented some post-transplant HCV infections following liver transplantation in both currently HCV-infected and uninfected patients (81, 82). However, HCV is often able to evade antibody recognition. The high mutation frequency, especially in HVR1 in the E2 protein where the tolerance for mutations is high, can lead to escape mutants. The envelope proteins are also highly glycosylated, and the virus is also be shielded by apolipoproteins (83, 84).

1.3.4 IFN-\(\gamma\) INDUCIBLE PROTEIN 10 kDa

Chemokines are cytokines working as chemoattractants for migrating cells. Functional T cell responses are necessary for clearing an HCV infection, and chemokines responsible for their recruitment are of considerable interest. One interesting chemokine, studied extensively in hepatitis C, is IFN-\(\gamma\) inducible protein 10 kDa (IP-10 aka CXCL10). High systemic and intra-hepatic levels of this chemokine is associated with treatment failure following IFN-based therapy, and the patients are also less likely to spontaneous resolve acute infection (85-90). IP-10 binds to the CXC chemokine receptor 3 (CXCR3), and consequently recruits CXCR3 expressing T cells, NK cells and monocytes to the site of infection (91). IP-10 is degraded into an antagonistic form of the full-length IP-10 protein by dipeptidyl peptidase-IV (DPPIV) also known as CD26 (92, 93). One study suggested that the lower rate of spontaneous resolution of acute HCV infection seen in patients with higher baseline levels of IP-10 is a due to a systemic build-up of truncated IP-10, thus possibly impairing migration of CXCR3 expressing cells to the infected liver. Furthermore, the study also showed patients failing IFN-based therapy to have higher baseline plasma DPPIV activity (90), which was subsequently confirmed in another study (94).
1.4 PURINE NUCLEOTIDE SYNTHESIS

The canonical purine nucleotides are adenosine triphosphate (ATP) and guanosine triphosphate (GTP). Besides building the DNA and RNA chains together with the pyrimidines they also serve as energy currency in many cellular processes. Their intracellular concentrations are tightly regulated to prevent mutations, and new ATP and GTP are synthesized in two different pathways, either de novo synthesis or through the salvage pathway (95, 96). De novo synthesis of purines requires several reactions, but the compounds used are simple amino acids and bicarbonate. The starting molecule is ribose-5-phosphate (R5P) produced through the pentose phosphate pathway. R5P is phosphorylated into the important sugar 5-phosphoribosyl-1-pyrophosphate (PRPP) by PRPP synthetase. PRPP can also be used in pyrimidine de novo synthesis. The first committed step towards purine production is the displacement of the pyrophosphate in PRPP by ammonia. The nitrogen containing purine rings are then successively built in several steps requiring energy from ATP, amino acids and formyltetrahydrofolate. The first purine built is the inosine monophosphate (IMP), which thereafter follow the pathways for GTP and ATP. Oxidation of IMP by the enzyme inosine monophosphate dehydrogenase (IMPDH) produces xanthine monophosphate (XMP) and subsequently GMP synthetase adds an amino group forming GMP, figure 4.

Figure 4. De novo synthesis of purines. R5P, ribose-5-phosphate, IMP, inosine monophosphate, XMP, xanthine monophosphate, AMP, adenosine monophosphate, GMP, guanosine monophosphate.
Building a GMP requires energy from ATP. Production of AMP from IMP is facilitated by the enzyme adenylosuccinate synthetase, and requires the energy from GTP (97). Ribonucleotide reductase subsequently forms the deoxy forms of ribonucleotides.

Salvage pathways use free purine bases either present from turnover in the cell or from diet. This is both an energy efficient way of making new purines, in addition to minimizing the degradation end product uric acid, which in high concentrations causes the joint disease gout. Free guanine and hypoxanthine are attached to PRPP by an enzyme called hypoxanthine-guanine phosphoribosyl transferase (HGPRT) to form GMP and IMP. Adenosine can be deaminated by adenosine deaminase (ADA) to hypoxanthine or be directly attached to PRPP by the enzyme adenine phosphoribosyltransferase (APRTase) resulting in AMP (97).

Balance and maintenance of sufficient concentrations of nucleotides are of course important for our cells as well as for viral replication. In vitro, adenoviruses has been shown to induce the pentose phosphate pathway to create more R5P important in de novo and salvage pathways of nucleotide synthesis (98). Interestingly, Human Immunodeficiency Virus 2 (HIV-2) encodes a protein, vpx, which maintains high levels of dNTPs in myeloid cells. Vpx inhibits the effect of the ISG SAMHD1, that otherwise dephosphorylates dNTPs. Similarly, several bacteriophages, i.e. viruses infecting bacteria, are able to degrade large proportions of the bacterial chromosome in order to recruit sufficient levels of nucleotides for their own replication (99).

Pharmacological inhibition of enzymes in the purine synthesis pathways often have dramatic effects and are used in heavy immunosuppression and cancer treatment such as mycophenolate mofetil, azathioprine and 5-flourouracil. Interestingly these pharmaceuticals counterintuitively may have antiviral effects in vitro, while in vivo these effects often are counteracted by their immunosuppressive properties. For example, hepatitis E virus (HEV) has been shown to be inhibited by mycophenolate mofetil an IMPDH inhibitor and CMV is inhibited by azathioprine in vitro (100, 101).

### 1.4.1 INOSINE TRIPHOSPHATE PYROPHOSPHATASE (ITPASE)

Inosine triphosphate pyrophosphatase (ITPase) is an enzyme that has an important function in keeping the intracellular nucleotide pool free of unwanted non-canonical purines such as inosine triphosphate (ITP) and xanthine triphosphate (XTP), that otherwise might be incorporated during
transcription or replication (102). Experiment on human cell lines has shown that reduced ITPase activity is associated with increased DNA breaks (103). ITPase does not discriminate between ribose or deoxyribose nucleotides, and dephosphorylates them to their corresponding monophosphate forms, e.g. IMP and XMP, with equal efficiency. IMP as explained above can serve as substrate for AMP and GMP synthesis. Importantly, the enzyme has no effect on the canonical purines adenine and guanine (104). ITP can be produced in the purine biosynthesis pathway or by deamination of adenine or guanine nucleos(t)ides. ITPase is expressed in all major tissues, with the highest expression in humans being in the heart, thymus, liver and thyroid gland (105). Orthologs of ITPase exist in all domains of life, and also in some viruses, e.g. Cassava Brown Streak virus (106). Absence of ITPase activity is not compatible with life, as exemplified by mice, completely deficient in ITPase, die in utero or before weaning. Interestingly, knocking out the ortholog of ITPase in E.coli resulted in an approximately 10-fold increase of inosine containing RNA. However, combining the knockout of the ITPase ortholog together with knockout of the IMPDH ortholog in E.coli resulted in 100-fold increase in inosine containing nucleic acids (107). As ribavirin (RBV) reportedly inhibits IMPDH, this experimental set up in E.coli may bear some relevance to RBV treatment in patients with reduced ITPase activity, which comprise approximately a third of patients.

The inosine triphosphate pyrophosphatase (ITPA) gene, situated in chromosome 20, became highly interesting in the HCV field after a GWAS focusing on anemia in conjunction with IFN-based combination therapy. Two genetic variants, a missense variant in exon 2 (rs1127354, P32T) and a splicing-altering single nucleotide polymorphism (SNP) in intron 2 (rs7270101, IVS2) known to cause reduced ITPase enzyme activity, were demonstrated to protect against RBV-induced hemolytic anemia, figure 5 (108). This association has been confirmed in numerous studies including in RBV containing DAA treatment regimens (109-116). One suggested mechanism of action underlying the protection against anemia has been that the reduced ITPase activity induces higher intracellular ITP levels (117). ITP subsequently substitutes for GTP, depleted by RBV, as energy source in an early step in ATP synthesis by adenylosuccinate synthase, and in doing so prevents ATP depletion (117). ATP reduction is seen in erythrocytes during RBV treatment, but to a lower extent in patients with reduced pITPase. Erythrocytes rely on glycolysis as the primary source of ATP production since they do not contain any mitochondria, rendering them vulnerable to ATP
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depletion. ATP is essential as energy source in many intracellular processes (118).

![Figure 5. Genetic localization of ITPA and SNPs rs1127354 and rs7270101.](image)

Since the discovery of ITPase association with RBV-induced anemia, several post-hoc studies with HCV infected patients treated with IFN and RBV have confirmed this association. Several have also reported associations between genetic variations conferring reduced ITPase activity and higher likelihood of achieving a sustained virological response (SVR), mediated by reduced relapse rates (109-112). This reduction in relapse risk resembles the effect observed when RBV was added to IFN in the early era of HCV therapy (119). Since reduced ITPase activity protects against anemia, it also improves RBV adherence, which consequently may impact on outcome (115). Therefore, it is necessary to account for adherence in the SVR analysis. Contrasting the abovementioned studies, others have not been able to demonstrate any association between ITPA variants and SVR (108, 113-115). Potential explanations for these discordant results include differences in adherence monitoring, RBV dosing, underlying HCV genotype, etc.
1.5 HEPATITIS C VIRUS DISEASE

1.5.1 ACUTE HCV INFECTION

Acute HCV infection is defined as viral replication occurring during the first 6 months after presumed exposure. Viremia present after 6 months, by definition, entails a chronic infection. HCV RNA can be detected within a few days after exposure, and is followed by a rise in liver transaminases (76, 120). During the acute infection, viral RNA levels often fluctuates extensively, and may transiently become undetectable for relatively prolonged periods of time (121, 122).

The initial symptoms of acute HCV infection are absent or unspecific in more than two-thirds of patients (123). Patients who develop a symptomatic acute infection have typical symptoms of hepatitis, pain in the right upper quadrant, nausea, jaundice, and dark urine (124). These patients are more likely to spontaneously resolve their infection. Incubation periods vary between 2-26 weeks, but usually are approximately 7-8 weeks (125). In western countries, patients rarely develop fulminate hepatitis with acute liver failure (ALF), a syndrome with massive hepatic necrosis resulting in encephalopathy, coagulopathy, and often death (126). Interestingly, ALF is much more commonly associated with acute HCV in Asia as compared to Europe and USA, but the reasons for this remain unclear (127).

Spontaneous clearance of the infection rarely occurs after 6 months, and almost exclusively during the first 12 months of infection (128). The rate of spontaneous clearance depends on which cohort is being studied, but the overall clearance rate seems to be approximately 35% (48). There are several host factors associated with spontaneous clearance of acute HCV infection. A recent meta-analysis reported that female gender, non-black ethnicity, younger age, non-HIV coinfection, HBV coinfection, not having alcohol/drug related problems, and having a symptomatic acute infection were associated with increased likelihood of viral clearance (128). Also, there is a genetic basis for clearing infection summarized in the sections of innate and adaptive immune responses. Patients who previously were able to clear one infection, also reportedly have a better chance to clear a reinfection (77, 129).

As acute HCV infection is often asymptomatic, it is difficult to identify patients in this phase of infection. Thus, some studies use the discrepancy of anti-HCV
reactivity in combination with undetectable HCV RNA in the absence of antiviral treatment to define a resolved acute HCV infection, although this approach obviously will not identify individual spontaneously resolving without seroconversion or where antibodies levels wane over time to below cutoff thresholds. Longitudinal studies, however, offer a more reliable source of information. A way of identifying patients with acute HCV is by following patients with high-risk behavior, for example people who injects drugs (PWID), with consecutive prospective testing. Two such studies have been performed thus far in Sweden (77, 121). In the Stockholm needle exchange program cohort (NEP) HCV naïve patients had a clearance rate of 20%, and patients who were reinfected had a clearance rate of 44% (77). Similarly, in the cohort from the NEP in Malmö the one-year clearance rate was 32% in previously HCV naïve patients (121).

1.5.2 CHRONIC HCV INFECTION

In the majority of HCV infected patients, the immune system is unable to clear the virus resulting in a life-long infection unless cured by treatment. Spontaneous clearance after one year is infrequent, but may occur (128). Spontaneous resolution has been seen in patients with end-stage decompensated cirrhosis as well as hepatocellular carcinoma (130), although this is rare. The viral load is generally stable and high in patients with chronic infection, whereas the liver transaminases tend to fluctuate. Symptoms of chronic infection, with the exception of cirrhosis, generally are unspecific, with fatigue being the most commonly reported (131). However, curing the disease has been demonstrated to improve quality of life (132). There are also some important, but infrequent extra-hepatic manifestations of chronic HCV, such as essential mixed cryoglobulinemia, membranoproliferative glomerulonephritis, and sporadic porphyria cutanea tarda. Chronic HCV also seems to increase the risk of non-Hodgkin’s lymphoma (133). The main consequence of a prolonged chronic HCV infection is, however, the progressive accumulation of liver fibrosis, which characteristically begins with deposition of collagen and other proteins in the portal tracts, followed by bridging between tracts, and finally destruction of the normal hepatic parenchymal architecture that results in cirrhosis (134). The rate of fibrosis progression differ substantially with approximately a third of patients developing cirrhosis within 20 years, one third after 20-50 years, and the remaining may not develop cirrhosis within their lifespan (135). Once cirrhosis is established, patients are at marked increased risk of decompensation with liver failure, esophageal varices, encephalopathy, ascites, coagulopathy, and above all development of HCC. In cirrhotic patients, the risk for decompensation is 10-20% within 5 years and the annual risk of HCC is around 4% (136-138). HCV-associated HCC and decompensated cirrhosis (HCC and
liver failure with ongoing or past HCV infection) was until very recently the leading cause of liver transplantation in USA as well as in the Nordic countries (139, 140).

1.6 HCV TREATMENT

1.6.1 INTERFERON

Monotherapy with IFN-α for HCV infection was initially evaluated in a small clinical trial prior to the discovery of HCV, at the time referred to as non-A, non-B hepatitis, and an evident effect and sustained reduction of transaminase levels, indicating reduction of liver inflammation, was noted in some patients (141). Later, after the virus was characterized and HCV RNA analysis became available, randomized clinical trials showed SVR rates of only around 25% in these patients treated with IFN alone (142). When RBV was added to the therapy SVR rates improved substantially (119, 143), primarily by reducing the risk of relapse after treatment, rather than having any major impact on viral kinetics. Additional improvement of SVR rates were noted following the introduction of IFN-α slightly chemically altered by the addition of polyethylene glycol (PEG), which improved pharmacokinetics and allowed for less frequent dosing. The combination of pegIFN once weekly as subcutaneous injection and RBV twice daily orally was the standard-of-care (SOC) for many years. SVR rates for HCV genotype 2 and 3 were approximately 80% following 12- or 24-weeks treatment duration, whereas for genotype 1 only 40-50% cleared their infection despite treatment duration of 48 weeks or more (144, 145). IFN treatment causes significant adverse effects such as depression, fatigue, nausea, flu-like symptoms, elevated transaminases, and hematological toxicity (146). The receptor for type I IFN is ubiquitous whereas for type III IFN is restricted to cells of epithelial origin, and the effect on ISG is quite similar. This has led to development of a pegIFNλ1 drug, which has equivalent antiviral efficacy, less bone marrow suppression but more liver toxicity (147). The IFN pharmaceuticals are recombinantly produced and exert their mechanisms of action via induction of ISGs. ISGs create an intracellular antiviral state, but also have immunomodulatory effects such as activating NK cells, inhibiting T cell apoptosis, increasing cytokine signaling, and upregulating antigen presentation (148). For more information, please see the chapter 1.6.1 on innate immunity in this thesis. However, IFN-based HCV therapy differs from the endogenous IFN response as it achieves high systemic concentrations in blood. IFN treatment commonly causes a biphasic decline in HCV RNA concentrations. The first phase, seen during the first days of treatment, is characterized by a rapid viral decline thought to be caused by the
direct antiviral effect of IFN with decreased viral production. The second phase is slower and is believed to be associated more with the indirect effects of IFN such as death or clearance of HCV infected cells (149).

### 1.6.2 RIBAVIRIN

RBV is a guanosine analogue with a unique broad-spectrum in vitro activity against both RNA and DNA viruses (150, 151). Unlike many other anti-viral nucleoside/nucleotides, RBV triphosphate does not work as a chain terminator though the exact mechanism of action is unclear (152). It has mainly been used in HCV therapy, but to some extent also in severe respiratory syncytial virus (RSV) infections, viral hemorrhagic fevers such as Lassa and Crimean–Congo, and chronic HEV infections (153). RBV monotherapy has a modest effect on HCV RNA concentrations with around 0.5 log10 reduction, but markedly decreases liver transaminases and improves liver histology (154). The addition of RBV to IFN-based therapy strikingly increases cure rates (155, 156). However, following the introduction of IFN-free therapy for HCV, the use of RBV in HCV therapy has diminished. In some difficult-to-cure HCV infected patients, RBV still seems to be able to improve cure rates substantially. In the latest guidelines from European Association for the Study of the Liver (EASL), RBV is still recommended as an add-on to direct-acting antiviral (DAA)-treatment in patients with decompensated cirrhosis, where therapy with protease inhibitors is contraindicated, and may be considered in patients failing to achieve SVR after two treatments with a DAA combination containing either a protease inhibitor or an NS5A inhibitor (157).

RBV reaches steady state after around four weeks in patients with normal renal function treated with twice daily (1-1.2 g/day) RBV dosing for HCV infection. At steady state, plasma trough RBV concentrations reach approximately 8-12 µmol/L (158). Adverse events increase rapidly when trough concentrations exceed 15 µmol/L (159). Notably, intrahepatic concentrations of RBV seem to be roughly 30 times higher than in plasma (160). RBV is phosphorylated intracellularly to RBV mono-, di- and triphosphates (RMP, RDP, and RTP). The proportions of these nucleotides vary, but one report assessing sofosbuvir and RBV concentration in human liver transplant specimens demonstrated that unphosphorylated RBV and RMP were clearly most abundant followed by RDP and RTP. Importantly, the authors of this study emphasize, even though samples were frozen within 5 minutes ex vivo, that considerable dephosphorylation might have occurred (160). Results from primary hepatocytes, however, contradict this finding, and instead shows a clear predominance of RTP intracellularly, which is consistent with findings in red blood cells (RBC) and in peripheral blood mononuclear cells (PBMC) (161).
The uptake of RBV into cells relies largely on nucleotide transporter proteins where equilibrative nucleoside transporter 1 (ENT1) has been shown to be responsible for uptake in erythrocytes (162). This transporter is highly expressed on erythrocytes likely accounting for their very high intracellular RBV concentrations.

The major side effect of RBV is hemolytic anemia, with a mean decline in hemoglobin of about 20 g/L during treatment (159). This has been suggested to be secondary to oxidative stress, resulting from depletion of ATP in erythrocytes where, as stated above, RBV concentrations are very high (118, 163). Because of the risks associated with anemia in patients with a history of cardiovascular disease, RBV is contraindicated in these patients (159). Also, since RBV is secreted by the kidneys, pharmacokinetics are substantially altered in patients with renal impairment, demanding extra precautions when treating these patients (164). RBV is also associated with itching, rash, cough as well as neuropsychiatric side effects among which insomnia is most common (165). Importantly RBV has been shown to be teratogenic in animal models, but so far not in humans, and is thusly contraindicated during pregnancy and breastfeeding. Pregnancy must be avoided for 4 months after RBV treatment, and for 7 months for female partners to RBV treated men (166).

The antiviral mechanism of action of RBV is unclear but several have been proposed; i) GTP depletion by inhibition of inosine monophospate dehydrogenase (IMPDH), ii) direct inhibition of viral RNA-dependent-RNA polymerases, iii) viral mutagenesis leading to error catastrophe, iv) inhibition of mRNA capping and, v) immune modulation, figure 6 (148). All these theories have some evidence to support them, and possibly there are several mechanisms of action working together.

IMPDH is an enzyme that facilitate the oxidation of IMP to XMP, which is the rate limiting step of de novo biosynthesis of guanine nucleotides from IMP (167). The RMP form of RBV potently inhibits IMPDH already at low concentrations, subsequently resulting in depletion of intracellular GTP (168). Aside from being an essential purine nucleoside triphosphate needed for the synthesis of host and viral RNA/DNA during the transcription and replication, GTP is also a source of energy used in protein synthesis as well as gluconeogenesis. Moreover it is used as an energy source for the binding of a new amino-bound tRNA to the A site of the ribosome as well as for the translocation of the ribosome towards the 3’ end of the mRNA during elongation. Numerous signal transduction pathways, in particular with G-proteins, is dependent on GTP (169).
Most viral RNA-dependent RNA polymerases lack proofreading and may incorrectly incorporate RTP instead of GTP during replication. Unlike GTP, RTP is able to form base pairs with UTP or CTP with equal efficiency (170). In line with this, a RBV-induced increase in G-to-A and C-to-U single nucleotide variations (SNVs) has been reported for several HCV genotypes (171, 172). Additionally, recent in vivo studies indicate that similar RBV-induced mutagenesis appears in HEV and GB virus B (173). This RBV-induced mutagenesis could possibly push an already mutation prone virus into error catastrophe (174-176). For poliovirus, RBV induced a 9.7-fold increase in mutation rate which resulted in 99.3% loss in infectivity consistent with error catastrophe, in vitro (175).

RBP induced mutagenesis seems to occur at higher RBV concentrations (168, 176). The accumulation of RBV in the liver might, however, be sufficient to support even the direct mutagenesis theory (160).
The stability, transport and translation of eukaryotic mRNAs and many viral RNAs relies on methylation of a G base in their 5' end (known as the "five-prime cap"). Interestingly, RMP is able to competitively bind one of the capping enzymes instead of GMP, subsequently leading to impaired translation (177). HCV uses an IRES instead of 5'cap and its translation consequently would not be affected by this. Likewise it does not seem that RBV is able to directly inhibit the HCV RNA-dependent-RNA-polymerase (178).

Finally, RBV has also been proposed to have effect on innate and adaptive immunity. In a clinical study of chronic HCV, RBV monotherapy was shown to down-regulate the general expression of ISGs (179). A high ISG expression before IFN and RBV combination treatment is associated with a poor treatment response (180). It has been suggested that RBV restores IFN responsiveness in the liver of HCV infected patients, as pre-treatment with RBV leads to a greater increase in ISGs (181, 182). Several publications have reported that RBV modulate the Th cell balance favoring a Th1 skewed immune response (183-185).

1.6.3 DIRECT ACTING ANTIVIRALS

Recently, numerous DAAs have been developed aided by the discoveries of subgenomic replicons and the HCV cell culture system. They are tremendously superior to the previous IFN-based treatment both in efficacy and treatment induced adverse events. As the name implies, they interfere directly with the viral proteins thereby inhibiting its life cycle. There are three different classes currently used; the NS5B polymerase inhibitors with both nucleos(t)ide and non-nucleos(t)ide analogues, NS3/4 protease inhibitors, and the NS5A inhibitors used in different combinations and in certain really difficult-to-treat infections combined with RBV (157). The first DAAs approved for treatment were the first-generation NS3/4A protease inhibitors boceprevir and telaprevir. They were added to an IFN and RBV backbone, and were able to improve SVR rates in HCV genotype 1 infected patients from around 40-50% to 70-80%. Unfortunately they also increased side effects (186).

The next major breakthrough was the introduction of IFN-free treatment, which first appeared in 2014 limiting the treatment associated adverse events to a minimum or to levels equivalent to placebo. Today several different treatment regimens are available, with three being pangenotypic, i.e. not requiring HCV genotyping prior to initiation. Cure rates exceeding 95% generally are achieved, with almost all compliant patients being curable (157).
There are several different N3/4A protease inhibitors in use today; glecaprevir, voxilaprevir, and grazoprevir. However, they are contraindicated for use in patients with decompensated liver cirrhosis. Protease inhibitors previously had a relatively low barrier to resistance although this has improved in the last generation of inhibitors. The NS5A inhibitors also have a low barrier to resistance. The NS5A drugs used today are elbasvir, ledipasvir, pibrentasvir and velpatasvir. The polymerase inhibitor in use today is the uridine analogue inhibitor sofosbuvir, characterized by a high barrier to resistance and high potency.

There are 6 different drug combination used in Europe and recommended by EASL. Glecaprevir/pibrentasvir and sofosbuvir/velpatasvir are two fixed dose drug combination that are recommended for use in all genotypes. In difficult-to-cure patients, e.g. patients with relapse after an initial DAA therapy, voxilaprevir is often added to the latter regimen also in a fixed dose combination pill. The choice for suitable regimen often is based on price, HCV genotype/subtype, previous treatment experience, baseline viral load, and presence of baseline resistance associated substitutions (RASs) (157).
2 AIM

The overall aim of this thesis was to evaluate common genetic variations in *ITPA* and *IFNL4* with regards to treatment response, natural history and RBV pharmacology in patients infected with HCV.

2.1 SPECIFIC AIMS

- Does *ITPA* genotype impact on treatment response in patients infected with HCV genotype 2 or 3 treated with pegIFN-α and RBV?

- Does RBV in monotherapy for four weeks prior to combination treatment with pegIFN-α or 2 weeks of double dosing concomitantly with pegIFN-α affect anemia or treatment outcome in patients with HCV genotype 1?

- Does *IFNL4* genotype impact on HCV viral decline in patients on RBV monotherapy?

- Why does reduced predicted ITPase (pITPase) activity increase SVR rates in HCV infected patients treated with pegIFN-α and RBV?

- Does *ITPA* and *IFNL4* genotype affect spontaneous resolution of acute HCV infection?
Table 1. Baseline Characteristics for Patients and Cells in Paper I-IV

<table>
<thead>
<tr>
<th>Cohort (Paper)</th>
<th>RibavC (I)</th>
<th>NORDynamic (II)</th>
<th>Huh 7.5 cells (III)</th>
<th>NEP Malmö (IV)</th>
<th>NEP Stockholm (IV)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n=21</td>
<td>n=21</td>
<td>n=16</td>
<td>n=349</td>
<td>n=1</td>
</tr>
<tr>
<td>Age, mean (SD), years</td>
<td>46.5 (10.5)</td>
<td>44.2 (10.7)</td>
<td>47.0 (8.7)</td>
<td>41.9 (10.9)</td>
<td>57.0</td>
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<tr>
<td>Sex female n(%)</td>
<td>9 (43%)</td>
<td>9 (43%)</td>
<td>5 (31%)</td>
<td>142 (41%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>BMI, mean (SD), kg/m²</td>
<td>27.1 (3.9)</td>
<td>26.2 (3.5)</td>
<td>25.4 (4.4)</td>
<td>25.8 (4.3)</td>
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</tr>
<tr>
<td>Characteristics of HCV infection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV-RNA at baseline, log10 IU/mL</td>
<td>6.5 (0.6)</td>
<td>5.8 (0.7)</td>
<td>6.2 (0.6)</td>
<td>6.0 (0.9)</td>
<td>-</td>
</tr>
<tr>
<td>HCV genotype 1 n (%)</td>
<td>21 (100%)</td>
<td>21 (100%)</td>
<td>16 (100%)</td>
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<td>0 (100%)</td>
</tr>
<tr>
<td>HCV genotype 2 n (%)</td>
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<td>0</td>
<td>0</td>
<td>99 (28%)</td>
<td>1 (100%)</td>
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<tr>
<td>HCV genotype 3 n (%)</td>
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<td>0</td>
<td>0</td>
<td>260 (72%)</td>
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<tr>
<td>HCV genotype Unknown n (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>19 (14%)</td>
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<td>Liver disease severity</td>
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<tr>
<td>No significant fibrosis*, n(%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>151</td>
<td>-</td>
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<tr>
<td>Bridging fibrosis*, n(%)</td>
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<td>-</td>
<td>-</td>
<td>128</td>
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<td>Cirrhosis*, n(%)</td>
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<td>-</td>
<td>-</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>Unknown n(%)</td>
<td>21 (100%)</td>
<td>21 (100%)</td>
<td>16 (100%)</td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td>APRI-score, mean (SD), score</td>
<td>0.8 (0.6)</td>
<td>0.9 (0.8)</td>
<td>0.8 (0.6)</td>
<td>1.0 (1.2)</td>
<td>-</td>
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<tr>
<td>Treatment regimens and response</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12/24/48 week Peg-IFN + ribavirin,n(%)</td>
<td>0/2/19</td>
<td>0/7/14</td>
<td>0/15</td>
<td>0/168/181</td>
<td>-</td>
</tr>
<tr>
<td>Spontaneous clearance rate, n(%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>45 (32%)</td>
</tr>
<tr>
<td>Host genetics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITPase activity &lt;100%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>130 (37%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>IFNL4 rs12979860 genotype CC n (%)</td>
<td>7 (33%)</td>
<td>8 (38%)</td>
<td>7 (44%)</td>
<td>149 (44%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>IFNL4 rs130234815 genotype TTTT n(%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>59 (42%)</td>
</tr>
</tbody>
</table>

HCV, hepatitis C virus; APRI, aspartate aminotransferase; Peg-IFN, pegylated interferon; SD, standard deviation; SVR, sustained virologic response; SOC, standard of care; NEP, needle exchange program. *Liver fibrosis Stage by Ishak protocol.
3.1.1 NORDYNAMIC STUDY (PAPER I)

Paper I is a retrospective, post-hoc analysis of samples obtained from patients from the NORDynamIC study (145). The NORDynamIC study was a phase three, open-label, randomized, multicenter, investigator-initiated trial comparing 12 weeks vs. 24 weeks of treatment with pegIFN-α and RBV, where adherence to dosing of study medication was closely monitored by a patient diary. Importantly, the RBV dose was relatively low at 800 mg per day regardless of body weight, which at the time was the standard-of-care dosing for HCV genotype 2 or 3 infection. The study was carried out between 2004-2005 in Denmark, Finland, Norway and Sweden. Patients were chronically infected with HCV genotype 2 or 3. They were all adults, and had not received any previous treatment for HCV. Patients with decompensated cirrhosis, as IFN is contraindicated in this setting, as well as patient with HIV or HBV co-infection were excluded from study entry.

Three hundred eighty-two patients were included in the intention-to-treat (ITT) analysis. In paper I most of the analyses were performed on the 303 patients included in the per-protocol analysis (PP), i.e. patients having received at least 80% of the target dose of RBV and pegIFN-α for at least 80% of the planned treatment duration. Baseline characteristics for 349 patients from the NORDynamIC study, with samples available for the ITPA rs1127354 and rs7270101 genotyping, analyzed in paper I are presented in table 1.

3.1.2 RIBAC STUDY (PAPER II)

The RibaC study was an open-label, randomized, multicenter, investigator-initiated pilot study trial comparing different RBV treatment strategies. Between 2010-2012, patients with chronic HCV genotype 1 infection were recruited from centers in Sweden, Denmark, Finland and Norway. 61 patients met the inclusion criteria and 58 patients agreed to start medication and constituted ITT population. Only patients with compensated liver disease and those who were IFN treatment naïve as well as being seronegative for hepatitis B surface antigen (HBsAg) and HIV antibodies (anti-HIV) were included in the study.

Patients were randomized to one of three arms; arm A “loading” (n=21), arm B “priming” (n=21) and arm C “standard-of-care” (n=16). All patients received pegIFN α-2a 180 µg once weekly for either 24 weeks if they had undetectable HCV RNA, analyzed by their local laboratory four weeks after initiation of pegIFN-α therapy or 48 weeks if not. Additionally, Arm A “Loading” received ≥26 mg/kg/day (double dose) of RBV for two weeks followed by standard-of-
Impact of Genetic Variants in ITPA and IFNL4 on Natural History, Treatment Response and Ribavirin Pharmacology in Hepatitis C Virus Infection

care dosing (≥13 mg/kg/day). Arm B “Priming” received standard-of-care dosing of RBV without pegIFN-α for four weeks followed by standard-of-care therapy. Arm C received standard-of-care treatment. Stopping rules were applied for treatment discontinuation if the patients had ≤2 log_{10} reduction in HCV RNA by week 12 or detectable HCV RNA at week 24. Baseline characteristics stratified based on treatment arm are shown in table 1.

Primary endpoints in this study were the early virological responses measured by first and second phase decline, i.e. HCV RNA decrease day 0-3 and day 3-7, respectively. SVR rates were not selected as endpoints as RibaC was a pilot study with the aim of recruiting 120 patients, which was considered insufficient to reach sufficient power for such an endpoint. Secondary endpoints included assessing differences in hemoglobin, plasma HCV RNA, IP-10, IFNL4 genotype, RBV concentrations and also later on treatment responses in the different treatment arms.

3.1.3 THE COHORTS FROM THE MALMÖ AND STOCKHOLM NEPS (PAPER IV)

Paper IV is based on a post-hoc analysis of patients derived from two different prospective open cohort studies of patients from the NEPs in Malmö and Stockholm. Upon entry to the NEPs, the initial baseline serum samples were analyzed for anti-HIV, HBsAg and anti-HCV. All participants were requested to undergo prospective testing at three-six month intervals, which was accepted by a majority of participants.

Patients from Malmö were recruited between 1997-2005. During this period 332 patients lacking antibodies against HCV at the first baseline screening were followed. Of these patients 186 seroconverted indicating acute HCV infection. 150/186 anti-HCV seroconverters had complete series of pre-baseline, baseline and 1 year follow up HCV RNA testing and samples from 139 of these were available for genetic testing, constituting the study population. Their baseline characteristics are shown in table 1. In 18 cases viral genotype could not be determined because of low HCV RNA levels or absence of HCV RNA in the available samples. None of the patients had ongoing infection with HBV or HIV.

Patients from the Stockholm NEP were recruited between 2013-2016. From this cohort we analyzed all patients susceptible for HCV-infection, i.e. patients lacking detectable antibodies against HCV (HCV naïve) as well as untreated patients with anti-HCV antibodies but without detectable HCV RNA in serum
(sign of previously spontaneously cleared infection). In all, 584 patients were included in the study, 300 were HCV naïve and 284 had previous spontaneously cleared an HCV infection. 114/300 HCV naïve became infected during follow up and 93/284 of those with previous spontaneously cleared infection got reinfected. 68/114 of the HCV naïve patient and 57/93 HCV experienced patients with acute infection had a follow up test >5 months after infection and samples available for analysis. Baseline characteristics are shown in table 1.

Spontaneous viral clearance was defined as the absence of viremiae in one follow-up sample drawn approximately 12 months after the appearance of anti-HCV antibodies in the Malmö cohort, and as becoming HCV RNA negative in a sample obtained >5 months after a new HCV infection (in the HCV naive cohort) or after a reinfection (in the spontaneously cleared cohort) in the Stockholm cohort. Generally, a chronic infection is defined as HCV RNA positivity >6 months after infection. Since patients often were sampled at approximately 3 month intervals, a substantial number of patients were sampled a few days short of the 6-month limit. Thus, the 5-month limit was chosen for the Stockholm cohort.

### 3.2 METHODS

#### 3.2.1 HCV RNA QUANTIFICATION AND GENOTYPING

In paper I and II plasma were quantified and genotyped at both the local and central laboratory. For quantification at the central laboratory COBAS Ampliprep/COBAS Taqman were used with a limit of detection at \( \leq 15 \text{ IU/mL} \). Genotyping at the central laboratory was done using RT-PCR and Taqman probes targeting the 5’non-coding region of HCV.

HCV RNA from the patients from the NEPs in Malmö and Stockholm was detected using Ampliprep/COBAS Taqman but, for the Malmö patients, at a 10-fold dilution, increasing the limit of detection at \( \leq 150 \text{ IU/mL} \) and viral genotyping for the Malmö patients was performed using phylogenetic analysis of 321 nucleotides in the NS5B region (187).

#### 3.2.2 ITPA AND IFNL4 GENOTYPING

Polymorphisms were determined by allelic discrimination using Taq-Man SNP Assays (Life Technologies) for rs7270101 and rs1127354 in *ITPA*, and
rs368234815/ss469415590 and rs12979860 in IFNL4 using an in-house analysis as described previously (188). All SNPs were in Hardy-Weinberg equilibrium.

### 3.2.3 IP-10 QUANTIFICATION

Quantification of IP-10 was performed using Quantikine, a solid-phase enzyme-linked immunosorbent assay, on plasma samples.

### 3.2.4 JFH1/J6, HUH 7.5 CELLS AND SIRNA TREATMENT

Huh-7.5 cells were cultured in cell culture medium supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids and 1% penicillin-streptomycin. Huh-7.5 cell line is derived from primary liver cancer cells and are of rs1127354 C/C and rs7270101 A/A ITPA genotype corresponding to normal pITPase activity and TT/ΔG rs368234815 IFNL4 genotype, table 1. To mimic the reduced ITPase activity seen in patients we transfected Huh-7.5 cells with small inhibiting RNA (siRNA) directed against ITPA mRNA. Scrambled RNA was used as control.

HCV J6/JFH-1 is a cell culture adapted HCV virus of genotype 2 initially collected from a Japanese patient with fulminant hepatitis. The J6/JFH-1 plasmid was used for production of infectious HCV. First the plasmid was linearized and then transcribed to viral RNA using a T7 RNA polymerase. The viral RNA was transfected into Huh-7.5 cells using electroporation. After 96 hours culturing, infectious HCV virions was collected from the cell culture medium.

### 3.2.5 HCV CELL CULTURE SYSTEM

During HCV infection, siRNA treated Huh-7.5 cells were pre-treated with RBV +/- guanosine 2 hours prior to infection with J6/JFH-1 HCV virions. Cells were washed after four hours and medium or medium supplemented with RBV was added. Cells were analyzed 72 hours post infection. A RBV toxicity screen was performed where Huh-7.5 cells were cultured in medium supplemented with increasing concentration of RBV following ITPA siRNA-transfection.

### 3.2.6 MEASUREMENT OF HCV ANTIGEN

Antigen measurement, paper III: Supernatant from each HCV-infected well was analyzed in duplicate with Architect HCV Ag Assay for automated detection of HCV core antigen.


3.2.7 **HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY**

RBV drug concentration in plasma was measured just prior to the morning dose of RBV, *i.e.* trough concentrations in the RibaC study, by use of solid phase extraction and high-performance liquid chromatography (HPLC) followed by UV-detection (wavelength 215 nm) (**paper II**).

In **paper III**, Huh-7.5 cells were harvested and snap frozen in liquid nitrogen. Standard NTPs and intracellular RBV triphosphate (RTP) were extracted and measured in a LaChrom Elite HPLC system with a Partisphere SAX column. To enable measurement of RTP, detection was carried out at both 215 nm and 260 nm. The level of each nucleotide is expressed as % of the total sample NTPs.

3.2.8 **NEXT GENERATION SEQUENCING**

Isolated RNA was reverse transcribed to DNA using HCV-specific primers in NS4A and NS5B. The cDNA was amplified in a nested PCR. The PCR product was visualized on a 1% agarose gel and concentrations were measured with Nanodrop 2000. The 5’ and 3’ PCR products were pooled, barcoded and size selected to 200 base pairs using the Ion Xpress plus fragment library kit together with the AB Library Builder System. The generated libraries were then amplified and purified according to protocol before quantification and quality assessed using the High Sensitivity D1000 screen tape kit together with the Agilent 2200 TapeStation system. Six barcoded libraries were pooled before being amplified, conjugated to sphere particles and loaded on an Ion 314™ Chip using the Ion Chef. The samples were sequenced on an Ion PGM System. The results were analyzed with CLCbio, where each sample was quality checked. The quality trim threshold was set at 0.01 (PHRED score >25) and sequences above 30 nucleotides in length were aligned to the J6/JFH-1 strain. All sequences had a read depth of at least 1,000X. The aligned sequences were analyzed using low-frequency variation. To validate reproducibility and to check for PCR errors, the first PCR and the nested PCR were sequenced for five samples, resulting in a comparable number of mutations, insertions and deletions in the two PCRs run from the same sample.

3.2.9 **ITPASE ENZYMATIC ASSAY**

Dephosphorylation of ITP, RTP and GTP was analyzed using PiColorLock Gold Phosphate Detection System. A system that detect the presence of
phosphates. Recombinant ITPase together with GTP, ITP and RTP were incubated 37° C for 10 minutes to allow the enzyme cleave the pyrophosphate. The pyrophosphates were then degraded into single phosphates in room temperature for 30 minutes resulting in a color shift. The plates were measured at 620 nm on Multiskan FC.

### 3.3 STATISTICS

All statistics was performed in either Prism (Version 6.0c, GraphPad Software, La Jolla, CA) or SPSS (Version 20.0.0, IBM Corp, Armonk, NY, USA) software. All reported P values are two-sided, and P values < 0.05 were considered significant.

When comparing proportion of patients achieving a certain endpoint in paper I-IV either Fisher’s exact test or \( \chi^2 \)-test was done based on anticipated group sizes. When comparing continues variables between groups Man-Whitney U test was used in Paper II. In paper III both Student T-test and one-way analysis of variance followed by Dunnett test on, if needed, logarithmic, values were implemented. Also, differences in number of viral mutations was assessed using Poisson’s distribution test. In paper IV, one-way ANOVA of variance with Dunnett’s test for multiple comparisons was used to determine differences in age in different subgroups and Welch’s T-test for comparing age in two different groups. Correlations were evaluated using Spearman’s rank-order correlation in paper I and II. The choice of statistical methods was based on normal distribution, linearity and differences in distribution between groups. In paper I, logistic regression was done to evaluate the association between pITPase activity accounting for the 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, RBV concentrations day 29, and treatment duration. In paper IV, bivariate logistic regression was done to evaluate \( IFNL4 \) genotypes in relation to outcome accounting for covariates; pITPase activity, age at seroconversion, viral genotype and gender. In a separate analysis viral genotype was excluded and the different cohorts was included instead.

### 3.4 ETHICS

All studies were performed in accordance with 1975 Helsinki declaration. Written informed consent was obtained for patients in the NORDynamIC and RibaC studies and these studies were registered in the national institute of health (NIH) trial registry (ClinicalTrials.gov Identifier: NCT00143000 and
phosphates. Recombinant ITPase together with GTP, ITP and RTP were incubated 37°C for 10 minutes to allow the enzyme cleave the pyrophosphate. The pyrophosphates were then degraded into single phosphates in room temperature for 30 minutes resulting in a color shift. The plates were measured at 620 nm on Multiskan FC.

3.3 STATISTICS
All statistics was performed in either Prism (Version 6.0c, GraphPad Software, La Jolla, CA) or SPSS (Version 20.0.0, IBM Corp, Armonk, NY, USA) software. All reported P values are two-sided, and P values < 0.05 were considered significant. When comparing proportion of patients achieving a certain endpoint in paper I-IV either Fisher's exact test or χ²-test was done based on anticipated group sizes. When comparing continues variables between groups Man-Whitney U test was used in paper II. In paper III both Student T-test and one-way analysis of variance followed by Dunnets test on, if needed, logarithmic, values were implemented. Also, differences in number of viral mutations was assessed using Poisson´s distribution test. In paper IV, one-way ANOVA of variance with Dunnett´s test for multiple comparisons was used to determine differences in age in different subgroups and Welch´s T-test for comparing age in two different groups. Correlations were evaluated using Spearman's rank-order correlation in paper I and II. The choice of statistical methods was based on normal distribution, linearity and differences in distribution between groups. In paper I, logistic regression was done to evaluate the association between pITPase activity accounting for the 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, RBV concentrations day 29, and treatment duration. In paper IV, bivariate logistic regression was done to evaluate IFNL4 genotypes in relation to outcome accounting for covariates; pITPase activity, age at seroconversion, viral genotype and gender. In a separate analysis viral genotype was excluded and the different cohorts was included instead.

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4 RESULTS

4.1 ITPA GENETIC POLYMORPHISMS IN PEGIFNα AND RBV TREATMENT FOR HCV GENOTYPE 2 AND 3

A post-hoc analysis was performed using data from samples collected from the patients enrolled in the NORDynamIC study. The effect of SNPs in ITPA at rs1127354 and rs7270101 causing reduced enzyme activity, on virological endpoints as well as RBV induced anemia was evaluated. The majority of patients (63%) were homozygous for the major allele at both positions, CC at rs1127354 and AA at rs7270101, corresponding to a fully functional enzyme. The combined allele frequency distribution in the study population and pITPase activity based on the combined genotype is reported in table 2.

![Table 2. Distribution of the compound ITPA rs1127354 and rs7270101 genotype in the NORDynamIC study and also estimated ITPase activity based on genotype. Adapted from Rembeck et al. Hepatology 2014, with permission.](image)

The main finding in this study was that SNPs causing reduced ITPase activity were significantly associated with increased likelihood of achieving SVR, in the per protocol (PP) group (odds ratio = 6.4 for completely reduced activity, p=0.0003). This finding was significant also when the patients were subdivided based on treatment duration (12 versus 24 weeks), HCV genotype, fibrosis stage and IFNL4 genotype (rs12979860). Furthermore, the association with SVR rates continuously improved as pITPase activity decreased (OR 2.3 for 100% versus ≤60% and OR 4.4 100% versus 30%). Interestingly, reduced pITPase activity had no impact on early viral responses and all patients in the PP group had undetectable HCV RNA at the end of treatment. Consequently, the difference in SVR rate was explained by reduced relapse rates, figure 7, similar to the effect previously observed when adding RBV to IFN-α therapy.
for HCV. In the ITT population reduced pITPase activity was still associated with treatment response, although slightly attenuated.

The well-documented protective effect of reduced ITPase activity against RBV-induced hemolytic anemia could once again be verified in this study, figure 8A.

As the pattern of reduced pITPase activity on treatment responses much resemble the effect of adding RBV to IFN treatment combined with the observation that reduced pITPase activity also confers protection against anemia, the most common cause of RBV dose reduction, obviously adherence to therapy needed to be thoroughly accounted for. However, there was no significant association between reduced pITPase activity and RBV dose reduction. Also, in a logistic regression analysis, anemia and RBV adherence did not alter the significant association between SVR rates and pITPase activity. To further account for this potential bias, RBV concentrations at treatment week 4 were compared based on pITPase activity. Surprisingly, patients with reduced pITPase activity had significantly lower RBV concentrations in plasma, the opposite as would have been the case if patients with reduced pITPase activity were more adherent to RBV therapy, figure 8B.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{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.}
\end{figure}
Of note is that the previously published lack of association between IFNL4 rs12979860 and SVR from the NORDynamIC study, was a significant predictor in this study, despite the contribution of pITPase activity, in the multivariate analysis for explaining SVR where the TT variant of rs12979860 was a negative predictor of SVR (OR=0.46, p=0.004). IFNL4 had to be forced into the statistical model as the univariate relationship with SVR was far from significant.

4.2 IMPACT OF DIFFERENT RBV DOSING STRATEGIES ON VIRAL RESPONSES, RBV CONCENTRATION AND ANEMIA

Paper II summarises the result of the RibaC trial, which aimed at exploring the effect of different treatment regiments of RBV. 58 HCV genotype 1 infected patients received either (i) 2 weeks RBV double-dosing concomitant with pegIFN-α (arm A, “loading”), (ii), 4 weeks RBV mono-therapy prior to adding pegIFN-α (arm B, “priming”) or (iii) standard-of-care (SOC) (arm C).
No statistical difference was seen between the treatment regiments on first and second phase decline. However, significantly more patients in the RBV mono-therapy “priming” arm achieved a very rapid virologic response (VRVR), i.e. HCV RNA below 1000 IU/mL 1 week weeks after initiation of pegIFN-α and rapid virological response (RVR), i.e. undetectable HCV RNA 4 weeks after initiation of pegIFN-α. VRVR and RVR were predefined secondary study endpoints. There were no significant differences in later on-treatment responses or SVR between the three study arms, figure 9.

Patients in the “loading” arm had as expected significantly higher mean plasma RBV concentrations compared to "SOC" at day 3 (4.3 vs. 3.0 µmol/L for “loading” and "SOC" respectively; P=0.002), day 7 (6.9 vs. 4.2 µmol/L; P<0.0001), and day 14 (9.6 vs. 5.2 µmol/L; P<0.0001). After 14 days of RBV double-dosing in the “loading” arm, RBV was reduced to standard dosing. The difference in plasma RBV concentration could still be observed at day 28 (7.6 vs. 6.2 µmol/L for “loading” and "SOC" respectively; P=0.04, Mann-Whitney U test). In the “priming” group, RBV concentrations reached steady state after 4 weeks of monotherapy, figure 10A. Patients in the “loading” arm had a more pronounced mean hemoglobin decline from day 0 to day 14 compared to the
"SOC" arm (-2.18 vs. -1.38 g/dL; P=0.03), figure 10C. One patient in the “loading” arm required a RBV dose reduction, other than the planned halving at day 14, during the first 28 days of therapy. This patient developed severe anemia, leading to discontinuation of RBV after 14 days, and of all therapy one week later. Three patients in the “loading” arm, two patients in the "SOC" arm, and one patient in the priming arm had a hemoglobin concentration below 10 g/dL during the first 28 days of dual therapy. Importantly, no patients in the “priming” group had a hemoglobin level below 10 g/dL during RBV monotherapy.

Figure 10. Impact of “loading” (2 weeks of RBV double-dosing concomitant with pegIFN-α), “priming” (4 weeks RBV mono-therapy prior to adding pegIFN-α), and “standard-of-care” on plasma RBV concentrations (A), hemoglobin (B), decline in HCV RNA (C), and ALT (D). Mean with standard error of the mean shown. Waldenström et al Plos One 2016, open access.

4.3 RBV MONOTHERAPY REDUCES VIRAL LOAD IN ASSOCIATION WITH IFNL4 GENOTYPE AND REDUCES IP-10 PLASMA CONCENTRATION

The RibaC study offered an almost unique opportunity to study the effect of RBV monotherapy on both virus and host. Twenty-eight days of monotherapy resulted in a mean HCV RNA level decrease of 0.46 log_{10} IU/mL, from 5.83
Impact of Genetic Variants in \textit{ITPA} and \textit{IFNL4} on Natural History, Treatment Response and Ribavirin Pharmacology in Hepatitis C Virus Infection

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SOC arm (\(-2.18\) vs. \(-1.38\) g/dL; \(P=0.03\)), figure 10 C. One patient in the loading arm required a RBV dose reduction, other than the planned halving at day 14, during the first 28 days of therapy. This patient developed severe anemia, leading to discontinuation of RBV after 14 days, and of all therapy one week later. Three patients in the loading arm, two patients in the SOC arm, and one patient in the priming arm had a hemoglobin concentration below 10 g/dL during the first 28 days of dual therapy. Importantly, no patients in the priming group had a hemoglobin level below 10 g/dL during RBV monotherapy.

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One of the proposed mechanisms of action for RBV is modulation of the expression of ISGs. IP-10 is an ISG that is of interest in the field of HCV biomarkers, as low systemic concentration of this chemokine is associated with both spontaneous resolution of acute infection as well SVR rates in pegIFN and RBV treated patients. Interestingly, 4 weeks of RBV mono-therapy reduced mean IP-10 concentration from 548 to 345 pg/mL (\(P<0.001\)), and a significant reduction could be seen already after one week of therapy (548 to 466 pg/mL, \(P=0.003\)). When pegIFN-\(\alpha\) was added to the therapy, an anticipated dramatic increase in IP-10 concentration was noted in all three study arms. \textit{ITPA} genotyping at rs1127354 and rs7270101 was performed for patients in the “priming” arm, but minor allele carriers were too few, making any reliable statistical analysis challenging.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure11.png}
\caption{Impact in arm B “priming” (i.e. 4 weeks RBV monotherapy prior to adding pegIFN-\(\alpha\)) of interleukin 28B (IL28B) genetic variant CC (n=8) vs. CT/TT (n=13) on decline in HCV RNA (A) and RBV concentration day 0 (B), as well as correlation between decline in HCV RNA and RBV concentration day 0 (C). Blue squares showing IL28B CC patients and red dots showing CT/TT carriage. Mean with standard deviation in (A and B). \(P\) values obtained using Mann-Whitney U test/Welch T test (3A), Mann-Whitney U test (3B) and Spearman correlation (3C). Waldenström et al Plos One 2016, open access.}
\end{figure}
4.4 IMPACT OF RBV AND ITPA SIRNA TREATMENT ON VIRAL REPLICATION, MUTAGENESIS AND NUCLEOTIDE CONCENTRATIONS IN VITRO

To further investigate a potential interaction between RBV and ITPase, we set up an in vitro experiment with the HCVcc system, paper III. Huh-7.5 cells were used for culturing the virus and were also subjected to small interfering RNA (siRNA) treatment targeting ITPA mRNA or random siRNA used as control. The siRNA treatment reduced ITPA mRNA and protein expression by approximately 70-80%, roughly the same decrease as noted in some of the patients with reduced ITPase activity, figure 12 C. The cells were then treated with increasing concentrations of RBV prior to infection with the J6/JFH1 HCV. The concentrations of RBV used were 0, 1, 10, 100, 200 µM. The trough concentration of RBV in plasma of treated HCV infected patients is roughly 8-10 µM, figure 10. Huh-7.5 cells tolerated the treatment well, figure 12 E.

Figure 12 The effect of ITPA siRNA transfection and RBV treatment on HCV replication (A and B), efficiency of ITPA siRNA transfection (C and D) and toxicity of RBV and siRNA treatment (E). Statistical significance was determined by t-test on logarithmic values (** p<0.01, *** p<0.001). Nystrom et al. Journal of Virology, 2018, Open Access.
The effect of RBV and *ITPA* siRNA treatment on viral replication, measured as both viral RNA and also concentration of HCV core antigen, was investigated. The result showed that replication was only affected in cells subjected to 100 and 200 µM of RBV. Importantly the antiviral effect was clearly more pronounced in ITPA siRNA treated cells, **figure 12A-B**.

The virus from the same experiments as above was analyzed using next generation sequencing to evaluate the effect of RBV and reduced ITPase activity on viral mutagenesis. *ITPA* siRNA treatment caused a dramatic increase in mutagenesis in cells treated with 100 and 200 µM of RBV. This was the case irrespective if we looked on all SNVs or G-to-A and C-to-U separately, **figure 13 A-C**.

![Figure 13. Single nucleotide variations (SNVs) (A), G-to-A (B) and C-to-U (C) mutations across the HCV genome in HCV infected Huh-7.5 cells treated with RBV and transfected with ITPA or negative control siRNA. Statistical significance determined by Poisson distribution (***, p<0.001). Nystrom et al Journal of Virology 2018, Open Access.](image)

RBV is known to inhibit IMPDH which is an enzyme, downstream of ITPase, involved in the purine metabolism with effects on the intracellular nucleoside triphosphate (NTP) pool. In this study intracellular concentrations of the canonical NTPs (ATP, GTP, UTP, CTP), but also RTP were evaluated using HPLC. Unfortunately, this method was unable to detect any ITP present. Already at 10 µM of RBV, the reduction of GTP levels was evident in relation to the other NTPs, with a decrease from 6.3% to 2.5%, p=0.0001. The ATP fraction decreased, whereas CTP and UTP increased, but the differences were modest. The canonical nucleotides were not affected by ITPA siRNA treatment, but interestingly RTP level was. As expected intracellular RTP levels followed the increase in RBV treatment concentration. Unexpectedly, ITPA siRNA further increased RTP concentration, and the difference was significant already at 10 µM of RBV treatment, suggesting that RTP might be a substrate for ITPase, **figure 14**. This hypothesis was validated by using recombinant ITPase together with RTP in an assay detecting released phosphates.
Indeed, this assay could confirm that ITPase dephosphorylates RTP in vitro, only slightly less efficiently than it does ITP, whereas no effect was seen on GTP dephosphorylation. Our initial hypothesis regarding the underlying mechanism of action explaining the impact of reduced pITPase activity on SVR was that ITP in slightly higher concentrations, as theoretically expected in patients with reduced ITPase activity, potentially could be incorporated into the HCV genome.

Therefore, we analyzed the impact of pITPase activity in IFN- and RBV-free therapy for HCV. Two different such studies were analyzed, i.e. UNITY-1 and ALLY-1. Overall no effect was observed, likely secondary to the high SVR rates achieved with these highly effective DAAs. However, when evaluating difficult-to-cure patients with baseline, pre-treatment RASs directed against NS5A in the HCV genome, reduced pITPase activity was associated with higher SVR-rates in both studies. The result was, however, only significant when patients from both studies were evaluated together because of the small sample size, figure 15.
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Figure 15. Percentage of patients with normal (blue) or reduced ITPase activity (red) having baseline, pre-treatment NS5A resistance associated substitutions (RASs) that achieved SVR following treatment with daclatasvir and sofosbuvir without RBV for 12 weeks in the ALLY-3 study (HCV genotype 3 infected patients with non-cirrhosis or cirrhosis) and with daclatasvir, asunaprevir and beclabuvir without RBV for 12 weeks in the UNITY-1 trial (HCV genotype 1 infected non-cirrhotic patients). Statistical significance using Fisher’s exact test (* p<0.05). Nystrom et al Journal of Virology 2018, Open Access.

4.5 INFL4 AND PREDICTED ITPASE ACTIVITY IN ACUTE HCV INFECTION

The results from ALLY-3 and UNITY-1 suggested a possible effect of reduced pITPase activity on treatment response even in the absence of RBV. To further pursue this idea, we retrospectively analyzed two large prospectively followed cohorts of PWID from the Malmö and Stockholm NEPs, with documented onset of acute HCV. Baseline characteristics of the participants in these cohorts are shown in table 1. The gender distribution as expected was skewed towards fewer women. HCV from 121/139 patients from the Malmö cohort were genotyped; 62 subjects were infected with HCV genotype 1, 11 with genotype 2, 48 with genotype 3 and in 18 cases sequences could not be obtained. Reinforced patients were approximately 10 years older at time of infection as compared to previously HCV naïve subjects. Clearance rate for HCV-naïve patients from the Malmö NEP was 32% and corresponding figure for the
Stockholm cohort was 26%. Spontaneous clearance was seen in 67% of reinfected patients. IFNL4 TT/TT rs1368234815 was seen in 45% of previously HCV naïve and 67% of reinfected subjects. Carriage of an allele causing reduction in pITPase activity was seen in 33% and 37% among previously anti-HCV IgG negative patients in the Malmö and Stockholm cohort respectively.

### 4.5.1 IFNL4 AND SPONTANEOUS RESOLUTION OF HCV INFECTION

In HCV naïve patients at enrolment in the study with IFNL4 TT/TT rs1368234815 genotype, 40 of 91 (44%) spontaneously cleared their infection vs. only 23/116 (20%) in patients carrying ΔGrs1368234815 allele(s) (p<0.001, Odds ratio 3.2 (95% confidence interval (CI) 1.7 to 5.9) (Figure 2A, paper IV). Similar results were seen in the Malmö and Stockholm cohorts when analyzed separately, but the result was only statistically significant in the larger Malmö cohort. The difference remained significant also when the result was stratified according to HCV genotype in the Malmö cohort. In genotype 1 infected subjects 8/21 (38%) for TT vs. 5/41 (12%) ΔG cleared their acute infections, p=0.02. Importantly this was also true for non-genotyp-1 infected subjects, which was seen when genotype 2 and 3 infected subjects were analyzed together. 11/28 (39%) of IFNL4 TT/TT rs1368234815 vs. 4/31 (13%) ΔGrs1368234815 carriers had a spontaneous resolution of infection, p=0.03 (Figure 2A, paper IV). Subjects with reinfection had a clearance rate of 67%. There was a trend towards higher rates among IFNL4 TT rs1368234815 homozygotes vs. ΔGrs1368234815 carriers (74% vs. 53%, p=0.1). Also, there was a statistically significant accumulation of rs368234815 TT/TT allele configuration in patients with favorable responses; HCV naïve patients without spontaneous clearance (35%), reinfected patients unable to clear their second infection (53%), naïve patients clearing their first infection (63%) and last patients clearing at least two infections (74%), p<0.001 (Figure 2B, paper IV). To further analyze the impact of host genetic variants on spontaneous clearance, a multivariate analysis was performed including gender, age at seroconversion for HCV, pITPase activity (<100% or 100%), previous HCV experience (HCV naïve and previous clearance), and IFNL4 rs368234815 genotype (TT homozygote or ΔG carriage). Only IFNL4 TT rs368234815 homozygosity (p=0.002, adjusted odds ratio 2.9 (95% CI 1.7-4.9) and previous HCV experience (p=0.0003, adjusted odds ratio 1.9 (95% CI 1.4-2.8) were significantly associated with spontaneous clearance of incident HCV infection.
4.5.2 PREDICTED ITPASE ACTIVITY AND SPONTANEOUS RESOLUTION OF HCV-INFECTION

Overall, spontaneous clearance was not associated with pITPase activity. However, in male subjects lacking a functional IFNL4 gene, i.e. rs368234815 TT/TT genotype, reduced pITPase activity was highly significantly associated with increased likelihood of resolution, (15/22 (68%) vs. 12/42 (29%), p=0.003), figure 3A paper IV. This difference was significant when restricting the analysis to male participants from the Malmö cohort, 9/13 (69%) vs. 10/32 (31%), p=0.04. The HCV naïve patients from the somewhat smaller Stockholm NEP cohort were used as a validation cohort, and the similar trend observed supported the initial finding from the Malmö cohort with male participants with rs368234815 TT/TT genotype and reduced pITPase activity having higher spontaneous clearance rates compared to those with normal pITPase activity (6/9 (67%) vs. 2/10 (20%), p=0.07), (Figure S1, in paper IV).

4.5.3 IMPACT OF IFNL4, PREDICTED ITPASE ACTIVITY ON AGE AT SEROCONVERSION FOR HCV

Subjects with reduced pITPase activity were significantly older than those with normal pITPase activity at the time of seroconversion (30.5 vs. 27.8 years respectively, p=0.02). No such significant difference was observed for IFNL4 rs368234815. Again, we stratified according to gender, and the result was only evident in male participants, where age at seroconversion differed both depending slightly on IFNL4 rs368234815 genotype (31.9 ± 8.6 vs. 29.2 ± 9.2, for TT/TT and ΔG, respectively, p<0.05), but more pronounced for pITPase activity of 100% or <100% ( 31.6 ± 8.8 vs. 28.0 ± 6.5 respectively, p=0.006). In contrast, no such difference was observed among female participants. Interestingly, the combination of IFNL4 rs368234815 TT/TT or ΔG and pITPase activity above or below 100% had a more pronounced association with age at seroconversion. In a ANOVA analysis including all genders and both cohorts, the groups differed highly significantly (F (3, 203 = 4.3, p=0.006). Again, this was only observed among male participants (F (3,151 =8.2, p<0.0001), and not females (F (3, 47 = 0.53, p=0.67), figure 3B, paper IV. Reported age of debut of illicit intravenous drug use was available in the Malmö cohort, and this did not differ significantly based on the combination of IFNL4 genotype and pITPase activity, but recall bias is inevitably a potential risk when evaluating self-reported data.
Impact of Genetic Variants in ITPA and IFNL4 on Natural History, Treatment Response and Ribavirin Pharmacology in Hepatitis C Virus Infection

5 DISCUSSION

5.1 DOES ITPA GENOTYPE IMPACT ON TREATMENT RESPONSE IN PATIENTS INFECTED WITH HCV GENOTYPE 2 AND 3 TREATED WITH PEGIFN α AND RBV?

In the NORDynamIC study with 354 peg-IFN α2a and RBV treated chronic HCV genotype 2 or 3 infected patients, we were able to demonstrate that SNPs in ITPA reducing the activity of ITPase was highly significantly associated with reduced relapse rates in a dose dependent manner (paper I). This association remained significant in a logistic regression model including relevant covariates. The most important confounders to account for in this study were RBV treatment adherence and RBV dose reductions. Reduced predicted ITPase activity has been, repeatedly and convincingly, reported to protect against the most important dose limiting treatment induced adverse event, namely RBV-induced hemolytic anemia (114, 115, 189). ITPA has also previously been shown to be associated with RBV dose reductions (40, 190). As the observed effect of reduced ITPase activity was similar to an augmented RBV effect, i.e. no impact on early viral responses and reduction of relapse rates, these confounders were crucial to account for in order to obtain a reliable result. One major advantage with this study was the use of a low dose of 800 mg RBV daily (standard-of-care dosing for HCV genotype 2 or 3 infection at the time), as well as close monitoring of adherence to and dosing of the study medication. This regimen was very well tolerated and dose reductions were uncommon. To minimize the confounding effect of adherence, we only analyzed the per protocol group, that is participants taking at least 80% of their RBV and pegIFN α for at least 80% of the time. Patients kept diaries for missed doses, and dose reduction recommended by the treating physician was documented. The association between SVR and pITPase activity also remained highly significant when dose reductions were included in the multivariate analysis. RBV concentration measured at day 29 was actually significantly lower among patients with reduced p ITPase activity. This, however, could be secondary to an increase in total distribution volume by means of irreversible intracellular accumulation of RTP intracellularly, as shown in paper III. Taken together, there was no evidence from the NORDynamIC study to suggest that the association between pITPase activity and SVR was caused by increased adherence to RBV therapy.
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Several but far from all studies, have reported associations between presence of SNPs associated with reduced ITPase activity, i.e. homo- or heterozygosity for ITPA $A_{\text{rs1127354}}$ or $C_{\text{rs7270101}}$, and higher likelihood of achieving SVR, mediated by reduced relapse rates. In support of our finding, an Italian study enrolling 70 HCV genotype 2 or 3 infected patients treated with 800 mg RBV daily and 123 HCV genotype 1 or 4 infected patients treated with 1000-1200 mg RBV in combination with pegIFN-$\alpha$, reported that reduced ITPase activity was associated with increased SVR rates also by means of reduced relapse rates (111). This finding was significant despite the inclusion of RBV dose reductions and anemia in the multivariate analyses, but still the authors could not confidently rule out adherence as a confounder. A GWAS of Japanese patients with HCV, treated with RBV and pegIFN reported interesting findings. Firstly, ITPA SNP at rs7270101 was found to be monoallelic, and secondly the minor $A_{\text{rs1127354}}$ alleles was associated with SVR in a univariate analysis but not when accounting for covariates (191). However, several Japanese studies have since then reported the minor $A_{\text{rs1127354}}$ ITPA allele to be associated with SVR secondary to reduced relapse rates. In these studies, the RBV dose was weight-based and ranged from 600-1000 mg/day (192-194). Results from two different Scandinavian clinical trials with a total of 457 HCV genotype 2 or 3 infected patients receiving 14 or 24 weeks of pegIFN and RBV treatment using the compound genotype of rs1127354 and rs7270101 could not see any association with SVR (114). This study was very similar to the NORDynamIC study, but differed in that weight-based RBV dosing was used and monitoring of adherence by diary was not performed. Instead of 800 mg as in NORDynamIC study, the North-C study used a higher weight-based dose between 800-1400 mg/day. Similarly, two large studies from Australia and USA with HCV genotype 1 infected patients treated with pegIFN and weight-based RBV with a daily dose ranging from 1000-1200 mg could not detect any association with SVR (113, 115). One explanation for the discordant results might be the RBV dosing used in these studies as higher doses might lead to sufficient intracellular concentrations of RBV regardless of ITPA variants.

We have not directly measured ITPase activity in our studies since this requires fresh erythrocytes which is impossible to obtain in retrospective studies. Direct measurement of ITPase is complicated with many technical difficulties making the indirect ITPA SNPs preferential in larger studies.
5.2 WHY DOES REDUCED ITPASE ACTIVITY INCREASE SVR RATES IN HCV INFECTED PATIENTS TREATED WITH PEGIFNα AND RBV?

Based on the similarities between the effect observed when RBV was added to IFN and the effect of reduced ITPase activity as well as their involvement in the purine metabolic cascade, we hypothesized that reduced ITPase activity had either (i) an own RBV-like effect by means of increased intracellular ITP concentrations or (ii) that it could increase the antiviral efficiency of RBV. ITPase dephosphorylates ITP to IMP, resulting in high intracellular ITP levels. ITP could cause an increase in mutagenesis and if incorporated in HCV RNA, would likely be immunostimulatory and structure altering. IMP is further metabolized by IMPDH to XMP and then to GTP. Also, ITPase and IMPDH cooperate to cleanse the nucleotide pool and to salvage purines, and IMPDH is inhibited by RBV-monophosphate, figure 16 (106). Thus, the notion of a RBV enhancing or similar effect of reduced ITPase activity in theory seems plausible.

In an effort to clarify the relation between ITPase and RBV, we performed an in vitro experiment with a hepatoma cell line (Huh-7.5) in which ITPase mRNA was degraded by the use of siRNA, reducing the concentration by approximately 80% as compared to mock siRNA treated cells, paper III. The
cells were then treated with different concentrations of RBV and infected with the J6/JFH1 HCV strain. siRNA silencing of *ITPA* resulted in increased RBV-induced reduction in HCV RNA and HCV core antigen, increased mutagenesis in the presence of higher concentrations of RBV, and increased intracellular levels of RBV-triphosphate (RTP). In *vitro*, we were also able to show, for the first time, that ITPase is able to dephosphorylate RTP, which likely explained the higher intracellular levels of RTP observed in ITPA siRNA treated cells. In a study on RTP in erythrocytes from 177 RBV treated patients, a similar result on reduced ITPase activity and RTP levels was noted. Patients with an estimated ITPase activity of 30% had an approximately two-fold increase in RTP in whole blood, supporting the notion that our *in vitro* observations likely also occur *in vivo* (195).

In the cell culture experiment we could only see an effect on viral replication in cells treated with a high dose of RBV, that is 100-200 µM. Typically, in patients treated for HCV with pegIFN and RBV, the plasma concentration is approximately 10 µM (158). Patients, however, are exposed to RBV for a considerably longer time than the 72 hours the cultured cells can be exposed. Additionally, the Huh-7.5 cell line is a hepatoma cell line and may thus differ from primary hepatocytes with regards to RBV metabolism. We only measured RTP and other NTPs as relative percentages, because absolute intracellular concentrations regrettably could not be measured by the method used. Hepatocytes have a high expression of ENT1 responsible for transport of RBV into the cells (196). Biopsies from the livers of RBV treated patients also demonstrates a pronounced accumulation of RBV with values well in excess of 200 µM, and comparing these concentrations with the estimated theoretical concentrations needed for RTP insertion into the HCV genome indicates that incorporation of RTP likely may occur *in vivo* (160). Also, several studies have shown a mutational pattern indicative of RTP insertion with G-to-A and C-to-U single nucleotide variants (SNVs), which is also the pattern we observed *in vitro* (170, 197, 198).

The increased SVR rate observed in patient with reduced ITPase activity in the NORDynamIC study, based on these results, is likely at least in part caused by higher RTP concentrations in their hepatocytes with subsequent increased mutagenesis and decreased replicative fitness. An indirect support for this in the NORDynamIC study was the lower plasma RBV concentrations seen in patients with reduced pITPase, indicating that a bigger fraction was likely accumulated in the cells. Lower RBV concentrations in patients with reduced ITPase activity has also been reported in IFN-free DAA studies (116). Once phosphorylated, RBV is trapped intracellularly and if less dephosphorylation occurs, as could be the case in patients with reduced ITPase activity, a larger proportion of RBV will remain intracellularly. Another
possible explanation for the lower concentrations seen in patients with reduced ITPase activity could, however, be the well-known protection from anemia, with a larger number of erythrocytes present that are capable of accumulating more RBV, thereby increasing the distribution volume. Importantly, we have not directly been able to demonstrate an incorporation of RTP or ITP into the HCV genome in our in vitro study. Incorrect incorporation of RTP into the viral genome, however, has been demonstrated by others for both poliovirus and HCV (170, 197, 198).

We observed a relatively major reduction of intracellular GTP concentrations already at low RBV levels, which is in line with the potent inhibition of IMPDH by RMP (199). Since the reduction of GTP was unrelated to ITPA siRNA treatment whereas viral reduction clearly was, it does not appear that GTP depletion could be the sole explanation for the antiviral activity of RBV. However, it is likely that the reduction of GTP with a simultaneous increase in the guanosine analogue RBV favors the latter insertion into the HCV genome. Low GTP levels might also impact on the level of cellular gene transcription as previously proposed (179, 181, 183, 200, 201), which may at least in part explain the impact of RBV on immune responses.

The combined effect of IMPDH inhibition and reduced ITPase activity have been shown to act synergistically in incorporation of ITP into the genome of E.coli and Saccharomyces cerevisiae (107). Based on this finding, we speculated whether this could also be the case in humans, thereby explaining the apparent antiviral effect of reduced ITPase activity. In such case RBV would act to increase a pre-existing antiviral defense in patients with reduced ITPase activity. We could, however, not detect any ITP in our ITPA siRNA treated liver tumor cells despite repeated attempts even when they were treated with high doses of the IMPDH inhibitor RBV, suggesting very low relative concentrations. Since ITPase seems to be able to metabolize RTP (paper III), it is reasonable that this is the main reason for the higher SVR rates among patients with reduced ITPase activity in the NORDynamIC study, where patients received 800 mg flat daily dosing of RBV irrespective of weight. These results thus favor a RBV enhancing effect rather than a RBV like effect.

In paper III we were, however, able to demonstrate an antiviral effect of reduced ITPase activity in a RBV-free setting. Reduced ITPase activity seemed to increase cure rates of HCV infection in patients treated with IFN- and RBV-free DAAs, if patients were infected with a substantial proportion of HCV strains with decreased sensitivity to the NS5A-inhibitor administered in the DAA regimen. Among 25 HCV genotype 1 infected patients treated with daclatasvir, asunaprevir and beclabuvir for 12 weeks in the UNITY-1 trial (202), with pre-treatment RASs directed against the HCV protein NSSA, a
non-significant trend towards improved outcome was observed in patients having genetic variants entailing reduced ITPase activity. A similar trend was observed among HCV genotype 3 infected patients with baseline NS5A RASs treated with daclatasvir and sofosbuvir without RBV for 12 weeks in the ALLY-3 study (203). Together these studies demonstrate that in the presence of baseline NS5A RASs, HCV-infected patients with reduced pITPase activity were significantly more likely to achieve SVR when treated with DAA therapy without RBV. This finding indicate an effect of ITPase in HCV infection other than solely mediated by means of RBV metabolism. However, these findings should be interpreted cautiously as they stem from post-hoc sub-analyses, which would require prospective validation. As the efficacy of DAA therapy has continuously evolved, currently used regimens cure almost all adherent patients even in the presence of pretreatment NS5A RASs, and thus such a prospective study is no longer possible. Further supporting the role of an antiviral effect of reduced ITPase activity is the fact that ITPA orthologs have been found in two different plant viruses; cassava brown streak virus (CBSV) and euphorbia ring spot virus (euRSV) (204-206). It has been speculated that these genes offer protection from insertion of non-canonical nucleotides such as XTP and ITP into the viral genome. To further explore the antiviral effect of reduced ITPase activity we analyzed a large number of subjects from two different NEPs and the results are discussed in section 5.4.

5.3 DOES RBV IN MONOTHERAPY FOR FOUR WEEKS OR 2 WEEKS OF DOUBLE DOSING AFFECT TREATMENT OUTCOME, ANEMIA OR RBV KINETICS?

The RibaC study (paper II) was initiated prior to the introduction of the DAA based HCV therapy, but the prospect of DAAs soon reaching the market impeded recruitment of participants, which resulted in an underpowered study in relation to predefined endpoints. Also, the virological response endpoints of the study became clinically less relevant since the vastly improved efficacy as well as markedly decreased adverse events of DAA based treatment largely abolished pegIFN and RBV combination treatment for HCV infection (157). Despite this, the study provided useful information on RBV dosing and the antiviral effects of RBV in monotherapy.

Neither a 4-week “priming” with RBV mono-therapy nor 2 weeks double-dosing had any effect on the primary study endpoints, i.e. first and second phase decline in HCV RNA, or on SVR. This is consistent with some previous studies, but in contrast with others noting an effect on early viral kinetics (35,
36, 46). RBV, reportedly has been associated with a steeper second phase viral decline and relapse (207). Therefore, the lack of impact particularly on the second phase decline was somewhat surprising. This could potentially be secondary to relatively small sample size in the study or the relatively high RBV weight based dose used in all treatment arms. However, more patients receiving 4 weeks of RBV mono-therapy achieved VRVR and RVR, resulting in a greater proportion of patients qualifying for shortened treatment duration from 48 to 24 weeks of combination therapy. Unfortunately, despite randomization, patients in the “priming” arm had a lower baseline viral load. This together with the viral decline caused by RBV monotherapy likely impacted on the likelihood of achieving VRVR and RVR.

The concentration of RBV seems to be especially important early during combination therapy with IFN, but higher RBV concentrations also increase the risk of hemolytic anemia (208, 209). RBV double-dosing (26 mg/kg/day) for two weeks resulted in a more rapid increase in RBV concentration compared to standard dosing (13 mg/kg/day). However, this was coupled to a more pronounced decline in hemoglobin from day 0 to day 14. Indeed, one patient required discontinuation of treatment because of anemia. Double dosing did not increase cure rates and this arm actually had the lowest SVR rates. The RibaC study was not powered to detect differences in SVR rates even if the planned number of recruited patients was reached. A lack of impact on SVR should, therefore, not be interpreted as if these different regiments do not have any effect on cure rates. One important finding was that patients in the double-dosage arm achieved RBV concentrations at day 7 that were in parity with levels achieved after 28 days among control patients receiving standard-of-care dosing, i.e. in proximity of target steady-state concentrations, without any major impact on hemoglobin concentrations. By day 14, patients in the “loading” arm reached peak RBV concentrations, and levels subsequently decreased upon reverting to standard RBV dosing, i.e. 13 mg/kg/day. Thus, retrospectively it appears that 14 days of “loading” might have been excessive resulting in a high, maybe unnecessary, peak in concentration and anemia after two weeks of therapy, and that one week of double dosing would have been preferential in order to rapidly achieve steady state.

The RibaC study, in theory, offered a way to study the RBV and ITPase joint effect on HCV in vivo since one treatment arm with patients received RBV monotherapy. However, the arm receiving RBV monotherapy only had 21 participants, and unfortunately the number of ITPA alleles corresponding to reduced activity were too few to calculate any reliable statistics
5.4 **DOES IFNL4 GENOTYPE IMPACT ON VIRAL DECLINE DURING RBV MONOTHERAPY?**

Four weeks of RBV mono-therapy decreased HCV RNA by 0.46 log$_{10}$ IU/mL, which is consistent with previous studies (182, 210, 211). The reduction was associated with $IFNL4_{rs12979860}$ genotype, with CC genotype entailing a greater viral decline as compared to CT/TT. This contrasts previous reports where no such association has been reported (182, 210). $IFNL4$ polymorphisms in HCV infection is a known prognostic markers of responsiveness to IFN and RBV treatment, resolution of acute HCV infection and likely also for responsiveness in DAA-based HCV therapy (62, 212, 213). Thus, it is reasonable that a similar effect could be detected in RBV monotherapy, especially as there is an ongoing endogenous IFN response, even in the absence of exogenously administered IFN-α.

RBV has been proposed to have several antiviral actions; GTP depletion and viral mutagenesis as seen in paper III, inhibition of the viral polymerase, but also modulating the immune defense (148). Among other effects on the immune system, RBV has been suggested to alter the expression of ISGs. IP-10 is a well-studied ISG in the field of HCV, with higher systemic concentrations having been demonstrated to be associated with a poor response to IFN-based HCV therapy as well as with decreased likelihood of spontaneous resolution of acute HCV infection (89, 214). We observed a correlation between the fold change of IP-10 and HCV RNA decline during 4 weeks of RBV therapy, and similar results has also been reported by others following RBV monotherapy as well as RBV containing DAA therapy (179, 215). One possible explanation could be that the viral decline drives the reduction in IP-10. However, a similarly plausible alternative mechanism could be a RBV-powered modulation of ISGs leading to restored IFN responsiveness, which has been reported both in vivo and in vitro (181, 201).

HCV RNA decline correlated with RBV concentration after 4 weeks of treatment, which is line with another study (211). $IFNL4_{rs12979860}$ CC carriers also had higher RBV concentrations after 4 weeks of treatment, but the association with viral decline remained significant in a statistical model accounting for this. To our knowledge, the association between $IFNL4$ genotype and RBV concentration has not been reported elsewhere.
5.5 **DOES IFNL4 AND ITPA GENOTYPE AFFECT SPONTANEOUS RESOLUTION OF ACUTE HCV INFECTION?**

In the two cohorts of PWID at two different NEPs, i.e. Malmö and Stockholm, we observed that inability to produce IFN-λ4 (TT rs368234815 homozygosity or CC rs12979860) was clearly associated with increased likelihood of spontaneous resolution following acute, incident exposure to HCV, both in univariate and multivariate analysis, **paper IV**. This finding is uncontroversial, and has been noted in several other studies since the discovery of its importance in a GWAS in 2009 (38). However, our study contributed additional data regarding its effect in HCV genotypes, other than genotype 1, which has most extensively been evaluated. Since both cohort studies were longitudinal, rather than cross-sectional, it was possible to obtain RNA sequences to determine HCV genotype, and the effect of IFNL4 was seen in not only the most common genotype 1, but also in genotypes 2 and 3. Although rare, there are some studies on non-1 infected subjects where, similar to our findings, absence of functional IFNL4 improved likelihood of spontaneous clearance among HCV genotype 2 or 3 infected participants (216, 217).

In the cohort of reinfected subjects from the Stockholm NEP, IFNL4 rs368234815 TT/TT carriage, i.e. lack of functional IFNL4, was associated with a clearance rate of 74% as compared to 53% if one or two ΔG alleles were present, although this difference was not significant. We could also see an enrichment of IFNL4 rs368234815 TT/TT carriage among individuals with more favorable responses, with the highest proportion in subjects clearing a reinfection. A similar, borderline significant association with higher clearance among patients with IFNL4 rs12979860 CC genotype among 28 reinfected subjects has previously been published from a very well defined cohort (129). Thus, it is very likely that lack of functional IFNL4 also is favorable in the setting of HCV reinfection.

In contrast to IFNL4, we could not observe any overall association between spontaneous clearance and ITPA compound genotype. In **paper I**, reduced ITPase activity increased cure rates in patients on pegIFN in combination with RBV, and in **paper III** we noted increased cure rates in patients following RBV-free DAA therapy infected with HCV with pre-treatment NS5A RASs. However, similar to the improvement noted when RBV was added to IFN therapy, we hypothesized that reduced ITPase activity could enhance the impact of the absence of functional IFNL4. RBV unaided does not cure any patients and when given as monotherapy, has only a modest effect on HCV RNA concentrations, but markedly improves SVR rates when added to IFN.
In theory, ITPase activity could thus be important in patients with an effective endogenous IFN response. Indeed, in male patients with the favorable IFNL4 TT/TTrs368234815 genotype reduced pITPase activity significantly improved the spontaneous clearance rate to 68% compared to 29% in those with normal pITPase activity. This finding remained significant when sub-analyzing the Malmö cohort, and a similar, albeit non-significant trend was noted in somewhat smaller Stockholm cohort. Thus, both RBV and reduced ITPase activity similarly appear to be able to deal a final blow to HCV after the viral infection has been weakened by strong exogenous or endogenous IFN-induced immune responses.

The gender difference in the abovementioned finding is rather striking, and supports the notion that female gender is often associated with favorable outcome following many viral infections, as recently exemplified in the ongoing COVID-19 pandemic by a higher mortality among male patients infected with SARS-CoV-2 (129, 218).

When further analyzing differences in covariates, we found a peculiar and to our knowledge previously unreported association between the host genetic variants and humoral antibody response as measured by age at the time HCV seroconversion. Male patients with genetic variations resulting in normal pITPase activity were significantly older at seroconversion than those with reduced pITPase. Also, male patients with absence of functional IFNL4 (i.e. TTrs368234815 homozygotes) were significantly older compared to ΔG carriers. When the analysis was stratified based on the combination of IFNL4 genotype and pITPase activity (100% or <100%), male TTrs368234815 homozygotes with normal pITPase activity had a mean age at seroconversion that was 5 years higher than the remaining patients, and this difference was highly significant. This difference was only observed among male participants, whereas the difference between females was only one year and non-significant.

It might initially appear counterintuitive that the age at anti-HCV IgG seroconversion is associated with host genetics, and also that this association is seen only in male participants. This finding obviously warrants replication in other settings, and there is also a need for preclinical studies to find a possible explanation. However, it is important to bear in mind that the absence of detectable antibodies is automatically not equivalent to not having previously been exposed to HCV (219). Additional explanations could include that subjects spontaneous resolve one or more HCV infection without ever developing detectable antibodies, or more likely develop an antibody response that wanes over time, which has been reported to occur in some subjects decades after infection (75).
Some similar associations on humoral immunity and \textit{IFNL4} previously has been reported in the setting of other viral infections. For example, decreased seroconversion has been observed following influenza vaccination in patients with T/T\textsuperscript{rs8099917} genetic configuration, i.e. lacking functional \textit{IFNL4}. Rs8099917 is a SNP located in close to \textit{IFNL4} and T/T\textsuperscript{rs8099917} is associated with the TT/TT\textsuperscript{rs368234815} configuration and defective \textit{IFNL4}. Also, influenza stimulated B-cells from rs8099917 T/T carriers produced lower amounts of the Th2 associated interleukin-4 (220). Similarly, TT/TT\textsuperscript{rs368234815} has been associated with lower antibody titers following measles vaccination (221). There are also studies suggesting that \textit{INFL} generally favors a Th1 skewed immune response, but data on \textit{IFNL4}, which is the most recently found member of the IFNL family, is rare (222).

Reduced ITPase activity likely results in an increase in unusual triphosphate nucleotides (e.g. XTP and ITP), that potentially could be incorporated into the HCV genome. Inosine containing single stranded RNA results in an up-regulation of several cytokines including interleukin-6, which promotes plasma cell survival (223, 224). Thus, normal pITPase activity causing less inosine containing HCV-RNA might cause a weaker antibody response that wanes over time. The higher age in patients with normal ITPase activity might then be explained by a higher proportion of patients being re-infected with HCV without being able to mount a detectable anti-HCV IgG response.

The associations between age at time of HCV seroconversion and \textit{IFNL4} genotype as well as pITPase activity in our study was only noted among male patients, and this does not appear to be secondary to insufficient power in the female cohort. There are multiple prior well-documented similar gender differences in antibody responses following vaccination (225).

Some limitations in the study should be noted. Spontaneous clearance would have been more reliable if two interspersed samples were required. Patients in the Malmö cohort showed very fluctuating patterns of HCV viremia during their first year of infection (121). Therefore, we cannot say with certainty that patients classified as viral clearers were not patients with a temporary viral suppression. This was especially true among patients from the Stockholm cohort where HCV-RNA negativity >5 months past infection was used as the definition of spontaneous clearance. The sera from the Malmö subjects also was diluted 1/10 prior to HCV RNA analysis, which might have caused an overestimation of clearance rate. The clearance rate in naïve infected and reinfected persons from the Stockholm NEP was higher than reported in the initial study. This is explained by more follow up samples present at the time of this new study and because of patients without a follow up sample taken > 5 months since reinfection were excluded from the study. Importantly, this
study was not performed to study clearance rates, as this has already been published (77, 121). Rather, this study aimed at evaluating the effects of IFNL4 and ITPA genetic variants on spontaneous resolution.
6 CONCLUSION

- Genetic variations resulting in reduced ITPase activity are associated with reduction in RBV-induced hemolytic anemia, lower plasma RBV concentrations and improved likelihood of achieving SVR by means of reduced relapse risk in HCV genotype 2 and 3 infected Nordic patients treated with pegIFN and RBV.

- ITPase is able to dephosphorylate RTP. Additionally, decreased ITPase activity in HCV infected and RBV treated liver tumor cells results in increased viral load reduction, RBV-induced mutagenesis and intracellular concentrations of RTP. These findings combined offer a plausible explanation for the increased cure rates seen in patients with reduced ITPase activity treated with pegIFN and RBV.

- Both RBV in monotherapy given for four weeks as well as double dosing of RBV for two weeks concomitantly with pegIFNα results in high RBV concentrations early in IFN-based combination therapy for HCV infection. Two weeks double dosing is associated with a more pronounced anemia, and likely one week would be sufficient to achieve steady state RBV concentrations. The antiviral effect of RBV is stronger in patients with favorable IFNL4 genotype. RBV also decreases the IFN inducible chemokine IP-10, important for recruitment of NK cells, monocytes and T cells to the site of infection.

- IFNL4 genetic variants resulting in inability to produce IFN-λ4 are linked with spontaneous clearance in HCV genotypes 1 and 2/3 in Swedish PWID. Men lacking functional IFNL4 and having reduced pITPase activity have an augmented immune response against HCV, as exemplified by higher rates of spontaneous resolution and younger age at anti-HCV seroconversion.
7 FUTURE PERSPECTIVES

Host prognostic markers of treatment response in patients with HCV have been of clinical significance during the pegIFNα and RBV era. DAA therapy has now revolutionized HCV therapy, and offers an almost certain cure with limited side effects for compliant patients. This obviously has limited the need for prognostic markers on treatment response.

Genetic variations resulting in reduced ITPase activity are present in approximate a third of humans without any major concern to health, and are associated with improved RBV efficacy as well as decreased likelihood of RBV-induced hemolytic anemia. Thus, partial inhibition of ITPase could thus be a potential future pharmaceutical to boost RBV treatment. Today RBV in high doses is recommended for treatment in some hemorrhagic fevers and in normal doses in chronic HEV infection as well as in HCV infection in patients with decompensated cirrhosis. These patients need the full potential of RBV but are also more vulnerable to anemia.

Our finding of older age at seroconversion to HCV in male PWID lacking functional IFNL4 but having normal ITPase activity age warrants further investigation. This might be of importance in vaccination against other infectious diseases, and should be assessed in studies evaluating the longevity of antibody responses. Also, our results regarding the effect of reduced ITPase activity in combination with absence of functional IFNL4 on spontaneous resolution of HCV infection in men needs to be replicated in more patients as well as in other infections, especially those where gender differences in outcome have been noted, e.g. COVID-19.

The association between genetic variations in IFNL4 and spontaneous resolution of acute HCV is strong and previously well-documented. The mechanisms underlying this dramatic effect remain unclear, and their discovery likely will have major importance for our understanding of the immune system. The notion that the ancestral ability to produce IFN-λ4, present in all other primates, results in reduced cure rates for some infectious diseases, as exemplified by HCV, is at present counterintuitive, and warrants further investigation.
Impact of Genetic Variants in ITPA and IFNL4 on Natural History, Treatment Response and Ribavirin Pharmacology in Hepatitis C Virus Infection

Host prognostic markers of treatment response in patients with HCV have been of clinical significance during the pegIFN α and RBV era. DAA therapy has now revolutionized HCV therapy, and offers an almost certain cure with limited side effects for compliant patients. This obviously has limited the need for prognostic markers on treatment response.

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Impact of Genetic Variants in \textit{ITPA} and \textit{IFNL4} on Natural History, Treatment Response and Ribavirin Pharmacology in Hepatitis C Virus Infection

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