Immunological and Genetic Markers Predicting Treatment Outcome in Hepatitis C Virus Infection

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To my family
ABSTRACT

Hepatitis C is a blood-borne infection caused by the hepatitis C virus (HCV). A chronic infection, which develops in most infected subjects, may lead to liver cirrhosis with ensuing liver dysfunction and liver cancer. The current standard therapy in chronic hepatitis C is a combination of pegylated interferon-α (peg-IFN) and ribavirin (RBV) for 24-48 weeks. Eradication of HCV (i.e. sustained virological response, SVR) is achieved in 50-80% of patients, albeit with significant side-effects. Further understanding of host factors that determine the effectiveness of treatment may provide diagnostic tools to distinguish patients who will be cured from those in whom treatment is likely to be futile.

The aim of this thesis was to identify biomarkers to predict outcome of combination therapy in chronic HCV infection. The biomarkers studied included IP-10, soluble CD26 (sCD26), and single nucleotide polymorphisms (SNPs) in proximity of genes encoding cytokines of the IFN-γ family.

Interferon-γ-inducible protein 10 kDa (IP-10 or CXCL10) is a chemokine that attracts mononuclear blood cells to sites of infection. IP-10 is produced by several cell types, including hepatocytes, and blood levels of IP-10 at onset of therapy are reportedly elevated in patients infected with HCV of genotypes 1 or 4 who do not achieve SVR. In the studies included in this thesis, it was observed that IP-10 in plasma is mirrored by intrahepatic IP-10 mRNA expression, and strongly predicts the reduction of HCV RNA in blood already during the first days of peg-IFN/RBV therapy for all HCV genotypes. Additionally, it was observed that a combined assessment of systemic IP-10 and IL28B-related SNPs further enhances the prediction of early viral decline and the final treatment outcome among HCV genotype 1-infected patients.

Serum dipeptidyl peptidase IV, also known as CD26, cleaves a dipeptide from the N-terminal region of IP-10, generating a truncated, competitive antagonist form of IP-10. Recent reports demonstrated that serum IP-10 in HCV patients is dominated by truncated IP-10. In this setting, the specific sCD26 activity was found to predict the effectiveness of peg-IFN/RBV therapy in chronic hepatitis C, and enhance the value of established outcome predictors.

Keywords: IP-10, CXCL10, CD26, hepatitis C virus, interferon, ribavirin, treatment, IL28B
Hepatit C är en leverinflammation orsakad av hepatit C-virus (HCV), ett RNA-virus som främst smittar via blod. Sjukdomen förlöper i en akut fas (som ofta är asymptomatisk) och en kronisk fas. Cirka 80% av infekterade utvecklar kronisk sjukdom med långvarig produktion av HCV i leverceller. Den kroniska leverinfektionen leder ofta till levercirrhos och leversvikt, och är en ledande orsak till hepatocellulär cancer.


I flera tidigare arbeten identifierades att blodnivåer av det kemotaktiska proteinet IP-10 förutsätter utfall av behandling vid kronisk hepatitis C-virusinfektion. IP-10 bildas av immuncellerna, epitelia cellerna och HCV infekterade hepatocyter och anses bidra till att attrahera mononukleära celler såsom monocyter, NK-cellerna och andra lymfocyter till infekterad vävnad. IP-10-nivåer i blod är av särskilt prognostiskt värde vid genotyp 1-infektion och hos patienter med andra negativa prognostiska faktorer såsom begynnande leverskada och hög kroppsvikt. Resultaten i denna avhandling visar att låga IP-10-nivåer före behandlingsstart förutsätter en tydlig reduktion av virusmängden i blod redan under de första dagarnas behandling, både för vilket i sin tur är förenat med gynnsamt utfall på lång sikt.

Tidigare studier har visat att enzymet CD26 klyver IP-10 till en inaktiv form, och det har spekuleras att patienter som svarar sämre på behandling har förhöjda nivåer av den klyvda, inaktiva formen av IP-10. Resultaten i avhandlingen visar att CD26-klyvningens specifika klyvingsaktivitet är, oberoende av IP-10, en prediktiv markör för behandlingsutfall.

Vidare visar resultaten i avhandlingen att gynnsam genetisk variation (polymorfism) nära IL28B-genen på kromosom 19, predikterar kraftig virusreduktion kort efter behandlingsstart. Både gynnsamma IL28B-polymorfismer och låga IP-10-nivåer före behandlingsstart konstaterades vara starka och av varandra oberoende prediktiva markörer för hur effektivt virus elimineras under de första dagarnas behandling. I avhandlingen påvisas även att dessa prediktiva faktorer i kombination kan identifiera vilka patienter som kommer att botas av behandling.
LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV):


* The authors have contributed equally to this work


IV. Askarieh G, Lagging M, Lindh M, Hellstrand K, and Söderholm J; for the King-1 and TTG1 Study Groups. Specific CD26 activity predicts treatment efficacy in chronic hepatitis C. In manuscript.
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### ABBREVIATIONS

<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>CXCR3</td>
<td>chemokine receptor 3</td>
</tr>
<tr>
<td>DAA</td>
<td>direct-acting antivirals</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EVR</td>
<td>early virological response</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
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<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IP-10</td>
<td>interferon-gamma-inducible protein 10 kDa</td>
</tr>
<tr>
<td>ISGs</td>
<td>interferon-stimulated genes</td>
</tr>
<tr>
<td>IU/mL</td>
<td>international units per milliliter</td>
</tr>
<tr>
<td>peg-IFN</td>
<td>pegylated interferon</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>RBV</td>
<td>ribavirin</td>
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<tr>
<td>RVR</td>
<td>rapid virological response</td>
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<tr>
<td>SNPs</td>
<td>single nucleotide polymorphisms</td>
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<td>SVR</td>
<td>sustained virological response</td>
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Liver inflammation, hepatitis, gives rise to symptoms of varying severity including jaundice, nausea, fatigue, itching, malaise, and liver dysfunction [5]. This liver inflammation may be caused by metabolic disorders, toxins, bacteria, alcohol, and pharmaceuticals, but viral infections are a dominant cause. Many viruses, including cytomegalovirus, Epstein-Barr virus, and herpes simplex virus are associated with transient and usually mild hepatitis during the acute infection. Viruses that mainly or exclusively target liver tissue are called hepatitis viruses with six major groups (A to E) [6]. The virus classification and hierarchical structure of these viruses differ significantly. The hepatitis C virus (HCV) belongs to the family Flaviviridae, which also includes dengue virus and yellow fever virus. These viruses are structurally similar and contain positive single-stranded RNA surrounded by a protective lipid bilayer. The Flaviviridae family is divided into three genera, Flavivirus, Pestivirus, and Hepacivirus where the hepatitis C virus (HCV) is included [7].

The main route of transmission for HCV is through parenteral exposure to infected blood. In developing countries HCV infection is foremost spread nosocomially, e.g. through transfusions with inadequately screened blood or use of unsterilized medical equipment. In developed countries the main source of infection is infected needles shared by intravenous drug abusers, but nosocomial transmission may also occur, e.g. through exposure to contaminated needles or contamination of multi-dose ampules [8].

The first six months post infection comprise the acute phase of hepatitis C. During this phase, approximately 20% of infected patients spontaneously clear the virus. However, for the majority of those infected the disease progresses into a chronic stage, which is defined as detectable HCV RNA in blood six months post infection [9], and often becomes a life-long infection if not treated. Chronic HCV infection frequently causes liver fibrosis, i.e.
abnormal accumulation of connective tissue in the liver, which affects the normal functions and structure of the organ. The end-stage of fibrosis with replacement of normal liver architecture, extensive scar tissue and regeneration nodules, is termed cirrhosis and may be lethal. Approximately 20% of chronically infected patients develop cirrhosis after 20 years of infection [10]. HCV-infected patients with liver cirrhosis are at high risk for the development of hepatocellular carcinoma (HCC), and HCC is a leading cause of death among these patients [9, 11, 12], in addition to liver insufficiency. End-stage liver failure caused by HCV is a major indication for liver transplantation.

Before the discovery of HCV in 1989, it was known that a form of chronic hepatitis of presumed viral origin – referred to as hepatitis non-A and non-B (NANBH) [13] – was prevalent among intravenous drug abusers and patients who had received blood transfusions. The availability of tests for the screening of antibodies against HCV has revealed that HCV infection is a major global health problem. In 1999 the World Health Organization (WHO) estimated that approximately 170 million people were chronically infected with HCV worldwide.

Patients with chronic hepatitis C, which is typically diagnosed by screening for HCV antibodies followed by the demonstration of HCV RNA in blood, are currently treated with a combination therapy of α-interferon (IFN-α) and the antiviral drug ribavirin (RBV). While details regarding the mechanism of action of these compounds remain to be established, the drugs appear to affect both the host immune system and the virus. Severe adverse effects follow the therapy e.g. flu-like symptoms, bone marrow suppression and neuropsychiatric morbidity including depression, thus severely compromising the quality of life of treated patients and reducing treatment adherence. However, since the standard therapy leaves 20-50% of the patients uncured, new drugs directly targeting the virus lifecycle are currently undergoing clinical evaluation.

This thesis aimed to establish biomarkers of relevance for the diversity of treatment response among patients with chronic HCV infection on standard
combination therapy. These pretreatment markers include IP-10, a cytokine attracting immune cells to the infection site, genetic polymorphisms near the \textit{IL28B} gene on chromosome 19, and the sCD26 enzyme, which truncates IP-10. These biomarkers may be utilized in optimizing therapy and may shed further light of the mechanisms of viral decay in HCV infection, but could also be important for encouraging patients with favorable baseline factors to initiate and to adhere to intended treatment doses.
1.1 The Hepatitis C Virus

1.1.1 The HCV Genome

The HCV is an enveloped, 9.6 kb long single-stranded positive-sense RNA virus, (+) ssRNA, containing one open reading frame (ORF) flanked by untranslated (UTR) regions in the 5´- and 3´-ends [14]. The ORF, which is schematically shown in Figure 1, encodes both structural and non-structural (NS) proteins. The 5´-UTR is a highly conserved region [15, 16], and contains the internal ribosome entry site (IRES) [17], that mediates binding of host ribosomes to the viral genome and facilitates the translation process. Replication is further facilitated through the interaction between 3´-UTR the NS5B RNA-dependent RNA polymerase (RdRp), a polymerase lacking proofreading activity.

The HCV structural proteins are the core protein (C), and the envelope glycoproteins 1 and 2 (E1 and E2). The core protein is an RNA-binding protein that is assumed to be involved in the viral capsid formation [18]. The highly glycosylated transmembrane proteins E1 and E2 are necessary for the initial viral attachment to host cells facilitating cell entry [19]. E2 contains hypervariable regions [20] where up to 80% of the sequence can differ between genotypes, subtypes, with extensive variation also within individuals [21].

The non-structural proteins are p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. p7 has proven to be important for HCV infectivity, and some studies suggest that it functions as a calcium ion channel [22]. The C-terminal end of NS2 and the N-terminal end of NS3 compose serine protease activity, cleaving the junction NS2/3, which is required for HCV replication. The serine protease domain of NS3 interacts further with NS4A, to anchor NS3 to the endoplasmic reticulum (ER), resulting in further cleavage of the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions [7].
NS4B is thought to play an important role in organizing the membrane-bound replication complex and the formation of “membranous web”, a membrane change that serves as a scaffold for the HCV replication complex. The replication complex is composed of viral proteins, replicating RNA, and altered cellular membranes derived from Golgi, ER and lysosomes [23]. Studies have shown that the NS5A region, also known as the interferon sensitivity domain region (ISDR), is prone to mutations, which is in turn correlated to the effectiveness of α-IFN-based therapy. The NS5A region is highly conserved among both hepaciviruses and pestiviruses, suggesting an important function for the viral lifecycle. NS5B RdRp is conserved and encodes a viral polymerase, which is one of the key components for HCV replication [7, 23-25].

Figure 1 The hepatitis C virus consisting of an envelope, transmembrane envelope glycoproteins, a nucleocapsid, and viral RNA. The 9.6 kb (approximately 9600 nucleotides) genome encodes one large open reading frame with ten proteins as shown in the lower part of the figure. Host proteases can cleave the C/E1, E1/E2, E2/p7, and p7/NS2 junction. NS2/NS3 viral protease cleaves the NS2/NS3 junction and NS3/4A protease cleaves the remaining junctions [2].
As mentioned above, the 5′-UTR and NS5B regions are highly conserved within genotypes. These regions are in turn used for sequencing and determining HCV genotypes and subtypes [26, 27].

1.1.2 The HCV Life Cycle and Replication

HCV, like other viruses, is dependent on a host cell for replication. In brief, the major steps in the viral life cycle are attachment, entry, translation, replication, packaging, assembly, and release of new infectious virions.

The initial steps of infection include attachment and internalization of the virus into the host cell, mediated by the viral envelope glycoproteins E1 and E2. Several host cellular receptors such as CD81, SR-BI claudin-1, and occludin have reported to be necessary for this process [28, 29]. After entry, the HCV nucelocapsid is uncoated (decapsidation) followed by release of the viral RNA into the host cytoplasm. Being positive stranded, the HCV RNA can immediately serve as messenger RNA (mRNA), and translation is initiated following ribosomal binding mediated by the HCV IRES domain [30, 31]. The polyprotein is then cleaved; the structural proteins are processed by host peptidases, and the non-structural proteins are cleaved by the virally encoded proteases [32], as previously outlined.

The replication step for HCV is thought to occur in a semiconservative manner [33], where the positive-strand RNA serves as a template, and the process is facilitated by the viral NS5B RdRp and other NS proteins. This step takes place in a replication complex formed in the membranous web. Maturation and assembly of the virus particles, which is thought to occur in the ER, in the Golgi apparatus, and in lipid droplets [34] take place after the replication and translation steps. Newly produced virions, consisting of the core protein, E1/E2 glycoproteins, and the viral RNA, are released from the host cell through the secretory pathway [35]. HCV can be found as virions or in association with lipoproteins in sera [36] where the latter form has been associated with increased host cell binding ability and more infectious forms of the virus.
The replication process of HCV is prone to mutations and errors due to the rapid replication process and lack of proofreading mechanisms. Approximately $10^{12}$ new HCV particles, with a half-life of 3 hours, are produced in an infected patient per day [37, 38]. The different steps of the viral life cycle and the involved viral proteins are possible targets in drug development, aiming at further improving the efficiency of treatment. For example, two protease inhibitors, boceprevir and telaprevir, were recently approved by the Food and Drug Administration in the United States for use in chronic HCV infection of genotype 1 (cf. below) [39].

### 1.1.3 HCV Genotypes

Due to diversities in the HCV nucleotide sequence, the virus is divided into seven genotypes, categorized 1 through 7 [40] and several subtypes named with the letters a, b, c etc. Among different genotypes, the diversity at the nucleotide level is estimated at approximately 30% and at 20-25% between subtypes [41]. Within infected individuals the HCV particles of different genotypes vary slightly, due to the propensity of the HCV to mutate [42]. These intra-host variations are called quasispecies, referring to the multitude of different variations of HCV sequences produced every day in each patient. This heterogeneous viral population may rapidly select for treatment-resistant clones, thus possibly reducing the treatment efficiency of the new direct-acting antiviral (DAA) drugs recently approved for treating HCV [43].

The geographical distribution of different genotypes is quite distinct, where for instance genotypes 1, 2, and 3 are distributed worldwide with a higher prevalence in Europe, North and South America, and Japan. HCV genotype 3 infections are also found in Southeast Asia, India, and Australia, whereas genotype 4 infections are prevalent in Africa and the Middle East [44]. As for treatment and curability, HCV genotypes 1 and 4 are considered as difficult-to-cure with the current standard of care whereas in comparison, genotypes 2 and 3 have higher cure rates [45]. Length and dosage of the current standard treatment also varies depending on genotype [44]. The most common HCV
genotype infection in Sweden is genotype 1, accounting for approximately 45% of all HCV infections. Approximately 35% are infected with genotype 3, 15% with genotype 2, and < 5% with genotype 4 [46].
1.2 Antiviral Therapies and Viral Kinetics

1.2.1 Treatment

*Alpha interferon and ribavirin therapy.* Infected patients with detectable HCV RNA in serum should be considered for the standard-of-care (SOC) therapy with pegylated interferon-α (peg-IFN-α) and ribavirin (RBV) [47]. The current regimen for HCV infection is a once weekly subcutaneous injection of peg-IFN-α combined with oral doses of RBV twice daily. IFN-α exerts antiviral activity through intracellular cascades, creating an antiviral state as well as down-regulating viral protein synthesis. In addition, IFN-α exerts multifaceted immunomodulatory effects such as activating natural killer (NK) cells, inducing cytokine production, preventing T-cell apoptosis, and increasing antigen presentation by up-regulating production of major histocompatibility complex class II (MHC II) on antigen-presenting cells [4].

The combination of IFN-α with the guanosine analogue RBV has improved SVR rates in hepatitis C, as compared to IFN-α monotherapy [48]. However, the mechanism of action of RBV in the treatment of hepatitis C remains unclear. One proposed mechanism is an immunomodulatory effect where a T-helper-1 (T_{H1}) rather than a T-helper-2 (T_{H2}) immune response is promoted [49]. Furthermore, RBV may cause mutations in the HCV genome, leading to production of defective HCV particles with reduced infectivity [4]. In addition, positive effects on the second phase viral decline, which is assumed to reflect immune-mediated elimination infected hepatocytes (cf. below), have also been observed as a result of the combination therapy of IFN/RBV as opposed to monotherapy with IFN [45, 47].

Of note, the standard therapy of IFN-α/RBV is related to severe adverse effects such as flu-like symptoms, gastrointestinal disturbances, depression, and bone marrow suppression [50-52], which severely compromise the quality of life during treatment and largely affect adherence to therapy.
**DAA therapy.** New antiviral agents, referred to as “Direct-Acting Antivirals” (DAA) have recently been approved for treatment of HCV infection, and several new drugs are under development in various phases of clinical trials [53, 54]. These compounds are primarily used for treating difficult-to-cure HCV genotype 1-infected patients. DAA compounds, e.g. NS3/4 protease, NS5A, and NS5B polymerase inhibitors, are designed to directly target virally encoded proteins. These inhibitors are currently combined with the standard peg-IFN-α/RBV therapy, and reportedly yield higher rates of favorable treatment outcome [55]. Also, trials using combinations of two or more DAAAs for an interferon-free treatment have reported preliminary promising results [56].

### 1.2.2 Virological Response

Duration of therapy is often determined by the viral kinetic response to treatment, which in turn is highly dependent on HCV genotype. Current therapy guidelines for genotypes 2 and 3 comprise 12 to 24 weeks of peg-IFN-α/RBV treatment, and for patients with HCV genotype 1 and 4 infection, 24 to 48 weeks, schematically shown in Figure 2. However, the cure rate for genotype 1 infection on peg-IFN/RBV combination treatment alone is lower and new therapeutic guidelines include addition of DAA therapy.

Plasma levels of HCV RNA are commonly monitored at baseline and at week 4, 12, and 24 after initiation of treatment to determine the virological response. Sustained virological response (SVR) is defined as undetectable HCV RNA 24 weeks after the completion of treatment, after which the risk of recurrence of HCV infection is small. Patients achieving a rapid virological response (RVR), defined as undetectable HCV RNA (i.e. ≤50 IU/mL) 4 weeks into treatment [1], have shown higher probability of achieving SVR, regardless of HCV genotype [57]. Furthermore, early virological response (EVR) is defined as undetectable HCV RNA 12 weeks into treatment, and delayed virological response (DVR), previously referred
to as partial early virological response (pEVR), as a reduction of HCV RNA by $\geq 2 \log_{10}$, from the baseline viral load, at week 12 of treatment [58]. Patients achieving a reduction of HCV RNA $< 1 \log_{10}$ at week 12 of treatment are referred to as null-responders, and have a very low likelihood of achieving SVR [59].

Virological responses are indicative of treatment responsiveness and provide useful tools for tailoring treatment duration and assessing the end-point. The SVR rate in patients infected with HCV genotypes 2 and 3, on peg-IFN/RBV therapy, is 70-80% compared to approximately 40-50% in the case of genotype 1 infections [1].

![Figure 2](image)

**Figure 2** Treatment scheme for combination therapy with peg-IFN/RBV for HCV genotypes 1, 2, 3, and 4. Figure adapted from [1].
Several pretreatment viral and host factors have been proven instrumental in the prediction of response and SVR. High viral load and genotype 1 and 4 infections are examples of unfavorable baseline viral factors [60], and some of the recognized unfavorable host baseline factors include high body mass index (BMI), African descent, male gender, higher age, and pronounced liver fibrosis [61]. Other important host factors that enhance predictiveness include baseline levels of the proinflammatory chemokine IP-10 and IL28B single nucleotide polymorphisms (SNPs), which will be further discussed in detail in this thesis.

1.2.3 Viral Kinetics during Therapy

Studies on the *in vivo* turnover rate of HCV during peg-IFN/RBV therapy have resulted in several mathematical models aiming to describe the viral decay. Pretreatment evaluation of viral RNA in serum represents steady-state conditions, i.e. the rate of HCV RNA production from infected hepatocytes and RNA degradation in the blood is in relative equilibrium. Furthermore, HCV dynamics studies suggest an early exponential decrease of HCV RNA after initiation of antiviral treatment in patients responding to therapy, from which information of the HCV RNA viral kinetics can be obtained [62, 63]. An early and rapid viral decline (first phase) is observed during the first few days of treatment. This is followed by a significantly slower (second phase) viral decline approximately 2-14 days into treatment [38, 64], as shown in Figure 3 when using unpegylated IFN-α. With the introduction of peg-IFN-α, which has a longer half-life, the first phase is prolonged and the second phase generally is similarly delayed.

Neumann *et al.* (1998) suggested that the first phase slope reflects reduced virion production by the infected cells or less *de novo* infection of susceptible cells, as a direct antiviral effect of interferon, whereas the slower second slope reflects the death rate of infected cells or the rate of clearance of HCV from infected cells. A rapid first phase decline (with an approximate 1-2 log_{10} HCV RNA reduction) has proven to be predictive of a higher likelihood of
achieving SVR. Studies have shown slow viral decay in patients infected with HCV genotype 1 and 4, which serves to explain the lower rate of SVR as compared to genotype 2 and 3 [65-67].

Figure 3 A rapid first phase viral decline the first few days on α-interferon therapy is followed by a slower second phase in subsequent weeks. Figure adapted from [4].
1.3 HIV/HCV Co-infection

1.3.1 Human Immunodeficiency Virus

The human immunodeficiency virus (HIV) is a member of the family Retroviridae and the genus Lentivirus. The retroviruses are RNA viruses with virally encoded reverse transcriptases that are responsible for producing DNA from an RNA template, and this DNA in turn can be incorporated in the genome of the infected cell. In 2008, WHO estimated that approximately 33 million people were infected in HIV [68].

HIV enters and infects cells expressing CD4 and either of the co-receptors CCR5 or CXCL4 [69, 70] eventually causing the acquired immunodeficiency syndrome (AIDS) [71]. Cells mainly infected and damaged by HIV include macrophages and CD4+ T cells. AIDS is characterized by immunodeficiency, depletion of CD4+ cells, increasing the susceptibility of the host to opportunistic infections, due to the existing HIV infection. Needle sharing among intravenous drug abusers is a common route of transmission for both HIV and HCV, and an estimated 4-5 million people worldwide are co-infected with HIV and HCV [72]. In Sweden an estimated 15% of HIV patients have antibodies against HCV [73].

1.3.2 Treatment and Hepatitis C Disease Progression

HIV is treated with various combinations of antiretroviral drugs, also known as highly active anti-retroviral therapy (HAART) [74], which have significantly reduced HIV morbidity and mortality. As for HCV treatment in patients co-infected with HIV/HCV, these patients are treated according to the standard recommendations mentioned previously for HCV monoinfected patients [75]. Torriani et al. (2004) noted that complications related to HCV-associated liver disease in co-infection with HIV are significant causes of morbidity among these patients, and with the effective antiretroviral therapy for HIV now available, chronic HCV infection is one of the leading causes of
mortality in co-infected patients. Reports have shown that HCV viral load is higher in co-infected than in HCV monoinfected patients, which may partially account for the lower SVR rates observed among co-infected patients. Aside from a higher baseline HCV viral load, the metabolism of the antiretroviral drugs constituting HAART may cause hepatotoxicity, which additionally contributes to the accelerated fibrosis progression noted among co-infected patients [76], compared to HCV monoinfected patients [77, 78].
1.4 Immune Response during HCV Infection and Biomarkers Predicting Outcome of Therapy

Uncertainty prevails concerning the proportion of hepatocytes that are infected during HCV infection. It has been reported that 15-81% of hepatocytes are infected during chronic HCV infection [79].

Upon viral entry, the host immune response is triggered through recognition of patterns in the viral RNA, known as pathogen-associated molecular patterns (PAMPs), by host PAMP receptors. A poly-uridine motif located in the 3’UTR-end and dsRNA stem-loop motifs in the 5’UTR-region of the HCV genome are examples of PAMPs that can trigger the PAMP receptors retinoic acid inducible gene I (RIG-I), Toll-like receptor 3 (TLR3), and TLR7/8 to induce endogenous IFN-α/β production with subsequent stimulation of interferon stimulated genes (ISGs) [3, 80]. These early mechanisms trigger the initial antiviral defense. The hepatitis C virus interferes with the immune response and a balance between the innate and the adaptive response is pivotal in not only progression of the disease, but also eradication of HCV [81]. Some of the essential mechanisms are mentioned in this thesis (cf. below).

1.4.1 Innate Immune Response and HCV

Interferons and interferon stimulated genes. The name interferon comes from the interfering properties with viral replication [82, 83]. The induction of IFN-α/β, just hours after infection, is a mechanism by which a direct antiviral environment is established in both infected and uninfected cells. IFNs are cytokines produced by many cells in response to pathogens, including HCV infected hepatocytes and liver infiltrating plasmacytoid dendritic cells, T cells or NK cells during HCV infection. There are different
types and classifications of IFNs. IFN-α/β/ω are referred to as type I [84], IFN-γ as type II, and IFN-λ as type III interferons [85]. In general, these cytokines signal to adjacent cells in the infected tissue, informing of a pathogenic state. As for HCV, IFN-α/β production is induced in the infected cell through RIG-I and mitochondrial antiviral signaling protein (MAVS) [86], but also through TLR3 as outlined above. An antiviral state is created downstream of these signals through interferon regulatory factor 3 (IRF3) and IRF7, inducing transcription of type I IFNs [87]. The signaling pathway that follows the release of IFN occurs through binding to the IFN-α/β receptor (IFNAR). The antiviral activity is exerted through the intracellular janus-activated kinase 1 (Jak1) tyrosine kinase 2 (Tyk2) and signal transducer and activator of transcription proteins 1 and 2 (STAT1/2) pathway. These in turn up-regulate the transcription of ISGs, genes regulated by interferons [88]. These events are summarized in Figure 4.

ISGs have different antiviral properties, such as inhibition of viral replication, inhibition of viral protein synthesis, and degradation of viral RNA [89-91]. In turn, the up-regulation of ISGs mediates the initial antiviral action of IFN [92].

Functional genomic studies have reported an association between HCV infection and hepatic expression of ISGs, and possibly also to fibrosis stage in the infected liver tissue during the infection (see review [3]). Studies on baseline expression of hepatic ISGs thus have demonstrated that the gene expression profiles prior to peg-IFN-α/RBV therapy is predictive of treatment outcome in that high baseline expression of some ISGs predict a less favorable response, particularly in difficult-to-cure genotype 1 and 4 infections [93, 94].
Additionally, IFN-α has immunomodulatory effects directing the immune reaction towards a TH1 response, which is considered predictive of resolution of HCV infection. A continued antiviral state is attained through additional cytokine production (e.g. IFN-γ) and the cytotoxic action of activated CD8⁺ cells and NK cells in the infected liver [95]. In addition, up-regulation of ISGs and the attraction of inflammatory cells to the tissue link the innate and adaptive immune system. The administered IFN-α used in the current standard therapy for HCV infection mimics the antiviral effects of endogenously produced IFN-α.
1.4.2 Adaptive T Cell Immune Response and HCV

It has been reported that the development of HCV-specific T cell responses are essential for the resolution of HCV infection [96] and that late viral clearance, reflected by the second phase of viral decline, coincides with the development of cellular adaptive responses. Several studies emphasize the importance of functional HCV-specific CD8\(^+\) T cells (cytotoxic lymphocytes) [97] and CD4\(^+\) T cells (T helper cells) for clearance of acute HCV infection [98], and an impaired or weak CD4\(^+\) T cell response has been associated to continued viremia and the establishment of a chronic infection [99]. CD4\(^+\) T cells recognize antigens bound to MHC II presented on antigen-presenting cells. This interaction induces the production of a repertoire of cytokines, in an attempt to create and maintain the antiviral state by attracting inflammatory cells to the infected tissue, inducing both T\(_{H1}\) and T\(_{H2}\) responses. During a T\(_{H1}\) response CD4\(^+\) T cells activate CD8\(^+\) T cells and this interplay is critical for the function of CD8\(^+\) T cells, whereas a T\(_{H2}\) response facilitates B cells into differentiating into antibody producing plasma cells [100]. CTLs are attracted to the site of infection where they eliminate infected cells upon recognition of viral antigens presented on MHC I [101].

The high replication rate of HCV, as well as impaired CD4\(^+\) T cell function, may contribute to CD8\(^+\) T cell exhaustion [81]. This state is characterized by loss of CD8\(^+\) T cell cytotoxic functions, such as diminished cytokine production, lower rate of proliferation, and dysfunctional memory T cells as is often the case in chronic HCV infection [102].

The hepatic innate immunity is triggered directly upon pathogen recognition, interferons are produced, and cells are engaged and migrate toward the infected liver. For the adaptive immunity, the antigen presentation to T cells \textit{i.e.} priming of T cells, occurs in the hepatic lymph nodes. These primed cells then enter the blood stream in order to re-enter the liver [103, 104].

Studies suggest that an inadequate hepatic defense may be a determining factor for the establishment of a chronic HCV infection. The level of early expression of ISGs, following the interferon production, varies largely
between infected patients [105]. Low expression of hepatic ISGs due to the absence of hepatic type I IFN production have been reported in chronically infected patients [106].

It has also been reported that an HCV infected liver is infiltrated by NK cells and HCV non-specific T cells [107], producing $T_{H1}$ proinflammatory cytokines. This argues for the importance of a functional innate response during HCV infection in the liver. Lymphocyte recruitment occurs through hepatic sinusoids or portal areas, and the inflammatory milieu, caused by the attraction of leukocytes to the infected tissue, has been hypothesized to be the main cause of liver damage in HCV infection rather than the virus itself [108].

Due to the onset of inflammatory processes, activation of hepatic stellate cells (cells involved in liver development and regeneration) and migration of inflammatory cells to the infected liver, severe damage is caused to the tissue resulting in the progression of hepatic fibrosis, commonly observed in chronically infected patients [109].

### 1.4.3 Evasion of the Immune System by HCV

The hepatitis C virus uses different mechanisms to evade host immunity. For instance, studies have shown that the NS3/4A protease can inhibit the TLR3 and RIG-I signaling pathway by cleaving IRF3 and inhibiting MAVS, thus interfering with the initial antiviral IFN-α/β production and downstream pathways related to this [110, 111]. HCV RNA replication, a step highly prone to error due to the lack of proofreading activity in the NS5B polymerase and the high viral replication rate, generates a highly divergent swarm of quasispecies contributing to viral escape. This evasion is maintained through continuous presentation of new viral epitopes to the immune system that are not instantly recognized [3, 37, 112]. Similarly, hypervariability in the E2 domain, driven by the immune system, complicates the recognition of the virus by the immune defense.
As mentioned previously, the new DAA therapy acts directly on the hepatitis C virus. The propensity of the virus to mutate and escape the immune system and also the different HCV genotypes, has not only hampered the development of vaccines, but could entail resistance to the new antiviral therapies [113].

1.4.4 \textbf{Interferon-γ-inducible Protein (IP-10)}

Elevated plasma levels of the ISG interferon-γ-inducible protein 10 kDa (IP-10 or CXCL10) is common in HCV infection. IP-10 is a chemokine produced by endothelial cells, activated T cells and hepatocytes during HCV infection, exerting its effects mainly through a G protein-coupled receptor CXCR3 [114, 115]. IP-10 signaling through CXCR3 [116] induces different effects depending on the site of receptor binding. One central effect of IP-10 is trafficking of activated, IFN-γ producing NK cells [117], T cells [118, 119] and monocytes to the site of infection, promoting a T_{H1} response [120]. It is also reported that CXCR3 stimulation can induce downstream intracellular signaling pathways (ERK phosphorylation), calcium mobilization and CXCR3 internalization [121, 122].

Studies show that intrahepatic levels of IP-10 mRNA are elevated during HCV infection compared to healthy controls, and IP-10 is in fact not detected when stained for in healthy liver biopsies [115]. Correspondingly, Zeremski et al. (2008) presented a significant association between levels of IP-10 and the grade of liver lobular and portal inflammation as well as the stage of fibrosis. This suggests that the CXCR3^+ immune cells attracted toward the HCV infected liver, infiltrating the tissue in the path of eliminating the infection, cause severe damage locally [123].

Apart from HCV disease, elevated levels of IP-10 have been observed in other inflammatory diseases such as multiple sclerosis [124]. High levels of other activators of CXCR3, the chemokines CXCL9 and CXCL11, have also been observed in HCV infection [125]. These cytokines have different
binding sites on CXCR3, and display different effects compared to IP-10 [121].

As outlined in detail in papers I and II included in this thesis and as shown in previous studies, baseline plasma and intrahepatic IP-10 levels are not only associated, but also predictive of treatment outcome and viral kinetics in chronic HCV infection [126-128]. These studies show that chronic HCV infected patients with low baseline IP-10 levels tend to respond more favorably, with increased probability of achieving SVR, to the standard peg-IFN/RBV treatment compared to patients with high baseline levels of the pro-inflammatory cytokine. This finding was also confirmed for HIV/HCV co-infected patients [129].

1.4.5 Single Nucleotide Polymorphisms Near the IL-28A, IL28B, and IL29 Genes (IFN-λs)

Recently several independent genome-wide association studies have reported that single nucleotide polymorphisms (SNPs) within chromosome 19, in the vicinity of the IL29, IL28A, and IL28B genes, encoding type III IFN-λ cytokines (IFN-λ1, IFN-λ2, and IFN-λ3, respectively) are significantly associated with treatment responsiveness and outcome following acute HCV infection. Three SNPs related to these genes rs8099917, rs12980275, and rs12979860, are discussed in this thesis, where previous studies have shown that variants of these are predictive of HCV treatment response [130-132], (schematical representation in Figure 5). The favorable genotypes, significantly predicting higher SVR rate, are CCrs12979860 regardless of race [133], AArs12980275, and TTrs8099917.
Immune cells produce IFN-λ in response to viral infections [134, 135]. Receptors and pathways involved for type III IFNs differ from those of type I IFNs. Similar to IFN-α, IFN-λ1-3 signal through the JAK/STAT pathway, but via the IL28-R/IL-10R receptor complex expressed on hepatocytes and epithelial cells [136] as opposed to IFNAR that are widely expressed in numerous tissues. However, the relation between IFN-λ production and SNPs in close proximity of IL28 remains unclear [137]. IFN-λ based drugs are conceivable candidates for treating HCV infection, and are currently being evaluated in clinical trials. The adverse effects appear less pronounced than for IFN-α particularly regarding bone marrow suppression, possibly because fewer cells carry the receptor, thus allowing for a more targeted therapy [138].

As presented in paper III in this thesis, lower baseline serum IP-10 levels were significantly associated with the favorable SNP variants CC.rs12979860, AA.rs12980275, and TT.rs8099997 of the IL28B gene [139]. Lagging et al. (2011) also reported that the favorable SNP variants and lower baseline IP-10 levels are independent predictors and when combined augment the prediction of
first phase decline and therapy outcome for chronic HCV genotype 1 infection.

1.4.6 Dipeptidyl Peptidase IV (DPPIV/CD26)

The membrane-bound protein dipeptidyl peptidase IV (DPPIV), also known as CD26, cleaves penultimate proline or alanine (X-Pro, X-Ala) from the N-terminal protein end of peptides [140, 141]. CD26 is also present in a soluble, non-membrane bound form in blood plasma, often referred to as soluble CD26 (sCD26) [142]. IP-10 is one of many naturally occurring targets cleaved by CD26. Reports have shown that upon truncation, the chemotactic activity of IP-10 is lost [143].

The CD26 molecule is expressed by several cell types e.g. T cells, NK and B cells, but also in tissues such as kidney, liver and intestines. The function of CD26 is not fully understood, but studies have shown that CD26 is a marker of a T_H1 response [144, 145]. Furthermore, it has been reported that adenosine deaminase (ADA), which is involved in metabolizing the nucleoside adenosine to inosine, can attach to CD26. This complex binding can act as a T cell co-stimulation [146] inducing T cell activation and IL-2 production, thus stimulating T cell proliferation. Also, adenosine inhibits both T cell [147] and NK cell [148] mediated cytotoxicity. In addition, elevated serum levels of CD26 have been observed in liver diseases and tissue degradation (and regeneration), although the natural source of serum CD26 (sCD26) is unknown (see review [149]).

CD26 has proven important in regulating blood levels of incretins, i.e. hormones responsible for lowering blood glucose. Hence, CD26 inhibitors are used in the treatment of diabetes mellitus (for review, see [150]). Other studies have shown that the peptidase activity caused by dipeptidyl proteases may be of relevance in hepatitis C [151]. Other groups and Casrouge et al. (2011) demonstrated the presence of a cleaved, antagonistic form of IP-10 (3-77 aa) as a complement to the full-length (1-77 aa), agonistic form, and compared the biological effects of full-length and truncated IP-10 [152]. It was found that the full-length chemokine, as opposed to the truncated form,
mobilizes CXCR3+ cells and internalizes the CXCR3 receptor upon binding, and that the truncated IP-10 acts as a competitive antagonist. In addition, Casrouge et al. furthermore also demonstrated that the truncated IP-10 dominates in NR (non-responders) patients compared to patients achieving SVR. Their finding thus may explain why elevated levels of pretreatment IP-10 prognosticate less favorable treatment outcome in patients with chronic HCV infection.
2 AIM

The aim of this thesis was to identify biomarkers of relevance to the outcome of therapy in chronic hepatitis C.

The specific aims were:

**Paper I**

To clarify whether baseline intrahepatic IP-10 mRNA expression reflects baseline plasma IP-10 levels in predicting first phase viral decline and sustained virological response during peg-IFN-α/RBV treatment in HCV infection.

**Paper II**

To elucidate the association between baseline plasma IP-10 levels and HCV RNA kinetics for HIV/HCV co-infected patients on interferon/ribavirin therapy, and the utility of IP-10 in prognosticating HCV treatment outcome in co-infected patients.

**Paper III**

To explain the impact of IP-10, in the setting of *IL28B* polymorphisms, on reduction of HCV RNA during therapy.

**Paper IV**

To assess the impact of CD26-mediated truncation of IP-10 on treatment outcome in HCV infection.
3 MATERIALS AND METHODS

Patients, Treatment and Sampling

**DITTO-HCV Study.** Between February 2001 and November 2003, 270 patients (180 men and 90 women) were recruited in a phase III, open-label, randomized, multi-center trial conducted by the DITTO-HCV study group at 9 centers in France, Germany, Greece, Israel, Italy, Netherlands, Spain, Sweden, and Switzerland. All patients were adults, had compensated liver disease, were treatment naïve for hepatitis C, and fulfilled the following inclusion criteria: a positive test for anti-HCV antibody, an HCV RNA level greater than 1000 IU/mL, and two serum alanine aminotransferase values above the upper limit of normal within 6 months of treatment initiation. Two hundred and sixty four patients had pretreatment plasma available for IP-10 analysis, and 73 of these patients had liver biopsies from which RNA could be extracted for further evaluation. All serum samples were stored at -80 °C before analysis.

All patients in the DITTO trial were initially treated for 6 weeks with 180 µg peg-interferon α-2a subcutaneous once weekly (Pegasys, F. Hoffmann-LaRoche, Basel, Switzerland) and ribavirin orally twice daily (Copegus, F. Hoffmann-LaRoche) at a total daily dose of 1000 mg for patients weighing less than 75 kg and 1200 mg daily for above 75 kg. After six weeks of therapy, half of the patients were randomized based on their viral kinetic classification to receive individualized therapy or to continue on standard combination therapy for a total of 48 weeks. Thus only reductions of HCV RNA during these first 6 weeks are included in the analyses in this study.

**NORDynamIC Trial.** Pretreatment plasma samples were obtained from 382 treatment naïve genotype 2/3 infected patients at 31 centers in Denmark, Finland, Norway, and Sweden who were randomized to 12 or 24 weeks of peg-interferon α-2a 180 µg/week plus ribavirin 800 mg/day during the NORDynamIC trial. Three hundred and fifty nine of these patients had
pretreatment plasma available for IP-10 analysis. All samples were stored at -80 °C before analysis.

In the NORDynamIC trial patients were randomized at study entry to either 12 or 24 weeks of therapy with 180 µg peg-interferon α-2a subcutaneous once weekly (Pegasys, F. Hoffmann-La Roche, Basel, Switzerland) and ribavirin twice daily (Copegus, F. Hoffmann-La Roche) at a total daily dose of 800 mg daily.

**DICO Study.** Between September 2004 and November 2006, 21 patients with HIV-HCV co-infection were included in a phase III, open-label, multi-center investigator-initiated trial conducted by the DICO study group in Denmark and Sweden.

All patients were anticipated to receive 48 weeks of therapy with 180 µg peg-interferon α-2a once weekly and ribavirin twice daily at a total daily dose of 800 mg for HCV genotypes 2 and 3, and 1000/1200 mg daily below or above 75 kg body weight for HCV genotype 1.

**KING1 and TTG1 Study.** Samples from 76 patients with HCV genotype 1 infections were included. The patients participated in either of two clinical studies assessing the viral kinetics of HCV genotype 1 infection during treatment with peg-IFN-alpha 2a (180 µg weekly) and ribavirin (1000 mg or 1200 mg depending on weight below or above 74 kg). In the first study (the KinG1 trial), conducted between 2003 and 2005, 53 patients with treatment-naïve HCV genotype 1 infection and a clinical indication for treatment were treated for 48 weeks, discontinuing if HCV RNA had not declined by at least 2 logs after 12 weeks. In the second study (the TTG1 trial, conducted between 2008 and 2010), 106 patients were randomized to standard-of-care or tailored treatment. The treatment duration in the standard-of-care group was 24, 48, or 72 weeks depending on whether HCV RNA was undetectable after 4, 12, or 24 weeks of treatment with the similar stopping rule as for the KinG1 trial. All patients were treatment-naïve and negative for hepatitis B surface antigen and anti-HIV. For the present study, the 76 patients treated at the Sahlgrenska University Hospital and adjacent units were included.
Written informed consent was obtained from each patient. The study protocols were approved by the Regional Ethical Review Board in Gothenburg, Sweden.

Blood samples (Study 1: serum in the KinG1 study; Study 2: plasma in the TTG1 study) were obtained at baseline and after 1, 2, 3, 4, 7-8, 12, 16, 20, and 24 weeks of treatment. Serum samples from healthy controls were obtained from buffy coats from Sahlgrenska and Kungälv Hospital.

**IP-10/CXCL10 ELISA**

Serum samples were gently thawed at room temperature before conducting the assay. IP-10 was quantified using Quantikine Human CXCL10/IP-10 Immunoassay (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s protocol, with the difference that serum samples were diluted 1:4. In brief, 25 µl serum was diluted in 75 µl RD6Q before 75 µl RD1-56 was added. Double rows of supplied standard samples (ranging from 500 pg/mL to 7.8 pg/mL) were added to each plate. After 2 hours incubation at room temperature, the wells were washed with 400 µl Wash Buffer, followed by addition of 200 µl IP-10 Conjugate. The plate was covered and incubated for further 2 hours at room temperature. A repeated wash step followed by addition of 200 µl Substrate Solution, for 30 minutes at room temperature for developing the wells, was performed. The wells were stopped with a Stop Solution and read at $\lambda = 450$ nm (corrected at 650).

**CD26 ELISA**

Serum and plasma samples obtained for the KING 1-study were thawed gently at room temperature before conducting the assay. An enzyme-linked Immunosorbert Assay for quantitative detection of human serum CD26 (sCD26) (eBiosciences, San Diego, CA, USA) was used and the assay was
conducted according the manufacturer’s protocol. Briefly, the supplied microstrip wells were washed with 400 µl Wash Buffer. Standards (ranging from 500 ng/ml to 15.6 ng/ml) were added in duplicate to the standard well on each plate. 100 µl Sample Diluent was added to each well and 80 µl Sample Diluent together with 20 µl sample to the sample wells. A prepared Biotin-Conjugated solution was added at a volume of 50 µl to each well. The plate was covered and incubated on a shaker set at 100 rpm, for 3 hours at room temperature. This was followed by a washing step, addition of 100 µl Streptavidin-HRP solution, and incubation on a shaker for 1 hour at room temperature. The washing step was once again repeated followed by addition of TMB Substrate Solution, for 10 minutes at room temperature, for developing the wells. The assay was stopped with a Stop solution and the plates read at λ= 450 nm (corrected at 620 nm).

**Total CD26 Enzymatic Activity**

The enzymatic activity of sCD26 was measured with DPPIV-Glo™ Protease Assay (Promega, Madison, WI, USA). This assay is based on the cleavage of a pre-obtained substrate (Gly-Pro-aminoluciferin) with CD26, followed by light production measured as luciferase activity. The luminescent signal recorded for 0.1 seconds, defined as relative light units (RLU), is proportional to the total amount of sCD26 activity in each sample. For the assay, 50 µL sample and 50 µL freshly prepared CD26/DPPIV-Glo was used, followed by an incubation time of 30 minutes with CD26/DPPIV-Glo™ reagent. 50 µL PBS was used as negative control.

**HCV Genotyping**

Genotyping of HCV was performed using the Inno-LiPA (Line Probe Assay) HCV II assay (Innogenetics NV, Ghent, Belgium). Briefly, amplified and probe labeled 5’-UTR sequences are added to a precoated nitrocellulose
membrane. The membrane holds various complementary binding areas specific for different genotypes. Hybridization occurs on the specific area/line matching the correct genotype, and the line is visualized through color development.

**HCV RNA Quantification**

**DITTO-HCV Study.** HCV RNA was determined by reverse transcription polymerase chain reaction (RT-PCR) using Cobas Amplicor HCV Monitor version 2.0 (Roche Diagnostics, Branchburg, NJ), and quantified on days 0, 1, 4, 7, 8, 15, 22, and 29, at the end of treatment, and 24 months after the completion of treatment in the DITTO-HCV trial.

**NORDynamIC Trial.** HCV RNA was determined by RT-PCR of plasma using Cobas AmpliPrep/Cobas TaqMan HCV Test (Roche Diagnostics, Branchburg, NJ) on days 0, 3, 7, 8, and 29, weeks 8 and 12, at the end of treatment, and 24 weeks after the completion of therapy.

**DICO Study.** Plasma HCV RNA was determined using the COBAS AmpliPrep/COBAS TaqMan HCV Test (Roche Diagnostics, Branchburg, NJ, USA; limit of detection ≤15 IU/ml) on days 0, 1, 2, 7, 14, 28, weeks 8, 12, 24, 36, 48 after treatment onset, and 24 weeks after completion of therapy.

**HIV-RNA Quantification**

**DICO Study.** Plasma HIV RNA was determined using the Roche COBAS Amplicor HIV-1 Monitor Test version 1.5 (Roche Diagnostics, Branchburg, NJ, USA; limit of detection ≤50 copies/ml).
Liver Biopsies and Scoring

**DITTO-HCV Study.** Liver biopsies were obtained from patients in the DITTO-HCV trial within 12 months prior to inclusion in the study, and liver biopsy samples were processed for both histological evaluation (≥1.5 cm) and for RNA analysis (≥1 cm). The biopsy material for RNA analysis was immediately immersed in RNAlater (Ambion, AMS Technology, Cambridgeshire, UK) and stored at -70°C until assayed. In total, RNA from 72 liver biopsies could be retrieved and evaluated. Only biopsies with a length exceeding 1.5 cm and containing more than six portal tracts were evaluated. In total, liver biopsies from 228 infected patients in the DITTO-HCV trial were retrieved and evaluated. For each biopsy, a hematoxylin-eosin and a Sirius Red stain were centrally staged and graded by two independent observers experienced in pseudo-numerical scoring of liver biopsies in a blinded fashion according to the Ishak protocol. Equivocal issues were debated after the independent scores were noted, and a consensus score was obtained. In addition, steatosis was graded as follows: absent = 0, less than 30% of hepatocytes involved = 1, 30%-70% of hepatocytes involved = 2, and >70% of hepatocytes involved = 3.

IP-10 mRNA Quantification

**DITTO-HCV Study.** Total RNA was isolated from liver biopsies using the RNeasyMini Kit (Qiagen) and subsequently treated with deoxyribonuclease I. RNA integrity was assessed using RNA 6000 nanochips with an Agilent 2100 Bioanalyzer. First-strand cDNA was synthesized from 500 ng purified RNA using the SuperScript II RNase H(-) reverse transcriptase (Invitrogen) and random hexadeoxynucleotides. For real-time PCR, the Human SYBR Green QuantiTect Primer Assay for IP-10 (CXCL10, cat. no. QT01003065) was used (Qiagen). Reactions were performed using a 7900HT Real-Time PCR System (Applied Biosystems) and all samples were assayed in triplicate. Optical data obtained were analyzed using the default and variable parameters.
available in the Sequence Detection Systems software (SDS, version 2.2.2; Applied Biosystems). Expression level of target gene was normalized using as endogenous control genes the eukaryotic translation elongation factor 1 alpha 1 (forward 5'-AGCAAAAATGACCCACCAATG-3', reverse 5'-GGCCTGGATGGTTCAGGATA-3') and the beta glucuronidase (forward 5'-CCACCAGGGACCATCCAAT-3', reverse 5'-AGTCAAAATATGTGTTCTGGACAAAGTAA-3').

The expression level of IP-10 mRNA in the first of the 72 liver biopsy evaluated was chosen as the reference, and assigned 1.0 arbitrary units (AU).

**DNA Extraction**

*DITTO-HCV Study.* DNA from peripheral blood mononuclear cells was isolated using the QIAamp DNA mini kit (Qiagen) and quantitated on the NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific).

**IL28B Genotyping**

*DITTO-HCV Study.* DNA samples from patients and controls were genotyped for the IL28B rs8099917, rs12979860 and rs12980275 polymorphism with TaqMan SNP genotyping assays (Applied Biosystems Inc, Foster City, CA), using the ABI 7500 Fast real time thermocycler, according to manufacturers recommended protocols. TaqMan probes and primers were designed and synthesized by Applied Biosystems Inc. Automated allele calling was performed using SDS software from Applied Biosystems Inc. Positive and negative controls were used in each genotyping assay. The primers and probes utilized were:
1. NCBI dbSNP ID rs8099917 Applied Biosystems (AB) reference: C_11710096_10

2. NCBI dbSNP ID rs12979860 Forward primer: TGTACTG-AACCAGGGAGCTC, Reverse primer: GCGCGGAGT-GCAATTCAAC,
   Vic probe: TGGTTCCGCGCCTTC, Fam probe: TGGTTACGCCTTC

3. NCBI dbSNP ID rs12980275
   Forward primer: GTGCTG-AGAGAAGTCAAATTCC,
   Reverse primer: CCGCTA-CCGGCAATATT,
   Vic probe: AGACACGTCTGTTTC- TA, Fam probe: ACACGTCCGTTTCTA

**Statistical Methods**

The statistical methods used in analyzing the data included the Mann-Whitney U-test, Spearman’s rank correlation coefficient r_s, Kaplan-Meier cumulative survival plots, log-rank tests, Kruskal-Wallis, Chi-square test, or Fisher’s exact test. Univariate and multivariate analyses were also performed between patient groups. All reported p-values are two-sided.
4 RESULTS AND DISCUSSIONS

Paper I-II

Earlier studies have shown that patients with chronic hepatitis C frequently have high levels of IP-10 in the circulation [128]. In paper I, we assessed plasma IP-10 and expression of IP-10 mRNA in liver biopsies from untreated infected patients to test the hypothesis that liver cells may constitute a significant source of systemic IP-10. As shown in Figure 6, a significant association was observed between intrahepatic IP-10 mRNA and plasma IP-10 ($r_s = 0.707; P<0.0001$).

We next asked whether ongoing formation of IP-10 in liver tissue, as reflected by the level of expression of IP-10 mRNA, prognosticated the outcome of peg-IFN/ribavirin treatment in these patients. A significant association was observed between pretreatment intrahepatic IP-10 mRNA and the first time-point of undetectable HCV RNA (HCV RNA $\leq 50$ IU/mL), measured over the first 6 weeks of therapy. Patients were dichotomized as having IP-10 mRNA expression below or above the median level, 0.8
arbitrary units (AU). The results showed a significant association between patients with low IP-10 expression levels (<0.8 AU) and achieving HCV RNA levels below detection limit (P=0.015 for all patients; P=0.017 for patients infected with genotype 1 or 4 and P=0.028 for patients infected with genotype 2 or 3; log-rank tests).

The results presented in paper I also showed a significant association between or pretreatment intrahepatic IP-10 mRNA expression and a rapid first phase HCV RNA decline (defined here as the first 24 h on therapy) along with a similar association for systemic IP-10. These conclusions were based on results achieved when patients were grouped as having above or below 1.0 log_{10} viral reduction (the median reduction) in the DITTO-HCV study. As seen in Figure 7, patients with a rapid viral decline had significantly lower intrahepatic IP-10 expression regardless of genotype (genotype 1/4 median 0.58 versus 1.30, P=0.01; genotype 2/3 median 0.53 versus 1.48, P=0.02; Mann-Whitney U-test).
Figure 7 Box plots displaying the 10th, 25th, 50th, 75th, and 90th percentiles of pretreatment intrahepatic (A-C) IP-10 mRNA or (D-F) plasma IP-10 for patients achieving greater than or less than 1.0 log₁₀ decline (i.e., the median reduction) in HCV RNA (IU/mL) during the first day of therapy in the DITTO-HCV trial. IP-10 mRNA levels are expressed as arbitrary units. Data are presented for all patients and for patients grouped by genotype (1/4 and 2/3). P values were obtained using the Wilcoxon-Mann-Whitney U-test.

The association between low plasma IP-10 levels and a pronounced reduction of viral RNA during the first day of therapy remained significant regardless of stage of fibrosis, steatosis or BMI. A similar, although non-significant, trend was observed for the second phase viral decline (HCV RNA reduction during day 8 and 29 on therapy). A separate analysis of IP-10 levels versus first day decline of HCV in an additional trial (the NORDynamIC trial,
n=382) confirmed that high IP-10 levels entail a poor elimination of HCV-RNA during the first day of therapy.

In summary, intrahepatic IP-10 mRNA expression mirrors plasma IP-10 levels, and systemic levels of plasma IP-10 as well as intrahepatic expression of IP-10 mRNA predict first phase viral decline during therapy.
In paper II, the association between baseline plasma IP-10 levels and HCV RNA kinetics among HIV/HCV co-infected patients on interferon/ribavirin therapy was studied. The results, based on the study of 21 co-infected patients, showed that low baseline plasma IP-10 levels (<150 pg/mL) were associated with favorable HCV RNA elimination, as the case for HCV monoinfected patients presented in paper I. Additionally, the results in paper II also showed that rapid first phase HCV RNA decline (defined here as the first 48 h of treatment) was significantly associated with low baseline plasma IP-10 levels ($r_s=-0.68$, $P=0.004$) for all HCV genotypes (1, 2, and 3) in the study, as seen in Figure 8. A weaker, but significant association was also observed between low baseline plasma IP-10 levels and the second phase viral reduction (HCV RNA reduction during day 7 and 28 on combination treatment).

Additionally, plasma IP-10 levels were measured during treatment and the results showed that IP-10 levels peaked 24 h after therapy initiation, regardless of baseline levels. Patients were grouped as having baseline IP-10 concentrations of <150, 150–600, or >600 pg/mL.

Furthermore, 24 weeks after treatment discontinuation, plasma IP-10 levels were significantly lower for patients who had achieved SVR than those who did not (median 114 pg/mL versus 356 pg/mL, $P=0.039$; Mann Whitney U-test), as shown in Figure 9. In contrast to previous studies [128], in HCV-monoinfected patients 3 out of 8 co-infected patients achieving SVR
continued to have IP-10 $>$ 150 pg/mL 6 months after the completion of HCV therapy.

![Figure 9](image-url)

The results presented in papers I and II thus imply that low baseline IP-10 levels predict favorable treatment outcome for HIV/HCV co-infected patients as well as for HCV monoinfected patients. In paper II, IP-10 not only predicted a rapid first phase decline but was also associated with a slower second phase decline. A rapid first phase viral decline during standard therapy reflects blocking of virion production as a result of antiviral effectiveness of interferon [38, 153]. This phase also predicts the slower second phase decline and final treatment outcome [154-156]. The second phase decline reflects the death rate of infected hepatocytes or the rate of clearance of HCV from infected cells and is in general much slower than its preceding phase. In line with this, the findings in paper I and II suggest that baseline IP-10 levels interfere with the antiviral properties of interferon and to a lesser extent with its immune activating properties.

In paper II, it was observed that IP-10 levels increased markedly one day after initiation of treatment, regardless of baseline level. It may be speculated that a more pronounced induction of IP-10 after treatment start reflects a more pronounced impact on the immune system. Patients with baseline plasma IP-10 levels $<$ 150 pg/mL measured approximately a 10-fold increase...
in IP-10 production, 24 hours after onset of therapy, compared to 1.5-fold increase for patients with baseline plasma IP-10 levels >600 pg/mL. Studies supporting these findings, suggest that the ongoing transcription of genes prior to treatment predicts unfavorable treatment response [94, 157], and that ISGs, including IP-10, in pretreatment liver biopsies are elevated in HCV non-responders. With this data, HCV monoinfected as well as HIV/HCV co-infected patients suffering from the adverse effects HCV treatment, yet having low levels of pretreatment IP-10 levels should be encouraged to continue therapy.
In paper III, the impact of IP-10 on eliminating HCV RNA during standard treatment for patients with favorable or unfavorable genotypes of $IL28B$ SNPs was investigated. The results, obtained using samples from the DITTO-HCV study, showed that homozygous carriers of SNPs that predict successful outcome of therapy such as $CC_{rs12979860}$, $AA_{rs12980275}$, and $TT_{rs8099917}$, had significantly lower baseline IP-10 levels than those heterozygous or homozygous for the risk allele, for all HCV genotypes (1-4) (at rs12979860 median 189 versus 258 pg/mL, $P=0.02$ Mann-Whitney U-test; at rs12980275 median 189 versus 158 pg/mL, $P=0.01$; and at rs80999917 median 224 versus 288 pg/mL, $P=0.04$), as seen in Figure 10.

Furthermore, we assessed HCV viral kinetics during treatment for patients infected with HCV genotype 1 and 2/3 to evaluate the impact of $IL28B$ variability on the effectiveness of HCV therapy. The results showed that among HCV genotype 1 patients who were homozygous for the favorable alleles (as listed above) had a pronounced HCV RNA reduction during the first phase (days 0 to 4 on treatment) compared to the patients carrying the risk alleles (mean 2.0/0.9/0.6 log$_{10}$ IU/mL for $rs12979860\ CC/CT/TT$; $rs12980275\ AA/AG/GG$; $rs8099917\ TT/AG/GG$).

Figure 10 Tenth, 25th, 50th, 75th, and 90th percentiles of pretreatment IP-10 in relation to $IL28B$ variants.
As shown in paper I, low baseline IP-10 levels were significantly associated with a rapid first phase decline. The results in paper III show that IP-10 levels were predicted the first phase decline in homozygous or heterozygous carriers for the favorable alleles, as seen in Figure 11. Similarly among genotype 2/3 infected, carriage of CC_{rs12979860} was significantly associated with a rapid first phase decline. The results also showed that baseline IP-10 levels for CT_{rs12979860} were correlated with efficient HCV RNA elimination. Neither IL28B-related SNPs nor baseline IP-10 predicted second phase viral decline.

Among homozygous carriers of the rs12979860 and rs8099917 alleles, a lower baseline IP-10 significantly predicted RVR. None of the patients with IP-10 >600 pg/mL achieved RVR regardless of IL28B genotype, and RVR was in turn the only independent predictor of SVR.

SNPs near IL28B reportedly predict spontaneous clearance of HCV as well as SVR following combination treatment in particular for HCV genotype 1-infected patients [130, 158, 159]. Furthermore, studies on IL28B mRNA expression (IL28A, IL28B, and IL10) and polymorphisms near the IL28B encoding region have shown that there is no significant correlation between genetic variability in these regions and the related protein expressions [160]. The finding that IL28B polymorphisms are associated to the first phase viral reduction, thus suggests that genetic variability in this location is directly linked to the antiviral effectiveness of interferon and ribavirin.
Systemic and intrahepatic levels of IP-10, as discussed in papers I and II, are elevated in HCV genotype 1 and 4-infected patients, which also have the lowest rate of SVR [128]. The finding in paper III that IL28B-related polymorphisms and baseline systemic IP-10 levels are independent predictive
factors, and thus augmenting the accuracy of response prediction in chronic HCV infection has subsequently been confirmed by others [161]. Moreover, in paper III modest elevation of baseline IP-10 was observed among patients carrying the risk alleles compared to patients with favorable allelic distributions. Elevated baseline levels of ISGs have been reported in patients with risk alleles [162, 163], which could be a hypothesis explaining our results.

In summary, paper III shows that baseline plasma IP-10 is significantly associated with \textit{IL28B}-related SNPs. IP-10 and \textit{IL28B} polymorphisms are independent predictors of the first phase HCV RNA elimination, RVR, and final treatment outcome, SVR.
Paper IV

In paper IV, plasma/serum baseline sCD26 concentration and sCD26 cleaving activity was measured in chronic HCV infected patients from two independent studies. In line with previous studies, an association between baseline IP-10 levels and first phase viral decline, as outlined in papers I and II, was confirmed in this study (where the first phase viral decline was defined as HCV RNA reduction between day 0 and 7). Furthermore, the degree of first phase viral decline significantly predicted SVR (P<0.0001). Considering that CD26 truncates IP-10, as shown in previous studies [152], the results presented in paper IV showed that baseline sCD26 levels and baseline IP-10 levels were correlated (Study 1 r_s=0.39 P=0.02; Study 2 r_s=-0.33 P=0.05). Also, there was a significant trend towards a negative correlation between sCD26 activity and baseline IP-10 levels (Study 1 r_s=-0.06 P=0.8; Study 2 r_s=-0.47 P=0.003).

Measuring sCD26 concentration and sCD26 activity revealed that patients achieving SVR had lower sCD26 concentration (Study 1 P=0.05; Study 2 P=0.02) but higher sCD26 activity (Study 1 P=0.06; Study 2 P=0.02). In line with these findings, a negative correlation was observed between sCD26 concentration and sCD26 activity in both studies (Study 1 r_s=-0.44 P=0.008; Study 2 r_s=-0.32 P=0.05). However, the absolute values of enzyme amount and activity varied considerably between studies.

To minimize the inter-study variation, the quotient of sCD26 cleaving activity per sCD26 molecule was calculated as *specific sCD26 activity*. It was found that patients achieving SVR had significantly higher specific sCD26 activity (high sCD26 activity and low sCD26 concentration) compared to non-SVR patients (n=71, P=0.0001), as shown in Figure 12.
The data also showed that IP-10 levels and the specific sCD26 activity were significantly inversed correlated (n=71, P=0.02) i.e. low IP-10 levels were correlated with high specific sCD26 activity. Interestingly, the results also showed that patients with high specific sCD26 activity displayed a higher rate of HCV RNA clearance during therapy measured over 48 weeks compared to patients with low specific sCD26 activity (P=0.0004), see Figure 13.

We did not observe a significant association between the specific sCD26 activity and the first and second phase viral decline. Of note, the amount of HCV RNA in blood at onset of therapy ("viral load") was significantly lower in patients with high specific sCD26 activity achieving SVR (r_s=-0.3 P=0.01).
However, the specific CD26 activity predicted SVR also within the group of patients with high (above median) viral load at baseline in patients with specific CD26 activity above or below median, $P=0.013$) with a trend also within the group with low viral load ($P=0.056$).

In the setting of $IL28B$ polymorphisms, the combination of high specific sCD26 activity with favorable allelic distributions $TT_{rs8099917}$ or $CC_{rs12979860}$ yielded a 93% positive predictive value for SVR ($P=0.0003$ and $P=0.01$, respectively).

In summary, the results in paper IV imply that the specific sCD26 activity may be an accurate predictor of treatment outcome in chronic hepatitis C.
5 CONCLUSIONS

Pretreatment biomarkers predicting therapy outcome in chronic HCV infection are useful tools elucidating the infection course of the virus. Most importantly, these tools could give information valuable in treatment optimization for patients undergoing combination treatment with interferon and ribavirin.

The main conclusions in this thesis are:

• Baseline IP-10 levels are predictive of first phase HCV RNA decline and SVR in HCV monoinfected and in HIV/HCV co-infected patients. Furthermore, systemic IP-10 levels mirror intrahepatic IP-10 mRNA expression.

• Genetic variability near the IL28B encoding region predicts early HCV RNA elimination during combination therapy and is independent of baseline IP-10 levels. Favorable IL28B genotypes and baseline IP-10 levels in combination enhance the predictive value of treatment outcome.

• The specific enzymatic activity of sCD26 is predictive of SVR, and is independent of IP-10 levels. In combination, the predictiveness of treatment outcome during combination therapy is augmented.
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