Hepatitis C virus: epidemiology and immunology
van den Berg, C.H.S.B.

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Chapter 1

Introduction
Hepatitis C virus

Hepatitis C virus (HCV) is a single-stranded RNA virus that belongs to the family of Flaviridae. HCV was first discovered in 1989. Seven major genotypes and over 80 subtypes of HCV are recognized worldwide. According to estimates dating from 1999 of the World Health Organization (WHO), approximately 170 million people are infected with HCV worldwide (Figure 1.1). The number of HCV-infected individuals in The Netherlands is only roughly known, and is estimated to be 15,000-65,000 infected individuals corresponding to a prevalence of 0.1-0.4% in the general population.

Figure 1.1 Estimated prevalence of HCV infection by WHO region. Reproduced from reference 4 with permission from the author.

Epidemiology

Transmission of HCV occurs mainly via exposure to infected blood. Therefore, injecting drug users (DU) are at high risk through the sharing of needles, syringes and other (injecting) drug use paraphernalia. Other risk groups for HCV infection are individuals who received a blood transfusion or blood products when screening of blood and blood products was not yet available (i.e., before 1991). Nosocomial transmissions through needle stick injuries, renal dialysis and infected equipment, contamination of injectable medication or flush solutions, or transplanted tissue also occur. In addition, household, mother-to-child and sexual transmission have been described. In the last years several reports have been published on outbreaks...
of acute HCV presumably transmitted via sexual exposure among human immunodeficiency virus (HIV)-positive men who have sex with men (MSM). The incidence of transfusion-related HCV transmission has drastically declined in developed countries after the introduction of HCV screening of blood and blood products in 1991. On the other hand, HCV transmission among injecting DU remains highly frequent. And since injecting drug use is reported in most countries in the world, most new HCV infections occur in DU. And HCV prevalence in DU populations is very high, ranging from 44 to >95%. HCV incidence is highest shortly after start of injection drug use, probably due to the highest injecting risk behaviour around initiation. HCV incidence in high income countries ranges between 2 and 25/100 person years (PY).

Since there is no prophylactic vaccine available for HCV, the most important measures to reduce HCV incidence in DU are prevention programmes aimed at reducing (injecting) risk behaviour, early diagnosis, and treatment to reduce the pool of chronically HCV-infected individuals. Harm reduction measures like methadone provision and needle exchange programs have proven successful for prevention of HIV infections in DU. However, HCV is more easily transmitted parenterally than HIV, not only via contaminated needles and syringes, but also via other (injecting) drug use paraphernalia. Hence, it is thought that the various harm reduction measures that have been effective in decreasing HIV incidence may not have had such a large effect on HCV incidence. Although it is biologically plausible to assume that harm reduction measures like needle exchange programs and opiate substitution treatment have an effect on the HCV incidence in DU, it has been difficult to prove this. And although declining prevalence of HCV was reported after the introduction of needle exchange programs, only few studies were able to describe the effect of either program on HCV incidence.

Natural history

Acute HCV infection is usually asymptomatic, but in approximately 20% of cases aspecific flulike symptoms like nausea, fever and/or abdominal pain occur. Only in a small proportion of cases jaundice is the presenting symptom. Chronic infection develops in 60-80% of cases, and is usually defined as persistence of HCV-RNA six months after acute infection. Factors associated with higher rates of viral persistence are male sex, older age, being immunocompromised (e.g., in HIV co-infection), and being of African-American race. It is important to realize that many of these studies were cross-sectional among prevalent HCV cases and therefore these are subject to selection bias: in cross-sectional studies among hospital patients, symptomatic patients are more likely to present and to be included than individuals that have cleared HCV. This would result in an underestimation of the rate of spontaneous viral clearance. On the other hand, cross-sectional studies among long-standing HCV-infected DU in the community might overestimate the rate of spontaneous HCV viral clearance, since those who developed chronic HCV are more likely to have deceased before the study start than those who cleared HCV. After spontaneous viral clearance individuals do not seem to be fully protected from a new HCV infection, since re-infection after clearance and superinfection in chronically infected DU has been described. Epidemiological studies in injecting DU have suggested that protective immunity occurs after a spontaneously cleared HCV infection, but other studies have shown contradicting results. However, some partial (cross-reactive) immunity might occur in DU after clearance, as evidenced by
lower peak HCV-RNA titres in re-infections compared to the peak HCV-RNA titre in primary HCV infection.45

It is estimated that in a minority of patients (approximately 20%) chronic HCV infection can eventually lead to liver fibrosis, liver cirrhosis and/or hepatocellular carcinoma in the decades after infection. These estimates are mostly based on hospital-based cohorts and not on population cohorts that have been followed up since HCV infection.48,49 Data from an Irish cohort of HCV-infected women also suggested that disease progression might be slower.50 Known risk factors for faster progression to liver fibrosis are alcohol abuse, older age at infection, and HIV or hepatitis B virus (HBV) co-infection.48,49

Due to shared routes of transmission of HCV and HIV, co-infection often occurs in high risk populations.8 Almost all HIV-infected DU and haemophiliacs are co-infected with HCV, whereas almost all HCV-infected MSM are co-infected with HIV.6,33 HIV infects CD4+ T cells and in the course of HIV infection the number of CD4+ T cells declines, which eventually leads to acquired immunodeficiency syndrome (AIDS). Although the effect of HCV co-infection on HIV progression remains controversial,51,52 HIV-infection clearly has an impact on HCV disease progression. Firstly, HIV co-infection during acute HCV infection is associated with lower rates of HCV clearance.42,53 Secondly, HIV-infected individuals have higher levels of HCV viremia.54 Thirdly, progression to liver fibrosis, liver cirrhosis and end stage liver disease is faster in co-infected individuals compared to HCV mono-infected individuals.55 Finally, HIV/HCV co-infected injecting DU are at higher risk of dying from a liver related cause of death than HCV mono-infected injecting DU.56

**Virology**

HCV is a single-stranded RNA virus that infects hepatocytes (liver parenchymal cells). The HCV genome consists of approximately 9,600 base pairs. Within the hepatocytes internal ribosome entry site (IRES)-mediated translation yields a polyprotein precursor that is subsequently cleaved by viral and host-cell proteases into the different structural (Core, E1, E2, p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins. (Figure 1.2)57 Core, E1 and E2 form the nucleocapsid of the HCV virion. P7 belongs to a family of proteins known as viroporins, which homo-oligomerise to form aqueous pores in cellular membranes, thereby enhancing membrane permeability in order to promote virus budding.58,59 The different nonstructural proteins are involved either in viral replication or in polyprotein processing.50 In short, NS2-NS3 is the zinc-dependent metalloproteinase that cleaves at the NS2/NS3 cleavage site. NS4A is the co-factor of the NS3 serine proteinase that releases the remaining HCV proteins of the polyprotein. NS4B protein is known to induce intracellular membrane changes which called a 'membranous web', which is a membrane-associated replication complex.61 NS5B is the RNA-dependent RNA polymerase and the function of NS5A is still unknown.60

Humans are the only natural host for HCV infection. For years, the research on the HCV lifecycle was hampered because there was no cell culture or small animal model available. Chimpanzees were the only available animal model, which has its limitations since the natural history of HCV is different in humans. Nowadays, it is possible to replicate HCV in a cell culture system (HCVcc) or in a subgenomic replicon system.57,62-65
Figure 1.2  The HCV polyprotein and processing, reproduced from reference 57 with permission from the author.57 NCR: non-coding region, IRES: internal ribosome entry site. Amino-acid numbers are shown above each protein (HCV H strain; genotype 1a; GenBank accession number AF009606). Solid diamonds show the cleavage sites of the HCV polyprotein precursor by the endoplasmic reticulum signal peptidase. The open diamond indicates further C-terminal processing of the core protein by signal peptide peptidase. Arrows indicate cleavages by the HCV NS2–3 and NS3–4A proteases. Dots in E1 and E2 indicate the glycosylation of the envelope proteins.

The replication rate of HCV is very high, each day up to $10^{12}$ virions are produced in an infected individual. Moreover, HCV replication is highly error prone, due to the lack of proofreading function of its RNA-dependent RNA polymerase, NS5B. The high viral turnover and the error prone replication process, result in rapid evolution of HCV within an infected host. The swarm of highly similar viral variants that develop within one host are called quasispecies and provide one of the mechanisms by which HCV evades host immune surveillance and establishes chronic infection.

Immunology

The innate immune system is the first non-specific defence system of the human body against foreign pathogens like viruses. The main functions of the innate immune system are activation of the complement system and triggering the adaptive immune system. After transmission of HCV, the virus infects hepatocytes most likely via receptor-mediated endocytosis, followed by release of HCV RNA in the cytoplasm. Plasmacytoid dendritic cells (pDC) are among the first cells of the innate immune system to encounter HCV. Pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRR) on the outer cell membrane, or on intracellular membranes of the pDC, like Toll-like receptors (TLRs) and other molecules/enzymes/receptors (e.g., RIG-I and MDA5) able to detect single- or double-stranded (viral) RNA. This activation triggers many intracellular events, including synthesis and release of type I interferons (IFN, α and β). Secretion of these type I IFN induces an antiviral state in the...
cell and also in neighbouring cells (thereby creating a time window for the host to develop an adaptive immune response). By interrupting the IFN pathway, HCV facilitates its own chronic course of infection. Many of the HCV proteins have been implicated to play a role in disrupting intracellular signalling. For example, Core protein is a suppressor of silencing interfering RNA (siRNA) and might thereby assist chronic evolution of HCV infection. NS3/NS4A disrupts the classical intracellular pathway for IRF-3 activation by cleaving MAVS (also known as Cardif, IPS-1 or VISA) and TRIF, which leads to less IFN gene translation. 

Adaptive immune responses in HCV infection

The adaptive immune system consists of the humoral and cellular immune response. Most individuals who get exposed to HCV develop antibodies in the course of infection, but these antibodies do not always offer protection from development of chronic HCV infection, re- or superinfection.

The main players in the adaptive cellular immune system are CD4+ T-helper cells and CD8+ cytotoxic T cells (CTL). CD4+ T cells recognize viral peptides presented by major histocompatibility complex (MHC) class II molecules on professional antigen presenting cells (APC). High and broadly targeted HCV-specific CD4+ T cells have been shown to play a major role in spontaneous resolution of HCV, both in chimpanzee and human studies. In contrast, the development of viral persistence has been associated with a weak and dysfunctional HCV-specific T-cell response. T-cell responses directed against HCV Core protein seem to be associated with persistent viremia, while T-cell responses directed against nonstructural (NS) proteins have been associated with viral clearance.

HCV-specific CTL recognize viral peptides presented by MHC class I molecules on the surface of infected hepatocytes. Each individual can express up to 6 different MHC class I molecules on his or her cells. Each MHC class I molecule can present viral peptides with a specific molecular signature. Many associations between MHC molecules and disease have been described, also for HCV. For instance, individuals expressing the HLA-B27 molecule are more likely to clear HCV spontaneously. A vigorous and multispecific HCV-specific CD8+ T-cell response during acute infection has been associated with a rise in alanine aminotransferase (ALT) levels and a drop in HCV-RNA titres. This suggests that HCV-specific CTL are effective in killing infected hepatocytes in acute HCV infection. Waning of these responses has been associated with an increase of HCV-RNA levels and subsequent development of chronic HCV infection. HCV replication is not only abrogated by CTL-mediated killing of HCV-infected hepatocytes, but also non-cytolytic inhibition of viral replication by IFN-γ occurs. HIV co-infection negatively influences HCV-specific T-cell responses. In HCV/HIV co-infected individuals the rate of spontaneous HCV clearance is lower than in HIV-uninfected individuals most likely due to a hampered development of HCV-specific adaptive immune response. Furthermore, HCV viral load is higher than in HCV mono-infected individuals, also suggesting loss of immune pressure.

HCV treatment

The current standard treatment for chronic HCV mono-infection consists of weekly pegylated interferon (PEG-IFN) and daily ribavirin. The aim of treatment is to eradicate viral RNA. Treatment success is defined as sustained virological response (SVR): undetectable HCV RNA 6 months after stop of treatment. HCV genotype is the
most important baseline predictor of SVR, the rate of SVR is much lower in genotype 1 and 4 infected patients (50-60%) than in genotype 2 and 3 infected patients (80-90%). The most important predictor of SVR during treatment is the so-called rapid virological response (RVR), defined as undetectable viral load at week 4 of treatment. Standard therapy duration is 48 weeks for individuals that are chronically infected with HCV genotypes 1 or 4 and 24 weeks for those infected with genotypes 2 or 3. Treatment is being more and more individualized, with patients who achieve RVR receiving shorter therapy, while those with a slow viral decline are treated longer. Both PEG-IFN and ribavirin have many side effect, like flu-like symptoms, depression, pancytopenia, and fatigue, causing dose reduction or discontinuation of treatment in a substantial proportion of patients. HCV treatment is more effective when initiated shortly after acute HCV infection than in the chronic phase of HCV infection.

Interferons are endogenous proteins with antiviral and immunomodulatory properties. PEG-IFN is recombinant interferon coupled to a polyethylene glycol (PEG) molecule. Ribavirin is a nucleoside analogue with broad-spectrum antiviral activity. The exact mode of action of ribavirin is unknown. It is thought that both PEG-IFN and ribavirin have immunomodulatory properties.

During treatment of HCV with PEG-IFN and ribavirin the viral decline is biphasic. Mathematical modelling has shown that the first decline can mainly be explained by blocking production of new virions, while the second slope is determined by the half-life of infected hepatocytes (i.e., killing of HCV-infected hepatocytes by CTL). In mono-infected individuals higher proliferative capacity of HCV-specific CTL at the start of therapy has been associated with successful treatment, which indeed suggests a role for CTL in forced viral clearance under influence of PEG-IFN and ribavirin. However, only one study showed an augmentation of HCV-specific T-cell responses during combination therapy, while other studies examining the dynamics of HCV-specific T-cell responses during HCV treatment with PEG-IFN and ribavirin did not. HIV co-infection negatively influences HCV treatment outcome: side effects of HCV treatment are more common and more severe, and SVR rates are lower compared to HIV-uninfected individuals.

Until recently most DU were not treated for their chronic HCV infection, partly because feasibility of treating DU was often questioned by clinicians. They perceived that lack of adherence and risk of re-infection would not make HCV treatment worthwhile. Since 2005 DU in the Amsterdam Cohort Study among DU are screened for HCV and offered treatment when found to be chronically infected. Preliminary results from this so-called DUTCH-C study (an acronym for drug users on treatment for chronic hepatitis C infection) are promising and show that treatment is realistic in DU when a multidisciplinary approach is taken. Hepatologists, addiction specialists, and research staff collaborate closely, and treatment is directly observed and combined with methadone provision.

Currently new therapeutic concepts are being developed which directly target viral enzymes, or influence host-virus interactions. Preclinical studies produced encouraging results, but the initial enthusiasm has been hampered by toxicity issues and rapid selection of resistance. Despite this, several of these new compounds are very promising and are expected to be registered within the next three years. Two protease inhibitors, telaprevir (VX-950) and boceprevir (SCH503034) have recently entered phase III clinical trials. Treatment regimens that include one of these new-generation anti-HCV drugs, referred to as STAT-C (specifically targeted antiviral therapy for HCV) have achieved SVR up to 65-75% and 50% in treatment-naïve patients and treatment-experienced patients who were nonresponsive to interferon/ribavirin,
respectively. Future treatment of chronic HCV will probably more effective and shorter and consist of a combination of pegylated interferon and ribavirin together with one or more new drugs.

There is no vaccine available for HCV. However, studies on natural clearance of HCV have shown that a robust, multispecific and lasting T-cell response is very important. Furthermore, it has been shown in a cohort of German women that were infected by a batch of infected anti-D immunoglobulins, early development of broadly targeted neutralizing antibodies was associated with viral clearance. Therefore, for an HCV vaccine to be successful, it will most likely have to elicit both a T-cell response and a humoral response. Both prophylactic and therapeutical vaccines (phase 1 and 2) are currently under study.

Outline of this thesis

The studies in this thesis were performed to improve our understanding of the epidemiology and natural history of HCV infection in DU. Furthermore, we aimed to get insights into the immunology of HCV infection during acute and chronic HCV infection and the influence of HIV co-infection hereon.

Most studies described in this thesis were performed within the framework of the Amsterdam Cohort Studies (ACS). The ACS among men having sex with men (MSM) was started in 1984 to investigate the prevalence, incidence, and risk factors of infections with HIV-1 and other blood-borne and/or sexually transmitted infections, as well as the effects of intervention. The Amsterdam Cohort Study among DU started a year later in December 1985, recruitment is ongoing and in recent years has been directed in particular to young DU. Participants in the ACS visit the Amsterdam Health Service every 4-6 months and each visit standardized questionnaires on health, socio-demographic situation, sexual and (injecting) drug use related risk behaviour are filled in. Also, each visit blood is drawn for prospective HIV testing and storage. Peripheral blood mononuclear cells (PBMC) are stored for all HIV-positive participants and for selected HIV-negative participants.

In chapter 2 the epidemiology of HCV in DU is studied. In chapter 2.1, the prevalence, incidence and risk factors for HCV infection in the ACS among DU are described for a 20-year period. Furthermore, the incidence of HCV is compared with the incidence of HIV. To further understand the decline of HCV and HIV incidence described in chapter 2.1, the effect of harm reduction measures like needle exchange programs and methadone on the incidence of both blood borne viruses is examined in chapter 2.2. Since the HCV prevalence found in never-injecting DU at entry in the ACS is much higher than the estimated HCV prevalence in the general Dutch population, chapter 2.3 describes determinants of HCV positivity in combination with molecular epidemiology among never-injecting DU from the ACS.

In chapter 3, 2 studies on the natural history of HCV are described. In chapter 3.1, the rate and determinants of spontaneous viral clearance of HCV are studied in HCV seroconverters. And in chapter 3.2, the mortality of HCV mono-infected DU is compared with the mortality in HCV/HIV co-infected DU and DU without HCV and HIV.
Chapter 4, consist of immunological studies of acute HCV in DU and MSM. The first chapter (chapter 4.1) describes longitudinal HCV-specific T-cell responses in injecting drug users with acute HCV infection. In chapter 4.2, the effect of acute HIV co-infection on the development of HCV-specific T-cell responses is studied in DU with acute HCV infection. In addition, longitudinal responses before and after HIV seroconversion in already HCV-infected DU are examined. Chapter 4.3 describes that HCV-specific T-cell responses are present before actual HCV viremia and HCV seroconversion took place in 3 HIV-infected MSM. In chapter 4.4 the longitudinal responses during acute HCV infection in HIV-infected MSM are studied.

Chapter 5 describes HCV-specific T-cell responses in the chronic phase of HCV infection. Chapter 5.1 describes whether increased exposure to HCV has an effect on the HCV-specific T-cell response. Currently standard HCV treatment consists of pegylated interferon (PEG-IFN) and ribavirin, in chapter 5.2, we show that the decline of HCV-specific t-cell responses parallels the decline in HCV viral load in genotype 1 and 3 HCV/HIV co-infected patients during treatment with PEG-IFN and ribavirin, suggesting a limited role for these responses in forced viral clearance.

In chapter 6, the general discussion, the main findings of the studies presented in this thesis are discussed and related to recent literature. Furthermore, recommendations for future research are presented.
Chapter 1
Introduction

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