HEPATITIS C VIRUS

EPIDEMIOLOGY AND IMMUNOLOGY

academisch proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties
ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op vrijdag 26 juni 2009, te 12:00 uur

door

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geboren te Wageningen
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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.\(^1\) Seven major genotypes and over 80 subtypes of HCV are recognized worldwide.\(^2\) According to estimates dating from 1999 of the World Health Organization (WHO), approximately 170 million people are infected with HCV worldwide (Figure 1.1).\(^3,4\) 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.\(^5,7\)

![Figure 1.1 Estimated prevalence of HCV infection by WHO region. Reproduced from reference 4 with permission from the author.](image)

Epidemiology

Transmission of HCV occurs mainly via exposure to infected blood.\(^8,9\) Therefore, injecting drug users (DU) are at high risk through the sharing of needles, syringes and other (injecting) drug use paraphernalia.\(^10,11\) 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).\(^12\) Nosocomial transmissions through needle stick injuries, renal dialysis and infected equipment, contamination of injectable medication or flush solutions, or transplanted tissue also occur.\(^13-17\) In addition, household, mother-to-child and sexual transmission have been described.\(^12,14,18-23\) 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.\textsuperscript{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 hepato-cellular 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.\textsuperscript{48,49} Data from an Irish cohort of HCV-infected women also suggested that disease progression might be slower.\textsuperscript{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.\textsuperscript{48,49}

Due to shared routes of transmission of HCV and HIV, co-infection often occurs in high risk populations.\textsuperscript{8} Almost all HIV-infected DU and haemophiliacs are co-infected with HCV, whereas almost all HCV-infected MSM are co-infected with HIV.\textsuperscript{6,33} HIV infects CD4\textsuperscript{+} T cells and in the course of HIV infection the number of CD4\textsuperscript{+} T cells declines, which eventually leads to acquired immunodeficiency syndrome (AIDS). Although the effect of HCV co-infection on HIV progression remains controversial,\textsuperscript{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.\textsuperscript{42,53} Secondly, HIV-infected individuals have higher levels of HCV viremia.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{58,59} The different nonstructural proteins are involved either in viral replication or in polyprotein processing.\textsuperscript{60} 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.\textsuperscript{61} NS5B is the RNA-dependent RNA polymerase and the function of NS5A is still unknown.\textsuperscript{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.\textsuperscript{57,62-65}
The HCV polyprotein and processing, reproduced from reference 57 with permission from the author. 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
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...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-\(\gamma\) 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 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.\textsuperscript{101} 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.\textsuperscript{100,102}

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.\textsuperscript{103} Therefore, for an HCV vaccine to be successful, it will most likely have to elicit both a T-cell response and a humoral response.\textsuperscript{104,105} Both prophylactic and therapeutical vaccines (phase 1 and 2) are currently under study.\textsuperscript{101,106}

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.\textsuperscript{107} 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.\textsuperscript{108} 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.
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Chapter 2

Epidemiology
Chapter 2.1

Major decline of hepatitis C virus incidence rate over two decades in a cohort of drug users

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Eur J Epidemiol 2007;22:183-193.
Abstract

Injecting drug users (DU) are at high risk for hepatitis C virus (HCV) and HIV infections. To examine the prevalence and incidence of these infections over a 20-year period (1985-2005), the authors evaluated 1276 DU from the Amsterdam Cohort Studies who had been tested prospectively for HIV infection and retrospectively for HCV infection. To compare HCV and HIV incidences, a smooth trend was assumed for both curves over calendar time. Risk factors for HCV seroconversion were determined using Poisson regression. Among ever-injecting DU, the prevalence of HCV antibodies was 84.5% at study entry, and 30.9% were co-infected with HIV. Their yearly HCV incidence dropped from 27.5/100 person years (PY) in the 1980s to 2/100 PY in recent years. In multivariate analyses, ever-injecting DU who currently injected and borrowed needles were at increased risk of HCV seroconversion (incidence rate ratio 29.9, 95% CI 12.6-70.9) compared to ever-injecting DU who did not currently inject. The risk of HCV seroconversion decreased over calendar time. The HCV incidence in ever-injecting DU was on average 4.4 times the HIV incidence, a pattern seen over the entire study period. The simultaneous decline of both HCV and HIV incidence probably results from reduced risk behavior at the population level.
Introduction

The most important mode of hepatitis C virus (HCV) transmission is through exposure to infected blood.\cite{1,2} Therefore injecting drug users (DU) are at high risk for HCV infection. Their main route of transmission is the sharing of needles or other injecting equipment.\cite{3}

In this population, the reported prevalences of HCV range from 40 to 85% in Europe and North America.\cite{1,4-11}

Under the threat of AIDS, DU reduced their injecting risk behaviour and consequently their incidence of HIV infection in the mid-1980s.\cite{12,13} However, their HCV incidence appears to be less affected by this decreased risk behavior, perhaps because HCV is more transmissible than HIV. This hypothesis is confirmed by several studies that show a high and stable prevalence of HCV antibodies in this population.\cite{14-17} In recent years, we reported a high but declining HCV prevalence among young DU in Amsterdam,\cite{18} whereas others still report high and stable HCV incidence among young DU who have recently started injecting.\cite{15,17,19,20}

The open and ongoing Amsterdam Cohort Studies (ACS) among DU started in 1985, and stored serum was retrospectively tested for HCV antibodies. Therefore, the ACS has the unique potential to present HCV incidence data for DU over two decades. The objectives of our study were to measure the HCV incidence over this long period, to evaluate risk factors associated with HCV seroconversion, and to compare the HCV incidence to the HIV incidence in this cohort over the same period.

Materials and Methods

The ACS is an open, prospective cohort study initiated to investigate the prevalence, incidence, and risk factors of infections with HIV-1 and other blood-borne and/or sexually transmitted diseases, as well as the effects of intervention.\cite{21} The DU cohort was initiated in 1985; recruitment is ongoing and in recent years has been directed in particular to young DU.

Participation in the ACS is voluntary, and informed consent is obtained for every participant at intake. ACS participants visit the Health Service of Amsterdam every 4-6 months. At every visit, they complete a standardized questionnaire about their health, risk behaviour, and socio-demographic situation. Questions about current behaviour refer to the period between the present and the preceding ACS visit. Questions at baseline refer to the period since 1980 or since the start of regular use of hard drugs. Blood is drawn for laboratory testing and storage.

Laboratory methods

To study HIV prevalence and incidence, all ACS participants since 1985 (n=1640) were prospectively tested for HIV antibodies by enzyme linked immunosorbent assays (ELISA), with confirmation by Western blot (since 1986: HIV Blot version 2.2, Genelab diagnostics).

To study the HCV prevalence and incidence, all participants with at least two visits between December 1985 and November 2005 (n=1276) were retrospectively tested for HCV antibodies, using the first sample available in each case. Third generation ELISA tests were used to detect HCV antibodies (AxSym HCV version 3.0; Abbott, Wiesbaden,
Germany). Individuals who were HCV negative at ACS entry were tested for HCV antibodies at their most recent ACS visit. On finding HCV seroconversion, samples taken in between these two visits were tested to identify the moment of seroconversion.

Statistical analyses

The date of HCV or HIV seroconversion was estimated as the midpoint between the last seronegative and the first seropositive ACS visit. The median duration of the HCV seroconversion interval between visits was 4.0 months, interquartile range (IQR) 3.7, 5.1 months. Using the Kaplan-Meier method, we examined the time elapsed from the start of injecting drugs to HCV seroconversion. Only HCV-negative DU were included and they were considered to be at risk from their start of injecting. Those who had started injecting before ACS enrolment entered the risk set at their date of ACS entry (i.e., left truncation). Those who did not seroconvert or who were lost to follow up were censored at their last ACS visit or ultimately 1 November 2005. We stratified the dates of starting injection into two decennia to investigate differences in HCV-free survival according to decade of starting injection.

Incidence rate curves were calculated by person-time methods. Poisson regression was used to test for the trend in HCV incidence over time and to determine risk factors for HCV seroconversion. All variables subject to change were treated as time-dependent variables. Due to the relatively long time-period between the point of infection and the appearance of HCV antibodies, the most probable moment of infection was assumed to have occurred around the last seronegative visit. Therefore, we assigned the risk behaviour reported at that visit to the HCV seroconversion period. However, for nine participants who reported starting injection at the first HCV antibody-positive visit, we set back the report of injecting risk factors from this visit to the last HCV antibody-negative visit. Multivariate models were built using forward-stepwise techniques, and variables with a univariate p-value <0.20 were considered as potential independent determinants. A p-value <0.05 was considered statistically significant. Interactions in the final model were checked.

Variables related to general characteristics, drug use, and sexual risk behaviour were examined as potential determinants of HCV seroconversion. General characteristics included sex, body mass index, calendar year of study visit, nationality, ethnicity, age, homelessness, hospitalization, and HIV status. The drug use variables included current injecting and the calendar period of starting injection. For current injectors, we also examined the frequency of injecting, the main type of drug injected, whether they injected mainly at home or borrowed needles, and needles obtained through a needle exchange program (NEP). Because there was a very strong association between current injecting and current borrowing of needles, we combined these two variables as follows: no current injecting; current injecting but no current borrowing of needles; current injecting and current borrowing of needles. Sexual behaviour included having a steady sexual partner, injecting drug use of the steady partner, having unprotected sex (with an injecting partner), and current prostitution (women only).

To compare the HCV and HIV incidence, we assumed that the observed data (i.e., the number of new infections per year) follows a Poisson distribution. We adopted a Bayesian approach. The logarithm of the incidence over calendar time was modelled using penalized splines. In this way, the incidence of both HCV and HIV was allowed to vary smoothly and nonlinearly over time. If the trends have the same pattern, then the difference between the incidences on a logarithmic scale is a constant.
Results

General characteristics and HCV prevalence

In total, 1640 DU have been enrolled in the ACS since December 1985. Of these, 1259 DU met the follow up criteria of at least two visits before November 2005 and also had enough stored serum to allow HCV testing. Of these participants, 803/1259 (63.8%) were male and 937/1259 (74.5%) had a Dutch nationality. The median age at ACS entry was 30.5 years (IQR 26.5, 35.8) (Table 2.1.1).

Table 2.1.1 General characteristics of drug users in the Amsterdam Cohort Study (*=at entry).

|                          | Total     | Ever-injecting DU | Never-injecting DU |
|--------------------------|-----------|-------------------|--------------------|
| Total number of participants | 1259      | 952               | 307                |
| Median age * (IQR)       | 30.5 (26.5, 35.8) | 29.84 (26.0, 36.0) | 30.6 (26.8, 35.7) |
| % Male sex               | 63.8      | 61.3              | 71.3               |
| % Dutch nationality      | 74.7      | 86.0              | 71.0               |
| Median duration of follow up (IQR) | 6.95 (3.56, 12.1) | 7.33 (3.84, 12.6) | 5.41 (2.60, 10.4) |
| Median age at start of injecting drugs (IQR) | - (17.8, 26.0) | - | - |
| Main drugs injected (%) * |          |                   |                    |
| cocktail, heroin/cocaine | - 40.0   | -                 |                    |
| heroin                   | 12.2      | 4.4               | 41.0               |
| cocaine                  | 8.9       | 31.5              | 3.0                |
| Main other drugs used (%) * |          |                   |                    |
| cocktail, heroin/cocaine | - 4.4    | 26.7              | 4.2                |
| heroin                   | 31.5      | 41.0              |                    |
| cocaine                  | 3.0       | 3.0               |                    |
| Frequency of injecting (%) |          |                   |                    |
| no current injecting     | - 28.5   | -                 |                    |
| daily                    | 34.0      |                   |                    |
| weekly                   | 30.7      |                   |                    |
| monthly                  | 4.4       |                   |                    |
| Number of recently borrowed needles(%) * |          |                   |                    |
| 0                        | - 44.9    | -                 |                    |
| 1-10                     | 7.6       |                   |                    |
| >10                      | 0.9       |                   |                    |
| unknown                  | 46.4      |                   |                    |
| % HCV-antibody positive * | 63.8      | 82.2              | 6.5                |
| HCV seroconversions during follow up | 59 | 58 | 1 |
| % HIV-positive *          | 20.4      | 5.8               | 3.6                |
| HIV seroconversions during follow up | 95 | 90 | 5 |

Ever-injecting DU: DU who had injected before ACS entry (n=905) or started injecting during follow up (n=47). Current/recently: in previous six months.
Of the 1259, 952 participants were ever-injectors: DU who had ever injected drugs before entry (n=905) or who had started injecting drugs during follow up (n=47). The median age at start of injection was 21.7 years (IQR 17.8, 26.0). The median ACS follow up time for ever-injectors was 7.3 years (IQR 3.8,12.6), whereas it was 5.4 years (IQR 2.6, 10.4) for never-injectors. In ever-injectors, the main drugs recorded at ACS entry were a cocktail of heroin and cocaine (40.0%), and most participants had injected daily or more frequently in the preceding 6 months (34.0%).

Of the 1259 DU, 803 (63.8%) had HCV antibodies at entry; of these, 30.6% (246/803) were HIV-co-infected. The prevalence at entry of HCV antibodies in ever-injectors varied from 92.9% in 1986 to 69.2% in 2001. The prevalence among never-injectors was 6.5% over the total study period and varied from 0 to 22.2% per calendar year. When evaluating HCV prevalence at entry by the time elapsed since start of injection, such prevalence was 59/99 (59.6%) for participants who had injected for less than two years before entry vs. 137/164 (82.5%) for participants who had injected for three to five years before entry. Among participants with >10 years of injecting drug use before ACS entry, the HCV prevalence was 327/346 (94.5%).

**HCV incidence**

Of the 456 DU seronegative for HCV at ACS entry, 59 seroconverted during follow up, of whom 58 injected and 1 did not. Among ever-injectors, the incidence declined from 27.5/100 PY in the late 1980s to approximately 2/100 PY in recent years (Figure 2.1.1B). There was a significant downward trend in HCV incidence over calendar time (IRR 0.86 per calendar year; 95% CI 0.82-0.90, p<0.001) (Figure 2.1.1B).

In line with the decline of the HCV incidence, the time since starting injection until HCV seroconversion has lengthened in more recent calendar periods. In 1980-1989, the median interval was 2.27 years (IQR 1.2, 5.6 years), whereas in 1990-1999, the median was 9.10 years (IQR 2.1, ∞ years) (Figure 2.1.2).

When restricting our analysis to DU who reported injecting since the preceding visit, a higher incidence but similar pattern was observed. In 1985-1990, the incidence rate in this group was extremely high, between 50–80/100 PY, but it dropped to 5-10/100 PY in 1990-1999.

**Comparison of HCV and HIV incidence**

Of 1276 DU, those HIV-negative at entry numbered 1013, of whom 95 (including 90 ever-injectors) seroconverted for HIV during follow up. The HIV incidence rate among ever-injectors dropped from 8.52/100 PY in 1986 to approximately 0 since 2000, with a slight increase in 2005 (Figure 2.1.1A).

When the observed HCV and HIV incidence curves and their fitted smooth curves are plotted in one graph with two scales, the curves look similar in shape. When we plotted the differences between the logs of the fitted model, we found no convincing evidence for a difference in pattern. The mean value of the differences on a log-scale over the twenty years is 1.48; hence the scale factor is estimated to be 4.4 (data not shown). The observed and fitted incidence patterns for both HCV and HIV with 95% confidence intervals are shown in Figure 2.1.2C.
Risk factors for HCV seroconversion

Time since start injecting can be seen as a proxy for the duration of exposure time, and preliminary analysis showed a very strong association between time since start of injecting and the time point of HCV seroconversion (IRR 0.80 per year, 95% CI 0.74-0.86) (Table 2.1.2). Therefore, in bivariate analysis, to adjust for variation in time from start of injecting (and thus time of exposure), all other variables were adjusted for time from start of injecting as a time-updated variable.

After correction for time since starting injection, the following risk factors were found to be significantly associated with an increased risk of HCV seroconversion: the combined variable of current injecting and current borrowing of needles, earlier calendar year of visit, use of needle exchange programs (NEPs), type of drugs injected, frequency of injecting drug use, and earlier decennium of starting injection (Table 2.1.2).
Interestingly, in univariate analysis persons were more at risk for HCV if they had seroconverted for HIV (IRR 5.68; 95% CI 2.27-14.2) or were chronically infected with HIV (IRR 3.12; 95% CI 0.76-12.8) than if they were HIV-negative. The type of drugs injected, and frequency of injection were associated with an increased risk of HCV infection, their effect is attributable to current injecting drug use itself. In fact, when evaluating these variables among only DU injecting drugs within the past six months we found no association between NEP use, the type of drug injected, or injection frequency and HCV infection.

![Figure 2.1.2 Kaplan-Meier estimates of the cumulative proportion of DU who remain without HCV infection since starting injection, grouped per decennium: the 1980s and 1990s. Curves were truncated when fewer than 10 persons remained at risk for HCV (thin line). Persons who started injecting before 1980 or after 2000 are not depicted in this figure, because at any moment in those periods, less than 10 persons were at risk for HCV.](image)

Figure 2.1.2  Kaplan-Meier estimates of the cumulative proportion of DU who remain without HCV infection since starting injection, grouped per decennium: the 1980s and 1990s. Curves were truncated when fewer than 10 persons remained at risk for HCV (thin line). Persons who started injecting before 1980 or after 2000 are not depicted in this figure, because at any moment in those periods, less than 10 persons were at risk for HCV.

In multivariate analysis, we found that current injecting combined with current borrowing of needles was a major risk for HCV seroconversion; the IRR was 29.9 (95% CI 12.6-70.9) for current injecting and borrowing compared to no injecting in the preceding period. The longer the time between start of injecting and study visit, the smaller the risk of HCV infection: IRR 0.89; 95% CI 0.83-0.96) Table 2.1.2. Calendar year remained significantly associated with a decreased risk of HCV infection when it was evaluated continuously in the model (IRR 0.87; 95% CI 0.82-0.93).
| Potential Risk Factor | UNIVARIATE ANALYSIS | BIVARIATE ANALYSIS | MULTIVARIATE ANALYSIS |
|-----------------------|---------------------|-------------------|----------------------|
|                       | HCV sc PY Incidence rate (per 100 PY) IRR 95% CI p value | IRR 95% CI p value | IRR 95% CI p value |
| Methadone dosage      | 0.18                | 0.92              |                      |
| 0 mg                  |                     |                   |                      |
| 1-59 mg methadone     |                     |                   |                      |
| >60 mg                |                     |                   |                      |
| HIV status            | 0.006               | 0.25              |                      |
| HIV-negative          |                     |                   |                      |
| HIV primary infection |                     |                   |                      |
| HIV chronically infected |                 |                   |                      |
| Decennium of starting injection | 0.002 | 0.13 |                      |
| 1970-79               |                     |                   |                      |
| 1980-89               |                     |                   |                      |
| 1990-99               |                     |                   |                      |
| 2000-present          |                     |                   |                      |
| Use of NEPs           |                     |                   |                      |
| No current injecting  |                     |                   |                      |
| Current injecting, no NEP |                 |                   |                      |
| Current injecting, irregular NEP |             |                   |                      |
| Current injecting, always NEP |           |                   |                      |
| Age (per 10 years)    |                     |                   |                      |
| Type of drugs mainly injected |         |                   |                      |
## Chapter 2.1

### Epidemiology

#### Univariate Analysis

| Variable                                      | HCV sc | PY Incidence rate (per 100 PY) | IRR  | 95% CI      | p value | IRR  | 95% CI      | p value |
|-----------------------------------------------|--------|---------------------------------|------|-------------|---------|------|-------------|---------|
| **Frequency of injecting**                    |        |                                 |      |             |         |      |             |         |
| No current injecting                         | 10     | 623                             | 1.60 | 1           | <0.001  | 1    |             |         |
| More times per day                           | 17     | 49                              | 34.7 | 21.7        | (9.92, 47.3) | 10.4 | (4.54, 23.9) | <0.001  |
| Once daily                                    | 1      | 4                               | 25.5 | 15.9        | (2.03, 124.3) | 12.2 | (1.55, 95.9) |         |
| More times per week                          | 19     | 66                              | 28.8 | 18.0        | (8.36, 38.6) | 10.5 | (4.75, 23.3) |         |
| Once weekly                                   | 1      | 12                              | 8.23 | 5.13        | (0.66, 40.1) | 5.27 | (0.67, 41.2) |         |
| More times per month                         | 3      | 37                              | 8.13 | 5.07        | (1.40, 18.4) | 2.71 | (0.72, 10.1) |         |
| Once monthly                                  | 2      | 13                              | 15.2 | 9.46        | (2.07, 43.2) | 8.18 | (1.79, 37.4) |         |
| Less than once monthly                       | 4      | 48                              | 8.36 | 5.21        | (1.83, 16.6) | 4.19 | (1.31, 13.4) |         |
| **Frequency of non-injecting drug use**       |        |                                 | 0.35 |             |         | 0.39 |             |         |
| More times per day                           | 21     | 249                             | 8.43 | 0.73        | (0.25, 2.14) | 0.60 | (0.20, 1.74) |         |
| Once daily                                    | 2      | 38                              | 5.26 | 0.45        | (0.08, 2.48) | 0.45 | (0.08, 2.47) |         |
| More times per week                          | 16     | 235                             | 6.81 | 0.59        | (0.20, 1.77) | 0.60 | (0.20, 1.80) |         |
| Once weekly                                   | 2      | 68                              | 2.94 | 0.25        | (0.05, 1.39) | 0.20 | (0.04, 1.09) |         |
| More times per month                         | 2      | 47                              | 4.26 | 0.37        | (0.07, 2.03) | 0.47 | (0.09, 2.58) |         |
| Less than once monthly                       | 0      | 17                              | 0.00 | 1           |         | 1    |             |         |
| **Type of drugs mainly used (non-injecting)** |        |                                 | 0.98 |             |         | 0.81 |             |         |
| Heroin                                       | 20     | 311                             | 6.43 | 1           |         | 1    |             |         |
| Cocaine                                      | 24     | 332                             | 7.23 | 1.12        | (0.62, 2.03) | 1.33 | (0.73, 2.41) |         |
| Cocktail, heroin/cocaine                     | 2      | 32                              | 6.25 | 0.96        | (0.22, 4.11) | 1.07 | (0.25, 4.58) |         |
| Amphetamines                                 | 1      | 13                              | 7.69 | 1.16        | (0.16, 8.06) | 1.48 | (0.20, 11.0) |         |
| **Having a steady partner**                  |        |                                 | 0.52 |             |         | 0.10 |             |         |
| No                                           | 36     | 496                             | 7.26 | 1.19        | (0.70, 2.02) | 1.55 | (0.91, 2.64) |         |
| Yes                                          | 22     | 360                             | 6.11 | 1           |         | 1    |             |         |
| **Injecting drug use of the steady partner** |        |                                 | 0.10 |             |         | 0.99 |             |         |
| No                                           | 12     | 255                             | 4.71 | 1           |         | 1    |             |         |
| Yes                                          | 10     | 105                             | 9.52 | 2.04        | (0.88, 4.71) | 0.99 | (0.42, 2.33) |         |
### Declining HCV Incidence in Drug Users from the ACS

#### UNIVARIATE ANALYSIS

|                      | HCV sc | PY  | Incidence rate (per 100 PY) | IRR   | 95% CI       | p value | IRR   | 95% CI       | p value |
|----------------------|--------|-----|----------------------------|-------|--------------|---------|-------|--------------|---------|
| **Homelessness**     |        |     |                            |       |              |         |       |              |         |
| No                   | 52     | 800 | 6.50                       | 0.61  | (0.26, 1.43) | 0.67    | (0.29, 1.57) |
| Yes                  | 6      | 57  | 10.5                       | 1     |              | 1       |       |              |         |
| **Hospitalized in past 6 months** |        |     |                            |       |              |         |       |              |         |
| No                   | 56     | 817 | 6.85                       | 1.34  | (0.33, 5.50) | 1.36    | (0.33, 5.59) |
| Yes                  | 2      | 39  | 5.13                       | 1     |              | 1       |       |              |         |
| **Current prostitution (females only)** |        |     |                            |       |              |         |       |              |         |
| No                   | 24     | 243 | 9.88                       | 1     |              | 1       |       |              |         |
| Yes                  | 2      | 47  | 4.26                       | 0.43  | (0.10, 1.81) | 1.19    | (0.28, 5.07) |
| **Current injecting and borrowing needles** |        |     |                            |       |              |         |       |              |         |
| No current injecting | 10     | 623 | 1.61                       | 1     |              | 1       |       |              |         |
| Current injecting, no current borrowing of needles | 25     | 159 | 15.7                       | 9.80  | (4.71, 20.4) | 6.26    | (2.94, 13.3) |
| Current injecting and current borrowing of needles | 12     | 23  | 52.2                       | 32.7  | (14.1, 75.7) | 21.4    | (9.17, 50.1) |
| **Time since start of injecting** |        |     |                            |       |              |         |       |              |         |
| 58                   | 856    |     | 6.8                        | 0.80  | (0.74, 0.86) | <0.001  | NA    |              | <0.001  |
| **Year of visit**    |        |     |                            |       |              |         |       |              |         |
| 58                   | 856    |     | 6.8                        | 0.86  | (0.82, 0.90) | <0.001  | 0.94  | (0.89, 0.99) | 0.009   |
| **Sex**              |        |     |                            |       |              |         |       |              |         |
| Male                 | 32     | 566 | 5.65                       | 1     |              | 1       |       |              |         |
| Female               | 26     | 200 | 8.97                       | 1.59  | (0.95, 2.66) | 1.28    | (0.76, 2.16) |

NA = not applicable

* = adjusted for time since start of injection.

** = analyses were not adjusted for time since start of injection and decennium of start, because the decennium can be derived from the time since start of injection and calendar year of visit.
Discussion

This study describes the prevalence and incidence of HCV in a large group of DU in Amsterdam, the Netherlands, over two decades. Findings show that the HCV incidence dropped considerably in that period. Interestingly, when we compared the HCV incidence rate to the HIV incidence rate in the same group of DU that have ever injected, the decrease was similar for the two infections. In line with the decline of the HCV incidence, the time from the start of injecting drugs until HCV seroconversion is longer at present than in the past.

To our knowledge, this is the first study to document among DU, over such a long period, a decline in HCV incidence that is not only strong but also comparable to the decline in HIV incidence. Our finding of a decline in HCV incidence contrasts with other studies that show a stable HCV incidence.

One explanation may be that those studies analyzed the HCV incidence over a shorter time interval, which might have been insufficient to show a significant decline. In Baltimore, USA, a significant decline of the HCV incidence was found in injecting DU followed between 1988 and 1996, but in contrast to our study with ongoing recruitment of participants, this decline was observed in a closed cohort study and a saturation effect probably has contributed to this decline.

In addition, the risk behavior of the total group of DU included in the ACS has substantially declined over time in Amsterdam. This finding suggests that a decline in risk behavior at the population level has contributed to the simultaneous decline of HCV and HIV incidence. The decreasing HCV incidence in Amsterdam DU, as opposed to high incidences in DU elsewhere, may likewise be partly explained by a larger reduction in injecting risk behavior in Amsterdam, compared to reductions elsewhere. The impact of methadone provision and NEP on this decline of risk behavior is very important and should be a focus of future studies. Methadone and NEP were readily available throughout the study period, and the median prescribed daily methadone dose increased during this period. Murray et al. demonstrated by mathematical modeling that the level of risk behavior determines whether HCV incidence decreases. They calculated that if injecting risk behavior is sufficiently decreased (through intense needle exchange programs and/or harm reduction strategies), then HCV incidence will accordingly decline.

Mathematical models have additionally shown the natural course of an epidemic might bring a decline in the incidence of infection. When a new infectious agent enters a population, the number of infected individuals and the incidence soon increase. Thereafter, as the number of susceptibles decreases, the chance for an infected individual to come into contact with an uninfected individual decreases as well. When the density of uninfected persons reaches a threshold below which the number of susceptibles cannot sustain an ongoing epidemic, incidence peaks and then starts to decline. In this light, the decrease in HIV incidence observed shortly after the introduction of HIV in Amsterdam in the early 1980s was due to the depletion of susceptibles, along with a reduction in risk behaviour. However, such depletion is less likely to be the case for HCV, which has existed among DU since the 1960s and possibly even before. This implies that the decrease in injecting risk behaviour might have an even greater impact on HCV than on HIV.

The contrast in study findings may be explained in part by the HCV test used. We used third-generation ELISA tests to measure HCV antibodies, whereas studies from the late
Declining HCV incidence in drug users from the ACS

1980s/early 1990s used first- or second-generation ELISA tests, which were more inclined to give false positive test results.\textsuperscript{36} The HCV prevalence among DU at ACS entry varies between 70-90%, with lower prevalence rates in recent years. This is consistent with what was described among DU in Amsterdam in the early 1990s\textsuperscript{28} and among recently starting injectors in Amsterdam and elsewhere.\textsuperscript{18,36} The HCV prevalence in never-injecting DU is much lower than in ever-injectors but still much higher than in low-risk populations (e.g., blood donors) or the general populations in Western countries,\textsuperscript{1,37} household transmission, rare sexual transmission, and reliability/unreliability of answers given in interviews may contribute to this finding among never-injecting DU.

Among DU in Amsterdam who have injected in the past 6 months, incidence rates were extremely high in the 1980s (50–80/100 PY). Similarly high incidence rates have been described by Smyth et al. among young, DU who have recently started injecting in Ireland, in the 1990s.\textsuperscript{10}

A possible limitation of our study is its lack of confirmatory testing for positive results of HCV antibody testing. However, such results in a high-risk population are likely to be true positives,\textsuperscript{35} and 232/803 (28.9%) of the positive participants were tested at two study visits or more, all with consistent HCV-positive test results. Therefore we believe the lack of confirmatory testing did not influence our results. Furthermore, although the ACS is an open, prospective cohort study, the influx of new participants in recent years has been lower than in earlier years. Lower risk DU could be overrepresented due to the decease of high-risk DU. However, the most recent HCV seroconversions took place in young drug users who entered the cohort after 1994.

Our risk factor analysis showed that HCV seroconversion is associated not only with current injecting and borrowing needles, as expected, but also with calendar year and time since start of injecting. The majority (70%) of HCV infections could have been prevented by eliminating the borrowing of needles. This might partly reflect the effect of the use of NEP, which were always available during the study period, but individual factors also might play a role in the decision to use NEP.

In conclusion, HCV incidence in our cohort showed a sharp decline in the past two decades, similar to the decline in HIV incidence, most likely due to a decrease in injecting risk behavior. We found that those who started injecting in a recent calendar period are at lower risk of HCV infection, presumably due to prevention activities. Thus it is important to continue and enhance such activities among DU and others at risk of starting injection, especially because the HCV risk is highest just after the start of injecting, when probably injectors are inexperienced.

Although we did not find an independent effect from either participation in a methadone program or from the use of needle exchange programs, these prevention measures in combination are likely to have contributed to the decline in risk behavior related to drug use at the population level. Therefore, it is important to evaluate the possibilities for harm reduction worldwide. During the late 1980s many acute HCV infections occurred, so there might have been more DU with high HCV-RNA levels associated with acute HCV infection. Therefore, in that period there may have been more and/or easier transmission of HCV. Higher HCV-RNA levels have also been associated with HIV co-infection.\textsuperscript{38} However, we believe that because the HCV prevalence remained relatively high and the pattern of the HIV and HCV incidence was comparable during the study period, on population level the HCV-RNA level varied only little over time, also because treatment prescription for HCV was very limited in our cohort.
Finally, it is important to decrease the prevalence of chronic HCV carriers and thus reduce the possibility for HCV transmission. DU should therefore be systematically screened for HCV infection, and those chronically infected should be treated.
Declining HCV incidence in drug users from the ACS

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Chapter 2.2

Full participation in harm reduction programs is associated with decreased risk for HIV and HCV: evidence from the Amsterdam Cohort Studies among drug users

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Addiction 2007;102:1454–1462.
Abstract

Objectives
To investigate the impact of harm-reduction programs on HIV and hepatitis C (HCV) incidence among ever-injecting drug users (DU) from the Amsterdam Cohort Studies (ACS).

Methods
The association between use of harm reduction and seroconversion for HIV and/or HCV was evaluated using Poisson regression. 714 DU were at risk for HIV and/or HCV during follow up. Harm reduction was measured by combining its two most important components — methadone dose and needle exchange program (NEP) use — and looking at 5 categories of participation, ranging from no participation (no methadone in the past 6 months, injecting drug use in the past 6 months, and no use of NEP) to full participation (≥ 60 mg methadone/day and no current injecting or ≥ 60 mg methadone/day and current injecting but all needles exchanged).

Results
Methadone dose or NEP use alone were not significantly associated with HIV or HCV seroconversion. However, with combination of these variables and after correction for possibly confounding variables, we found that, full participation in a harm reduction program (HRP) was associated with a lower risk of HIV and HCV infection in ever-injecting DU, compared to no participation (incidence rate ratio 0.43 (95% CI 0.21-0.87) and 0.36 (95% CI 0.13-1.03), respectively).

Conclusions
In conclusion, we found that full participation in HRP was associated with a lower incidence of HCV and HIV infection in ever-injecting DU, indicating that combined prevention measures -- but not the use of NEP or methadone alone -- might contribute to the reduction of the spread of these infections.
Impact of harm reduction on HCV and HIV incidence in drug users

Introduction

Injecting drug users (DU) are at high risk for blood-borne infections, including HIV and HCV, through the sharing of needles and injection equipment. Various approaches to deal with the consequences of hard drugs have been taken; some countries aim to ban illicit drug use completely, whereas The Netherlands and others take a harm reduction approach. This harm reduction approach may have had a major impact on the HIV and HCV epidemic. The ultimate goal of harm reduction is to stop DU from using drugs, but until this is possible, the policy is to minimize the damage DU inflict on themselves and the society at large. Diverse programs (with a low, medium or high threshold) started in The Netherlands at the end of the 1970s, providing methadone in combination with social-medical care and needle-exchange facilities. They have no waiting lists and are relatively easy to enter and re-enter. Ongoing drug use during participation is tolerated in low- and medium-threshold programs. Low-threshold programs have been operated since 1982 by the Amsterdam Health Service. For clients who have regulated their drug use, methadone can be prescribed in a medium-threshold program via their general practitioner. Clients who are willing to detoxify can receive methadone in a high-threshold program through an outpatient addiction clinic. Circulation between the different programs is permitted and ‘promotion’ to higher-threshold programs is encouraged. With the harm reduction approach, the Amsterdam methadone programs reached an estimated 2,700 of the 3,500 to 4,000 opiate users in Amsterdam. All services are free of charge for residents of The Netherlands. The effects of methadone provision or needle exchange programs (NEP) separately on HIV incidence have been examined, with conflicting results. Very few studies describe the effect of either program on HCV incidence, although declining prevalence of HCV was reported after the introduction of NEP.

The Amsterdam Cohort Study (ACS) among DU comprises a large group of DU who are prospectively tested for HIV. We tested their stored sera for HCV, retrospectively, and therefore had the unique opportunity to document the effect of harm reduction on the incidence of both HIV and HCV over a long time period.

Materials and Methods

Study population and design

The Amsterdam Cohort Study (ACS) among DU is an open, prospective cohort study initiated to investigate the prevalence, incidence, and risk factors of infections with HIV and other blood-borne and/or sexually transmitted infections, as well as the effects of interventions. It has collected detailed information on the participation in harm reduction programs (HRPs). The DU cohort was initiated in 1985; recruitment is ongoing and in recent years has been directed in particular toward young DU. ACS participation is voluntary, and informed consent is obtained for every participant at intake. ACS participants visit the Amsterdam Health Service every 4-6 months. At intake and every visit, they give blood for HIV testing and storage; they also complete a standardized questionnaire about their health, drug use and sexual risk behaviour, and socio-demographic situation. At intake, questions about current behaviour refer to the preceding six months and/or to the period since 1980 or since the start of regular use of
hard drugs (i.e., heroin, cocaine, amphetamines and/or methadone at least three times per week). At follow up visits, questions refer to the time between the present and the preceding visit.

Laboratory methods

All ACS participants since 1985 (n=1640) were prospectively tested for HIV antibodies by enzyme linked immunosorbent assays (ELISA). All participants with at least two visits between December 1985 and November 2005 (n=1276) were retrospectively tested for HCV antibodies, using the first sample available in each case. Third generation ELISA tests were used to detect HCV antibodies (AxSym HCV version 3.0; Abbott, Wiesbaden, Germany). Individuals who were HCV-negative at ACS entry were tested for HCV antibodies at their most recent ACS visit. On finding HCV seroconversion (defined as the presence of HCV antibodies in a previously seronegative individual), we tested samples taken in between these two visits to indicate the seroconversion interval.

Statistical analyses

HIV and/or HCV-negative ever-injecting drug users entered the risk set at study entry or at their start of injecting drug use during follow up, and were followed up until seroconversion for respectively HIV or HCV, or until end of follow up, ultimately at 1 November 2005. The date of HCV or HIV seroconversion was estimated as the midpoint between the last seronegative and the first seropositive ACS visit. Poisson regression was used to determine the effect of harm reduction on HCV and HIV incidence. Incidence rates and incidence rate ratios (IRR) with their corresponding 95% confidence intervals (95% CI) were calculated. We evaluated the potential confounding effect of all variables listed below and evaluated interaction between variables included in the final model. Multivariate models were built using forward-stepwise techniques, and variables with a univariate p-value ≤0.10 were considered as potential independent determinants. All variables subject to change were treated as time-dependent variables, these variables refer to the six months prior to the visit. A p value ≤0.05 was considered statistically significant.

To study the impact of harm reduction on HIV and HCV seroconversion, we combined injecting drug use, use of NEP and methadone dosage into one variable with five categories (Table 2.2.1). Because higher doses of methadone are more effective than lower doses in lowering the prevalence of injecting drug use risk behaviour, we considered ≥60 mg methadone per day an adequate minimum dosage for opioid replacement therapy and used that dose as cut-off value for our definition of adequate harm reduction.11-13

General characteristics of persons evaluated included sex, nationality, age, HIV status in cases of HCV as outcome, HCV status in cases of HIV as outcome, HIV status of the steady partner, homelessness, and hospitalization. The drug use variables included current injecting (yes or no), frequency of injecting, the main type of drug injected, the time elapsed since start of injecting drug use, the frequency of non-injecting drug use, and the type of drug mainly used as non-injecting drug.
Table 2.2.1 Definition of five levels of harm reduction used to evaluate the effect of harm reduction on HIV and HCV incidence in the Amsterdam Cohort Studies.

| Level of Harm Reduction | Definition |
|-------------------------|------------|
| No harm reduction       | No methadone in the past six months, injecting drug use in the past six months, and no use of NEP |
| Incomplete harm reduction | Any dose of methadone daily in the past six months, injecting drug use in the past six months and irregular* or no use of NEP; OR 0-59 mg methadone daily in the past six months, injecting drug use in the past six months, and always use of NEP |
| Full harm reduction     | ≥60 mg methadone daily in the past six months and no injecting drug use in the past six months; OR ≥60 mg methadone daily, injecting drug use in the past six months, and always use of NEP |
| Limited dependence on harm reduction | 1-59 mg methadone daily in the past six months and no injecting drug use in the past six months |
| No dependence on harm reduction | No methadone in the past six months and no injecting drug use in the past six months |

*Irregular use of NEP=1-99% of needles used in the past six months obtained via NEP. Always use of NEP=100% of needles used in past six months obtained via NEP.

Results

General characteristics

In total 1640 DU were enrolled in the ACS, 1276 DU had at least 2 visits. DU with more than 1 visit were older (median 31.4 (interquartile range (IQR) 31.0-31.8) years vs. 28.7 (28.1-29.4) years), more often male (63.9% vs. 56.9%), more often of Dutch nationality (74.5% vs. 60.2%) and more often HIV positive (20.6% vs. 16.2%) when compared to DU with only 1 visit to the ACS.

952 DU were so called ever injecting DU: DU who had ever injected drugs before ACS entry (n=905) or who started injecting drugs during follow up (n=47). 714 of these ever-injecting DU were HIV and/or HCV negative at study entry and were at risk for HIV and/or HCV during follow up. 164 DU (22.9%) were negative for both infections at study entry, 546 DU (76.5%) were HIV-negative and HCV-positive, and 4 DU (0.6%) were HCV-negative and HIV positive. The HIV prevalence among HCV-negative DU was 2.4% at entry, while the HCV prevalence among HIV-negative DU was much higher (76.2%). The DU included were mainly of Dutch nationality and mainly male (Table 2.2.2).

HIV-negative DU had a longer median time since starting injection than HCV-negative DU (respectively, 7.4 and 2.4 years). Furthermore, the proportion of DU who had recently injected (i.e., in the past 6 months before ACS entry) was larger for the HIV-negative DU than for HCV-negative DU. HIV-negative DU injected more often than HCV-negative DU, and HCV-negative DU used non-injecting drugs more often than their HIV-negative counterparts (Table 2.2.2). The median follow up time was 3.56 years (IQR 1.15-7.91 years) for DU at risk for HCV and 8.13 years (IQR 4.25-13.0 years) for DU at risk for HIV.
Table 2.2.2  General characteristics at entry and during follow up of 710 HIV negative and 168 HCV negative ever-injecting DU included in HIV and HCV analysis respectively.

|                      | HIV                  | HCV                  |
|----------------------|----------------------|----------------------|
| At entry             |                      |                      |
| HIV/HCV infection (n at risk) | 710 % 168 %         |                      |
| Prevalence HIV infection at entry risk set | - 4 | 2.4 |
| Prevalence HCV infection at entry risk set | 541 76.2 | - |
| Overall HIV incidence (per 100 PY) | 1.65 |                      |
| Overall HCV incidence (per 100 PY) | 6.78 |                      |
| General characteristics |                      |                      |
| Steady partner at entry | 333 46.9 | 77 45.8 |
| Median age at entry risk set (years (IQR)) | 30.0 (27.0-36.0) | 29.0 (25.0-33.0) |
| Female | 274 38.6 | 56 33.3 |
| Dutch nationality | 526 74.1 | 147 87.5 |
| Western European ethnicity | 602 84.8 | 139 82.7 |
| Injecting drug use |                      |                      |
| Median time since start injecting (years (IQR)) | 7.21 (3.04-12.1) | 2.43 (0.06-7.16) |
| Injecting in the past 6 months | 524 73.8 | 100 59.5 |
| Among recent injectors |                      |                      |
| Injecting more than 1 time a week | 429 82.3 | 53 54.6 |
| Main drug injected |                      |                      |
| heroin | 94 17.9 | 33 33.0 |
| cocaine | 77 14.7 | 14 14.0 |
| speedball (i.e., combination of heroin and cocaine) | 271 51.7 | 37 37.0 |
| other | 82 15.6 | 16 16.0 |
| Non-injecting drug use |                      |                      |
| Non-injecting drug use in the past 6 months | 497 70.0 | 149 88.7 |
| Frequency of non injecting drug use |                      |                      |
| 1 or more times daily | 190 38.2 | 77 51.7 |
| 1 or more times weekly, but less than 1 or more times daily less | 188 37.8 | 61 41.0 |
| than weekly |                      |                      |
| Main non injecting drug use at entry |                      |                      |
| heroin | 239 48.2 | 66 44.2 |
| cocaine | 215 43.3 | 73 49.0 |
| other | 42 8.5 | 10 6.7 |
| Follow up |                      |                      |
| Median number of visits at risk (IQR) | 17 (8-29) | 15 (8-28) |
| Median number of PY (IQR) | 8.13 (4.25-13.0) | 3.56 (1.15-7.91) |
| Median number of days between follow up visits (IQR) | 128 (118-168) | 128 (119-166) |

Under study, 90/710 DU at risk for HIV seroconverted and 58/168 at risk for HCV. The median duration of the HIV and HCV seroconversion interval between visits was 4.0
months (IQR 3.7-6.0 months) and 4.0 months (IQR 3.7-5.1 months), respectively. The HIV incidence ranged from 8.5/100 PY in the late 1980s to approximately 0 in the most recent years, whereas HCV incidence was very high in the late 1980s (27.5/100 PY) and declined to around 2/100 PY in more recent years.\(^{14}\)

**Effect of harm reduction participation on HIV and HCV incidence**

When evaluating the separate effects on HIV and HCV seroconversion of methadone dose or NEP we found that having any prescribed dose of methadone was associated with lower incidence rates of HIV and HCV infection, but not to a statistically significant degree (\(p=0.084\) and \(p=0.21\), respectively). Use of NEP was associated with a higher risk of HIV and HCV seroconversion, but with restriction of this variable to injecting drug use in the preceding six months, the IRR changed towards one and no longer reached statistical significance (data not shown). However, when methadone dose and NEP were combined as described in Table 2.2.1, full participation in a HRP was associated with a two- to threefold reduction in the risk of HIV seroconversion and with a six- to sevenfold reduction in the risk of HCV seroconversion (Table 2.2.3).

In univariate analysis the following variables were also associated with a higher risk of HIV or HCV: injecting drug use in the past six months, borrowing needles in the past six months, more recent onset of injecting drug use, a higher frequency of injecting drugs, mainly injecting speedball, younger age, and having an HIV-positive steady partner. A change in methadone dosage in the past six months was associated with a higher risk for HCV seroconversion but not HIV seroconversion. DU who were chronically HIV-infected or had an acute HIV infection in the six months preceding the visit were at increased risk for HCV seroconversion (Table 2.2.3).

In multivariate analysis we found that after correcting for having an HIV-positive steady partner and a smaller number of years since starting injection (both factors being independently associated with HIV seroconversion), the combined harm reduction variable remained independently associated with HIV seroconversion (Table 2.2.4). That is, DU fully participating in HRPs were at a decreased risk of HIV seroconversion compared to DU not fully participating in a HRP (IRR 0.43, 95% CI 0.21-0.87).

In multivariate analysis for HCV, we found that with correction for time elapsed since start of injecting, DU fully participating in a HRP were at decreased risk of HCV seroconversion compared to DU not participating in a HRP (IRR 0.36, 95% CI 0.13-1.03). As with HIV, DU who recently started injecting drug use were at increased risk of HCV seroconversion. The effect of HIV status of the steady partner on HCV incidence had the same direction as its effect on HIV incidence (Table 2.2.4).

In sensitivity analyses, we found that the effects of harm reduction on HIV and HCV seroconversion did not substantially change when analysis was restricted to the years after 1989 (i.e., when a methadone dose of \(\geq 60\) mg daily was more readily available for DU). Also, when the lower limit of adequate methadone dosage was adjusted to \(\geq 80\) mg daily, the effects of harm reduction on HIV and HCV seroconversion did not substantially change.
Table 2.2.3 Univariate associations between general characteristics, drug use characteristics, sexual risk behaviour characteristics, and HIV and HCV seroconversion among DU in the ACS. Sc = seroconversion, PY = person years, IRR = incidence rate ratio, 95% CI = 95% confidence interval.

| Harm reduction                              | HIV | C    | Incidence (sc/100 PY) | PY | IRR     | 95% CI   | p value | HIV | C    | Incidence (sc/100 PY) | PY | IRR    | 95% CI   | p value |
|---------------------------------------------|-----|------|-----------------------|----|---------|----------|---------|-----|------|-----------------------|----|--------|----------|---------|
| Level of harm reduction                     |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| No harm reduction                           |     |      |                       |    |         |          | <0.001  |     |      |                       |    |         |          | <0.001  |
| Incomplete harm reduction                   |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Full harm reduction                         |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Pre-ultimate goal                           |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Ultimate goal                               |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Methadone dosage                            |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| 0 mg                                        |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| 0-60 mg                                     |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| ≥60 mg                                      |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Needle exchange programme (% of needles obtained via) |     |      |                       |    |         |          | <0.001  |     |      |                       |    |         |          | <0.001  |
| No recent injecting                         |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| 0 %                                         |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| 1-99 %                                      |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| 100 %                                       |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Change in methadone dosage compared to previous visit |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| No change                                  |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Increase                                   |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Decrease                                   |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Unknown                                    |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| General characteristics                     |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Sex                                         |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Male                                       |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Female                                     |     |      |                       |    |         |          |         |     |      |                       |    |         |          |         |
| Age (per 10 years increase)                |     |      |                       |    |         |          | <0.001  |     |      |                       |    |         |          | <0.001  |

(definitions described in table 2.2.1)
### Impact of harm reduction on HCV and HIV incidence in drug users

| Variable                                      | No Homelessness | Yes Homelessness | HIV Incidence (100 PY) | HCV Incidence (100 PY) | p-value | 95% CI  |
|-----------------------------------------------|-----------------|-----------------|------------------------|------------------------|---------|---------|
| Hospitalization in preceding 6 months        | 1.58            | 2.74            | 0.76                   | 4.59                   | 0.18    | (0.70-3.79) |
| HC/IV status at visit                         | 1.56            | 2.90            | 0.69                   | 5.11                   | 0.29    | (0.76-12.6) |
| Acute infection in previous 6 months          | 1.73            | 4.64            | 1.91                   | 3.91                   | 0.0055  |         |
| Injecting in past 6 months                   | 2.83            | 20.74           | 23.15                  | 12.9                   | <0.001  | (6.54-25.6) |
| Borrowing of needles                          | 0.38            | 1.60            | 1.60                   | 1.60                   | <0.001  | (6.54-25.6) |
| Frequency of injecting in previous 6 months   | 3.66            | 34.72           | 23.15                  | 12.9                   | <0.001  | (6.54-25.6) |
| Drug use variables                            |                |                 |                        |                        |         |         |
| Injecting in past 6 months                   | 3.05            | 15.19           | 23.15                  | 12.9                   | <0.001  | (6.54-25.6) |
| Borrowing of needles                          | 1.53            | 8.36            | 8.36                   | 8.36                   | <0.001  | (6.54-25.6) |
### Epidemiology

#### HIV

| Main drug injected in previous 6 months | Incidence (/100 PY) | Incidence (/100 PY) | 95% CI          | p value | 95% CI          | p value |
|----------------------------------------|---------------------|---------------------|-----------------|---------|-----------------|---------|
| No injecting drug use in previous 6 months | 0.38                | 10                  | 2633.8          | 1       | 1.60            | 10      | 623.4 | 1       |
| Heroin                                 | 2.26                | 13                  | 574.2           | 5.96    | (2.61-13.6)     | 19.62   | 9     | 45.9   | 12.2   | (4.97-30.1) |
| Cocaine                                | 1.81                | 8                   | 442.7           | 4.76    | (1.88-12.1)     | 24.31   | 10    | 41.1   | 15.2   | (6.31-36.4) |
| Speedball                              | 3.41                | 48                  | 1408.6          | 8.97    | (4.54-17.7)     | 18.30   | 21    | 114.7  | 11.4   | (5.37-24.2) |
| Amphetamines                           | 1.87                | 4                   | 213.9           | 4.92    | (1.54-15.7)     | 7.45    |        |        |        | (1.63-34.0) |
| Methadone                              | 3.02                | 4                   | 132.6           | 7.95    | (2.49-25.3)     | 21.3    |        |        |        | (5.87-77.5) |
| Other                                  | 4.94                | 3                   | 60.8            | 13.0    | (3.58-47.2)     | 26.92   | 8     | 29.7   | 44.5   | (12.2-161.6) |
| Time since start injection drug use (years) | 0.93                | (0.91-0.96)         | <0.001          | 0.90    | (0.74-0.86)     | <0.001  | 0.59   | 0.020  | 0.0013 | 0.45-1.30) |

#### Sexual risk behaviour

| Sexual risk behaviour | Incidence (/100 PY) | Incidence (/100 PY) | 95% CI          | p value | 95% CI          | p value |
|-----------------------|---------------------|---------------------|-----------------|---------|-----------------|---------|
| Heterosexual risk behaviour in previous 6 months | 0.87                | (0.72-1.66)         | 0.0013          | 0.59    | 0.77            | (0.45-1.30) |
| No                    | 1.59                | 52                  | 3270.9          | 1       | 7.57            | 36      | 475.9 | 1       |
| Yes                   | 1.73                | 38                  | 2192.5          | 1.09    | (0.72-1.66)     | 5.79    | 22    | 380.0  | 0.77   | (0.45-1.30) |
| HIV status of steady partner | 0.0013             |                      | 0.20            |         | 0.37            | (0.33-0.81) |
| No steady partner     | 1.63                | 67                  | 4122.5          | 1       | 7.83            | 53      | 676.7 | 1       |
| HIV positive          | 6.90                | 10                  | 145.0           | 4.24    | (2.18-8.25)     | 10.71   | 2     | 18.7   | 1.37   | (0.33-5.81) |
| HIV negative          | 1.14                | 11                  | 963.7           | 0.70    | (0.37-1.33)     | 2.62    | 3     | 114.6  | 0.33   | (0.10-1.07) |
| Unknown HIV status    | 0.98                | 2                   | 203.8           | 0.60    | (0.15-2.46)     | 0.00    | 0     | 34.0   |        | (0.10-1.07) |
Table 2.2.4  Multivariate analysis of the effect of participation in harm reduction programs on HIV and HCV seroconversion.

|                  | HIV                  |  |  | HCV                  |  |  |
|------------------|----------------------|---|---|----------------------|---|---|
|                  | IRR  | 95% CI  | p value | IRR  | 95% CI  | p value |
| No harm reduction| 1    | <0.001  | 1       | 1    | <0.001  | 1       |
| Incomplete harm reduction | 0.87 | 0.50-1.52 | 1.17 | 0.59-2.31 |
| Full harm reduction      | 0.43 | 0.21-0.87 | 0.36 | 0.13-1.03 |
| Limited dependence on harm reduction | 0.046 | 0.006-0.35 | 0.044 | 0.006-0.35 |
| No dependence on harm reduction  | 0.20 | 0.078-0.50 | 0.13 | 0.044-0.40 |
| Time since start injection drug use (per year) | 0.95 | 0.92-0.98 | <0.001 | 0.87 | 0.81-0.93 | <0.001 |
| No steady partner     | 1    | 0.004   | 1       | 0    | 0.026   | 1       |
| HIV positive steady partner | 4.53 | 2.23-9.21 | 3.49 | 0.84-14.5 |
| HIV negative steady partner | 0.82 | 0.43-1.57 | 0.42 | 0.13-1.37 |
| Steady partner with unknown HIV status         | 0.75 | 0.18-3.06 |

IRR=incidence rate ratio, 95% CI=95% confidence interval.

Discussion

Our data suggest that the combination of adequate methadone therapy and full participation in NEP substantially contributed to the reduction of the incidence of HIV and HCV in DU in Amsterdam, although a statistically significant effect was not seen when methadone dose or NEP were considered separately. It is likely that Amsterdam’s comprehensive program, in which methadone treatment and NEP are combined, explains the reported decline of HIV and HCV incidence.

We found no evidence that the effect of harm reduction was larger on HCV incidence than on HIV incidence, since our risk estimates for the different levels of harm reduction participation were comparable. One explanation might be that the Amsterdam harm reduction approach, which maintains contact with as many DU as possible, has an effect not only on injecting but also on sexual risk behaviour due to counselling and condom distribution. Our findings are in line with the reduction of sexual and drug-related risk behaviour seen in the ACS since the mid 1990s. Having an HIV-positive steady partner was associated with a higher risk of HIV infection, showing that HIV is more effectively transmitted sexually than HCV.7

The evaluation of HRPs is complicated, because it is hard to link participation in HRPs to outcome variables such as the incidence of blood borne infections. In some observational studies, methadone programs and NEP have been shown to reduce the incidence of HIV but not HCV.5,6,15,16 Ecological studies have shown a declining HCV prevalence after the introduction of NEP, while HCV incidence remained high.17-20 To our best knowledge, our study describes the combined effect of methadone therapy and NEP on HCV incidence, and over the longest period of time. The ACS among DU is a well-defined open cohort study with ongoing recruitment, that has been followed over the past 20 years. On average, 90% of participants that visited the ACS a given calendar year returned the next year as well. Despite its great strengths, ACS is not a randomized controlled trial and therefore a causal association between harm reduction participation and risk for HIV or HCV infection can not be proven. However, we could
not think of any unmeasured confounder both affecting harm reduction participation and HIV or HCV infection. Although NEP and methadone prescription were not available at the study setting, we cannot exclude that a cohort effect might partially explain the observed decrease in HIV and HCV incidence and injecting behaviour we observed in our cohort. Furthermore, risk behaviour was self-reported, and bias toward socially desirable answers could cause underestimation of the proportion engaged in risk behaviour. Although the data on HRP participation were also self-reported, Langendam *et al.* studied the harm reduction measures in the ACS and matched the self-reported methadone doses to the central methadone registry (CMR) and they found no clear difference in the self-reported dose and the dose at the CMR. As expected, DU not injecting drugs in the past 6 months and taking a low dose of methadone daily (i.e., with limited dependence on harm reduction) and DU not injecting drugs in the past 6 months and not receiving any methadone (i.e., with no dependence on harm reduction) were at lower risk for HIV and HCV seroconversion than were DU fully participating in a HRP. Interestingly, the limited-dependence group were at lower risk for HIV and HCV seroconversion than the no-dependence group, although the difference was not statistically significant. It could be that, because DU receiving a low dose of methadone are still surrounded by the social-medical care network associated with the methadone therapy, they might return more easily to a higher dose of methadone or call for other help in case of problems than DU who have completely stopped methadone and are out of the network.

The most important implication of our study is that only when methadone is combined with provision of needles and syringes through exchange programs is there a significant reduction of HIV and HCV incidence. Our finding is most important for countries with recent and sometimes explosive outbreaks of HIV and/or HCV among DU, like in the former Soviet Union and Asia. To provide only needles and syringes or only methadone will not be sufficient to curb the rapid spread of these and other blood borne infections among DU. It is essential to offer a comprehensive program in which both measures are combined, preferably also with social-medical care and counselling.
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Chapter 2.3

Never injected, but hepatitis C virus-infected:
A study among self declared never-injecting drug users from the Amsterdam Cohort Studies

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*J Viral Hepatitis. In press.*
Abstract

The aim of this study was to gain insight in transmission routes of hepatitis C virus (HCV) infection among never-injecting drug users (DU), by studying incidence, prevalence, determinants, and molecular epidemiology of HCV infection. From the Amsterdam Cohort Studies among DU, 352 never-injecting DU were longitudinally tested for HCV antibodies. Logistic regression was used to identify factors associated with antibody prevalence. Part of HCV NS5B was sequenced to determine HCV genotype and for phylogenetic analyses, in which sequences were compared with those from injecting DU. HCV antibody prevalence was 6.3% and HCV incidence was 0.49/1,000 person years. HIV-positive status, female sex, and starting injection drug use during follow up (a putative marker of past injection drug use), were independently associated with HCV prevalence. The main genotypes found were genotype 3a (50%) and 1a (30%). Phylogenetic analysis revealed that HCV strains in never-injecting DU did not cluster together and did not differ from HCV strains circulating in injecting DU. We found a higher HCV prevalence in never-injecting DU than in the general population. Phylogenetic analysis shows a strong link with the injecting DU population. The increased risk could be related to underreporting of injecting drug use or to household or sexual transmission from injectors to non-injectors. Our findings stress the need for HCV testing of DU who report never injecting, especially given the potential to treat HCV infection effectively.
Introduction

Acute hepatitis C virus (HCV) infection is usually asymptomatic, and leads to chronic infection in 50-80% of patients.\(^1\) Decades of chronic HCV infection can lead to liver cirrhosis and, in 1-5% of these patients, eventually to hepatocellular carcinoma as well.\(^2\) In recent years, treatment success rates have substantially improved.\(^3\) The most important mode of HCV transmission is through exposure to infected blood,\(^1,4\) and although sexual and household transmission have been described, they appear to happen only occasionally.\(^5-7\)

While never-injecting drug users (DU) do not share needles and/or syringes, their HCV prevalence is still higher than in the general population. Some studies suggest that HCV infection in never-injecting DU is associated with the sharing of drug-use paraphernalia, especially utilities used for consumption of crack, but others could not confirm these findings (reviewed in ref. 8). Alternatively, never-injecting DU might become infected with HCV through needle-stick accidents, household transmission, or sexual exposure. Recent review of research describing HCV among non-injecting DU points to a substantial gap in our knowledge of HCV in never-injecting DU, as no uniform risk factors could be identified.\(^8\)

The Amsterdam Cohort Study (ACS) among DU comprises a large group of never-injecting DU. It was designed to evaluate the sexual and blood borne transmission of HIV, other blood borne pathogens, and sexually transmitted diseases, as well as the determinants of transition to injecting drug use. This design has the potential to determine prevalence, incidence, and risk factors for HCV infection among never-injecting DU. Additionally, we used phylogenetic analysis to investigate whether HCV strains isolated from never-injecting DU were closely related to strains circulating among injecting DU, or whether separate introductions had occurred through unrelated modes of transmission.\(^9\)

Methods

The ACS among DU is an open, prospective cohort study initiated in 1985.\(^9\) Participation in the ACS is voluntary, and informed consent is obtained for every participant at intake. Recruitment is ongoing and in recent years has been directed in particular towards young DU. Both injecting and non-injecting DU are included and visit the Amsterdam Health Service every 4-6 months. Each study visit standardized questionnaires on (injecting) drug use and sexual risk behaviour are administered by trained research nurses and blood is drawn for prospective HIV testing and storage of serum. To study HCV prevalence and incidence we retrospectively tested stored serum from all participants having at least two visits between December 1985 and November 2005 (n=1276), using the first available sample in each case. Individuals who were HCV negative at ACS entry were tested for HCV antibodies at their last ACS visit before November 2005. On finding HCV seroconversion (defined as the presence of HCV antibodies in a previously seronegative individual), we tested samples taken between these two visits to determine the moment of seroconversion (defined as the midpoint between the last HCV seronegative sample and the first seropositive visit).\(^10\) Third generation commercial microparticle EIA system tests were used to detect HCV antibodies (AxSym HCV version 3.0; Abbott, Wiesbaden, Germany). 28.9% of the
seropositive participants were tested at two study visits or more, all with consistent positive HCV-antibody test results. Presence of HCV antibodies in all never-injecting DU was confirmed with Western blot (Deciscan HCV Plus immunoblot; BioRad). All ACS samples were stored at -80°C. All ACS participants since 1985 (n=1640) were tested for HIV antibodies by enzyme linked immunosorbent assays (ELISA), since 2003 AxSym HIV Ag/Ab Combo (Abbott) at each study visit. Results were confirmed by Western blot, since 1986, by HIV Blot version 2.2 (Genelab diagnostics).

Statistical analyses

Anti-HCV antibody prevalence and incidence were calculated. Follow-up time was calculated from HCV-negative study entry through HCV seroconversion, the moment of starting injecting drug use, or November 2005, whichever occurred first. Risk factors for the presence of HCV antibodies at study entry were examined using logistic regression. All risk factors refer to the past 6 months, unless stated otherwise. They included: general and demographic factors (sex, nationality, ethnicity, calendar year of visit); drug use-related risk factors (ever-injecting drug use, years of regular heroin/cocaine/amphetamines use, start of injecting drug use during ACS follow up, alcohol use) and specifically cocaine-use-related factors (years of regular cocaine use/cocaine snorting/basing of cocaine); sexual risk behaviour (having sex with injecting DU/commercial sex workers/men who have sex with men since 1980, main sexual preference since 1980, number of commercial sexual contacts since 1980, having a steady sexual partner, having an injecting steady sexual partner, HIV status of the steady sexual partner, condom use (with steady sexual partner/casual partner/commercial contacts) and other clinically relevant variables (subjects’ history of HIV, jaundice, blood transfusion, tattoo, piercing).

Multivariate logistic regression models were built using forward stepwise techniques. All variables with a p-value ≤0.10 in univariate analysis were considered for entry into the model. Statistical analysis was performed by use of STATA (version 9.2; StataCorp) and SPSS (version 15.0; SPSS Inc.) software. All statistical tests were two-sided; a p-value ≤0.05 was considered to be statistically significant. Interaction and confounding were checked between the variables in the final models and all variables with a univariate p-value ≤0.20.

Reverse-transcription polymerase chain reaction (RT-PCR) methods

After HCV antibody screening, HCV-seropositive samples were additionally tested for the presence of HCV RNA. RNA isolation was performed on 100 μl of serum using the TriPure method (Roche Diagnostics). Each RNA isolate was used as input for two nested multiplex RT-PCRs. The first PCR, which targets the conserved HCV core region, was devised as a genotyping system to differentiate genotypes 1a, 1b, 2a, 2b, 3a, 4, 5a and 6a. The second RT-PCR, which targets the NS5B region, was used for phylogenetic analysis. Conditions and primers for both PCRs have been described elsewhere.11

Sequencing and phylogenetic analysis

The sequencing reaction and analysis were performed as described earlier.11 Briefly, NS5B PCR products were ethanol precipitated. Sense and antisense strands were
separately cycle-sequenced using the BigDye Terminator system (version 1.1; Perkin Elmer). Sequence products were purified using DyeEx spin kits (Qiagen) and analyzed on an ABI-310 automated sequencer (Applied Biosystems). Sequence alignment of the 436-bp NS5B fragment was performed using the BioEdit software package.\(^\text{12}\) Viral genotype was confirmed after phylogenetic analysis of the NS5B sequences obtained from subjects (GenBank accession numbers EU410492 to EU410507) along with established GenBank reference sequences.\(^\text{13}\) Mega software (version 3.1; available at: http://www.megasoftware.net) was used to construct a phylogenetic tree by the neighbour-joining method, using the Tamura-Nei substitution model with \(\gamma\)-distribution (\(\alpha=0.40\)). Bootstrap values (n=1,000) were calculated to analyze the stability of tree topology. HCV sequences obtained from DU who reported never injecting were compared to all known HCV sequences from injecting DU participating in the ACS (unpublished data).\(^\text{11,14}\)

**Results**

**General characteristics**

Among the 1276 DU who participated in the ACS and had two or more visits between December 1985 and November 2005, 364 DU reported never having injected drugs before study entry. Of these 364, 352 (96.7%) had serum available for HCV testing. They were mainly male (69.3%) and of Dutch nationality (305/352, 86.6%); of the 305 Dutch participants 101 (33.1%) were of Surinamese ethnicity. Of 352 never-injecting DU, 154 preferred cocaine as their main type of non-injected drugs (43.8%). Of the 352, 22 (6.3%, 95% CI 3.9–9.4%) were HCV antibody-positive at study entry and 14/352 (4.0%, 95% CI 2.2-6.6%) DU were HIV-positive (Table 2.3.1). The total HCV-negative and never-injecting follow up time was 2005 person years (PY); the median follow up time per participant was 6.4 years (interquartile range (IQR) 3.01-11.3 years). Only one never-injecting DU seroconverted for HCV during follow up; the HCV incidence was 0.049 per 100 PY (95% CI 0.01–0.35 per 100 PY). However, 47 never-injecting DU started injecting during follow up, of whom 7 were HCV-positive at study entry and 23 seroconverted for HCV after starting injection.

In addition to the observed HCV incidence, we calculated an estimated incidence using prevalence data, assuming that the duration of regular hard-drug use before study entry equals the time of exposure to HCV. Information on the number of years of regular cocaine/regular heroin use was available for 285/352 individuals (81.0%), including 20/22 HCV positive never-injecting DU. The duration of regular use of heroin or cocaine was used as the time of exposure. These 285 individuals had a total of 2,539 person years of regular drug use. The estimated time of HCV infection was defined as the midpoint of years of duration of regular use of hard drugs, yielding an estimated incidence of 0.79 per 100 PY. Assigning the estimated time of HCV infection to either the start of regular hard drug use before study entry (maximum estimated HCV incidence) or at study entry (minimum estimated HCV incidence), changed the estimated HCV incidence only slightly to 0.82 or 0.76 per 100 PY, respectively.
Table 2.3.1 General characteristics of never-injecting drug users (DU) at entry in the Amsterdam Cohort Studies among DU.

| General drug use and HCV related characteristics | HCV + n=22 | HCV - n=330 |
|--------------------------------------------------|-----------|------------|
| Median age (IQR) | 30 (26-37) | 30 (26-36) |
| Female sex | 12/22 (54.4%) | 96/330 (29.1%) |
| Dutch nationality | 19/22 (86.4%) | 286/330 (86.7%) |
| Homeless in the past 6 months | 0/14 (0%) | 45/262 (17.2%) |
| Main type of drug used in the past 6 months | | |
| Heroin | 6/20 (30%) | 137/300 (45.7%) |
| Cocaine | 13/20 (65%) | 141/300 (47%) |
| Heroin and cocaine together | 1/20 (5%) | 15/300 (5%) |
| Other | - | 7/300 (2.3%) |
| HIV-positive (%) | 3/22 (13.6%) | 11/330 (3.33%) |
| Ever tattoo | 6/14 (43%) | 91/194 (47%) |
| Ever piercing | 2/14 (14%) | 20/194 (10%) |
| Jaundice (ever) | 2/8 (25%) | 4/68 (6%) |
| Blood transfusion (ever) | 2/8 (25%) | 5/67 (7.5%) |
| Follow up characteristics | | |
| Median number of visits to ACS (IQR) | 15 (6-25) | 12 (5-22) |
| Median years follow up in ACS (IQR) | 7.58 (4.58-14.1) | 6.13 (2.99-11.1) |
| Number of HCV seroconversions | - | 1 |
| HCV viral characteristics | | |
| HCV RNA positive | 15 (68%) | NA |
| Genotypes mainly related to injecting drug use | | |
| 1a | 4 (26.7%)* | |
| 3a | 8 (53.3%) | |
| Genotypes mainly related to other risks | | |
| 1b | 2 (13.3%)* | |
| 2a | 1 (6.7%) | |
| NA = not applicable; *% among all HCV RNA positive individuals. |

Associations with the presence of HCV antibodies

In univariate logistic regression (Table 2.3.2), the following variables were significantly associated with the presence of HCV antibodies at entry in the ACS: female sex (OR 2.93, 95% CI 1.22-7.00) and starting injection during follow up, a putative marker of past injection drug use (OR 3.38, 95% CI 1.30-8.80). Although the association had only borderline significance, HIV-positive participants had a higher risk of being HCV-positive (OR 4.58, 95% CI 1.18-17.8, p=0.053) (Table 2.3.2). No significant association of HCV with crack use was found, although the OR for cocaine compared to heroin as the main type of drug used was 2.11 (95% CI 0.78-5.70), and the OR for one or more times daily cocaine use was higher compared to less frequent cocaine use in the six months preceding ACS entry.

In multivariate logistic regression, HIV-positive status (OR 5.07, 95% CI 1.21-21.3), female sex (OR 2.85, 95% CI 1.15-7.05) and starting injection during follow up in ACS (OR 2.78, 95% CI 1.03-7.47), were independently associated with the presence of HCV antibodies.
Table 2.3.2  Univariate and multivariate logistic regression. Determinants of HCV in never-injecting drug users (DU) at entry in the Amsterdam Cohort Studies among DU.

|                                | Proportion | Univariate |          |       |          | Multivariate |          |       |
|--------------------------------|------------|------------|----------|-------|----------|--------------|----------|-------|
|                                |            | OR         | 95% CI   | p value | OR       | 95% CI       | p value  |       |
| **Demographic variables**      |            |            |          |        |          |              |          |       |
| Age (per 10 years of increase) | 1.29       | 0.75-2.23  | 0.36     | 0.017  | 1.023    |               |          |       |
| Sex                           |            |            |          |        |          |              |          |       |
| Male                          | 10/244     | 1          | 0.017    | 0.23   |          |              |          |       |
| Female                        | 12/108     | 2.93       | 0.12-7.00| 0.28   | 0.15     | 1.15-7.05    | 0.023    |       |
| Year of visit                 |            |            |          |        |          |              |          |       |
| 1985-1992                     | 13/125     | 1          | 0.063    |        |          |              |          |       |
| 1993-1998                     | 4/114      | 0.31       | 0.10-1.00|        | 0.017    | 0.14-1.16    | 0.023    |       |
| 1999-2005                     | 5/113      | 0.40       |          |        |          |              |          |       |
| Nationality                   |            |            |          |        |          |              |          |       |
| Dutch                         | 19/305     | 0.97       | 0.28-3.43| 0.36   |          |              |          |       |
| Non-Dutch                     | 3/47       | 0.97       | 0.07-4.69| 0.36   |          |              |          |       |
| Years of education after primary school | 3/31 | 1 | 0.07-4.69 | 0.36 |          |              |          |       |
| <3                            | 3/31       | 1          |          |        |          |              |          |       |
| 3                             | 4/35       | 1.20       | 0.25-5.86|        |          | 0.07-1.96    |          |       |
| 4-5                           | 3/78       | 0.37       | 0.07-1.96|        |          | 0.02-1.87    | 0.32     |       |
| >5                            | 3/69       | 0.42       | 0.081-2.23|       |          | 0.07-1.87    |          |       |
| Alcohol use in the past 6 months | 0.38 |           |          |        |          |              |          |       |
| No                            | 12/139     | 1          |          |        |          | 0.27-1.64    |          |       |
| Yes                           | 9/151      | 0.67       | 0.07-4.69| 0.36   |          |              |          |       |
| **Drug use related risk factors** | |            |          |        |          |              |          |       |
| Main type of non-injecting drug used in past 6 months | 0.32 |          |          |        |          |              |          |       |
| Heroin                        | 6/143      | 1          |          |        |          | 0.07-4.94    |          |       |
| Cocaine                       | 13/154     | 2.11       | 0.78-5.70|        |          | 0.17-13.5    | 0.70     |       |
| Cocktail of heroin/cocaine (i.e., speedball) | 1/15 | 1.62 | 0.17-13.5 | 0.70 |          |              |          |       |
| Frequency of non-injecting drug use (main drug used) in past 6 months | 0.70 |          |          |        |          |              |          |       |
| Multiple times daily          | 11/137     | 1          |          |        |          |              |          |       |
| Once daily                    | 1/20       | 0.60       | 0.07-4.94|        |          | 0.13-13.5    | 0.70     |       |
| Several times weekly, but less than daily | 5/113 | 0.53 | 0.18-1.57 | 0.70 |          |              |          |       |
| Several times monthly, but less than weekly | 1/20 | 0.60 | 0.07-4.94 | 0.70 |          |              |          |       |
| Once monthly                  | 1/4        | 3.81       | 0.37-39.9|        |          | 0.07-4.69    | 0.36     |       |
| Less frequent                 | 1/11       | 1.14       | 0.13-9.79|        |          | 0.13-9.79    | 0.36     |       |
| Non-injecting drug use of steady partner | 0.35 |          |          |        |          | 0.07-4.94    |          |       |
| Not applicable, no steady partner | 13/169 | 1 |          |        |          | 0.13-9.79    | 0.36     |       |
| No, never                     | 4/35       | 1.55       | 0.47-5.06|        |          | 0.13-9.79    | 0.36     |       |
| Yes, now or ever              | 4/91       | 0.55       | 0.17-1.74|        |          | 0.13-9.79    | 0.36     |       |
| Start of injecting drug use during follow up | 0.02 |          |          |        |          | 0.13-9.79    | 0.36     |       |
| No                            | 15/305     | 1          |          |        |          | 0.13-9.79    | 0.36     |       |
| Yes                           | 7/47       | 3.38       | 1.30-8.60| 0.10   |          | 1.03-7.47    | 0.043    |       |
| Years of regular heroin use   |            |            |          |        |          |              |          |       |
| Less than 6 months (or never start) | 1/49 | 1 |          |        |          | 0.13-9.79    | 0.36     |       |
| 6 months-5 years              | 3/66       | 2.29       | 0.23-22.6|        |          | 0.13-9.79    | 0.36     |       |
| ≥5 years                      | 16/170     | 4.99       | 0.64-38.6|        |          | 0.13-9.79    | 0.36     |       |
Chapter 2.3

### Epidemiology

#### Univariate

| Proportion HCV+ | Univariate OR | 95% CI | p value | Multivariate OR | 95% CI | p value |
|-----------------|---------------|--------|---------|----------------|--------|---------|
| **Years of regular amphetamines use** |              |        |         |                |        |         |
| Less than 6 months (or never start) | 18/242 | 1 |        |                |        |         |
| 6 months or more | 2/43 | 0.67 | 0.15-3.02 |
| **Cocaine related risk factors** |              |        |         |                |        |         |
| Years of regular cocaine use |              |        |         |                |        |         |
| Less than 6 months (or never start) | 3/45 | 1 |        |                |        |         |
| 6 months-5 years | 6/112 | 0.79 | 0.19-3.32 |
| ≥5 years | 11/128 | 1.31 | 0.35-4.95 |
| Frequency of cocaine use in 6 months before ACS entry |              |        |         |                |        |         |
| No cocaine use | 1/38 | 1 |        |                |        |         |
| Once or more times monthly | 1/28 | 1.37 | 0.082-22.9 |
| Once or more times weekly | 5/87 | 2.26 | 0.25-20.0 |
| Once or more times daily | 6/61 | 4.04 | 0.47-34.9 |
| **Sexual risk behaviour** |              |        |         |                |        |         |
| Sex with injecting DU since 1980 |              |        |         |                |        |         |
| No | 8/149 | 1 |        |                |        |         |
| Yes | 4/54 | 1.41 | 0.41-4.89 |
| Sex with commercial sex workers since 1980 |              |        |         |                |        |         |
| No | 5/94 | 1 |        |                |        |         |
| Yes | 7/114 | 1.16 | 0.36-3.80 |
| Sex with MSM since 1980 |              |        |         |                |        |         |
| No | 8/172 | 1 |        |                |        |         |
| Yes | 4/36 | 2.56 | 0.73-9.02 |
| Main sexual preference since 1980 (excluding contacts with commercial sex workers) |              |        |         |                |        |         |
| Exclusively heterosexual | 15/285 | 1 |        |                |        |         |
| Not exclusively heterosexual | 5/47 | 2.14 | 0.74-6.20 |
| Number of prostitution contacts in the 6 months preceding ACS entry (males and females) |              |        |         |                |        |         |
| No prostitution contacts | 1/20 | 1 |        |                |        |         |
| 1-9 | 10/159 | 1.27 | 0.15-10.5 |
| ≥10 | 8/95 | 1.75 | 0.21-14.8 |
| Prostitution contacts in the 6 months preceding ACS entry (males and females) |              |        |         |                |        |         |
| No | 8/155 | 1 |        |                |        |         |
| Yes | 4/51 | 1.56 | 0.45-5.43 |
| Steady partner in the 6 months preceding ACS entry |              |        |         |                |        |         |
| No | 13/202 | 1 |        |                |        |         |
| Yes | 9/137 | 1.02 | 0.42-2.46 |
| Steady partner that injects/injected drugs in the 6 months preceding ACS entry |              |        |         |                |        |         |
| Steady partner injects/injected drugs | 2/35 | 1 |        |                |        |         |
| Steady partner does/did not inject drugs | 7/105 | 1.18 | 0.23-5.96 |
| Not applicable, no steady partner | 13/202 | 1.13 | 0.24-5.26 |
Epidemiology of HCV in never-injecting drug users

| Last HIV test result of steady partner | Proportion HCV+ | Univariate OR 95% CI p value | Multivariate OR 95% CI p value |
|---------------------------------------|-----------------|-------------------------------|------------------------------|
| Not applicable, no steady partner in the 6 months preceding ACS entry | 20/283 | 1 | |
| Positive | 1/10 | 1.46 | 0.18-12.1 |
| Negative | 0/37 | - | - |
| Unknown | 1/20 | 0.69 | 0.088-5.44 |
| Always use of condoms with steady partner | 0.33 |
| Not applicable, no steady partner in the 6 months preceding ACS entry | 2/15 | 1 | |
| No | 17/254 | 0.47 | 0.097-2.24 |
| Yes | 3/83 | 0.24 | 0.037-1.60 |
| Always use of condoms with casual partners | 0.07 |
| Not applicable, no casual partners in the 6 months preceding ACS entry | 1/51 | 1 | |
| No | 18/212 | 4.64 | 0.60-35.6 |
| Yes | 3/89 | 1.74 | 0.18-17.2 |
| Use of condoms with prostitution partners | 0.47 |
| Always use of condoms | 3/36 | 1 | |
| Not always use of condoms | 3/26 | 1.43 | 0.27-7.75 |
| Not applicable, no prostitution partners | 16/289 | 0.64 | 0.18-2.33 |

**Other risk factors**

| HIV status | Proportion HCV+ | Univariate OR 95% CI p value | Multivariate OR 95% CI p value |
|------------|-----------------|-------------------------------|------------------------------|
| Negative | 19/338 | 1 | 1 |
| Positive | 3/14 | 4.58 | 1.18-17.8 | 5.07 | 1.21-21.3 |
| Tattoo (ever) | 0.77 |
| No | 6/97 | 1 | |
| Yes | 8/111 | 1.18 | 0.39-3.52 |
| Piercing (ever) | 0.65 |
| No | 12/186 | 1 | |
| Yes | 2/22 | 1.45 | 0.30-6.95 |
| Jaundice (ever) | 0.11 |
| No | 6/70 | 1 | |
| Yes | 2/6 | 5.33 | 0.80-35.4 |
| Blood transfusion (ever) | 0.16 |
| No | 6/68 | 1 | |
| Yes | 2/7 | 4.13 | 0.66-26.1 |

OR=odds ratio, 95% CI=95% confidence interval.

**HCV RNA and phylogenetic analysis**

Of 22 HCV-antibody positive never-injecting DU at ACS entry, 15 (68.2%) had detectable HCV RNA. The most frequent HCV genotype found was 3a (53.3%), followed by genotype 1a (26.7%) (Table 2.3.1). HCV genotypes 1a and 3a are generally associated with injecting drug use, and in injecting DU in the ACS they account for 252/317 (79%) of HCV infections for which genotyping was performed. Hence, the proportion of injection-related HCV genotypes was comparable among injecting DU and never-injecting DU.11,14,unpublished data Figure 2.3.1 shows a phylogenetic tree of HCV
genotype 3a, comprising the eight NS5B sequences obtained from never-injecting DU together with all available genotype 3a NS5B sequences (n=65) from injecting DU.\textsuperscript{11,14,unpublished data} Comparable to a pedigree, a phylogenetic tree illustrates the evolutionary relationships between genes or organisms or, in our case, the relationship among aligned NS5B sequences of several HCV genotype 3a viral variants. The more related two sequences are, the smaller the horizontal distance between those sequences in the tree. Based on phylogenetic analysis, sequences from never-injecting DU could not be distinguished from those of injecting DU. Sequences derived from never-injecting DU were interspersed with those of injecting DU, and they were not distinct phylogenetic isolates, nor did they form separate never-injecting DU clusters. This was observed also in HCV genotype 1a sequences (data not shown). The three never-injecting DU not infected with HCV genotype 1a or 3a harboured distinct strains of genotype 1b and 2a, which in the Netherlands and Belgium are linked to blood transfusion and nosocomial transmission rather than injecting drug use.\textsuperscript{15,16} The proportion of never-injecting DU infected with these types (20%) was somewhat larger than the proportion observed among injecting DU (9%) in the ACS, but the difference was not statistically significant ($p=0.26$, Pearson Chi square). Interestingly, only one never-injecting (male) DU seroconverted during follow up despite denying injecting drug use. He has regularly reported a steady sexual relationship with an injecting (female) DU who also participates in the ACS. She is a known injecting DU and became chronically infected with HCV genotype 2b at least 2.7 years before her male never-injecting DU sexual partner seroconverted for HCV. When comparing their two HCV sequences, the sequences were 100% identical (data not shown), making accidental exposure during household contacts or sexual transmission the likely route of transmission in this couple.
Figure 2.3.1 NS5B Phylogenetic tree of prevalent HCV genotype 3a infections among never-injecting drug users (DU) (shaded) and ever-injecting DU in Amsterdam, using the neighbour-joining method based on Tamura-Nei substitution with $\gamma$-distribution ($\alpha=0.40$). Each isolate code contains the year of sampling.
Discussion

In this cohort of never-injecting DU, the HCV prevalence was 6.3% (95% CI 3.7-8.8%). Although much lower than the prevalence in injecting DU in the same cohort (83.5%), this is substantially higher than in the general population in The Netherlands (estimated to be 0.1-0.4%). In literature, the HCV prevalence in never-injecting DU ranges from 2.3 to 35.3%. However many studies were not specifically designed to measure HCV prevalence in never-injecting DU and often did not include questions on non-injection drug use risk factors for HCV.

The observed HCV incidence was very low at 0.049/100 PY, sixteen-fold lower than the HCV incidence estimated from the prevalent cases at study entry (0.79/100 PY). This suggests underreporting of past injecting drug use, which may have led to misclassification of injecting DU as never-injecting DU. However, this estimated HCV incidence has limitations: it does not take into account losses to follow up in the unknown cohort that the prevalence sample is supposed to represent. Nor does it take differential recruitment of rates of healthy and infected subjects into account. However, when interpreted with caution, it could support our hypothesis of underreporting of injection drug use. Especially when injecting was incidental or stopped before entry in the ACS, participants may deny past risk behaviour, as has been described for HCV-positive blood donors in the Netherlands.

Starting injection later during follow up was independently associated with a higher prevalence of HCV antibodies at entry. Of 352 never-injecting DU, 47 switched to injecting drug use after a median of 56 months (IQR 20-58 months). Of the 47, 7 were among the 22 found to be HCV seroprevalent at entry. Again, this finding could suggest that some injecting DU were misclassified as never-injecting DU. They might have given socially desirable answers and denied injecting, since it is perceived among DU as damaging to their appearance and as overstepping a limit in the drug-using scene in Amsterdam. Alternatively, the DU who started injecting during follow up were already actively participating in the scene of injecting DU and were therefore more likely to become exposed to HCV through routes other than injecting drug use, such as needle stick accidents. Since HIV and HCV share transmission routes, the finding that HIV-positive never-injecting DU had a higher HCV prevalence at entry compared to HIV-negative participants, could imply that HIV-positive status is an indicator of unreported injecting drug use. On the other hand, HIV is transmitted sexually much more efficiently than HCV, and HCV might be transmitted more easily to and/or from HIV-positive individuals, compared to HIV-negative individuals, since HIV co-infection is associated with higher HCV RNA viral load.

Phylogenetic analysis revealed that the HCV sequences of never-injecting DU did not cluster together, suggesting that they were not a uniform group that became infected through sharing of non-injection drug use paraphernalia. In contrast, the non-injecting DU clustered together with the sequences found in injecting DU in the ACS (Figure 2.3.1), indicating that they have close links with injecting DU and possibly underreport injection drug use. So although these DU did not report injecting drug use, they were infected from the pool of injecting DU. Although self-reported data on methadone prescription in this cohort have been investigated and shown to be consistent with data from the Dutch Central Methadone Registration, self-reported data on sexually transmitted diseases (STD) were shown to be less consistent with diagnosis of such diseases. In this study, based on the findings from logistic regression and
phylogenetic analysis, some misclassification of ever-injecting DU seems likely in this never-injecting DU population. Female sex was also associated with a higher HCV prevalence at entry, possibly indicating that women having sex with an HCV-positive partner are at higher risk for sexual transmission than men, as has been shown for HIV. However, this gender difference has not yet been described for HCV. We did not find an association between the presence of HCV antibodies and sexual behaviour. Furthermore, we observed only one HCV seroconversion during >2,000 person years of follow up, indicating that the risk of sexual transmission -- and also household transmission -- is very small as has been demonstrated in partner studies among discordant heterosexual couples. Unfortunately we were not able to perform risk factor analysis based on just one HCV seroconversion, but such analysis of incident cases in a longitudinal study would be more robust than a cross-sectional analysis of prevalent cases.

HCV has been detected on drug-use paraphernalia, and it has been hypothesized that HCV can be transmitted via these utilities (e.g., straws used for cocaine snorting). In line with our phylogenetic finding of non-clustering of never-injecting DU, we did not find statistically significant associations between cocaine use and the presence of HCV antibodies. However, questions on snorting paraphernalia were not included in the ACS questionnaires used in our study period. Some questions (e.g., having a tattoo, having a piercing) were added to the questionnaires in 2001 and thus yield data for only a portion of participants included in this study. A similar limitation holds true for the data on having received a blood transfusion, a question not asked after 1989, shortly before HCV screening of donor blood was introduced in developed countries. Moreover, never-injecting DU might potentially have received a blood transfusion when travelling to countries where transfusion is not yet safe. Although the direction of the effect of having received a blood transfusion was as expected (i.e., higher risk for those who have received a blood transfusion compared to those who did not), the main HCV genotype related to transmission by blood transfusion is genotype 1b, whereas the main genotypes circulating among never-injecting and injecting DU are 1a and 3a. Remarkably, in The Netherlands between 1997-2002, genotypes 1a and 3a, were found in 9/18 (50%) of HCV RNA-positive new donor candidates who most likely acquired HCV through a contaminated blood transfusion in the past.

In conclusion, although the incidence of HCV was very low in this study among never-injecting DU, the prevalence was much higher than in the general population. In the methadone outposts of the Amsterdam Health Service, HCV screening is offered every year irrespective of recent injecting drug use. Although, we could not distinguish whether the increased risk of HCV infection in never-injecting DU was related to underreporting of injection or to household or sexual transmission, HCV strains of never-injecting DU cluster with those found among injecting DU. HCV treatment has improved substantially since 2000 and is effective in up to 80-90% of patients. Therefore, whatever the route of transmission, it is clear that routine HCV testing and treatment should be extended to both never-injecting and injecting DU.
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Chapter 3

Natural history
Chapter 3.1

Female sex is the strongest predictor of spontaneous viral clearance in a cohort of hepatitis C virus seroconverters

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Submitted.
Abstract

Background & Aims
Although acute hepatitis C virus (HCV) infection is usually asymptomatic and rarely recognized, predictors should be identified to guide treatment, since acute HCV is more responsive to treatment than chronic HCV.

Methods
Subjects were participants in the Amsterdam Cohort Study (ACS) among DU and included prospective HCV seroconverters (n=55) and recent HCV-positive participants who started injecting drug use within two years before ACS entry (n=51). Presence of HCV RNA was determined at a minimum of two time-points shortly following HCV infection to identify viral clearance. Logistic regression was used to examine potential determinants of HCV clearance.

Results
The rate of spontaneous viral clearance was 33.0% (95% confidence interval (CI) 24.2-42.8%). Women were more likely than men to spontaneously clear HCV infection (odds ratio 3.13, 95% CI 1.35-7.25). No HCV virological or sociodemographic characteristics were significantly associated with spontaneous HCV clearance, but HIV and HBV co-infection might play a role.

Conclusions
Since female sex was the strongest predictor of spontaneous HCV clearance, treatment of acute HCV might be postponed in HIV-negative women. However, since HCV-treatment outcome is less favourable in HIV co-infected individuals, HCV treatment should not be postponed in individuals and groups who are HIV-infected or at high risk of HIV co-infection.
Female sex predicts spontaneous HCV clearance

Introduction

Hepatitis C virus (HCV) is mainly transmitted through exposure to infected blood.\(^1\) Acute infection is usually asymptomatic and leads to chronic infection in an estimated 75% of individuals.\(^2,3\) Chronic HCV infection can in time lead to liver fibrosis and cirrhosis, end-stage liver disease, and hepatocellular carcinoma.\(^4\) Treatment during acute HCV with pegylated interferon (with or without ribavirin) is associated with higher treatment success rates when compared to treatment during chronic infection.\(^5,7\) To be able to decide whether early treatment is indicated, early predictors of spontaneous viral clearance are urgently needed. The majority of the studies on spontaneous viral clearance have been conducted among persons for whom the duration of HCV infection is unknown. These seroprevalent studies are subject to selection bias, which can potentially lead to biased rates of viral clearance and risk estimates. Studies conducted among acute HCV cases are less likely to suffer from methodological flaws. However, the potential to examine the rate and determinants of spontaneous viral clearance of acute HCV is restricted, since acute infection is usually asymptomatic and therefore rarely recognized. The limited published data show that 14-38% of persons with acute HCV cleared the virus, and that clearance is associated with symptomatic acute HCV, female sex, non-black race, lower peak HCV-RNA titre, induction of neutralizing antibodies early in HCV infection, and high and broad HCV-specific CD4\(^+\) and CD8\(^+\) T-cell responses.\(^5,8-14\) However, sample size and data collection of these studies are often limited and definitions of viral clearance differ between the studies.

Since the prospective Amsterdam Cohort Study (ACS) among drug users (DU) has retrospectively identified a substantial number of incident HCV infections,\(^15\) it provided an unique opportunity to study the spontaneous HCV clearance rate and its potential determinants, measured before and around acute HCV infection, in a population that includes asymptomatic acute HCV cases.

Materials and methods

Study population

The Amsterdam Cohort Study (ACS) among DU is an open, prospective cohort study initiated in 1985 to investigate the prevalence, incidence, and risk factors of HIV infections and other blood-borne and/or sexually transmitted diseases, as well as the effects of intervention.\(^16\) Recruitment is ongoing and in recent years has been directed in particular toward young DU. Participation in the ACS is voluntary, and informed consent is obtained for every individual at intake. ACS participants visit the Amsterdam Health Service every 4-6 months, each visit they complete a standardized questionnaire about their health, risk behaviour, and sociodemographic situation. Questions at ACS entry refer to the six months preceding the visit; questions at follow up refer to the interim since the preceding visit. Blood is drawn each visit for laboratory testing and storage.
Screening for HCV, HBV and HIV

To identify HCV seroconverters, we retrospectively tested stored serum from all participants having at least two visits between December 1985 and November 2005 (n=1276), using the first available sample in each case. Individuals who were HCV antibody negative at ACS entry were tested for antibodies at their last ACS visit before November 2005. On finding seroconversion, we tested samples taken between these two visits to determine the moment of seroconversion (third generation commercial microparticle EIA system, AxSym HCV version 3.0; Abbott, Wiesbaden, Germany). All HCV seroconverters were included in the present study (n=59). Also included were DU who were HCV antibody positive at ACS entry and had started injecting drug use within two years before entry. These individuals most probably represent recent HCV infections, since we have shown that approximately 50% of injecting DU in the ACS become infected within two years after starting injection drug use.15

To assess hepatitis B virus (HBV) status, stored blood samples were retrospectively tested for anti-HBc by the same algorithm as for HCV (AxSym Core, Abbott, Germany and Hepanostika; Organon Technika, the Netherlands). To identify individuals with active HBV infection, the presence of HBV surface antigen (HBsAg) was determined (AxSym HBsAg, Abbott, Germany) in serum. All ACS participants since 1985 (n=1,640) were prospectively tested for HIV antibodies by enzyme linked immunosorbent assays (ELISA) at each ACS visit. Results since 1986 have been confirmed by Western blot using HIV Blot version 2.2, Genelab diagnostics (Singapore).

Reverse-transcription polymerase chain reaction (RT-PCR) methods

For each seroconverter, HCV RNA was measured at a minimum of four time-points when samples were available: the last visit before HCV seroconversion, i.e., the last HCV antibody negative visit (t=-1), two visits shortly after HCV seroconversion (t=1 and t=2), and a visit approximately one year after HCV seroconversion (t=3) (Figure 3.1.1A). In those who were positive at entry, HCV RNA was measured at two time-points (t=1 and t=2): the first two visits following entry (most probably corresponding to t=2 and t=3 in prospective seroconverters). All serum samples were tested for the presence of HCV RNA using an in-house quantitative real-time RT-PCR based on the conserved 5'-UTR, as described by Van de Laar et al.17 Briefly, RNA extraction was performed using the Boom method, in which 200 μl of serum and 15 μl of internal control were added to 900 μl of lysis buffer and 20 μl of size-fractioned coarse silica particles.18 RNA was eluted in a volume of 100 μl and transcribed to cDNA as detailed elsewhere.19 Realtime PCR mixes (25 μl total volume) contained 12.5 μl of 2x LC480 probes master, 0.6 μM of forward and reverse primers (described in Van de Laar et al.).17 Real-time PCR was performed on a Roche LC480 platform. Quantification of viral RNA was performed by using standard curves which were produced by linear regression analysis of dilution series of plasmid DNA containing the 5’-UTR sequence.

In addition, genotyping was performed as described in Van de Laar et al., cDNA was used as input for one nested multiplex RT-PCR based on the NS5B region.17 Conditions and primers have been described elsewhere.17,20
Statistical analyses and definitions

Date of HCV seroconversion was estimated as the midpoint between the last HCV antibody-negative visit and the first HCV antibody-positive visit in prospectively identified HCV seroconverters. In prevalent HCV cases who started injection ≤2 years prior to enrolment, the date of seroconversion was estimated as the midpoint between start of injection drug use and entry in ACS. In this prospective group, spontaneous HCV clearance was defined as two consecutive HCV RNA-negative test results, at least four months apart, after HCV seroconversion. In the prevalent group, clearance was defined as two consecutive HCV RNA-negative test results after ACS entry (time points 1 (prospective seroconverters only), 2 and 3 in Figures 3.1.1A and 3.1.1B).

Logistic regression was used to evaluate the associations between spontaneous clearance of HCV and sociodemographic variables at the first HCV antibody-positive visit (sex, age at infection, ethnicity, calendar year of infection); drug use related variables (recent injecting drug use, frequency of injecting, main type of drug injected, frequency of non-injecting drug use, main type of non-injected drug, daily methadone dose, alcohol use, having a steady sexual partner that injects drugs); ongoing drug use [i.e., continuing drug use after HCV seroconversion], standard collected data on clinical symptoms (jaundice, severe tiredness, fever, night-sweating, diarrhea); HCV characteristics at first visit after seroconversion or study entry (HCV genotype, HCV viral load), co-infections (HIV co-infection, CD4 and CD8 count, HBV co-infection) (see Figure 3.1.1, associations were assessed using the first HCV seropositive visit for seroconverters and for recent prevalent cases (t=1)). All questions regarding (injecting) drug use and clinical symptoms refer to the six months preceding ACS entry or the period since the last visit. Multivariate logistic regression models were built using backward stepwise techniques. All variables with a p-value ≤0.20 in univariate analysis were considered for entry into the model. Statistical analysis was performed by use of STATA (version 9.2; StataCorp) and SPSS (version 15.0; SPSS Inc.) software. All statistical tests used were two-sided; a p-value ≤0.05 was considered to be statistically significant. Interaction and confounding were checked between the variables in the final models and all variables with a univariate p-value ≤0.10.

Using Poisson regression, we also examined whether the incidence of clinical symptoms was higher for visits of injecting DU who cleared HCV shortly after acute infection compared with those who did not. Regarding the prospective group, we used the last visit before HCV seroconversion and the first three visits following HCV seroconversion for these analyses. Since DU could contribute more visits and events, Generalized Estimating Equations (GEE) was used to correct for repeated measurements.

In a sensitivity analysis, analyses were repeated using only the prospectively identified acute HCV cases who had a small seroconversion interval (i.e., no more than six months between last HCV antibody-negative visit and first HCV antibody-positive visit) (n=43).
Figure 3.1.1  Schematic representation of two types of HCV seroconverters. A) prospectively identified HCV seroconverters and B) recent HCV-seropositive participants at entry in the Amsterdam Cohort Study among DU who were included in the study within two years after starting injection drug use.

Results

General characteristics

Sufficient follow up and serum were available to assess outcome of acute HCV infection for 55 out of 59 DU with documented HCV seroconversion (based on HCV antibody-negative visit followed by HCV antibody-positive visit) and for 51 of 58 DU who were HCV antibody-positive at entry in ACS, and started injection drug use within two years before entry. The median interval between last negative and first positive visit was 4.0 months (IQR 3.7-5.0 months) for the 55 HCV seroconverters. The median duration of injection drug use before study entry for DU with recent HCV was 1.12 years (interquartile range (IQR) 0.33-1.50 years). Of all 106 participants, 41.5% were female, and the majority was of west-European ethnicity (84.9%). The median age at HCV infection was 28.7 years (25.6-34.8 years). Of 106 participants, 93 (87.7%) reported recent injecting drug use, of whom 50.5% reported daily injecting and 34.6% reported recent sharing of needles. The median follow up time after HCV seroconversion was 9.4 years (IQR 3.9-13.4 years).

Of those that were HCV-RNA positive at the first available visit after seroconversion or ACS entry (n=72), 37.5% had genotype 1, 33.3% genotype 3, 8.3% genotype 2, and 6.9% genotype 4. For all samples in which HCV genotype could not be determined, HCV viral load was <1,000 IU/ml (n=10). The median log viral load (IQR) did not differ significantly among genotypes (p=0.25, Kruskal-Wallis) being 4.40 (3.38-5.42), 5.73 (4.64-6.14), 4.36 (3.19-5.30) and 4.69 (3.00-5.66) for genotypes 1, 2, 3, and 4, respectively.
Rate and determinants of spontaneous viral clearance

According to our definition of two consecutive HCV-RNA negative test results shortly after HCV seroconversion, the infection was spontaneously cleared in 35 of the 106 DU (33.0%, 95% CI 24.2-42.8%).

Sociodemographics and behaviour

In univariate analysis, women had threefold higher odds of spontaneous viral clearance. Of 38 HIV-negative women, 50.0% cleared HCV spontaneously, in contrast to only 25.5% HIV-negative men (14/55). Having a steady partner, injecting or non-injecting, was also significantly associated with higher odds of HCV clearance compared to not having a steady partner (twofold and threefold higher odds, respectively). After considering all variables with a univariate p-value ≤0.20 in a backward stepwise multivariate analysis, only female sex was an independent predictor of spontaneous viral clearance (OR 3.13, 95% CI 1.35-7.25).

In a sensitivity analysis including only prospectively identified HCV seroconverters with a seroconversion interval ≤6 months (n=43), the rate of spontaneous clearance was 38.6% (95% CI 24.4-54.5%). HCV clearance was still significantly associated with female sex (OR 5.43, 95% CI 1.42-20.7).

Clinical symptoms

Of all HCV cases, 36.8% reported at least one of the clinical symptoms (jaundice, severe tiredness, fever, night-sweating, diarrhoea) in the 4-6 months preceding the first HCV antibody-positive visit, but none of the examined symptoms were significantly associated with HCV viral clearance in univariate analysis (Table 3.1.1). Although fever was non-significantly associated with viral clearance, those who reported fever were more likely to clear HCV than those who did not (OR 2.29, 95% CI 0.73-7.13). This association was independent of female sex (after adjustment for gender aOR 2.40, 95% CI 0.73-7.86).

Table 3.1.1 Univariate analysis of factors associated with HCV clearance in a cohort of 106 individuals with acute HCV acquired through injection drug use.

|                      | N  | Clearance rate (%) | OR      | P value |
|----------------------|----|--------------------|---------|---------|
| Sociodemographics    |    |                    |         |         |
| Age (per 10 year increase) | 106  | 33.0               | 0.62 (0.32-1.18) | 0.13 |
| Sex                  |    |                    |         |         |
| Male                 | 62  | 22.6               | 1       |         |
| Female               | 44  | 47.7               | 3.13 (1.35-7.25) | 0.0068 |
| Ethnicity            |    |                    |         |         |
| Western European     | 90  | 31.1               | 1       |         |
| Non-Western European | 16  | 43.8               | 1.72 (0.58-5.09) | 0.33 |
| Calendar year of infection |    |                    |         | 0.67    |
| ≤1988                | 36  | 33.3               | 1       |         |
| 1989-1991            | 27  | 37.0               | 1.18 (0.41-3.34) |         |
| 1992-1994            | 22  | 22.7               | 0.59 (0.17-1.98) |         |
| ≥1995                | 21  | 38.1               | 1.23 (0.40-3.77) |         |
### Natural history

#### Clinical symptoms

| Symptom        | No | Yes | Unknown | Clear (rate %) | OR (95% CI)      | P value |
|----------------|----|-----|---------|---------------|-----------------|---------|
| Jaundice       | 32 | 9   | 65      | 32.3          | 1.10 (0.23-5.31)| 0.97    |
| Severe tiredness| No | 26  | 77      | 30.8          | 1.22 (0.47-3.16)| 0.69    |
| Fever          | No | 92  | 14      | 30.4          | 2.29 (0.73-7.13)| 0.16    |
| Night-sweating | Yes| 26  | 77      | 30.8          | 0.82 (0.32-2.14)| 0.69    |

#### HCV-related

| Variable                                    | N  | Clear (rate %) | OR (95% CI)      | P value |
|---------------------------------------------|----|---------------|-----------------|---------|
| HCV genotype (among those with detectable viral load) |    |               |                 | 0.41    |
| 1                                           | 26 | 7.69          | 1               |         |
| 2                                           | 6  | 0             |                 |         |
| 3                                           | 23 | 13.3          | 1.71 (0.26-11.3)|         |
| 4                                           | 5  | 20.0          | 3.00 (0.22-41.4)|         |
| Untypable (due to low viral load)            | 10 | 30.0          | 5.15 (0.71-37.2)|         |
| Log HCV viral load (among those with detectable viral load at the first HCV antibody positive visit) |    |               |                 | 0.071   |
| <3                                          | 18 | 27.8          | 9.23 (0.97-87.6)|         |
| 3-4.9                                       | 25 | 4.00          | 1               |         |
| >4.9                                        | 29 | 10.3          | 2.77 (0.27-28.5)|         |
| HIV-1                                        |    |               |                 | 0.13    |
| Absence of HIV-1 antibodies                  | 93 | 35.5          | 3.03 (0.63-14.5)|         |
| Presence of HIV-1 antibodies                 | 13 | 15.4          | 1               |         |
| Hepatitis B co-infection                     |    |               |                 | 0.68    |
| Anti-HBc-negative                            | 49 | 32.7          | 1               |         |
| Anti-HBc-positive, HBsAg-negative            | 36 | 30.6          | 0.91 (0.36-2.29)|         |
| Anti-HBc-positive, HBsAg-positive            | 19 | 42.1          | 1.50 (0.50-4.46)|         |
| Having a steady partner that injects drugs   |    |               |                 | 0.033   |
| No steady partner                           | 66 | 24.2          | 1               |         |
| Steady partner who injected drugs now or ever| 14 | 42.9          | 2.30 (0.69-7.62)|         |
| Steady partner who never injected drugs      | 25 | 52.0          | 3.06 (1.18-7.95)|         |
| Alcohol use (any daily)                      |    |               |                 | 0.30    |
| No                                          | 44 | 38.6          | 1               |         |
| Yes                                         | 62 | 29.0          | 0.65 (0.29-1.47)|         |
| Injecting drug use in the previous 6 months  |    |               |                 | 0.66    |
| Yes                                         | 93 | 32.3          | 1               |         |
| No                                          | 13 | 38.5          | 1.31 (0.40-4.35)|         |
| Continuation of injection drug use after HCV seroconversion (i.e., injecting drug use at first and second HCV antibody positive visit) |    |               |                 | 0.77    |
| No                                          | 32 | 34.4          | 1               |         |
| Yes                                         | 73 | 31.5          | 0.88 (0.36-2.12)|         |
To further evaluate the association between viral clearance and clinical symptoms that might have occurred around the time of HCV seroconversion, we determined the incidence rate ratios of clinical symptoms on different visits around and following HCV seroconversion (see methods section). The incidence rate of each of symptom did not significantly differ between those individuals who spontaneously cleared HCV and those individuals who developed chronic infection (data not shown).

**Co-infections**

At the time of HCV seroconversion (or ACS entry in those already HCV-positive), 13 DU were HIV-co-infected. In univariate analysis, spontaneous HCV clearance was more likely in HIV-negative individuals than in HIV-positive individuals (OR 3.03, 95% CI 0.63-14.5), although the difference did not reach statistical significance. The effect of HIV co-infection was independent of female sex (adjusted OR 3.48, 95% CI 0.70-17.4). CD4 and CD8 T cell counts were available for 51 of the 106 HCV seroconverters (range 230-2510 CD4 T cells/ml and 20-170 CD8 T cells, respectively), and only 1 DU had a CD4 count below 350 cells/ml. The median CD4 and CD8 count in those who cleared HCV was not higher than in those who developed chronic HCV infection (935 (IQR 710-1180) and 990 (IQR 770-1180) CD4 T cells/ml, and 60 (IQR 45-85) and 70 (IQR 40-70) CD8 T cells/ml, respectively). Although of borderline significance, HIV-negative women tended to have higher CD4 counts than HIV-negative men (median 1070 and 930 CD4 cells/ml, respectively (p=0.071)). For those with detectable HCV viral load at the first available sample after ACS entry or HCV seroconversion and who developed persistent viremia, HCV viral load was somewhat lower in women than in men, but this was not statistically significant (median log 3.43 (IQR 3.0-4.70) and 4.36 (3.0-5.43), respectively (p=0.14)).

Of all patients with acute HCV, 36 had evidence of cleared HBV infection, (i.e., they were anti-HBc-positive and HBsAg-negative), and 19 had an active HBV infection (i.e., were HBsAg-positive and anti-HBc-positive). In univariate analysis, those with active HBV infection were more likely to clear HCV spontaneously (OR 1.50, 95% CI 0.50-4.46) than those with cleared HBV infection (OR 0.91, 95% CI 0.36-2.29), or those never exposed (reference), although this effect did not reach statistical significance.

**Discussion**

To our knowledge, this is the largest longitudinal study to present data on factors associated with HCV viral clearance in individuals with drug-use-related acute HCV infection. The clearance rate and factors influencing clearance were assessed in a prospective cohort of retrospectively identified DU with acute HCV, regardless of their clinical presentation at the time of acute HCV infection. The rate of spontaneous HCV clearance was 33.0%.

Women were more likely to clear HCV spontaneously than men (OR 3.13, 95% CI 1.35-7.25), in line with other studies. We believe that, the differential expression of steroid hormones is a likely explanation, particularly since the HCV clearance rate does not differ between children of opposite sex. Interestingly, it seems that steroid hormones indeed influence the immune system, since women have higher CD4 and CD8 counts than men, independent of HIV status. In addition, typically women have
lower HIV loads than men.\textsuperscript{23} To date, however there are no indications that this difference is clinically relevant.

To address possible study limitations, the rate of clearance we found is higher than observed in studies among acute clinical cases (reviewed by Micallef in ref. 24), but may still be underestimated in injecting DU.\textsuperscript{24} Many injecting DU experience repeated exposure to HCV and HCV re-infection after their initial HCV seroconversion, due to continuing risk behavior (i.e., injecting drug use and borrowing of needles/syringes).\textsuperscript{17} Such re-infection after clearance might result in persistent HCV viremia, leading to an underestimation of the clearance rate. Especially in the presence of acute HIV co-infection, persistence of HCV re-infection is likely. However, in this cohort of DU with acute HCV, we did not find an association between ongoing risk behaviour and viral clearance shortly after the initial HCV infection.

HIV infection has been associated with loss of viral control of HCV, as evidenced by a higher HCV viral load in HIV co-infected individuals.\textsuperscript{25} However, since HCV usually precedes or coincides with HIV infection in DU, almost all HIV co-infected DU in our study retained high CD4 counts at the point of HCV infection and HCV viral load was not significantly different between co-infected and mono-infected individuals. Although the effect we found was not significant, its direction suggests that HCV clearance might be less likely in HIV co-infected individuals. In line with other studies, we have shown in a subgroup of injecting DU that acute HIV co-infection hampers the especially beneficial HCV-specific CD4 T-cell responses targeting nonstructural proteins.\textsuperscript{26-31}

Patients with spontaneous viral clearance of chronic HCV after HBV-superinfection have been described,\textsuperscript{32} and cross-sectional studies have shown that HBsAg-positive HIV-infected individuals are more likely to be HCV-RNA-negative than HBsAg-negative HIV-infected individuals.\textsuperscript{33-35} Although our population-based sample was larger than samples in other studies on viral clearance after acute HCV infection, it was too small to demonstrate a significant effect. A larger group of individuals with acute HCV is needed to precisely evaluate the effect of HBV and HIV co-infection on HCV clearance.

Having defined viral clearance as two consecutive HCV-RNA-negative visits after HCV seroconversion, we defined HCV viral persistence as the presence of HCV RNA at one or two of these visits, regardless of HCV strain present. Therefore, we did not distinguish between viral persistence of one strain and superinfection by another. Furthermore, although HCV clearance is believed to take place within the first six months after acute infection, evidence shows that clearance might take much longer.\textsuperscript{13, 36} Since we regularly measured HCV RNA only in the first two years after HCV infection, we recognize that measurements of longer duration would be necessary to evaluate possible late clearance and its predictors. In our cohort, only 5 out of 71 (7.0\%) individuals who did not clear HCV spontaneously within the first three visits after HCV seroconversion were HCV-RNA-negative at the last study visit before November 2005 or the penultimate visit preceding death, indicating that late clearance might occur (data not shown).

In contrast to other studies, age at the time of HCV infection was not associated with the rate of HCV clearance, possibly because the age distribution of our study population is relatively homogeneous. We also cannot distinguish whether inoculum size or route of transmission influences the rate of HCV clearance, since all but one DU in this study were injecting DU.

Others have suggested that individuals presenting with clinical symptoms after exposure to HCV after needle-stick injury or presenting at an outpatient clinic are more likely to spontaneously resolve acute HCV infection,\textsuperscript{5,37} but self-reported clinical symptoms were not associated with HCV viral clearance in this cohort of DU. A possibility is that DU are
Female sex predicts spontaneous HCV clearance

more likely to have aspecific physical symptoms than the general population, due to co-morbidity and a different standard of hygiene, and that these symptoms impede their ability to detect changed symptoms associated with viral clearance. In conclusion, behavioural factors and also clinical symptoms measured around and shortly after HCV seroconversion seem unable to discriminate between individuals who will resolve HCV and those that will develop persistent viremia, at least not in this cohort of DU. Since female sex is the only strong predictor of spontaneous viral clearance, the decision to start HCV treatment might be postponed in HIV-negative women with acute HCV. On the other hand, since HCV treatment in the acute phase is superior to treatment in the chronic phase, and outcome is less favourable in HIV-co-infected individuals, HCV treatment should not be postponed in individuals and groups who are HIV-infected or at high risk of subsequent HIV co-infection.38-40
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Chapter 3.2

Increased risk of hepatitis related death among HCV/HIV co-infected drug users compared to mono HCV infected drug users, a 20-year prospective study

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*J Acquir Immune Defic Syndr* 2008;47:221-225.
Abstract

Background
Progression of liver related disease is accelerated in individuals co-infected with HIV and hepatitis C virus (HCV). Because the life expectancy of HIV-infected drug users (DU) improved after the widespread use of highly active antiretroviral therapy (HAART), HCV related death is likely to become more important. To disentangle the effects of HCV and HIV, we compared the overall and cause-specific mortality between HCV/HIV-infected DU and HCV-infected DU and DU without HCV or HIV, followed up between 1985-2006.

Methods
A total of 1295 participants in the Amsterdam Cohort Study were included. Cause-specific hazard ratios (CHR) were estimated for the eras before (<1997) and since HAART (≥1997) within and among serologic groups.

Results
The risk of dying decreased for most causes of death (COD) ≥1997, this decrease was not the same for the different serologic groups. Among HCV/HIV co-infected DU, the risk of hepatitis/liver-related death did not substantially change over time (CHR: 0.87, 95% CI:0.21-3.58), whereas the risk of AIDS-related mortality decreased. Compared with DU solely infected with HCV, HCV/HIV co-infected DU were at increased risk of dying from hepatitis/liver-related disease (CHR: 7.15, 95% CI: 1.98-25.8), other natural causes (CHR: 3.09, 95% CI: 1.41-6.79), and non-natural causes (CHR: 2.30, 95% CI: 1.07-4.95) in the HAART era.

Conclusions
HCV/HIV co-infected DU remain at increased risk of dying from hepatitis/liver-related death in the HAART-era compared to HCV mono-infected DU. This risk did not change in HCV/HIV co-infected DU after HAART was introduced, suggesting that in the HAART era, HIV continues to accelerate HCV disease progression. Efforts should be made to establish effective treatment for HCV infection in HCV/HIV co-infected individuals.
Introduction

The progression of liver disease associated with hepatitis C (HCV) is known to be accelerated in HIV-infected persons.\(^1\) In the mid-1990s, highly active anti-retroviral therapy (HAART) became available and improved their survival. In the HAART era, mortality from HCV is therefore likely to take on a greater significance among these individuals,\(^2\) and several studies have indeed shown an increase in liver-related deaths.\(^3,4\) Drug users (DU) with HIV also benefit from HAART at the population level, although their improvement is less than observed for heterosexual and homosexual individuals.\(^7,8\) Because HIV-infected DU are almost universally co-infected with HCV, HCV/HIV co-infection has a major impact on their mortality.\(^9\) Indeed, an earlier study showed that the risk of dying from hepatitis- and liver-related disease among HIV-infected DU is increased in the HAART era compared with the pre-HAART era.\(^10\) The Amsterdam Cohort Study (ACS) among DU includes non-HIV-infected DU and HIV-infected DU, with or without HCV co-infection; information on causes of death is available, providing the unique opportunity to disentangle the effects of HIV and HCV on cause-specific mortality in DU with one or both viruses. In addition, their mortality can be compared with that of uninfected DU who are nevertheless at increased risk of dying compared to the general population. In the present study, we therefore compared the cause-specific mortality between HCV/HIV co-infected DU, HCV mono-infected DU and DU without HCV or HIV. They were followed between 1985 to 2006, to compare the pre-HAART and HAART-era.

Methods

Study population

The prospective ACS among drug users began in December 1985 and is still ongoing,\(^11\) with 1640 DU included as of 1 January 2006. Recruitment is by means of local methadone outposts, sexually transmitted diseases clinics, and word of mouth. Both injecting and noninjecting DU, using hard drugs (i.e., heroin, cocaine, and/or methadone) at least three times per week, are invited to participate. DU return for their ACS follow up visit every four to six months at the Health Service of Amsterdam. At each visit, a standardized questionnaire is administered by trained nurses and blood is drawn for laboratory testing and storage. HIV-positive DU undergo a clinical examination by a physician. The ACS has been conducted in accordance with the ethical principles set out in the declaration of Helsinki and written informed consent is obtained before data collection.

Serological testing

After each ACS visit, blood samples are prospectively tested for HIV antibodies by enzyme-linked immunosorbent assay (ELISA), and positive results are confirmed using Western blot (since 1995: HIV Blot version 2.2, Genelab diagnostics). For HIV-infected DU, CD4 cell counts and HIV RNA plasma levels are determined. In the present study, stored samples from DU with at least two cohort visits were retrospectively tested for HCV antibodies, starting with the blood samples collected at the first cohort visit in each
case. A third generation ELISA assay was used to detect HCV antibodies (AxSYM HCV version 3.0; Abbott, Wiesbaden, Germany). DU who were HCV-negative at their first cohort visit were tested for HCV-antibodies at their last cohort visit. If this blood sample was HCV positive, samples taken in between the first and last visit were tested to identify the approximate moment of seroconversion. All blood samples collected at the first visit were retrospectively tested for hepatitis B core antigen (HBc) antibodies.

Specific causes of death

Information about vital status was obtained by matching the ACS data against the local and national registries. To obtain information on the cause of death (COD), we reviewed medical records from the hospitals, methadone clinics, and general practitioners. Causes were grouped into five categories: AIDS/HIV related, liver-related (including HCV- and hepatitis B-related death, and liver disease) and non-natural causes of death (including: overdose, accidents, suicide and homicide), other natural causes of death and unknown. When more than one cause of death was recorded, the most likely cause was scored according to the following hierarchy: non-natural as most likely, followed by AIDS/HIV related, HCV/liver related and natural.

Statistical analyses

Of the 1640 DU participating in the ACS, 1295 had at least two cohort visits and were included in this study. Follow up was calculated from ACS entry until the earliest of the following: death, one year after the last visit, or censoring date 1 January 2006. Using calendar time as a proxy for the introduction and widespread use of HAART, we defined two calendar periods: before 1997 and 1997 onwards, to reflect the pre-HAART and HAART eras, respectively.

Four serologic groups were defined: (1) HCV-positive/HIV-positive, (2) HCV-positive/HIV-negative, (3) HCV-negative/HIV-negative and (4) HCV-negative/HIV-positive. Individuals could switch between groups (time updated covariate) when they acquired an infection during follow up.

The date of HIV or HCV seroconversion was estimated as the midpoint between the last seronegative and first seropositive ACS visit.

Using the Kaplan Meier method, we estimated the time from ACS entry to death by any cause for each serologic group. Cause-specific hazard ratios (CHR) were estimated within and between serologic groups using a Cox proportional hazards model. All analyses were adjusted for age, sex, hepatitis B status at ACS entry, and duration of injecting. The confounding effect of current injecting, alcohol intake and homelessness was also evaluated. All variables subject to change, such as age, alcohol intake, duration of injection and current injecting, were treated as time-updated variables. The number of individuals (n=17) and deaths (n=5) for the HCV-negative/HIV-positive serologic group was too small to estimate any CHR. Also in the HCV-/HIV- group the number of deaths for some specific COD was too small to estimate the CHR for these COD. Finally, a sensitivity analysis was conducted by excluding those DU who had never injected drugs.
Results

Baseline characteristics of the DU are presented in Table 3.2.1. The median age of the 1295 DU with at least two cohort visits was 30 years, and 64% were male. At baseline, 621 DU (72%) had ever injected drugs, and 31 noninjecting DU started to inject during follow up. At ACS entry, 20% had an HCV/HIV co-infection, 44% were mono-infected with HCV, 36% of the DU were not infected with HIV or HCV, and 1% were solely infected with HIV. During follow up, 95 HIV and 59 HCV seroconversions occurred, and 272 DU died. A specific COD was available for 252 death cases.

HCV/HIV co-infected DU and HCV mono-infected DU were more often from Dutch origin, had higher anti-HBc prevalence rates, and had more often a history of injecting drugs. Less than 1% of the HCV-infected patients received HCV treatment.

Table 3.2.1 Baseline and follow up characteristics among drug users with at least two cohort visits in the Amsterdam Cohort Study, 1985-2006.

|                   | Total | HCV+/HIV+ | HCV+/HIV- | HCV-/HIV- | HCV-/HIV+ |
|-------------------|-------|-----------|-----------|-----------|-----------|
| **Baseline:**     |       |           |           |           |           |
| Number (%)        | 1295 (100) | 256 (20) | 565 (44) | 457 (36) | 17 (1)    |
| Men (%)           | 828 (64) | 156 (61) | 340 (60) | 318 (70) | 14 (82)   |
| Age (median, IQR) | 30 (26-36) | 32 (28-36) | 31 (27-36) | 29 (26-35) | 28 (26-33) |
| Dutch nationality | 973 (76) | 206 (80) | 482 (85) | 274 (60) | 11 (64)   |
| Anti-HBc antibodies | 663 (51) | 213 (83) | 359 (64) | 85 (19) | 6 (35)    |
| Ever-injecting    | 921 (72) | 250 (98) | 535 (95) | 127 (28) | 9 (53)    |
| Time since first injecting (median, IQR) | 8 (4-13) | 10 (6-14) | 8 (4-14) | 3 (0-8) | 7 (4-12)  |
| Homeless          | 123 (10) | 16 (6) | 46 (8) | 58 (13) | 3 (18)    |
| Current daily alcohol intake (drinks/day): | | | | | |
| 0                 | 428 (33) | 85 (33) | 186 (33) | 148 (32) | 9 (53)    |
| 1-4               | 153 (12) | 19 (7) | 58 (10) | 76 (17) | 0 (0)     |
| >4                | 128 (10) | 20 (8) | 58 (10) | 48 (11) | 2 (12)    |
| missing           | 586 (45) | 132 (52) | 263 (47) | 185 (41) | 6 (35)    |
| CD4 cell counts at entry* (cells/l) (median, IQR) | 410 (290-700) | | | | 600 (420-710) |
| Follow up:       |       |           |           |           |           |
| Follow up time (median, IQR) | 7 (4-13) | 9 (5-14) | 9 (5-14) | 6 (3-11) | 7 (4-12)  |
| Total deaths      | 272 | 172 | 70 | 24 | 6    |
| Causes of death: (%) | | | | | |
| AIDS related      | 75 (28) | 70 (40) | 0 | 0 | 5 (83) |
| Hepatitis related | 21 (28) | 18 (10) | 3 (4) | 0 | 0 |
| Non-natural       | 86 (32) | 37 (22) | 39 (56) | 10 (42) | 0 |
| Natural           | 70 (26) | 36 (22) | 20 (29) | 13 (54) | 1 (17) |
| Unknown           | 20 (7) | 11 (6) | 8 (11) | 1 (0.4) | 0 |
| Seroconversions:  |       |           |           |           |           |
| HCV               | 59 | 15 | 44 | | |
| HIV               | 95 | 89 | | | 6 |

* only available for the HIV infected participants , IQR: interquartile range
Overall mortality

The all-cause mortality was highest among DU infected with both HCV and HIV: after 10 years of follow up, 49% (95% confidence interval (CI): 42-54) had died (Figure 3.2.1). HIV mono-infected DU show a slightly lower death rate: 10 years after ACS entry 43% (95% CI: 2-66) had died. All-cause mortality was lowest among DU without HIV or HCV infections and among those with mono HCV infection 7% (95% CI: 3-11) and 13% (95% CI: 10-16) had died, respectively, after 10 years of follow up.

Risk of dying

We compared the risk of dying from each specific COD in the pre-HAART era with the HAART era. Overall, the risk of dying decreased in the HAART era for almost all COD, but the effect of calendar time was not the same for each serologic group. Therefore the CHRs and their 95% CI are shown for the serologic groups separately in Table 3.2.2. Within each group the risk of dying in the HAART era is compared to that in the pre-HAART era. Because we wanted to know the impact of HCV infection on mortality, we likewise compared separately the risk of dying from specific causes among the co-infected and uninfected DU (HCV-positive/HIV-positive and HCV-negative/HIV-negative) with the risk among HCV mono-infected DU (reference) for the pre-HAART era and HAART era.
Liver-related mortality in HCV and HCV/HIV infected drug users

Table 3.2.2 The adjusted* cause-specific hazards (CHR) and their 95% confidence intervals for each cause of death. CHR are estimated within each serologic group and in the pre-HAART and HAART era separately, compared to the risk of dying among HCV+/HIV-infected drug users.

|                      | AIDS/HIV related causes | Hepatitis/liver related causes | Non-natural causes | Natural causes | Unknown |
|----------------------|-------------------------|--------------------------------|--------------------|---------------|---------|
| **Within serologic groups (HAART-era vs. pre-HAART era)** |                         |                                |                    |               |         |
| HCV+/HIV-            |                         |                                |                    |               |         |
| Pre HAART            | *                       | 1                              | 1                  | *             |         |
| HAART-era            | 0.77                    | 0.55                           | (0.37-1.63)        | (0.15-1.99)   |         |
| HCV+/HIV+            |                         |                                |                    |               |         |
| Pre HAART            | 0.37                    | 0.87                           | 0.57               | 0.90          | 5.65    |
| HAART-era            | (0.19-0.72)             | (0.21-3.58)                     | (0.22-1.47)        | (0.31-2.67)   | (1.42-22.5)|
| HCV-/HIV-            |                         |                                |                    |               |         |
| Pre HAART            | *                       | 1                              | *                  |               |         |
| HAART-era            | 0.33                    |                                | (0.03-3.85)        |               |         |
| **Between serologic groups in the pre-HAART era** |                         |                                |                    |               |         |
| HCV+/HIV-            |                         |                                |                    |               |         |
| HCV+/HIV+            | *                       | 1                              | 1                  | 1             |         |
|                         |                         |                                | (1.22-7.58)        | (0.75-2.37)   | (0.27-6.91)|
| HCV-/HIV-            |                         |                                | *                  | 0.58          | *       |
|                         |                         |                                |                    | (0.13-2.60)   |         |
| **Between serologic groups in the HAART era** |                         |                                |                    |               |         |
| HCV+/HIV-            |                         |                                |                    |               |         |
| HCV+/HIV+            | 7.15                    | 3.09                           | 2.30               | 8.70          |         |
|                         | (1.98-25.8)             | (1.41-6.79)                     | (1.07-4.95)        | (2.89-26.42)  |         |
| HCV-/HIV-            | 1.11                    | 0.92                           | 1.22               |               |         |
|                         | (0.34-3.61)             | (0.25-3.43)                     | (0.12-11.68)       |               |         |

^ Adjusted for: age, sex, hepatitis B status at ACS entry and duration of injecting.
* Results not presented due to small numbers

Changes in the risk of death within serologic groups

In the HAART era compared to the pre-HAART era, in DU infected with both HCV and HIV, we observed a significant reduction in the risk of dying from AIDS-related death (CHR adjusted for age, sex, hepatitis B status at ACS entry and duration of injection: 0.37, 95% CI: 0.19-0.72). In this group the risk of dying from liver related death did not significantly change (CHR: 0.87, 95% CI: 0.21-3.58).

No significant reductions in the risk of dying for all COD were observed in HCV mono-infected and the uninfected serologic groups. The risk of dying specifically from hepatitis or liver-related death could not be estimated for DU solely infected with HCV because, no hepatitis/liver-related deaths were observed in the pre-HAART era.

Comparison of the risk of death among serologic groups

When comparing the risk of dying in HCV/HIV co-infected DU with the risk of dying among HCV mono-infected DU, those co-infected had a significantly higher risk of dying.
Chapter 3.2

Natural history

from nonnatural CODs (CHR: 3.03, 95% CI: 1.22-7.58) in the pre-HAART era. The same was true for the HAART era (CHR: 2.30, 95% CI: 1.07-4.95). In the HAART era, the co-infected DU had a significantly higher risk of dying from hepatitis/liver-related death than HCV mono-infected DU (CHR: 7.15, 95% CI: 1.98-25.8) and from natural CODs (CHR: 3.09, 95% CI: 1.41-6.79). No major differences were seen between DU without infections and HCV mono-infected DU, except that in the pre-HAART era, the noninfected DU had a nonsignificantly lower risk of dying from natural CODs (CHR: 0.58, 95% CI: 0.13-2.60).

Adjustment for homelessness, alcohol intake and current injecting did not affect the results. When including ever-injectors only (n=952) in a sensitivity analysis, we found that the risk of dying from hepatitis/liver-related disease within the HCV/HIV co-infected group was somewhat higher in the HAART era than in the pre-HAART era (CHR: 1.23, 95% CI: 0.18-8.26) and the effect was opposite when compared to the CHR in the total study population. The effect remained nonsignificant, however. In the HAART era, the increased risk of dying from hepatitis/liver-related disease was smaller than observed in the total population for co-infected DU compared to HCV mono-infected DU (CHR: 3.94, 95% CI: 0.59-26.22). The other CHRs were comparable to the results in the total population.

Discussion

This study describes the cause-specific mortality in a large group of DU over a 20-year period. The risk of dying was highest among DU solely infected with HIV or co-infected with HCV/HIV. Although the risk of dying substantially decreased for almost all causes in the HAART era, the decrease was not the same for all serologic groups. The risk of dying from hepatitis/liver-related disease did not change significantly over time among HCV/HIV co-infected DU, but this study demonstrates a strongly increased risk of their dying from hepatitis/liver-related disease compared to DU solely infected with HCV in the HAART era. This suggests that in the HAART-era, HIV co-infection continues to accelerate HCV disease progression.

One might argue that DU have not benefited from HAART; however, comparing the risk of dying among DU infected with HCV and HIV between the pre-HAART and HAART eras shows that the risk of dying from AIDS-related causes decreased over time. This is in line with other studies and indicates that DU indeed benefit from HAART, although their uptake of HAART is lower than seen in other HIV risk groups.7-10 Although several studies have shown an increase in liver-related mortality among HIV-infected individuals in the HAART era, the impact of HCV co-infection on HIV disease progression remains contradictory.11-17 When mortality was compared between DU infected with HCV and/or HIV and DU without an infection, a study found higher overall mortality rates among HCV/HIV co-infected DU versus non-HCV/HIV infected DU, but did not compare cause-specific mortality.9 In an earlier study, no liver-related deaths occurred among HCV mono-infected individuals, whereas 10% of the HCV/HIV co-infected DU developed liver decompensation.18 This study was analyzed cross-sectionally, however. In the present longitudinal ACS study, we had the unique opportunity to evaluate the effect of the time-updated HIV and HCV status of all DU on cause-specific mortality. In addition, we were able to correct for duration of injecting
drugs, which served as a proxy for duration of HCV infection since most DU get infected with HCV within two year after start injecting.\textsuperscript{19}

The results of this study show an increased risk of dying from hepatitis- and liver-related death in the era of HAART among those infected with both HIV and HCV compared with those solely infected with HCV. Theoretically, the increase could be explained by HBV infection, which was highest among those DU who were co-infected with HCV/HIV. We adjusted for anti-HBc status at study entry, however. This adjustment may be a limitation of the study, since an anti-HBc-positive test result is a marker for past HBV infection but not for chronic HBV infection. In the general population, 5-10% of the HBV infections become chronic, whereas a higher percentage become chronic in HIV-infected individuals.\textsuperscript{20} Therefore, we might have overestimated the effect of anti-HBc, but this overestimation would be smaller for those DU infected with HIV.

In this study, HCV treatment was not taken into account, but it occurred very sporadically and only recently in our cohort (1%) and would therefore only marginally affect our results.

The risk of dying from non natural causes (i.e. overdose, suicide, homicide and accidents) was increased in HCV/HIV co-infected DU compared to HCV mono-infected DU in both the pre-HAART and HAART eras, whereas no differences were seen between HCV mono-infected DU and DU without HCV and HIV. This finding suggests that HCV/HIV co-infected DU had been taking more risk in general with respect to drug use.

Although the impact of HCV co-infection on HIV disease progression is still debated, this study shows higher all-cause mortality among HCV/HIV co-infected DU than in the other serologic groups. When they are compared with HCV mono-infected DU, their risk of hepatitis- and liver-related death remains higher in the HAART era, suggesting that HIV continues to alter HCV disease progression. Although HCV treatment among HCV/HIV co-infected individuals is complicated, our results highlight its importance and the need to establish effective treatment for HCV in HCV/HIV co-infected individuals. We believe that, next to reducing risk behavior related to drug use, daily observed therapy for DU with HIV and/or HCV is likely to increase their uptake, adherence, and therapy success.
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Chapter 4

Immunology in acute HCV
Chapter 4.1

HCV-specific T-cell responses in injecting drug users: evidence for previous exposure to HCV and a role for CD4⁺ T cells focussing on nonstructural proteins in viral clearance

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*J Viral Hepat.* 2008;15:409-420.
Abstract

To understand parameters associated with resolved Hepatitis C Virus (HCV)-infection, we analyzed HCV-specific T-cell responses longitudinally in 13 injecting drug users (IDU) with a prospectively identified acute HCV infection. Seven IDU cleared HCV and six IDU remained chronically infected. T-cell responses were followed in the period needed to resolve and a comparable time span in chronic carriers. Ex-vivo T-cell responses were measured using IFN-γ Elispot assays after stimulation with overlapping peptide pools spanning the complete HCV genome. CD4+ memory T-cell responses were determined after 12-day stimulation with HCV proteins. The maximum response was compared between individuals.

The T-cell responses measured directly ex vivo were weak but significantly higher in resolvers compared to chronic carriers, whereas the CD4+ memory T-cell response was not different between resolvers and chronic carriers. However, HCV Core protein was targeted more often in chronic carriers compared to individuals resolving HCV infection. CD4+ T-cell responses predominantly targeting non-structural proteins were associated with resolved HCV-infection. Interestingly, the observation of memory T-cell responses present before documented HCV seroconversion suggest that reinfection in IDU occurs often, while the presence of these responses were not predictive for the outcome of infection. However, a transition of the HCV-specific CD4+ memory T-cell response from targeting Core to targeting non-structural proteins during onset of infection was associated with a favourable outcome. Therefore, the specificity of the CD4+ memory T-cell responses measured after 12-day expansion seems most predictive of resolved infection.
Introduction

Hepatitis C virus (HCV) is a positive-stranded RNA virus which is mainly transmitted through blood-contact. HCV infection persists in the majority of individuals resulting in liver fibrosis, cirrhosis and/or hepatocellular carcinoma. Acute HCV infection, however, is difficult to identify because it is usually asymptomatic and most infections occur among injection drug users (IDU).

Studies in humans have shown that spontaneous clearance of acute HCV infection is associated with strong and sustained CD4+ and CD8+ T-cell responses against several HCV-derived antigens. The mechanism by which the immune response fails to control the virus remains, however, unclear. Because identification of acute HCV infection is rare, only in a limited number of persons a longitudinal follow up of HCV-specific T-cell responses from the early phase of HCV infection has been reported. Most of these responses have been studied using a limited number of epitopes, mostly restricted by the HLA A2 haplotype. It is likely that this approach underestimates the complete T-cell immune response.

To study why some HCV-infected IDU fail to clear the virus, we analysed the HCV-specific immune response between IDU with a prospectively identified acute HCV infection who eventually resolved HCV or remained chronic. We followed ex vivo T-cell responses using HCV overlapping peptide pools covering the complete HCV genome. In addition, we measured memory CD4+ T-cell responses after stimulation with HCV proteins, using a sensitive and reproducible expansion assay, in the period needed to resolve and a comparable time span in eventual chronic carriers.

Methods

Study subjects

Study subjects were recruited from the Amsterdam Cohort Studies (ACS) among drug users, an open, ongoing cohort study that started in December 1985 to study the epidemiology of HIV/AIDS and other blood borne or sexually transmitted diseases, which was carried out in accordance with the Helsinki declaration and approved by the institutional Review Board. Participants visit the Amsterdam Health Service every 4-6 months to fill in a detailed questionnaire on injecting drug use and other risk behaviour. In addition, blood is drawn for prospective HIV-testing and storage of PBMC. By screening for HCV antibodies in stored serum, Beld et al. retrospectively established the HCV status of 358 drug users included between December 1985 and March 1996 and identified 19 HCV seroconverters among those at risk. Four were HIV positive before HCV seroconversion and two experienced an acute HIV infection at the time of HCV infection. Seven of 19 (39%) HCV seroconverters resolved HCV-infection. All HCV seroconverters were studied longitudinally for the presence of HCV RNA. Conversion from a negative to positive HCV-RNA test could be documented in 18 of them making it possible to estimate the date of infection (EDI). Thirteen HIV-negative acute HCV infected out of 18 HCV-RNA converters were selected for our study (Table 4.1.1).
Table 4.1.1  Clinical and laboratory characteristics.

| Subject (pubID) | Subject study ID | Age at EDI | Sex | Total follow up | Follow up since EDI | Interval of HCV-RNA conversion | HCV genotype | Resolving phase (months) | Resolving phase (months) | HLA-class I A/B alleles |
|-----------------|------------------|------------|-----|-----------------|---------------------|-------------------------------|--------------|--------------------------|--------------------------|------------------------|
| 19854           | R1               | 38 y       | M   | 171 mo          | 146 mo              | 3.5 mo                       | 3a           | 14.6                     | A3, 25                   | B27, 51                 |
| 18915           | R2               | 37 y       | F   | 110 mo          | 15 mo               | 4 mo                         | 1a           | 4.2                      | A2                       | B44, 62                 |
| 16991           | R3               | 26 y       | F   | 137 mo          | 41 mo               | 4 mo                         | 3a           | 4.0                      | A2, 3                    | B7, 60                  |
| 16994           | R4               | 29 y       | F   | 56 mo           | 46 mo               | 4 mo                         | 1a           | 6.0                      | A24, 34                  | B49, 53                 |
| 18885           | R5               | 24 y       | M   | 182 mo          | 171 mo              | 4 mo                         | 1a           | 4.8                      | A26, 66                  | B27, 41                 |
| 18787           | R6               | 26 y       | M   | 119 mo          | 108 mo              | 5 mo                         | 3a           | 24.2                     | A30, 68                  | B18, 62                 |
| 12905           | R7               | 28 y       | M   | 183 mo          | 142 mo              | 3.5 mo                       | 1a           | 3.6                      | A3, 29                   | B44, 55                 |
| 18898           | C1               | 34 y       | F   | 177 mo          | 154 mo              | 3 mo                         | 1a           | chronic                  | A1, 28                   | B7, 27                  |
| 18866           | C2               | 20 y       | F   | 146 mo          | 139 mo              | 5 mo                         | 1a           | chronic                  | A2, 32                   | B14, 44                 |
| 18877           | C3               | 34 y       | M   | 165 mo          | 142 mo              | 3.5 mo                       | 3a           | chronic                  | A2, 28                   | B7, 8                   |
| 16941           | C4               | 25 y       | F   | 137 mo          | 99 mo               | 3.5 mo                       | 3a           | chronic                  | A24, 69                  | B7, 60                  |
| 12970           | C5               | 28 y       | F   | 153 mo          | 134 mo              | 39 mo                        | 3a           | chronic                  | A2, 23                   | B49, 53                 |
| 19927           | C6               | 27 y       | F   | 143 mo          | 100 mo              | 23 mo                        | 1a           | chronic                  | A2, 3                    | B7                      |

Immunology in acute HCV
Seven of these 13 subjects resolved acute HCV infection and six remained chronically infected. During follow up, two of seven resolvers became re-infected with HCV and HIV-1, after which they remained chronically HCV-HIV co-infected. As all 13 individuals with an acute HCV infection were identified retrospectively from the ACS, designed for prospective follow up of HIV-1 infection, no active examination of the occurrence of an acute hepatitis was done. Examination of the medical files did not show any indication of symptomatic acute HCV infection.

Definitions

HCV infection was defined as the conversion from a negative to a positive HCV-RNA test (bDNA HCV 3.0 Bayer, lower limit of detection 615 IU/ml or HCV-RNA assay by transcription-mediated amplification (TMA), Versant HCV-RNA Qualitative assay, Bayer, lower limit of detection 5 IU/ml), documented over two consecutive visits in combination with HCV seroconversion (presence of antibodies to HCV by 3rd-generation Enzyme Immunoassay, EIA 3.0 Abbot Laboratories).

Estimated date of infection (EDI) was defined as the midpoint between the last negative and first positive HCV-RNA test date. EDI was determined with a variation of \( \pm 2 \) months in 11 of 13 subjects. In two subjects, EDI had a variation of \( \pm 19 \) months and \( \pm 12 \) months respectively.

Date of HIV seroconversion was defined as the midpoint between the last negative and first positive HIV antibody test (commercial EIA, Abbot) and confirmed by Western blot (Diagnostic Biotechnology, Belgium)). HIV-RNA plasma concentration was determined by NASBA technology (lower quantification limit of \( 10^3 \)HIV-RNA copies/ml).

Resolved infection was defined as two consecutive visits with negative qualitative HCV-RNA assays after onset of HCV-infection.

The resolving phase was defined as the phase after EDI in which spontaneous clearance was possible and calculated as the time elapsed between EDI and the midpoint between the last positive and first negative HCV-RNA time point.

HCV genotyping

RNA was isolated using the TriPure method (Roche Diagnostics, Almere, the Netherlands) and subsequently amplified and genotyped using a nested RT-PCR based on the conserved Core region of the HCV genome as described by Ohno et al.22 Genotypes were confirmed by sequencing part of the NS5B region of the HCV genome.23

Peptides, peptide pools and proteins

As peptide pools for stimulation of both CD4+ and CD8+ T-cell responses, panels of overlapping peptides (provided by NIH Aids Research Reagent Program) spanning the complete HCV genome corresponding to the HCV 1a genotype (H77 sequence, Genbank access AF009606) with a length of 18 amino acids (overlapping adjacent peptides by 11 aa) derived from the following HCV proteins were used: Core/envelope polyprotein (Core, E1, E2, p7 protein, aa 1-805, consisting of 116 peptides), NS2 protein (aa 806-1022, consisting of 31 peptides), NS3 protease/helicase (aa 1023-1645, consisting of 50 peptides), NS4 protein (aa 1646-1967, consisting of 49 peptides), NS5A protein (aa 1968-2415, consisting of 67 peptides) and NS5B protein (aa 2416-3011, consisting of 87 peptides). Peptides were dissolved in DMSO and 1 µg of total peptide pool mix (each peptide present in a representative amount, i.e. in a concentration of
1 µg divided by the number of peptides in the pool) was used in stimulations. The DMSO concentration never exceeded 1% in the final stimulation. Expansion of CD4\(^+\) T cells was performed using the HCV proteins Core, NS3, NS4 and NS5 (provided by Chiron).

### PBMC separation and storage

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation on Ficoll-Hypaque and cryopreserved using a computerized freezing system in liquid nitrogen within 24 hours of collection.

### Elispot assay for single cell IFN-\(\gamma\)-release

IFN-\(\gamma\) producing antigen-specific T cells were enumerated using overnight IFN-\(\gamma\) specific Elispot assays as previously described using the anti-IFN-\(\gamma\) antibodies from Mabtech (Stockholm) and streptavidin poly-HRP from Sanquin (Amsterdam). PBMC were stimulated directly \emph{ex vivo} in triplicate wells at 1 x 10\(^5\) cells/well in the absence or presence of 1 µg/ml peptide pools. To provide us with the highest sensitivity, we have optimized our assay in such a way that the ratio background to specific response level is optimal at 100,000 cells input. Individual cytokine-producing cells were detected as dark purple spots after a reaction with TMB substrate (Sanquin, Amsterdam) and counted using the A.EL.VIS automated spot analyzer. The number of specific T cell responders per 10\(^6\) PBMC was calculated after subtracting two times negative control values, which leads to the highest specificity as validated in our lab. A response of 50 spots/10\(^6\) PBMC was regarded as positive (after subtraction of negative control values), based on values in healthy blood bank donors.

### Expansion of HCV-specific CD4\(^+\) T cells

As direct responses towards HCV proteins did not result in detectable responses, we used an expansion assay prior to measurement of effector function. To expand HCV-specific CD4\(^+\) T cells, 3x10\(^6\) PBMC were cultured for 12 days as previously described in the presence of the HCV proteins Core, NS3, NS4 and NS5. Culture medium consisted of RPMI 1640 (Gibco Life technologies, Breda, The Netherlands) supplemented with penicillin/ streptomycin and 10% human pool serum. Cells were cultured at 2x10\(^5\) PBMC/well in 100 µl medium in 96-well round bottom plates, at 37\(^\circ\)C and 5% CO\(_2\). Protein (2 µg/ml) was added on days 0 and 6. IL-2 was added at 10 U/ml on days 3, 6, and 9. On day 12, cells were pooled, washed, counted and rested overnight in complete medium. On day 13 cells were restimulated for six hours with overlapping peptide pools, corresponding to the HCV proteins used to expand T cells, to assess effector function (IFN-\(\gamma\) production).

### Detection of IFN-\(\gamma\)-producing HCV-specific T cells after re-stimulation

IFN-\(\gamma\) producing cells after re-stimulation with overlapping peptide pools were enumerated by intracellular cytokine staining (ICCS). As higher numbers of IFN-\(\gamma\) producing cells are expected after expansion than directly \emph{ex vivo}, ICCS was chosen for better visualization. Briefly, 10\(^6\) PBMC were stimulated for six hours with HCV Core/envelope (also including E1,E2 and p7 peptides), NS3, NS4 and NS5 peptide pools (at 2 µg/ml of each peptide) and both \(\alpha\)CD28 (2 µg/ml) and \(\alpha\)CD49d (1 µg/ml) as
co stimuli, in the presence of 1:1000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA). As a negative control, PBMC were stimulated with medium and co stimulation alone. As a positive control PBMC were stimulated with 10 ng/ml PMA and 2 μg/ml ionomycin. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD4, CD8 and IFN-γ (BD). After fixation (Cellfix, BD) 200,000 events were acquired on a FACSCalibur flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using the software program CELL Quest (BD). Reponses were scored as positive when twice above the medium control value and expressed as the percentage of IFN-γ producing CD4+ T cells.

**Calculation of the number of IFN-γ producing CD4+ T cells/10^6 PBMC input**

To enable comparison of donors and patients with different CD4+ T cell numbers, a more absolute number of HCV-specific CD4+ T cells was determined by calculating the number of HCV protein-specific IFN-γ-producing CD4+ T cells recovered out of 10^6 PBMC put into culture on day 0. This is the combination of the initial number of specific cells present and their ability to survive, proliferate and differentiate in vitro. To this end we counted the number of cells after culture (the ones that survived and proliferated) and calculated the number of IFN-γ producing T cells grown out as a function of the input (initial frequency of specific cells) using the following equation: (number of cells grown out/number of input cells x % IFN-γ) x 10,000 = number of cells per 10^6 input PBMC). As this calculation takes into account all variables potentially influencing the end result, it results in a more reliable number than just the percentage of responding cells after 12 days (as previously shown in ref 17).

Addition of no stimulus or control proteins (Chiron) during the 12-day culture did not lead to recovery of specific T cells after 12 days. In addition, stimulation with a mismatch antigen (e.g., HIV peptides), did not lead to detectable HCV-specific T-cell responses (either CD4 or CD8) after re-stimulation with HCV peptides.

**Statistical analysis**

Data are presented as medians with minimum and maximum values. Comparisons between the peak T-cell responses of resolvers and chronic HCV infected patients were done by Mann-Whitney test using Statistical Product and Service Solutions (SPSS) for Windows, version 9.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**Clinical and laboratory findings**

We studied 13 HIV-negative IDU with documented onset of HCV infection (Table 4.1.1). Seven of these resolved HCV infection after a median of 4.8 months (the resolving phase of HCV-infection, interquartile range 3.6-24.2 months) (Table 4.1.1). Six of 13 subjects remained chronically infected as evidenced by the presence of high levels of HCV-RNA plasma concentrations beyond two years (Figure 4.1.1B). After the estimated date of infection (EDI), resolvers had lower HCV-RNA concentrations (median 3.53 log10 IU/ml, range 2.78-6.9) than chronic carriers (median 5.57 log10 IU/ml, range 4.34-6.12) (p=0.051, Figure 4.1.1). Onset of HCV infection was always associated with
self-reported injecting drugs-use (Figure 4.1.1A,B). In most cases injecting drug use continued during follow up, which may lead to reexposure or reinfection with HCV.

T-cell analyses

T-cell responses were analysed in the time span needed to clear the acute infection, which we refer to as the resolving phase of HCV infection. To make the two groups, resolvers and chronic carriers, comparable, we have measured T-cell responses in chronic carriers within a comparable time span (within the first two years after EDI). The first time point analysed during follow up had a median of 3.5 months after EDI for resolvers (interquartile range 1-10 months) as well as chronic carriers (interquartile range 1-16 months). Comparison between groups was performed using the highest observed T-cell response. The median time to development of the highest observed T-cell response was 11 months (range 4-23) in resolvers and 19 months (range 15-24) in chronic carriers (Figure 4.1.1).

Resolvers have higher ex vivo HCV-specific T-cell responses than chronic carriers

Using an IFN-γ-Elispot assay after stimulation with pools of overlapping peptides, the total HCV-specific T-cell response directly ex vivo was estimated by the sum of the separate responses towards the different HCV-peptide pools (Figure 4.1.1A,B). At the time point at which the highest T-cell response was observed, resolvers had significantly higher HCV-specific T-cell responses (median 640 spots/million PBMC, range 83-5445) than chronic carriers (median 74 spots/million PBMC, range 0-230) (p=0.035) (Figure 4.1.2A). At that time point, a corresponding HCV-RNA measurement could be performed in 6 of 7 resolvers and all of them had plasma concentrations below 615 IU/ml (Figure 4.1.1A).

Next, we identified which antigens/peptide pools were targeted. Resolvers targeted a median of 5 antigens, (range 3-6) while chronic carriers targeted a median of two antigens, (range 0-5), suggesting that a larger breadth of the T-cell response was associated with resolved infection (Figure 4.1.2A). No differences for the specificity of the HCV-specific T-cell response to a particular peptide pool (cluster) was observed between resolvers and chronic carriers directly ex vivo.

We were able to analyse long-term HCV-specific T-cell responses in three resolvers and five chronic carriers, some at multiple time points. (see Figure 4.1.1) These long-term HCV-specific T-cell responses (median follow up 124 months, range 59-135) were repeatedly low in both groups (0 spots and median of 9 spots/million PBMC (range 0-47), respectively) (Figure 4.1.2B).

Total CD4⁺ memory T-cell responses are not significantly different between resolvers and chronic carriers

HCV-specific CD4⁺ T cells were quantified using a 12-day in vitro expansion assay, which was shown to give a proper reflection of a memory CD4⁺ T-cell response.17,28 Representative FACS plots of HCV-specific CD4⁺ T cells in one chronic carrier (Figure 4.1.3A) and one resolver (Figure 4.1.3B) after 12-day expansion with HCV proteins and re-stimulation with peptide pools show the percentage of CD3⁺CD4⁺ T cells producing IFN-γ (total CD4⁺ T-cell response 16.23% and 25.17% respectively). Subsequently the number of IFN-γ producing CD4⁺ T cells that grew out after 12 days was calculated in relation to the number of cells put into culture at day 0.
Follow up of injecting drug use and HCV-specific T-cell responses in 13 IDU. Follow up (in months), relative to estimated date of HCV infection at t=0 months (vertical dashed line) is shown for seven resolvers (R1-R7, A) and six chronic carriers (C1-C6, B). Resolvers R6 and R7 became chronic HCV carriers after re-infection in combination with HIV-1. Viral RNA plasma concentrations are shown on a logarithmic scale on the y-axis (A, B upper panels) with lower limits of quantification of 615 IU/ml for HCV (*) and 1000 cp/ml for HIV-1 (○). A negative HCV RNA that was positive or negative in a qualitative assay is indicated with + or - respectively. Self-reported injecting drug use is indicated by □ on the x-axis (A, B upper panels). The sum of HCV-specific T-cell responses as measured by IFN-γ Elispot assay against different HCV-peptide pools are shown as a solid vertical bar. For resolver R3, the time point with the highest observed HCV-specific T-cell response is indicated with *. HCV-specific T-cell responses that were measured but found to be less than 50 spots/10⁶ PBMC or undetectable are indicated with # on the x-axis (A, B lower panels).
**Chapter 4.1**

**Immunology in acute HCV**

### A IFN-γ producing of HCV-specific T cells directly *ex vivo*

![Histogram](image1.png)

**Figure 4.1.2.** HCV-specific T-cell responses. The number of IFN-γ producing T cells, as measured by IFN-γ Elispot assay after stimulation with overlapping HCV peptide pools, are shown for 7 resolvers (2A, left panel) and 6 chronic carriers (2A, right panel) during acute HCV-infection. The T-cell response for resolver R3 is shown at a different y-axis (indicated on the right and encircled by a dashed line) (left panel) to be able to show the protein specificities of all resolvers clearly. HCV-specific T-cell responses <50 spots/10⁶ PBMC are indicated with * (right panel). The kinetics of HCV-specific T-cell responses during long-term follow up (months) is shown for resolvers (2B, left panel) and chronic carriers (2B, right panel). The vertical dashed line at t=0 indicates estimated date of HCV infection.

This number of IFN-γ producing CD4⁺ T cells/million PBMC is a composite of the frequency of HCV-specific T cells within the total T cell pool and the ability of the cells to survive and proliferate *in vitro*.

Although higher HCV-specific CD4⁺ T-cell responses were observed in resolvers (median 10663 IFN-γ producing CD4⁺ T cells/million PBMC, range 93-74197) than in chronic carriers (median 2604 IFN-γ producing CD4⁺ T cells/million PBMC, range 1278-15730), the differences were not significant (p=0.534) (Figure 4.1.4A). After a median follow up of 114 months (range 59-154 months), an HCV-specific CD4⁺ T-cell response could be assessed in one resolver (160 IFN-γ producing cells/million PBMC) and in five chronic carriers (median 3844 IFN-γ producing cells/million PBMC, range 484-25652) (data not shown).
Figure 4.1.3 HCV-specific CD4+ T-cell responses after a 12-day expansion period.

Representative FACS plots of the percentage of HCV-specific IFN-γ-producing CD4+ T cells after 12-day expansion with NS3, NS4, NS5 or Core in a representative chronic carrier (C12, panel A) and resolver (R5, panel B). The percentage of CD3+CD4+ T cells producing IFN-γ (y-axis) upon 6-hour restimulation with NS3, NS4, NS5 and Core/envelope peptide pools is shown after 12-day expansion using proteins. In the upper right quadrants of each FACS plot the percentage of IFN-γ-producing CD4+ T cells is shown.
Resolvers have low CD4⁺ Core responses and high CD4⁺ nonstructural protein responses

We analyzed CD4⁺ T-cell responses that were directed against Core, NS3, NS4 and NS5 separately. The breadth of the CD4⁺ T-cell response against the separate proteins was the same in resolvers (median 3 proteins, range 1-4) and chronic carriers (median 3 proteins, range 2-4) (Table 4.1.2). However, the strength of the responses against the separate proteins (Core versus NS proteins) was different between resolvers and chronic carriers (Figure 4.1.4B). Resolvers had a significantly lower percentage of CD4⁺ T cells directed against Core (median 1%, range 0-14%) compared to chronic carriers (median 47%, range 2.3-68; p=0.008) (Figure 4.1.4C).

In resolvers the highest CD4⁺ T-cell response measurable was targeted against a cluster of 2 or 3 nonstructural proteins and in one (R7) against a single nonstructural protein (NS3) (Table 4.1.2). In contrast, Core protein was the most or second most dominantly targeted protein in four of six chronic carriers (Table 4.1.2). Noteworthy, a chronic carrier (C8) who generated a relative strong CD4⁺ T-cell response (8285 IFN-γ producing cells/million PBMC) against nonstructural proteins (98% of total response) (Table 4.1.2) during the early phase of chronic infection (Figure 4.1.1B), was capable of suppressing HCV-RNA plasma concentrations (<615 IU/ml). However, HCV-RNA levels became detectable again, despite a sustained magnitude of the total CD4⁺ T-cell response (7101 IFN-γ producing cells/million PBMC). However, the quality of the CD4⁺ T-cell response had changed by increasing the Core protein response from 2% to 22% (Figure 4.1.4B, right panel), with a concomitant decrease against the nonstructural proteins (data not shown).

| Subject | Core % | abs. | NS3 % | abs. | NS4 % | abs. | NS5 % | abs. |
|---------|--------|------|--------|------|--------|------|--------|------|
| R1      | 1      | 221  | 20     | 5212 | 16     | 3999 | 63     | 16158|
| R2      | 0      | 0    | 0      | 77   | 0      | 8537 | 23     | 2595 |
| R3      | 0      | 0    | 0      | 0    | 57     | 892  | 43     | 682  |
| R4      | 9      | 7113 | 19     | 13903| 40     | 29287| 32     | 23894|
| R5      | 3      | 272  | 27     | 2867 | 42     | 4498 | 28     | 3026 |
| R6      | 14     | 436  | 70     | 2186 | 0      | 0    | 16     | 492  |
| R7      | 0      | 100  | 0      | 93   | 0      | 0    | 0      | 0    |
| C8      | 2      | 187  | 44     | 3812 | 19     | 1604 | 35     | 2882 |
| C9      | 68     | 1949 | 0      | 0    | 27     | 791  | 5      | 2877 |
| C10     | 37     | 851  | 12     | 296  | 51     | 1183 | 0      | 0    |
| C11     | 44     | 567  | 0      | 0    | 56     | 711  | 0      | 0    |
| C12     | 33     | 689  | 0      | 0    | 46     | 972  | 21     | 454  |
| C13     | 54     | 8514 | 28     | 4368 | 12     | 1820 | 6      | 1028 |
Figure 4.1.4  HCV-specific CD4\(^+\) T cell response.
A. The number of IFN-\(\gamma\) producing CD4\(^+\) T cells after 12 days of expansion calculated from the input PBMC (IFN-\(\gamma\) producing CD4\(^+\) T cells/10\(^6\) PBMC) as measured by ICCS after re-stimulation, is shown for 7 resolvers (left panel) and six chronic carriers (right panel) before and after acute HCV infection. The CD4\(^+\) T-cell response is shown for resolver R3 at the right y-axis (encircled a dashed line) (left panel), as this individual has a much higher T-cell response. The low CD4\(^+\) T cell peak response in resolver R7 (68 IFN-\(\gamma\) producing cells/10\(^6\) PBMC) is indicated with # (A, left panel). Separate protein-specific responses are shown by gray tones in the bars.
B. The kinetics of HCV-specific CD4\(^+\) T-cell responses directed against Core protein (as percentage of IFN-\(\gamma\) producing CD4\(^+\) T cells/10\(^6\) PBMC) is shown for resolvers (left panel) and chronic carriers (right panel) over onset of HCV infection and long term follow up. Estimated date of HCV infection is indicated by a vertical dashed line at month=0.
C. HCV-specific CD4\(^+\) T-cell responses against Core and NS-proteins, presented as a percentage of the total HCV-specific CD4\(^+\) T cell response (y-axis), are compared between resolvers and chronic carriers (x-axis) at the moment of the highest T-cell response.
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HCV-specific T-cell responses before and after EDI

In four individuals (R2, 3, 7, C11) we were able to assess HCV-specific CD4+ memory T cells and ex vivo T-cell responses before onset of infection at month -27, -52, -5, -14, respectively. A relative long interval before EDI was chosen in order to prevent analyses of HCV-specific T-cell responses during the window phase of an acute HCV infection. Surprisingly, ex vivo HCV-specific T-cell responses were easily detectable before EDI (in resolvers median 167 spots/million PBMC, range 55 to 255 and in the chronic carrier 200 spots/million PBMC). After onset of infection, HCV-specific T-cell responses increased in resolvers (varying from 2- to 20-fold) but decreased to undetectable levels in the chronic carrier during the same time span (Figure 4.1.1). These easily detectable T-cell responses before EDI coincided with self-reported injecting drug use in most IDU, but HCV RNA remained undetectable (Figure 4.1.1A,B). Noteworthy, in resolver R3 the T-cell response became undetectable when injecting drug use had stopped during the period before EDI.

Also HCV-specific CD4+ T-cell memory responses after 12-day expansion were easily detected well before EDI (median 2570, range 1025-17249 IFN-γ producing cells/million PBMC), while no CD4+ T-cell responses were detectable after stimulating four healthy individuals who served as a control group (data not shown). Interestingly, Core protein-specific CD4+ T-cell responses, which dominated before EDI, decreased over onset of infection to very low levels in all resolvers (Figure 4.1.4B, left panel) with a concomitant increase in responses against nonstructural proteins (data not shown). In contrast, after onset of infection the CD4+ T-cell response to Core protein increased in the chronic carrier (Figure 4.1.4B, right panel).

Discussion

Based on studies of acute hepatitis C in humans, clearance of hepatitis C infection is thought to be associated with the ability to mount strong T-cell responses. Our study shows that, although T-cell responses in general were low, resolvers had higher ex vivo HCV-specific T-cell responses during the resolving phase of HCV-infection compared to chronic carriers in a comparable time frame. In contrast, we show that the magnitude of the total HCV-specific memory CD4+ T-cell response after expansion did not differ significantly between resolvers and chronic carriers. However, HCV-specific CD4+ NS protein responses were significantly higher in resolvers compared to chronic carriers.

Using stimulation with overlapping peptide pools spanning the entire HCV genome, our study among IDU with a prospectively sampled HCV-infection confirms previous studies (which were often cross-sectional and in health care workers) that direct ex vivo T-cell responses are weak. But resolved HCV infection is associated with relatively stronger HCV-specific T-cell responses. Long-term HCV-specific T-cell responses observed directly ex vivo were weaker than reported by others. Obviously, our group of individuals may have been different in many ways compared to other groups. For one the frequency of (injecting) drug use, which by itself may have a negative influence on the cellular immune responses. In those in whom we assessed HCV-specific T-cell responses from before onset of infection, we observed that an increase of these responses over EDI was associated with a favourable outcome of infection.
As CD4+ T-cell responses in our hands were undetectable directly \textit{ex vivo} using protein stimulation, we studied the role of CD4+ T-cell responses in HCV infection using a recently developed 12-day expansion assay.\textsuperscript{17} This assay was previously shown to detect antigen-experienced T cells that are able to proliferate and exert their function by cytokine production upon re-encounter with the antigen. It was shown in both HCV-infected\textsuperscript{28} and \textit{Plasmodium falciparum}-infected individuals\textsuperscript{31} that protection against infection and/or clearance of the pathogen was associated with IFN-\(\gamma\) producing CD4+ T cells measured after \textit{ex vivo} expansion.

We found that most resolvers had higher HCV-specific CD4+ T-cell responses and most chronic carriers had lower CD4+ T-cell responses, confirming the general picture of robust CD4+ T-cell responses in self-limiting HCV-infections.\textsuperscript{32} However, the difference in magnitude of the CD4+ T-cell response between resolvers and chronic carriers was not significant. This suggests that the outcome of HCV infection may not be determined by the magnitude of IFN-\(\gamma\) producing CD4+ T cells, as previously suggested in a study in chimpanzees.\textsuperscript{33} Other qualitative aspects of the HCV-specific CD4+ T-cell response could play a role in the outcome of HCV-infection. Interestingly, we found that HCV-specific CD4+ T-cell responses against nonstructural proteins were significantly higher in resolvers compared to chronic carriers. In addition, over onset of infection, the CD4+ T-cell responses against separate proteins showed an increasing CD4+ T-cell response against nonstructural proteins in resolvers. Moreover, recurrence of HCV-RNA after temporary control of HCV infection during the early phase of infection, was also associated with a shift towards a more dominant CD4+ Core protein response, suggesting that a chronic carrier state is associated with the appearance of a relative strong CD4+ Core protein response.

In an earlier study, a trend for CD4+ T-cell responses to Core protein was found to be more common in individuals who evolved to chronic hepatitis\textsuperscript{5} and in a more recent cross-sectional study it was found that T-cell responses in resolvers were more commonly targeting nonstructural proteins.\textsuperscript{34} However, other studies using similar techniques failed to support this finding and showed that CD4+ T-cell responses were broad, but not specifically targeting only NS proteins.\textsuperscript{35} In that light the Core protein-responses detected prior to seroconversion in subsequently resolving individuals is of interest as Core protein-responses are more abundant before onset of infection and shift to dominant NS-responses over seroconversion.

We assume that differences in reactivity of CD4+ T cells against Core during HCV infection is caused by differences in Core-antigen presentation to CD4+ T cells.\textsuperscript{36} We did observe lower HCV-RNA plasma concentrations in resolvers compared to chronic carriers, but it remains speculative whether higher HCV-RNA concentrations lead to more circulating Core protein. It has been suggested that serum of HCV-infected individuals contains virus particles with HCV Core epitopes exposed on their surfaces\textsuperscript{27} and that serum may contain free circulating Core proteins.\textsuperscript{36} In addition, HCV Core is thought to play a role in modulating immune responses by affecting the function of virus-specific T cells.\textsuperscript{36,39,40} Higher HCV replication could lead to higher quantities of Core antigen in plasma and this may negatively influence the development or maintenance of an effective T-cell response. This may further facilitate HCV replication\textsuperscript{41}, potentially pushing the balance between clearance and persistence into the direction of a chronic HCV infection.

Traditionally, acute hepatitis C is considered to run a course of approximately six months, which is based on the observation that most patients who spontaneously clear HCV, do so within the first three to four months of infection.\textsuperscript{52} However, most
observations were done in symptomatic patients who were subsequently referred to medical centres. Consequently, most analyses of a successful immune response were done in symptomatic HCV-infected patients, whom we assume were not repeatedly exposed to HCV, once they received medical care during the first six months of infection. In contrast, we assume that some of our asymptomatic injecting drug users were repeatedly re-infected with HCV during the acute phase of infection. As a consequence of this repeated and prolonged HCV exposure, some IDU probably needed more time to resolve HCV infection (Figure 4.1.1). On the other hand, we may have overestimated the time to viral clearance due to some wide HCV-RNA testing intervals. Late viral clearance (>24 months) has been reported before.

A possible caveat in our study might be the use of HCV peptide pools based on the genotype 1a consensus sequence, as 6 out of 13 patients were infected with genotype 3a strains, which may result in underestimation of T-cell responses in these patients. On the other hand genotype 3a was equally divided over the two groups studied. However, three of the chronic carriers who carried genotype 3a strains lacked responses against NS5 (Table 4.1.3). Whether this is due to their genotype difference or due to escape mutations has to be elucidated. The fact that the resolvers infected with genotype 3a did not show NS5 responses, suggests that the three genotype 3a chronic carriers may have obtained additional mutations making them unresponsive to NS5 stimulation.

In addition, we were not able to investigate the T-cell response at the same time point in each individual. Although most individuals were studied within three months after EDI, three of the resolvers and two of the chronic carriers were studied after more than ten months. To adjust for this potential bias we compared the highest T-cell response instead of the earliest time point measured. However, these different approaches led to similar results. (This thesis, chapter 4.3) Furthermore, one chronic carrier had a long interval between last negative and first positive RNA samples and thus the EDI has a larger improbability. We cannot exclude that this has interfered with the results.

An unexpected finding was the detection of HCV-specific memory T-cell responses in resolvers as well as a chronic carrier before EDI, despite undetectable HCV RNA and HCV antibodies. These responses suggest that exposure to HCV occurs much more often than previously thought and can lead to induction of a cellular immune response without consistently detectable viremia or seroconversion, and may influence subsequent outcome of infection. Indeed, in one of our subjects it was previously shown that HCV seroconversion after a “first” HCV infection was followed by loss of detectable antibodies after which re-seroconversion occurred after a “second” HCV infection. Clearance of the “second” HCV infection in R3 was associated with very strong T-cell response, suggestive of protective immunity and strikingly paralleling periods of intermittent injecting drug use. Alternatively, the dominant Core responses before acute HCV infection may be the result of exposure to Core particles instead of infectious virus. Conversely, we cannot assume that individuals in whom we could not demonstrate potential previous infection have truly not been in contact with HCV before follow up.

Intriguingly, the detectable T-cell responses before EDI in chronic carrier C11 were apparently not protective, suggesting that a CD4+ T-cell responses before EDI does not predict an effective immune response after re-exposure to high levels of HCV RNA. It has been reported that IDU who were previously infected were less likely to develop persistent HCV viremia than individuals infected for the first time, indeed suggesting that protective immunity may be acquired. However, a more recent study did not confirm
this and calculated the re-infection rate to be 41/100 PY. Therefore, the observed T-cell responses before onset of infection may be merely a reflection of exposure to the virus, as has been reported in homosexual men who seroconverted for HIV despite detectable HIV-specific cytotoxic T-lymphocyte (CTL) responses well before HIV seroconversion. Most likely T-cell responses decrease after clearance and can only be detected after recent (re)-infection. Interestingly, in resolver R3 the T-cell response became undetectable when injecting drug use had stopped during the period before EDI.

Surprisingly, in the chronic carrier and the resolvers, the memory responses measured before onset of infection were in part directed against Core protein. One would expect the memory response in the individuals whom resolve after (re)-infection, to resemble a protective response, which would be directed predominantly against nonstructural proteins. In resolvers the response became mainly NS-focused only after (re)-infection, suggesting that a rapid transition to a predominantly NS-response would provide the ability to gain a protective response.

In conclusion, during the resolving phase of HCV infection higher ex vivo HCV-specific T-cell responses and memory HCV-specific CD4+ T-cell responses targeting mainly nonstructural proteins are observed in resolvers compared to in chronic carriers. Memory T-cell responses present before documented HCV-seroconversion suggest that re-infection in IDU occurs often, while the presence of these responses were not predictive for the outcome of infection. Persistent HCV viremia was associated with increasing HCV-specific CD4+ T-cell responsiveness against Core protein, implicating a role for Core protein in negative modulation of the CD4+ T-cell response, which may have implications for the design of HCV vaccines.
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Chapter 4.2

Comprehensive longitudinal analysis of hepatitis C virus (HCV)-specific T-cell responses during acute HCV infection in the presence of existing HIV-1 infection

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*J Viral Hepatitis;* 2009;16:239-248.
Abstract

Objective
To study the development of HCV-specific T cell immunity during acute HCV infection in the presence of an existing HIV-1 infection in four HIV-1 infected men having sex with men (MSM).

Methods
A comprehensive analysis of HCV-specific T-cell responses was performed at two time points during acute HCV infection using a T-cell expansion assay with overlapping peptide pools spanning the entire HCV genome.

Results
Three patients with (near) normal CD4⁺ T cell counts (range 400-970x10⁶/l) either resolved (n=1) or temporary suppressed HCV RNA. In contrast, one patient with low CD4⁺ T cell counts (330x10⁶/l), had sustained high HCV RNA levels. All four patients had low HCV-specific CD8⁺ T-cell responses, and similar magnitudes of CD4⁺ T-cell responses. Interestingly, individuals with resolved infection or temporary suppression of HCV-RNA had HCV-specific CD4⁺ T-cell responses predominantly against nonstructural (NS) proteins. While the individual with high HCV RNA plasma concentrations had CD4⁺ T-cell responses predominantly directed against Core.

Conclusions
Our data show that an acute HCV infection in an HIV-1 infected person can be suppressed in the presence of HCV-specific CD4⁺ T-cell response targeting nonstructural proteins. However further research is needed in a larger group of patients to evaluate the role of HIV-1 on HCV-specific T-cell responses in relation to outcome of acute HCV infection.
Introduction

Hepatitis C virus (HCV) leads to a chronic infection in the majority of patients. HCV-specific CD8+ T cells play a central role in the control of hepatitis C. A multi-specific CTL response during the first six months of infection is associated with resolved infection and the presence of strong polyclonal CTL responses is associated with lower HCV RNA plasma concentrations. Several studies have shown a close correlation between polyclonal, multi-specific proliferative CD4+ T-cell response and clearance of acute HCV infection. Furthermore, HCV-specific memory CD8+ and CD4+ T-cell responses were shown to be important for the control of re-infection with HCV in a serially infected chimpanzee. It has been suggested that such a protective HCV-specific memory response may exist in injecting drug users and that an effective HCV-specific immune response may be lost after co-infection with HIV-1. There is a general believe that HIV-1 infected patients have a lower clearance rate of acute HCV infection compared to persons with an acute HCV mono-infection. Therefore, we hypothesized that HIV-1 may influence HCV infection by impairing HCV-specific T-cell responses.

As a read-out of functionality of T cells several parameters can be analyzed. The ability of HCV-specific T cells to produce effector cytokines (e.g., IFN-γ and IL-2) after direct stimulation was shown to be low. Therefore we used a recently described sensitive and reproducible assay combining specific in vitro expansion and effector T cell analysis to determine the number of HCV-specific memory T cells. This assay is not just a measure of proliferation, but detects a combination of the survival of specific T cells, proliferative capacity of these cells and ability to differentiate into effector T cells and produce IFN-γ. Numbers of antigen specific T cells, capable of proliferation and subsequent IFN-γ production in response to re-exposure to antigen were shown to correlate with protection against HCV and malaria.

To our knowledge, there are no longitudinal data available at this moment on the effect of already existing chronic HIV-infection on HCV-specific immunity after acute HCV infection. We had the unique opportunity to identify four men having sex with men (MSM) with an acute HCV infection in presence of an already existing HIV-1 infection and performed a comprehensive analysis of HCV-specific CD4+ and CD8+ T cells after stimulation with HCV overlapping peptide pools.

Materials and Methods

Subjects

Between 2002 and 2004, four Caucasian men having sex with men (MSM I, II, III, IV) with known HIV-1 seropositivity varying from 1.5 to 10 years were identified with an acute hepatitis, as evidenced by markedly elevated liver enzymes, by primary HIV providers in Amsterdam, the Netherlands (Table 4.2.1). Three of four male subjects (I, III, IV) received highly active anti-retroviral treatment (HAART, range 15-118 weeks) when this acute hepatitis occurred. Because of the markedly elevated ALT levels and hence initial suspicion of HAART toxicity, HAART was stopped in 2 MSM (I, III) and HIV-RNA levels became detectable after 12 and 14 weeks. Because of continuing high ALT levels after cessation of HAART, suspicion on acute HCV arose and patients were tested for HCV antibodies and presence of HCV RNA. After confirmation of acute HCV
infection, earlier stored plasma samples were retrospectively tested for HCV antibodies and HCV RNA and HCV seroconversion was documented. The patients were followed regularly at the outpatient clinic. An alanine-aminotransferase (ALT) peak (median 1,056 IU/l, range 697-1,949) was observed a median of seven weeks (range 4-9 weeks) after the onset of HCV infection (Figure 4.2.1). All patients gave informed consent.

Definitions

Acute HCV infection was defined as detectable serum HCV RNA in the setting of documented seroconversion to HCV antibody positivity within the past six months. The onset of HCV infection was retrospectively determined and estimated to have occurred between the last HCV RNA negative and first positive serum sample, at the moment of sexual risk behaviour or the presence of a sexual transmitted disease. Resolved HCV infection was defined as undetectable HCV RNA (qualitative HCV RNA, TMA) at two consecutive visits, during a period of six months or more.

HCV-serology and RNA analyses

HCV antibody testing was performed with a third-generation enzyme immunoassay (Ortho Diagnostics, Rochester, NY). Serum HCV RNA was quantified by bDNA (Versant™ HCV 3.0, Bayer Diagnostics with a lower limit of detection of 615 IU/ml). Subsequently, HCV RNA was measured by means of a qualitative transcription mediated amplification (TMA) assay (Versant™ HCV RNA Qualitative assay, Bayer Diagnostics, with a lower limit of detection of 5 IU/ml), when HCV RNA was below the detection limit of the bDNA assay (615 IU/ml). For HCV genotyping RNA was isolated using the TriPure method (Roche Diagnostics, Almere, the Netherlands) and subsequently amplified using a nested RT-PCR based on the conserved Core region of the HCV genome as described by Ohno et al. Genotypes were confirmed by sequencing a part of the NS5B region of the HCV genome.

Assessment of HCV-specific T-cell responses

HCV-specific T-cell responses were assessed at two time points during the first 30 weeks after onset of acute HCV infection (first time point, range 6-14 weeks, second time point range 10-28 weeks). Panels of overlapping peptides spanning the complete HCV genotype 1a genome were used as stimulation (provided by NIH Aids Research Reagent Programme). Peptides had a length of 18 amino acids (overlapping adjacent peptides by 11 aa) derived from the following HCV proteins: Core polyprotein (Core, E1, E2, p7 protein, aa 1-805), NS2 protein (aa 806-1,022), NS3 protease/helicase (aa 1,023-1,645), NS4 protein (aa 1,646-1,967), NS5A protein (aa 1,968-2,415) and NS5B protein (aa 2,416-3,011). Peptides were pooled in a way that each single peptide in the pool was present at a concentration of 1 mg/ml.

ELIspot assay for single cell IFN-γ release

IFN-γ producing HCV-specific T cells were enumerated using IFN-γ specific ELIspot assays as previously described using the anti-IFN-γ antibodies from Mabtech (Stockholm) and streptavidin poly-HRP from Sanquin (Amsterdam). PBMC were stimulated in triplicate wells at 100,000 cells/well in the absence or presence of 2 μg/ml
of peptide pools, which are the optimal conditions to provide the best ratio between detectable and background responses for this assay. Individual cytokine-producing cells were detected as dark purple spots after a reaction with TMB substrate (Sanquin, Amsterdam) and counted using the A.EL.VIS automated spot analyzer. The number of specific T-cell responders per 10⁶ PBMC was calculated after subtracting two times negative control values. Based on values in healthy controls, a response of 50 spots/10⁶ PBMC was regarded as positive.

Expansion of HCV-specific T cells

To expand HCV-specific T cells, PBMC were cultured for 12 days as previously described in the presence of overlapping peptide pools corresponding to the Core, NS2, NS3, NS4 and NS5 proteins. Peptide pools (2 μg/ml) were added on day 0 and 6. IL-2 was added at 360 IU/ml on days 3, 6, and 9. On day 12, cells were pooled, washed, counted and rested overnight in complete medium. On day 13 cells were restimulated to determine the number of IFN-γ-producing effector HCV-specific T cells.

Figure 4.2.1 Follow up of MSM during acute HCV infection. Kinetics of both HCV-RNA load (filled circles) and ALT-levels (open squares) are depicted in time (weeks) for four MSM (I-IV) who experienced acute HCV infection. Estimated date of acute HCV infection is depicted with the dotted vertical line. Periods on HAART (horizontal bars) or PEG-IFN/ribavirin therapy (vertical solid lines) are indicated in the figures.
Table 4.2.1 General characteristics of the study population.

| MSM   | Age | Time since HIV-1 infection | HAART* | Duration of HAART* | HIV-RNA* | HCV genotype | CD4 count$^b$ | Nadir CD4 counts$^a$ | Outcome HCV-infection | HCV therapy | Outcome therapy |
|-------|-----|-----------------------------|--------|-------------------|----------|---------------|----------------|--------------------|-----------------------|--------------|----------------|
| I     | 37  | 3 years                     | yes    | 2 years           | <50 cp/ml| 1             | 640            | 340                | resolved             | No           | NA             |
| II    | 37  | 1 year                      | no     | -                 | <50 cp/ml| 4             | 970            | 550                | chronic              | No           | NA             |
| III   | 45  | 5.5 years                   | yes    | 5 years           | <50 cp/ml| 1             | 400            | 250                | chronic +28 wk       | Relapse      | +28 wk Relapse |
| IV    | 50  | 2 years                     | yes    | 6 months          | <50 cp/ml| 1             | 260            | 130                | chronic +14 wks      | Relapse      | +14 wks Relapse |

*Men who have sex with men (MSM), study subject; *age in years at onset acute HCV infection; & at onset acute HCV infection; $^a$ CD4$^+$ T-cell numbers (x10$^{6}$/l), before onset of acute HCV infection; $^b$ nadir CD4$^+$ T-cell numbers (x10$^{6}$/l), during acute HCV infection; $^+$ First HIV-RNA during acute HCV.

Table 4.2.2 Proliferation data CD4$^+$ T-cells after 12 day expansion.

| Patient | HCV antigen | Percentage IFN-γ$^+$ | Proliferation rate | number of IFN-γ producing CD4$^+$ T cells/million PBMC input | % Core of total response |
|---------|-------------|----------------------|--------------------|-------------------------------------------------------------|--------------------------|
| MSM I   | CORE        | 0.23                 | 0.089              | 204                                                         | 5.5                      |
|         | NS2         | 0.10                 | 0.050              | 50                                                          |                          |
|         | NS3         | 0.29                 | 0.113              | 328                                                         |                          |
|         | NS4         | 0.85                 | 0.079              | 668                                                         |                          |
|         | NS5A+5B     | 2.20                 | 0.112              | 2,473                                                       |                          |
| TOTAL   |             |                      |                    | 3,723                                                       |                          |
| MSM II  | CORE        | 0.89                 | 0.057              | 507                                                         | 33                       |
|         | NS2         | 0.03                 | 0.067              | 20                                                          |                          |
|         | NS3         | 0.21                 | 0.086              | 181                                                         |                          |
|         | NS4         | 1.19                 | 0.054              | 637                                                         |                          |
|         | NS5A+5B     | 0.26                 | 0.070              | 182                                                         |                          |
| TOTAL   |             |                      |                    | 1,527                                                       |                          |
| MSM III | CORE        | 0.53                 | 0.303              | 1,607                                                       | 56                       |
|         | NS2         | 0.11                 | 0.082              | 91                                                          |                          |
|         | NS3         | 0.76                 | 0.059              | 452                                                         |                          |
|         | NS4         | 0.07                 | 0.102              | 682                                                         |                          |
|         | NS5A+5B     | 0.04                 | 0.086              | 34                                                          |                          |
| TOTAL   |             |                      |                    | 2,866                                                       |                          |
| MSM IV  | CORE        | 1.29                 | 0.102              | 1,319                                                       | 85                       |
|         | NS2         | 0.04                 | 0.075              | 30                                                          |                          |
|         | NS3         | 0.04                 | 0.066              | 26                                                          |                          |
|         | NS4         | 0.36                 | 0.049              | 178                                                         |                          |
|         | NS5A+5B     | 0.00                 | 0.081              | 0                                                           |                          |
| TOTAL   |             |                      |                    | 1,553                                                       |                          |

Percentage IFN-γ$^+$: percentage of IFN-γ producing cells after 12 day expansion and restimulation with peptide pools minus percentage of IFN-γ producing cells restimulated with medium; proliferation rate: number of cells recovered after 12 days of culture/number of cells put in to culture at day 0; number of # IFN producing CD4$^+$ T cells/million PBMC input: the product of the percentage of IFN-γ-producing T cells and the proliferation rate (x10.000 to express this number as a number per million PBMC input).
Detection of IFN-γ producing HCV-specific T cells after re-stimulation

IFN-γ producing cells after re-stimulation with overlapping peptide pools were enumerated by intracellular cytokine staining (ICCS). Briefly, 10^6 PBMC were stimulated for six hours with HCV Core, NS2, NS3, NS4 and NS5 peptide pools (2 μg/ml) and both αCD28 (2 μg/ml) and αCD49d (2 μg/ml) as co-stimuli, after one hour 1:1,000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA) was added. As a negative control, PBMC were stimulated with medium and co-stimulation alone. As a positive control PBMC were stimulated with 10 ng/ml PMA and 2 μg/ml ionomycin. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD4, CD8 and IFN-γ (BD). After fixation (Cellfix, BD) min. 20,000 events were acquired on a FACS Calibur or LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analysed using the software program CELLQuest (BD).

Results were expressed as the percentage of IFN-γ producing T cells, which was calculated by subtracting the percentage of IFN-γ producing T cells in medium from the percentage of IFN-γ producing T cells in peptide pool restimulated conditions (Table 4.2.2). To determine the number of HCV-specific T cells per individual, enabling comparison of individuals with different T cell numbers, we calculated the number of HCV-protein-specific IFN-γ-producing T cells recovered out of 10^6 PBMC put into culture on day 0. To this end we determined the proliferation rate, i.e., the total number of cells recovered after 12 days of expansion divided by the total number of cells put in to culture at day 0 (Table 4.2.2). We called this rate a proliferation rate, since it is a measure of proliferation of HCV-specific T cells. However, another important determinant of this proliferation rate is the death rate of non-HCV-specific T cells, as can be seen in Table 4.2.2: the number of cells declines on average a factor 10 after 12 days of culture.

The number of HCV-specific T cells is the product of the percentage of IFN-γ-producing T cells and the proliferation rate. This number of HCV-specific effector T cells shown in Table 4.2.2, is therefore a combination of (memory) cells initially present in the PBMC pool and the ability of these cells to survive, proliferate and differentiate to (IFN-γ producing) effector T cells during the 12-day culture period. Thereby this assay is not restricted to measuring only one quality of (memory) T cells, but it is a combination of factors contributing to its effector function. It has been shown that Epstein-Barr virus (EBV)-specific cells measured after 12 days expansion were inversely correlated with antigen burden in case of EBV-infection.17

Results

Acute HCV infection and HIV-related determinants

Four MSM with acute HCV in the presence of HIV-1 infection were included, of whom three were infected with genotype 1. HIV-1 viral load was undetectable in all four MSM (Table 4.2.1). After onset of HCV infection in these four MSM, three different patterns in the course of HCV RNA levels were identified. First, resolved HCV infection in one MSM (I), as evidenced by undetectable HCV-RNA plasma concentrations (TMA) from week 14 onwards (Figure 4.2.1). Approximately half a year later MSM I became re-infected.
with HCV genotype 4, in the presence of high HIV-1 RNA levels and remained chronically HCV-HIV co-infected thereafter. A second pattern was characterized by transiently suppressed HCV RNA levels (<615 IU/ml, MSM II and III) during a period varying between four and ten weeks. The third pattern (MSM IV) was characterized by sustained high levels of HCV RNA since onset of infection (Figure 4.2.1). The number of CD4\(^+\) T cells prior to onset of HCV infection and during acute HCV infection (nadir) were highest in MSM who could resolve (I) or temporarily suppress HCV RNA (II, III) and lowest in MSM IV, in whom we observed sustained high HCV RNA levels (Table 4.2.1, Figure 4.2.1).

### HCV-specific T-cell responses during acute HCV infection

HCV-specific T-cell responses were assessed after stimulation with HCV peptide pools using Elispot assay for IFN-\(\gamma\) production directly ex vivo at two different time points in MSM I, II and III. Overall, low to undetectable HCV-specific T-cell responses were observed at the earliest time point (range 6-11 weeks, range 0-307 spots/million PBMC, which equals 0-0.031%) (Figure 4.2.2A) and last assessed time point during acute infection (range 14-28 weeks, range 0-13 spots/PBMC, 0-0.0013%) (data not shown). Because of these low HCV-specific T-cell responses measured by Elispot-assay, we analyzed HCV-specific T cells after a 12 day expansion period using overlapping peptide pools spanning the entire HCV genome. Especially CD4\(^+\) T cells were shown to grow out during the 12-day stimulation period, which made analyses of CD8-responses more difficult.

Overall, HCV-specific CD4\(^+\) T-cell responses were stronger than CD8\(^+\) T-cell responses and ranged from 1.73 to 3.67% IFN-\(\gamma\) producing CD4\(^+\) T cells (Table 4.2.2). When taking the proliferation rate into account (Table 4.2.2), numbers ranged from 1,527-3,723 IFN-\(\gamma\) producing CD4\(^+\) T-cells/million PBMC at the first time point (Figure 4.2.2C, Table 4.2.2) and from 1,164-3,862 IFN-\(\gamma\) producing CD4\(^+\) T cells/10\(^6\) PBMC at the second time point during acute HCV infection (data not shown).

After restimulation the percentage of HCV-specific CD8\(^+\) T cells varied between 0 and 0.29% at the earliest assessed time point, and between 0 and 0.45% at the last assessed time point (data not shown). When incorporating the proliferation rate and thereby calculating the number of HCV-specific T cells (see section Methods), IFN-\(\gamma\) producing CD8\(^+\) T cells ranged from 0 and 318 at the earliest assessed time point (Figure 4.2.2B) and varied between 17 and 237 at the last assessed time point (data not shown).

### Resolved HCV infection is associated with an HCV-specific CD4\(^+\) T-cell response against nonstructural proteins

By comparing CD4\(^+\) T-cell responses directed against HCV proteins separately at the earliest assessed time point, MSM I, who resolves HCV infection showed an HCV-specific CD4\(^+\) T-cell response almost completely targeted against nonstructural proteins (3,520 IFN-\(\gamma\) producing CD4\(^+\) T cells/10\(^6\) PBMC, 95% of total CD4\(^+\) T-cell response) (Figure 4.2.2C). MSM II and III, who could suppress HCV RNA temporarily during the early phase of acute HCV infection, showed more pronounced CD4\(^+\) T-cell responses directed against Core protein (507 IFN-\(\gamma\) producing CD4\(^+\) T cells/10\(^6\) PBMC, 33% of total CD4\(^+\) T-cell response and 1,607 IFN-\(\gamma\) producing CD4\(^+\) T cells/10\(^6\) PBMC, 56% of total CD4\(^+\) T-cell response, respectively). While in MSM IV, in whom elevated HCV RNA levels persisted, a CD4\(^+\) T-cell response that was predominantly targeted against Core-
protein (1,319 IFN-γ producing CD4+ T cells/10^6 PBMC, 85% of total CD4-response) (Table 4.2.2, Figure 4.2.3). When analysing a later time point during acute HCV infection (range 14-28 weeks), CD4+ T-cell responses show a similar pattern as the earlier assessed time point with relative low CD4+ T-cell Core responses in MSM I with resolving HCV infection (13%) compared to higher CD4+ T-cell Core responses in chronic carriers MSM II (59%), MSM III (77%) and MSM IV (60%) (data not shown).

During follow up of MSM I infected with HCV genotype 1, HCV RNA levels remained undetectable with normalized ALT levels until week 41. Thereafter, HCV RNA became detectable again, however, from a different genotype (genotype 4). In the absence of anti-HIV therapy during this HCV re-infection, MSM I developed chronic infection as evidenced by persistent high levels of HCV RNA (Figure 4.2.3). In contrast to a predominantly nonstructural protein directed CD4+ T-cell response, as observed during the first acute infection, the development of chronic HCV infection was associated with a CD4+ T-cell response predominantly directed against Core protein (range 58-69% of total CD4+ T-cell response) (Figure 4.2.3).

**Figure 4.2.2**  HCV-specific T cells during acute HCV infection.

HCV-specific T cells measured after stimulation with HCV overlapping peptide pools corresponding to the Core (black) and NS-protein regions (grey tones) are indicated in MSM I, II, III and IV. A) HCV-specific T cells (spots per 10^6 PBMC) measured directly ex-vivo using IFN-γ Elispot assay B) HCV-specific CD8+ T cells and C) HCV-specific CD4+ T cells measured after 12 days expansion and expressed as number of IFN-γ producing T cells/10^6 PBMC input.
T-cell responses in MSM I and IV.

58% - 69% PBMC (γprod cells/10^6 PBMC) (genotype 4) is indicated by the double dotted line. Onset of acute HCV re-infection (genotype 4) is indicated by the double dotted line. A) Onset of acute HCV infection in MSM I (genotype 1) is depicted by the dotted line. Onset of acute HCV re-infection (genotype 4) is indicated by the double dotted line. B) PEG-IFN/ribavirin therapy is indicated with the double dotted area.

T-cell responses during anti HCV-therapy

Because of persistently elevated HCV-RNA levels, MSM III and IV were treated for acute HCV infection with pegylated interferon-α (PEG-IFN) plus ribavirin (RBV). Treatment was not successful in either of them. In MSM IV we assessed HCV-specific T-cell responses during treatment. Throughout temporary suppression of HCV RNA a low CD4+ T-cell response (640 IFN-γ producing CD4+ T cells/10^6 PBMC), only targeting nonstructural proteins (NS3 75%, NS4 25%) was observed (Figure 4.2.3). However,
HCV RNA relapsed during treatment and after re-appearance of HCV RNA the HCV-specific CD4+ T-cell response had shifted back towards Core (1,034 IFN-γ producing CD4+ T cells/10^6 PBMC, 47% of total CD4 response) (Figure 4.2.3). MSM III was treated for HCV after (immunological) follow up in this study.

Discussion

To our knowledge this is the first study in which HCV-specific T-cell responses were analyzed during acute HCV infection in the presence of an already existing HIV-1 infection. HIV-1 seropositivity itself did not seem to be a dominant factor for the outcome of acute HCV infection. However, continuous HIV-1 replication seemed associated with a negative outcome of acute HCV infection, which may suggest that uncontrolled HIV-1 infection may impair the development of an effective HCV-specific T-cell response.

We found that the individuals with permanent or transient suppression of HCV RNA during acute HCV had higher CD4+ T cell numbers, which may suggest that low CD4+ T cell numbers impaired the development of an effective immune response. This finding seems in line with recent cross-sectional studies in which the magnitude and breadth of HCV-specific CD8+ T-cell response depended on CD4+ T cell count in HIV/HCV co-infected individuals. However, in our study only low ex vivo HCV-specific T-cell responses were observed. It has been shown that HCV-specific T cells during acute infection are impaired in IFN-γ production. Although it is possible that these cells make other cytokines, like IL-2, this is not likely in the light of a bulk of reports in both mice and human showing that mainly IL-2 production (and consequently proliferative capacity) is disturbed during chronic viral infections, and that IFN-γ responses are actually the last to wane in the face of chronic high antigen levels. As CD8+ memory T cells measured after expansion were also low, this suggests that either also proliferative capacity is impaired or that high frequencies of antigen specific CD8+ T cells may not be necessary in order to resolve acute HCV infection in HIV co-infected patients. Recent data on the occurrence of HCV-specific T-cell responses in HIV-HCV co-infected patients suggest however that HCV-specific immune responses occur in frequencies not different from HCV mono-infected patients. The assay we use is based on a combination of proliferative capacity and initial frequency of memory T cells and thereby may analyze a different function of T cells compared to other studies. We have chosen this assay as it has proven to be reproducible, specific and sensitive, being able to detect HCV-specific T-cell responses in individuals with low direct ex vivo responses. Furthermore, by taking into account a combination of functional properties instead of analysing only one function of memory T cells, like proliferation (e.g., CFSE dye dilution or ³H uptake) or cytokine production (e.g., IFN-γ production), it provides better insight in the capacity of memory T-cell responses to control infection.

Using this assay we also studied HCV-specific T-cell responses before and after acute HCV mono-infection in injecting drug users. The height of CD4+ T-cell responses in the MSM in this study were comparable to that in mono-infected injecting drug users that develop chronic HCV infection. Similarly, the transition of the HCV-specific CD4+ memory T-cell response from targeting Core to targeting nonstructural proteins during onset of infection was associated with a favourable outcome in both IDU and MSM. However, we believe it is difficult to make a comparison between these two groups with acute HCV infection. The mode of transmission is very different, and possibly also the
size of the inoculum. While injecting drug users get frequently (re-)exposed to HCV parenterally during sharing of needles and/or syringes, the mode of transmission in MSM is still unclear. Acute HCV in MSM has been associated with co-infection with ulcerative sexually transmitted diseases (e.g., lymphogranuloma venereum and syphilis) and with rough sexual techniques, suggesting that HCV may be transmitted sexually. Furthermore, HCV infection in MSM seems to occur mainly within the HIV-positive population, which further complicates comparisons. In this setting, the dramatic drop of the number of CD4⁺ T cells, especially in the mucosa of the gut, has also been suggested to play a significant role in the susceptibility to HCV in HIV-1 infected MSM.

A maintained CD4⁺ T-cell response is a prerequisite for prolonged HCV RNA suppression and protection from secondary infection. We observed CD4⁺ T-cell responses of comparable magnitudes in these MSM. However, in individuals who resolved HCV infection or temporarily suppressed HCV RNA a HCV-specific CD4⁺ T-cell response mainly targeted nonstructural proteins, and the individuals developing chronic HCV infection or HCV RNA relapse a relative strong CD4⁺ T-cell response against Core polyprotein was seen, as we have previously also shown in Ruys et al. and as was also reported by others. Qualitative differences in reactivity of CD4⁺ T cells against Core during acute infection may be caused by higher levels of Core-antigen presentation to CD4⁺ T cells, possibly because of higher quantities of Core-antigen in plasma. In addition, HCV Core is thought to play a role in modulating immune responses by affecting the function of virus-specific T cells. It has been reported that HCV-HIV co-infected patients have higher HCV-RNA plasma concentrations compared to HCV mono-infected patients, in theory leading to more Core-antigen presentation and potentially causing a stronger negative effect on the development of an effective T-cell response. An ineffective T-cell response may further facilitate HCV replication. Recent studies in HCV mono-infected and HCV/HIV co-infected patients suggest that early treatment of acute HCV is much more effective compared to treatment in a later and chronic phase. We observed disappointing results of early treatment of HCV infection in our two treated patients which is in line with another recent study in HCV/HIV co-infected patients. It has to be noted that in MSM IV HCV viral load in the first 10 weeks of infection was comparable to HCV viral load in MSM II and III. Therefore this individual might have been able to clear HCV spontaneously or temporarily suppress HCV RNA in the absence of treatment early after infection. In one of the treated individuals we were able to analyze T-cell responses during treatment. CD8⁺ T-cell responses became undetectable soon after the treatment was started in line with a recent study in which HCV-specific CD8⁺ T-cell responses also decreased with successful treatment and increased transiently with recrudescence of viremia in those who failed to achieve a sustained viral response. The small size and heterogeneity of these patients will not allow for firm conclusions, however, these longitudinal findings add to the limited data available on HCV-specific T-cell responses during acute HCV in HIV-1 co-infected persons and encourage the search to identify these patients to analyze larger groups. It is important to understand immunological factors associated with viral clearance, especially since HCV/HIV co-infection is associated with lower rates of HCV treatment success and with more complications during HCV treatment. Furthermore, the spread of HCV in HIV infected MSM is continuing, and is becoming a major public health problem.

Our data may suggest that an acute HCV infection in an HIV infected person can be at least temporarily suppressed in the presence of HCV-specific CD4⁺ T-cell response
targeting nonstructural proteins, despite low HCV-specific CD8⁺ T-cell responses. However, further research is needed in a larger group of patients to evaluate the role of HIV on HCV-specific T-cell responses in relation to the outcome of acute HCV infection.
Chapter 4.2
Immunology in acute HCV

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Chapter 4.3

HCV-specific CD4+ T-cell responses in HIV and HCV seroconverters are influenced by the sequence of HIV and HCV infection and outcome of previous HCV infection

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Submitted.
Abstract

Objective
To elucidate the role of HIV infection on HCV-specific CD4+ T-cell responses in acute HCV infection.

Design
We analyzed HCV-specific CD4+ T cells in 14 injecting drug users (IDU) from the prospective Amsterdam Cohort Study among drug users shortly after HCV seroconversion in the presence or absence of acute HIV infection. In addition, we analyzed the influence of HIV infection on pre-existing HCV-specific CD4+ T-cell responses in already HCV-infected IDU who subsequently acquired HIV.

Methods
HCV-specific CD4+ T-cell responses were analyzed after 12 days of expansion using recombinant HCV proteins. Interferon-γ was measured as read-out of effector function.

Results
CD4+ T-cell responses against nonstructural (NS) HCV proteins, especially T-cell responses against NS5, were higher among HCV mono-infected IDU who clear HCV infection (n=6) compared to IDU with persistent HCV viremia both in the presence (n=4) and absence of HIV co-infection (n=4). Interestingly, IDU with acute co-infection who were previously able to clear HCV mono-infection (n=2) had higher HCV-Core-specific CD4+ T-cell responses than those without evidence of previous exposure to HCV (n=2).

Conclusions
Both acute HCV/HIV co-infected IDU who were previously exposed to HCV and mono-infected IDU who developed chronic HCV infection tended to have higher responses to Core protein compared to IDU that clear HCV. Furthermore, the HCV-specific T-cell responses were also skewed to unfavourable Core in 3/5 IDU after HIV seroconversion. These results suggest that the effect of HIV infection on HCV-specific T-cell responses is not clear-cut, but is influenced by the sequence and outcome of previous HCV infection.
Introduction

HIV and hepatitis C virus (HCV) infection are both highly prevalent in injecting drug users (IDU) due to shared routes of transmission, and HIV/HCV co-infection is common.\(^1\,^2\) CD4\(^+\) T cells play an important role in controlling acute HCV infection in humans and have been shown to be crucial for HCV clearance in chimpanzees.\(^3\,^4\) High and broad HCV-specific CD4\(^+\) T-cell responses are associated with clearance of acute HCV mono-infection in humans.\(^5\,^8\) Cross-sectional studies have shown that HIV co-infection is associated with loss of control of HCV viremia,\(^9\,^11\) suggesting that HIV co-infection negatively influences HCV-specific T-cell responses. Indeed it has been shown that chronic HIV co-infection is associated with lower HCV-specific CD4\(^+\) T-cell responses in acute HCV.\(^12\) However, to our knowledge no studies have been done comparing HCV-specific CD4\(^+\) T-cell responses in IDU with acute HIV/HCV co-infection and in IDU with acute HCV mono-infection.

There are different assays one can use to measure antigen-specific T cells by fluorescence-activated cell sorting (FACS).\(^13\,^14\) One of the frequently used assays to measure effector function is intracellular cytokine staining (ICCS) directly ex vivo after short-term (6-hour) stimulation. Unfortunately, HCV-specific CD4\(^+\) T cell frequencies measured directly ex vivo are very low.\(^12\,^15\) Another frequently used assay is the carboxyl fluorescent succinimidyl ester (CFSE) dye dilution assay, with which proliferative capacity of antigen-specific T cells can be visualized by FACS analysis after 6-day stimulation with antigen. Furthermore, a previously described assay in which T cells were expanded for 12 days can be used to measure effector function after expansion. This assay has been shown to be a reproducible and sensitive assay for measuring Epstein-Barr virus-specific memory T-cell responses, and has also been used for detection of HCV-specific responses.\(^16\,^17\) In this paper we compared results from this T cell line-based assay with direct stimulation and expansion assays with a stimulation period comparable to CFSE dye dilution (i.e., six days).

To study factors associated with viral clearance and the influence of HIV co-infection, we examined HCV-protein-specific CD4\(^+\) T-cell responses using the T cell line based expansion assay shortly after acute HCV infection in the presence or absence of acute HIV infection in IDU with documented HCV seroconversion. To further explore the influence of HIV infection during chronic HCV infection, we also examined HCV-specific CD4\(^+\) T-cell responses longitudinally in HCV infected IDU who later acquire HIV infection.\(^17\)

Methods

Study population

From the prospective Amsterdam Cohort Studies among drug users (ACS)\(^18\), we have studied IDU with documented HCV infection and HCV seroconversion during follow up.\(^19\) HCV infection was estimated as the midpoint between a negative and a positive HCV-RNA test independent of the presence of HCV antibodies, documented over two consecutive visits (bDNA HCV 3.0 Bayer or HCV RNA assay by transcription-mediated amplification (TMA), Versant HCV RNA Qualitative assay, Bayer Diagnostics). Date of HIV or HCV seroconversion (i.e., appearance of antibodies to HIV, EIA; Abbot
Laboratories, confirmation by Western blot (Diagnostic Biotechnology, Herent, Belgium) or appearance of antibodies to HCV, EIA 3.0 Abbot Laboratories, respectively) was estimated as the midpoint between the last negative and first positive HIV or HCV antibody test.

We analyzed HCV-specific CD4⁺ T-cell responses in 14 IDU with acute HCV infection in the presence (n=4) or absence (n=10) of acute HIV infection. The IDU were divided into three groups: IDU who became infected with HIV and HCV at approximately the same time and who all developed chronic HCV viremia (group co-CH, n=4), HCV mono-infected IDU who cleared HCV (group mono-CL, n=6) and HCV mono-infected IDU who developed chronic infection (group mono-CH, n=4). HCV clearance was defined as two consecutive visits with negative qualitative HCV-RNA assays after the onset of HCV infection. In group co-CH, two IDU previously cleared HCV without HIV co-infection (CO-1 and CO-2) and two IDU had acute co-infection without evidence of an earlier HCV infection (CO-3 and CO-4). None of these IDU were treated with antiretroviral therapy or anti-HCV treatment during acute HCV infection.

To study the influence of HIV infection on pre-existing HCV-specific memory CD4⁺ T-cell responses, we studied five IDU who acquired HCV infection a considerable period before HIV seroconversion, and who had PBMC available before and after HIV seroconversion. Characteristics of the study population are shown in Tables 4.3.1A and 4.3.1B.

**Direct stimulation**

To measure effector function directly *ex vivo*, cryopreserved PBMC were thawed and stimulated for six hours in the presence of overlapping HCV peptide pools, corresponding to the Core/E1/E2/p7, NS2, NS3, NS4, and the NSSAB regions of the HCV genome (peptides were 18-mer peptides, overlap 11 amino acids; kindly provided by NIH AIDS Research Reagent Program).

| Group | Number of IDU | Median age (range)* | Median CD4 count (range)* | HCV genotype (%)# | Median log HCV viral load (copies/ml)# |
|-------|---------------|---------------------|---------------------------|-------------------|---------------------------------------|
| CO    | 4             | 25 (27-32)          | 830 (810-980)             | 1a (75%), 3a (25%)| 2.7 x 10⁵                               |
| CL    | 6             | 31 (23-38)          | 1115 (600-1580)           | 1a (33%)          | 1.9 x 10³                               |
| CH    | 4             | 26 (21-33)          | 750 (590-1550)            | Undetectable/ND (67%) | 3.8 x 10⁵                               |

*: at time of acute HCV infection, #: at time of T cell analysis, ND: not done.
HCV-specific T-cell expansion

To expand HCV-specific memory T cells, cryopreserved PBMC were thawed and subsequently expanded during a period of six or twelve days using recombinant HCV proteins Core (C22-3), NS3 (C33c), NS4 (C100-3) and NS5A/B (NS5) (2 μg/ml; kindly provided by M. Houghton and K. Crawford, Chiron) in the presence of IL-2 (180 IU/ml). After expansion, cells were pooled, washed, counted and rested overnight. On day 7 or 13, cells were restimulated using peptide pools to assess effector function (i.e., IFN-γ production).

Detection of IFN-γ producing HCV-specific T cells after (re-)stimulation

As a read-out of effector function, IFN-γ-producing CD4+ T cells were enumerated using intracellular cytokine staining. Briefly, PBMC were stimulated for six hours with HCV Core/E1/E2/p7, NS2, NS3, NS4 and NS5 peptide pools (2 μg/ml) and both αCD28 (2 μg/ml) and αCD49d (2 μg/ml) as co-stimuli. After one hour 1:1000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA) was added. As negative control, PBMC were stimulated with medium and co-stimulation alone. As positive control PBMC were stimulated with 10 ng/ml PMA, 2 μg/ml ionomycin and co-stimulation. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD4, CD8 and IFN-γ (BD). After fixation (Cellfix, BD) min. 20,000 events were acquired on a FACS Calibur or LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using CELLQuest software (BD).

Results were expressed as the percentage of IFN-γ producing CD3+CD4+ T cells, which was calculated by subtracting the percentage of IFN-γ producing T cells in medium from that in peptidepool restimulated conditions. To enable comparison of IDU with different CD4+ T cell counts, we did not only compare the percentages of IFN-γ-producing CD4+ T cells, but we also determined the proliferation rate, which we defined as the total number of cells recovered after six or twelve days of expansion divided by the total number of cells put in to culture at day 0 (Table 4.3.2). Subsequently we calculated the number of HCV-specific effector T cells as the product of the percentage of IFN-γ-producing T cells and the proliferation rate. For easier understanding of this number, we expressed the number of IFN-γ producing T cells per million PBMC. This number (as shown in Table 4.3.2), is therefore a combination of (memory) cells initially present and the ability of these cells to survive, proliferate and differentiate to (IFN-γ-producing) effector T cells during the 12-day culture period. It has been shown that these cells were inversely correlated with antigen burden in case of EBV infection.¹⁷
Comparison of different assays for measuring HCV-specific CD4⁺ T cells in two IDU (CL2 and CH2) with different outcome of primary HCV infection, clearance vs. persistent viremia. After short term stimulation (i.e., six hours) or short time culture (i.e., six days) no HCV-specific T-cell responses can be detected. However, after 12-day expansion there is clear proliferation and effector function in response to HCV proteins.

| Antigen | CH2 | IFN-γ producing CD4⁺ T cells* | Number |
|---------|-----|-------------------------------|--------|
| Core    | 0.00| 0.00                          | 0.03   |
| NS3     | 0.00| 0.00                          | 0.12   |
| NS4     | 0.00| 0.00                          | 0.08   |
| NS5     | 0.00| 0.00                          | 0.03   |

| Antigen | CL2 | IFN-γ producing CD4⁺ T cells* | Number |
|---------|-----|-------------------------------|--------|
| Core    | 0.00| 0.00                          | 0.04   |
| NS3     | 0.00| 0.00                          | 0.11   |
| NS4     | 0.01| 0.00                          | 0.01   |
| NS5     | 0.00| 0.00                          | 0.14   |

*: Expressed as percentage IFN-γ producing CD3⁺CD4⁺ T cells of total number of PBMC. Data for six hour stimulation with NS2 peptide pool not shown, no recombinant protein available for NS2.

Statistical analysis

All statistical tests were two-sided. A p-value ≤0.05 test was considered to be statistically significant. Non-parametric tests (Mann-Whitney (two groups) or Kruskal-Wallis (>2 groups)) were used to test differences between groups. All statistical analysis were performed using SPSS software version 15.

Results

General characteristics

To study factors associated with viral clearance and the influence of HIV infection hereon, we examined HCV-specific CD4⁺ T-cell responses in 14 IDU with acute HCV infection in the presence or absence of acute HIV infection. The median interval between first HCV-RNA positive visit and visit at which PBMC were analyzed was 4.3 months (range 0-32.3 months). Six IDU cleared HCV and eight stayed chronically HCV infected. The three groups were comparable in age, CD4 count and genotype at the estimated moment of HCV infection. However, HCV-RNA load in group mono-CL was lower than in the other two groups (p=0.011) (Table 4.3.1A).

Furthermore, we examined HCV-specific CD4⁺ T-cell responses in five HCV-infected IDU before and after HIV seroconversion to evaluate the effect of HIV infection on earlier established HCV-specific CD4⁺ T-cell responses. The median interval between HCV and HIV infection was 2.4 years, 3/5 IDU had chronic HCV infection and were infected with HIV thereafter, 2/5 IDU first cleared HCV and got re-infected with HIV/HCV at approximately the same time and subsequently developed chronic co-infection (Table 4.3.1B). At the time of HIV infection all had HCV genotype 1 infection, except IDU CO-2.
who was infected with HCV genotype 3a. CD4 counts were all ≥450/mm³ at the moment of acute HCV infection.

**Comparison of different assays to measure HCV-specific T cells**

To compare sensitivity of several assays to measure virus-specific proliferative and effector responses, we performed 6-hour, 6-day and 12-day stimulation assays. Direct *ex vivo* (6-hour stimulation) or after expansion (6 or 12 days) cells were restimulated to assess effector function with IFN-γ as read-out. Due to limited availability of PBMC, these experiments were performed in two IDU (CL-2 and CH-2).

As shown in Table 4.3.2 and in line with previous experiments, after 6-hour stimulation or 6-day expansion and subsequent restimulation no HCV-specific response could be detected in these IDU as the percentage of IFN-γ-producing CD4⁺ T cells in the peptide stimulated conditions did not exceed the percentage of IFN-γ producing CD4⁺ T cells in unstimulated conditions. However, after 12 days we could clearly distinguish an IFN-γ-producing CD4⁺ T cell population (range 0.03-0.14% of total PBMC) which is HCV-specific, since there was no IFN-γ production after restimulation with medium and co-stimulation alone (Table 4.3.2). Additional control experiments (including culture and restimulation of cord blood and PBMC from healthy blood-bank donors with HCV proteins, and culture with HCV-NS3 protein and restimulation with HCV-NS4 peptide pool) showed that the observed IFN-γ production after 12 days in HCV-infected individuals indeed was HCV specific (data not shown). Because we show here that this assay is capable of detecting HCV-specific CD4⁺ T cells in individuals that do not show HCV-specific T-cell responses after shorter stimulation and/or culture, we have used this assay to examine the effect of HIV co-infection on the quality and quantity of HCV-specific CD4⁺ T-cell responses in IDU.

Next to IFN-γ production after 12 days, we calculated the proliferation rate (see methods section). This estimate not only depends on the true proliferation of cells, but also on death of cells. At day 0, the proliferation rate is per definition one as the number of cells put into culture at that moment is the same as the number of cells recovered. After six days the median proliferation rate decreased to 0.26 and 0.22 for IDU CL-2 and IDU CH-2, respectively (Figure 4.3.1). After 12 days the proliferation rate had increased again. Interestingly, the proliferation rate in IDU CL-2 --able to spontaneously clear HCV-- increased to 0.74 in contrast to IDU CH-2 --not able suppress HCV-- in which the proliferation rate increased to 0.52 (Figure 4.3.1). These data indicate that most proliferation took place after the sixth day of culture (Figure 4.3.1).

**Comparison of HCV-specific CD4⁺ T-cell responses in acute HCV infection in the presence or absence of acute HIV infection**

Using the assay based on 12-day expansion and subsequent restimulation, we measured HCV-specific T-cell responses in three groups of IDU. The total HCV-specific T-cell response was higher in IDU that cleared HCV infection (mono-CL, median 9,474 IFN-γ producing CD4⁺ T cells/million PBMC input) when compared to IDU with chronic HCV infection (mono-CH, median 2,223 IFN-γ producing CD4⁺ T cells/million PBMC input) or group IDU with HIV and HCV infection at approximately the same time (co-CH, median 2,749 IFN-γ producing CD4⁺ T cells/million PBMC input), although this did not reach statistical significance (Figure 4.3.2A). Interestingly, only 1/4 co-infected and none of the chronically infected IDU had responses against more than three proteins, in contrast to IDU who cleared HCV, of whom 4/6 had responses against more than three
proteins (Figure 4.3.2B). Furthermore, responses against Core protein tended to be lower in mono-CL than in mono-CH (p=0.087). All IDU in group mono-CL had higher T-cell responses against nonstructural proteins than against Core protein in the same IDU (Figure 4.3.1A). In contrast, in group mono-CH the responses against nonstructural proteins were lower than responses against Core protein in the same IDU (Figure 4.3.1A). Responses against NS3 protein tended to be higher in co-CH than in mono-CH (p=0.076). Responses against NS3 protein tended to be higher in mono-CL than in mono-CH (p=0.062), but not higher than in co-CH. Median responses against NS4 protein were higher in mono-CL then in the other groups, although this was not statistically significant. Responses against NS5 protein were significantly lower in co-infected IDU and in mono-CH than in mono-infected IDU who eventually clear HCV infection (mono-CL) (p=0.010 and p=0.019, respectively) (Figure 4.3.1B). Thus, HCV-specific CD4⁺ T-cell responses against nonstructural proteins tended to be higher in mono-CL when compared to mono-CH and co-CH. And of note, IDU with acute HIV/HCV co-infection showed no to very limited HCV-specific responses to NS5.

In Figure 4.3.3a, the individuals who contract HIV and HCV at approximately the same time (CO-1, CO-2, CO-3 and CO-4) are shown in more detail including their injection drug use. It can be seen that injection drug use and HIV and HCV seroconversion coincide. In the second panel, log10 HCV and HIV viral load are shown, HCV load is always higher than HIV load. In the third panel, the decline of the CD4 count is shown in relation to the moment of HIV seroconversion. The CD4 count drops to ≤350 cells/mm³ in varying time periods (range 0.62-10.4 years). In the lowest panel HCV-specific CD4⁺ T-cell responses are shown.

![Figure 4.3.1](image_url)  
**Figure 4.3.1** Proliferation rate as measured directly *ex vivo* and after six or twelve days culture in the presence of HCV pepitope pools (*ex vivo*) or proteins (for 6-day or 12-day culture) in patients CL2, who recovers spontaneously from HCV infection and CH2, who develops persistent HCV viremia. The proliferation rate was calculated as the number of cells recovered out of culture on day 6 or 12 divided by the number of cells put into culture on day 0. Therefore the proliferation rate for direct stimulation is per definition 1.
Figure 4.3.2A Total number of HCV-protein-specific IFN-γ producing CD4⁺ T cells/million PBMC input as measured by intracellular cytokine staining after 12 days culture with IL-2 and HCV recombinant proteins in injecting drug users (IDU) with acute HCV infection in the presence or absence of acute HIV infection. Abbreviations: CO=IDU with HIV and HCV infection at approximately at the same time; CL=IDU with acute HCV mono-infection who clear HCV infection; CH=IDU with acute HCV mono-infection who develop chronic HCV infection.

Figure 4.3.2B Number of HCV-protein specific IFN-γ producing CD4⁺ T cells/million PBMC input as measured by intracellular cytokine staining after 12 days culture with IL-2 and HCV proteins in injecting drug users (IDU) with acute HCV infection in the presence or absence of acute HIV infection. Abbreviations: CO=IDU with HIV and HCV infection at approximately at the same time; CL=IDU with acute HCV mono-infection who clear HCV infection; CH=IDU with acute HCV mono-infection who develop chronic HCV infection.
Interestingly, CO-1 and CO-2 were previously able to clear HCV infection in the absence of HIV infection (clearance indicated with *). However, they were not able to clear HCV infection again in the presence of HIV infection. After HIV infection, we observed higher Core responses (2,629 and 934 IFN-γ producing CD4+ T cells/million PBMC input) compared to CO-3 and CO-4 (0 and 245 IFN-γ producing CD4+ T cells/million PBMC input; Figure 4.3.2B). In contrast, despite the high responses directed against NS3 observed in CO-3 and CO-4, these IDU were not able to control HCV viremia (Figure 4.3.2B and 4.3.3). These data suggest that the influence of HIV co-infection on the HCV-specific T-cell response depends on the order and outcome of precedent HCV infection(s).

HCV-specific CD4+ T-cell responses before and after HIV infection

To study the direct influence of acute HIV co-infection on HCV-specific CD4+ T-cell responses, we studied T-cell responses longitudinally in five IDU. Two IDU from group co-CH were included, CO-1 and CO-2, and three additional IDU, CO-5, CO-6 and CO-7 (Figure 4.3.3A and 4.3.3B). The latter three IDU became HIV infected after having contracted earlier chronic HCV infection. PBMC ranging from 1.9 to 7.6 months before and 1.9 to 13.3 months after HIV seroconversion were analyzed. In IDU with a prior chronic HCV infection, we observed lower HCV-specific CD4+ T-cell responses (537, 623 and 1174, respectively) compared to IDU who were able to clear an earlier HCV infection (3114 and 3745 CD4+ T cells, respectively (Figure 4.3.1C). Interestingly, in IDU previously able to clear HCV infection, we observed higher responses against Core protein after HIV infection, suggesting that HIV infection negatively influences HCV-specific CD4+ T-cell responses by skewing the response away from nonstructural proteins. However, in acute co-infection without evidence of an earlier HCV infection, we do not observe this skewing of the HCV-specific T-cell response towards Core protein, suggesting that the effect of HIV is not straightforward, but is influenced by the host’s history of previous HCV infection. After HIV seroconversion we observed a decline of responses against nonstructural proteins in 4/5 IDU and a decline in overall height of HCV-specific CD4+ T-cell responses in 3/5 IDU (Figure 4.3.3).
Figure 4.3.3 IDU with documented HIV and HCV seroconversion in whom HCV-specific CD4⁺ T-cell responses were studied longitudinally. Grey vertical line represents estimated HCV infection, grey dotted vertical line represents estimated HIV seroconversion. In the upper panel injection drug use is represented by gray bars. In the second panel HIV viral load (in copies/ml, indicated by dotted line and grey dots) and HCV viral load (in IU/ml, indicated black line and white dots) are shown on a log scale. In the third panel CD4 count is shown (10⁸ cells/ml) over calendar time. In the last row CD4⁺ T-cell responses as measured by 12-day culture and subsequent restimulation are shown.
Figure 4.3.3 Part 2.
### Table

|                | CO5       | CO6       | CO7       |
|----------------|-----------|-----------|-----------|
|                | 19927     | 18783     | 14956     |

### Diagram

![Graphs showing HCV-specific T cells in HIV-infected MSM](image)

**Figure 4.3.3 Part 3.**
Discussion

High and broad HCV-specific CD4\(^+\) T-cell responses during acute HCV infection have been associated with viral clearance.\(^6,8,21,22\) We also observed that mono-infected IDU who cleared HCV infection tended to have higher CD4\(^+\) T-cell responses and responses against a larger number of proteins than IDU that develop chronic HCV viremia in the presence or absence of HIV infection. Interestingly, most IDU in this study that cleared HCV infection had NS3-specific memory CD4\(^+\) T-cell responses, while only one IDU from the mono-CH group had a NS3-specific memory CD4\(^+\) T-cell response. NS3 has been described as highly conserved and immunodominant and responses against NS3 are associated with viral clearance;\(^23-25\) however two IDU that were simultaneously infected with HCV and HIV were not able to clear HCV despite high NS3 responses. In addition, we observed a striking absence of T-cell responses against NS5 protein --specifically targeted in IDU that cleared infection-- in IDU with acute HIV/HCV co-infection that develop chronic HCV infection and also in mono-infected IDU that develop chronic HCV viremia. NS5 is a variable region of HCV, and development of chronic infection might be due to the inability to raise T-cell responses against NS5. A possible explanation for this may be that the primary responses against NS5 cannot be maintained and chronic HCV infection develops because of escape mutations away from the original NS5 sequence.\(^26\)

Higher responses against Core protein were observed in acute phase of infection in IDU that were not able to spontaneously control HCV replication. This suggests that responses against Core are either associated with a less favourable outcome of infection or with exposure to higher levels of Core. HIV co-infection is associated with higher HCV viral load and HCV viral load is associated with the amount of Core protein in serum.\(^15,24,27-29\) Also in IDU previously able to clear HCV, we observe an increase of Core response and a decrease of responses against nonstructural proteins after HIV seroconversion indicating that HIV infection influences HCV-specific T-cell responses in an unprofitable way. In IDU that were chronically HCV infected before HIV seroconversion, we did not observe similar kinetics. This could be due to repeated exposure to HCV at the moment of infection with HIV, evoking HCV-specific memory CD4\(^+\) T-cell responses next to development of new strain-specific T-cell responses. Alternatively, virus characteristics might explain these findings.

In contrast to the findings by Lauer et al., who describe the absence of proliferative CD4\(^+\) T-cell responses against HIV and HCV in chronically co-infected individuals,\(^30\) we observed low --but measurable-- T-cell responses in acutely co-infected patients who later develop chronic infection. This may be explained by the fact that in contrast to Lauer et al. we analyzed PBMC shortly after HCV infection and not in the later course of chronic infection, when it has been shown that both proliferative responses and the capacity to secrete IFN-\(\gamma\) are reduced.\(^30\) Even more, ongoing antigenic exposure has been shown to diminish antigen-specific T-cell responses and for example during HAART the HIV-specific proliferative responses were shown to increase again.\(^31,32\) An alternative explanation may be a difference in sensitivity of the assay used. We show that the used assay can pick up HCV-specific responses in individuals that do not show IFN-\(\gamma\) production after direct ex vivo stimulation or after restimulation following six days culture. The difference in sensitivity of the assays can be explained by the assumption that cells that are not HCV-specific will die in the first days of culture, while HCV-specific cells will survive and start to proliferate. After addition of extra antigen on day 6 in the
presence of IL-2 HCV-specific cells will expand further and differentiate to an effector phenotype capable of IFN-γ production upon re-encounter with antigen. Although our study was performed in only a limited number of patients, they present an exceptional chance to study the role of HCV-specific CD4⁺ T cells in acute HCV infection, because of the prospective sampling which was independent of clinical symptoms. This is very different from patients sampled after presentation at a clinic weeks to months after the start of clinical symptoms, which could introduce a delay in sampling and selection bias, since clinical symptoms have been associated with a more vigorous immune response. Indeed, Thimme et al. described five health care workers that were sampled prospectively from the moment of exposure and found that the symptomatic infected patient who cleared HCV had vigorous T-cell responses.²² In conclusion, in this small group of IDU we show that HCV mono-infected IDU who clear HCV infection have higher memory CD4⁺ T-cell responses against NS5 protein compared to IDU that develop chronic HCV infection in the presence or absence of HIV during acute infection. Furthermore, we show that the effect of HIV infection on HCV-specific CD4⁺ T cells in IDU is dependent on the history and outcome of previous HCV infection. To enable a clearer understanding of the complex interaction between HIV and HCV and their corresponding immune responses, future studies should address this issue with a focus on prospectively identified infected individuals --preferably with information on previous exposure to HCV and/or HCV infection-- to minimize selection bias towards those with clinical symptoms.
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Chapter 4.4

Detectable HCV-specific T cells in men having sex with men years before manifest HCV infection

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Manuscript in preparation.
Introduction

Since 2000 there have been reports on outbreaks of acute hepatitis C virus (HCV) in HIV-infected men having sex with men (MSM).\textsuperscript{1-4} Phylogenetic analyses indicate that there has been introduction of multiple HCV strains, and that the introductions have occurred between 1995 and 2000.\textsuperscript{1,2} These ongoing epidemics are becoming a major public health problem.\textsuperscript{5} Adaptive immunity plays a major role in spontaneous viral clearance of HCV, however the exact correlates of protection are still unknown.\textsuperscript{6} HIV co-infection and probably the weakening of the immune system, is associated with loss of control of viral load and with lower HCV-treatment success rates.\textsuperscript{7,8} Here, we examined HCV-specific CD4\textsuperscript{+} T-cell responses in three HIV-infected MSM before HCV-antibody seroconversion and subsequent chronic HCV infection took place. Unexpectedly, HCV-specific T-cell responses were already present years before HCV seroconversion in all three MSM, indicating that these individuals had been exposed to HCV, but did not get (chronically) HCV infected until years later. These findings have implications for our understanding of correlates of protection and for future vaccine development.

In risk groups with parenteral exposure risk to HIV and HCV (like drug users (DU)), HCV usually precedes HIV infection. In the recently described HCV outbreaks among MSM mainly HIV-infected individuals are affected. HCV-specific T-cell responses seem to develop differently in the context of HIV co-infection, and it has been suggested that the development of the T-cell response is hampered by HIV co-infection.\textsuperscript{9} Previously, we and others have reported on HCV-specific T-cell responses in HIV-uninfected DU before actual HCV infection occurred, suggesting previous exposure to HCV.\textsuperscript{10-13} We were interested whether such exposure without apparent infection also occurs in MSM before they acquire HCV infection through sexual transmission. This would be unexpected, since the HCV prevalence and incidence in injecting DU populations is very high, while --until recently-- in MSM prevalence and incidence remained low.\textsuperscript{14} Therefore, the probability of exposure to HCV per ‘risky’ behaviour is much higher in DU than in MSM (e.g., sharing needles or syringes vs. unprotected high-risk sexual behaviour) and therefore also the probability of identifying multiple exposed uninfected individuals. Furthermore, most individuals were already HIV-infected at time of HCV seroconversion and it has been shown that HIV co-infection negatively influences HCV-specific T-cell responses.\textsuperscript{9,15,16}

Materials and Methods

Study population

In the ACS among MSM, eight HCV seroconverters in HIV-infected MSM were identified before 2003.\textsuperscript{1,17} From these men, three had cryopreserved peripheral blood mononuclear cells (PBMC) available before HCV seroconversion (MSM 1, 2, and 3).

HCV antibody, RNA and genotyping

HCV seroconversion was defined as occurrence of HCV antibodies in a previously negative individual. HCV antibodies were detected using a third generation EIA system (AxSym HCV version 3.0; Abbott, Wiesbaden, Germany). RNA detection and genotyping based on phylogenetic analysis was performed as described in Van de Laar et al.\textsuperscript{1}
HCV peptide pools

Synthetic peptides (14-18-mers, overlapping by 11 amino acids; Mimotopes, Australia) were dissolved in DMSO and then pooled. The peptides were divided in six pools, corresponding to core, NS2, NS3, NS4, NS5A and NS5B regions of HCV. The peptides were dissolved in such a way that the final concentration of each individual peptide was 1 mg/ml in the pool.

HCV-specific T cell expansion

Since the frequency of HCV-specific T cells directly ex vivo is very low, we use an assay for detection of HCV-specific T-cell responses based on expansion of these cells for 12 days in the presence of recombinant interleukin-2 (IL-2) and peptide pools. In short, cryopreserved PBMC were thawed, washed and counted. After that cells (2×10^6 cells/ml) were put into culture in a 96-wells plate for 12 days in the presence of overlapping HCV peptide pools and IL-2 (360 IU/ml). On day six extra peptide pool was added. On day two, six and eight extra IL-2 was added.

Detection of IFN-γ producing HCV-specific T cells after (re-)stimulation

IFN-γ-producing CD4^+ T cells were enumerated using intracellular cytokine staining (ICCS). Briefly, after 12-day expansion cells were pooled, washed and counted. PBMC were stimulated for six hours with HCV Core, NS2, NS3, NS4, NS5A and NS5B peptide pools (2 μg/ml) and αCD28 (2 μg/ml) and αCD49d (2 μg/ml) as co-stimuli. After one hour 1:1,000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA) was added. As negative control, PBMC were stimulated with medium and co-stimulation alone. As positive control PBMC were stimulated with 10 ng/ml PMA, 2 μg/ml ionomycin and co-stimulation. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with fluorescently labelled monoclonal antibodies specific for CD3-pacific blue, CD4-PE-Cy7, CD8-APC-Cy7 and IFN-γ-FITC (CD3, CD4 and CD8 monoclonal antibodies from eBioscience, IFN-γ-FITC from BD). After fixation (Cellfix, BD) at least 25,000 events were acquired on an LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using FACSDiva software (BD).

Results were expressed as the percentage of IFN-γ producing CD4^+ or CD8^+ T cells, which was calculated by subtracting the percentage of IFN-γ producing T cells in medium from that in peptide pool restimulated conditions. Next to the percentage of IFN-γ-producing T cells, we also determined the proliferation rate, which we defined as the total number of cells recovered after 12 days of expansion divided by the total number of cells put in to culture at day 0. Subsequently we calculated the number of HCV-specific effector T cells as the product of the percentage of IFN-γ-producing T cells and the proliferation rate. For easier understanding of this number, we expressed the number of IFN-γ-producing T cells per million PBMC input. This number is a combination of (memory) cells initially present in the PBMC pool and the ability of these cells to survive, proliferate and differentiate to (IFN-γ-producing) effector T cells during the 12-day culture period.

The observed response after expansion and restimulation is HCV-specific since we do not observe IFN-γ production after restimulation with medium. Furthermore, when expanding PBMC from a healthy (HCV-negative) blood bank donor or cord blood, expansion is less and we do not observe IFN-γ production. Also when expanding PBMC from HCV-infected individuals using peptide pool corresponding to the NS3 region of the
HCV genome and restimulating with NS4 peptide pool no IFN-γ production is observed (data not shown).

Results

In the Amsterdam Cohort Study among MSM, eight individuals with HCV seroconversion during follow up were identified by Van de Laar et al. Of these eight individuals, three had PBMC available before HCV seroconversion. To see whether HCV-specific T-cell responses were present in these men, we measured memory function of HCV-specific T cells approximately one year before HCV seroconversion. General characteristics of the MSM are shown in Table 4.4.1. The median age was 33 years, and the median duration of HIV infection at the time of HCV infection 5.3 years. Although the men were HIV-infected for some time, CD4+ T cell numbers were relatively high at time of HCV seroconversion (median 580 cells/ml). MSM 1 and 2 were on different regimens of HAART and HIV-1 viral load was undetectable in MSM 2 (Table 4.4.1). The presence of HCV-specific CD4+ and CD8+ T-cell responses was studied using a sensitive assay for detection of antigen-specific responses with a low frequency ex vivo. This assay is based on in vitro expansion of antigen-specific T cells in the presence of overlapping peptide pools and IL-2 and subsequent restimulation with peptide pools (see methods section).

To our surprise, we observed both CD4+ and CD8+ HCV-specific T-cell responses in all three MSM. In Figure 4.4.1, a representative dotplot of HCV-specific IFN-γ production after 12 days expansion is shown.

![Figure 4.4.1](image_url)

Figure 4.4.1 Representative dot plot of IFN-γ production after restimulation with Core peptide pool. HCV-specific T cells were first expanded for 12 days in the presence of overlapping HCV peptide pools and interleukin-2. After that cells were pooled, washed, counted and rested overnight. On the next day cells were restimulated with co-stimulation and a. medium, b. PMA/ ionomycin or c. corresponding peptide pool.
Table 4.4.1 Univariate associations between general characteristics, drug use characteristics, sexual risk behaviour characteristics, and HIV and HCV seroconversion among DU in the ACS.

| HCV genotype | Last HCV RNA negative sample | First HCV RNA positive sample | Estimated date of seroconversion | Time of sampling before first positive sample (months) | Age at first positive sample (years) | Duration HIV infection (years) | CD4 count (cells/ml) | Lowest CD4 count during follow up (cells/ml) | HIV viral load (copies/ml) |
|---------------|-----------------------------|-------------------------------|--------------------------------|-------------------------------------------------------|------------------------------------|----------------------------|------------------|---------------------------------------------|--------------------------|
| MSM 1 4d      | 13-8-2001                   | 4-2-2002                      | 8-11-2001                      | 9.10-2000                                              | 15.9                               | 41                        | > 9.3 (prev pos) | 420                                         | 200                      | 33319                                   |
| MSM 2 3a      | 2-10-2001                   | 21-1-2002                     | 26-11-2001                     | 26-3-2001                                              | 9.9                                | 33                        | 5.3 (sc)        | 610                                         | 210                      | <50                                     |
| MSM 3 1b      | 8-5-2001                    | 20-8-2001                     | 29-6-2001                      | 4-4-2000                                               | 16.5                               | 33                        | 3.3 (sc)        | 580                                         | 420                      | 35000                                   |
Restimulation with Core peptide pool or PMA/ionomycin and co-stimulation (α-CD28 and α-CD49d) reveals clear IFN-γ production by both CD4\(^+\) and CD8\(^+\) T cells after 12 days expansion, whereas there is no IFN-γ produced after restimulation with medium and co-stimulation. Although there is a large variation in the magnitude of both CD4\(^+\) (MSM 1: 1,046; MSM 2: 8,088; and MSM 3: 1,243 IFN-γ producing CD4\(^+\) T cells/million PBMC) and CD8\(^+\) T-cell responses (MSM 1: 1,319; MSM 2: 14,133; and MSM 3: 643 IFN-γ producing CD8\(^+\) T cells/million PBMC) (Figure 4.4.2a and 4.4.2b), the CD4\(^+\) T-cell response in MSM 1 and 3 is comparable to the response after seroconversion in 4 HIV-infected MSM with acute HCV co-infection we described earlier (range 1527-3723, Figure 4.4.3).\(^{18}\)

In contrast, in MSM 2 we observed a much higher response that is comparable to HCV-specific T-cell response in HIV-uninfected individuals with acute HCV (range 93-74197 IFN-γ producing CD4\(^+\) T cells/million PBMC).\(^{10}\)

Interestingly, we observed high Core-specific CD4\(^+\) T-cell responses in MSM 1 and MSM 2 (37.2% and 32.9% of the total HCV-specific CD4\(^+\) T-cell response, respectively), although these responses have been associated with viral persistence after HCV seroconversion. Possibly this Core-specific response is triggered by repeated exposure to HCV. Unfortunately we do not have additional information on sexual risk behaviour (as a proxy for continuing exposure to HCV) in these MSM.

Figure 4.4.2  A: HCV-specific CD4\(^+\) T-cell responses expressed as the number of IFN-γ producing CD4\(^+\) T cells/million PBMC input.
B: HCV-specific CD8\(^+\) T-cell responses expressed as the number of IFN-γ producing CD8\(^+\) T cells/million PBMC input.
HCV-specific T cells in HIV-infected MSM

Figure 4.4.3  Total HCV-specific CD4⁺ T-cell responses expressed as the number of IFN-γ producing CD4⁺ T cells/million PBMC input in relation to HCV-specific CD4⁺ T-cell responses expressed as the number of IFN-γ producing CD4⁺ T cells/million PBMC input from other studies. HIV-infected MSM with acute HCV infection earlier described in Van den Berg et al., injecting drug users with acute HCV infection earlier described in Ruys et al.

Discussion

HCV-specific T-cell responses have been reported previously in persistently antibody and RNA negative individuals that were exposed to HCV sexually. Also HIV-specific T-cell responses before infection have been described in MSM and Kenyan female sex workers with high-risk sexual behaviour who appeared resistant to HIV infection. We can only speculate on the reason why the enormous increase in HCV prevalence in HIV-infected MSM occurred now (recently an HCV prevalence of 18% was found in HIV-infected MSM in a cross-sectional study from the Amsterdam outpatient clinic for sexually transmitted infections), while HCV has always circulated at a low level in the MSM population. There are different potential explanations for the recent outbreaks of HCV, and most likely it has been a combination of the following options. Biological explanations include the simultaneous increase in prevalence of ulcerative sexually transmitted infections (e.g., lymphogranuloma venereum (LGV), syphilis) which might facilitate HCV transmission by breaking down the mucosal barrier of the gut. It has also been described that shortly after HIV infection, CD4⁺ T cells are depleted especially in the gut. Furthermore, introduction of more virulent HCV strains or HCV strains that are more easily transmitted sexually might contribute to the rapid spread of HCV, although this seems less likely since there have been introductions of multiple strains at
approximately the same point in time. In addition, a change in risk behaviour might contribute to the increased incidence of HCV. Interestingly, some HIV-infected individuals have a very slow HCV antibody seroconversion, and HCV-specific T cells might be triggered long before HCV antibody seroconversion. Although most individuals described by Thomson et al. had detectable levels of HCV viremia, there was an association between height of viremia and time to seroconversion with individuals with lower viral load taking longer to seroconvert. So it is possible that some individuals in our study had very low level HCV viremia --as has been described for HIV in high-risk seronegative MSM-- inducing an antigen-specific T-cell response but not (yet) an antibody response. Larger cohorts of HIV-infected individuals at risk for HCV infection are needed to further disentangle factors associated with protection from HCV and the role of HCV-specific T-cell responses before HCV-antibody seroconversion. Furthermore, identification of additional patients like those described here may enhance our understanding on why the outbreak of sexually transmitted HCV occurred since 2000.
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Chapter 5

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Skewing of hepatitis C virus (HCV)-specific CD4+ T cells to Core protein is associated with the presence of HCV RNA

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Submitted.
Abstract

Individuals with a HCV-specific T-cell response mainly directed against Core protein seem more likely to develop chronic HCV infection. We hypothesized that persistent exposure to HCV by continuing risk behaviour leads to higher exposure to Core protein and may skew the HCV-specific T-cell response towards Core. To test this, we studied HCV-specific T-cell responses in HCV-seropositive drug users (DU) including HCV-RNA+ (n=10) and HCV-RNA- (n=8) injecting DU with a high frequency of injection drug use and sharing of needles and HCV-RNA+ DU that denied ever-injecting (n=9). HCV-specific CD4+ T-cell responses were measured using a sensitive assay based on expansion of specific memory T cells by HCV recombinant proteins and interleukin-2 (IL-2) and measuring interferon (IFN)-γ production after restimulation. The total HCV-specific CD4+ T-cell response was lower in never-injecting DU compared to both RNA+ and RNA- injecting DU (p=0.034). In addition, the response against nonstructural (NS) proteins was higher in injecting DU compared to never-injecting DU (p=0.045). Interestingly, the percentage of individuals with a CD4+ response directed against Core was lower in individuals without detectable HCV RNA (0%) than in those with HCV RNA (40-44%) (p=0.049). In conclusion, ongoing injecting risk behaviour is associated with higher overall CD4+ HCV-specific T-cell responses, but detectable HCV RNA is associated with a higher T-cell response directed against Core protein.
Exposure to HCV and Core-specific T-cell responses

Introduction

Hepatitis C virus (HCV) is mainly transmitted via infected blood. After acute infection approximately 60-80% of individuals develop persistent viremia, which can lead to liver-related morbidity and mortality after years of chronic infection. Recently, we and others have described that HCV-specific T-cells directed against nonstructural proteins of HCV are associated with a favourable outcome of acute HCV in both injecting drug users (IDU) and men having sex with men (MSM). Individuals with a response mainly directed against Core protein are more likely to develop chronic HCV infection, and also in the chronic phase of infection a higher fraction of HCV-specific T cells are directed towards Core. Next to its role in formation of the HCV virion, Core has been shown to have immunomodulatory functions, thereby possibly supporting viral persistence. Thus Core is important in immunomodulation and it seems to induce T-cell responses which are not effective in clearance of the virus. Although we observe a higher proportion of the HCV-specific T-cell response to be focussed on Core protein in the majority of chronically HCV-infected patients, we do not observe this in all patients. We hypothesized that persistent exposure to HCV –by continuous risk behaviour, like injecting drug use or repeated sexual exposure– leads to enhanced exposure to mainly structural proteins and therefore leads to skewing of the HCV-specific T-cell response towards Core protein. Therefore, we here studied the association between the frequency of recurrent exposure to HCV (measured as frequency of injecting drug use and number of shared needles in the previous 6 months) and the focus of the HCV-specific T-cell response as measured after 12 day expansion.

Materials and Methods

Study population

To evaluate the association between the HCV Core-specific CD4+ T-cell response and continuous exposure to HCV, we included HCV-infected injecting drug users (DU) from the Amsterdam Cohort Studies (ACS) among DU with the highest frequency of recent injecting drug use (i.e., in the past 6 months) and the highest number of shared needles in the past 6 months (n=18). Next to these, we included recently identified HCV-infected self-reported never-injecting DU (n=9).

Virological assays

All ACS participants with at least two visits between 1985 and November 2005 were tested for presence of HCV antibodies using a third generation commercial microparticle EIA system test (AxSym HCV version 3.0; Abbott, Wiesbaden, Germany). After HCV antibody screening, HCV-seropositive samples were additionally tested for the presence of HCV RNA. RNA isolation was performed on 100 μl of serum using the TriPure method (Roche Diagnostics, Almere, the Netherlands). Each RNA isolate was used as input for two nested multiplex RT-PCRs. The first PCR, which targets the conserved HCV core region, was devised as a genotyping system to differentiate subtypes 1a, 1b, 2a, 2b, 3a, 4, 5a and 6a. The second RT-PCR, which targets the NS5B region, was
used for genotype confirmation. Conditions and primers for both PCR have been described elsewhere. All ACS participants since 1985 (n=1640) were tested for HIV antibodies by enzyme linked immunosorbent assays (ELISA) at each study visit. Results were confirmed by Western blot (since 1986, by HIV Blot version 2.2, Genelab diagnostics, Singapore).

Immunological assays

Phenotyping of T cell subsets

Surface staining was performed to evaluate various T cell subsets present. To examine the presence of cytotoxic granules in CD8+ T cells, cryopreserved peripheral blood mononuclear cells (PBMC) were stained with a combination of monoclonal antibodies against CD3, CD4, CD8, perforin and granzyme B. The amount of immune activation was determined by staining PBMC with a combination of monoclonal antibodies against CD3, CD4, CD8, HLA-DR, CD38, PD-1 and CD57. Furthermore, we examined the proportion of naïve, memory and effector T cells in the PBMC pool, by staining with monoclonal antibodies against CD27, CD127, CCR7 and CD45RO. In short, PBMC were thawed, washed and stained with the different sets of fluorescently labelled monoclonal antibodies. In these analyses, PBMC from 10 healthy controls (unpaid blood bank donors) were included for comparison.

HCV-specific CD4+ T cell expansion

HCV-specific CD4+ T-cell responses were measured using a sensitive assay based on short-term culture of peripheral blood mononuclear cells (PBMC) previously described for detection of HCV, but also other antigen-specific T-cell responses and has been shown to be more sensitive than assays with a shorter stimulation period, like Elispot or CFSE. In short, PBMC were thawed and cultured for 12 days in the presence of recombinant HCV proteins (Core (C22-3), NS3 (C33c), NS4 (C100-3) and NS5A/B (NS5)) (kindly provided by Chiron/Novartis) and recombinant interleukin-2 (IL-2) (Chiron, Uxbridge, UK). After 12 days, cells were washed, pooled and rested overnight. On day 13, cells were restimulated using overlapping peptide pools (18-mers, 11 overlapping amino acids, Mimotopes, Australia) and the HCV-specific response was quantified by analysis of IFN-γ production using intracellular cytokine staining.

Quantification of IFN-γ production by HCV-specific T cells

As a read-out of effector function, IFN-γ-producing CD4+ T cells were enumerated using intracellular cytokine staining (ICCS) after HCV-specific T cell expansion. Briefly, PBMC were stimulated for six hours with HCV Core/E1/E2/p7, NS2, NS3, NS4 and NS5 peptide pools (2 μg/ml) and both αCD28 (2 μg/ml) and αCD49d (2 μg/ml) as co-stimuli, after 1h 1:1,000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA) was added. As a negative control, PBMC were stimulated with medium and co-stimulation alone. As a positive control PBMC were stimulated with 10 ng/ml PMA, 2 μg/ml ionomycin and co-stimulation. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD4, CD8 and IFN-γ (BD). After fixation (Cellfix, BD) min. 50,000 events were acquired on LSR II flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using FACSDiva software (BD).
Results were expressed as the percentage of IFN-γ producing CD4+ T cells, which was calculated by subtracting the percentage of IFN-γ producing T cells in unstimulated conditions from the percentage of IFN-γ producing T cells in peptide pool restimulated conditions. To enable comparison of DU with different CD4+ T cell counts, we did not only compare the percentages of IFN-γ-producing CD4+ T cells, but we also determined the proliferation rate, which we defined as the total number of cells recovered after six or twelve days of expansion divided by the total number of cells put in to culture at day 0.\textsuperscript{17} The number of HCV-specific effector T cells was calculated as the product of the percentage of IFN-γ producing T cells and the proliferation rate.

Statistical analyses

Non-parametric tests (Kruskal-Wallis) were used to compare medians between groups, if there was a significant difference Dunns’ post-test was used to compare medians between groups. Pearson $\chi^2$ was used to compare proportions between groups. All performed statistical tests were two-sided. A p-value $\leq 0.05$ was considered to be statistically significant. All statistical analyses were performed using Graphpad and SPSS (Graphpad version 5.0, SPSS version 15.0.1, SPSS Inc.).

Table 5.1.1 Demographic characteristics of drug users (never-injecting and injecting).

|                          | Injecting DU HCV RNA- | Injecting DU HCV RNA+ | Never-injecting DU HCV RNA+ |
|--------------------------|-----------------------|------------------------|-----------------------------|
| n                        | 8                     | 10                     | 9                           |
| Median age (range)       | 29 (21-39)            | 28 (24-38)             | 30 (24-38)                  |
| Male sex (%)             | 6 (75)                | 5 (50)                 | 5 (55.6)                    |
| HIV prevalence (%)       | 0 (0)                 | 2 (20)                 | 2 (22.2)                    |
| HCV genotype distribution| -                     | 4                      | -                           |
| 1a                       |                       | 4                      | -                           |
| 1b                       |                       | 1                      | 2                           |
| 3a                       |                       | 4                      | 7                           |
| 4d                       |                       | 1                      | -                           |
| At least daily injection drug use in the past six months (%) | 7 (88)            | 6 (60)                 | -                           |
| Frequency sharing needles in the six months preceding entry ACS (range) | 9 (6-700)        | 25 (5-180)             | -                           |

Results

Study population and HCV RNA presence and genotype

In total, 22 HCV-antibody positive never-injecting DU were identified.\textsuperscript{13} Of these 22, 15 (68.2%) were HCV-RNA+ and 9/15 had PBMC available for T cell analyses. Furthermore, we included 18 injecting DU that reported recent injecting drug use (i.e., in the past 6 months). Most injecting DU reported at least daily injecting drug use (72.2%) and the median number of shared needles was 10 (5-700) in the six months preceding ACS entry. For those reporting injecting drug use at entry, the median time since first
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Injection was 7.3 years (IQR 3.5-9.1 years). Surprisingly, from these injecting DU only 55.6% (10/18) was HCV-RNA positive.

In DU with detectable HCV RNA, genotype 3a was most common (7/9 never-injecting DU and 4/10 injecting DU). In the HCV-RNA negative injecting DU, no individuals were HIV positive, while in the two groups where HCV RNA could be detected, the HIV prevalence was 20-22% (ns). Other general characteristics were similar in the three groups (Table 5.1.1).

Immune activation, as measured by HLA-DR and CD38 expression on CD4+ and CD8+ T cells, did not differ significantly between the three groups of DU. Furthermore, the distribution of CD4+ and CD8+ T cell subsets (defined by CD45RO and CD27 expression) was not different between individuals with HCV RNA and those without detectable HCV RNA or between the three groups (Table 5.1.2).

Table 5.1.2 Immune activation and subset differentiation of CD4+ and CD8+ T cells in HCV-RNA positive never-injecting DU, HCV-RNA positive IDU, and HCV-RNA negative IDU from the Amsterdam Cohort Studies.

|                  | Never IDU, RNA+ | IDU, RNA+ | IDU, RNA- |
|------------------|-----------------|-----------|-----------|
|                  | Median (IQR)    | Median (IQR) | Median (IQR) |
| CD4+ T cells     |                 |           |           |
|                  | HLA-DR+/CD38+   | 0.7 (0.49-2.19) | 2.3 (0.98-10.1) | 0.93 (0.41-1.88) |
| Phenotyping      |                 |           |           |
| Memory (CD27+/CD45RO+) | 10.9 (5.66-17.5) | 15.7 (9.56-24.4) | 11.2 (10.4-19.8) |
| Naive (CD27+/CD45RO+) | 7.81 (6.84-35.8) | 25.6 (17.7-34.7) | 22.3 (20.0-27.6) |
| Effector (CD27+/CD45RO+) | 26.2 (7.11-54.3) | 16.6 (8.16-27.9) | 13.5 (6.55-26.9) |
| Memory effector (CD27-/CD45RO+) | 31.8 (26.9-69.3) | 38.2 (16.4-48.9) | 44.3 (39.2-62.3) |
| CD8+ T cells     |                 |           |           |
|                  | HLA-DR+/CD38+   | 1.17 (0.91-2.31) | 1.39 (0.76-2.32) | 1.16 (0.88-1.72) |
| Phenotyping      |                 |           |           |
| Memory (CD27+/CD45RO+) | 23.3 (11.1-26.0) | 13.3 (11.8-17.2) | 16.3 (9.97-25.4) |
| Naive (CD27+/CD45RO+) | 31.4 (19.9-41.6) | 36.0 (24.3-48.0) | 34.0 (28.9-46.4) |
| Effector (CD27+/CD45RO+) | 2.94 (1.83-6.51) | 1.18 (0.61-3.87) | 3.10 (1.27-6.34) |
| Memory effector (CD27-/CD45RO+) | 39.8 (31.0-64.7) | 42.3 (32.1-53.5) | 38.1 (30.0-57.8) |

HCV Core-specific CD4+ T-cell response is associated with presence of HCV RNA

To study the association between ongoing risk behaviour and skewing of the T-cell response to Core, we measured HCV-specific CD4+ and CD8+ T-cell responses in DU with high-risk behaviour and never-injecting DU. An HCV-specific CD4+ T-cell response could be detected in 20/27 DU (using a cut-off value of 150 IFN-γ producing CD4+ T cells/million PBMC). Irrespective of HCV-RNA presence, injecting DU had higher total HCV-specific CD4+ T-cell responses (median 1372 and 1010 IFN-γ producing CD4+ T cells/million PBMC) compared to never-injecting DU (median 294 IFN-γ producing CD4+ T cells/million PBMC, p=0.012) (Figure 5.1.1a). Especially, the NS5-specific CD4+ T-cell response was higher in injecting DU compared to never-injecting DU (p=0.0006). An HCV-specific CD4+ T-cell response was present in 6/9 HCV RNA-positive never-injecting DU (66.7%).
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in 9/10 (90%) HCV RNA-positive injecting DU, and in 5/8 HCV RNA-negative injecting DU (62.5%). The breadth of the HCV-specific CD4⁺ T-cell response was not different between groups. Interestingly, none of the HCV RNA-negative injecting DU had detectable response against Core protein, compared to 4/10 HCV RNA-positive injecting DU and 4/9 HCV RNA-positive never-injecting DU. The median Core-specific T-cell response in those with a detectable response was 28.7% (range 11.9-71.2%) of the total HCV-specific response, and was not different in RNA-positive injecting DU compared to RNA-positive never-injecting DU (Figure 5.1.1B). All 4 DU that were HIV co-infected had detectable HCV RNA. Only one of them had a low, but detectable, HCV-specific CD4⁺ T-cell response (targeting NS4, 237 HCV NS4-specific CD4⁺ T cells/million PBMC) (Figure 5.1.1A, HIV-infected DU indicated with asterisk). CD4 counts ranged from 210 to 900 CD4⁺ T cells/mm³.

Figure 5.1.1A  HCV-specific CD4⁺ T-cell response per drug user (* indicates HIV-infected DU).
Here we studied the association between the frequency of recurrent exposure to HCV (measured as frequency of injecting drug use and number of shared needles in the previous six months) and the focus of the HCV-specific T-cell response as measured after 12 day expansion. We show that HCV-seropositive injecting DU with a high frequency of injection and sharing of needles have higher total HCV-specific CD4⁺ T-cell responses compared to HCV-infected never-injecting DU irrespective of the presence of HCV RNA. Furthermore, we show that the presence of HCV RNA is associated with a higher HCV Core-specific CD4⁺ T-cell response in both injecting and never-injecting DU compared to HCV-RNA negative injecting DU. Thus, continuous exposure does not influence the focus of the T-cell response, but rather the presence of HCV RNA affects the focus of the T-cell response to HCV.

Frequent injecting and sharing of needles in IDU can be seen as a proxy for frequent exposure to HCV. Although HCV re-infection may not necessarily have occurred after each shared needle, it is plausible to assume that the chance of exposure was very high, since HCV prevalence ranged between 63 and 93% in Amsterdam in the 20 years since the start of the ACS.²⁰ This continuous exposure is likely to have boosted the immune system, which has led to higher HCV-specific T-cell responses in injecting DU.

Although the frequency of injecting drug use and the number of shared needles was very high in the selected DU, 44.4% of them were HCV-RNA negative at the first ACS visit, suggesting they were able to control HCV. However, longitudinal HCV-RNA testing is necessary to conclude they had spontaneously cleared HCV. Furthermore, it is possible that this high HCV-RNA negative proportion is due to survival bias, since these DU had a long history of injection drug use. And although, 44.4% is a high proportion of spontaneous clearance, it is still within the range of spontaneous clearance we found in HCV seroconverters in the ACS (clearance rate 33.0%, Pearson χ², p=0.35).²¹ Larger
numbers are needed to evaluate whether continued exposure is associated with viral clearance.

The HCV Core protein is synthesized as a polypeptide of ~22 kDa and is one of the structural proteins of HCV. It functions as the nucleocapsid of the HCV virion. The amino acid sequence of Core is highly conserved among different HCV genotypes. It has been shown that nonenveloped HCV nucleocapsids occur naturally in the serum of HCV-infected individuals and that Core protein is abundantly present in serum of HCV-infected patients (and chimpanzees). The amount of Core protein present in serum is correlated with the height of HCV viral load. Therefore, targeting of Core protein by the adaptive immune system might be associated with unfavourable outcome of infection, since HCV-RNA and HCV Core protein are higher in individuals with persistent infection compared to those with a self-limiting course of infection. Indeed, we show here that HCV-specific T cells targeting Core protein were only present in individuals with detectable HCV RNA.

Presence of HCV RNA in serum implies active viral replication in the liver, which means that there is a continuous antigen production. When more Core antigen is produced, this will probably results in skewing of the T-cell response. Since HCV is translated as a polyprotein, it is unlikely that more Core protein than NS proteins is produced. However, since it has been shown that nonenveloped HCV nucleocapsids can be present in serum, T cells might be more exposed to Core than to NS proteins.

Since all individuals in this study with supposed continuous exposure to HCV did all show a high CD4 T-cell-response targeting HCV nonstructural proteins and did not show a significant response targeting Core protein, future vaccine development should focus preferably on nonstructural proteins.
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Chapter 5.2

Stronger decline in HCV-specific T-cell response in genotype 3 compared to genotype 1 HCV/HIV co-infected patients during treatment with pegylated interferon and ribavirin suggests no role for HCV-specific T cells in forced viral clearance

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Submitted.
Abstract

It has been suggested that HCV clearance during treatment with pegylated interferon (PEG-IFN) and ribavirin (RBV) is dependend on killing of HCV-infected hepatocytes by HCV-specific T cells. In this study we analyzed the role of HCV-specific CD4+ and CD8+ T cells in viral clearance during therapy in HIV co-infected patients, in whom lower rates of sustained viral responses have been reported, with different HCV genotypes. HCV-specific T-cell responses were measured in the first 12 weeks of treatment using a sensitive assay for detection of antigen-specific memory T cells using overlapping peptide pools corresponding to the infecting genotype of the patient.

In total, 28 HCV/HIV co-infected patients were included, of whom 18 were infected with HCV genotype 1 and 10 were infected with HCV genotype 3. During the first 12 weeks of HCV treatment, we observed a decline in both magnitude and breadth of HCV-specific CD4+ and CD8+ T-cell responses. Even more, genotype 3 infected patients tended to have a stronger decline in HCV-specific T-cell responses than genotype 1 infected patients, which paralleled the decline in viral load. This indicates that even in individuals infected with a genotype associated with a good response to therapy the immune response is not enhanced. In addition, a higher percentage of perforin-expressing CD8+ T cells at baseline in genotype 3 patients was observed, suggesting there is a difference in general host immune effector function between genotype 1 and 3 patients.

Although the number of patients in our study is small and does not allow for definite conclusions, augmentation of HCV-specific T cells does not appear to play a major role in forced viral clearance.
Introduction

Spontaneous viral clearance of hepatitis C virus (HCV) occurs in 15-40% of individuals. Treatment of chronic HCV infection with pegylated interferon (PEG-IFN) and ribavirin (RBV) leads to sustained viral response (SVR, defined as undetectable HCV-RNA load 6 months after cessation of therapy) in 50-90% of patients. The most important baseline predictor for SVR known so far is HCV genotype; both HCV mono-infected and HCV/HIV co-infected individuals with genotype 2 and 3 respond better to therapy than those with genotype 1 and 4. The underlying mechanism is not yet understood. Decline of HCV-RNA levels during combination therapy is biphasic and mathematical modeling of HCV viral kinetics during therapy has suggested that the first slope of HCV-RNA decline is most likely determined by the half life of free virus (i.e., blocking of new virus production), while the second slope is determined by the half life of infected cells (i.e., killing of infected hepatocytes by cytotoxic T lymphocytes (CTL)). Individuals with an impaired immune system, like those co-infected with HIV, have higher rates of treatment failure suggesting that the host immune system plays an important role in treatment-induced viral clearance. HCV-specific CTL can recognize infected hepatocytes and have different mechanisms to kill them. Next to Fas-mediated apoptosis, CTL can induce apoptosis using cytotoxic granules containing perforin and granzymes. In HCV mono-infected patients it has been shown that higher proliferative capacity of HCV-specific CD8+ T cells before therapy and higher pretreatment IFN-γ production by CD4+ T cells were associated with SVR, which may reflect better capacity to kill infected target cells during forced clearance of HCV.

Approximately 30% of hepatitis C virus-infected patients are HIV co-infected due to shared routes of transmission. HCV/HIV co-infection is associated with lower rates of SVR after treatment of HCV with PEG-IFN and RBV. Furthermore, HCV/HIV co-infection is associated with faster progression to liver-related morbidity and mortality. Interferons play an important role in the human antiviral response. In acute HCV it has been shown that a strong HCV-specific T-cell response (predominantly IFN-γ production) targeting multiple HCV proteins is associated with spontaneous viral clearance. We aimed to investigate whether HCV-specific T-cell responses play a role in 'forced' viral clearance during treatment, and whether these responses are inferior in HCV/HIV co-infected individuals which may explain the lower response rates. Since there is a large disparity in efficacy of PEG-IFN and RBV treatment between HCV genotypes, we studied individuals infected with HCV genotypes that respond good (i.e., HCV genotype 3) and genotypes that are less responsive to HCV treatment (i.e., HCV genotype 1) to examine whether these individuals display different T-cell responses.

Materials and Methods

Patients and HCV treatment

To analyze the dynamics of HCV-specific T-cell responses in HCV/HIV co-infected individuals, 28 HIV-infected patients with chronic HCV genotype 1 or 3 co-infection were studied prospectively. Of those, 18 patients were included in a randomized, open label, pilot study and were treated for chronic HCV infection during HIV co-infection with combination therapy consisting of PEG-IFN and RBV. 10 patients were treated with
PEG-IFN-α2b (Peginteron, Schering-Plough) 1.5 μg/kg/wk (standard-arm) and 10 patients were treated with induction PEG-IFN-α2b (regimen: 3.0 μg/kg/wk during the first four weeks, 2.0 μg/kg/wk during the next four weeks and 1.5 μg/kg/wk during the remaining 40 weeks) plus oral RBV at a daily dose of 1000 mg when bodyweight was less than 75 kg or 1200 mg when it was equal or more than 75 kg. The remaining 8 patients were included from another study (Privicop) that was designed for immunological and viral studies during HCV treatment in which patients were treated with standard doses PEG-IFN-α2a (180 μgr/wk, Pegasys, Roche) and weight-based RBV. Institutional review boards at participating centers approved the protocol. All patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Viral assays

Serum HCV RNA was quantified by bDNA (Versant™ HCV 3.0, Bayer Diagnostics) during screening, at baseline and thereafter at week 2, 4, 8, 12, 24, 48 and 72 as long as HCV RNA remained detectable. Below the detection limit of the bDNA assay (615 IU/ml), HCV RNA was measured by means of a qualitative transcription mediated amplification (TMA) assay (lower limit of detection 10 IU/ml, Versant™ HCV RNA Qualitative assay, Bayer Diagnostics). In Privicop patients, samples below the detection limit were measured using a qualitative Roche Amplicor polymerase chain reaction (PCR) assay (lower limit of detection 50 IU/ml, Roche Molecular Systems, Pleasanton, California).

Response definitions

Rapid viral response (RVR) was defined as undetectable viral load at week four of treatment using a qualitative assay. Early viral response (EVR) was defined as undetectable viral load or at least 2 log HCV-RNA load decline at week 12 of treatment compared to baseline. Sustained viral response (SVR) was defined as undetectable HCV RNA at week 24 after stopping of treatment.

Flow cytometry and phenotyping of PBMC

Six color fluorescence analysis was performed. Cryopreserved peripheral blood mononuclear cells (PBMC) were thawed and up to 1 x 10⁶ cells were stained using different sets of monoclonal antibodies. At least 100,000 events were acquired using a LSRII flowcytometer (BD Biosciences). Lymphocytes were gated by forward and sideward scatter and data analyzed using the software program FACSDiva (BD Biosciences).

Degranulation activity and cytotoxic granules (containing perforin and granzymes) generally define CD8⁺ T cells with cytotoxic function. Perforin expression was determined by an intracellular staining. In short, cryopreserved PBMC were thawed, washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD8 and perforin (eBioscience). After fixation (Cellfix, BD) min. 50,000 events were acquired on a LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using FACSDiva software (BD).
HCV peptide pools

For stimulation and expansion of HCV-specific T cells, pools of peptides were used. To study differences in T-cell responses between HCV genotype 1 and 3 infected individuals, pools of overlapping peptides spanning the complete HCV genotype 1a and 3a were used, respectively (kindly provided by www.beiresources.com). The pools consisted of 14- to 18-mers, overlapping by 11 amino acids. Peptides were pooled in six pools (corresponding to Core, NS2, NS3, NS4, NS5A and NS5B region of HCV) in such a way that each peptide was present in the final pool at a concentration of 1 mg/ml.

HCV-specific T-cell expansion

Since the frequency of HCV-specific T cells in HCV/HIV co-infected patients is usually very low, we used an assay that has proven to be very sensitive to show the presence of HCV- and other antigen-specific T cells. Furthermore, to study the difference in T-cell response between individuals infected with genotypes 1 and 3, we stimulated PBMC with overlapping peptide pools from the corresponding HCV genotype. To expand HCV-specific memory T cells, cryopreserved PBMC were thawed and subsequently expanded during a period of 12 days using overlapping HCV peptide pools corresponding to Core, NS2, NS3, NS4 and NS5B region of the HCV genome (2 μg/ml) in the presence of recombinant interleukin-2 (rIL-2, 360 IU/ml). After 12 days, expanded cells were pooled, washed, counted and rested overnight. On day 13 cells were restimulated using peptide pools from the corresponding HCV genotype to assess effector function (i.e., IFN-γ production).

Detection of IFN-γ producing HCV-specific T cells after (re-)stimulation

As a read-out of effector function, IFN-γ producing T cells were enumerated using intracellular cytokine staining (ICCS). Briefly, PBMC were stimulated for six hours with HCV Core, NS2, NS3, NS4 and NS5 peptide pools (2 μg/ml) and both αCD28 (2 μg/ml) and αCD49d (2 μg/ml) as co-stimuli, after 1 hour 1:1,000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, USA) was added. As a negative control, PBMC were stimulated with medium and co-stimulation alone. As a positive control PBMC were stimulated with 10 ng/ml PMA, 2 μg/ml ionomycin and co-stimulation. After stimulation, cells were washed, permeabilized (FACS Permeabilizing Solution, BD), washed again and stained with antibodies specific for CD3, CD4, CD8 and IFN-γ (eBioscience). After fixation (Cellfix, BD) min. 25,000 events were acquired on a LSRII flow cytometer (BD). Lymphocytes were gated by forward and sideward scatter and data analyzed using FACSDiva software (BD).

Results were expressed as the percentage of IFN-γ producing T cells, which was calculated by subtracting the percentage of IFN-γ producing T cells in medium from the percentage of IFN-γ producing T cells in peptide pool (re-)stimulated conditions. To enable comparison of patients with different baseline CD4+ T cell counts, we did not only compare the percentages of IFN-γ-producing T cells, but we also determined the proliferation rate, which we defined as the total number of cells recovered after 12 days of expansion divided by the total number of cells put in to culture at day 0. Subsequently we calculated the number of HCV-specific T cells as the product of the percentage of IFN-γ-producing T cells and the proliferation rate. For easier understanding of this number, we expressed the number of IFN-γ producing T cells per
million PBMC. This number of HCV-specific T cells is a combination of (memory) cells initially present and the ability of these cells to survive, proliferate and differentiate to (IFN-γ-producing) effector T cells during the 12-day culture period. 8

Statistical analysis

All tests performed were 2-sided. A p-value ≤ 0.05 was considered to be statistically significant. Pearson $\chi^2$ test was used for comparison of proportions. Non-parametric tests were used for comparison between groups (Kruskal-Wallis). Statistical analyses were performed using SPSS and STATA (v9.2, Statacorp, Collegestation, Texas, USA).

Results

Baseline characteristics of the patient population

In total, 28 HIV-infected patients were included. Of those, 18 were co-infected with HCV genotype 1 and 10 were co-infected with HCV genotype 3. The patients were mainly male, and mainly of Caucasian ethnicity. In each group, five patients were treated with induction treatment and the remaining patients were treated with standard dose regimens of PEG-IFN-α2a or PEG-IFN-α2b. The median log baseline HCV viral load was 6.23 IU/ml (interquartile range (IQR) 5.58-6.41 IU/ml), and was somewhat lower in HCV genotype 3 infected individuals compared to HCV genotype 1 infected individuals (median (IQR) 5.78 (5.39-6.23) and 6.34 (5.97-6.48), respectively, p=0.069). For 13/28 patients, liver biopsies were performed prior to baseline. Of these patients, 6 (46.2%) showed significant fibrosis. ALT levels were lower in the group of patients that were treated with PEG-IFN-α2a, median 55 IU/ml than in the groups treated with PEG-IFN-α2b median 101 IU/ml and 115 IU/ml for standard and induction arm, respectively (p=0.048). Of the 28 HIV-infected patients, 19 were treated with highly active antiretroviral therapy (HAART). The median CD4 count for all included patients was 430 cells/ml (IQR 280-740 cells/ml), and was somewhat higher in patients not receiving HAART (median 477 cells/ml, IQR 390-655 cells/ml) compared to those on HAART (372 cells/ml, IQR 270-740 cells/ml) (p=0.44, data not shown).

Outcome of therapy

Of 28 included patients, 22 (78.6%) achieved EVR and of those, 11 (50%) achieved SVR. As expected, patients infected with HCV genotype 3 were more likely to achieve EVR (10/10 genotype 3 infected patients vs. 12/18 genotype 1 infected patients, p=0.039) and SVR (6/10 genotype 3 infected patients vs. 5/18 genotype 1 infected patients, p=0.090) compared with individuals infected with HCV genotype 1 (Table 5.2.1). The median decline of log10 viral load in the first four weeks of treatment was somewhat lower in genotype 1 infected patients (2.11 (IQR 1.66-3.56)) than in genotype 3 infected patients (3.10 (IQR 2.15-4.39), p=0.17), in the first 12 weeks of treatment this was 3.62 (IQR 2.13-4.46) and 4.50 (3.49-4.89), respectively (p=0.15).
Baseline HCV-specific T-cell responses

Since in HCV mono-infected individuals it has previously been shown that both baseline proliferative capacity of HCV-specific CTL and IFN-γ production of CD4+ T cells was associated with SVR,7,8 we analyzed HCV-specific T-cell responses using a previously described sensitive assay that measures a combination of these properties.10,21 The experiments could be carried out in 21 of 28 (75%) patients that had sufficient PBMC available. HCV-specific CD4+ T-cell responses could be detected in 13/21 (61.9%) and HCV-specific CD8+ T-cell responses in 14/20 (70.0%) individuals. 11/20 (55.0%) individuals had both HCV-specific CD4+ and CD8+ T-cell responses. 7/13 (53.8%) HCV genotype 1 infected individuals had both HCV-specific CD4+ and CD8+ T-cell responses, compared to 4/7 (57.1%) HCV genotype 3 infected individuals (p=0.89, χ²).

The median height of the total HCV-specific CD4+ T-cell response was not different between treatment groups (median (IQR) 685 (0-3544), 1006 (0-4175), and 468 (144-1164) IFN-γ producing CD4+ T cells/million PBMC for standard PEG-IFN-α-2a, standard PEG-IFN-α-2b and induction PEG-IFN-α-2b, respectively (Kruskall Wallis, p=0.98)) (Figure 5.2.1A). The median height of the total HCV-specific CD8+ T-cell response was also similar for patients in the different treatment groups (median (IQR) 412 (8-1361), and 1242 (180-2823) IFN-γ producing CD8+ T cells/million PBMC for standard PEG-IFN-α-2a and induction PEG-IFN-α-2b, respectively (Kruskall Wallis, p=0.32)) (Figure 5.2.1A). Although ALT levels were lower in the group treated with standard PEG-IFN-α-2a than the other groups, baseline HCV-specific T-cell responses were comparable, therefore we subsequently analyzed the groups together. Baseline HCV viral load did not differ

### Table 5.2.1 Baseline characteristics of included patients.

| Characteristic                                | HCV genotype 1 | HCV genotype 3 | P value * |
|-----------------------------------------------|----------------|----------------|-----------|
| General characteristics                        |                |                |           |
| Female sex (%)                                | 4 (22.2%)      | 1 (10%)        | 0.42      |
| Median age (IQR)                              | 42 (34-46)     | 42 (39-47)     | 0.81      |
| Treatment related characteristics             |                |                |           |
| Standard of care (n)                          | 13             | 5              |           |
| PEG-IFN-α2-a (Pegasys ®)                      | 8              | -              |           |
| PEG-IFN-α2-b (Pegintron ®)                    | 5              | 5              |           |
| Induction arm (n)                             | 5              | 5              |           |
| At least treated up to week 12 (%)            | 83.3%          | 100%           | 0.17      |
| EVR (%)                                       | 12 (66.7%)     | 10 (100%)      | 0.039     |
| SVR (%)                                       | 5 (27.8%)      | 6 (60.0%)      | 0.004     |
| HCV/liver-related characteristics             |                |                |           |
| Median baseline HCV viral load (IQR)          | 6.34 (5.97-6.48) | 5.78 (5.39-6.23) | 0.069    |
| Median ALT (IU/ml) (IQR)                      | 68 (51-88)     | 126 (85-152)   | 0.25      |
| F3-F4 liver fibrosis in biopsy                | 5/8            | 1/5            | 0.22      |
| HIV-related characteristics                   |                |                |           |
| Median CD4 count (IQR)                        | 430 (370-740)  | 280 (250-540)  | 0.37      |
| Treated with HAART (n)                        | 11             | 9              | 0.11      |
| Undetectable HIV viral load (n)               | 11             | 8              | 0.31      |

* Pearson χ² for proportions, Kruskall-Wallis for continuous variables.
significantly between groups, however HCV viral load tended to be lower in genotype 3 infected patients (p=0.069).

In the analyzed patients, only three individuals did not achieve an EVR. In patients that did achieve an EVR the observed median CD4+ T-cell responses (686 IFN-γ producing CD4+ T cells/million PBMC (IQR 45-2482)) were similar to those who did not (144 IFN-γ producing CD4+ T cells/million PBMC (0-10371), p=0.87) (Figure 5.2.1B). The HCV-specific CD8+ T-cell response was not significantly different between those who achieved EVR (median 884 IFN-γ producing CD8+ T cells/million PBMC (IQR 116-3613) and those who did not achieve EVR (1912 IFN-γ producing CD8+ T cells/million PBMC (IQR 0-1958), p=0.91) (Figure 5.2.1B). The median height of the total HCV-specific CD4+ T-cell response was 685 IFN-γ producing CD4+ T cells/million PBMC (IQR 45-3528). This was not significantly different between HCV genotype 1 and HCV genotype 3 infected patients (345 IFN-γ producing CD4+ T cells/million PBMC (IQR 0-3114) and 1006 IFN-γ producing CD4+ T cells/million PBMC (IQR 57-4175), respectively, p=0.39) (Figure 5.2.1c). The median HCV-specific CD8+ T-cell response for all included patients was 1100 IFN-γ producing CD8+ T cells/million PBMC (IQR 116-3613). This did not significantly differ between genotype 3 infected individuals: median 1912 (IQR 429-12477) and 658 (IQR 54-1242) IFN-γ producing CD8+ T cells/million PBMC, respectively, p=0.10) (Figure 5.2.1C).

Since in spontaneous viral clearance there is an association with breadth of the HCV-specific T-cell response, we evaluated how many HCV proteins were targeted per patient. At baseline of treatment a median of two proteins was targeted by HCV-specific CD4+ T-cell responses, while HCV-specific CD8+ T cells targeted a median of 1.5 proteins (data not shown). The median number of targeted proteins was not significantly different between individuals infected with genotype 1 compared with those infected with genotype 3. Only a minority of individuals had a detectable CD4+ or CD8+ HCV-specific T-cell response targeting Core protein (5/21 and 6/20, respectively) (Figure 5.2.2B and 5.2.2C).
Figure 5.2.1

A) Baseline HCV-specific CD4⁺ and CD8⁺ T-cell response as measured after 12 day expansion in the three different study groups: standard arm: treated with PEG-IFN-α2b and RBV, induction arm: treated with PEG-IFN-α2b induction scheme and RBV, and Privicop: treated with PEG-IFN-α2b and RBV.

B) Baseline HCV-specific CD4⁺ and CD8⁺ T-cell response as measured after 12 day expansion in patients achieving an early viral response (EVR) and those who do not.

C) Baseline HCV-specific CD4⁺ and CD8⁺ T-cell response as measured after 12 day expansion in patients infected with HCV genotype 1 and 3.
Fate of HCV-specific T-cell responses during treatment

It is likely that differences in augmentation of HCV-specific T-cell responses will be most obvious shortly after initiation of treatment when these responses are boosted by PEG-IFN and RBV treatment. Therefore, longitudinal HCV-specific T-cell responses were examined at baseline, week 4 and week 12 of treatment, corresponding to time points of RVR and EVR, respectively.

During HCV-treatment both HCV-specific CD4⁺ T cell and HCV-specific CD8⁺ T-cell responses declined. This decline was clear in both HCV genotype 1 infected and genotype 3 infected individuals (Figure 5.2.2A and 5.2.2B). In genotype 1 infected patients the HCV-specific CD4⁺ T-cell responses declined from 468 to 68 CD4⁺ T cells/million PBMC (IQR 0-912 CD4⁺ T cells/million PBMC) at week 4 and 900 CD4⁺ T cells/million PBMC (IQR 22-2794) at week 12. The HCV-specific CD8⁺ T cells declined from 1912 to 103 CD8⁺ T cells/million PBMC (IQR 0-1144 CD8⁺ T cells/million PBMC) at week 4 and 173 CD8⁺ T cells/million PBMC (IQR 0-1995 CD8⁺ T cells/million PBMC) at week 12 in genotype 1 infected patients (Figure 5.2.2A).

In genotype 3 infected patients, the decline in the first four weeks was more pronounced, the HCV-specific CD4⁺ T-cell responses declined from 922 to 214 CD4⁺ T cells/million PBMC (IQR 58-350 CD4⁺ T cells/million PBMC) at week 4 and to 50 CD4⁺ T cells/million PBMC (IQR 33-70) at week 12. HCV-specific CD8⁺ T cells in genotype 3 infected patients declined from 658 to 517 CD8⁺ T cells/million PBMC (IQR 307-747 CD8⁺ T cells/million PBMC) at week 4 and 243 CD8⁺ T cells/million PBMC (IQR 24-372 CD8⁺ T cells/million PBMC) at week 12 (Figure 5.2.2B).

Interestingly, in HCV genotype 1 infected individuals HCV-specific CD4⁺ and CD8⁺ T-cell responses (≥200 IFN-γ producing T cells/million PBMC) could still be detected after 12 weeks of treatment in five and three patients, respectively (Figure 5.2.2A). This in contrast to HCV genotype 3 infected patients, where in all individuals HCV-specific T cell had declined to undetectable levels (Pearson χ² for CD4⁺ p=0.037 and for CD8⁺ p=0.59, Figure 5.2.2B). The total HCV-specific CD4⁺ and CD8⁺ T-cell response at week 12 was lower for genotype 3 infected patients (50 (IQR 33-70) IFN-γ producing CD4⁺ T cells) compared to genotype 1 infected patients (900, IQR 22-2794 IFN-γ producing CD4⁺ T cells), although this not reach statistical significance (Figure 5.2.3) Although a CD8⁺ T-cell response above the detection limit was observed more often in individuals with genotype 3, no difference in median CD8⁺ T-cell responses was found between genotype 1 (173, IQR 0-1995 IFN-γ producing CD8⁺ T cells/million PBMC) and genotype 3 infected patients (243, IQR 24-372 IFN-γ producing CD8⁺ T cells/million PBMC) (Figure 5.2.1C).

Not only the magnitude of the total HCV-specific T-cell response declined over time, but also the breadth of the response decreased in most individuals with a detectable HCV-specific CD4⁺ (n=13) or CD8⁺ (n=14) T-cell response at baseline. The breadth of the CD4⁺ T cell decreased in nine individuals and remained the same in four individuals, while the breadth of the CD8⁺ T-cell responses decreased in six, remained equal in four and increased in one patient. In patients who achieved an EVR and had a detectable baseline response, the number of proteins targeted by the CD4⁺ and CD8⁺ T-cell response decreased, in 8/9 and 6/10 patients, respectively. (Figure 5.2.2B and 5.2.2C)

Interestingly, patient C19 showed a decline in HCV-specific CD4⁺ T-cell responses at week 4, however the HCV-specific CD4⁺ T cell response rebounded to baseline level at week 12. This patient showed an initial viral response to treatment (with detectable HCV-RNA, but under the detection limit of the bDNA assay (615 IU/ml) at week 4) however HCV-RNA relapsed and was above the bDNA limit from week 8 onwards. In
HCV-specific T cells during HCV treatment

the genotype 3 group, while all patients decreased considerably, patient C17 showed still detectable T-cell responses at week 4. Although the T-cell response declined afterwards to undetectable levels, this patient had a relapse after stop of therapy.

Figure 5.2.2A Fate of HCV-specific CD4+ T (upper panel) and CD8+ T (middle panel) cells in genotype 1 infected patients as measured after 12 day expansion at baseline, week 4 and week 12. In the lower panel log10 viral load is shown. ND=not done.
Figure 5.2.2B  Fate of HCV-specific CD4$^+$ (upper panel) and CD8$^+$ T (middle panel) cells in genotype 3 infected patients as measured after 12 day expansion at baseline, week 4 and week 12. In the lower panel log10 viral load is shown. ND=not done.
HCV-specific T cells during HCV treatment

Figure 5.2.3 Median height and interquartile range of HCV-specific CD4⁺ and CD8⁺ T-cell response at week 12 after start of treatment.

Figure 5.2.4
A) Representative dotplot of perforin staining in CD3⁺ T cells as measured by intracellular cytokine staining in four patients.
B) Perforin expression at HCV therapy baseline as measured by intracellular cytokine staining. Shown are median and range.
Perforin content in CTL

Perforin is an important molecule for CTL to mediate killing of infected cells and is upregulated after activation in effector T cells. Since PEG-IFN and RBV treatment is believed to enhance immune responses, we also analyzed perforin expression of T cells directly \textit{ex vivo}. In Figure 5.2.4A, representative dotplots of perforin staining in two patients with HCV genotype 1 infection and two patients with HCV genotype 3 infection are shown. Median perforin expression in 10 HCV-uninfected healthy donors was 0.97% (IQR 0.42-4.0%) (data not shown). Interestingly, the median percentage of perforin$^+\text{CD8}^+$ T cells was higher in both genotype 1 (3.10%, IQR 1.50-11.3%) and genotype 3 (22.3%, IQR 8.20-31.6%) infected patients. Surprisingly, the perforin content in CD8$^+$ T cells in genotype 3 infected patients was higher compared to patients infected with genotype 1 (p=0.019, Figure 5.2.4B). No decline in perforin expression was observed during treatment (data not shown).

Discussion

Based on mathematical modeling of HCV viral kinetics during treatment with PEG-IFN and RBV, it was proposed that the second phase of the decline of HCV is caused by (HCV-specific) CTL.\textsuperscript{6} However, so far only one study was able to show an augmentation of HCV-specific T-cell responses during treatment with PEG-IFN and ribavirin\textsuperscript{21}, the difference between this study by Kamal et al. and other studies is that it was performed on fresh PBMC.\textsuperscript{7,8,22} Other studies have shown associations between SVR and either proliferative capacity of HCV-specific CD8$^+$ T cells at baseline,\textsuperscript{7} or pretreatment IFN-\gamma secretion by HCV-specific CD4$^+$ T cells.\textsuperscript{8} However, no augmentation of HCV-specific T-cell responses was observed.\textsuperscript{7,8,23} Since HIV/HCV co-infection is associated with lower treatment success rates, we conducted a thorough analysis of the HCV-specific T-cell responses early during treatment to investigate whether immunological predictors for EVR, which is associated with achieving SVR could be identified. Furthermore, since HCV genotype 3 is more responsive to treatment with PEG-IFN and RBV, we analyzed HCV-specific T-cell responses using overlapping peptide pools corresponding to the infecting genotype of the patient.

Although the used assay has proven to be very sensitive for the detection of low frequencies of antigen-specific T cells,\textsuperscript{16,19,20,24} we did not observe an increase in HCV-specific T-cell responses during treatment. On the contrary, we observed a decrease of HCV-specific T cells during treatment in nearly all included patients. This suggests that the immunomodulatory properties of PEG-IFN and RBV on HCV-specific T cells might actually be limited in forced viral clearance, at least in HIV/HCV co-infected patients. Even more, we observed undetectable HCV-specific T-cell responses more often in HIV/HCV genotype 3 compared to HIV/HCV genotype 1 infected patients at week 12 after start of treatment. This implies that the immune response declines in parallel with the viral load, and that there is no boosting of the immune response in these patients. This is not completely unexpected, since HIV-specific T-cell responses have also been shown to decrease after initiation of HAART.\textsuperscript{29}
Interestingly, we observed a higher percentage of CD8+ T cells expressing perforin in HCV genotype 3 than in HCV genotype 1 HIV co-infected patients. This cytotoxic quality of CTL was measured in the total CD8+ T cell population, and not in HCV-specific T cells, therefore the observed difference might not be HCV specific. The fact that we do not observe a concurrent decline of perforin with HCV viral load, also suggests that it is a reflection of the general immune activation state of the patients. Interestingly, HCV genotype 3 infection has been associated with higher prevalence of steatosis of the liver, and perhaps the high perforin expression in T cells is associated with this development of steatosis, but larger longitudinal studies are needed to explore this association. Furthermore, in our study we observed higher ALT levels in genotype 3 infected patients, however, this seemed to be caused by lower ALT levels in patients treated with standard PEG-IFN-α2-a, who were all infected with HCV genotype 1. Possibly this disparity is related to a different distribution of fibrosis and/or inflammation in both treatment studies. Since we have only liver biopsies in 14 out of 28 patients, we cannot exclude this. Furthermore, different HAART regimens in both treatment studies could have led to the observed difference in ALT levels.

Although chronic HCV infection has been associated with HCV-specific T-cell responses targeting Core protein in acute and chronic HCV infection, this was not observed in our cohort of hospital-recruited patients. Perhaps skewing of HCV-specific T cells towards Core protein is caused by higher exposure to the virus due to continuing risk behaviour (e.g., injecting drug use or sexual risk behaviour) in combination with high HCV-RNA load (i.e., antigenic pressure). These hospital-recruited patients may only have high antigenic pressure. This should be a focus of further study.

The experiments described in this study were performed on peripheral T cells, it would be interesting to study the dynamics of intrahepatic T cells, especially since Neumann-Haefelin et al. have shown an enrichment of HCV-specific T cells in the liver compared to the peripheral compartment. Although liver biopsies are performed in the Netherlands according to AASLD and national guidelines, a biopsy is not mandatory before start of treatment. Future studies might consider fine needle aspiration biopsies, which are much less invasive for the patient.

In conclusion, we did not observe an enhancement of HCV-specific T-cell responses in this cohort of HIV/HCV genotype 1 and 3 co-infected patients. We did observe different kinetics of the HCV-specific T-cell responses in genotype 1 and 3 infected individuals, and these responses paralleled the decline in HCV viral load. Furthermore, we observed a higher perforin content of CD8+ T cells at baseline in genotype 3 patients, possibly indicating that there is a difference in general host immune activation between HCV genotype 1 and 3 infected patients, for which the cause is unknown, but which may be associated to the degree of liver inflammation. Although the number of patients in our study is small and does not allow us to draw firm conclusions, augmentation HCV-specific T cells does not appear to play a major role in forced viral clearance.
Chapter 5.2

Immunology in chronic HCV

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Chapter 6

General discussion
HCV infection in injecting DU

Injecting drug users (DU) are at high risk for acquiring hepatitis C virus (HCV) infection via exposure to infected blood through the sharing of needles and syringes.\textsuperscript{1,2} HCV prevalence in injecting DU populations can be up to 90%.\textsuperscript{1,3,4} By using a statistical method that was based on coalescent theory, it was estimated that HCV has been circulating in DU populations in Europe since the 1960s, and that the introduction of HCV was possibly even earlier than that.\textsuperscript{5,6} Although HCV prevalence remained high and relatively stable in ever-injecting DU from the ACS (69-93% between 1985 and 2005), we found that the HCV incidence declined substantially in the past two decades, from 27.5/100 person years (PY) in the late 1980s to 2/100 PY in more recent years.\textsuperscript{7} The decline of HCV incidence was comparable with the decline in HIV incidence observed in the Amsterdam Cohort Studies (ACS) in the same period, although HCV incidence was always higher than the HIV incidence.\textsuperscript{7,8} This can be partially explained by the natural course of an epidemic: after the introduction of a pathogen in a certain population, the number of infected individuals and the incidence increases. While this happens, the number of individuals at risk decreases, and therefore the chance for an infected individual to transmit the pathogen to uninfected individual also decreases. It is therefore the natural course of an epidemic that when the density of people at risk reaches a certain threshold below which the number of susceptible individuals cannot sustain an ongoing epidemic, incidence peaks and then starts to decline.\textsuperscript{9} The observed decrease in HIV incidence in the ACS among DU was most likely due to depletion of individuals at risk, along with a reduction in risk behaviour. For HCV on the other hand, which had already been circulating for a longer time than HIV in DU in Amsterdam, it is less likely that the natural course of the epidemic was the only cause for the decline of the incidence.

There are several other important factors that most probably influenced the declining HCV incidence: the observed decrease in injecting risk behaviour at the population level might have had a greater impact on HCV than on HIV. The number of new injectors declined, which decreased the size of the susceptible population.\textsuperscript{8} Furthermore, since HCV is mainly transmitted via blood-blood contact, and not via sexual contact, the number of susceptible individuals for HCV decreased more than for HIV. In addition, mortality of the highest risk individuals in the population, who were often co-infected with HIV, may have contributed to the declining HCV transmission, since they were no longer a source for new infections.

Within the ACS, there is the opportunity to investigate factors that determine the time from start of injection drug use to HCV seroconversion. We found that this window period is longer for injecting DU that started injection drug use in the 1990s than in the 1980s.\textsuperscript{7} Knowledge on determinants of the window period between start of injection drug use to HCV infection is important, in view of the fact that prevention measures should be applied and are only effective in this period.

Harm reduction

While HCV incidence decreased in Amsterdam, HCV incidence remained high in DU populations in many developed countries.\textsuperscript{1,4} Probably the prevalence of injecting risk
behaviour declined more in Amsterdam compared to elsewhere. Murray et al. demonstrated by mathematical modelling that the level of risk behaviour determines whether HCV incidence decreases.\textsuperscript{10} They calculated that if injecting risk behaviour is sufficiently decreased (through intense needle exchange programs and/or harm reduction strategies), then HCV incidence and prevalence will accordingly decline.

Methadone and needle exchange programs in Amsterdam and the rest of The Netherlands are incorporated in the so-called harm reduction approach and were readily available in Amsterdam since the end of the 1970s. The ultimate goal of harm reduction is to stop drug use, but until this is possible, the policy is to minimize the damage DU inflict on themselves and the society at large. This comprehensive harm reduction approach has probably had a major impact on the HIV and HCV epidemic in Amsterdam. We found that only methadone provision or only participation in needle exchange programs did not decrease the risk of HCV or HIV infection, while combining the two measures resulted in a two- to threefold reduction in risk of acquiring HCV and/or HIV.\textsuperscript{11} This implies that harm reduction programs should be comprehensive, widespread, and easily accessible. This has major implications for countries with new and sometimes explosive outbreaks of both HCV and HIV in DU, like China and countries of the former Soviet Union,\textsuperscript{12-14} since providing only methadone or only needle-exchange facilities will not be enough to curb these epidemics, but such measures have to be combined with social and medical care.

\textit{Non-injecting DU}

Self-reported never-injecting DU in the ACS had a much higher HCV prevalence (6.3\%) than the general Dutch population.\textsuperscript{15,16} Several studies have shown an association between HCV infection and non-injection drug use paraphernalia, indicating that HCV might also be transmitted via this route in non-injecting DU.\textsuperscript{17} However, we could not confirm this in the ACS.\textsuperscript{15} Phylogenetic analysis showed that the HCV strains circulating in HCV-infected never-injecting DU are interspersed with strains derived from HCV-infected injecting DU, indicating that they were probably infected from the same pool. The incidence in never-injecting DU was very low, with only one HCV seroconversion in >2,000 person years of follow up.\textsuperscript{15} This increased risk of prevalent HCV infection and the discrepancy between HCV prevalence and incidence in self-reported never-injecting DU could be related to underreporting of injecting drug use, next to household or sexual transmission from injectors to non-injectors. Whatever the route of transmission is in never-injecting DU, these findings stress the need for HCV testing of DU who report regular hard drug use, especially given the potential to treat HCV infection effectively. It should be noted that the never-injecting DU included in this study, were DU with regular use of hard drugs (i.e., heroin, cocaine, methadone or amphetamines at least three times per week) and with a high prevalence of polydrug use.\textsuperscript{15}

Although there have not been many new HCV infections in DU in recent years, there is still a large pool of chronically HCV-infected DU. Most chronically HCV-infected DU have not yet been treated for their chronic HCV, as many physicians question the effectiveness of HCV treatment in DU due to the perceived lack of adherence and the risk of re- and superinfection, which may negatively influence treatment outcome. However, it is important to treat DU not only in their own interest, but also since they can be a source of HCV, even though it is unknown how much transmission there actually is from DU to the general population. Within the ACS it has been shown that HCV treatment for chronically infected DU is feasible using a multidisciplinary approach in which directly observed therapy is combined with methadone provision and where there...
is a close collaboration between research staff, hepatologists, addiction specialists and psychiatrists.\textsuperscript{18}

Natural History

 Estimates of spontaneous HCV clearance

Acute hepatitis C virus infection is asymptomatic in many patients, and therefore it is hard to study factors associated with spontaneous viral clearance.\textsuperscript{19} Furthermore, there are many factors that can influence the estimates of spontaneous viral clearance. Several of these factors are discussed below.

Definition spontaneous viral clearance

Chronic HCV infection is usually defined as persistence of detectable HCV RNA at least six months after acute HCV infection and it is generally accepted that spontaneous clearance of HCV takes place within six months after clinical presentation or HCV antibody seroconversion.\textsuperscript{20,21} However, definitions of spontaneous clearance of HCV can vary between studies, which makes it difficult to compare them. Since HCV-RNA levels can fluctuate around the level of detection of the used assay,\textsuperscript{22} multiple samples should be tested for the presence HCV RNA before chronic infection can be ruled out. Whether undetectable HCV-RNA levels in serum really means that HCV is fully eradicated from the body, remains unclear. There are some studies that suggest that several immune cells can remain HCV infected even after spontaneous clearance or successful treatment with pegylated interferon (PEG-IFN) and ribavirin (RBV).\textsuperscript{23} Whether this very low-grade persistence of HCV-RNA has clinical consequences has to be analyzed in prospective longitudinal studies of individuals that spontaneously cleared HCV or successfully treated individuals.

Late clearance

Although HCV clearance is supposed to occur within six months after infection, late spontaneous clearance has been described: up to 24 months after HCV infection, and even after that in Alaskan-natives.\textsuperscript{24-26} To evaluate the prevalence and determinants of late clearance in the ACS, more longitudinal data on HCV-RNA load are needed. The comparison between individuals with and without late clearance, and the evaluation of determinants of late HCV clearance might add to the knowledge how to turn the insufficient innate immune response and HCV-specific immunity around into an adequate immune response which is capable of eradicating the virus.

Continuous risk behaviour increases risk of super- and re-infection, and HIV co-infection

In retrospectively identified HCV seroconverters from the ACS among DU the overall rate of spontaneous viral clearance was 33.0\%. However, we know that the rate of HCV re- and superinfection is high in DU with continuous injection drug use in this and other cohorts.\textsuperscript{27,28} Therefore, the observed rate of spontaneous viral clearance might actually be an underestimation of the ‘true’ clearance rate. To have a better estimate of spontaneous viral clearance, one would need a prospective cohort of HCV seroconverters with preferably shorter sampling intervals and sequencing of (part of) the HCV genome for phylogenetic analysis, which provides the possibility of discrimination between different HCV strains.
Another factor complicating estimations of viral clearance in active injecting DU is that continuous risk behaviour not only increases the risk on HCV re- and superinfection, but also increases the risk for subsequent HIV infection which is associated with lower clearance rate and with HCV superinfection. Continuous risk behaviour might result in an underestimation of the viral clearance rate. In the ACS, originally designed for HIV research, frequent longitudinal HCV-RNA measurements have not yet been established. Currently, HCV-RNA measurements are available for all HCV seroconverters and injecting DU included within two years after start of injection drug use around seroconversion or entry and at the end of follow up. However, more longitudinal measurements are needed to answer important questions regarding the natural history of HCV. These questions include the natural kinetics of HCV RNA during acute and subsequent chronic HCV infection, variation per genotype and whether there is an association between viral load and the progression to liver disease or liver-related death. Furthermore, a more accurate estimate of the incidence of HCV re- and superinfection in comparison to incidence of initial infection would be possible.

Factors influencing HCV clearance

So far, no reliable predictors for spontaneous viral clearance have been defined, but female sex, non-African race, younger age, high and broad HCV-specific T cells, certain HLA haplotypes, and symptomatic presentation have been associated with spontaneous viral clearance. These associations were mainly derived from cross-sectional studies, which may have biased the results. In the ACS among DU, we had the unique opportunity to study factors associated with viral clearance in retrospectively identified HCV seroconverters. Below the main factors associated with viral clearance are discussed.

Female sex

For many infectious diseases it has been described that the severity of disease is less in women compared to men. In addition, women are more likely to suffer from many auto-immune diseases, conditions in which the immune system reacts against self, implying a more reactive immune system in women than in men. In the ACS, female sex was the most important predictor of spontaneous viral clearance in HCV seroconverters. This is in line with a high rate of spontaneous viral clearance that was found in pregnant women that had received contaminated anti-D immunoglobulins. For HIV, it has been described that women have lower HIV-RNA loads compared to men. Interestingly, this effect disappears after the menopause. Although a difference in HCV viral load between men and women has not yet been described, our results suggest that women tended to have a lower HCV viral load than men, but this did not reach statistical significance. It has been shown that HCV clearance is comparable in girls and boys, which suggests that sex hormones might be important in HCV viral suppression. It would be very interesting to address this question in further research.

HIV and hepatitis B virus co-infection

As expected, HIV co-infection at the moment of HCV infection was associated with a lower odds of spontaneous clearance of HCV in HCV seroconverters from the ACS among DU, however this effect did not reach statistical significance. HIV infects CD4+ T cells, and during the natural course of HIV infection these cells are depleted. This depletion is attributed to heightened immune activation, and it is thought that microbial
translocation (i.e., increased systemic translocation from microbes and/or microbial products without evident bacteraemia from the lumen of the gut) contributes to this systemic immune activation.\textsuperscript{40} We and others have found indications that HIV co-infection leads to impairment of the development of a fully competent HCV-specific T-cell response, even before clinically overt immunodeficiency (as evidenced by the occurrence of opportunistic infections) has developed.\textsuperscript{41-44} Furthermore, we have shown in a small group of DU that especially HCV-specific CD4\textsuperscript{+} T-cell responses directed against non-structural HCV proteins are negatively influenced by HIV infection.\textsuperscript{45} However, larger numbers of prospectively sampled patients with HIV and HCV seroconversion are necessary to clarify the influence of HIV on HCV-specific T-cell responses.

In several cross-sectional studies that looked at the effect of hepatitis B virus (HBV) co-infection on HCV clearance in both HIV-infected and -uninfected individuals,\textsuperscript{26,46-51} presence of HBV surface antigen (HBsAg) was associated with higher odds of undetectable HCV RNA in only two studies.\textsuperscript{46,52} However, the cross-sectional design of these studies limits the ability to draw causal relations. One small longitudinal study and two case reports of chronically HCV-infected individuals with subsequent acute HBV infection show that some individuals cleared both HBV and HCV infection after acute HBV.\textsuperscript{53-55} We found that chronic HBV infection (as evidenced by positive HBV-Core antibodies and HBV-surface antigen expression) was associated with higher odds of spontaneous clearance of HCV, although this did not reach statistical significance.\textsuperscript{35} The interaction between these two hepatotropic viruses is interesting, and deserves further study. Since both viruses infect hepatocytes, possibly there is a direct competition for target cells in the liver. It could also be hypothesized that the local inflammatory environment that is induced by the second infection gives a local immunological boost and thereby enhances the capability of the immune system to eradicate not only HBV-, but also HCV-infected cells. It has not been reported whether HCV superinfection in HBV-infected individuals is also associated with viral clearance of both viruses. Furthermore, interaction between viral proteins from the two viruses might be an explanation, in which one viral protein inhibits a viral protein crucial for replication of the other virus.

**Clinical symptoms and HCV clearance**

In other persistent viral infections (e.g., Epstein-Barr Virus and HIV), severe clinical symptoms during acute infection are related to clinical problems later in life, including Hodgkins’ lymphoma, and a faster progression to AIDS and death for HIV, respectively.\textsuperscript{56,57} Clinical symptoms in acute EBV infection (infectious mononucleosis, a self-limiting lymphoproliferative disorder), are thought to be caused by massive expansion of virus-specific CD8\textsuperscript{+} T cells. Interestingly, in acute HCV a large expansion of HCV-specific T cells seems advantageous and is associated with spontaneous viral clearance.\textsuperscript{19,58-66} It has been shown that clinical jaundice and alanine aminotransferase (ALT) rise in exposed individuals was coincident with expansion of HCV-specific T cells,\textsuperscript{66,67} which suggests that the symptoms in acute HCV are mediated by the HCV-specific immune response, although numbers of patients were very small. We did not find evidence for the previously described association between clinical symptoms and HCV clearance.\textsuperscript{35} This is in line with Okayama et al. (14 HCV seroconverters, none reported jaundice), Cox et al. (62 HCV seroconverters, none reported jaundice),\textsuperscript{68,69} and in contrast to hospital-based studies by Corey et al. (that reported high occurrence of symptoms in 28 HCV infections in 24 patients), as did Tilman Gerlach et al. (60 acute
HCV, no asymptomatic patient cleared HCV. These findings, together with our observation that individuals with spontaneous viral clearance do not have higher CD8 counts than individuals that develop chronic HCV, suggests that the importance of clinical presentation as a predictor of viral clearance might be overrated. However, as the numbers in the studies mentioned are small, future studies should include more patients or pool data from several observational cohorts to gain statistical power.

HCV-specific T-cell responses

Both HCV-specific CD4+ and CD8+ T cells play an important role in spontaneous viral clearance in acute HCV infection. Depletion experiments in chimpanzees have shown that HCV clearance could not be achieved after CD4+ T cell depletion and subsequent re-infection. Since acute HCV infection is usually asymptomatic, patient numbers have usually been limited in studies on HCV-specific T cell immunity during acute HCV, ranging from 3 to 38 patients. However, spontaneous clearance has also been described in the absence of a strong CD4+ or CD8+ T-cell response. In conclusion, the exact correlates of immunological control over HCV are still unknown, and future research should focus on the quantity and quality of HCV-specific T cells, preferably in a larger cohort of patients. Furthermore, standardization of used assays would allow for better comparison between different laboratories and different patient cohorts.

We and others have shown that HCV-specific T-cell responses mainly targeting nonstructural HCV proteins are beneficial, and are associated with spontaneous viral clearance. However, spontaneous clearance has also been described in the absence of a strong CD4+ or CD8+ T-cell response. In conclusion, the exact correlates of immunological control over HCV are still unknown, and future research should focus on the quantity and quality of HCV-specific T cells, preferably in a larger cohort of patients. Furthermore, standardization of used assays would allow for better comparison between different laboratories and different patient cohorts.

We and others have shown that both injecting DU, and MSM can have HCV-specific T-cell responses before HCV antibody seroconversion. However, it is still unclear whether these responses are due to exposure to the virus, or due to actual infection, replication and successive clearance of the virus. Furthermore, it needs to be elucidated whether these responses play a role in protection against HCV infection and if so, what qualities of these T cells are important for the protection.

Remodelling of HCV-specific T-cell responses during chronic HCV infection and supposed re-exposure

Usually, professional antigen presenting cells (APC) like dendritic cells (DC) take up antigen in the periphery, and then migrate to a lymph node. There, naive CD8+ T cells are primed by the APC, after which they differentiate in cytotoxic T lymphocytes (CTL) with effector functions and memory CD8+ T cells. These CTL are released to the peripheral compartment and home to the site of inflammation to find the antigen they are primed for and kill the infected cells when they recognize one. When the HCV-specific T-cell response develops and quickly wanes or does not develop at all in acute HCV infection, viral persistence usually evolves. This waning of the immune response can have several causes. First of all, HCV seems to influence DC by suppressing CCR7 expression, which is a lymphoid tissue homing marker and thereby ‘trapping’ the DC in the liver. When T cells are not properly primed by DC, the T-cell response will be less, favouring HCV persistence. Viral escape mutations may render the HCV-specific T cells that are present non-functional, since they do not recognize the epitope anymore. Although the HCV genome is highly variable, Neumann-Haefelin et al. have shown that only half of the T-cell responses found in the liver were associated with viral escape mutations, and thereby also showed that the other half of the HCV-specific T cells recognized epitopes that were still present in the circulating virus.
During chronic HCV infection and supposed re-exposure by continuous risk behaviour, antigen-specific T-cell responses are constantly evoked and boosted. We show in chapter 5.1 that skewing of hepatitis C virus (HCV)-specific T cells to Core responses in chronic HCV infection in injecting drug users is associated with the presence of HCV RNA. Since these Core-specific T-cell response are associated with viral persistence, future studies should focus on whether targeting of Core by T cells is less efficient than targeting NS proteins or whether the observed skewing of these responses can be attributed to high antigenic pressure, with more presentation of Core to T cells and thereby skewing of the HCV-specific T-cell response.

During chronic HCV infection, liver fibrosis and -cirrhosis can develop. Development of liver-related morbidity and mortality is multifactorially determined, some of these factors are known, for instance alcohol consumption and HIV and HBV co-infection, while others remain to be elucidated. It is assumed that the development of liver fibrosis and -cirrhosis is mediated by chronic inflammation of the liver. Although there are many competing causes of death in the ACS, there are individuals that die of liver-related causes. Although liver-related death occurs only in a limited number of HCV seroconverters, a comparison between longitudinal HCV-specific T-cell responses in those who progress to HCV-related causes of death and those who do not, might identify host (T cell) characteristics that are associated with progression, especially since it is possible to correct for confounding factors like alcohol consumption and HIV co-infection within the ACS.

**Forced viral clearance during treatment of HCV with PEG-IFN and ribavirin**

Our findings on female sex and HIV co-infection suggest that HCV treatment can be postponed in HIV-negative women to await spontaneous viral resolution, unless the patient wants to be treated immediately. However, although the optimal regimen for treatment of acute HCV in HIV-infected individuals is unknown, treatment should not be postponed in individuals and groups who are HIV-infected, since treatment outcome is less favourable in HIV co-infected individuals. Furthermore, it has been shown in HCV mono-infected patients that the chance of achieving an SVR declines significantly within a year after acute infection, and there is no reason to assume that this will not occur in HCV/HIV co-infected individuals.

HCV genotype is the most important baseline predictor of HCV-treatment outcome, however, the underlying mechanism for this is yet unknown. Factors negatively influencing HCV treatment outcome are HIV co-infection, higher baseline viral load, and African-American race. It is thought that during treatment of HCV with PEG-IFN and RBV, HCV-specific T-cell responses are enhanced. However, so far only one study has been able to show this, while others did not find a boost of HCV-specific T cells during treatment. In chapter 5.2, we examined HCV-specific T-cell responses in HIV co-infected patients with HCV genotype 1 and 3, and we observed that HCV-specific T-cell responses at week 12 after start of treatment were more often undetectable in genotype 3 than in genotype 1 infected patients. Although this study was performed in a limited number of patients, this decline of HCV-specific T-cell responses suggests that the observed HCV-specific T-cell responses are dependent on continuous antigenic stimulation, which is in line with Capa et al. This implies that the role of HCV-specific T cells in viral clearance during treatment is limited and that forced HCV clearance is mainly achieved by the antiviral effectiveness of PEG-IFN and RBV.
Future perspectives of antiviral drugs

Next to the available PEG-IFN and RBV treatment, several 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 genotype 1 infected and treatment-experienced patients who were nonresponsive to PEG-IFN/RBV, respectively. Future treatment of chronic HCV will probably be more effective and shorter and consist of a combination of PEG-IFN and RBV together with one or more new drugs. During treatment with these new compounds the viral decline of HCV is very fast. In analogy to HIV, it has been shown that HIV-specific T-cell responses weaken very fast after initiation of HAART. Since there is no antigen present, there is no need for HIV-specific T cells anymore. It can be expected that this will happen to HCV-specific T cells during STAT-C as well. However, so far no studies have been done that support this hypothesis.

At present, 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, that 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.
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Summary
Summary

In this thesis the epidemiology of hepatitis C virus (HCV) in drug users (DU) and the immunology of HCV in DU, men who have sex with men (MSM) and hospital-recruited patients are described. This thesis starts with an overview of the epidemiological, viral, immunological and clinical characteristics of HCV (chapter 1).

Epidemiology

Injecting DU are at high risk for HCV and human immunodeficiency virus (HIV) infections through the sharing of needles and syringes. Within the Amsterdam Cohort Studies (ACS) among DU, originally initiated to investigate the epidemiology of HIV/AIDS, all participants are prospectively screened for HIV infection. To study the epidemiology of HCV, all participants with at least two visits were retrospectively screened for the presence of HCV antibodies.

In chapter 2.1, HCV prevalence, incidence and risk factors were studied in ever-injecting DU. HCV prevalence was very high, ranging from 63 to 93%. The yearly HCV incidence dropped substantially from 27.5/100 person years (PY) in the 1980s to 2/100 PY in recent years. Current injecting drug use and borrowing of needles was the main risk factor for HCV infection. The decline of HCV incidence was comparable to the decline in HIV incidence.

This declining HCV incidence led us to the investigation of the impact of harm-reduction programs on HIV and HCV incidence among ever-injecting DU from the ACS. Harm reduction participation was categorized by combining its two most important components (methadone dose and needle exchange program (NEP) use) and looking at 5 categories of participation, ranging from no participation to full participation. Methadone substitution treatment or NEP use alone were not significantly associated with HCV or HIV seroconversion. However, when these variables were combined, we found in chapter 2.2 that full participation in a harm reduction program was associated with a lower risk of HIV and HCV infection in ever-injecting DU, compared to no participation.

In self-reported never-injecting DU from the ACS, we found a higher prevalence of HCV (6.3%) compared to the general Dutch population (chapter 2.3). We studied incidence, prevalence, determinants, and molecular epidemiology of HCV infection to gain insight in transmission routes of HCV among never-injecting DU. HCV incidence was very low (0.49/1,000 PY). Risk factors for prevalent HCV infection were HIV-positive status, female sex, and starting injection drug use during follow up (a putative marker of past injection drug use). Using phylogenetic analysis, we found that HCV strains in never-injecting DU did not differ from HCV strains circulating in injecting DU, which implies a strong link with the injecting DU population. The increased risk could be related to underreporting of injecting drug use or to household or sexual transmission from injectors to non-injectors.

Natural history

Many aspects of the natural history of HCV are not yet well known, since acute HCV infection is usually asymptomatic and rarely recognized. In chapter 3.1 we show that spontaneous clearance occurred in 33.0% of HCV seroconverters in the ACS and that female sex was the most important predictor of clearance. No HCV viral or other
sociodemographic characteristics were significantly associated with spontaneous HCV clearance, but HIV and HBV co-infection might play a role. Progression to liver-related death is accelerated in HIV/HCV co-infected individuals. Since the life expectancy of HIV-infected DU improved after the widespread use of highly active antiretroviral therapy (HAART), HCV-related death is likely to become more important. In chapter 3.2 we describe the overall and cause-specific mortality between DU with HIV/HCV co-infection, HCV mono-infection, HIV mono-infection and DU without HIV or HCV. We show that HIV/HCV co-infected DU remain at increased risk of dying from liver-related death in the HAART era compared with HCV mono-infected DU.

Immunology in acute HCV

To understand parameters associated with resolved HCV infection, we analyzed HCV-specific T-cell responses in injecting DU with HCV seroconversion in chapter 4.1. We observed that the specificity of the CD4⁺ memory T-cell responses measured after 12-day expansion was most predictive of clearance: CD4⁺ T-cell responses predominantly targeting nonstructural proteins were associated with resolved HCV infection. Interestingly, we observed memory T-cell responses present before documented HCV seroconversion, suggesting that exposure had occurred before actual HCV infection.

In chapter 4.2, we describe the development of HCV-specific T-cell responses in 4 HIV-infected men having sex with men (MSM) with acute HCV infection. We show that an acute HCV infection in an HIV-1 infected individual can be suppressed in the presence of an HCV-specific CD4⁺ T-cell response targeting nonstructural proteins. To elucidate the role of HIV infection on HCV-specific CD4⁺ T-cell responses in acute HCV infection, we analyzed HCV-specific CD4⁺ T cells in 14 injecting DU shortly after HCV seroconversion in the presence or absence of acute HIV infection (chapter 4.3). In addition, we analyzed the influence of HIV infection on pre-existing HCV-specific CD4⁺ T-cell responses in already HCV-infected DU who subsequently acquired HIV. We show that both acute HCV/HIV co-infected DU who were previously exposed to HCV and mono-infected DU who developed chronic HCV infection tended to have higher responses to Core protein compared to DU that clear HCV. Furthermore, the HCV-specific T-cell responses were also skewed to unfavourable Core in 3/5 DU after HIV seroconversion. These results suggest that the effect of HIV infection on HCV-specific T-cell responses is not clear-cut, but is influenced by the sequence and outcome of previous HCV infection.

In chapter 4.4, we describe the unexpected finding that HCV-specific CD4⁺ T-cell responses were present in 3/3 HIV-infected MSM more than one year before HCV antibody seroconversion, indicating that these individuals had been exposed to HCV, but did not get (chronically) HCV infected until later.

Immunology in chronic HCV

A disadvantageous effect of HCV-specific T-cell responses directed against HCV Core protein in relation to spontaneous HCV clearance was observed in DU and MSM in chapters 4.1, 4.2 and 4.3, but we did not observe Core-specific T-cell responses in all individuals with chronic HCV infection. In chapter 5.1 we examined the relation between persistent exposure to HCV (by continuing risk behaviour) and skewing of the HCV-specific T-cell response towards Core in injecting DU with a high frequency of injecting
drug use and sharing of needles and self-reported never-injecting DU. We observed higher HCV-specific CD4\(^+\) T-cell responses in injecting DU compared to never-injecting DU, suggesting that continuous exposure leads to boosting of the immune response. Interestingly, none of the HCV-RNA negative injecting DU had a detectable Core response, while such a response was present in 40-44% of HCV RNA-positive DU. This suggests that continuous presence of HCV RNA affects the T-cell response to HCV.

It has been suggested that HCV clearance during treatment with pegylated interferon (PEG-IFN) and ribavirin (RBV) is caused by killing of HCV-infected hepatocytes by HCV-specific T cells. HIV/HCV co-infection is associated with lower sustained viral response rates after HCV treatment. In chapter 5.2, we describe how HCV-specific CD4\(^+\) and CD8\(^+\) T-cell response evolve during treatment in HIV co-infected patients with HCV genotypes 1 and 3. During the first 12 weeks of HCV treatment, we did not observe an augmentation of these responses, on the contrary we observed a decline of the height and the breadth of HCV-specific CD4\(^+\) and CD8\(^+\) T-cell responses that paralleled the decline in viral load. This suggests that enhancement of HCV-specific T cells does not appear to play a major role in forced viral clearance. Furthermore, we observed a higher perforin content of CD8\(^+\) T cells at baseline in genotype 3 patients, possibly indicating that there is a difference in general host immune activation between HIV-infected genotype 1 and 3 patients, for which the cause is unknown, but which may be associated to degree of liver inflammation.

Finally, in chapter 6, the main findings from our studies are discussed.
Samenvatting
Samenvatting

In dit proefschrift worden de epidemiologie van hepatitis C virus (HCV) in druggebruikers (DU) en de immunologie van HCV in DU, homomannen en ziekenhuispatiënten beschreven. Het proefschrift begint met een algemene inleiding over de epidemiologische, virologische, immunologische en klinische kenmerken van HCV infectie.

Epidemiologie

Injecterende DU hebben een verhoogd risico om HCV en humaan immunodeficiëntie virus (HIV) op te lopen door het delen van spuiten en naalden. In de Amsterdamse Cohort Studie (ACS) onder DU, die oorspronkelijk geïnitieerd werd om de epidemiologie van HIV en AIDS te bestuderen, werden alle deelnemers prospectief getest op de aanwezigheid van HIV. Om de epidemiologie van HCV te bestuderen, zijn alle deelnemers met tenminste 2 bezoeken retrospectief getest op de aanwezigheid van HCV antistoffen.

In hoofdstuk 2.1 worden de HCV prevalentie, incidentie en risicofactoren voor het oplopen van HCV beschreven in DU uit de ACS. De HCV prevalentie in DU die ooit drugs hebben geïnjecteerd was hoog, variërend tussen de 63% en 93% per kalenderjaar in de periode van 1985-2005. De jaarlijkse HCV incidentie daalde substanstieel van 27,5/100 persoonsjaren in de jaren '80 tot 2/100 persoonsjaren in meer recent kalenderjaren. Injecterend druggebruik en het daarbij delen van naalden was de belangrijkste risicofactor voor HCV infectie. De daling van de HCV incidentie was vergelijkbaar met de daling van de HIV incidentie in de ACS.

De observatie van een dalende HCV en HIV incidentie bracht ons ertoe het effect van zgn. ‘harm reduction’ programma’s op de incidentie van beide infecties te onderzoeken onder DU die rapporteerden ooit gespoten te hebben. Deelname aan ‘harm reduction’ programma’s werd in categorieën verdeeld door de meest belangrijke componenten (methadondosering en deelname in spuitomruilprogramma’s) te combineren. Op deze manier konden er vijf niveaus van deelname aan ‘harm reduction’ programma’s worden gedefinieerd, variërend van geen deelname tot volledige deelname. Methadondosering of deelname aan spuitomruilprogramma’s alleen waren afzonderlijk niet significant geassocieerd met het niet oplopen van HCV of HIV. In hoofdstuk 2.2 vonden we daarentegen dat DU die ooit hadden geïnjecteerd een lager risico hadden op HIV en HCV wanneer beide ‘harm reduction’ strategieën werden toegepast in vergelijking met DU die niet of onvolledig deelnamen aan ‘harm reduction’ programma’s.

In DU die rapporteerden dat zij nooit gespoten hadden bij intrede in de ACS vonden we een hogere prevalentie van HCV (6.3%) dan in de algemene Nederlandse populatie (hoofdstuk 2.3). We bestudeerden incidentie, prevalentie, determinanten en de moleculaire epidemiologie van HCV om inzicht te krijgen in de verspreiding van HCV onder niet-injecterende DU. De HCV incidentie was erg laag (0.49/100 persoonsjaren). Determinanten van een prevalente HCV infectie waren HIV infectie, vrouwelijk geslacht en het starten van injecterend druggebruik tijdens follow up in de ACS (een mogelijke marker van injecterend druggebruik in het verleden). Met behulp van fylogenetische analyse toonden we aan dat in niet-injecterende en injecterende DU (deels) dezelfde HCV virussen circuleren. Dit impliceert dat de verspreiding van HCV onder niet-injecterende DU gelinkt is aan de epidemie in injecterende DU. Het verhoogde risico op HCV infectie in DU die rapporteren nooit geïnjecteerd te hebben zou verklaard kunnen
worden door het onderrapporteren van injecteren of door transmissie van HCV van injecterende naar niet-injecterende DU. Bovendien zou er sprake kunnen zijn van huishoudelijke of seksuele transmissie van injecterende naar niet-injecterende DU.

Natuurlijk beloop van HCV infectie

Omdat acute HCV meestal onopgemerkt blijft en asymptomatisch verloopt, zijn veel aspecten van het natuurlijk beloop van HCV nog onbekend. In hoofdstuk 3.1 laten we zien dat 33.0% van de HCV seroconverters in de ACS het virus spontaan klaart. In onze studie blijkt vrouwelijk geslacht de belangrijkste voorspeller van spontaan klaren van HCV. Geen andere virale of socio-demografische kenmerken waren significant geassocieerd met spontaan klaren van HCV, maar de rol van co-infecties met HIV of hepatitis B virus ten aanzien van het spontaan klaren van HCV dienen verder worden uitgezocht.

De progressie van levergerelateerde ziekte is versneld in HIV/HCV co-geïnfecteerde individuen. De invoering van highly active antiretrovirale therapie (HAART) heeft de levensverwachting van DU met HIV verbeterd, waardoor het aannemelijk is dat HCV gerelateerde sterfte in deze groep belangrijker wordt. In hoofdstuk 3.2 beschrijven we de verschillen in overall en doodsoorzaakspecifieke sterfte in DU met HIV/HCV co-infectie, HCV mono-infectie, HIV mono-infectie en DU zonder HIV of HCV. We laten zien dat HIV/HCV co-geïnfecteerde DU een verhoogd risico op levergerelateerde sterfte houden in het HAART tijdperk vergeleken met HCV mono-geïnfecteerde DU.

Immunologie tijdens acute HCV infectie

In hoofdstuk 4.1 hebben we HCV-specifieke T celresponsen geanalyseerd in DU met HCV seroconversie met als doel beter inzicht te krijgen in factoren die geassocieerd zijn met spontaan klaren van HCV. We zagen dat de specificiteit van de HCV-specifieke CD4⁺ memory T celrespons het beste spontaan klaren van HCV voorspelt: een HCV-specifieke CD4⁺ T celrespons die met name tegen niet-structurele eiwitten van HCV gericht was geassocieerd met spontaan klaren van HCV. Verder observeerden we de aanwezigheid van HCV-specifieke memory CD4⁺ T celresponsen vóór de gedocumenteerde HCV seroconversie. Dit impliceert dat er reeds blootstelling aan HCV was zonder dat er een daadwerkelijke HCV infectie optrad.

In hoofdstuk 4.2 beschrijven we de ontwikkeling van HCV-specifieke T celresponsen gedurende acute HCV infectie in vier HIV geïnfecteerde mannen die seks hebben met mannen (MSM). We laten zien dat acute HCV infectie onderdrukt kan worden in HIV geïnfecteerde personen met HCV-specifieke CD4⁺ T celresponsen gericht tegen niet-structurele eiwitten van HCV.

Om de invloed van HIV infectie op HCV-specifieke CD4⁺ T celresponsen in acute HCV infectie beter te begrijpen, hebben we in hoofdstuk 4.3 HCV specifieke T celresponsen gemeten in DU met acute HCV mono- of HCV/HIV co-infectie. Verder hebben we gekeken naar de invloed van HIV op bestaande HCV specifieke CD4⁺ T celresponsen door deze te meten voor en na HIV seroconversie in DU die al HCV geïnfecteerd waren. We laten zien dat zowel mono-geïnfecteerde DU die een chronische infectie ontwikkelen als DU met acute HIV/HCV co-infectie die eerder in contact kwamen met HCV een hogere T celrespons tegen Core eiwit hebben dan DU die HCV spontaan klaren. Verder werd na HIV seroconversie de HCV specifieke T celrespons in 3/5 DU ongunstig beïnvloedt en meer tegen Core gericht. Deze resultaten geven een aanwijzing dat het effect van HIV infectie op HCV specifieke T celresponsen niet
eenduidig is, maar wordt beïnvloed door de volgorde van de infectie en de uitkomst van een eventuele eerdere HCV infectie.

In hoofdstuk 4.4 beschrijven we de onverwachte bevinding dat er reeds HCV-specifieke CD4⁺ T celresponsen aanwezig waren in 3/3 MSM voordat er HCV seroconversie optrad. Dit is een indicatie dat deze individuen blootgesteld zijn geweest aan HCV, maar nog niet (chronisch) geïnfecteerd raakten tot een later tijdstip.

Immunologie tijdens chronische HCV infectie

In de hoofdstukken 4.1, 4.2 en 4.3 observeerden we een ongunstig associeatie van HCV-specifieke T celresponsen gericht tegen het Core eiwit van HCV en het ontwikkelen van chronische HCV infectie. We zagen echter niet in alle individuen met chronische HCV Core-specifieke T celresponsen. In hoofdstuk 5.1 hebben we daarom gekeken naar de relatie tussen herhaalde blootstelling aan HCV (door persisterend risicogedrag) en de focus van de HCV-specifieke CD4⁺ T celrespons in injecterende DU met een hoge frequentie van injectorend druggebruik en delen van naalden versus DU die rapporteerde nog nooit te hebben gespoten bij intrede in de ACS. We observeerden hogere HCV-specifieke CD4⁺ T celresponsen in injecterende DU dan in niet-injecterende DU, wat suggereert dat herhaalde blootstelling de immuunrespons verhoogd. Geen van de HCV-RNA negatieve DU had een detecteerbare HCV-specifieke CD4⁺ T celrespons, in tegenstelling tot 40-44% van de HCV-RNA positieve DU. Dit suggereert dat de continue aanwezigheid van RNA de T celrespons beïnvloedt.

Het precieze werkingsmechanisme van HCV therapie is onbekend. Het wordt gesuggereerd dat het uiteindelijk klaren van HCV tijdens therapie met gepeglyeerd interferon (PEG-IFN) en ribavirine HCV specifieke cytotoxische T cellen kunnen stimuleren en op deze manier HCV geïnfecteerde levercellen kunnen opruimen. HIV/HCV co-infectie is geassocieerd met een lagere kans op succesvolle behandeling van HCV. In hoofdstuk 5.2 beschrijven we het beloop van de HCV-specifieke CD4⁺ en CD8⁺ T celrespons tijdens HCV therapie in HCV genotype 1 en 3 geïnfecteerd patiënten met HIV. Gedurende de eerste 12 weken van therapie zagen we geen toename van de HCV-specifieke T celrespons, maar juist een afname van de breedte en de hoogte van de respons. Deze afname verliep parallel aan de daling van de hoeveelheid HCV RNA gedurende antivirale therapie. Dit suggereert dat HCV-specifieke T cellen geen belangrijke rol spelen in het geforceerd klaren van HCV onder antivirale therapie. Verder zagen we dat patiënten die geïnfecteerd waren met HCV genotype 3 een hogere perforine expressie in CD8⁺ T cellen bij start van therapie hadden dan patiënten die geïnfecteerd waren met HCV genotype 1. Dit zou een aanwijzing kunnen zijn dat er een verschil is in totale immuunactivatie tussen patiënten met HCV genotype 1 en 3 die tevens een HIV infectie hebben.

Uiteindelijk worden in hoofdstuk 6 de resultaten uit dit proefschrift besproken en in een bredere context geplaatst.
List of publications
List of publications

CHSB van den Berg, C Smit, M Bakker, B Berkhout, S Jurriaans, RA Coutinho, KC Wolthers, M Prins. Major decline of hepatitis C virus incidence rate over two decades in a cohort of drug users. Eur J Epidemiol 2007;22:183-193.

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CHSB van den Berg, TA Ruys, NM Nanlohy, SE Geerling, JT van der Meer, JW Mulder, JA Lange, D van Baarle. Comprehensive longitudinal analysis of hepatitis C virus-specific T-cell responses during acute HCV infection in the presence of existing HIV-1 infection. J Viral Hepat 2009;16:239-248.

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CHSB van den Berg, TA Ruys, NM Nanlohy, TJW van de Laar, MGHM Beld, M Prins, D van Baarle. HCV-specific CD4⁺ T-cell responses are influenced by the sequence (and outcome) of previous HIV and HCV infection. Submitted.

TJW van de Laar, R Molenkamp, CHSB van den Berg, CJ Schinkel, MGHM Beld, M Prins, RA Coutinho, SM Bruisten. Frequent HCV reinfection and superinfection among injecting drug users argue against protective immunity against hepatitis C virus. Submitted.
CHSB van den Berg, CJ Schinkel, TJW van de Laar, R Molenkamp, D van Baarle, RA Coutinho, M Prins. Female sex is the strongest predictor of spontaneous viral clearance in a cohort of hepatitis C virus seroconverters. Submitted.

CHSB van den Berg, NM Nanlohy, TJW van de Laar, M Prins, D van Baarle. Skewing of hepatitis C virus (HCV)-specific CD4^+ T cells to Core protein is associated with the presence of HCV RNA. Submitted.

CHSB van den Berg, JE Arends, NM Nanlohy, TA Ruys, JA Lange, IM Hoepelman, D van Baarle. Stronger decline in HCV-specific T-cell response in genotype 3 compared to genotype 1 HCV/HIV co-infected patients during treatment with pegylated interferon and ribavirin suggests no role for HCV-specific immune responses in forced viral clearance. Submitted.

FAE Lambers, IG Stolte, CHSB van den Berg, RA Coutinho, M Prins. Harm reduction intensity - Its role in HAART adherence among drug users in Amsterdam. Submitted.
Dankwoord
Dankwoord

’The sky is the limit’, dat leek af en toe het motto van mijn project en was het grote voordeel van als één van de eerste HCV AIO’s te beginnen bij de GGD: alles wat leuk was kon, en mocht. Nadat ik de vacature in de yellow pages had gelezen, bedacht had dat die vacature mij op het lijf geschreven was en ook daadwerkelijk werd aangenomen, heb ik de afgelopen 4 jaar met heel veel plezier aan dit proefschrift gewerkt. Maar het was er waarschijnlijk niet in deze vorm geweest als ik niet veel hulp (en gezelligheid) van anderen had gehad in deze periode, en daarom wil ik iedereen die heeft bijgedragen aan dit boekje vanaf deze plek bedanken.

Mijn begeleiding door promotor en co-promotores was buitengewoon en bovenal erg aangenaam: Roel, Maria, Debbie, ik vond het een eer door jullie opgeleid te worden en ik heb waanzinnig veel van jullie geleerd. Dankjulliewel voor jullie kritische blik, het enorme tempo waarmee jullie stukken becommentarieerden en voor het motiveren op momenten dat ik het even niet meer zag zitten. Roel, ik waardeer het enorm dat je me altijd mijn eigen gang hebt laten gaan. Hoewel je sinds het begin vaak sceptisch was over de immunologische kant van dit boekje – en dan met name over het feit dat de kant er ooit zou komen – heb je me altijd laten doorgaan. Maria, het feit dat jij altijd open stond en dat jij altijd tijd maakte (ook als jij die eigenlijk niet had en zelfs als jij aan de andere kant van de aarde zat) om te overleggen en te brainstormen over nieuwe, wilde HCV plannen, hebben me enorm geïnspireerd en gemotiveerd de afgelopen jaren. Debbie, jouw gedrevenheid en onuitputtelijke positieve inslag werken enorm aanstekelijk, en ook al ben ik op mijn enthousiasme aangenomen en niet op mijn genialiteit, hoop ik dat je tevreden bent met het resultaat dat er ligt.

De beoordelingscommissie van dit manuscript: prof. dr. Miedema, beste Frank, bedankt voor het plaatsnemen in de beoordelingscommissie, jammer dat je niet bij de verdediging kon zijn. Ook de overige leden van de beoordelingscommissie, prof. dr. Ten Berge, prof. dr. Brinkman, prof. dr. Janssen, en prof. dr. Levi, wil ik bedanken voor het beoordelen van dit proefschrift. Dr. Fontanet, dear Arnaud, thank you very much for reading the manuscript and for coming to Amsterdam.

Nening en Marleen, mijn paranimfen, ik ben blij dat jullie naast me staan op deze bijzondere dag. Lieve Nening, dankjewel voor alles (praktische en emotionele!) steun de afgelopen 3,5 jaar in het lab. Ik zal ‘onze’ flow (en het foute uur) gaan missen! Lieve Marleen, dankjewel voor het doorsturen van de vacature, voor alle geweldige vakanties (waarvan eentje inclusief je collega’s!), en ik weet zeker dat jij hier volgend jaar ook staat, succes met die laatste stukken!

Al mijn (oud)-collega’s bij de GGD Amsterdam wil ik heel erg bedanken voor hun bijdrage aan dit proefschrift. Allereerst mijn kamernootjes: Akke en Karen, ‘kamer 150 oude stijl’, wat hebben wij veel gelachen daar! De aangrenzende kamer met verpleegkundigen (in chronologische volgorde): Joke, Ans, Janneke, Bettina, zuster Marjolein en Laura: dankjulliewel voor de gezelligheid en alle hulp. Sandra, helaas hoeven we nu nooit meer met z’n tweeën met een GGD-autotoe op pad om levens te redden... Titia, af en toe leek het wel Herberg De Zoete Inval op B1.10A, maar het was
vooral heel productief en gezellig! Thijs en Robin, dankjullie wel voor alle koppen koffie en lunches binnen of buiten de deur. Het was fijn dat jullie bleven hangen op de GGD! Colette, dat was nog eens een manier om te leren analyseren! Anneke, dankjewel voor het beantwoorden van al mijn vragen over de (in opzet briljante!) D-data, ik ben zo blij dat ik nauwelijks iets met de H-data heb hoeven doen!!! Anouk, het motto “bij twijfel, kopen” houden we er zeker in. Freke, als jij het niet goedkoper kan vinden op internet, dan kan het niet! Wanneer gaan we nou naar Marrakech (en dat geldt ook voor jou, Jannie)!

Christine, jij leerde ons druggebruikers te behandelen voor HCV, de DUTCH-C is door Karen en jou een enorm succes! Ineke, zó gezellig dat je weer terug kwam na de afdeling onderzoek! Maarten, hoewel je af en toe wat sarcastisch uit de hoek komt, ben je geloof ik een aanwinst voor de afdeling. In ieder geval weet je veel van STATA! Bart, volgend jaar ben ik er weer bij met carnaval! Geen afdeling zonder secretariaat: Carol Ann en Will (als jij het niet weet…). Verder natuurlijk ook alle andere collega’s van de afdeling onderzoek: Daniela, Dick, Femke (nu ook HCV!), Irene (soms was een full time begeleider misschien toch handiger), Marc, Maria X, Marion, Marlies, Martijn, Rik (die snoeppot was killing!!), Ronald (dankjewel voor al je commentaar en hulp bij alle (statistische) stukken), Udi (zoals jij is er maar één), en Reinier. Officieel geen GGD, maar erg gezellig op congressen: Joep en Bart, San Francisco en Washington waren top, wanneer gaan we weer?

In het UMC Utrecht hadden we binnen de afdeling immunologie onze eigen ‘Groep Debbie’: Ana (HLA rules!), Anne (ook bij jou werd de immunologie aan het einde echt leuk), Bente, Dan, Dena, Evelien, Floor (nog eens nadenken over interactie tussen EBV en HCV?), Ingrid en Jolanda (fijn dat we in de laatste fase van onze promolies lotgenotencontact konden hebben, het is allemaal toch goed gekomen), Joop (onze joined effort heeft toch twee mooie papers opgeleverd! ), Karlijn (de core blijft Core!), Leonie (zouden we nu het af is vaker kunnen gaan sporten?), Ronald en Wouter (het blijft een briljant plan!). In het UMC Utrecht hadden we binnen de afdeling immunologie onze eigen ‘Groep Debbie’: Ana (HLA rules!), Anne (ook bij jou werd de immunologie aan het einde echt leuk), Bente, Dan, Dena, Evelien, Floor (nog eens nadenken over interactie tussen EBV en HCV?), Ingrid en Jolanda (fijn dat we in de laatste fase van onze promolies lotgenotencontact konden hebben, het is allemaal toch goed gekomen), Joop (onze joined effort heeft toch twee mooie papers opgeleverd! ), Karlijn (de core blijft Core!), Leonie (zouden we nu het af is vaker kunnen gaan sporten?), Ronald en Wouter (het blijft een briljant plan!).

AIO-kamer 3 in Utrecht heeft een enorm verloop gehad de afgelopen jaren, wat resulteerde in niet minder dan 22 kamergenoten in 3,5 jaar tijd! En misschien vergeet je dan zelfs nog iemand… Hoewel jullie allemaal hebben bijgedragen aan een gezellige, productieve, aan het einde van de middag bloedhete kamer, wil ik een aantal van jullie apart noemen, waaronder de laatste bezetting: Annelieke, Arjen, Ellen, Kees (niks mis met iedere dag kroketten!), Peter, Rogier, Sabina, en Suzan (de term “woest aantrekkelijk” is niet meer uit mijn vocabulaire te krijgen). And of course I would also like to thank my English speaking roommates Hakim and Vica. Verder wil ik nog twee mensen van de oude garde bedanken, Jantine, Lydia (succes bij het IRAS!). Naast AIO-kamer 3, waren er nog twee AIO-kamers, een hoop stafkamers en een complete andere verdieping waar veel collega’s van zaten en zitten die ik wil bedanken, ik zou niet durven om een uitputtende lijst te maken, maar ik wil in ieder geval Ankje (kom maar door met die 2e pannenkoek), Christine, Eva, Ineke (ook eindelijk klaar!), Gerrit, Kiki (en toen zat je ineens in mijn commissie, super!), Linde, Nienke, Sigrid en Marieke noemen. En ook hier mag het secretariaat niet ongenoemd blijven: Saskia and Yvonne, dankjullie wel.

Verder wil ik alle deelnemers aan de Amsterdamse Cohort Studies bedanken, zonder wie veel van de studies in dit proefschrift niet mogelijk waren geweest. Naast het feit dat mijn project één grote samenwerking was, zijn veel stukken in dit proefschrift het
resultaat van samenwerking met andere afdelingen dan de twee waar ik heb gewerkt. Ik wil alle co-auteurs bedanken voor hun belangrijke inbreng in dit proefschrift, met name Margreet Bakker, Janke Schinkel, Marcel Beld, en Richard Molenkamp.

Zonder vrienden die accepteren dat je soms liever achter je pc blijft zitten dan met ze af te spreken, en die je ook nog leuk vinden als je vlak vóór de deadline van je manuscript zit, is het denk ik heel lastig om te promoveren. Lieve Carline en Jasper, bij jullie mocht ik altijd crashen, en ik ben heel blij met jullie relativeringsvermogen (en kookkunsten!). Cindy, vriendinnetje van het eerste uur, ook al woon je ver weg en ben je nu mama, we gaan elkaar nog vaak zien! Sytske, het was me het jaar wel, er is veel veranderd, maar eigenlijk is alles hetzelfde gebleven. Fonnet en Fleur, van samen co-assistent op de chirurgie in Hilversum tot vriendinnetjes en lotgenoten: de een promoveert een week eerder dan ik en de ander volgend jaar, dankjulliewel voor de koppen koffie, de etentjes en natuurlijk het tripje naar Turijn! Imelda, wat een heerlijke vakanties en wat kun jij goed forel vissen…

En dan als laatste diegenen die voor mij het allerbelangrijkst zijn: lieve papa en mama, dankjulliewel voor jullie onvoorwaardelijke liefde en steun, voor jullie ongelooflijke vertrouwen in mij en voor het op de poezes passen als ik weer eens 3 weken vakantie aan een congres moest vast plakken! Ik hou van jullie.

Charlotte
Curriculum Vitae
Curriculum Vitae

Charlotte Heleen Sophie Betty van den Berg is op 20 augustus 1978 geboren in Wageningen. Na vier jaar in Bussum te hebben gewoond, vertrok het gezin Van den Berg naar Maastricht. Nadat zij daar het ongedeeld VWO heeft afgerond op het Sint Maartenscollege, werd zij uitgroot voor de studie geneeskunde. Hoewel ze vol goede moed begon aan de 1e kandidatuur geneeskunde aan de Katholieke Universiteit Leuven, heeft ze het daarop volgende jaar toch weer meegeloot voor de opleiding geneeskunde in Nederland, ditmaal succesvol. In 1997 begon zij met de studie geneeskunde aan het AMC/Universiteit van Amsterdam.

Tijdens de studie was er niet alleen tijd voor studeren, zo heeft zij in het derde jaar van het doctoraal geneeskunde een jaar als penningmeester in het bestuur van de MFAS gezeten. Verder heeft zij een facultatieve klinische stage op de afdeling interne geneeskunde gedaan in het RSUP Manado, Sulawesi, Indonesië. Tijdens de wetenschappelijke stage heeft zij onderzoek gedaan naar het voorkomen van uniparentele disomie bij patiënten met het Beckwith-Wiedeman syndroom op de afdeling klinische genetica in het AMC (begeleiders J. Bliek, dr. M. Mannens en prof. dr. N. Leschot).

Na haar co-schappen, ondanks een keuze- én een oudste co-schap kinder-geneeskunde, heeft zij uiteindelijk toch voor interne geneeskunde gekozen. Na een half jaar klinische ervaring te hebben opgedaan in het Kennemer Gasthuis (opleider prof. dr. R. ten Kate), is zij begonnen als beleids-AIO van het AMC. Begeleid door prof. dr. R. Coutinho, dr. M. Prins en dr. D. van Baarle, heeft zij promotieonderzoek gedaan bij de afdeling onderzoek van de GGD Amsterdam en de afdeling immunologie van het UMC Utrecht. De resultaten van dat onderzoek staan beschreven in dit proefschrift. Sinds 1 april 2009 is ze in opleiding via het AMC tot internist in het Flevoziekenhuis in Almere (opleiders prof. dr. P. Speelman en dr. S. Peters).