Epidemiology and diagnosis of acute hepatitis C virus infection
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INTRODUCTION AND OUTLINE OF THIS THESIS
HEPATITIS C VIRUS - A BRIEF HISTORY

After the development of diagnostic tests for hepatitis B virus (HBV) in 1963 and hepatitis A virus in 1973, it was clear that most cases of blood transfusion-associated hepatitis were not caused by these or other known viruses [1]. The causative agent of this blood-borne, non-A, non-B hepatitis virus was designated the hepatitis C virus (HCV) after its discovery in 1989. HCV is a small (50 nm), spherical, enveloped, positive sense, single-stranded RNA virus, and belongs to the genus Hepacivirus of the Flaviviridae family [2]. Soon after HCV was identified, genome sequences that became available showed a high level of genetic diversity. Currently, 7 genotypes have been described, covering at least 67 subtypes (e.g., 1a, 1b, 1c) [3,4]. HCV is considered a serious global health problem; an estimated 2.6-3.1% of the world’s population (equivalent to 169-202 million people) is chronically infected and is therefore at risk of developing liver disease [5–7]. Currently, over 350,000 deaths are attributed to chronic HCV infection each year worldwide [8]. By 2007, mortality associated with HCV infection had surpassed that from human immunodeficiency virus (HIV) infection in the USA [9].

EPIDEMIOLOGY

Worldwide
In many developed countries, the prevalence of HCV infection in the general population is less than 2% (figure 1). In several countries in Latin America, the former Soviet Union, Africa, the Middle East, and Southern Asia, the prevalence is higher (>2%). Egypt has the highest prevalence of at least 15%, due to unsafe community-wide parenteral therapy against schistosomiasis from the 1920s until the 1980s [10–13]. India has a lower prevalence of approximately 0.5-1.5% due to a (historically) underdeveloped healthcare system [14,15], but because India is the second most populous country in the world with currently over 1.2 billion inhabitants, the disease burden is among the highest [15].

The Netherlands
The most recent HCV seroprevalence estimate in the Netherlands was 0.22% (range: 0.07-0.37), corresponding to approximately 28,100 individuals (range: 9,600-48,000) [16]. The majority (i.e., 41%) of HCV positive individuals is estimated to be first generation migrants from high-endemic countries. In the Netherlands, HIV-infected men who have sex with men (MSM) are currently the main group at risk for HCV infection [17,18], while HCV seroprevalence is highest among people who inject drugs (PWID); estimates range from 46.7-78.4% for HIV-negative PWID, up to 91.6-95.9% for HIV-positive PWID [16]. The estimated prevalence of chronic HCV infection among ever-injecting PWID in Amsterdam was 80.7% in 2010 [19]. Currently, very few incident infections occur among PWID in the Netherlands, mostly because the total number of PWID, the number of new PWID, as well as injection risk behavior has
been decreasing since 1986 [20,21]. One of the major contributors to the observed decrease in incidence is a high competing mortality rate due to HIV infection in HCV-infected PWID [19]. A declining trend is also seen in the US [22], and in Australia (after 2003) [23], although the decline is not as profound as in the Netherlands.

**Figure 1** Global prevalence and genotype distribution of HCV infection, by Global Burden of Disease (GBD) region. The size of the pie charts is proportional to the number of seroprevalent cases [7]. Copyright © 2014 Messina et al., reprinted with permission of the publisher, John Wiley & Sons, Inc.

**TRANSMISSION**

The three main transmission routes along which HCV currently travels are: (1) sharing of injecting equipment among PWID [24], (2) nosocomial transmission, and (3) sexual transmission, which is mostly observed among HIV-infected MSM [25,26]. Mother-to-child transmission rates are estimated to be 5-7% [27,28], and are up to four times higher among HIV/HCV-coinfected mothers [28,29]. Coinfection with HIV and HCV is relatively common because both viruses share the same transmission routes; up to one third of HIV-infected individuals are estimated to be co-infected with HCV [30–32].

**People who inject drugs**

PWID are considered to be at the highest risk of HCV infection [33–35]. Worldwide, over 75% of incident infections occur among PWID [36]. Because transmission of HCV is more than 10 times more likely to occur through blood-blood exposure than transmission of HIV [37], HCV infection usually precedes HIV infection among PWID.

**Nosocomial exposure**

Before 1992, no serological screening tools were available for HCV. Patients who had a blood
transfusion (e.g., hemophiliacs) or organ transplantation prior to this year were therefore at increased risk of HCV infection. In developed countries, incidence of HCV infection after a transplant or transfusion decreased from 5.0-13.0% before 1986, to 1.5-9.0% between 1986-1990, to 0.6-3.0% thereafter [5]. In developing countries, nosocomial transmission is mostly caused by receiving blood(products) from unscreened donors, or other unsafe medical procedures [36].

Sexual transmission
Among serodiscordant monogamous heterosexual couples, sexual transmission of HCV is considered negligible [38]. However, in the early 2000s multiple outbreaks of HCV were reported among HIV-infected MSM in Australia [39], France [40], Germany [41], the Netherlands [42,43], Switzerland [44], the UK [45,46], and the USA [47]. Analysis of multiple, mostly European, cohorts in a collaborative study known as the Concerted Action on SeroConversion to AIDS and Death in Europe (CASCADE), revealed that the incidence of HCV among HIV-infected MSM appeared to be slightly elevated already during the mid-1990s, but the main expansion started after 2002 [48]. Whether the increase in HCV incidence was due to injecting drug use of these men was, for a long time, debated [49]. The HCV epidemic among MSM seems to be largely restricted to those infected with HIV [50,51]. This may partly be a reflection of higher sexual risk behavior among MSM who acquire HIV infection compared to MSM who do not. The practice of MSM selectively having unprotected sex with men of the same HIV-status, referred to as serosorting [52], may have added to the spread of HCV infection among HIV-infected MSM [53]. However, an enhancing effect of HIV infection itself on susceptibility and/or infectivity for HCV may also exist [54]. In addition, HIV may be more efficiently transmitted through sexual contact than HCV and the prevalence of HIV was, at least at the start of the HCV epidemic, lower for HCV than HIV; HIV-negative MSM with risky sexual behavior would therefore be more likely to first acquire HIV, and later on HCV [43]. Having concomitant other, especially ulcerative, sexually transmitted infections (STI), may also increase transmissibility of HCV [50].

VIROLOGY

Genome organization
HCV consists of a single open reading frame with approximately 9,400 nucleotides, that encode for a single, polyprotein that contains slightly over 3000 amino acids (depending on the genotype). This polyprotein is post-translationally cleaved into three structural proteins (core, E1 and E2), seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and is flanked by two highly conserved untranslated regions (UTR; figure 2). The 5’UTR consists of four highly structured domains and contains the internal ribosome entry site (IRES). The IRES is essential for the virus to use the ribosomes in the host cell for the initiation of translation. The 3’UTR consists of stem-loop structures and an internal poly(U/UC) tail [55].
Life cycle

Human hepatocytes are the main target of HCV. Viral entry occurs through receptor mediated endocytosis. Virus receptors and co-receptors on the host cell that are involved in cell entry include tetraspanin CD81, scavenger receptor class B type I (SR-B1), claudin-1, occludin, Niemann-Pick C1-like 1 (NPC1L1), and low-density lipoprotein (LDL) receptor [55–58]. Mannose-binding lectins DC-SIGN and L-SIGN may also facilitate internalization [59,60]. After viral entry, translation takes place at the ribosomes located at the endoplasmic reticulum (ER), after which the polyprotein is cleaved by activity of both cellular and virally encoded proteases, the latter including \textit{NS2}, \textit{NS3}, and co-factor \textit{NS4A}. The \textit{NS5B} protein serves as an RNA dependent RNA polymerase [57].

Replication takes place in the so-called ‘membranous web’, a web-like structure which is formed through \textit{NS5A}-induced alterations of the membrane, and consists of ER-derived vesicles. All HCV proteins accumulate on the membranous web to form a viral replication complex [61]. New positive sense RNA strands are generated by the \textit{NS5B} polymerase through transcription of an intermediate negative sense RNA template strand. The lack of a proofreading mechanism of the viral polymerase is the cause of a large intra-host genetic diversity, sometimes referred to as quasispecies [3]. Excess positive sense RNA may be used (again), either for translation, or as templates for replication [57]. Finally, virus particles are assembled by recruiting envelope
(E1 and E2) complexes and budding at the ER membrane. Mature virions are released by exocytosis [62].

**Diagnosis**

Standard HCV testing is performed by screening for the presence of HCV-specific immunoglobulin G (IgG) antibodies in serum. Commercial assays that can detect IgG response of HCV antibodies (anti-HCV) are enzyme immunoassays (EIA) that express recombinant proteins (i.e., core, NS3, NS4, and NS5). Anti-HCV is usually detectable within 8-20 weeks of infection [63]. Conflicting results regarding delayed seroconversion due to HIV co-infection have been published; this topic is addressed in part III of this thesis. The recombinant immunoblot assay (RIBA) was developed as a supplemental assay to enhance specificity of positive anti-HCV EIAs [64]. Currently, the primary purpose of RIBA testing is to distinguish between resolved HCV infection (EIA positive, RNA negative, RIBA positive) and false-positivity of the EIA (EIA positive, RNA negative, RIBA negative). RIBAs are rarely used in clinical practice for some years now, because clinical relevance is determined by the presence or absence of HCV RNA rather than a false-positive result of the EIA. Therefore, a positive anti-HCV test is more likely followed by an HCV RNA test. Commericially available quantitative HCV RNA assays are very sensitive and can have a lower limit of detection (LLOD) of 15 international units (IU) ml⁻¹. Another HCV-specific diagnostic assay targets the core antigen. The currently available core antigen assay is less sensitive than HCV RNA assays (LLOD: 500-3000 IU ml⁻¹) but, because it can be up to four times cheaper and results may be available sooner than RNA testing, it may be a relevant alternative for low-resource countries. The core antigen assay may also be used as a screening tool for HCV infection among HIV-infected individuals, as low HCV viral loads are rarely reported in HIV/HCV-coinfection [65]. A non-specific way of screening for HCV includes regular assessment of serum alanine aminotransferase (ALAT) concentration; elevated liver enzyme concentrations may indicate hepatic inflammation [66], even when the elevation is considered ‘mild’ [67].

**MOLECULAR EPIDEMIOLOGY**

**Diversity among HCV strains**

For HCV, inter- and intra-genotype sequence similarities are around 65-70% and 70-85%, respectively, depending on the genetic (sub)region of HCV [4]. When genetic similarity between different viral strains is larger, it is likely that a common source of infection and/or transmission route exists [68]. Genotype distribution differs by both geographic region (figure 1) and transmission route. Worldwide and in the Netherlands, the prevalence of HCV genotype 1 is highest, followed by genotypes 2 and 3. Genotype 4 is most prevalent in Africa and the Middle East, genotype 5 in Southern sub-Saharan Africa, and genotype 6 in South-East Asia [69,70]. Regarding differences in genotype distribution by transmission route, subtypes 1a, 3a, and
4d are commonly found among PWID [71,72], while subtypes 1b, 2a and 2b are associated with contaminated blood products [70,73–75]. Among HIV-infected MSM, subtypes 1a and 4d are the dominant genotypes [41,71,76]. In order to determine the genotype or subtype of a diagnosed infection, part of the genome can be sequenced; regions that are particularly distinctive are the core [77] and NS5B regions [78]. Alternatively, a commercial line probe assay (LiPA) [79] and a RealTime HCV genotype assay [80] are available that target the conserved 5’UTR and core regions.

**Phylogenetic analysis**

Because of the genetic diversity that is apparent among different genotypes and subtypes, phylogenetic analysis can be used as a tool to study the evolutionary relationship between different variants, which can be used to identify potential transmission networks. For this, the genetic distance (or divergence) between different viral strains is represented in an evolutionary tree. The absolute difference between two sequences, often referred to as p-distance, underestimates the number of (synonymous and non-synonymous) nucleotide substitutions [81]. Therefore, more complex substitution models have been developed that correct for differences in base frequency, transversion and transition rates (including reversals) [82–87]. When the divergence between sequences is small, a monophyletic cluster may be visible; a group of sequences that share a common ancestor. To determine the robustness of the inferred phylogenies, bootstrapping is a frequently used method [88]. In order to perform molecular epidemiological studies, sociodemographic information and preferably also data on risk behavior from each study subject is combined with the phylogeny of a gene of interest. For HCV, NS5B, core, and/or envelope sequences are often used for investigating its molecular epidemiology [41,71,78,89–92].

**NATURAL HISTORY**

**Clinical course of infection**

The majority of acute HCV infections (>70%) pass by clinically silent; among patients who do experience symptoms during acute infection, those most frequently reported are not specific for HCV infection: loss of appetite, fatigue, abdominal pain, nausea, and rarely icterus (jaundice) [93,94]. Approximately 25% of acute HCV infections are spontaneously cleared, usually within the first six months of infection [95–97], but clearance rates ranging from 11-49% have been reported [98,99]. While the underlying mechanisms of spontaneous clearance are not fully understood, several associations are known to positively affect the ability to clear HCV: younger age at time of infection [100,101], female gender [102–107], HBV co-infection, HIV-negative status [107], elevated bilirubin, elevated ALAT, and elevated interferon-γ-inducible protein (IP)-10 levels [108,109], favorable host single-nucleotide polymorphisms rs12979860 and/or rs8099917 in the interferon-lambda (IFNL) 3 gene
(formerly known as IL28B) [107,110], and among individuals infected with HIV: higher CD4+ T-cell count [108]. Patients that progress to chronic infection are at risk of accumulation of hepatic fibrosis and may develop cirrhosis (10-20% after 20-30 years of infection) without therapeutic intervention [95]. Hepatic fibrosis is the result of collagen production by hepatic stellate cells. Normally, hepatic stellate cells are dormant and store the majority of the body’s vitamin A, but liver damage will lead to an activated state of these cells [111]. Cirrhosis is a condition in which the architectural organization of the liver is significantly altered due to hepatic fibrosis; this may lead to impaired liver function, and portal hypertension. Once the liver has become cirrhotic, risk of hepatocellular carcinoma and hepatic decompensation are respectively 1-5 and 3-6% per annum [112]. The level of fibrosis is often scored using either the METAVIR [113] or the Ishak [114] scale; these scales range from F0 or 0 (i.e., no fibrosis) to F4 or 6 (i.e., cirrhosis), respectively. HIV/HCV-coinfected individuals have an accelerated progression of liver disease and increased mortality [30,32,115]. The exact mechanisms behind the associations with accelerated disease progression in coinfected individuals compared to HCV-monoinfected individuals have not been fully elucidated. Hypotheses include a direct effect of HIV-infection on the hepatocytes and/or stellate cells, and HIV-induced immunologic dysregulation, e.g., diminished HCV specific T-cell responses, increased immune activation, and increased hepatocyte apoptosis [30].

**HCV reinfection**

A previously cleared infection (either spontaneously or treatment-induced) does not protect against reinfection, although partial protective immunity against viruses similar to the primary infection may be developed [68,91,116–118]. Chimpanzees that were re-exposed to HCV in reinfection studies have (but not consistently) shown cross-genotype immunity (i.e., protection against heterologous virus strains) [119]. Among HIV/HCV-coinfected MSM that spontaneously cleared, or were successfully treated for acute HCV infection, the incidence of reinfection has been estimated to be between 8.0-15.2 per 100 person-years (PY) of follow-up [120,121]. Among PWID treated for chronic infection, incidence of reinfection was 0.8-4.7 per 100 PY [68], and among PWID that cleared acute infection, reinfection incidence was 1.8-46.8 per 100 PY [118,122]. Reinfection is usually supported by the observation that a different viral strain is present after a patient had an undetectable viral load. Usually, sequence analysis of the NS5B gene will suffice to determine this [68]. However, in a (relatively) recent outbreak setting as is the case for HIV-infected MSM, reinfection with the same HCV genotype may only be determined (or excluded) using sequence analysis of a more variable region, e.g., the hypervariable region 1 (HVR1; also see figure 2), located in the E2 gene [91,121]. This part of the HCV genome allows for more detailed analysis because it has a faster evolution rate, driven by the host immune response, than the NS5B region.

Dual or mixed infection with two (or more) distinct HCV variants may, depending on the timing of these infections, be classified as coinfection or superinfection. Dual infection
refers to infection with two or more heterologous HCV variants simultaneously, or within a time frame in which no immunologic response to the first virus has yet been established. Superinfection is defined as infection with a heterologous virus after persistent infection of the first variant has already developed [123,124]. Few studies have examined the frequency of superinfection; three small studies among PWID reported frequencies ranging from 0% to 24% [124]. However, the impact of dual/mixed HCV infection or superinfection on clinical outcomes of the disease remains unclear [124,125].

TREATMENT

Successful treatment is indicated by a sustained virological response (SVR), meaning that no detectable HCV viral load is present 24 weeks after treatment cessation; this is regarded as a cure. Currently SVR12 is often reported, meaning 12 weeks after end of treatment (EOT) no HCV viral load can be detected. Viral relapse is defined as having a detectable HCV RNA at 24 weeks following EOT, without detectable HCV RNA at EOT. To exclude reinfection (discussed hereafter), this treatment outcome is generally supported by confirmation that the same viral strain was present before and after EOT.

Because the number of treatment options has increased enormously since 2013 and will continue to do so in the coming years, this paragraph was updated until 1 December 2015 using the Dutch HCV treatment guidelines, published by the NIV (Dutch internists association), NVHB (Dutch association for HIV care providers), NVMDL (Dutch association of gastrointestinal and liver doctors), NVH (Dutch society of hepatology), and NVZA (Dutch association of hospital pharmacists). In addition, www.hcvguidelines.org was accessed; this website is a cooperative effort from the American Association for the Study of Liver Diseases (AASLD), the Infectious Diseases Society of America (IDSA), and the International Antiviral Society-USA (IAS-USA) to constantly update recommendations for testing, managing, and treating HCV.

An increasing number of different treatment regimens can be used to treat an HCV infection. In the late 1980s, interferon α was used to treat chronic HCV infection [126,127]. The US Food and Drug Administration (FDA) approved interferon α-con1 for treatment of HCV infection in patients with compensated liver disease in 1997. Ribavirin was initially developed for treatment of HIV infection [128], but was more effective for treatment of flaviviruses. Therefore, studies were conducted to evaluate the effect of ribavirin therapy on patients with chronic HCV infection [129,130]. Ribavirin was found not to affect the HCV RNA load, but led to a decrease in ALT. Subsequently conducted studies showed that the combination of interferon α and ribavirin was more effective for treatment of chronic HCV infection than was therapy with either of these agents [131]. Ribavirin was approved by the FDA in 1998.
Pegylated (peg) interferon was developed in the late 1990s and was shown to be more effective than interferon [132]. Peg-interferon α-2b and peg-interferon α-2a were approved in 2001 and 2002, respectively. Since then, treatment for chronic HCV mono-infection consisted of peg-interferon and ribavirin for 12-24 weeks for genotypes 2 and 3 (response rate: 76%-80% SVR), or 24-48 weeks for genotypes 1 and 4 (response rate: 46%-60% SVR [133–135]. In 2002, ribavirin was also approved for combination therapy in HIV/HCV-coinfected patients. SVR rates obtained among HIV/HCV-coinfected patients were higher using dual therapy, but were lower than among HCV mono-infected patients [136]. Outside clinical trials, acute HCV infection among HIV-infected patients are currently treated with peg-interferon and ribavirin for 24 weeks, irrespective of the genotype [137,138]. While therapy with peg-interferon is highly effective for treatment of recently acquired HCV, the optimal timing of treatment initiation, regimen and influence of host factors remain unclear [139]. Adverse effects of treatment with peg-interferon and ribavirin may be clinically significant, and may require discontinuation of treatment; these adverse effects include severe depression, insomnia, suicide, and pancytopenia [140,141].

Recently, a new era of HCV treatment has begun, with medication known as direct acting antiviral drugs (DAA) [142]. DAAs directly target key components of the viral life cycle. When the life cycle cannot be completed, no viable virus can be produced. Viral targets of HCV DAA are: NS3 (protease inhibitor (PI); all have the extension -previr) [143], but also inhibitors of the viral replicase complex, e.g., (non-) nucleoside and nucleotide NS5B polymerase inhibitors (all have the extension -buvir), and NS5A inhibitors (all have the extension -asvir) [143]. In April 2012, two PIs were licensed in the Netherlands: telaprevir and boceprevir [144–147]. These drugs were administered in combination with peg-interferon and ribavirin and improved SVR rates, but also significantly increased side effects like rash, pruritis and anemia [148,149]. Both boceprevir and telaprevir have already become obsolete due to the development of more favorable DAAs. However, treatment of acute HCV infection among HIV/HCV-coinfected patients may be shortened to 12 weeks when triple therapy with peg-interferon, ribavirin, and telaprevir or boceprevir is given [150,151]. Currently, sofosbuvir, simeprevir, daclatasvir, ombitasvir-paritaprevir-ritonavir with or without the addition of dasabuvir, and ledipasvir-sofosbuvir have been approved for treatment of chronic HCV infection in the Netherlands, regardless of METAVIR stage [152–154]. Ombitasvir-paritaprevir-ritonavir, and daclatasvir-asunaprevir-beclabuvir are the next regimens in line for approval. The chosen regimen (and the addition of ribavirin) depends, among others, on the HCV genotype, prior response to treatment, HIV treatment regimen, the degree of fibrosis, and renal insufficiency [144,155–161].

DAAs allow for shortened, simplified treatment and are better tolerated than interferon-based treatment regimens. The proportion of patients that achieve an SVR following DAA therapy is about 95%, but may be lower for patients infected with HCV genotype 3 [162,135,149,163].
At the moment, a 12-week course of sofosbuvir-ledipasvir is, at a cost of €50,000 in the Netherlands, around 13 times more expensive than 24 weeks peg-interferon and ribavirin. Because the costs of these novel drugs are high, cost effectiveness and budget impact remain an important barrier for widespread implementation [164–170]. Drug-drug interactions with HIV PIs, as well as substance use [171], can be problematic and may require close monitoring; the latest updates on drug-drug interactions can be obtained via the website for hepatitis drug interactions [172].

**PREVENTION**

Currently implemented and effective primary prevention activities for HCV are: screening donors of blood, plasma, organ, and tissue; virus inactivation of plasma-derived products; sterilization of surgical and dental instruments; high coverage of comprehensive harm reduction programs including needle and syringe exchange and opiate substitution programs for PWID [173–175]. In response to the recent epidemic among HIV-infected MSM, HCV awareness campaigns have been launched. In addition, secondary prevention efforts include identification, counseling, and testing especially those who are at increased risk of infection. Timely diagnosis may be beneficial by preventing ongoing transmission through behavioral adaptation, contact tracing, and treatment initiation (i.e., treatment as prevention) [176,177].

**Preventive efforts among PWID**

The risk of HIV- and HCV-infection decreased notably among PWID in Amsterdam since 1990; probably partly because of implemented harm reduction programs [174,175,178]. These harm reduction programs consisted of needle exchange programs (started in the mid-1980s), opiate substitution therapy (methadone programs started in 1981) and risk education programs [179]. Programs with high uptake and a combined multi-component approach have the best potential to decrease onwards transmission of HCV [180] as well as reinfection [181]. The beneficial effect of integrated interventions in PWID on HCV was also shown in studies conducted outside the Netherlands [182–187]. Treatment of HCV with peg-interferon and ribavirin has become increasingly available for PWID and, while expensive, is considered cost-effective [174,188,189]. However, while studies have shown that response rates are comparable with other populations, HCV treatment uptake is still limited among PWID [190,191].

**Preventive efforts among MSM**

Several risk reduction strategies are applied by MSM to prevent infection with sexually transmitted infections (STI). Condom use is the most known and most widely practiced way of protection against STI. HIV-specific risk reduction strategies that are applied and do not include condoms are strategic positioning (top/bottom), withdrawal before ejaculation,
and serosorting [52, 192]. However, especially the latter is thought to have (and have had) a significant effect on the transmission of HCV (and other STI) among HIV-infected MSM [50]. The impact of treatment as prevention among MSM is likely to be important in reducing the number of incident HCV infections, as suggested in two modelling studies [193, 194]. In addition, awareness of HCV and knowledge of HCV complications increased over time [195], as well as the number of MSM that know their status by getting tested.

In the Netherlands, awareness of HCV among MSM has been created, among others, by the ‘Man tot Man’ (i.e., Male to Male) initiative. This initiative of Soa Aids Netherlands and the Public Health Services of Amsterdam and Rotterdam-Rijnmond, aims to provide information on homosexuality, sexual health, sexually transmitted infections, etc. Their website [196] also includes an extensive summary of current knowledge on HCV: e.g., symptoms, risk factors, and options for testing and treatment. Also Poz&Proud, part of the Dutch HIV Association, has information on their website about HCV [197] and flyers are distributed since August 2009. International examples of information providers are the Hepatitis C Trust from the UK [198], the Centers for Disease Control and Prevention (CDC) [199], and the National AIDS Treatment Advocacy Project (NATAP) from the USA [200], and Canada’s Source for HIV and Hepatitis C Information (CATIE) [201].

Vaccine development

Currently no vaccine exists for the prevention of infection with HCV. The development of a vaccine has been hampered by various obstacles, such as the high level of genetic variability, the relatively poor immune response of the host, and the absence of small animal models to test candidate vaccines [202]. However, a cell culture system that allows studying virus neutralization in vitro has been introduced in 2005 [203] and more recently, full genome HCV replication in human hepatoma cell lines has been shown [204]. The availability of these systems will likely increase the pace in which vaccine candidates can be identified. Earlier studies have shown that an effective vaccine would be capable of inducing cross-neutralizing antibodies against all major HCV genotypes, as well as broad HCV-specific CD4+ T helper and CD8+ cytotoxic responses [205]. The most promising results so far have been obtained with the use of an adenovirus-based vector vaccine comprising envelope glycoproteins gpE1/gpE2 [206, 207].
DATA USED IN THIS THESIS

The studies described in this thesis rely on data that were collected in the initiatives summarized in Table 1. The background of each of these cohorts is stated here, in order of appearance in this thesis:

Amsterdam Cohort Studies
The Amsterdam Cohort Studies on HIV infection and AIDS (ACS) is a collaboration between the Public Health Service of Amsterdam, the Academic Medical Center, Sanquin Blood Supply Foundation, DC Klinieken, and the Stichting HIV Monitoring (SHM). It comprises two open and ongoing cohort studies: one among MSM that started in 1984 [208], and one among injecting and non-injecting drug users (DU) that started in 1985 [209]; these studies aimed to investigate the epidemiology, psychosocial determinants, course of infection, and pathogenesis of HIV infection, and of blood-borne infections and STI other than HIV, and to evaluate the effects of interventions. As of 31 December 2014, these cohorts consisted of 2,649 MSM and 1,680 DU. Every three to six months, participants complete a standardised questionnaire regarding demographics, medical history, sexual and drug use behaviour, underlying psychosocial determinants, healthcare use, depression, and psychological disorders. HIV-infected participants undergo a medical examination (in the past, also HIV-negative DU were examined), and blood is collected for diagnostic tests and storage.

DUTCH-C project
In December 2004 the Drug Users Treatment for Chronic Hepatitis C (DUTCH-C) project was initiated to evaluate the feasibility of HCV testing and treatment among DU. This multidisciplinary treatment unit was a collaboration between the Academic Medical Center and the Public Health Service of Amsterdam. Opiate substitution therapy was integrated, and provided by a major clinic located close to the Public Health Service of Amsterdam. The DUTCH-C project started with HCV screening of 449 ACS participants, 267 of whom were anti-HCV positive, and 134 were HCV-RNA positive [210]. Because of the successful treatment results that were obtained thereafter (65% obtained SVR in an intention-to-treat analysis), the project was extended in 2007 to also provide HCV treatment to non-ACS DU. In March 2014, the approach changed; HCV care for DU is coordinated by a social nurse of the Mental Health department of the Public Health Service of Amsterdam (MGGZ). Treatment is provided by liver specialist in the hospital in close collaboration with the MGGZ department.

MSM network study
The MSM network study recruited 2,492 MSM between August 2008 and July 2009, at the STI outpatient clinic of the Public Health Service of Amsterdam and the HIV treatment centre of the Academic Medical Center Amsterdam. All were aged ≥18 years and could understand written Dutch or English. In order to determine the spread of STIs among MSM, participants
were tested for STI and completed a detailed questionnaire. Urethral, rectal and pharyngeal *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, hepatitis B and C, and HIV were tested according to the STI clinic protocol; participants could opt-out for HIV testing. The questionnaire was administered online and regarded demographics of themselves, their steady partner and/or up to three other partners during the preceding 6 months. Overall sexual behavior was assessed, as well as sexual behavior with each of the mentioned partners.

**MOSAIC study**

The MSM Observational Study for Acute Infection with hepatitis C (MOSAIC) study was initiated in December 2008 and is still ongoing. The original main objectives were to identify the frequency, clinical consequences and determinants (viral and host factors) of acquiring acute HCV infection (primary, re- or superinfection), to assess HCV treatment outcome, and to study the impact of acute HCV infection on the morbidity and mortality. The study population comprises HIV-infected MSM aged ≥18 years old that were prospectively or retrospectively diagnosed with acute HCV, and HIV-monoinfected controls (only included for prospectively identified cases) [137]. Since the revision of the research protocol in January 2015 inclusion is extended to HIV-negative MSM and HIV-infected heterosexuals with acute HCV, and the objectives (mentioned earlier) were also extended to these populations. In addition, identification of the driving factors of the HCV epidemic among MSM has been added to the objectives of the study, as well as studying the role of HIV/HCV-coinfected MSM in the ongoing HIV epidemic. Blood samples are stored and clinical outcomes, including treatment and fibroscan results, are collected during inclusion and follow-up visits that take place every six months, or more frequently during HCV treatment. Questionnaires on risk behaviour and quality of life are self-administered and also collected at each study visit. The data collection takes place during regular patient care visits at HIV outpatient clinics in Amsterdam at the Academic Medical Center, Onze Lieve Vrouwe Gasthuis (OLVG Hospital), Slotervaart Hospital, in Utrecht at the University Medical Center, and in Rotterdam at the Erasmus University Medical Center. Up to May 2015, 156 HIV-infected MSM with acute HCV infection (cases; 105 of whom were prospectively identified), and 166 HIV-infected MSM with no history of HCV infection (controls) have been included. The MOSAIC study will continue to include and follow-up participants at least until mid-2018.

**ATHENA national observational HIV cohort**

The AIDS Therapy Evaluation in the Netherlands (ATHENA) project was initiated in 1996 to investigate the effects of early adoption of the (then) newly introduced combination antiretroviral therapy for HIV infection. The valuable results obtained among 3,600 HIV-infected patients, led to the continuation and extension of the research; the Stichting HIV Monitoring (SHM) was established in 2001 to include all HIV-infected patients in the Netherlands. By May 2015, the ATHENA national observational HIV cohort comprised data from over 24,263 HIV-infected individuals, followed longitudinally in 26 HIV treatment centres in the Netherlands and one in Curaçao [211].
Bi-annual HIV surveys at the STI clinic
From 1991 until 2012, cross-sectional and anonymous bi-annual surveys were performed at the STI outpatient clinic from the Public Health Service of Amsterdam (in Dutch also known as the ‘DWAR’ studies). These surveys aimed to gain insight into the heterosexual spread of HIV infections in Amsterdam [212]. Recruitment during each survey took place until at least 1,000 STI clinic attendees (men and women; heterosexual, bisexual, and homosexual) were included and interviewed about risk factors for STI and blood-borne infections. From all participants, blood was drawn, tested and stored. Because a high HCV prevalence was found among HIV-infected MSM in May 2007 (14.6%), anti-HCV testing became available for all MSM with HIV-positive or unknown status attending the STI outpatient clinic in November 2007 [18]. Unfortunately, this service was stopped because of financial constraints in May 2014.

AIMS AND OUTLINE OF THIS THESIS

In several studies presented in this thesis, epidemiological and molecular approaches were combined to gain insight into possible linkage between HCV infections. This gives an extra dimension to the outcomes measured in our studies, as the phylogenetic profile of the HCV infections could be added to the analyses. Other studies included longitudinal data from observational cohorts, in which incident HCV (re)infection and possible predictors for HCV infection could be evaluated. Blood samples collected during multiple cross-sectional studies were used to evaluate the utility of an HCV antigen test for screening purposes. The studies in this thesis were initiated with the ultimate goal to increase our understanding of primary and recurrent acute HCV infection among two risk groups in the Netherlands: PWID and HIV-infected MSM. The results of these studies in particular may contribute to improved prevention programs, and may inform clinical decision making and testing policies (e.g., whom to test, and which test(s) should be used).

In part I the current state of the HCV epidemic among MSM and PWID is illustrated. Chapter 2 shows updated estimates of the incidence of primary HCV infection among MSM from the ACS. In Chapter 3, the incidence of HCV reinfection during follow-up among active drug users that were successfully treated in the DUTCH-C project is studied.

Part II focuses on sexual transmission of HCV among HIV-infected MSM. In chapter 4, data from the MSM network study were analysed to determine whether HCV circulated in identifiable high-risk MSM subcultures. Chapter 5 describes risk factors for HCV infection by comparing the baseline questionnaires and clinical data of cases and control patients from the MOSAIC study. Data from the MOSAIC study were combined with data from the ATHENA observational cohort in chapter 6, in which the existence of HIV-transmission networks with increased risk for HCV infection was studied using phylogenetic analysis of both HIV and HCV sequences.
Part III describes the possible use of assays for screening and diagnosis of acute HCV (re)infection in various settings. In chapter 7 HCV antibody dynamics following acute (re)infection were investigated among participants of the MOSAIC study. Chapter 8 presents a case study of an HIV-infected man with seronegative HCV infection, who also had no signs of hepatic inflammation. Chapter 9 evaluates the use of an HCV antigen assay for screening for acute and chronic HCV infection among HIV-infected MSM. For this study, data and sera collected at the bi-annual surveys at the STI outpatient clinic were used.

Finally, chapter 10 discusses the main findings of this thesis, relates these to recent literature, and gives recommendations for future studies.
Table 1 Overview of data sources used for the studies described in this thesis.

<table>
<thead>
<tr>
<th>Data source</th>
<th>Study (sub-)population used in the study that is presented in this thesis</th>
<th>Data used from study period</th>
<th>Chapter(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS among MSM [208]</td>
<td>HIV-infected and HIV-uninfected MSM, followed-up at the Public Health Service of Amsterdam</td>
<td>1984-2011</td>
<td>2</td>
</tr>
<tr>
<td>DUTCH-C project [210]</td>
<td>HCV-mono-infected active drug users from the ACS that were treated for HCV</td>
<td>2005-2011</td>
<td>3</td>
</tr>
<tr>
<td>MSM network study [213]</td>
<td>HIV-infected MSM with and without HCV infection, recruited at the STI clinic of the Public Health Service of Amsterdam and at the HIV outpatient clinic of the AMC</td>
<td>2008-2009</td>
<td>4</td>
</tr>
<tr>
<td>MOSAIC study [137]</td>
<td>HIV-infected MSM with and without acute HCV infection, recruited at the HIV outpatient clinics of the AMC, QLVG Hospital, Slotervaart Hospital, UMC Utrecht, and Erasmus UMC</td>
<td>2009-2014</td>
<td>5-7</td>
</tr>
<tr>
<td>ATHENA national observational HIV cohort study [211]</td>
<td>HIV-infected individuals MSM, recruited at 26 HIV treatment centers in the Netherlands</td>
<td>1996-2014</td>
<td>6</td>
</tr>
<tr>
<td>Bi-annual HIV surveys [212]</td>
<td>HIV-infected MSM, recruited at the STI outpatient clinic of the Public Health Service of Amsterdam</td>
<td>2009-2012</td>
<td>9</td>
</tr>
</tbody>
</table>

Abbreviations: ACS: Amsterdam Cohort Studies; AMC: Academic Medical Center; DUTCH-C: Drug Users Treatment for Chronic Hepatitis C; HCV: hepatitis C virus; HIV: human immunodeficiency virus; MOSAIC: MSM Observational Study of Acute Infection with hepatitis C; MSM: men who have sex with men; STI: sexually transmitted infection; UMC: University Medical Center.
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